ANALYSIS OF *ANAPLASMA MARGINALE* STRAINS GROWN *IN VITRO*

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Par

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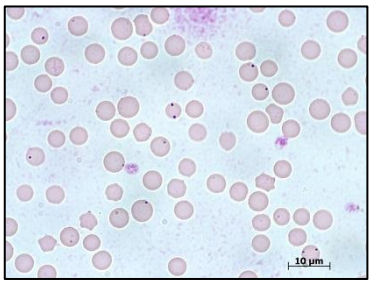

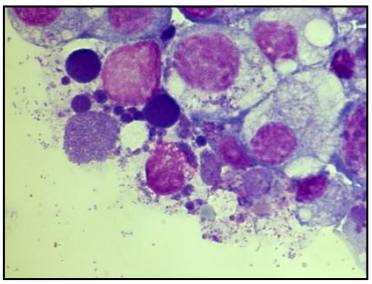

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"Analysis of *Analplasma marginale* strains grown in vitro"

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PREFACE

This PhD project was conducted at the Institute of Comparative Tropical Medicine and Parasitology at Ludwig-Maximilians-Universität, München, Germany.

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Key words: *Anaplasma marginale,* tick cell cultures, Percoll gradients, Major Surface Proteins, 2D-DIGE, IDE8

Mots clés: *Anaplasma marginal*e, culture cellulaire tique, Percoll gradients, Major Surface Proteins, 2D-DIGE, IDE8

Abstract

Anaplasma marginale is a tick-borne pathogen that affects ruminants worldwide, causing a disease called anaplasmosis. The disease is endemic in tropical and subtropical regions of the New World, Europe, Africa, Asia and Australia where it causes large economic losses in the cattle industry.

A. marginale is an obligatory intracellular bacterium that multiplies only within tick cells or ruminants' erythrocytes. Many differences among *A. marginale* strains have emerged, which were probably driven by continuous exposure to different host immune systems during the transition of bacteria between ticks and vertebrates. The vast majority of studies aiming at elucidating differences between strains were conducted on the genomic level, and little is known about protein expression. Thus, this thesis investigates differences in protein regulation among *A. marginale* strains.

A. marginale cultivated *in vitro* are in general an excellent source of organisms for experimentation. Furthermore, culture-derived organisms offer an alternative to the use of experimental animals.

Many studies require intracellular organisms free from host cell debris. Therefore the use of Percoll gradients for the separation of *A. marginale* was evaluated. Bacteria isolated in this way contained only minimal amounts of IDE8 cell stroma but most importantly they retained their viability. *A. marginale* purified this way can be used directly for proteomic studies or for vaccination trials.

In this thesis three geographical *A. marginale* strains grown *in vitro* have been partially characterized by gene and serological analyses. The differences on the proteomic level have been assessed by the 2D-DIGE technique, indicating that many antigenic membrane proteins are differentially regulated among the strains examined. Some of these proteins are also known to be virulence-associated.

Increasing the number of strains in continuous *in vitro* cultivation, and improving purification methods for rickettsia, allow researchers to investigate differences in protein expression between *A. marginale* strains, and therefore identify proteins which could be incorporated into an improved vaccine against anaplasmosis.

Résumé

Anaplasma marginale est un agent pathogène issu des tiques qui affectent les ruminants dans le monde entier, causant une maladie appelée anaplasmosis. La maladie est endémique dans les régions tropicales et subtropicales du Nouveau Monde, d'Europe, d'Afrique, d'Asie et d'Australie où elle cause d'importantes pertes économiques dans l'industrie du bétail.

A. marginale est une bactérie obligatoirement intracellulaire qui ne se multiplie que dans les cellules des tiques ou des érythrocytes des ruminants. De nombreuses différences parmi les souches de *A. marginale* sont apparues, probablement à cause d'une exposition continue à différents systèmes immunitaires d'hôtes lors du passage de la bactérie des tiques aux vertébrés. Un grand nombre d'études génétiques ayant pour but d'élucider les différences entre les souches ont été réalisées.

Malheureusement, peu de résultats concernant l'expression des protéines d'*A. marginale* ont été obtenus. Alors que cette thèse prouve des différences dans la régulation des protéines parmi les souches de *A. marginale.*
A. marginale s'étant reproduites *in vitro* sont en général une excellente source d'organismes pour les expérimentations. De plus, les organismes issus de ces cultures sont une excellente alternative aux animaux de laboratoire.

De nombreuses études cliniques requièrent des organismes intracellulaires débarrassés de tout débris de la cellule hôte. Pour ce faire, l'utilisation de gradients Percoll pour la séparation de *A. marginale* a été pratiquée. Les bactéries isolées de cette manière ne contenaient qu'une quantité infime de IDE8 cellules, mais plus intéressant elles conservaient leur viabilité. *A. marginale* purifiées de cette manière peuvent être utilisées directement pour des études protéomiques ou pour des études ayant pour but le développement de nouveaux vaccins.

Trois souches séparées géographiquement de *A. marginale* élevées *in vitro* ont été partiellement caractérisées par des analyses génétiques et sérologiques. Les différences au niveau protéomique ont été mesurées au moyen de la technique 2D-DIGE, indiquant que de nombreuses membranes protéines antigéniques sont

différemment dosées dans les souches étudiées. Certaines de ces protéines sont aussi connues pour avoir un facteur de virulence.

L'augmentation du nombre de souches dans de continuelles cultures *in vitro* et l'amélioration des méthodes de purification des bactéries ont permis aux scientifiques de rechercher les différences dans l'expression protéine des souches *A. marginale* et ainsi d'identifier quelles protéines pourraient être utilisées pour un vaccin plus efficace contre anaplasmosis.

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CHAPTER 1

1. Literature review

1.1. Anaplasma marginale - historical background

Anaplasma marginale was first described in the early 1900s by Sir Arnold Theiler who observed “marginal points” in erythrocytes of cattle suffering from gallsickness (galsiekte) (Theiler, 1910, 1911, 1912). Although two decades earlier the microorganism had already been discovered by other investigators, it had been erroneously considered as a part of the *Babesia bigemina* life cycle (Smith and Kilborne, 1893). Yet, Theiler demonstrated that babesiosis and anaplasmosis can often co-exist in the same animal; he then succeeded in separating the two agents and produced a “pure infection” with only *A. marginale*. He indicated that the “marginal points” differ from any known blood parasite and named the new pathogen *Anaplasma marginale*.

The scientific name proposed by Theiler was based on the microscopic observation of the pathogen in infected blood smears. “Anaplasma” stands for the absence of a stained cytoplasm, and “marginale” for its marginal localization in infected erythrocytes. Furthermore, Theiler (1911) also described a subspecies of *A. marginale*: located in the centre of erythrocytes. *Anaplasma centrale*, is less pathogenic than *A. marginale* and causes only a slight attack of the disease. An *A. centrale*-based, blood-derived, live vaccine was exported from South Africa to other parts of the world, i.e. Australia, Israel and Latin America, where it has been in use for over a century (Kocan et al., 2010b).

1.2. Epidemiology

A. marginale is one of the most prevalent tick-borne pathogens of cattle in tropical and subtropical areas worldwide (~40° N to ~32° S) (Aubry and Geale, 2011). It is endemic in the New World, Central and South America, Australia and some regions of Asia and Africa (Kocan et al., 2010b). In the USA anaplasmosis is enzootic throughout the southern states, but due to the movement of cattle, anaplasmosis has now been reported in almost every state (Kocan et al., 2010a). In some countries it is

considered to be a foreign animal disease e.g. in Canada, where an outbreak of anaplasmosis resulted from mechanical transmission of the organism from imported cattle (Boulanger et al., 1971). In Europe, it is found mainly in Mediterranean countries like Italy (de la Fuente et al., 2005e; de la Fuente et al., 2005f; Torina et al., 2008) and Spain (de la Fuente et al., 2005d), although few isolated cases have also been reported in Hungary (Hornok et al., 2012) and Austria (Baumgartner et al., 1992).

The wider distribution and increase in outbreaks of the disease result from transport of asymptomatic carrier animals, which are reservoirs for subsequent mechanical or biological transmission to susceptible cattle in non-endemic areas. Wildlife may be possible reservoir hosts, and represent a source of infection for free-ranging cattle (Kocan et al., 2010a; Kocan et al., 2010b). Furthermore, factors such as climate, host abundance, tick-host diversity and topography have been all shown to have an impact on the epidemiology of *A. marginale* (Estrada-Pena et al., 2008). Changes in climate influence the distribution, physiology and behavior of many different arthropod vectors (Jonsson and Reid, 2000). The possible introduction of new tick species into areas where they did not exist before may complicate the control and prevention of tick-borne diseases.

1.3. Classification

In 2001 Dumler et al. proposed a reclassification of Rickettsiales based upon genetic analysis of 16S rRNA and *groESL* genes. Organisms of this taxon were then assigned to one of the two families: Rickettsiaceae and Anaplasmataceae. All bacteria classified within these families are intracellular pathogens. However, unlike the Rickettsiaceae, which grow freely within the host cytoplasm or nucleus, members of Anaplasmataceae are found exclusively within membrane-bound vacuoles in the cytoplasm of the host cells. Moreover, almost all organisms assigned to the family Anaplasmataceae multiply in both vertebrate and invertebrate hosts (Kocan et al., 2010a).

Following the phylogenetic analysis, four genera were formed within the Anaplasmataceae family, namely: *Ehrlichia*, *Neorickettsia, Anaplasma* and *Wolbachia.* All are gram-negative bacteria, demonstrating two morphological structures: large

reticulate forms, or smaller dense forms with condensed protoplasm. Anaplasmataceae infect canids, humans, ruminants and rodents. Formerly the genus *Anaplasma* consisted of *A. ovis*, *A. marginale* (**Table 1.1**) and a less pathogenic subspecies of *A. marginale*, *A. centrale* (*A. marginale* ss*. centrale*). Following the reclassification, *A. bovis* (formerly *Ehrlichia bovis*), *A. phagocytophilum* (formerly *Ehrlichia phagocytophila*, *E. equi* and the human granulocytic ehrlichiosis (HGE) agent), *A. platys* (formerly *Ehrlichia platys*) and *Aegyptianella* (genus *incertae sedis* due to the lack of sequence information) have also been included into the *Anaplasma* genus (Dumler et al., 2001).

Table 1.1. *Anaplasma marginale* classification according to Dumler et al. (2001).

Class:	Alphaproteobacteria
Order:	Rickettsiales
Family:	Anaplasmataceae
Genus:	*Anaplasma*
Species:	*Anaplasma marginale*

1.4. Life cycle

The life cycle of *A. marginale* is coordinated with the tick feeding cycle (**Figure 1.1**) (Kocan et al., 2004; Kocan et al., 1992a). Ticks become infected when feeding on *A. marginale*-infected animals.

The likelihood of ticks acquiring at least one organism is higher in the acute phase (95–100 %) of infection when compared to the chronic phase (27–84 %) (Eriks et al., 1993). Infected erythrocytes are ingested by ticks with the blood meal, providing the source of *A. marginale* infection for tick gut cells (**Figure 1.2.A**). Afterwards extensive replication occurs within other tick tissues, including the salivary glands (**Figure 1.2.B**).(Ge et al., 1996; Kocan et al., 1992a; Kocan et al., 1992b).

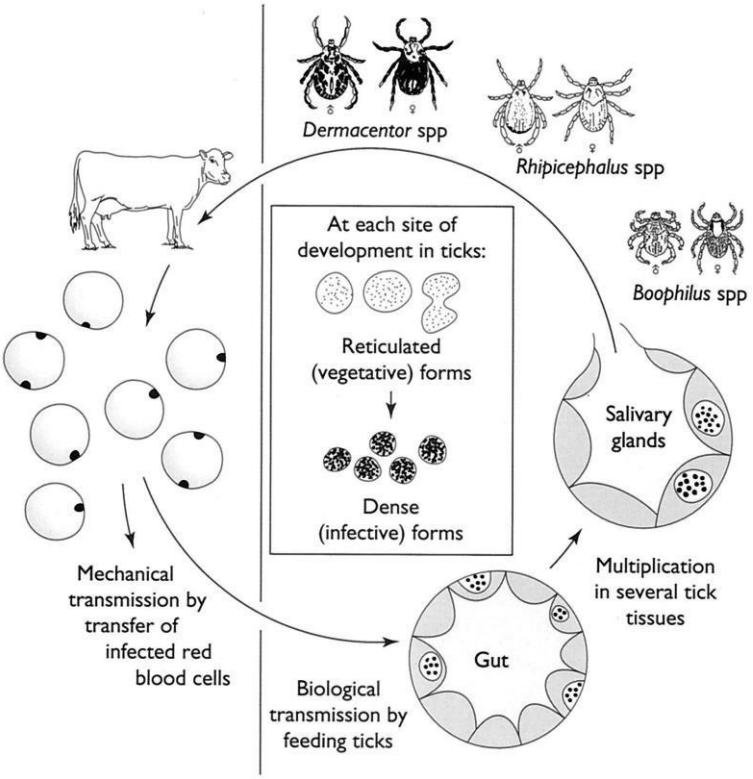


Figure 1.1. Developmental cycle of *A. marginale* in cattle and ticks (taken from Kocan et al. 2003).

The level of *A. marginale* organisms in adult male *Dermacentor andersoni* ticks can reach approximately 10^5 organisms per salivary gland (Kocan et al., 1992a) regardless of the rickettsemia level in the blood during acquisition feeding (Eriks et al., 1993). During subsequent feeding rickettsiae are transmitted via the salivary glands of the tick to vertebrate hosts (Kocan et al., 1992a).

At each infection site within the tick, *A. marginale* develops within membrane-bound vacuoles, forming colonies. The first form seen within *A. marginale* colonies is the reticulated (vegetative) form, which divides by binary fission (**Figure 1.3**, asterisk) and results in the formation of large colonies containing hundreds of organisms. The reticulated forms are then transformed into dense forms (0.5-0.8 µm) (**Figure 1.3**, arrow), which are the infective forms. They can survive for a short time outside cells (Kocan et al., 2008).

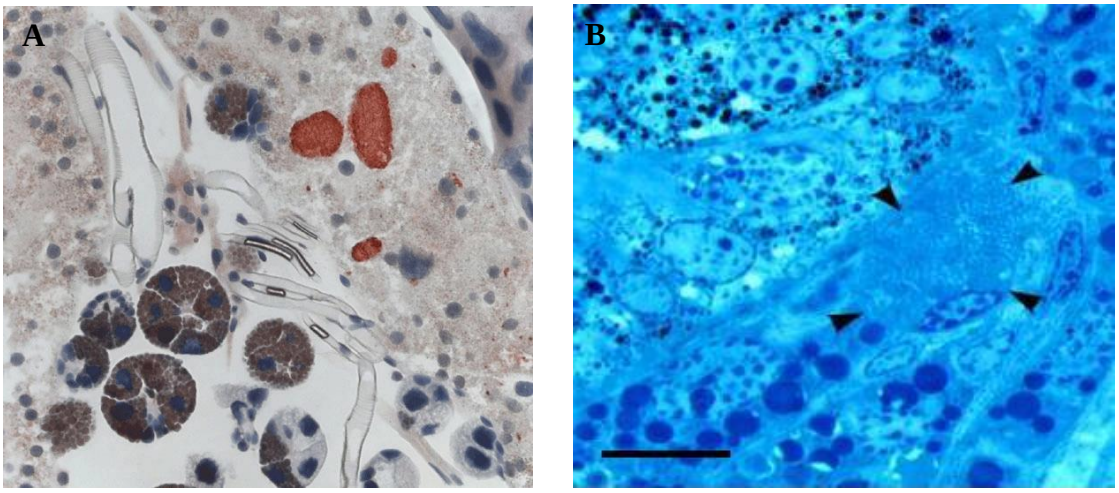
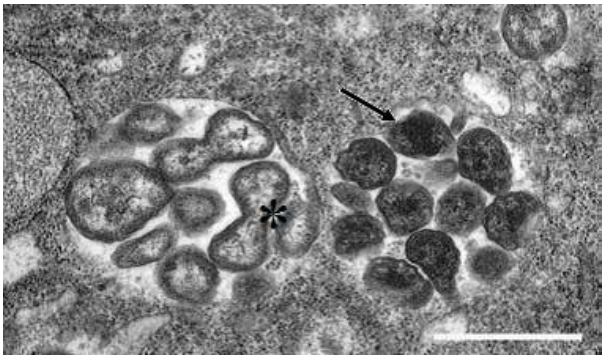


Figure 1.2. *A. marginale* development within tick tissues. A). Colonies (red) in *D. andersoni* midgut cells, (taken from "Livelihood hazards" M. Sebaihia, N. R. Thomson, L. Crossman and J. Parkhill); B). *A. marginale* colonies (arrowheads) in *Dermacentor reticulatus* salivary gland cell (taken from Zivkovic et al. 2007).

Cattle become infected when the dense form is transmitted during tick feeding via the salivary glands (Kocan et al., 2004). The tick cell culture model has been used for studying entry and exit mechanisms of the rickettsia from IDE8 cells (Blouin and Kocan, 1998). Host cell invasion is initiated by the adhesion of the dense form to the tick cell membrane, and the rickettsia subsequently enters into cells by endocytosis.

While leaving the cell, colony and cell membranes fuse, allowing the rickettsia exit without host cell injury. Blouin and Kocan (1998) suggested that the same mechanism occurs within naturally infected tick cells, which may facilitate high infection rates without pathological changes in ticks.

Figure 1.3. Electron micrograph of the developmental stages of *A. marginale* within colonies in tick cells. Reticulated forms within a colony divide by binary fission (asterisk), dense forms (arrow). Bar =1 mm (taken from Kocan et al. 2004).



1.5. Transmission

Transmission of *A. marginale* to vertebrate hosts occurs in two main ways: biologically by ticks, and mechanically by biting flies or by blood contaminated fomites. Transplacental transmission to the calf fetus has also been reported (Grau et al., 2013; Maldonado et al., 2012; Rey Valeiron et al., 2003; Zaugg, 1985).

Various tick species have been reported to be vectors of *A. marginale* in different regions of the world (**Table 1.2**) (Kocan et al., 2004). *A. marginale* DNA has been identified in many tick species or in ticks which transmitted the disease experimentally. However, this does not necessarily imply that they are able to transmit the organisms under natural conditions (Shkap et al., 2009; Zivkovic et al., 2007). In addition, recent analysis suggests that in some regions tick species which have not previously been considered as vectors may also transmit *A. marginale (*de la Fuente et al., 2005d; Fyumagwa et al., 2009; Zahang et al., 2013). Above all, *Rhipicephalus* (*Boophilus)* spp. are the most prevalent vectors of anaplasmosis in most tropical and subtropical countries. In the United States, however, *Dermacentor* spp., including *D. variabilis*, *D. andersoni* and *D. albipictus*, are the major vectors of anaplasmosis (de la Fuente et al., 2001c; Kocan et al., 1981), probably because a compulsory acaricide-treatment program in the 1940s (Stiller et al., 1989) led to the eradication of the *R.* (*B.) microplus* tick.

Transmission occurs by one stage (intrastadial) or from stage to stage (inter- or transstadial). Intrastadial transmission is effectuated mainly by male ticks (Kocan et al., 2010a). Serial transmission by male *D. andersoni* ticks to five consecutive cattle has been demonstrated (Kocan et al., 1992a). However, it has been shown that *A. marginale* was not transmitted from infected to uninfected adult *Dermacentor* spp. ticks during co-feeding on the same cattle (Kocan and de la Fuente, 2003). Interstadial transmission e.g. ingestion by nymphs and inoculation by adults has been demonstrated by *R.(B.) annulatus,* a single-host tick (Shkap et al., 2009), and by *D. andersoni,* a three-host tick (Kocan et al., 1981).

Table 1.2. Tick species transmitting *Anaplasma marginale* (modified after Kocan et al. (2004).

Tick species	References
Ixodid Ticks	
*Amblyomma gemma**	(Fyumagwa et al., 2009)
Dermacentor albipictus	(Lankester et al., 2007)
Dermacentor andersoni	(Anthony and Roby, 1966; Kocan et al., 1992a; Kocan et al., 1981; Lankester et al., 2007)
Dermacentor hunteri	(Stiller et al., 1999)
Dermacentor occidentalis	(Anthony & Roby, 1966)
Dermacentor reticulatus	(Zivkovic et al., 2007)
Dermacentor variabilis	(Anthony & Roby, 1966; Kocan et al., 1981; Lankester et al., 2007; Stich et al., 1989)
Hyalomma asiaticum	(Zahang et al., 2013)
Hyalomma excavatum	(Shkap et al., 2009)
Hyalomma marginatum rufipes	(Potgieter, 1979)
Ixodes scapularis	(Rees, 1934)
Ixodes ricinus	(Helm, 1924)
Rhipicephalus (Boophilus) annulatus	(Samish et al., 1993)
Rhipicephalus appendiculatus	(Fyumagwa et al., 2009)
Rhipicephalus bursa	(Sergent et al., 1945)
Rhipicephalus (Boophilus) calcaratus	(Sergent et al., 1945)
*Rhipicephalus compositus**	(Fyumagwa et al., 2009)
Rhipicephalus (Boophilus) decoloratus	(Potgieter, 1979; Theiler, 1912)
Rhipicephalus (Boophilus) microplus	(Futse et al., 2003)
*Rhipicephalus praetextatus**	(Fyumagwa et al., 2009)
*Rhipicephalus pulchellus**	(Fyumagwa et al., 2009)
Rhipicephalus sanguineus	(Shkap et al., 2009)
Argasid Ticks	
Argas persicus	(Howell et al., 1941)

* Tick species in which *A. marginale* DNA has been detected.

The occurrence of transovarial transmission of few tick-borne pathogens e.g. *Babesia* spp. by single-host *R. Boophilus* spp. is well known (Howell et al., 2007). Yet, transmission of *A. marginale* from one tick generation to the other remains controversial, although Theiler (1912) and few other authors have suggested that this type of transmission does occur (Anthony and Roby, 1962; Rees and Avery, 1939; Stich et al., 1989). Interestingly, multiplication of *A. marginale* within the tissues of engorged *R.(B.) microplus* females has been confirmed (Ribeiro and Lima, 1996). Moreover, in eggs and larvae derived from *R. B. microplus* ticks collected from infected cattle, *A. marginale* specific DNA fragments have been amplified. Yet, the transmission of *A. marginale* by these larvae to animals has never been proven (Moura et al., 2003). Some authors suggested that *Ehrlichia* spp. and *Anaplasma* spp. are not transmitted transovarially due to the lack of the aldolase/adducing domain protein (Dunning Hotopp et al., 2006).

Mechanical transmission of the pathogen occurs when infected blood is transferred to susceptible animals by contaminated fomites: needles, dehorning saws, nose tongs, tattooing instruments, ear tagging devices and castration instruments (Kocan et al., 2004) . Additionally, different species of hematophagous diptera e.g. *Tabanus* spp. flies (Hawkins et al., 1982), *Stomoxys calcitrans* (stable fly) (Potgieter et al., 1981) or mosquitoes have been demonstrated to have the ability to disseminate *A. marginale*. Although biological transmission has been shown to be more efficient (Scoles et al., 2008), mechanical transmission is the major route of infection in areas where the strains are not tick-transmissible or appropriate tick vectors do not occur (de la Fuente et al., 2001c).

1.6. Pathogenesis

A. marginale is very host specific and under natural conditions infects only ruminants. Although clinical anaplasmosis occurs most often in cattle, other ruminants like water buffalo (*Bubalus bubalis*), American bison (*Bison bison*), white-tailed deer (*Odocoileus virginianus*), black-tailed deer (*Odocoileus hemionus columbianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*), black wildebeest (*Connochaetes gnou*), blesbuck (*Damaliscus pygargus phillipsi*) and duiker (*Sylvicapra grimmia*) can also become infected (Aubry and Geale, 2011; Kocan et al., 2010b; Kuttler, 1984).

The only known site of *A. marginale* development in cattle is within erythrocytes (**Figure 1.4.A**). Interestingly, because bacteria can be propagated in a bovine endothelial cell line (Munderloh et al., 2004), it has been suggested that endothelial cells may serve as a site of initial replication after tick attachment, or as a reservoir for *A. marginale* during persistent infection. After experimental infection of calves with the *A. marginale* St. Maries strain, Carreno et al. (2007) observed the infection of endothelial cells by dual fluorescence microscopy. In contrast, Wamsley et al. (2011) did not detect *A*. *marginale* within endothelial cells after tick-feeding transmission to immunocompetent cattle either in dermal samples of tick attachment sites or in post-mortem tissues. In addition, they also did not observe seroconversion or clinical anaplasmosis in calves, when *A. marginale* grown in the endothelial cell line (RF/6A) was used for the experiments. At the moment *in vivo* infection of endothelial cells remains controversial.

A. marginale enters erythrocytes by endocytosis and resides within small membrane-bound inclusions, referred to as initial bodies, where it divides by binary fission (Kocan et al., 1978b). The membrane-bound vacuole derives from the erythrocyte membrane and can contain four to eight organisms (**Figure 1.4.B**). In acute anaplasmosis multiple infections of single erythrocytes are observed. *A. marginale* has rarely been observed free of erythrocytes. Interestingly treatment of cells with a calcium ionophore induced bacteria exit, suggesting a mechanism that is dependent on the mobilization of calcium (Brown et al., 2006).

Clinical disease in cattle is directly related to the number of infected erythrocytes. During the initial infection, there is a geometric increase phase when the number of infected red blood cells doubles nearly every 24 h (Miller, 1956). In the acute phase up to 70 % of erythrocytes can be infected (Kieser et al., 1990; Kocan et al., 2010a), although the first symptoms can occur as soon as only 15 % of erythrocytes are infected. The incubation period varies with the number of organisms in the infective dose and ranges from 7 to 60 days (Kocan and de la Fuente, 2003).

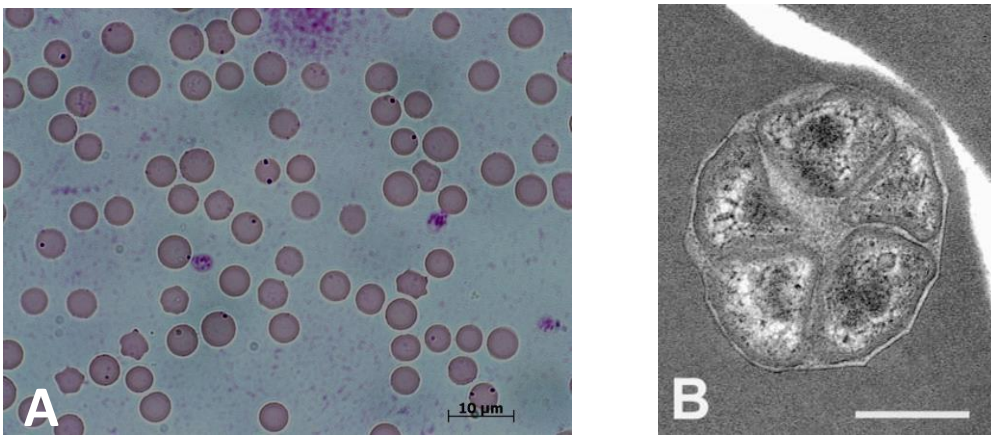


Figure 1.4. Bovine erythrocytes infected with *A. marginale.* A). Organisms are seen as black, irregular shaped dots, usually at the edge of infected red blood cells, Giemsa staining, B). An infected erythrocyte with five *A. marginale* inclusion bodies. Electron microphotograph, bar 0,5µm (taken from Kocan et al. 2004).

During the course of infection, erythrocytes become chemically altered by the bacteria. Subsequently, „marked" erythrocytes are recognized by reticuloendothelial cells and removed from the circulation, which results in anemia and icterus (Kocan et al., 2003).

The acute phase of the disease includes symptoms such as fever, weight loss, icterus, abortion, lowered milk production and even death (Kuttler, 1984). Differences in virulence between *Anaplasma* strains and the level and duration of the rickettsemia play a role in the severity of clinical manifestations. Although cattle of all ages can become infected with *A. marginale*, the severity of disease is age dependent. Calves under 6 months of age are much more resistant to disease (although not infection) than older cattle. In older calves mild or acute, but rarely fatal disease develops, while in cattle over 2 years of age, the disease often is fatal (Kocan et al., 2003). Cattle that recover from anaplasmosis remain lifelong carriers serving as a reservoir of the rickettsia (Kieser et al., 1990). During the carrier state, the rickettsemic cycles occur at approximately seven weeks intervals, with peaks of 10^7 rickettsia per ml of blood (Eriks et al., 1993; French et al., 1998; Kocan et al., 2010b). The chronically infected cattle are generally immune to further clinical disease; however, they can relapse to anaplasmosis, for example when infected with other pathogens.

1.7. Differences within strains

Initially a small number of *A. marginale* strains was recognized on the basis of morphological characteristics, geographical origin, whether they were cross- protective in cattle or infectious and transmissible by ticks. Presently strains are characterized not only on the basis of the above characteristics, but additionally by either level of virulence or variation in membrane surface proteins (MSPs).

1.7.1. Morphology

Two morphological forms of *A. marginale* are known, one with (**Table 1.3**) and one without an inclusion appendage. Inclusion appendages, are also called "tails", "bands" or "filaments". They usually occur in the form of a tapering tail, a loop, a disk or a ring, and can only be visualized through immunological or ultrastructural techniques. With traditional staining, only the “head portion” of the tailed *Anaplasma* is visible (Carson et al., 1974).

The inclusion appendages observed under the electron microscope are not directly attached to the bacterium and are not surrounded by an inclusion membrane (**Figure 1.5**) (Kocan et al., 1984). In cattle erythrocytes, the tailed strain appears as a spherical marginal body, or as a “comet – shaped” organism, which contains a head, body and tail. In *D. andersoni* nymphs the inclusion appendages were observed in midgut tissues till 10 days after repletion from infected cows. Following day 15, appendages were found free in the midgut lumen or attached to the cell membrane of midgut epithelial cells (Kocan et al., 1984).

The tails are composed of polymerized F-actin filaments with a diameter of 7-10 nm and contain no parasite DNA (Stich et al., 1997). Ferritin-conjugated anti-*A. marginale* sera react with bacterial-specific antigens, indicating that appendages are recognized by the host’s immune system (Kocan et al., 1978a; Kocan et al., 1978b). Stich et al. (1997) have also shown that the inclusion appendage contains host actin filaments. Interestingly, unlike the classic pattern in which actin is assembled on the bacterial surface, the *A .marginale*-associated appendage assembles on the external vacuolar

surface and does not have to be secreted across the bacterial membrane and the membrane surrounding the parasitophorous vacuole. A new polymorphic appendage-associated protein has been identified, designated as *A. marginale* appendage associated protein (AAAP), however, its role in invasion or replication within the host cell is still unknown (Stich et al., 2004).

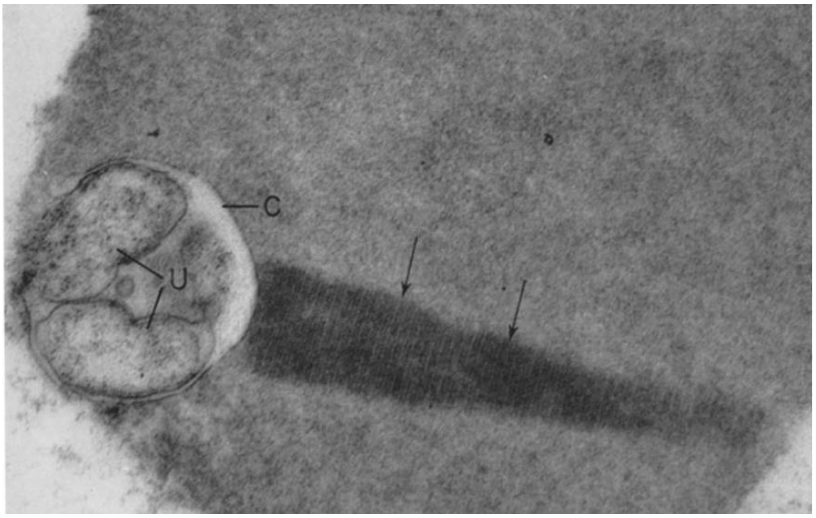


Figure 1.5. Electron micrograph of tailed *Anaplasma marginale* in bovine erythrocytes containing a comet-like (arrows) inclusion appendage (C) containing two subunits (U) (taken from Simpson et al. 1965).

The function of the *A. marginale* appendage in the infection of ticks and erythrocytes is unclear. At first, Kocan et al. (1984) suggested that the appendage may play a role in infection of tick gut cells, as they observed that only the tailed Virginia isolate, and not the Florida isolate (without appendage), infected *D. andersoni* ticks. Two years later, Smith et al. (1986) verified that an inclusion appendage is not responsible for infectivity, as only one of the two tailed *Anaplasma* strains tested, was readily transmitted by *D. variabilis* ticks. In some viruses and bacteria, the appendage has been shown to influence motility, which improve their propagation and enhance the spread of infection (Cossart and Lecuit, 1998). Stich et al. (1997) suggested that appendage increase *A. marginale* motility enhancing contact with the tick gut epithelium or bovine erythrocytes.

Table 1.3. *Anaplasma marginale* isolates with an inclusion appendage.

***A. marginale* isolate**	**Reference**
Illinois	(Smith et al., 1986)
Virginia	(Smith et al., 1986)
UFMG1 (Brazil)	(Ruiz et al., 2005)
California	(Potgieter et al., 1981)
Texas	(Franklin and Redmond, 1958)
Oregon	(Pilcher et al., 1961)
Mexico	(Simpson et al., 1965)
Oklahoma	(Kocan et al., 1978b)
Washington	(Barbet et al., 1983)
Israeli T	(Palmer et al., 1988)

Interestingly, *A. marginale* with an appendage was initially named *Anaplasma caudatum* (*caudatum* - tailed) (Boone et al., 2005; Kreier and Ristic, 1963). Moreover, in 1974 the creation of a new genus *Paranaplasma* has been proposed, due to serological and immunological differences between isolates with and without an appendage (Kreier and Ristic, 1974). Nonetheless, according to the work of Smith et al. (1986) this classification has been abandoned and presently *A. marginale* with an inclusion is not considered a separate species.

1.7.2. Major Surface Proteins

The surface of tick-borne intracellular bacteria consists of many proteins which are remodeled during the transmission of the pathogen between vertebrate and invertebrate hosts. They mediate functions which are necessary for survival, replication and transmission. Their expression changes, in order to facilitate bacterial survival in different hosts.

Six major surface proteins (MSPs) namely: MSP1a, MSP1b, MSP2, MSP3, MSP4 and MSP5 have been identified on *A. marginale* (Alleman et al., 1997; Barbet and Allred, 1991; McGarey and Allred, 1994; McGuire et al., 1994; Palmer et al., 1994;

Palmer et al., 1985) and are being used for phylogenetic analyses of *A. marginale* strains (as reviewed by de la Fuente et al., 2005b). These MSPs are involved in host–pathogen interactions and may evolve more rapidly than other nuclear genes because of selective pressure exerted by the host's immune system.

The *A. marginale* MSP1 complex is composed of a heterodimer of two structurally unrelated polypeptides: MSP1a and MSP1b, linked by disulfide bonds (Vidotto et al., 1994). The MSP1a is encoded by the single-copy gene, *msp1α* and varies in size among different geographic isolates due to the changing number of 23-31 amino acid tandem repeat peptides in the N-terminal part of the protein (Allred et al., 1990; as reviewed by Cabezas-Cruz et al., 2013; de la Fuente et al., 2003; de la Fuente et al., 2001b; de la Fuente et al., 2002c). The N-terminal part of MSP1a is highly glycosylated (Garcia-Garcia et al., 2004a). The expression of MSP1a in *A. marginale* from tick cell cultures is downregulated in comparison with bacteria derived from bovine erythrocytes (Garcia-Garcia et al., 2004b).
Formerly, the *msp1α* gene was widely used for phylogenetic studies, as it did not appear to undergo antigenic variation in cattle or ticks (Bowie et al., 2002). However, while phylogenetic studies of MSP1a repeat sequences provided evidence of *A. marginale*-tick coevolution, they could not provide phylogeographic information on a global scale because of the high level of MSP1a genetic diversity among geographic strains (Estrada-Pena et al., 2009).

MSP1b is encoded by members of the *msp1 β* multigene family (Barbet and Allred, 1991), which are polymorphic between different isolates of *A. marginale*. In contrast to MSP1a which has been shown to be an adhesin for bovine erythrocytes and tick cells (cultured and native), MSP1b is an adhesin only for bovine erythrocytes (de la Fuente et al., 2001a; McGarey and Allred, 1994).

MSP2 and MSP3 unlike other outer membrane proteins in *A. marginale*, have a single expression site but multiple alleles distributed throughout the chromosome (Brayton et al., 2003).

MSP2 is an immunodominant outer membrane protein, encoded by a polymorphic gene family (Palmer et al., 1994). The MSP2 expression is under the control of a single operon consisting of a promoter and four open reading frames. The *msp2*

gene consists of nine pseudogenes, which play a substantial role in achieving multiple antigenic variations (Brown et al., 2003).

MSP3 is also an immunodominant antigen, encoded by a polymorphic multigene family whose exact function is unknown (Alleman et al., 1997). Recently, it has been shown that simple variants of MSP3 are expressed in early mammalian infection and within the tick vector, and multiple antigenic variants emerge only under selective immune pressure during persistent infection (Palmer and Brayton, 2013).

Antigenic variation of MSP2 and MSP3 has been proposed as a likely mechanism by which *A. marginale* evades the host immune system, resulting in lifelong persistence in the mammalian host (French et al., 1999; French et al., 1998; Palmer et al., 2000).

MSP5 and MSP4 are immunodominant proteins, encoded by single gene copies, which remain conserved in different *A. marginale* strains, as well as in *A. centrale* (Molad et al., 2004; Oberle et al., 1993; Visser et al., 1992). At present, the role of these proteins is not well defined, however; the fact that they remain conserved suggests that they are important in the *Anaplasma* life cycle. Phylogenetic analysis indicated that MSP4 is not a good genetic marker for global analysis, but it can provide some information about strain differences within geographic regions (de la Fuente et al., 2005b).

The recombinant MSP5 protein and monoclonal antibodies against it are used for detection of anti-*Anaplasma*-specific antibodies by ELISA (de Echaide et al., 2005; Ewing et al., 1997). Although the *msp5* gene is widely used for the detection of *A. marginale* carrier cattle by nested PCR (Bock and de Vos, 2001), the MSP5 ELISA is the recommended method for confirming infection, as rickettsemia can drop below PCR-detectable levels.

1.7.3. Tick transmission

A. marginale strains differing in their tick transmissibility and in general infectivity for ticks may serve as useful tools to identify the genetic requirements for tick transmission. It has been hypothesized that differences in tick transmission

efficiency are due to genetic variability within *A. marginale* strains, which confer a tick transmission phenotype. Nevertheless, after comparison of genomes of five strains differing in tick transmissibility, no specific genes were determined (Dark et al., 2009). Therefore Dark et al. (2009) suggested that the differences exist most likely in shared genetic elements: either in coding or regulatory regions.

More promising results have been obtained with proteomic approaches which aimed at elucidating proteins responsible for colonization of tick cells. When proteomes of *A. marginale* strain from tick cell culture and erythrocytes were compared, a set of up-regulated proteins was identified (Noh et al., 2008; Ramabu et al., 2011). Although the functions of most of these proteins are still unclear, of particular interest is the ankyrin-repeat containing protein, Am638, as ankyrin-repeat motifs are thought to mediate protein-protein interactions (Ramabu et al., 2011). Further proteomic analysis comparing more *A. marginale* strains from naturally infected cattle and ticks are required in order to find key proteins involved in tick transmissibility.

The MSP1a tandem repeats were shown to be necessary for adhesion of *A. marginale* to tick and mammalian cells, which was attributed to differences in amino acid sequences of individual repeats. The negatively charged amino acids, aspartic acid (D) and glutamic acid (E) at position 20 were shown to be essential for binding of MSP1a to tick cell extracts. When glycine (G) was located at position 20, binding was not observed (de la Fuente et al., 2003). Recently, it has been confirmed that the 2-D conformation of MSP1a protein also correlates with tick transmissibility (Cabezas-Cruz et al., 2013). In most cases the α-helix conformation was found in abundance in strains transmitted by ticks.

1.7.4. Virulence

Several isolates of *A. marginale* have been identified which differ in virulence (Bastos et al., 2010; Rodriguez Camarillo et al., 2008). The identification of strains with low pathogenicity is very important as they could be used as live vaccines. Live vaccines result in persistent, life-long infections, providing protection against homologous and heterologous strains.

There are many reasons for differences in pathogenicity, one of which is protein glycosylation. Several glycoproteins of Gram-negative bacteria have already been shown to play a role in adhesion, invasion and pathogenesis. Most bacterial glycoproteins appear to be either associated with the surface of the organism or to be secreted into the environment, suggesting their role in the interaction with the host. Although the function of protein glycosylation is unclear in many cases, it has been proven to be essential for the attachment and infectivity of *Chlamydia trachomatis* elementary bodies and also to be responsible for the binding of blocking antibodies in *Neisseria meningitidis* (as reviewed by Benz and Schmidt, 2002). Remarkably, the MSP1a protein of *A. marginale,* which serves as an adhesin for host cells, is highly glycosylated (Garcia-Garcia et al., 2004a). The exact role of MSP1a glycosylation in invasion of host cells or pathogenicity has not been entirely explained. Most likely, since it is a surface protein and is directly exposed to the host cells, the bacteria may modulate MSP1a expression/glycosylation in order to evade the host's immune response.

Besides glycosylation, *A. marginale* inclusion appendages may also play a role in pathogenicity. Appendages have been associated with serological or immunological variances (Kuttler and Winward, 1984). Differences in pathogenicity have been observed between two Brazilian *A. marginale* strains, which correlated with the presence of the inclusion appendage (Bastos et al., 2010). The low pathogenic, tailed UFMG1 strain (Ribeiro et al., 1997), provided protection against the highly pathogenic non-tailed UFMG2 strain (Bastos et al., 2010).However in vaccination trials using a heterologous geographical strain from Israel as a challenge, cattle vaccinated with UFMG1 were not protected from the disease (Kenneil et al., 2013).
Interesting results have been obtained with two U.S. strains: the tailed Virginia and non-tailed Florida isolates (Kuttler et al., 1984). Kuttler at al. (1984) observed that cattle vaccinated with the commercial killed vaccine were resistant to a challenge with the Virginia isolate, whereas a 47 % mortality has been observed when the cattle were challenged with the Florida isolate. However, there was no cross-protection when cattle were vaccinated with Virginia strain and afterwards cross-challenged with Florida, or the other way round.

In trials conducted in Mexico vaccination with live *A. marginale* Yukatan strain

provided immunity against challenge with other Mexican strains (Rodriguez Camarillo et al., 2008). However, experiments with different geographical strains are required, to confirm the low virulence of this strain.

Although immense progress has been made in molecular biology and in *in vitro* cultivation of *A. marginale*, at present animal experimentation to measure the severity of the disease is the only way of assessing strain pathogenicity.

1.8. Anaplasmosis control methods

Presently used control methods, consisting of antibiotic treatment, vaccination and arthropod control, have not significantly changed for many years and depend on the geographical area, availability, cost and feasibility of application (Kocan et al., 2000).

1.8.1. Antibiotics

Three types of antibiotics are used for the treatment of anaplasmosis: tetracyclines, fluoroquinolones and imidocarb dipropionate (Aubry and Geale, 2011). Chemotherapeutic treatment is effective in decreasing bacterial numbers, but the efficacy in clearing infection and thus preventing the establishment of a pathogen reservoir is variable (Coetzee et al., 2005; Reinbold et al., 2010; Wallace et al., 2007). Recently, treatment of persistently infected steers with oral chlortetracycline for 80 days cleared *A. marginale* infections (Reinbold et al., 2010). Nevertheless, treatment of clinically affected animals is expensive. Moreover, it is becoming less acceptable, as antibiotic resistance rises in pathogens. In addition, some countries restricted the use of imidocarb due to its prolonged retention in the edible tissues of animals for slaughter (Kocan et al., 2010b).

1.8.2. Arthropod control

Treatment of animals with acaricides reduces the number of ticks, thus indirectly decreasing anaplasmosis transmission. However, the use of acaricides for vector

control is becoming a concern due to increasing acaricide-resistance among tick populations (George et al., 2004), the pollution of the environment and the contamination of milk and meat products (Graf et al., 2004).

The modern approach for arthropod control is based on the use of anti-tick vaccines, which have the benefits of being cost-effective, reducing environmental contamination and preventing the development of acaricide-resistant ticks. Two vaccines Gavac (Vargas et al., 2010) and TickGARD (Odongo et al., 2007) containing recombinant *R. (B.) microplus* gut antigens Bm86 and Bm95 are currently being used.

Another promising antigenic protein involved in the modulation of tick feeding and reproduction, subolesin, has been tested (de la Fuente et al., 2005a). Preliminary experiments have shown, that the number of ticks infected with *Anaplasma* spp. were reduced when ticks were injected with subolesin double- stranded RNA before being fed on cattle with ascending rickettsemia (de la Fuente et al., 2006). Furthermore, cattle immunized with recombinant subolesin were protected against *R. (B.) microplus* infestations due to a decrease in tick survival and reproduction rates (Almazan et al., 2003; Merino et al., 2011).

1.8.3. Vaccination

A long-lasting immunity induced by vaccination is an economical and effective way to prevent and control bovine anaplasmosis. Mass vaccination programs can significantly reduce the use of acaricides and antibiotics thus preventing an emergence of resistant ticks or pathogens. At present, two types of vaccines are used and are the vaccines of choice: live vaccines and inactivated formulations. They induce protection from severe clinical symptoms, but do not prevent infection, so that cattle after infection may remain carriers of *A. marginale* (Kocan et al., 2003).

1.8.3.1. Live vaccines

The use of live vaccines for anaplasmosis control began in the early 1900s, with the

isolation of *A. centrale* (Theiler, 1911). They consist of less pathogenic *A. centrale* or attenuated strains of *A. marginale*.

The immune response induced by such vaccines is similar to a natural infection and animals develop persistent infections with the vaccine strain. However, preimmunization with one strain has been shown not to provide cross-protection in widely separated geographic areas (Kenneil et al., 2013; Kuttler et al., 1984). Live vaccines consist of infected blood, taken from splenectomized, quarantined calves inoculated with the selected vaccine strain. These vaccines carry the risk of transmitting other "silent" pathogens and despite the global impact of anaplasmosis, their use is forbidden in the US (Rogers et al., 1988).

Immunization of cattle with less pathogenic subspecies i.e. *A. centrale* is in routine use in several countries: South Africa, Zimbabwe, Malawi, Australia, Israel, Uruguay and Argentina (Shkap et al., 2009).

It is noteworthy that some African and Latin American isolates of *A. marginale* can overcome an *A. centrale* induced immunity (Bock et al., 2003; Brizuela et al., 1998).

The second type of live vaccine consists of attenuated *A. marginale* strains. These vaccines were used in South America and California, although severe reactions have been observed in adult cattle after vaccination (Henry et al., 1983). In calves, however, these vaccines produce mild infections and lead to immunity against clinical anaplasmosis, although not in widely separated geographic areas (Kocan et al., 2003). The attenuation of *A. marginale* can be achieved by two methods. The first involves irradiation and subsequent multiple passages through deer and sheep (Ristic and Carson, 1977). The second consists of numerous passages through splenectomized calves followed by passages through splenectomized sheep (Jorgensen et al., 1993). Yet, it has been reported that attenuated *A. marginale* vaccines reverted to virulence after successive passages through cattle or ticks (Kocan et al., 2000).

1.8.3.2. Inactivated vaccines

An inactivated vaccine comprising of non-living *A. marginale* was developed in the United States and was used effectively till its withdrawal from the market in 1999

(Kocan et al., 2003). Although inactivated vaccines are also produced in splenectomized animals, it is less likely that any other pathogens contaminating the vaccine will remain viable and infectious after the inactivation process. However, extensive purification is required to remove bovine cell stroma as only partial purification resulted in the development of erythrocytic isoantibodies in vaccinated cattle. The inactivated vaccines reduced clinical disease and mortality, yet did not always provide cross-protection (Kuttler and Winward, 1984). For this reason inactivated vaccines are most likely to be useful when produced from locally isolated strains.

1.8.3.3. Culture-derived vaccines

Bacteria grown in tick cell cultures are being investigated as an alternative source of *A. marginale* for live vaccine production. This technique has the advantage of allowing the inclusion of multiple strains, ease of standardization, freedom from bovine red blood cells and pathogens and does not require the use of expensive, splenectomized calves (Kocan et al., 2003). Cattle immunized with a cell culture-derived *A. marginale* strains develop protective immunity and do not develop clinical signs of anaplasmosis after challenge. However, as with most anaplasmosis vaccines, infection with the challenge strain is not prevented (Bastos et al., 2010; de la Fuente et al., 2002b; Kocan et al., 2001).

1.9. Cell culture systems

For a long time the lack of an *in vitro* culture system has been the major impediment to anaplasmosis research and infected cattle served as the only source of *A. marginale*. Although bovine erythrocytes can be used for maintaining bacteria in culture, they are not suitable for continuous propagation (Blouin et al., 2002a; Waghela et al., 1997). Since the establishment of the first tick cell line in 1975 (Varma et al., 1975), the number of continuous tick cell lines has increased to over 50 derived from both ixodid and argasid species (Bell-Sakyi et al., 2007; as reviewed by Passos, 2012). Currently, most of the available tick cell lines have been deposited in the Tick Cell Biobank (pirbright.ac.uk/research/tickcell/Default.aspx).

More than 200 *A. marginale* isolates have been reported worldwide (as reviewed by Cabezas-Cruz et al., 2013), but only few of them have been propagated in tick cell cultures, namely: from Brazil UFMG1 and UFMG2 (Bastos et al., 2010; Bastos et al., 2009), from the USA Virginia, Oklahoma (Blouin et al., 2000; Munderloh et al., 1996), St. Maries (Hammac et al., 2013) and Oregon (Kocan et al., 2004) and an isolate from Israel (unpublished work).

Bacteria are propagated mostly in cell lines derived from *Ixodes scapularis* IDE8 and ISE6 (Munderloh et al., 1994) although some strains grow also in cell lines from *R. (B). microplus* or *D. andersoni* ticks (Oliva Chavez et al., 2012). *Anaplasma* colonies from tick cell cultures are similar to those observed in ticks (Blouin and Kocan, 1998) and remain infective for ticks and cattle after continuous passages in tick cell cultures (Blouin et al., 2000).

In addition to tick cell lines, *A. marginale* infects and grows in several mammalian cell lines: Vero (kidney epithelial) and RF/6A (retina choroid endothelium), as well as in primary cultures of bovine vascular endothelial cells (Munderloh et al., 2004; Oliva Chavez et al., 2012; Wamsley et al., 2011).

A. centrale used as a live vaccine is still produced in cattle, because all attempts to propagate this vital subspecies *in vitro* have failed.

In vitro cultures provide an excellent source of *A. marginale* organisms which can be used for serological diagnosis (Saliki et al., 1998), screening of antibiotics (Blouin et al., 2002b), vaccines development (de la Fuente et al., 2002b; Hammac et al., 2013) or proteome profiling (Noh et al., 2008). Furthermore, culture systems allow the study of pathogen–host cell interactions and pathogen variations in response to a changing host cell environment (Blouin et al., 2002a). Additionally great quantities of bacteria can be obtained in less time at reduced costs, and most importantly without the use of experimental animals. Therefore, more effort should be put into establishing additional *A. marginale* strains *in vitro*.

1.10. Recent interests in anaplasmosis research

While research carried out in the last two decades has contributed greatly to our knowledge of the antigenic composition of *A. marginale*, it did not lead to the development of effective vaccines which could provide cross-protection worldwide. The availability of *in vitro* grown bacteria together with novel genomic, transcriptomic and proteomic techniques, has great potential for vaccine development. Currently, two trends can be observed in anaplasmosis research. The first is developing effective tools to induce immunity in cattle through vaccination. The second is preventing transmission of *A. marginale*, by yet undiscovered methods of blocking tick transmission of pathogens. In general, new approaches for anaplasmosis control focus on the use of outer membrane proteins (OMPs) in novel vaccines. OMPs are not only essential for the bacterium but also serve as major targets for the immune system of the host.

Kocan et al. (1996) suggested that the vaccination of cattle with *A. marginale* OMPs, should not only aim at preventing the bacterial infection in animals but also at preventing the transmission of *A. marginale* by infected ticks. The rationale of this idea was the fact that some bovine immunoglobulins can cross the tick midgut epithelium and enter the hemolymph. Feeding ticks are exposed to antibodies present in host serum for a relatively long time. Therefore, cattle immunized against ticks and/or against stages of hemoparasites within ticks would produce antibodies which would be taken up by a tick with the bloodmeal, thus affecting the vector and/or the parasite. Preliminary experiments, however, have not shown any difference in development or transmission of *A. marginale* in ticks fed on vaccinated cattle.

Selected epitopes of immunogenic sub-dominant proteins are being tested in vaccination trials. Subdominant antigens tend to be less variable, as parasites allow the host to mount an immune response against them, therefore they are likely irrelevant for the survival of the organism (Brown et al., 2006). It has been shown, that cattle immunized with the *A. marginale* MSP1 protein complex presented a protective humoral immune response, however, its efficacy was variable. Similarly, mice vaccinated with chemically synthesized critical motifs of MSP1a functional epitope (Santos et al., 2012) essential for antibody recognition,

were protected against *A. marginale* challenge (Santos et al., 2013). Although the protective immunity in cattle vaccinated with subdominant OMP AM779 alone was not sufficient to induce protection, slightly greater T-cell responses were observed when compared to animals vaccinated with OMPs (Albarrak et al., 2012). Therefore Albarrak et al. (2012) suggested that subdominant antigens should still be considered individually and collectively for vaccine development.

Using sera from cattle vaccinated with *A. marginale* OMP complexes, Lopez et al. (2005) identified 24 immunodominant *A. marginale* proteins, resolved on 2-dimensional gels. As expected all identified proteins were membrane-associated. These included the well characterized surface-exposed OMPs like MSP2, MSP3 and MSP5, as well as recently identified appendage-associated proteins. Additionally, among the 21 newly described antigenic proteins, type IV secretion system proteins and members of the MSP2 superfamily were identified. Similar antigens with some additional OMPs were detected in an experiment when sera from *A. centrale*-immunized cattle were used (Agnes et al., 2011).

Two sets of individual OMPs were tested by Noh et al. (2013) as immunogens: a complex of OMPs linked by covalent bonds or treated with dithiothreitol (DTT) which reduces disulfide bonds. Although both immunogens induced protective immunity, the antibody response induced by the linked immunogen was much better. These findings suggest that cross-linking enhances immunogenicity and could minimize the dose of antigen required for the induction of protective immunity.

Parallel to proteomics, comparative genomic analyses are also being run. They similarly aim at elucidating immunodominant proteins, which are identical within strains and so may be used as vaccine antigens (Dark et al., 2011; Dark et al., 2012; Palmer et al., 2012) Notably, the results are similar to those obtained with proteomic techniques i.e. comparing the genome of 10 U.S. *A. marginale* strains Dark et al. (2011) selected 19 conserved antigens, mostly OMPs and Type 4 Secretion Proteins.

1.11. Project outline

Anaplasma marginale affects ruminants worldwide, causing anaplasmosis in tropical and subtropical regions. Considerable effort is made to control the disease, yet strategies have only minimally advanced over the past few decades even though our knowledge of *A. marginale* has increased considerably within this time.
On the one hand, the problem arises from *A. marginale* itself, as differences in isolates, mainly in protein expression lead to a lack of cross-protection among geographically separated strains. On the other hand, drug resistance increases not only in bacteria, but also in the tick vectors due to the ungoverned use of acaricides. Furthermore, the only vaccine on the market, *A. centrale*, is forbidden in many countries due to the risk of transmitting other blood-borne pathogens.

This thesis aims to shed light onto differences within geographical *A. marginale* strains grown in the IDE8 tick cell line. In order to address this question the main objectives of the project were:

- to develop a practical and effective approach for the purification of intact and viable *A. marginale* from infected tick cell cultures

- to check if there are differences in genes/proteins within geographical *A. marginale* strains propagated *in vitro*, differing in morphology, protein sequence and pathogenicity

Few *A. marginale* strains can be propagated in tick cell cultures, yet, due to its obligatory intracellular nature, separation of bacteria from host cell materials is problematic. In **Chapter 2** various purification methods are reviewed. The use of Percoll gradients for separation of intact and viable *A. marginale* from IDE8 cells is examined, with the focus on the easiness, reproducibility and lack of toxicity of the method.

The functional and immunological relevance of MSP1a protein from 224 *A. marginale* strains were analyzed. Additionally, the consistent nomenclature based on the MSP1a structure was proposed, is covered in **Chapter 3**.

In **Chapter 4** some molecular and immunological characteristics of different geographical *A. marginale* strains grown in IDE8 tick cell culture are depicted with the particular focus on MSPs and proteins participating in response to stress conditions.

In **Chapter 5** differences in protein expression among these strains are evaluated with the use of 2D-DIGE technique and Mass Spectrometry. The possibility of inclusion of selected proteins into novel vaccines is also discussed.

The major findings and conclusions of this thesis are summarized in **Chapter 6.**

CHAPTER 2

Use of Percoll gradients to purify *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) from tick cell cultures

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2. Use of Percoll gradients to purify *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) from tick cell cultures

2.1. Abstract

Anaplasma marginale (Rickettsiales: Anaplasmataceae) is an obligate intracellular bacterium that multiplies exclusively within membrane-bound vacuoles in the cytoplasm of the host cells. A number of *A. marginale* isolates can be propagated in the *Ixodes scapularis* IDE8 tick cell line, which provides a reliable source of antigens for a wide variety of studies. However, because of its intracellular nature, separation of bacteria from host cell materials remains an important constraint for researchers. In the present study we evaluated the use of Percoll gradients for purification of two Brazilian strains of *A. marginale* grown in IDE8 tick cells. The purified *A. marginale* monitored in Giemsa-stained smears contained only minimal amounts of IDE8 cell stroma. The total protein yields were 1.2 mg and 1.7 mg, while the DNA titres quantified with real-time PCR were 6.4 x 10^9 for UFMG1 and 4.87 x 10^9 for UFMG2 copies in the purified material, respectively. Additionally, we confirmed the viability of purified bacteria by infecting tick cells after being freshly purified and after retrieval from long-term storage. Importantly, the viability of the organisms is preserved after use of this separation method and therefore the purified organisms can be used in enzymatic assays and other research approaches where living organisms would be preferred.

Key words: *Anaplasma marginale*, intracellular bacteria, Percoll gradients, purification

2.2. Introduction

Anaplasma marginale is a tick-borne pathogen of cattle that causes bovine anaplasmosis worldwide (Kocan et al., 2004). This organism is classified in the belongs to the order Rickettsiales of which members are all obligate intracellular organisms, found exclusively within membrane-bound vacuoles in the cytoplasm of host cells (Dumler et al., 2001).

The establishment of continuous tick cell lines derived from *Ixodes scapularis*, such as IDE8 (Munderloh et al., 1994), led to the development of *in vitro* culture systems for cultivation of *Anaplasma* spp., in which several strains *A. marginale* have been propagated successfully (Bastos et al., 2009; Blouin et al., 2000; Blouin & Kocan, 1998; Munderloh et al., 1996). *A. marginale* colonies observed in cultured tick cells were morphologically similar to those described in *Dermacentor* tick cells (Blouin & Kocan, 1998) and remain infective for ticks and cattle (Munderloh et al., 1996). Therefore, the *in vitro* culture system provides an excellent source of antigens for development of serodiagnostic tests or vaccines (de la Fuente et al., 2002; Noh et al., 2013; Saliki et al., 1998).

However, due to the intracellular nature of these organisms, their release from host cells can be quite problematic, complicating the frequent aim of obtaining undamaged and viable organisms, deprived of host cell debris. Several methods to purify different Rickettiales from their host cells have been described. The most common separation method is based on Renografin gradients, as described by Weiss et al. (1975). However, separation was found to be disruptive, and thus decreased the yield of viable bacteria (Carlyon, 2005; Emelyanov, 2009; Hanson et al., 1981).

Other methods consist of filtration through different-pore-size filters (Ge & Rikihisa, 2007; Mottaz-Brewer et al., 2008; Noh et al., 2008) or separation on sucrose density gradients (Li & Wu, 2004; Ogawa et al., 2007), yet, the former has mostly been used for cell organelles separation e.g. outer membranes (Lopez et al., 2005).

Percoll gradients have been used for purifications of numerous rickettsial pathogens

maintaining good viability (Hajem et al., 2009; Tamura et al., 1982; Yuksel et al., 2006). Although, Percoll separation has been practical for the purification of *A. marginale* from infected bovine erythrocytes (McCorkle-Shirley et al., 1985), this method of purifying *A. marginale* from cultured tick cells has not been reported previously.

In the present study, we evaluated the use of the Percoll gradients for purification of *A. marginale* grown in IDE8 tick cells. Additionally, we reported the viability and the yield of purified bacteria. This easily reproducible technique, allowing the isolation of intact, free of host cell components, organisms, will provide an alternative to other purification methods that are commonly in use.

2.3. Materials and methods

2.3.1. Cell cultures

The IDE8 tick cell line derived from *Ixodes scapularis* embryos (Munderloh et al., 1994) was used. Uninfected cultures were maintained in L-15B medium (Munderloh & Kurtti, 1989), supplemented with 5% heat-inactivated fetal bovine serum, 10 % tryptose phosphate broth, 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.1 % bovine lipoprotein concentrate (MP Biomedicals; Irvine CA, USA); the pH was adjusted to approximately 6.8. Cultures were propagated in 75 cm^2 plastic flasks at 32 °C. For infected IDE8 cell cultures, the L-15B medium was further modified by the addition of 0.1 % $NaHCO_3$ and 10 mM HEPES and the pH was adjusted to approximately 7.5 (Munderloh, et al., 1996). The cultures were propagated at 34 °C in 75 cm^2 plastic culture flasks in 15 ml of the medium. Growth of the infected cell cultures was assessed weekly by examination of Giemsa-stained cytospins smears. The bacteria were harvested from cell cultures when the infections levels were approximately 80 %.

2.3.2. *A. marginale* strains

Two Brazilian strains of *A. marginale* were used for the experiments: UFMG1

and UFMG2; both have been previously established and propagated in IDE8 tick cell cultures (Bastos et al., 2010; Bastos et al., 2009).

2.3.3. Preparation of Percoll density gradient

Bacteria were purified using Percoll density gradient centrifugation, as described previously by Yuksel et al. (2006) with some modifications. All purification steps were carried out at 4 ºC. Briefly, suspensions of infected IDE8 cells were transferred into polycarbonate tubes (Nalgene) and centrifuged at 10.000 x *g*, for 20 min at 4 ºC (Sorvall SS-34, Thermo Scientific). The resulting pellets containing bacteria and tick cells were resuspended in 4 ml of Tris-sucrose buffer (33 mM Tris, 250 mM Sucrose, pH 7.4), homogenised with a Dounce tissue grinder and subsequently vortexed for 1min. The homogenate was centrifuged at 210 x g for 10 min at 4 ºC, to eliminate cell debris and remaining intact cells (semi-purified fraction). Four ml aliquots of the supernatants were loaded onto 27 ml of a 30 % (v/v) Percoll (GE Healthcare, Uppsala, Sweden) solution and centrifuged at 25.000 x *g* for 60 min, at 4 ºC. The upper band comprising cell debris was carefully removed, while the lower band containing bacteria was diluted with cold, sterile Phosphate-Buffered Saline (PBS) and centrifuged at 20.000 x g for 30 min, at 4ºC. Two additional PBS washes were performed to eliminate the remaining Percoll solution. The resulting supernatant was removed and the volume was brought to 200 µl with fresh PBS. One hundred microliter of purified bacteria has been preserved for viability experiments. In order to identify the density at which the host cells form a band, uninfected IDE8 cells were processed in the same way. Additionally, the marker beads (1.051 g ml^{-1},1.068 g ml^{-1}, 1.100 g ml^{-1}) (GE Healthcare, Uppsala, Sweden) were used to determine densities through the Percoll gradient.

Proteins were extracted from 50 µl of purified bacteria with lysis buffer (150 mM sodium chloride, 1% Triton X-100, 50 mM Tris pH 8) containing protease inhibitors (Complete, Mini, EDTA-free, Roche) and sonicated (5 s/cycle, 5 cycles; 0 ºC). After centrifugation at 13.000 x g for 30 min at 4 ºC, the supernatant was collected and the protein concentration was determined with DC Protein Assay (Bio-Rad).

2.3.4. Cryopreservation and viability of purified bacteria

The viability of bacteria was assessed by inoculation of uninfected IDE8 cell cultures with 10 µl of the Percoll purified *A. marginale* suspended in PBS. For long-term storage, 100 µl of purified bacteria were re-centrifuged at 10,000 x g for 10 min at 4 °C. The remaining PBS was carefully removed and the pellet was resuspended in sucrose-phosphate-glutamate freezing buffer (SPG) (Bovarnick et al., 1950). For each bacterial isolate six aliquots, 1 ml each, were frozen overnight at -80 °C, using a NALGENE® Frosty™ Cryo 1 °C freezing container, which resulted in a continuous decrease of temperature at a rate of one degree per minute. Afterwards, 3 cryotubes of each isolate were transferred into liquid nitrogen and the other 3 replicates were stored at -80 °C. Six months later, cryopreserved vials were thawed rapidly at 37 °C and the content was transferred immediately into culture flasks (25 cm^2) of uninfected IDE8 cells, containing 5 ml of modified L15-B medium. The medium was replaced twice a week and the growth of the infected cell cultures was assessed weekly by examination of Giemsa-stained cytospin smears.

2.3.5. Quantification of *A. marginale* DNA by real-time PCR

DNA was extracted from 20 µl of Percoll purified bacteria, using QIAamp DNA Mini Kit (Qiagen, Germany) and eluted in 200 µl of AE buffer, following the manufacturer's instructions. The *msp1β* gene was amplified, as described previously (Molad et al., 2006), cloned into pGEM®-T easy vector (Promega, Madison, USA) and propagated in competent *Escherichia coli* cells, following the manufacturer's instructions. Plasmid DNA was purified with QIAprep Spin Miniprep Kit (Qiagen, Germany) and sequenced. To plot the standard curve, serial 10-fold dilutions of plasmids in nuclease-free water were prepared, ranging from 10 to 2.5 x 10^9 copies of DNA in μl^{-1}.

The quantification, targeting a 95-bp fragment of *A. marginale msp1β* gene, was performed with a Real-time PCR System (Applied Biosystems 7500, Germany), as previously described by Carelli et al. (2007). The reaction mixture (25 µl) contained 20 µl of master solution and 5 µl of template or plasmid DNA dilutions. Each sample was tested in triplicate.

2.4. Results

2.4.1. Percoll density gradients and quantifications

A 30 % (v/v) Percoll solution was used to purify *A. marginale* from IDE8 tick cells (**Figure 2.1.A**). After separation of the semi-purified homogenate (**Figure 2.1.B**) on Percoll gradient, a diffuse, white band of bacteria was formed between 1.051 g ml^{-1}

and 1.070 g ml^{-1}. The Giemsa-stained bacteria recovered from this band, were verified by light microscopy to contain primarily concentrated *A. marginale* with minimal host cell debris (**Figure 2.1.C**). The cell debris fraction was formed at a density of about 1.100 g ml^{-1}, as determined by a control experiment, in which uninfected cells were applied to the gradient. The amount of protein in preparations from bacteria purified on 30 % Percoll was 1.7 mg for UFMG1 and 1.2 mg for UFMG2 in one flask.

Ten-fold dilutions of the plasmid DNA were used in order to create the standard curve. The detection limit of the real-time PCR assay was 1.3 x 10^1 copies of DNA standard in 5 µl of sample, what corresponded to dilution 10^{-11}. On average, the number of *A. marginale* DNA copies in μl^{-1} of extracted material was 3.2 x 10^6 for UFMG1 and 2.5 x 10^6 for UFMG2. The total titres of *A. marginale* DNA in 200 µl of purified material were 6.4 x 10^9 for UFMG1 and 4.87 x 10^9 for UFMG2 copies, respectively.

2.4.2. Viability of purified bacteria

Organisms remained infective for IDE8 cells after the purification process. The first bacterial colonies were observed within 10 days after inoculation of IDE8 cells with 10 µl of freshly purified *A. marginale*.

After long-term storage, all replicates of purified bacteria, either cryopreserved in liquid nitrogen or in a -80 °C freezer, were infective for monolayers of cultured IDE8 cells. First bacterial colonies were visible between 14 to 17 days after culture initiation.

2.5. Discussion

Propagation of intracellular rickettsia, such as *A. marginale*, in tick cell lines provided an important alternative to in vivo studies, providing a defined and controlled environment in which to study pathogen-host cell interactions. Furthermore, large quantities of *A. marginale* can be propagated in cultured cells which are free of bovine red blood cell stroma. However, before any analysis, bacteria purification from host cell components is required.

A modification of the method described by Yuksel et al. (2006), based on the use of 30 % (v/v) Percoll, was evaluated in this study to purify *A. marginale* grown in IDE8 tick cells. To our knowledge, this is the first time this separation method was used for purification of tick cell culture-derived *A. marginale.* Although, diverse concentrations of Percoll have been evaluated for different rickettsia grown *in vitro* (Hajem et al., 2009; Tamura et al.,1982; Yuksel et al., 2006), the 30 % Percoll gradient has been found to be best suited for the isolation of *Piscirickettsia salmonis*. In comparison to other Percoll concentrations, recovered bacteria appeared to retain their intracellular structure. Moreover, the viability and yield of recovered bacteria was higher when 30 % Percoll was used (Yuksel, et al., 2006).

In our studies the tested protocol generated linear and reproducible gradients, confirmed with the use of density marker beads. The bacterial band was diffused between densities of 1.051 g ml^{-1} and 1.070 g ml^{-1}, similarly to previously reported data (Yuksel et al., 2006). The host cell debris band formed at the density around 1.100 g ml^{-1}, which was determined from a control gradient of uninfected IDE8 cells. The release of rickettsia from host cells is often incomplete due to the obligate intracellular nature of the pathogen, therefore in Giemsa-stained smears of the purified *A. marginale* minimal amounts of IDE8 cell debris can be observed (**Figure 2.1.C**). Yuksel et al. (2006) obtained the best purity of *P. salmonis* with 30 % Percoll, when compared to 40 % and 50 % concentrations. Additionally, when analyzed with transmission electron microscopy, purified material from 30 % Percoll were mainly composed of whole-cell *P. salmonis* with only small amounts of fragmented host cell material present. Hajem et al. (2009) reported that after

analyses of proteins from *Rickettsia helvetica* purified on a 40 % Percoll gradient, they did not find any proteins with similarity to Vero host cells or mammalian cells. Similarly, we expect that bacteria purified on 30 % Percoll might contain only small amounts of host cell proteins, which would make them appropriate for proteomic analysis.

Since Percoll is non-toxic, purified specimens can be transferred directly into cultured cells (Pertoft, 2000). The viability of both purified bacterial isolates was tested immediately after the purification protocol and after 6 months for cryopreserved specimens. In the first case, *A. marginale* colonies were detected in Giemsa-stained cytospin smears ten days after culture inoculation, while resuscitation after 6 months of cryopreserved Percoll-purified bacteria required 2 weeks, which correspond to the interval of time when fresh material from infected culture is used for the inoculation of new cells (Blouin and Kocan, 1998). These results demonstrated that Percoll did not kill the bacteria and the organisms were infective for cell cultures.

The total protein yields obtained after purification of bacteria from one flask (15 ml) of IDE8 cells were 1.7 mg and 1.2 mg for UFMG1 and UFMG2 strains, respectively. The yields reported by Weiss et al. (1975) was 18 mg protein after purifying *Rickettsia typhi* from LM3 cells cultured in 40 flasks (approx. 475 ml each) by Renografin density gradients.

The number of bacteria was quantified using the real-time PCR method, based on *msp1β* gene fragment. The assay has been previously shown to be highly sensitive, as it has been able to detect 3 x 10^1 DNA copies in ml^{-1} of *Anaplasma*-infected erythrocytes (Carelli et al., 2007). In Percoll purified preparations we detected on average 2.9 x 10^6 *A. marginale* DNA copies in μl^{-1} of sample. Instead, in DNA standards, the assay detected as few as 1.3 x 10^1 copies in 5 µl of sample. The number of copies obtained from cell culture was 10^3 more in 1 µl of purified samples, when compared to the number of bacteria in infected animals that were ranging 6.30 x 10^1 to 3.24 x 10^5 DNA copies per µl of blood (Carelli et al., 2007; Decaro et al., 2008).

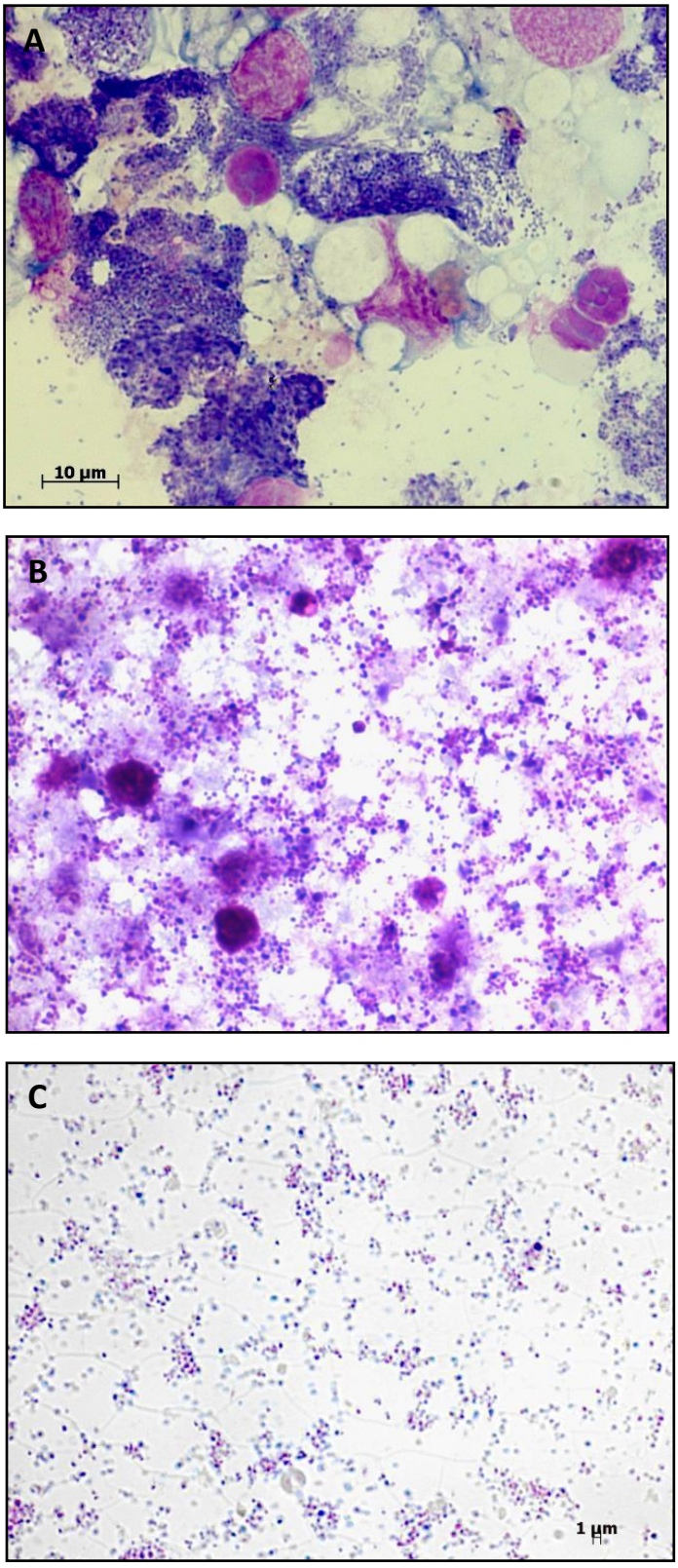

Figure 2.1. The purity of *A. marginale* assessed during different steps of the purification process by Giemsa staining: A)**.** *A. marginale* heavily infected IDE8 cells, B)**.** The semi-purified bacteria containing cell debris after homogenisation and low speed centrifugation**,** C)**.** Percoll purified bacteria.

In conclusion, the use of 30 % Percoll separation is a quick and reproducible method for the purification of *A. marginale* from IDE8 tick cells. *A. marginale* purified from cultured tick cells could be useful for many types of studies that were not possible previously. However, while *A. marginale* harvested from tick cell cultures are free of bovine erythrocyte stroma which was a problematic contaminant of previous whole-organisms killed vaccines, subsequent studies have shown that gene expression of *A. marginale* differs in erythrocyte and tick cell culture-derived organisms. Most notably, as reported previously (de la Fuente et al., 2001; Garcia-Garcia et al., 2004), MSP1a was found to be downregulated in *A. marginale* cultivated in ticks cell lines. Therefore, use of *A. marginale* from cultured tick cells differs, which should be taken into consideration when they are targeted for use in specific applications. Furthermore, Percoll did not decrease the viability of *A. marginale*, even when bacteria were cryopreserved for up to 6 months. Because of the retention of *A. marginale* viability, this Percoll method appears to be superior to previous ones such as Renografin or sucrose gradients.

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CHAPTER 3

Functional and immunological relevance of *Anaplasma marginale* major surface protein 1a sequence and structural analysis

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3. Functional and immunological relevance of *Anaplasma marginale* major surface protein 1a sequence and structural analysis

3.1. Abstract

Bovine anaplasmosis is caused by cattle infection with the tick-borne bacterium, Anaplasma marginale. The major surface protein 1a (MSP1a) has been used as a genetic marker for identifying A. marginale strains based on N-terminal tandem repeats and a 5′-UTR microsatellite located in the msp1a gene. The MSP1a tandem repeats contain immune relevant elements and functional domains that bind to bovine erythrocytes and tick cells, thus providing information about the evolution of host-pathogen and vector-pathogen interactions. Here we propose one nomenclature for A. marginale strain classification based on MSP1a. All tandem repeats among A. marginale strains were classified and the amino acid variability/frequency in each position was determined. The sequence variation at immunodominant B cell epitopes was determined and the secondary (2D) structure of the tandem repeats was modeled. A total of 224 different strains of A. marginale were classified, showing 11 genotypes based on the 5′-UTR microsatellite and 193 different tandem repeats with high amino acid variability per position. Our results showed phylogenetic correlation between MSP1a sequence, secondary structure, B-cell epitope composition and tick transmissibility of A. marginale strains. The analysis of MSP1a sequences provides relevant information about the biology of A. marginale to design vaccines with a cross-protective capacity based on MSP1a B-cell epitopes.

3.2. Introduction

Bovine anaplasmosis, caused by the intraerythrocytic rickettsia *Anaplasma marginale* (Rickettsiales: Anaplasmataceae), is an economically important disease of cattle

which is endemic in tropical and subtropical regions of the world [1], [2]. This obligate intracellular pathogen can be transmitted biologically by ticks, mechanically by transfer of infective blood on fomites or the mouthparts of biting insects [1], [2], and, less commonly, by transplacental transmission from dams to their calves [3].

Many geographic strains of *A. marginale* have been identified worldwide which differ in morphology, protein sequence, antigenic characteristics and their ability to be transmitted by ticks [1], [2], [4]–[15]. The genetic diversity of *A. marginale* strains derived from bovine erythrocytes has been characterized based on the sequence of major surface protein (MSP) genes, several of which have been shown to be involved in host cell/pathogen interactions [16]. MSP1a, one of six MSPs described previously on *A. marginale*, is a 70–100 kDa protein encoded by a single-copy gene, *msp1a*, which is conserved during the multiplication in cattle and ticks [17]. MSP1a is involved in adhesion of *A. marginale* to bovine erythrocytes and tick cells and therefore is a determinant of infection for cattle and transmission of *A. marginale* by ticks. MSP1a has also been shown to be involved in development of bovine immunity against *A. marginale* [3]. Strains of *A. marginale* were originally identified by differences in the molecular weight of MSP1a because of variable number of 23–31 amino acid serine-rich tandem repeats located in the N-terminal region of the protein which is continuous with a highly conserved C-terminal region [6], [11], [14]. Because the number and sequence of tandem repeats remained the same in a given strain, the *msp1a* gene was recognized as a stable genetic marker for geographic strain identity [9], [12], [15], [18]–[20]. Phylogenetic analyses of *A. marginale* strains using MSPs were reported by de la Fuente et al. [14], [21]–[23]. While sequence analysis of MSP4 provided phylogeographic information, MSP1a did not prove to be as suitable for these studies [24]. However, MSP1a repeat sequence analysis contributed to the understanding of the genetic diversity of *A. marginale* within specific regions, as well providing insight into the evolution of host–pathogen-vector interactions [14], [21]–[23], [25].

MSP1a also contains neutralization sensitive T- and B-cell epitopes required for development of a protective immune response [8], [10], [26]–[29]. One B-cell epitope within the MSP1a tandem repeat ((Q/E)ASTSS) was recognized by a monoclonal antibody that neutralized *A. marginale in vitro* [6]. This neutralization-sensitive epitope was found to be conserved among heterologous *A. marginale* strains [29],

[30]. An additional linear B-cell epitope (SSAGGQQQESS) was found to be immuno dominant [26], [28], [31]. Cattle immunized with MSP1 were partially protected against challenge with homologous and heterologous strains [32]–[34]. Furthermore, MSP1a antibodies reduced the infectivity of *A. marginale* for cultured tick cells [35] and infection and transmission of *A. marginale* by *D. variabilis* [1].

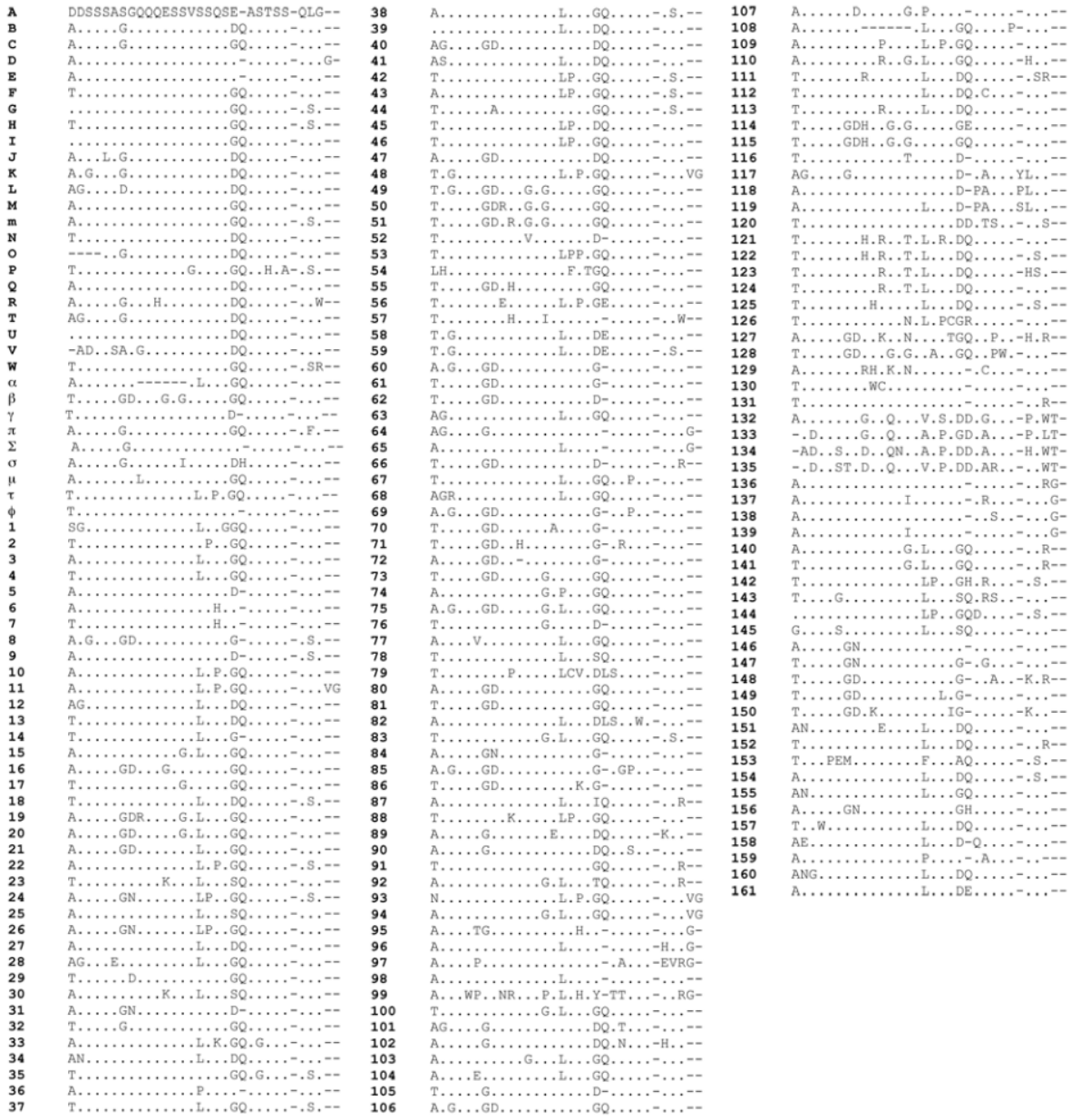


Figure 3.1. MSP1a tandem repeat sequences in *A. marginale* strains. The one letter amino acid code was used to depict MSP1a repeat sequences. Dots indicate identical amino acids and gaps indicate deletion/insertions. The ID of each repeat form was given following the nomenclature proposed by de la Fuente et al. (2007) [14].

MSP1a is relevant to many facets of *A. marginale* research. Strain classification enables a comprehensive study of the extensive worldwide diversity of *A. marginale*. As reported herein, development of an unified nomenclature of MSP1a from *A. marginale* strains based on all available sequence data allowed for review and characterization of the worldwide genetic diversity of *A. marginale*. The information generated from these studies will be fundamental toward understanding the functional and immune relevance of *A. marginale* MSP1a and in formulating vaccines that will be cross-protective among these diverse strains.

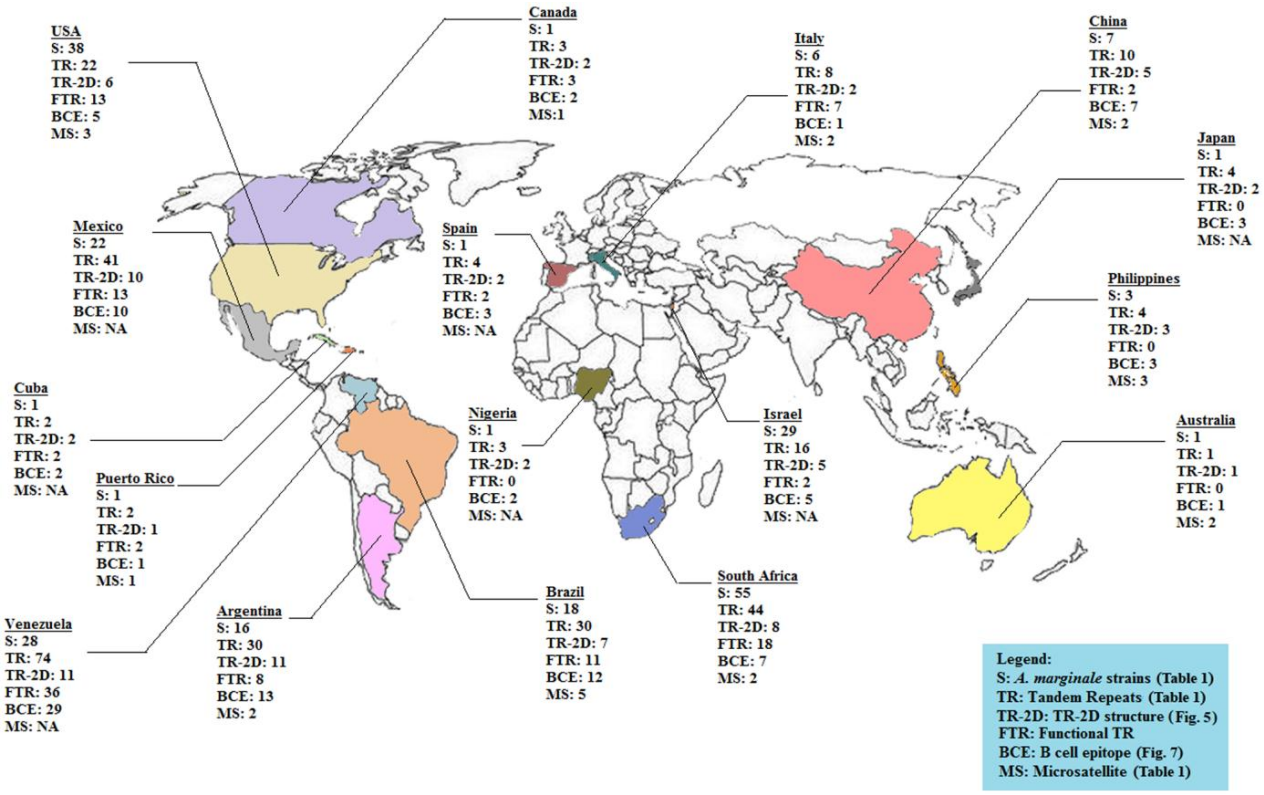


Figure 3.2. World *A. marginale* MSP1a molecular map. The worldwide molecular characterization of *A. marginale* MSP1a sequences is shown. The number of *A. marginale* strains (S), tandem repeats (TR) and microsatellites (MS), tandem repeat 2D structures (TR-2D), functional tandem repeats (FTR) containing D and E at position 20 and B cell epitope types (BCE) are represented for each country. Primary data is depicted in figures 1, 3 and 6. The information on 5' UTR microsatellites is not available (NA) for some sequences.

3.3. Results and Discussion

3.3.1. Classification of *A. marginale* strains using MSP1a sequence data

In this study we propose a unified nomenclature for the classification of *A. marginale* strains based on the sequences of the MSP1a tandem repeats and the 5′-UTR microsatellite. This approach was supported by the following considerations: (i) the availability of numerous *A. marginale* MSP1a sequences in GenBank, (ii) the fact that MSP1a is encoded by a single-copy gene [1], (iii) the tandem repeat structure and sequence vary among strains from different geographic locations, while the remaining portion of the protein is highly conserved [14], (iv) the tandem repeats structure is a stable genetic marker that is conserved within a strain during the acute and persistent chronic phases of the *A. marginale* infection in cattle and after passage and transmission by ticks [1], (v) the tandem repeats contain functional domains that serve as adhesins for bovine erythrocytes and tick cells, a prerequisite for infection of host cells [10], [36], (vi) the tandem repeats contain relevant B cell epitopes and neutralization epitopes important for natural or induced immune protection in cattle [6], [31], and (vii) a microsatellite which has been implicated in the regulation of MSP1a expression levels is located in the 5′-UTR of the msp1a gene [25].

In this study, 193 different MSP1a tandem repeats were identified, 79 of which were published in GenBank but not formally classified (**Figure 3.1**). Two new microsatellite structures were described in our analysis and named J and K (J: m = 1, n = 8, d = 21; K: m = 2, n = 8, d = 25) after Estrada-Peña et al. [25]. Unique *A. marginale* strains (224; 77% of all sequences found) are based on differences in geographic location, the number and structure of the MSP1a tandem repeats and microsatellites when available. These *A. marginale* strains came from 17 world regions providing a global MSP1a diversity (**Figure 3.2**), and were classified following our proposed nomenclature (**Table 3.S1**). The majority of *A. marginale* strains had more than one MSP1a tandem repeat and the maximum number of repeats was 10. No strains were reported with 9 tandem repeats (**Table 3.S1** and **Figure 3.3**). **Table 3.1** provide a list of the most commonly reported strains and tandem repeats. The majority of strains

Table 3.1. Geographical occurrence of the most common *A. marginale* strains**.**

Strains	Structure of MSP1a tandem repeats	Number of strains	World occurrence
Most common	τ 22 13 18	7	4x Argentina, 3x Mexico
	α β β β Γ	7	4x Argentina, 2x Mexico, 1x Taiwan
Second common	34 13 4 37	6	6x South Africa
Third common	B B M	5	5x Argentina
	F M M	5	4x Argentina, 1x Mexico

The most frequent *A. marginale* strains and their geographical occurrence are shown. The most common tandem repeats found among all the *A. marginale* strains are underlined and there were found more than 60 (M), 80 (β) and 90 (B) times.

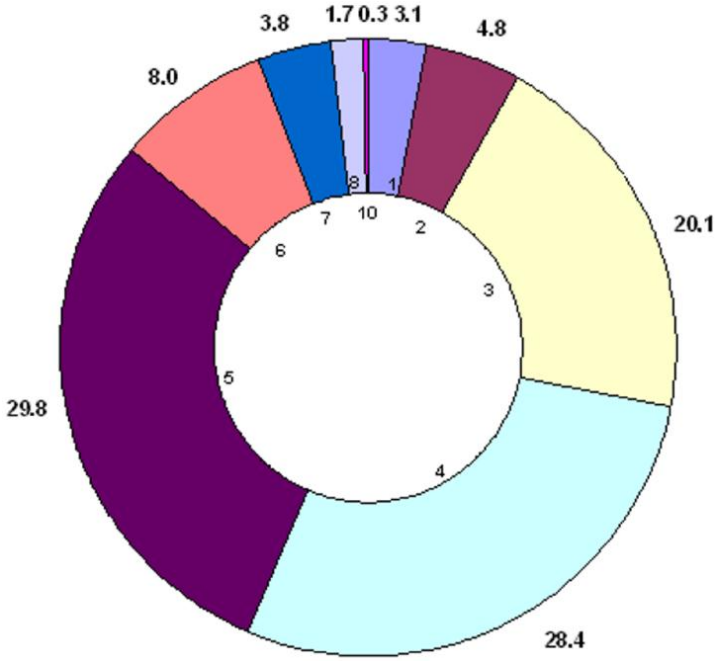
were seen in only a given region, although several strains were isolated from multiple South American countries (Argentina/Chaco/− (τ, 22, 13, 18) from Argentina and Mexico; Brazil/Parana/− (τ, 10, 15) from Brazil and Argentina; Mexico/Pichucalco/E - (α, β, β, Γ^2) from Argentina, Brazil and Mexico; and Mexico/Tamaulipas/− (64, 65, D, 65, E) from Mexico and Venezuela). The strain, Argentina/Santa Fe/− (α, β^3, Γ), was the only strain found in more than one continent, and was reported in Argentina, Mexico, and Taiwan. Most of the MSP1a tandem repeats were shared between different strains, and repeat B, the most common tandem repeat sequence, occurred in 43 strains (**Table 3.1**). While some tandem repeats were unique to one country (repeat 72 was only reported in Brazil) or continent (repeat B was found throughout the American continent), some repeats appeared to be distributed worldwide (repeat M was reported in Israel, Italy, USA and South America). This weak association between specific tandem repeat sequences and particular geographic regions was reported previously by de la Fuente et al. [14] and may be attributed to worldwide cattle movement, among other factors. Notably, in Australia, in which introduction of cattle has been limited, only one MSP1a genotype has been reported [37].

3.3.1. The biological implications of sequence variation of MSP1a tandem repeats.

The tandem repeated portion of the N-terminal region of the *A. marginale* MSP1a has been shown to be an adhesin for bovine erythrocytes and tick cells, and thus are involved in pathogen infection of host cells and transmission by ticks [10], [36], [38].

In contrast, the MSP1a N-terminal tandem repeats are absent in *A. marginale* subsp. centrale. Although *A. centrale* can be transmitted by *Rhipicephalus simus*, the tick species from which this organisms was initially isolated, this *Anaplasma* sp. cannot be transmitted by other tick species that are known to be *A. marginale* vectors [20], [39].

Figure 3.3. Number of tandem repeats among *A. marginale* strains. The total number



of strains classified in our study were organized by the number of MSP1a tandem repeats. The percent of *A. marginale* strains (external numbers) containing different number of tandem repeats (internal numbers) is shown. The most common numbers of MSP1a tandem repeats among strains were 3 (yellow), 4 (light blue) and 5 (violet).

These analyses provided information on the range and frequency of variations in the *A. marginale* MSP1a tandem repeats. Herein, we present the sequence variation data and discuss biological implications of these findings, including O-glycosylation, amino acids at position 20 for binding to tick cell extract (TCE), protein conformation, pathogen-environmental relationships, and combination of these factors.

3.3.2.1. O-glycosylation

MSP1a tandem repeats were found to have a high variability across almost all the 31

amino acid positions, suggesting considerable evolutionary pressure on this molecule (**Figure 3.4.A**). Four positions were totally conserved: serine (S)4 and S25, alanine (A)22 and Glicine 31 (**Figure 3.4.A**). MSP1a has been shown to be O-glycosylated, with S/threonine (T) regions present in the tandem repeats as the target site for this type of glycosylation [31]. Furthermore, the binding capacity of MSP1a to tick cells diminished after deglycosylation [31]. The conservation of S4 and S25 among all the tandem repeats included in this study could indicate that the O-glycosylation at these two positions is highly relevant for *A. marginale* infection. Several bacterial glycoproteins have also been reported to play a role in bacterial adhesion, invasion and pathogenesis [40].

3.3.2.2. Relevance of amino acids at position 20 for binding to tick cell extract (TCE)

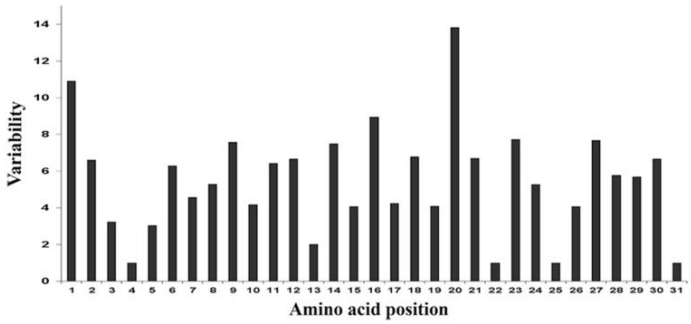
Within the MSP1a tandem repeats, the negatively charged amino acids, aspartic acid (D) and glutamic acid (E), at position 20 were shown to be essential for binding of MSP1a to TCE. When glycine (G) was located at position 20, binding was not observed [10]. This result suggested that the amino acid at position 20 may be essential for *A. marginale* binding to tick cells, a prerequisite for pathogen infection and transmission by ticks. In fact, previous experiments confirmed the existence of both tick-transmissible and not transmissible *A. marginale* strains and, at least for some strains, the presence of TCE-binding with tandem repeats correlated with strains that were transmissible by *Dermacentor sp*. ticks [10]. In all strains, the first MSP1a tandem repeat (R1) contained 67 (34.7%) different sequences. However, R1 tandem repeats had less amino acid variability and 6 conserved positions when compared to non-R1 tandem repeats, in which only 4 conserved amino acid positions were found (**Figure 3.4.B**). These results suggested that the R1 tandem repeat may play a role in *A. marginale* infection and transmission. We found 87 tandem repeats containing D20 (71%) or E20 (29%) (**Figure 3.1**). In total, 161 *A. marginale* strains contained one of these tandem repeats at least once and in 114 (71%) of these strains, the D20 or E20 was found in the R1 tandem repeat. Surprisingly, the highest variable amino acid was at position 20 (**Figure 3.4.A**), suggesting greater

evolutionary pressure at this amino acid position. From our findings, G was the most frequent amino acid at position 20 (**Figure 3.4.C**), in both R1 and non-R1 tandem repeats (data not shown), but only 4 amino acids were found at position 20 in R1 (from highest to lowest frequency: G, D, E and serine [S]) while 7 different amino acids were found at position 20 in non-R1 tandem repeats (G, D, E, S, T, isoleucine [I] and tyrosine [Y]) (**Figure 3.4.C**). In previous experiments, non-R1 tandem repeats had a phylogenetic correlation with tick-transmissible strains, but this correlation was not seen with R1 tandem repeats [9]. We propose that non-R1 tandems are also involved in *A. marginale*-tick interactions which require more genetic variability, because more than 20 different tick species have been reported to transmit *A. marginale* [24].

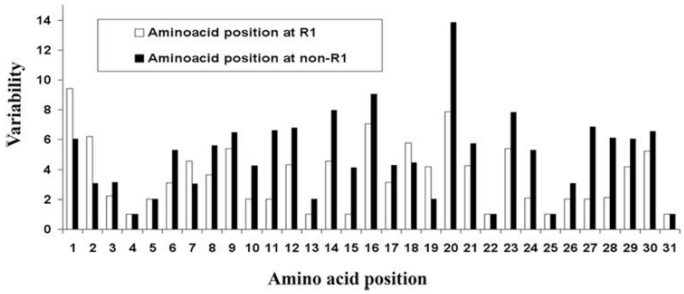
3.3.2.1. Protein conformation

As proposed previously both amino acid sequence and protein conformation may contribute to the function of MSP1a as adhesin [10]. Herein, we explored this hypothesis by predicting the 2-D structure of all the MSP1a tandem repeats. We found that 14 models explained all of the variability of 2-D structure among the 193 tandem repeats (**Figure 3.5**). Three α-helical 2-D structure models, differing in the length and amount of α helixes in the tandem repeat, described 68% of the 2-D structure variation (presented as A, σ and F in **Figure 3.5**). The analysis revealed that the amino acid at position 20 correlated with specific 2-D structure changes in the tandem repeat. When D or E amino acids were at this position, the structure of the tandem repeat was predominantly long α-helical structures (Model types 39, A, 13 and σ), but when a G was in this position, the repeat was a short α helix, β-strand or coiled 2-D structure (Model types 4, 10, α and 48) (**Figure 3.5**). The other four amino acids that were found at lower frequencies at position 20, (I, Y, T and S; **Figure 3.4.C**), except for Y, retained the α-helical 2-D structure (**Figure 3.5**).

A



B



C

Amino acid position	Frequency of amino acid per position																	
	W	P	V	Q	K	C	Y	F	I	R	A	T	D	L	S	N	G	H	E
1											0,54	0,39	0,04	0,005	0,005	0,005			
2													0,9		0,005	0,005	0,07	0,005	0,005
3										0,005					0,92		0,06		
4															1				
5	0,005													0,005	0,98				
6		0,01	0,005								0,95	0,01			0,005				0,01
7													0,005		0,65		0,33		
8											0,005		0,21			0,02	0,75		
9				0,92						0,02				0,005		0,005	0,01	0,02	0,01
10	0,005			0,96						0,01								0,02	
11		0,005		0,93	0,005	0,005				0,03								0,01	
12			0,005	0,01	0,01											0,005	0,05		0,89
13															0,99				0,005
14		0,005							0,023			0,029			0,8	0,017	0,12		
15			0,98								0,005						0,005		0,005
16		0,01	0,01								0,01			0,4	0,56				
17		0,04						0,005			0,005				0,94				
18		0,06		0,88	0,01					0,01					0,005			0,02	
19						0,005						0,01			0,98		0,005		
20							0,005		0,005			0,005	0,3		0,02		0,5		0,14
21		0,016		0,89						0,008			0,04					0,008	0,03
22											1								
23						0,011				0,011	0,02	0,011			0,9	0,005	0,02		
24		0,02								0,005		0,94			0,01			0,005	
25															1				
26	0,005	0,005									0,005				0,98				
27		0,017	0,005	0,91	0,005		0,005								0,017			0,03	
28				0,01				0,005		0,005				0,86	0,11				
29	0,029									0,052				0,034	0,005		0,89		
30			0,16									0,2			0,04		0,6		
31																	1		

Figure 3.4. Amino acid variability and frequency in *A. marginale* MSP1a tandem repeats. The amino acid variability (A), comparison of the variability between tandem repeats at positions R1 and non-R1 (B) and frequency (C) were calculated per amino acid position in the MSP1a tandem repeats using the formula Variability = number of different amino acids at a given position / frequency of the most common amino acid at that position [50]. The one letter amino acid code was used to name the amino acids in (C) and the most frequent amino acids per position are colored in gray.

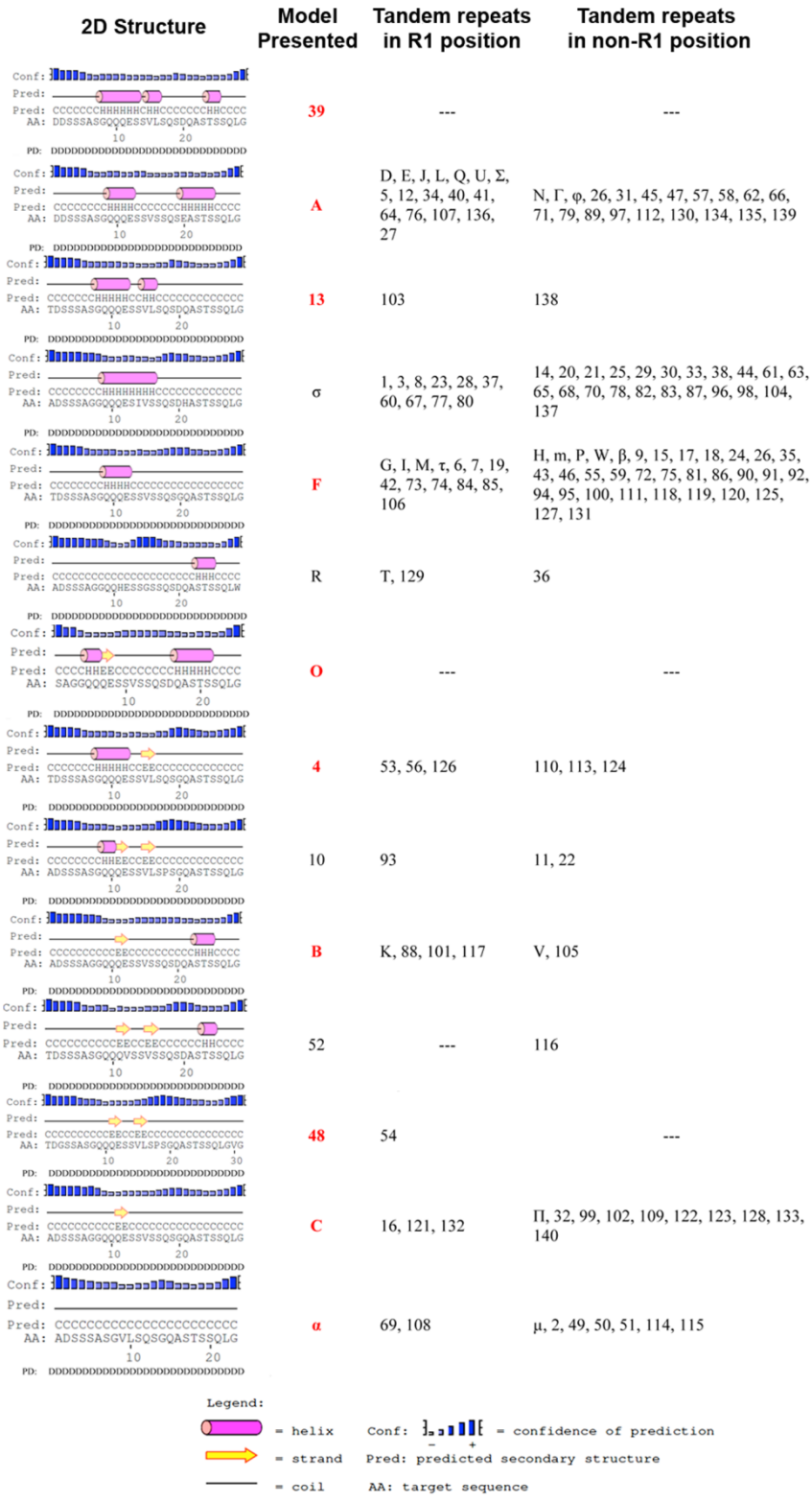


Figure 3.5. Changes in putative 2D structure and disorder analysis of *A. marginale* MSP1a tandem repeats. The PSIPRED web server was used to predict the 2D structure. The tandem repeats were grouped into fourteen 2D structure models. Tandem repeats shown represent prototypes of corresponding tandem repeat 2D structures. In the second column (model presented) is shown the ID of the tandem repeat presented as prototype. Models ID in red represent tandem repeats in R1 position (first tandem in the

MSP1a sequence).

Our results suggest that the MSP1a tandem repeat 2-D structure also correlated with tick transmissibility (**Table 3.2**). Strains reported previously that were not transmitted by *Dermacentor sp***.** had a predominant pattern for 2-D structure of tandem repeats of β strand, short α-helix or coiled structures, regardless of whether or not they had TCE-binding tandem repeats (**Table 3.2**). In contrast, abundant α-helices were found in tandem repeats of strains transmitted by ticks (**Table 3.2**). In the last case, as shown for the USA/Florida/G - (A, B^7) strain, the presence of all seven TCE-binding tandem repeats did not correlate with tick-transmissibility; this Florida isolate was clearly shown to be non-infective for ticks or cultured tick cells (**Table 3.2**).

Table 3.2. Effect of putative MSP1a tandem repeat 2-D structure on *A. marginale* tick transmission phenotype.

Strains	MSP1a tandem repeats 2D structure	Transmission by ticks		
		Dermacentor spp.	*R. sanguineus* or *R. microplus*	*H. excavatum*
USA/Idaho/ C - (D^5, E)	(α-α, α-α, α-α, α-α, α-α, α-α)	Yes (*)	ND	ND
Puerto Rico/Puerto Rico/ C - (E, φ^5)	(α-α, α-α, α-α, α-α, α-α, α-α)	Yes (***)	Yes (***)	ND
USA/Virginia/ G - (A, B)	(α-α, β-α)	Yes (*)	ND	ND
USA/St.Maries/ G - (J, B^2)	(α-α, β-α, β-α)	Yes (*)	Yes (***)	ND
USA/Oklahoma/ G - (U)	(α-α)	Yes (+)	ND	ND
USA/Missisippi/ D - (D^4, E)	(α-α, α-α, α-α, α-α, α-α)	Yes (*)	ND	ND
USA/Rassmusen/ - (A, F, H)	(α-α, α-c, α-c)	Yes (*)	ND	ND
USA/Kansas/ - (E, M, φ)	(α-α, α-c, α-α)	Yes (-)	ND	ND
Nigeria/Zaria/ - (54, 55, F)	(β-β, α-c, α-c)	Yes (**)	ND	ND
Israel/Israel tailed/ F - (1, F, M, 3)	(α-c, α-c, α-c, α-c)	ND	Yes (****)	Yes (****)
Israel/Israel non tailed/ G - (1, 4)	(α-c, α-β)	ND	Yes (****)	No (****)
USA/Florida/ G - (A, B^7)	(α-α, β-α, β-α, β-α, β-α, β-α, β-α, β-α)	No (*)	ND	ND
USA/California/ G - (B^2, C)	(β-α, β-α, β-c)	No (*)	ND	ND
USA/Okeechobee/ G -(L, B, C, B,C)	(α-α, β-α, β-c, β-α, β-c)	No (*)	ND	ND
USA/Illinois/ G - (M, N, B, M, H)	(α-c, α-α, β-α, α-c, α-c)	No (*)	ND	ND

The information about transmission of A. marginale strains by ticks was collected from (*) de la Fuente et al. (2003) [10], (**) Zivkovic et al. (2007) [65], (***) Futse et al. (2003) [44], (****) Shkap et al. 2009 (****) [39], (-) Leverich et al. (2008) [66], and (+) Barbet et al (2001) [67]. TCE-binding tandem repeats are underlined. Abbreviation: ND, not determined.

However, the 2-D structure appeared to be a determinant for the biological transmission of *A. marginale*, because the Israel/Israel tailed/F - (1, F, M, 3) strains, while not having TCE-binding repeats but did have α-helices as 2-D structure, were tick transmissible (**Table 3.2**). As listed in **Table 3.2**, the data collected thus far regarding *A. marginale* transmissibility by ticks is related to the major vector

Dermacentor sp. The complexity of the relationship between the 2-D structure, TCE-binding repeats and tick transmissibility was also seen with the Brazil/Minas Gerais/E strain–(13, 42, 13, 18) which does not contain β strands and is not transmissible by *Rhipicephalus (Boophilus) microplus* [13]. This example demonstrated a different pattern as that observed with *A. marginale* that are not transmissible by *Dermacentor sp.* The 2-D structure data presented in the present study is in agreement with an analysis performed recently on *A. marginale* MSP2 variants in tick or mammalian cells [41]. The 2-D structure analysis using PSIPRED demonstrated that MSP2 variants expressed in ticks were predominantly α-helices, while β-strands were present in MSP2 variants expressed only in mammalian cells [41], [42].

3.3.2.1. Pathogen-environmental relationships

A. marginale was recorded in four eco-region clusters defined in our study (**Table 3.3**). Eco-region Cluster 1 extended over large areas of central Africa and central South America, primarily Argentina and southern Brazil, and was a region with medium to high Normalized Difference Vegetation Index (NDVI) values and a well-defined seasonal decrease between June and September. The highest recorded temperature and annual rainfall of approximately 1,000 mm occurs in Eco-region Cluster 1. Eco-region Cluster 2 included vast areas of the Mesoamerican corridor, northern South America and a small territory of eastern South Africa, and included zones with high NDVI throughout the year without seasonal variability. The temperature values in Eco-region Cluster 2 were similar to those in Eco-region Cluster 1, but with an annual rainfall of approximately 1,500 mm. Eco-region Cluster 3 extended over central South Africa and scattered parts of the southern USA and Mexico, and had the lowest NDVI values with minimal change across the year. This eco-region had lower temperature values and minimum rainfall. Finally, Eco-region Cluster 4 extended over large areas of the USA and had a clear NDVI signature that was low between November and March and then rose to maximum levels in July. This area was the coldest among the four eco-region clusters, with an annual rainfall of approximately 800 mm/year. The results of this study demonstrated that 82% of

MSP1a R1 unique sequences were associated with only one eco-region cluster (**Table 3.3**). Seventeen R1 unique sequences (27% of the total number of R1 sequences) were reported exclusively in Eco-region Cluster 1 and shared 16 out

Table 3.3. Association of *A. marginale* MSP1a R1 sequences with world ecoregions.

Ecoregion	R1 sequences[(a)]	Other R1 sequences[(b)]
1: central Africa and central South America, primarily Argentina and southern Brazil	M, 4, 8, 12, 16, 56, 60, 64, 67, 69, 72, 78, 93, 132, γ, π, τ	A, B, D, T, 13, 23, α
2: Mesoamerican corridor, northern South America and a small territory of eastern South Africa	E, F, 28, 37, 48, 53, 54, 84, 85, 101, 117, 121, 126, 129, 136, ε	A, B, L, T, 13, 23, α
3: central South Africa and scattered parts of southern USA and Mexico	M, O, Q, U, 1, 3, 5, 6, 7, 27, 33, 34, 39, 40, 42, 74, 77, 82, 141, 142, 143, 147, 151, 154, 155	A, D
4: USA	I, J, K, O, U, 19,	A, B, L, α

World ecoregions were built upon temporal series of NDVI values. (a) R1 sequences recorded in one ecoregion only. (b) R1 sequences that have been reported in other ecoregions.

of 31 amino acids (51.6% of the total number of amino acids) (**Table 3.3**). Sixteen R1 unique sequences (17%) were reported only in Eco-region Cluster 2 which had 64.5% identical amino acids (**Table 3.3**). Twenty-five R1 unique sequences (32%) were only found in Eco-region Cluster 3, of which 64.5% of their amino acids were shared (**Table 3.3**). Only five R1 sequences were exclusively associated with Eco-region Cluster 4, which had 77.4% identical amino acids (**Table 3.3**). Eight R1 sequences, were found simultaneously in more than one of the eco-region clusters (**Table 3.3**). These results confirmed that *A. marginale* MSP1a R1 sequences clustered according to a pattern of abiotic (climate) factors, and are related to both the species of tick vector and the performance of this tick vector in the eco-region [25]. Higher variability in R1 repeat sequences appeared in areas where several tick species are candidate vectors (i.e. USA and Canada) or where mechanical transmission is common (i.e. central Argentina). Remarkably, only one *A. marginale* MSP1a genotype has been recorded in Australia (**Table 3.S1**) along with a single tick vector species, *Rhipicephalus australis* [43]. As reported previously, the hypothesis of strain geographic association was rejected [25]. Mantel's test on R1 sequences was 0.82 ($P<0.001$) when applied to eco-region clusters using only unique sequences. The same test provided a value of 0.31 ($P = 0.145$) for the distances matrix based on

geographical association of strains. All the *A. marginale* MSP1a R1 sequences within each eco-region cluster appeared to be under positive selection as shown by dN/dS indexes of 1.83, 1.61, 1.54 and 1.21 for Eco-region Clusters 1 to 4, respectively. Therefore, these results confirmed the hypothesis that *A. marginale* strains are associated with factors that drive the biological performance of ticks vectors in each region [25].

3.3.2.2. Influence of a combination of factors

A phylogenetic correlation was found among *A. marginale* strains between MSP1a tandem repeats 2-D structure, transmissibility by ticks and the presence of TCE-binding tandem repeats (**Figure 3.6**). Notably, cluster β contains all non-tick-transmissible *A. marginale* strains, abundant β-strand tandem repeat 2-D structure, and a low proportion of TCE-binding repeats (**Figure 3.6**). The exception to this rule is the USA/St. Maries/G – (J, B^2) strain, which is tick-transmissible [34], [44] but falls into this cluster. This position of the USA/St. Maries/G – (J, B^2) strain in the phylogenetic tree suggests that *A. marginale* tick-transmissible strains may evolve from non-tick-transmissible strains. The cluster α-2 contains tick-transmissible strains with the highest proportion of α-helices and all TCE-binding tandem repeats. In contrast, strains in cluster β-α-c have a more variable 2-D structure and a high proportion of TC non-binding tandem repeats. The high β-strand content and short α-helixes in MSP1a tandem repeats appears to be associated with a non-tick-transmissible phenotype, similar to the results reported recently with MSP2 sequence study [41]. However, variable 2-D structures such as those in cluster β-α-c may be required in order to bypass the absence of TCE-binding tandem repeats and maintain the tick-transmission phenotype. The presence of TCE-binding tandem repeats could contribute to the organization of the MSP1a molecule, as seen in cluster α-1, where high content of α-helices correlated only with the presence of TCE-binding tandem repeats. Additionally, the analysis using the GeneSilico Metaserver predicted that tandem repeats have a protein disorder across the whole tandem repeat (data not shown). Intrinsically disordered proteins demonstrated better molecular recognition due to a higher specificity, larger interacting surfaces and different folding patterns

upon binding [45].

3.3.2.1. Analysis of B cell epitope in MSP1a tandem repeats

Variation in *A. marginale* outer membrane proteins, such as MSP1a, is a major challenge in developing vaccines that can provide cross-protection between the diversity of strains worldwide. MSP1a has long been investigated as a vaccine candidate [68], [32]–[34] due to the presence of a conserved neutralization-sensitive B-cell epitope at position 20–26 of tandem repeats [6], [29]. However, a study [31] of the the antibody response to the strain USA/Oklahoma/G - (K, C, H), demonstrated that after vaccination with whole *A. marginale* or recombinant MSP1a, a different

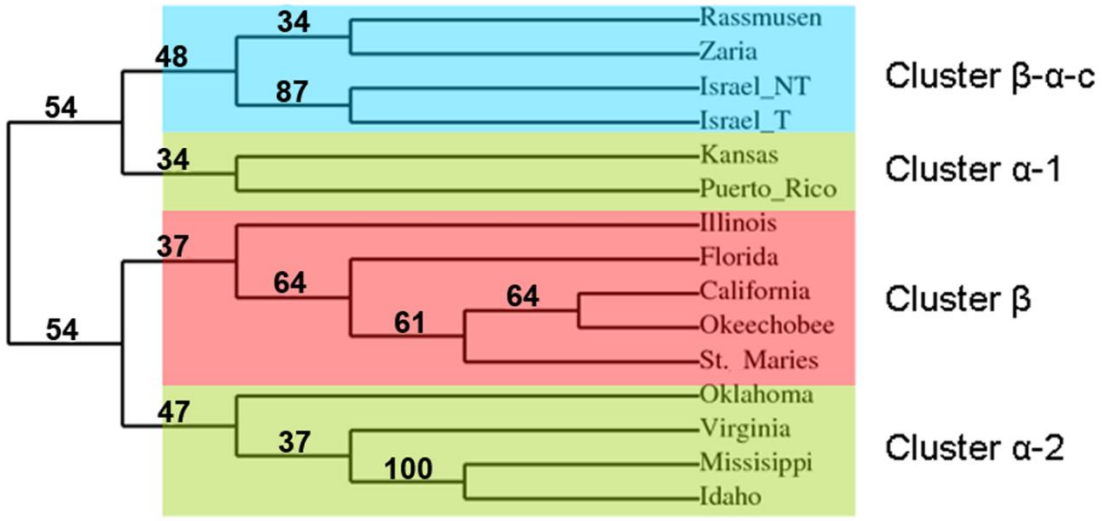


Figure 3.6. Phylogenetic tree based on MSP1a tandem repeat amino acid sequences.
The MSP1a sequences from tick-transmissible and non-transmissible strains (**Table 3.2**) were included in the phylogenetic analysis. The phylogenetic tree was reconstructed using the neighbor joining and maximum likelihood methods. Reliability for internal branch was assessed using the bootstrapping method with 1000 bootstrap replicates. Bootstrap values are shown as % in the internal branch. The tree shows four phylogenetic clusters containing different patterns of MSP1a tandem repeat 2D structures. Cluster β-α-c (blue), cluster α-1 and cluster α-2 (beige) contain tick-transmissible *A. marginale* strains while in cluster β (red) fall the non-tick-transmissible strains.

MSP1a B-cell epitope was immunodominant, SSAGGQQQESS, a linear epitope at

amino acid positions 4 to 14 of the tandem repeat. As the antibody response is of principal importance in anaplasmosis, strain to strain variation in tandem repeat B-cell epitopes would be an important consideration in development of an MSP1a recombinant vaccine [46]–[48]. We therefore characterized the diversity of the immunodominant position 4–14 B-cell epitope among sequenced strains.

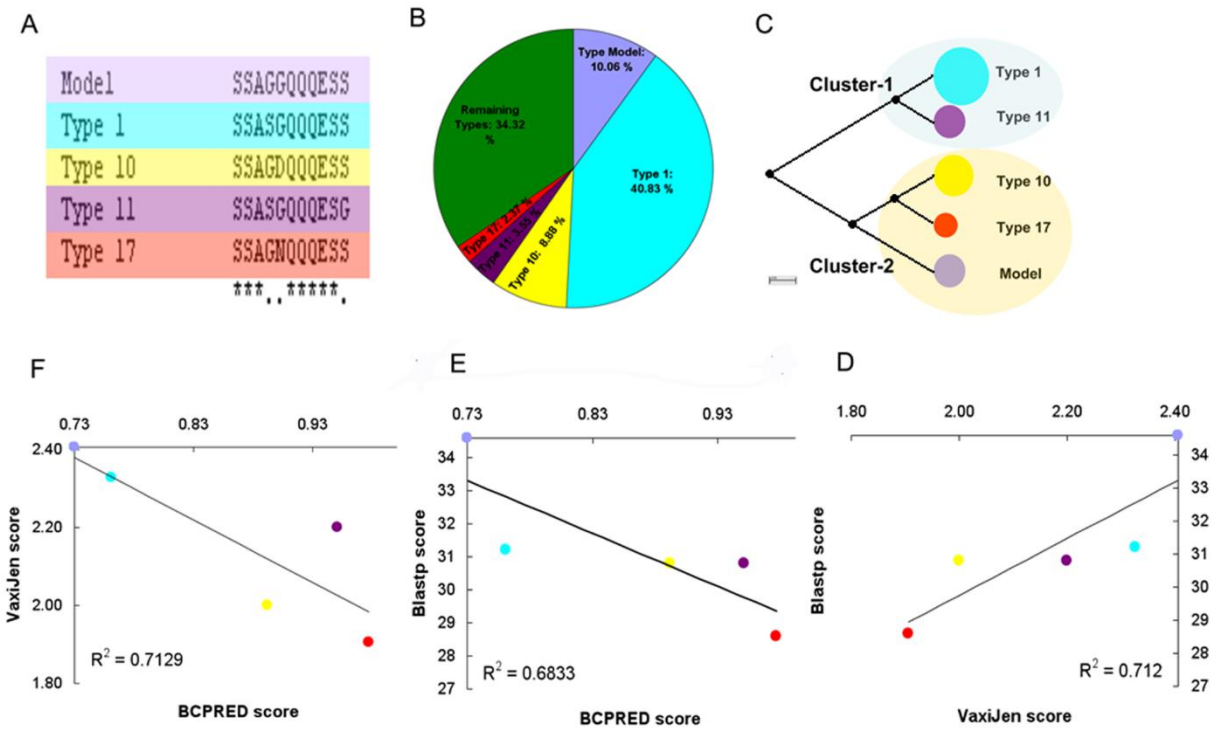


Figure 3.7. B-cell epitope analysis in *A. marginale* MSP1a tandem repeats.

The B-cell epitopes were predicted using BCPRED server. The type 1 B-cell epitope was used as reference (Model) for comparisons. (A) Clustalw alignment and amino acid changes in the 5 more represented MSP1a tandem repeat B cell epitopes. B-cell epitope types model (light violet), 1 (blue), 10 (yellow), 11 (dark violet) and 17 (red) are shown. (B) Percent of tandem repeats containing each type of B cell epitopes. (C) Neighbor joining phylogenetic tree based on B cell epitope amino acid sequences showing the two clusters formed by the 5 more represented B cell epitopes. Cluster-1: Types 1 and 11 and Cluster-2: Types Model, 10 and 17. Correlations between VaxiJen/Blastp (D), BCPRED/Blastp scores (E) and VaxiJen/BCPRED (F) scores are shown. These correlations suggest that the epitopes with higher homology (Blastp score) share in common the immunogenic properties represented by VaxiJen/BCPRED.

This epitope showed high sequence variability among all MSP1a sequences reported to date (**Figure 3.4.A**). From the 172 MSP1a tandem repeats included in the B-cell epitope analysis, 53 sequence variants were found; nevertheless 5 of those variants covered 64% of the total epitope variability (**Figures 3.7.A** and 3.**7.B**). These 5 variants formed 2 phylogenetic clusters (**Figure 3.7.C**); variants in cluster 2 share the same antibody recognition site, while those in cluster 1, types 1 and 11, have different antibody recognition sites (data not shown). All B-cell epitope types were surface exposed (data not shown) as was previously predicted for the Type 1 B-cell epitope using the TMHMM2 algorithm [31].Seven of the 53 B-cell epitope variants gave a 0 score in both B-cell epitope prediction servers BCEPRED and BCPREDS (data not shown), suggesting that some amino acid changes in the immunodominant B-cell epitope (amino acids 4–14) could be the determining factor for the loss of this epitope. Analysis by VaxiJen, a predictor of protective antigens [49], demonstrated that the highest VaxiJen score belongs to the type model B-cell epitope, while types 1, 10, 11 and 17 have VaxiJen scores lower than the type model but higher than the average for all 53 epitopes (**Figure 3.7.D**). Among the main types of B-cell epitopes, a linear but negative correlation was observed between VaxiJen and BCPREDS scores and between Blastp and BCPREDS scores (**Figures 3.7.E** and 3.**7.F**), suggesting a relationship between sequence identity and immune properties among the B-cell epitopes. Overall, these results suggested that different immune properties exist among the different MSP1a types of the B-cell epitopes.

As this is an immunodominant epitope [31], tandem repeats with epitopes predicted to be recognised by different antibodies could be a factor in the frequent lack of cross-protection between heterologous strains. Conversely, strains which share the same type of antibody recognition site may be more likely to be cross-protective.

A correlation (R^2 = 0.69) was found between the number of 2-D structure models present in a given geographic location and the amount of B-cell epitope types in the same region (**Figure 3.2**). Therefore, we explored the hypothesis that there was a link between 2-D structure and B-cell epitopes among the MSP1a tandem repeats. An α-helical structure was seen in 88% of the tandem repeats containing type 1 B-cell epitopes and in 100% of tandem repeats containing types 10, 11 or 17 B-cell epitopes. In contrast, 69% of the tandem repeats containing type model B-cell

epitopes had β-strand structures. Interestingly, a correlation was found between tick transmissibility and the type of B-cell epitopes present on MSP1a repeats, possibly due to these structural differences between epitope types. 71% of the MSP1a tandem repeats present in non-tick-transmissible *A. marginale* strains were found to have type model B-cell epitopes, whereas 87% of the tandem repeats in tick transmissible strains contained type 1 B-cell epitopes. This data suggest antigenic differences between tick-transmissible and not-transmissible *A. marginale* strains, and agrees with the finding that both type 1 and model type epitopes fall into different phylogenetic clusters (**Figure 3.7.C**) presenting different putative antibodies recognition sites. Both epitopes had the highest VaxiJen and BCPRED scores among the 5 most common B-cell epitopes, but shared low identity as shown by Blastp score (data not shown).

Collectively, the results of these studies demonstrate that the unified nomenclature proposed herein using MSP1a sequences provides information about *A. marginale* strain world distribution, transmissibility by ticks, infective potential, antigenic variability and putative utility for MSP1a vaccine development. The structural and immune analyses of MSP1a revealed a phylogenetic correlation between *A. marginale* tick transmissibility, 2-D structure adopted by the tandem repeats and the type of B-cell epitopes present in the tandem repeats. These results are fundamental information for design of MSP1a structure-based vaccines which would be cross protective against multiple *A. marginale* strains, and for development of serodiagnostic methods based on differential B-cell epitopes, for epidemiological characterization of field strains.

3.4. Methods

3.4.1. *Anaplasma marginale* strains classification

A total of 289 A. marginale MSP1a sequences with complete tandem repeat regions included in this study were obtained from published research and the GenBank sequence database [http://www.ncbi.nlm.nih.gov/]. These sequences were analyzed

and classified, and the tandem repeats were named (or renamed) following the nomenclature proposed by Allred et al. [6] and de la Fuente et al. [14]. When microsatellite sequences were included in the *msp1a* published nucleotide sequence, they were used to assign a genotype following the system of Estrada-Peña et al. [25]. Briefly, the 5′-UTR microsatellite located between the putative Shine-Dalgarno (SD) sequence (GTAGG) and the translation initiation codon (ATG), GTAGG (**G/A TTT**) m (**GT**) n **T** ATG (microsatellite sequence is shown in bold letters) and the SD-ATG distance (d) calculated in nucleotides as (4 × m)+(2 × n) +1 were used. We propose one nomenclature for *A. marginale* strains based on MSP1a with the following structure: country/locality/microsatellite genotype - (structure of tandem repeat), and all MSP1a sequences were classified using this nomenclature. When multiple strains had 100% amino acid sequence similarity across tandem repeats, they were listed under one strain name, with geographical information taken from the isolate with the most complete information. When this information was equal between isolates, information was used from the isolate first submitted to GenBank.

3.4.2. Amino acid variability within MSP1a tandem repeats

Tandem repeat sequences were aligned using Clustalw, and each amino acid position was numbered from 1 to 31. The amino acid variability was determined using the formula of Kuby et al. [50]. The variability was equal to the number of different amino acids at a given position/frequency of the most common amino acid at that position.

3.4.3. Correlation analysis between MSP1a tandem repeats and world ecological regions

The analysis was conducted as described previously, assuming that (i) eco-regions could be delineated by quantitative abiotic characters based on well-recognized and repeatable attributes and (ii) *A. marginale* strains were associated with each eco-region and subjected to different environmental conditions that could be analyzed by multivariate geographic clustering [25]. The feature selected to build the eco-regions was the NDVI, which is a variable that reflects vegetation stress and summarizes information about the ecological background for the performance of tick populations

[25]. A 0.1° resolution series of monthly NDVI data was obtained for the period 1986–2006. The 12 averaged monthly images were subjected to Principal Components Analysis (PCA) to obtain decomposition into the main axes representing the most significant, non-redundant information. The strongest principal axes were chosen using Cattell's Scree Test [25]. The PCA analysis retained three principal axes, including 92% of the total variance. A hierarchical agglomerative clustering on PCA values was then used to classify multiple geographical areas into a single common set of discrete regions. Mahalanobis distance was used as a measure of dissimilarity and the weighted pair-group average was used as the amalgamation method. A value of 0.05 was used as the cut-off probability for assignment to a given eco-region.

3.4.4. Bioinformatics

Secondary structure was predicted using the position-specific scoring matrices method [51] from the PSIPRED server [52], and protein disorder was predicted using the GeneSilico Metaserver [53].

The immunodominant B-cell epitope SSAGGQQQESS (amino acid positions 4–14), previously mapped in the A. marginale strain USA/Oklahoma/G - (K, C, H) MSP1a sequence [31] will be referred to as epitope “Type 1″. The variability among MSP1a tandem repeats within this B-cell epitope (amino acid positions 4–14) was evaluated. The percent of amino acid identity and Blastp score among the B-cell epitopes had a linear correlation (R^2 = 0.85), so the Blastp score was used as an identity index in the analysis. Prediction/score of B-cell epitope was determined using BCPREDS server [54] and the protective potential of the B-cell epitope was predicted using the VaxiJen server [55]. Prediction of physicochemical properties of the B-cell epitope was assayed using BCEPRED server [56]. PepSurf algorithm [57], implemented in the PEPITOPE server [58], was used to determine the structure/position of the affinity-selected B-cell epitopes in a model protein. The 3D analysis of MSP1a tandem repeat B-cell epitopes was performed using a model of the crystal structure of the Fv corresponding with the anti-blood group A antibody AC1001 (PDB ID: 1JV5) [59].

For phylogenetic analysis, sequences were aligned with MUSCLE (v3.7) configured for the highest accuracy [60]. After alignment, ambiguous regions (i.e., containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b) [61]. The phylogenetic tree was reconstructed using the neighbor joining (NJ) and maximum likelihood methods implemented in PHYLIP package (v3.66), NJ distances were calculated using FastDist [62], [63]. Reliability for internal branch was assessed using the bootstrapping method (1000 bootstrap replicates). Graphical representation and editing of the phylogenetic tree were performed with TreeDyn (v198.3) [64].

Supporting Information: Table 3.S1. Classification of *A. marginale* strains based on the proposed nomenclature.

A total of 289 MSP1a sequences were analyzed. *A. marginale* 224 unique strains were classified using the nomenclature proposed in our study: Country/Locality/microsatellite genotype - (structure of tandem repeat). The 5′UTR microsatellite genotype was included when available. The structure of tandem repeats was represented following the nomenclature previously proposed [14] (**Figure 3.1**). When the same repeat was present more than one time, a super-index was used to represent copy number for this repeat.

Strain Name	Accession No. / Reference	Tandem repeat structure	No. Tandem repeats
Argentina/Chaco/ - (α, β, Γ^2, β^2 ,Γ)	DQ833264	α β Γ Γ β β Γ	7
Argentina/Chaco/ - (τ, 11, 10^2, 11, 10, 15)	DQ833266	τ 11 10 10 11 10 15	7
Argentina/Chaco/ - (τ, 22, 13, 18)	DQ833263	τ 22 13 18	4
Argentina/Cordoba/ - (23, 24, 25, 26, 27^2)	DQ833261	23 24 25 26 27 27	6
Argentina/Corientes/ - (53, 10^2)	DQ833273	53 10 10	3
Argentina/Corientes/ - (48, 11, 10)	DQ833274	48 11 10	3
Argentina/Corientes/ - (α, 49, 50, 51, 52)	DQ833275	α 49 50 51 52	5
Argentina/Corrientes/ - (α, β^3)	DQ833272	α β β β	4
Argentina/Entre Rios/ - (F, M^2)	DQ833249	F M M	3
Argentina/Mercedes/ - (23, 30, 31^3)	DQ833271	23 30 31 31 31	5
Argentina/Quitilipi/ - (28, 29, M, 29, M, F)	DQ833270	28 29 M 29 M F	6
Argentina/Salta/ G - (B^2, M)	AF428093	B B M	3
Argentina/Santa Fe/ - (α, β^3, Γ)	DQ833253	α β β β Γ	5
Argentina/Virasoro/E - (Σ, B, Q, B, C)	AF428094	Σ B Q B C	5
Australia/Northern Territory/J - (8)	AF407542	8	1
Australia/Western Australia/ E - (8)	AF407545	8	1
Brazil/Minas Gerais/ B - (13, 27^3)	JX844209	13 27 27 27	4
Brazil/Minas Gerais/ C - (α, β, τ, M)	AY283199	α β τ M	4
Brazil/Minas Gerais/ D - (72, 62, 61)	JX844216	72 62 61	3
Brazil/Minas Gerais/ D - (C, F, N)	AY283198	C F N	3
Brazil/Minas Gerais/ E - (13, 159^2)	Bastos et al.2010	13 159 159	3

Brazil/Minas Gerais/ E - (72, 62, 61)	JX844210	**72**	**62**	**61**			3
Brazil/Minas Gerais/ E - (78, 24^2,25, 31)	JX844206	**78**	**24**	**24**	**25**	**31**	5
Brazil/Minas Gerais/ E - (α, β^2, 13)	JX844215	**α**	**β**	**β**	**13**		4
Brazil/Minas Gerais/ E - (α, β^2, N)	AY283200	**α**	**β**	**β**	**N**		4
Brazil/Minas Gerais/ E - (α, β^2, φ)	JX844207	**α**	**β**	**β**	**φ**		4
Brazil/Minas Gerais/ E - (τ, 57, 13, 18)	JX844213	**τ**	**57**	**13**	**18**		4
Brazil/Minas Gerais/ E - (τ, 57, β^2, φ)	JX844205	**τ**	**57**	**β**	**β**	**φ**	5
Brazil/Minas Gerais/ E - (13, 27^2)	EU676175	**13**	**27**	**27**			3
Brazil/Minas Gerais/ E - (13, 42, 13, 18)	EU676176	**13**	**42**	**13**	**18**		4
Brazil/Minas Gerais/ G - (B, Q, B, M)	JX844208	**B**	**Q**	**B**	**M**		4
Brazil/Minas Gerais/ G - (B^2,Q, σ, μ)	AF428092	**B**	**B**	**Q**	**σ**	**μ**	5
Brazil/Parana/ - (τ, 10, 15)	AY998121	**τ**	**10**	**15**			3
Brazil/Parana/ E - (16, F, 17, 13, 18)	AY998120	**16**	**F**	**17**	**13**	**18**	5
Canada/Saskatchewan/ E - (D, Q^2, R)	AY253141	**D**	**Q**	**Q**	**R**		4
China/HBA8/ - (19, 20, 19, 21)	DQ811774	**19**	**20**	**19**	**21**		4
Cuba/Habana/ - (A, B^4)	AY489564	**A**	**B**	**B**	**B**	**B**	5
Israel/ Israel round / - (1, F, M, 3^2)	AY355282	**1**	**F**	**M**	**3**	**3**	5
Israel/Golan Heights/ - (1, 4, 3^3)	EU678758	**1**	**4**	**3**	**3**	**3**	5
Israel/Golan Heights/ - (1, F, M, 3^3)	Molad et al, 2009	**1**	**F**	**M**	**3**	**3**	**3**	6
Israel/Golan Heights/ - (1, F, M^2, 3)	Molad et al, 2009	**1**	**F**	**M**	**M**	**3**		5
Israel/Golan Heights/ - (1, F, M^2, 3^2)	Molad et al, 2009	**1**	**F**	**M**	**M**	**3**	**3**	6
Israel/Golan Heights/ - (3, M, 3^3)	Molad et al, 2009	**3**	**M**	**3**	**3**	**3**		5
Israel/Golan Heights/ - (74^3, 73^2)	EU678765	**74**	**74**	**74**	**73**	**73**		5
Israel/Golan Heights/ - (74^3, 76)	EU678756	**74**	**74**	**74**	**76**			4
Israel/Golan Heights/ - (77, 25, 3^2)	EU678755	**77**	**25**	**3**	**3**			4
Israel/Golan Heights/ - (77, 25, 3^4)	Molad et al, 2009	**77**	**25**	**3**	**3**	**3**	**3**	6
Israel/Golan Heights/ - (77, 25^2, 3^2)	Molad et al, 2009	**77**	**25**	**25**	**3**	**3**		5
Israel/Golan Heights/ - (77, 3, 4, 3)	Molad et al, 2009	**77**	**3**	**4**	**3**			4
Israel/Golan Heights/ - (77, 3^4)	EU678757	**77**	**3**	**3**	**3**	**3**		5

Israel/Israel non tailed/ G - (1, 4)	AF352559	**1**	**4**						2
Israel/Israel tailed/ - (1, 2, M, 3)	AY295077	**1**	**2**	**M**	**3**				4
Israel/Israel tailed/ F - (1, F, M, 3)	AF352560	**1**	**F**	**M**	**3**				4
Israel/Jerusalem/ - (77, 3^6)	Molad et al, 2009	**77**	**3**	**3**	**3**	**3**	**3**	**3**	7
Israel/Jerusalem/ - (78, 3^2)	Molad et al, 2009	**78**	**3**	**3**					3
Israel/Jerusalem/ - (78, 3^3)	EU678759	**78**	**3**	**3**	**3**				4
Israel/Or-Haner/ - (M, F^2)	AY355284	**M**	**F**	**F**					3
Israel/Upper Galilee/ - (7, E^2, M, 3^2)	EU678761	**7**	**E**	**E**	**M**	**3**	**3**		6
Israel/Upper Galilee/ - (7, E^4)	EU678760	**7**	**E**	**E**	**E**	**E**			5
Israel/Upper Galilee/ - (M, F^3)	AY846868	**M**	**F**	**F**	**F**				4
Israel/Western Galilee/ - (4, 3, 4, 3)	Molad et al, 2009	**4**	**3**	**4**	**3**				4
Israel/Western Galilee/ - (74^2, 76)	Molad et al, 2009	**74**	**74**	**76**					3
Israel/Western Galilee/ - (75, 73)	EU678763	**75**	**73**						2
Israel/Western Galilee/ - (77, 3, 38)	Molad et al, 2009	**77**	**3**	**38**					3
Israel/Western Galilee/ - (77, 4, 3)	EU678762	**77**	**4**	**3**					3
Israel/Western Galilee/ - (78, 25^2, 15^2)	EU678764	**78**	**25**	**25**	**15**	**15**			5
Italy/Sicily/ C - (5, Γ^3)	AY702928	**5**	**Γ**	**Γ**	**Γ**				4
Italy/Sicily/ C - (5, φ^3)	AY702929	**5**	**φ**	**φ**	**φ**				4
Italy/Sicily/ G - (6, 7^3)	AY702932	**6**	**7**	**7**	**7**				4
Italy/Sicily/ G - (M^3, Q)	AY702926	**M**	**M**	**M**	**Q**				4
Italy/Sicily/ G - (Q, M, Q^2, M)	AY702930	**Q**	**M**	**Q**	**Q**	**M**			5
Italy/Sicily/ G - (Q, N^3)	AY702931	**Q**	**N**	**N**	**N**				4
Japan/Okinawa/ - (80, 73, 81, 73, M)	FJ226456	**80**	**73**	**81**	**73**	**M**			5
Mexico/ Santa Martha/ - (α, β^3, Γ)	EF053268	**α**	**β**	**β**	**Γ**	**β**			5
Mexico/ Tamaulipas/ - (69, 61, 70, 71, 61)	EU283852	**69**	**61**	**70**	**71**	**61**			5
Mexico/Aguascalientes/ E - (4, 9, 10, 11, 9)	DQ501243	**4**	**9**	**10**	**11**	**9**			5
Mexico/Morelos/ E - (α, β^2, Γ)	AF345869	**α**	**β**	**β**	**Γ**				4
Mexico/Pichucalco/ E - (α, β, $\beta\Gamma^2$)	DQ501244	**α**	**β**	**β**	**Γ**	**β**	**Γ**		6
Mexico/Playa Vicente/ - (T, C, B^2, C, B, C)	JN564636	**T**	**C**	**B**	**B**	**C**	**B**	**C**	7

Mexico/Puente de Ixtla/ G - (12, 13, 14)	DQ501242	**12**	**13**	**14**					3
Mexico/Tamaulipas/ - (28, 29, 104, 29, M, F)	JN564643	**28**	**29**	**104**	**29**	**M**	**F**		6
Mexico/Tamaulipas/ - (4, 63^2, 27, 12)	EU283850	**4**	**63**	**63**	**27**	**12**			5
Mexico/Tamaulipas/ - (4, 9, 10^2, 9)	EU283848	**4**	**9**	**10**	**10**	**9**			5
Mexico/Tamaulipas/ - (56, 22, 58, 59)	EU283847	**56**	**22**	**58**	**59**				4
Mexico/Tamaulipas/ - (60, 61^2, 62, 61)	EU283849	**60**	**61**	**61**	**62**	**61**			5
Mexico/Tamaulipas/ - (64, 65, D, 65, E)	EU283853	**64**	**65**	**D**	**65**	**E**			5
Mexico/Tamaulipas/ - (67, 68, 63, 27, 12)	EU283851	**67**	**68**	**63**	**27**	**12**			5
Mexico/Tamaulipas/ - (D, 65, D, 65, E)	EU283854	**D**	**65**	**D**	**65**	**E**			5
Mexico/Tepic/ - (α, β^2, Γ)	JN564639	**α**	**β**	**β**	**Γ**				4
Mexico/Tlapacoyan/ - (103, C, F)	JN564638	**103**	**C**	**F**					3
Mexico/Tlapacoyan/ - (108, β^3, Γ)	JN564641	**108**	**β**	**β**	**β**	**Γ**			5
Mexico/Tlapacoyan/ - (T, C, B^2, C^2)	JN564644	**T**	**C**	**B**	**B**	**C**	**C**		6
Mexico/Yucatan/G - (T, C, B^2,C, B, π)	AF345871	**T**	**C**	**B**	**B**	**C**	**B**	**π**	7
Nigeria/Zaria/ - (54, 55, F)	EU106083	**54**	**55**	**F**					3
Puerto Rico/Puerto Rico/ C - (E, φ^5)	AY191826	**E**	**φ**	**φ**	**φ**	**φ**	**φ**		6
Republic of the Philippines/Cebu/ E - (46, 88,46)	JQ839006	**46**	**88**	**46**					3
Republic of the Philippines/Cebu/K-(107, M, 107, M^2)	JQ839007	**107**	**M**	**107**	**M**	**M**			5
Republic of the Philippines/Cebu/D - (46^2)	JQ839005	**46**	**46**						2
Republica of China/Taiwan/ - (106, F^2, M^3,F, M)	FJ188386	**106**	**F**	**F**	**M**	**M**	**M**	**F**	**M**	8
Republica of China/Taiwan/ - (M, β, Γ, β^5,)	FJ195757	**M**	**β**	**Γ**	**β**	**β**	**β**	**β**	**β**	8
Republica of China/Taiwan/ - (α)	FJ188391	**α**							1
South Africa/EC22/G – (27, 13, 4^2, 37)	KC470183	**27**	**13**	**4**	**4**	**37**			5
South Africa/EC23/G – (151, 152, 4^2, 153)	KC470184	**151**	**152**	**4**	**4**	**153**			5
South Africa/GP_C1/ - (82, 13, 4^2, 37)	KC470167	**82**	**13**	**4**	**4**	**37**			5
South Africa/GP_C2/ - (34, 27, 3, 38, 13, 3, 38)	KC470168	**34**	**27**	**3**	**38**	**13**	**3**	**38**	7
South Africa/GP_C5/ - (3, 4^3, 37)	KC470169	**3**	**4**	**4**	**4**	**37**			5
South Africa/GP-C1112105/G – (34, 37)	KC470170	**34**	**37**						2
South Africa/GP-C1817105/G – (34, 13^2)	KC470172	**34**	**13**	**13**					3

South Africa/GP-C4117/G – (3, 36, 38)	KC470171	**3**	**36**	**38**						3
South Africa/GP-C7117105/ - (34, 13, 37)	KC470173	**34**	**13**	**37**						3
South Africa/KZN_14/ E- (142, 43, 25, 31)	KC470179	**142**	**43**	**25**	**31**					4
South Africa/KZN_19/ E – (141, 140^2)	KC470180	**141**	**140**	**140**						3
South Africa/KZN_D/ - (42, 43, 25, 163, 31)	KC470177	**42**	**43**	**25**	**157**	**31**				5
South Africa/KZN_F/ - (42, 43, 25, 31^2)	KC470174	**42**	**43**	**25**	**31**	**31**				5
South Africa/KZN_Y/ - (143, 144, 145, 146)	KC470178	**143**	**144**	**145**	**146**					4
South Africa/KZN51/E - (147)	KC470182	**147**								1
South Africa/LP_10/G – (27, 13, 3)	KC470156	**27**	**13**	**3**						3
South Africa/LP34/G – (34, 13, 3, 38)	KC470159	**34**	**13**	**3**	**38**					4
South Africa/LP37/G – (27, 13^2, 37)	KC470153	**27**	**13**	**13**	**37**					4
South Africa/LP46/G – (3, 38)	KC470154	**3**	**38**							2
South Africa/MP_C2/ - (34, 13, 158, 37)	KC470160	**34**	**13**	**158**	**37**					4
South Africa/MP_C5/ - (15^2,100, 83)	KC470161	**15**	**15**	**100**	**83**					4
South Africa/Northeastern Free State/ - (27, 13, 4, 44)	DQ813555	**27**	**13**	**4**	**44**					4
South Africa/Northeastern Free State/ - (27, 18)	DQ813559	**27**	**18**							2
South Africa/Northeastern Free State/ - (27, 37)	DQ813557	**27**	**37**							2
South Africa/Northeastern Free State/ - (27, 4, 13, 4^2)	DQ813554	**27**	**4**	**13**	**4**	**4**				5
South Africa/Northeastern Free State/ - (3, 36, 3, 36^2, 3, 36, 38)	DQ813564	**3**	**36**	**3**	**36**	**36**	**3**	**36**	**38**	8
South Africa/Northeastern Free State/ - (3^2, 38)	DQ813547	**3**	**3**	**38**						3
South Africa/Northeastern Free State/ - (33, 35^2)	DQ813545	**33**	**35**	**35**						3
South Africa/Northeastern Free State/ - (34, 13, 4, 37)	DQ813536	**34**	**13**	**4**	**37**					4
South Africa/Northeastern Free State/ - (34, 4, 37)	DQ813542	**34**	**4**	**37**						3
South Africa/Northeastern Free State/ - (34, F, 4, H)	DQ813544	**34**	**F**	**4**	**H**					4
South Africa/Northeastern Free State/ - (39, 37, 13^4, 37)	DQ813560	**39**	**37**	**13**	**13**	**13**	**13**	**37**		7
South Africa/Northeastern Free State/ - (40, Q^2)	DQ813561	**40**	**Q**	**Q**						3
South Africa/NW_C2/ - (27, 13, 4^2, 37)	KC470162	**27**	**13**	**4**	**4**	**37**				5
South Africa/NW_C4/ - (27, 13, 4, 37)	KC470163	**27**	**13**	**4**	**37**					4
South Africa/NW_C5/ - (82, 13, 79, 4, 37)	KC470164	**82**	**13**	**79**	**4**	**37**				5

South Africa/NW-C1/G – (34, 13, 3, 36, 38)	KC470165	**34**	**13**	**3**	**36**	**38**				5
South Africa/NW-C4/G – (34, 36, 38, 3)	KC470166	**34**	**36**	**38**	**3**					4
South Africa/Southwestern Free State/ - (27, 13, 4, 13, 4)	DQ813553	**27**	**13**	**4**	**13**	**4**				5
South Africa/Southwestern Free State/ - (27, 4, 13^2, 37)	DQ813551	**27**	**4**	**13**	**13**	**37**				5
South Africa/Southwestern Free State/ - (27, 4^3, 37)	DQ813552	**27**	**4**	**4**	**4**	**37**				5
South Africa/Southwestern Free State/ - (3, 13, 4^2, 37)	DQ813550	**3**	**13**	**4**	**4**	**37**				5
South Africa/Southwestern Free State/ - (34, 13^2, 37)	DQ813543	**34**	**13**	**13**	**37**					4
South Africa/Southwestern Free State/ - (34, 45^2, 46, 37)	DQ813556	**34**	**45**	**45**	**46**	**37**				5
South Africa/Southwestern Free State/ - (41, 13^3, 4, 37)	DQ813562	**41**	**13**	**13**	**13**	**4**	**37**			6
South Africa/Southwestern Free State/ - (42, 43, 25, 31)	DQ813563	**42**	**43**	**25**	**31**					4
South Africa/WC10/G – (154)	KC470190	**154**								1
South Africa/WC11/G – (40, Q^5, 37)	KC470191	**40**	**Q**	**Q**	**Q**	**Q**	**Q**	**37**		7
South Africa/WC12/G – (27, 13, 37)	KC470192	**27**	**13**	**37**						3
South Africa/WC13/G – (M, Q, M, Q, M)	KC470193	**M**	**Q**	**M**	**Q**	**M**				6
South Africa/WC14/G – (155, 36, 38)	KC470194	**155**	**36**	**38**						3
South Africa/WC16/G – (34, 13, 4, 13^2, 4, 37)	KC470196	**34**	**13**	**4**	**13**	**13**	**4**	**37**		7
South Africa/WC4/ - (40, Q^2, m)	KC470186	**40**	**Q**	**Q**	**m**					4
South Africa/WC6/G – (3, 4^2, 37)	KC470187	**3**	**4**	**4**	**37**					4
South Africa/WC7/G - (M^4)	KC470188	**M**	**M**	**M**	**M**					4
Spain/Va48/ - (40, 47^2, 32, C^2)	DQ811775	**40**	**47**	**47**	**32**	**C**	**C**			6
Taiwan/Nantou/ E - (α, β^2, Γ, 105)	EU677384	**α**	**β**	**β**	**Γ**	**105**				5
Taiwan/Taichung/ J - (α, β^3, Γ, 105)	FJ188389	**α**	**β**	**β**	**β**	**Γ**	**105**			6
USA/California/ G - (B^2, C)	AY010242	**B**	**B**	**C**						3
USA/Cushing/ G - (L, C, B, C)	AY127056	**L**	**C**	**B**	**C**					4
USA/Cushing2/ G - (K, N^2, F, H)	AY127057	**K**	**N**	**N**	**F**	**H**				5
USA/Florida/ G - (A, B^7)	M32871	**A**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	8
USA/Glencoe1/ G - (K, F, N, F, H)	AY127053	**K**	**F**	**N**	**F**	**H**				5
USA/Glencoe2/ G - (B, M, F, H)	AY127054	**B**	**M**	**F**	**H**					4
USA/Glencoe3/ G - (T, B, C)	AY127055	**T**	**B**	**C**						3

USA/Idaho/ C - (D^5, E)	M32868	D	D	D	D	D	E					6
USA/Illinois/ G - (M, N, B, M, H)	AF345867	M	N	B	M	H						5
USA/Kansas/ - (B^2)	Palmer et al, 2004	B	B									2
USA/Kansas/ - (B^3)	Palmer et al. 2004	B	B	B								3
USA/Kansas/ - (B^5)	Palmer et al. 2004	B	B	B	B	B						5
USA/Kansas/ - (B^6)	Palmer et al. 2004	B	B	B	B	B	B					6
USA/Kansas/ - (D^2, E)	Palmer et al. 2004	D	D	E								3
USA/Kansas/ - (D^5)	Palmer et al. 2004	D	D	D	D	D						5
USA/Kansas/ - (D^6, E)	Palmer et al. 2004	D	D	D	D	D	D	E				7
USA/Kansas/ - (D^9, E)	Palmer et al. 2004	D	D	D	D	D	D	D	D	D	E	10
USA/Kansas/ - (E, M, φ)	Palmer et al. 2004	E	M	φ								3
USA/Mississippi/ D - (D^4, E)	AY010243	D	D	D	D	E						5
USA/Missouri/ G - (B^4)	AY127052	B	B	B	B							4
USA/NewCastle/ G - (L, B, C, B)	AY127063	L	B	C	B							4
USA/Okeechobee/ G - (L, B, C, B, C)	AY010244	L	B	C	B	C						5
USA/Oklahoma/ G - (U)	AY127059	U										1
USA/Okmulgee/ G - (K, B, V, C)	AY127060	K	B	V	C							4
USA/Oregon/ - (G)	Palmer et al, 2001	G										1
USA/Pawhuska/ G - (I, H)	AY127064	I	H									2
USA/Pawhuska/ G - (K, B, M, F, W)	AY253144	K	B	M	F	W						5
USA/Rassmusen/ - (A, F, H)	AF293064	A	F	H								3
USA/St.Maries/ G - (J, B^2)	AY010245	J	B	B								3
USA/Stigler/ G - (T, B^2, C)	AY127058	T	B	B	C							4
USA/Stillwater/ G - (K, F^3, H)	AY127061	K	F	F	F	H						5
USA/Stillwater/ G - (L, B, C^2)	AY127062	L	B	C	C							4
USA/Stillwater68/ - (K, B, M, F, H)	DQ811776	K	B	M	F	H						5
USA/Texas/ G - (O, B, M, P)	AF428091	O	B	M	P							4
USA/Texas198/ - (B^2, m, B, m)	DQ811778	B	B	m	B	m						5
USA/Virginia/ G - (A, B)	AY010246	A	B									2

USA/Washington/ G - (B[3], C)	M32869	**B**	**B**	**B**	**C**				4
USA/Wetumka/ G - (K, C, H)	AY010247	**K**	**C**	**H**					3
Venezuela/Barinas/ - (101, B[2], 102)	JF957801	**101**	**B**	**B**	**102**				4
Venezuela/Barinas/ - (α, β[4])	JF957813	**α**	**β**	**β**	**β**	**β**			5
Venezuela/Lara/ - (117, 118[2], 119, 120)	JF957796	**117**	**118**	**118**	**119**	**120**			5
Venezuela/Lara/ - (121, 122, 123, 124, 125)	JF957787	**121**	**122**	**123**	**124**	**125**			5
Venezuela/Lara/ - (126, 127, 128)	JF957798	**126**	**127**	**128**					3
Venezuela/Lara/ - (129, 130, 131, φ, 57)	JF957792	**129**	**130**	**131**	**φ**	**57**			5
Venezuela/Lara/ - (13, 111, 112, 113)	JF957797	**13**	**111**	**112**	**113**				4
Venezuela/Lara/ - (13, 18[2], 13)	JF957805	**13**	**18**	**18**	**13**				4
Venezuela/Lara/ - (136, 137, 138, 139, 95[2])	JF957810	**136**	**137**	**138**	**139**	**95**	**95**		6
Venezuela/Lara/ - (60, 62, 61, 62, 61, 62, 61)	JF957807	**60**	**62**	**61**	**62**	**61**	**62**	**61**	7
Venezuela/Lara/ - (60, 62[2], 61)	JF957808	**60**	**62**	**62**	**61**				4
Venezuela/Lara/ - (64, 96, 97, 99)	JF957809	**64**	**96**	**97**	**99**				4
Venezuela/Lara/ - (64, 98, F[3])	JF957806	**64**	**98**	**F**	**F**	**F**			5
Venezuela/Lara/ - (84, 62[2], 61)	JF957793	**84**	**62**	**62**	**61**				4
Venezuela/Lara/ - (85, 86, 61, 66)	JF957790	**85**	**86**	**61**	**66**				4
Venezuela/Lara/ - (E, φ[3], β, 100)	JF957803	**E**	**φ**	**φ**	**φ**	**β**	**100**		6
Venezuela/Lara/ - (T, B[3])	JF957788	**T**	**B**	**B**	**B**				4
Venezuela/Lara/ - (T, M)	JF957800	**T**	**M**						2
Venezuela/Lara/ - (T, β[3], 116)	JF957789	**T**	**β**	**β**	**β**	**116**			5
Venezuela/Lara/ - (α, β, 114, 115, Γ)	JF957791	**α**	**β**	**114**	**115**	**Γ**			5
Venezuela/Merida/ - (13, 63[2], 27, 63[3],27)	JF957802	**13**	**63**	**63**	**27**	**63**	**63**	**63**	**27**	8
Venezuela/Merida/ - (T, B, 90, C, 91)	JF957804	**T**	**B**	**90**	**C**	**91**			5
Venezuela/Merida/ - (T, B, C, F, 89)	JF957814	**T**	**B**	**C**	**F**	**89**			5
Venezuela/Tachira/ - (37, 87, 92,15)	JF957794	**37**	**87**	**92**	**15**				4
Venezuela/Yaracuy/ - (132, 133, 134, 135[2])	JF957812	**132**	**133**	**134**	**135**	**135**			5
Venezuela/Yaracuy/ - (64, 65, D, E)	JF957811	**64**	**65**	**D**	**E**				4
Venezuela/Yaracuy/ - (93, 10, 94, 109, 110)	JF957799	**93**	**10**	**94**	**109**	**110**			5

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CHAPTER 4

Molecular and immunological characterization of three strains of *Anaplasma marginale* grown in cultured tick cells

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Ticks and Tick-borne Diseases (*in press*)

4. Molecular and immunological characterization of three strains of *Anaplasma marginale* grown in cultured tick cells

4.1. Abstract

Anaplasma marginale is an economically important tick-borne pathogen of cattle that causes bovine anaplasmosis. A wide range of geographic strains of *A. marginale* have been isolated from cattle, several of which have been characterized using genomics and proteomics. While many of these strains have been propagated in tick lines, comparative analyses after propagation in tick cells have not been reported. The overall purpose of this research therefore was to compare the degree of conservation of selected genes after propagation in tick cell culture among *A. marginale* strains from the U.S. (the Virginia strain) and Brazil (UFMG1 and UFMG2 strains). The genes studied herein included those which encode the proteins HSP70 and SODB involved in heat shock and stress responses, respectively, and two genes that encode major surface proteins MSP4 and MSP5. Strain identities were first confirmed by sequencing the tandem repeats of the *msp1a* gene which encodes for the adhesin, MSP1a. The results of these studies demonstrated that the genes encoding for both stress response and heat shock proteins were highly conserved among the three *A. marginale* strains. Antibodies specific for MSP4, MSP5, SODB and HSP70 proteins were used to further characterize the *A. marginale* strains, and they reacted with all of these strains propagated in tick cell culture, providing further evidence for antigenic conservation. Although antigenic differences were not found among the three *A. marginale* strains, multi-locus sequence analysis (MLSA) performed with nucleotide sequences of these genes demonstrated that the *A. marginale* Brazilian and U.S. strains fall in different clades. These results showed that phylogenetically distant strains of *A. marginale* are antigenically conserved, even after several *in vitro* passages, supporting the use of some of the above conserved proteins as candidates for universal vaccines.

Keywords: *Anaplasma marginale*, *hsp70*, IDE8 tick cell line, major surface proteins, *sodb*, stress response proteins

4.2. Introduction

Anaplasma marginale, a gram-negative bacterium in the genus *Anaplasma,* is an obligate intracellular pathogen that multiplies within a parasitophorous vacuole in the cytoplasm of both vertebrate and invertebrate hosts cells (Dumler et al., 2001). In ruminants, *A. marginale* develops within erythrocytes which, when taken up by ticks during feeding, first infect gut cells. After development in tick gut cells, other tissues become infected, including the salivary glands from where the rickettsia is transmitted to cattle during tick feeding (Ge et al., 1996; Kocan et al., 1992a; Kocan et al., 1992b). The genetic heterogeneity of *A. marginale* strains is diverse throughout the world (Cabezas-Cruz et al., 2013; de la Fuente et al., 2007; Mutshembele et al., 2014). Therefore, the molecular and immunological characterization of *A. marginale* strains is fundamental toward the discovery of conserved proteins that may be candidate antigens for development of effective vaccines for control of bovine anaplasmosis.

Six major surface proteins (MSPs) of *A. marginale* have been described and shown to be involved in host-pathogen interactions, including MSP1 a & b and MSPs 2-5 (as reviewed by Kocan et al., 2003), and were shown to be important for the infection, development and survival of the rickettsia in vertebrate and tick hosts, as well as for evading host immunological defences. MSP1a, MSP4, and MSP5 have been useful for molecular characterization of *A. marginale* strains (as reviewed by Aubry & Geale, 2011; Bowie et al., 2002). MSP1a is an adhesin for bovine erythrocytes and tick cells (Blouin et al., 2003; McGarey & Allred, 1994) and contains relevant immune epitopes (Allred et al., 1990; Garcia-Garcia et al., 2004). While MSP1a varies in molecular weight among geographic isolates because of varying number of tandem repeats located in the N- terminal region of the protein (Allred et al., 1990; Cabezas-Cruz et al., 2013; de la Fuente et al., 2001b), this MSP is strain specific and conserved throughout the rickettsia's developmental cycle in cattle and ticks (Bowie et al., 2002). In contrast, the *msp5* gene is highly conserved among all *Anaplasma* spp. and its presence provides pathogen identity as an *Anaplasma* but does not confirm the species. Although the function of MSP4 is unknown, previous analysis of *A. marginale* isolates demonstrated that sequence

variation of MSP4 was sufficient for strain identification and phylogeographic studies (de la Fuente et al., 2003b).

Less is known about the variability of other *A. marginale* proteins among strains. For example, the *hsp70* gene, identified in the genome of *A. marginale* (Brayton et al., 2005), encodes for a heat shock protein (HSP) involved in protein folding under physiological and stress conditions. Another protein, superoxide dismutase B (SODB), an enzyme which is implicated in reactive oxygen species detoxification, has been reported in Rickettsiales (Dunning Hotopp et al., 2006; Ohashi et al., 2002). Yet there is only one publication available which evaluated *sodb* in *A.marginale* Pernambuco-Zona da Mata isolate with different strains (Junior et al., 2010). To our knowledge no data are available for other *A. marginale* isolates or *in vitro* grown strains.

Knowledge of the genetic diversity and immunological characteristics of *A. marginale* strains is fundamental for epidemiological and virulence studies, and also for development of improved vaccines. Although many antigenic (Barbet et al., 1999; Oberle et al., 1988; Oliveira et al., 2003; Vidotto et al., 1994) and genetic (Cabezas-Cruz et al., 2013; de la Fuente et al., 2005a; de la Fuente et al., 2007; de la Fuente et al., 2002b) differences have been reported among *A. marginale* isolates from cattle, comparative analyses have not been done for geographical strains after propagation in tick cell lines.

IDE8 tick cell line derived from *Ixodes scapularis* cells provides the perfect system for *A. marginale* propagation (Munderloh et al., 1996). For a long time the lack of an *in vitro* culture system has been the major impediment to anaplasmosis research and infected cattle served as the only source of bacteria. In particular *in vitro* system provides controlled conditions for investigations, as using the same cell culture reduces variability that arises when each strain is propagated in a separate animal. Additionally, it enables quick and easy production of material for experimentation without the use of experimental animals.

Although more than 200 *A. marginale* strains are recognized worldwide (Cabezas-Cruz et al., 2013), less than ten have been reported to be propagated *in vitro*

(Bastos et al., 2010; Bastos et al., 2009; Blouin et al., 2000; Hammac et al., 2013; Kocan et al., 2004; Munderloh et al., 1996).

Herein, we report studies on MSP4, MSP5 and HSP70 and SODB proteins, in order to determine their conservation among U.S. and Brazilian strains of *A. marginale* propagated in tick cell culture. In addition, nucleotide sequences of *msp4*, *msp5,* and fragments of *msp1α*, *hsp70* and *sodb* genes were determined and studied by multi-locus sequence analysis (MLSA).

4.3. Materials and methods

4.3.1. *Anaplasma marginale* strains

The three strains of *A. marginale* for these studies were obtained from naturally infected cattle in the U.S. and Brazil and then used to infect the IDE8 tick cell line (**Table 4.1**). The isolation of the Brazilian *A. marginale* strains UFMG1 (Bastos et al., 2009) and UFMG2 (Bastos et al., 2010), and the U.S. Virginia strain (Munderloh et al., 1996) was done as described previously. The organisms were propagated in IDE8 tick cell cultures at 34°C in L-15B (Munderloh & Kurtti, 1989) medium supplemented with 5% heat-inactivated fetal calf serum (FCS), 10 % tryptose phosphate broth (TPB), 0.1 % bovine lipoprotein concentrate (MP Biomedicals; Irvine CA, USA), 0.1 % $NaHCO_3$ and 10 mM HEPES, and the pH was adjusted to 7.5. The three *A. marginale* strains were each propagated in IDE8 cells in 75 cm^2 plastic culture flasks.

4.3.2. Bacteria purification and protein extraction

When greater than 80 % of tick cells were infected, the bacteria were purified on a Percoll gradient as described previously (Lis et al., 2014), and all purification steps were carried out at 4 ºC. In order to remove the remaining Percoll, purified bacteria

Table 4.1. *A. marginale* strains used in the study.

Isolate	Origin	GeneBank accession numbers				
		msp1α	***msp4***	***msp5***	***hsp70***	***sodb***
UFMG1	Minas Gerais State, Brazil	EU676176	KM624516	KM624518	KM624513	KM587713
UFMG2	Minas Gerais State, Brazil	EU676175	KM624517	KM624519	KM624514	KM587714
Virginia	USDA, Beltsville, Virginia	AY010246	AY010254	KM624520	KM624515	KM587715

were resuspended in cold PBS and centrifuged at 20.000 x g for 30 min, at 4 ºC. The supernatant was removed and the pelleted organisms were washed two more times with cold PBS. Proteins from the purified bacteria and uninfected IDE8 cells were extracted with lysis buffer [150 mM sodium chloride, 1 % (v/v) Triton X-100, 50 mM Tris, pH 8.0, containing protease inhibitors (Complete, Mini, EDTA-free, Roche, Switzerland)] and sonicated (5 s/cycle, 5 cycles, 0 ºC). The lysate was centrifuged at 14,000 x g for 30 min at 4 ºC, after which the supernatant was collected and proteins were precipitated in ice-cold acetone (1:4) for 4 h at -20 °C. The samples were then centrifuged at 12.000 x g for 30 min at 4 ºC, and pellets were resuspended in solubilization buffer [1 % (w/v) SDS, 100 mM Tris-HCl, pH 9.5]. The protein concentration was determined using the BCA Protein Assay (Thermo Scientific, San Jose, CA, USA) using BSA as the standard.

4.1.1. Antibodies

The recombinant *A. phagocytophilum* NY18 proteins MSP4 (AFD54597), HSP70 and SOD were produced in *E. coli* BL21 cells (Champion pET101 Directional TOPO Expression kit, Carlsbad, CA, USA), induced with IPTG and purified using the Ni-NTA affinity column chromatography system (Qiagen Inc., Valencia, CA, USA) following manufacturer's recommendations. Purified proteins were used to immunize rabbits and IgGs from preimmune and immunized animals were purified (Montage Antibody Purification Kit and Spin Columns with PROSEP-A Media, Millipore, Billerica, MA, USA) and used for analysis (Ayllon et al., 2013). Mouse monoclonal anti-*A. phagocytophilum* MSP5 antibody was obtain from VMRD, Inc. (Pullman, WA, USA),

while the secondary antibodies, goat anti-mouse and goat anti-rabbit conjugated with HRP, were obtained from Sigma (Madrid, Spain).

4.1.2. Western blot analysis

Ten micrograms of proteins extracted from purified bacteria were separated on mini-polyacrylamide gels and then transferred to a nitrocellulose membrane in MiniTrans blots (Bio-Rad, Hercules, CA, USA). The membranes were then washed with TBST [0.1 % Tween 20 in TBS: 0.5 M NaCl, 0.02 M Tris pH 7.5] and blocked overnight at 4°C with 5 % skimmed milk. The membranes were then incubated for 2 h at 37°C with the primary antibodies diluted 1:200, after which they were washed five times with TBST and then incubated for 1 h at room temperature with the secondary HRP-conjugated antibodies diluted 1:10.000 in TBST. The membranes were washed again and the color was developed using TMB Stabilized Substrate for HRP (Promega, Madison, WI, USA).

4.1.3. DNA isolation and amplification

The primers used for amplification of the *msp1α*, *msp4*, *msp5* genes were described previously and are listed in the **Table 4.2**. The Primer Blast program (ncbi.nlm.nih.gov/) was used to design primer sets for fragments of the *hsp70* and *sodb* genes. Sequence information of the north eastern Brazil strain of *A. marginale* (GenBank accession number, GU991630; (Junior et al., 2010) was used to design *sodb* primers and *A. phagocytophilum* sequence data (GenBank accession number, AF029321) were used for selecting the *hsp70* gene primers.

For each PCR reaction an amplification mix contained 5–10 ng of purified genomic DNA as a template, 1 µl of each primer (final concentration of 10 pmoles) and 25 µl of PCR Master Mix (Promega, Madison, USA). The volume was made up to 50 µl with nuclease-free water. The amplification of the *sodb* and *hsp70* genes was done as follows: 2 min at 94 °C followed by 35 cycles of 30 sec at 94 °C, 30 sec at 58 °C, and 1 min at 72 °C with a final extension step of 5 min. Amplification of the *msp1α, msp4*

and *msp5* genes was done as described by Lew et al. (2002), de la Fuente et al. (2003), and Singh et al. (2012), respectively. All PCR reactions were done using tAB GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA). The PCR products were stained with 6X DNA Loading Dye (Fermentas, Erlangen, Germany) and visualized in 1.5 % agarose mini-gels.

Table 4. 2. Primers used in the study for the amplification of *A. marginale* genomic DNA.

Target gene	Upstream/downstream primer sequences (5′- 3′)		References
msp1α	F	TGTGCTTATGGCAGACATTTCC	(Lew et al., 2002)
	R1	TCACGGTCAAAACCTTTGCTTACC	
	R2	AAACCTTGTAGCCCCAACTTATCC	
msp4	F	GGAGCTCCTATGAATTACAGAGAATTGTTTAC	(de la Fuente et al., 2003)
	R	CCGGATCCTTAGCTGAACAGGAATCTTGC	
msp5	F	GCATAGCCTCCGCGTCTTTC	(Singh et al., 2012)
	R	TCCTCGCCTTGGCCCTCAGA	
sodb	F	CGTCACTATAACGGGCACCA	This study
	R	CGCGGTTTCAAGCCTCTGTA	
hsp70	F	GGTTTACCAGGGTGAGCGAA	This study
	R	GACGGCCCCCTCTAAAAACA	

4.1.1. Sequencing of PCR products

The amplified fragments of *msp1α, msp4*, *msp5*, *hsp70* and *sodb* genes from the three *A. marginale* strains were purified using MiniElute PCR Purification kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions and sequenced. Consensus sequences were obtained for all PCR products by comparing sense and antisense strands using ClustalW algorithm.

4.1.2. Phylogenetic analysis

Multi-locus sequence analysis (MLSA) was done using the five loci, *hsp70*, *sodb*, *msp1α*, *msp4* and *msp5* for each of the three strains. The nucleotide sequences of *hsp70*, *sodb*, *msp1α*, *msp4* and *msp5* were also obtained from four *A. marginale* strains for which the genomes have been sequenced and are available in Genbank: *A. marginale* str. St. Maries (genome accession number: NC_004842.2), *A. marginale*

str. Florida (genome accession number: NC_012026.1), *A. marginale* str. Gypsy Plains (genome accession number: NC_022784.1) and *A. marginale* str. Dawn (genome accession number: NC_022760.1). The nucleotide sequences were concatenated and aligned using MAFFT (v7), configured for the highest accuracy (Katoh & Standley, 2013). After alignment, regions with gaps were removed manually and 2360 gap-free sites were used to reconstruct phylogenetic trees using maximum likelihood (ML), neighbor joining (NJ) and bayesian inference (MB) methods as implemented in PhyML (v3.0 aLRT) (Anisimova & Gascuel, 2006; Guindon & Gascuel, 2003), PHYLIP (v3.66) (Felsenstein, 1989) and MrBayes (v3.1.2) (Huelsenbeck & Ronquist, 2001), respectively. The reliability for the internal branches of ML was assessed using the bootstrapping method (1000 bootstrap replicates) and the approximate likelihood ratio test (aLRT – SH-Like) (Anisimova & Gascuel, 2006). Reliability for the NJ tree was assessed using bootstrapping method (1000 bootstrap replicates). 10,000 generations of Markov Chain Monte Carlo (MCMC) chains were run for MrBayes.

The phylogenetic analysis of the *msp4* gene was performed as described above using the maximum likelihood method. The *msp4* sequences of *A. marginale* isolates from Brazil and USA are as follow (GenBank accession numbers in brackets): Brazil (AY714546), Brazil12 (AY283197), Brazil10 (AY283196), Brazil9 (AY283195), Brazil8 (AY283194), Brazil7 (AY283193), Brazil6 (AY283192), Brazil5 (AY283191), Brazil4 (AY283190), Brazil3 (AY283189), Brazil (AF428082) and the U.S. Virginia (AY010254), Pawhuska (AY127078), NewCastle (AY127077), Forida (AAC36877), Stillwater (AY127076), Okmulgee (AY127074), OklahomaCity (AY127073), Stigler (AY127072), Cushing2 (AY127071), Cushing1 (AY127070), Glencoe3 (AY127069), Missouri (AY127066), Oregon (AY127065), Okeechobee (AY010253), St.Maries (AY010249), California (AY010248), Mississippi (AY010251), Idaho (AY010250), Oklahoma (AY010252), USA1 (AF428088), USA (AF428081) were used for comparisons and creation of a phylogenetic tree.

4.1. Results and Discussion

4.1.1. Propagation of *A. marginale* strains in tick cell culture

A. marginale multiplies exclusively within membrane-bound vacuoles in host cells, erythrocytes in the vertebrate host and various cells in tick cells. While *A. marginale* infections in erythrocytes have been maintained in whole erythrocyte culture for short periods, a continuous system has not been reported (Blouin et al., 2002). Tick cell lines derived originally from embryos of *Ixodes scapularis* (IDE8, ISE6) cell lines supported continuous propagation of *A. marginale* (Munderloh et al., 1994). Propagation of *A. marginale* in cultured tick cells provided the opportunity for comparative analysis and this *in vitro* system reduces variability that may result among indivdual cattle. In addition, large quantities of bacteria can be produced in a short time. The three *A. marginale* strains, the Virginia from the U.S and two Brazilian UFMG1 and UFMG2 strains, were each grown in IDE8 cell line, and the organisms purified from these infected cultures were used for these studies.

4.1.2. Molecular and immunological characterization of HSP70 protein from the *A. marginale* strains.

The main objective of this study was the molecular and immunological characterization of major surface and stress response proteins of three geographical *A. marginale* strains grown in the IDE8 tick cell line. Heat shock proteins, also called chaperons, participate in the cellular response to heat stress, and comprise five major and broadly conserved families: HSP100s, HSP90s, HSP70s, HSP60s, and small heat shock proteins (sHSPs) (as reviewed in Richter et al., 2010). Many of these HSPs were identified in *A. marginale* after whole genome analysis (Brayton et al., 2005), but thus far have not been used for characterization of geographic strains. In this research we focused on the *hsp70* gene because it has been demonstrated to be one of the most highly conserved among other HSPs (Richter et al., 2010). Under stress conditions

HSP70 prevents the aggregation of unfolded proteins, while under normal physiological conditions it is primarily involved in the *de novo* folding of proteins (as reviewed in Mayer & Bukau, 2005).

Table 4.3. Percentage of identity between the *hsp70* gene fragment of *A. marginale* strains grown *in vitro* compared to other members of Anaplasmataceae family.

Organism (strain)	GeneBank	% Identity
Anaplasma marginale Florida	CP001079	99
Anaplasma marginale St. Maries	CP000030	99
Anaplasma centrale Israel	CP001759	85
Anaplasma phagocytophilum JM	CP006617	75
Ehrlichia chaffeensis Arkansas	CP000236	74
Ehrlichia ruminantium Welgevonden	CR925678	73
Ehrlichia canis Jake	CP000107	72
Neorickettsia sennetsu	CP000237	69
Neorickettsia risticii Illinois	CP001431	67

The predicted *A. marginale* HSP70 protein consists of 645 amino acids (aa) (Brayton et al., 2005). Using primers designed for this study, a single band resulted for all three strains after PCR. (**Figure 4.1.A**). The amplified gene fragment had a 100 % identity with Florida and St. Maries isolates, and 95 % identity with *A. centrale* (**Table 4.3**). Moreover, they had a high percentage of identity when aligned with other organisms classified in the family Anaplasmataceae, including *A. phagocytophilum* 75 %*; Ehrlichia* spp., 73 %; and *Neorickettsia* spp., 68 % (**Table 4.3**). This *hsp70* gene fragment encoded for 200 aa of the C-terminus of the protein. The putative protein sequences of UFMG2 and Virginia strains were identical with the Florida and St. Maries isolates, while one substitution was found at the position 572 (arginine for lysine) in UFMG1. In Western Blot analysis the polyclonal anti-*Anaplasma* HSP70 antibody reacted with a single 70 kDa band of all three *A. marginale* strains (**Figure 4.2.A**). This band was also observed in uninfected IDE8 cells that served as controls (data not shown) and confirms previous reports of HSP70 being one of the most highly conserved

chaperones. The prokaryotic homolog shares an approximate 60 % sequence identity with eukaryotic HSP70 protein (as reviewed in Richter et al., 2010).

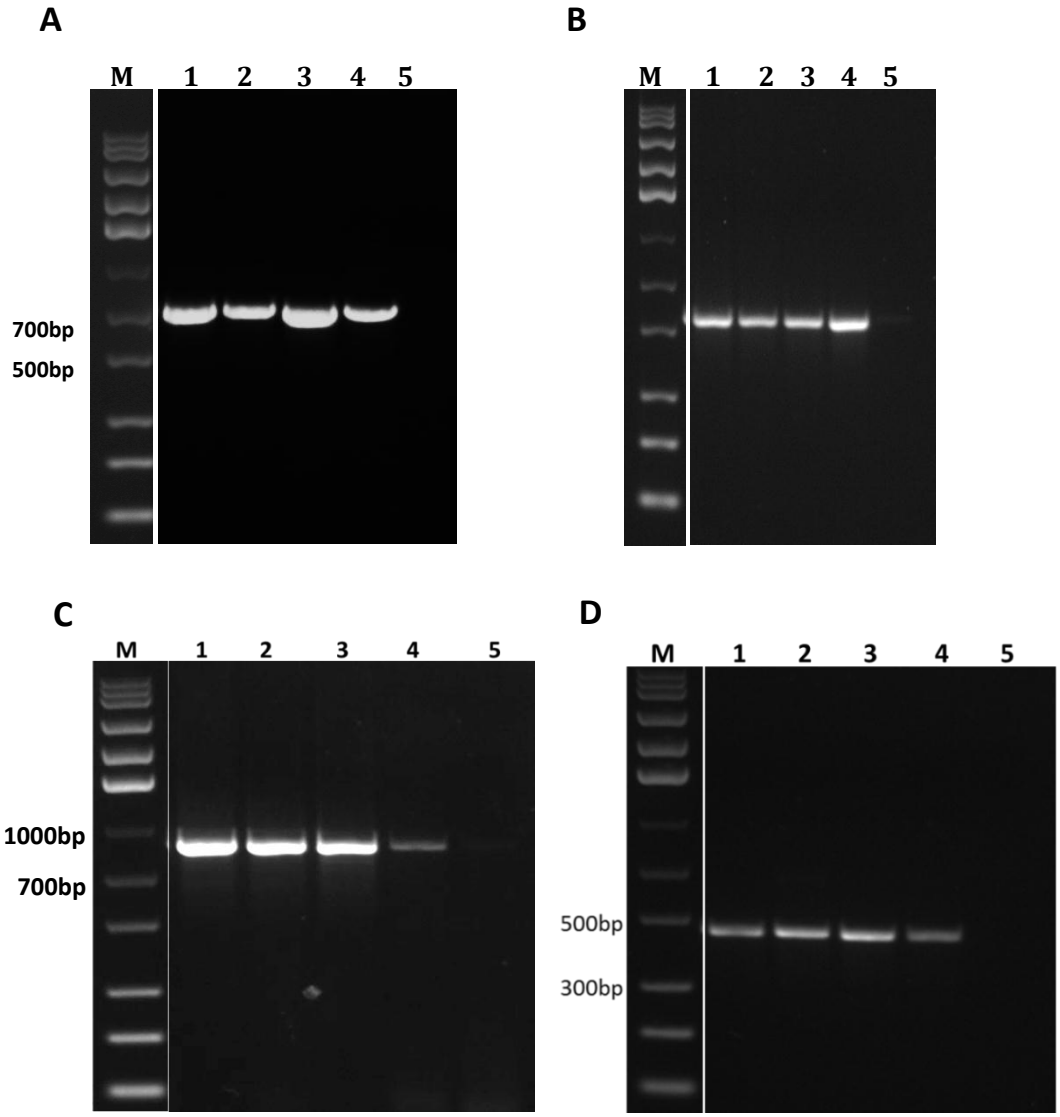

Figure 4.1. *A. marginale* PCR products separated in 1,5% TBE-agarose. A: *hsp70* gene; B: *sodb* gene; C: *msp4* gene; D: *msp5* gene. Lanes: M – DNA ladder, 1. UFMG1, 2. UFMG2, 3.Virginia, 4. positive control, 5. negative control.

4.1.1. Molecular characterization of *sodb* gene and putative proteins from different isolates of *A. marginale*

After pathogen infection, professional phagocytes produce reactive oxygen species (ROS), which are known to damage nucleic acids, proteins and lipids (Morel et al.,

1991). Superoxide dismutases (SODs) are stress response proteins implicated in ROS detoxification, which catalyse dismutation of superoxide radicals to hydrogen peroxide and oxygen (Morel et al., 1991). Three types of SODs have been reported in bacteria (SODA, SODB and SODC), classified according to their corresponding metal co-factor (Grace, 1990), yet only SODB has been reported in Rickettsiales so far (Dunning Hotopp et al., 2006; Junior et al., 2010; Ohashi et al., 2002). In our study, primers were designed using the sequence of the NE-Brazil strain of *A. marginale* (GenBank accession number: GU991630) as a template, and used to amplify the *sodb* gene fragment from the three strains. A single band of ~ 520 bp (**Figure 4.1.B**) resulted and sequencing revealed that the 483 bp *sodb* gene fragment was identical in the three strains. The predicted putative 160 aa protein fragment was identical when compared to NE-Brazil strain (GenBank accession number: D6Q019) and Florida strain (GenBank accession number: B9KIY9), while one substitution was observed when compared to the St. Maries strain (GenBank accession number: Q5PAD7). Compared with *A. centrale* (GenBank accession number: D1AUA7) demonstrated only 87 % identity in the amino acid sequences (data not shown). These results are consistent with those obtained previously by Junior et al., (2010), in which a high level of *sodb* gene sequence identity was found among different strains of *A. marginale*.

The *sodb* gene in *Anaplasma spp.* probably encodes FeSOD protein of ~ 21 kDa (Carlyon et al., 2004; Ohashi et al., 2002). In Western Blot analysis the polyclonal antibody reacted with a single band around ~ 50 kDa in all strains (**Figure 4.2.D**). This is not uncommon as in many prokaryotic organisms SODs are present as dimers (Brydges & Carruthers, 2003; Cooper et al., 1995; Hassett et al., 1993; Lynch & Kuramitsu, 2000). The fact that all strains reacted with the same antibody suggests that they share common epitopes.

Many of *A. marginale* proteins undergo antigenic variation what impede they use as a vaccine subunits. *Sodb* gene is highly conserved among strains, therefore it should be considered as a component of future vaccines as conserved proteins provide cross-protection against heterologous strains. In Anaplasmataceae SODB is co-transcribed with components of the type IV secretion system (Ohashi et al., 2002), therefore it is believed not only to play a role in defence against peroxide stress, but to participate in pathogenesis.

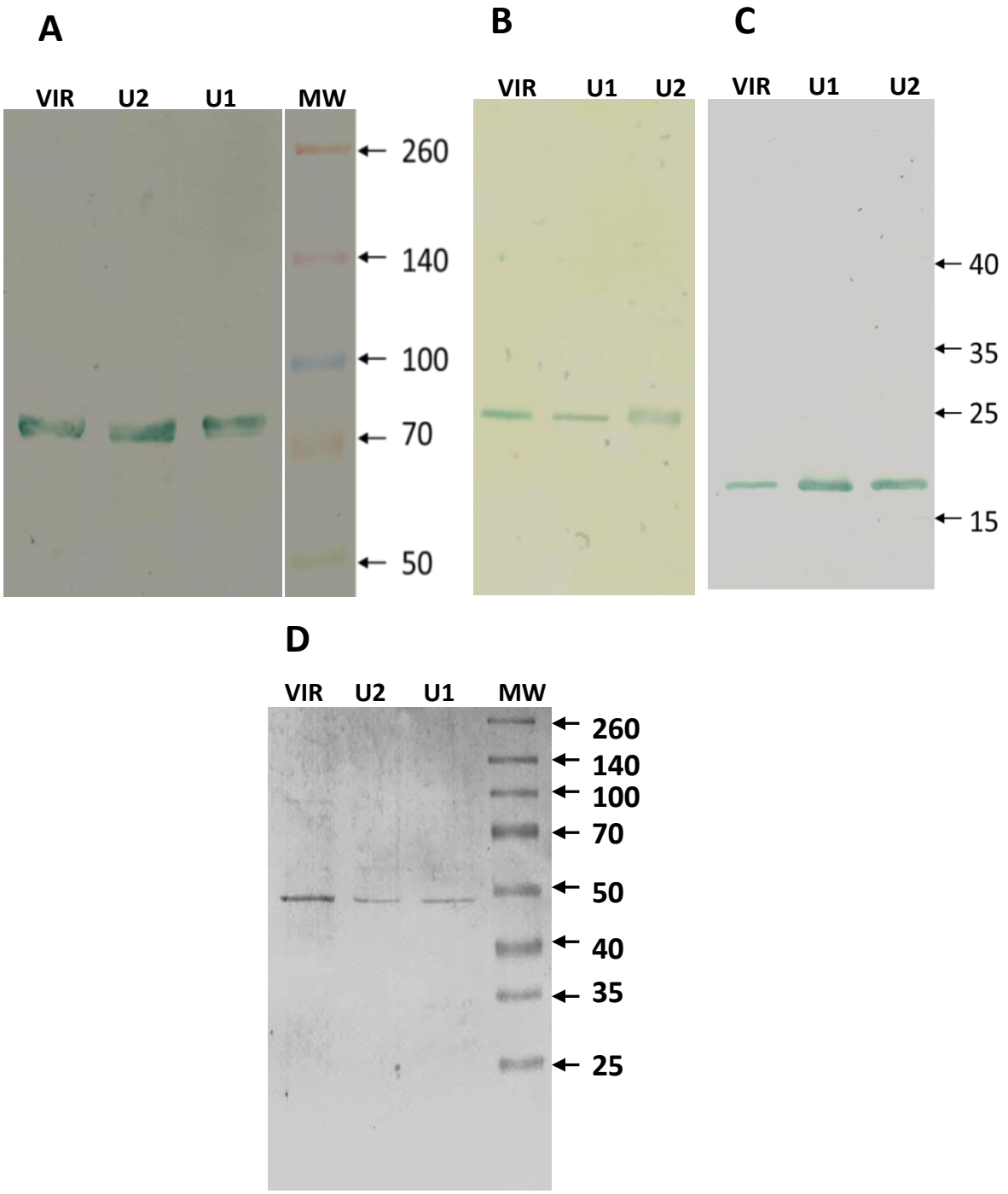

Figure 4.2. Conservation of epitopes between geographical *A. marginale* strains grown *in vitro*. Proteins obtained from purified initial bodies were separated on 7 % gel (A) and 12 % gels (B, C, D) and probed with A: anti-HSP70 polyclonal antibody; B: with anti-*A. marginale* MSP4; C: with anti- *A. phagocytophilum* MSP5, D: with anti-SODB. Lanes: VIR- Virginia, U2 – UFMG2, U1 – UFMG. Molecular size markers (MW) are in the right margin.

4.1.1. MSP1a genotype and tick cells infection phenotype

More than 230 different antigenic types based on the MSP1a protein exist (as rewieved in Cabezas-Cruz et al., 2013; de la Fuente et al., 2001b). While the *msp1*α gene has a conserved portion among *A. marginale* strains, the 5' end region, which encodes the tandem 23-31 amino acid repeats (TR) is variable (as reviewed in Cabezas-Cruz et al., 2013). In this study, strain identity of UFMG1, UFMG2 and Virginia strains was confirmed using the portion of the *msp1α* gene sequences encoding for tandem repeats that were obtained from GeneBank (**Table 4.1**). The number and sequence of tandem repeats of the *msp1α* gene have remained the same after many years of cultivation in tick cell cultures and were identical to the sequences that had been deposited previously in GeneBank. The MSP1a had previously been shown to be conserved over successive passages in IDE8 cell culture (Barbet et al., 1999; Bastos et al., 2009; Blouin et al., 2000), and was also conserved during propagation in the RF/6A endothelial cell line (unpublished data). MSP1a was shown to be an adhesin for cultured and native tick cells (de la Fuente et al., 2001a; McGarey & Allred, 1994), and amino acids found at position 20 within tandem repeats were found to play an essential role in this protein's adhesion properties (de la Fuente et al., 2003a). Furthermore, the negatively charged amino acids, aspartic (D) and glutamic acid (E), were shown to be essential for protein binding to tick cell extract (TCE), while adhesion was not observed when glycine (G) was found at this position (de la Fuente et al., 2003a). The presence of TCE binding aa was shown to correlate with transmissibility by *Dermacentor* sp. (Cabezas-Cruz et al., 2013; de la Fuente et al., 2003a). *A. marginale* isolates that were not infective for ticks also were not infective for IDE8 cells (Bell-Sakyi et al., 2007; Blouin et al., 2002; Kocan et al., 2004). Interestingly, the Brazilian UFMG1 strain, which has D at position 20 in two out of four tandem repeats was also shown to be refractory to infection and transmission by *Rhipicephalus microplus* (Ruiz et al., 2005), even though this strain can be propagated in the cell line derived from this same tick species (Esteves et al., 2009). While tick transmission data are unavailable for the UFMG2 isolate, its propagation in IDE8 tick cell culture, as well as the presence of four tandem repeats with D present at position 20, suggests that it is likely to be tick transmissible. The Virginia isolate, which is tick transmissible (Kocan et al., 1992b), meets all of the requirements by having TCE-binding amino acids

in the tandem repeats and is infective for tick cell lines derived from multiple tick species, including *I. scapularis, R. microplus* and *D. andersoni* (Oliva Chavez et al., 2012).

4.1.2. Molecular and immunological characterization of MSP4 and MSP5 proteins from the three *A. marginale* strains

MSP4 and MSP5, immunodominant proteins encoded by single genes (Oberle et al., 1993; Visser et al., 1992), are conserved on *A. marginale* during the acute and chronic phases of the infection in cattle, as well as during propagation in tick cell culture (Barbet et al., 1999; Knowles et al., 1996; Visser et al., 1992).

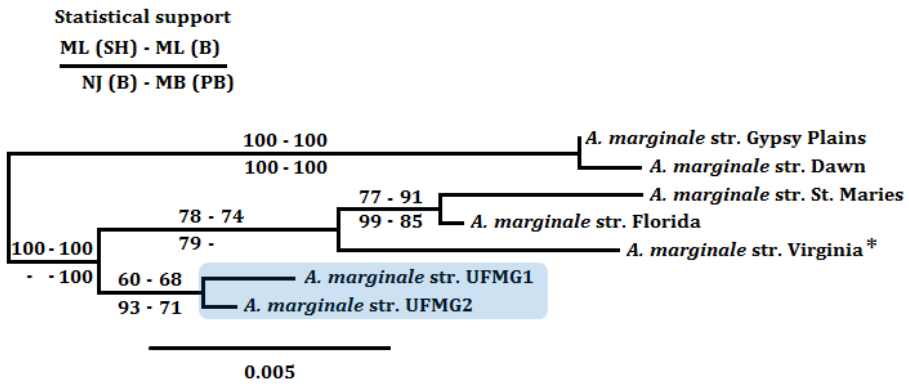

Figure 4.3. Multilocus sequence analysis of *A. marginale* strains. In this study the

The multilocus sequence analysis (MLSA) was performed using the nucleotide sequences of the selected genes (*hsp70*, *sodb, msp1a*, *msp4* and *msp5*). The sequences were concatenated, aligned, and phylogenetic trees constructed using ML, NJ and MB. The two strains from Brazil (box) form a clade separated from the strains isolated in USA. The strain Virginia is also shown (asterisk). Numbers on internal branches are the values of the different statistic tests as shown in the figure (above left) (see Materials and Methods). Only values higher than 50 are presented.

msp4 gene was amplified and expressed in *E. coli* using the primers used by de la Fuente et al. (2003b) (**Table 4.2**). The 847 bp consensus sequences were obtained for the three strains (**Figure 4.1.C**). In both Brazilian strains six variable nucleotides resulted in two substitutions at the protein level, glycine for aspartic acid at position 79 and threonine for asparagine at position 99, when compared with the Florida isolate.

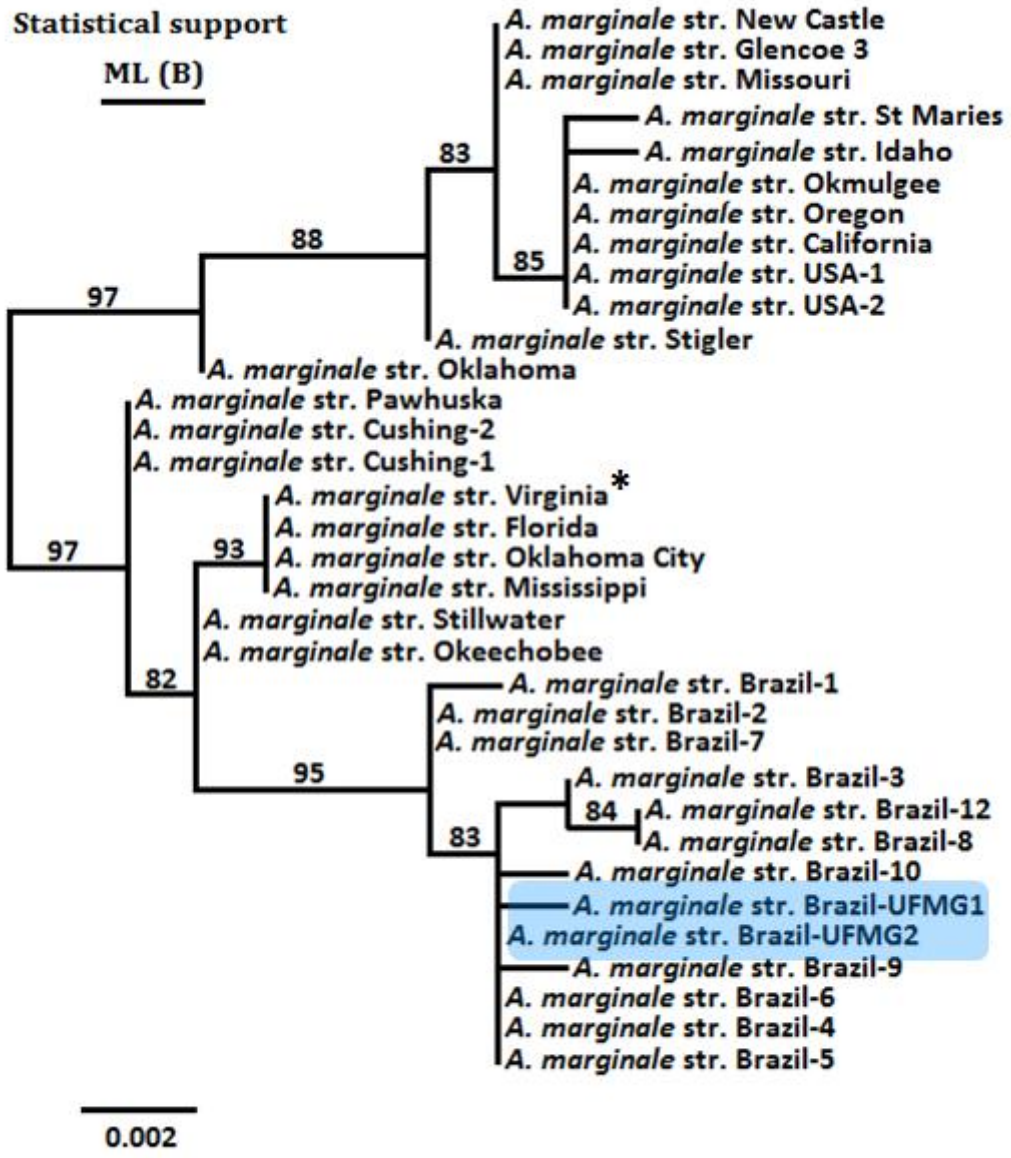

Figure 4.4. Phylogenetic unrooted tree based on the *A. marginale msp4* gene sequences from the U.S and Brazil.
The tree shows relationship of *A. marginale* strains propagated *in vitro* with geographical isolates from the same countries. Bootstrap values are shown as % in the internal branch. Only bootstrap values equal or higher than 50 % are shown. The GenBank accession numbers of *A. marginale* geographical isolates used for comparison are displayed.

The *msp4* gene sequence of the Virginia strain, which has been passaged for many years in the IDE8 tick cell culture, was identical to the one deposited previously in the GeneBank AY010254 (de la Fuente et al., 2001c), as well as the reference Florida sequence. While MSP4 is highly conserved among other *Anaplasma* spp., sufficient

sequence variation was found to support its use in phylogeographic studies. A phylogenetic tree was built using the *msp4* sequences of the Brazilian and U.S isolates available in GenBank (**Figure 4.4**). The UFMG1 and UFMG2 strains of *A. marginale* grown in IDE8 tick cell culture were found to be in the same clade as other Brazilian isolates, while the Virginia strain was in clade that contained the U.S isolates. These results were also confirmed by MLSA (**Figure 4.3**).

The *msp5* gene fragment was amplified using a set of external primers as described by Singh et al. (2012) and sequenced directly (**Figure 4.1.D**). *msp5* gene fragments differed in a few base change, but shared 98 % identity when compared to Florida isolate. Similar results were obtained by Torioni de Echaide et al. (1998), in which a 95 % identity in the 30 analyzed sequences when compared with the Florida isolate. Western blot analysis of *A. marginale* purified proteins using monoclonal antibody against the *A. phagocytophilum* MSP5 protein produced a single band ~ 19 kDa in all strains (Fig. 2 C). Similarly, a single band of 30 kDa was observed when reacted with polyclonal anti-MSP4 antibody (**Figure 4.2.B**). These results confirmed the conservation of MSP4 and MSP5 among the *Anaplasma*, including *A. marginale*, *A. centrale*, *A. ovis* and *A. phagocytophilum* (de la Fuente et al., 2002a; de la Fuente et al., 2005b; Molad et al., 2004; Visser et al., 1992). Although the role of both MSP5 and MSP4 have not been determined, the fact that these proteins are highly conserved suggests that they play a fundamental role in the biological cycle of *Anaplasma* sp.

4.1. Conclusions

Development of the tick cell culture system has contributed to *A. marginale* research by providing a venue for *in vitro* studies from which consistent samples can be produced for comparative analyses, thus eliminating the variation that may result in cattle. Using tick cell culture derived organisms, we demonstrated that the stress response proteins, HSP70 and SODB, as well as MSP4 and MSP5 proteins, are highly conserved among distantly related *A. marginale* strains. This SODB may likely be a candidate antigen for development of a vaccine for control of bovine anaplasmosis because this protein is highly conserved, co-transcribed with components of the type IV secretion system, and may be implicated in the pathogenesis of *A. marginale* infection (Ohashi et al., 2002).

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CHAPTER 5

5. Identification of differentially expressed proteins among geographical *A. marginale* strains grown *in vitro* by 2D- DIGE

5.1. Introduction

Current control measures for anaplasmosis vary with geographic locations and include combinations of immunization, chemoprophylaxis and vector control (Kocan et al., 2000). The use of the most efficient and economical vaccine, consisting of live *A. centrale* organisms, is forbidden in many countries, as it increases the risk of contamination with other "silent" blood-borne pathogens (Kocan et al., 2000; Rogers et al., 1988). A further concern is that despite immunization, a few anaplasmosis outbreaks have been reported in vaccinated populations (Bock and de Vos, 2001; Brizuela et al., 1998). Nevertheless, due to the increasing resistance of pathogens and ticks against antibiotics, the acaricides control strategies should also include some form of vaccination (George et al., 2004; Rosario-Cruz et al., 2009).

Many investigations have focused on abundant, highly immunodominant, outer membrane proteins which could be used in novel vaccines (Dark et al., 2011; Dark et al., 2012; Palmer et al., 2012). Although native or recombinant outer membrane proteins are immunogenic, they provided complete to weak protection among animals (Noh et al., 2008; Tebele et al., 1991). *A. marginale* strains differ in their membrane surface proteins (Brayton et al., 2003; Cabezas-Cruz et al., 2013; Palmer et al., 1994). Since, genes are regulated in response to environmental factors, it cannot be predicted if or how a particular protein will be expressed under natural conditions (Berghoff et al., 2013). We have a broad understanding of the antigenicity of *Anaplasma*-proteins, but we need to gain knowledge about the variability of protein expression, as some proteins may not be expressed at a sufficiently high level to elicit a protective immune response. Yet, the data concerning the variability of protein expression among *A. marginale* strains from geographically diverse regions was

missing.

Here, two dimensional Difference Gel Electrophoresis (2D-DIGE) followed by RP-LC-MS/MS analysis were performed in order to elucidate the differences in protein expression profiles of *A. marginale* propagated in IDE8 tick cell cultures. Strains from Brazil and USA, that differ in virulence, morphology and transmissibility (Bastos et al., 2010; Ribeiro et al., 1997; Ruiz et al., 2005; Smith et al., 1986) were used for the experiments. It was demonstrated that many of the immunogenic outer membrane proteins, as well as proteins involved in stress defense, are differentially regulated among the strains.

This is the first study reporting an insight into differentially expressed proteins of *A. marginale* strains from geographically diverse regions.

5.2. Materials and methods

5.2.1. Anaplasma marginale strains

Three geographical *A. marginale* strains UFMG1, UFMG2 and Virginia were used for experiments. Brazilian strains differ in pathogenicity UFMG2 is highly pathogenic (Bastos et al., 2010) while UFMG1 is characterized by low pathogenicity and the presence of an inclusion appendage (Ribeiro et al., 1997). The Virginia isolate also has an inclusion appendage (Smith et al., 1986).

5.2.2. Bacteria purification and protein extraction

Bacteria were propagated and purified as described previously (Lis et al., 2014). Purified bacteria were resuspended in ice-cold lysis buffer [40 mM tris, 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 60 mM DTT, the protease inhibitor cocktail (Complete, Mini, EDTA-free, Roche, Germany)] and sonicated in a cooled ultrasonic bath until the lysate was clear. The lysates were centrifuged at 13 000 x g for 20 minutes at 4 °C to pellet insoluble material and supernatant was collected as the protein sample. Before subsequent analysis, samples were concentrated and

desalted using the 3 kDa Ultrafree-0.5 Centrifugal Filter Device (Merck, Millipore) according to manufacturer's protocol and protein concentration was determined by using the BCA Protein Assay (Thermo Scientific, San Jose, CA, USA). Samples were stored at -80 °C until use for labeling.

5.2.3. 2D-DIGE

The 13-cm immobilized pH gradient (IPG) strips with a pH range 3-11 NL were rehydrated overnight in DeStreak Rehydration Solution (GE Healthcare) containing 2 % (v/v) IPG buffer (GE Healthcare). 50 µg of protein from each bacterial sample was labeled with 400 pmol of Cy3 or Cy5 fluorochromes dissolved in anhydrous DMF (Sigma, St. Louis, MO, USA). The labeling was performed for 30 min on ice, following the random labeling scheme. Reactions were quenched by adding 1 µL of 10 mM lysine followed by incubation for 10 min on ice, in the dark. An internal standard was prepared by mixing equal aliquots of all samples and the resulting mixture was labeled with Cy2 dye (Figure 1). The six individual labeled samples corresponding to two biological replicates from each *A. marginale* strain were distributed randomly across DIGE gels together with the internal standard in each separation.

Cy3	Cy5	Cy2
Virginia	UFMG1	IS
UFMG1	UFMG2	IS
UFMG2	Virginia	IS

Table 5.1. Randomization scheme of samples' labeling. Cy2, Cy3, Cy5 – cyanine fluorochromes used.

An equal volume of 2 x sample buffer [7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 2 % (w/v) DTT and 2 % (v/v) IPG buffer, pH 3-11] was added to combined samples and the resulting mixture was applied on rehydrated IPG strips via anodic cup loading. IEF was performed according to Gorg et al. (2000) on Ettan IPGphor 3 (GE Healthcare) at 20 °C under following conditions: 300 V for 3 h, 300-1000 V for 6 h,

1000-10000 V for 3 h, 10000 V for 3 h and 500 V for 4 h. Second dimension SDS-PAGE was performed on homogeneous 12 % gels casted in low fluorescence glass plates. Electrophoresis was carried out at 20 °C and 0.5 W/gel for 30 min followed by a second step at 15 W/gel for 4 h.

5.2.4. Image acquisition and data analysis

The fluorescence signals of the Cy-labeled protein samples were imaged using an Ettan DIGE Imager (GE Healthcare) and image analysis was performed with DeCyder 2 D Software, version 7.0 (GE Healthcare). In total 9 gel images were considered for the analysis, 6 corresponded to the different samples labeled with Cy3 and Cy5 and 3 corresponded to sample pool labeled with Cy2. Spots detection, normalization and volume ratio calculations were carried out using Differential In-Gel Analysis (DIA) module. For Biological Variation Analysis (BVA), all gel images were distributed in 4 groups; 3 groups of different *A. marginale* strains and an internal standard group. The most representative standard image with average quality was assigned as the master image. Paired comparisons were carried out between different *A. marginale* strains and protein spots with ≥ 2.5-fold change and $p<0.05$ by Student's t-test, were considered as differentially expressed between the samples. Spots of interest were excised manually from preparative gels made as described above, but using 150 µg of unlabeled sample for each strain and stained with Sypro Ruby (Molecular Probes, Invitrogen, Eugene, OR, USA) and subjected to mass spectrometric protein identification.

5.2.5. In-gel digestion and mass spectrometry

Manually excised gel plugs were subjected to in-gel trypsin digestion according to Shevchenko et al. (1996). The extracted peptides were desalted on OMIX Pipette tips C18 (Agilent Technologies, Santa Clara, CA, USA), dried-down and stored at −20 °C until mass spectrometry analysis. The protein digest was resuspended in 0.1 % formic acid and analyzed by RP-LC–MS/MS using an Agilent 1100 LC system (Agilent Technologies) coupled to a linear ion trap LTQ-Velos mass spectrometer (Thermo

Scientific). The peptides were concentrated (on-line) by reverse phase chromatography using a 0.1 mm × 20 mm C18 RP precolumn (Thermo Scientific), and then separated using a 0.075 mm × 100 mm C18 RP column (Thermo Scientific) operating at 0.3 µl/min. Peptides were eluted using a 40-min gradient from 5 to 35 % solvent B (Solvent A: 0.1 % formic acid in water, solvent B: 0.1 % formic acid in acetonitrile). ESI ionization was done using a Fused-silica PicoTip Emitter ID 10 µm (New Objective, Woburn, MA, USA) interface. Peptides were detected in survey scans from 400 to 1600 amu (1 µscan), followed by three data dependent MS/MS scans (Top 3), using an isolation width of 2 mass-to-charge ratio units, normalized collision energy of 35 % and dynamic exclusion applied during 30 s periods.

5.2.6. Protein analysis

Protein identification was carried out using the SEQUEST algorithm (Proteome Discoverer 1.3, Thermo Scientific). The obtained MS/MS raw files were searched against the Anaplasmataceae databases with the following constraints: tryptic cleavage after arginine and lysine, up to two missed cleavage sites, tolerances of 1 Da for precursor ions and 0.8 Da for MS/MS fragment ions and methionine oxidation and cysteine carbamidomethylation was allowed. A false discovery rate (FDR) < 0.01 was considered as a condition for successful peptide assignments during subsequent protein identification. The theoretical pI and the MW of the proteins were calculated using Protein Identification and Analysis Tools on the ExPASy Server (expasy.org/compute_pi).

5.2.7. In silico analysis of hypothetical proteins

To predict the subcellular localization, presence of signal sequences and transmembrane helices, the following algorithms were used: CELLO (cello.life.nctu.edu.tw) (Yu et al., 2006), PSORTb (psort.org/psortb) (Yu et al., 2010), SignalP 4.0 (cbs.dtu.dk/services/SignalP) (Petersen et al., 2011), the transmembrane hidden Markov model (TMHMM) (cbs.dtu.dk/services/TMHMM) (Krogh et al., 2001) and Phobius (phobius.sbc.su.se) (Kall et al., 2007). Furthermore, protein similarity

searches were performed for protein products using BLAST program (ncbi.nlm.nih.gov). The protein-protein interaction networks were predicted by STRING v9.1 (string-db.org) (Franceschini et al., 2013).

5.3. Results and Discussion

In the present study, the 2D-DIGE technique coupled with RP-LC–MS/MS was used to identify differences in protein regulation among *A. marginale* strains from Brazil and the U.S. Up to now the comparative protein expression profiles of *in vitro* grown bacteria from different geographical strains was not available. For our analysis, we took advantage of access to *A. marginale* strains grown in IDE8 tick cell culture, derived from different continents, varying in virulence and morphology.

5.3.1. Proteome analysis

The whole proteome of four different *A. marginale* strains has been resolved, using a non-linear pH gradient of 3–11 (**Figures 5.1** and **5.2**). 24 protein spots with significant higher spot volume (at least 2.5 times different) and a *p-value* below 0.05 were found and subsequently analyzed by RP-LC-MS/MS (**Table 5.2**). Nineteen spots were previously annotated, yielding a total of 17 different proteins. Based on the information available for *A. marginale* and other members of Anaplasmataceae family in the UniProt database, the identified proteins were grouped according to their predicted function.

The highest number of differentially expressed protein spots was identified in *A. marginale* UFMG2 strain (12), most of them were putative proteins (**Table 5.2**). The least number of differentially regulated proteins (5) was found in the UFMG1 strain.

5.3.2. Outer membrane proteins

Nine differentially regulated protein spots encoded for outer membrane proteins: Major Surface Protein 2 (MSP2), Major Surface Protein 4 (MSP4), Outer Membrane Protein 8 (OMP8), Outer Membrane Protein 11 (OMP11), VirB9-2 (formerly VirB9), VirB9-1 (formerly Conjugal Transfer Protein) (Sutten et al., 2010) and Elongation

factor Tu (EF-Tu) (**Table 5.2**).

5.3.3. Major Surface Proteins

5.3.3.1. MSP2

Major Surface Proteins are the most abundant among other OMPs (Palmer and McGuire, 1984). Two MSP2s variants differing in molecular weight and isoelectric point were identified (**Table 5.2**). Different MSP2 variants have been shown to arise not only in different cell types e.g. tick vs. mammalian (Oliva Chavez et al., 2012), but also within one cattle during different phases of *A. marginale* infection (French et al., 1999).

MSP2 is encoded by a multigene family composed of a single expression site and nine pseudogenes dispersed throughout the chromosome (Palmer et al., 1994). During the recombination of *msp2* pseudogenes, up to 9^4 variants of MSP2 protein can be expressed (Brayton et al., 2002). Interestingly, N- and C-terminal regions are highly conserved (French et al., 1999) and although not surface exposed, contain conserved $CD4^+$ T lymphocyte epitopes (Abbott et al., 2005; Brown et al., 2003).

This property of the MSP2 protein enables bacteria to evade the host immune system and to establish persistent infection, as developed antibodies are directed only towards a specific variant (Brayton et al., 2003). Additionally, although vaccination with MSP2 induces immunity, it does not confer protection since antibodies recognize conserved membrane domains, but not the surface-exposed, variable regions which are relevant for protective immunity (Agnes et al., 2011).

5.3.3.2. MSP4

MSP4 was identified as differentially regulated in the UFMG2 strain (Table 1). The MSP4 protein is very conserved not only between *A. marginale* strains, but also among different *Anaplasma* spp. (de la Fuente et al., 2002a; de la Fuente et al., 2005c; Molad et al., 2004).

Many researchers indicate that due to the fact that MSP4 remains conserved and is surface exposed it could provide cross-protection in cattle (Dark et al., 2011; Kawasaki et al., 2007; Molad et al., 2004). Agnes et al. (2011) and Lopez et al. (2005) suggested, that it is probably not very antigenic to induce a relevant immunity in cattle. The function of the MSP4 protein is still unknown.

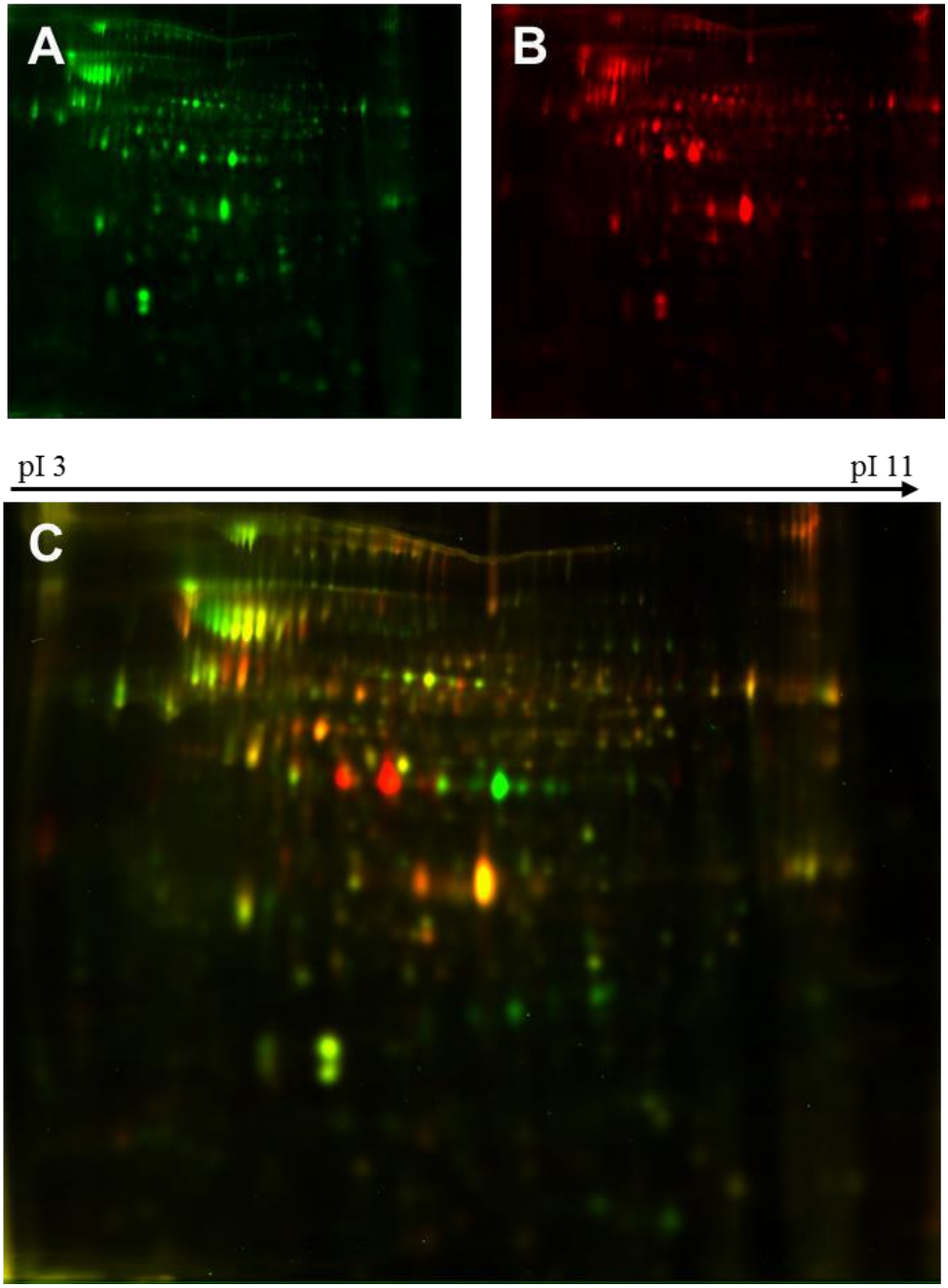

Figure 5.1. A representative 2D-DIGE gel image of CyDye-labelled *A. marginale* proteins. Cy3- and Cy5-labelled proteins from Virginia (A) and UFMG1 (B) strains, respectively. Overlay of the two images (C).

5.3.3.3. Type IV secretion system proteins

EF-Tu together with VirB9-2 and VirB9-1 are the components of the type IV secretion system (T4SS) (Lopez et al., 2007). The T4SS is a 1.1 MDa protein complex, made up of 12 interacting VirB/D membrane proteins that span the outer and inner bacterial membranes.

The T4SS complex subunits are highly conserved not only among *A. marginale* strains, but also with orthologous proteins in *A. phagocytophilum, E. chaffeensis* and *E. canis* (Junior et al., 2010; Lopez et al., 2007; Morse et al., 2012; Sutten et al., 2010; Vidotto et al., 2008). In Anaplasmataceae family members T4SS is known to be important for survival and pathogenicity as they transport proteins, DNA and nucleoproteins across the bacterial cell envelope in Gram-negative bacteria (Gillespie et al., 2010; Ohashi et al., 2002). Native as well as recombinant T4SS proteins have been demonstrated to induce an antibody response in cattle (Araujo et al., 2008; Lopez et al., 2007).

Elongation factor-Tu (EF-Tu) belongs to the family of hydrolases involved in protein synthesis, which promote chain elongation during polypeptide synthesis in the ribosome. Although for a long time EF-Tu was believed to be a cytoplasmic protein, recent analyses have demonstrated that in *A. marginale* and in some other bacteria it is membrane-associated (Araujo et al., 2008; Granato et al., 2004; Jacobson and Rosenbusch, 1976; Lopez et al., 2005). EF-Tu has been demonstrated to mediate bacterial attachment to host cells (Granato et al., 2004; Jacobson and Rosenbusch, 1976).

5.3.4. Other outer membrane proteins

Outer membrane protein 8 (OMP8) and OMP11 were found to be up-regulated in Virginia and UFMG2 *A. marginale* strains, respectively (**Table 5.2**). OMP8 is encoded by a single gene, while OMP11 is encoded by a complex gene family (Agnes et al., 2011). Both proteins are highly conserved among *A. marginale* strains

and elicit immune responses in cattle (Junior et al., 2010).

These OMPs together with other highly conserved proteins i.e. T4SS were suggested to be good candidates as vaccine antigens (Agnes et al., 2011; Dark et al., 2011; Palmer et al., 2012).

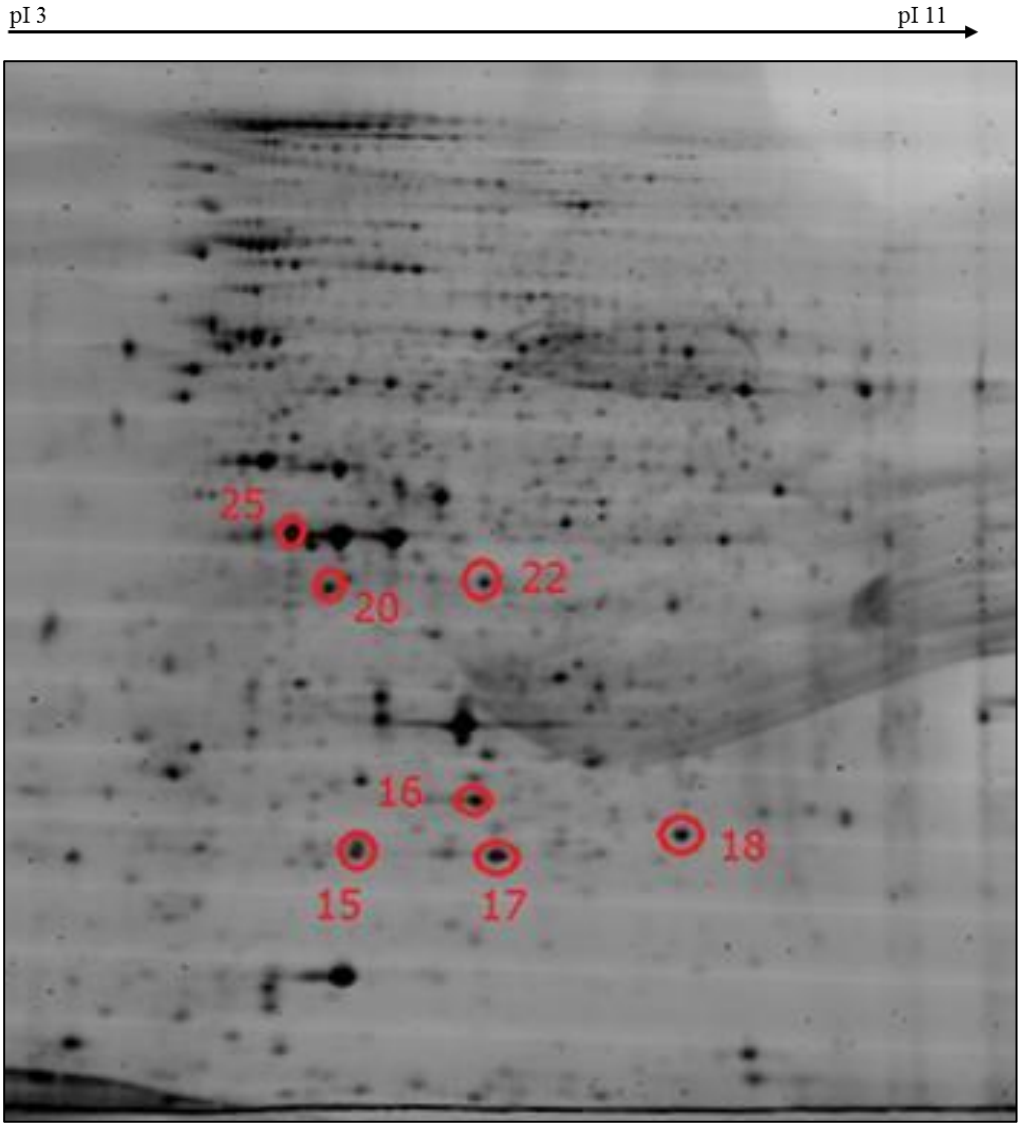

Figure 5.2. Preparative gel of *A. marginale* strain Virginia stained with Sypro Ruby. The pH gradient is given at the top of the figure. Circles and numbers correspond to differentially expressed proteins. Identified proteins are listed in Table 5.2.

5.3.5. Protein biosynthesis

Four proteins involved in transcription and the protein biosynthesis processes, generally classified as housekeeping proteins, were found differentially regulated i.e. transcription regulation factors and ribosomal subunits (**Table 5.2**). No data is

Table 5.2. *A. marginale* proteins differentially expressed among strains.

Strain	Spot No.	Protein name	Gene name	Accession number UniProt	Organism	Theoretical		No. of peptides in nanoLC MS/MS	References
						Mass (kDa)	pI		
Outer membrane proteins									
U1	22	Major surface protein 2 variable region	*msp2*	Q9L757	*A. marginale*	16,6	4,9	2	(Rurangirwa et al., 2000)
V	25	Major surface protein 2	*msp2*	Q84CN8	*A. marginale*	42,3	6,0	9	(Brown et al., 2003)
U2	17	Major surface protein 4	*msp4*	Q8G8H4	*A. marginale*	26,9	5,9	29	(de la Fuente et al., 2002b)
U2	18	Major surface protein 4	*msp4*	Q2LCL7	*A. marginale*	26,1	5,7	76	(de la Fuente et al., 2002b)
V	22	Outer membrane protein 8	*omp8*	Q2V9L5	*A. marginale*	43,1	8,7	5	(Lohr et al., 2002)
U2	1	Outer membrane protein 11	*omp11*	Q5P9I8	*A. marginale*	19,5	4,4	10	(Brayton et al., 2005)
U2	24	VirB9-2*	*virB9*	E3UVW3	*Anaplasma* sp.	16,7	6,8	2	(Lopez et al., 2007)
U2	12	VirB9-1*	*trbG*	D6Q020	*A. marginale*	29,7	5,6	7	(Junior et al., 2010)
U2	38	Elongation factor Tu	*tuf1*	Q5PBH1	*A. marginale*	42,9	5,3	42	(Brayton et al., 2005)
Protein biosynthesis									
V	15	Transcriptional regulator	*tr1*	Q8G8W0	*A. marginale*	21,2	5,3	7	(Lohr et al., 2002)
U1	4	50s ribosomal protein L9	*rplI*	B9KHP7	*A. marginale*	23,5	4,5	1	(Dark et al., 2009)
U2	2	50s ribosomal protein L7/L12	*rplL*	Q5PBG5	*A. marginale*	14,1	4,7	10	(Brayton et al., 2005)
V	20	Elongation factor Ts	*tsf*	B9KIW9	*A. marginale*	30,9	5,4	20	(Dark et al., 2009)
Stress induced proteins									
U1	12	Thioredoxin peroxidase 1	*tdpX1*	Q5PAZ1	*A. marginale*	22,8	5,9	3	(Brayton et al., 2005)
V	16	Thioredoxin peroxidase 1	*tdpX1*	Q5PAZ1	*A. marginale*	22,8	5,9	13	(Brayton et al., 2005)
V	17	Superoxide dismutase	*sodB*	D6Q019	*A. marginale*	25,3	6,2	2	(Junior et al., 2010)

Intermediary metabolism									
U1	13	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	*ispF*	Q3YT02	*E. canis*	19,1	8,1	1	(Mavromatis et al., 2006)
V	18	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	*ispF*	Q3YT02	*E. canis*	19,1	8,1	1	(Mavromatis et al., 2006)
U2	20	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	*ispF*	Q3YT02	*E. canis*	19,1	8,1	1	(Mavromatis et al., 2006)
U1	14	Putative uncharacterized protein	AM613	Q5PAS0	*A. marginale*	57,1	9,0	1	(Brayton et al., 2005)
U2	3	Putative uncharacterized protein	AM936	Q5PA38	*A. marginale*	13,9	4,9	3	(Brayton et al., 2005)
U2	36	Putative uncharacterized protein	AM1080	Q5P9U1	*A. marginale*	52,5	5,5	6	(Brayton et al., 2005)
U2	40	Putative uncharacterized protein	AM778	Q5PAG6	*A. marginale*	58,1	9,0	3	(Brayton et al., 2005)
U2	41	Putative uncharacterized protein	AM778	Q5PAG6	*A. marginale*	58,1	9,0	10	(Brayton et al., 2005)

U1 – UFMG1, U2- UFMG2, V-Virginia.

*) VirB9-2 formerly VirB9, VirB9-1 formerly Conjugal Transfer Protein (Sutten et al., 2010

available about their surface exposure. Genes encoding for these proteins being essential for all organisms are highly conserved which makes them unlikely targets for drug/vaccine development (Dunning Hotopp et al., 2006; Ogawa et al., 2007).

Interestingly it has been demonstrated that the proportion and number of ribosomal proteins and other proteins related to translation are higher in intracellular bacteria when compared to other bacteria (Ogawa et al., 2007). For this reason Ogawa et al. (2007) suggested, that although other bacteria have orthologous genes, these proteins may be essential for obligate intracellular bacteria.

5.3.6. Stress induced proteins

Enzymes involved in stress defense, thioredoxin peroxidase 1 (alkyl hydroperoxide reductase; AhpC/Prx1) and superoxide dismutase (SODB), were up-regulated in Virginia and UFMG1 strains (**Table 5.2**). These defensive proteins scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS), which at high quantities can cause severe damage to nucleic acids, proteins and lipids, leading to the induction of apoptosis (Morel et al., 1991).

Peroxiredoxins (Prxs) (EC 1.11.1.15) are an ubiquitous and highly expressed family of cysteine-based peroxidases, which contain an absolutely conserved active site cysteine (Nelson et al., 2011; Poole et al., 2011). AhpC/Prx1 is one of the subfamilies distinguished within the Prxs family (Nelson et al., 2011). The cellular localization of this protein is not clear. The AhpC/Prx1 protein from *Entamoeba histolytica,* that belongs to the same group as *A. marginale* AhpC/Prx1 (Nelson et al., 2011), has been demonstrated by some authors to be located in the cytoplasm (Tachibana et al., 1991), by others, to be cell surface- associated (Choi et al., 2005).

SODs, together with Prxs, protect bacteria from the effect of ROS produced by the host's professional phagocytes upon stimulation with microbes (Junior et al., 2010; Morel et al., 1991; Tu et al., 2012). Prokaryotic SODs are often dimers, and each respective enzyme subunit typically uses a single atom of its metal

cofactor: iron (Fe), manganese (Mn), copper (Cu) or zinc (Zn) in its active site (Lynch and Kuramitsu, 2000). Gram-negative bacteria commonly synthesize two cytoplasmic isozymes: MnSOD and FeSOD (Imlay, 2008). Based on the deduced amino acid sequence, the *sodb* gene of *Anaplasma* spp. probably encodes an iron-containing SOD (FeSOD) (Dunning Hotopp et al., 2006; Ohashi et al., 2002), which is conserved among *A. marginale* strains (Junior et al., 2010; Chapter 4). Other SOD isozymes in A. marginale have yet to be demonstrated.

Bacterial SODs in addition to detoxification of endogenously produced superoxides, play a role in the pathogenicity of animal and plant pathogens (Battistoni, 2003; Dhar et al., 2013; Lynch and Kuramitsu, 2000). The possible role of *A. marginale* SODB in pathogenesis and surface localization supports the fact, that in *Anaplasma* spp. it is co-transcribed with components of the T4SS (Brayton et al., 2005).

The mechanisms by which *A. marginale* is able to invade and grow within the host erythrocytes are not completely elucidated. The scavenging proteins, like SODs or Prxs, protecting bacteria from host-derived reactive oxygen species are likely to play a key role during invasion and survival by counteracting oxidants generated by host cells. The maintenance of redox homeostasis is essential in living organisms, and therefore makes these proteins potential target candidates for novel drugs. Hence, the exact cellular localization of stress response proteins in *A. marginale* should be confirmed experimentally. On the other hand, the development of specific inhibitors could be an obstacle as these enzymes are strongly conserved within organisms (Gretes et al., 2012).

5.3.7. Hypothetical proteins

Among differentially expressed protein spots, we found five proteins annotated as hypothetical uncharacterized, because their exact role in *A. marginale* has not been established yet. By applying *in silico* analysis tools their subcellular localization, as well as the presence of signal peptides and transmembrane domains was predicted (**Table 5.3**).

Table 5 . 3. Characteristics of putative uncharacterized proteins differentially expressed among *A. marginale* strains.

Strain	Spot No.	Gene name	Accession number UniProt	Length (aa)	CELLO v. 2.5	PSORTb v. 3.0.2	Phobius	TMHMM v. 2.0 & SignalP 4.1
U1	14	AM613	Q5PAS0	506	cytoplasmic	unknown	1th	-
U2	3	AM936	Q5PA38	130	cytoplasmic	unknown	1th	-
U2 U2	40 41	AM778	Q5PAG6	531	outer membrane	outer membrane	1th/signal	signal
U2	36	AM1080	Q5P9U1	473	cytoplasmic/inner membrane	periplasmic	2th	1th

The subcellular localization was predicted by CELLO (Yu et al., 2006), and PSORTb (Yu et al., 2010). The transmembrane helices and signal sequences were predicted by Phobius (Kall et al., 2007)., TMHMM 2.0 (Krogh et al., 2001) and SignalP 4.0 (Petersen et al., 2011), (aa – amino acid, th – transmembrane helix, U1 – UFMG1 and U2 - UFMG2 strains).

The Search Tool for the Retrieval of Interacting Genes (STRING v 9.1) was used to predict possible interactions of these uncharacterized putative proteins. The program quantitatively integrates interaction data from genomic context, high-throughput experiments and co-expression available in online resources for different organisms, in order to predict proteins' functional and physical associations (Franceschini et al., 2013). Predictions based on the nearest neighbor in the genome indicated only one interacting protein for AM613 and AM936, AM612 and fumarate hydratase (fumC), respectively (data not shown). Both proteins are probably cytoplasmic as indicated by the Subcellular Localization Predictive System CELLO, yet whether they are anchored in the cell membrane is not clear (**Table 5.3**).

AM613 has been reported to be up-regulated in *A. marginale* from tick cell lines when compared with those from bovine erythrocytes, yet its function is unknown (Ramabu et al., 2010).

The interactions of AM778 and AM1080 proteins are presented in **Figures 5 . 3.A** and **5.3.B.**

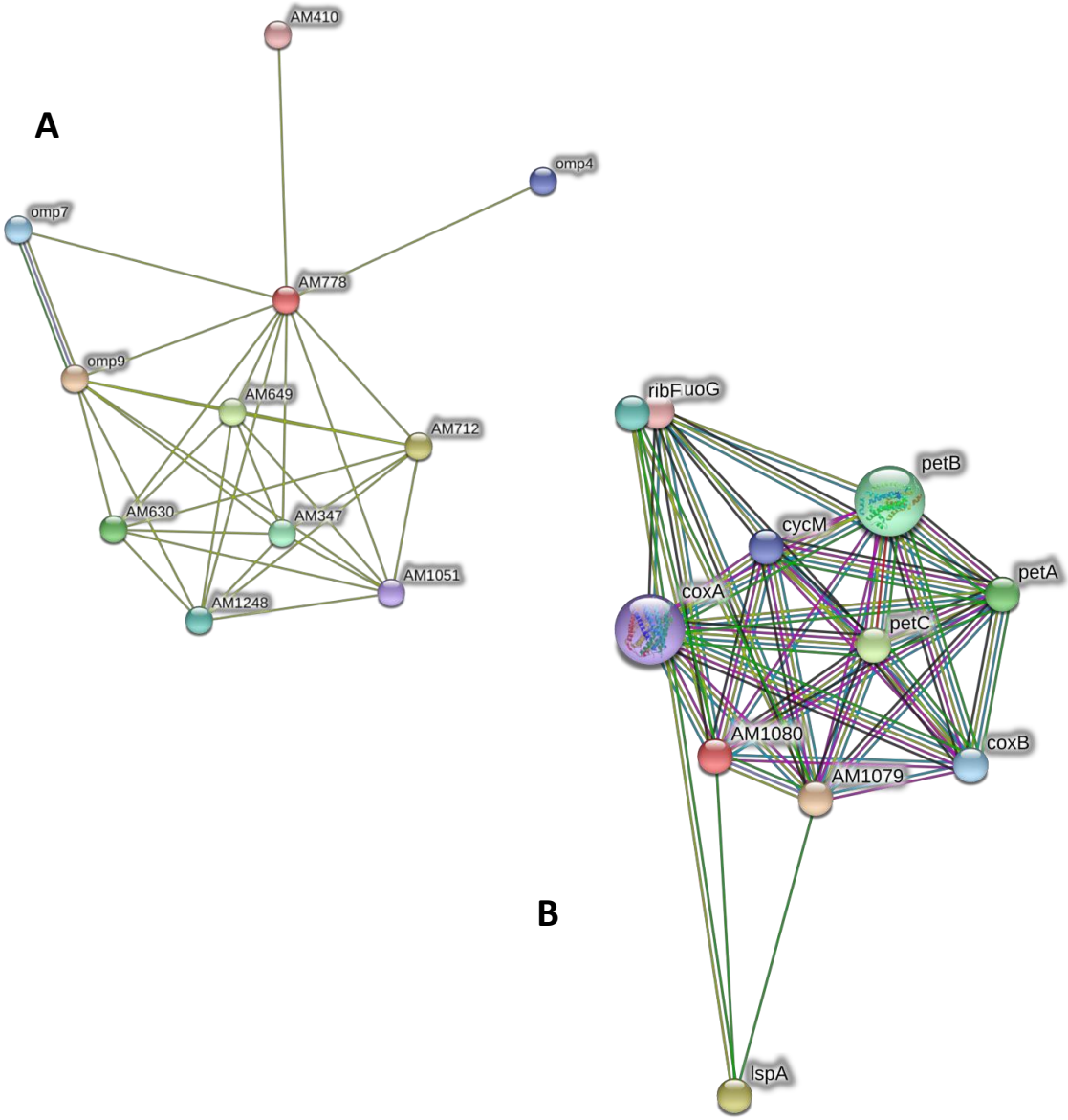

Figure 5.3. Protein-protein interaction networks predicted by STRING v 9.1 algorithm for A). AM778 and B). AM1080 (indicated as red balls).The interactions depicted by different line colours are predicted based on related proteins, database and literature search and the nearest neighbour in genome. The more lines connecting a protein, the stronger evidence for its possible interaction are available.

AM778 is weakly associated with many uncharacterized proteins: AM410, AM712, AM649, AM630, AM347, AM1248 and AM1051, as well as with different outer membrane proteins: OMP4, OMP7 and OMP9 (**Figure 5.3.A**). The fact that it strongly interacts with OMPs suggest that it may be membrane-associated. Additionally, *in silico* analysis indicated AM778 as an outer membrane protein

possessing one transmembrane domain (**Table 5.3**).

AM778 is encoded by a locus that also encodes AM779 and AM780, which are highly antigenic surface expressed proteins (Agnes et al., 2011; Albarrak et al., 2012; Dark et al., 2011; Lopez et al., 2005; Noh et al., 2008). The AM778 protein is conserved among other members of the Anaplasmataceae family, and up to now has only been found in bacteria isolated from tick cells (Noh et al., 2008). For this reason, Noh et al. (2008) suggested that AM778 may play a role in the colonization of the tick vector. Remarkably, we found AM778 to be up-regulated in the UFMG2 strain, which in contrast to non-tick transmissible UFMG1 strain, is transmissible by the only known biological vector of *A. marginale* in Brazil, *B. (R.) microplus* tick (Ruiz et al., 2005).

Based on database searches, nearest neighbor in the genome, related proteins and their interactions from other species and literature search, AM1080 was predicted to be strongly interconnected with different proteins, in particular with cytochromes: cytochrome C (cycM), cytochrome B (petB), cytochrome c1 (petC), cytochrome c oxidase subunit I (coxA) and II (coxB), cytochrome B6-F complex iron-sulfur subunit (petA) (**Figure 5.3.B**). It is also associated with NADH dehydrogenase subunit G (nuoG), riboflavin kinase (ribF) and lipoprotein signal peptidase (lspA).

AM1080 is a conserved protein which belongs to the metallopeptidases family M16. Interestingly, many cytochrome bc1 complex proteins, which were predicted to interact with this protein (**Figure 5 . 3.B**), are also classified within this family. The complex III consists of three common subunits: cytochrome b, cytochrome c1 and the Rieske [2Fe-2S] protein, which are involved mainly in electron transfer processes (Travaglini-Allocatelli, 2013). Since all complex III proteins are transmembrane, it is very probable that AM1080 protein is also membrane associated, as predicted by different algorithms (**Table 5.3**).

The BLAST database search indicated AM108 protein as insulinase from peptidase M16 family, with homologues in other members of the Anaplasmataceae family e.g. *A. phagocytophilum.*

Other proteins, namely AM613, AM936, AM778 did not have a significant BLAST score to any putative protein.

5.3.8. Intermediary metabolism

2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECDP) was differentially regulated in among strains (**Table 5.2**). MECDP is an important enzyme of non-mevalonate pathway (MEP) in which isopentenyl diphosphate (IPP), an isoprenoid compound, is synthesized (**Figure 5.5**) (Lange et al., 2000). The biosynthesis of IPP in mammals proceeds through an alternative mevalonate pathway (MAV).

Isoprenoids are indispensable for many cellular functions, i.e. electron transport or carbohydrate carriers in the biosynthesis of peptidoglycan (Heuston et al., 2012). Yet, some *Rickettsia* spp. are believed to exploit IPP from host cells, as they lack genes for IPP synthesis (Lange et al., 2000; Sangari et al., 2010). In some bacteria isoprenoids have been suggested to be involved in response to oxidative stress and therefore to allow adaptation to the host environment (as reviewed in Heuston et al., 2012)

Enzymes involved in IPP synthesis represent an attractive target for potential drugs (Odom, 2011). For example fosmidomycin, an inhibitor of one of the MEP pathway enzymes (**Figure 5.5**) has shown activity in killing *Plasmodium falciparum*, a parasite that similarly to *A. marginale* resides in erythrocytes (Heuston et al., 2012; Steinbacher et al., 2003). MECDP is predicted to be cytoplasmic by subcellular localization algorithms, which makes it rather unsuitable as a vaccine target.

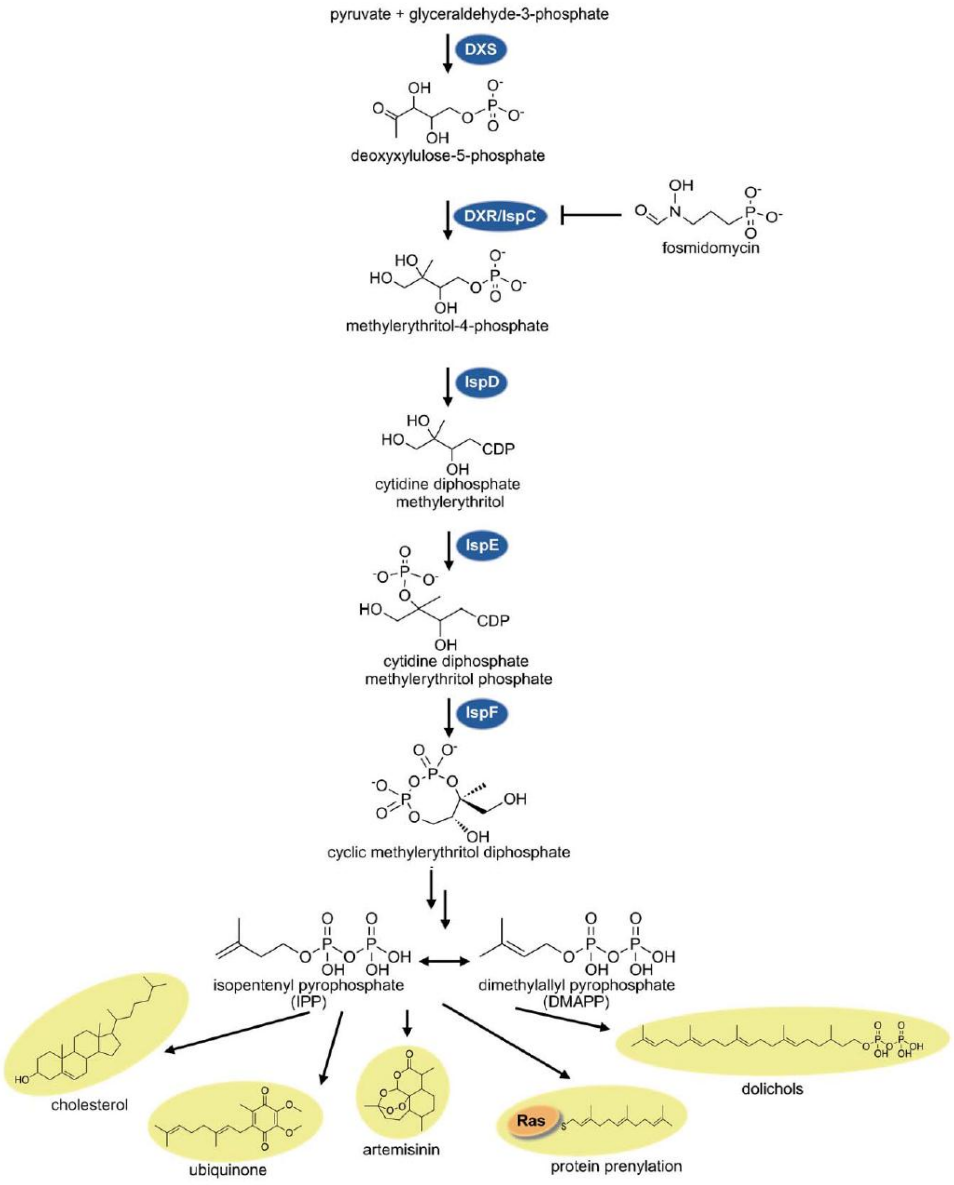

Figure 5.5. The non-mevalonate MEP pathway of isoprenoid biosynthesis. Taken from Odom (2005).

In the MEP pathway, IPP and DMAPP are generated from pyruvate and glyceraldehyde 3-phosphate. Enzymes of this pathway are named here according to their *E. coli* homologs. IspF (2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase) was found differentially regulated among *A. marginale* strains. Fosmidomycin inhibits the DXR/IspC enzyme and blocks isoprenoid biosynthesis *in vivo*.

Although 2D-DIGE coupled with mass spectrometry analysis is a potent high-throughput technique, we failed to identify a few of protein spots (data not shown). This may be due to low protein amounts, or due to PTMs like glycosylation which can hinder protein identification by Mass Spectrometry. Although the bacteria were purified, one cannot exclude contamination with host cells proteins, which could explain why some proteins did not produce hits when searched against *A. marginale* or Anaplasmataceae family databases.

Two *A. marginale* strains used for analysis, namely UFMG1 and Virginia are known to possess an inclusion appendage (Ribeiro et al., 1997; Smith et al., 1986). The inclusion appendage can be observed in *A. marginale*-infected erythrocytes (Chapter 1). In ticks, appendages were found free in the midgut lumen or attached to the cell membrane of midgut epithelial cells (Kocan et al., 1984). The fact that none of the appendage associated proteins were identified, suggest that they might be not expressed in bacteria derived from tick cell cultures, as they have been identified by 2-DE in bacteria from infected erythrocytes (Agnes et al., 2011; Lopez et al., 2005). It is also possible that there was no significant difference in these proteins expression among strains, or that they were not sufficiently abundant to be identified by RP-LC-MS/MS.

Among differently regulated proteins we did not identify MSP3, which undergoes antigenic variation similar to MSP2 (Brayton et al., 2003). We were also expecting to identify MSP1a, which varies among different *A. marginale* strains in molecular weight, because of a varying number of tandem repeat (TR) peptides of 23-31 amino acids in the N-terminal region (Oberle et al., 1988). The domain that follows the TR part is highly conserved among different strains (de la Fuente et al., 2007). The Virginia strain has only two TR while both Brazilian strains consist of four TR (see also Chapter 3). These differences should be easily recognizable on 2D-DIGE, unless MSP1a protein has been hampered by other proteins having exactly the same pI and MW.

Interestingly, MSP1a has never been identified in bacteria from erythrocytes by mass spectrometry analysis of immunoreactive spots, although it should have been recognized by antibodies from animals vaccinated with *A. marginale* OMPs (Lopez et

al., 2005). One of the explanations suggested by Lopez et al. (2005) was that MSP1a protein had not been sufficiently abundant to be detected by LC– MS/MS.

The sequence of the MSP1a protein may hamper its identification by MS. The first obstacle is the fact that none of the *A. marginale* strains analyzed here contains lysine (K) or arginine (R) in the tandem repeats (TR) region (see Chapter 3). Due to the fact that for spots digestion trypsin, that cleaves proteins on the C-terminal side of K and R was used, the first peptide that could be obtained from Virginia MSP1a would be 57 aa long (*nota bene* it still would be the shortest when compared to those from other three strains). The mass spectrometer is most efficient at obtaining sequence information from peptides that are between 7-20 residues (Steen and Mann, 2004). Sequence stretches longer than 20 aa gives rise to signals appearing outside the recorded mass-to-charge interval, while peptides shorter than 7 aa match more than one protein in the database.

Secondly, PTMs and in particular glycosylations add to the molecular weight of the protein. The TR part of MSP1a protein (Garcia-Garcia et al., 2004a), as well as the highly conserved domain, contain a high number of serine/threonine residues that can be glycosylated (de la Fuente et al., 2001b). After prediction of possible trypsin cleavage sites of whole sequence of MSP1a from the *A. marginale* Florida strain with PeptideCutter (web.expasy.org/peptide_cutter/), it returns 46 expected peptides. Yet, only two of them that are between 7-20 aa long, do not have any possible glycosylation sites. Therefore, a search based too tightly around an experimental measurement of the molecular weight of the MSP1a protein probably fails to find a correct match.

A. marginale proteins expressed in cultured ticks cells and bovine erythrocytes differ in the expression of some proteins, such as MSP1a (de la Fuente et al., 2001a) or AM613 (Ramabu et al., 2010). For this reason, the results obtained here should be further tested in bacteria derived from bovine erythrocytes. However, using the same tick cell line for cultivation of different *A. marginale* strains, with the known exception of MSP2, we excluded some of the variations that occur when growing each strain separately in different animals.

5.4. Conclusions

For the first time the quantitative proteomic technique 2D-DIGE was used to analyze proteome level differences among geographical *A. marginale* strains grown in IDE8 tick cells. Here we demonstrated that apart from many OMPs, various stress-associated proteins and enzymes were also differently expressed among strains. Some of the proteins had only been predicted by whole genome screening, despite not being identified by other techniques. We could also confirm that OMP11 is expressed in bacteria derived from tick cell lines, although it was thought to be expressed only in bacteria from bovine erythrocytes. Furthermore, we identified some uncharacterized proteins which, by *in silico* analysis, were predicted to be associated with or protruding from cell membranes. Such proteins are of special interest, because they are known to interact with the immune system of the host. To summarize, our results provide the first evidence of intra-strain differences in protein expression which is of fundamental importance. In addition, the identification of novel, potentially antigenic proteins may provide new drug/vaccine targets to be tested in future.

6. Conclusions and recommendations

Infected tick cell lines are an excellent source of bacteria, as they support the replication of bacteria to a very high titre within a very short time. However, due to the obligatory intracellular nature, bacteria have to be purified from host cell debris before subsequent analyses. The use of easily reproducible Percoll gradients for the purification of *A. marginale* from IDE8 cells was evaluated and described in Chapter 2. We demonstrated that Percoll preserves the viability of *A. marginale*, even when they were cryopreserved in liquid nitrogen for up to 6 months. Because of this feature, the Percoll purification method appears to be superior to those previously reported such as Renografin gradients. Furthermore, Percoll is non-toxic, therefore bacteria purified from tick cell cultures are useful for vaccination trials and many other studies which were not possible previously.

The MSP1a protein contains tandem repeats that vary in number and sequence among different *A. marginale* strains. Yet, they do not appear to undergo antigenic variation in cattle, tick or tick cell lines. MSP1a plays an important role in the adhesion of the bacteria to erythrocytes and tick cells. It also contains neutralization sensitive epitopes required for the development of an immune response in cattle.
Analysis of all *A. marginale* sequences available at the time point in GeneBank and the proposal of uniform nomenclature for *A. marginale* strains are presented in Chapter 3. Additionally, the role of glycosylation, protein conformation as well as the relevance of amino acids at the position 20 are discussed.

Bacteria propagated *in vitro* are probably the most reliable source to assess strain differences on the proteomic level. In this system variations which arise during the propagation of each strain in an animal are eliminated, as different strains are grown in the same cell line and if necessary, uninfected cells can be included as negative control.
In Chapter 4 and 5 the comparison of four *A. marginale* strains grown *in vitro* was described. The Virginia strain is well characterized, but data about two Brazilian *A. marginale* strains UFMG1 and UFMG2 was missing. Some molecular and

serological characterizations of these strains were done and are presented in Chapter 4. The identity of the strains was confirmed by analysis of the *msp1α* gene part encoding for tandem repeats. Next, we presented evidence that MSP4, MSP5, SODB and HSP70 are highly conserved across *A. marginale* strains when examined by gene sequencing and western blot analysis.

In Chapter 5, the hypothesis that *A. marginale* strains differ in their protein expression was tested by the 2D-DIGE technique. We demonstrated that not only housekeeping proteins are differentially regulated, but more importantly, also proteins which are known to be highly antigenic. Most of them were membrane- associated proteins like those from the T4SS complex, VirB9-1, VirB9-2 and EF-Tu, as well as MSP4, OMP8 and OMP11. Besides these, proteins involved in stress response SODB and AhpC/Prx1 and putative uncharacterized proteins AM613, AM778, AM936, AM1080 were also up-regulated in some strains. The exact role of the latter ones is unknown, yet they were predicted by *in silico* analysis to be associated with or protruding from cell membranes. It would be of additional interest to carry out studies on these proteins, as they are likely to interact with the host immune system. Since many of identified up-regulated proteins are known to play a role in virulence-associated functions, they may contribute to differences in pathogenicity across *A. marginale* strains.

The outcome of our studies represent the first insight into intra-strain differences in protein regulation among *A. marginale* grown *in vitro*. Since many proteins are differently expressed among bacteria from *in vitro* vs. *in vivo* models, further analysis of *A. marginale* derived from infected erythrocytes are necessary to confirm the results presented here. It is highly probable that *in vivo* some proteins may not be expressed at a sufficiently high level to elicit a protective immune response. Such experiments would additionally allow to *A. centrale* to be included in comparisons, since despite many attempts, it has never been established *in vitro* and is propagated only in cattle.

LIST OF ABBREVIATIONS

2DE	two dimensional electrophoresis
2-D DIGE	2-D Fluorescence Difference Gel Electrophoresis
aa	amino acids
AhpC	alkyl hydroperoxide reductase
amu	atomic mass units
BVA	biological variation analysis
bp	base pairs
BSA	bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1- propanesulfonate
C-terminal	carboxy terminal
Cy	cyanine dye
DIA	differential in-gel analysis
DIGE	difference gel electrophoresis
DMF	dimethylformamide
dNTP	deoxynucleotide tri-phosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
FCS	foetal calf serum
FDR	false discovery rate
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HRP	horseradish peroxide
IEF	isoelectric focusing
IgG	immunoglobulin G
IPG	immobilized pH gradient
IPP	isopentenyl diphosphate
kDa	kilodalton
LC-MS/MS	liquid chromatography tandem mass spectrometry
M	molar
MS	mass spectrometry
MW	molecular weight
NADH	nicotinamide adenine dinucleotide - hydrogen (reduced)
NL	non linear
N- terminal	amino terminal
OMP	outer membrane protein
PBS	phosphate buffered saline
PBST	phosphate buffered saline Tween
PCR	polymerase chain reaction

pI	isoelectric point
pmol	pikomol (10^{-12})
PTM	post translational modification
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RP-LC	reversed phase liquid chromatography
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPG	sucrose potassium glutamate
TEMED	N,N,N,N-tetramethylendiamine
U	enzyme unit
V	volt
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

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