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Faculty of Science

Analysis of the Lipoprotein Domain from the Hemelipoglycoprotein of the Tick *Dermacentor marginatus*.

Bachelor thesis

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Annotation:

The study aim is to clone the hemelipoglycoprotein lipoprotein domain (LD) of the tick *Dermacentor marginatus*. The lipoprotein domain will be prepared as a recombinant protein and purified. The subunits of native hemelipoglycoprotein will be isolated and the presence of lipoprotein domain in the subunits will be determined.

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1. Introduction and Objectives

The majority of the so-called carrier proteins (CP) is known for their ability to bind, store, and transport heme. Furthermore, tick carrier proteins are presumable transporters of lipids and proteins. The antioxidation activity can be expected from all CPs due to the binding of heme. The CP of the tick *Dermacentor marginatus*, hemelipoglycoprotein (HLGP), is one of the most abundant proteins of *D. marginatus* hemolymph. Its expression varies among tick species and depends also on developmental stage and blood-feeding behavior. However, in general, CPs are non-sex-linked and are present in unfed ticks as well. Therefore, the ability to sequester heme is probably not the only important function of these proteins.

A common part of carrier proteins is the lipoprotein N-terminal domain. These domains occur in insects as well as in vertebrates, although their sequences suggest, that there is no relation between lipoprotein domains of the two groups. This domain is assumed to be conserved among Ixodida and to be responsible for binding lipids, carbohydrates, and metals. However, the putative functions of this domain have never been proven.

The objective of this work is to isolate the hemelipoglycoprotein subunits from the hemolymph of the tick *D. marginatus* and to determine the presence of the lipoprotein domain (LD) in the subunits. The hemelipoglycoprotein LD should be cloned, prepared as a recombinant protein, and purified.

2. Literature review

2.1 Tick classification and general characterization

The members of the order Ixodida are generally called ticks. Ixodida belong into the phylum Arthropoda, subphylum Chelicerata, class Arachnida, subclass Acari and superorder Parasitiformes and can be further classified into three families: Ixodidae (hard ticks, 702 species), Argasidae (soft ticks, 193 species), and Nuttalliellidae (1 species) (Guglielmone et al., 2010). Ticks are obligate hematophagous ectoparasites of mammals, birds, and reptiles.

The family Nutalliellidae contains a single species *Nuttalliella namaqua* representing the most basal tick´s lineage connecting the two major families Ixodidae and Argasidae (Mans et al., 2011). These two families differ significantly in their physiology and life strategy.

Argasidae representatives are characterized by their wrinkle leathery integument and by the absence of a sclerotized scutum, which allows them fast body enlargement during feeding. Their life cycle takes about 2-3 years and they spend the whole time in the immediate vicinity of the host (in dens, nests etc.). Also, the physiology of Argasidae species is adapted to life in a dry environment. They feed on blood repeatedly in short time periods (minutes to hours) and in small volumes on multiple hosts. The developmental cycle consists of larval stage, up to 5 nymphal stages (depending on the host accessibility and blood-feeding opportunity), and the adult stage. The genera *Argas*, *Ornithodoros*, and *Otobius* have major veterinary and/or medical impact.

Ixodidae are characterized by the presence of a sclerotized scutum, serving as a defense system against mechanical defense response of the host. Furthermore, this scutum demonstrates sexual dimorphism. Male body is usually fully covered by the scutum, while female body is covered only to approximately one half of the body. The prominent capitulum with hypostome stands out forward from the megascopic gnathosoma. The developmental cycle of Ixodidae usually comprises larval, nymphal, and adult stage, and may include 1-3 different hosts and takes between 2 to 6 years (Süss, 2003). Larvae, nymphs, and adult females feed once to engorgement and their feeding period can last up to several days. Ticks have developed variety of life strategies, including different types of life cycles. One-host life-cycle is typical by the complete development of the individual from larval to adult stage on one host. Two-host ticks moult on the first host from the larval to the nymphal stage, the engorged nymphs then drop off

and moult to the adult stage in the environment. The adult tick then has to find a new host to feed on. The three-host ticks feed on different host in each developmental stage and never moult on the host. In all the above described types of life-cycles, adult females drop off the host after mating and feeding and lay a large batch of eggs (the number of eggs is the greater the more hosts are necessary to complete the life cycle). The most important genera with the highest impact on public-health or world economy are genera *Ixodes*, *Rhipicephalus* (including the former genus *Boophilus*), *Dermacentor, Amblyomma*, *Hyalomma*, and *Haemaphysalis* (Jongejan and Uilenberg, 2004).

2.1.1 The genus *Dermacentor*

The genus *Dermacentor* of the family Ixodidae consist of 34 species (including one species of the former genus *Anocentor*) (Guglielmone *et al.*, 2010) inhabiting all continents except for Australia. *Dermacentor* species usually follow the three-host life-cycle. These species have eyes, short palpae, and distinct ornate scutum. The species *D. marginatus* and *D. reticulatus* are of particular importance due to transmission of diseases and causing economical losses on livestock in Eurasia, while in North America *D. variabilis* and *D. andersoni* play significant role. Pathogens transmitted by *Dermacentor* species are either viral, causing e.g. Colorado tick fever (CTFV) and tick-borne encephalitis, or bacterial causing e.g. Rocky Mountain Spotted Fever (*Rickettsia rickettsii*), tularemia (*Francisella tularensis*) and Siberian tick typhus (*R. siberica*) (Estrada-Peña and Jongejan, 1999). Moreover, these tick may also transmit diseases of protozoan origin like canine babesiosis (*Babesia canis canis*) to animals (Sréter et al. 2005). The bite of *Dermacentor* ticks can cause tick paralysis in rare cases (Gregson J. D., 1958; Hadwen S., 1913).

2.1.1.1 Dermacentor marginatus

Dermacentor marginatus, also known as ornate sheep tick, is one of the two european *Dermacentor* species (next to *D. reticulatus*). This species is typically found in woods or on meadows. *D. marginatus* is one of the main Palearctic species reported attacking man. Furthermore, *D. marginatus* along with *D. reticulatus* mainly infest livestock and game animals in Eurasia. It is the main vector of the pathogen *R. slovaca* (Rehacek *et al*., 1990; Beati *et al.*, 1993). The second important pathogen transmitted by *D. marginatus* is the Omsk Hemorrhagic

Fever virus (OHFV) (Nuttall and Labuda, 1994). The presence of another virus, Crimean–Congo Hemorrhagic Fever virus (CCHFV), in *D. marginatus* has been shown as well (Kondratenko, 1976, Markeshin *et al*., 1992). *D. marginatus* capability of transmission of many other pathogens has been demonstrated. Some of the examples are: tick-borne encephalitis virus (TBEV) (Kozuch a Nosek, 1971), Bhanja virus (BHAV) (Hubalek *et al*., 1988; Labuda *et al*., 1997), West Nile virus (Hubalek a Halouzka, 1999), *R. sibirica*, *R. conorii, Borrelia burgdorferi* (Smetanová *et al*., 2006), *F. tularensis* (Hubalek *et al*., 1990), *Coxiella burnetii* (Jongejan and Uilenberg, 2004) and the protozoa of the genus *Babesia* (Pietrobelli *et al*., 2007). The bite of *D. marginatus* may also cause crustaceous or necrotic lesion, surrounded by an erythema (erythema migrans-like) and painful regional lymphadenopathies, which are, in fact, probably caused by *R. slovaca* infection (Oteo *et al.*, 2004).

2.1.1.2 Dermacentor variabilis

Dermacentor variabilis, also called American dog tick, is a nearctic species occurring throughout the USA and in parts of Canada and Mexico. According to study of Campbell and Bowles (1994), *D. variabilis* is a very active tick found on man throughout the whole area of USA. It is the primary vector of *R. rickettsii* causing Rocky Mountain Spotted fever (RMSF) in the eastern parts of the USA and transmits *Anaplasma marginale* causing bovine anaplasmosis (Jongejan and Uilenberg, 2004). Another significant pathogen, *F. tularensis* causing tularemia, is also transmitted by this species (Hopla and Hopla, 1994). *D. variabilis* also produces paralysis toxin in saliva, which can endanger both humans and animals (Jongejan and Uilenberg, 2004). In North America, *D. variabilis* belongs to important ectoparasites of livestock.

2.2 Ticks as parasites and vectors

Generally, ticks can be found in all important bio-geographical regions, although single species can be restricted to specific areas. Species of public-health interest are found on each continent except for Antarctica. Around 10 % of currently known tick species act as pathogen vectors. Ixodidae are generally much more important for pathogen transmission than Argasidae (Jongejan and Uilenberg, 2004). In addition to the possibility of disease transmission, the hosts are also endangered by the tick blood-feeding behavior itself, which can trigger immune responses like irritations, allergies, or even paralysis caused by toxins in tick saliva. The public

and scientific interest is focused on species endangering humans, companion animals, and livestock. Although reliable data reporting the annual damages on the livestock caused by ticks are missing, their annual worldwide economical and medical impact is considered to be high. In the northern hemisphere, the significant majority of the total number of disease cases of human comprises Lyme borreliosis (caused by bacteria of *B. burgdorferi* sensu lato complex) and tick-borne encephalitis (caused by the tick-borne encephalitis virus) (Sumilo et al., 2008; Rizzoli et al., 2011). The majority of health problems of cattle and small ruminants is caused by protozoan and rickettsial diseases (Jongejan and Uileneberg, 2004).

2.3 Blood digestion

Ticks are fully dependent on their single nutrition source – the blood. The primary nutritive component and major protein of the host blood is hemoglobin released from erythrocytes. Apart from hemoglobin, also all other blood-proteins serve as nutrition source for anabolic processes such as vitellogenesis or egg production. The blood-feeding usually takes several days and is preceded by the process of attachment to the host, which may take few hours, during which the tick produces various biochemical substances from its salivary glands, e.g. cement for stronger attachment or anticoagulant proteins. The actual blood digestion begins in the tick midgut (Coons et al. 1986; Tarnowski and Coons, 1989). Hemoglobin is digested by a complex hemoglobinolytic system of enzymes (see Horn et al., 2009) and the released heme is stored in hemosomes (Lara et al., 2003). Heme can be up-taken and transported by hemelipoglycoprotein or other heme-binding proteins in hemolymph (Gudderra et al., 2001; Maya-Monteiro et al., 2000; Thompson et al., 2007).

2.4 Heme and its sequestration

The ability to sequester, store, and transfer heme is vital in blood-feeding arthropods ingesting large amounts of hemoglobin. Free heme is cytotoxic and triggers the formation of reactive oxygen species (Hamza et al., 1998). This iron-containing molecule is usually found as a prosthetic group in hemoproteins (Donohue, 2008). Many strategies how to utilize heme and at the same time to mitigate its toxicity have evolved crosswise the Arthropoda. Interestingly, while hematophagous insect, *Rhodnius prolixus*, retained functional heme biosynthetic pathway, the hard tick *Rhipicephalus microplus* lost the ability to synthesize heme and is fully dependent

on reuse of the ingested heme (Braz et al. 1999). Although the study of Braz and colleagues (1999) is limited to a single tick species, it is probably not only *R. microplus* lacking heme biosynthetic pathway. Therefore the tick carrier proteins, being able to bind, store, and transport heme molecules, seem to be a vital adaptation to hematophagy in ticks. The major hemolymphal storage proteins binding heme are hemelipoglyco-carrier protein (CP) (Guderra et al. 2001, 2002a, b), HeLp (Maya-Monteiro et al. 2000, 2004) or hemelipoglycoprotein (Dupejova et al., 2011). The primary transporter of non-heme bound iron is ferritin 2 (Hajdusek et al., 2010).

2.5 Tick hemolymph proteins

The complex liquid called tick hemolymph has various functions in the tick body. Most importantly it transports a variety of molecules through the tick body. In addition to other components of the hemolymph, proteins, enzymes, hormones, and nutrition molecules are present in large amount. Furthermore, its important function is to regulate osmotic pressure within the tick body and thus to support the stability of different tissues (Sonenshine, 1991).

Hemolymph consists of hemocytes (circulating cells) and plasma. Proteins are the major (by weight) soluble component of tick plasma. According to Sonenshine (2001), hemolymph proteins of *D. variabilis* contribute to the crude hemolymph weight by 11.5-14.3 %.

Despite the obvious importance of major hemolymphal proteins, any further studies of these molecules are missing (see the review Gudderra et al., 2002b). The only thoroughly investigated protein so far, has been vitellogenin (Vg) (Thompson et al., 2005). Next to the major types of tick hemolymph proteins, other protein groups worth mentioning are: macroglobulins, lectins and antimicrobial peptides.

Two representatives of α-2-macroglobulin (α2M) protease inhibitors have been identified so far. First one, tick α-2-macroglobulin (TAM) (Kopacek et al., 2000), has been isolated from plasma of *O. moubata* and serves as a universal protease inhibitor (Hoffman et al., 1999). The second one is α2M IrAM-An from the tick *I. ricinus* contributing, eventually directly serving, as a trigger for certain type of bacterial phagocytosis (Buresova et al., 2009).

Lectins have been investigated in more detail as they managed to attract the attention of researches by their expected role in pathogen-parasite transmission and/or infection immunity responses. First tick lectins were found in soft ticks (*O. tartakovskii*, *O. papilipes* and *A. polonicus*) (Grubhoffer and Matha, 1991; Grubhoffer et al., 1991; Veres and Grubhoffer, 1991).

According to the inhibitory assays, these lectins demonstrate high affinity to sialic acid with extended binding affinity for *N*-acetylamino-D-hexosamines and D-galactose (Grubhoffer et al., 1991). Next, a variety of lectins was isolated also from other tick species (Kuhn et al., 1996; Grubhoffer and Kovar, 1998; Kovar, 2000; Huang et al., 2007), all of them showing sialic acid-binding activity. Several important lectins from the group of the so-called FReP (fibrinogen-like proteins) have been identified as well (see Rego et al. 2005).

The only fully characterized and purified tick lectin is Dorin M (Kovar et al., 2000; Rego et al., 2005, 2006).

Lectin molecules of different species containing fibrinogen-like domain generally demonstrate high amino-acid sequence similarity in these motifs (Rego et al., 2005). Furthermore, it is generally accepted that the fibrinogen-like domains may play significant role in vector pathogen transmission and recognition.

2.5.1 Vitellogenins/vitellins

Vitellogenin (Vg) and vitellin (Vn) of ticks are extensively investigated hemolymph proteins. Tick Vgs and Vns can be characterized as heme-glycolipo-proteins (Chinzei et al., 1983; Rosell and Coons, 1991; James and Oliver, 1997; Gudderra et al., 2001). Vgs and Vns of insects are similar in their amino acid and lipid composition to ticks, however, insect Vgs and Vns do not contain heme (Gudderra et al., 2001). Also Vn of tick closer relative, twospotted spider mite (Tetranychus urticae), does not bind heme (Cabrera et al., 2009).

Investigation of Vgs in insects and ticks demonstrated high similarity in these proteins, while the Vgs of crustaceans show slightly higher divergence (see Guddera et al., 2002a).

Tick vitellogenin, a precursor of the egg yolk protein, is synthesized by the fat body during vitellogenesis, it circulates in the hemolymph, and is incorporated into eggs as Vn (Chinzei et al., 1983; Rosell and Coons, 1992; James and Oliver, 1996, 1999; Taylor and Chinzei, 2001). Vg is known for its ability to bind heme and also to act to some extent as antioxidant (Logullo et al., 2002). Vitellogenin and vitellin have been isolated and partially characterized in several species, e.g. in *O. moubata, D. variabilis,* and *I. scapularis* (Taylor and Chinzei, 2001; Rosel and Coons, 1992; James and Oliver, 1997).

In *D. variabilis*, Vg consist of two native proteins – VgA (330 kDa) and VgB (320 kDa). Furthermore the whole protein contains seven subunits ranging from 22 to 215 kDa in size

(Sullivan et al., 1999). The isolelectric point (pI) is different for VgA (6.55) and VgB (6.65) (Gudderra et al., 2002b). Vn of *D. variabilis* comprises two proteins as well: Vn A (480 kDa) and Vn B (370 kDa) (Rosell and Coons, 1991). The DvVg expression in part-fed females was initiated by mating (Thompson et al., 2005).

2.5.2 DvCP, OpCP, HeLp, and HLGP

The other major hemolymph proteins of ticks are so called carrier proteins or hemelipoproteins. The nomenclature varies in time and according to the source. Donohue and colleagues (2008) tried to unify the nomenclature to "carrier proteins" (CP), which would be prefixed by the abbreviation of the specie name, e.g. DvCP means *D. variabilis* carrier protein.

Some of the studied proteins posses the ability to bind, store, and transport heme (Gudderra et al., 2001; Maya-Monteiro et al., 2000). This ability shows to be vital for species without functional heme-biosynthetic pathway, e.g. for *R. microplus* (Braz et al., 1999). It is supposed that most of these proteins play significant role in the transport of proteins and lipids. Their occurrence and abundance in tissues differ mainly between soft and hard ticks. The main carrier protein fully characterized so far is hemelipoglyco-carrier protein (CP) of the American dog tick, *D. variabilis* (thus named DvCP). CP seems to be highly conserved among Ixodidae. Its form was probably derived from an ancestral Vg gene (Donohue et al., 2008). Unlike Vg, CP is a non-sex-linked protein and appears to be ubiquitous through the whole developmental cycle of the tick.

2.5.2.1 HeLp

A carrier protein, the major hemolymph protein of *R. microplus*, has been characterized as heme lipoprotein (HeLp) (Maya-Monteiro et al., 2000). Its newly promoted appellation is RmCP. Its molecular weight is 354 kDa and consist of two apoproteins (103 and 92 kDa). Its density is 1.28 g/ml and its pI is 5.8. The protein contains 33 % of lipid out of which 35 % consist of cholesterol ester and the remaining are mainly neutral lipids or phospholipids. HeLp also contains 3 % of sugars, out of which the major contributor is mannose (up to 90 %). One HeLp molecule can bind two molecules of heme and one additional heme-binding site is created when incubated with hemin. HeLp was detected in both males and females (fully engorged and partially fed ones), however, it has not been found in mature eggs of *R. microplus*. As this

species is known for its missing heme-synthetic pathway, dietary heme binding, transport, and storage are vital for its survival. It was demonstrated by radioactively labeled HeLp (^{55}Fe-heme), that heme is transported (by this protein) to ovaries where it is incorporated. The results of Maya-Monteiro and colleagues (2000, 2004) suggest that HeLp in *R. microplus* plays a key role in heme recycling and transport and could be the major adaptation to hematophagy. Furthermore, it was proven that HeLp acts as an antioxidant against the heme-induced oxidative damage (Maya-Monteiro et al., 2004). As the addition of heme to the phosphatidylcholine liposomes leads to lipid peroxidation, dangerous free radicals are released. Phospholipids oxidation was significantly reduced by addition of HeLp. Therefore HeLp can inhibit heme induced oxidative reactions within the cell and also prevent red blood cell lysis caused by heme (Maya-Monteiro et al., 2004).

2.5.2.2 DvCP

Another characterized hemelipoglyco-carrier protein (formerly lipoglycoheme-carrier protein) (DvCP) is a major hemolymphatic protein from *D. variabilis* (Gudderra et al., 2001). In partially fed females, DvCP represents 60-80 % of the hemolymph proteins. Its total molecular weight differs according to a detection method. Its MW reaches 200 kDa by native-PAGE, but 340 kDa by gel filtration chromatography. Using SDS-PAGE, two polypeptide chains were identified with molecular weights of 98 and 92 kDa. Native DvCP contains lipids (cholesterol and phospholipids), mono- and tri-acylglycerides and free fatty acids. The exact role of this heme-carrier protein is unknown. DvCP was found in whole body homogenates of fed and unfed larvae, fed nymphs and in eggs (Gudderra et al., 2001). The plasma of fed and unfed adult tick also demonstrates the presence of DvCP. In partially fed or fully engorged females, the CP was detected in hemolymph, salivary glands, muscles, and ovaries as well as in the fat body (Gudderra et al., 2002a). The CP concentration in different tissues varies during feeding suggesting a change in protein expression during vitellogenesis. Unlike DvVg, DvCP appearance in the hemolymph is not initiated by mating, but by the attachment to the host and initiation of blood feeding (Gudderra et al, 2001). Therefore carrier proteins appear to be the major storage protein in ticks before vitellogenesis. Furthermore, during vitellogenesis, heme is transported to vitellogenin either directly and/or via CP (Thompson et al., 2007).

Up to date, DvCP is the only isolated, characterized and completely sequenced tick carrier

protein (Gudderra et al., 2001, 2002a, 2002b; Donohue et al., 2008). Transcript of the carrier protein of the lone star tick *Amblyomma americanum* has been sequenced as well (GenBank: ABK40086.2). The DvCP transcript is coding for two subunits containing three motifs: a lipoprotein N-terminal domain, a domain of unknown function and a von Willebrand factor type D domain. Similar motifs are included in tick Vgs and are also expected in heme-binding proteins of hematophagous insects. However, no significant sequence similarity with tick heme-binding proteins was found in insects studied so far. The lipoprotein domain is generally considered to be part of proteins binding lipids, carbohydrates, and metals (Donohue et al., 2008) and thus appears to be the most important part of the transport protein. The domain with an unknown function consist of several large open beta sheets. Lastly, the von Willebrand domain appears to play a role in multimerization of the protein units. The whole mRNA sequence (GenBank: DQ422963) of 4951 bp in length contains an open reading frame (4641 bp) encoding a protein precursor of 1547 amino acids (from bp 44 to bp 1684) (Donohue et al., 2008).

The two subunits of DvCP are separated by a cleavage site RXXR common to storage proteins and Vg. This cleavage site is placed from amino acids (a.a.) 746 to 749 in the first half of the domain of unknown function. The presence of the cleavage site in the middle of the domain suggests, that it no domain. Otherwise it would remained conserved and non-cleaved. The smaller subunit correspond to the smaller polypeptide chain (92 kDa) and consists of 734 a.a. in total (a.a. 16 to 749 within the sequence of the protein) and contains the lipoprotein N-terminal domain (a.a. 16 to 622). The bigger subunit corresponds to the bigger polypeptide chain (98 kDa) and consists of 798 a.a. in total (a.a. 750 to 1547) and includes the von Willebrand factor type D domain (a.a. 1351 to 1522) at the carboxy-terminus (Donohue et al., 2008).

Thanks to the knowledge of mRNA and amino acids sequences, the evolutionary relationship of DvCP with other proteins was investigated (Donohue et al., 2008). According to currently known sequences in the GenBank database, DvCP was determined as closely related to vitellogenins.

2.5.2.3 OpCP

Another high molecular weight carrier protein was found in *O. parkeri* (Gudderra et al.,

2002a). Its molecular weight is 668 kDa. SDS-PAGE demonstrated that this lipoprotein consists of two major peptide chains (114 and 93 kDa) and one minor (48 kDa). The 93 kDa chain is similar in molecular weigh to the smaller subunit of DvCP. When compared to DvCP, OpCP demonstrated expression in coxal fluid instead of fat body and salivary glands. Even more importantly, OpCP did not contain heme under identical experimental conditions. This suggests, that the species evolved different strategies how to utilize carrier proteins in heme sequestration. CPs could be utilized mainly as protein and lipid transporters in some species.

2.5.2.4 HLGP

Recently isolated and characterized major hemolymphal protein from *D. marginatus* named hemelipoglycoprotein (HLGP) demonstrates similarity to DvCP (Dupejova et al., 2011). Its properties classify this protein into a group of carrier proteins, thus the nomenclature offers an alternative: DmCP. HLGP molecular weight is 290 kDa. Its two polypeptide chains separated by SDS-PAGE were estimated to be 95 and 100 kDa in size. This CP tends to form high molecular weight complexes with putative fibrinogen-related proteins or other proteins of unknown character (Dupejova et al., 2011). HLGP was recognized by antibodies against tick lectin Dorin M, which would suggest the presence of some fibrinogen-like or lectin motif in the HLGP structure. However, DvCP, a related carrier protein, does not contain any of these motifs.

This protein was detected in plasma and hemocytes of unfed and partially fed females and males. Furthermore, the presence of HLGP was confirmed in salivary glands and gut of fed females. Two forms of HLGP were described, glycosylated and non-glycosylated one. Its glycosylated form carries primarily high-manose glycans and also complex *N*-glycans (Dupejova et al., 2011). HLGP demonstrates hemagglutination activity. Further experiments proved high galactose- and weaker mannose-binding activity, fucose was not recognized by the protein.

2.5.3 N-terminal lipoprotein domain

The N-terminal amino acid sequence of the protein is probably conserved among Ixodida (Maya-Monteiro et al., 2000; Guddera et al., 2001). The cleavage site exists between the secretion signal and the mature N-terminus of DvCP sequence, which secures that the mature CP starts with sequence FEVGKEYV in DvCP (Guddera et al., 2001) or in apoHeLp-B (Maya-

Monteiro et al., 2000). Lipoprotein N-terminal domain is, according to Donohue (2008), a common element of proteins binding lipids, carbohydrates and metals.

3. Materials and methods

3.1 Material

3.1.1 Ticks

Ticks of the species *Dermacentor marginatus* were obtained from the breeding facility of the Institute of Parasitology, Biology Centre, ASCR. Males and females after metamorphosis were separated and kept in moist environment at 26°C. Partly fed females (6 days blood-feeding on guinea pigs) were used for the hemolymph collection. Females were also used for isolation of mRNA.

3.1.2 Primers

All the primers were designed using the DvCP sequence (ABD83654.1) as a model and were analyzed using the OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA, eu.idtdna.com/analyzer/applications/oligoAnalyzer). The synthesized primers were supplied by GENERI BIOTECH (Hradec Králové, Czech Republic).

Table 1: Primers(F for forward, R for reverse)

NAME	SEQUENCE	ANNEALING TEMPERATURE (T predicted by OligoAnalyzer 3.1 / T predicted by the supplier GENERI BIOTECH)
HLGP LD F1	GCTTCGAGGTCGGCAAGGAGTAT	61°C / 50-55°C
HLGP LD R1	GCCAGGGCGCGGAAAGCG	66°C / 50-55°C
HLGP LD F2	AAGATTGGCTTCGAGGTCGG	57°C / 45-55°C
HLGP LD R2	GCCCGGTTAGCCAGGGCGCGGAAA	70°C / 45-55°C
HLGP LD F3	GACGACGACAAGATTGGCTT	55°C / 50-55°C
HLGP LD R3	GAGGAGAAGCCCGGTTAGCCAGG	64°C / 50-55°C
PRE HLGP LD F	TTCTGTGGCTATCGTTGCTCG	57°C / 47-49°C
PRE HLGP LD R	GGGTCAAGGCGCCGTAGT	60°C / 47-49°C
HLGP LD GFP F	TTGGGATCCTTCGAGGTCGGCAAG	63°C / 54°C
HLGP LD GFP R	GCTCCATGGCTGATGACGAAAGCG	63°C / 54°C
Control-F1	GACGACGACAAGATTCACGACGACAAGG	62°C / 55-58°C
Control-R1	GAGGAGAAGCCCGGTATCGTGGTCGCA	67°C / 55-58°C
Control-F2	GACGACGACAAGATACGCGAAATCGCCGA	65°C / 56-58°C
Control-R2	GAGGAGAAGCCCGGTATACGAGGTCCAGAT	66°C / 56-58°C

3.1.3 Plasmids

3.1.3.1 pRSET/EmGFP Vector (Life Technologies)

pRSET/EmGFP vector is a bacterial expression vector containing Emerald green fluorescent protein. It´s size is 3.6 kbp and carries an ampicillin resistance gene.

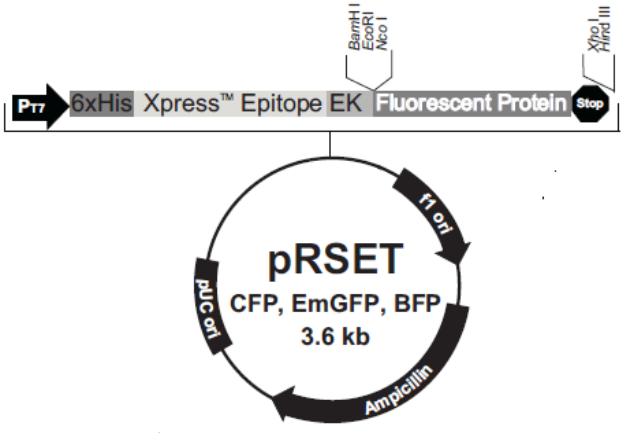

Figure 1: pRSET vector (Invitrogen, Manual for pRSET/FP Vectors)

It contains 6xHis-tag for purification of recombinant proteins on metal chelating columns, T7 promoter, and T7 forward and reverse priming sites. Further, it contains f1 origin, which allows single strand rescue of DNA,

and pUC origin, which ensures high copy replication. For the directional cloning, restriction sites for BamHI and NcoI were used.

3.1.3.2 pET-41 Ek/LIC Vector (Novagen)

pET-41 Ek/LIC vector is used for rapid directional cloning and following expression. It´s size is 5.9 kbp and carries a kanamycin resistance gene. It carries GST-tag and S-tag on the N-terminus of the insert and His-tag on both the N- and the C-termini. The f1 origin is also present.

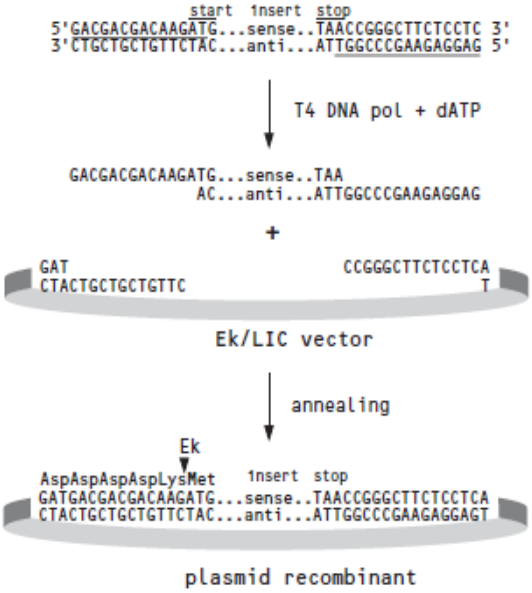

Figure 2: Cloning strategy for Ek/LIC vector (Novagen manual TB317 0806)

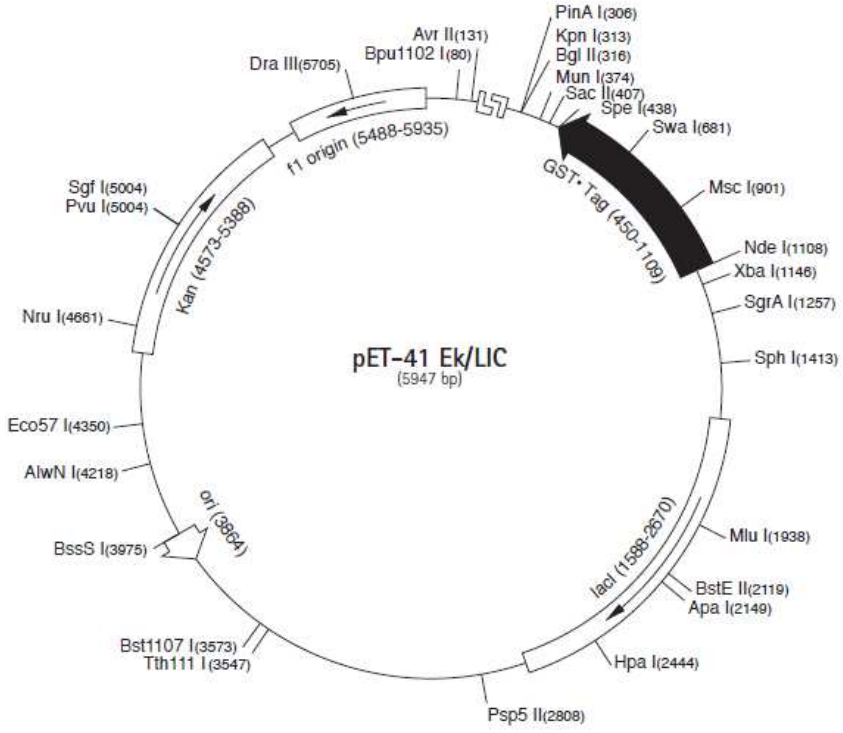

Figure 3: pET-41 Ek/LIC vector (Novagen manual TB317 0806)

3.1.3.3 11AALPPP_HLGP_LD_pMK-RQ (Life Technologies)

The sequence of the DvCP lipoprotein N-terminal domain was assembled by a commercial service according to the DvCP sequence from NCBI database (Life Technologies, 11AALPPP_HLGP_LD_pMK-RQ manual). This sequence was cloned into pMK-RQ vector using SfiI cloning sites. The plasmid carries kanamycin resistance gene. The sequence of the construct was verified by sequencing.

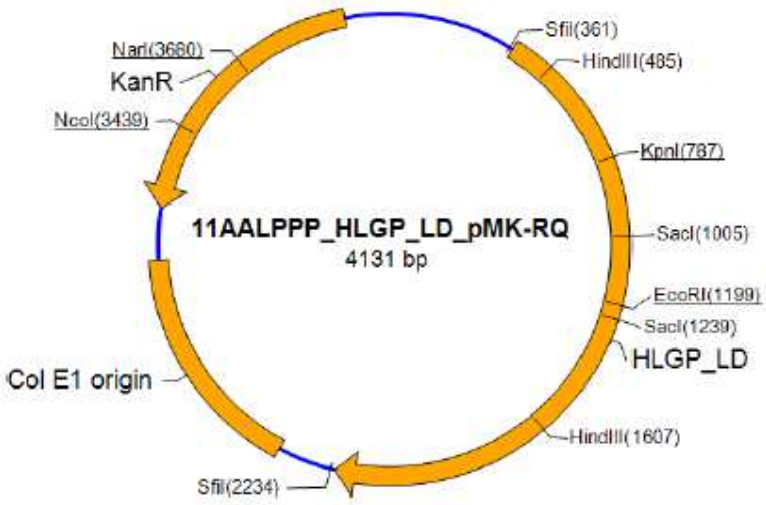

Figure 4: 11AALPPP_HLGP_LD_pMK-RQ vector scheme(Life Technologies, GeneArt Services, ref. no. 1146673)

3.1.4 *E. coli*

NovaBlue GigaSingles or Singles Competent Cells from Novagen; or One Shot TOP 10 Chemically Competent *E. coli* from Life Technologies were used for bacterial transformation.

GigaSingles competent cells feature greater than $1x10^9$ cfu/μg transformation efficiency, as well as the One Shot TOP 10 cells.

3.1.5 Solutions and Buffers

Table 2: Prepared buffers and solutions

HEMOLYMPH COLLECTION	PBS	14 mM NaCl, 0.15 mM KH_2PO_4, 1.8mM Na_2HPO_4, 0.27 mM KCl, pH 7.4
GEL ELECTROPHORESIS (SDS-PAGE)	Electrode buffer (5x)	0.125 M Tris, 0.96 M glycine, 0.5% SDS
	10% SDS	10% SDS in distilled water
	10% APS	10% APS in distilled water
	Separation buffer (4x)	1.5 M Tris-HCl, pH 8.8, 0.4% SDS
	Stacking buffer (4x)	1 M Tris-HCl, pH 6.8, 0.4% SDS
ELECTROELUTION	Electroelution buffer	200 mM glycin, 25 mM Tris-HCl, 0.025% SDS, pH 8.5
WESTERN BLOTTING	Blotting buffer	20% methanol, 25 mM Tris-base, 150 mM glycin
IMUNOBLOTTING	5% milk	5% non-fat dried milk in PBS
	PBS-Tween 20	0.05% Tween 20 v PBS
	Developing solution	0.1 M Tris, 0.05 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 9.5; NBT/BCIP solution
	Primary antibodies	mouse immune serum against HLGP (Dupejova, 2011)(Laboratory of Molecular Biology of Vectors and Pathogens Parasitology Institute, BC AS CR)
BACTERIAL TRANSFORMATION	LB medium with ampicilin	10 g tryptone, 5 g yeast extract, 10 g NaCl in 1l of dH_2O, 100 µg/ml of ampicilin
	LB medium with kanamycin	10 g tryptone, 5 g yeast extract, 10 g NaCl in 1l of dH_2O, 50 µg/ml of kanamycin
	agar plate with ampicilin	10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar in 1 l of dH_2O, 100 µg/ml of ampicilin
	agar plate with kanamycin	10 g tryptone, 5 g yeast extract, 10 g NaCl , 15 g agar in 1l of dH_2O, 50 µg/ml of kanamycin
PLASMID ISOLATION	TEG buffer	50 mM glucose, 10 mM EDTA, 25 mM Tris
	NaOH/SDS solution	0.2 M NaOH, 0.1% SDS
	3 M KAC buffer	120 ml 5 M CH_3COOK (pH=7), 23 ml ice cold acetic acid, 57 ml H_2O
	Fenol-chloroform solution	fenol-chloroform-isoamylalcohol in 25:24:1 ratio (pH 7)
	TE buffer	10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8.3)

3.1.6 Kits and Chemicals

Table 3: Kits and Chemicals

HEMOLYMPH COLLECTION	Protease Inhibitor Single-Use Coctail (Thermo Scientific)
GEL ELECTROPHORESIS (SDS-PAGE)	PageRuler Unstained Protein Ladder(Thermo Scientific)
	PageRuler Prestained Protein Ladder (Thermo Scientific)
	Prestained Protein Molecular Weight Marker (AppliChem)
	4x Loading dye + 0,8 M DTT (Thermo Scientific)
	5x Loading dye (Thermo Scientific)
	30% Acrylamide (Merck)
	TEMED (SERVA)
	PageBlue Protein Staining Solution (Thermo Scientific)
ELECTROELUTION	ElutaTube Protein, DNA and RNA Extraction and Dialysis Kit (Thermo Scientific)
PROTEIN CONCENTRATION	Acetone (Merck)
IMUNOBLOTTING	Secondary antibody: goat anti-mouse IgG, labeled with alkaline phosphatase (Vector Labs)
	Methanol (Merck)
REVERSE TRANSCRIPTION	First Strand cDNA Synthesis Kit (Thermo scientific)
PCR	TrueStart Hot Start Taq buffer (Thermo Scientific)
	TrueStart Hot Start Taq DNA polymerase (Thermo Scientific)
	Taq buffer (Thermo Scientific)
	$MgCl_2$ (Thermo Scientific)
	2 mM dNTP (Thermo Scientific)
	Taq polymerase (Thermo Scientific)
	PCR water (Top Bio s.r.o.)
	DEPC water
	Combi PPP Master Mix (Top Bio s.r.o.)
	GoTaq polymerase (Thermo Scientific)
	Green GoTaq buffer (Thermo Scientific)
	KAPA 2G ROBUST polymerase (KAPA BIOSYSTEMS)
	KAPA 2G buffer A (KAPA BIOSYSTEMS)
	KAPA 2G buffer B (KAPA BIOSYSTEMS)
	KAPA 2G buffer GC (KAPA BIOSYSTEMS)
	KAPA enhancer (KAPA BIOSYSTEMS)
	$MgCl_2$ buffer (KAPA BIOSYSTEMS)
	DMSO (Thermo Scientific)

DNA ELECTROPHORESIS	O´GeneRuler 1kb DNA ladder (Thermo Scientific)
	GeneRuler 1 kb DNA ladder (Thermo Scientific)
	GeneRuler 100 bp Plus DNA ladder (Thermo Scientific)
	6x DNA Loading Dye (bromophenol blue and xylene cyanol FF) + SYBR Green (Amresco)
	6x Orange DNA Loading Dye (xylene cyanol FF and orange G) + SYBR Green (Thermo Scientific)
	50 x TAE electrophoresis buffer (MERCK)
	Agarose (SERVA Electrophoresis)
DNA ELUTION FROM THE AGAROSE GEL	illustra GFX PCR DNA and gel bound purification kit (GE Healthcare Life Sciences)
	Gel extraction kit (QIAGEN)
	DNA extraction kit (Fermentas/Thermo Scientific)
CLONING	SfiI restrictase (Fermentas/Thermo Scientific)
	1x Buffer G (Fermentas/Thermo Scientific)
	T4 DNA polymerase (Novagen)
	BamHI restrictase (Thermo Scientific)
	NcoI restrictase (Thermo Scientific)
	1x Tango buffer (Thermo Scientific)
	10x T4 DNA ligase buffer (Thermo Scientific)
	T4 DNA ligase (Thermo Scientific)
BACTERIAL TRANSFORMATION	S.O.C. medium (Life Technologies)
BACTERIAL STOCK	80% glycerol (Lach:ner)
PLASMID ISOLATION	GenJET Plasmid Miniprep Kit (Thermo Scientific)
	ethanol 96%

3.2 Methods

3.2.1 Hemolymph collection

Hemolymph from partly fed females was sucked in a pipette tip after cutting off part of the front leg. Drops of the hemolymph were collected into PBS (prepared using pure chemicals and HPLC-grade water) containing proteases inhibitors. Hemolymph of ca 8 ticks was mixed together in the PBS volume of 50 µl.

3.2.2 Polyacrylamide gel electrophoresis (SDS-PAGE)

In the first experiment, SDS-PAGE was used for separation of hemolymph proteins of *D.*

marginatus according to their molecular weight. For separation of high molecular proteins, where HLGP belongs, 8% polyacrylamide gels were used. The components of the gels were mixed according to the Tab. 4 and the gels polymerized for 30 minutes. Hemolymph of semi-engorged females (collected according to chapter 3.2.1) was diluted 1:5 in PBS and then mixed with corresponding amount of 5x Loading Dye (non-reducing LD). After addition of LD, samples were shortly and gently vortexed and heated at 95°C for 3 minutes.

In the second experiment, SDS-PAGE was used to separate the HLGP polypetide chains after reduction with dithiothreitol (DTT). The pure HLGP protein dissolved in 50 µl of MilliQ water and was mixed with corresponding amount of 4x Loading Dye with DTT (reducing LD). After addition of LD, samples were shortly and gently vortexed and heated at 95°C for 5-10 minutes. When the protein after reduction remained unreduced, further addition of DTT was needed.

The samples prepared as described above were loaded onto the 8% gel placed in the electrophoretic apparatus (Bio-Rad) and the whole apparatus was immersed in the electrode buffer. The electrophoresis run at 130 V for approximately 1-1.5 hour. The gels were either stained by PageBlue Protein Staining Solution according to the manufacturer instructions or used for immunoblotting. The HLGP protein bands in the SDS-PAGE gels were cut off the gel and were directly analyzed by mass-spectrometry or were electro-eluted in order to be further reduced into polypeptide chains.

Table 4: Composition of the 8% SDS-PAGE gel

	8% gel (ml)	stacking gel (ml)
30% acrylamide	1.3	0.17
separation buffer (4x)	1.25	-
stacking buffer (4x)	-	0.25
distilled water	2.4	0.57
10% APS	0.05	0.01
TEMED	0.003	0.001

3.2.3 Immunoblotting

PVDF membrane was incubated in methanol for 5-10 minutes and then placed into the blotting buffer for 1 minute. The SDS-PAGE gel was washed with blotting buffer. The blotting sandwich (from the top: blotting paper, gel, membrane, blotting paper) was assembled between

the electrodes of the apparatus TRANS-BLOT® SD SEMI DRY TRANSFER (Bio-Rad). Separated proteins were transferred onto the membrane during 1 hour at 20 V or overnight at 2 V.

The membrane was washed in distilled water and blocked for up to 1 hour at room temperature (RT) in 5% solution of non-fat milk in PBS. The membrane was then incubated for 1 hour at RT with primary antibodies diluted 1:500 in 5% milk in PBS. PBS-Tween was used to wash the membrane, three times for 5-10 minutes. The incubation with secondary antibody (diluted 1:1000 in 5% milk in PBS) was carried out at RT for 1 hour. The washing step with PBS-Tween was repeated three times. The development of alkaline phosphatase signal was performed in 10 ml of developing solution with 200 μl 1x BCIP/NBT solution (no shaking). The developing reaction was stopped by rinsing the membrane in distilled water.

3.2.4 Protein electroelution

The electroelution of proteins from the SDS-PAGE gel was carried out in the ElutaTubes. The protein identified as HLGP by immunoblotting was cut out of the gel and placed into the ElutaTube. The tube was then filled with electroelution buffer (cca 800 μl), closed and placed into the electrophoretic apparatus filled with electroelution buffer. The tubes were oriented so that the current was flowing through the membranes in the tube walls. The electroelution run at 100 V for at least 3 hours, maximum overnight. At the end, the current was reversed for 40 s in order to allow the protein release from the membrane into the buffer. Next, the polyacrylamide gel was pulled out of the tube, while the buffer remained in the ElutaTube. The same ElutaTube was further used for dialysis of the electroelution buffer with the protein against PBS (pH 7.4). The tube was placed into the holder and immersed into the PBS in 1L volume beaker. The dialysis was performed overnight at 4°C under constant mixing of the PBS. After the dialysis, the solution of the protein was mixed with 4x volume of ice-cold acetone and placed into a freezer for at least 1 hour. HLGP precipitated and was centrifuged at 13 000 rpm for 5 minutes. The liquid was removed and the protein dried in a vacuum concentrator. The protein pellet was then dissolved in 50 μl of MilliQ water. HLGP protein was stored at -20°C and was further reduced by reducing Loading Dye in order to separate its polypeptide compartments by SDS-PAGE.

3.2.5 Mass-spectrometry

The protein bands identified as HLGP by immunoblotting were cut off the gel and sent for mass-spectrometry analysis into the Laboratory of Mass Spectrometry, Faculty of Science, University of South Bohemia. The procedure was following: 1) sample digestion (trypsin); 2) UPLC (Ultra Performance Liquid Chromatography) using Nano Acquity UPLC (Waters); 3) Peptide Mass Fingerprinting and fragment analysis (MS/MS) using Q-Tof Premiere Mass Spectrometer (Waters); 4) Protein identification using the PLGS 2.3 software; and 5) Uniprot and NCBI public protein databases search.

3.2.6 Reverse transcription

First Strand cDNA Synthesis Kit (Thermo Scientific) was used for reverse transcription. mRNA of female *D. marginatus* was used as a template. This mRNA was isolated by the supervisor, Ján Štěrba, 6 months ago and stored at -80°C. Three different cDNAs were produced using three primers: A) oligo(dT)18 primer, B) random hexamer and C) gene specific primer HLGP LD R1 (Tab. 1). The procedure was carried out according to manufacturer's instructions.

3.2.7 PCR

3.2.7.1 cDNA D. marginatus and HLGP LD F1-R1, F2-R2 and PRE HLGP LD primers

All the types of cDNA of *D. marginatus* obtained by reverse transcription were used as the DNA template in PCR reactions in order to obtain the DNA sequence encoding for HLGP lipoprotein domain, suitable for cloning into pET-41 Ek/LIC Vector.

PPP master mix (Tab. 5), Taq polymerase (Tab. 6), GoTaq polymerase (Tab. 7) and KAPA 2G ROBUST polymerase (Tab. 8) were used for the reaction with HLGP LD F1-R1 primers. GoTaq polymerase and KAPA 2G ROBUST polymerase were used for the reaction with F2-R2 primers (Tab. 9, Tab. 10), where the cDNAs were used as the DNA template. Optimization of the reaction conditions with different $MgCl_2$ concentrations was also performed. The preliminary PCR reaction with PRE HLGP LD primers was performed with KAPA 2G ROBUST polymerase, where $MgCl_2$ concentration changes were also applied (Tab 11). This PCR served for preliminary amplification of the desired sequence, which was prolonged on both ends, thus preventing the F1-R1 primers annealing to other section of the cDNA.

Table 5: PCR with PPP master mix (F1-R1 primers)

	volume (µl)
PPP master mix	10
primer F1	1
primer R1	1
cDNA	0.1
PCR water	Till 20

Table 6: PCR with Taq polymerase (F1-R1 primers)

	volume (µl)
buffer Mg-	2
$MgCl_2$	1, 1.5, 2
2 mM dNTP	2
primer F1	1
primer R1	1
cDNA	0.5
Taq polymerase	0.1
PCR water	Till 20

Table 7: PCR with GoTaq polymerase (F1-R1 primers)

	volume (µl)
Buffer Green	4
2 mM dNTP	2
$MgCl_2$	0; 2
primer F1	1
primer R1	1
cDNA	1
GoTaq polymerase	0.2
PCR water	Till 20

Table 8: PCR with KAPA 2G ROBUST polymerase (F1-R1 primers)

	volume (µl)	volume (µl)	volume (µl)	volume (µl)
KAPA 2G	4 (A)	4 (B)	4 (GC)	4 (GC)
Enhancer 1	-	-	-	4
2 mM dNTP	0.4	0.4	0.4	0.4
primer F1	1	1	1	1
primer R1	1	1	1	1
cDNA	1	1	1	1
KAPA 2G ROBUST polymerase	0.2	0.2	0.2	0.2
PCR water	Till 20	Till 20	Till 20	Till 20

Table 9: PCR with GoTaq polymerase (F2-R2 primers)

	volume (µl)
Buffer Green	4
2 mM dNTP	2
$MgCl_2$	0; 2
primer F2	1
primer R2	1
cDNA	1
Go Taq polymerase	0.2
PCR water	Till 20

Table 10: PCR with KAPA 2G ROBUST polymerase (F2-R2 primers)

	volume (µl)
KAPA 2G B	4
2 mM dNTP	0.4
primer F2	2
primer R2	2
cDNA	0.2
KAPA 2G ROBUST polymerase	0.2
PCR water	Till 20

Table 11: PCR with KAPA 2G ROBUST polymerase (PRE HLGP primers)

	volume (µl)
KAPA 2G A	4
$MgCl_2$	0; 0.5
2 mM dNTP	0.4
PRE HLGP LD F	1
PRE HLGP LD R	1
cDNA	1
KAPA 2G ROBUST polymerase	0.2
PCR water	Till 20

The reactions were performed according to the given program with annealing temperature gradient (Tab. 12).

Table 12: PCR temperature and time program

		F1-R1		F2-R2		PRE HLGP	
	initialisation	95°C	5 min	95°C	5 min	95°C	5 min
29 x ↑	denaturation	95°C	0.5 min	95°C	0.5 min	95°C	0.5 min
	annealing	48-64°C	1 min	50-64°C	1 min	47-54°C	1 min
	elongation	72°C	1 min	72°C	1 min	72°C	1 min, 2 min
	final elongation	72°C	3 min	72°C	3 min	72°C	3 min
	final hold	14°C	infinity	14°C	infinity	14°C	infinity

3.2.7.2 Plasmid 11AALPPP_HLGP_LD_pMK-RQ and HLGP LD F1-R1, F2-R2, F3-R3 primers

The plasmid containing the sequence encoding for DvCP lipoprotein domain was used as a template for PCR with HLGP LD primers. Firstly F1 and R1 primers were used, after this reaction the PCR product size was determined via electrophoresis, using 1kbp marker (Thermo Scientific). The PCR product band was cut off the gel and the product was eluted (see chapter 3.2.9) and used as a template for PCR with F2 and R2 primers. Lastly, product of this second reaction was used after electrophoresis and elution from the gel as a DNA template for PCR with F3-R3 primers.

The F2-R2 primers were also used in PCR with the plasmid directly, without previous amplification using the F1 and R1 primers. This was enabled by overlap of the primers with the lipoprotein domain.

The first PCR was always carried out with PPP master mix (Tab. 13). When the results were not satisfying, the PCR reaction was modified until unambiguous results were obtained (Tabs. 14, 15, 16, 17). The PCR using master mix with F2-R2 primers was also additionally modified by addition of DMSO (gradient of 0.2-2 µl in 0.2 µl steps).

Table 13: PCR using PPP master mix

	volume (µl)
PPP master mix	12.5
primer F1 or F2 or F3	1
primer R1 or R2 or R3	1
DNA	0.5-2
PCR water	Till 25

Table 14: PCR using Taq polymerase

	volume (µl)
combination buffer	2
2 mM dNTP	2
primer F1 or F2 or F3	1
primer R1 or R2 or R3	1
DNA	0.5
Taq polymerase	0.1
PCR water	Till 20

Table 15: PCR using TrueStart HotStart Taq polymerase

	volume (µl)
10x TrueStartTaq buffer	2.5
2 mM dNTP	2.5
primer F2	1
primer R2	1
$MgCl_2$	0.8-3
DNA	2
TrueStart HotStart Taq polymerase	0.2
PCR water	Till 25

Table 16: PCR using Pfu polymerase

	volume (µl)
Buffer Pfu	2
10 mM dNTP	0.4
primer F3	1
primer R3	1
PCR water	Till 20
DNA	1
Pfu polymerase	0.2

Table 17: PCR using KAPA 2G ROBUST polymerase

	volume (µl)	volume (µl)	volume (µl)	volume (µl)
KAPA 2G	4 (A)	4 (B)	4 (GC)	4 (GC)
Enhancer 1	-	-	-	4
2 mM dNTP	0.4	0.4	0.4	0.4
primer F3	1	1	1	1
primer R3	1	1	1	1
DNA	1	1	1	1
KAPA 2G ROBUST polymerase	0.2	0.2	0.2	0.2
PCR water	Till 20	Till 20	Till 20	Till 20

The reactions were performed according to the given program with annealing temperature gradient (Tab. 18).

Table 18: PCR temperature and time program

		F1-R1		F2-R2		F3-R3	
	initialisation	95°C	5 min	95°C	5 min	95°C	5 min
30 x ↑	denaturation	95°C	0.5-1 min	95°C	0.5 min	95°C	0.5-1 min
	annealing	51-56°C	1 min	50-64°C	1 min	45-60°C	1 min
	elongation	72°C	1-3 min	72°C	1-3 min	72°C	1-3 min
	final elongation	72°C	5-10 min	72°C	5-10 min	72°C	5-10 min
	final hold	14°C	infinity	14°C	infinity	14°C	infinity

3.2.7.3 D. marginatus cDNA and HLGP LD GFP primers

All the cDNAs of *D. marginatus* obtained by reverse transcription were used as the DNA template in these PCR reactions in order to obtain the product encoding for HLGP lipoprotein domain, suitable for cloning into pRSET/EmGFP Vector.

PPP master mix was used in the PCR reaction (Tab. 19) and the PCR was carried out in the thermal cycler following the program in the Tab. 20.

Table 19: PCR using PPP master mix

	volume (µl)
PPP master mix	12.5
primer HLGP LD GFP F	1
primer HLGP LD GFP R	1
cDNA	2
PCR water	Till 25

Table 20: PCR temperature and time program

		HLGP GFP primers	
	initialisation	95°C	5 min
30 x ↑	denaturation	95°C	1 min
	annealing	53°C	1 min
	elongation	72°C	3 min
	final elongation	72°C	3 min
	final hold	14°C	infinity

3.2.7.4 Plasmid 11AALPPP_HLGP_LD_pMK-RQ and HLGP LD GFP primers

The plasmid encoding for DvCP lipoprotein domain was used as the DNA template in PCR reactions in order to obtain product encoding for DvCP lipoprotein domain, suitable for cloning into pRSET/EmGFP Vector.

PPP master mix was used in the PCR reaction (Tab. 21) and the PCR was carried out in thermal cycler following the scheme in the Tab. 22.

Table 21: PCR using master mix

	volume (µl)
PPP master mix	12.5
primer HLGP LD GFP F	1
primer HLGP LD GFP R	1
cDNA	2
PCR water	Till 25

Table 22: PCR temperature and time program

		HLGP GFP primers	
	initialisation	95°C	5 min
30 x ↑	denaturation	95°C	1 min
	annealing	50-60°C	1 min
	elongation	72°C	3 min
	final elongation	72°C	3 min
	final hold	14°C	infinity

3.2.7.5 Control PCR with control F1-R1, F2-R2 primers

The primers Control-F1-R1 and F2-R2 are detecting short sequences within the lipoprotein domain. PCR using PPP master mix with these primers was used to determine, whether the PCR product contains the desired sequence. The PCR mixture was mixed according to the PPP master mix manufacturer's instructions and carried out in thermal cycler according to the program in the Tab. 23. As a positive control, plasmid 11AALPPP_HLGP_LD_pMK-RQ was used as a template.

Table 23: PCR temperature and time program

		Control-F1-R1		Control-F2-R2	
	initialisation	95°C	5 min	95°C	5 min
30 x ↑	denaturation	95°C	1 min	95°C	1 min
	annealing	55°C	1 min	55°C	1 min
	elongation	72°C	1 min	72°C	1 min
	final elongation	72°C	10 min	72°C	10 min
	final hold	14°C	infinity	14°C	infinity

3.2.8 DNA electrophoresis

1% agarose gel was used for separation and size determination of large PCR products and plasmids. 2% agarose gel was made for separation and size determination of small control PCR products. The samples were mixed with DNA loading dye (Thermo Scientific) with SYBR Green (Amresco) and loaded on the gel. The electrophoresis run at 130 V for approximately 40 minutes in the case of 1% gel and 90 minutes in the case of 2% gel. The DNA size was determined according to the DNA ladder.

3.2.9 DNA extraction from the agarose gel

DNA was eluted from the gel with one of the DNA elution kits: illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences), Gel Extraction Kit (QIAGEN) or DNA Extraction Kit (Thermo Scientific). The DNA extraction was carried out according to the manufacturers' instructions. MilliQ water was used as a final elution buffer. The purified DNA was either sent for sequencing or used for further PCR or cloning.

3.2.10 Cloning

3.2.10.1 pRSET/EmGFP Vector

The amplified DNA sequence encoding for lipoprotein domain of DvCP (PCR product of 11AALPPP_HLGP_LD_pMK-RQ and HLGP LD GFP primers) was cleaved by BamHI and NcoI restrictases.

The double digestion of PCR product (the insert) was carried out as folowing: BamHI (5 µl), NcoI (1 µl), buffer Tango (2 µl) and the DNA (12 µl~0.25 µg) were mixed and incubated at 37°C for 14 hours. The reaction was stopped by inactivation of the enzymes at 80°C for 20 minutes.

The double digestion of the plasmid was carried out as following: BamHI (5 µl), NcoI (1 µl), buffer Tango (2 µl) and the DNA (2 µl ~ 1 µg) were mixed and incubated at 37°C for 14 hours. The reaction was stopped by inactivation at 80°C for 20 minutes.

The cleaved plasmid and insert were ligated in 1:1 and 3:1 ratio. The 1:1 ratio mixture was prepared as following: linear vector DNA (1 µl), insert DNA (2 µl), 10x T4 DNA ligase buffer (2 µl), T4 DNA ligase (0.2 µl) and DEPC water (14.8 µl). The 3:1 ratio mixture is following: linear vector DNA (1 µl), insert DNA (6 µl), 10x T4 DNA ligase buffer (2 µl), T4 DNA ligase (0.2 µl), and DEPC water (10.8 µl). The ligation mixtures were incubated at 16°C for 24 hours. The ligation product was used for bacterial transformation and remaining volume stored at -20°C.

3.2.11 Bacterial transformation

3.2.11.1 NovaBlue GigaSingle Competent Cells

The cells were stored at -70°C, handled carefully and never vortexed. The tube was handled always on the very top in order to prevent unwanted warming of the cells. Cells were thawed on ice for 5 minutes and were flicked several times to be evenly resuspended. 2 µl of the plasmid DNA were added to the cells and the mixture was gently stirred with the pipette tip. The cells were incubated on ice for 5 minutes before the 90 s heat-shock (42°C) was applied to the cells. Afterward, the cells were quickly placed on ice and let to recover for 2 minutes. 250 µl of pre-warmed S.O.C. medium was added to the cells while keeping the cells on ice. The cells were placed in a shaking incubator for 1 hour at 37°C. Then, the cells were plated on an agar plate

(with an appropriate antibiotic) in 50, 100 and 200 µl volumes. The cells were spread out on the plate with sterile bended glass rod and placed into the 37°C incubator, where the cells grew overnight. The grown single colonies were transferred into liquid medium with the appropriate antibiotic and cultivated for at least 10 hours before harvesting.

3.2.11.2 One Shot TOP10 Chemically Competent Cells

The cells were stored at -70°C, handled carefully, always hold on the very top of the tube and never vortexed. Cells were thawed on ice until the complete resuspension visible. 2 µl of the plasmid DNA were added to the cells and the mixture was gently stirred. The cells were incubated on ice for 30 minutes before placing the cells into water bath (42°C) for 30 s. Afterwards, the cells were quickly placed on ice and let to recover for 2 minutes. 250 µl of pre-warmed S.O.C. medium was added to the cells while keeping them on ice. The cells recovery took place in a shaking incubator for approximately 1 hour at 37°C. Then, the cells were plated on agar plates containing appropriate antibiotic. 50, 100 and 200 µl volume of the cells was used for plating. The cells were spread out on the plate with sterile bended glass rod and placed into the 37°C incubator, where the cells grew overnight. The grown single colonies were transferred into liquid medium with appropriate antibiotic and cultivated for at least 10 hours before harvesting.

3.2.12 Plasmid isolation

Plasmids from small volume culture were isolated using GeneJET Plasmid Miniprep Kit (Thermo Scientific). The instructions of the manufacturer were followed without any changes.

Plasmids from bacterial volume of 25 ml were isolated by the alkaline lysis method. Bacterial pellet, obtained from 20 ml bacterial culture, was resuspended in 1 ml of ice-cold TEG solution. 2 ml of freshly prepared NaOH/SDS solution were added, which lysed the cells. 1.5 ml of ice-cold 3M KAC solution was added, mixed and incubated on ice for 10 minutes in order to precipitate proteins, cell membranes, SDS and chromosomal DNA while the plasmid DNA remained in the solution. The precipitate was centrifuged for 10 minutes at 7 000 g and the supernatant was transferred into a new 15 ml tube. The organic phase of the same volume (phenol-chloroform solution) was added to the supernatant, the mixture was shaken and centrifuged for 5 minutes at 5000 g in order to precipitate the remaining protein in the

interphase. The water phase was transferred into a new tube and 3x volume of ice-cold ethanol was added. The mixture was let to precipitate for 20 minutes in the freezer and the DNA precipitate was harvested afterward by centrifugation at 7000 g for 20 minutes. The pellet containing plasmid DNA was washed in ethanol, resuspended in 0.5 ml of TE buffer and stored for further use at -20°C. Plasmids were also sent for sequencing.

3.2.13 Sequencing

The plasmids (75-150 ng) or PCR products (25 ng) were mixed with primers (2.5 pmol), water was added to 7.5 μl and the mixture was sent for sequencing. The plasmids and PCR products were sequenced in Laboratory of Genomic, BC AS CR in České Budějovice on the machine ABI PRISM 3130xl (Applied Biosystems).

4. Results

4.1 HLGP subunits

The hemolymph proteins concentration was determined by NanoPhotometer Pearl (Implen) to be 3491 μg/ml. The hemolymph proteins were separated on 8% SDS-PAGE gel using reducing (Fig 5., lane 7, Fig. 6, lane 4) and non-reducing loading dye (Fig 5., lane 8, Fig. 6, lane 5). The hemelipoglycoprotein was identified via immunoblotting with primary mouse antibodies against HLGP and secondary goat anti-mouse antibodies with alkaline phosphatase (Fig. 6). In agreement with the previous study of Dupejova and colleagues (2011), a strong protein band of a size of ~260 kDa (Fig. 5., lane 8, A1; Fig. 6., lane 5, A1) was identified. However, other protein bands were also recognized by the antibody (Fig. 5., lane 8, A2 and A3; Fig. 6., lane 5, A2 and A3). The protein bands A1 and A2 were sent for mass-spectrometric (MS) analysis and the identity of both was confirmed to be DvCP related protein, thus probably HLGP. According to the study of Dupejova and colleagues (2011), the larger protein A2 (~290 kDa) could be the glycosylated form of HLGP. Protein band labeled A3 (Fig. 5., lane 3, A3; Fig. 6., lane 3, A3) does not have sharp ends. Therefore it may contain also other proteins apart from HLGP and therefore it has not been sent to MS analysis.

The non-reduced HLGP band A1 was cut off the gel and electro-eluted. The extracted HLGP had generally concentration about 400 μg/ml. HLGP was reduced with reducing loading dye and the subunits were separated on 8% polyacrylamide gel. Two larger HLGP polypeptide chains resembling non-reduced HLGP (~260 kDa, ~270 kDa) (Fig. 5., lanes 3-5, B1 and B2) and two reduced peptide chains of the approximate size ~95 kDa and ~100 kDa were visible on the gel (Fig. 5., lanes 3-5, B3 and B4). Furthermore, in some cases, a less visible short chain appeared (~68 kDa) (Fig. 5., line 5, B5). The immunoblotting analysis with anti-HLGP primary antibodies detected three polypeptide chains (Fig. 6). The protein bands B1-B5 were sent for MS analysis.

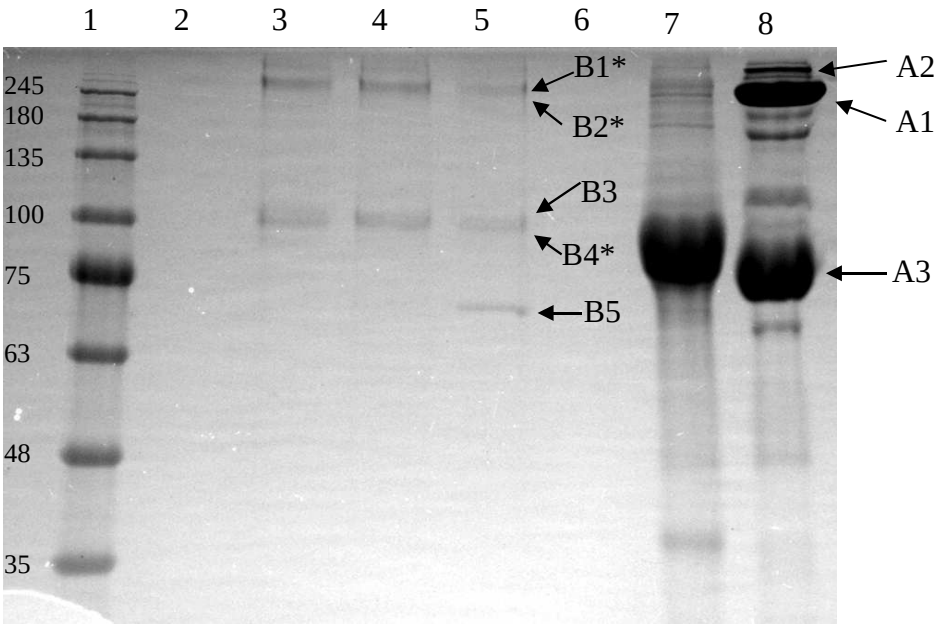

Figure 5: SDS-PAGE 8% gel stained with CBB G-250 dye: 1-pre-stained Protein Ladder, 2-empty, 3-5-reduced HLGP, 6-empty, 7-reduced Hemolymph, 8-non-reduced Hemolymph; the stars indicate proteins containing only LD peptides

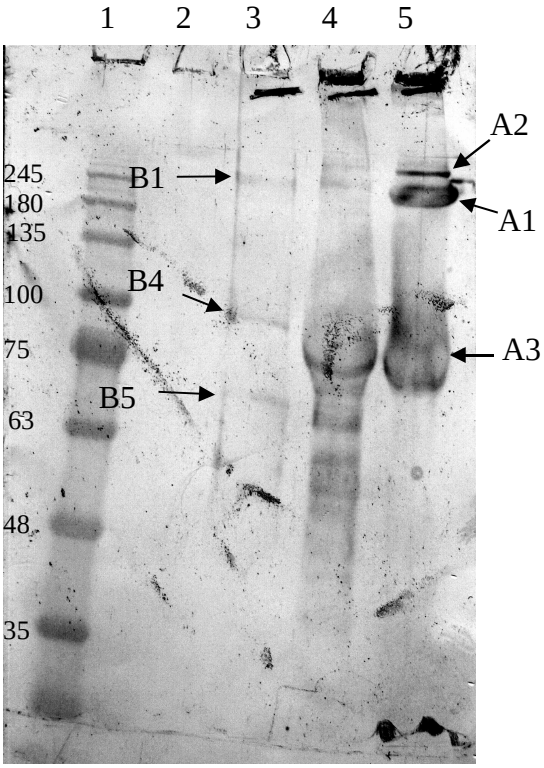

Figure 6: PVDF membrane after signal development: 1-prestained Protein Ladder, 2-empty, 3-reduced HLGP, 4-reduced hemolymph, 5-non-reduced hemolymph

The amino-acid sequences identified by MS were compared to the known DvCP amino acid sequence and classification of peptides into the two subunits was performed.

MS analysis identified A1 and A2 (Fig. 5, line 8, Fig. 6, line 5) bands to be closely related proteins to the DvCP of *D. variabilis*. Thus, the protein is most probably hemelipoglycoprotein (*D. marginatus*, Dupejova et al. 2011). The detected peptides from these bands were analyzed in order to find to which subunits they belong. The non-reduced HLGP protein bands A1 and A2 (Fig. 5, line 8) contained peptides from both subunits (Tab. 24, Fig. 7). The different sizes (260 and 290 kDa) of apparently the same protein could be caused by glycosylation (Dupejova et al., 2011).

Table 24: Examples of peptides detected by MS

	A1	A2
peptides from the subunit containing LD	(K)HNELSVDDADRFYNK(L)	(R)EMVTEPSDQVVAFVSSAFR(S)
	(R)TLDLEEEHDAATTDTTLVEAGEK(E)	(K)GVLSLFQLDLVK(G)
	(R)MAALWALK(Q)	(R)LSLSIFGK(T)
	(Y)GDEDIKEIDNK(E)	(K)NLWNFMGR(R)
peptides from the larger subunit	(K)VFIHTTKLEILPVTEDSGLIVR(V)	(K)ELDIDISYK(F)
	(R)EHGIPFNYYCMKFLR(H)	(K)FTHTDEDEQQLAAAAEGK(A)
	(K)TFYLSHDMTYLNPTELGVPVFFDFK(Q)	(R)YSFNHDLFNHK(L)
	(K)VTLAPLEGK(L)	(R)LAPYYAK(R)
subunit present	both subunits	both subunits

B1 and B2 bands are according to their size (cca 260 kDa) and according to the MS analysis apparently the same peptide chains since all of these were found to be closely related to the DvCP of *D. variabilis*. In these HLGP bands, all the detected peptides belong to lipoprotein N-terminal domain (Tab. 25, Fig. 8, Fig. 5-samples marked with the star). The protein of the size of cca 95 kDa (B4) was analyzed and was identified to be a closely related protein to DvCP. The detected peptides from this samples are also constituents of the lipoprotein N-terminal domain (Tab. 25, Fig. 5-samples marked with the star).

Protein bands B3 and B5 were not identified as tick-related.

Table 25: Examples of peptides detected by MS

	B1	B2	B4
peptides from the subunit containing LD	(K)GVLSLFQLDLVK(G)	(R)EMVTEPSDQVVAFVSSAFR(S)	(R)TLDLEEEHDAATTDTTLVEAGEK(E)
	(R)YVLPLWETNPR(F)	(K)GVLSLFQLDLVK(G)	(R)YVLPLWETNPR(F)
	(R)TLDLEEEHDAATTDTTLVEAGEK(E)	(K)ASSHLIISSTYSPK(Y)	(K)GVLSLFQLDLVK(G)
	(K)HNELSVDDADRFYNK(L)	(R)YVLPEWDHDTR(F)	(K)FDEGKLEEFSIGK(N)
peptides from the larger subunit	X	X	X

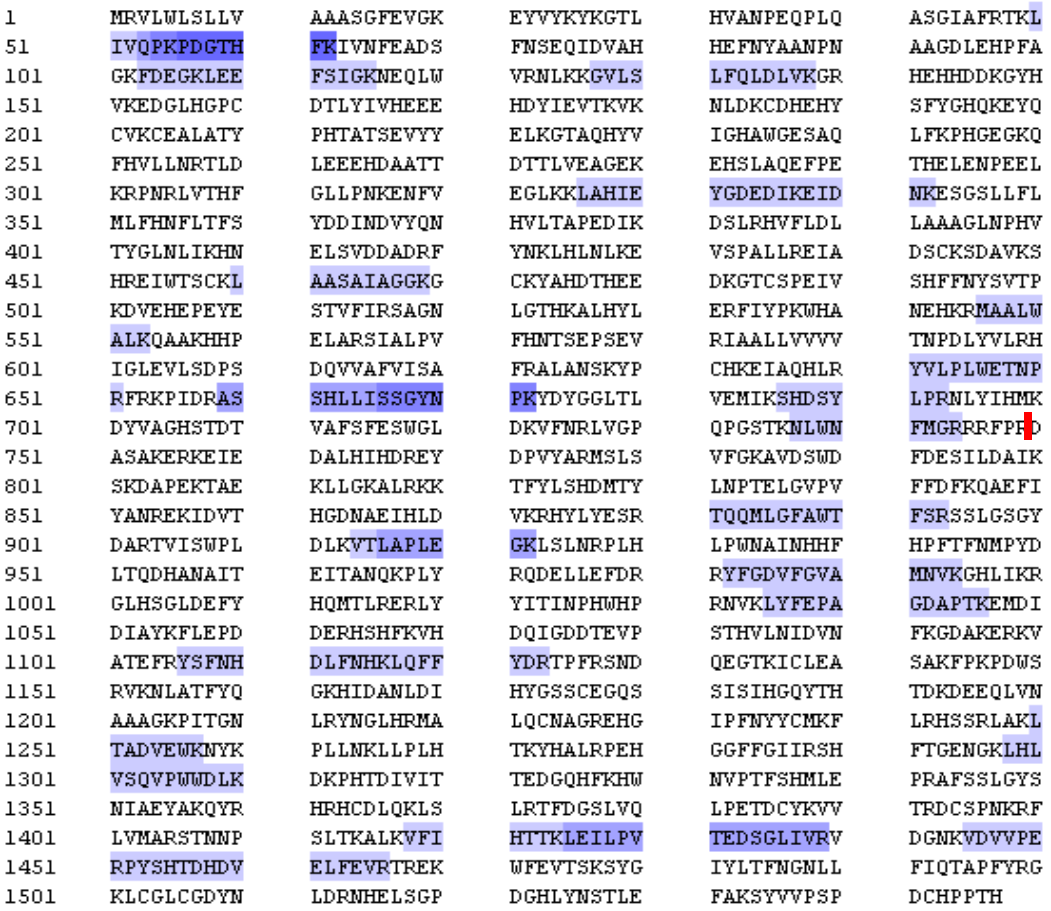

Figure 7: The DvCP a.a. sequence and the coverage map of unreduced HLGP (A1 band from the Fig. 5, line 8); the red line highlights the cleavage site between the two subunits

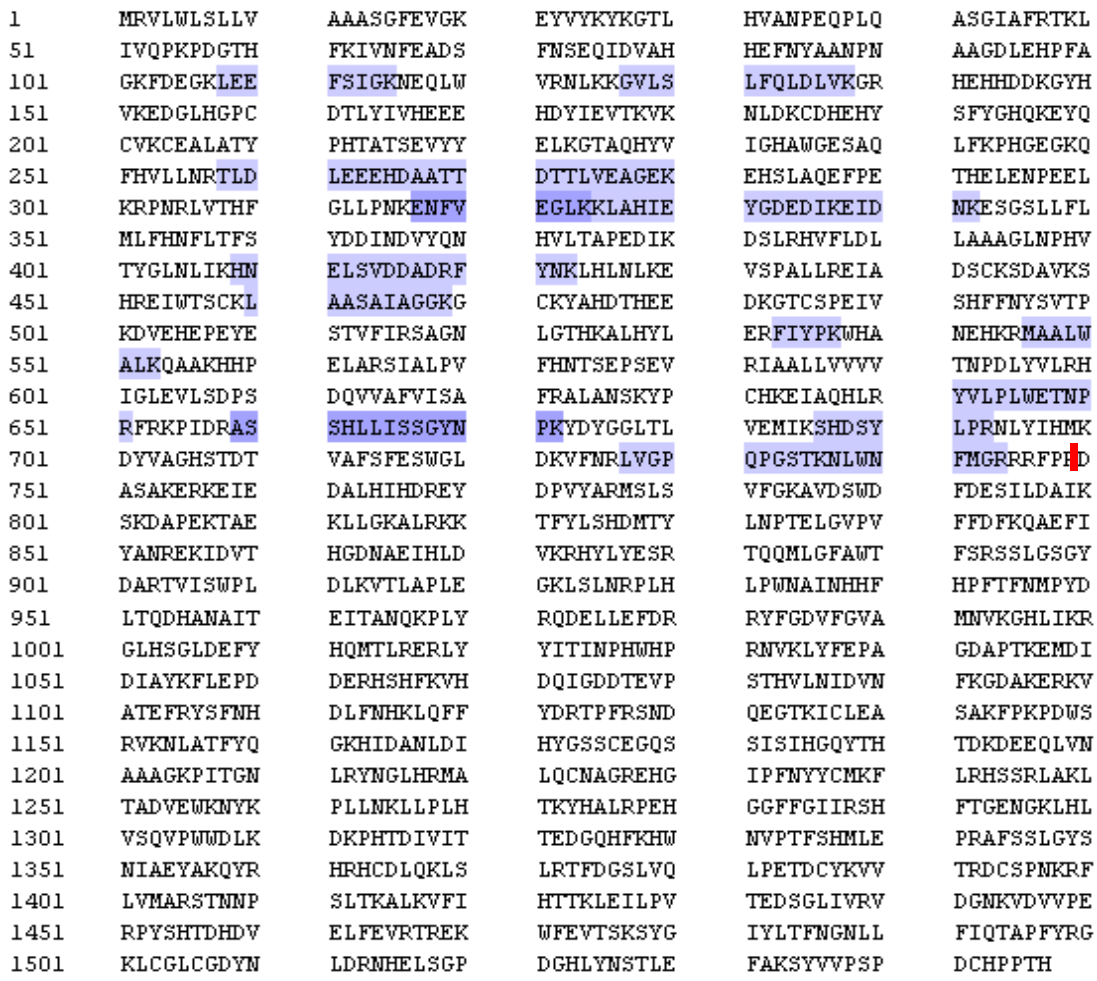

Figure 8: The DvCP a.a. sequence and the coverage map of reduced HLGP (B1 band from the Fig. 5, line 5); the red line highlights the cleavage site between the two subunits

4.2 Recombinant Lipoprotein N-terminal Domain

4.2.1 Cloning with pET-41 Ek/LIC Vector

4.2.1.1 Cloning of D. marginatus DNA sequence encoding for HLGP into pET-41 Ek/LIC Vector

The three cDNAs of *D. marginatus,* obtained from reverse transcription using (A) oligo(dT)18 primer, B) random hexamer and C) gene specific primer HLGP LD R1, were used as a template for PCR with HLGP LD F1-R1 primers. With F1-R1 primers, various polymerases (Taq, GoTaq, KAPA 2G ROBUST) and various cycling programs were tried. Despite all optimization efforts all PCRs provided products of inappropriate sizes.

PCRs with PRE HLGP LD primers resulted mostly in smeared PCR products.

Primers F2-R2 were examined, whether these could anneal directly without previous F1-R1 amplification. The PCR with GoTaq and KAPA 2G ROBUST polymerase and with cDNAs as a template resulted in a smear.

As no DNA encoding for HLGP was obtained, no HLGP recombinant could have been prepared.

4.2.1.2 Cloning of the DvCP sequence (incorporated in 11AALPPP_HLGP_LD_pMK-RQ plasmid) into pET-41 Ek/LIC Vector

Since we were not able to amplify the HLGP sequence, the construct 11AALPPP_HLGP_LD_pMK-RQ carrying nucleotide sequence for DvCP was used for NovaBlue GigaSingles cells transformation. The plasmid was propagated and isolated in high amount (cca 5 mg in 0.5 ml TE).

For the following PCRs, 11AALPPP_HLGP_LD_pMK-RQ plasmid propagated by bacteria was used as the template. PCR-1 with HLGP LD F1-R1 primers, using PPP master mix, resulted in the single PCR product of desired size (Fig. 9, line 2). Therefore, the reaction mixture from PCR-1 was used as a template for PCR-2 with F2-R2 primers. This reaction resulted in PCR products of several sizes including desired size of the product (cca 1800 bp), which was still sufficiently strong (Fig. 10, lines 1-8), therefore, the reaction mixture from PCR-2 was still used as a template for the final PCR-3 with F3-R3 primers. The PCR-3 product of the desired size

was only weak band on the gel (Fig. 11, lines 1-8). Therefore, the whole procedure was repeated with additional purification steps of PCR products in between the PCRs.

PCR-1 product (Fig. 9, line 2) was eluted from the agarose gel. The success of elution was checked by electrophoresis. Purified PCR-1 product was used for control PCR with Control primers for short peptide sequences. The control products corresponded with the expected size, (control products of sizes 164 bp and 95 bp; Fig. 13, lines 1-3, lines 5-7), thus the PCR-1 product was the desired one.

PCR-1 product was used for PCR-2, however, no PCR-2 product was observed (Fig. 12, lines 1-8). The same PCR-2 was tried with TrueStart HotStart Taq DNA polymerase with $MgCl_2$ gradient, with identical results. The same PCR-2 using PPP master mix and DMSO gradient was set up and no PCR products were observed. Therefore we concluded, the F2-R2 primers were not able to anneal to the purified PCR-1 product.

PCR-2 product (from PCR, where PCR-1 reaction mixture was a template; Fig. 10, product marked with the arrow) was eluted from the agarose gel and used for PCR-3. The success of elution was checked by electrophoresis. This purified PCR-2 product was used for control PCR with Control primers, which verified the sequence to be the desired one (Fig. 15). No PCR-3 product was observed (Fig. 14, lines 1-8). Therefore, the F3-R3 primers were probably not able to anneal to the purified PCR-2 product. We did not obtain the desired sequence suitable for cloning into the pET-41 Ek/LIC vector.

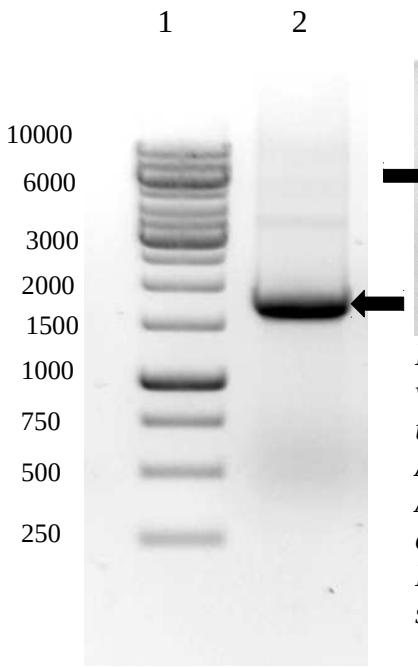

Figure 9: 1% gel, samples stained with SYBR Green: PCR-1 with F1-R1 primers: 1- 1kb DNA ladder, 2-PCR product (annealing temperature=50°C), the arrow shows the desired product-size (cca 1800 bp)

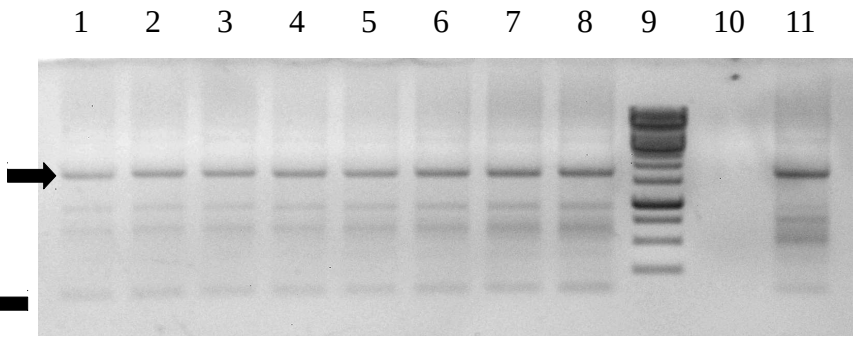

Figure 10: 1% gel, samples stained with SYBR Green: PCR-2 with F2-R2 primers, where PCR-1 product was used as a template; 1-annealing temperature (AT)=45°C, 2-AT=46°C, 3-AT=47.8°C, 4-AT=50.7°C, 5-AT=54.2°C, 6-AT=56.9°C, 7-AT=58.8°C, 8-AT=60°C, 9-1kb DNA ladder, 10-negative control (no DNA template), 11-positive control (PCR with F1-R1 primers and PCR-1 product as a template), the arrow shows the desired product-size

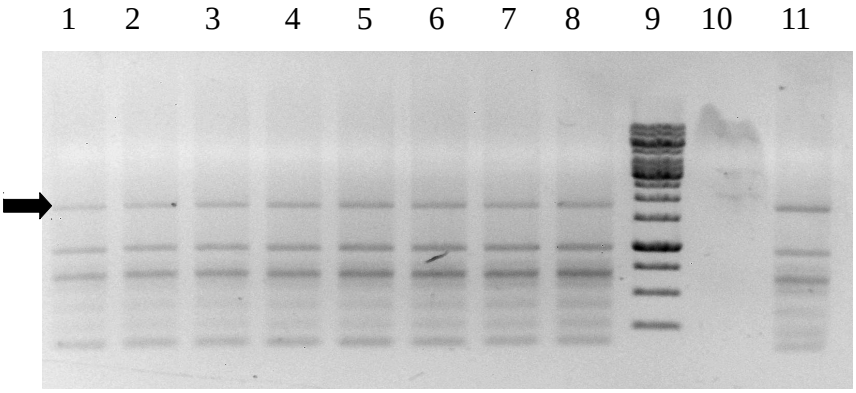

Figure 11: 1% gel, samples stained with SYBR Green: PCR-3 with F3-R3 primers, where PCR-2 product was used as a template; 1-annealing temperature (AT)=45°C, 2-AT=46°C, 3-AT=47.8°C, 4-AT=50.7°C, 5-AT=54.2°C, 6-AT=56.9°C, 7-AT=58.8°C, 8-AT=60°C, 9-1kb DNA ladder, 10-negative control (no DNA template), 11-positive control (PCR with F1-R1 primers and PCR-2 product as a template), the arrow shows the desired product-size

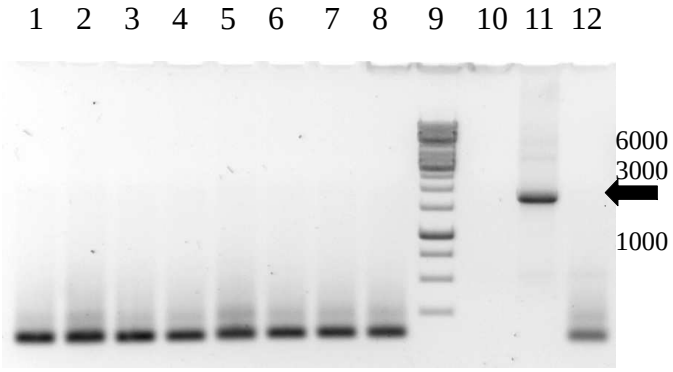

Figure 12: 1% gel, samples stained with SYBR Green: PCR-2 with F2-R2 primers, where purified PCR-1 product was used as a template; 1-AT=45°C, 2-AT=46.4°C, 3-AT=48.8°C, 4-AT=52.6°C, 5-AT=57.1°C, 6-AT=60.9°C, 7-AT=63.4°C, 8-AT=65°C, 9-1kb DNA ladder, 10-negative control (no DNA template), 11-positive control (PCR with F1-R1 primers and 11AALPPP_HLGP_LD_pMK-RQ plasmid as a template), 12-control (PCR with F1-R1 primers); arrow shows the desired product-size

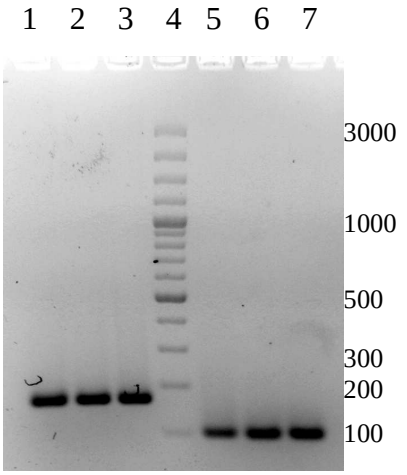

Figure 13: 2% gel, samples stained with SYBR Green: control PCR, where purified PCR-1 product was used as a template; 1-Control-1 primers (AT=50°C), 2- Control-1 primers (AT=55°C), 3- Control-1 primers (AT=58°C), 4-100 bp Plus DNA ladder, 5-Control-2 primers (AT=50°C), 6-Control-2 primers (AT=55°C), 7-Control-2 primers (AT=58°C)

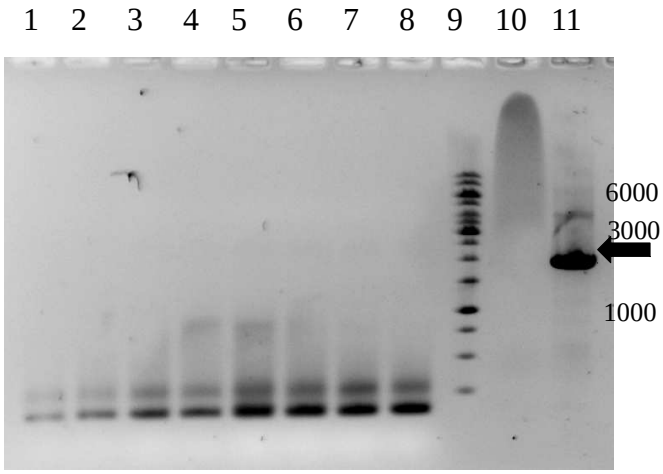

Figure 14: 1% gel, samples stained with SYBR Green: PCR-3 with F3-R3 primers, where purified PCR-2 product was used as a template; 1-AT=45°C, 2-AT=46°C, 3-AT=47.8°C, 4-AT=50.7°C, 5-AT=54.2°C, 6-AT=56.9°C, 7-AT=58.8°C, 8-AT=60°C, 9-1kb DNA ladder, 10-negative control (no DNA template), 11-positive control (PCR with F1-R1 primers and 11AALPPP_HLGP_LD_pMK-RQ plasmid as a template), the arrow shows the desired product-size

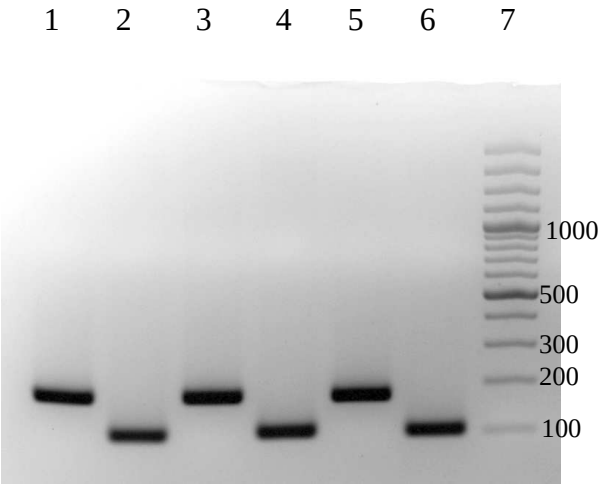

Figure 15: 2% gel, samples stained with SYBR Green: control PCR, where purified PCR-2 product was used as a template; 1-Control-1 primers (AT=50°C), 2-Control-2 primers (AT=50°C), 3-Control-1 primers (AT=55°C), 4-Control-2 primers (AT=55°C), 5-Control-1 primers (AT=58°C), 6-Control-2 primers (AT=58°C), 7-100 bp Plus DNA ladder

4.2.2 Cloning with pRSET/EmGFP Vector

4.2.2.1 Cloning of D. marginatus DNA sequence encoding for HLGP into pRSET/EmGFP Vector

The PCR with HLGP LD GFP primers used A, B, and C cDNAs of *D. marginatus*. No product was observed on the gel (Fig. 16, lines 1-3).

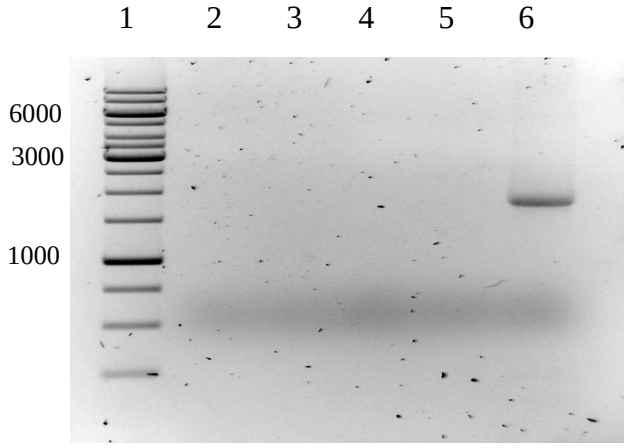

Figure 16: 1% gel, samples stained with SYBR Green: PCR with cDNA of D. marginatus, using HLGP LD GFP primers: 1-1 kb DNA ladder; 2-A cDNA; 3-B cDNA; 4-C cDNA; 5-negative control; 6-positive control

4.2.2.2 Cloning of the DvCP sequence (incorporated in 11AALPPP_HLGP_LD_pMK-RQ plasmid) into pRSET/EmGFP Vector

For the following PCRs, 11AALPPP_HLGP_LD_pMK-RQ plasmid propagated by bacteria was used as the template. The PCR with HLGP LD GFP primers provided single band PCR products of correct size (1838 bp) (Fig. 17, line 2 and 3). The lipoprotein domain nucleotide sequence was amplified using primers with restriction site for BamHI restrictase (forward) and with restriction site for NcoI restrictase (reverse). The PCR products were eluted from the gel and their concentrations were measured on NanoPhotometer (Implen). The control PCR with Control-F1-R1 and F2-R2 primers confirmed, that the product contains the DNA sequences encoding peptides from the LD domain (Fig. 18, lines 1-3, lines 6-8).

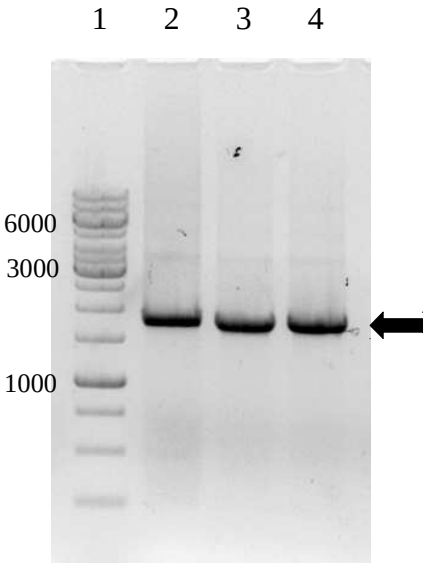

Figure 17: 1% gel, samples stained with SYBR Green: 1-1kb DNA ladder, 2-AT=56°C, 3-AT=56°C, 4-positive control (PCR with F1-R1 primers and original 11AALPPP_HLGP_LD _pMK-RQ plasmid as a template), the arrow shows the desired product-size

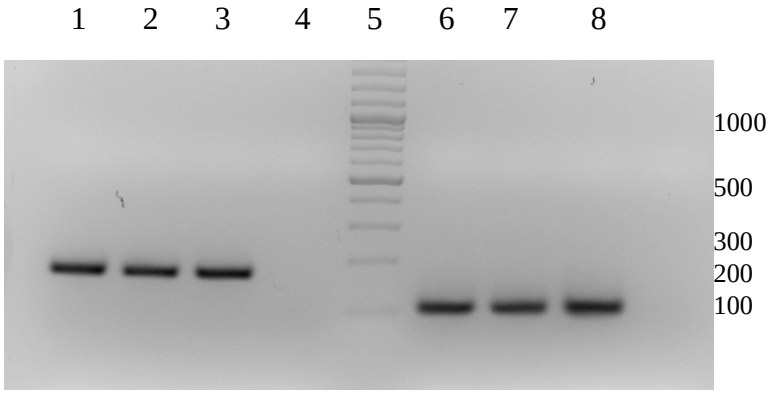

Figure 18: 2% gel, samples stained with SYBR Green: control PCR, where purified PCR product was used as a template; 1-Control-1 primers (AT=50°C), 2-Control-1 primers (AT=55°C), 3-Control-1 primers (AT=58°C), 4-negative control, 5-100 bp Plus DNA ladder, 6-Control-2 primers (AT=50°C), 7-Control-2 primers (AT=55°C), 8-Control-2 primers (AT=58°C), 9- negative control

The purified PCR product encoding for LD of DvCP was double digested with BamHI and NcoI. The pRSET/EmGFP plasmid was also digested with these enzymes. The ligation of the insert with the plasmid was carried out in 3:1 and 1:1 ratio. The restriction took 14 hours and the optimal ligation 24 hours. The ligation mixture was used for transformation and the remaining solution was stored in -20°C for two weeks. The transformation of NovaBlue Singles cells with 1:1 and 3:1 ligation mixture resulted in only three colonies on agar plates with ampicilin resistance. Out of these colonies only one produced plasmid of the desired size of 5.4 kb (Fig. 19, line 10). The parallel transformation of NovaBlue Singles with plasmid 11AALPPP_HLGP_LD_pMK-RQ (carrying kanamycin resistance) was carried out for control, which resulted in 7 colonies. The plasmids isolated from these colonies should have the size of 4.1 kb, however no plasmid of this size was visible (Fig. 19, line 1-7). PCR with HLGP LD GFP

primers using the isolated plasmids as templates demonstrated positive result (Fig. 20, lines 3, 4, 5) and the control PCR with HLGP I 1 and 2 primers showed positive results as well. The pRSET/EmGFP plasmid incorporating LD sequence was sent to sequencing. However good the PCR results seemed to be, the results of the sequencing were negative; no sequence was detected at all.

The transformation of TOP 10 cells with the same ligation mixture was carried out two times with different times of heating shock (30 sec and 90 sec), but both resulted in no colonies at all. The last performed transformation with NovaBlue GigaSingles cells resulted in many single colonies. Twenty of them were cultivated and their plasmids isolated, but none of them contained the desired plasmid (Fig. 21). This suggests possible antibiotic degradation in the media and agar plates. Another experiment will be carried out using freshly prepared antibiotic solution in the near future.

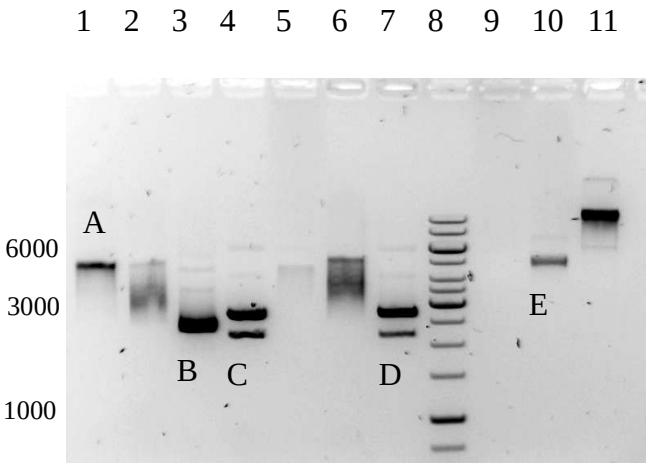

Figure 19: 1% gel, samples stained with SYBR Green: Plasmids isolated from transformed NovaBlue Singles: 1-7 plasmids from the control transformation with 11AALPPP_HLGP_LD_pMK-RQ plasmid, plasmids A-E are tested using PCR (Fig. 23); 8-1kb DNA ladder; 9-plasmid from 3:1 ligation mixture transformation, 10 and 11-plasmids from 1:1 ligation mixture transformation

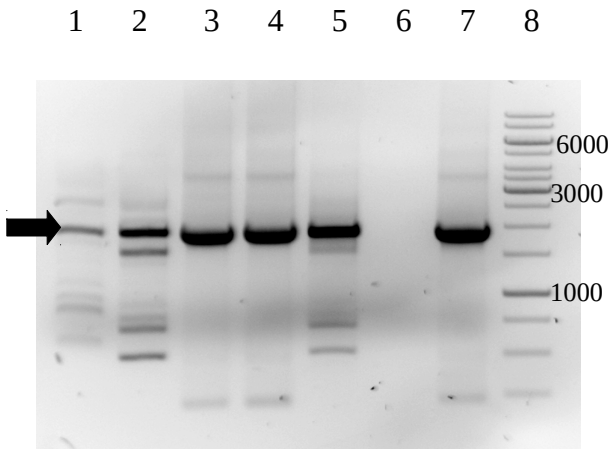

Figure 20: 1% gel, samples stained with SYBR Green: PCR with GFP primers (AT=53°C); isolated plasmids as templates: 1-plasmid A as the template, 2-plasmid B as the template , 3-plasmid C as the template, 4-plasmid D as the template, 5-plasmid E as the template, 6-negative control (no DNA template), 7-positive control (PCR with F1-R1 primers and 11AALPPP_HLGP_LD_pMK-RQ plasmid as a template), 8-1kb DNA ladder; the arrow shows the desired product-size

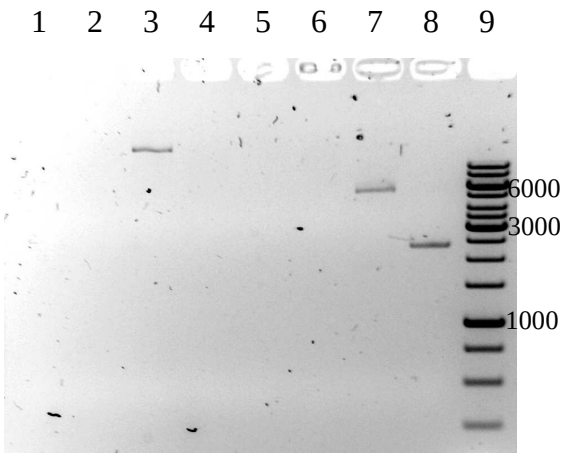

Figure 21: 1% gel, samples stained with SYBR Green: Plasmids isolated from transformed NovaBlue GigaSingles: 1-5 -plasmid from 1:1 ligation mixture transformation; 6,7 - plasmids from the control transformation with 11AALPPP_HLGP_LD_pMK-RQ plasmid; 8-isolated E plasmid; 9-1kb DNA ladder

5. Discussion

This work was focused on the lipoprotein N-terminal domain (LD) of a tick hemelipoglycoprotein (HLGP) (eventually DvCP). The LD is one of the three domains present in tick carrier proteins. The other two domains are von Willebrandt domain type D and a domain with unknown function. The lipoprotein N-terminal domain is expected to be able to bind lipids, carbohydrates, and metals (Donohue et al., 2008). The DvCP has two subunits (92 and 98 kDa) produced by cleaving in the cleavage site a.a. 746-749 (RXXR) (Donohue et al., 2008). The native HLGP contains two polypeptide chains (95 and 100 kDa) as identified by SDS-PAGE, but the presence of the two subunits was not proven yet definitively (Dupejova et al., 2011). The smaller subunit of DvCP and presumably HLGP contains the lipoprotein domain, which consists of 607 a.a..

The unreduced hemolymph proteins of *D. marginatus* were separated by SDS-PAGE and anti-HLGP antibodies detected two proteins of size cca 260 and 290 kDa. These protein sizes correspond with the sizes of the non-glycosylated and glycosylated form of HLGP, respectively (Dupejova et al., 2011). These HLGP forms were analyzed by MS in this work and both proteins contain peptides from both subunits.

The non-glycosylated HLGP was further reduced with reducing Loading Dye in order to separate the reducible parts of the protein via SDS-PAGE.

Certain part of the HLGP remained unreduced with the size of cca 260 kDa. According to MS analysis, this unreduced HLGP contains solely peptides from the smaller subunit containing LD. No peptides from the second slightly bigger subunit were detected. The presence of unreduced HLGP (260 kDa) after reduction with DTT suggests, that there are other forces/bonds than disulfide bonds enabling association of the smaller subunits only. One possible explanation would be inter-subunits connection via covalent dityrosin bonds, that can occur in tyrosin-rich proteins or can be induced by incubation with peroxidases (Atwood et al., 2004; Mai et al., 2011). Another possibility would be the failure of reducing using DTT because of the sample redox conditions. For example, the presence of Ca^{2+} stabilizes the disulfide bonds (Dirlam-Schatz and Attie, 1998).

The reduced HLGP chain appeared as cca 95 kDa size protein band on 8% gel. According to MS results, this chain contained peptides of the smaller subunit including LD. No other

polypeptide chain was observed at all. However, Dupejova and colleagues (2011) detected two HLGP chains of size 95 and 100 kDa. As it is very difficult to visually distinguish protein bands of such similar size, slight differences in separation conditions may result in lack of resolution in this case. It is possible, that our samples were not resolved sufficiently due to usage of 8% gel, while Dupejova and colleagues (2011) used gradient gels.

In this thesis, peptides from the smaller subunit, corresponding to the shorter polypeptide chain (95 kDa), has been present in both reduced and non-reduced samples, whereas peptides from the bigger subunit were present only in non-reduced HLGP. In compliance with the HLGP size (260 or 290 kDa), it is probable, that at least three polypeptide chains create the native protein. It is possible, that HLGP contains different chains combined in various ways. Furthermore, it seems that small subunits represent the majority in non-reduced HLGP structure. Similarly, the vitellin of *D. variabilis* is an oligomer (possibly dimer), which is comprised of mixture of uncleaved monomers and its subunits (Thompson, 2007). I hypothesize that the bigger subunit was not recognized either on the gel by CBB staining or by MS in the reduced form due to its insufficient amount in the samples.

The irregular occurrence of 68 kDa sized peptide chain between the reduced HLGP chains, revealed in the study, may indicate even wider variability. This small chain could have been also just the result of HLGP contamination with other proteins when this was cut off the gel. However, this 68 kDa chain was detected on the membrane using anti-HLGP antibodies, therefore the mistake is not a likely possibility.

From results of this thesis it seems, that the smaller subunit corresponding to the shorter peptide chain including the LD domain is probably the basic building block of the protein. On the other hand, DvVg, which also contains LD (on the N-terminus) and von Willebrand domain (on the C-terminus), has the cleavage site RXXR placed in two thirds of the lipoprotein domain (Thompson, 2007). The position of a cleavage site directly in the presumed LD may indicate a different function (lack of function) of the domain in Vgs compared to CPs. As the LD is part of tick (Thompson et al., 2007), as well as insect vitellogenins (Chen et al., 1996), and the vitellogenin receptors belong to the low-density lipoprotein receptor family (in insect: Sappington et al., 1998; in ticks: GenBank: AAZ31260.3), it comes forward that the lipoprotein domain may serve for protein recognition and that some CP receptors will be revealed in the future. However, the lipoprotein N-terminal domain contained in the small subunit of CPs seems

to be the necessary part of the heme-binding proteins among ticks.

The original intention to clone the DNA encoding for LD of HLGP from *D. marginatus* and prepare it in recombinant form using primers designed according to the known DvCP nucleotide sequence was based on assumption, that DvCP from *D. variabilis* and HLGP from *D. marginatus* are closely related and thus their sequences should be very similar. Since the LD is also present in heme-binding vitellogenins, we assume this part of the carrier proteins to be conserved. Therefore, based on this similarity assumption, all the primers were designed according to the known DvCP sequence (Donohue et al., 2008).

As all the PCRs using cDNA of *D. marginatus* failed, either the cDNA was degraded or the sequences encoding for HLGP and DvCP differ in at least the beginning and the end of the LD sequence. Although this seems unlikely, the vitellogenin sequences of *D. variabilis* and *O. moubata* also differ in the N-terminus and partly in C-terminus (Gen Bank: AAW78557.2 and BAH02666.2).

Next, the vector carrying the nucleotide sequence encoding for DvCP was used as a template for PCRs with HLGP LD 1, 2 and 3 primers. Despite various PCR modifications, no product was obtained. The primers 1 and 2 were able to anneal to the vector sequence and the PCR product identity was confirmed with control PCR. However, the PCR products showed to be inappropriate template for all the primers as none of these could anneal to its own PCR product. The possible inhibition of the PCR by formation of secondary structures in the template was excluded, because the addition of DMSO into the PCR reaction did not change the result. Therefore the possibility of multiple prolongation of the sequence in order to create the suitable cloning sites was rejected.

Different PCR with the vector carrying the nucleotide sequence encoding for DvCP LD was carried out in order to clone the LD sequence into the pRSET/EmGFP vector. The difference against the above described experiment is only in the number of primers. While for cloning into pET vector, three primers were needed, for cloning into pRSET only one pair of primers is needed. There were therefore no problems with obtaining the desired product sequence (Fig. 17). The cloning via restriction sites was done in order to maintain the correct orientation of the insert in the plasmid. The bacterial transformation was carried out with several types of competent cells, none to provide generally very good efficiency. The mixed results of no

colonies and many colonies, carrying no plasmid, suggest combination of low transformation efficiency combined with antibiotic degradation in media resulting in lack of selection. No pRSET/EmGFP plasmid with inserted LD sequence, validated by sequencing, was obtained so far.

6. Conclusion

The aim of this thesis was to isolate the HLGP subunits and to determine whether the lipoprotein N-terminal domain is present in the subunits. Next, the LD should be prepared as recombinant protein and purified.

The HLGP protein was detected in the hemolymph of *D. marginatus* in the putative glycosylated (290 kDa) and non-glycosylated form (260 kDa). The non-glycosylated HLGP was reduced, its polypetide chains were identified by immunoblotting and analyzed by MS. The size of the shorter chain was estimated to be 95 kDa. The longer chain (detected in studies of Dupejova and colleagues, 2011) was not detected at all. Part of the reduced HLGP resembled non-reduced protein (260 kDa) and was analyzed with MS.

Both subunits were identified in both glycosylated and non-glycosylated HLGP isolated from the hemolymph. The smaller subunit containing the lipoprotein N-terminal domain was the only identified subunit in the protein after reduction (in both 95 kDa polypeptide chain and the 260 kDa whole HLGP).

The recombinant HLGP lipoprotein domain could not be obtained from cDNA of *D. marginatus* because of probable sequence differences between the actual HLGP sequence and the known DvCP sequence.

The sequence encoding for DvCP was cloned into pRSET/EmGFP plasmid and transformed into several kinds of competent cells for propagation. No plasmid with the insert verified by sequencing was obtained yet.

7. References

Atwood C. S., Perry G., Zeng H., Kato Y., Jones W. D., Ling K. Q., Huang X., Moir R. D., Wang D., Sayre L. M., Smith M. A., Chen S. G., Bush A. I. (2004), Copper mediates dityrosine cross-linking of Alzheimer's amyloid-beta. Biochemistry 43(2): 560-8.

Angelov L., Dimova P., Berbencova W. (1996), Clinical and laboratory evidence of the importance of the tick *D. marginatus* as a vector of *B. burgdorferi* in some areas of sporadic Lyme disease in Bulgaria. European Journal of Epidemiology, Oct;12(5): 499-502.

Beati L., Finidori J. P. and Raoult R. (1993), First isolation of *Rickettsia slovaca* from *Dermacentor marginatus* in France. The American Journal of Tropical Medicine and Hygiene 48: 257–268.

Braz G. R. C., Coelho H. S. L., Masuda H. and Oliveira P. L. (1999), A missing metabolic pathway in the cattle tick *Boophilus microplus.* Current Biology 9: 703-706.

Buresova V., Hajdusek O., Franta Z., Sojka Z., Kopacek P. (2009), IrAM-An alpha2 macroglobulin from the hard tick *Ixodes ricinus*: characterization and function in phagocytosis of a potential pathogen *Chryseobacterium indologenes*. Developmental and Comparative Immunology 33: 489-98.

Cabrera A. R., Donohue K. V., Khalil S. M. S., Sonenshine D. E., Roe R. M. (2009), Characterization of vitellin protein in the twospotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae). Journal of Insect Physiology 55: 655-661

Chen J, Sappington T, Raikhel A (1997), Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. Journal of Molecular Evolution 44: 440-451

Chinzei, Y., Chino, H., Takahashi, K. (1983), Purification and properties of vitellogenin and vitellin from a tick, *Ornithodoros moubata*. Journal of Comparative Physiology 152B: 13–21.

Dirlam-Schatz K., Attie A. D. (1998), Calcium induces a conformational change in the ligand binding domain of the low density lipoprotein receptor. The Journal of Lipid Research 39: 402-411.

Coons L. B., Rosell-Davis R., Tarnowski B. I. (1986), Bloodmeal digestion in ticks. In: Sauer, J.R. and Hair, J.A., editors. Morphology, Physiology and Behavioral Biology of Ticks, pp. 248-279. Ellis Horwood, Chinchester, England.

Donohue K. V., Khalil S. M. S., Mitchell R. D., Sonenshine D. E., Roe R. M. (2008), Molecular characterization of the major hemelipoglycoprotein in ixodid ticks. Insect Molecular Biology 17: 197-208.

Dupejova J. (2011), diploma thesis: Hemlipoglykoprotein z hemolymfy klíštěte *Dermacentor marginatus*: purifikace a biochemická charakterizace. Hemelipoglycoprotein from the *Dermacentor marginatus* hemolymph: purification and biochemical characterization. Mgr. Thesis, in Czech. University of South Bohemia in České Budějovice, Faculty of Science

Dupejova J., Sterba J., Vancova M., Grubhoffer L. (2011), Hemelipoglycoprotein from the ornate sheep tick, *Dermacentor marginatus*: structural and functional characterization. Parasites & Vectors, 4: 4

Estrada-Peña A. and Jongejan F. (1999), Ticks feeding on humans: a review of records on human-biting *Ixodoidea* with special reference to pathogen transmission. Experimental and Applied Acarology 23: 685–715

Gregson J. D. (1958), Host susceptibility to paralysis by the Tick *Dermacentor andersoni* Stiles (*Acarina: Ixodidae*). The Canadian Entomologist, 90: 421-424

Grubhoffer L., Mat'ha V. (1991), New lectins in invertebrates. Zoological Science 8: 1001–1003.

Grubhoffer L., Kovar V. (1998), Arthropod lectins: affinity approaches in analysis and reparation of carbohydrate binding proteins. In: Wiesner, A., Dunphy, G.B., Marmars, V.J., Morishima, I., Sugamaran, M., Yamakava, M., editors. Techniques in Insect Immunology FITC-5., pp. 47-57. SOS Publications, New Haven USA.

Grubhoffer L., Veres J., Dusbabek F. (1991). Lectins as the molecular factors of recognition and defence reaction of ticks. In: Dusbabek, F., Bukva, V. (Eds.) Modern Acarology, Vol. 2. Academia, Prague, pp. 381–388.

Guddera N. P., Neese P. A., Sonenshine D. E., Apperson C. S., Roe R. M. (2001), Developmental profile, isolation, and biochemical characterization of a novel lipoglycoheme-carrier protein from the American dog tick, *Dermacentor variabilis* (Acari: Ixodidae) and observations on a similar protein in the soft tick, *Ornithodoros parkeri* (Acari: Argasidae). Insect Biochemistry and Molecular Biology 31: 299-311.

Guddera N. P, Sonenshine D. E., Apperson, C. S. and Roe R. M. (2002a), Tissue distribution and characterization of predominant hemolymph carrier proteins from *Dermacentor variabilis* and *Ornithodoros parkeri*. Journal of Insect Physiology 48: 161-170.

Guddera N. P, Sonenshine D. E., Apperson C. S. and Roe R. M. (2002b), Hemolymph proteins in ticks. Journal of Insect Physiology 48: 269-278.

Guglielmone A. A., Robbins R. G., Apanaskevich D. A., Petney, T. N. Estrada-Peña, A. Horak, I. G. Shao, R. and Barker S. C. (2010), The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. Zootaxa, 2528: 1–28

Hadwen S. (1913), On "tick paralysis" in sheep and man following bites of *Dermacentor venustus.* Parasitology, 6: 283-297

Hajdusek O., Almazán C., Loosova G., Villar M., Canales M., Grubhoffer L., Kopacek P., de la Fuente J. (2010), Characterization of ferritin 2 for the control of tick infestations. Vaccine 28: 2993–2998

Hamza I., Chauhan S., Hassett R., O'Brian M. R. (1998), The bacterial IRR protein is required for coordination of heme biosynthesis with iron availability. Journal of Biological Chemistry 273: 21669-21674.

Hoffmann J. A., Kafatos F. C., Janeway C. A. J. R., Ezekowitz R. A. B. (1999), Phylogenetic perspectives in innate immunity. Science 284: 1313–1318.

Horn M., Nussbaumerova M., Sanda M., Kovarova Z., Srba J., Franta Z. et al. (2009), Hemoglobin digestion in blood-feeding ticks: mapping a multipeptidase pathway by functional proteomics. Chemistry & Biology 16: 1053–1063

Huang X., Tsuji N., Myioshi T., Nakanuta-Tsuruta S., Hiabayashi J., Fujisaki K. (2007), Molecular characterization and oligosaccharide-binding properties of a galectin from the argasid tick *Ornithodoros moubata*. Glycobiology 17: 313-23.

Hubalek Z., Halouzka J. (1999), West Nile fever – a reemerging mosquito-borne viral disease in Europe. Emerging Infectious Disseases 5: 643-50.

Hubalek Z., Juricova Z., Halouzka J. (1990), *Francisella tularensis* from ixodid ticks in Czechoslovakia. Folia Parasitologica. 37: 255-60.

Hubalek Z., Mittermayer T., Halouzka J., Cerny V. (1988), Isolation of "exotic" Bhanja virus (Bunyaviridae) from ticks in the temperate zone. Archives of Virology 101: 191-7.

James A.M., Oliver J.H. (1996), Vitellogenin concentrations in the hemolymph and ovaries of *Ixodes scapularis* ticks during vitellogenesis. Experimental and Applied Acarology 20: 639–647.

James A. M., Oliver J. H. (1997), Purification and partial characterization of vitellin from the black-legged tick, *Ixodes scapularis*. Insect Biochemistry and Molecular Biology 27: 639–649.

James A. M., Oliver J. H. (1999), Localization of vitellogenin production in the blacklegged tick, *Ixodes scapularis* (Acari: Ixodidae). Invertebrate Reproduction and Development 35: 81–87.

Jongejan F. and Uilenberg G. (2004), The global importance of ticks. Parasitology 129: S3–S14.

Kondratenko V. F. (1976), Importance of ixodid ticks in transmission and preservation of the causative agent of Crimean-Congo hemorrhagic fever in the foci of infection. Parazitology 10: 297–302. (In Russian, English translation NAMRU3 T116).

Kopacek P., Weise C., Saravanan T., Vitova K., Grubhoffer, L. (2000), Characterization of an macroglobulin-like glycoprotein isolated from the plasma of the soft tick *Ornithodoros moubata*. European Journal of Biochemistry 267: 465–475.

Kovar V., Kopacek P., Grubhoffer L. (2000), Isolation and characterization of Dorin M, a lectin from plasma of the soft tick *Ornithodoros moubata*. Insect Biochemistry and Molecular Biology 30: 195-205.

Kozuch O., Nosek J. (1971), Transmission of tick-borne encephalitis (TBE) virus by *Dermacentor marginatus* and *D. reticulatus* ticks. Acta Virologica 15: 334.

Kuhn K. H., Uhlir J., Grubhoffer L. (1996), Ultrastructural localization of a sialicspecific hemolymph lectin in hemocytes and other tissues of the hard tick *Ixodes ricinus* (Acari:Ixodidae). Parasitology Research 82:215-221.

Labuda M., Alves M. J., Elecková E., Kozuch O., Filipe A. R. (1997), Transmission of tick-borne bunyaviruses by cofeeding ixodid ticks. Acta Virologica 41: 32-328

Lara F. A., Lins U., Paiva-Silva G., Almeida I. C., Braga C. M., Miguens F. C., Oliveira P. L. and Dansa-Petretski M. (2003), A new intracellular pathway of haem detoxification in the midgut of the cattle tick *Boophilus microplus*: aggregation inside a specialized organelle, the hemosome. The Journal of Experimental Biology 206: 1707–1715.

Logullo C., Moraes J., Dansa-Petretski M., Vaz JR. I.S., Masuda A., Sorgine M.H.F., Braz G.R., Masuda H., Oliveira P.L. (2002), Binding and storage of heme by vitellin from the cattle tick, *Boophilus microplus*. Insect Biochemistry and Molecular Biology 32:1805-1811.

Mai K., Smith N. C., Feng Z.-P, Katrib M., Šlapeta J. Šlapetova I., Wallach M. G., Luxford C., Davies M. J., Zhang X., Norton R. S., Belli S. I. (2011), Peroxidase catalysed cross-linking of an intrinsically unstructured protein via dityrosine bonds in the oocyst wall of the apicomplexan parasite, *Eimeria maxima.* International Journal for Parasitology 41: 1157-1164

Mans B. J., de Klerk D., Pienaar R. and Latif A. A. (2011), *Nuttalliella namaqua*: A living fossil and closest relative to the ancestral tick lineage: Implications for the evolution of blood-feeding in ticks. PLoS ONE 6(8): e23675.

Markeshin S. I. A., Smirnova S. E., Evstafev I. L. (1992), An assessment of the status of natural foci of Crimean-Congo hemorrhagic fever in the Crimea. Journal of Microbiology Epidemiology and Immunobiology 4: 28-31.

Maya-Monteiro C. M., Daffre S., Logullo C., Lara F. A., Alves E. W., Capurro M. L., Zingali R., Almeida I. C., Oliveira P. L. (2000), HeLp, a heme lipoprotein from the hemolymph of the cattle tick, *Boophilus microplus.* Journal of Biological Chemistry 275: 36584-9.

Maya-Monteiro C. M., Alves L. R., Pinhal N., Abdalla D. S. P. and Oliveira P. L. (2004), HeLp, a heme-transporting lipoprotein with an antioxidant role. Insect Biochemistry and Molecular Biology 34: 81-87.

Miyoshi T., Tsuji N., Islam M. K., Huang X., Motobu M., Alim M. A., Fujisaki K. (2007), Molecular and reverse genetic characterization of serine proteinaseinduced hemolysis in the midgut of the ixodid tick *Haemaphysalis longicornis*. Journal of Insect Physiology 53: 195–203.

Nuttall P. N. and Labuda M. (1994), Tick-borne encephalitis subgroup. In: Sonenshine and Mather, editors. Ecological dynamics of tickborne zoonoses, pp. 351–481. Oxford University Press, Oxford.

Oteo J. A., Ibarra V., Blanco J. R., Martı´nez de Artola V., Ma´rquez F. J., Portillo A., Raoult D. and Anda P. (2004), *Dermacentor*-borne necrosis erythema and lymphadenopathy: clinical and epidemiological features of a new tick-borne disease. Clinical Microbiology and Infection 10: 327–331

Pietrobelli M., Cancrini G., Moretti A., Tampieri M.P. (2007), Animal babesiosis: an emerging zoonosis also in Italy? Parassitologia 1: 33-8.

Rehacek J., Kovacova E., Lisak V. and Rumin W. (1990), Occurrence of *Coxiella burnetii*, *Rickettsia slovaca* and organisms resembling bacillary *rickettsiae* in their natural foci in Slovakia 20 years after their first detection. Folia Parasitologica 37: 285–286.

Rizzoli, A., Hauffe H.C., Carpi, G., Vourc'h G.I., Neteler, M., & Rosà, R. (2011), Lyme borreliosis in Europe. Eurosurveillance 16(27): 19906.

Rosell R., Coons L.B. (1991), Purification and partial characterization of vitellin from the eggs of the hard tick, *Dermacentor variabilis*. Insect Biochemistry 21,: 871–885.

Rosell R., Coons L.B. (1992), The role of fat body, midgut and ovary in vitellogenin production and vitellogenesis in the female tick, *Dermacentor variabilis*. International Journal of Parasitology 22: 341–349.

Sanger F. S., Nicklen S. and Coulson A. E. (1977), DNA sequencing with chain-termination inhibitors. Proceedings of the National Academy of Sciences USA 74: 5463-5467.

Sappington T. W., Raikhel A. S. (1998), Molecular characteristics of insect vitellogenins and vitellogenin receptors. Insect Biochemistry and Molecular Biology 28: 277-300

Smetanova K., Schwarzova K., Kocianova E. (2006), Detection of *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Rickettsia* spp., and *Borrelia burgdorferi* s. l. in ticks, and wild-living animals in western and middle Slovakia. Annals of the New Your Acadeny of Sciences 1078: 312-5.

Sonenshine D.E. (1991), Biology of ticks. Vol. 1. Oxford University Press, New York.

Sullivan C. D., Rosell R. C., Coons L. B. (1999), Partial characterization of vitellogenin from the ixodid *Dermacentor variabilis*: Preliminary results. In: Needham, G.R., Mitchell, R., Horn, D.J., Welbourn, W.C., Editors. Acarology IX, Volume 2, Symposia, July 17 - 22, 1999. pp. 477–480. Ohio Biological Survey, Columbus, Ohio.

Sréter T., Széll Z., Varga I. (2005), Spatial distribution of *Dermacentor reticulatus* and *Ixodes ricinus* in Hungary: evidence for change? Veterinary Parasitology 128: 347-351

Sumilo D., Bormane A., Asokliene L., Vasilenko V., Golovljova I., Avsic-Zupanc T., Hubalek Z. and Randolph S. E. (2008), Socio-economic factors in the differential upsurge of tick-borne encephalitis in central and Eastern Europe. Reiews in Medical Virology 18: 81–95.

Süss J. (2003), Epidemiology and ecology of TBE relevant to the production of effective vaccines. Vaccine 21: S1 16-35

Taylor, D., Chinzei, Y. (2001), Vitellogenin and its synthesis in the soft ticks. In: Halliday, R.B., Walter, D.E., Proctor, H.C., Norton, R.A., Colloff, M.J., Editors. Acarology: Proceedings of the 10th International Congress, Canberra, Australia, July 5-10, 2001. pp. 622–627, CSIRO Publishing, Collingwood, Australia.

Tarnowski B. I., Coons L. B. (1989), Ultrastructure of the midgut and bloodmeal digestion in the adult tick *Dermacentor variabilis*. Experimental and Applied Acarology 6: 263-289.

Thompson D. M., Khalil S. M. S., Jeffers L. A., Ananthapadmanaban U., Sonenshine D. E., Mitchell R. D., Osgood C. J., Apperson C. S., Michael Roe R. (2005), *In vivo* role of 20-hydroxyecdysone in the regulation of the vitellogenin mRNA and egg development in the American dog tick, *Dermacentor variabilis* (Say). Journal of Insect Physiology 51: 1105-1116.

Thompson D. M., Khalil S. M. S., Jeffers L. A., Sonenshine D. E., Mitchell R. D., Osgood C. J., Michael Roe R. (2007), Sequence and the developmental and tissue specific regulation of the first complete vitellogenin messenger RNA from ticks responsible for heme sequestration. Insect Biochemistry and Molecular Biology 37: 363-74.

Veres J., Grubhoffer L. (1991), Detection and partial characterisation of a new plasma lectin in the hemolymph of the tick *Ornithodoros tartakovskyi*. Microbiology Letters 45, 61–64.

Winter C. E., Penha C., Blumenthal T. (1996), Comparison of a vitellogenin gene between two distantly related rhabditid nematode species. Molecular Biology and Evolution 13: 674-684