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IDENTIFICATION OF SALIVARY ANTIGENS
FROM THE DEER TICK *IXODES SCAPULARIS*
AS NOVEL VACCINE CANDIDATES

BY
MARTIN BECKER

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

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UNIVERSITY OF RHODE ISLAND

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MASTER OF SCIENCE
OF
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2013

Abstract

Ticks act as vectors for a number of different pathogens such as *Borrelia burgdorferi* the causative agent of Lyme disease. The most prominent tick vector in the United States is the deer tick *Ixodes scapularis*. Tick bites are of special public health concern since there are no vaccines available against most tick transmitted pathogens. Based on the observation that host animals such as guinea pigs or humans can develop adaptive immune responses to tick bites, anti-tick vaccination is a potential approach to mitigate health risks associated with tick bites.

The study presented here aimed at identifying immunogenic salivary proteins from *I. scapularis* recognized by human immune sera. To identify these potential antigens, which later on need to be characterized with regards to their vaccination potential, a phage display approach was utilized. Antigen libraries derived from salivary gland mRNA of 18 h fed *I. scapularis* were screened with human immune sera.

Screening with an antigen library derived from nymphal ticks led to identification of a metalloprotease. This enzyme has been described previously and appears promising as a novel vaccine candidate. Furthermore, it has close homologs in other ixodid species raising its potential as a universal vaccine.

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List of Abbreviations

(v/v)	Volume per volume
(w/v)	Weight per volume
°C	Degree celsius
µg	Micro gram
µl	Micro liter
µm	Micro meter
2xYT	Two times yeast tryptone medium
2xYT-AK	2xYT medium supplemented with ampicillin and kanamycin
2xYT-GA	2xYT medium supplemented with glucose and ampicillin
2xYT-GK	2xYT medium supplemented with glucose and kanamycin
2xYT-T	2xYT medium supplemented with tetracycline
APS	Ammonium persulfate
<i>B. burgdorferi</i>	<i>Borrelia burgdorferi</i>
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CIP	Calf intestinal phosphatase
CPCR	Colony polymerase chain reaction
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
g	Gram
gDNA	Genomic deoxyribonucleic acid
h	Hour
HRP	Horseradish peroxidase
<i>I. pacificus</i>	<i>Ixodes pacificus</i>
<i>I. ricinus</i>	<i>Ixodes ricinus</i>
<i>I. scapularis</i>	<i>Ixodes scapularis</i>
Ig	immunoglobulin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilo bases
kDa	Kilo dalton
kV	Kilo volts
l	Liter
M	Molar
mA	Milli ampere

Metis	Metalloprotease Ixodes ricinus
min	Minute
ml	Milli liter
mM	Milli molar
M-MLV	Murine leukemia viruses
MP1	Metalloprotease 1
MPBST	Milk powder phosphate buffered saline tween 20
mRNA	Messenger ribonucleic acid
MTP	Microtiter plate
NaAc	Sodium acetate
NBT	Nitro blue tetrazolium
NCBI	National center for biotechnology information
ng	Nano gram
NHS	<i>N</i> -Hydroxysuccinimide
nm	Nano meter
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween 20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
rpm	Rounds per minute
rRNA	Ribosomal ribonucleic acid
s	Second
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGH	Salivary gland homogenate
SOC	Super Optimal broth with catabolite repression
ssDNA	Single stranded deoxyribonucleic acid
TAE	Trisbase, acetic acid, EDTA
TEMED	Tetramethylethylenediamine
tHRF	Tick Histamine Release Factor
TMB	3,3',5,5'-Tetramethylbenzidine
Tris	Tris(hydroxymethyl)aminomethane
U	Unit
UTR	Untranslated region
V	Volt
x g	Times gravity
α	Anti

1 Introduction

1.1 Ticks act as vectors for a number of disease causing agents

Pathogen transmission by ticks is a worldwide problem that causes significant health risks and economic losses (Wikel and Alarcon-Chaidez, 2001). The hard tick *Ixodes scapularis*, which is prevalent in the United States especially in the north east, functions as a vector for a number of pathogens such as the Lyme disease spirochete *Borrelia burgdorferi*, as well as the agents of human anaplasmosis (*Anaplasma phagocytophilum*) (Dai et al., 2010), human babesiosis (*Babesia microti*) and selected flaviviruses (Schuijt et al., 2011b) such as an encephalitis causing virus (Turtle et al., 2012) amongst other examples. There are currently no vaccines available for most of these tick-transmitted infections frustrating prevention of health risks posed by tick-bites.

Ticks are obligate ectoparasites that require blood meals which can be taken from a vast array of different hosts such as mammals, birds or even reptiles (Francischetti et al., 2009). Furthermore, the blood meal is essential for the tick with respect to life cycle progression. Ticks traverse through three different life cycle stages starting as larvae that hatch from eggs. After acquisition of their first blood meal, most commonly on small rodent hosts, the larvae overwinter and finally molt into nymphs. Nymphs require another blood meal before molting into adults (Brunner et al., 2011). Adult female *Ixodes scapularis* ticks take one last blood meal which is essential before oviposition and eventually die after the eggs have been laid (Francischetti et al., 2009).

Interestingly, the rates of transovarian pathogen transmission from the adult tick to the eggs/larvae are very low, at least with regards to bacterial pathogens. Thus, larvae become infected when obtaining their first blood meal on a host that already carries the

pathogen (Brunner et al., 2011; Mather et al., 1989). Consequently, nymphal ticks receive a high significance when considering pathogen transmission to humans.

Pathogen transmission can be classified as a bidirectional process in which tick feeding on a host is required, defining the tick as a vector for the respective pathogen. Additionally, the phenomenon of infection among co-feeding ticks has been observed in which the pathogen transfers from the infected tick to a non-infected tick feeding in close proximity (Jones et al., 1989). Many different studies outline the central role tick saliva plays in efficient feeding as well as pathogen transmission, strengthening the interest in saliva in vector biology. Also, the way in which the pathogen is sustained and keeps spreading becomes obvious as it utilizes the tick as a carrier to get from one host to another. In the case of *B. burgdorferi*, transmission via ticks is the only known mechanism by which humans become infected (Tilly et al., 2008).

1.2 Saliva plays a central role in successful tick feeding

Saliva plays a central role in tick-feeding and has been shown to have anti-hemostatic and anti-inflammatory effects as well as immuno-modulatory properties which impair the host's innate and adaptive immune system. Saliva constitutes a highly complex pharmacological cocktail which contains numerous bioactive lipids and proteins that also act in redundant ways (Brossard and Wikel, 2004; Carvalho et al., 2010). Brossard and Wikel (2004) provide a very detailed description of tick saliva compounds and their actions in tick feeding. Amongst other examples they describe IL-8 binding proteins that inhibit binding of IL-8 to its receptor, prostaglandins that shift the expression pattern of cytokines towards a Th2 response, inhibitors of T cell activation like Salp15 or immunoglobulin binding proteins that prevent effector functions.

Strengthening the importance of tick saliva in tick-borne disease transmission, studies have shown that pathogen transmission using its natural vector is more efficient than

artificial injections with a needle and syringe underlining the fact that saliva actively benefits pathogen transmission (Frischknecht, 2007). Additionally, pathogen transmission from the host to the tick is promoted by tick salivary proteins like the glutathione peroxidase Salp25D. Knock-down experiments for this protein have shown a decreased transmission of *B. burgdorferi* from infected hosts to the tick vector (Narasimhan et al., 2007b). Furthermore, immunization of mice with recombinant Salp25D impaired pathogen transmission from the tick to the host (Das et al., 2001).

A recent study using yeast-surface display identified five immunogenic salivary proteins from fully engorged *I. scapularis* nymphs capable of immunizing rabbits when given as a cocktail (Schuijt et al., 2011b). Results obtained by the same group revealed the importance of another salivary protein, tick histamine-release factor (tHRF), during late phases of the feeding process. When the tHRF gene was silenced by RNA interference, tick blood feeding success was impaired significantly (Dai et al., 2010). Since histamine is a pro-inflammatory cytokine, the effect achieved by tHRF seems to be counterintuitive. However, this finding leads to another important property of the salivary proteins: gene expression in the salivary glands during feeding is highly dynamic and adapts to the tick's current requirements (McNally et al., 2012; Narasimhan et al., 2007a). This also leads to the necessity of clearly defining feeding time-points in studies of the feeding process and salivary gland expression dynamics (Ribeiro et al., 2006; Valenzuela et al., 2002). The dynamics of gene expression in the salivary glands also are represented by the fact that the feeding process can be divided into a total of nine different phases including host seeking, several different feeding stages as well as detachment (Anderson and Magnarelli, 2008). Of special interest are the early phases of feeding in which the tick has to establish its bite site. It has been stated that in the first 24 h of the feeding process (which can last up to one week) only minute amounts of blood are taken up by the tick and that little to no pathogen transmission occurs (Dai et al., 2010).

1.3 Acquired tick-bite immunity

Given that there are more than 20,000 new infections just of *B. burgdorferi* solely in the United States every year (Marques, 2010), the threat that ticks pose to human and animal health becomes abundantly clear. Nevertheless, the tick faces an impressive array of host defense mechanisms which need to be overcome in order to successfully obtain a blood meal (Brossard and Wikel, 2004; Francischetti et al., 2009). In addition to innate mechanisms like hemostasis or inflammation (Francischetti et al., 2008) many hosts have been shown to acquire specific tick-bite immunity (Ribeiro, 1995).

The first scientific report of acquired immunity against ticks in guinea pigs was published over 70 years ago (Trager, 1939). This report describes that upon repeated infestation with the dog tick, *Dermacentor variabilis*, guinea pigs developed inflammatory reactions leading to impaired tick feeding and increased tick mortality rates (Trager, 1939). Similar results using other tick species and hosts such as rabbits or bovines have confirmed those early findings (Kashino et al., 2005; Wikel, 1996; Willadsen and Jongejan, 1999).

Acquired tick immunity can manifest itself in many different ways. Among the valid definitions listed in the literature are decreased numbers of ticks engorging on a host or decreased weight of the ticks, as well as prolonged feeding, premature drop-off, decreased oviposition or even death of the tick as well as hypersensitivity reactions of the host (Wikel, 1996; Willadsen and Jongejan, 1999).

Studies in mice have revealed critical components of the innate and adaptive immune system with respect to acquired tick bite immunity. A study focusing on basophils and mast cells as well as different Ig receptors on their surface showed that these cell types as well as immunoglobulins of the IgG and the IgE class are essential for acquired tick immunity (Wada et al., 2010).

Immunization studies using bovines have confirmed the importance of immunoglobulins of the IgG class (Kashino et al., 2005). IgE antibodies have also repeatedly been reported as

central players in tick-bite hypersensitivity, making them exciting probes for immunogenic salivary protein discovery (Brossard and Wikel, 2004; Matsuda et al., 1990).

Furthermore, acquired tick immunity has also been related to impaired pathogen transmission (Nazario et al., 1998; Wikel et al., 1997) leading to a simple but genuine idea: Preventing successful tick feeding on a host may prevent pathogen transmission.

1.4 Vaccination against ticks

The finding that acquired tick-bite immunity also affects pathogen transmission led to the idea of anti-tick vaccination. To date, there is only one commercially available anti-tick vaccine which is used in cattle and based on the midgut protein Bm86 from the cattle tick *Boophilus microplus* (de la Fuente et al., 2007a; de la Fuente et al., 2007b). For humans, there are currently no vaccines available against most of the pathogens transmitted by ticks. An efficient vaccine against tick vectors, however, would not only lower the vaccination burden for patients but also protect against a larger array of tick-borne diseases. Furthermore, it could provide an opportunity to decrease the application of acaricides which will have beneficial effects on the environment and agriculture. Also compromising the use of acaricides is the fact that some species of ticks have been observed to quickly develop resistance (Willadsen, 2004). The quest to identify immunogenic antigens that reveal a high efficacy in vaccination has proven difficult. Willadsen (2004) listed three different approaches that have been utilized to identify such potential antigens. First, he names the use of blood sera from tick immune animals as probes. A second approach is presented by analyses of proteins that are essential to the tick's function and third, biochemical fractionation can be used to determine the minimal protein mixture that elicits a protective response when given as a vaccine (Willadsen, 2004).

Salivary proteins appear to be of special interest when it comes to identifying vaccine candidates and there are a number of different reports that have successfully identified immunogenic salivary proteins (Das et al., 2001; Schuijt et al., 2011a; Schuijt et al., 2011b). It has been suggested that targeting salivary proteins of the early feeding phase (< 24 h), in which it is also known that almost no pathogen transfer occurs, is sufficient to establish a sound immune response (Narasimhan et al., 2007a). However, limiting the search of novel vaccine candidates to salivary glands might appear somewhat shortsighted, since as it was stated above, the only commercially available vaccine is based on a midgut protein, revealing the importance of such concealed antigens (Nuttall et al., 2006). Host antibodies appear capable of traversing the host-tick interface where they can bind their respective antigens, for example in the tick's gut, leading to damage of the tick after it has engorged on blood (Willadsen, 2004). Salivary protein antigens represent an excellent potential target for disrupting tick feeding and in particular, the pathogen transmission and infection process should pathogens reach host tissue.

Besides immunogenicity, cross-species reactivity would be another desirable trait for an identified vaccine candidate. With more than 900 different tick species identified to date, a vaccine that protects against multiple species would be highly advantageous. In accordance with this notion it can be stated that there are a significant number of homologous proteins described between different species, a finding that confirms hopes for effective cross-species vaccines (Parizi et al., 2012).

Finally, it has to be noted that tick-host interactions are typically specific, and that findings in one tick/host system cannot be extended to others without appropriate experimentation (Lawrie and Nuttall, 2001; Willadsen and Jongejan, 1999). This statement illustrates the highly complex interactions and reactions to different stimuli and also further complicates the identification of vaccine candidates, especially if laboratory animals are used to identify vaccines for future use in humans.

1.5 Phage Display

Phage display is a robust technique suited for high-throughput screenings of specific and high-affinity interactions between biomolecules, especially proteins and peptides such as antibody fragments and/or antigens (Schirrmann et al., 2011). The technique was developed in the mid 1980s by George P. Smith (Smith, 1985). Since then, many improvements and modifications have been introduced such that phage display has become one of the most important affinity screening methods.

The major strength of the system is established by the intrinsic connection of genotype and phenotype. This connection becomes clear after briefly considering the biology of filamentous phage which are primarily used for displaying proteins (Sidhu, 2005). Filamentous phage can be grouped into different strains such as M13, fd or f1. They are comprised of an outer protein capsid consisting primarily of the major coat protein pVIII which is responsible for up to 87% of the phages' mass (Smith and Petrenko, 1997). The tips of the 1 µm long, rod shaped phage are built up by the minor coat proteins pIII and pXI on one end and pVII and pIX on the other end. Inside the phage particle, the ssDNA encoding all phage proteins is located. Filamentous phage infect their host *E. coli* via the F pilus present on the surface of the bacterial cell. pIII is essential for infection as its N-terminus initiates the contact to the F pilus, eventually translocating the viral ssDNA into the cytoplasm. Here, the complementary DNA strand is synthesized by host enzymes to yield the replicative form, from which phage proteins are expressed. Finally, new phage particles are assembled and leave the host cell via a non-lytic mechanism (Barbas, 2001). Phage particles can now be utilized to display foreign proteins on their surface by fusion of the respective gene to the gene encoding one of the surface proteins, preferably pIII (Georgieva and Konthur, 2011). The result is a peptide::pIII fusion protein whose genetic information is directly encoded by the DNA located inside the phage particle. Thus, the connection of genotype and phenotype is achieved (Hust et al., 2006). The feature of

foreign peptides such as antibody fragments or potential antigens displayed on the phage surface permits efficient screening for interactions of these peptides with other molecules of interest. Such a screening is called biopanning (Konthur and Crameri, 2003).

There are different strategies available for fusion of the gene of interest with the gene encoding the coat protein. Fusion directly within the phage genome is one opportunity, or use of specialized plasmids, called phagemids, which carry the respective coat protein gene and regulatory elements, can be utilized (Qi et al., 2012).

Phage Display has been shown previously to be highly efficient with respect to screening large libraries containing antibody fragments and identifying binders to many different antigens (Colwill and Graslund, 2011; Hust et al., 2011). Conversely, the same methodology can be used to construct antigen libraries to identify potentially immunogenic proteins from various organisms (Kugler et al., 2008; Meyer et al., 2012; Naseem et al., 2010). One major disadvantage of cDNA or gDNA expression libraries are non-functional inserts that are cloned in the wrong orientation, are not in frame with the gIII gene or leader peptide, or contain stop codons or parts of the untranslated regions (Georgieva and Konthur, 2011).

An elegant way to tackle this issue is the introduction of a phagemid/helperphage system in which the helperphage (called Hyperphage) lacks a functional gIII (Rondot et al., 2001). In this case, the only functional pIII, which is essential for phage packaging, results from the phagemid and thus requires in-frame cloning of an insert that does not contain a stop codon. In the process of phage packaging these constructs are directly selected, leading to an enrichment of open reading frames (ORFs). Accordingly, the pHORF/Hyperphage system that has been published previously (Hust et al., 2006; Kugler et al., 2008; Meyer et al., 2012) was utilized in the study presented here. **Figure 1-1** illustrates the whole process of library construction, packaging including ORF enrichment, biopanning and subsequent screening for immunogenic peptides.

Figure 1-1: Illustration of the construction of an antigen phage display library with subsequent packaging and ORF enrichment (top panel). After packaging of the library into phage particles a biopanning is performed using immobilized capture antibody and immune sera to enrich phage particles displaying immunogenic peptides. After three rounds of affinity enrichment a screening ELISA is performed to identify those phages displaying immunogenic peptides. Adapted from Meyer et al., 2012

1.6 Goal of the study

The goal of this master thesis research was to identify immunogenic proteins from the salivary glands of the deer tick, *Ixodes scapularis*. The criterion applied to classify a protein as immunogenic was its recognition by antibodies from human blood sera. Human subjects contributing blood sera were previously known to have strong immune responses to tick bites.

To identify these salivary proteins, a phage display approach based on an antigen library constructed from mRNA of ticks, fed for 18 hours on a rodent host, was used. This antigen library was packaged into phage particles and screened against the human sera available as illustrated in **Figure 1-1**.

Proteins selected in this analysis are predicted to function as novel vaccine candidates with special emphasis to application in humans.

2 Materials and Methods

2.1 Equipment

For the research carried out in this study the following equipment and devices were used.

Table 2-1: Equipment used in this study

Apparatus	Model	Manufacturer
Blotting Machine	Trans-Blot Turbo	Bio-Rad, Munich
Centrifuges	5415 D	Eppendorf, Hamburg
	5819 R	Eppendorf, Hamburg
	Sorval RC6 Plus	Kendro, Langenselbold
	Multifuge 3 S-R	Heraeus, Hanau
Cleanbenches	Hera safe	Heraeus, Hanau
	MSCAdvantage	Thermo Fisher Scientific, Bonn
Electrophoresis chamber	Biotechnology Model 40-0708	Peqlab, Erlangen
	Mini Protean III for SDS PAGE	Bio-Rad, Munich
ELISA Reader	Sunrise	Tecan, Crailsheim
ELISA Washer	Columbus Pro	Tecan, Crailsheim
Gel Documentation	Get Jet Imager	Intas, Göttingen
Incubators	BE 400	Memmert, Schwabach
	Certomat BS-1	B. Braun Biotech, Melsungen
	Infors HT (shaking incubator)	Infors, Einsbach
	I-series 24	New Brunswick Scientific, Enfield, CT
	6251	Thermo Scientific, Asheville, NC
Pipettes	Research	Eppendorf, Hamburg
Power Supplies	Electrophoresis Power Supply 301	Amersham Bioscience, Freiburg
	Electrophoresis Power Supply 601	
Rocker	GFL 3013	Omnilab Laborzentrum, Bremen
Rotating Shaker	Rotator RS-24	Kisker Biotech, Steinfurt
Scales	Analytic 1205MP	Sartorius, Göttingen
	Fine scale laboratory	
Thermocycler	S1000 Thermal Cycler	Bio-Rad, München
	Mastercycler 621	Eppendorf, Hauppauge, NY, USA
Thermomixer	Compact	Eppendorf, Hamburg
	Comfort	Eppendorf, Hamburg

Tissue Lyser	Tissue Lyser II	Qiagen, Valencia, CA, USA
Ultrasound Sonicator	Sonoplus Sonicator 3000	Bandelin Misonix, Farmingdale, NY, USA
Vortex Water Installation	REAX2000 Arium 611	Heidolph, Schwabach Sartorius, Göttingen

2.2 Consumable Supplies

Consumable supplies utilized in this study are listed in the following table.

Table 2-2: Consumable supplies used in this study

Material	Manufacturer
Dynabeads M270 Carboxylic Acid	Invitrogen Dynal AS, Oslo, Norway
Disposable Cuvettes	Brand, Wertheim
Electroporation cuvettes 1 mm	Bio-Rad, Munich
Inoculation loops	VWR, Darmstadt
MTP 96 Well Flat Bottom	Sarstedt, Nümbrecht
MTP 96 Well Polypropylene, U-shaped	Greiner-Bio-one, Frickenhausen
MTP 96 Well Polystyrole, High Binding	Costar, USA
MTP cover foil AeraSeal	Excel Scientific, Victorville, CA, USA
MTP cover foil Sealplate	
PCR stripes	Greiner-Bio-one, Frickenhausen
Petri dishes (7 cm, 15 cm)	Greiner-Bio-one, Frickenhausen
Petri dishes, squared (24.5 cm)	Sigma, Munich
Pipette tips	Sarstedt, Nümbrecht
Pipette tips, filtered	Greiner-Bio-one, Frickenhausen
PVDF membrane	Carl Roth, Karlsruhe
Reaction tube (1.5 ml, 2 ml)	Sarstedt, Nümbrecht
Reaction tube (15 ml, 50 ml)	Greiner-Bio-one, Frickenhausen
Screw-top micro caps	Sarstedt, Nümbrecht
Spatulas	VWR, Darmstadt
Sterile filter (0.2 µm, 0.45 µm)	Sartorius, Göttingen

2.3 Kits and Markers

The following kits were used for isolation and purification of nucleic acids.

Table 2-3: Kits used in this study

Kit	Manufacturer
Illustra mRNA purification Kit	GE Healthcare, Piscataway, NJ, USA
NucleoSpin Gel and PCR Clean-up	Macherey-Nagel, Düren
peqGOLD MINIprep Kit I	Peqlab, Erlangen
PlusOne Silver Staining Kit protein	GE Healthcare, Piscataway, NJ, USA
RNeasy MiniElute Clean-up Kit	Qiagen, Valencia, CA, USA

Markers used in gel electrophoretic analyses (DNA and protein) are listed in the following table.

Table 2-4: DNA and protein markers used in this study

DNA Marker	
GeneRuler 1 kb Plus Ladder 100 bp DNA ladder	Fermentas, St. Leon-Rot NEB, Frankfurt a. M.
Protein Marker	
Precision Plus Protein dual color	Bio-Rad, Munich
Precision Plus Protein unstained	Bio-Rad, Munich

2.4 Chemicals

Unless noted differently, all chemicals were obtained from the following companies: Sigma-Aldrich Chemie GmbH, Fermentas GmbH, Merck KGaA, Riedel-de Haen-Honeywell Specialty Chemicals Seelze GmbH, Roche Diagnostics GmbH, Carl Roth GmbH & Co.KG. Media supplements were obtained from Difco-Voigt Global Distribution Inc.

For all solutions, media and buffers prepared, cleaned and completely desalted water was used (Arium 611, Sartorius AG).

2.5 Enzymes

Enzymes used in this study are listed in the following table.

Table 2-5: Enzymes used in this study

Enzyme	Manufacturer
AfeI	NEB, Frankfurt a. M.
Alkaline Phosphatase (CIP)	NEB, Frankfurt a. M.
AluI	NEB, Frankfurt a. M.
CviKI-1	NEB, Frankfurt a. M.
<i>E. coli</i> DNA Ligase	NEB, Frankfurt a. M.
<i>E. coli</i> DNA Polymerase I	NEB, Frankfurt a. M.
ExTaq DNA Polymerase	Takara
GoTaq DNA Polymerase	Sigma, Munich
Large Klenow Fragment	NEB, Frankfurt a. M.
MMLV Reverse Transcriptase	NEB, Frankfurt a. M.
PmeI	NEB, Frankfurt a. M.

RNAse H	Invitrogen
Superscript II Reverse Transcriptase	Invitrogen
T4 DNA Polymerase	NEB, Frankfurt a. M.
T4 Ligase	Promega
Trypsin	Sigma, Munich

2.6 Antibodies

Antibodies used in this study are listed in the following table. Dilutions were always prepared in 2% MPBST unless noted otherwise.

Table 2-6: Antibodies used in this study

Antibody	Dilution	Manufacturer
Mouse α -M13 IgG (fd, F1), monoclonal	1:1000	Progen, Heidelberg
Mouse α -human IgG (Fc specific), monoclonal	1:5000	Sigma, Munich
Goat α -human IgG (Fc specific)- Peroxidase conjugated, polyclonal	1:70000	Sigma, Munich
Mouse α -M13-Peroxidase conjugated, monoclonal	1:40000	GE Healthcare
Goat α -human IgG polyvalent	1:6700	Sigma, Munich
Goat α -human IgG (Fc specific) phosphatase conjugated	1:5000	Dianova, Hamburg

2.7 Bacteria, Bacteriophages and Plasmids

Bacterial strains as well as bacteriophages used in this study are listed in the following table.

Table 2-7: Bacteria and helperphages used in this study

Bacterial Strain	Genotype	Source
<i>E. coli</i> XL1-Blue-MRF'	K12: Δ (mcrA)183 Δ (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacIqZ Δ M15 Tn10 (TetR)	Stratagene
<i>E. coli</i> Top10F'	K12: F- mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 nupG recA1 araD139 Δ (ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ -	Invitrogen
<i>E. coli</i> DH5 α 409-1 (K02) Helperphage Hyperphage		Rondot et al., 2001 AG Dübél, TU Braunschweig

Plasmids used in this study are listed in the following table.

Table 2-8: Plasmids used in this study

Plasmid	Description	Source
pHORF3	Phagemid vector, Cloning of antigen-libraries, Phage-Display, PmeI restriction site	AG Dübél, TU Braunschweig

2.8 Ticks

2.8.1 Housing

Pathogen-free *Ixodes scapularis* nymphs were reared using standard methods (Mather and Mather, 1990). All unfed nymphs were generated from larvae derived from field-collected adult females blood fed in the laboratory. Larvae were blood fed on pathogen-

free hamsters, collected and allowed to molt under environmental conditions of 23°C, >90% relative humidity, and a 14 h light/10 h dark photoperiod.

Adult female *Ixodes scapularis* used in this study were field collected and housed under environmental conditions of 23°C, >90% relative humidity, and a 14 h light/10 h dark photoperiod.

2.8.2 Feeding

In all feeding experiments performed in this study, ticks were allowed to feed on a rodent host (mouse or hamster) for 18 hours. After 18 hours ticks were pulled off the host and stored in a glass vial with a wet paper towel until subsequent dissection. All animal studies were performed in accordance with protocols AN04-10-006 and AN08-04-017 under the University of Rhode Island, Institutional Animal Care and Use Committee (IACUC).

2.8.3 Dissections

For dissections, fed ticks were immobilized on a glass slide previously prepared with double sided sticky tape. 15 µl of PBS were placed on the tick and the animal was opened using a razor blade. Salivary glands were dissected using fine needles and transferred either into ice cold PBS or mRNA Extraction buffer at room temperature depending on the experiment.

2.8.4 Preparation of salivary gland homogenate (SGH)

Salivary glands dissected previously and stored in PBS (see section 2.8.3) were placed on ice. Samples were sonicated three times for 20 s using the Sonicator 3000 at an intensity of 5.0. Between each sonication step the sample was centrifuged at 10,000 x g and 4°C for 2 min. After the third sonication step the samples were centrifuged for 10 min and all resulting supernatants were pooled. This pool was centrifuged again for 10 min at 10,000 x g and 4°C and the resulting supernatant was stored at -80°C.

2.9 Media

2.9.1 Culture media for *Escherichia coli*

Unless noted otherwise, *E. coli* were cultured in fully buffered two times yeast-tryptone media (2xYT). For solid media 15 g agar per liter were added.

Table 2-9: Recipe for 2xYT medium

2xYT Medium	
Bacto-Yeast Extract	1.0 % (w/v)
Bact-Tryptone	1.6 % (w/v)
NaCl	0.5 % (w/v)

Table 2-10: Recipe for SOC medium

SOC Medium	
Bacto-Yeast Extract	0.5 % (w/v)
Bact-Tryptone	2.0 % (w/v)
NaCl	0.05 % (w/v)
Glucose	1.8 % (w/v)

2.9.2 Media Supplements

Media supplements were prepared as concentrated stock solutions and autoclaved or filter sterilized. Addition of supplements to the media was performed under sterile conditions. For solid media supplements were added after media had reached a temperature below 55°C.

Table 2-11: Media supplements used in this study

Supplement	Stock solution	Final concentration
Ampicillin	100 mg/ml	100 µg/ml
Kanamycin	50 mg/ml	50 µg/ml
Tetracycline	10 mg/ml	20 µg/ml
Glucose	2 M	100 mM
IPTG	1 M	0.5 mM

2.10 Buffers and Solutions

For all solutions and buffers prepared, cleaned and completely desalted water was used (Arium 611, Sartorius AG). Concentrations listed are in water unless noted otherwise.

Table 2-12: Buffers and solution used in this study

Acrylamide Mix				
Acrylamide	30	%	(w/v)	
Bisacrylamide	0.8	%	(w/v)	
Agarose Gel				
Agarose	1.0	%	(w/v)	in TAE Buffer
Ammonium Peroxosulphate (APS-) Solution				
APS	10	%	(w/v)	
Blotting Buffer				
Tris	25	mM		
Glycine	192	mM		
Carbonate Buffer				
NaHCO ₃ , pH 9.6	50	mM		
ELISA Blocking Buffer				
Milk powder	2	%	(w/v)	in PBS
ELISA Washing Buffer				
Tween20	0.05	%	(w/v)	in PBS
Ethidium Bromide Solution				
Ethidium Bromide	0.1	%	(w/v)	
Laemmli Buffer (5x)				
SDS	10	%	(w/v)	
Glycerol	50	%	(w/v)	
Bromophenol Blue	0.02	%	(w/v)	
β-Mercaptoethanol	15	%	(v/v)	
MPBS-T				
Milk Powder	2	%	(w/v)	in PBS-T
PBS				
NaCl	0.8	%	(w/v)	
KCl	0.02	%	(w/v)	
Na ₂ HPO ₄ x 2 H ₂ O	0.144	%	(w/v)	
KH ₂ HPO ₄	0.024	%	(w/v)	
PBS-T				
Tween20	0.05	%	(v/v)	in PBS
PEG/NaCl				
PEG 6000	20	%	(w/v)	
NaCl	2.5	M		
Phage Dilution Buffer				
Tris	10	mM		
NaCl	20	mM		
EDTA	2	mM		
Stacking Gel Buffer (for SDS Gel)				
Tris-HCl, pH 6.8	1.0	M		
SDS-PAGE Running Buffer				
Tris	25	mM		

Glycine	192	mM	
SDS	0.1	%	(w/v)
SDS Solution			
SDS	10	%	(w/v)
TAE Buffer			
Tris-HCl	4	mM	
Acetic acid	2	mM	
EDTA	1	mM	
TMB Reagent			
Solution A, pH 4.1			
Potassium Citrate	30	mM	
Citric acid	0.5	M	
Solution B			
Tetramethylbenzidine	10	mM	
Acetone	10	%	(v/v)
Ethanol	90	%	(v/v)
Hydrogen peroxide	0.3	%	(v/v)
Separating Gel Buffer (for SDS Gel)			
Tris	1.5	M	
Western Blot Substrate Buffer			
Tris HCl	100	mM	
MgCl ₂	0.5	mM	

2.11 Oligonucleotides

Primers used for PCR and reverse transcription experiments are listed in the following table.

Table 2-13: Oligonucleotides used in this study

Primer Name	Sequence 5' → 3'
MHLacZPro_f	GGCTCGTATGTTGTGTGG
MHgIII_r	CTAAAGTTTTGTCGTCTTTCC
Random Hexamers	Obtained from Invitrogen
3' SMART CDS Primer II A	AAGCAGTGGTATCAACGCAGAGTACT ₍₃₀₎ AT
SMART II A Oligonucleotide	AAGCAGTGGTATCAACGCAGAGTACGCGGG
5' PCR Primer II A	AAGCAGTGGTATCAACGCAGAGT

2.12 Software and Databases

Software and Databases used in this study are listed in the following table.

Table 2-14: Software used in this study

Software	Application
ExPASy Prot Scale	Online Tool to generate protein hydrophobicity plots http://web.expasy.org/protscale/
Geneious	Vector Management, Sequence analysis, virtual cloning
MultAlin Interface	Online Sequence alignment tool http://multalin.toulouse.inra.fr/multalin/
NCBI	Literature research, sequence comparisons (BLAST), gene and protein sequence information http://www.ncbi.nlm.nih.gov/

2.13 Microbiological Methods

2.13.1 Liquid and solid culture media

All media were sterilized by autoclaving (121°C, 20 min, excess pressure 1 bar). Heat sensitive supplements were filter sterilized (pore size 0.2 µm) and added to the media under sterile conditions. For solid media Agar-Agar (Bacto) was added before autoclaving.

2.13.2 Culture Techniques

2.13.2.1 Plating

After transformation or transduction of *E. coli*, cell suspension was plated on a 2xYT Agar plate supplemented with 0.1 M glucose and the respective antibiotic with a spatula. Incubation was performed at 37°C overnight. Plates were stored at 4°C for up to four weeks after sealing with Parafilm.

2.13.2.2 Liquid cultures

Starter cultures were prepared in 5 ml or 30 ml scale in 2xYT media supplemented with 0.1 M glucose and the respective antibiotic. Inoculation was performed either from a single colony taken from a plate or directly from a glycerol stock. Cultures were incubated at 37°C and 250 rpm for 16 to 18 hours in a shaking incubator.

Main cultures of volumes between 50 and 400 ml were inoculated from the starter culture with OD₆₀₀ adjusted to 0.05 to 0.09. Composition of the medium was generally set up as for the starter culture. Incubation of the main culture was performed at 37°C and 250 rpm in a shaking incubator until OD₆₀₀ of 0.5 was reached.

2.13.3 Preparation and transformation of *E. coli* TOP10F' electrocompetent cells

2.13.3.1 Preparation

1200 ml 2xYT medium were inoculated with 20 ml of an *E. coli* TOP10F' starter culture to reach an OD₆₀₀ = 0.06 and incubated at 37°C and 250 rpm until an OD₆₀₀ of 0.5 was reached. Next, the culture was incubated on ice for 30 min followed by a centrifugation step at 4000 x g, 4°C for 15 min. The cell pellets were resuspended in 800 ml ice-cold dH₂O followed by another centrifugation step at 4000 x g, 4°C for 20 min. This wash step was repeated once. Following, the cell pellets were resuspended in 200 ml ice-cold 10% glycerol and centrifuged at 3220 x g, 4°C for 10 min in 50 ml reaction tubes. Resuspension of the cell pellets was performed in 1.6 ml ice-cold 10% glycerol and aliquots of 200 µl were stored at -80°C until use.

2.13.3.2 Transformation

Prior to electroporation the electrocompetent *E. coli* TOP10F' cells were thawed on ice. DNA derived from a ligation was added to 50 µl electrocompetent cells and incubated on ice for 5 min. Subsequently the DNA-cell suspension was filled into pre-chilled cuvettes and electroporation was performed with a pulse of 1.7kV. 1 ml SOC medium with a temperature of 37°C was added immediately and the cells were incubated at 37°C and 600 rpm for 1 h. Finally the whole cell suspension was plated on 2xYT agar plates supplemented with 0.1 M glucose and the respective antibiotic. The plate was incubated over night at 37°C.

For construction of antigen libraries, the resulting colonies were floated off the agar with 40 ml 2xYT medium. The resulting cell suspension was centrifuged at 3220 x g, 4°C for 15 min. The pellet was resuspended in 2xYT medium containing 20% glycerol and the sample was stored at -80°C.

2.13.4 Preparation of *E. coli* cell lysate

A 40 ml starter culture was centrifuged at 3220 x g for 15 min and the pellet was resuspended in 40 ml PBS. The pellet was washed two more times with 40 ml PBS. Cell lysis was performed by the application of ultrasound to the sample with a power output of 65% (Sonotrode MS73) for 5 cycles. The resulting suspension was stored at -20°C.

2.13.5 Affinity-Selection of antigen-phage from libraries

2.13.5.1 Production of the helperphage Hyperphage

A starter culture of *E. coli* DH5 α 409-1 (K02) was incubated over night at 37°C and 250 rpm in 2xYT-GK medium. 400 ml 2xYT-GK were inoculated with 200 μ l starter culture and induction of Hyperphage production was achieved by addition of 0.5 mM IPTG. The culture was incubated at 30°C and 250 rpm for 24 hours. Afterwards, the cell suspension was centrifuged at 6000 x g, 4°C for 20 min and the supernatant was transferred into a fresh tube. For precipitation of the phage 1/5 volume PEG/NaCl was added to the suspension which was subsequently incubated on ice at 4°C over night. The phage were then pelleted by centrifugation at 10,000 x g, 4°C for 1 h. The supernatant was discarded and the pellet was resuspended in 20 ml phage dilution buffer and filter sterilized (pore size 0.45 μ m). Again 1/5 volume PEG/NaCl was added and the sample was incubated on ice for 1 h followed by a centrifugation at 20,000 x g, 4°C for 20 min. The resulting pellet was resuspended in 5 ml phage dilution buffer and the Hyperphage suspension was stored at 4°C and subject to titration.

2.13.5.2 Packaging of antigen-phage libraries

400 ml 2xYT-GA medium were inoculated with 1 ml from a glycerol stock containing the respective library and the culture was grown at 37°C and 250 rpm until an OD₆₀₀ of 0.5 was reached. 25 ml from that culture were transferred to 50 ml reaction tubes and infected with 2.5×10^{11} CFU Hyperphage. Infection was achieved by incubating at 37°C for 30 min followed by a second incubation for 30 min at 37°C and 250 rpm. After centrifugation at 3220 x g for 10 min the cell pellet was resuspended in 400 ml 2xYT-AK and the culture was incubated over night at 30°C and 250 rpm. Precipitation of the phage particles was performed as described above for the production of Hyperphage. The packaged library was stored at 4°C in 2 ml screw top vials and subject to titration.

2.13.5.3 Determination of phage titers

50 ml 2xYT-T medium were inoculated with 200 µl of an *E. coli* XL1 blue MRF' or an *E. coli* TOP10F' starter culture and the culture was grown at 37°C and 250 rpm until an OD₆₀₀ of 0.5 was reached. To determine the phage titers after packaging of a library or of the eluted phage after a panning round, 10 µl of the phage sample diluted in phage dilution buffer were added to 50 µl *E. coli* culture and the samples were incubated at 37°C for 30 min. Dilutions were prepared starting at 10^{-2} and continuing in steps of two orders of magnitude until a dilution of 10^{-10} was reached. After the 30 min incubation 10 µl of each dilution were plated in triplicate, air-dried and incubated at 37°C over night. Additionally, a positive control, as well as negative controls containing cells only, cells plus phage dilution buffer and phage only, were plated.

2.13.5.4 Panning

For the specific selection of immunogenic antigen-phage mouse α-human IgG (Fc specific) monoclonal antibody was diluted 1:5000 in carbonate buffer and 100 µl were immobilized

in the well of a MTP (Costar) at 4°C overnight. In parallel, human blood serum was pre-incubated at 4°C overnight as outlined in the following table.

Table 2-15: Composition of the serum pre-incubation mixture

Component	Concentration
Patient serum	1:10
Hyperphage	1×10^{11} phage/ml
<i>E. coli</i> cell lysate	10%
2% MPBST	To adjust volume

After coating overnight all wells of the MTP were emptied and blocked with 350 µl 2% MPBST for 1.5 h at room temperature followed by three washing steps with PBST using an ELISA washer. Next, the pre-incubated patient serum was filled into the wells and incubated at room temperature for 2 h followed by three washing steps with PBST. After capturing the serum antibodies, 200 µl of the packaged antigen phage from the library or the previous panning round were filled into the wells and the MTPs were incubated for 2 h at room temperature. This incubation was followed by 10 x N stringent washing steps with PBST where N = number of the panning round. Specifically bound phage particles were eluted by addition of 200 µl Trypsin solution (10 µg/ml) to each well and incubation at 37°C for 30 min. 190 µl of the eluted phage were used to reinfect *E. coli* TOP10F' and amplify the phage. The remaining 10 µl were used for titration of the eluted phage. In each selection experiment three rounds of panning were performed.

2.13.5.5 Infection of *E. coli* and production of antigen-phage

To amplify eluted phage 50 ml 2xYT-T medium were inoculated with 200 µl of an *E. coli* TOP10F' starter culture and the culture was grown at 37°C and 250 rpm until an OD₆₀₀ of 0.5 was reached. To 20 ml of that culture the eluted phage were added and the mixture was incubated at 37°C for 30 min followed by a second incubation for 30 min at 37°C and 250 rpm. Next, the re-infected cells were pelleted by centrifugation at 3220 x g for 10 min.

The pellet was resuspended in 250 µl 2xYT medium and plated on a 15 cm 2xYT-GA agar plate. The plate was incubated at 37°C overnight.

Colonies were floated off the agar plate using 5 ml 2xYT medium. To 1 ml of the cell suspension 300 µl 80% glycerol were added and the sample was stored at -80°C. Approximately 250 µl of the cell suspension were used to inoculate 50 ml 2xYT-GA medium to an OD₆₀₀ of 0.05 to 0.09. The culture was subsequently grown at 37°C and 250 rpm until an OD₆₀₀ of 0.5 was reached. 5 ml of this culture were infected with 5×10^{11} Hyperphage particles by incubation at 37°C for 30 min followed by a second incubation for 30 min at 37°C and 250 rpm. The cells were pelleted at 3220 x g for 10 min and the pellet resuspended in 30 ml 2xYT-AK followed by an incubation at 30°C and 250 rpm overnight.

To precipitate the phage particles, cells were pelleted at 3220 x g for 10 min and the supernatant was mixed with 1/5 volume PEG/NaCl and incubated on a rocker on ice for 1 h. After centrifugation at 20,000 x g, 4°C for 1 h the pellet was resuspended in 10 ml phage dilution buffer and filter sterilized (pore size 0.45 µm). Again 1/5 volume PEG/NaCl was added and the suspension incubated on ice on a rocker for 1 h followed by a centrifugation step at 20,000 x g, 4°C for 1 h. The pellet containing the phage particles was resuspended in 500 µl phage dilution buffer and stored at 4°C until it was used in the next panning round. 10 µl were used to titer the amplified phage.

2.13.5.6 Production of antigen-phage in microtiter plates

The wells of a microtiter plate (96 well, flat bottom, Sarstedt) were filled with 150 µl 2xYT-GA medium and each well was inoculated with a single colony resulting from the third panning round of an antigen-phage selection experiment. As negative control the wells H3 and H6 were not inoculated (master plate). A previously validated positive control was not available. The plate was incubated overnight at 37°C and 300 rpm. A new microtiter plate (96 Well, flat bottom, Sarstedt) was filled with 150 µl 2xYT-GA medium and each well was inoculated with 10 µl from the respective well of the master plate and incubated for exactly

2 h at 37°C and 300 rpm. Next, 5×10^9 Hyperphage particles were added to each well and the plate was incubated at 37°C for 30 min followed by a second 30 min incubation at 37°C and 300 rpm. Subsequently the bacteria were pelleted by centrifugation at 3220 x g for 10 min and the supernatant was discarded. The pellets were resuspended in 150 µl 2xYT-AK and the plate was incubated at 37°C and 300 rpm overnight.

Bacteria in the master plate were pelleted at 3220 x g for 10 min and the supernatant was discarded. The pellets were resuspended in 2xYT-GA medium containing 20% glycerol and the master plate was stored at -80°C.

The production plate was centrifuged at 3220 x g for 10 min and the supernatants were transferred to a fresh microtiter plate (96 Well, flat bottom, Sarstedt). 40 µl PEG/NaCl were added to each well and the plate was incubated on ice at 4°C overnight. Phage particles were pelleted by centrifugation at 3220 x g for 1 h at 4°C. The supernatants were discarded and the phage pellets resuspended in 150 µl phage dilution buffer. The plate was centrifuged again at 3220 x g for 10 min to pellet remaining bacteria and supernatants were transferred to a fresh microtiter plate (96 Well Polypropylene, U-shaped, Greiner Bio-one).

2.14 Molecular Biological Methods

2.14.1 Preparation of Plasmid DNA from *E. coli*

Preparation of plasmid DNA was performed using the peqGOLD Miniprep Kit I (peqlab, Erlangen). *E. coli* carrying the plasmid of interest were cultured in 5 ml 2xYT-GA medium at 37°C and 250 rpm overnight. The cells were harvested by centrifugation at 5000 x g for 10 min and plasmid DNA was isolated according to the supplier's instructions.

2.14.2 DNA Amplification via Colony Polymerase Chain Reaction (CPCR)

A colony PCR was performed after construction of the antigen libraries to obtain an overview of the distribution of different insert sizes of each library. The DNA template originated from a single bacterial colony which was picked with a sterile pipette tip and directly inoculated into the aliquoted PCR master mix. The polymerase used was GoTaq-DNA-Polymerase. PCR approaches were 10 µl in volume containing one-fifth of the 5x GoTaq Buffer as well as 200 µM dATP, dCTP, dGTP and dTTP. The final concentration of each primer used was 500 nM and each approach contained 0.025 U GoTaq-DNA-Polymerase. The PCR program is summarized in the following table.

Table 2-16: PCR program used for colony PCR analysis

Cycles	Temperature (°C)	Description	Duration
1	95	Melting of the DNA	120 s
24	94	Melting of the DNA	15 s
	56	Primer Annealing	20 s
	72	Elongation	90 s
1	72	Final Elongation	300 s
1	16	Storage	∞

2.14.3 Preparation of mRNA from tick salivary glands

Preparation of mRNA was performed using the Illustra mRNA purification Kit (GE Healthcare). Adult or nymphal *Ixodes scapularis* were allowed to feed on a rabbit or rodent host for 18 h. Subsequently, the salivary glands were extracted as outlined in section 2.8.3 and directly transferred into mRNA Extraction Buffer (GE Healthcare). The tissues were homogenized in two cycles of 2 min each at a frequency of 20 Hz using the Tissue Lyser II (Qiagen). Subsequently, the mRNA was isolated according to the supplier's instructions. The mRNA obtained was further purified and concentrated using the RNeasy Mini Elute clean-up Kit (Qiagen) according to the supplier's instructions. The mRNA was eluted in a total volume of 13 µl.

2.14.4 Conventional cDNA synthesis

To synthesize the first strand cDNA, 13 µl salivary gland mRNA were mixed with 7.5 µg random hexamer primers and 1.25 µl dNTPs (10 mM). The volume was brought to 16.5 µl with nuclease free water and the sample was incubated at 70°C for 5 min followed by a 5 min incubation on ice. Next, 5 µl First Strand Buffer (Invitrogen), 2.5 µl DTT (0.1 M) and 1 µl Superscript II Reverse Transcriptase (Invitrogen) were added and the sample was incubated at 25°C for 10 min followed by 50 min at 42°C. After a final incubation for 15 min at 70°C the first strand cDNA was either stored at 4°C or directly used for second strand synthesis.

To synthesize the second strand, the whole first strand sample containing the RNA-DNA hetero-duplex was mixed with 10 U *E. coli* DNA Ligase, 40 U *E. coli* DNA Polymerase I and 2 U RNase H. 15 µl 10x *E. coli* Ligase Buffer were added and the volume was adjusted to 150 µl with nuclease-free water. The sample was vortexed gently and incubated at 16°C for 2 h. After addition of 10 U T4 DNA Polymerase, the sample was incubated at 16°C for another 5 min followed by addition of 10 µl of 0.5 M EDTA. The double stranded cDNA was isolated from this mixture by adding 160 µl phenol:chloroform:isoamyl alcohol (25:24:1) and thorough shaking. After centrifuging for 5 min at 14,000 x g, the upper aqueous layer was removed and the DNA precipitated by addition of 70 µl 3 M Sodium Acetate (pH 5.2) and 500 µl ice-cold ethanol. After centrifuging for 20 min at 14,000 x g, the supernatant was discarded and the pellet washed once with ice-cold 70% ethanol. Finally the pellet was resuspended in 14 µl nuclease-free water.

2.14.5 SMART cDNA synthesis

Tick salivary gland derived mRNA was used to generate double-stranded cDNA for subsequent cloning into pHORF3. To prepare first strand cDNA, 13 µl mRNA (at least 25 ng) were mixed with 1 µl 3' SMART CDS Primer II A (12 µM) and 1 µl SMART II A

Oligonucleotide. The mixture was incubated at 72°C for 2 min to abrogate secondary structures within the mRNA, followed by an incubation on ice for 2 min. After a brief centrifugation 4 µl 5x First Strand Buffer (Invitrogen), 0.2 µl DTT, 1 µl dNTP Mix (10 mM each) and 1 µl M-MLV reverse transcriptase (Invitrogen) were added and the mixture was incubated at 37°C for 1 h yielding first strand cDNA. For the synthesis of the second strand a long distance PCR was performed using the following master mix:

Table 2-17: Master Mix prepared for the long-distance PCR performed during SMART cDNA synthesis

Component	Volume	
10x ExTaq Buffer	5.00	µl
dNTP Mix (2.5 mM each)	4.00	µl
5' PCR Primer II A (12 µM)	2.00	µl
ExTaq DNA Polymerase	0.25	µl
dH ₂ O	36.75	µl
Total Volume	48.00	µl

Aliquots of 48 µl master mix were filled into PCR tubes and 2 µl of first strand cDNA were added. The PCR program for the synthesis of the second strand is summarized in the following table.

Table 2-18: PCR program used for the long-distance PCR

Cycles	Temperature (°C)	Description	Duration
1	95	Melting of the DNA-RNA Hybrid	60 s
	95	Melting of the DNA	15 s
24	65	Primer Annealing	30 s
	72	Elongation	6 min
1	72	Final Elongation	10 min
1	4	Storage	∞

2.14.6 Restriction Digest of DNA

To eliminate untranslated regions from the full length transcripts obtained from the SMART cDNA synthesis and to generate blunt ended DNA fragments suitable for cloning into pHORF3, a restriction enzyme digest using the blunt end cutters Afel, Alul and CviKI-1 was performed. 30 µl SMART cDNA (~1.7 µg) were mixed with 1.25 U of each of the

restriction enzymes named above as well as NEBuffer 4 and BSA in a total volume of 100 μ l. The sample was incubated at 37°C for 10 min followed by a heat inactivation at 65°C for 20 min.

The phagemid vector pHORF3 was linearized by digestion with the blunt cutting enzyme PmeI. Incubation was performed at 37°C overnight followed by heat inactivation at 65°C for 10 min. To avoid religation, the vector was dephosphorylated with 0.5 μ l alkaline phosphatase (calf intestinal phosphatase, CIP) at 37°C for 30 min. Linearized and dephosphorylated vector was cleaned up using the NucleoSpin Kit (Macherey Nagel) and stored at -20°C.

2.14.7 DNA polishing

To obtain blunt ended DNA fragments for cloning of cDNA from the conventional cDNA synthesis into pHORF3, the DNA needed to be polished. To achieve this, the cDNA was mixed with 1mM dNTPs, 15 U T4 DNA Polymerase and 5 μ g BSA. Also 5 μ l NEBuffer 2 was added and the volume brought to 50 μ l. The sample was incubated at 25°C for 10 min followed by the addition of 12.5 U Large Klenow Fragment and another incubation at 25°C for 10 min followed by a 2 h incubation at 16°C. The blunt-ended DNA was cleaned up using the NucleoSpin Kit (Macherey Nagel).

2.14.8 Agarose Gel Electrophoresis

In an agarose gel electrophoresis, DNA fragments were separated in an electric field, proportional to the negative logarithm of their molecular mass. Thus, 1% (w/v) agarose gels were supplemented with 10 μ l ethidium bromide solution per 35 ml of gel and the separation was performed at a constant voltage of 120 V for 30 min in TAE buffer. Documentation of the DNA fragments was performed under UV radiation (λ = 312 nm) using a video camera.

2.14.9 DNA Ligation

For ligations, generally 500 ng of linearized, dephosphorylated pHORF3 vector were used with a 10-fold molecular excess of insert. The amount of insert needed to achieve this 10-fold excess was calculated using the following formula.

$$Insert [ng] = \frac{ng\ Vector \times kb\ Insert}{kb\ Vector} \times 10$$

Ligations were performed in total volumes of 60 µl using 3 U of T4 DNA Ligase (Promega). The samples were incubated overnight at 16°C and subsequently cleaned using the NucleoSpin Kit (Macherey Nagel).

2.15 Biochemical Methods

2.15.1 Bead Coupling Assay

To enrich serum antibodies specific for salivary gland homogenate (SGH), 2×10^7 Carboxy Beads (Dynabeads, Invitrogen) were washed twice with 1 ml PBST and twice with 0.5 ml NaAc buffer (pH 4.5) by rotating for 5 min. Next 100 µl EDC and 100 µl NHS were added and the beads were rotated for 10 min at room temperature followed by another two wash steps with 0.5 ml NaAc buffer (pH 4.5) and 5 min of rotation each. 20 µg SGH ($1 \mu\text{g}/10^6$ beads) were diluted in 200 µl NaAc buffer and incubated with the beads under rotation for 20 min at room temperature. Subsequently, the beads were washed three times with 1 ml PBST followed by a 2 h rotation with 1 ml 100 mM ethanol amine. After this blocking step the beads were washed three times with 1 ml PBST, once with 1 ml PBS followed by an equilibration in 1 ml 0.1 M glycine/HCl pH 2.2. The beads were washed another three times with 1 ml PBS and incubated with 400 µl of a 1:1 serum-PBS dilution under rotation at 4°C over night. Next, the beads were washed three times with 1 ml PBS and the serum antibodies were eluted by incubation with 150 µl 0.1 M glycine/HCl pH 2.2 under rotation

for 15 min at room temperature. The supernatant was neutralized by addition of 45 μ l 0.5 M Tris-HCl pH 8 and brought to a total volume of 300 μ l by adding PBS. The eluted antibodies were stored at 4°C until they were used in a subsequent capture or titration ELISA.

2.15.2 Enzyme Linked Immunosorbent Assay (ELISA)

2.15.2.1 Capture ELISA

Capture antibody goat α -human IgG, polyvalent (Sigma I1761) was diluted 1:6700 with carbonate buffer and 100 μ l were coated in each well of a MTP (Costar) overnight at 4°C. All wells were emptied and blocked with 350 μ l 2% MPBST for 1.5 h at room temperature followed by three washing steps with PBST using an ELISA washer. Next, a dilution series of a protein N standard in 2% MPBST was prepared according to the supplier's instructions and 100 μ l of the respective dilution were filled into the wells and incubated at room temperature for 2 h. At the same time different volumes of eluted serum antibodies from the bead coupling assay diluted with 2% MPBST were also incubated in wells with immobilized capture antibody. After three washing steps with PBST using an ELISA washer, goat α -human IgG (Fc specific) peroxidase conjugated antibody (Sigma, A0170) was diluted 1:70,000 in 2% MPBST and aliquots of 100 μ l were incubated in each well for 1 h at room temperature followed by three washing steps with PBST using an ELISA washer. Finally 100 μ l substrate solution (TMB-A and TMB-B, ratio 19:1) were added to each well. The staining reaction was allowed to take place for 5 to 20 min at room temperature and was stopped by addition of 100 μ l 1 N H₂SO₄.

2.15.2.2 Screening ELISA

A screening ELISA was performed to analyze the interaction of patient serum with the antigen displaying phage obtained after three rounds of panning. Hence, mouse α -M13 capture antibody (Progen) was diluted 1:400 in carbonate buffer and aliquots of 100 μ l

were incubated in the wells of a MTP (Costar) overnight at 4°C. At the same time a serum pre-incubation mixture was set up as outlined in section 2.13.5.4 and incubated at 4°C overnight. All wells of the MTP were emptied and blocked with 2% MPBST for 1.5 h at room temperature followed by three washing steps with PBST using an ELISA washer. Next, 50 µl monoclonal antigen displaying phage, produced in MTPs (see section 2.13.5.6) were diluted with 50 µl 2% MPBST, filled into the wells and incubated at room temperature for 2 h. As negative controls 1×10^{10} Hyperphage (instead of antigen displaying phage) were added to the wells H3 and H6. After three washing steps with PBST using an ELISA washer 100 µl pre-incubated serum were filled into each well and incubated for 2 h at room temperature. After another three washing steps with PBST goat α -human IgG (Fc specific) peroxidase conjugated detection antibody was diluted 1:70,000 in 2% MPBST and 100 µl aliquots were filled into each well. As positive control mouse α -M13 peroxidase conjugated detection antibody (GE Healthcare) was diluted 1:40,000 in 2% MPBST and 100 µl were filled into wells H9 and H12 and incubated at room temperature for 1 h. After three washing steps with PBST using an ELISA washer 100 µl substrate solution (TMB-A and TMB-B, ratio 19:1) were added to each well. Staining reactions are allowed to take place for 5 to 20 min at room temperature and were stopped by adding 100 µl 1 N H₂SO₄.

2.15.2.3 Titration ELISA

200 ng salivary gland homogenate (SGH) from 18 h fed *Ixodes scapularis* nymphs diluted in carbonate buffer were immobilized in the wells of a microtiter plate (Costar) at 4°C overnight. Wells were blocked with 350 µl 2% MPBST for 1.5 h at room temperature followed by three washing steps with PBST using an ELISA washer. As negative controls, wells were solely blocked without the immobilization of SGH. Patient serum was diluted in 2% MPBST (range of 1:10 to 1:10,000) and incubated in aliquots of 100 µl in each well for 2 h at room temperature followed by three washing steps with PBST using an ELISA washer. Goat α -human IgG (Fc specific) peroxidase conjugated antibody (Sigma, A0170)

was diluted 1:70,000 in 2% MPBST and incubated in aliquots of 100 μ l in each well for 1 h at room temperature followed by three washing steps with PBST using an ELISA washer. As an additional negative control the detection antibody was incubated in a well with immobilized SGH that had previously not been incubated with patient serum. Finally, 100 μ l substrate solution (TMB-A and TMB-B, ratio 19:1) were added to each well and staining reactions were allowed to take place for 5 to 20 min at room temperature before being stopped by adding 100 μ l 1 N H₂SO₄.

2.15.3 Protein analysis via SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The discontinuous SDS-PAGE was performed as outlined by Laemmli (Laemmli, 1970) under consideration of the modifications made by Righetti (Righetti, 1990) using a stacking gel to focus the proteins with a subsequent separation in a separating gel. The protein samples were mixed with SDS sample buffer followed by an incubation at 95°C for 5 min. After a short incubation on ice, the samples were run on a 12% polyacrylamide gel. The electrophoresis was performed at 200 V and 400 mA for 45 min. The composition of the stacking and separating gel is summarized in the following tables.

Table 2-19: Recipe for a SDS-PAGE stacking gel

Component	Volume	
dH ₂ O	1.00	ml
Acrylamide solution	0.26	ml
Tris	0.20	ml
SDS solution	15	μ l
APS solution	15	μ l
TEMED	2	μ l

Table 2-20: Recipe for a SDS-PAGE separating gel

Component	Volume
dH ₂ O	1.3 ml
Acrylamide solution	1.6 ml
Tris	1.0 ml
SDS solution	40 µl
APS solution	40 µl
TEMED	2 µl

2.15.4 Western Blot

To transfer proteins previously separated in a SDS-PAGE to a PVDF membrane, a Western Blot was performed. Hence, a piece of filter paper was soaked in blotting buffer and placed on the blotting machine. On the filter paper the PVDF membrane, which was previously equilibrated in methanol, was placed followed by the gel and another piece of soaked filter paper. The protein transfer from the gel to the PVDF membrane was achieved by application of an electric field at 20 V and a maximum of 770 mA for 35 min.

2.15.5 Immunostain

After the transfer of proteins to the PVDF membrane, an immunostain was performed. First the membrane was blocked for 1 h in 2% MPBST on a rocker to avoid unspecific binding. Next, the membrane was incubated with goat α -human IgG (Fc specific) phosphatase conjugated (Dianova) diluted 1:5000 in 2% MPBST for 1 h at room temperature. After two wash steps with substrate buffer the membrane was incubated in 10 ml substrate buffer supplemented with 100 µl NBT and 100 µl BCIP for 15 min at room temperature. The reaction was stopped by discarding the substrate solution and two subsequent wash steps with water. Finally, the membrane was dried between paper towels and documented using a digital scanner.

3 Results

3.1 Analysis of human blood sera

3.1.1 Determination of the abundance of antibodies specific for salivary gland homogenate (SGH)

For this study, human blood sera from seven different individuals were analyzed to assess their titers of antibodies specific for salivary gland homogenate (SGH) of 18 hour fed *Ixodes scapularis* nymphs by titration ELISA. Prior to this analysis, patients were grouped as either tick bite sensitized by multiple tick bites or tick bite naïve. Grouping was performed according to the patient's own statements and is summarized in the following table.

Table 3-1: Summary of human patient sera available for this study and tick bite status according to the patient's own statement

Patient	Tick bite status
1	naïve
2	sensitized
3	sensitized
4	sensitized
10	naïve
11	sensitized
12	sensitized

To analyze serum IgG binding to SGH at different serum dilutions, a titration ELISA was performed. As a negative control, serum at the lowest dilution of 1:10 was administered to blocked wells without SGH.

Results depicted in **Figure 3-1** reveal two important facts about the human blood sera analyzed. For all sera analyzed the signal decreased very rapidly with further dilution of the sample, indicating low titers of SGH specific antibodies of the IgG isotype.

Secondly, it has to be noted that different sera reveal very different signal-to-noise ratios. A high background signal, referring to higher unspecific binding capacity, would be

undesirable in subsequent phage-display panning since this might lead to amplification and selection of non-immunogenic phage clones. Among the serum samples analyzed, patient 2 and patient 12 exhibited the highest signal to noise ratio and appeared most promising for further analyses to identify immunogenic tick salivary proteins. Nevertheless, additional information verifying the presence of anti-SGH antibodies needs to be obtained.

Figure 3-1: Titration ELISA to assay the reactivity of IgG present in human blood sera to SGH from 18 h fed *I. scapularis* nymphs

3.1.2 Specific enrichment of anti-SGH antibodies

To enrich antibodies from the human blood sera that are specific for SGH, a bead coupling assay was performed. In this approach, SGH obtained from 18 h fed *I. scapularis* nymphs was coupled to carboxy beads (Dynabeads, Invitrogen). To analyze the immobilization of SGH on the beads, the protein sample was visualized on an SDS-Polyacrylamide gel with subsequent silver staining before and after incubation with the magnetic beads.

Figure 3-2: SDS-PAGE of different amounts of SGH with subsequent silver staining, Lane M: Precision Plus unstained Protein Marker, Lane 1: 1 µg, lane 2: 500 ng, lane 3: 250 ng, lane 4: 100 ng, lane 5: 50 ng, lane 6: 0 ng

The silver staining (**Figure 3-2**) visualized different amounts of SGH on the SDS gel revealing the majority of proteins having a molecular mass between 25 kDa and 75 kDa. Importantly, it can be observed that the amount of 1 µg of SGH protein can be visualized very efficiently with this type of assay, thus providing the basis for the concentration of SGH needed for carboxy bead incubation. In the case of unsuccessful binding of SGH to the beads, the concentration in the supernatant needs to be high enough to reveal unsuccessful binding by silver gel analysis. Accordingly, the carboxy beads were incubated with 0.1 µg/µl SGH. 16 µl of the resulting supernatants were analyzed on a gel (**Figure 3-3**). Additionally, control beads were prepared without SGH. SDS-PAGE and silver staining analysis of the supernatants (**Figure 3-3**) confirmed successful binding of SGH to the carboxy beads since the supernatants showed no remaining protein. This suggests that the whole amount of SGH was coupled to the beads.

Figure 3-3: SDS-PAGE of supernatants from the immobilization of SGH to carboxy beads. Lane M: Precision Plus unstained Protein Marker, Lane 1+2: supernatant from SGH coupling, Lane 3+4: supernatant from control beads without SGH

Following successful immobilization of SGH, carboxy beads were incubated overnight with human blood serum pooled from patients 2 and 12, then washed stringently and resuspended in PBS. From this bead suspension, distinct volumes were analyzed for antibody binding by ELISA using peroxidase conjugated α -human IgG (Fc specific) detection antibody.

Figure 3-4: ELISA on magnetic beads that were previously coupled with SGH and incubated with pooled human blood serum from patient 2 and 12. Control beads were solely blocked with ethanol amine prior to incubation with human blood serum. Human IgG bound to the beads were detected using an α -human IgG (Fc specific) HRP conjugated antibody.

The ELISA performed directly on the magnetic beads (**Figure 3-4**) confirmed the accumulation of serum antibodies on beads previously coupled with SGH. Antibody binding to the control beads also could be observed, although to a far lesser extent, suggesting that antibodies specifically recognizing SGH were enriched on the SGH coupled beads.

In the next step, the antibodies captured on the beads were eluted with an acidic solution of 0.1 M glycine/HCl, pH 2.2. After a short incubation, the supernatant was separated from the magnetic beads and neutralized. The resulting aqueous solution containing the eluted antibodies was analyzed on a silver gel as well as on a western blot. On an SDS gel (**Figure 3-5**) different amounts of eluate from either SGH coupled beads or control beads previously incubated with human serum were analyzed. In this SDS-PAGE, performed under reducing conditions, the bands for IgG antibodies were expected to run at 50 kDa (heavy chain) and 25 kDa (light chain), as clearly observed for the positive control.

Figure 3-5: Silver gel analysis of antibodies eluted from SGH coupled beads as well as control beads. Different volumes of eluate were applied to the gel. Lane M: Precision Plus unstained Protein Marker, Lane 1: 16 μ l eluate from SGH beads, Lane 2: 10 μ l eluate from SGH beads, Lane 3: 5 μ l eluate from SGH beads, Lane 4: 16 μ l eluate from control beads, Lane 5: 10 μ l eluate from control beads, Lane 6: 5 μ l eluate from control beads, Lane (+): IgG positive control, 126 ng

To detect the eluted antibodies, the characteristic IgG bands can most prominently be observed for the largest amounts of eluate applied to the gel (Lanes 1 and 4 in **Figure 3-5**). It also can be noted that the amount of antibodies eluted from the SGH coupled beads is larger than the amount eluted from the control beads. Western blot analysis of the same samples led to detection of IgG bands at the expected height of the 50 kDa marker band (**Figure 3-6**).

In conclusion, the results obtained from the bead coupling assay validate its application for enriching antibodies from human blood sera that specifically bind salivary gland homogenate from 18 hour fed *I. scapularis* nymphs. The assay can be used to directly analyze the content of SGH specific antibodies in various serum samples.

To justify the subsequent use of human blood sera in phage display pannings for identifying immunogenic tick salivary proteins, the bead coupling assay was used to verify the presence of anti-tick salivary protein antibodies in the serum samples. Due to limitations in the availability of SGH, only two different sera could be analyzed. Thus,

patient 2 and patient 12 were selected, according to the findings from the titration ELISA (Figure 3-1).

Figure 3-6: Western blot analysis of antibodies eluted from SGH coupled beads as well as control beads. Different volumes of eluate were applied to the gel. Lane M: Precision Plus Dual Color Protein Marker, Lane 1: 16 μ l eluate from SGH beads, Lane 2: 10 μ l eluate from SGH beads, Lane 3: 5 μ l eluate from SGH beads, Lane 4: 16 μ l eluate from control beads, Lane 5: 10 μ l eluate from control beads, Lane 6: 5 μ l eluate from control beads, Lane (+): IgG positive control, 126 ng. After blotting the antibodies were detected using an α -human IgG (Fc specific) peroxidase conjugated detection antibody.

3.1.3 Analysis of selected human blood sera for specific anti-tick antibodies

The bead coupling assay outlined above was repeated using sera from patients 2 and 12, respectively. The amount of antibodies eluted from SGH coupled- and control beads after incubation with serum from either patient 2 or patient 12 was determined by a capture ELISA and compared to a Protein N Standard. Additionally, the IgG content of the crude serum was determined. Results of the capture ELISA are summarized in **Table 3-2**.

Table 3-2: Determination of the IgG concentrations of crude serum, eluate from SGH coupled beads and eluate from control beads of patients 2 and 12.

Serum	Antibody Concentration of		
	Crude serum	Eluate from SGH coupled beads	Eluate from control beads
Patient 2	9.39 mg/ml	85.63 ng/ml	42.82 ng/ml
Patient 12	11.04 mg/ml	375.50 ng/ml	104.07 ng/ml

Determining exact concentrations of IgG antibodies permitted normalization of the amounts of the respective antibodies applied in a repeated titration ELISA on SGH. This assay was used to reveal the specific binding capacity of enriched antibodies from the SGH coupled beads as compared to the crude serum and the eluate from the control beads. This assay (**Figure 3-7**) confirmed the successful enrichment of anti-SGH antibodies from the sera of patients 2 and 12. When the same amount of antibodies from either crude serum or the control eluate was applied to SGH, no significant signal could be observed. Thus, it can be concluded that both sera analyzed do contain SGH specific antibodies. This finding justifies use of the sera for subsequent phage display screenings to identify immunologically relevant antigens.

Figure 3-7: Titration ELISA of normalized crude serum as well as eluted antibodies from SGH coupled beads and control beads from patient 2 (A) and patient 12 (B) on salivary gland homogenate

3.2 Analysis of an antigen library from adult *Ixodes scapularis*

3.2.1 Construction of an antigen library from adult *Ixodes scapularis*

For the construction of an antigen library based on transcripts from salivary glands of adult *I. scapularis*, female ticks were allowed to feed on a rabbit host for 18 h. After removing ticks from the rabbit, tick salivary glands were dissected followed by mRNA extractions and conventional cDNA synthesis using random hexamer primers. The blunt ended cDNA was analyzed on an agarose gel and subsequently cloned into the phagemid vector pHORF3 to yield the library. *E. coli* TOP10F' were transformed with this library by electroporation. After determination of the primary diversity, the library was packaged into phage particles using the helperphage M13K07ΔgIII (Hyperphage) to enrich for open reading frames.

Figure 3-8: Based on mRNA from salivary glands of 18 h fed adult *I. scapularis* cDNA was synthesized using random hexamer primers (A). This cDNA was blunt ended, cloned into pHORF3 and *E. coli* TOP10F' were transformed with this library; from the resulting colonies 20 random clones were picked and analyzed in a PCR, (B) M: Marker, (-): empty pHORF3 as negative control

Examining the double-stranded cDNA on an agarose gel (**Figure 3-8 (A)**) showed a smear ranging from 200 bp to approximately 1500 bp. This broad distribution of cDNA fragments satisfies criteria for antigen library construction with respect to yielding a high diversity of different antigens. A broader the distribution of different inserts indicates higher diversity of the library. Based on this, the insert sizes of 20 randomly-picked clones were analyzed and revealed a broad distribution of different insert sizes (**Figure 3-8 (B)**). Also, nearly 100%

of the clones appeared to carry inserts, further fulfilling the criteria for antigen library construction.

To further assess the characteristics of the library, some 20 random clones were subjected to sequence analysis to provide insight into what is encoded within the library. A BLASTn analysis using these sequences revealed that most hits referred to database entries from *Ixodes scapularis* designated as “tandem repeats”, and no known function could be linked to those sequences. Furthermore, the library contained some rRNA sequences, which was unwanted due to their non-protein-coding nature. There were some sequences that encoded putative salivary proteins, giving the library its significance. The most important characteristics to evaluating the quality of the library are summarized in **Table 3-3**.

Table 3-3: Summary of important characteristics to evaluate the quality of the antigen library derived from salivary glands of adult ticks

Ticks needed	Diversity	Titer after packaging	Effort	Insert (%)	Random sequencing
500 - 700	1×10^6	5×10^9 to 2×10^{11}	high	>95%	Many “tandem repeat” sequences Some rRNAs

3.2.2 Panning of an antigen library from adult *Ixodes scapularis*

To identify immunogenic polypeptides from tick salivary glands of adult *I. scapularis*, the antigen library was screened against human blood serum. For this purpose, the library discussed above was packaged into phage particles that display the respective salivary protein on their surface. Primary packaging of the library with Hyperphage yielded a titer of 5×10^9 CFU/ml. A total of three panning rounds was performed to enrich for immunogenic peptides. The pannings were performed in MTPs in which IgG antibodies from patient sera were captured, followed by incubation with the antigen displaying phage and stringent washing. Prior to their application, the human blood sera had been pre-incubated with *E. coli* cell lysate and Hyperphage to prevent enrichment of unspecific phage due to antibodies recognizing the phage particle itself and not the peptide displayed. Over the

course of the three panning rounds, the titer of eluted phage after each round was monitored for an indication of successful enrichment.

The first two pannings performed with the antigen library derived from adult *I. scapularis* salivary glands resulted in titers of less than 10^3 eluted phage after the first panning round. Consequently, these pannings were cancelled. Packaging of the library into phage particles was repeated and yielded an increase in titer of almost two orders of magnitude as compared to the first packaging (also see **Table 3-3**).

The newly packaged library was used for a new panning and the titers of eluted phage were monitored after each panning round (**Figure 3-9**). The titers of eluted phage increased with subsequent panning rounds, suggesting an enrichment of phage particles displaying potentially immunogenic peptides.

Figure 3-9: Increasing titers of eluted phage from the adult tick antigen library after each panning round. The increase of eluted phage after each round indicates an enrichment of specific phage clones.

After the third panning round, 94 random clones were picked and grown in a microtiter plate (MTP) to subsequently produce monoclonal antigen-phage for a screening ELISA. The wells H3 and H6 remained empty as negative controls. Clones inoculated into wells

H9 and H12 were later on not used for screening with patient serum but as positive controls for a successful phage production.

A screening ELISA performed to test for immunogenic polypeptides displayed on the phage particles was performed in two different ways. In the first approach, monoclonal antigen-phage were immobilized in the wells of a MTP directly. Subsequently, pre-incubated patient serum was added to the phage. After stringent washing, human antibodies were detected by application of peroxidase -conjugated α -human IgG (Fc specific) antibody.

In the second approach, the monoclonal phage particles were not coated directly to the wells of a MTP but instead were captured using a monoclonal mouse α -M13 antibody. The remaining procedure of the incubation and detection was essentially the same.

For negative controls in the screening ELISA, Hyperphage was added to the respective wells (H3 and H6) instead of monoclonal antigen-displaying phage particles. Thus, the only difference between the negative control and the experimental approaches was the display of the respective tick polypeptide on the phage surface. As positive control, the presence of phage particles was assayed by applying an peroxidase-conjugated α -M13 detection antibody. Positive controls were not incubated with human blood serum.

Figure 3-10: Results of the screening ELISA from the randomly picked clones resulting from the third panning round of the antigen library derived from adult *I. scapularis* salivary glands. In (A) the phage particles were coated directly whereas in (B) the phage particles were captured. Negative Control: Hyperphage; Positive control: detection of phage particles

Results of the screening ELISA revealed no immunogenic peptides displayed on any of the phage analyzed (Figure 3-10). However, production and immobilization of the monoclonal antigen-phage was successful as confirmed by the positive control. One finding that can be derived from the experiments shown is that there is a difference for this assay with regards to the comparison of directly coated or captured phage. In the case of captured phage particles, it can be noted that the general background signal is lower. This phenomenon also was observed previously (Havlik, personal correspondence) and is confirmed here. Consequently, for all screening ELISAs performed hereafter, the phage particles were always captured. To solve the issue of unsuccessful panning the component that was to be changed most easily was addressed and a new antigen library was constructed.

3.3 Analysis of an antigen library from nymphal *Ixodes scapularis*

3.3.1 Construction of an antigen library from nymphal *Ixodes scapularis*

Since nymphal ticks are of higher relevance with respect to transmission of pathogens, primarily due to their smaller size, an antigen library based on nymphal tick salivary glands was constructed. The general procedure was the same as for the adult antigen library (section 3.2.1), however, a different strategy for cDNA synthesis was chosen to overcome a limitation in the availability of salivary gland mRNA.

Ixodes scapularis nymphs were allowed to feed on a mouse or hamster host for 18 hours. After ticks were pulled off their host, tick salivary glands were dissected and subjected to mRNA extractions. The mRNA extracted in this process was used to synthesize double-stranded cDNA following the protocol for SMART cDNA synthesis (Clontech, CA). Briefly, an oligo(dT) Primer with an adapter is used together with a reverse transcriptase. The reverse transcriptase used needs to have two advanced activities. First, terminal transferase activity is necessary which leaves a small number of cytosine residues at the 3' end of the newly synthesized first strand cDNA. Second, template switching activity is needed. The reaction mixture contains a second primer comprised from 5' to 3' of the adapter sequence followed by a small number of guanidine residues. This primer recognizes the C residues synthesized by the reverse transcriptase and subsequently acts as new template to add the adapter sequence to the first-strand cDNA. Finally, a PCR can be performed using a specific primer for the adapter sequence which allows amplification of all transcripts. The SMART cDNA that was synthesized was visualized on an agarose gel.

This protocol, however, leads to full length transcripts containing the stop codon as well as 5' UTR and 3' UTR, all of which are unwanted for subsequent cloning into pHORF3. To avoid these issues, the SMART cDNA was subjected to a restriction enzyme digest using the three blunt-end cutters Afel, Alul and CviKI-1, making the digested cDNA was directly

available for cloning. After cloning into pHORF3, the resulting antigen library was transformed into *E. coli* TOP10F' by electroporation. After determining the primary diversity, the library was packaged into phage particles using the helperphage M13K07ΔgIII (Hyperphage) to enrich for open reading frames.

Figure 3-11: Based on mRNA from salivary glands of 18 h fed *I. scapularis* nymphs, cDNA was synthesized following the SMART cDNA synthesis protocol (A). This cDNA was subject to a restriction enzyme digest, cloned into pHORF3 and *E. coli* TOP10F' were transformed with this library; from the resulting colonies 20 random clones were picked and analyzed in a PCR, (B) M: Marker, (-): empty pHORF3 as negative control

Examination of the SMART cDNA visualizes the size of cDNA fragments ranging from 200 bp to more than 1500 bp (**Figure 3-11 (A)**) revealing a satisfying distribution of different fragments. Additionally, using the SMART protocol, it was possible to synthesize μ g amounts of cDNA from only ng amounts of mRNA, overcoming the limitation of low availability of the desired starting material. The distribution of different insert sizes after cloning into pHORF3 was analyzed by PCR and subsequent agarose gel electrophoresis (**Figure 3-11 (B)**). This analysis revealed a satisfying distribution of different inserts as well as an insert percentage of close to 100%. Furthermore, the clones also were subjected to sequence analysis. Performing a BLASTn analysis using the sequences obtained from randomly picked clones, most sequences could be linked to previously annotated database entries of *Ixodes scapularis* salivary proteins. None of the sequences analyzed referred to the “tandem repeat” entries encountered when analyzing the antigen library derived from adult ticks (see section 3.2.1). Also, no sequences referring to rRNAs were found, confirming the specific reverse transcription of poly (A) tailed RNAs only. In

conclusion, the parameters analyzed to evaluate the quality of the library appear to be very promising. An overview of the important parameters is given in **Table 3-4**.

Table 3-4: Summary of important characteristics to evaluate the quality of the antigen library derived from salivary glands of nymphal ticks

Ticks needed	Diversity	Titer after packaging	Effort	Insert (%)	Random sequencing
~50	1×10^6	2×10^{11}	low	>95%	Mostly annotated tick sequences

3.3.2 Panning of an antigen library from nymphal *Ixodes scapularis*

3.3.2.1 Panning using pooled human blood sera

To identify immunogenic tick salivary proteins, three rounds of panning were performed using the nymphal antigen library and pooled human blood sera from all patients classified as sensitized (see **Table 3-1**). The pooled serum was pre-incubated in a mixture containing Hyperphage and *E. coli* cell lysate to prevent enrichment of unspecific phage due to antibodies recognizing the phage particle itself and not the peptide displayed. Over the course of the three panning rounds, the titers of eluted phage after each round were monitored to give an indication of successful enrichment (**Figure 3-12**). Two pannings were performed in parallel.

Figure 3-12: Development of the titers of eluted phage from the nymphal antigen library after each panning round. The increase of eluted phage after each round indicates the enrichment of specific phage clones.

For both pannings, titers increased with subsequent rounds indicating successful enrichment of potentially significant clones. To identify immunogenic peptides displayed by phage, a screening ELISA was performed. After the third panning round 94 random clones from each panning performed were picked and grown in MTPs to produce monoclonal antigen-displaying phage. The results of the screening ELISAs are shown in **Figure 3-13**.

It has to be noted that, despite the promising increase in titers of eluted phage after each panning round, the screening ELISA did not reveal a positive clone in either of the two pannings performed. Moreover, none of the signals obtained was significantly higher than that of the negative control. As with the adult antigen library, unsuccessful production of the monoclonal phage can be excluded to be the issue, since the positive control gave a strong signal.

Two critical points for ensuring a successful panning are the amount of anti-tick specific antibodies in the sera as well as the quality of the antigen library. Since with the nymphal antigen library all parameters considered to evaluate the quality of the library appear to be very good (see **Table 3-4**), it suggested that changes in the use of human blood sera might

lead to a successful panning. Thus, a second panning was performed using individual instead of pooled sera.

Figure 3-13: Screening ELISAs of the randomly picked clones after the third panning round. Panning was performed using the nymphal antigen library and pooled human blood serum from patients previously classified as sensitized. Negative Control: Hyperphage; Positive control: detection of a random antigen displaying phage using an α -M13 peroxidase conjugated antibody.

3.3.2.2 Panning using single human blood sera

To identify immunogenic tick salivary proteins, an alternative panning strategy was utilized in which human blood serum from only one individual was used instead of a serum pool. Besides this variation, the strategy pursued was essentially the same as outlined previously. For the following analyses sera from patients 2, 11, 12 and patient X were used. As outlined for all previous pannings performed, the titers of eluted phage after each panning round were monitored (**Figure 3-14**). In all cases a prominent increase in eluted

phage can be observed, suggesting the successful enrichment of phage particles displaying potentially immunogenic peptides.

Figure 3-14: Development of the titers of eluted phage from the nymphal antigen library after each panning round using individual human blood sera. The increase of eluted phage after each round indicates the enrichment of specific phage clones.

As outlined for previous pannings, after the third panning round, 94 randomly picked clones were grown in MTPs to produce monoclonal phage particles displaying tick salivary peptides. The resulting monoclonal phage were used to perform a screening ELISA to assess for successful enrichment of phage displaying immunogenic peptides.

Results of the screening ELISAs are shown in **Figure 3-15**. While the pannings performed with sera from patient 11 (**Figure 3-15 (B)**) and patient X (**Figure 3-15 (D)**) resemble the results seen in all previously performed screening ELISAs, pannings performed with sera from patient 2 (**Figure 3-15 (A)**) and patient 12 (**Figure 3-15 (C)**) led to clones with signal intensities higher than that of the negative control containing Hyperphage. Signals being one and a half to twice as high as the signal of the negative control can be considered as potential clones of interest and eight clones from the panning with patient 2 serum as well as five clones from the panning with patient 12 serum were selected and subject to sequence analysis. The sequences obtained were analyzed using the BLASTn algorithm. The eight clones obtained from patient 2 serum revealed four different inserts. The most

abundant clone which occurred four times among the eight clones analyzed was carrying the genetic information for a fragment of Metalloprotease 1 from *I. scapularis* but also had significant similarity to metalloproteases from *I. pacificus* and *I. ricinus*. The five clones obtained from panning with patient 12 serum revealed three different inserts. The results are summarized in **Table 3-5**.

Table 3-5: Summary of the sequencing analysis of positive clones obtained from panning with individual patient sera. The inserts found were subject to a BLASTn analysis to determine the nature of the peptide displayed on the phage.

Derived from Patient	Frequency	Insert size	Accession Number	Description	Identity
2	1/8	-	-	Sequencing failed	-
2	1/8	64 bp	XM_002406537.1	<i>Ixodes scapularis</i> DNA ligase, putative, mRNA	100%
2	4/8	118 bp	AY264367.1	<i>Ixodes scapularis</i> salivary gland metalloprotease mRNA	92%
2	1/8	43 bp	XM_002403042.1	<i>Ixodes scapularis</i> 60S ribosomal protein L14, putative mRNA	100%
2	1/8	79 bp	XM_002416616.1	<i>Ixodes scapularis</i> alpha tubulin, mRNA	99%
12	2/5	-	-	Sequencing failed	-
12	1/5	43 bp	XM_002403042.1	<i>Ixodes scapularis</i> 60S ribosomal protein L14, putative, mRNA	100%
12	1/5	139 bp	XM_002434300.1	<i>Ixodes scapularis</i> E3 ubiquitin protein ligase Bre1, putative, mRNA	99%
12	1/5	79 bp	XM_002416616.1	<i>Ixodes scapularis</i> alpha tubulin, mRNA	99%

Figure 3-15: Screening ELISAs of the randomly picked clones after the third panning round. The panning were performed using the nymphal antigen library and individual human blood sera. (A) Patient 2, (B) Patient 11, (C) Patient 12, (D) Patient X; Negative Control: Hyperphage; Positive control: detection of a random antigen displaying phage using an α -M13 peroxidase conjugated antibody.

3.3.2.3 Verification of binders identified in the pannings

To verify binders that were identified in pannings with individual patient sera and listed in **Table 3-5**, the respective phage were newly packaged and subject to a repeated ELISA using the respective patient serum. As outlined previously, the patient sera were pre-incubated with *E. coli* cell lysate and Hyperphage. As negative control, Hyperphage were captured by an α -M13 antibody instead of antigen displaying phage particles. The clones that could not be sequenced successfully were designated as “unknown” and included in the verifying ELISA. The results of the verifying ELISA are shown in **Figure 3-16**. For all clones analyzed, it can be observed that the signal is significantly stronger than that of the negative control. However, signal intensities in general are comparably low as they were throughout all previously performed analyses.

Figure 3-16: ELISA to verify the specific recognition of antigen displaying phage identified in Section 3.3.2.2 by the respective patient serum; (A) Patient 2; (B) Patient 12; Hyperphage serves as negative control as previously outlined for the screening ELISA analyses.

4 Discussion

Due to their capacity for pathogen transmission, ticks pose significant risks to humans, domestic animals and cattle, resulting in public health concerns as well as economic losses (Wikel and Alarcon-Chaidez, 2001). Current treatments to relieve tick burdens in endemic areas are mostly based on the application of acaricides. This strategy, however, has negative impacts on the environment and agriculture. Additionally, certain tick species quickly develop insecticide resistance (Willadsen, 2004). Another strategy gaining greater attention over the past two decades is the idea of anti-tick vaccination (de la Fuente et al., 2007b; Nuttall et al., 2006). Based on the phenomenon of acquired tick bite immunity (Trager, 1939), vaccination with molecules present in tick saliva appears to be most promising when pursuing an anti-tick vaccination approach (Willadsen, 2004).

The goal of this study was to identify *Ixodes scapularis* salivary antigens that might serve as novel vaccine candidates for humans. To identify these proteins, a phage display approach was employed. The screening was designed to identify *I. scapularis* salivary antigens that are recognized by antibodies present in human blood sera.

4.1 Analysis of human blood sera

Human blood sera from seven different individuals were available for this study. These patients had been grouped as being either tick bite sensitive or naïve according to their own statement. This first classification of the serum samples already states a critical point when evaluating the sera. Individuals previously bitten by ticks can provide sound information that they have been in close contact with ticks, thereby imparting significance to their sera with respect to the presence of anti-tick antibodies. Conversely, individuals reporting to be tick-bite naïve may still have been bitten by ticks, even multiple times, but may never have recognized it. An additional issue with the sera used in this study is that it

is unknown when sensitized patients had last been in contact with ticks. This is an important factor for the presence of anti-tick specific antibodies in the serum, since titers decrease after prolonged elapsed time with no tick contact. A titration ELISA was performed to further assess the reactivity of serum antibodies with SGH. Results obtained from this assay did not produce a clear distinction between tick bite sensitive and tick naïve patients, leading to a major limitation: the lack of a validated serum negative control. In addition to the indistinguishability between tick bite sensitive and naïve blood sera, low titers of serum antibodies specific for SGH, in general, were another limitation. Following examination by the titration ELISA the most promising sera were obtained from patients 2 and 12, especially due to their relatively high signal-to-noise ratios. Continued analyses of these sera using the bead coupling assay to enrich SGH specific antibodies and the subsequent titration ELISA with these enriched antibodies on SGH confirmed the presence of SGH specific antibodies. Nevertheless, low antibody titers in general were a limitation to the experimental design.

The study presented here is the first investigation aiming to identify immunogenic tick salivary proteins using human immune sera. All studies reviewed in the literature used sera from laboratory animals or cattle. The advantage of laboratory animals becomes clear when considering that rabbits for example can be challenged with ticks multiple times in intervals of two weeks and that serum samples can be taken at the peak point of an antibody response (Das et al., 2001; Narasimhan et al., 2007a; Schuijt et al., 2011b). Such highly reactive sera cannot be obtained so easily from humans, somewhat limiting the usability of human sera. Nevertheless, the observed presence of SGH specific antibodies together with the patient's reports of immune responses to tick bites, justifies use of the serum samples available.

4.2 Construction of salivary gland antigen libraries

Salivary gland antigen libraries constructed in this work are based on mRNA from the salivary glands of 18 hour fed *I. scapularis*. Since these tissues are very small, especially during early stages of the feeding process, mRNA yields are a limiting factor. Even though the salivary glands grow and change in morphology during the feeding process (Kaufman, 1989), the early time points are of higher significance with respect to identifying potential vaccine candidates (Narasimhan et al., 2007a).

Two different approaches for cDNA synthesis from salivary gland mRNA were utilized in this study. First, conventional cDNA synthesis was used to construct an antigen library based on adult ticks. In this approach the amount of cDNA synthesized is approximately equal to the amount of mRNA available making this strategy very laborious with respect to the collection of starting material. This is also the primary reason why adult ticks were used, since their salivary glands are significantly larger than those of nymphal ticks. For synthesis of a sufficient amount of cDNA a total of 500 to 700 adult ticks were dissected. A comparable study reported dissection of more than 1000 nymphs in order to construct an antigen library (Schuijt et al., 2011b). The resulting cDNA was analyzed by agarose gel electrophoresis revealing a sufficiently broad distribution of different fragments (**Figure 3-8**). In this analysis a distinct band at approximately 450 bp was very prominent. A possible explanation for this finding could be that the reverse transcriptase used for cDNA synthesis has an optimum for synthesizing fragments of this size. This single band could still be comprised of different fragments even though they have the same size.

Sequence analysis of random clones from this library revealed inserts encoding rRNA among other sequences. This result reflects the unspecific reverse transcription of any kind of RNA present in the reaction mixture, making this protocol susceptible to contamination. It also demonstrates that, even though an mRNA extraction kit was used, some rRNA contamination occurred diluting the diversity of the library.

The second approach, using a SMART cDNA synthesis protocol, proved to be much more efficient. Salivary glands of only 50 nymphs were required in order to synthesize large amounts of cDNA. The distribution of different fragments obtained with this approach was sufficiently broad. Furthermore, no rRNA contamination was observed, probably due to the use of an oligo (dT) primer, which creates a second, strong selection for poly (A) tailed RNAs. SMART cDNA synthesis has been used previously in tick salivary gland transcriptomics (Ribeiro et al., 2006; Valenzuela, 2002; Valenzuela et al., 2002) and the use of such cDNAs in phage display approaches also has been addressed previously (Georgieva and Konthur, 2011). These obvious advantages, however, should not belie the fact that there are some disadvantages of this method as well. Utilization of the two adapter primers and subsequent PCR amplification could introduce a bias towards certain transcripts. Additionally, the full-length transcripts need to be fragmented which was achieved by the action of three different restriction enzymes. This step introduces a bias to the cDNA fragments since occurrence of the restriction sites will not be completely random. Finally the ratio of highly abundant to less abundant transcripts is unlikely to be preserved during the PCR amplification step.

In conclusion, according to the parameters employed to evaluate the two antigen libraries, SMART cDNA synthesis proved to be the better strategy to construct a library of desired quality.

4.3 Screening for immunogenic peptides

Successful phage display screenings seeking to identify immunogenic proteins rely primarily on two factors. First, the antigen library needs to be of good quality with respect to diversity and display of antigenic peptides. Second, the availability of antibodies used to probe the library is crucial. The low titers of anti-tick antibodies in the human blood sera present a major limitation. The panning strategy pursued in this study has been utilized

successfully in previous reports (Kugler et al., 2008; Meyer et al., 2012; Naseem et al., 2010), however, convalescent sera from recently infected animals provided higher antibody titers in those studies.

For the study presented here, two different ways of employing human blood sera were assayed. When all sera previously classified as tick sensitized were pooled, screening with either of the two antigen libraries constructed was unsuccessful. This could be explained by the idea that each of the sera contains tick-specific antibodies. However, different individuals might recognize different salivary antigens. In this case, pooling of the sera further dilutes the tick-specific antibodies, thus impeding successful enrichment of the corresponding antigens. None of the results obtained in the study presented here contradict this notion. The idea that serum pooling and subsequent antibody dilution could be a limitation is further underlined by the fact that panning, using individual patient sera led to identification of immunogenic salivary proteins. Another critical point of the experimental setup is performance of the screenings as library-vs-library approaches in which two complex mixtures are screened against each other. This double-sided high complexity limits the number of possible specific interactions exacerbating identification of immunogenic polypeptides.

It is noteworthy that, despite the fact whether or not a panning was judged successful, the titers of eluted phage after each panning round increased. Thus, in all cases phage have been enriched, however, this includes enrichment of unspecific phage particles as shown in the respective screening ELISAs. This effect might be due to remaining serum antibodies that recognize phage coat proteins even though the sera were pre-incubated with Hyperphage. Presence of such antibodies also would explain the general background signal in all screening ELISA analyses. Another explanation could be that enriched phage particles display peptides promoting some sort of selection advantage. Factors influencing this advantage could be an increased infectivity of the phage due to the peptide displayed,

thereby leading to a higher production rate of a specific clone. Additionally, unspecific binding might be caused by interactions between phage and other protein components present in the screening setup (Vodnik et al., 2011). This last point is of special significance when considering the very high background signals of the different sera. Interestingly, antigens identified in this study resulted from screenings with those sera that previously had been mentioned to reveal the best signal-to-noise ratios, confirming that general serum stickiness is an important criterion.

4.4 Metalloprotease 1 and its homologs

Among the salivary proteins identified in this work, the metalloprotease appears to be of greatest interest. This is primarily due to the fact that it is the only extracellular and actively secreted protein recognized by the human immune sera.

Previous reports state that Metalloprotease 1 (MP1) of *Ixodes scapularis* is synthesized as a pre-pro-enzyme containing a signal peptide as well as a pro-peptide which are both cleaved off in order to get the active protein (Francischetti et al., 2003). MP1 has a theoretical molecular weight of 37 kDa, a conserved zinc-binding domain as well as 12 highly conserved cysteine residues. Furthermore, it has been reported to have fibrin(ogen)lytic activity as well as gelatinase activity (Francischetti et al., 2003). Thus, this enzyme is hypothesized to act as an anti-hemostatic in tick saliva. Interestingly, it has been noted that *Borrelia* spirochetes upregulate expression of metalloproteases in their vertebrate hosts, potentially enhancing degradation of extracellular matrix proteins to improve spreading of the pathogen (Gebbia et al., 2001). This fact presents one possible route on which tick feeding can actively promote pathogen transmission, raising the significance of the observation made in this study to suggest MP1 as a novel vaccine candidate.

Figure 4-1: Sequence alignment of protein sequences of the peptide displayed on the phage identified (Insert) and the protein sequences of metalloproteases from *I. scapularis* (AY264367.1), *I. pacificus* (AY674268.1) and *I. ricinus* (AM747806.1).

Increasing the potential of MP1 as an anti-tick vaccine is the finding that the protein has close homologs in other tick species such as *I. pacificus* and *I. ricinus*. The sequence alignment shown in **Figure 4-1** demonstrates the close homology between metalloproteases of these tick species. Additionally, the immunogenic peptide displayed on the phage in this work, reveals high identity with all three proteins, raising hopes for a more universal, multi-species vaccine candidate (Parizi et al., 2012).

The insert identified in the phage display screening covers a sequence which coincides exactly with the beginning of the cysteine rich region and just nine amino acids away from the zinc-binding domain. Binding of an antibody to this region thus might interfere in binding of the metalloprotease to proteins of the extracellular matrix, as this has been a suggested function of the cysteine rich region (Francischetti et al., 2003). Furthermore, binding of an antibody this close to the active site might interfere with the catalytic function of the enzyme, for example by steric hindrance of substrate binding.

Of utmost interest for the evaluation of MP1 as a novel vaccine candidate are reports about salivary gland metalloproteases in *I. ricinus*, termed Metis. (Decrem et al., 2008a; Decrem et al., 2008b). Metis 1 is a close homolog to *I. scapularis* MP1 (see **Figure 4-1**). In

the studies presented by Decrem et al., the vaccination potential of Metis 1 was evaluated and showed a negative effect on weight gaining and oviposition in ticks that fed on vaccinated hosts. Vaccination with Metis 1 elicited an antibody response against Metis 1 and 2 in rabbits. Furthermore, knock-down of Metis 1 by RNA interference led to an increased mortality rate in ticks (Decrem et al., 2008a; Decrem et al., 2008b). Since the Metis proteins were analyzed because rational considerations suggested them as immunologically interesting enzymes, the findings in this work confirmed the potential relevance of metalloproteases in anti-tick vaccination.

This relevance is further underlined by vaccination experiments with a metalloprotease from *Haemaphysalis longicornis* in rabbits even though there is little sequence identity to metalloproteases from Ixodes species (Imamura et al., 2009).

4.5 Further binders identified

Except for the metalloprotease, all other binders identified in this work are intracellular proteins. While it might seem appropriate to exclude non-secreted proteins as potential immunogenic targets, host contact with these proteins is still possible and a tick ribosomal protein has recently been suggested as a vaccine candidate (Rodriguez-Mallon et al., 2012). Salivary glands change dynamically in gene expression and morphology during feeding (Kaufman, 1989) with cell proliferation as well as cell death as expected outcomes. From these cells, intracellular components can leak into the surrounding fluids and thus, reach the host where an adaptive immune response can be initiated.

However, as determined by sequence analysis, the likelihood that all binders identified are artifacts appears significant. This is primarily due to the fact that none of the inserts are incorporated into the vector in the right frame. In the case of DNA ligase, ribosomal protein and ubiquitin ligase, a high identity sequence exists in the opposite direction to the coding sequence of the signal peptide and the gene encoding pIII. Additionally, none of the inserts

contain a stop codon in the +3 reading frame, explaining why the respective phage could be packaged.

In the case of α -tubulin, the orientation of the insert is correct; however the reading frame is shifted as compared to the surrounding genetic elements of the vector. Furthermore, the insert does not reveal a stop codon in any of the possible reading frames and the corresponding tubulin sequence has a 100% identity to human α -tubulin. This last finding, especially, excludes α -tubulin as a significant binder.

Sequence analysis of the peptides actually displayed on the phage particles revealed two interesting facts. First, three of the four sequences contain a cysteine residue in the C-terminal half of the peptide. Recent findings report the coupling of protein toxins to antibodies via cysteine residues of the hinge region (M. Hust, personal correspondence) giving room for speculations regarding the occurrence of cysteine residues in the peptides displayed and their selection by serum antibodies.

Second, three of the four sequences show large hydrophobic stretches and only few hydrophilic areas when analyzed in a hydrophobicity plot (data not shown). Thus, one could speculate that the respective peptides have been selected due to unspecific hydrophobic interactions. This also could be brought into context again with the general stickiness of the human blood sera. Furthermore, due to the limited number of desired immunogenic interactions (as outlined above) the possible number of unspecific interactions is increased, leading to an enrichment of the undesired clones found in this study.

4.6 Conclusion

The study presented here was seeking to identify salivary antigens from *Ixodes scapularis* that were recognized by antibodies from human blood sera utilizing a phage display approach. In order to perform a successful screening two main limitations had to be

overcome. First, the low abundance of serum antibodies specific for salivary proteins was handled by using individual serum samples instead of serum pools. Additionally, enrichment of anti-tick specific antibodies was successful, however, only in very small amounts.

Second, the availability of mRNA as starting material for the construction of antigen libraries was limiting. This issue was resolved by pursuing a SMART based cDNA synthesis approach which includes an amplification step.

Finally, the human blood sera and antigen libraries available were successfully screened and led to identification of a metalloprotease from *I. scapularis* that has close homologs in other ixodid species as well. Previous reports about the function of this enzyme together with vaccination trials of one homolog in *I. ricinus* impart high potential to this protein with respect to acting as a novel vaccine candidate.

4.7 Outlook

The results presented in this work suggest Metalloprotease 1 from *Ixodes scapularis* as a novel anti-tick vaccine candidate. However, further validation of this protein with respect to its potential for triggering immune reactions is needed. The only validation provided so far is based on an ELISA using human blood serum and the peptide displaying phage. Cloning, expression and purification of the whole protein followed by ELISA analysis with different human blood sera is required. For this examination a validated negative control serum needs to be identified.

Furthermore, once the protein has been produced recombinantly, vaccination studies in a suitable model organism such as a humanized mouse model can provide deeper insights into vaccination potential of MP1.

Using the bead coupling assay presented in this work, phage display screenings could be repeated using the enriched serum antibodies to improve antigen detection. Additionally,

with the optimized protocol for library construction, different tick tissues such as the midgut as well as different time points of the feeding process could be addressed for antigen identification. Finally, the protocols used in this study also can be utilized in analyses of other tick species to extend our knowledge of tick-host interactions with particular focus on humans.

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