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DIPLOMARBEIT

Design and development of an immune dependent system for the detection of antibodies against *R. conorii, R. helvetica and R. slovaca* and screening for specific antibodies.

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1. Abbreviations

ADP	adenosine diphosphate
AluI	restriction enzyme
AMP	adenosine monophosphate
APS	ammonium persulfate
ATP	adenosine triphosphate
Bp	base pairs
CCHF	crimean-Congo hemorrhagic fever
DNA	deoxyribonucleic acid
DTT	dithiothreitol
e.g.	*exempli gratia*
ELISA	enzyme-Linked ImmunoSorbent Assay
GC	guanine-cytosine
GTP	guanine triphosphate
IFA	immunofluorescent antibody assay
IgG	immunoglobulin G
IGM	immunoglobulin M
IMAC	immobilized metal ion affinity chromatography
kDa	kilo-Dalton
km	kilometer
M	molarity
mA	milliampere
mg	milligram
min	minutes
ml	millilitre
mm	millimetre
MRL	buffer
MSF	mediterranean spotted fever
MST	multi spacer typing
NAD+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NCBI	national Center for Biotechnology Information
Ni-NTA	Ni^{2+}- Nitrilotriacetic acid
Nk	natural killer cell
nm	nanometre
nrdb	non-redundant data base
PAGE	polyacrylamid-gelelektrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	a measurement of the concentration of hydrogen ions
PPE	rickettsial palindromic element

RFLP	restriction fragment length polymorphism
RMSF	rocky mountain spotted fever
rRNA	ribosomal RNA
RT	room temperature
SAM	*S*-Adenosyl methionine
SDS	sodium dodecylsulfate
sec	second
SFG	spotted fever group
SOD	super oxide dismutase
STG	spotted typhus group
Tab	table
TBS	tris buffered saline
TCA	trichloracetic acid
TEMED	N,N,N',N'-Tetramethylethylendiamine
TG	typhus group
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
UTP	uridine triphosphate
v/v	volume/volume
VDC	voltage
w/v	weight/volume
WB	western blotting
µl	microlitre
µm	micrometre

2. Abstract

Design and development of an immune dependent system for the detection of antibodies against *R. conorii, R. helvetica, R. slovaca* and screening for specific antibodies.

The rickettsiae are bacteria which share an obligate intracellular habitat. The rickettsiae comprise intracellular parasites as well as intracellular symbionts. The parasitic rickettsiae are associated with a transmission vector, usually arthropods, during their infectious cycle to reach a vertebrate host. Rickettsiae are the causative agents for many different diseases with various disease courses. Rickettsioses are regarded as emerging diseases since the end of the 20th century.

This diploma thesis deals with three rickettsial species namely *Rickettsia slovaca, Rickettsia helvetica* and *Rickettsia conorii*. These rickettsiae are transmitted by hard ticks. *R. conorii* as well as *R. helvetica* were found in Austrian ticks. *R. slovaca* was found in ticks of several countries of central Europe including Austria [142].

The diploma thesis is divided into two major parts. The first part describes a project which is intended to develop an assay for the detection of human antibodies to the three rickettsial species. The detection assay shall specifically determine the genus and the species of rickettsiae. The ELISA technique was chosen as detection system. The second part concerns the elvaluation of sera from tick-exposed persons and from persons of the average population.

For the ELISA design the genomes of the three *Rickettsia* species were screened for a suitable antigen which bares epitopes that are recognized by the human immune system. The antigen was reduced to a single region which has proven antigenic features to minimize the size of the antigen used in the following ELISA. Each chosen antigen region was amplified by PCR and checked by sequencing. The sequence data for *R. slovaca* and *R. helvetica* concerning the chosen antigen was not available. Therefore the primers designed for *R. conorii* were also used for the other two strains. The amplified antigen region was cloned in an expression vector (Quiagen pQE 30 UA 6x Ni-NTA tag), the protein was purified by affinity chromatography and checked by SDS gel, Coomassie staining and dot blot.

Sera from hunters and from persons of the average population were matched according to age. The sera were tested with a *Rickettsia conorii* IFA test for specific IgG and IgM, by the Weil-Felix agglutination assay and by real time PCR designed for *R. conorii* and *R. helvetica*.

The PCR amplification of the antigenic region lead to differently sized fragments of the rickettsiae tested. The *R. conorii* fragment was a 1194 bp fragment, the *R. slovaca* a 1300 bp fragment and the *R. helvetica* a 900 bp fragment. The controll sequencing showed correct relation to known sequences of the database.

The IFA test showed positive IgG results with sera from four women and four men of the blood donor group, and one woman and seventeen men from the hunter group. The Weil-Felix results did not show a countable result. The Real Time PCR yielded two positive results, one for *R. helvetica* and one for *R. conorii*.

Cloning will be performed in an ongoing study with a new vector and restriction cloning in order to receive positive expression clones. Positive real time PCR result for *R. conorii* corresponded with positive IFA serology for IgG. The Weil- Felix test results were all negative.

3. Introduction

3.1.History of rickettsiology

3.1.1. Rickettsia rickettsii agent of the Rocky Mountain Spotted Fever

The first report on cases of Rocky Mountain Spotted Fever (RMSF) was published in 1896 by Major Marshall H. Wood, an army physician, in Boise, Idaho [3]. Three years later in 1899, Maxey described cases of RMSF in the Snake River valley of Idaho [4]. Later Wilson and Chowning observed seven deaths due to RMSF in the Bitterroot valley [5] in the spring of 1902. A very small number of people got infected with the RMSF at that time in the United States, but in Bitterroot Valley and Snake River valley more than 100 cases were reported in 1902, this indicated that a highly virulent strain of the causative agent had to be present and highly transmitted in this valley. They studied 111 cases of RMSF, the majority of which were on the west side of the Bitterroot River and 69% of the cases were fatal. On the basis of the history of tick exposure and the seasonality of the disease peaks, they concluded that RMSF was spread by wood ticks [5]. Today the Selway-Bitterroot Wilderness is a protected wilderness area in the states of Idaho and Montana, in the northwestern United States. At one point three million acres (5,300 km²), it is one of the largest designated wilderness areas in the United States. It spans the Bitterroot Mountain Range, on the border between Idaho and Montana. It covers parts of Bitterroot National Forest, Clearwater National Forest, Lolo National Forest, and the Nez Perce National Forest. Several rivers like the Frank Church-River, the Lochsa and the Selway rivers that join to form the Clearwater River are flowing in the area. This is a perfect habitat for the vector of the RMSF. Howard Taylor Ricketts (professor of Pathology at the University of Chicago and with an undergraduate degree in zoology from the University of Nebraska) was born in February the 9th 1871 in Findley, Ohio. Before Howard Ricketts started his research in this valley the research of Wilson and Chowning [5] went on for three years without a scientific proof of the agent causing the RMSF. Howard Ricketts initiated a new study of the spotted fever in 1906 [6]. While working in the Bitterroot valley in 1906, Dr. Howard Taylor Ricketts demonstrated tick transmission of RMSF to guinea pigs, and showed that the agent of RMSF was present in blood obtained from infected humans, and that it could be removed by filtration. Ricketts reported the presence of ‘‘minute polar staining bacilli’’ in freshly laid eggs of infected ticks [4]. Although Ricketts observed a very small bacillus, he was unable to isolate and culture the causative agent to fulfill the Koch's Postulate using contemporary laboratory techniques. Nonetheless,

his work suggested that bacterial diseases could be biologically transmitted from parasites to humans and animals alike. He published his findings in the *Journal of the American Medical Association* under the title "A Micro-Organism Which Apparently Has a Specific Relationship to Rocky Mountain Spotted Fever: A Preliminary Report" in 1909. The following year a lack of funding prevented Ricketts from returning to Montana. Four years after these classic investigations, Ricketts died of epidemic typhus in Mexico City, but only after having described the distinctive microbe (later known as *Rickettsia prowazekii*) he found in the lice and blood from patients carrying the disease. In 1916, Wolbach published two papers in which he described the appearance of *Rickettsia rickettsii,* which he visualized in blood vessels by using the Giemsa stain technique [7]. In 1919, he reported that *R. rickettsii* is an intracellular pathogen and he described the vasculitic lesion caused by that pathogen [8]. The rickettsiae used in the present study were described several years after H. Ricketts worked. *R. conorii* was first described 1910, *R. slovaca* in 1968 and *R. helvetica* in 1979.

3.2.Phylogeny of rickettsia

3.2.1. Genus Rickettsia

Rickettsiae are bacteriae which are members of the phylum proteobacteria. Proteobacteria are divided into five sections the – alpha-, beta-, gamma-, delta-, epsilonproteobacteria. The section alpha proteobacteria is comprised entirely of obligate intracellular symbionts and contains the rickettsiae. The section alphaproteobacteria is divided into three families the anaplasmataceae, the rickettsiaceae and the holosporaceae. The family rickettsiaceae contains the rickettsiae which are divided into two genera the rickettsia and the orientia. The genus rickettsia contains two groups, the spotted fever group and the typhus group. Sometimes ago a third group was also added to the genus *Rickettsia* the scrub typhus group which is nowadays excluded (Wolbachia). The genus *Rickettsia* understands 27 described species (Tab. 1) and one still unclear candidate [9].

Genus *Rickettsia*			
Name	date of confirmed description	reference	validation
Rickettsia prowazekii	1916	[10]	
Rickettsia aeschlimannii	1997	[11]	
Rickettsia africae	1996	[12]	
Rickettsia akari	1946	[13]	
Rickettsia asiatica	2006	[14]	
Rickettsia australis	1950	[15]	
Rickettsia bellii	1983	[16]	
Rickettsia canadensis	1967	[17]	
Rickettsia conorii	1932	[18]	
Rickettsia felis	2001	[19]	renamed [20]
Rickettsia heilongjiangensis	2006	[21]	renamed [22]
Rickettsia helvetica	1993	[23]	
Rickettsia honei	1998	[24]	
Rickettsia japonica	1992	[25]	
Rickettsia massiliae	1993	[26]	
Rickettsia montanensis	1965	[27]	renamed [28]
Rickettsia parkeri	1965	[29]	
Rickettsia peacockii	1997	[30]	
Rickettsia prowazekii	1916	[31]	
Rickettsia raoultii	2008	[32]	
Rickettsia rhipicephali	1978	[33]	
Rickettsia rickettsii	1922	[34]	
Rickettsia sennetsu	1956	[35]	
Rickettsia sibirica	1948	[36]	
Rickettsia slovaca	1998	[37]	
Rickettsia tamurae	2006	[38]	
Rickettsia tsutsugamushi	1931	[39]	
Rickettsia typhi	1943	[40]	

Tab. 1 Characterised *Rickettsia* species (2008 *Approved Lists of Bacterial Names in IJSEM Online*)

3.2.2. Phylogenetic markers pf rickettsiae and techniques

The best known phylogenetic rickettsial markers are the 16SrRNA, the citrate synthase gene *gltA* and the rOmpA [41]. The 16SrRNA marker is used to determine if the bacteria of interest is located in a near relation to the rickettsiae. This marker is not suitable to determine the relation on the strain or genus level of rickettsia because of the essential function in the translation process the 16SrRNA locus is highly conserved in the genus rickettsia and is target of a high evolutionary pressure leading to an even stronger conservation [42]. To determine the relation of the rickettsiae in the genus *Rickettsia* or in the two groups the citrate synthase gene, the rOmpA or other genes were tested. These loci had to fulfill a certain conservation of the sequence but need to be different from each other [43, 44].

3.2.3. RFLP

Restriction fragment length polymorphisms of the rOmpA and the rOmpB were studied to differentiate rapid between the two groups of the rickettsiae and the strains as well. The RFLP is based on the fact that genes or gene fragments amplified by PCR can directly be digested by restriction enzymes and will according to the insertion of repeats differ in the length of the resulting fragments. This method should lead to a unique profile of fragments for every strain tested. But the results showed that just *Rickettia massilae* and *Rickettsia japonica* did show unique fragments and no other strain could be differentiated by this method. And some fragments from the Vero host cells did show similar fragments to some rickettsiae tested [45, 46].

3.2.4. MST

Multi-spacer typing is a technique used to identify rickettsiae at species level and below. The MST is based on the fact that intergenic spacers, which are less subject to evolutionary pressure than coding sequences. These spacers are located between the coding sequences and are common in all the bacteriae. It was demonstrated that the spacers if compared between different strains are conserved but also different enough to allow a species differentiation. Six

spacer regions were compared to achieve an identification result which was reliable and reproducible [47].

3.2.5. A phylogenetic tree for rickettsiae

This tree (Fig. 1) is based on the estimated phylogenies of ten rickettsial taxa based on 731 core proteins which sequences are known and listed in the PATRIC database. The first tree was calculated with the Byesian analysis (Tree A). Three MCMC chains were primed with a neighbor-joining tree and run independently for 25000 generation in model-jumping mode. Burnin was attained by 2500 generations for all chains, and a single tree topology with exclusive use of the Jones substition model was observed in post-burning data. The consensus tree shown here thus has a 100 % support for every branch. Branch support is from the distribution of posterior probabilities from all trees minus burn-in. The second tree was calculated by using an exhaustive search and using most likely parsimony (Tree B). Branch support is from one million bootstrap replicates [41, 42]. The Cladogramm shows that *Rickettsia bellii* is outstanding of both rickettsial groups, the TG and the SFG. Its relation is high enough to be counted as a member of the rickettsiae but it does not support a classification for any of the existing taxonomic groups. To this day it is unclear if *R .belli* is a member of a third rickettsial group.

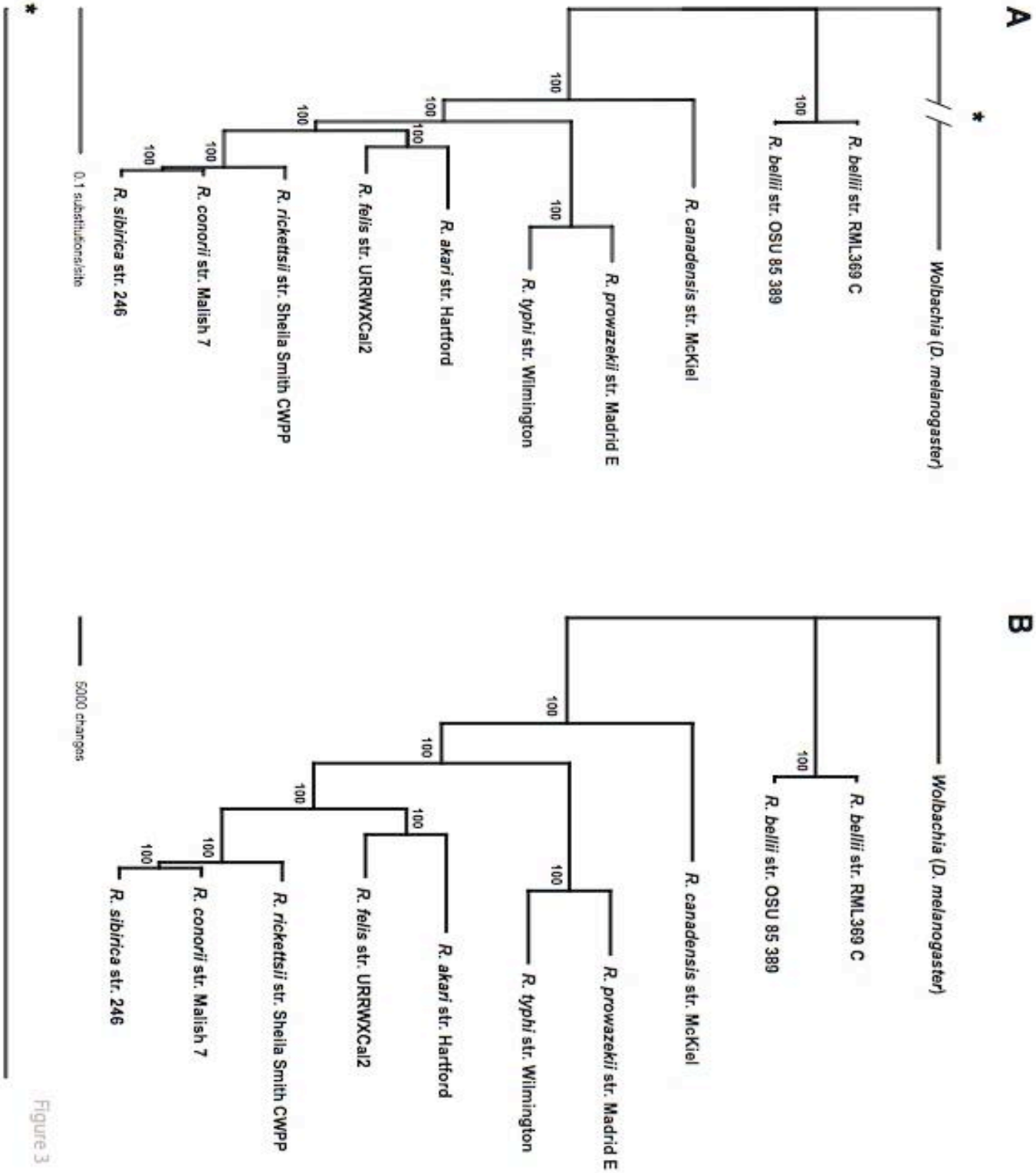


Figure 3

Fig. 1 Phylogenetic tree for rickettsiae calculated from the PATRIC database

3.3. Morphology

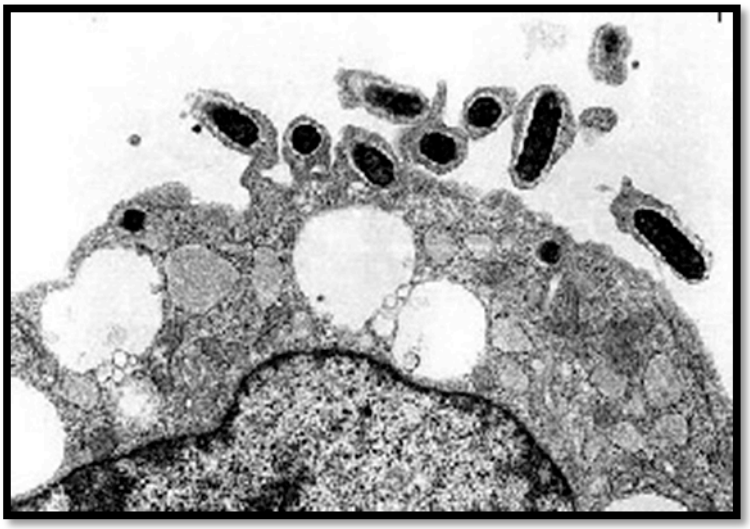


Fig. 2 Rickettsiae leaving a necrotic host cell

Rickettsiae are gram negative, coccoid or rod shaped and sometimes pleomorphic proteobacteriae. They share a typical bacterial cell wall structure, no flagella and multiplying via binary fission inside the host cell of the vector. Their size ranges from 0.3 to 0.7µm wide and 1 to 2 µm long. Rickettsiae or rickettsia-like bacteriae are found as pathogenic agents in animals, humans and plants and as endosymbionts in invertebrates. Some members of the genus rickettsiae are intranucular living endosymbionts of vertebrates. The endosymbiontic living Rickettsiae are associated with sometimes life maintaining importance for the host. For example we shall use a *Rickettsia* found in the booklouse *Liposcelis bostrychophila* which performs reproduction via parthenogenesis. This *Rickettsia* stays in specialized tissue cells of its host and if removed from it the early oocyte development is stopped and no reproduction may be performed. This *Rickettsia* is a member of the SFG and shares some genetically similarities with mycetomic bacteria which indicates a long co-evolution with the host away from the original relation neighbours [48].

The majority of the rickettsiae are obligate parasitic living microorganisms which are at some point in their life cycle linked with a blood-sucking arthropod as transmission vector and/or as secondary host. The transmission vector comprises blood-sucking arthropods such as ticks, fleas, mites, chiggers and some mammals. The secondary hosts are often mammals like rodents, birds, dogs and many more which fulfill the function of a reservoir. The pathogenic, parasitic living rickettsiae are transmitted by the saliva or the faeces of their specific transmission vector. The in the case of a saliva transmission the feeding act establishes a

direct entrance port for the microorganism and the faeces enters for example the respiratory organs on aerosols or is rubbed in a vector-caused wound [1]. The rickettsiae are not able to traverse intact epithelic tissues but may enter through unharmed mucosal epithelias like lungs. Rare cases of transmission by blood transfusion are known and transmission by organ transplantation is believed to be possible but no case of transmission by sexual contact or by placental contact is known [2]. Rickettsiae are the causative agents of a variety of diseases in humans and animals, of which the most important are typhus fever and Rocky Mountain Spotted Fever. The severity of the diseases caused by members of the rickettsiae ranges from mild Rickettsial-Pox to life threatening RMSF. The duration can vary from self limiting MSF to chronic Q-fever or recrudescent Brill-Zinser disease. Rickettsiae infect host cells and produce great amounts of toxins, thus damaging the host cells and inducing cell death by stimulating necrosis [1] (Fig. 2).

3.3.1. Mitochondrial ancestor role of the genus Rickettsia

Phylogenetic studies of the rickettsiae and the eukaryotic mitochondria lead to some major similarities between these two. The small subunit of the rRNA was the first target for this comparison. The loss of several genes for the gain of better adaption to the eukaryotic host was similar to the reduced mitochondria genome which lost its genes to the host genome [49]. The phylogenetic analysis of the small subunit of the rRNA and the chaperonin 60 sequence lead to the hypothesis that the mitochondria and the rickettsia are descendants of the same ancestor organism. The genus *Rickettsia* involves some *Rickettsia*-like bacteria which if taken into count by doing the phylogenetic analysis are the nearest existing relatives to this hypothetical ancestor group [50]. By comparing the bioenergetic level of the most similar candidate to the ancestor of both in the genus *Rickettsia* is *Rickettsia prowazekii* but *Rickettsia belli* is the rickettsia sharing the most genome similarities to a hypothetical ancestor for the Mitochondrion and the genus *Rickettsia.* Most of the genes supporting mitochondrial activities are nuclear. Many of the 300 proteins encoded in the nucleus of for example yeast (*Saccharomyces cerevisiae*) which are destined for service within the mitochondrion are close homologues of their counterparts in *R. prowazekii*. Nearly one-quarter of these proteins are required for bioenergetic processes and another one-third of them are required for the expression of the genes encoded in the mitochondrial genome. In total, more than 150 nucleus-encoded mitochondrial proteins share significant sequence homology with *R.*

prowazekii proteins. The mitochondrial genome of the early diverging, freshwater protozoan *Reclinomonas americana* is more like that of a bacterium than any other mitochondrial genome sequenced so far [51]. This genome contains 67 protein-coding genes, most of which provide components of genetic processes and the bioenergetic system. For example the genes *rplKAJL* and *rpoBC* are identically organized in *R. prowazekii* and the mitochondrial genome of *Reclinomonas americana*. Likewise, the genes encoding the S10, *spc* and the ∝-ribosomal protein operons are organized similarly in the two genomes [52]. Furthermore the *R. prowazekii* genome contains genes encoding components of the tricarboxylic acid cycle and for some electron transport systems similar to the mitochondrial system. But it lacks genes to support the necessary glycolysis. The genome contains five genes coding for adenine nucleotide translocators which are important for the obligate intracellular parasite to exploit the cytoplasmatic ATP cycle of its host for energy gain but there is also a similar pathway in the *Rickettsia prowazekii* of generating ATP as there is in the mitochondria. The rickettsia ATP/ADP translocases are monomers with twelve trans-membrane regions each, whereas the mitochondrial translocases are dimers with six trans-membrane regions per dimer. No relationship between the primary structures of the mitochondrial and *Rickettsia* ATP/ADP translocases were found, indicating that these transport systems may have originated independently [52].

3.3.2. Cell wall structure

Rickettsia as member of the gram-negative bacteria has a similar structure of the cell wall it contains diaminopimelic acid and lacks teichoic acid (Fig. 3). The cell wall is divided into three major layers. Starting at the innermost is the cytoplasamtic membrane which is an electron dense rigid membrane, coated by a peptidoglycan layer. After that follows the periplasmatic space in which several porins and other proteins reach. Then follows the outer membrane which contains the majority of porins and the different uptake and excrete systems as well as different receptors for the interaction with the environment. A thin layer coats the outer membrane. The S-layer it is build using different proteins and glycoproteins. The S-layer functions as the first defense against the harmful environmental influences as the pH, bacteriophages and phagocytosis. The outer membrane and the S-layer host as well the immune dominant antigens, in the case of rickettsiae the *ompA*, *ompB* and the *sca1*. A general

antigen, which is shared by the majoritiy of the bacteria, LPS is attached to the outer membrane and is important for the fixation of the S-layer on the cell surface [1].

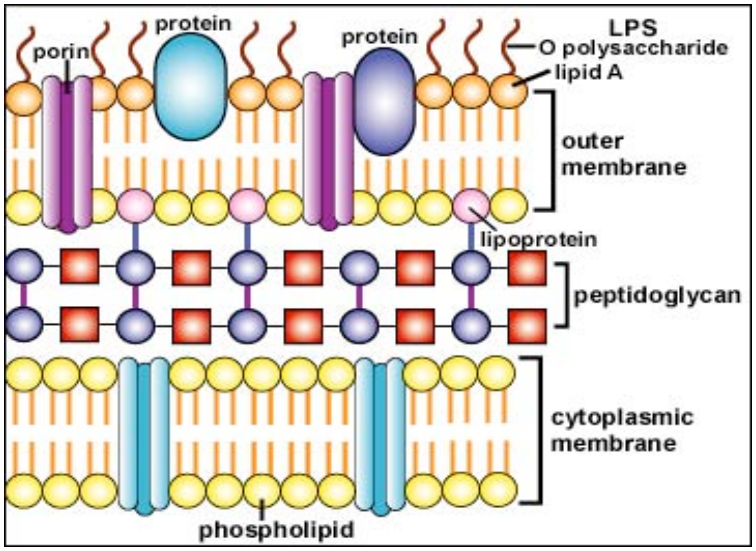


Figure 3 Cell wall structure of gram negative bacteriae

3.3.3. Replication mechanism

The generation time of rickettsiae under favorable host cell like conditions is approximately 8 hours. Under these conditions rickettsiae multiply by transverse binary fission. If the conditions change to a not favorable but viable state providing poor nutrition sources, the rickettsiae cease dividing and grow into long filamentous forms, which stop divison. When new nutrition sources are available the rickettsiae start to multiply rapidly several times into typical small coccoid shaped daughter cells. The rickettsiae start to move in the host cell cytoplasma, immediately after the division is finished. The movement will be described in the following paragraphe. The host cell is normally able to handle the movement and the growth of about 6-8 rickettsial cells before is ruptures [1].

C. burnetii differs from other rickettsiae in that it is enclosed in a persistent vacuole during growth and division. Six to ten daughter cells will form within a host cell before the cell ruptures and releases them [1].

3.3.4. Cell migration

Cell migration is a central process in biological systems. Migration is a genetically old skill the majority of cells posses e.g. bacteriae mainly for acquisition of nutrition and avoiding of unfavorable habitat changes, protozoa also mainly for acquisition of nutrition and avoiding negative habitat changes, amoeba for dispersion in the habitat and to consume particles, spermatozoa a direct movement to fulfill the reproduction purpose and in eucaryotic cells. In eucariotic cells the migration is very important for the immune competent cells to reach the place of infection, for tissue cells to reach their designated location in the tissue and many other cells to fulfill their imprinted purpose. Every migration machine is based on the use of different proteins as motor. Eukaryotic cells are mainly using actine as motor for their migration. In bacteriae very often a flagellum is used to achieve migration. In eukaryotic cells a flagellum and cilliae are used to achieve migration but it is important to mention that the flagella of the bacteriae are not comparable to the flagella of the eukaryotic cells.

In the case of rickettsiae the bacteriae are moving by using the host cell migration machine. This mechanism is well studied in the *Listeria*, *Shigella* and the vaccinia virus [53, 54].

The host cell migration machine works by using a constant flow of energy to build a treadmill of actin monomers. The actin monomers are attached to other actin monomers at the only free binding point and build a string of actin monomers. This string is as long stable as long there are not too much actin monomers attached to it. If the string reaches the point of too much monomers the first monomer of the string is lost. This leads to a constant flow of actin monomers attaching themselves to the monomer string and getting lost. The overall size of the actin string depends on the stabilizing molecules of the cell and the place it is used. Actin monomers that are located in the migration front of the cell will just form short actin strings at a maximum of 20 monomers at a time attached to each other [55].

In the case of *Listeria* the protein Arp2/3 which is a host cell protein is important for the nucleating and generating of a Y-branched array of actin filaments during host membrane extension and the locomotion of the pathogen. The pathogen is pushed by the constantly generating branched array of actin filaments through the host cell. The pathogen is able to exploit this actin motor to move through the host cell and to leave the host cell. Arp2/3 requires some sort of protein presented by the pathogen activated, this is the WASP-family or the *ActA* of listeria. The actin tails pushing *R. conorii* through the host cell are made of long

unbranched actin filaments indicating the use of another pathogen activation protein. In vitro studies did show that the rickettsial *RickA* does induce the Arp2/3 complex. But the inducing is less efficiently then the WASP or *ActA* inducing. This actin based motility is also a virulence mechanism for the listeriae and the rickettsiae. The more efficiently building listeria *ActA* complexes do lead to actine filaments which are more unstable than the longer and slower *RickA* built filaments [56]. If the *RickA* protein is expressed in the host cell it induces the formation of lobopodia which need long unbranched actin filaments to be functional [57].
The *RickA* protein (Fig. 4) contains a N-terminal domain for binding monomeric actin (G domain), a central proline-rich region believed to play a role in binding to WASP-like proteins and the *WCA* region including WASP-homology 2 (*WH2*), central (*C*) and acidic (*A*) domains that should interact with the Arp2/3-complex of the host cell [55, 56].

Fig. 4 Schematic domains of the *RickA* gene

By comparing *R .conorii* of the SFG and *R. typhi* of the TG and so far the only TG rickettsiae that performs an actin based motility regarding the actin filament usage and the locomotion *R. conorii* is more active and does build longer actine tails between 0.33 and 15 microns. *R. typhi* does build actin tails as well but just a small percentage of the bacteriae and shorter tails [60].The movement of the *R. typhi* seems to be deficient (or inefficient) in its directional control of motility since its movement occurs in circular paths with many changes in speed and direction, unlike the SFG rickettsiae, which tend to move in straight paths with constant speeds [61]. This indicates a possible cause for the different cytopathic effect of these two rickettsial strains [62].The hypothesis is supported by the fact that *R. rickettsii* one of the most cytopathic rickettsiae do activate with its *RickA* proteins the unbranched and the the branched actin filament building, this leads to a higher efficiency of exploiting the migration mechanism by using short Y actin filaments and long actin tails [63].
Genetic comparisons of the *RickA* gene locus in several rickettsial strains did lead to 12 rickettsial strains which posses a functional *RickA* locus all these twelve rickettsial strains are members of the SFG (e.g. *R. conorii* and virulent and avirulent strains of *R. rickettsii*, *R. montanensis*, *R. parkeri*, *R. australis* and *R. monacensis*). It is believed that the TG lost this ability over the course of evolution [64]. But *R. peacockii*, a member of the SFG which is

closely related to *R. rickettsii*, is unable to form actin-tails. This indicates a evolutionary disruption of the *RickA* gene locus [65, 58, 59].
The genetic locus of the twelve SFG rickettsiae did show some differences. Proline-rich repeats were inserted in different amounts in the in the locus, amino acids were changed and the WASP-homology region were presented in different amounts as well [66].

3.4. Metabolism

As obligatory intracellular living cells many rickettsiae possess a highly specific energy metabolism which is highly adapted to the host cell interior which functions as a metabolite-replete niche. In salt solutions with buffered conditions the isolated rickettsiae are unstable, losing both metabolic activity and infectivity for animal cells. The medium for growing rickettsiae in vitro needs to be enriched with potassium, serum albumin and sucrose to support viable rickettsiae for some hours [1]. By adding ATP to the medium the rickettsiae start to metabolize and consume oxygen. In general rickettsiae can oxidize only glutamate or glutamine and cannot oxidize glucose or organic acid as electron donors. It possesses a respiratory chain complete with cytochromes and is able to carry out electron transport phosphorylation, using NADH as electron donor. They are able to synthesize some of the small essential molecules but obtain the majority from the host cell metabolism. So unlike highly adapted parasites they maintain some sort of independent metabolic function, which are used if the host cell milieu changes to unfavorable conditions [1].

3.4.1. Energy Transport in rickettsia

3.4.1.1. ATP/ ADP transport

ATP is a necessary free energy source for the carbohydrate cycle, the lipid cycle and some other energy consuming reactions in the metabolism of rickettsiae. It has also the function of an adenylate source for some bacteriae. Rickettsiae are not able anymore to synthesize this energy molecule or to rebuild ATP from already used ADP so the rickettsiae possess an ATP and ADP transporter system which is carrier mediated in the case of the *R. prowazekii* the genetically oldest rickettsia known so far. The genome of *R. prowazekii* contains five genetic

sequences which are believed to be translocases, the Tlc family. But only the *tlc1* is actually an ATP/ADP translocase, the others are transporters for CTP, UTP and GDP. The system is used to exchange rickettsial ADP for host cell ATP which is available at high concentrations in the host cell cytoplasma. The host ATP is used as main energy source and not as may be regarded as adenylate source for the bacteria. The system is dependant on the phosphate concentration in the host cell, the pH level at a functional maximum of seven and the concentration of the other energy molecules means that ADP inhibits ATP transportation and *vice versa*. The carrier system is highly selective for ATP and ADP but can transport sugar modified ATP and ADP molecules like dATP, ddATP without a loss of efficiency. Other energy molecules like AMP were neither transported nor did function as inhibitors for this transporter molecule [67, 68, 69, 70].

3.4.1.2. AMP transport

Rickettsiae posses a selective exchange and transport system for AMP. AMP is exchanged with other daenosine phosphates like ATP or ADP from the host cell by different translocases than the ATP band ADP is. The selective carrier is inhibited by a high concentration of ATP, AMP and ADP. The system is dependant on the phosphate and magnesium concentration of the host cell cytoplasma; it is also temperature-dependant, which means an optimum of input at 34°C and a loss of input at 0°C. Compared to the before described ATP/ADP exchange system, the AMP input is not as fast and not as efficient as that system [71].

3.4.1.3. NAD+ transport

NAD^+ is an electron carrier for the cell metabolism. Many enzymes carry a NAD^+ binding site like dehydrogenases for pyruvate, a part of the glycolysis. It can be generated as metabolite from AMP with the help of other molecules like glutamine. Several steps are needed to synthesize the functional NAD^+ from the AMP. Rickettsiae are able to perform an input of the NAD^+ from the host cell cytoplasma. But the NAD+ needs to be hydrolyzed before the inpu can be performed. The membrane of the rickettsiae contains a pyrophosphatase which may perform a hydrolysis of ATP to AMP and in the same step generating NAD^+ from host NADH. As described before the AMP, ATP or ADP which is

generated during the process of NAD^+ hydrolysis will be transported as well into the rickettsial cell. High concentrations of AMP in the host cell do inhibit the uptake and hydrolysis reaction, but the rickettsia is also able to perform the hydrolysis of NADH inside its cytoplasma by using host cell ATP [72].

3.4.2. Polyamine metabolism in rickettsiae

Polyamines are ubiquitary products from the cell metabolism of eucaryotes and microorganisms. State of knowledge show that the polyamines putrescine, spermidine and spermine are essential factors needed for the growth and differentiation of tissues in eucryotic organisms and for growth and division in microorganisms [73]. The host of the rickettsiae synthesizes its own polyamines endogenous, but there is the possibility of an intake by other means like the nutrition of the host. For example human tissue is able of synthesizing and of uptake from the nutrition of the essential polyamines [74, 75]. The ornithin carboxylase is the main enzyme in the polyamine synthesis pathway. Rickettsiae are able to grow in an ornithin carboxylase mutant cell line which is fed with putrescine but not with the other two polyamines. The rickettsiae which had been grown in these cell lines contained all three polyamines but did not show a significant uptake or transport of spermidine, spermine and putriscine. The rickettsiae did perform the de novo synthesis of the polyamines by using arginine as source for their arginin decarboxylase. 100% of the rickettsial polyamines were synthesized from host cell arginine [76]. An important component of the de novo synthesis of the polyamines is the S-adenosylmethionine which plays important roles in many metabolic pathways as part of the ATP usage as energy source. Rickettsia is able to transport SAM in its cytoplasma by a selective transport system. The genome of the rickettsia contains a *metK* gene coding for the SAM production cascade enzyme adenosylmethionine decarboxylase. This would indicate that SAM is not necessary for the rickettsial growth but in fact the SFG and the Madrid E. strain of *R. prowazekii* do inherit a mutation in the *metK* gene and suffer a loss of function but not all members of the TG which do inherit a functional *metK* gene [52]. These members of the TG are able to grow even without a host support of SAM. This indicates further more that the SAM pathway genes are in the process of genetic degradation and that the SFG orthologs are more degraded than the TG orthologs.

3.4.3. Amino acid transport and metabolism in rickettsiae

Amino acid transport systems in free-living bacteria such as *E. coli* have characteristically been highly specific, with high affinity and capable of scavenging amino acids in low concentrations from the medium [77, 78].

Rickettsiae are not able to synthesize all of the needed amino acids but can transport the needed amino acids from the host cell in its cytoplasma. This is accomplished by a very highly specific influx transport system for the amino acids the bacterium needs for its metabolism. This feature probably allows the rickettsiae, which is in a rich but highly competitive environment, to compete effectively for free amino acids with the metabolic machinery of the cytoplasm of its host cell.

3.4.3.1. Proline

In the case of proline an amino acid which is not essential but is the only amino acid with a secondary amino group and with a ring formation, rickettsiae are not able to synthesize it. Just the biological active L-proline is incorporated by the rickettsia. The proline is not used as energy source to drive the hemolysis and not used for the deriving of CO_2 as carbon source. And the enzymes pyroline-5-carboxylate-reductase and pyrroline-5-carboxylate dehydrogenase, the major proline metabolism enzymes, were not active in rickettsia [79]. This indicates that rickettsiae do not have a functional proline metabolism. The influx is not compared to a specific efflux which leads to the hypothesis that the rickettsiae do have an unknown carrier molecule for the incorporated proline and some unknown use for the incorporated proline.

3.4.3.2. Serine

In the case of serine the rickettsiae depend on the host provided amino acid pool. The pool of this amino acids is increasing during an infection with rickettsiae in tested Vero cells. Without this amino acid the rickettsial growth is inhibited. The activity of the serine hydroxymethyltranserase, which catalyses the conversion of serine and tetrahydrofolate into glycine and methylenetetrahydrofolate, was detected in a rickettsial population which

indicates a functional Serine metabolism of the rickettsiae [80]. The gene for this molecule was also found in the genome of *R. prowazekii* [52].

3.4.3.3. Glutamine

In the case of glutamine, rickettsiae lack the necessary coding sequences for the nitrogen metabolism which allows the synthesis of glutamine, indicating that glutamine must be acquired directly from the host cell cytoplasma [52].

3.4.3.4. Other amino acids

Several genes associated with the lysine biosynthesis (*lysC*, *asd*, *dapA*, *dapB*, *dapD*, *dapE* and *dapF*) are present in *R. prowazekii*. The biosynthetic pathways leading to lysine, methionine and threonine share the first two of these genes the *lysC* and the *asd*. But none of the downstream genes for threonine biosynthesis or fragments of these genes are found in *R. prowazekii*. The lysine pathway itself is missing *lysA*, which encodes the enzyme that converts meso-diaminopimelate to lysine [52].

3.4.4. Glycolysis and TCA cycle in rickettsia

Glucose can be metabolized to pyruvate anaerobically to synthesize ATP through entering the glycolytic pathway. Glucose is metabolized to fructose 6-phosphate which is metabolized by phosphofructogenase to fructose 1,6-bisphosphate which enters a long cascade of metabolic changes and ends as phosphoenolpyruvat. The phosphoenol pyruvat is metablolized by apyruvate kinase to pyruvate. Glycolysis brings just a fraction of the ATP outcome possible from the glucose molecule. Glucose may also enter an aerobic pathway as well, the citric acid cycle. In this metabolical pathway a glucose derivate pyruvate is reduced to acetyl Coa which is completely reduced to CO_2 and this produces the energy to synthesize or recycle larger amounts of ATP.

In the case of rickettsiae the transport of the glucose intermediate uridine 5'-diphosphoglucose is possible but not of glucose. The function of the required enzymes hexokinase and phosphoglucomutase for the glucose metabolism is missing [81]. Several genes coding for the glycolysis pathway are missing in the rickettsial genome supporting the thesis of the non functional glycolytic pathway to oxidize glucose [52]. But the uptake of the glucose derivate described before indicates that rickettsia is able to perform another metabolic pathway to oxidize the glucose derivate in a pathway, the TCA cycle. This is supported by the fact that rickettsia has encoding genes for the enzymes required for pyruvate metabolism indicating a possible pyruvate transport system. A well described mechanism for this transport is the phosphoenol pyruvate (PEP) translocase [82]. The TCA cycle is also important for the amino acid metabolism by inserting some amino acids in the deamination pathway which leads to the TCA cycle. Several genes encoding for this steps are located in the rickettsia genome for example *aatA*, encoding aspartate aminotransferase, which catalyses the degradation of aspartate to oxaloacetate and glutamate, *tdcB* encodes threonine deaminase, which converts threonine into ∝-ketobutyrate, *ilvE* encodes branched-chain-amino-acid aminotransferase, which converts leucine, isoleucine or valine into glutamate and *pccA* and *pccB* encode propionyl-CoA carboxylase, which converts propionyl-CoA, an intermediate in the breakdown of methionine, valine and isoleucine, into succinyl-CoA [52].

3.4.5. Potassium acquisition in rickettsia

The uptake of potassium depends on the metabolic needs of the rickettsiae if the rickettsiae are in the state of erythrocyte lysis or the lysine transport the uptake is increased to a significant level [83]. Other salts play an important role as well in the rickettsiae. The uptake from the host or artificial medium does influence the growth rate and the stability [84]. Different salt concentrations do not influence different rickettsiae [85].

3.4.6. Genome of rickettsiae

To understand the pathogenicity, physiologies and evolutionary mechanisms, different species of rickettsiae are sequenced and are on the way to be sequenced. Up to now the 11 following

Rickettsia species (Tab. 2) are completely sequenced [42]. The genome of *R. prowazekii* as an example, is composed of a singular circular chromosome, which contains 1.111.523 base pairs and 834 orf`s which are coding for proteins were investigated before. Important to mention that 24% of the genome is built by non - coding sequences; this is a big amount compared to other genomes of microorganisms. This may indicate the adaption and co-evolution to the host organism. As mentioned before, no genes for anaerobic glycolysis are present but genes coding for the TCA cycle and a respiratory chain complex [52].

Species	Sequence Type	Topology	Size(bp)	GC%	Protein orf's
Rickettsia bellii	chromosome	linear	1528980	31.61%	728
Rickettsia canadensis	chromosome	circular	1159772	31.05%	782
Rickettsia conorii	chromosome	circular	1268755	31.55%	706
Rickettsia sibirica	chromosome	circular	1250021	32.47%	611
Rickettsia africae	chromosome	circular	1276710	32.40%	639
Rickettsia massiliae	chromosome	circular	1360898	32.54%	694
Rickettsia akari	chromosome	linear	1231060	32.33%	728
Rickettsia felis	chromosome	circular	1485148	32.45%	881
Rickettsia prowazekii	chromosome	circular	1111523	29%	621
Rickettsia typhi	chromosome	circular	1111496	28.92%	629
Rickettsia rickettsii	chromosome	linear	1257710	32.47%	603

Tab. 2 Topology, sequence type and siz of several genomes of rickettsiae

All rickettsiae share the features of a single chromosome but not all share the circular conformation of it. The conformation is not different in the two separated groups of rickettsia; TG member and SFG member share the circular and the linear chromosome conformation.

The size of the genome does not differ much in the rickettsiae sequenced so far. Plasmids are common in some of the rickettsiae but no relation to infectivity or symbiontic life style could be proved [86].

The GC content is important for the heat stability of the translated protein of the rickettsiae, which is not as important as it is for organisms living in extrem heat habitats. But the overall genome GC content does not refer to the heat stability of the organism like the *Streptomyces coelicolor* shows which have a GC content of 72% and is a soil living fungus like bacteria. An organsims with a very low GC content compared to other known organsims is the Plasmodium falciparum with a GC content about 20% of the whole genome. The GC content is also used as a taxonomic characteristic to classifiy organisms.

3.4.6.1. Rickettsia bellii

The genome of *R. bellii* does not share the co-linearity of the other rickettsial genomes. It exhibits many genes highly similar to homologues in intracellular bacteria of amoebae but unique to the genus rickettsiae. Sex pili-like cell surface appendages on *R. bellii* were observed. It was also found that *R. bellii* multiplies very efficiently in the nucleus of eukaryotic cells and can survive in the phagocytic amoeba, *Acanthamoeba polyphaga* [87].

3.4.6.2. Selfish repeats

In bacteriae interspersed DNA repeat sequences are usually confined in the intergeneic region, which do not code for a specific protein. Rickettsiae genomes carry special types of repeats in the coding sequences which are unique for them. Theses repeats are inserted in the orf's in frame and so are translated into the protein structure. For example *R. conorii* does carry 656 repeats. Proteins which carry these repeats do not suffer a loss of function or a gain of function. It seems that these repeats are linked to an evolutionary adaption mechanism to avoid host defense mechanisms, they can be inserted very fast, one generation, change the surface of the protein and do not interfere with the overall protein function. These repeats were separated into 10 families, three of them are palindromic repeats which show a progressive loss of the palindromic property in the functional folded protein [88]. The copy number of the repeats ranges from 5 to 223- every family of repeats carry a consensus part as well as a variable part. Of these 11 families (RPE-1 to RPE-11) 10 are specific to rickettsiae and the remaining family contains two directly orientated RS3 core motifs which are normally found in neisseria genomes [89]. All repeat families share a relative GC rich sequence compared to the overall GC content of the *R. conorii* genome. The genome of *R. prowazekii* contains tenfold less repeats then the *R. conorii* genome, but despite this they show a clear co-linearity of the genes. The difference in the amount of repeats indicates some sort of cleaning mechanism lost or dysfunctional in *R. conorii*.

3.4.6.3. Evolution in rickettsiae or reductive genome

The genome of organisms which started the evolutionary road of adaption to a niche or the symbiontic evolution is target of another evolutionary pressure than the free living organism

genome was. The co-evolution to fit the niche, the symbiontic partner or the host is a stronger pressure on the genome than, for example, the need of nutrition acquisition from the habitat, because the relation does provide nutrition easier than it would be in the host-outside competition. This means that genes formally needed for survival are rendered expendable and as a consequence these genes become vulnerable to obliteration by mutation. And this does lead over a long time of co-evolution, to an obligate need of the partner, host or niche. If the bacteria which is entering a habitat that is closed from outside influences and genetic diversion it will accumulate these non fatal mutations of the former essential genes over time and generation changes. The selection in the habitat is not able to remove these mutations from the clonal population; this is referred to as “Muller's ratchet” or “near-neutral evolution”. The mutations start with a diminishing of function and lead, to a total loss of function of non essential genes over time [52]. The cascade started by the first mutation of inactivation is likely to initiate a sequence of events in which subsequent mutations freely transform it, by degrees, from a pseudogene, to unrecognizable sequence, to small fragments, to extinction. A strong indicator for the ongoing degeneration of the genome of non essential genes is the nucleotide substitution it does reflect the mutation bias of the genome. This bias can be estimated roughly by counting the frequencies of third-position bases in the codons (Wobble hypothesis).

Rickettsiae as obligate intracellulary or intranuculary parasites are a good example for this theory. Genes in rickettsiae which may have been target of this evolutionary degeneration are, for example, genes required for the amino-acid biosynthesis, nucleoside biosynthesis and the anaerobic glycolysis. The partial loss of these genes and the in some rickettsiae still functional or just silenced sequences indicate that the progenitor of the rickettsia was a free living bacteria. As example for the bias of a rickettsia, *R. prowazekii*, is 18% GC compared to the 29% of overall GC content- this means that the evolution of the genome will decrease the overall GC content to the third base bias of 18%. Approximatley one-quarter of the *R. prowazekii* genome is composed of non coding sequences with an average GC content lower than the coding sequences, 25% in non-coding to 30% in coding sequences [90]. This indicates that the majority of the non-coding sequences are the left-overs of genes and are in the process of being eliminated. Another mechanism is also presented in rickettsiae which are able to rearrange genetic sequences, an intrachromosomal recombination mechanism. This mechanism is able to duplicate sequences, rearrange flanking sequences and add or take fragments from sequences resulting in deletions. This may have been the case for one unlinked copy of *rrs* and *rrl*, both of which are surrounded by new flanking sequences [91].

3.4.6.4. The *metK* gene in the process of degeneration

As described before in the polyamine synthesis paragraph, the gene encodes for *S* - adenosylmethionine synthetase [92], which catalyses the biosynthesis of SAM. The *R. prowazekii* has received a stop codon in a highly conservated region of the *metK* gene which is the cause of the loss of function. In many other rickettsiae, mainly members of the SFG, defects, such as termination codons, insertions, and a preponderance of small deletions, have also been observed in the *metK* genes [93]. This random distribution of lethal mutations among some *metK* alleles from different rickettsia species indicates that the gene may have just entered the extinction process, which is supported by the existence of a SAM transport system [91].

3.4.6.5. Evolution of the rickettsial mother

Comparing the bias, amount of functional orf's, non-coding pseudogenes, repeats and duplications of different rickettsiae may lead to the ancestral genome of this family. Following the thesis of neutral evolution the rickettsial ancestor already possessed all or the majority of the genes the present rickettsiae possess and more that were lost in the course of the co-evolution [94]. The genome of *R. prowazekii* did not contain a high incidence of neo-genes, this indicates that the rickettsial ancestor exhibited just a few gene transfers before it entered the future host cell. In the host cell the habitat did not support the survival of two different bacteriae minimizing the possible later gene transfer [52]. To get a reliable result for the rickettsial ancestor genome the five major sub groups were compared on the genome level by using genomes of *R. conorii* [18], *R. prowazekii* [10], *R. typhi* [40], *R. belli* [16], *R. massilae* [15], *R. africae* [12] and *R. felis* [19]. These species contain approximately 39,5 RNA genes, and between 807 and 1285 protein coding sequences. The 704 core proteins that are common in rickettsiae are not all known today- 546 of these genes are associated with a known biochemical function and the remaining 158 are unknown but 40 of these unknown are unique to rickettsiae.

The amount of lost genes in the rickettsiae ranges from 50 in *R. felis* to 306 in *R. typhi*. The rickettsial ancestor possessed approximately 1254-1700 genes and 1252 protein coding sequences [94]. The result indicates that the TG genomes were shaped from the reductive ancestral genome. 611 cases of not comparable genes loci remained in the pool of genomes.

211 of these were unique to *R .bellii* supporting its classification neither for the TG nor the SFG. 404 cases were common in the SFG and the TG but not in the hypothetical ancestor, this strongly indicates gene transfer. The gene transfer is performed by a plasmid which was found in *R. felis* [87]. The plasmid carried some genes usually associated with gene transfer. This indicated a possible gene transfer in the rickettsial ancestor. *R. bellii* which does not exhibit the colinearity observed between other rickettsial genomes, and encodes a complete set of putative conjugal DNA transfer genes most similar to homologues found in *Protochlamydia amoebophila* UWE25, an obligate symbiont of amoebae. This suggests that amoeba-like ancestral protozoa could have served as a genetic "melting pot" where the ancestors of rickettsiae and other bacteria promiscuously exchanged genes, eventually leading to their adaptation to the intracellular lifestyle within eukaryotic cells.

3.5. Clinical relevance and pathogenesis

3.5.1. Pathogenesis of rickettsiae

Intracellular living parasitic rickettsiae are in need of several virulence mechanisms to maintain their survival in the host cell, to invade new host cells and to interact with the immune system of the vector and the host challenging them. The genetic determinants required for the rickettsiae to invade a host and to establish a stable infection are not well understood at the moment. Different complete sequence assays of rickettsiae lead to about 40-50 genes involved in the pathogenesis of the rickettsiae [86, 52].

3.5.2. Adherence to the host cell

Adherence to the host cell is the first step of rickettsial pathogenesis. The adhesins are presumed to be outer membrane proteins. The outer membrane protein OmpA has been implicated in adherence of *R. rickettsii* because antibodies to OmpA have been shown to block adherence [95]. The major antigens are called OmpA, OmpB and *sca1-11*. Other important antigens are lipoproteins like *nlpD* and an ortholog of a *Salmonella enterica* serovar typhimurium virulence factor *mviN* [96]. The host cell receptor for any rickettsia has yet to be identified. Although the main target cells of rickettsia in vivo are endothelial cells, rickettsiae

can infect virtually every cell line in vitro [91]. Thus, either the receptor for rickettsia is ubiquitous among cells, or rickettsiae can bind to different receptors.

3.5.3. Invasion of host cells

After attaching to the host cell membrane, rickettsiae are phagocytosed by the host cell. The rickettsiae are believed to induce host cell phagocytosis because they can enter cells that normally do not phagocytose particles [97]. The rickettsiae escape the phagosome and enter the cytoplasma shortly after entering them.

R. prowazekii contains a dinucleoside polyphosphate hydrodolase that is believed to play an important role in the maintenance of the favorable cell environment for the rickettsial growth in the host cell [98]. This protein hydrolysis toxic dinucleoside oligophosphates within the host cell to produce ATP, thus providing an environment better suited for the rickettsial growth [99]. Cellular dinucleoside oligophosphates are necessary by-products of the reactions of aminoacyl-tRNA synthetases [100] and the amount present in prokaryotic and eukaryotic cells is small [101]. The exact function of these molecules is still not well understood but some studies indicate that the dinucleoside oligophosphates may be signaling molecules that can increase more than 100-fold when cells are exposed to conditions such as heat shock and oxidative stress, and this increase can lead to cellular toxicity which will decrease the possible growth of the rickettsiae [102, 103].

The new invasion of host cells or the invasion during ongoing infection induced due to the cell injury performed by the rickettsiae oxidative stress in the host cells. The host cell starts a defense cascade of reactive oxygen molecules which damages the rickettsiae [104]. Orthologs of iron-associated superoxide dismutases are present in rickettsiae [52] which fulfill the same function as the eukaryotic SOD do, they catalyze the dismutation of reactive oxygen molecules into oxygen and hydrogen peroxide.

To leave the infected cells the rickettsiae use not only the previously described movement. The exit of the cells, the double vacuole and the phagolysosome, which surrounds them when they enter a new cell, is performed by using a phospholipase A2 [105]. The TG, *R. typhi*, seems to use a patatin famlily like protein as phospholipase. Patatin is the main storage protein found in potatoes but also has phospholipase activity [106, 107]. In the SFG, *R*

.*conorii*, another gene seems to encode for a phospholipase D protein [108]. An ortholog of the SFG phospholipase D is also present in the TG.

Hemolysis may play an important role in parthogenesis of the TG rickettsiae but not of SFG rickettsiae [95, 96]. The genome of a TG *R. prowazekii* contains coding sequences for two potential hemolysins [52]. The hemolysis is performed with blood cells and end in the destruction of them. The rickettsiae are able to infect many different cells in the host but the first contact host cells are the blood cells because they are transmitted by blood sucking arthropods or by the feces of arthropods which is rubbed in a wound [109].

3.5.4. Medical relevance

Rickettsiae are ubiqitary distribted over the world. Rickettsial infections have been important diseases during several centuries in the western civilization. The epidemic typhus has been known since 400 years and was associated with war and crisis. Several wars were influenced by the outbreak of the epidemic typhus and lead to estimated more than 1,000,000 deaths in the two world wars. The epidemic typhus is transmitted by body lice (e.g. *Pediculus humanus corporis*) and can be transmitted fast from people to people by using unwashed cloths, it is caused by *R. prowazekii*. The modern hygiene prevents an outbreak of epidemic typhus in the western civilization. But ongoing increasment of the poor people in the western civilization does increase the risk of a reoccurring of the epidemic typhus because of poor sanitary conditions which do enhance louse proliferation [110]. A recent outbreak of epidemic typhus in Burundi in a jail showed the emerging course of the disease [111].The RMSF which is caused by the *R. rickettsii* is distributed in North and South America. The cases reported in the USA since 1920 were decreasing and from 1200 per year to 250 and estimating the not reported cases 400 to 700 per year in the USA. But in 2007 a fatal case in Panama [112] which was the first reported case since 50 years this indicates a still stable number of not reported cases. The MSF which is distributed around the Mediterranean Sea and south Russia the fatality of the disease seems to be increasing over the past 20 years [113]. MSF is caused by *R. conorii*.

These and several other rickettsial caused diseases were emerging in the past 20 years. The caused disease and the distribution of some members of the genus *Rickettsia* is summarized in Table 3.

	TG			SFG	
organism	disease	distribution	organism	disease	distribution
R. rickettsii	Rocky Mountain spotted fever	Western hemisphere	R. prowazekii	Epidemic typhus, Recrudescent typhus, Sporadic typhus	South America and Africa, Worldwide, United States
R. akari	Rickettsialpox	USA, former Soviet Union	*R. typhi*	Murine typhus	Worldwide
R. conorii	Boutonneuse fever	Mediterranean countries, Africa, India, Southwest Asia			
R. sibirica	Siberian tick typhus	Siberia, Mongolia, northern China			
R. australis	Australian tick typhus	Australia			
R. japonica	Oriental spotted fever	Japan			

Tab. 3 Geographical distribution of human pathogens of the genus *Rickettsia*

3.5.5. Treatment of rickettsioses

Rickettsia species are susceptible to the broad-spectrum antibiotics, doxycycline, tetracycline, and chloramphenicol; they were first shown to be effective in the treatment of RMSF during the late 1940s. Prevention of exposure to infected arthropods offers some protection as well. A vaccine exists for epidemic typhus but is not readily available. Tests from 1998 [114] showed that of 13 tested antibiotics (doxycycline, thiamphenicol, rifampin, amoxicillin, gentamicin, co-trimoxazole, ciprofloxacin, pefloxacin, ofloxacin, erythromycin, josamycin, clarithromycin, and pristinamycin) by using two in vitro cell culture methods, the plague assay and the microplague colorimetric assay [115], for 27 rickettsial species ,the

susceptibility of rickettsiae to doxycycline, thiamphenicol, and fluoroquinolones were proven. Beta-lactams, aminoglycosides, and cotrimoxazole showed no significant antirickettsial activity. Typhus group rickettsiae were susceptible to all macrolides tested, whereas the spotted fever group rickettsiae, *R. bellii*, and *R. canada* were more resistant, with josamycin, a safe alternative for the treatment of MSF, being the most effective compound. Strain Bar 29, *R. massiliae*, *R. montana*, *R. aeschlimannii*, and *R. rhipicephali*, which are members of the same phylogenetic subgroup, were more resistant to rifampin than the other rickettsiae tested.

3.6. Aim

As described before in this study three species of rickettsia were used (*R. helvetica*, *R. slovaca* and *R. conorii*) to get a suitable antigen to design a detection system which may differentiate genus and species. The antigen which was used to obtain the differentiation is the *omp1* which holds six regions in the protein five repeats and a single antigenic region which shares some homology with antigens from influenca. The cross reactivity should be low because of the missing repeats in the protein.

3.6.1. Rickettsia conorii

In 1910 seven cases of a fever were studied by Conor and Bruch in Tunisia. The fever was described as "fièvre boutonneuse de Tunisie". The causative agent was unknown at that time [116]. Soon thereafter several cases around the Mediterranean basin were reported. The disease was renamed in Mediterranean boutonneuse fever. But the typical manifestation of a popular rash in the majority of the cases changed the name into spotted fever. The black rash which is one of the main symptoms of the spotted fever was described in 1925 [117] in France. The transmission vector was described in 1930 by an experiment in which homogenized dog ticks were inoculated in humans [118]. In 1932 Brumpt isolated the rickettsial agent causing the MSF and named it after Conor [119].

R. conorii is the etiologic agent of boutonneuse fever or Mediterranean spotted fever. It is believed to be the most geographically dispersed spotted fever group member [120]. *R. conorii* is transmitted by the brown dog tick *Rhipicephalus sanguineus* but just by the larvae and the nymphs [121]. Characteristic symptoms of the infection are fever, rash and a

vasculitis. Fatal outcomes are occurring in endemic areas of a high vector population [122, 123]. The interaction of the *R. conorii* and the immune system is well studied and the immunity is based on an IFN-y production and active T lymphocytes [124, 125]. Nk-cells and macrophages are involved in the innate immune response. *R. conorii* is able to infect macrophages but it can be controlled in the macrophages by three different mechanisms of the cell nitric oxide synthesis, hydrogen peroxide production and tryptophan degradation. The *Rickettsia* is able to interact here with different previously - described control genes. The first interaction of innate immunity and rickettsial virulence mechanisms at the inoculation point is still poorly understood [126, 127]. *R. conorii* has been described in France as previously stated, in Greece [128], in Italy [129], in Israel [130], in Russia [131] in Spain [132] and in many other countries.

3.6.1.1. Rickettsia conorii complex

Mediterranean spotted fever and its variants-Astrakhan fever, Israeli tick typhus, and Indian tick typhus-are caused by *R. conorii* and are transmitted by dog ticks. The variants are counted as members of the *R. conorii* complex but not as subspecies. The four subspecies were separated on the base of genetic and serological methods [133].

3.6.2. Rickettsia helvetica

The first sign of the *R. helvetica* or Swiss agent appeared 1979 in a publication from Burgdorfer [134]. This paper described a survey for ticks and rickettsial agents in some parts of Switzerland. In 11.7% of the surveyed ticks (*Ixodes ricinus*) this “Swiss agent” of a spotted fever agent was present. The rickettsial-like bacteria developed intracellular and were located in the germative cells as well as in the ovaries. The "Swiss agent" appeared to be nonpathogenic for guinea pigs, domestic rabbits, and Swiss mice, but in male meadow voles (*Microtus pennsylvanicus*) it produced a microscopically detectable infection in the tunica vaginalis. Further characterization by Beati et al. [135] suggested that this *Rickettsia* represented a new member of the spotted fever group of rickettsiae and suggested the name *R. helvetica*. In 1996 a rickettsiae of the spotted fever group were detected in an *Ixodes ricinus* in Sweden. This was the first time that a *Rickettsia* species was recorded as being indigenous to

Scandinavia [136]. A preliminary characterization, including amplification and sequencing of almost the entire 16S rRNA gene, showed 100% homology with the published sequence of *R. helvetica*. On the other hand, digestion of the approximately 380-bp fragment of the citrate synthase gene with *Alu*I revealed a pattern with distinct differences from the published restriction fragment length polymorphism (RFLP) pattern of *R. helvetica* [137]. Results of a serosurvey of forest workers from an area in France where the patient lived showed a 9.2% seroprevalence against *R. helvetica.* The serosurvey were performed with 379 serum samples from forest workers from Alsace, all of whom were state employees. This population was composed of 377 men and 2 women, 20 to 59 years of age; 360 (95.5 %) reported frequent tick bites but were clinically asymptomatic; the remaining 19 reported no tick bites. After informed consent from the patients, antibodies to *R. helvetica, R. conorii, R. slovaca,* and *"R. mongolotimonae"* were determined. [138].

In the year 2000 *R. helvetica* has already been isolated from *Ixodes ricinus* collected in Switzerland, France, Slovenia, and Sweden. These ticks, which readily bite humans, are also the vectors of *Borrelia burgdorferi* and *Anaplasma phagocytophila,* the agents of Lyme borreliosis and human granulocytic ehrlichiosis, all over Europe. In 2002 109 *I. ricinus* ticks collected in north and central Italy were collected and the PCR examinations were performed and nine ticks positive for rickettsia were found. Among them, no less than three different spotted fever groups of rickettsiae were revealed, including *R. helvetica* [139]. *R. helvetica* is distributed all over Europe but several findings as in Morocco [140] were made and indicate a worldwide distribution of *R. helvetica*.

3.6.2.1. First confirmed cases of Rickettsioses by R.helvetica

In 1997 a patient was hospitalised in eastern France who showed a febrile illness apparently caused by bacteria but the causative agent could not be determined. This patient sero-converted to *R. helvetica* four weeks after a succsessful antibiotic treatment [138].

In 1999 it was demonstrated that *R. helvetica* may play a role in the development of perimyocarditis and sudden death in two young patients in Sweden. The examination showed chronic interstitial inflammation and the presence of *Rickettsia*-like organisms predominantly located in the endothelium [139].

3.6.3. Rickettsia slovaca

R. slovaca was first isolated from *Dermacentor marginatus* ticks collected in Slovakia in 1968 [140]. At that time the organisms was not counted to be a member of the pathogenic rickettsiae. A technique used to identify *R. slovaca* is the RLFP which was performed by using a genus specific primer pair of the citrate synthase gene or the antigens like the ompA and ompB followed by the the AluI, PstI and RsaI digestion [141]. *R. slovaca* has been reported in several countries since the first description in Slovakia for example in France [142] or in Armenia [143]. The isolated *R. slovaca* strains were collected from *D. marginatus* and from *Argas persicus* ticks. The presence of the *R. slovaca* was confirmed by using a PCR [141]. Several ticks collected in Germany in 1975 were screened for rickettsial strains and four were isolated. One of these rickettsial strains was similar to *R. slovaca* but it was not confirmed to be [144]. Studies performed on the antibody distribution in *R. sanguineus* showed that antibodies against *R. slovaca* were present. The ticks were collected in the outskirts of Rome in Italy [145]. It was also isolated in Hungary and in Portugal in 1995 from adult *D. maginatus* [146]. It was also detected in Austria in 1977 from *D. marginatus* [147] and in *I. ricinus* in Lithuania in 1981 [148]. In 2001 the distribution of the *R. slovaca* was extended by the finding of it in *Dermacentor* ticks in the Russian Federation and Kazakhstan [149].

3.6.3.1. First confirmed cases with Rickettsia slovaca

The first occurrence of an illness which was suspected to be related to a *R. slovaca* infection was in 1998. The symptomes were hemiparesis, meningitis, and transient skin inflammation at the site of the tick bite from a *Dermacentor* tick. Its etiology was confirmed serologically by a significant increase of complement-fixating antibodies against *R. slovaca*. The identification of the species was not made with appropriate methods, just the serological evidence remained. The rickettsial antibodies are known to result in many cross reaction in between the SFG or the TG [143]. In 1987 a patient was observed with an unusual crustaceous scalp reaction after receiving a tick bite [150]. The most general symptoms include enlarged and sometimes painful lymph nodes in the region of the tick bite, characteristically in the occipital region and or behind the sternocleidomastoideal muscle as well as fever, fatigue, dizziness, headache, sweat, myalgia, arthralgia, sweat and loss of appetite. Besides the local reaction, the most pronounced symptom was enlarged lymph

nodes, which led to the clear visible symptoms but may not have been a *R. slovaca* infection [151, 152, 153].

4. Material and Methods

4.1. Genomic material

The genomic DNA samples used in this study were ordered from the "WHO Collaborative Center for rickettsial Reference and Research" in Marseille. Genomic DNA samples of *R. helvetica, R. conorii* and *R. slovaca* were used. The Malish 7 strain of *R. conorii* used in this study was first sequenced in 2002, the other strains are not sequenced up to date. Primers and probes for PCR and Real Time PCR applications were in silico by using the NCBI database designed and ordered from the MWG Company. The DNA used as calibration standard, in the case of *R. conorii,* was received from Invitrogen.

4.1.1. Human serum samples

Serum samples for screening studies and positive control sera were obtained from the serum collection of the Hygiene Institute, including a collection of sera from hunters and from blood donors. Sera from hunters were collected in collaboration with the Burgenländischer Jagdverband and blood donor sera were kindly provided by the Austrian Red Cross.

4.2. Methods

4.2.1. In silico methods

4.2.1.1. Selecting the antigen of interest for the primer design

The number of described antigens in the NCBI database for *R. conorii* (so far the only strain of the 3 strains in this study that has a fully sequenced genome) was 13, two of them were AAL03825, a 17kD and 159aa antigen and an outer membrane protein precursor, possibly for the *ompA*. No further information was available about domains and antigenic regions so it did

not fulfill the premises: AAL02557, *Sca1* [51] a 5000 bp and 1891aa antigen too provided no specific antigenic region but a firmly described function as an autotransporter in the outer membrane it was not taken because of the size of the possible cloning sequence: AAL03169, a 256aa and 771bp antigen, which was described as hypothetical antigen but a pair of primers were already provided by the reference paper [70]. The hypothetical antigen was not clearly studied: AAL02740. 1 outer membrane protein (*omp1*) a 2307 bp and 768 aa had 6 predescribed protein domains. Five of the 6 domains were repeats with possible antigenic characteristics (Pfam07244) but the last domain was an antigenic region with some homology to other antigens like that of Influenza. The domain was blasted and the results showed no significant similarity to other bacteria than rickettsia strains. According to the reference paper [70] the gene is no split-gene like some other rickettsial surface protein genes.

4.2.1.2. Assorting a panel of test sera

The sera used in this study were taken from a collection of sera of the Hygiene Institute. The sera were matched by age to get a wide range of time of exposure to ticks. Hunters which did actively hunt for a long time were taken preferently before hunter samples with a shorter tick exposure time but the same age. The age of the hunters ranged from 18-64 years. Altogether, 100 samples were selected in the age limits of the oldest to youngest donor (18-62 years of age). Serum samples from 24 female and 76 male hunters and from 33 female and 67 male blood donors were included. The blood donor sera were taken mainly from persons who lived in the same geographical area as the hunters but who were not professionally exposed to infected ticks.

4.2.1.3. Biostatistics

The null hypothesis was formulated theoretically and based on the experimental study. The choice of samples and the collection of experimental data were performed to verify the hypothesis. The null hypothesis Ho was formulated to be rebutted by the alternative hypothesis Ha which could be verified by the experimental data with an calculable risk of a false result.

For example one of the Ho hypotheses in the present study was that the characteristic gender does influence the amount of sero-positive patient samples.

The correlated Ha alternative hypothesis must be that gender does not influence the amount of sero-positive patient samples.

The qualitative, discrete and nominal scaled characteristics were matched by frequency and the CHI^2 test was performed. The median was used to calculate the degree of freedom and the bias.

The independent, quantitative and ordinal scaled characteristics were down scaled on the nominal scale to allow a match in 2 by 2 tables. The accuracy and the frequency were calculated for these characteristics using the mean. The t-test for independent characteristics was used to calculate the p-value.

4.2.2. DNA manipulation techniques

4.2.2.1. PCR

The antigenic region that was chosen for this study was obtained and amplified by a Hot Start PCR. Several sets of primers were designed and tested for the pfam01103 region on the *omp*1 gene sequence obtained from the NCBI Database. The PCR program used started with 95°C for 15 minutes to unfold the Hot start polymerase followed by 95°C for 5 minutes, 42 °C for 2 minutes and 72 °C for 3minutes, this cycle was repeated 30 times. The reaction mix for a single reaction contained 5 µl of the chosen forward and 5µl of the reverse primer, $MgCl_2$ (for *R. helvetica* 5 µl, 3.5 µl for *R. slovaca* and 4 µl for *R. conorii*), 5 µl of the ten times reaction buffer supplied by Applied Biosystems, 1µl of the dNTP's, 0,5 µl of the hot start polymerase, 3 µl of the genomic DNA and deionized water up to a total volume of 50 µl.

4.2.2.2. Real time PCR

In this study a real time PCR (Tab. 4) primer and probe set labeled with the fluorescence dye FAM specific for the *gltA* (citrate synthase gene) was used [154].

	forward	reverse	probe
Rickettsia conorii	TCG-CAA-ATG-TTC-ACG-GTA-CTT-T	CAC-AAT-GGA-AAG-AAA-TGC-ACG-A	TGC-AAT-AGC-AAG-AAC-CGT-AGG-CTG-GAT-G
Rickettsia helvetica	TCG-CAA-ATG-TTC-ACG-GTA-CTT-T	CAC-AAT-GGA-AAG-AAA-TGC-ACG-G	TGC-AAT-AGC-ACG-AAC-CAT-AGG-CTG-GAT-G

Tab. 4 RT-PCR primers and probes

The reaction mix was mixed at least for 5 reactions at a time. It contained 25µl Sensi dT mix supplied by Stratagene, 2µl forward and reverse primer, 2 µl of the fluorescence labeled probe, 1 µl of the studied blood sample and deionized water up to a total volume of 50 µl. The temperature program started with an initial holding of 50°C for 3 minutes, followed by 95°C for 5 minutes, 60 cycles of 95°C for 20 seconds and 60°C for 40 seconds. The calibration graph was obtained from two reference concentrations of genomic DNA with two replicas in the case of *R .helvetica* and a pure form of the *gltA* gene in the case of *R. conorii* in this case as well with two replicas. The DNA concentrations were measured by a Nanodrop Photometer.

4.2.3. DNA purification and separation methods

4.2.3.1. Separation by agarose gel electrophoresis

According to the following upstream applications for the DNA fragments, sequencing, separation by size and fragement size, an agarose gel (Tab. 5) was prepared by combining the agarose powder and the one time TAE buffer in a 500 ml Ehrlenmeyer flask, and heated in a microwave for 2 up to 4 minutes until the agarose was completely dissolved. After the solution was cooled at approximately 60 °C EtBr was added depending on the Gel percentage 5 up to 10 µl. The liquid gel was then poured out onto a taped plate with the comb already attached to it. The polymerization took about 30 min at 4°C. The samples were then mixed with loading buffer (Tab. 4) and transferred into the slots. The running conditions for the Gel were 120 VDC and 70 mA. After 1 up to 2 hours the gel was observed in an UV radiation chamber. As reference marker a step ladder containing 17 fragments from 50 to 3000 bp was used.

Agarose concentration	0.7%	1.0%	3%
Agarose	1.05 g	1.5 g	3.0 g
1x TAE	7.5 ml	7.5 ml	7.5 ml
ddH2O	142.5 ml	142.5 ml	142.5 ml
EtBr (5 mg/ml)	5µl	7 µl	10 µl
total vol	150 ml	150 ml	150 ml

Tab. 5 Agarose gel composition with different gel concentrations

4.2.3.2. Purification of DNA from agarose gel

The slice of agarose containing the DNA fragments was mixed with 10µl capture buffer per 10 mg of gel slice, up to a maximum of 900 mg gel slice. The sample was stored at 4°C overnight to improve the capture buffer activity. The samples were then heated at 60°C until the agarose was dissolved completely. The mix was centrifuged shortly to collect the DNA at the bottom of the tube. 600 µl of the capture buffer DNA mix was transferred into a micro spin column provided by the kit as well as the buffers (Ge Healthcare). The sample was incubated on the column for 1 min at room temperature. Then the column was centrifuged at 16000 g for 30 sec, the flow through was discarded and 500 µl of the washing buffer was through was discarded. 20 µl of elution buffer 1 or 2 (depending on the further use) was transferred into the column to elute the sample, incubated at room temperature for 1 minute and centrifuged at 16000 g transferred on the column. Again it was centrifuged at 16000 g for 30 sec and the flow was again centrifuged at 16000 g for 1 minute. The final flow through was collected and stored at -20°C (in general, about 70% of the DNA purified from the gel could be recovered after the purification).

4.2.3.3. Purification of plasmidic DNA from cell culture

To obtain enough plasmidic DNA for the purification (Qiagen Miniprep) a single colony of cloning candidates were picked and grown in 10 ml LB medium with kanamycin (25µg/ml) and ampicillin (100 µg/ml) over night at 37°C with shaking. On the next morning 10ml of the overnight culture was centrifuged at 8000 rpm 3 minutes at room temperature and the cell pellet was resuspended in 250 µl buffer P1, then 250µl of buffer P2 were added at this point the solution should change color to blue. Then 300 µl buffer N3 was added and the color should disappear. The whole solution was then centrifuged at 13,000 rpm the supernatant applied to a micro spin column and again centrifuged at 13,000 rpm for 60 seconds. The flow through was discarded, 750 µl buffer PE added on the column and centrifuged at 13,000 rpm for 60 seconds. The plasmidic DNA absorbed by the silica membrane was incubated at room temperature for 1 minute and was after that eluted in water by centrifugation at 13,000 rpm for 1 minute. The eluted plasmidic fraction was stored at -20°C and the plasmidic pellet from the overnight culture was stored at -80°C.

4.2.3.4. Sequencing of the DNA fragments

The sequencing was started with a sequencing PCR. The reaction mix contained 2 µl AB mix (provided by ABI prism), 1 µl forward or reverse Primer, 2µl DNA fragments and 5 µl water. The PCR program started with 96°C for 30 seconds, 30 cycles of 96°C for 10 minutes, 50°C for 5 minutes and 60°C for 4 minutes. After the PCR was completed the product was centrifuged at 3000 pm for 1 minute and mixed in a 500µl tube with 1 µl potassium acetate. After that 33µl of 100% EtOH was added and incubated on ice for at least 20 minutes. After the incubation the mix was shortly and carefully mixed by vortexing or inverted by shaking. Then the mix was centrifuged at 20600g for 30 minutes at 4 °C. The supernatant was carefully discarded and 90µl of 70% EtOH was added to the pellet followed by another centrifugation step at 20600g for 10 minutes at 4°C. After that the supernatant was discarded and the DNA pellet was air dried at room temperature. At this point 20 µl of HiDi Formamide was added and the mix was incubated at room temperature for 5 minutes. Then the mix was incubated another 5 minutes at 95°C and centrifuged at 3000 rpm for 1 minute. After that the mix was incubated on ice at least 5 minutes. The results were observed by using the online sources Genedoc or ClustalX.

4.2.4. Cloning and expression techniques

4.2.4.1. A-Addition

To increase the cloning efficiency of the UA-cloning system Qiagen provides an A-Addition kit, which adds adenosines to both ends of the PCR fragments. The mix of 1 up to 8µl DNA fragments depending on the amount and purity of the PCR product, 2µl 5 times master mix provided by the kit and deionized water up to 10µl was incubated at 37°C for 30 min with shaking.

4.2.4.2. Ligation with pQE-30 UA

The 2x Ligation Master-Mix, the pQE-30 UA expression cloning vector DNA, and the deionized water (provided by the kit) were thawed on ice for approximately 2 hours. The solutions were mixed carefully before use to avoid localized concentrations of salts. The ligation-reaction mixture were mixed by adding 1 µl Vector DNA, 1 up to 4 µl PCR product depending on the size of the fragment, water up to 5µl total volume and 5 µl 2 times Ligation Master mix provided by the kit (Qiagen). The ligation-reaction mix was mixed briefly by inverting it by hand followed by spinning it shortly and incubated at 16°C for 2 hours.

4.2.4.3. Bacterial strain

The bacterial strain used in this study was *Escherichia coli* M15. *E. coli* M15 was grown in LB broth medium (table2) at 37°C with shaking over night. The following antibiotics, kanamycin (150 µg/ml) and ampicillin (100 µg/ml) were added as selective antibiotics. The *E. coli* host strain contained both the expression (pQE) and the repressor (pREP4) plasmid. The M15 strain was derived from *E. coli* K12 and has the phenotype *NaIS, StrS, RifS, Thi–, Lac–, Ara+, Gal+, Mtl–, F–, RecA+, Uvr+, Lon+*. The *E. coli* strain M15 does not contain a chromosomal copy of the *lacIq* mutation, so the pREP4 had to be maintained by a constant selection for the Kanamycin resistance.

4.2.4.4. The vector plasmid and the inhibition plasmid of the expression system

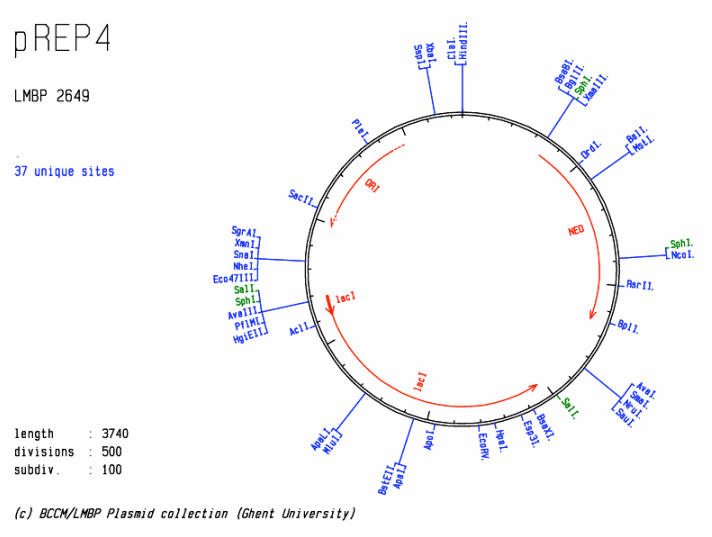


Fig. 5 pREP4 plasmid vector (Qiagen)

The pREP4 vector (Fig. 5) yields the inhibition system for the cloned expression vector and the inhibition can be inhibited by adding IPTG. It holds the kanamycin resistence gene. The pREP 4 plasmid holds several cleavage sites as well as *Xbal* 3641, *ClaI* 3736, *HindIII* 1, *BglII* 321, *BalI* 567and *Ncol* 917.

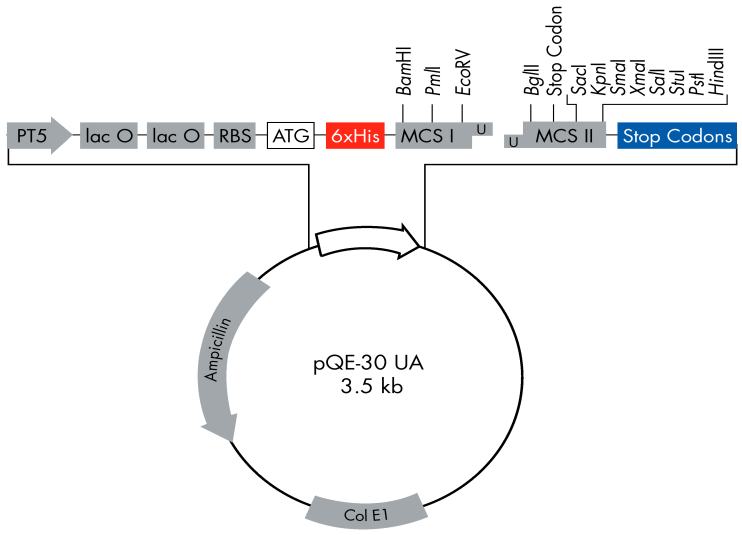


Fig. 6 pQE-30 cloning vector (Qiagen)

The pQE-30 UA cloning vector (Fig. 6) holds several operation and cleavage sites like XhoI (CTCGAG) at 1-6 bp, the T5 promoter/lac operator element at 7-87 bp, the T5 transcription start at 61 bp, the 6xHis tag coding sequence at 127-144 bp, the Multiple cloning site I at 145-173 bp, the Cloning site for PCR product at 174-175 bp, the multiple cloning site II at 176-235 bp, the lambda *t0* transcriptional termination region at 251-345 bp, the *rrnB* T1 transcriptional termination region at 1107-1205 bp, the ColE1 origin of replication at 1681 bp and the ß-lactamase coding sequence at 3299-2439bp.

4.2.4.5. Preparation of competent E. coli

A trace of the M15[pREP4] cells from the vial was removed with a sterile filter tip, and stroke out on a LB agar (Tab. 6) plate containing 25 µg/ml kanamycin followed by an incubated at 37°C overnight. A single colony was picked and inoculated in 10 ml of LB medium with kanamycin (25 µg/ml) and was grown overnight at 37°C. 1 ml of the overnight culture was mixed with 100 ml of prewarmed LB medium containing 25 µg/ml kanamycin in a 250 ml

flask, and shaken at 37°C until an OD_{600} of 0,5 was reached (approximately for 2 hours). After the incubation the mix was cooled on ice for at least 5 minutes and the culture was transferred to a centrifuge tube. The collection of the cells was performed by centrifugation at 4000g for 5 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in cold TFB1 buffer (30 ml for a 100 ml culture Tab. 6). The suspension was incubated on ice for an additional 90 minutes. Then the cells were again collected by centrifugation at 400g for 5 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in 4ml cold TFB2 buffer (Tab. 6). Then Aliquots of 200 µl were prepared frozen at -80°C and stored in liquid Nitrogen.

4.2.4.6. Transformation of competent M15 cells

The ligation mix (10 µl) was transferred into a cold 1.5 ml micro centrifuge tube, and kept on ice. The competent *E.Coli* cells were thawed on ice at the same time. 100µl of the competent cells were placed inside the tube with the ligation mix and incubated on ice for at least 20 minutes. Thereafter the tube was placed on a 42°C water bath for 90 sec. Following the heat shock 500 µl of psi broth were transferred into the ligation mix tube and it was incubated for 90 minutes at 37°C with shaking. After that 50 and 100 µl aliquots were plated out on LB-agar plates containing 25µg/ml Kanamycin and 100µg/ml Ampicillin. The plates were incubated at 37°C overnight. A negative control was performed by transforming 20 µl of water and 200 µl of this negative control was plated out on the appropriate antibiotic plates.

4.2.4.7. E.coli culture growth and inducing of expression

A single colony of the positive clone candidates was picked and inoculated in 20 ml of LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. The cell suspension was grown at 37°C overnight with vigorous shaking. 1 liter culture (LB, 100 µg/ml ampicillin, 25 µg/ml kanamycin) was inoculated with 10 ml of the overnight culture and grown at 37°C with vigorous shaking until an OD600 of 0.6 was reached. Expression was induced by adding IPTG to a final concentration of 1 mM to the cells. The culture was incubated for an additional 5 hours to perform sufficient expression. The cells were harvested by centrifugation at 4000 x *g* for 20 minutes at room temperature and stored at -20°C.

4.2.5. Protein separation and purification methods

4.2.5.1. Affinity chromatographic separation

A stored induced cell pellet was thawed for 15 min on ice and resuspended in lysis buffer pH 8 (Tab. 6) at 5 ml per gram wet weight. The cells suspension was incubated for 60 min at room temperature to improve the lysis reaction. To complete the lysis reaction the cell suspension were freezed at -80°C and thawed at room temperature. The mix was centrifuged at 10,000 x *g* for 30 minutes at room temperature to pellet the cellular debris. The supernatant was transferred on a Ni NTA column and the flow through was stored. The column was then washed with denaturating washing buffer pH 6.3 (Tab. 6) 2 times with 4 ml per washing step, the flow through was stored. The column was then filled with 2 times 1 ml of denaturating elution buffer pH 4.5 (Tab. 6). The columns were reused 2 times until the resin turned brown.

4.2.5.2. Resin washing

The Ni NTA column was constantly flooded for 30 minutes with 5N NaOH solution and washed with 30% EtOH three times. The resin was stored in 30% EtOH to prohibit growth of possibly contaminating microorganisms.

4.2.5.3. SDS gel

The gel plates were assembled with spacers and inserted into the pouring chamber. 0.75 mm thick Gels as well as 1.0 mm thick Gels were used depending on the gel chamber size. The 12.5 % separation gel was mixed by adding 5 ml water, 2.5 ml lower Tris (Tab. 6), 2.5 ml 50% polyacrylamid, 50 µl APS and 5 µl TEMED together. The stacking gel was mixed by adding 3.25 ml water, 1.25 upper Tris (Tab. 6), 0.5 ml 50% Polyacrylamid, 40µl APS and 5 µl TEMED. The running conditions for the gels were 80VDC until the samples reached the separation gel and then 120VDC, 90 mA until the dye front reached the bottom of the gel, a standard electrophoresis running buffer (Tab. 6) was used. The protein samples were mixed 1:1 with sample Laemmli buffer (Tab. 6) and heated at 95°C for at least 5 minutes.

4.2.5.4. Coomassie staining

The gels containing the separated proteins were incubated in coomassie stainig solution (Tab. 6) for 1h up to overnight. The destaining reaction was done with a standard destaining solution (Tab. 6) 20 min up to 1 hour depending on the blue background coloration. The gels were observed and filmed under normal light.

4.2.5.5. Western Blot

At first, the protein samples were separated by the usage of SDS gel. The gel was incubated in Bjerum buffer (Tab. 6) for 1 hour and the nitrocellulose membrane, the filter pads were incubated with the sponge pads also 1 hour in Bjerum buffer. The assembling of the filter paper, membrane and gel was performed following the blotting manual provided by the manufacturer. The blotting was performed 2 hours with two cooling pads. The positively blotted membrane was dried at room temperature for at least 1 hour. The membrane was incubated in PBS/BSA buffer for 1 hour. After that 10 µl of serum was added and incubated over night at 4 °C. The next day the membrane was washed three times 7 minutes with PBS tween (Tab. 6) and the antibody conjugate was transferred on the membrane and incubated 2 hours followed by three times 7 minutes washing with PBS Tween. Then the substrate for the HRP (Tab. 6) was transferred on the membrane and incubated until the results were visible. The reaction was stopped by rinsing it with distilled water.

4.2.5.6. TCA precipitation of proteins

The protein sample was diluted in a 1:1 ratio with 10% Acetone TCA and incubated at -20°C for 20 minutes up to overnight. The solution was then centrifuged at 20,900g for 15 minutes. The resulting pellet was washed with ice cold Acetone 2 times and centrifuged as before. The so formed pellet was dried at room temperature and resuspended in SDS sample buffer.

4.2.5.7. Dot Blot

A small stripe of nitrocellulose was prepared with one dot, 10 µl per dot, with the searched protein candidate, the primary antibody, a flow through fraction from the cloned cells and the secondary antibody. The membrane was air dried for at least 1 hour and then blocked with TBS/milk (Tab. 6) powder for 1 hour on the rocker. The blocked membrane was incubated in the primary antibody solution (TBS Antibody 1:2000 table2) for 1 hour on the rocker. Three washing steps with TBS 10 min each followed the incubation. Then the membrane was incubated in the secondary antibody solution (TBS secondary antibody 1:100) for 1 hour. Another three washing steps with TBS 10 min each were performed. The visualization was performed like described above in the Western Blot paragraph.

Bjerum buffer	48 mM Tris, pH 9.2;39 mM glycine 20% (w/v) methanol;0.0375% SDS
TFB1	100 mM RbCl, 50 mM MnCl2, 30 mM potassium acetate, 10 mM CaCl2, 15% glycerol, pH 5.8;
TFB2	10 mM MOPS, 10 mM RbCl, 75 mM CaCl2, 15% glycerol, adjust to pH 6.8 with KOH, sterile filter
Lysis solution	100 mM NaH2PO4 13.8 g NaH2PO4·H2O (MW 137.99 g/mol) 10 mM Tris·Cl 1.2 g Tris base (MW 121.1 g/mol) 8 M urea 480. 5 g (MW 60.06 g/mol) Adjust pH to 8.0 using NaOH.
Denaturating washing solution	100 mM NaH2PO4 13.8 g NaH2PO4·H2O (MW 137.99 g/mol) 10 mM Tris·Cl 1.2 g Tris base (MW 121.1 g/mol) 8 M urea 480.5 g (MW 60.06 g/mol) Adjust pH to 6.3 using HCl.
Denaturating elution solution	100 mM NaH2PO4 13.8 g NaH2PO4·H2O (MW 137.99 g/mol) 10 mM Tris·Cl 1.2 g Tris base (MW 121.1 g/mol) 8 M urea 480.5 g (MW 60.06 g/mol) Adjust pH to 5.9 using HCl.
Destain solution	40% ethanol; 10% glacial acetic acid
Staining solution	0.05%Coomassie brilliant blue R-250; 40% ethanol; 10% glacial acetic acid
PBS TWEEN	8 g of NaCl,0.2 g KCl,1.44 g Na_2HPO_4,0.24 g KH_2PO_4; Adjust the pH to 7.4 with HCl; add H_2O to 1 liter; add 0.2 ml Tween
HRP substrate	30% H_2O_2, PBS
Electrophoresis running buffer	0.5M Tris base; 1.92M Glycine; 0.5% SDS; pH 8.8

Laemmli buffer	0.09M TrisCl, 20% glycerol; 2% SDS; 0.02% bromphenol blue; 0.1 DTT
LB agar	10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl 15 g/liter agar
LB broth	10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl 4 mM MgSO4, 10 mM KCl
Upper Tris	4x_36.4g Tris base,0.8g SDS add 150 ml H_2O pH to 8.8 and add 200ml H_2O
Lower Tris	4x_12,11g Tris Base 0.8g SDS 150ml pH at 6.8pH and add H_2O to 200 ml
Loading buffer	4% SDS 20% glycerol 10% 2-mercaptoethanol 0.004% bromphenol blue 0.125 M Tris HCl
PBS/BSA	Like PBS but add 0.5g BSA
TBS milk powder	Tris Base 6.1 g Sodium Chloride 8.8 g Distilled water 1000 ml add milk powder
TBS primary Antibody solution	Tris Base 6.1 g Sodium Chloride 8.8 g Distilled water 1000 ml add 1:1000 antibody solution

Tab. 6 Composition of buffers and solutions used in this study

4.2.6. Immunological methods

4.2.6.1. Immunofluorescence assay (IFA)

In immune fluorescence tests antigens will be bound by a specific fluorescein labeled antibody or set of antibodies from immunized species. The immunofluorescence assay in this study was specific for *R. conorii* (IgG and IgM) (Focus Diagnostics, USA).

In the IgM detection approach the serum sample was diluted 1:8 in MRL-buffer and after that diluted with yolk sac diluent 1:8, to clear the IgG antibodies and other non specific antibodies from the sample.

For IgG the sample was only diluted with MRL-buffer 1:64. Twenty five microlitres of both sample approaches were placed on a ready to use slide. Every slide holds eight prepared wells, coated with antigens of *R. conorii*. The slides with the samples were placed into a wet chamber and inoculated for 30 minutes at 37°C. After the inoculation the slides were rinsed in

distilled water for a few seconds and thereafter placed in a MRL-buffer solution for about ten minutes to remove all unbound or unspecific bound serum antibodies. It was not necessary to dry the slides after the washing steps but possible to dry them at room temperature for about five minutes. In the second stage of the assay the wells on the slides were filled with fluorescein-labeled antibodies to human IgG or IgM. So each well was covered with 25 micro liters of the detection solution (labeled antibodies). Now the slide was incubated at 37°C for another 30 minutes and like mentioned before protected from totally drying. The slide was rinsed in distilled water for a few seconds and placed in MRL-buffer solution for ten minutes. Then it was necessary to dry the slide entirely for the microscopical examination. The slide were analyzed by using fluorescence microscopy (Nikon DS-Fi 1 camera and Nikon super high pressure mercury lamp HB 10101AF) and the program NIS Elements BR 2.30. The slides were examined by 800 ms exposure time and 40x Magnification. A second microscope was also used to check the slides, a Euroimmun Eurostar with a CP-Achromat 40x Magnification (but without a camera).

4.2.6.2. WEIL-Felix reaction

The blood sera were diluted geometrically up to 1:128 with PBS-buffer and mixed in micro titer plates with the *Proteus* antigens OX-2, OX-19 and OX-K. The plates were incubated at 37C° overnight and the agglutination reaction was read with the naked eye.

5. Results

5.1. DNA manipulation techniques

5.1.1. PCR

For this study four different primers were designed, two forward primers, two reverse primers and all of them checked for self complementary base pairs or potential hairpin structures. The sequence of the gene of interest , the *omp*1 gene, was stored in the NCBI data base in a -1 frame and because of that the primers were also designed in -1 as well as in +1 frame and both frames were tested. The primers were as well tested at different melting temperatures,

45°C and 50°C to obtain a sufficient amount of the PCR product and a minimum of unspecific DNA bands. The primers were also tested with different $MgCl_2$ concentrations for the same reason. The targeted gene fragment of *R. conorii* was 966 bp long and with both primers the PCR result for *R. conorii* had to be 1194 bp long. The Gene fragment size of *R. helvetica* and *R. slovaca* was unknown because of a lack of a complete sequence in the NCBI database. The complete primer sequences for the first PCR assays are shown in (Tab. 7).

	name	direction	sequence	bp	Tm °C	GC %
Frame +1	10178	forward	CCG-AAG-ACC-CTG-TTA-TAA-GA	20	50	45
	10051	forward	AGC-TCC-GAC-TAA-AGCTAA-AG	20	50	45
	11361	reverse	GAC-TTG-AAC-GCG-GTA-TCT-AA	20	50	45
	11371	reverse	CGT-TCA-AGT-CGC-GGT-ATG-AC	20	54	55
Frame -1	10178	forward	CCG-AAG-ACC-CTG-TTA-TAA-GA	20	50	45
	10051	forward	AGC-TCC-GAC-TAA-AGCTAA-AG	20	50	45
	11361	reverse	GAC-TTG-AAC-GCG-GTA-TCT-AA	20	54	55
	11371	reverse	CGT-TCA-AGT-CGC-GGT-ATG-AC	20	50	45

Tab. 7 Primer results

The DNA stock solution of *R. helvetica* contained 141.6 ng/µl genomic DNA, the genomic DNA solution of *R. slovaca* 99.5 ng/µl, and the genomic DNA solution of *R. conorii* 133 ng/µl. The purity of the genomic DNA was controlled by spectrophotometry (NanoDrop® ND-1ßßß, 220-750 nm Specktrum, PEQLAB Deutschland). A possible RNA contamination was assessed for *R. conorii* at a ratio of 1.87 (260/280), for *R. helvetica* at a ratio of 1.90 (260/280) and for *R. slovaca* at a ratio of 1.85 (260/280); thus, the RNA contamination was not significant for all three DNA solutions. A possible protein contamination was also checked. The ratio for *R. conorii*, *R. slovaca,* and for *R. helvetica* was 2.01, 1.94 and 2.14, respectively, indicating no protein contamination. The PCR reactions were performed with different dilutions of the stock solutions. Standard dilution for *R. conorii* was 1:1000 and yielded a stable amplification product (Fig. 7). A 1:100 dilution for *R. helvetica* and a 1:10 dilution for *R. slovaca* yielded also stable results (Fig. 7, Tab. 8). This may be due to the *R.conorii* primers because the sequences are not identical but share high homology regions. To save genomic DNA this was attempted with already amplified bands. The *R. conorii* bands

which resulted from the amplified bands showed a high amount of the bands sought for but several unspecific bands as well (Fig. 8). These unspecific bands required a separation by agarose gel gradient 2d electrophoresis in order to get just a single band of the wanted size. The *R. helvetica* bands from a PCR result performed with already amplified bands showed two very weak signals of unspecific bands which were smaller than the searched band (Fig. 8). *R. slovaca* did not lead to a result with the amplified fragments. The primers seemed to be too unspecific to bind the amplification at the temperature and the $MgCl_2$ concentration used, and the amount of cycles

The amount of DNA output from the PCR reaction was measured by purification in an agarose gel. 10µl of DNA fragments were checked on the agarose gel and purified as described before. In the case of *R. conorii* the first purified band from the first PCR assay lead to 24.4 ng/µl of DNA. Later attempts with better adjusted PCR conditions lead to an increasing output, namely 27.6 ng/µl, 65 ng/µl, 19.02 ng/µl, 22.9 ng/µl, 44.5 ng/µl, 51.17 ng/µl and 122.41 ng/µl. The 122.41 ng/µl purified PCR product was chosen for the cloning assay and the control sequencing which showed a very low RNA and protein contamination, ratios of 2.01 (260/280) and of 2.56 (260/230). The first purified DNA product of *R. helvetica* yielded 31.61 ng/µl. Later attempts with adjusted PCR conditions lead to the following results; 30.27 ng/µl, 19.43 ng/µl, 18.6 ng/µl, and 24.45 ng/µl. In the following upstream applications the first purified band was chosen because it showed a ratio of 1.8 (260/280) and a ratio of 2.1 (260/230). The purified DNA product of *R. slovaca* showed an amount of 30.48 ng/µl. Later attempts yielded 18.70 ng/µl, 21.34 ng/µl, 14.38 ng/µl and 16.88 ng/µl. For the following upstream applications the first purification was used which had a 260/280 ratio of 1.48 and a 260/230 ratio of 1.8.

						$MgCl_2$ µl	50°C	
Rickettsia conorii	Frame	45 °C	50 °C	3	3,5	4	4,5	5
10051 - 11361	-1							
10178 - 11371	-1	1194 bp	1194 bp, 250 bp	250 bp	250 bp	1194 bp, 250 bp	1194 bp, 250 bp	smear
11361 - 10178	-1							
11371 - 10051	-1	1194 bp	1194 bp	250 bp	250 bp	1194 bp s	1194 bp w	1194 bp w
10051 - 11361	1		900 bp					
10178 - 11371	1	200 bp	1000 bp, 800 bp					
11361 - 10178	1		1050 bp					
11371 - 10051	1							
Rickettsia slovaca								
10051 - 11361	-1							
10178 - 11371	-1		1300 bp		1300 bp w	1300 bp w	1300 bp w	1300 bp s
Rickettsia helvetica								
10051 - 11361	-1							
10178 - 11371	-1		900 bp		900 bp s	900 bp w	900 bp w	900 bp, 100 bp
w: weak band	s: strong band							

Tab. 8 Resulting PCR bands with all the possible primers from Tab. 7

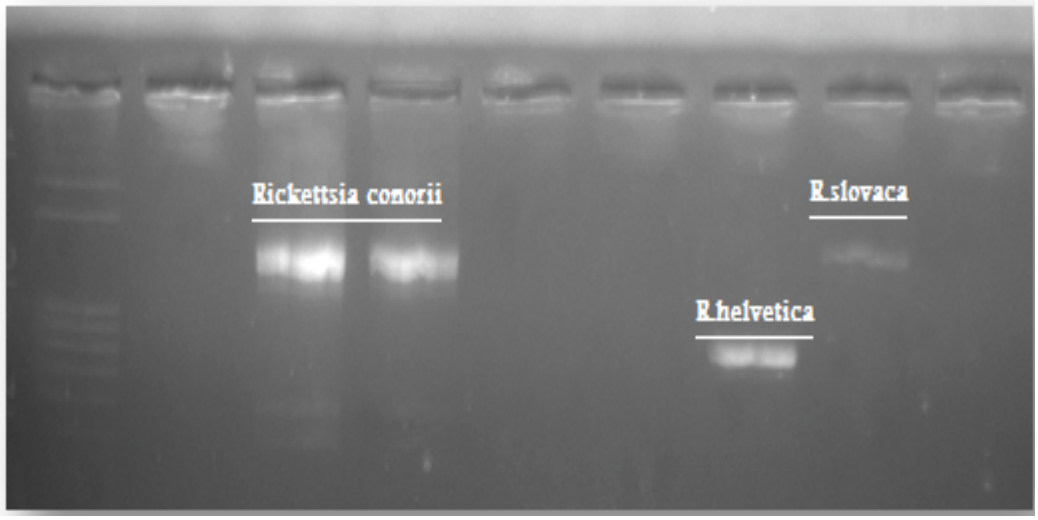


Fig. 7 Resulting PCR bands from genomic stock solution with the final primers

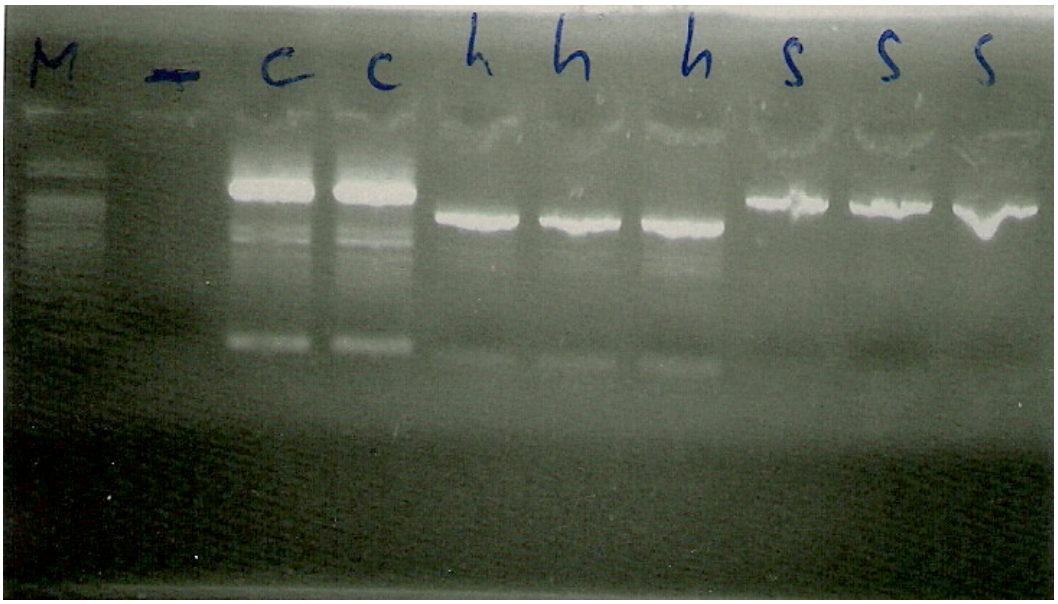


Fig. 8 Resulting PCR bands from genomic DNA with the final primers (c = *R. conorii*, h = *R. helvetica*, s = *R. slovaca*)

5.1.2. Real-time PCR

Real-time PCR analysis was performed with 100 sera from hunters and 100 from blood donors in order to get a qualitative insight if healthy and tick exposed persons or people of the average population carry rickettsial DNA in their peripheral blood. The serological results will follow in the paragraph of the IFA and Weil Felix test results. Two different real-time PCR systems were used in this study. First, the system of Corbett Rotor Gene (Stratagene, Agilent Technologies company) and the software Rotor Gene 1.7.75 (Stratagene, Agilent Technologies company) was used. 52 samples were exclusively tested for *R. helvetica* DNA sequences. The second program was done with the real-time PCR system 7,700 of Applied Biosystems (AB) and with the MXpro software (AB). 148 samples were tested, 48 for *R. helvetica* and 100 for *R. conorii*. Two standard dilutions with 2 replicas were tested per real-time PCR run in order to obtain a reliable standard calibration curve.

5.1.2.1. The results of the real-time PCR runs

Results for R. helvetica

In the AB assay the standard calibration was performed with dilutions of 1:2, 1:5, 1:100 of the *R. helvetica* genomic DNA stock solution (Fig. 9a, 9b).

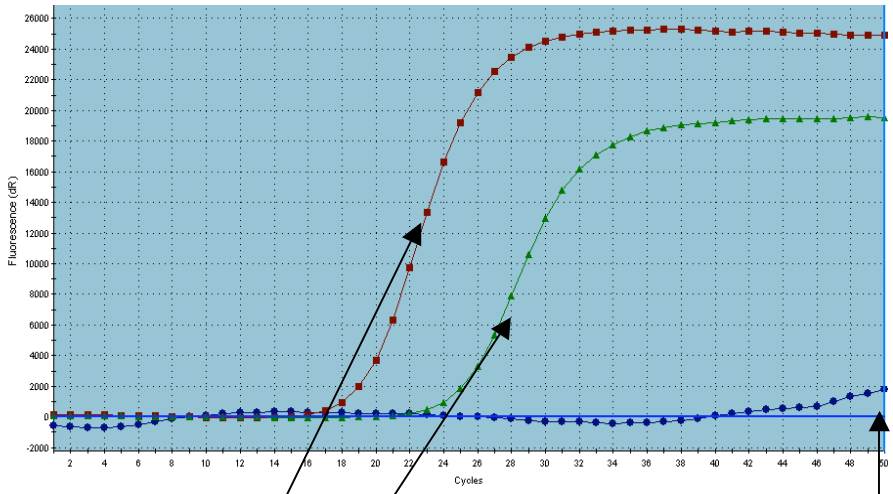


Fig. 9a Standard curve, amplification of *Rickettsia helvetica* DNA

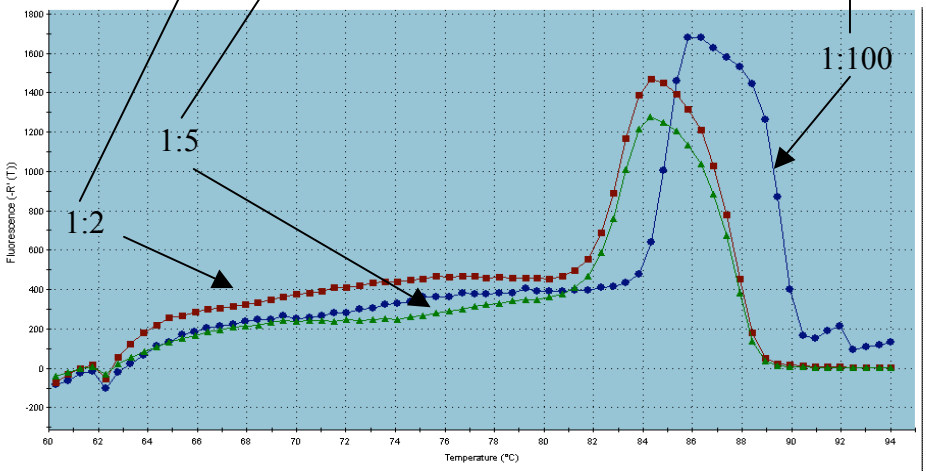


Fig. 9b Standard melting curve for *Rickettsia helvetica* DNA

In the Stratagene assay (Fig. 10) the standard calibration for *R. helvetica* was performed with genomic DNA of the non-diluted and the 1:10 diluted stock solution.

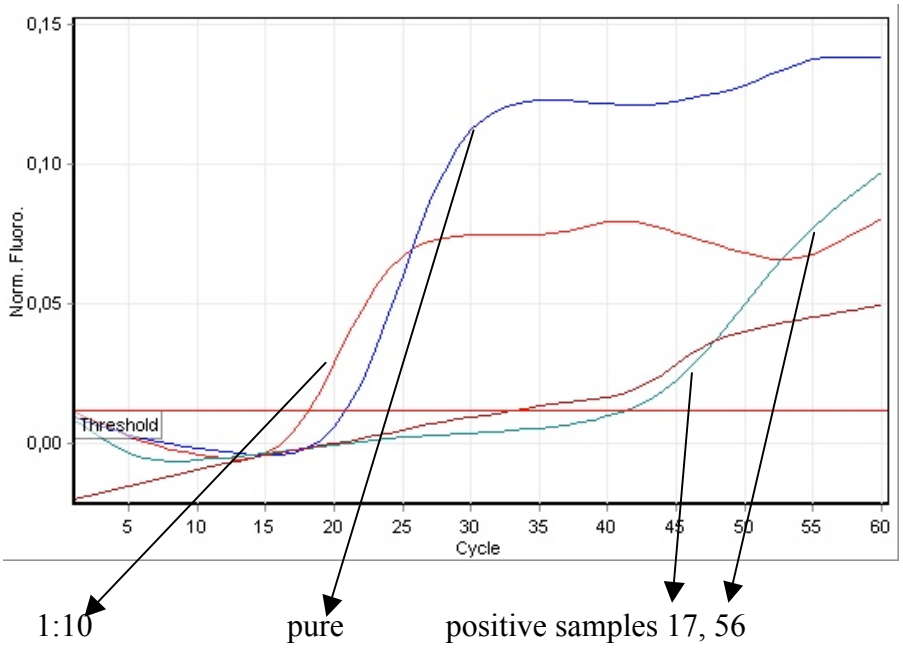


Fig. 10 Results with the Stratagene system, showing standard and positive results

The calibration results with the Stratagene assay were not suited to estimate the DNA concentration of the tested samples because of a too high threshold and of a not linearly starting measurement of the standards and the samples. But it was possible to determine the range of the amplification; the two positive results were then clearly in the range. The measurement was repeated several times and yielded best results. The positive samples were not tested in the AB system. The other tested samples in the AB system did not show a positive result.

Results with R. conorii

Calibration of the *R. conorii* was performed by a purified standard solution (Ingenetix). This standard contained a fragment of the citrate synthase gene *gltA*. It was diluted 1:20, 1:50 and 1:100 with 3 replicas per run to achieve at least 2 different results for the calibration graph (Fig. 11).

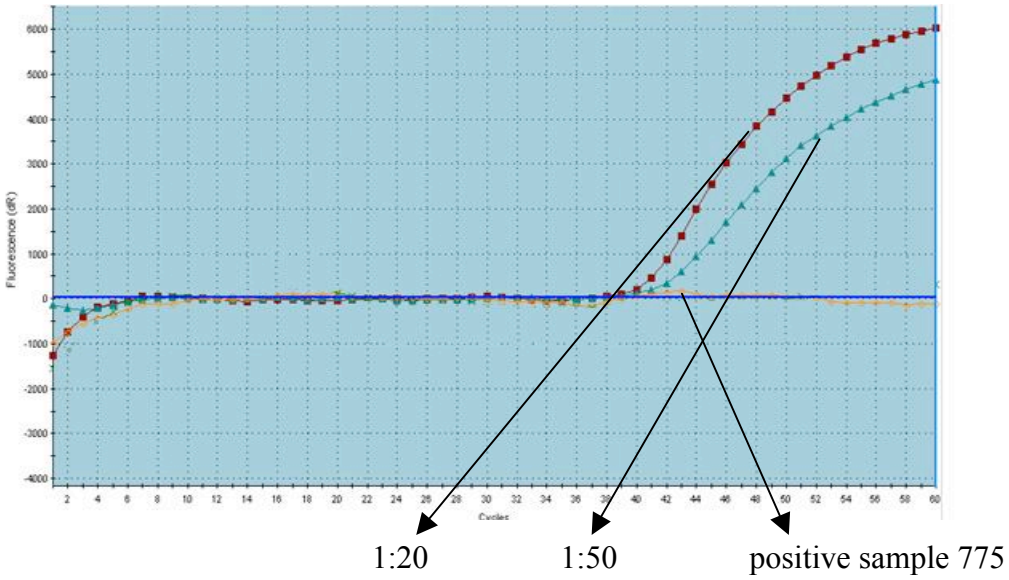


Fig. 11 Standard curves and positive results from the Stratagene system

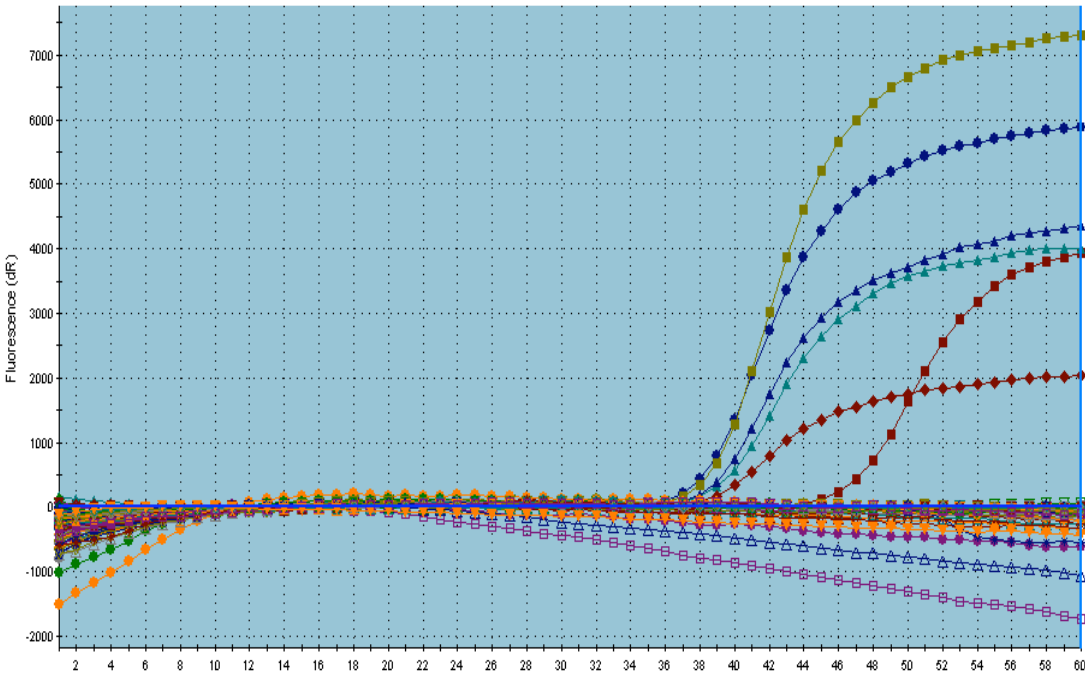


Fig. 12 Real-time PCR results with 96 samples AB system

The curves that increase significantly in their CT value at cycle 36 are standard samples and their replicas (Fig. 12). Table 9 shows the positive results for *R. conorii* and for *R. helvetica*.

	samples	Ct value	Treshold
R.helvetica	56	41,2	~ 10
R.helvetica	17	32,98	~ 10
R.conorii	775	39,58	32,04

Tab. 9 Samples and threshold values of the positive results with the Real-Time PCR

5.1.3. Sequencing results

A sequencing approach was performed to verify the PCR products of *R.conorii* as well as to obtain sequence information about the *R. helvetica* and *R. slovaca* sequences.

5.1.3.1. Results with Rickettsia conorii

The concentrations of the PCR fragment used for sequencing were121 ng/µl and 44.18 ng/µl. Forward and reverse primers were used. The forward primer did not lead to a positive sequencing result possibly due to the low melting temperature of the forward primer possibly because of the annealing temperature of the sequencing PCR which was higher than the melting temperature. With the reverse primer the sequencing results showed a positive match with the intended *R. conorii* sequence (Fig. 12).

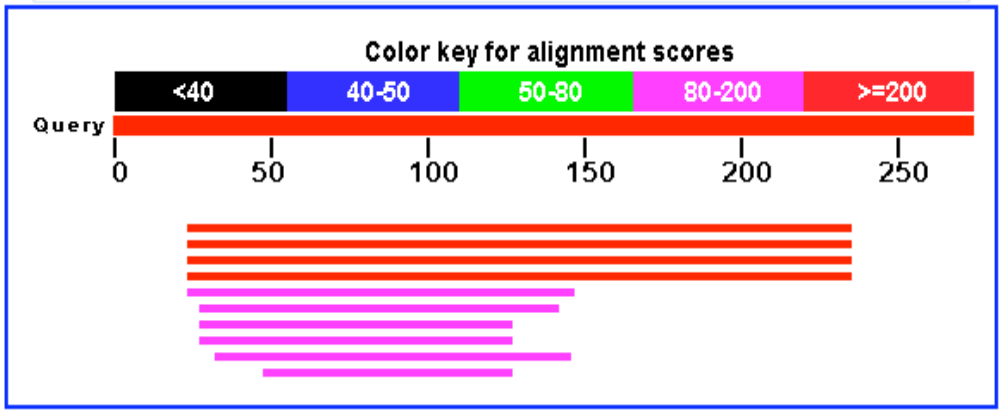
> c1r 121ng

GACAAAAGNGANATATACANTTNCGGCGTATAGTTTTATTTAGAAGATGG

TGCCAATGTGGGGATTTGAACCCCANACCNTACGCATTACAGAATGCGCCGCNTCTACCAGCATGAGCTACAT
CGGCGTG

AAATATGATTATATTATGCATGAGAATCTGAGTTCCCTACNGAGACTATGCTTTCGGTCTATTGCAGAGCAGAC
GACATG

CACAAGGGAGCGNTGGCAANCCAATTAANGAAATGTNTAACNATGGCNCAAAGNCNTCNTACG



Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AE008587.1	Rickettsia conorii str. Malish 7, section 19 of 114 of the complete	237	237	77%	2e-59	88%
CP000848.1	Rickettsia rickettsii str. 'Sheila Smith', complete genome	231	231	77%	9e-58	87%
CP000766.1	Rickettsia rickettsii str. Iowa, complete genome	226	226	77%	4e-56	87%
CP000683.1	Rickettsia massiliae MTU5, complete genome	206	206	77%	5e-50	85%
CP000847.1	Rickettsia akari str. Hartford, complete genome	158	158	45%	2e-35	90%
AJ235270.1	Rickettsia prowazekii strain Madrid E, complete genome; segmer	134	134	41%	3e-28	88%
CP000053.1	Rickettsia felis URRWXCal2, complete genome	132	132	36%	9e-28	90%
AE017197.1	Rickettsia typhi str. Wilmington complete genome	124	124	36%	2e-25	89%
CP000409.1	Rickettsia canadensis str. McKiel, complete genome	119	119	41%	7e-24	86%
CP000087.1	Rickettsia bellii RML369-C, complete genome	111	111	28%	1e-21	92%

Fig. 12 Sequencing result with *R. conorii*

5.1.3.2. Results with Rickettsia helvetica

The PCR fragment concentration used for the sequencing was 31.61 ng/µl and 44.18 ng/µl. The forward and the reverse primers were used to perform the sequencing. In case of *R. conorii*-sequencing the forward primer did not lead to a reliable sequencing result and the reverse primer did show useful sequencing results. The alignment of the sequences showed 299 base pairs which are presented in all of the sequenced fragments and 10 unidentified base pairs which are not shared by all of the sequenced fragments. The blast result showed a close relation to the *Rickettsia* spp. which was taken as positive result for the PCR fragment received from the *R. helvetica* assay (Fig. 13).

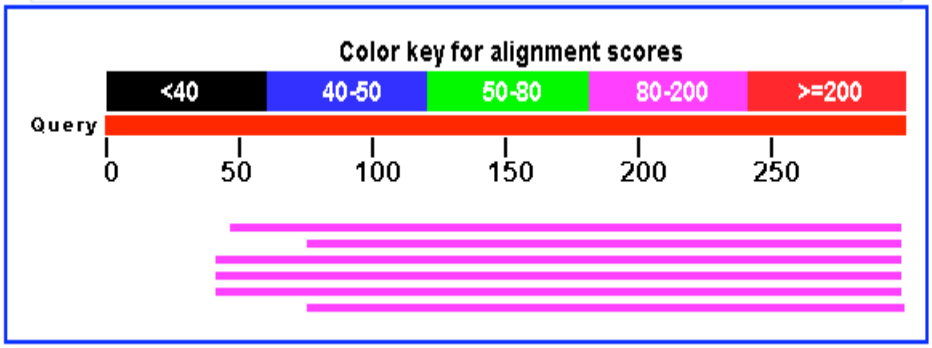
> h3r4

CCTTGTTGTTAGGATTATAATAACTGTATAGCCTCNTTGCACCTGNTTTTGGTTAGTTNCATTGATTAGGTGGATTTAAATTAG

TTTATTCCTGGCAGATTTANCAATCGATTTTAAAAGCGGATTGGCNACCGCCCTTTTAGCTTCCTAAATATACATTCCTG

CAAAGGTAATGCCGGCCATCTTTTCAAAGCCCCGANGNAAACNNGCCATACCCAGGAATTATCTAACNTTTAAATCCTT

TTACATAATGCATCAAGATTATATTTTGAGCCTTGGAAACATACCTTCAAGCCATC



Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP000683.1	Rickettsia massiliae MTU5, complete genome	187	187	83%	2e-44	81%
CP000847.1	Rickettsia akari str. Hartford, complete genome	185	185	74%	8e-44	82%
CP000766.1	Rickettsia rickettsii str. Iowa, complete genome	178	178	85%	1e-41	80%
CP000848.1	Rickettsia rickettsii str. 'Sheila Smith', complete genome	178	178	85%	1e-41	80%
AE008661.1	Rickettsia conorii str. Malish 7, section 93 of 114 of the complete	172	172	85%	6e-40	80%
CP000409.1	Rickettsia canadensis str. McKiel, complete genome	122	122	74%	6e-25	77%

Fig. 13 Sequencing result with *R. Helvetica*

5.1.3.3. Results with Rickettsia slovaca

The PCR fragment concentration used for the sequencing was 32.1 ng/µl. The forward and the reverse primer were used to perform the sequencing. As with *R. conorii* the forward primer did not lead to a reliable sequencing result and the reverse primer did show useful sequencing results. The alignments of the results lead to a sequence of 310 base pairs which are presented in all of the sequenced fragments and 6 unidentified base pairs which are not shared by all of the sequenced fragments. The Blast result showed a distant relation to the *Rickettsia* spp. group which maybe a result of the rearranging DNA repeats in the *omp*1 gene (Fig. 14).

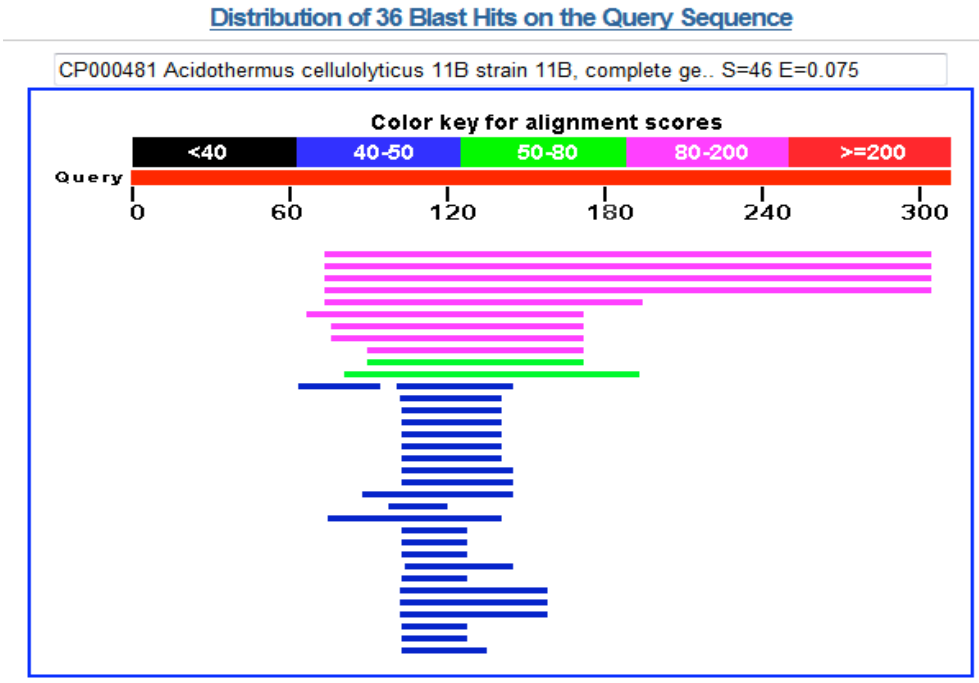
> bearb s3

CCGAAGCCGGCCCCCGCGCGGATAAAGCNTCCTTCCCGGNCCACCAATAAAGGTAATTCCCGCGTTAAATAAG
ATTTTAT

ATTAGAAGATGGTGCCAAGTGTGGGGATTTGAACCCCAGCACCTACGCATTACGAAGTGCGCCGCCTCCTACC
AGCCTGC

AGCNTACATCGGGGTGAAGATATGATTATATTATGCCTGAGCATACCGGAATTCCCTACCGAGACTATGNTTTC
GCCCAT

TGCGGAGCNGACGGCGTGACNAAGGNAGCCGCTGGCCAATCCAAATAAAAAAGGTTAAAATGCGTATAAC



Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AE008587.1	Rickettsia conorii str. Malish 7, section 19 of 114 of the complete	156	156	73%	6e-35	83%
CP000848.1	Rickettsia rickettsii str. 'Sheila Smith', complete genome	138	138	73%	2e-29	82%
CP000683.1	Rickettsia massiliae MTU5, complete genome	136	136	73%	5e-29	81%
CP000766.1	Rickettsia rickettsii str. Iowa, complete genome	132	132	73%	7e-28	81%
CP000847.1	Rickettsia akari str. Hartford, complete genome	100	100	38%	4e-18	85%
CP000053.1	Rickettsia felis URRWXCal2, complete genome	95.1	95.1	33%	2e-16	85%
AE017197.1	Rickettsia typhi str. Wilmington complete genome	86.0	86.0	30%	9e-14	85%
AJ235270.1	Rickettsia prowazekii strain Madrid E, complete genome; segmer	86.0	86.0	30%	9e-14	85%
CP000849.1	Rickettsia bellii OSU 85-389, complete genome	84.2	84.2	26%	3e-13	89%
CP000087.1	Rickettsia bellii RML369-C, complete genome	78.8	78.8	26%	1e-11	87%
CP000409.1	Rickettsia canadensis str. McKiel, complete genome	77.0	77.0	36%	4e-11	80%

Fig. 14 Sequencing result with *R. slovaca*

5.1.4. Cloning results

5.1.4.1. Ligation results

The ligation was performed with freshly prepared media and freshly prepared competent *E. coli* cells (M15 strain), 2 weeks old. The antibiotics kanamycin and ampicillin were prepared as stock solutions sterile filtered and stored as aliquots at -20°C for 2 weeks but freshly prepared for the last cloning attempt. All solutions were autoclaved or sterile filtered to guarantee the purity of the solution and to minimize the contamination with other microorganisms like fungi.

Ligation results

Rickettsia conorii *Rickettsia helvetica*

A 100µl LB plate of the *R. conorii* cloning assay supplemented with the ampicillin and kanamycin showing the growth of the clone candidates. A 50 µl LB plate was used for the *R. helvetica* and *R. slovaca* cloning assay.

Rickettsia slovaca

The ligation rate was increased by using the before described A-Addition kit. The PCR fragments were gel purified and checked by sequencing. The number of colonies which grew on the agar plate followed the amount of 50, 100 and 200 µl plated volume (Tab. 10). Cloning

was performed 5 times and the average number of clone candidates was taken in order to learn about the insertion rate of the PCR products.

R. conorii	cloning assay nr.				
Ligationmix used	1	2	3	4	5
50µl	21	3	8	5	9
100µl	32	7	15	7	11
200µl	57	14	20	19	16
R. helvetica	cloning assay nr.				
Ligationmix used	1	2	3	4	5
50µl	5	8	6	7	3
100µl	18	12	9	8	7
200µl	25	18	13	16	9
R. slovaca	cloning assay nr.				
Ligationmix used	1	2	3	4	5
50µl	0	2	6	5	3
100µl	5	3	9	6	5
200µl	7	8	11	14	9

Tab. 10 Growth results of 5 cloning assays shown as numbers of colonies

The first ligation reaction seemed to bare a contamination of the agar or medium because of the unsuspected high insertion rate of the expression plasmid or the high insertion of an unknown DNA fragment. The selection for positive clones was performed using a double antibiotic selection system of a blunt end cloning vector. The direction of the prelinearised vector prohibits a self annealing reaction and the PCR fragments were prepared with additional Adenines to make sure that they can be inserted during the ligase-reaction.

5.1.4.2. Growth rates of the cloning candidates

Two important growth steps were necessary between the transformation and the following expression of the cloning candidates. The time the picked clones needed for reaching an OD_{600} of 0.5 gave a hint about the possible toxicity of the inserted protein or the interaction of the expressed protein with the *E. coli* interior protein carrier systems of the strain both may lead to an extended amount of time to reach the begin of their exponential growth phase.

The growth phase of all five cloning assays was as predicted by the protocol for a well growing *E. coli* carrier strain. The OD_{600} of 0.5 for 10ml medium at 37°C was reached after 60 min of incubation. The second incubation to induce the expression lead to the OD_{600} of 0.5 after 5 h of incubation at 37°C.

These results were not predicted because the protein, although its sequence is known, inserted in the carrier cells is a surface protein and an antigen and thus the expression by *E. coli* will be difficult and the carrier cells will grow with a slower generation times. Surface antigens have several hydrophobic regions which may interact negatively with the building of the *E. coli* cell wall or with the cell interior mechanisms. The protein that was tried to be inserted in this study showed in silico four hydrophobic regions (Fig. 15, 16)

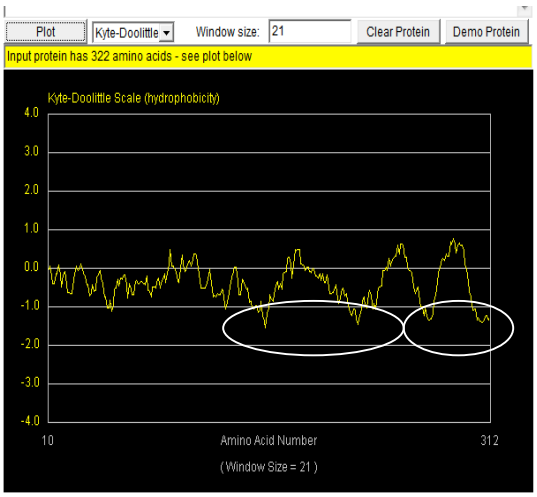


Fig. 15 Four hydrophobic regions

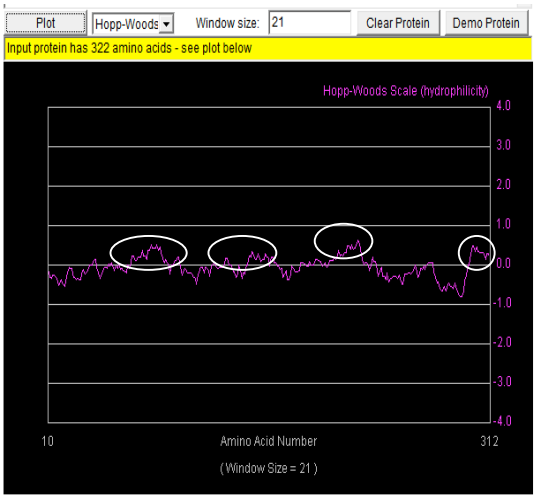


Fig. 16 Four antigenic regions

The Kyle Dolittle method of calculating the hydrophobic regions by comparing the single hydrophobic characteristics of the amino acids is based on the fact that peaks above zero are hydrophobic and at a window size of 21 (number of amino acids examined at a time) around -1.6. Four regions in the *R. conorii* protein used for this study are hydrophobic.

The Hopp-Woods method of reviewing the hydrophobic characteristics of the single amino acids was designed for predicting potentially antigenic regions exposed to the outside of the cell or on the outside of a highly folded polypeptide. Peaks above zero are likely to be hydrophilic. The four hydrophobic regions are predicted as they were in the Kyle Dolittle diagram.

5.1.4.3. Control sequencing of the clones

Before the sequencing was performed a PCR with the plasmidic DNA purified from the clone candidates was done to search for amplification and thus positive clones (Fig. 17). In this attempt one clone showed a positive signal at the predicted bp range. This did not match with the later in this chapter discussed proteomics results. One clone of the *R. conorii* fragment showed a positive result in the PCR with the self designed primers. The positive *R. conorii* result did as well show some of the observed unspecific bands from the control amplification. One clone for *R. helvetica* did show a result that can be interpreted as a positive signal.

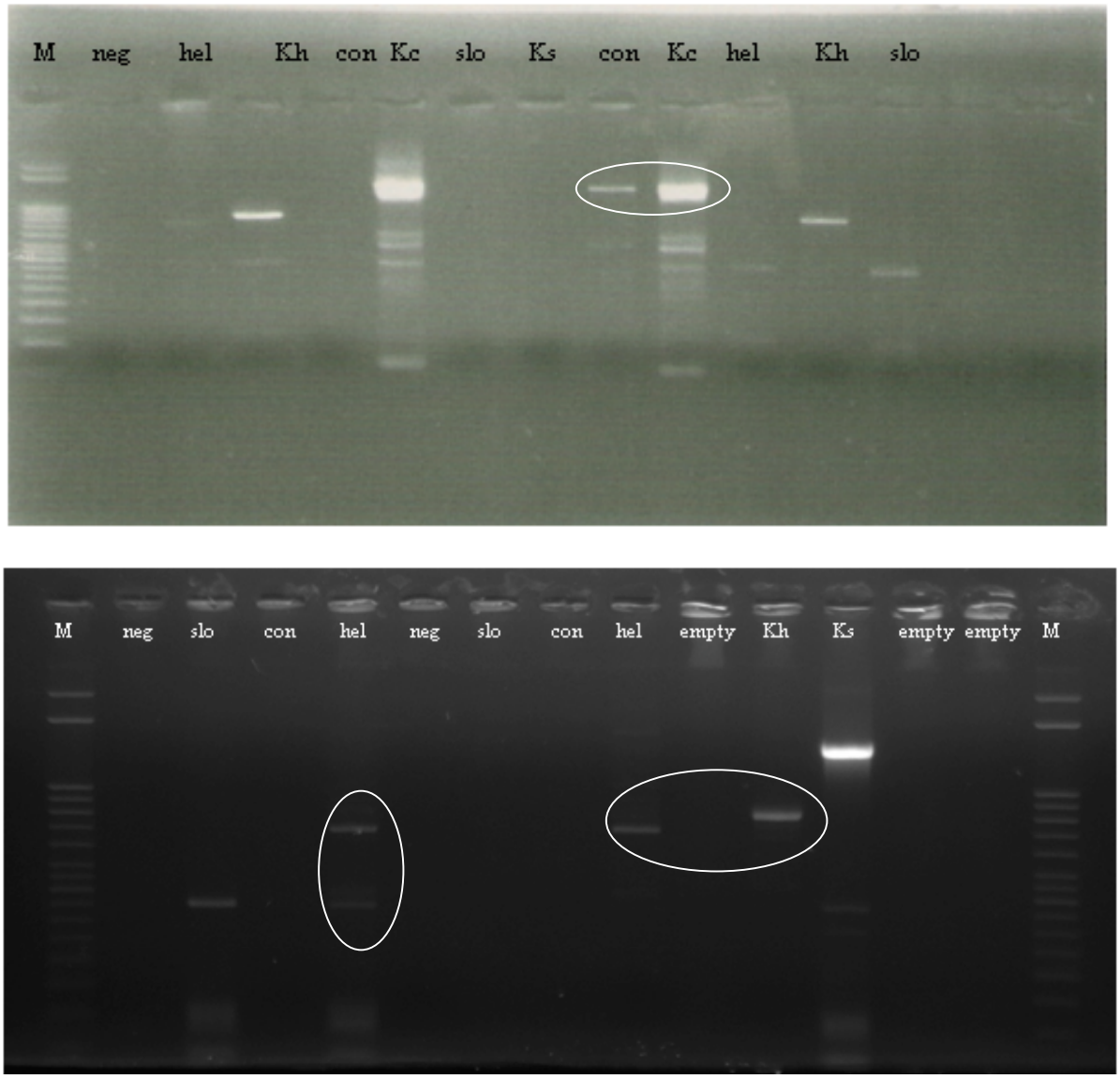


Fig. 17 10µl of the samples were loaded on the gel, 20µl of the marker, circeled are the possible positive clone fragments and the control fragments. M marker, neg negative control, slo *R. slovaca*, con *R. conorii*, hel *R. helvetica*, Ks control *R.*

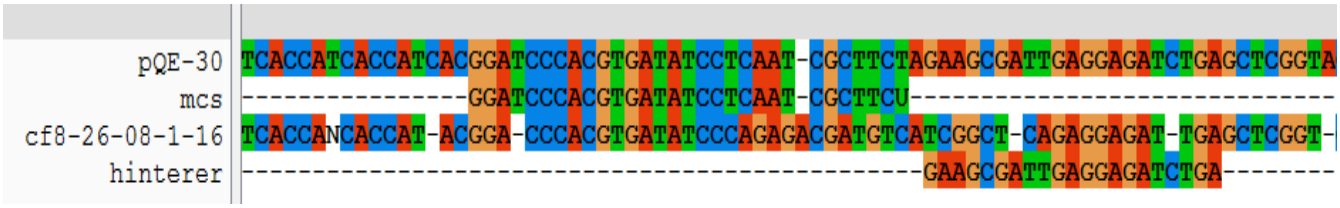
slovaca and Kh control *R. helvetica*. The samples were taken from a PCR performed at three different PCR cyclers.

The control band of *R. helvetica* is slightly larger than the sample bands obtained from the *R. helvetica* clone and an unpredicted smaller band of 350 bp appeared in some of the results. The result was unclear and a control sequencing approach was necessary in order to determine whether the fragments were correctly inserted.

The control sequencing was first performed with the self desigend primers previously used to get the DNA fragments from the genomic DNA of the three *Rickettsia* strains. The primers were used with the sequencing protocol described before. No result for all three strains was obtained from this sequencing attempt.

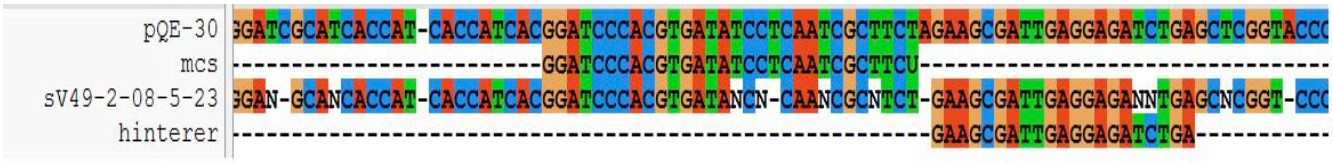
The second control sequencing was also performed to check the correct insert of the cloned DNA fragments. The sequences were checked with primers designed from Qiagen for sequencing cloned sequences in the PQe30 UA cloning vector. The forward and the reverse primers were used to obtain sequences. The sequences were aligned at the multiple cloning site divided at the ligation point and the PQe30 UA vector sequence. The sequences received were self aligned and the highest possible sequence was used for the alignment with the MCS.

Result with Rickettsia conorii



Result with Rickettsia helvetica

Result with Rickettsia slovaca



Mcs is the front part of the whole MCS site.

hinterer is the back part of the MCS site.

hV59-1-08-9-08, cf8-26-08-1-16 and sV49-2-08-5-23 are the sequence results obtained.

The results showed that no insertion occurred in the cloning attempt. It seemed that just a single adenine has been inserted in all the clone candidates.

5.1.5. Proteomics result

5.1.5.1. Results with SDS Gel

The SDS PAGE was performed as described in the method section. To get a rough idea of the protein of interests which may occur in the SDS page gel, the molecular weight was calculated by the amount of base pairs divided by three and the result multiplied with one hundred ten. The *R. conorii* protein which was translated from a 1194 bp DNA fragment should have an estimated molecular weight of 43.78 kDa. The *R. helvetica* fragment which was translated from an approximately 900 bp DNA fragment should lead to a protein with the molecular weight about 33 kDa. The *R. slovaca* protein was translated from a 1200 bp fragment of DNA so it would appear as a 44 kDa band on the SDS gel.

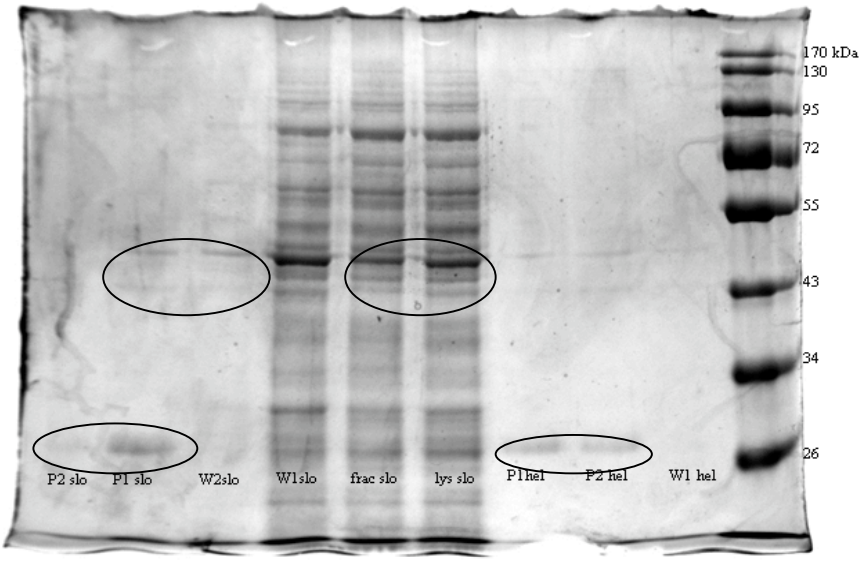


Fig. 18 Slo *R. slovaca*, con *R. conorii*, hel *R. helvetica* protein gel after the purification P2 second elution of the Protein, P1 first elution of the Protein, W1 washing step one, W2 washing step two, frac fraction after the inducing with IPTG, lys is the fraction after lysis

The purification was performed with an affinity chromatopgraphic column. The anchor for the protein in this column was a 6x histidine sequence which was inserted near the MCS and the expression system to the translated protein. It was not possible to predict exactly the change of size that would occur with the attached anchor but was estimated by plus minus 10 kDa. The fractions did show some protein bands that were eluted from the column in the end (Fig. 18) but it could not be shown if these bands were present before the inducing took place because some *E. coli* protein bands were present at the same height (Fig. 19). The expressed protein should have been expressed in larger amounts according to the instruction manual but as described before the protein of interest was a surface antigen and because of that possibly highly toxic to the expression cell system. This would explain the comparably low expression rate of the protein. Another possibility was that the expressed protein was degraded during the purification or the DNA was degraded during the transformation.

The result showed three protein bands for all cloned PCR fragments, the first at 50 kDa, the second at 45-44 kDa and the third at 27-30 kDa. This matched at some point with the predicted protein band size for the three fragments cloned consindering the possible changes in size mentioned before.

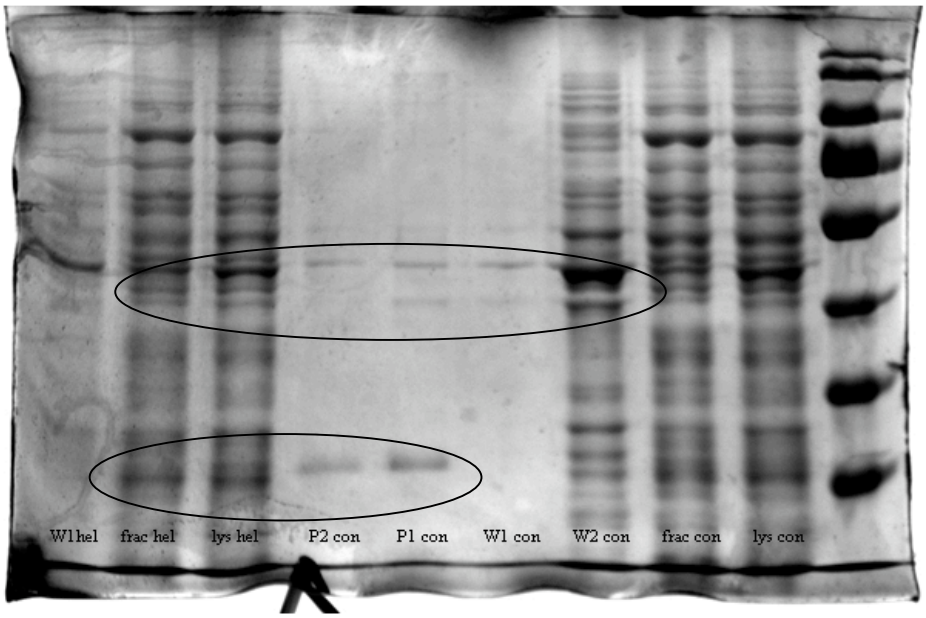


Fig. 19 The *E. coli* protein level before inducing the expression system and the purified bands

To eliminate the unspecific bands that occurred in the first purifications and to obtain just the band of the protein with the Ni-NTA anchor the expression and purification was redone under

changed circumstances for example working at 4°C to prevent degradation of the protein bands during the purification and adding imidazol at a concentration of 10 mM to the washing buffer to reduce the unspecific binding proteins.

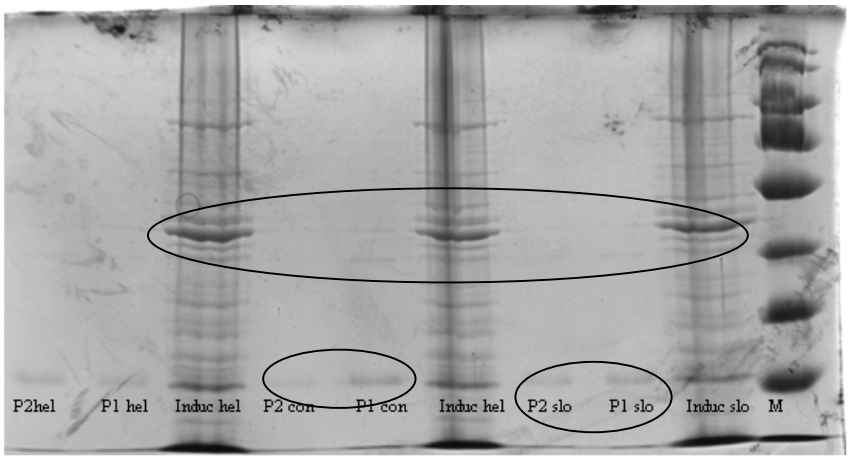


Fig. 20 The bands that were purified after the conditions were changed

As predicted, the unspecific bands were weaker than before but the protein band around 30 kDa was purified even under these conditions and the 30 kDa band which stayed unchanged in all of the purified cloning candidates was too small to be the predicted proteins of *R. slovaca* and *R. conorii* but could match the predicted *R. helvetica* result (Fig. 20). The induced fraction shows clearly that the band appears strong after inducing the expression system but the band is still weaker than it was expected with the strong T7 promotor system.

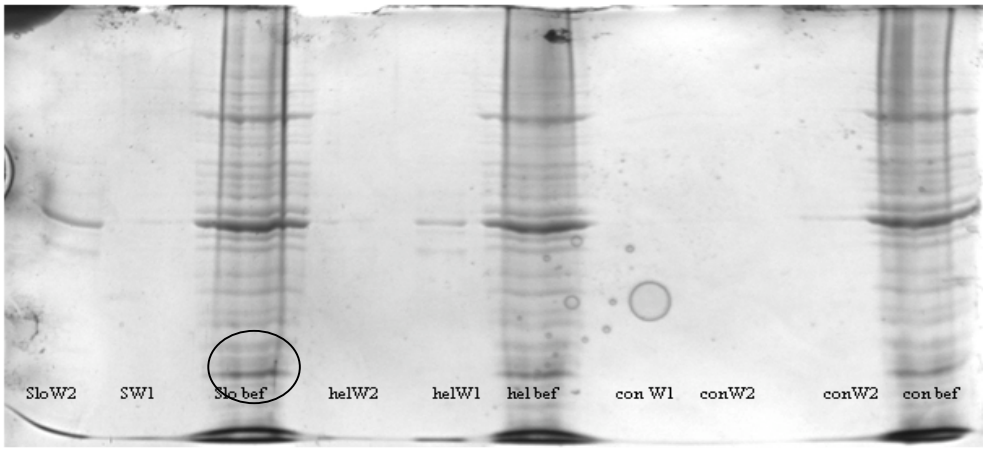


Fig. 21 Bef = before inducing with IPTG under imidazole washing

A rather strong 30 kDa band appeared in the fraction of the cells before the IPTG inducing under imidazol washing conditions (figure 21). This may indicate that the band is an *E. coli* protein that binds the Ni-NTA matrix; this was checked by performing a dot blot assay with the purified 30 kDa protein band.

5.1.5.2. Results with dot blot

The dot blot was performed with a fusion fraction from all the five cloning assays to increase the amount of protein.

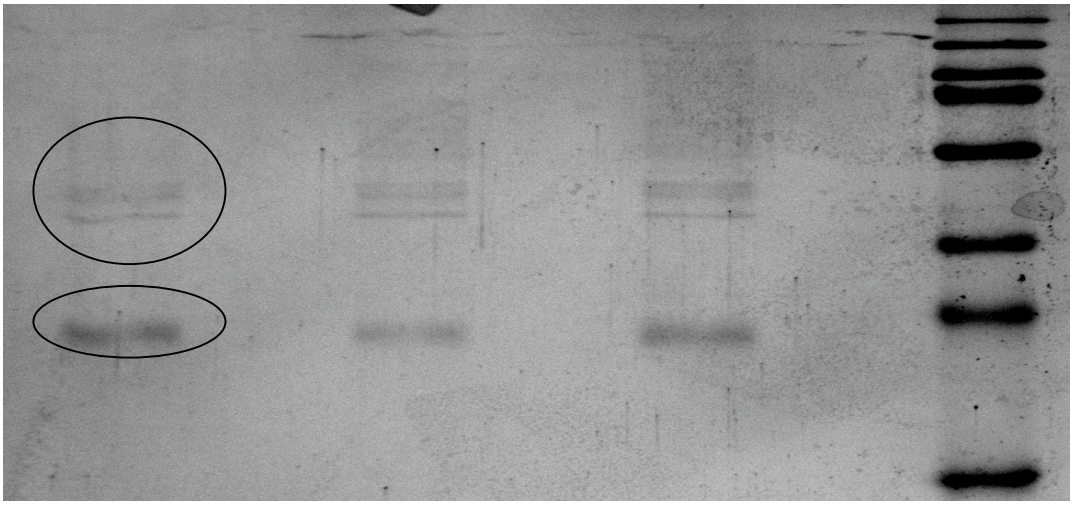


Fig. 22 Fusion protein on a protein gel

Even the fusion of five cloning assays did not lead to higher amounts of the protein (Fig. 22). The other bands shown here will be discussed in the discussion chapter.

The dot blot was loaded with 20 µl of the fusion proteins for the three tested strains, 20 µl of the primary antibody, 20 µl of the secondary antibody and a lysis fraction for all three strains to determine if the tagged protein was washed through the affinity column.

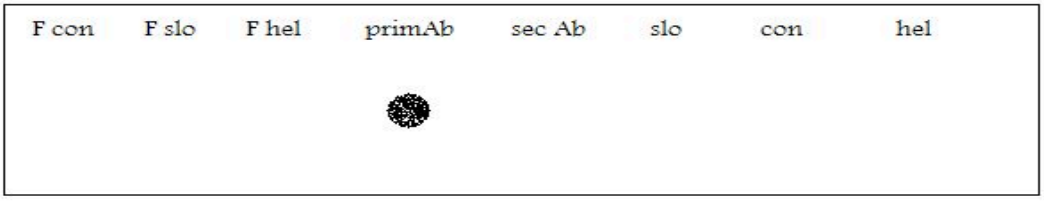


Fig. 23 Dot blot schematic F lysis fraction, primAb primary antibody, sec Ab secondary antibody

The result showed that the primary antibody does react specifically with the secondary antibody, reveiled by the color reaction (Fig. 23). The secondary antibody does not interact with the membrane or the other proteins. The missing signal in the lysis fraction indicates, besides the sequencing result described, that these proteins were *E. coli* proteins which bound unspecific but rather strong to the Ni-NTA matrix in the IMAC columns.

5.1.6. Immunological results

5.1.6.1. IFA results

The IFA used is specific for thedetection of IgG and IgM antibodies against *R. conorii*. The reaction is marked by a fluorescein-labeled antibody which binds to the specific serum antibodies that are bound to the respective rickettsial antigens. Reading was done against an IgG positive control.

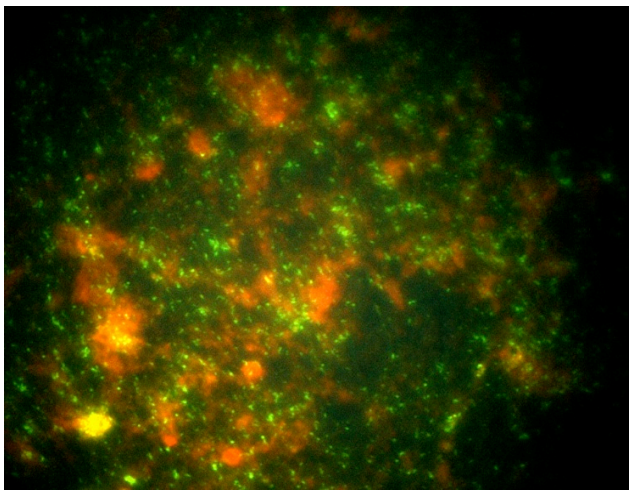


Fig. 24 IgG positive control from IFA

The yellow-green dots represent the immunological complexes of the antibodies with the antignes. The red coloured background represents the cells in which the rickettsiae were grown (Fig. 24).

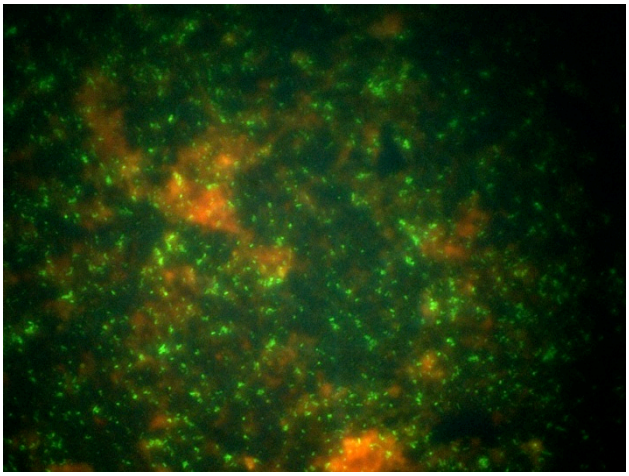


Fig. 25 IgG positive sample from IFA

Only one serum sample was probably positive for IgM but the signal was too weak and thus not counted as positive. The same sample was, however, positive for IgG (Fig. 25).

Of the 200 tested sera 27 were positive for anti rickettsial IgG. Seventeen of these sera were from male hunters and one of female a hunter. Four of the positive blood donor sera were taken from male and three from female hunters (Tab. 11 and Fig. 26) to calculate the risk factor and the distribution of anti rickettsial IgG by age.

n	♀ (bd)	♀ (hunter)	♂ (bd)	♂ (hunter)
100	97	96	96	86
0	4	1	4	17

	Blood donor	Hunter
♀	12.12%	4.2%
♂	5.9%	22.4%

Tab. 11 Numbers and percentage of seropositive samples from male hunters; (bd = blood donors)

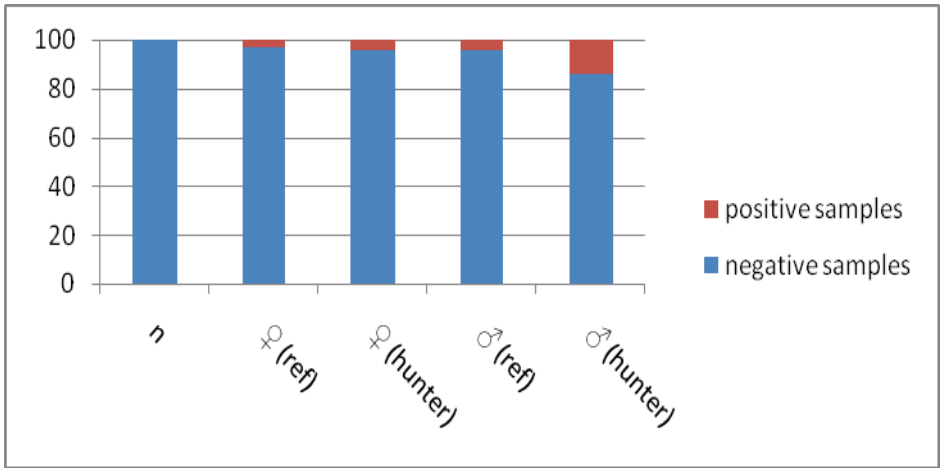


Fig. 26 100 samples per library were tested distribution of positive male and female samples; (ref = blood donor)

5.1.6.2. Results with the Weil-Felix Test

The Weil-Felix test was performed with the previously described dilutions of the sera; a positive serum control was included in each run. The MRL buffer was freshly mixed for every reaction. The first test was performed on the serum of a patient with acute rickettsiosis (Fig. 28).

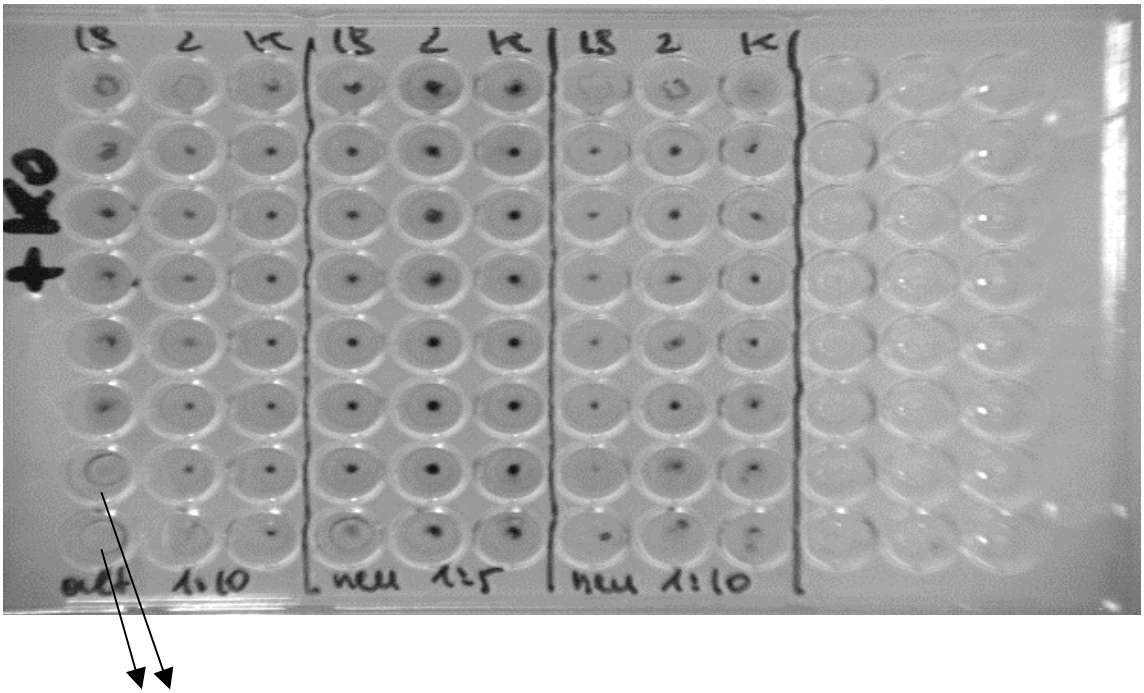


Fig. 28 Positive results with the positive control

From the 200 sera tested none was positive in the Weil-Felix test. The sera were tested twice. The Weil-Felix test shows usually a strong agglutination reaction with sera of patients with an acute rickettsial infection. The test uses antigens of *Proteus* OX strains which allow a heteroagglutination with antibodies directed against various rickettsial biovars as described before.

5.1.7. Statistic relations

Table 12 shows the results for gender and seropositivity. The amount of positive samples for the male and female donors were also predicted by statistically methods. The CH^2 value for the null hypothesis was measured with the SPSS program. The null hypothesis (H_0) states that there would be a relation between gender and seropositivity. The anti hypothesis (H_1) states that there would be no significant relation between the seropositivity and the gender of the sample donor.

Blood donor sera

			negativ	positive	total	CHI² p value
Gender	male	amount	63	4	67	0.286
		predicted	61,6	5,4	67	
	female	amount	29	4	33	
		predicted	30,4	2,6	33	
Total		amount	92	8	100	
		predicted	92	8	100	

Tab. 12 The statistic values for the relation between seropositivity and gender

The p value of the CHI^2 tests was calculated at a degree of freedom of 1 with an error rate of 5%. The p value was 0.286 and a positive result should have been at least 3.0 to 3.841 so there was no significant relation between gender and seropositivity, H0 was denied.

Hunter sera

Table 13 shows the results for gender and seropositivity. The amount of positive samples for the male and female donors were also predicted by statistically methods. The CH^2 value for the null hypothesis was measured with the SPSS program. The null hypothesis (H_0) states that there would be a relation between gender and seropositivity. The anti hypothesis (H_1) states

that there would be no significant relation between the seropositivity and the gender of the sample donor.

			negativ	positive	total	CHI² p value
gender	male	amount	59	17	76	0.043
		predicted	59.3	1.8	76.9	
	female	amount	23	1	24	
		predicted	23.4	0,9	24.5	
total		amount	82	18	100	
		predicted	82	18	100	

Tab. 13 The statistic values for the relation between seropositivity and gender

The p value of the CHI^2 tests was calculated at a degree of freedom of 1 with an error rate of 5%. The p value was 0.043 and a positive result should have been at least 3.0 to 3.841 so there was no significant relation between gender and seropositivity, H0 was denied.

The relation between age and seropositivity was calculated by the t-test.

Reference sera Table 14 shows the results for age and seropositivity. The amount of positive and negative samples were stated as N. The amount of the relative degrees of freedom was also calculated (Tf).The null hypothesis (H_0) states that there would be a relation between age and seropositivity. The anti hypothesis (H_1) states that there would be no significant relation between the seropositivity and the age of the sample donor.

t-test				
	IFA	N	P value	Tf
age	neg	92		
	pos	8	0.334	1.662

Tab. 14 The statistic values for the t test to calculate a realtion between age and seropositivity

The p value should have been 1.5 to 1.886 to be positive so no significant relation between age and seropositivity was proven statistically, H0 was denied.

Hunter sera Table 15 shows the results for age and seropositivity. The amount of positive and negative samples were stated as N. The amount of the relative degrees of freedom was also calculated (Tf).The null hypothesis (H_0) states that there would be a relation between age and seropositivity. The anti hypothesis (H_1) states that there would be no significant relation between the seropositivity and the age of the sample donor.

t-test				
	IFA	N	P value	Tf
age	neg	80		
	pos	18	0.76	1.662

Tab. 15 The statistic values for the t test to calculate a realtion between age and seropositivity

The p value should have been 1.5 to 1.886 to be positive so no significant relation between age and seropositivity was proven statistically, H0 was denied. But the p value for the hunter was closer to the positive value than the blood donor result.The following picture (Fig. 27) shows the age of persons with seropositive results for both groups.

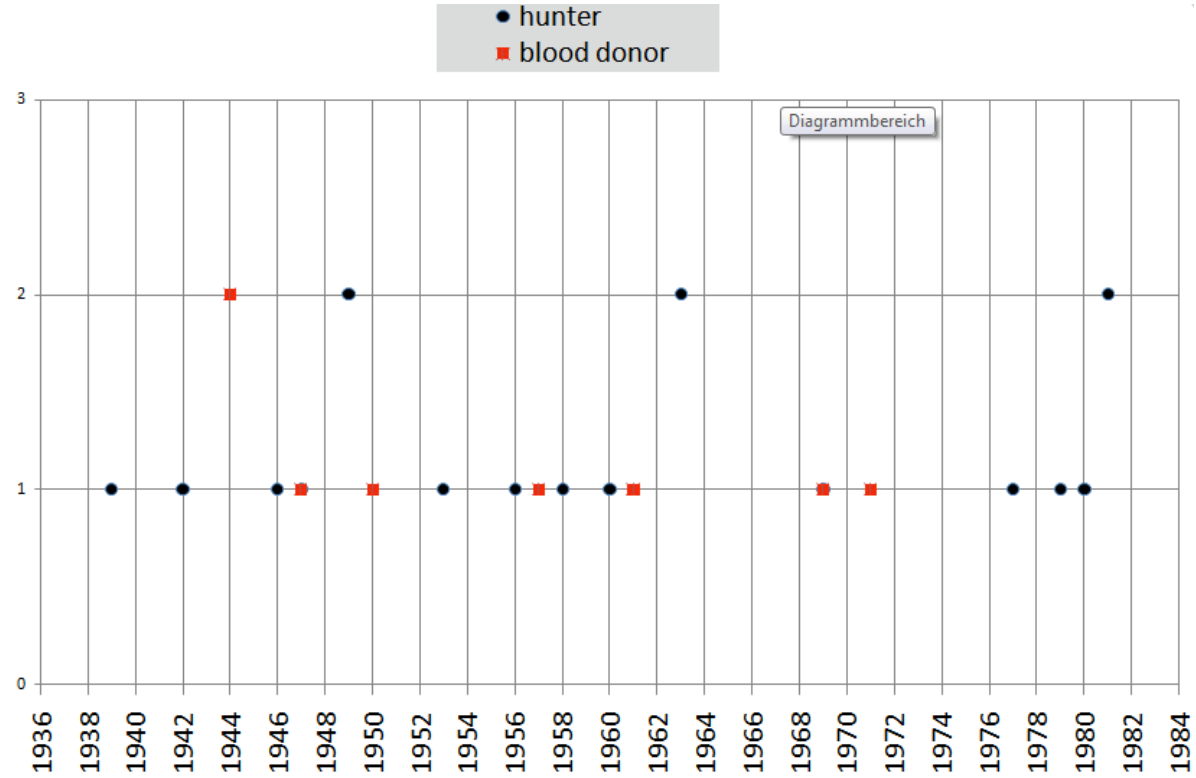


Fig. 27 Distribution of anti rickettsial positive samples compared to age

Correlation of time of hunting activity and seropositivity Table 16 shows the results for time of hunting activity and seropositivity. The amount of positive and negative samples were stated as N. The amount of the relative degrees of freedom was also calculated (Tf).The null hypothesis (H_0) states that there would be a relation between age and seropositivity. The anti hypothesis (H_1) states that there would be no significant relation between the seropositivity and the age of the sample donor.

t-test				
	IFA	N	P value	Tf
hunting time	neg	80		
	pos	18	0.991	1.2

Tab. 16 The statistic values for the t test to calculate a realtion between time of hunting activity and seropositivity

The p value should have been 1.5 to 1.885 to be positive so no significant relation between age and seropositivity was proven statistically, but the calculated value was 0.991. That indicates that higher degrees of freedom and a larger population of samples would match the p value and so match the H_1 hypothesis more exactly than it does now and so prove the statistic realtion.

6. Discussion

6.1. Omp1 of R. conorii, R. slovaca and R. helvetica

In the present study the omp1 antigen region of *R. conorii*, *R. slovaca* and *R. helvetica* it was successfully amplified and sequenced. The omp1 is one of the antigens which is not immune dominant but has antigenic potential which is indicated by the similarity with some already known and well studied antigens from the influenza virus for example the D15 surface Antigen of *Haemophilus influenza* [155]. The region was in silico chosen and amplified by self designed and evaluated primers. The antigen appears to play no important role in the pathogenicity of *R. conorii* or the other two rickettsiae. It is an autotransporter similar to an ABC transporter from other bacteriae and a membrane associated antigen. This indicates the contact to the human immune system and allows assuming the presence of specific antibodies in case of infection. The interaction of the immune system with the antigen of *R. conorii,* the causative agent of the MSF, is well studied. Several studies indicate that the major immune dominant antigens are not the only interaction partners of the immune system. The primers for the omp1 region of interest were designed only for *R. conorii* because the sequence data of the other two rickettsiae were not available. The resulting DNA fragments of *R. slovaca* and *R. helvetica* differ in size and similarity to the already sequenced DNA obtained from the NCBI or PATRIC database.

Several studies about the rickettsial evolution state that the genes of rickettsia are in a constant flow of degradation or loss of function if they are not needed for the ongoing survival or if they may be replaced by a more efficient gene cascade. No studies are available about the necessity of the not immune dominant antigens in rickettsiae. It is clear that the antigens do have a role in the rickettsiae besides of a role in virulence. In the case of the omp1 it is an autotransporter function for essential compounds. The fact that rickettsiae are intracellulary living organisms which do need the antigens or virulence factors all the time to maintain their survival indicates that the omp1 will not be degradated premature. This thesis is supported by the fact that the omp1 has 5 repeating antigenic domains which are conserved in several rickettsiae like *R. bellii*, *R. prowazekii* and *R. akari.* These conserved elements maybe rearranged to optimize the function of the antigen but they are conserved as region and will not be changed.

The different size of the amplified DNA fragments may be caused by the different incorporation of repeating DNA segments in the rickettsial genome [89]. These selfish repeats are incorporated in the genome of the rickettsiae in different amount and size depending on the repeat family. It may be the case that the selfish repeats are incorporated in different amounts into the two unknown rickettsiae sequences. Because of that the size of the fragments differs in the same antigen but the *R. conorii* primers did bind. The primers were designed from a highly conserved region of the omp1 in a repeating element at the 5´ side and a conserved promoter element at the 3´side. The selfish repeats vary in size from 5 to 223 bp so it is difficult to estimate the number of repeats inserted in the DNA fragments found with the *R. conorii* specific primer.

But the difference in size may also be caused by the different arrangement of the repeating antigenic regions in the omp1 of *R. conorii*. The omp1 starts with an antigenic region followed by five repeating elements and ends with the region of interest. The primers were set into these repeating elements because the omp1 of other rickettsiae were compared to the omp1 of *R. conorii.* The reference rickettsiae were *R. bellii*, *R. prowazekii* and *R. akari. A*ll these reference proteins showed the same amount of repeated antigenic regions. Because of that they were chosen to be the place of the primer start. The resulting DNA fragments should be starting in the conserved repeating elements and end in elements conserved like that of the following gene. The repeats were chosen by comparing with all the sequence of rickettsiae in the database to assure the unique proportion of the primer sequence. But it is possible that in the two unknown sequences of *R. helvetica* and *R. slovaca* these repeating elements are located at a different place of the genome. The sequences received were compared to each other and showed no 100 % similarity with each other indicating that the primer did work properly.

The sequencing results of the three omp1 region fragments were shown in the result chapter. The sequenced fragments were compared to the NCBI database for *R. helvetica* 299 bp with 10 unidentified bp were sequenced and for *R. slovaca* 310 with 8 unidentified bp were sequenced. The unidentified bases resulted from several sequencing assays by comparing the resulting sequences and keeping the bases with the highest incidence rate in all the sequences. As reference the *R. conorii* sequence was checked several times as well and the rate of unidentified bases was six.

The omp1 fragment of *R. helvetica* shares an 80% similarity to *R. akari, R. conorii, R. canadensis, R. rickettsii and R. massilae* if searched on a high relation level. This seems to be

a good result for the fragment. It is not possible to compare that relation to the phylogenetic trees available because these trees are based on all proteins of the sequenced rickettsiae or on the 16S, gltA and others but not on the omp1. There are older versions of phylogenetical trees that were based on the ompA, ompB and the sca1. This tree was not suited to show the phylogenetical relation of the rickettsiae because the conservation of these sequences was too high to determine the species but it was useful to determine the genus. These other trees may be used to determine the relation and the standing of the newly found sequences and indicate that the sequences actually are from the genus *Rickettsia*.

The omp1 fragment of *R. slovaca* shares an 80% similarity with *R. akari*, *R. conorii*, *R. rickettsii* and *R. massilae.* But the search of similarities on a high relation level did not lead to this result. On the high level no similarity was found for the fragment. The results for high similarity lead to no known sequence posted in the database. This may be caused by the self designed primers which were not as heat stable as the normal PCR primers should be. The case of different fragments sequenced in the sequencing assay was denied by the sequencing graphs showing that just one set of fragments was present in the sequencing. The search filter settings had to be set on a wider error rate of the compared base pairs and that lead to the shown similarities. This indicates that the *R. slovaca* sequence is still one of the more changed base pair sequence of the rickettsiae in the SFG. This is supported by several studies that state that *R. slovaca* is one of the less over all conserved rickettsiae and does not share many similarities with the other members of the SFG.

The conclusion of these results is that the evolutionary stable elements may be used to amplify an unknown sequence in between these stable elements in the outer membrane proteins. This technique is used in several other assays as well as the MST or the walking through the chromosome assay. This study showed that the antigenic repeating elements which are conserved in all sequenced rickettsial outer membrane proteins are suited for techniques using such stable elements. No study was available using these antigenic elements for MST to build a phylogenetic tree of the in between lying regions which do apparently differ in size. It is necessary to check other rickettsiae to verify this thesis. The three tested rickettsiae were all from the same phylogenetical rickettsial group of the SFG. It seems possible that members of the TG may differ more in size than the members of the SFG. The three tested rickettsiae strains did not share a close relation to each other in the phylogenetical group so this indicates that the other members of the SFG will share the difference in the sequence size.

1. Detection of human IgM

The Weil-Felix test was designed several years ago for the rapid detection of a rickettsial disease which should be fast and specific for the phylogenetical groups of rickettsia. The test is based on the fact that several of the *Proteus* OX antigens are bound by the same human antibodies as the rickettsial antigens are to a high rate homolog with these epitopes [156, 157]. The test system is working for the whole genus *Rickettsia* with the exception of *R. akari* [157]. The whole cell suspension of *Proteus* OX -2 does react strongly with antibodies against rickettsiae from the SFG except with antibodies against the RMSF caused by *R. rickettsii* and the whole cell suspension of *Proteus* OX-19 react with antibodies against the whole TG as well as *R. rickettsii*. *Proteus* OX-K does react with patients´sera with scrub typhus and *O. tsutsugamushi* as well as related infections [157]. The antibodies from rickettsial infection are believed to be still present up to two to three years after the actual disease took place. Studies of patients with *R. conorii* caused MSF showed that the antibody titer increased from about the tenth day to the fifteenth day of infection. On the fifteenth day about 100% of the infected patients have developed a significant antibody titer [158] but the raising of the titer was patient dependent and different in speed.

The antibodies are detectable by the assay after a significant titer is reached. The mainly reacting antibodies in the Weil-Felix agglutination are of the human IgM type one and two. These antibodies are mainly produced from the plasma cells during and a short time after the peak of infection and the peak of immune response or challenge [159, 160, 161, 162]. But some patients which do not suffer from MSF may show an unusual rise in IgM titers leading to possible false positive result [163]. But also cases are known in which the Weil-Felix result was positive without a significant IgM titer [164].This may be caused by another chronic infection or the ongoing immune reponse to such a disease. A study about primary biliary cirrhosis [165] showed that the IgM titer was rising even after the symptoms were cured. The test was compared to other immune dependant assays in several studies and the outcome was that the Weil-Felix test is not suited to detect MSF but other rickettsial infections [166].

In this study none of the tested sera showed a positive result in the Weil-Felix test with the antigen OX-K, 2 and 19. This should have detected all possible infections with rickettsiae from the three phylogenetical groups and some other related cross reacting epitopes of closely related agents. A possible cause for the missing reaction between the specific antibodies and the antigens from *Proteus* may have been the age of the sera. The hunter sera were taken in

2000 from asymptomatic persons and the blood donor sera were taken in 2006 from asymptomatic donors as well. Both groups were in question about possible rickettsial infections in their past and both sera were kept frozen at minus 20C°.
Only one of the 200 tested sera was possibly positive for IgM but the fluorescence signal was too weak to be considered positive and thus not counted as a positive result. All of the other tested sera showed no positive IFA result at all despite several changes in the method and several times doing the assay from aliquots to avoid refreezing. The IgG IFA assay did show several significant positive results and this compared to other studies [166, 159, 160, 161, 162] indicated that the Weil-Felix assay is not suited for non acute sera and does not react sufficiently with human anti rickettsial IgG.

A study from Germany in which 286 hunter sera from the whole country taken at the national hunting fair in Dortmund 2006 were tested for rickettsial antibodies [167]. The study was published in 2008 and the sera tested in the 2 years between the fair and the printing. The study described that some hunters did remember rickettsia-like symptoms in the past. Some hunters did also hunt in neighbouring countries or more exotic places. The serological test used was an IFA for IgM and IgG specific for different rickettsial species including the three species searched in this study (9 *Rickettsia* species: *R. conorii, R. slovaca, R. helvetica*, *R. massiliae, R. mongolitimonae, R. israelensis, R. aeschlimannii, R. felis,* and *R. typhi*). The results of the study showed several antibody titers in the hunter specimen including *R. helvetica*. The German hunters showed positive results for IgM and IgG. The results were shown in the dilution used to detect the titer. The tests were performed with antigens received from the unit of rickettsiology of the University of Marseille in France. The dilutions used were 1:128 IgG and 1:64 IgM for *R. conorii*. Two German hunters showed IgG and IgM and three showed just IgM. These results do not match with the results obtained from the Austrian hunters in the present study. The Focus diagnostics test does use another antigen for the detection of the IgM antibodies and the positive control did indicate a functional detection but the dilution used was the same and the dilution was changed in a second test attempt to be sure to detect the IgM antibodies. The German study did not describe the used method or antigens precisely. It seems that the IgM titer in the Austrian hunter population tested is too low to be detected by the Weil- Felix test and too low to be detected by the Focus IFA, or the antigen used in the German assay was more specific than the Focus antigen.
One may consider that the proportion of specific IgM detected in the German sera should have been detected in the Austrian serum samples as well. But taken into consideration that

the amount of IgM is decreasing and that the amount of memory IgM which is circulating in the blood is normally extremely low the IgM may have been from another source of infection and the results are just representing cross reactivity. But the memory IgM is the immune globuline which is produced at the highest amount in immunized individuals [168]. The immunization by *Rickettsia* is still not well understood because the vector does produce a host immune response free entrance port for the rickettsiae. The saliva of the ticks does inhibit the inflammation and does interact with the immunological signals on the interleukin base. After the act of feeding the normal immunological response would take place and the rickettsiae which have infected the first host cells in which they multiply would be extracted by the killer cells or by other first line defens mechanisms of the immune system. But this is not the case because the rickettsiae themselves influence the immune system by increasing the inflammation signals. These mediators enable the distribution of the pathogens in the tissue additionally by decreasing the apoptosis signals. This may interact negatively with the establishment of a relevant immun reaction against rickettsiae. This is supported by several studies [169] which showed in mouse models that animals inoculated with immunized serum of *R. conorii* or *R. australis* did not survive a challenge with a relatively high number of rickettsial cells.

In 1992 a study [170] was conducted in which a formalin-killed *R. rickettsii* strain was used as a vaccine in volunteers to study the development of immunity against a later challenge with *R. rickettsii.* After the challenge, six of the not vaccinated volunteers and 12 out of 16 of the vaccinated volunteers developed RMSF and in some of the vaccinated cases even with a shorter incubation period. Another immunization attempt was performed with recombinant parts of the ompA of *R. rickettsii* which was enhanced by recombinant parts of *Mycobacterium vaccae* did show a 55% survival of mice against a challenge with a lethal dose of *R. conorii* [171]. The immunization was just temporally and after several months the mice without the injection were died. This study showed further more that the level of IFN-y was increased indicating that the cellular immunity was stimulated. The memory IgM is produced by the memory B-cells in combination with the memory T-cells and that cycle is part of the humoral immunity. That indicates that the level of IgM of the memory T-cells and B-cells against rickettsiae may rise or fall as well during the normal house- keeping of the patient or that the titer is rising after a second contact with a rickettsiae infested tick if some sort of immunity is established by the patient. The role of the tick and the rickettsiae in the development of immunity stays unclear to this day.

The conclusion of these results is that the Weil-Felix agglutination test is not suited for the detection of specific antibodies in non acute sera of patients. The assay showed a detectable amount of IgM with the serum of an acute patient and also the IgG result was positive. This underlines that this agglutination assay preverably reacts with IgM antibodies. The sera from hunters in this study did not show a detectable amount IgM as well as the sera from blood donors. The German hunter sera showed positive results for IgM but no control group was screened. This indicates that the tests used may have some major difficulties with the specificity or the detection limit of the IgM. It would be elucidating to retest the sera with the method used by the German researchers in order to understand the difference in the results.

6.3.Species differentiation by antibody detection

Species differentiation by using antibodies which are specific against a rickettsial species is a difficult task. Several studies showed that the cross reaction rate of the immunological assays with rickettsial antigens is very high especially in between the phylogenetical groups, the TG and the SFG [46]. The studies were performed with monoclonal antibodies to determine the presence of epitopes and the reactivity. The tests were not performed by using polyclonal antibodies which are present in the serum of a patient who had contact with rickettsiae. Polyclonal antibodies may lead to a different and more specific result because the antigens are bearing several epitopes which are recognized by the antibodies in different speed and different recognition strength. And the plasma cells which are specificly chosen by the immunological proliferation mature in the ongoing challenge of the rickettsial infection. So it may be possible to receive a species determining result with the use of polyclonal sera. The polyclonal sera carry several different immune globulin types which recognize the antigen used as detection anchor. In this study the polyclonal antibodies of persons who had a possible contact with rickettsiae were tried to be used with a recombinant antigen, linear Epitop, to achieve possible species determination.

The serum of an acute patient who suffers from rickettsiosis contains high concentrations of IgG, IgM and antibodies of other immune globulin classes. Blood contains also a detectable amount of rickettsial DNA. The amount of DNA in the peripheral blood is decreasing over time after the end of the immunological challenge or the contact. But the rate in which the DNA is decreasing depends on the DNA characteristics.

A common feature in pathogenic DNA is the masking as eukaryotic DNA, carrying several signals which prevent the DNA from degradation. Furthermore the foreign DNA is stored in

small amounts in the memory cell system to provide the matrix for a next upcoming challenge. It is believed that free DNA in the blood without specific signals is degraded in four to eight hours via the metabolic pathways. But in the case of a chronic disease or a constant challenge like in the case of a fungal infection the amount of pathogenic organisms and so the amount of pathogenic DNA present in the blood stays constant at a rather small amount, depending on the number of pathogenic cells which are found per ml blood [172].

It is also known that small DNA fragments are common in plants which are not degraded because of their special signals which are incorporated in their sequence. This indicates that it should be possible to detect DNA with a real-time PCR specific for rickettsiae in persons who underwent a silent infection. The DNA stored in the memory B and T cells should be sufficient to detect the rickettsial challenge. In the present study the serum samples were tested with a primer probe set obtained from the literature as described before in the method chapter. These primers were tested and designed for *R. conorii* and *R. helvetica*. From the 200 tested sera three showed a positive result by real-time PCR. Two of them were *R. helvetica* positive and one *R. conorii* positive. These three positive samples did match with the IFA results which showed an anti rickettsial titer for IgG and one very weak for IgM. The detectable amount of rickettsial DNA or the specific primer amplified part of the sequence showed that the three positive samples from hunters must have been target of a rickettsiae infected tick bite less than three years [173]. Especially the hunter which did show a weak IgM result plus the strong IgG and the real time PCR result was very likely in contact with an infected tick in that time span. The probe set was not empirically tested in this study but the similarities were tested in silico. No possible false reaction should have occurred with the primer set, because neither human DNA was a possible target for the primer, nor any of the sequenced fungi. The samples were re-tested three times and the melting curves were taken into consideration as well in order to minimize the possibility of a false positive result or a wrong limit of detection. The results did not share similarities to normal noise signals.

The fact that one of the positive results was obtained with the *R. conorii* set of primers did indicate that the hunter had contact with a *R. conorii* infected tick. But the possibility of an autochthonous transmission of *R. conorii* to humans in Austria is not realistic. The majoritiy of the tick population in Austria belongs to the *I. ricinus* species which – to the state of knowledge - does not carry or transmit *R. conorii*. *R. conorii* is considered to be transmitted by the brown dog tick *Rhipicephalus sanguineus* only. *R. sanguineusis* an ubiquitary distributed tick that does live in the Austrian climatic situation. *R. conorii* needs a constant temperature to develop in the vector. The median temperature requirement is not met in

Austria at the time being. The climatic situation ranges from -30°C to 40°C with an annual median of 8.9 °C which does not support the constant warm temperatures which *R. conorii* needs to survive in the vector tick. In the last 5 years the winter temperatures did not reach the former minus temperature anymore in some regions which made them mild winters which gave the ticks a chance to survive and stay active even longer.

But also cases are known in which small micro habitats which are artificially warmed in winter and which provide a constant flow of hosts all the year showed rickettsiae infected ticks which carried also *R. conorii* [174]. These micro habitats are for example animal care stations and animal hotels which are getting more and more important nowadays with the increasing domestic pet numbers in Western Europe. In these animal hotels the dogs are held carefree in special rooms and are transported to every activity the pet owner payed for. These places for dog activities are visited by several dogs on a day and will provide a possible tick population enough time and host availability to attach for a blood meal. The hotel rooms may be cleaned sufficiently but ticks are very resistant arthropods which can survive long time spans without a host and survive several chemical treatments used in the cleaning process.

It is also important to take wild and pest rodents into consideration as well because they survive the relatively mild winters better than the normal winters and in their nests which are located near human cities or homes the ticks may survive as well, taking the rodent as intermediate host. One case in Germany showed that in an animal care center the tick population was quite big. This indicates that the positive hunter may have been in such a micro habitat in the last three years or his possible dog carried one of these ticks from such a habitat and the soft ticks feed several times before they reproduce or grow in the next phase of their life cycle. The hunter may also have been in an epidemic region for vacation. The vacation countries were taken into consideration but it is possible that a short trip to Italy was not stated in the anamneses of the patient and a dog may have been the carrier of the tick. [174].

Two serum specimens from hunters showed a positive real-time PCR result for *R. helvetica*. These sera contained also antibodies to rickettsiae. A study from 2007 in Austria showed for the first time that *R. helvetica* is present in *I. ricinus* ticks from all aprts of the country. OM Austria *I. ricinus* may also harbour different pathogens such the tick-borne or Central European encephalitis virus, *Borrelia burgdorferi, Francisella tularensis, R. monacensis, Anaplasma*, *phagocytophilum, Babesia divergens, Coxiella burnetii* and various *Babesia* species. Cross reacting antibodies between *R. helvetica, R. monacensis* and *Anaplasma phagocytophilum. R. monacensis* is closely related to *R. helvetica* and shares similarities in its

outer membrane antigens like described before. By real-time PCR *R. akari, R. australis, R. conorii, R. honei, "R. marmionii," R. japonica, R. massiliae, R. montanensis, R. rhipicephali, R. rickettsii, R. sibirica, R. slovaca,* and *R. parkeri* were detected with the same set of primers and probes but the *R. helvetica* set varies in three bases, two in the probe sequence and one in the reverse primer sequence. This indicates the risk of infection with *R. helvetica* by a bite of an infeced tick during hunting activity. A false positive PCR reaction is still possible because of the difference in the bases that do not influence the forward primer and may have influenced the amplification by unspecific binding. This is supported by the fact that the positive results showed very weak signals but the amplification did rise in the appropriate range like the reference standard dilutions did. But the signal was even after the amplification very weak. And a serum sample with a strong *R. helvetica* signal was not available for a positive control.

As discussed for the *R. conorii* detection in a serum sample, the travel history of the persons was not available and thus tick contact in another country may have occurred. But the presence of *R. helvetica* was already confirmed in several European countries like Switzerland, Germany, Polen and many more. This additionally pleads for an infection of the hunter with *R. helvetica* after tick contact in Austria.

The German study showed that six hunters were positive for *R. aeschlimannii* and one for *R. helvetica*. The cross reactivity was investigated by the German group by cross checking all specific antigens with each other and just using the sera reacting with only one of these antigens.

This method was not reliable for the rickettsiae tested and so the second characteristic used for the determining the species was the geographic origin of the infection, a rickettsial like disease reported by the hunters. The positiveresult with *R. aeschlimannii* was not definitive because of the possible cross reactivity with *R. helvetica* and *R. monacensis*, two rickettsial species common in Europe. Up to date the presence of *R. aeschlimannii* in Germany was not described. The sera of the present study were all tested with a *R. conorii* assay and the anti-rickettsial antibodies detected are possibly directed against *R. helvetica, R. monacensis* or *R. slovaca*. These are the three rickettsial species which are common in Austria [175].

In a study from Denmark [176] 259 serum samples were examined by the Weil-Felix test and by two IFA products (Focus Diagnostics as well as Fuller Laboratories). The results with all three test systems were compared with each other. Many sera showed a positive Weil-Felix result possibly the provided cut offs for the test seemed to low for study population. Of the

109 tested sera from blood donors 16% were positive for *R. conorii* at a 1:256 dilution. Furthermore cross reactions was observed with *R. conorii*, *R. helvetica* and *R. typhi*.
In the present study the 100 blood donors showed 8% seropositivity for *R. conorii* at a dilution of 1:256. The test was redone several times increasing the dilution or decreasing the dilution but the results did not vary [176].

In the Denmark study sera were also screened for antibodies against other bacterial antigens such as *Salmonella*, *Heliobacter*, *Coxiella*, *Mycoplasma*, *Campylobacter* and *Chlamydia*. Cross reaction was particularly observed with *Mycoplasma* and *Coxiella* antigens. Prevalence of antibodies to *Mycoplasma pneumonia,* causative agent of the atypical pneumonia, is a regular observation in adults. *Coxiella burnetti* causes a greater source of false positive rickettsial diagnostic results because it can be transmitted by air, and is commun in rural environment [175]. It is the causative agent of the Q-fever and shares some morphological and genetical similarities with the genus rickettsiae but is excluded into the new genus coxiella. The similarities to rickettsiae may be the cause of the cross reactivity of the anti rickettsial antibodies. This may also cause a super infection if both pathogens are transmitted by the same tick.

The conclusion of this discussion part is that the species differentiation is still difficult for rickettsial diseases. In several studies like a case report from Switzerland [177] in which a trekking tourist came back with a rickettsial infection but the exact species could not be determined. The causative agent may have been *Rickettsia sibirica* subsp. *sibirica* or *mongolitimonae* taken into consideration the travel route in Asia and the clinical diagnosis. The seroconversion of *R. conorii* antibodies in the tested patient did not lead to a clear determination of the causative agent because of the previously described cross reactivity. The real-time PCR assay from serum is not suited for rickettsial detection and species determination because of its very low sensitivity. The cross reaction between the *R. conorii* antigen and the *R. helvetica* antigen needs further testing to determine the possibility and amount of such cross reactions. This indicates that seropositive sera were mostly due to an infection with *R. helvetica.* It would be desirable to develop a specific assay in order to minimize cross reactions within the genus *Rickettsia* and the closely related genera like *Coxiella*. Furthermore, the cut off needs to be adjusted to the prevalence of specific antibodies in the population.

6.4.Contaminating proteins in the IMAC column system

The expression system used in this study produces a protein with a 6x His tag anchor which is important for the later purification with Ni-NTA matrix of the protein from the carrier cell lysate. The purification with the 6x His tag and the Ni-NTA matrix was described in other studies as simple and efficient purification for cloned proteins [178]. The strong ionic binding of the Ni-NTA anchor and the 6xHis tag can be broken by high concentrations of imidazole or a pH shift in the acidic or basic milieu. Several problems may cause the loss of the expressed protein for example the native conformation which masks the anchor and so the protein is washed in the washing fraction or the Ni-NTA anchor is unstable at the chosen peptide and the peptide is washed through the column or the imidazol concentration is too high and no bound peptides can rest inside the column during the washing steps [179].

In the present study the proteomics result from the expressed proteins did show some purified bands at 50, 45 and 30 kDa which would have matched the estimated size of 43,33 and 44 kDa of the cloned peptides (Fig. 19). The purification was performed several times changing the imidazol concentration, checking the pH of the used buffers, changing the buffers used and changing the overall working temperature. All these cloning assays and the performed purifications lead to the same three bands in the SDS-PAGE. These bands occurred in all of the three cloned peptides carrier *E. coli* strains and shared exactly the same size, which should have been differently in size. This indicated some possible *E. coli* proteins which may rest inside the column even through the stringent washing steps but which are eluted entirely by a pH change. The bands were also present in the lysis fraction (Fig. 20) but the lysis fraction was overloaded with proteins of the same size.

The increasing of the imidazol concentration in the washing buffer did lower the signal of the three bands especially the larger bands (50 and 45 kDa Fig. 21) and the larger bands occurred in the washing buffer fraction but the 30 kDa band stayed in the eluted fraction.

The unknown bands were further more checked by a dot blot assay with a Qiagen provided antibody against the Ni-NTA 6x His-tag. The dot blot showed that no Ni-NTA anchor molecule was present on the three bands which were eluted by the affinity chromatographic column. This may have been because of a possible loss of the anchor during the washing steps. This was checked by performing a Western blot with a highly positive serum against rickettsiae but this was negative as well. Additionally, whether the cloned antigen was immunogenic remains open.

To get more information about the insertion and the expressed protein the *E. coli* clones were at the same time sequenced to check the insert. This result showed that no insert was present in the clones and this excluded the possibility that one of the three proteins were an inserted peptide or a fragment of these.

All these tests indicated that the purified proteins were *E. coli* proteins which may bind in the Ni-NTA specific column. This is supported by some studies of a 27 kDa *E. coli* metal ion-regulated Peptidyl-prolyl *cis/trans*-isomerase the *SlyD*. This molecule is regulated by divalent metal ions like the Ni+ that is attached on the NTA matrix in the IMAC column [180]. The *SlyD* contains a C-terminal histidine-rich metal binding domain which shares some structural analogies with the NTA matrix used in the affinity IMAC columns and because of that also share the high affinity for the Ni+ ions [181]. In 2003 a study was presented that revealed *SlyD* as the only *E. coli* protein capable of contaminating IMAC NTA column systems which are used in native conditions. The *SlyD* can be overcome by using denatured conditions and a large amount of protein increasing the competition for the binding spots in the IMAC column. *SlyD* is present in high amounts in the *E. coli* metabolism at every stage of the cell because it plays an important role in the chaperon cascade to fold other proteins [182].

The conclusion of this discussion is that in the present study the missing of the expressed protein resulted in no competition for the *E. coli SlyD* for the IMAC binding sites and so even in denatured conditions the protein could be purified in the column. This indicates that the denatured conditions are less important than the amount of tagged protein for the purification without contamination. But the larger protein bands stayed unknown but are certainly *E. coli* contaminating proteins as well. These larger bands were not purified in large amounts and so it did not interfere with the result in presented study but it may interfere with recombinant proteins which share the same size.

6.5.Seroprevalence of Rickettsia in Austria and other countries

The previously described study from Germany [167] in which also hunter sera were tested showed that of the 286 tested hunter sera 26 had a positive antibody titer for rickettsiae. But the distribution ratio of male to female donors was different. The German hunter samples were composed of 252 male and 34 female hunters and the age ranged from 17-79 years; 26 (9.1%) were IgG and IgM positive. The present study showed 18 % sera positive for

rickettsial antibodies. This difference in results may be due to several reasons. First the different methods and the antigens used for the seroprevalence testing in the German study. This is particularly relevant for the IgM results since the sera were stemming from healthy hunters in the German and Austrian study; no hunter suffered from a rickettsial-like disease in last 5 years; and the number of tick bites in the German hunters was 4 per year on average. Annual tick bites were not evaluated among the Austrian hunters. The higher seropositivity in Austrian hunters may be the result of the higher infestation rate of rickettsiae in local ticks [183]. An epidemiological study from 2004 in Germany showed that in 8.9% of 1,187 *I. ricinus* rickettsial DNA was detected. In Austria roughly one third of 853 examined *I. ricinus* ticks showed rickettsial DNA. Since the tick contact of Austrian hunters will not be less than that of German hunters is also supported by the fact that the long term hunters show a significant correlation with seropositivity [184], different to the German study where no correlation between the total time of hunting activity and seropositivity was found. This missing correlation may be due to an unsuitable age distribution. No correlation was found between age, sex and seropositivity in both studies discussed.

Prevalence of rickettsial antibodies in humans was measured with an antigen of *R. conorii*. *R. conorii* is the agent of MSF which is endemic in many countries around the Mediterranean Sea. Reports from Italy, France, Greece, Turkey and several African countries document the presence of this pathogen and indicate that the number of cases is slightly increasing and observed even in other European countries. The test results from this work indicated a high cross reactivity of the antibody tests used with the rickettsiae that are known to exist in Austria; namely *R. slovaca, R. helvetica and R. monacensis*.

In Greece MSF was not reported since 1972 but a seroepidemiologic survey from 1991 of samples collected in three rural villages of central Greece showed that of the 254 tested sera 148 were positive for rickettsial IgG [185]. Western blot results confirmed a *R. conorii* infection in 115 cases. However, as outlined before, *R. conorii* shows high cross reactivity between rickettsial strains in the SFG [186]. In 2002 another seroepidemical survey was performed in northern Greece to estimate the distribution of *R. conorii* and *R. typhi*. Of 1,584 serum samples collected from persons living in rural, urban and sub urban area 125 (7.9%) tested positive in an assay using a *R. conorii* antigen. The prevalence in rural donors was higher and was slightly higher in man than in women [187].

In 2007 a seroepidemiologic survey in Israel performed on human and canine sera revealed IgG antibodiesagainst the *R. conorii* (Israeli strain 487) in 14 of b136 human sera and in 69 of

85 canine sera [188]. None of the fourteen sero positive humans was a reported MSF case. Surprisingly, 13 of the 14 IgG seropositive human sera tested also positive for IgM.The high seroprevalence in dogs is very likely due to the high exposure to the vector, the brown dog tick.

In 2004 in Spain the seroprevalence of *R. conorii* was tested in the province of Sevilla [189]. Sera of 504 persons were tested who were characterized by age, gender, medical history, and the living place. IgG was measured with the same Focus test system used in the present study. The results showed that 9.7% of the urban people, 6.4% of the sub urban and 10.8% of the rural people tested IgG positive. The older patients showed seroprevalence with *R. conorii* IgG antibodies. IgM was not tested.

In 1997 in Turkey 98 sera from persons living in the Antalya region near the coast line were tested for *R. conorii* antibodies; 13 specimens tested positive for IgG [190].

In 2004 in Tunisia the prevalence of antibodies against *R. conorii* was evaluated. In this study 500 sera from blood donors were tested by an IFA assay detecting IgG and IgM. The sera were reference tested by Western blotting assay; 9% were confirmed positive for antibodies to *R. conorii*. The results did not differ significantly between male and female donors and not between rural and urban people. [191].

In the present study 25 of the 200 sera tested with a *R. conorii* antigen IFA test were positive for rickettsial IgG antibodies and one weakly positive for IgM antibodies. Of 100 sera from hunters, 18 were seropositive. A significant correlation between age and seropositivity could be assessed. This indicates that older people living in urban areas are not highly in risk of tick bites and thus less frequently seropositive for rickettsiae. But being employed in a business that is connected to a rural environment increases tick exposure and also risk of acquiring a rickettsial infection. This did not entirely correlate with the results from the study in Spain [189] in which the older participants showed a higher sero prevalence than the younger ones. Of the 100 sera from the blood donors 8 were positive with an age range from 29 to 56 years.

Comparing the number of tested sera in the seven studies listed here with the percentage of positive results shows that in Spain 26.9%, in Tunisia 9%, in Turkey 13.3 %, in Israel 10.3 %, in Greece 7.8 %, in Germany 9.1 % and in Austria 13.5 % were IgG positive for *R. conorii*. The IgM percentage was only available from the study from Isreal in which it was 9.5 % compared to this study in which 0.5 % of the samples showed a positive result.

Comparing the different percentages of rickettsiae seropositives and the age distribution in the studies from the different countries may allow the hypothesis that the sociological behavior of the tested population has an important influence on the result. In the present study the spare time activitys and on going trend to more fitness and out door activity shared by some younger and older generation members result in higher expositions to ticks and in the region of Vienna the Vienna wood is highly covered in tick activity [192]. The study from Tunisia [191] did not show any significant difference between older and younger participants this correlates with the results obtained in this study.

7. Abstract in German

Design and development of an immune dependent system for the detection of antibodies against *R. conorii, R. helvetica, R. slovaca* and screening for specific antibodies.

Rickettsien sind Bakterien, die in einem obligatorisch intrazellularen Habitat leben. Zu ihnen gehören sowohl intrazelluläre Parasiten als auch Symbionten. Die parasitisch lebenden Rickettsien sind an einen Vektor gebunden, der die Übertragung gewöhnlich in einen Wirbeltierwirt vornimmt; hauptsächlich sind das Arthropoden. Zu den Rickettsien gehören auch Pathogene, die Auslöser vieler humaner Erkrankungen mit unterschiedlicher Schwere im Verlauf sind. Rickettsiosen werden seit Ende der 1990er Jahre zu den „emerging diseases" gezählt.

Die Diplomarbeit umfasst drei Rickettsien-Arten: *Rickettsia slovaca, Rickettsia helvetica* und *Rickettsia conorii*. Diese drei Arten werden von Zecken übertragen. *R. conorii* sowie *R. helvetica* wurden bereits in Mitteleuropa und so auch in Österreich nachgewiesen, ebenso *R. slovaca* [142].

Die Diplomarbeit wurde in Teilprojekte unterteilt. Das erste Projekt beschäftigt sich mit dem Aufbau und der Evaluierung eines Detektionssystems für Antikörper gegen die drei vorher genannten Rickettsien-Arten. Das Testsystem soll spezifisch zwischen Genus und Art unterscheiden können. Der zweite Teil der Diplomarbeit beschäftigt sich mit dem Testen von Seren zweier Populationen, der Risikogruppe Jäger, die oft Zecken ausgesetzt sind und einer Blutspendergruppe.

Für den ELISA Test wurden die drei zu untersuchenden Genome der Rickettsien nach einem passenden Antigen untersucht, das human immunogene Epitope enthält. Das Antigen wurde auf eine Region reduziert, um das für den ELISA zu nutzende Peptid klein zu halten. Jede in Frage kommende Region im gewählten Antigen wurde mittels PCR amplifiziert und durch Sequenzierung getestet. Die Sequenzdaten von *R. slovaca* und *R. helvetica* waren nicht erhältlich. Aus diesem Grund wurde das Primerpaar, welches für *R. conorii* entworfen wurde, auch für die anderen zwei Rickettsien-Arten genutzt. Das so amplifizierte Antigen wurde in einen Expressionsvektor (Quiagen

pQE 30 UA 6x Ni-NTA tag) kloniert, mittels Affnitäts-Chromatographie aufgereinigt und mittels SDS Gel, Dot blot und Coomassie Färbung überprüft.

Seren von Jägern und von Blutspendern wurden nach dem Alter abgeglichen. Die Seren wurden mit einem IFA für *R. conorii* IgG und IgM, mit einem Weil Felix Test und einem Real Time Protokoll für *R. helvetica* sowie *R. conorii* getestet.

Die PCR Amplifikation der drei Rickettsien führte zu unterschiedlich großen Fragmenten. Das *R. conorii*-Fragment war ein 1194 bp Fragment, das *R. slovaca* ein 1300 bp Fragment und das *R. helvetica* ein 900 bp Fragment. Kontrollsequenzierung zeigte Verwandtschaft zur erwarteten Organismusgruppe.

Der *R. conorii*-IFA Test war IgG positiv mit Seren von 4 Frauen und 4 Männern der Blutspender- und einer Frau sowie 17 Männern der Jägergruppe. Der Weil Felix Test zeigt kein verwertbares Ergebnis. Der Real Time PCR Test war mit 2 Proben einmal für *R. helvetica* und einmal für *R. conorii* positiv. Das positive Real Time PCR-Ergebnis korrelierte mit dem Ergebnis des *R. conorii* IFA Tests für IgG.

8. References

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