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„DETECTION OF PATHOGENIC *BARTONELLA* SPECIES IN AUSTRIAN *IXODES RICINUS* TICKS“

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“Life is a lesson, you learn it when you’re through!”

Limp Bizkit – Take a look around

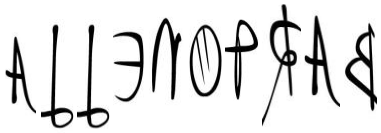
Zu Beginn meiner Arbeit möchte ich mich noch aufs herzlichste bei meinen Eltern, RENATE und PETER, für ihre lange Unterstützung bedanken!

Alternative Orthography: BARTONELLA

Hexadecimal (or equivalents, 770AD-1900s)

42 41 52 54 4F 4E 45 4C 4C 41

Leonardo da Vinci (1452-1519; backwards)



American Sign Language (origins from 1620-1817 in Italy and, especially, France)

Semaphore (1791, in France)

Braille (1829, in France)

Morse Code (1836)

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Dancing Men (Sir Arthur Conan Doyle, 1903)

Binary Code (1918-1938, probably earlier)

01000010 01000001 01010010 01010100 01001111 01001110 01000101 01001100 01001100 01000001

HTML Code (1990)

&#66 &#65 &#82 &#84 &#79 &#78 &#69 &#76 &#76 &#65

ISO 10646 (1991-1993)

0042 0041 0052 0054 004F 004E 0045 004C 004C 0041

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1. Abbreviations

Å	Ångström (internationally unit of length equal to 0.1 nanometre)
Am	Adult Male
Aw	Adult Female
BA	Bacillary Angiomatosis
bep	*Bartonella* Translocated Effector-proteins
BFP	Bundle-Forming-Pili
BPH	Bacillary Peliosis Hepatis
CSD	Cat Scratch Disease
Ct	Threshold Value
dNTP	DesoxyNucleoside-TriPhosphate
EDTA	EthyleneDiamineTetraAcetic
ELISA	Enzyme-Linked Immunosorbent Assay
Eppi	Eppendorf Tube
FAM	6-Carbox-Fluorescein
FRET	Fluorescence Resonance Energy Transfer
GFP	Green Fluorescent Protein
IF-Test	Immunofluorescence Test
IIF	Indirect Immunofluorescence
kDa	Kilo-Dalton
M	Marker, Step Ladder
LAN	Linker Arm Nucleotide
MM	Master-Mixture
mya	Million Years Ago
NC	Negative Control
NaOAc	Sodium Acetate
Rho	Signalling Protein of the Rho Family
RT	Room Temperature
T1SS	Type One Secretion System
Ta	Annealing Temperature
TAE	Tris Acetate EDTA Buffer

TAMRA	6-Carboxy-Tetramethyl-Rhodamin
Tm	Melting Temperature
VEGF	Vascular Endothelial Growth Factor
VirB/VirD4/bep	Pathogenicity Island (PAI) of *B. henselae* and *B. quintana*

2. Abstract

DETECTION OF PATHOGENIC *BARTONELLA* SPECIES IN AUSTRIAN *IXODES RICINUS* TICKS

Bartonella species are aerobic, Gram negative, facultative intracellular bacteria, which cause a variety of human and non-human diseases. They are pleomorphe, slightly curved rods belonging to the alpha-2 subgroup of the class Proteobacteria. Until now 24 *Bartonella* species have been described. At least 9 are pathogens for humans. They can cause Cat-scratch Disease, Carrion's disease, Trench fever, Bacillary angiomatosis and Endocarditis. Ticks are one main vector for *Bartonella* species. Therefor high-risk group like hunters and foresters should be emphasized. Further, *Bartonella* vectors beside ticks are cat fleas, sand flies and clothes louses.

In this study 4 strains of *Bartonella* (*B. clarridgeiae*, *B. grahamii*, *B. henselae*, and *B. doshiae*) were cultivated and used as positive controls for the design of a *Bartonella* specific PCR. Two different PCRs were established: a *Bartonella* specific one and one for species differentiation. Therefor two target sequences were chosen: the 16S rRNA gene and the 16-23S intergenic spacer region, whereas the primers for the ITS region were used for species differentiation. With these *Bartonella* PCRs a tick library has been screened. The tick library consists of 10.326 *Ixodes ricinus* ticks (adults, nymphs and larvae) collected at different locations in Austria. This screening was performed with two different PCR assays: a Real Time PCR and a Nested PCR. The results and the two analysing methods have been compared to each other.

In further studies two libraries of human sera have been screened for antibodies to *Bartonella* by an immuno fluorescence test (IIF-Test for *B. henselae* and *B. quintana* IgG, Euroimmun Biochip Kit). The first library consists of 891 hunter sera of the year 2002 and the second library was used as a reference consisting of 624 sera from blood donors to the red cross of the year 2006.

The screening of 216 tick samples, 24 samples of each province in Austria consisting of 8 Adults, 8 Nymphs and 8 Larvae, resulted in an infection rate of 7.4 in the case of the Real Time PCR and 10.7% for the Nested PCR.

Furthermore the results showed that the rate of infection increases from larva stage to adult ticks!

The established differentiation PCR with the target sequence 16-23S intergenic spacer region showed good results with the different *Bartonella* positive controls but no results could be obtained for the screened positive tick samples.

The indirect immuno fluorescence screening with the EUROIMMUN Kit for IgG antibodies against *Bartonella henselae* and *B. quintana* resulted in a slightly higher rate of infection within the hunters as high risk group. 13.5% seropositive of the Hunter serum library and 11.5% of the Blood donors (Red Cross) serum library.

3. Introduction

3.1. *Bartonellaceae*

3.1.1. Historical overview of the family *Bartonellaceae*

3.1.1.1. *Bartonella bacilliformis*

All started with the outbreak of an epidemic in the year 1540, which was caused by *Bartonella bacilliformis*. Conquistador Miguel de Estete described it as an epidemic with a biphasic process. So possibly it is the oldest described epidemic. But it took 365 more years till the species was described by Dr. Alberto Barton (Fig. 1) in 1905 [32]. To his honor the organism was named *Bartonella bacilliformis*, the agent of the Carrións disease. The course of the disease is biphasic starting with the Oroya fever followed by the chronicle second phase, the Verruga peruana (Fig. 2) which are vasculoproliferative lesions of the skin [1].
Furthermore typical morphological changes of a Verruga peruana were found in a re-hydrogenated mummy of the Inca-time [2]. This finding is a proof that this disease has existed in this region for hundreds of years.

Fig. 1 Dr. Alberto Barton

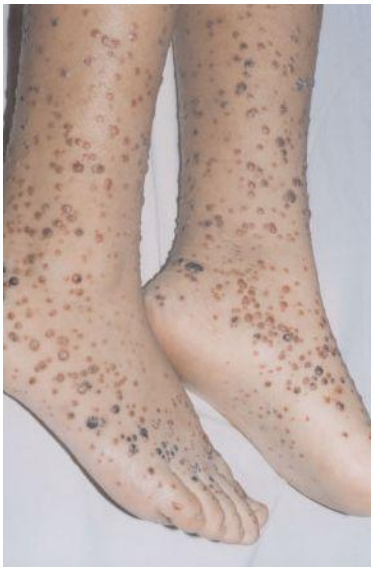


Fig. 2 Verruga Peruana

Many decades the family of the bartonellaceae consisted of only one species: *Bartonella bacilliformis*. This endemic pathogen can be found in the river valleys and canyons of the South-American Andes Mountains. The bacteria are transmitted through blood-sucking female sand flies of the genus *Lutzomya verrucarum*. These flies inhabit the Andes Valleys

between 500 and 3.000 meters elevation [3]. Especially in Peru and Colombia outbreaks of this disease could have been observed. 1870, short after the foundation of the republic of Peru the then government decided to build a railroad from the capital city Lima to the 5.000 meters higher situated mine settlement La Oroya. Therefor 10.000 workers from Chile and the coastal cities of Peru were engaged. This people came from a region where the Carrións disease was totally unknown. In the course of the construction work, especially in the area near the Rimac river, 8.000 people fell ill and died cause of high fever and severe anaemia. This phase of the disease was therefore called Oroya fever. Workers which survived the fever developed weeks to month later wart like skin disorders, called "verruga peruana" [5]. Then in August of the year 1885 a 26 year old medical student with the name Daniel Alcides Carrión wanted to prove with a self-experiment that the Oroya fever and the Verruga peruana are caused by the same pathogen. He inoculated secretion obtained of the skin lesion of a 14 year old Verruga peruana patient. 21 days after the inoculation Carrión sickened with high fever and a severe anaemia. 18 days later, on the 5th October 1885 he passed away [5]. To honor his heroic act the as "Bartonelloses" long known disease was also called "Carrión-disease".

With the knowledge of the vector for *Bartonella bacilliformis* a try was made to exterminate the sand flies in the endemic areas. The result was that sand flies could have been found in other regions with a higher density. As a consequence, in this endemic free areas endemics emerged [5, 6]. The last endemic was in the year 1998 in the region Urubamba in Peru [7].

3.1.1.2. *Bartonella quintana*

The next newly described Bartonellaceae was *Bartonella quintana* in 1917 by Schmincke [26] and Henry da Rocha Lima [11]. Schmincke named it *Rickettsia quintana* and da Rocha Lima, a Brazilian microbiologist, *Rickettsia pediculi*. When the bacterium was divided from *Rickettsia*, as an honor of da Rocha Limas work, it was named *Rochalimaea quintana* [27]. Finally in the year 1993 it was reassigned to the family Bartonellaceae with the name *Bartonella quintana* [25]. It's the agent which causes trench fever, bacillary angiomatosis and endocarditis. Trench fever was the first clinical manifestation of a *Bartonella* infection to be recognized. The name "trench fever" was coined because the disease came to prominence as an infection of troops in both the German and Allied armies during World War I. The troop

physicians His and Werner independently described a cyclic feverish progress of the disease. It was located in Wolhynia, a region in what is today Ukraine [8]. The disease was characterized by a 5-day relapsing fever, with severe and persistent pain in the shins, and although rarely fatal, it resulted in prolonged disability. It has been estimated that trench fever affected over 1,000,000 people [9]. The disease was named Five day fever (Werner) or Wolhynia fever (His). But it is also known under the name Werner-His disease or Trench fever [10, 11]. Under these victims of World War I also J.R.R. Tolkien (John Ronald Reuel Tolkien) could be found [12].

Short abstract of John Garth's book Tolkien and the Great War: The Threshold of Middle-earth:

> *"He writes that when war broke out, Tolkien was active in an Oxford literary society known as the Tea Club and Barrovian Society (TCBS), along with three of his closest friends. Finishing his degree before joining up, Tolkien served as a signal officer in the nightmarish Battle of the Somme in 1916, where two of those friends were killed. The ordeal on the Somme led to trench fever, which sent him home for the rest of the war and probably saved his life. It also influenced a body of Northern European-flavored mythology he had been inventing and exploring in both prose and verse before the war, toward its evolution into The Book of Lost Tales and in due course Lord of the Rings and The Silmarillion. This book could not pretend to be aimed at other than the serious student of Tolkien, and readers will benefit from a broad knowledge of his work (as well as a more than casual knowledge of WWI). But it also argues persuasively that Tolkien did not create his mythos to escape from or romanticize the war. Rather, the war gave dimensions to a mythos he was already industriously exploring."*

Although cases of trench fever were simultaneously reported on two fronts, the disease was supposedly conveyed from the eastern front to the western front by German soldiers in 1914 having been originally acquired from endemically infected Russian populations [10, 13, 14, 15]. Cause of the transferral of British troops to Mesopotamia and Salonika the disease was carried with them and spread in these regions [16, 17]. Subsequent investigations revealed that the Trench fever was known since the Middle Ages [10]. In Northern Ireland in the first

half of the nineteenth century [18] and also in Russia [14] a similar disease was described. After World War I cases have been monitored in Spain, Sweden, Ukraine, Georgia and Russia [10]. After the conclusion of the war, the incidence of trench fever and thus medical interest in the condition fell dramatically. But during World War II the disease reemerged and German troops [10] spread it throughout Norway, Finland [19] and Yugoslavia [20]. The next diseases have been monitored in Algeria, Egypt, Ethiopia, Japan, China and Mexico [13]. The recent outbreak was described in Burundi 1997 [21].

3.1.1.3. *Bartonella henselae*

The next big step was the identification of *Bartonella henselae* [22]. The first evidence of a new *Bartonella* species was provided by Dr. Relman in 1990. He managed to identify the pathogen which causes Bacillary Angiomatosis as a close relative of *Bartonella quintana* [23]. Almost at the same time Dr. Slater isolated a bacterium out of the blood sample of an immunosuppressed patient and also related it as a close relative to *Bartonella quintana* [24]. Finally Welch and colleagues isolated the bacterium from a (HIV)-infected patient with BA, septicaemia and parenchymal peliosis. They characterized and named it *Rochalimaea henselae* [22] which was renamed to *Bartonella henselae* and incorporated to the family bartonellaceae [25]. The species is spread worldwide. In cats it causes an asymptotic intraerythrocytic bacteremia. The disease transmission to humans happens through bites or scratches of cats and can also be transmitted through cat-fleas [28, 29]. Beside this disease, named cat scratch disease, *Bartonella henselae* can cause bacteremia and endocarditis. This involves immunocompromised as well as immunocompetent patients [30, 31].

3.1.2. Phylogeny of the genus *Bartonella* (formerly known as *Rochalimaea*)

Bartonella belong to the phylum proteobacteria. They are assigned to the class α2 proteobacteria, the order rhizobiales and the family bartonellaceae. The next kinships are *Brucella abortus* and *Agrobacterium tumefaciens* which are also counted among the order rhizobiales. Until now 24 *Bartonella* species and 3 subspecies have been described. At least 9

are pathogens for humans (Tab. 1). The last member of the genus *Bartonella*, *B. melophagi* is such a newly discovery that its name has yet to be formally accepted. So the genus *Bartonella* consists of 2 human specific species, 6 zoonotic species and 15 animal specific ones. The last two members have not been assigned to one of this groups until know. But in the case of *B. melophagi*, which was found in human blood samples, it is assumed to have potentials as a pathogen to humans.

The best markers which are used for detection and differentiation of *Bartonella* species are 16S rRNA, ribC (riboflavin synthase) gltA (citrate synthase), rpoB (beta subunit of RNA polymerase), 16-23s ITS (intergenic transcribed spacer region), groEL (heat shock protein), and ftsZ (cell division protein) sequences. This different target sequences were used for the comparison of neighbour-joining trees (Fig. 3 [52]).

Bartonella spp.	Year of novel describtion	Reservoir	Vektor	Diseases	Year of original describtion
human-specific spp.:					
B. bacilliformis	1905 [32]	human	sandflea	Carrion disease: Oroya fever and Verruga peruana	1870
B. quintana	1917 [26]	human	body louse	Trench fever Bacillary Angiomatosis Endocarditis	1917 1992 1993
zoonotic spp.:					
B. clarridgeiae	1996 [33]	cat	cat flea	Cat scratch disease	1997
B. elizabethae	1993 [34]	rat	unknown	Endocarditis	1993
				Neuroretinitis	1998
B. grahamii	1995 [35]	mouse, vole	unknown	Neuroretinitis	1999
B. henselae	1992 [22]	cat	cat flea	Cat scratch disease Bacillary Angiomatosis Endocarditis Bacillary Peliosis, Neuroretinitis, Bacteremia, fever	1950 1992 1993 -
B. vinsonii subsp. arupensis	1999 [36]	mouse	tick	Bacteremia, fever	1999
B. washoensis	2003 [37]	gopher	unknown	Myocarditis	-
				Endocarditis	2002
animal specific spp.:					
B. alsatica	1999 [38]	rabbit	unknown	not described	-
B. birtlesii	2000 [39]	mouse	unknown	not described	-
B. bovis (= B. weissii)	2002 [40]	cattle / cat	unknown	not described	-
B. capreoli	2002 [40]	deer	unknown	not described	-
B. chomelii	2004 [41]	cattle	unknown	not described	-
B. doshiae	1995 [35]	vole	unknown	not described	-
B. koehlerae	1999 [42]	cat	unknown	not described	-
B. peromysci	1942 [43]	deer, mouse	unknown	not described	-
B. schoenbuchii	2001 [44]	red deer	unknown	not described	-
B. talpae	1911 [45]	mole	unknown	not described	-
B. taylorii	1995 [35]	mouse, vole	unknown	not described	-
B. tribocorum	1998 [46]	rat	unknown	not described	-
B. vinsonii subsp. berkhoffii	1996 [47]	dog	tick	Endocarditis	2000
B. vinsonii subsp. vinsonii	1946 [48]	vole	mite	not described	-
newly discovered:					
B.rochalimae	2007 [49]	?	?	illness with features resembling Oroya fever	-
B.melophagi	2009 [50]	sheep ?	ked fly ?	not described	-

Tab. 1 List of so far known *Bartonella* species (effective 2009)

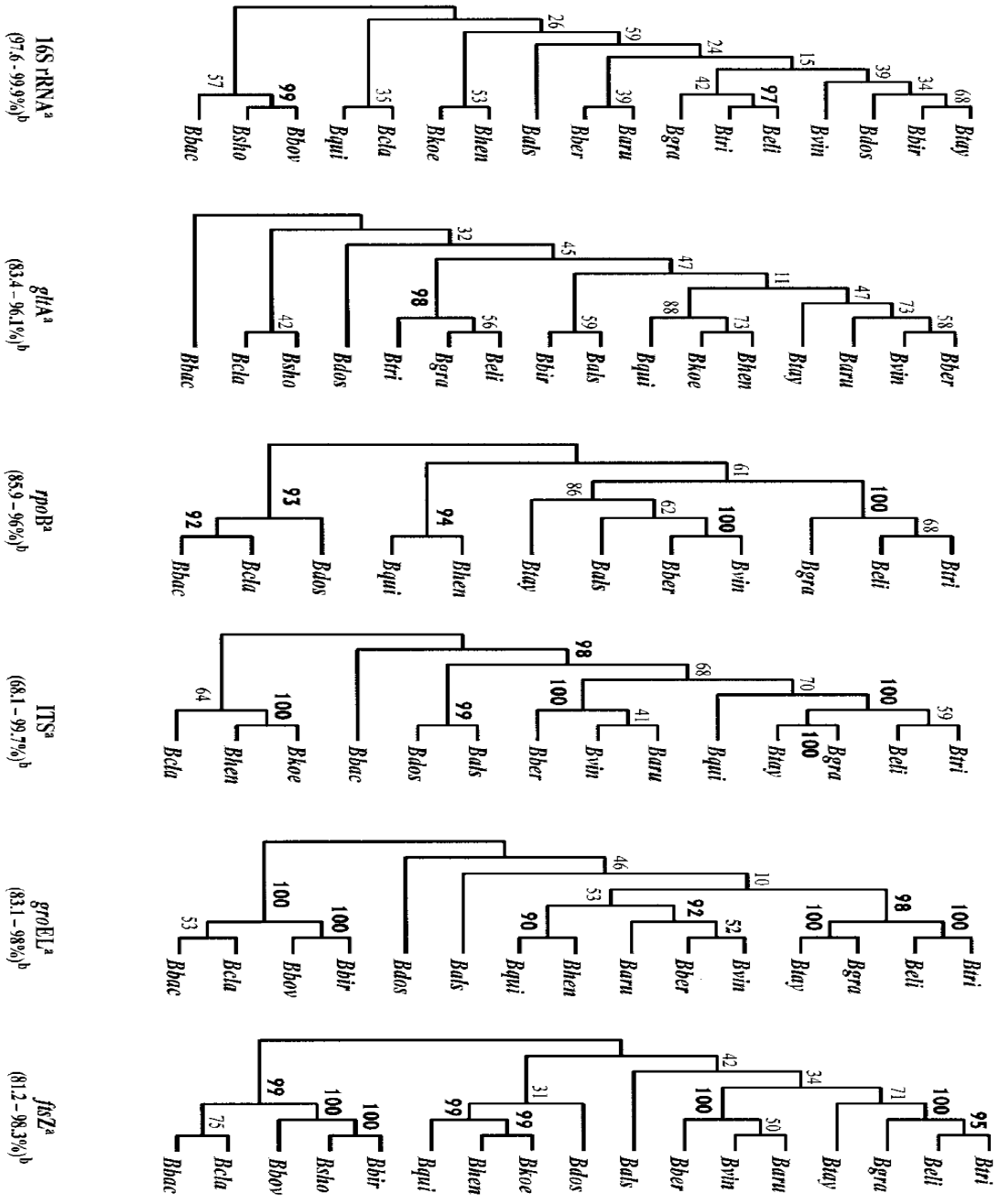


Fig. 3 Comparison of neighbour-joining trees based on 16S rDNA, gltA, rpoB, ITS, groEL, and ftsZ partial or complete sequences. Bootstrap values at tree nodes are based on 100 replicates; values of 90 are boldfaced. Trees were unrooted, and only topology was shown for these trees.

a, gltA, citrate synthase; rpoB, beta subunit of RNA polymerase; ITS, 16S–23S rRNA ITS; groEL, heat shock protein; ftsZ, cell division protein.

b, range of the level of DNA sequence similarity for each gene used. Designations for species and subspecies consist of the letter B (for *Bartonella*)

and the following abbreviations: ber, vinsonii subsp. berkhoffii; vin, vinsonii subsp. vinsonii; aru, vinsonii subsp. arupensis; tri, tribocorum; eli, elizabethae; gra, grahamii; tay, taylorii; als, alsatica; dos, doshiae; hen, henselae; qui, quintana; koe, koehlerae; cla, clarridgeiae; bir, birtlesii; sho, schoenbuchensis; bac, bacilliformis; bov, bovis

3.1.3. Biological characteristics of *Bartonellaceae*

Bartonella are spread worldwide. They are aerobic, Gram negative, facultative intracellular bacteria, which cause a variety of human and non-human diseases. These parasites can also infect healthy people but are considered especially important as opportunistic pathogens. They are pleomorphe, slightly curved rods with a size of 0.2-0.5 µm x 1.7-2.2 µm. Biochemical *Bartonellaceae* cannot convert glucose and indole. They are oxidase-, urease- as well as catalase- negative. Their genome size is between 1600 and 2100kb [53, 54, 55].
In culture *Bartonellaceae* grow very slow and need a complex medium with hemin added [56, 57]. The best growth results can be achieved in co-culture with endothelial cells. Easier and very effective too is the cultivation on Columbia blood agar plates. The medium consists of 5% sheep- or rabbit- blood and is incubated at 35 °C to 37°C (in the case of *B. bacilliformis* at 30°C) and a CO_2 concentration of 5%. Furthermore it is beneficial to use a water vapour saturated atmosphere. In primary cultures the grey to white colonies appear after 6 to 21 days in comparison to subcultures. There the first colonies can be found after 3 to 4 days [58].

3.1.4. Distribution and Reservoir

Bartonellaceae are spread worldwide with different core areas for the particular species (Tab. 2). Especially *Bartonella bacilliformis and B. rochalimae* can only be found in South America.
A further factor for this diverse distribution is the wide range of different reservoirs and vectors (Tab. 1). Most *Bartonella* species colonise the blood of different mammals. Cats and dogs, cattle and sheep as companion animals, mice and rats in close proximity to humans as well as moles, voles and deer are counted among this different reservoir mammals. So it seems that most of these *Bartonella* species have a defined and very tight host spectrum they colonise. So this specificity for the host or vector may explain the current geographic distribution of *Bartonella* species.

Species	Distribution
B. alsatica	Europe
B. bacilliformis	South America
B. bovis	Europe, Africa, North America
B. clarridgeiae	Europe, USA, Asia
B. doshiae	Europe
B. elizabethae	Europe, USA, Asia
B. grahamii	Europe, Canada, Asia
B. henselae	South America, Europe, USA, Africa, Asia
B. koehlerae	USA
B. quintana	South America, Europe, USA, Africa
B. rochalimaea	Peru
B. taylorii	Europe
B. vinsonii subsp. berkhoffii	Europe, USA
B. washoensis	USA

Tab. 2 Overview of the geographical distribution of *Bartonella* species

3.1.5. Infection of the reservoir host

Most of the *Bartonella* species colonize the blood of various mammals. Using the example of Schulein et al. *Bartonella tribocorum* – model the course of the infection could have been described very detailed [59, 60]. They infected Wistar rats through an intravenous injection with *Bartonella tribocorum*. After the injection it took only some hours so that all culturable organisms were cleared from the circulating blood. In addition the blood remained sterile for 3 to 4 days. It's unknown which kind of primary niche *Bartonella* is using in this time. It could be possible they use vascular endothelial cells as primary niche or other authors believe the bone marrow is likely [61]. After 4 to 5 days post inoculation (d.p.i.) the bacteremic phase started. The bacteria associated with circulating mature erythrocytes and subsequently invaded them (Fig.4). The next phase witch immediately follows the invasion process is the intracellular replication. From this state on *Bartonellae* persist for weeks within the erythrocytes and thereby increasing their chances for transmission by blood-sucking arthropods. The cessation of the bacteremia is an antibody-dependent process [62]

In contrary to infections of humans witch result in an illness the course of infection of the animal host mostly shows a long lasting, asymptomatic bacteremia. This hypothesis could have been proven through cat [63], rat [59] and mouse [62] experiments.

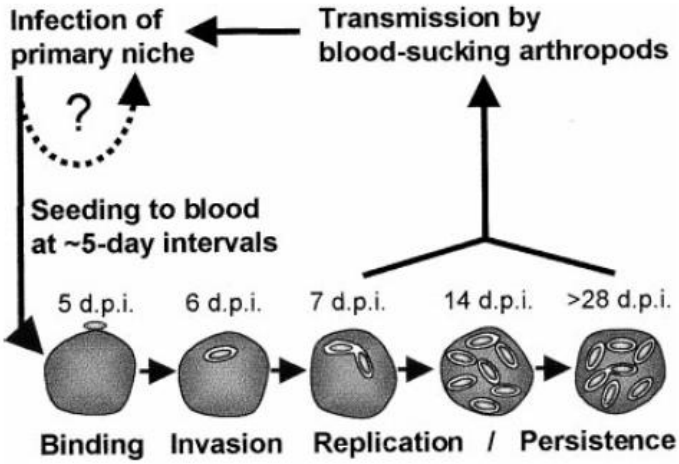


Fig. 4 Model of erythrocyte parasitism by *Bartonella* spp. [59]

Further studies on the course of *Bartonella* infections of the reservoir host were performed of the research groups Kosoy [64], Bermond [39] and Boulouis [65].

3.1.6. Pathogeneses

So the colonization of an organism with *Bartonella* can result in different kind of reactions: the asymptotic colonization in animal hosts on the one side and the acute or chronic disease upon the colonization of man on the other side. This is possible because *Bartonella* possess several very fascinating properties for pathogeneses. Two of these pathogenicity mechanisms are apparent: the invasion of endothelial cells and induction of neoformation of capillaries (angiogenesis) and the possibility of the invasion of mature human erythrocytes.

The invasion of endothelial cells and induction of angiogenetic processes is known for *B. baciliformis, B. henselae* and *B.quintana* [66, 67, 68, 69, 70, 71]. It could have been shown that the mechanism for the invasion of the endothelial cells was a bacterial-induced endocytosis. A second capability is the invasome-mediated internalisation of the bacterium into the endothelial cell. This is a unique process which has until now only been observed for *B. henselae*. The bacteria interact with endothelial cells resulting in bacterial aggregation on the cell surface and the subsequent engulfment and internalisation of the bacterial aggregate by the invasome structure. So the bacteria have the capability to restructure the actin-cytoskeleton for the internalisation [67, 68]. There are at least 5 membrane-proteins of *B. henselae* which can bind to endothelial cells [72].

After the invasion this 3 mentioned *Bartonellaceae* have the capability to induce proliferation of endothelial cells [73, 74, 69, 71]. This ability seems to be VEGF ("vascular endothelial growth factor") mediated and Rho dependent. Furthermore the anti-apoptotic activity of *Bartonella* can play a role in the VEGF mediated suppression of the caspase-activity and DNA-fragmentation [71, 75]. The clinically manifestation of endothelial cell proliferation is the Bacillary Angiomatosis, Veruruga peruana [1, 2, 5] and Peliosis hepatis.

3.1.6.1. The erythrocyte-invasion of *Bartonella*

A wide spread mechanism of many microorganisms to invade cells is the induction of a phagocytic process of the host. This process is essential for many intracellular occurring pathogens [76].

Beside *Bartonella* only some parasites like *Plasmodium* [77] or *Anaplasmataceae* [110] have the capability to invade mature human erythrocytes.
Generally the most widespread mechanism of many microorganisms to invade cells is the induction of a phagocytic process of the host cell. Simplified the phagocytoses can be divided in 3 stages: **the adhesion** – a receptor-mediated process which results in the restructure of the cell-cytoskeleton, **the internalisation** – the engulfment with the cell-plasma membrane forming an endocytotic vesicle and the uptake into the cell and **the evasion of the host defence and the escape.** This principle is used of many bacteria in different ways. Even for *Bartonella bacilliformis* [73], *B. henselae* [67, 68] and for *B. quintana* [66] it could have been shown *in vitro* (for *B. quintana* even *in vivo*) that these bacteria are incorporated in various epithelial and endothelial cells.
In the case of bacterial mature erythrocytes invasion there are some differences reasoned in the special properties of this cell type. Mature erythrocytes lack a cell nucleus and most organelles. So the cell can't be stimulated to start a phagocytic process nor does it feature the possibility of an actin-dependent formation of endocytotic vesicles. Furthermore the erythrocyte-membrane (Fig. 5) shows much more stability against shearforces than other cell membranes [78].
So the question is how does *Bartonella* manage to invade mature erythrocytes? The first guess was that *Bartonella* invade erythroid cells. But in the case of *B. tribocorum* this was not

verified [79]. It could have been shown that not until the fourth day of the bacteremic phase *B. tribocorum* could have been found intraerythrocytic. The invasion process and the involved factors have yet not been sufficiently studied.

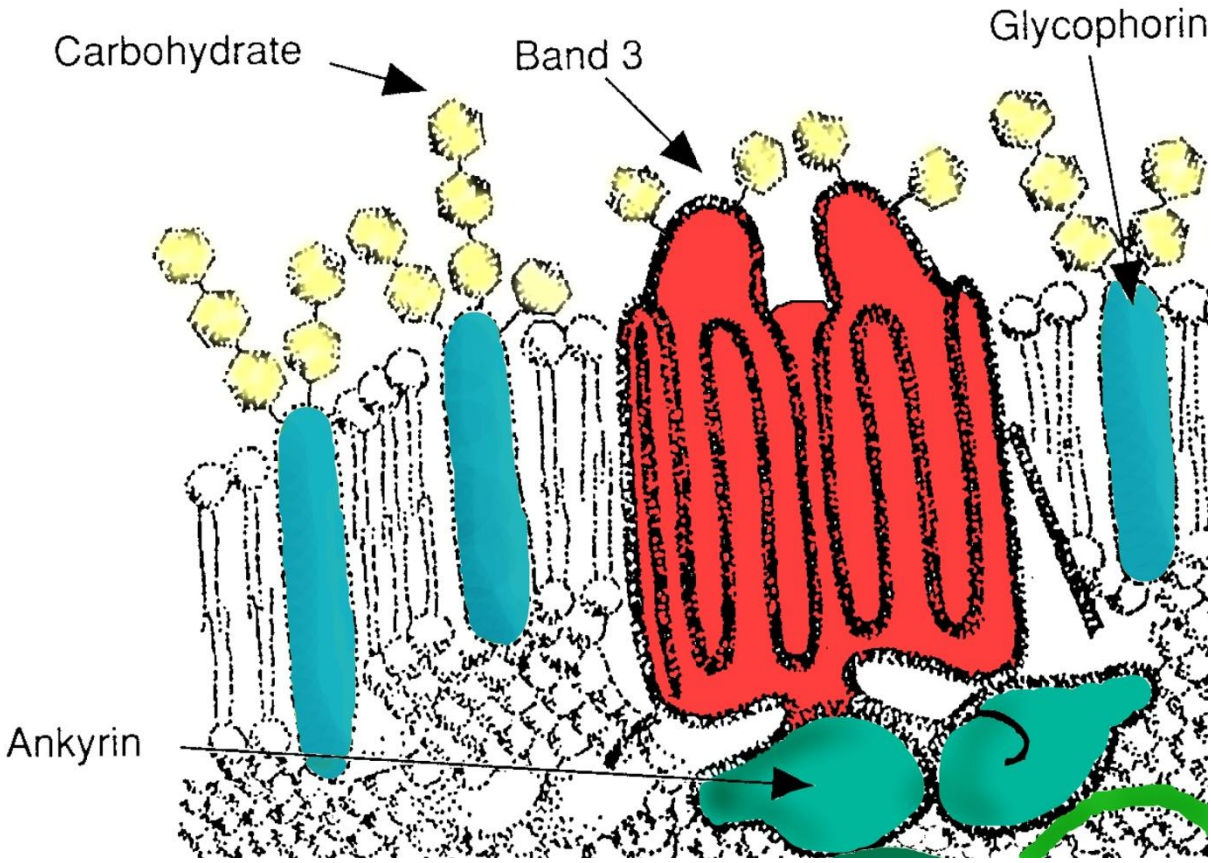


Fig. 5 Model of an erythrocyte cell membrane

Ankyrin - based macromolecular complex - proteins linking the bilayer to the membrane skeleton through the interaction of their cytoplasmic domains with Ankyrin.

Band 3 - Anion transporter, also an important structural component of the erythrocyte cell membrane, makes up to 25% of the cell membrane surface, each red cell contains approximately one million copies

3.1.6.1.1. Membrane-receptor-protein

There may be is an interaction of *B. bacilliformis* and *B. henselae* with identified proteins of human as well as animal erythrocytes. In the human erythrocyte membrane a 230 kDa protein could have been found independently by two different studies. This protein, assumed to be spectrin, is bound by *B. bacilliformis* even if it's cleaned up. Further proteins found to be involved were glycophorin A, glycophorin B and actin [80, 81]. So this special invasion

ability of *B. bacilliformis* seems to be attributed to a contact-dependent process [82]. Older postulations like of Walker and Winkler in 1981 [83] had the opinion that the binding-structure at erythrocytes are no glycoproteins but glycolipids.

3.1.6.1.2. Flagella, fimbriae and deformin

The first big step was the detection of a stable complex between *B. bacilliformis* and human erythrocytes *in vitro*. It was studied through an electron microscope by Benson et al. in 1986 [84]. *B. bacilliformis* as well as *B. clarridgeiae* and *B. schoenbuchensis* possess multiple unipolar **flagella**. In the case of *B. bacilliformis* it could have been shown that the inhibition of flagellin resulted in a reduced binding capability to the erythrocyte surface and an elimination of the invasion capability. So the 42 kDa flagellin protein which is needed to build flagella of *B. bacilliformis* is responsible for the bacterial mobility and therefore the formation of an aggregation [84, 85, 86, 87, 88, 89]. Since the most *Bartonella* species, like *B. henselae* [67], have no flagella but still invade erythrocytes there have to be further mechanisms for invasion.

Some *Bartonella* species possess bundle-forming-pili (BFP) also known as **fimbriae**. For many other bacteria this BFP play a major role in bacterial adhesion but in the case of *Bartonellaceae* it is unexplained [87].

The building of aggregations of *Bartonellaceae* on the surface of red blood cells went along with structural changes of these blood cells. This discovery was made by Benson et al. and related to **deformin** [84]. It causes morphological changes of the cell surface like folds, invaginations and the formation of intracellular vacuoles [86]. Deformin is a hydrophobic factor which was isolated from *B. bacilliformis* and *B. henselae* cultures harvested of red blood cells. Even after the removal of the bacteria and consequently of deformin the structural changes of the red blood cells maintained [84].

Further studies showed that also purified deformin causes deformation of the erythrocyte cell wall [81]. First assumptions pointed to a protein-like structure [90]. In further, more actual studies deformin was described as a small hydrophobic molecule with the following properties: a molecular weight of about 1.4 kDa [91] and soluble through albumin-binding [70]. A possible treatment was described by Mernaugh and Ihler with Vanadate, an ATPase-

inhibitor or the phospholipid Dilauroylphosphatidylcholine. Further on the intracellular calcium concentration can be increased [86].

3.1.6.1.3. Type IV secretion system

Many microorganisms have developed various systems to transport pathogenicity factors into the intracellular space. These transport systems of gram negative bacteria are divided in 6 classes: T1SS – T6SS [92, 93, 94, 95, 96].

The *Bartonella* type IV secretion systems consist of the VirB/VirD4 and the Trw system. The T4SS is homologous to the conjugation apparatus of bacteria and consists of several membrane associated and trans-membrane proteins (Fig. 6) [97-101].

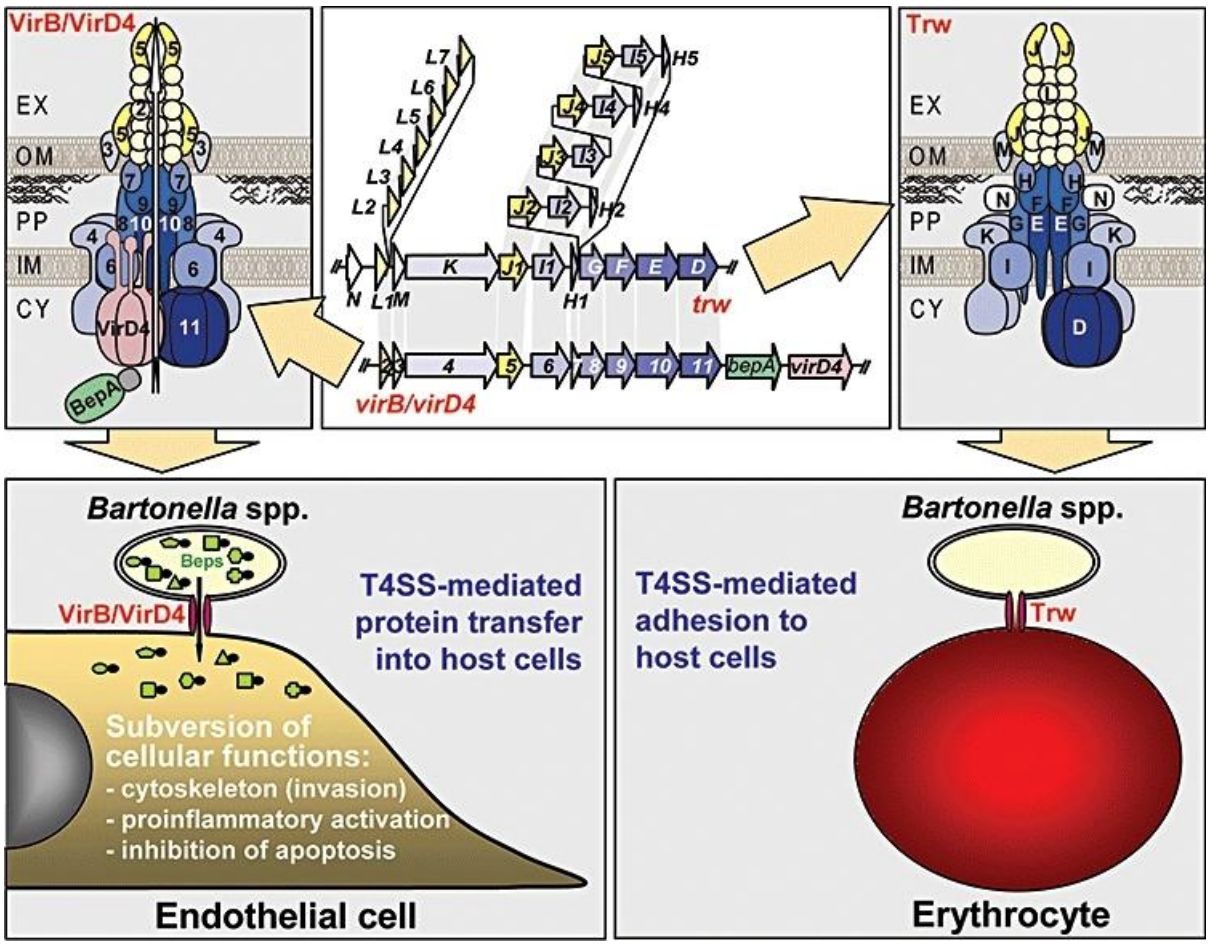


Fig. 6: The VirB/VirD4 and Trw type IV secretion system of *Bartonella* [98].

Of the two known *Bartonella* T4SS the Trw contributes to the development of an intraerythrocytic infection whereas VirB/VirD4 mediates a sub-function of the endothelial cell function [102].

So the Trw system is required for intraerythrocytic parasitism of *Bartonella*. It might not be required for infection of erythrocytes but rather for their colonization and/or for intraerythrocytic replication. The finding that the Trw system is upregulated during in vitro infection of erythrocyte leads to further suggestions. The bacteria might gain competence for subsequent erythrocyte infection during their transient inhabitation of red blood cells. [97, 103].

The Bartonella VirB and VirD4 genes are highly conserved between *B. henselae* and *B. quintana* in which the encoding loci have been sequence-analyzed. There is also a close phylogenetic relationship between the AvhB/Tra conjugation system of *Agrobacterium tumefaciens* and the VirB/VirD4/bep pathogenicity island (PAI) of *B.henselae* and *B. quintana* (Fig. 7) [97]. In contrast the bep genes (*Bartonella* translocated effector-proteins) differ widely between *Bartonella* species. Similar to VirB/VirD4, BepA–BepG are required for invasion, proinflammatory activation and antiapoptotic protection of erythrocytes. Thus, BepA–BepG are likely to represent the molecular effectors mediating VirB/VirD4-dependent cellular responses in infected erythrocytes [97, 104].

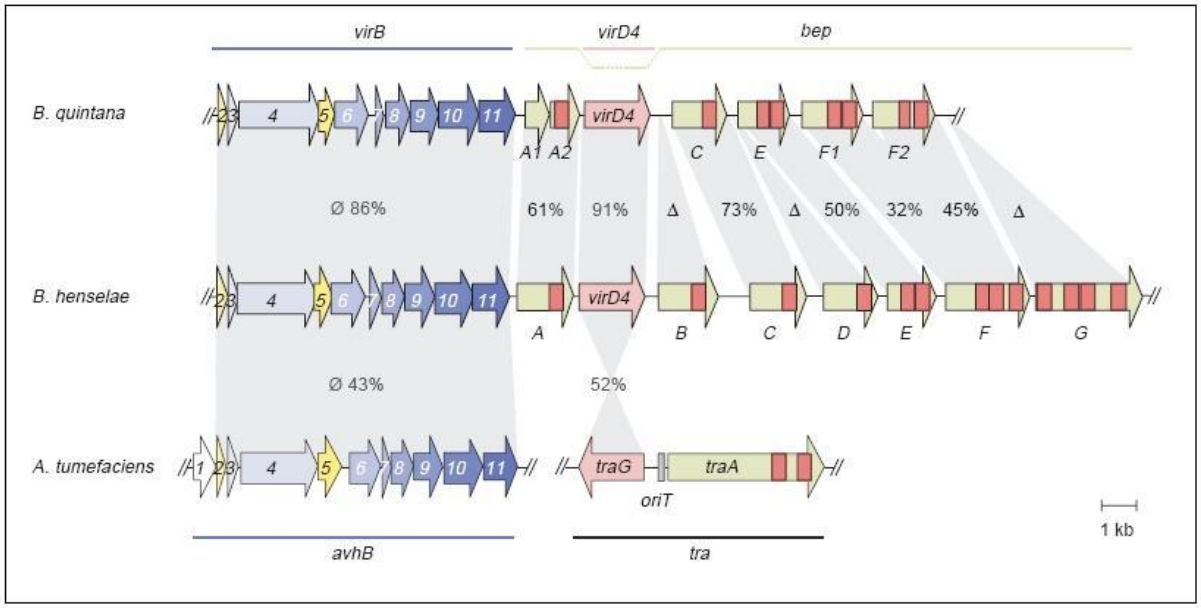


Fig. 7: PAI and AvhB/Tra conjugation system.

3.1.7. Diseases caused by *Bartonella* species pathogenic to humans

Immunocompetent people	
hemolytic-pyretic	
Bacteremia	*B. bacilliformis, B. henselae, B. quintana, B. clarridgeiae, B. elisabethae, B. vinsonii ssp. Arupensiss* [36]
Oroya fever	*B. bacilliformis*
Trench fever	*B. quintana*
inflammatory-granulomatous	
Cat scratch disease	*B. henselae, B. clarridgeiae*
Endocarditis	*B. henselae, B. quintana, B. elisabethae*
Lymphadenopathy	*B. henselae, B. quintana*
Neuroretinitis	*B. henselae, B. grahamii* [105]
vasoproliferative	
Verruga peruana	*B. bacilliformis*

Immunocompromised people	
hemolytic-pyretic	
Bacteremia, fever	*B. henselae, B. quintana, B. vinsonii ssp. arupensiss*
inflammatory-granulomatous	
Endocarditis	*B. henselae, B. quintana*
vasoproliferative	
Bacillary Angiomatosis	*B. henselae, B. quintana*
Bacillary Peliosis	*B. henselae*

Tab. 3 Human pathogens among the *Bartonella* species

Of the so far known 24 *Bartonella* species and 3 subspecies at least 9 are pathogens for humans (Tab. 1). The 3 most important pathogens of these 9 species are *B. bacilliformis, B. henselae* and *B. quintana* (Tab. 3). These *Bartonella* species can be transmitted by arthropods or by direct contact with infected mammals, and various diseases can occur after infection with these pathogens. Depending on the immune status of the patients the disease pattern can differ widely.

3.1.8. Diseases according to Bartonella species

3.1.8.1. *Bartonella bacilliformis*

Oroya-fever is a haemolytic disease and the first stage of the biphasic course of the Carrións disease caused by *Bartonella bacilliformis*. Transmitted through *Lutzomya verrucarum* (sandfly) the disease is endemic in the South American Andes [3]. After the infection through the sandfly it takes 2-14 weeks till the first symptoms appear. Oroya-fever is the acute form and comes along with high fever, severe haemolytic anaemia, hepatosplenomegaly, lymphadenopathy, paleness, tachycardia and circulatory collapse [4, 106]. The haemolytic anaemia is caused by the bacterial colonisation of the erythrocytes. In the acute phase 40-100% of the erythrocytes can be infected [107, 108]. Without treatment the disease is fatal in 40-85% of the cases.

Patients who survive the first phase show after 2-3 month the first signs of the second stage of the Carrións disease; it is called Verruga peruana (Fig. 2) which presents with vasculoproliferative lesions of the skin [1] belonging to the vasoproliferative group of diseases. These lesions, wart like papules, are similar to Kaposi's sarcoma. They emerge especially on the limbs and can concern the all layers of the skin [109, 13]. Three different forms can be differentiated: miliary nodules with 2-3mm in diameter, a few large nodules with a diameter of 1cm, or mular with painful, deep-seated lesions in the ankles. All of these forms show angioneogeneses and endothelial cell proliferation [109]. In most of the cases this pathological changes disappear after 1-2 month without therapy [111].

3.1.8.1. *Bartonella clarridgeiae*

1995 *Bartonella clarridgeiae* was isolated by Clarridge *et al* [112] and in 1996 it was characterised and described by Lawson *et al* [33]. The first evidence that *B. clarridgeiae* causes cat scratch diseases like *B. henselae* was found 1997 [113]. The patient, a veterinary, who got bitten by a cat, fell ill with symptoms like headaches, fever and swelling of the lymph node. At the site of the bite a papular lesion developed. Serologically only antibodies against *B. clarridgeiae* could be found. The next case of *B. clarridgeiae* cat scratch disease was described 1998 by Margileth *et al* [114]. So after these and further studies it could have been

shown that *B. clarridgeiae* is like *B. henselae* an agent that causes CSD. Further symptoms are weight loss and a possible chest-wall abscess [114].

3.1.8.2. *Bartonella henselae*

The most frequent disease caused by *Bartonella* species is the cat scratch disease (CSD). In cats it causes an asymptomatic intraerythrocytic bacteremia. The transmission of pathogens to humans occurs through bites or scratches of cats; they can also be transmitted through cat-fleas [28, 29]. CSD is an inflammatory-granulomatous disease with major differences of the symptoms between immunocompetent and immunocompromised people. Epidemiological and clinical features of CSD were first described by Debré *et al* in 1950 [115]. The causality of *B. henselae* in CSD has since been demonstrated by serological and molecular assays [23, 116, 117, 118, 119]. There are frequent CSD cases in North America and in Europe. Especially children in warm and humid areas [120, 121, 122, 123]. According to research studies in the USA in 1985 and 1997 the incidence rate of the disease was 9 cases per 100.000 inhabitants per year [124, 125]. Immunocompetent adults can fall ill but in most of the cases the infection is subclinical and so not diagnosed. The course of a typical CSD is in general self-restricted and lasts approximately 2 to 3 month. Three to 10 days after the bite or scratch small russet papule are arising at the wounded skin-area followed by inflammatory lymphadenopathy and sometimes suppuration and fever. The lymph node swelling reaches its mean size of 5cm diameter after 2 to 3 weeks, and lymph nodes of the axillia [124] and of the neck [126] are most frequently affected.

In atypical cases manifestations like oculoglandular syndrome of Parinaud, erythema nodosum, encephalopathy, osteolytic lesions, thrombocytopenic purpura, and erythema marginatum occur [124, 127].

In case of immunocompromised patients, like HIV-infected persons, the clinical manifestation is totally different and can be life-threatening [128]. Especially atypical CSD can be observed in immunocompromised patients [129, 130]. Beside CSD *Bartonella henselae* can cause two vasoproliferative diseases in immunocompromised persons, bacillary angiomatosis and bacillary peliosis [22, 131, 132, 133, 134, 135]. However, cases of bacillary angiomatosis have also been described in immunocompetent persons [136].

The cutaneous form of bacillary angiomatosis leads to vasculoproliferative lesions of the skin. These are papule and vascular tumours which resemble them of verruga peruana. If these lesions affect the bone marrow, the liver, the spleen or lymph nodes the disease is called bacillary peliosis hepatis [137, 138, 139]. It's an uncommon vascular condition characterised by randomly distributed multiple blood-filled cavities [140, 141, 142, 143].
Furthermore *Bartonella henselae* can cause endocarditis [144].
That's in the range of the most important diseases *B. henselae* can cause but there are case reports after which practically every human organ or tissue can be affected.

3.1.8.3. *Bartonella quintana*

There are many names for this haemolytic pyretic disease caused by *B. quintana*; shinbone fever, five day fever, quintan fever, the most common one is trench fever. All of these names describe characteristics of the disease. Symptoms are arthralgia especially of the shinbone, fever relapsing approximately every 5 days, strong headaches, anorexia, shivering, excessive sweating, tachycardia and myalgia [13, 145,146, 147].
Especially in cases of immunocompromised patients the infection manifested as bacillary angiomatosis, endocarditis or unspecific fever [148, 149; 150].

3.1.8.4. Bartonella elizabethae, B. grahamii and B. vinsonii subsp. arupensis

So far there are only two studies where *B. elizabethae* was detected in humans and related to endocarditis [151] and neuroretinitis [152].
With PCR methods *B. grahamii* was detected in ocular fluid of a patient with neuroretinitis [105]. Furthermore there have been indications that *B. grahamii* is the causative agent of bilateral occlusion of branches of the central artery of the retina [153].
Bartonella vinsonii subsp. *arupensis* was isolated from the blood culture of a cattleman [36]. The second finding was in 2003; a 79 year old man with endocarditis was tested positive for *B. vinsonii* subsp. *arupensis* [154].

3.1.9. Diagnosis

Many different genetic methods for the detection of *Bartonella* species have been developed in the last years. For instance site directed mutagenesis of *B. bacilliformis* [88] and *B. tribocorum* [60]. Or the construction of a suitable expression vector for protein synthesis in *B. henselae* and its use to establish green fluorescent protein (GFP) as a novel expression marker [155, 156]. So this emerging pathogen could be labelled in vivo by GFP and detected with fluorescence microscopy to study the genetics and the interactions with living host cells.

The detection of *B. bacilliformis*, which causes Oroya fever, is possible by blood cultures or blood smears stained with Giemsa. In lesions of verruga peruana the pathogens can be detected through antibody or DNA tests [106, 157, 158].

In case of a suspected CSD caused by *B. henselae* (Fig. 8) serological methods like immunofluorescence test, ELISA, Western Blot as well as PCR tests are used [58, 118, 159, 160, 161, 162]. The histological detection in biopsy material shows the best results with the Warthin–Starry silver nitrate-based staining method [163]. The cultivation of *B. henselae* out of biopsy material is a very lengthy and unreliable procedure and thus it is playing a minor role among diagnostics [163].

The diagnostic of a *B. quintana* infection is difficult because of its similarity to many other febrile diseases. So the case history of the patient is very important. Did he have been in endemic regions or had contact to lice. The infection can be detected serologically by detection of specific antibodies with IIF and Western Blot assays, microbiologically by blood cultures and by detection of specific nuclear sequences by PCR [106, 165, 166].

Similar diagnostic procedures may be helpful for the detection of infections by *B. clarridgeiae*, *B. elizabethae* and *B. grahamii*.

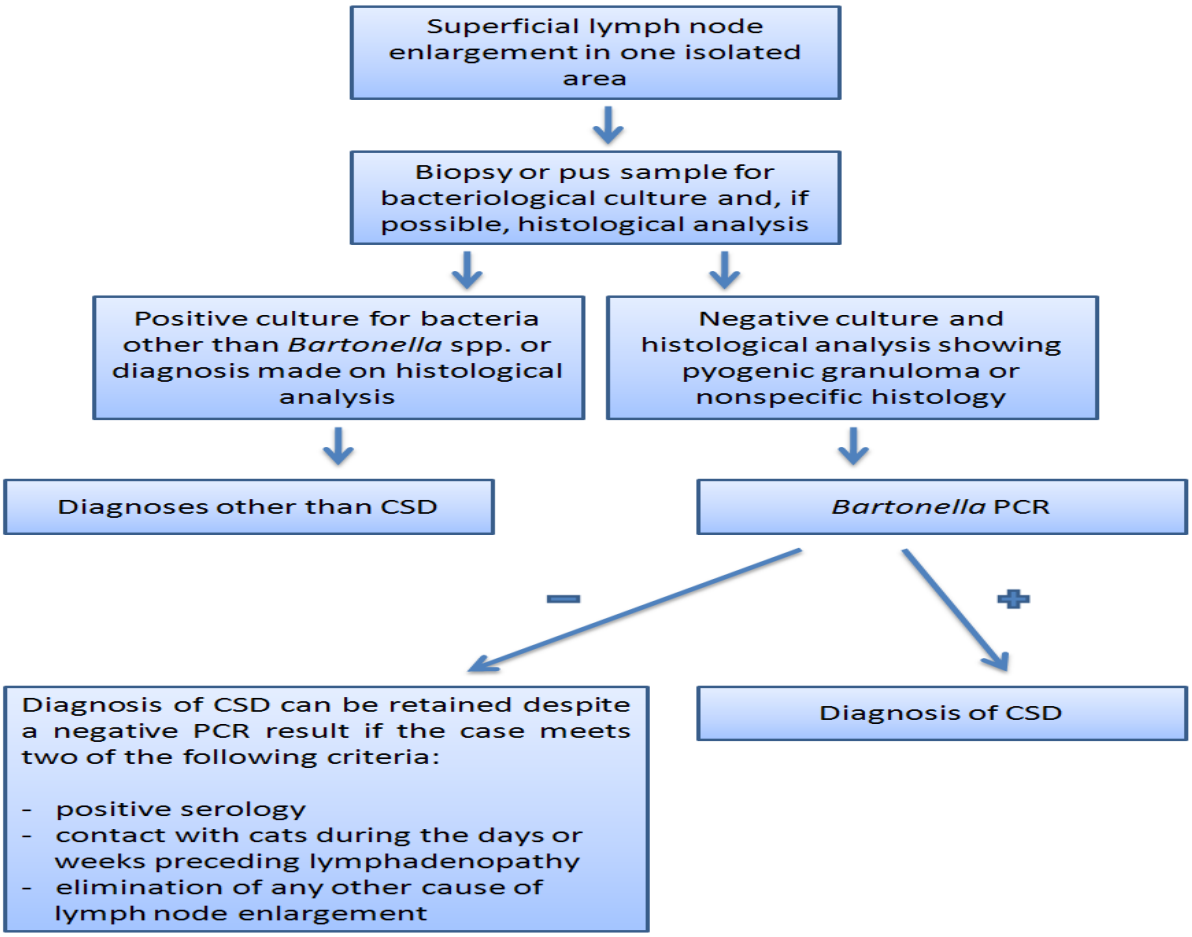


Fig. 8 Algorithm for CSD diagnosis [164]

3.1.10. Treatment

Bartonelloses are treated with antibiotics. The acute phase of Carrion´s disease is treated mainly with ciprofloxacin (or chloramphenicol). For verruga peruana rifampicin or macrolides are recommended [106, 111, 167]. *B. quintana* infections are treated with doxycycline, erythromycin or azithromycin [13, 168] and in case of an endocarditis additionally with aminoglycoside [169].

Bacillary angiomatosis caused by *B. henselae* is treated with erythromycin [22, 24, 148, 170]. CSD is most commonly treated with macrolide antibiotics such as azithromycin [125, 171]. It has been shown that there is a major difference between the in vivo and in vitro antibiotic susceptibility of Bartonella species. In vitro Bartonella are sensitive to almost every antibiotic tested. In vivo some antibiotics, like beta-lactams, are only poorly effective [172, 173]. In

order to prevent relapses the antibiotic treatment should be extended to last at least 6 weeks. In laboratory spontaneous resistances to antibiotics have occurred, e.g. cournemycin [174].

3.2. *Ixodes ricinus*, a possible tick vector

3.2.1. Systematic and morphology

The first phylogenetic tree for the ticks has been published by Hoogstraal and Aeschlimann in 1982 based on morphology, development, karyotype, reproduction and host phylogeny [175]. In the 1990s first phylogenetic tick studies with molecular characters were performed. Until now there have been more than 30 papers on the molecular phylogeny and evolution of ticks [175, 176, 177]. Historically there is only little information about the tick evolution so fossil ticks date back only 30 to 40 mya [180].

Ixodes ricinus ticks belong to the phylum arthropoda. They are assigned to the class arachnida, the order parasitiformes, the suborder ixodida and the family ixodidae (hard ticks). Their common name is "gemeiner Holzbock". These castor bean ticks are specialized as bloodsucking ectoparasites of mammals, birds, reptiles and amphibian [175, 178, 179].

The male tick is 2 – 3 mm long with a scutum on the dorsal side. It is a sclerotized carapace which overspreads the whole dorsal side (Fig. 9). In contrary unfed female ticks are 3 - 5 mm long and their scutum just covers the front part of the dorsal side. This ensures that the female can stretch its body during the blood meal to a size of up to 1 cm. The colour of the male is black-brown and of the female maroon. Fed females have a change in their colour to blue-grey. They have cutting mandibles (chelicerae) and a feeding tube (hypostome) which is covered with recurved teeth used for the blood-feeding process [178, 181, 182].

Fig. 9 *Ixodes ricinus* developmental stages: from left to right lava, nymph, adult tick male, adult tick female [EUCALB homepage, http://meduni09.edis.at/eucalb/]

3.2.2. Life cycle

The *I.* ricinus life cycle (Fig. 10) is dependent on several environmental factors like air temperature, atmospheric humidity and light as well as the disposability of hosts. Therefore the life cycle can last 2 to 6 years [181, 183]. There are 3 developmental stages and in every stage a blood meal is required to evolve to the next level. Out of the laid eggs six-legged larvae with a size of up to 0.6 mm hatch. After the blood meal it takes days to months till the larva moults to the nymph. The eight-legged nymph has like the adult male tick only a small scutum and can reach a size of up to 1 mm. After a 3 – 4 days blood meal the nymphs run through a gestation period of several weeks to reach the sexually mature adult stage. During the sucking action the female can soak up to 200-times of their normal weight in blood. In this time the fertilization takes place followed by the death of the male. The saturated and fertilized female falls of the host and lays up to 2000 eggs in the upper layers of earth before it dies [178, 182, 183, 184].

Ixodes ricinus ticks use a very wide host range, whereas larvae and nymphs prefer small mammals, reptiles and birds and adult ticks bigger mammals as host [185, 186, 187].

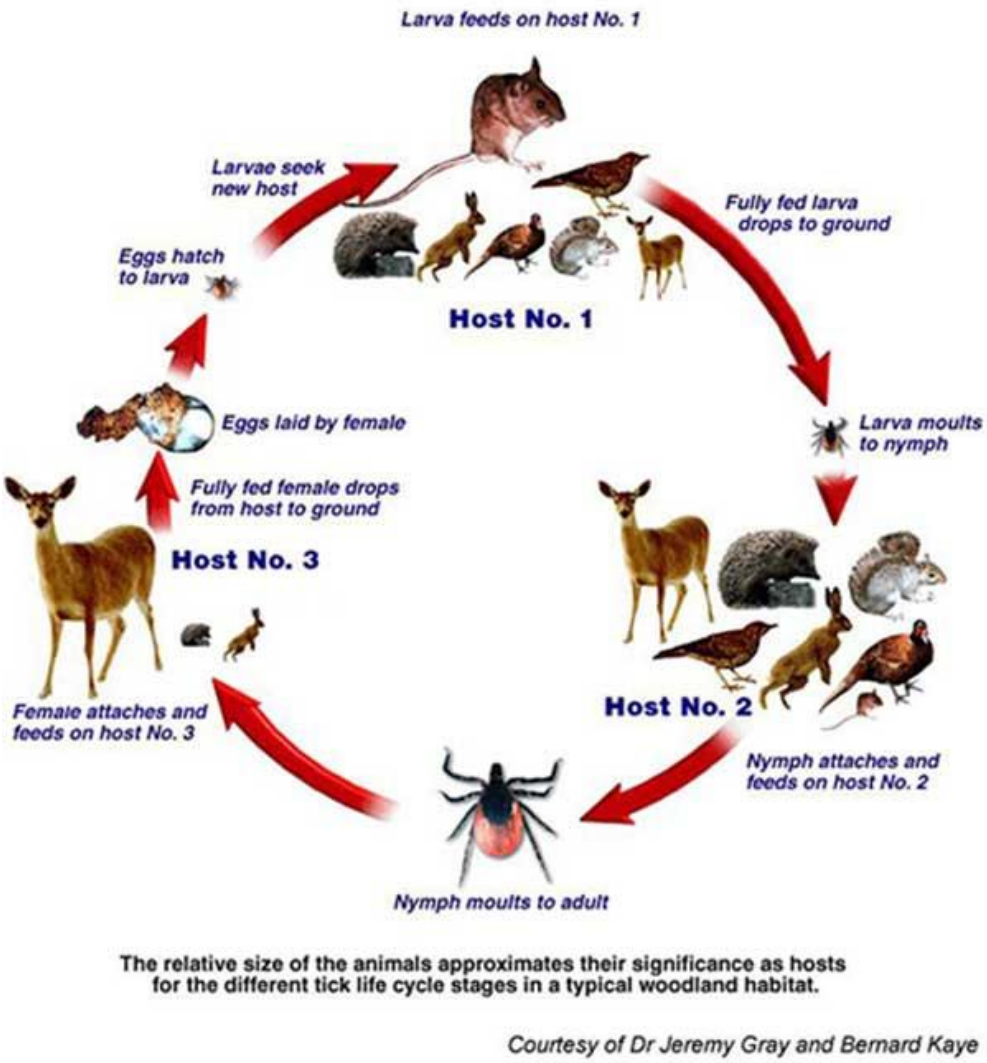


Fig. 10 *Ixodes ricinus* life cycle [EUCALB homepage, http://meduni09.edis.at/eucalb/]

3.2.3. Geographical distribution and activity

I. ricinus is the most frequent tick species in Central Europe [184]. Their distribution stretches from Portugal to the Caspian Sea and Southern Scandinavia to North Africa (Fig. 11). In these regions *I. ricinus* can be found in heights of 0 – 2000 m above sea level [184, 188]. The limitation of the geographical distribution of *Ixodes* ticks lie mainly within the climatic factors [181]. As habitat ticks are using the vegetation near the ground at transitions of meadows to dense undergrowth, wood glades, forest paths and runways as well as open areas without tree population [188, 189].

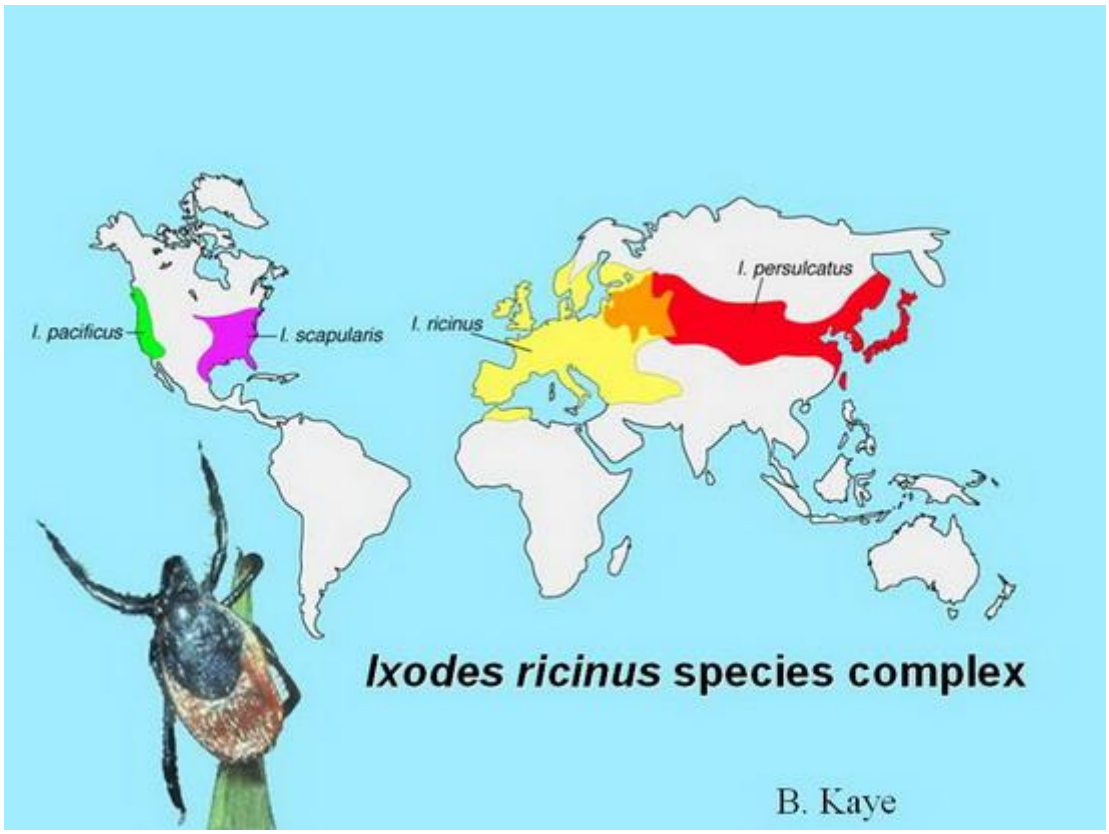


Fig. 10 Tick distribution [EUCALB homepage, http://meduni09.edis.at/eucalb/]

To analyse and predict the occurrence of ticks and tick-borne-diseases in a spatial location GIS (geographical information system) is used. It's a system of computer software, hardware and data and personal to manipulate analyse and present information [175].

The tick host-seeking activity is between March and October with different heights depending on their habitats and developmental stage. If there are really hot days the activity has its heights in the early morning, in the evening and at night. Ticks are located on exposed locations just under the top of the vegetation [178, 181]. The height depends on the developmental stage of the tick and consequently the size of the preferred host animal [188, 189].

3.2.4. Host finding, attack and sucking action

Larvae are located on grasses in up to 30 cm height, nymphs in about 100 cm height and adults can be found in heights up to 150 cm. All developmental stages have highly developed

sensory organs for the host finding. Through the hallers organ, paired sensory pits on the tarsus (foot) of the first pair of legs equipped with sensory hairs, the ticks can detect vibrations, scents and temperature. Light stimuli are detected through visual sensillia and chemical sensillia can detect tissue fluids. Furthermore ticks can detect heightened CO_2 and ammonia concentrations as well as pheromones and kairomones (interspecies information-bearing compound or mixture). After the host contact the tick needs only seconds for the adherence. Preferred for the blood sucking are humid, warm and well supplied with blood areas. In humans that are areas like thin skin parts, scalp, ears, throat, crook of the arm, knee bend, inguinal region as well as hands and feet. After the contact it takes before the blood sucking action starts. Than it takes 2 to 15 days till the tick finishes the sucking action [175, 181, 184, 189, 190, 191, 192, 193].

For the sucking action the ticks use their stingingly sucking mandibles and they harden their selves in the host skin with the hypostome. A further fixation is provided by the cement-like saliva. Furthermore the saliva has an anaesthetic virtue so that the tick bite remains unnoticed. The feeding period of female ixodid ticks can be divided into three phases: (1) **the preparatory feeding** phase occupies the first day, when the female tick anchors itself in place, (2) **the slow feeding** phase occurs over the subsequent 4-8 days, when the female mates and increases its weight approximately 10-fold, (3) **the rapid feeding phase** occupies only about 24 hours, during which the female increases its weight a further 10-fold and then detaches from the host [175, 178].

As pool feeders ixodid ticks create an interior wound cavity which contains blood, digested vessel walls, dissolved cells and tissue liquid. During the blood meal the blood is immediately condensed to maximise the substance absorption. The so resulting water is transported back into the host through specialised salivary gland cells. During the whole blood meal there is a constant saliva secretion into the host to sustain and improve the anchoring and because of the immunosuppressive, anti-haemostatic, anti-inflammatory and analgesic properties of the tick saliva. Because the tick intestine lacks digestive enzymes several potential pathogenic microorganisms are transmitted to the host [175, 178, 194]. That could be one reason why particularly ticks transmit a greater variety of pathogens than other arthropod groups [190].

3.2.5. Epidemiology

In Europe it is assumed that more than 95% of all tick-borne diseases are associated with *I. ricinus* and *I. persulcatus*. Important pathogens transmitted by tick vectors are species of *Anaplasma, Babesia, Borrelia, Erlichia*, and *Rickettsia* species and the FSME virus [175, 189, 193, 195]. In the case of *Bartonella* there have been publications which imply that ticks could play a role as vectors. Till now it remains premature to conclude that ticks might transmit *Bartonellae* to humans, but it is also premature to conclude otherwise [175, 196 – 203].

Relevant for the epidemiology of the *Ixodes* transmitted pathogens are the wide host spectrum, the reservoir function of hosts and ticks, the extent of tick infestation and the transfer mechanisms within tick populations [193]. Further importance lies on the capability of ticks to transmit pathogens transstadially and / or transovarially as well as the rare occurrence of passive transmission of bacteria through feeding in close proximity to other ticks (cofeeding) [184, 193, 204]. Moreover ticks have endosymbionts, like some *Rickettsia* and *Wolbachia* species, that indeed are transmitted transovarially and transstadially but after the current knowledge are non pathogenic [205]. So we are only talking about tick vectors if the carried microorganisms are transmitted "inoculative" to the host during the sucking action through continuous saliva secretion and occasional regurgitation of the intestinal contents [181].

3.3. Aims

Over time many species of *Bartonella* have been affecting human life. This can be traced back till the time of the Inca Empire. It has even been implied that *Bartonella quintana* could have been one possible cause of the decline of the Inca Empire [2, 206].

Up to the present day, and especially the last 20 years, many new species have been discovered and known ones showed knew abilities. So the number of known *Bartonella* species is rapidly growing. Some of them are responsible for distinct infectious diseases and show different prevalence and antibiotic susceptibility profiles. Not only have some vectors of *Bartonella* been fully characterized, but also intermediate hosts are actually much more numerous and diverse than previously thought. Furthermore there is only a limited arsenal of antibiotics effective in vivo on this peculiar intracellular pathogen. This facts show the

importance of a focused *Bartonella* research, especially in the case of more effective molecular methods [207, 208]. As mentioned earlier it is also unclear if ticks transmit *Bartonellae* to humans, but it would be also premature to conclude otherwise [203].
In this study 4 strains of *Bartonella* (*B. clarridgeiae*, *B. grahamii*, *B. henselae*, and *B. doshiae*) were cultivated and used as positive controls for the design of *Bartonella* specific PCRs. With these *Bartonella* PCRs a tick library has been screened. The second part of the study involves the screening of two libraries of human sera for antibodies to *Bartonella* by an immunofluorescence test.

3.3.1. Bartonella PCRs

Two different PCRs were established: a *Bartonella* specific one and one for species differentiation. Therefor two target sequences were chosen: the 16S rRNA gene and the 16-23S intergenic spacer region, whereas the primers for the ITS region were used for species differentiation.
Furthermore the aim was to compare two different PCR detection methods for *Bartonella* species. The comparison was made for a Nested / Seminested PCR and a Real Time PCR. The following screening was performed with a tick library consisting of 10.326 *Ixodes ricinus* ticks (adult, nymphs and larvae) collected at different locations in Austria.

3.3.2. IIF - Test

An indirect immune fluorescence test was used in order to detect IgG antibodies against B. henselae and B. Quintana, and was performed for two libraries. The first library consisted of 891 hunter sera (high risk group) of the year 2002. The second library was the reference group and consisted of 624 sera from blood donors of the year 2006.

4. Material and Methods

4.1. Biological material

4.1.1. Tick samples

From spring up to summer 2005 ticks from several locations in every province of Austria have been collected (Fig. 12). The collecting was performed with the flagging method. For this a white cotton blanket was dragged over the vegetation. The ticks attached to the blanket where collected with forceps, arranged according to the developmental stage and frozen at -20°C.

This collection consists of 10.433 ticks (Tab. 4).

	Ticks	
Total	**10.433**	**100%**
Larvae	2.652	25,4%
Nymphs	6.611	63,4%
Adults	1.170	11,2%
Percentage of *Ixodes ricinus* ticks	10.322	**98,94%**
Larvae	2.587	24,80%
Nymphs	6.573	63,00%
Adults	1.162	11,14%
Percentage of *Haemaphysalis concinna* ticks	111	**1,06%**
Larvae	63	0,60%
Nymphs	42	0,40%
Adults	6	0,06%

Tab. 4 Tick collection

For the screening only the *Ixodes ricinus* ticks were used and as a further differentiation the adult ticks were subdivided in male and female.

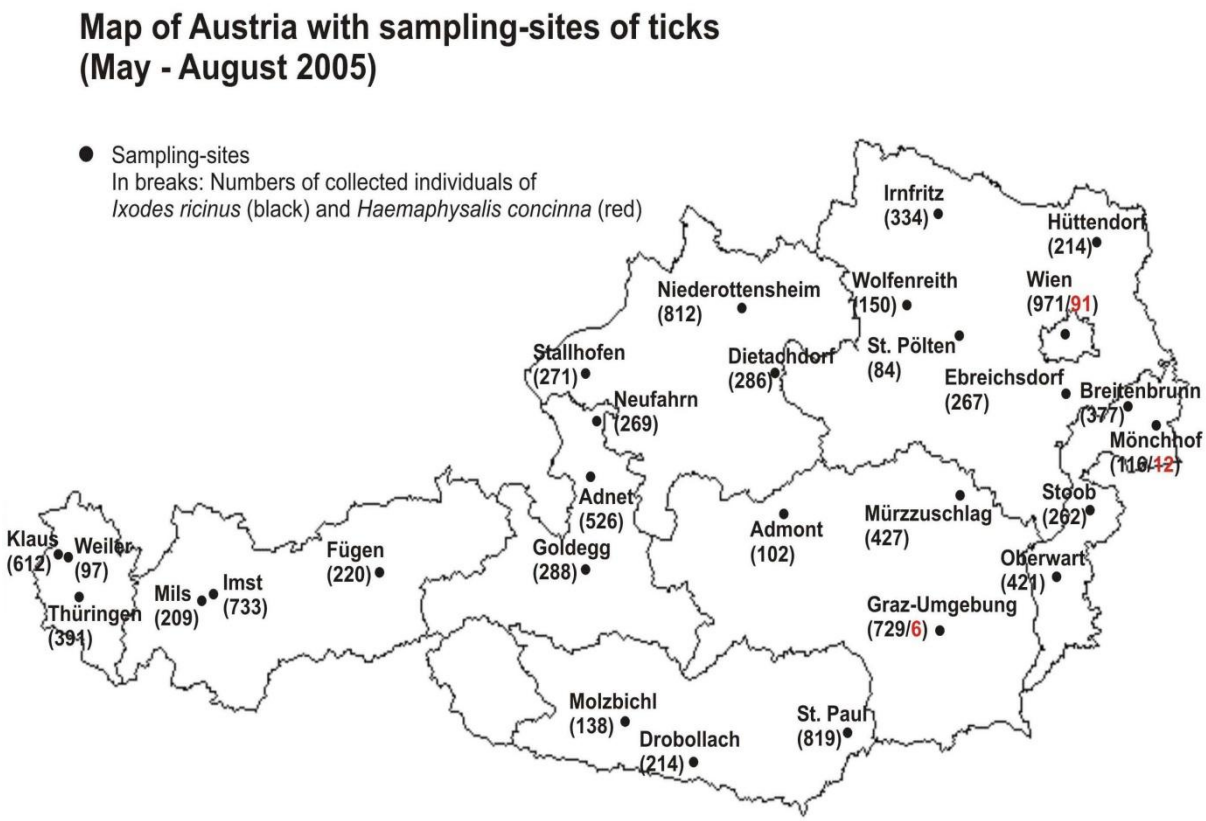


Fig. 12 Tick sampling sites

4.1.2. *Bartonella* strains

Four *Bartonella* species were obtained from the "Institute of Medical Microbiology of the Albert-Ludwigs-University Freiburg". These strains of *B. clarridgeiae, B. grahamii, B. henselae,* and *B. doshiae* were used as positive controls for the PCR assays. *B. doshiae* was the only species which is not a human pathogen (Tab. 5).

***Bartonella* species**	**Number**
Bartonella clarridgeiae	ATCC 51734
Bartonella grahamii	ATCC 700132
Bartonella henselae	ATCC 700693
Bartonella henselae	ATCC 49882
Bartonella doshiae	ATCC 700133

Tab. 5 Bartonella species

4.1.3. Human serum samples

Serum samples were obtained from the serum collection of the Hygiene Institute. As a high risk group a hunter serum library of the year 2002 was used. Sera were then collected in collaboration with the "Burgenländischer Jagdverband".
As a reference to this high risk group a second library of red cross blood donors of the year 2006 was used, kindly provided by the Austrian Red Cross.

Serum Library	Year of collection	Total sera
Hunters of Burgenland, Austria	2002	891
Red Cross Blood Donors, Austria	2006	624

Tab. 6 Serum Libraries

4.2. Solutions, reagents and Kits

Tick DNA extraction from ticks:	70% and 100% ethanol (Merck)
	DNeasy Tissue Kit (Qiagen)
	Proteinase K (Roche Molecular Biochemicals)
Isolation of DNA from bacteria:	DNeasy Mini Kit (Qiagen)
Primer and Probes:	p24 E and E2 with TaqMan Hydrolysis probe p24; N-p24 E and E2; 321s and as; BTNi F and R; Barton 1 and 2
PCRs:	10x Reaction buffer B (Solis Biodyne)
	dATP, dCTP, dGTP and dTTP (Solis Biodyne)
	Hot Fire Polymerase (Solis Biodyne)
	$MgCl_2$ (Solis Biodyne)
	SensiMix (Quantace)
Agarose gel electrophoresis:	10x Loading buffer (Eppendorf)
	50x TAE (Tris Acetat EDTA)
	Agarose (Biozym)
	Ethidium Bromide

	Step ladder (Sigma Aldrich)
Gel-DNA Purification:	GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience – GE Healthcare)
PCR and Sequencing:	1x EDTA (Ethylenediaminetetraacetic)
	70% and 100% ethanol
	AB Mix – Big Dye Terminator v 1.1 cycle Sequencing RR – 100 Kit (Applied Biosystems)
	Hidi Formamide
IIF-Test:	*Bartonella henselae* and *Bartonella quintana* IgG Biochip Kit (EUROIMMUN – Medical Labour Diagnostics)

4.3. Culture medium

Columbia agar + 5% sheep blood (BioMerieux – Clinical Diagnostics)
for deep freezing: Roti – Store cryo-vials (Carl Roth GmbH + Co. KG)

4.4. Methods

4.4.1. Tick DNA extraction

Every tick was separately incubated in 70% EtOH for at least 10 min and after that dried on a stage. Adults and nymphs were sliced with a sterile lancet and transferred into an Eppendorf tube (Eppi). Larvae were not sliced. So prepared the ticks were used for the DNA extraction with the DNeasy Tissue Kit.

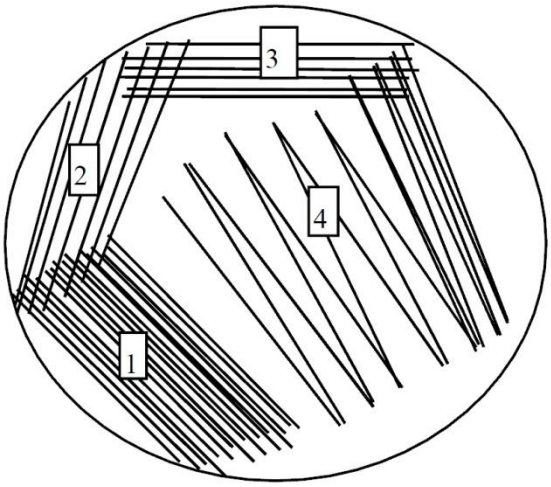
For the lysis 90µl of buffer ATL and 10µl of proteinase K (used the proteinase K of Roche Molecular Biochemicals and not the one provided from the kit; 14.4µg/µl water). The mixture was vortexed and incubated in a water quench at 60°C over night. The next step was the binding process. Therefor 100µl of buffer AL was added, followed by short vortexing and incubation at 70°C for 10 min. Than 100µl 96-100% EtOH were added, vortexed and spun for 5 min. at maximum speed (e.g. 14.000rpm). The supernatant was pipetted (about 300 µl) and

applied to a DNeasy column with a collection tube. The column was centrifuged at 8.000rpm for 2 min. and the collection tube was discarded. For the washing step a new collection tube was used with the column. 500µl buffer AW1 were added followed by centrifugation at 8.000rpm for 1 min. The collection tube was discarded again, the column was placed in a new collection tube and 500µl buffer AW2 were added. Centrifugation at 13.000rpm for 3 min. and the solution was discarded. This washing step with buffer AW2 was repeated and afterwards the column was spun for 3 min. at 13.000rpm to dry it. In the elution step the column was placed in an Eppi and 40µl, preheated to 67.5°C, AE buffer were added. The column was incubated for 5 min. at room temperature and afterwards spun 2 min. at 8.000rpm. The eluate was aliquoted and stored at -20°C.

4.4.2. Cultivation and Isolation of genomic DNA from *Bartonella*

4.4.2.1. *Bartonella* cultivation

The *Bartonella* strains were streaked onto blood agar plates with the streak plate procedure. An inoculation loop was sterilized through a Bunsen burner flame. With the cold sterilized loop a small amount of the bacterial growth was picked and immediately and gently streaked over a quarter of the agar plate (Fig. 13 area 1). The loop was flamed again and allowed to cool. Of the edge of area 1 which was just streaked, the streaks were extended into the second quarter of the plate (Fig. 13 area 2). The last step was repeated and the third dilution streak on the plate was produced (Fig. 13 area 3). Finally one further streak into the center of the plate was made (Fig. 13 area 4).



Inoculation of a streak plate

1. Area of initial inoculation and first streaks yields heavy growth.
2. Area of second streaks from area 1 yields less dense growth
3. Area of third streaks from area 2 yields weak growth
4. Area of fourth streaks from area 3 yields single colonies.

Fig. 13 Streak plate procedure

The inoculated streak plates were incubated at 37°C and a CO_2 concentration of 5%. The incubation time depends on the *Bartonella* species and lies between 3 and 12 days.

For deep freezing the grown and harvested *Bartonella* cultures were resuspended in cryo-medium and should at least have a measured OD_{660} of 2. The suspension was filled into a Roti – Store cryo-vial and stored at -80°C.

4.4.2.2. Isolation of genomic DNA from *Bartonella*

The bacteria were harvested from a culture plate with an inoculation loop. Only one colony should be picked and the spot position marked on the plate. The isolation was performed according to the DNeasy Mini Kit of Qiagen.

The harvested bacteria colony was suspended in 180µl of buffer ATL by vigorous stirring. 20µl proteinase K were added, vortexed for 15 sec. and incubated at 56°C for 3 hours on a rocking platform. Afterwards the Eppi was centrifuged at 3.000rpm for 1 min. 200µl buffer AL were added, the mixture vortexed 15 sec., incubated at 70°C for 10 min. and again briefly centrifuged at 3.000rpm for 1 min. In the next step 200µl 96-100% EtOH were added, vortexed 15 sec. and again briefly centrifuged at 3.000rpm for 1 min. The whole mixture was applied to a spin column with a 2 ml collection tube and spun at 8.000rpm for 2 min. The collection tube with the flow-through was discarded and the spin column was placed in a new tube. 500µl of buffer AW1 were added and spun at 8.000rpm for 2 min. Once again the tube

with the flow-through was discarded. To the spin column with a new collection tube 500µl of buffer AW2 were added, centrifuged at full speed (e.g. 14.000rpm) for 3 min. and the tube with the flow-through was again discarded. The spin column with a new collection tube was centrifuged again at 14.000rpm for 1 min. The now dried spin column was placed in a clean Eppi and 200µl buffer AE was added. The incubation takes place at room temperature for 5 min followed by the centrifugation at 8.000rpm for 1 min. The eluate was aliquoted in 1.5µl Eppis and stored at -20°C.

4.4.3. Primer and Probes

4.4.3.1. Primer

The primer length and sequence are of critical importance in designing the parameters of a successful amplification. It should be complex enough so that the likelihood of annealing to sequences other than the chosen target is very low. The chance of finding an A, G, C or T in any given DNA sequence is ¼, for every dinucleotide 1/16 and for every further nucleotide growing exponentially. So, a sixteen base sequence will statistically be present only once in every 4 billion bases: this is about the size of the human or maize genome, and 1000x greater than the genome size of E. coli. Thus, the association of a greater-than-17-base oligonucleotide with its target sequence is an extremely sequence-specific process. Consequently, 17-mer or longer primers are routinely used for amplification from genomic DNA of animals and plants [211].
The melting temperature (Tm) of a nucleic acid duplex increases both with its length and with increasing G + C content.

A simple formula to calculate the Tm is “The Wallace Rule”:
Tm = 4(G + C) + 2(A + T)°C.

Thus, the annealing temperature (Ta) chosen for a PCR depends directly on the length and composition of the primers. The Ta should be about 5°C below the lowest Tm of the primer pair to be used [209]. But there is also a more rigorous treatment of Ta [210]: they maintain

that if the Ta is increased by 1°C every other cycle, specificity of amplification and yield of the products <1kb in length are both increased.

So important rules that have to be incorporated are [210]:

1. The primers should be between 17 and 20 bases in length
2. Base composition should be 50 to 60 % of G + C
3. Primers should have a G or C, or a GC or CG on the 3' end to prevent "breathing" of the ends and so increase the efficiency of priming
4. Tm should be between 55 and 80°C
5. Runs of more than three Cs or Gs at the 3' end should be avoided because of the risk of mispriming at G or C rich sequences on the target strand
6. The 3' ends of the primers shouldn't be complementary cause of the formation of primer dimmers
7. Primer self-complementarity should be avoided to prevent the formation of secondary structures like hairpins
8. Run a computer search against the vector and insert DNA sequences to verify that the primers are unique.

4.4.3.1.1. Step by step procedure

Through all steps of the primer creation the 8 rules should be incorporated as well as in any way possible.

- The target sequence was chosen and download for every species to the program Clustal X 1.83 and a database was compiled. The sequences were aligned and swept for suitable areas for the primers. If the primer set should just detect the genus in this case *Bartonella* the amplicon sequence between the primer pairs can be complementary between the species. On the contrary for species differentiation the amplicon sequence has to be diverged.

- In the next step a second database was compiled with all close related genera of *Bartonella* and with all possible genera that could contaminate the PCR. This database was aligned with the primer sets. So false positive results of the PCR assays could have been prevented.
- The so found primer sets were specific for the genera, *Bartonella*, and were crosschecked with the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). With the "BLAST" tool, especially the nucleotide blast, the specificity of the primers were determined.
- Next was the control of the primer length and sequence. Therefor many free online tools were available, like "http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/". This tool checks the primer length, GC content, Tm (even Tm settings when needed), hairpin structures, self-dimers and hetero-dimers. A good tool to control the folding of the sequences is mfold, "http://mfold.bioinfo.rpi.edu/" of Prof. Michael Zuker.
- Finally the found primer sets were ordered at MWG (http://www.mwg-biotech.com/).

4.4.3.2. Probes

The TaqMan hydrolyses probe is a linear probe. The nucleotide acid sequence used for the probe is designed to bind between "sense" and "antisense" primer, so in the amplicon area. To produce a TaqMan probe several parts are needed. As already mentioned a specific nucleotide acid sequence with a length of 20 to 30bp. The length shouldn't drop below 20bp to avoid unspecific hybridisation and shouldn't exceed 30bp to avoid building of secondary structures. The guidelines for the sequence are much the same like for primers but there are some more points to be incorporated. Guanine bases on the 5' end of the sequence should be avoided because even after the hydrolysis of the reporter (fluorophore) the guanine has the ability to quench the signal (Fig. 14). One difference to the primer design is that the GC concentration should be between 20 and 80%. To provide a higher stability for the probe sequence – template – hybrid the Tm of the probe should be 5 to 10°C higher than the one of the primers. The optimal binding site of the 5' end of the probe is close to the 3' end of the template. Further the strand should be selected that gives the probe more Cs than Gs.

If the sequence complies the requirements a quencher can be attached at the 3' end. The most common quencher dye is TAMRA (6-carboxy-tetramethyl-rhodamin) with an emission wavelength of 580nm. The quencher is attached to the 3' end of the probe through a linker arm nucleotide (LAN). The 3' OH of the probe is blocked through a special phosphate.

Finally a reporter dye has to be attached covalently to the 5' end of the probe. The most common dye is FAM (6-carbox-fluorescein) with an emission wavelength of 518nm (Fig. 15) [212]. If the reporter dye and the quencher dye are in close proximity (10 to 100Å) the fluorescence-resonance-energy-transfer (FRET) occurs. That means a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon (quenching) [213]. The so excited acceptor (quencher dye) emits the received energy in its specific wavelength which is different to the one of the reporter dyes. During the PCR the single stranded parts of the probe are hydrolysed through the Taq polymerase. So the spatial distance between the reporter and the quencher dye increases and thus FRET does not occur (Fig. 14).

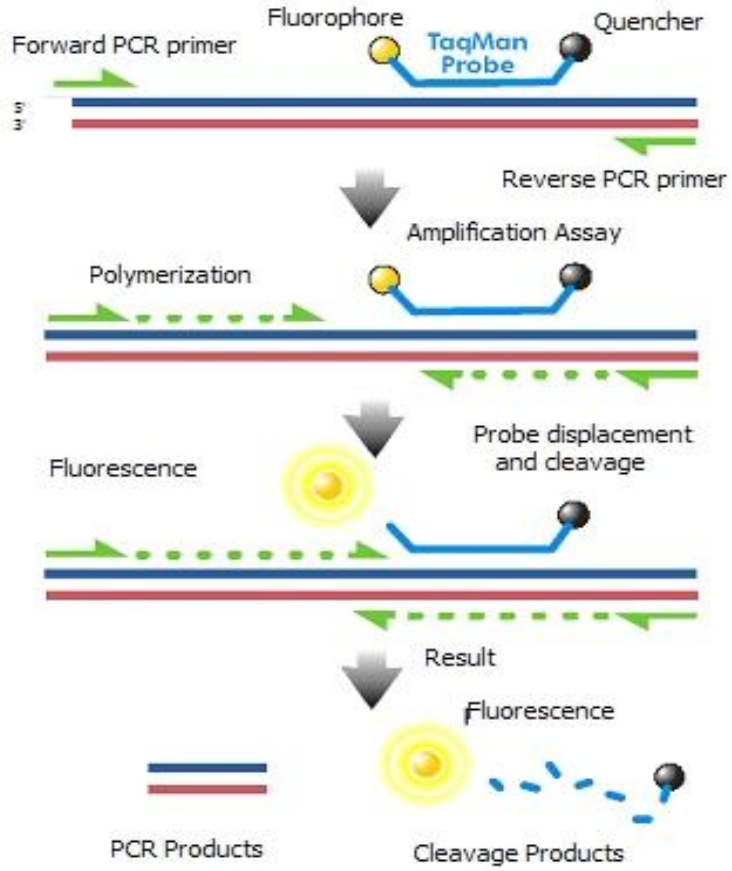


Fig. 14 Function principle of a TaqMan probe

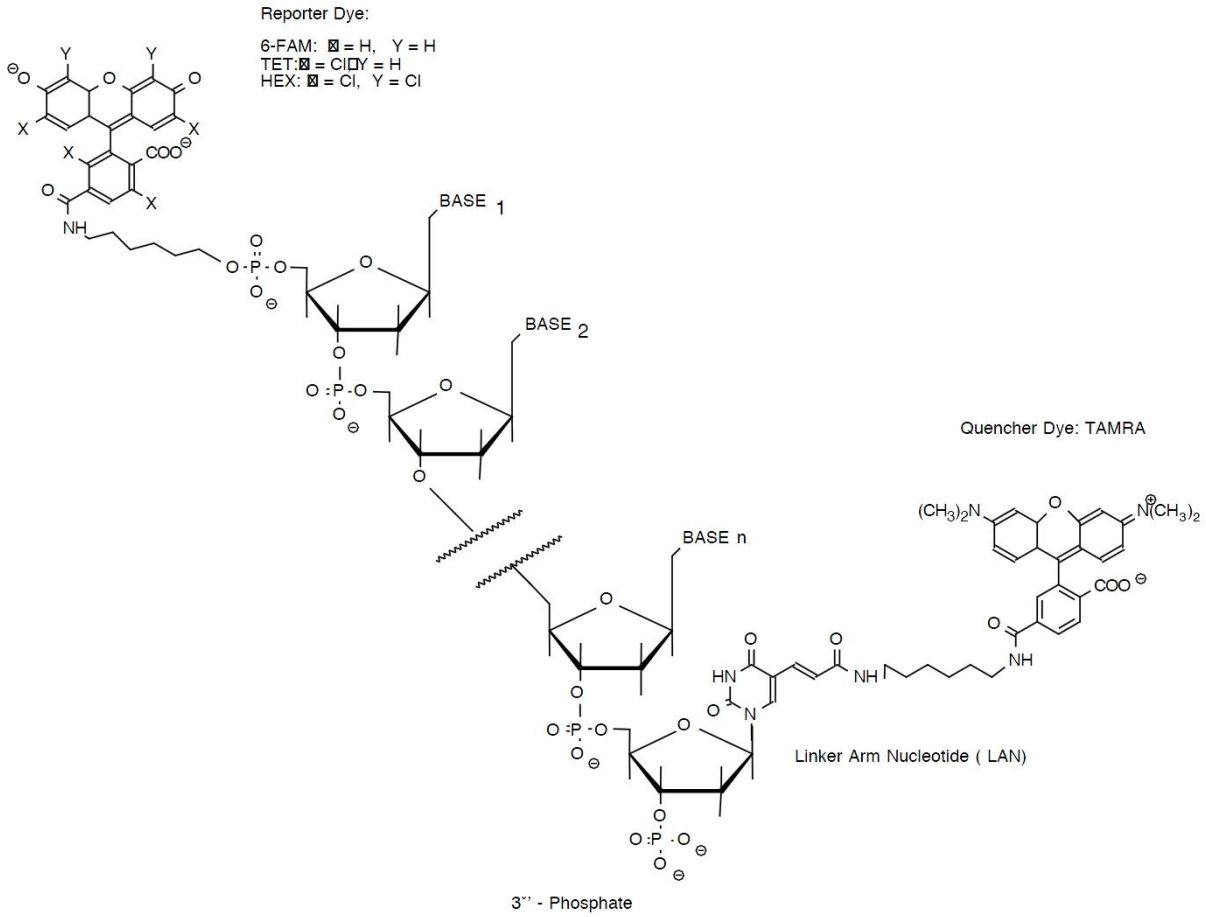


Fig. 15 Chemical structure of a TaqMan probe [212]

4.4.4. PCRs

4.4.4.1. PCR, Semi-nested PCR and Nested PCR

This molecular biological technique was founded by Kary Mullis in 1983. It is based on the denaturation of a double-stranded DNA (Fig. 16 - 1 denaturation), to which at the 5'- and 3'-end of the specific area to amplify oligonucleotide molecules bind (Fig. 16 – 2 annealing). The oligonucleotides will be elongated through a DNA-dependent DNA-polymerase, in the presence of free desoxynucleoside-triphosphate (dNTP) (Fig. 16 -3 elongation). The DNA-polymerase elongates the DNA double-strand till such time as it drops down the DNA or the reaction is interrupted. This interruption occurs when the incubation-temperature is raised to 95°C and is combined with the repeat of the denaturation. If the reaction mixture is cooled down to 40-60°C free oligonucleotides bind, dependent of their Tm values, to their

complementary DNA-matrix sequences. With this method the target sequence can be replicated exponentially [214].

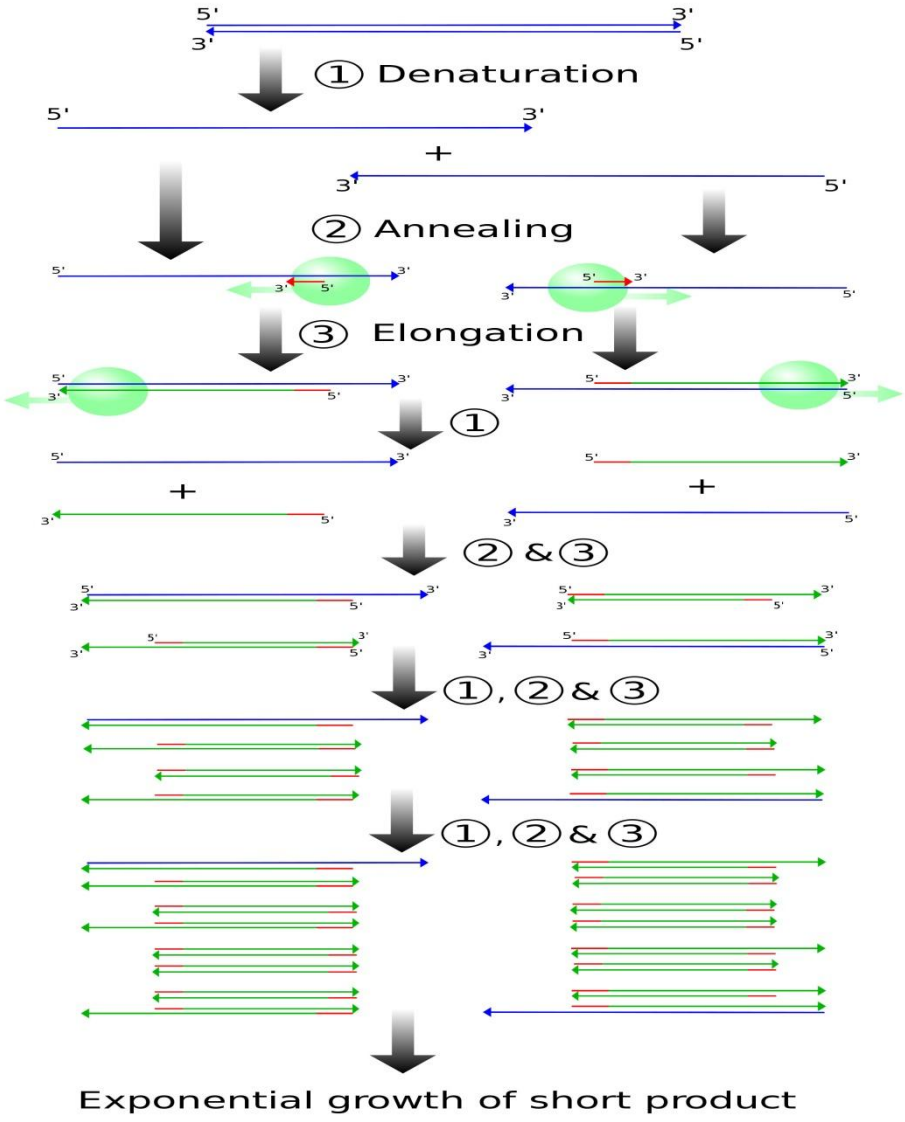


Fig. 16 Schematic PCR cycle

For the standard PCR one primer set is needed. A “forward” and a “reverse” primer with the binding site in between the target sequence.

In contrary the Semi-nested and Nested PCR consist of two succeeding amplifications. The first is the same like in the standard PCR with primers outside the wanted PCR-fragment. The second one, in the case of a Semi-nested PCR, uses one primer of the first amplification and for the second one a new primer in between the amplicon sequence of the first amplification. The second amplification for the Nested PCR is pretty much the same like the Semi-nested

one. The only difference is that both primers are in between the amplicon sequence of the first amplification. So in the second amplification the amplicons of the first one are used with the result of higher product specificity through the minimization of unspecific side-products [214].

The reaction mixture for a PCR consists of dH2O, the reaction buffer, $MgCl_2$, the primer set, dNTPs, the polymerase (Hot fire polymerase) and the target DNA.
The reaction buffer supplies the polymerase with all needed chemical components. So it provides an approximative optimal milieu for the enzyme at different temperature levels. The buffer consists of Tris-HCl, $(NH_4)_2SO_4$ and Tween-20.
Magnesium chloride is a metabolic cofactor for most DNA polymerases. The Mg^{2+} ions influence the enzyme activity, raise the dsDNA melting temperature and form a soluble complex with nucleotides, in what way the substrate is formed which is recognised by the polymerase. So the $MgCl_2$ concentration can influence the specificity and yield of the PCR. The standard concentration lies between 1.0 and 2.5 mM.
The dNTPs are the substrates for the DNA polymerase. The quality of the dNTPs lies in the content of base-modifications. The lower the content of modified bases the higher is the quality of the dNTPs for a PCR.
The Hot-start polymerases are modified versions of a normal polymerase with the advantage to be thermostable and are only activated by a 15 min. incubation step at 95°C. So during the preparation of the reaction mixture no unwanted amplification occurs [214].

4.4.4.1.1. Standard PCR Step by step procedure

The standard PCR was performed with 6 different primer sets: p24 E and p24 E2, BTNi F and BTNi R, Bart 2 and Bart 1R, 321 s and 321 as and Barton 1 and Barton 2. As already mentioned (see 4.4.3.1 Primers) the primers have been designed and constructed in accordance to the general rules of primer design [210, 214]. To adjust the PCR for *Bartonella* detection several runs were performed with positive controls of different *Bartonella* species with different concentrations. Through dilution series the proper concentration of the positive controls could have been found. For a normal run two standards were needed as controls: one Master-mix with the positive control and one with dH_2O as negative control.

So the first step was to pipet the Master-mix (Tab. 7). First the dH_2O was pipette, then the reaction buffer and the $MgCL_2$, next were the primers and the dNTPs and finally the polymerase. This Master-mix was used for all PCR-samples. The Master-mix was pipette to 500µl Eppis and combined with the different DNA templates which were tested and the different controls. The whole pipetting-procedure was performed on ice to prevent unwanted reactions before the PCR run has started. Because of this reason it was important to adhere to the pipetting order as described above.

Reaction Mixture Standard PCR		
Component	**Volume[µl]**	**Final Concentration**
dH_2O	11,0	----
Reaction Buffer	5,0	----
$MgCl_2$	5,0	5mM
Primer f	5,0	1µM
Primer r	5,0	1µM
dNTPs	1,0	400µM/dNTP
Polymerase	0,5	2,5U
DNA template	3,0	variable
dH_2O up to 50 µl	14,5	----
Total Volume	**50,0**	----

Tab. 7 Standard PCR Reaction Mixture

After the pipetting step the prepared Eppis were loaded to the PCR cycler. A program with the proper time and temperature steps was set (Tab. 8). The proper Ta's were determined through a temperature-gradient PCR.

Cycler Program Conditions Standard PCR			
Reaction	**Temperature[°C]**	**Time**	**Cycles**
Enzyme Activation Step	95	15 min	-
Denaturation	95	1 min	30x
Annealing	Primer Tm - 5°C	2 min	
Elongation	72	2 min	
Finishing Step	72	2 min	-
Hold	4	∞	-

Tab. 8 Cycler Program Standard PCR

4.4.4.1.2. Nested / Semi-Nested PCR Step by step procedure

The nested and semi-nested PCR is very much the same like the standard PCR. The first PCR run was performed with the external primer set p24 E and p24 E2 and the template DNA (Tab. 9).

Cycler Program Conditions Nested / Semi-Nested PCR Step I			
Reaction	**Temperature[°C]**	**Time**	**Cycles**
Enzyme Activation Step	95	15 min	-
Denaturation	95	1 min	30x
Annealing	Primer Tm - 5°C	2 min	
Elongation	72	2 min	
Finishing Step	72	2 min	-
Hold	4	∞	-
Cycler Program Conditions Nested / Semi-Nested PCR Step II			
Reaction	**Temperature[°C]**	**Time**	**Cycles**
Enzyme Activation Step	95	15 min	-
Denaturation	95	1 min	30x
Annealing	Primer Tm - 5°C	2 min	
Elongation	72	2 min	
Finishing Step	72	2 min	-
Hold	4	∞	-

Tab. 9 Cycler Program Nested / Semi-Nested PCR

The second run was performed with the amplicons of the first run and the internal primer set N-p24 E and N-p24 E2 in the case of the nested PCR and p24 E and N-p24 E2 in the case of the semi-nested PCR (see also 4.4.4.1 PCR, Semi-nested PCR and Nested PCR). The difference in the reaction mixture was that in the step II PCR the amplified sequences of the first run were used (Tab. 19).

Reaction Mixture Nested / Semi-Nested PCR Step I		
Component	**Volume[µl]**	**Final Concentration**
dH_2O	12,0	----
Reaction Buffer	5,0	----
$MgCl_2$	5,0	5mM
Primer f	5,0	1µM
Primer r	5,0	1µM
dNTPs	1,0	400µM/dNTP
Polymerase	0,5	2,5U
DNA template	2,0	variable
dH_2O up to 50 µl	14,5	----
Total Volume	**50,0**	----
Reaction Mixture Nested / Semi-Nested PCR Step II		
Component	**Volume[µl]**	**Final Concentration**
dH_2O	12,0	----
Reaction Buffer	5,0	----
$MgCl_2$	5,0	5mM
Primer f	5,0	1µM
Primer r	5,0	1µM
dNTPs	1,0	400µM/dNTP
Polymerase	0,5	2,5U
Amplicons of step I PCR run	2,0	variable
dH_2O up to 50 µl	14,5	----
Total Volume	**50,0**	----

Tab. 10 Reaction Mixture Nested / Semi-Nested PCR

4.4.4.2. Real-Time PCR

The main advantage to the standard-PCR is that the Real-Time PCR can measure the amplified matrixes directly (in real time). So this feature coins the name Real-Time-PCR [215]. For the detection of the amplicon amount fluorescent molecules (fluorophores) are used. This molecules are bound to the sequence specific oligonucleotides (see chapter 4.4.3.2 Probes). The principle of the detection relies on the extinction of the used fluorophore through short-wave light and the emission of longer-wave light (between 500 and 800nm). This emission is detected by an optical detection-unit per PCR cycle and in addition the fluorescence-intensity is measured [214].

The reaction mixture for a Real-time PCR is similar to the one of the standard PCR: dH2O, reaction buffer, $MgCl_2$, the primer set, dNTPs, the polymerase and the target DNA. Only one further thing has to be added if not working with SYBR green as fluorophore, the probe sequence.

4.4.4.2.1. Step by step procedure

Like the standard PCR the reaction mixture was pipette on ice. In addition the handling with the probe was performed fast to prevent long light exposure because the fluorophore of the probe is photosensitive. The probe final concentration should be about one third of the primer concentration (Tab. 11). The standards should be used in triplicates if possible. The cycler program differed from the normal PCR as can be seen in Tab. 12.

Reaction Mixture Real-Time PCR		
Component	**Volume[µl]**	**Final Concentration**
2x SensiMix	25,0	1x (3mM $MgCl_2$)
Primer f	2,0	200 nM
Primer r	2,0	200 nM
DNA template	10,0	variable
Probe	2,0	80nM
$MgCl_2$	----	----
dH_2O up to 50 µl	9,0	----
Total Volume	**50,0**	----

Tab. 11 Reaction Mixture Real-Time PCR

Cycler Program Conditions Real-Time PCR			
Reaction	**Temperature[°C]**	**Time**	**Cycles**
Enzyme Activation Step	95	10 min	-
Denaturation	95	10 sec	50x
Annealing-Elongation	60	45 sec	
Hold	RT	∞	-

Tab. 12 Cycler Program Real-Time PCR

4.4.5. Agarose gel electrophoresis

The 50x TAE stock solution was diluted with dH_2O to a 1x TAE solution. TAE has the function as electrophoretic running buffer. If the buffer concentration is too low, to less ions are available for the electric conductivity and the migration velocity of the DNA is very low. On the other hand side if the buffer concentration is too high, the good electric conductivity results in a heat generation which denaturants the DNA and melts the gel. So it's important to have a proper concentration of the running buffer. For a 3% small gel 100ml 1x TAE and 3g Agarose were mixed and shortly boiled up. During the cooling process down to room temperature the mixture was stirred continuously. At RT 5µl vortexed ethidium bromide was pipetted to the mixture and all together poured into the small gel rack. Then the comb was

inserted to produce the needed wells for the loading of the PCR products. Till the gel was cooled down and had become solid it was stored in a cold and light protected surrounding. The next step was to carefully remove the comb and put the gel with the rack into the 1x TAE filled tank. The wells were located on the side of the electrode that had the negative current. Because the ethidium bromide is very UV sensitive the gel was protected from light exposure as good as possible.

After the PCR run 27µl of the PCR product was pipetted in a new Eppi filled with 3µl of the 10x loading buffer consisting of Bromophenol Blue, Xylene Cyanol FF, EDTA, pH 8.0 and 50% Glycerol in dH_2O. This DNA gel loading buffer is intended to facilitate loading of the samples into the wells. It increases the density of the sample and gives it a colour, thus facilitating the loading procedure. In addition, the two dyes migrate in the same direction as the nucleic acids, serving as rough indicators of the electrophoretic progress. When the gel loading buffer was added to mixtures containing enzymes that require Mg^{2+} or Ca^{2+} for their reactivity, the EDTA in the buffer terminated these reactions.

One Eppi was prepared with a proper step ladder, depending on the fragment length of the amplicon sequences, to determine the size of the bands after the gel run. It was an electrophoresis marker for DNA containing 17 bands ranging from 50bp to 3000bp. 90µl step ladder and 10µl 10x loading buffer were mixed and 20µl were injected into the first well. If possible there should be one empty well between the controls and the samples to prevent false results through flowing of the mixtures into one another. After the loading process the power supply was turned to 75V with a run time counter of 60 to 65 minutes.

The analysis of the gel was performed with a gel-documentation equipment. The ethidium bromide, intercalated in the DNA, was exposed to UV-light resulting in a fluorescence cause of the phenanthridine moiety. The ethidium bromide in the gel was fluorescing too but through the intercalation and in further consequence the conformational change of the ethidium bromide the fluorescence of the intercalated agent was intensified almost 20 fold. So a photograph of the gel was taken under ultraviolet lighting conditions.

4.4.6. Gel-DNA Purification

Before the bands of interest in the agarose gel were used for sequencing they had to be purified. Therefor the "GFX PCR DNA and Gel Band Purification Kit" was used. The bands were cut out of the gel with a maximum weight of 900mg. For each 10mg gel slice 10µl capture buffer type 3 were added. The type 3 buffer contains a pH indicator that changes colour at various pH levels to identify whether the capture buffer plus sample mix is at the optimal pH for DNA to bind to the silica membrane. Than the Eppi was mixed by inversion and incubated at 60°C for 15-30 min. until the agarose was completely dissolved. Once the agarose was completely dissolved the capture buffer type 3-sample mix had to be yellow or pale orange in colour. If the colour differs a small volume of sodium acetate had to be added till the colour fits the requirements. After that, for every purification, a GFX MicroSpin column was placed into one collection tube. Up to 800µl of the capture buffer type 3-sample mix was transferred onto the assembled GFX MicroSpin column with the collection tube. It followed an incubation at RT for 1 min and a spinning at 16.000 x g for 30 sec. The flow through was then discarded and the GFX MicroSpin column with the empty collection tube was used for the washing and drying step. 500µl wash buffer type 1 were added to the GFX MicroSpin column to remove salts and other contaminations from the membrane bound DNA. The column with the collection tube was spun at 16.000 x g for 30 sec and then the collection tube with the flow through was discarded. The column was placed to a normal 1.5ml Eppi for the elution step. For the elution buffer type 6, sterile nuclease free water, was used. 40µl were added to the center of the membrane of the MicroSpin column and incubated at RT for 1 min. After that the column with the Eppi was spun at 16.000 x g for 1 min. to recover the purified, membrane bound DNA. The eluate was used for sequencing and could have been stored at -20°C for a short-time.

4.4.7. Sequencing PCR and Sequencing

The purified PCR product was used for a sequencing PCR. Therefor the AB-Mix was mixed with the PCR product, a forward or reverse primer and dH_2O (Tab. 13). The AB-Mix consisted of $MgCl_2$, reaction buffer, dNTPs and polymerase. Only one primer was needed because the classical chain-termination method after Sanger requires a single-stranded DNA

template [218]. The PCR product concentration in the reaction mixture depends on the length of the amplicon sequence. Between 100 and 200bp 1 to 3ng are needed, from 200 to 500bp 3 to 10ng are needed and between 500 and 1000bp 5 to 20ng have to be used. The mixture with a reaction volume of 10µl was stirred gently and centrifuged briefly at 3.000 rpm for 1 min. For the sequencing PCR a special cycler program was used (Tab. 14).

Reaction Mixture Sequencing PCR		
Component	**Volume[µl]**	**Final Concentration**
AB Mix	2,0	----
Primer f/r	1,0	1µM
Purified PCR Product	2,0	variable
dH_2O	5,0	----
Total Volume	**10,0**	----

Tab. 13 Sequencing PCR reaction mixture

Cycler Program Conditions Sequnencing PCR			
Reaction	**Temperature[°C]**	**Time**	**Cycles**
Enzyme Activation Step	96	30 sec	-
Denaturation	96	10 sec	30x
Annealing	50	5 sec	
Elongation	60	4 min	
Hold	4	∞	-

Tab. 14 Cycler program sequencing PCR

After the sequencing PCR the product was purified. Therefor the PCR Eppis with the product were centrifuged briefly at 3.000 rpm for 1 min. The 10µl of the reaction mixtures were pipetted to a 500µl Eppi containing 1µl vortexed NaOAc buffer. Then 33µl 100% EtOH were added and the mixture vortexed briefly and incubated on ice for 20 min. It followed a centrifugation step for 30 min. at 4°C and maximum speed. The supernatant was discarded,

90µl of 70% EtOH were added and centrifuged for 10 min. at 4°C and maximum speed. The supernatant was discarded and the sample dried at RT for 5 min. In this time the HiDi formamide was thawed and the thermo-mixer with dH_2O filled Eppis preheated to 95°C. 20µl of the thawed and vortexed HiDi formamide was pipetted to the sample and incubated at RT for 5 min. The HiDi formamide is a hygroscopic dissolvent and reductant with the purpose of stabilizing and denaturing of nucleic acids and so preventing the formation of hairpin structures. The next incubation step was in the prepared thermo-mixer water quench at 95°C for 5 min. Finally the sample was centrifuged briefly at 3.000 rpm for 1 min. and subsequently incubated on ice for at least 5 min. The so purified sample was used for the sequencing directly.

After sequencing the sample nucleotide sequences was identified through the NCBI database "BLAST" tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.4.8. Indirect Immunofluorescence-Test

The secondary or indirect immunofluorescence test uses two different antibodies. The first or primary antibodies against *B. henselae* or *B. quintana* recognise the target molecules which are *B. henselae*- and *B. quintana*- infected cells attached to the slides and bind to them. The second or secondary antibodies are fluorescein-labelled anti-human antibodies which bind to the primary antibodies and so staining them. The samples to be tested were diluted after the dilution series schema: 1:10, 1:32, 1:100, 1:320 and 1:1.000. For qualitative analysis the antibody titer of 1:320 was used as a reference [219].

4.4.8.1. Step by step procedure

For a standardized immunological analysis the EUROIMMUN Titerplane technique was used. 25µl of the diluted samples (see 4.4.8 Indirect Immunofluorescence-Test) were applied to the reaction fields of a reagent tray. The biochip-slides were then placed into the recesses of the reagent tray, where all BIOCHIPs of the slide come into contact with the fluids, and the individual reactions commence simultaneously. On every chip one negative and the positive controls for *B. henselae*- and *B. quintana* had to be added. The samples were incubated for 30

min. at RT. After the incubation follows the washing step where the biochip-slides were rinsed with a flush of PBS-Tween and afterwards were immersed immediately for at least 5 min. in a cuvette containing PBS-Tween. In the next step 20µl of fluorescein-labelled anti–human immunoglobulin was pipetted onto each reaction field of a cleaned reagent tray. Then one biochip-slide after another was removed from the PBS-Tween, blotted with a paper towel on the back and the long edges and immediately put into the recesses of the reagent tray. During the following incubation period of 30 min. at RT the slides had to be protected from direct light exposure. After the incubation follows the washing step where the biochip-slides were rinsed with a flush of PBS-Tween and afterwards were immersed immediately for at least 5 min. in a cuvette containing PBS-Tween. Also after the second incubation followed a washing step where the biochip-slides were rinsed with a flush of PBS-Tween and afterwards were immersed immediately for at least 5 min. in a cuvette containing PBS-Tween. After this step for every 150µl PBS-Tween 10 drops of Evans Blue were added for counterstaining. In the embedding step 10µl drops of glycerol per each reaction field were placed onto a cover glass. Then one biochip-slide after another was removed from the PBS-Tween, dried with a paper towel on the back and the edges as well as the surface around and put onto the prepared cover glasses. In the evaluation step the slides were analysed by using fluorescence microscopy.

5. Results

5.1.1. Tick DNA extraction

From the Austrian tick collection of 10.433 ticks (see Tab. 4), 24 ticks of every location have been picked for extraction. Each time 8 larvae, 8 nymphs and 8 adults whereas the adults consisted of 4 male and 4 female ticks (Tab. 15). The extraction resulted in eluates with 40µl AE buffer in each of them. They were aliquoted to two Eppis with 20µl and stored at -20°C.

Processed Ticks						
Processing Date	**Province**	**Location**	**Species**	**Larvae**	**Nymphs**	**Adults**
10.05.2008	Niederösterreich	Irnfritz	*I. ricinus*	8		
10.05.2008	Niederösterreich	St. Pölten	*I. ricinus*		8	8
10.05.2008	Salzburg	Goldegg	*I. ricinus*		8	
10.05.2008	Salzburg	Adnet bei Hallein	*I. ricinus*	8		8
11.05.2008	Kärnten	Faaker See/ Drobollach	*I. ricinus*	8	8	
11.05.2008	Kärnten	Egelsee bei Molzbichl	*I. ricinus*			8
11.05.2008	Oberösterreich	Niederottensheim bein Linz	*I. ricinus*	8	8	8
03.07.2008	Tirol	Imst	*I. ricinus*		8	8
03.07.2008	Tirol	Mils	*I. ricinus*	8		
08.07.2008	Vorarlberg	Klaus	*I. ricinus*		8	8
08.07.2008	Vorarlberg	Thüringen	*I. ricinus*	8		
22.07.2008	Burgenland	Breitenbrunn	*I. ricinus*		8	5
22.07.2008	Burgenland	Breitenbrunn II	*I. ricinus*			3
22.07.2008	Burgenland	Oberwart	*I. ricinus*	8		
22.07.2008	Steiermark	Admont	*I. ricinus*	8		
22.07.2008	Steiermark	Mürzzuschlag	*I. ricinus*		8	8
22.07.2008	Wien	Prater/ Lusthaus (Index 5)	*I. ricinus*	8	8	8
			Total Number	72	72	72
				216		

Tab. 15 Processed Ticks of Austria

5.1.2. Cultivation and Isolation of genomic DNA from *Bartonella*

5.1.2.1. *Bartonella* cultivation

As already mentioned four *Bartonella* species were used for cultivation and subsequently as positive controls for the PCR-assays (see Tab 5). The results of the cultivation showed that under equal conditions *B. clarridgeiae* needed a much longer start-up phase in comparison to the other species. Even after 7 days no colony could have been detected visually against what *B. grahamii*, *B. henselae*, and *B. doshiae* were close to their stationary phase. *B.* clarridgeiae showed the first visible colonies after 9 to 10 days (Fig. 17).

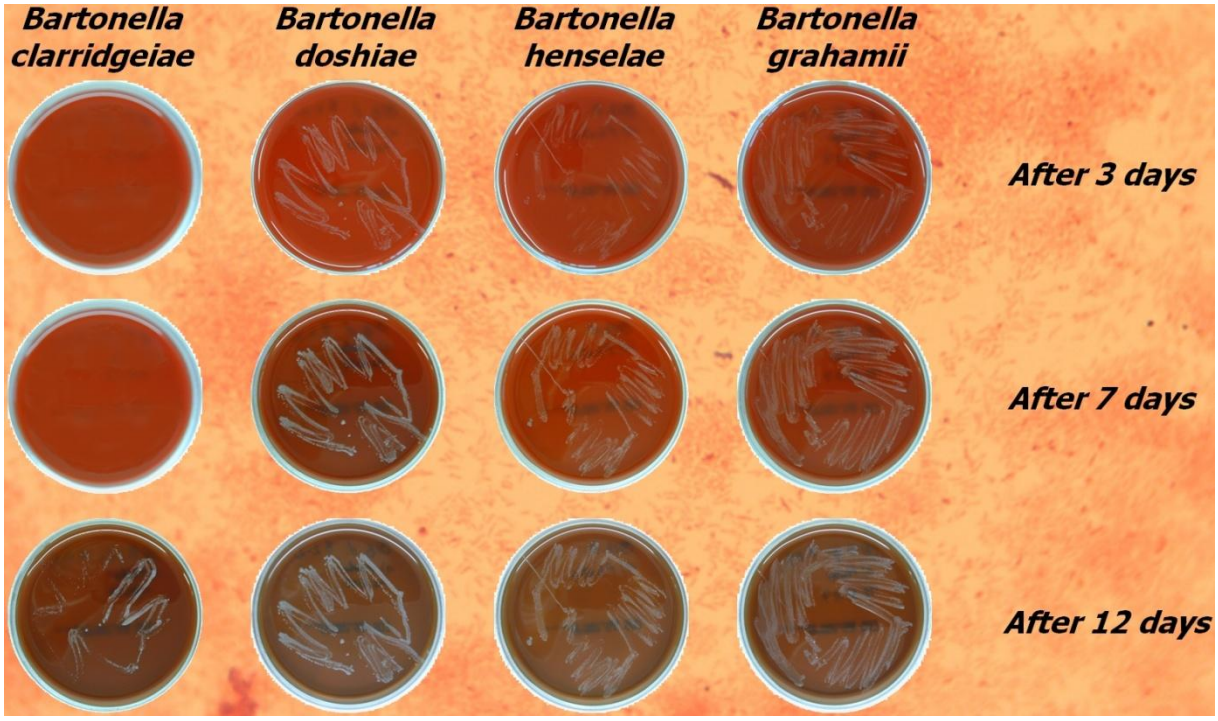


Fig. 17 *Bartonella* Cultivation on Columbia Blood-Agar Plates

5.1.2.2. Isolation of genomic DNA from *Bartonella*

Of this cultivated culture plates single colonies have been picked and new subculture plates were produced in duplicates. Of one of these subcultures the whole plate is harvested and deep frozen at -80°C with Roti–Store cryo-vials. The second subculture is used to pick a

single colony to isolate the genomic DNA with the DNeasy Mini Kit of Qiagen (Tab. 16). It resulted in eluates with 200µl AE buffer aliquoted to 4 Eppis and stored at -20°C.

Bacterial cultures of *Bartonella* on Sheep-blood Agar Plates						
Reference Number	Species	Subculture	Strain	Creation Date	Deep freezing (-80°C)	
					Number	Date
A 1	*Bartonella clarridgeiae*	original	ATCC 51734	24.08.2007		---
A 1-1	*Bartonella clarridgeiae*	1-1	ATCC 51734	06.09.2007	1	01.10.2007
A 1-2	*Bartonella clarridgeiae*	1-2	ATCC 51734	06.09.2007		---
A 2-1	*Bartonella clarridgeiae*	2-1	ATCC 51734	19.09.2007	2	01.10.2007
A 2-2	*Bartonella clarridgeiae*	2-2	ATCC 51734	19.09.2007		---
B 1	*Bartonella doshiae*	original	ATCC 700133	24.08.2007		---
B 1-1	*Bartonella doshiae*	1-1	ATCC 700133	29.08.2007		---
B 1-2	*Bartonella doshiae*	1-2	ATCC 700133	06.09.2007		---
B 2-1	*Bartonella doshiae*	2-1	ATCC 700133	06.09.2007		---
B 2-2	*Bartonella doshiae*	2-2	ATCC 700133	19.09.2007	3	01.10.2007
B 3-1	*Bartonella doshiae*	3-1	ATCC 700133	19.09.2007	4	01.10.2007
D 1	*Bartonella henselae*	original	ATCC 49882	24.08.2007		---
D 1-1	*Bartonella henselae*	1-1	ATCC 49882	29.08.2007		---
D 1-2	*Bartonella henselae*	1-2	ATCC 49882	29.08.2007		---
D 2-1	*Bartonella henselae*	2-1	ATCC 49882	06.09.2007		---
D 2-2	*Bartonella henselae*	2-2	ATCC 49882	06.09.2007		---
D 3-1	*Bartonella henselae*	3-1	ATCC 49882	19.09.2007	5	01.10.2007
D 3-2	*Bartonella henselae*	3-2	ATCC 49882	19.09.2007	6	01.10.2007
E 1	*Bartonella grahamii*	original	ATCC 700132	24.08.2007		---
E 1-1	*Bartonella grahamii*	1-1	ATCC 700132	29.08.2007		---
E 1-2	*Bartonella grahamii*	1-2	ATCC 700132	29.08.2007		---
E 2-1	*Bartonella grahamii*	2-1	ATCC 700132	06.09.2007		---
E 2-2	*Bartonella grahamii*	2-2	ATCC 700132	06.09.2007		---
E 3-1	*Bartonella grahamii*	3-1	ATCC 700132	19.09.2007	7	01.10.2007
E 3-2	*Bartonella grahamii*	3-2	ATCC 700132	19.09.2007	8	01.10.2007

Tab. 16 Bacterial cultures of *Bartonella* on Sheep-blood Agar Plates

5.1.2.3. *Bartonella* positive Controls

The *Bartonella* positive controls were used for the PCR-assays. So it was essential to measure the concentration of the stock solutions and create dilution series (Tab. 17). The focus for the dilution series was to find dilution levels were the Bartonella positive controls with different stock solutions have the same concentration and were close to the detection limit of the PCR-assay (Tab. 18). Through this dilution series the sensitivity of the PCR-assays could have been tested. The DNA concentration measurement was performed with a NanoDrop Spectrophotometer. Some data of the measurement had to be observed to be sure that the positive controls are suitable for the PCR's. The ratio of sample absorbance at 260/280nm is used to assess the purity of DNA and RNA. A ratio of about 2 is generally accepted as pure. The ratio of 260/230 is a further measure of nucleic acid purity with an optimal range between 1.8 and 2.2. At A260 is the absorbance of nucleic acids and at A280 the one of mainly proteins (Tab. 17). So the measurement curves should show a peak at 260nm and a lower value at 280nm (Fig. 18-21).

Further information about the positive control dilution series and the PCR sensitivities are provided at chapter 5.1.4 PCRs.

Report | Test type: Nucleic Acid

Report Name A 311007 | Report Full Mode: Ignore

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230
discarded	Default	31.10.2007	13:05	47,49	0,950	0,498	1,91	1,76
discarded	Default	31.10.2007	13:08	368,40	7,368	3,855	1,91	2,68
discarded	Default	31.10.2007	13:10	235,30	4,706	2,391	1,97	2,30
B. clarridgeiae stock	Default	31.10.2007	13:12	40,36	0,807	0,378	2,14	1,75
B. doshiae stock	Default	31.10.2007	13:13	233,50	4,670	2,405	1,94	2,15
B. henselae stock	Default	31.10.2007	13:14	48,76	0,975	0,508	1,92	1,77
B. grahamii stock	Default	31.10.2007	13:16	27,27	0,545	0,241	2,26	2,98

Tab. 17 Concentrations of the *Bartonella* positive controls measured with a NanoDrop Spectrophotometer

Bartonella stock concentrations and dilution series		
Bartonella Species	Dilution [1:x]	Concentration [pg/µl]
1 - Bartonella clarridgeiae 31.10.07	-	4,04E+04
	50	8,07E+02
	500	80,72
	2.000	20,18
	5.000	8,07
	12.000	3,36
	24.000	1,68
	120.000	0,34
3 - Bartonella doshiae 31.10.07	-	2,34E+05
	50	4,67E+03
	500	467,00
	2.000	116,75
	29.000	8,07
	70.100	3,36
	140.100	1,68
	700.500	0,34
5 - Bartonella henselae 31.10.07	-	4,88E+04
	50	9,75E+02
	500	97,52
	2.000	24,38
	6.000	8,07
	14.500	3,36
	29.000	1,68
	145.000	0,34
7 - Bartonella grahamii 31.10.07	-	2,73E+04
	50	5,45E+02
	500	54,54
	2.000	13,64
	3.400	8,07
	8.500	3,36
	16.500	1,68
	82500	0,34

Tab. 18 Concentrations of the *Bartonella* positive controls with the dilution series

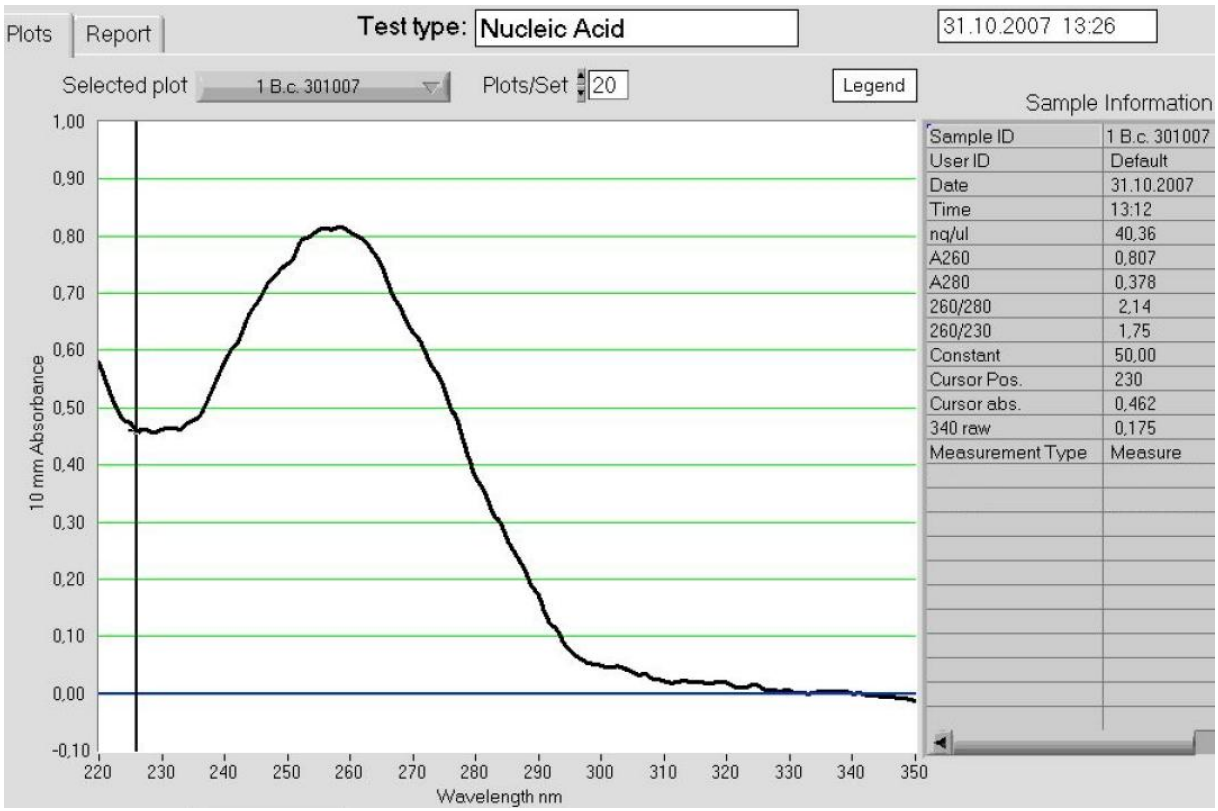


Fig. 18 Concentration measurement curve of the *B. clarridgeiae* positive stock solution

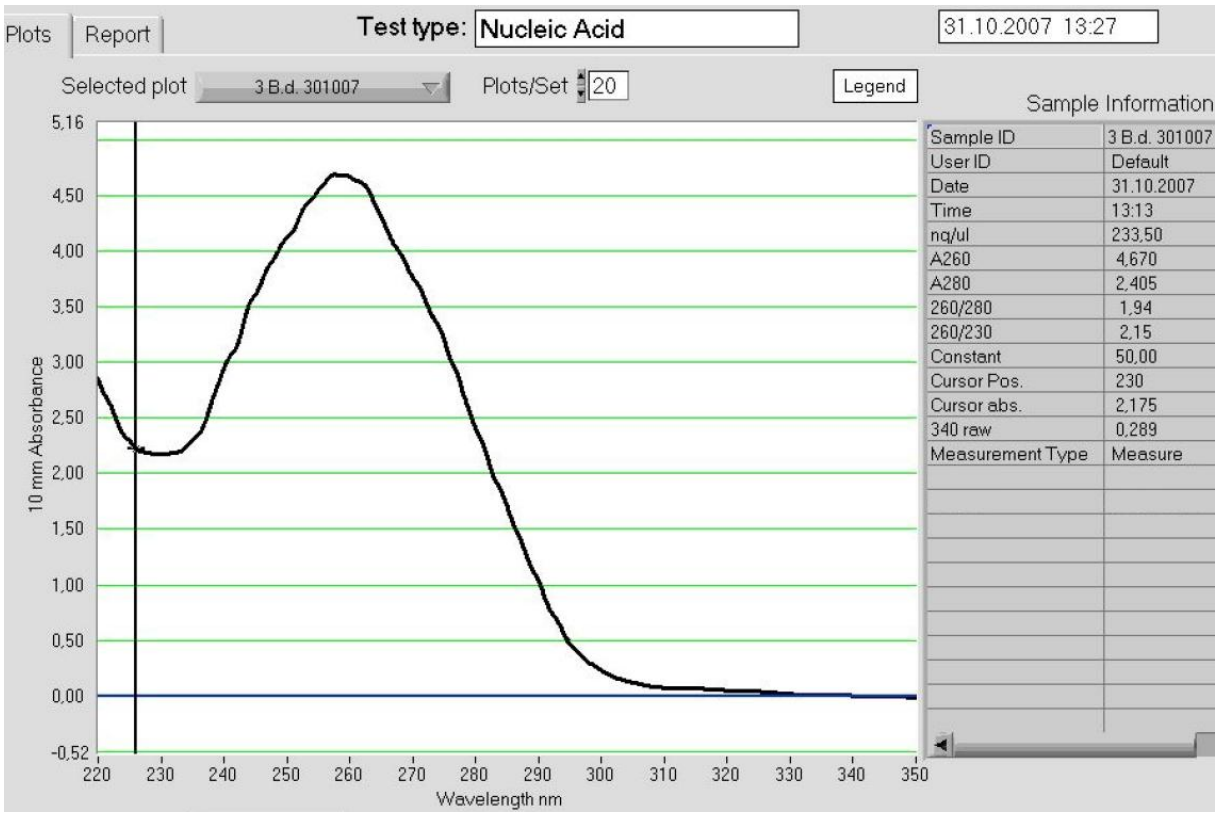


Fig. 19 Concentration measurement curve of the *B. doshiae* positive stock solution

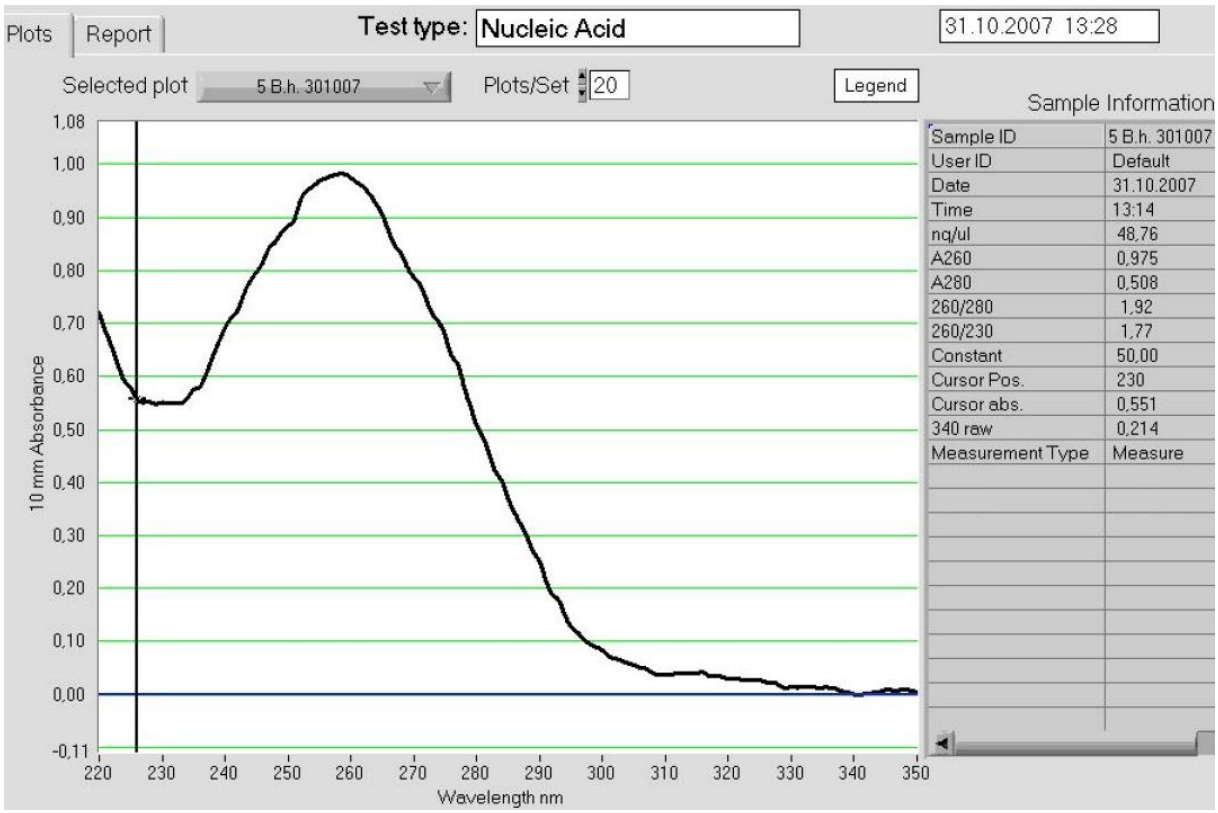


Fig. 20 Concentration measurement curve of the *B. henselae* positive stock solution

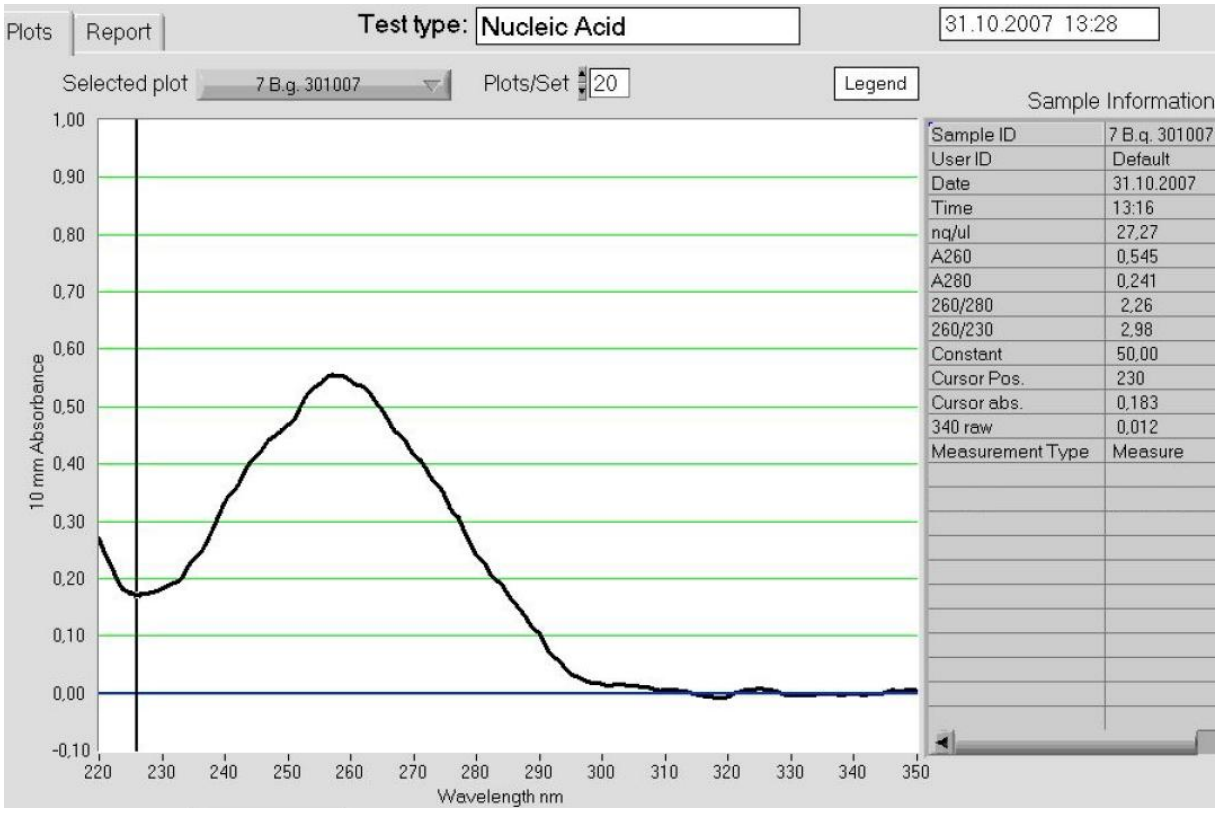


Fig. 21 Concentration measurement curve of the *B. quintana* positive stock solution

5.1.3. Primer and Probes

For the PCR-assay seven different primer sets have been found which fit the requirements and the rules (see chapter 4.4.3 Primer and Probes) for proper primer design (Tab. 19 and 20). In table 19 details of the location on the target sequence have been given. Therefore the sequences of the target gene of all *Bartonella* species have been aligned. Depending on these aligned sequences the locations of the primers were determined (Fig. 22-24). The same procedure was implemented for the Real-Time PCR probe. The second table (Tab. 20) shows the primer sets with their target sequences, the primer Tm values and the resulting mean Ta values and the measured optimal Ta values (see chapter 5.1.4 PCRs). This optimal Ta values have been determined through several PCR runs with a temperature gradient.

Primer	Nucleotide Sequence	Location on the Target Sequence and Length	Amplicon Length
p24 E [196]	5´- CCTCCTTCAGTTAGGCTGG -3´	980bp - 998bp = 19bp	437-438bp
p24 E2	5´- TCCTTGCGGTTAGCACAGCA -3´	1398bp - 1417bp = 20bp	
BTNi F [216]	5´- TTAGAGTGAGCGGCAAAC -3´	79bp - 96bp = 18bp	464-476bp
BTNi R	5´- TAAATATCCGCCTACATGCGC -3´	534bp - 554bp = 21bp	
Bart 2	5´- GTGAGGTCGGAGGTTCAAGT -3´	538bp - 557bp = 20bp	272bp
Bart 1R	5´- GAGCAGTAAACCGCCCGA -3´	792bp - 809bp = 18bp	
321 s [217]	5´- AGATGATGATCCCAAGCCTTCTGG -3´	384bp - 410bp = 24bp	194-307bp
321 as	5´- AATTGGTGGGCCTGGGAGGACT -3´	688bp - 709bp = 22bp	
Barton 1 [158]	5´- TAACCGATATTGGTTGTGTTGAAG -3´	324bp - 348bp = 24bp	587-590bp
Barton 2 [158]	5´- TAAAGCTAGAAAGTCTGGCAACATAACG -3´	885bp - 913bp = 28bp	
N-p24 E	5´- ACAGGTGCTGCATGGCTG -3´	1006bp - 1023bp = 18bp	390-391bp
N-p24 E2	5´- CTTCGGGTAAAACCAACTCC -3´	1377bp - 1396bp = 20bp	
p24 E [196]	5´- CCTCCTTCAGTTAGGCTGG -3´	980bp - 998bp = 19bp	123-124bp
p24 E2-RT	5´- CAACTHAATGCTGGCAACTAAGG -3´	1081bp - 1103bp = 23bp	
Probe	**Nucleotide Sequence**	**Location on the Target Sequence and Length**	
Probe p24	5´- ACAGGTGCTGCATGGCTGTCGTCAG -3´	1006bp - 1030bp = 25bp	

Tab. 19 PCR Primers Table I

Target Sequence	Primer	Tm	Annealing Temp.
1 - 16S rRNA Gen	p24 E [196]	58,8°C	mean value - 54,1°C
	p24 E2	59,4°C	optimal value - 56,4°C
2 - 16S rRNA Gen	BTNi F [216]	53,7°C	mean value - 50,8°C
	BTNi R	57,9°C	optimal value - 53,1°C
3 - 16S rRNA Gen	Bart 2	59,4°C	mean value - 53,8°C
	Bart 1R	58,2°C	----------
4 - 16-23S Intergenic Spacer Region	321 s [217]	62,7°C	mean value - 58,4°C
	321 as	64,0°C	optimal value - 56,0°C
5 - Riboflavin Synthase Gen	Barton 1[158]	57,6°C	mean value - 54,9°C
	Barton 2 [158]	62,2°C	optimal value - 54,3°C
6 - 16S rRNA Gen	N-p24 E	58,2°C	mean value - 52,8°C
	N-p24 E2	57,3°C	optimal value - 55,0°C
7 - 16S rRNA Gen	p24 E [196]	58,8°C	mean value - 54,2°C
	p24 E2-RT	59,5°C	optimal value - 56,4°C

Target Sequence	Probe	Tm
7 - 16S rRNA Gen	Probe p24	67,9°C

Tab. 20 PCR Primers Table II

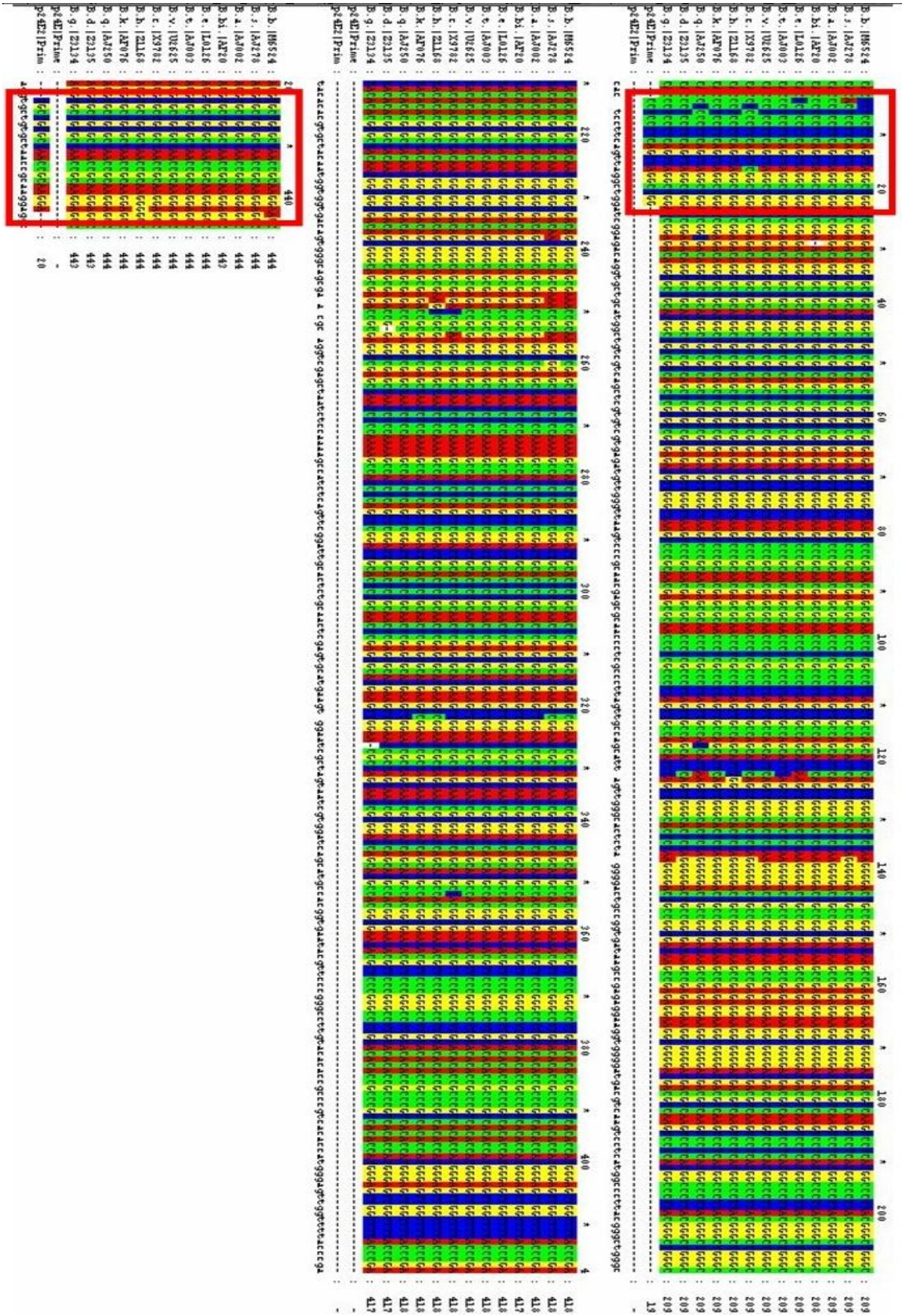


Fig. 22 Section of the 16S rRNA gene with the p24 E and E2 primer binding sites marked with a red rectangle

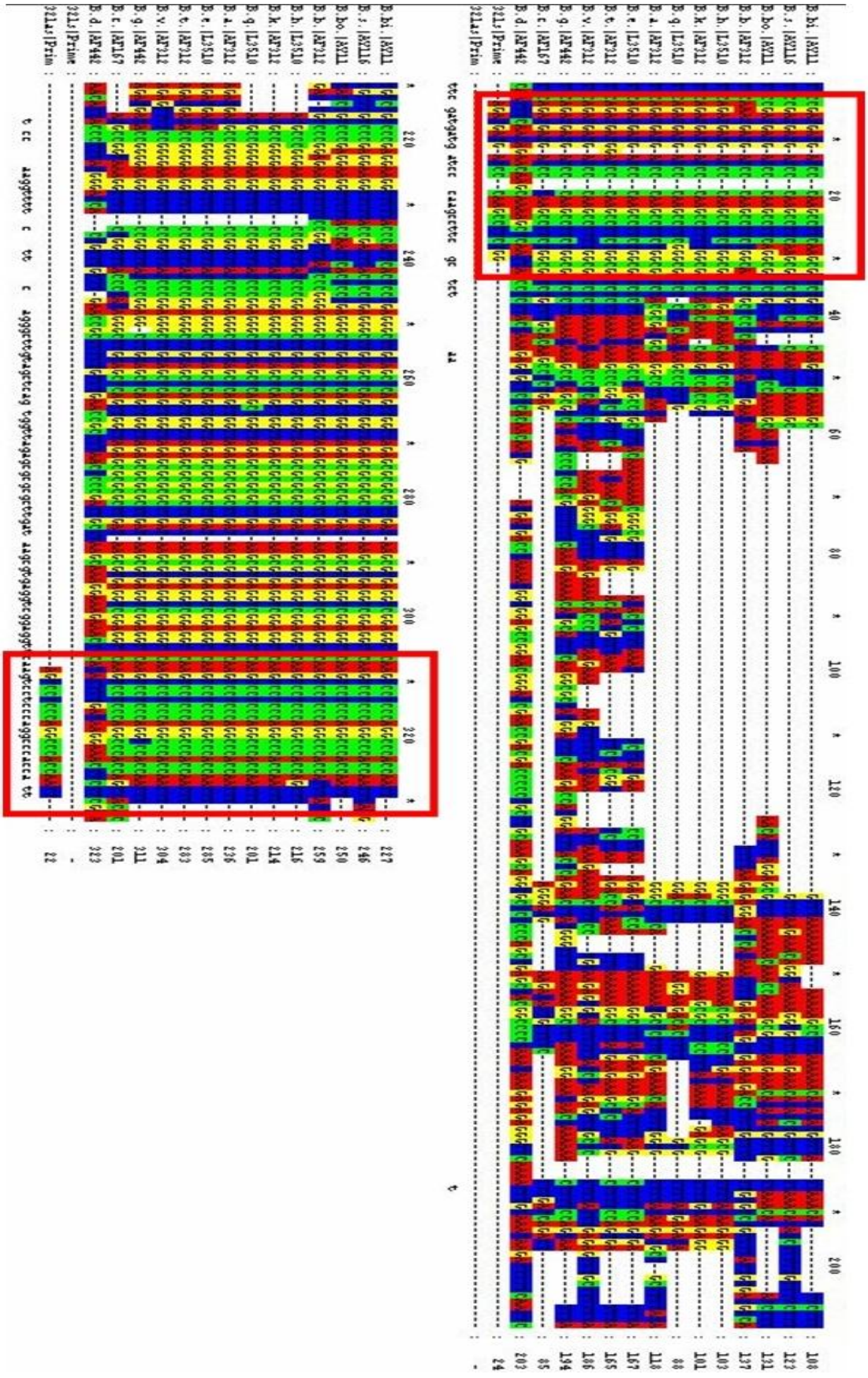


Fig. 23 Section of the 16-23S ITS region with the 321s and as primer binding sites marked with a red rectangle

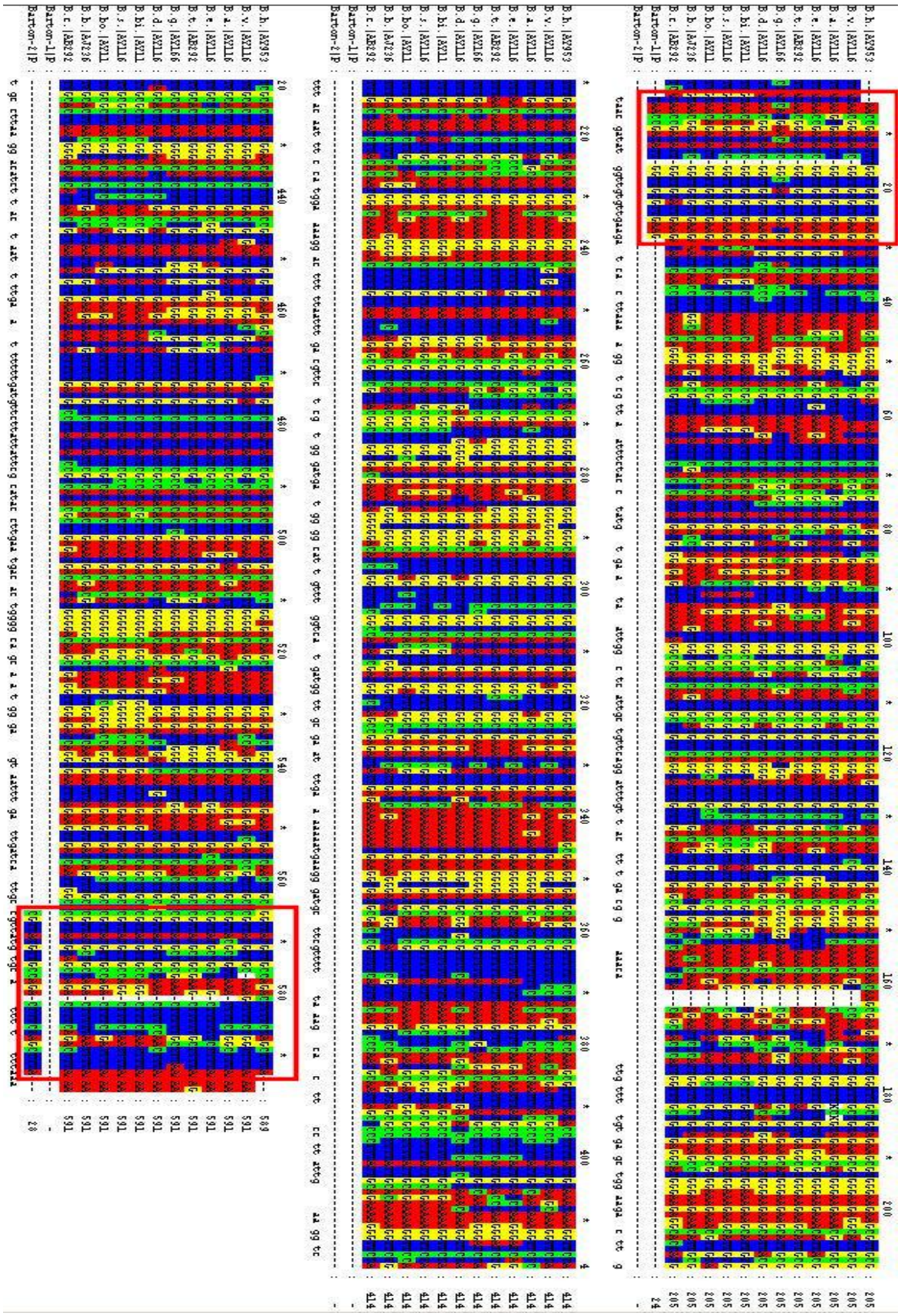


Fig. 24 Section of the ribC gene with the Barton 1 and 2 primer binding sites marked with a red rectangle

5.1.4. PCRs

5.1.4.1. PCR, Semi-nested PCR and Nested PCR

First step was to calibrate the PCRs. Therefor the primer sets were tested with a constant concentration of the positive control (Fig. 25). The gel shows the different amplicons of the primer sets. The differences in the intensity of the bands refer to the different dilution levels of the primers (Tab. 21). Through the marker the bands in lane 3 and 5 could have been estimated at 590bp, for lane 8, 9 and 11 at 220bp and the lanes 12, 13 and 14 at 430bp. So the results correspond with the expected sizes (see Tab. 19 PCR Primers Table I). Furthermore the results showed that the primers concentration has a lower limit at the dilution of 1:1000 (Lanes 7, 11, 14). This dilution corresponds roughly to 10nM of the primer final concentration in the master-mix. The lanes 16 to 18 were used to test the primer set Bart 1R and 2. Even after several further PCR runs with this primer set no bands could have been produced. Therefor this primer set was discarded.

Fig. 25 PCR gel with a constant positive control and different concentrations of the primers

Lanes	Mixture
1	Step Ladder
2	negative control with dH_2O
3	Primer set Barton 1 and 2 with a dilution of 1:10
4	blank
5	Primer set Barton 1 and 2 with a dilution of 1:100
6	blank
7	Primer set Barton 1 and 2 with a dilution of 1:1000
8	Primer set 321 s and as with a dilution of 1:10
9	Primer set 321 s and as with a dilution of 1:100
10	blank
11	Primer set 321 s and as with a dilution of 1:1000
12	Primer set p24 E and E2 with a dilution of 1:10
13	Primer set p24 E and E2 with a dilution of 1:100
14	Primer set p24 E and E2 with a dilution of 1:1000
15	blank
16	Primer set Bart 1R and 2 with a dilution of 1:10
17	Primer set Bart 1R and 2 with a dilution of 1:100
18	Primer set Bart 1R and 2 with a dilution of 1:1000

Tab. 21 PCR gel mixture table

The next step was to detect the proper Ta values. Therefor PCRs with a temperature gradient have been performed (Fig. 26 – 27 and Tab.22 - 23). In both gels one tested primer set was Bart 1R and 2 which, as already mentioned, showed no results. For p24 the optimal Ta value was at 56.4°C in lane 5. Lane 2 with 51.8°C was too far away of the mean Ta value and lane 3 and 4 showed unspecific amplification at about 900bp. In the case of Barton the optimal Ta value was at lane 12 with 54.3°C. The best Ta value for BTNi was at lane 5 with 53.1°C and for 321 at lane 14 with 56.0°C.

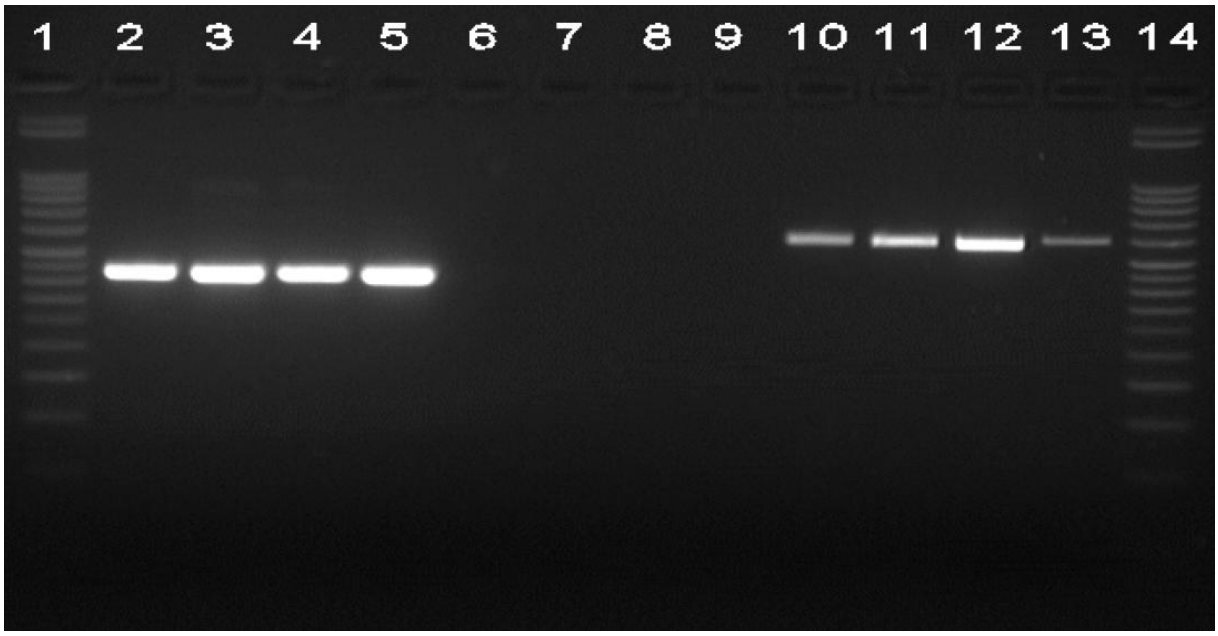


Fig. 26 PCR gel with a constant positive control and different Ta values I

Lanes	Mixture	Ta's [°C]
1	Step Ladder	-
2	p24 E and E2	51,9
3	p24 E and E2	53,8
4	p24 E and E2	54,3
5	p24 E and E2	56,4
6	Bart 1R and 2	51,2
7	Bart 1R and 2	53,5
8	Bart 1R and 2	54,3
9	Bart 1R and 2	56,4
10	Barton 1 and 2	50,6
11	Barton 1 and 2	52,7
12	Barton 1 and 2	54,3
13	Barton 1 and 2	55,7
14	Step Ladder	-

Tab. 22 PCR gel mixture table referring to Fig. 26

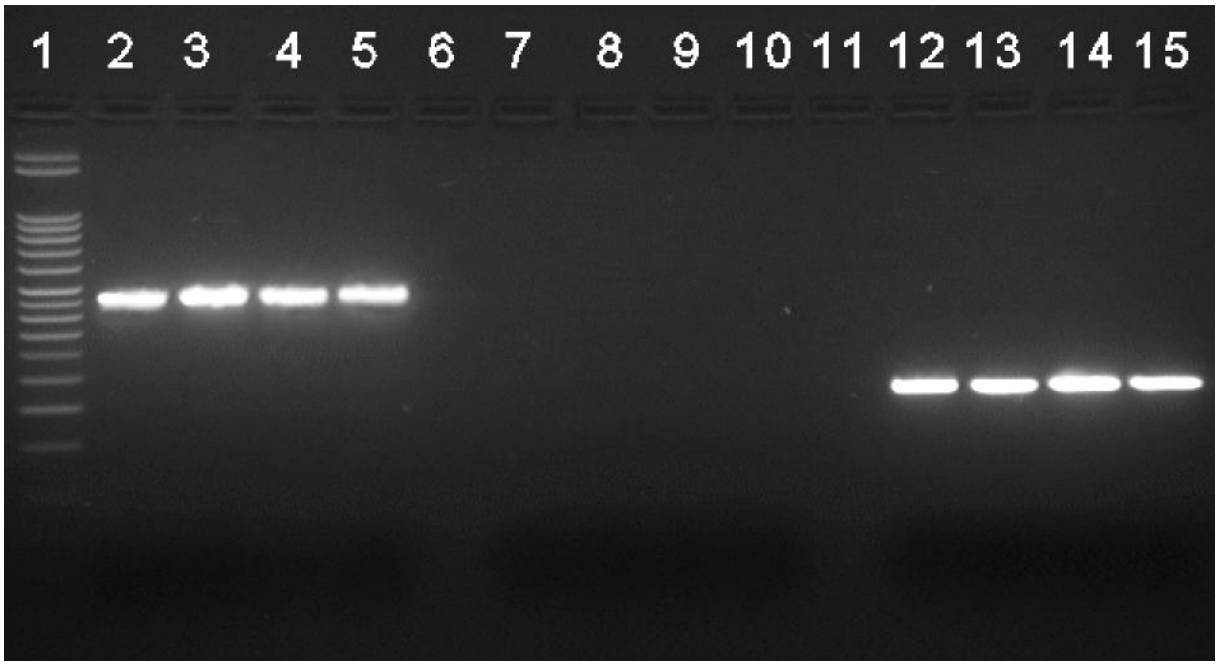


Fig. 27 PCR gel with a constant positive control and different Ta values II

Lanes	Mixture	Ta's [°C]
1	Step Ladder	-
2	BTNi F and R	48,8
3	BTNi F and R	51,0
4	BTNi F and R	52,1
5	BTNi F and R	53,1
6	blank	-
7	Bart 1R and 2	51,0
8	Bart 1R and 2	53,1
9	Bart 1R and 2	54,2
10	Bart 1R and 2	56,6
11	blank	-
12	321 s and as	53,1
13	321 s and as	55,2
14	321 s and as	56,0
15	321 s and as	56,9

Tab. 23 PCR gel mixture table referring to Fig. 27

The following PCRs were performed to test the detection limit. Therefor different concentrations of the positive controls were used (Fig. 28-30 and Tab. 24-26). As can be seen in the following gels the sensitivity limit of the PCR was detected at about 0.34pg/µl genomic DNA, lanes 4, 7, 10 and 13 in figure 30.

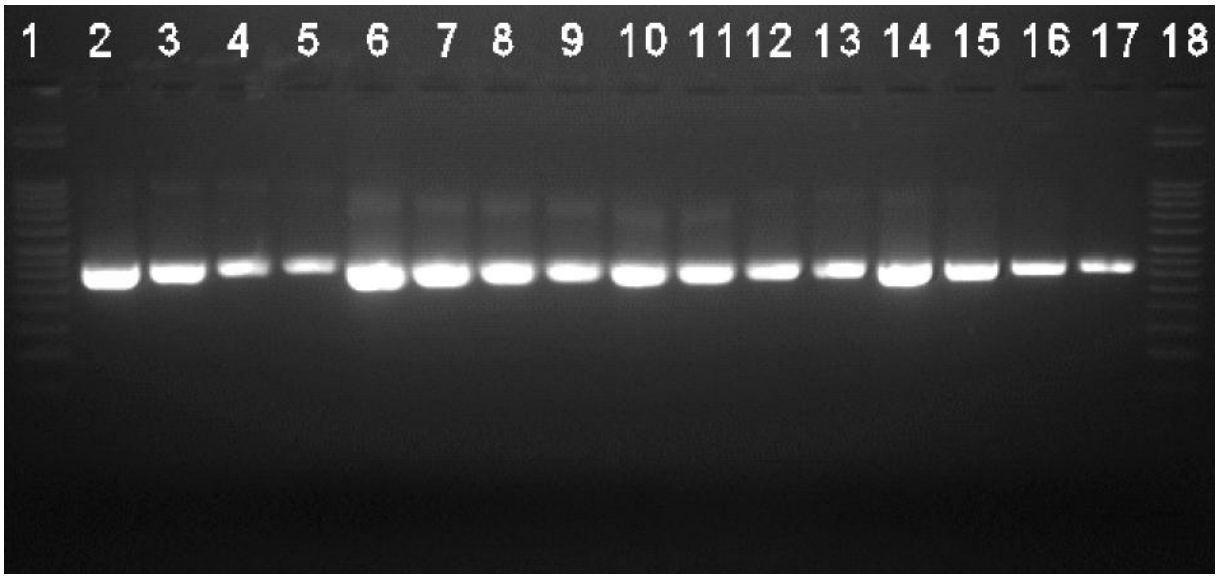


Fig. 28 PCR gel with different concentrations of the positive control I

Lanes	Primer	Positive Controls	
		Species	Concentration [pg/µl]
1	Step Ladder	-	-
2	p24 E&E2	*B. clarridgeiae*	4,04E+04
3	p24 E&E2		8,07E+02
4	p24 E&E2		4,04E+02
5	p24 E&E2		80,72
6	p24 E&E2	*B. doshiae*	2,34E+05
7	p24 E&E2		4,67E+03
8	p24 E&E2		2,34E+03
9	p24 E&E2		467,00
10	p24 E&E2	*B. henselae*	4,88E+04
11	p24 E&E2		9,75E+02
12	p24 E&E2		4,88E+02
13	p24 E&E2		97,52
14	p24 E&E2	*B. grahamii*	2,73E+04
15	p24 E&E2		5,45E+02
16	p24 E&E2		2,73E+02
17	p24 E&E2		54,54
18	Step Ladder	-	-

Tab. 24 PCR gel mixture table referring to Fig. 28

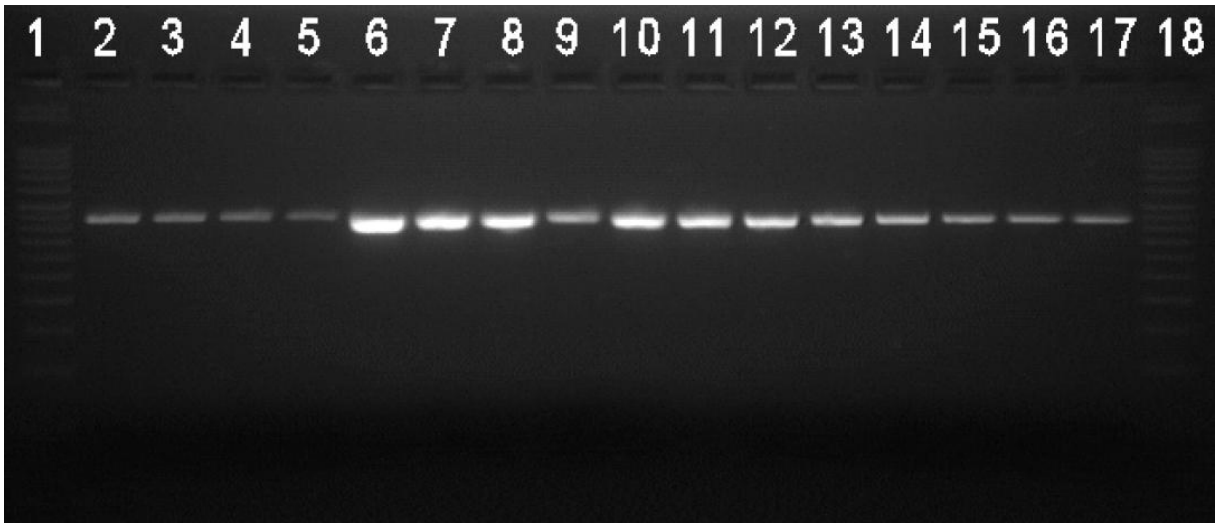


Fig. 29 PCR gel with different concentrations of the positive control II

Lanes	Primer	Positive Controls	
		Species	Concentration [pg/µl]
1	Step Ladder	-	-
2	p24 E&E2	*B. clarridgeiae*	20,18
3	p24 E&E2		13,45
4	p24 E&E2		10,09
5	p24 E&E2		8,07
6	p24 E&E2	*B. doshiae*	116,75
7	p24 E&E2		58,38
8	p24 E&E2		38,92
9	p24 E&E2		8,07
10	p24 E&E2	*B. henselae*	24,38
11	p24 E&E2		16,25
12	p24 E&E2		12,19
13	p24 E&E2		8,07
14	p24 E&E2	*B. grahamii*	13,64
15	p24 E&E2		9,09
16	p24 E&E2		8,52
17	p24 E&E2		8,07
18	Step Ladder	-	-

Tab. 25 PCR gel mixture table referring to Fig. 29

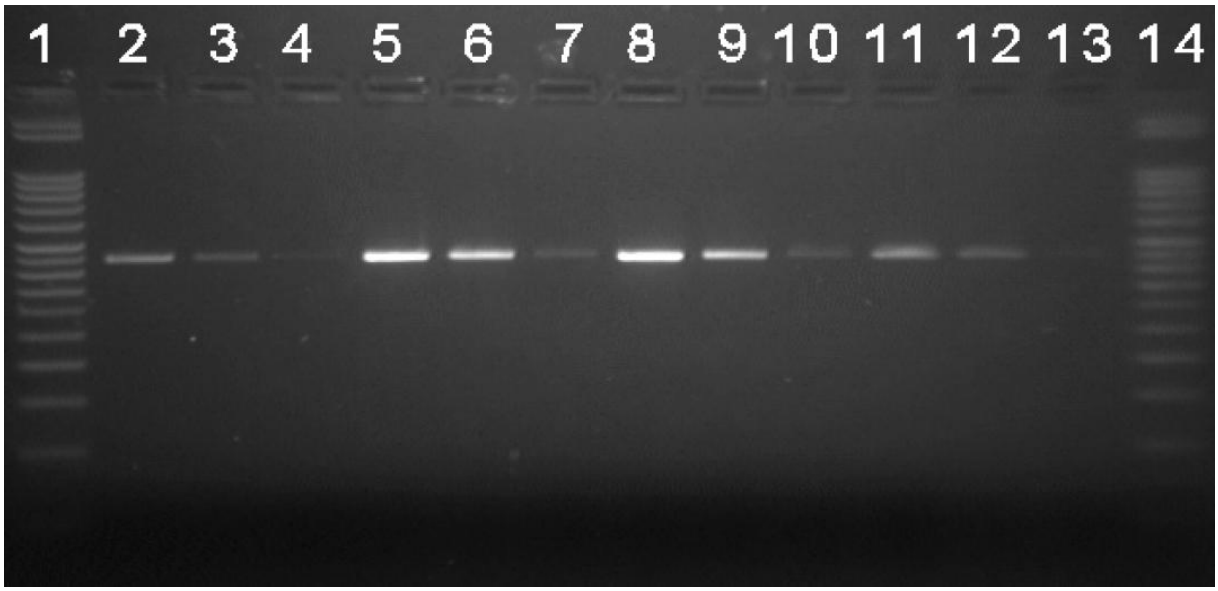


Fig. 30 PCR gel with different concentrations of the positive control III

Lanes	Primer	Positive Controls	
		Species	Concentration [pg/µl]
1	Step Ladder	-	-
2	p24 E&E2	*B. clarridgeiae*	3,36
3	p24 E&E2		1,68
4	p24 E&E2		0,34
5	p24 E&E2	*B. doshiae*	3,36
6	p24 E&E2		1,68
7	p24 E&E2		0,34
8	p24 E&E2	*B. henselae*	3,36
9	p24 E&E2		1,68
10	p24 E&E2		0,34
11	p24 E&E2	*B. grahamii*	3,36
12	p24 E&E2		1,68
13	p24 E&E2		0,34
14	Step Ladder	-	-

Tab. 26 PCR gel mixture table referring to Fig. 30

Finally the different primer sets were tested with dilution series of the different positive controls (Fig. 31 and Tab. 27). The results have been analysed and compared with the values

measured with Clustal X 1.83 (Tab. 28). Furthermore the band intensity has been verified through the results of the dilution series. In the end the best results have been achieved with the primer set p24 E&E2 so it was chosen for the screening of the tick library.

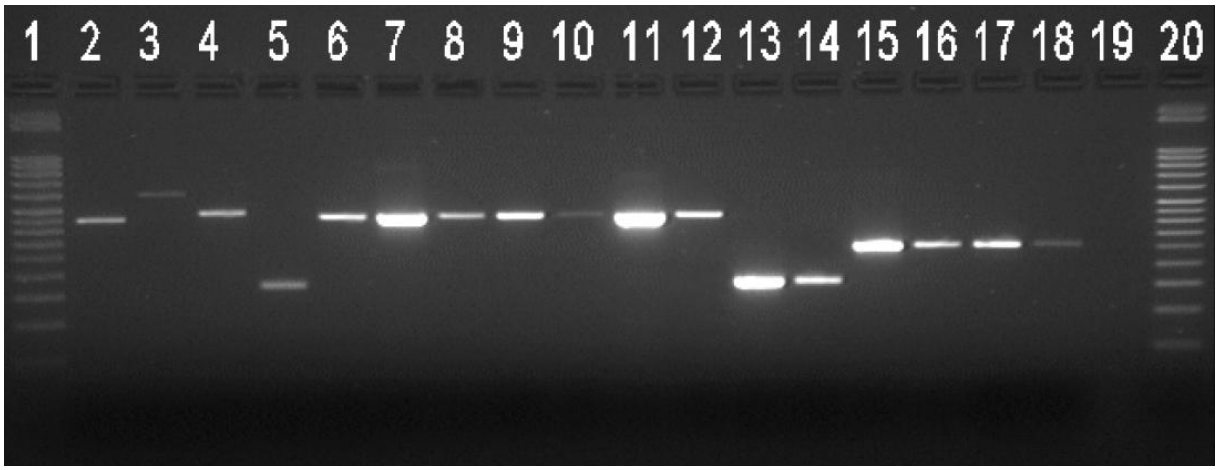


Fig. 31 Final calibration PCR gel with all primer sets and different concentrations of the positive controls

Lanes	Primer	Positive Controls	
		Species	Concentration [pg/µl]
1	Step Ladder	-	-
2	p24 E&E2	*B. henselae*	1,68
3	Barton 1&2	*B. henselae*	1,68
4	BTNi F&R	*B. henselae*	1,68
5	321 s&as	*B. henselae*	1,68
6	p24 E&E2	*B. clarridgeiae*	16,80
7	p24 E&E2	*B. grahamii*	168,80
8	p24 E&E2	*B. grahamii*	8,40
9	p24 E&E2	*B. grahamii*	16,80
10	p24 E&E2	*B. grahamii*	0,34
11	p24 E&E2	*B. doshiae*	168,80
12	p24 E&E2	*B. doshiae*	8,40
13	321 s&as	*B. henselae*	42,20
14	321 s&as	*B. henselae*	3,36
15	321 s&as	*B. grahamii*	168,80
16	321 s&as	*B. grahamii*	8,40
17	321 s&as	*B. grahamii*	16,80
18	321 s&as	*B. grahamii*	0,34
19	blank	-	-
20	Step Ladder	-	-

Tab. 27 PCR gel mixture table referring to Fig. 31

Lanes	Primer	Positive Controls	Amplicon Length [bp]	
			Clustal X 1.83	Measured
1	Step Ladder	-	-	-
2	p24 E&E2	*B. henselae*	438	450
3	Barton 1&2	*B. henselae*	590	600
4	BTNi F&R	*B. henselae*	465	475
5	321 s&as	*B. henselae*	212	250
6	p24 E&E2	*B. clarridgeiae*	438	450
7	p24 E&E2	*B. grahamii*	437	450
8	p24 E&E2	*B. grahamii*	437	450
9	p24 E&E2	*B. grahamii*	437	450
10	p24 E&E2	*B. grahamii*	437	450
11	p24 E&E2	*B. doshiae*	437	450
12	p24 E&E2	*B. doshiae*	437	450
13	321 s&as	*B. henselae*	212	250
14	321 s&as	*B. henselae*	212	250
15	321 s&as	*B. grahamii*	307	325
16	321 s&as	*B. grahamii*	307	325
17	321 s&as	*B. grahamii*	307	325
18	321 s&as	*B. grahamii*	307	325
19	blank	-	-	-
20	Step Ladder	-	-	-

Tab. 28 Final PCR gel results compared with Clustal X values

5.1.4.1.1. PCR Tick Screening

The tick screening was performed with a Nested PCR due to fact that with the Standard PCR and the Semi-Nested PCR no positive ticks could have been detected. This result didn't correlate with other studies. So it had to be assumed that the PCR was not sensitive enough to detect *Bartonella* DNA in ticks. Especially when these ticks have been frozen for several years and the DNA degraded over time. The alternative, to pool ticks was not used because of two main advantages of the Nested PCR. First through a Nested PCR higher product specificity as well as a minimization of unspecific side-products can be achieved. Especially

the minimization of the side-products is an advantage for the following sequencing procedure. The second reason is that every tick can be tested separately and so it's much easier to differentiate between male and female adult ticks.

In the first gel the ticks of the region Vienna have been screened (Fig. 32 and Tab. 29). In lane 1 and 20 the step ladder was loaded as a length marker with a 400bp band marked with the red rectangle. The expected bands at 390-391bp show the amplicon of the nested primer set N-p24 E and E2. So the tick samples in the lanes 4, 5, 6, 8, 11, 12, 13, 16, 17 and 19 could have been verified as *Bartonella* positives.

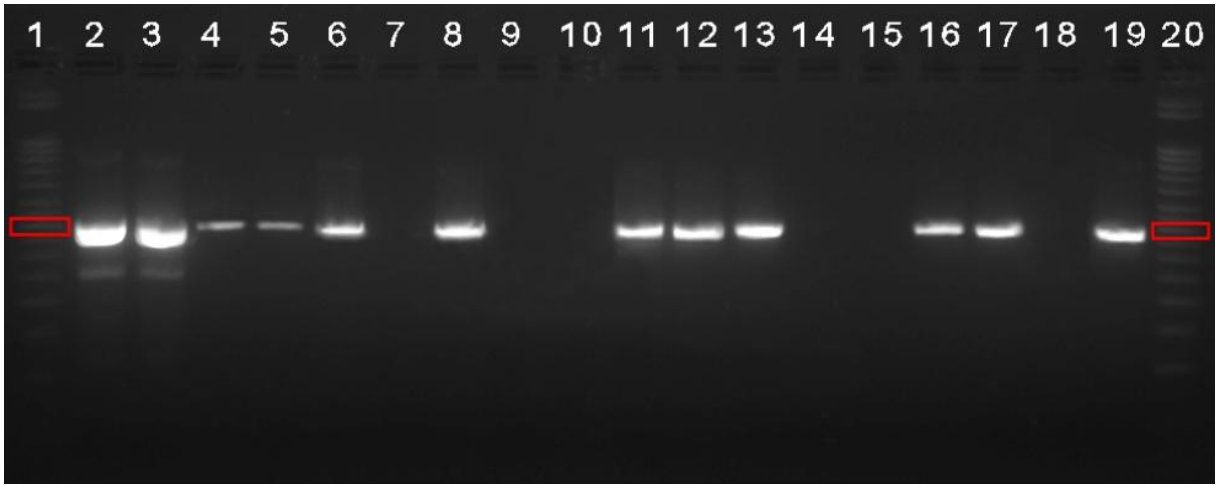


Fig. 32 Nested PCR tick screening of the region Vienna – Prater / Lusthaus (Index 5)

Lanes	Mixture	Concentration [pg/µl]	Sample Type
1	Step Ladder	-	-
2	*B. henselae* spiked pos. Control	48,76	Standard
3	*B. henselae* spiked pos. Control	24,38	Standard
4	Wien - Prater/ Lusthaus (Index 5) L1	-	Tick DNA Sample
5	Wien - Prater/ Lusthaus (Index 5) L2	-	Tick DNA Sample
6	Wien - Prater/ Lusthaus (Index 5) N1	-	Tick DNA Sample
7	Wien - Prater/ Lusthaus (Index 5) L3	-	Tick DNA Sample
8	Wien - Prater/ Lusthaus (Index 5) N2	-	Tick DNA Sample
9	Wien - Prater/ Lusthaus (Index 5) N3	-	Tick DNA Sample
10	Wien - Prater/ Lusthaus (Index 5) N4	-	Tick DNA Sample
11	Wien - Prater/ Lusthaus (Index 5) Am1	-	Tick DNA Sample
12	Wien - Prater/ Lusthaus (Index 5) Am2	-	Tick DNA Sample
13	Wien - Prater/ Lusthaus (Index 5) Am3	-	Tick DNA Sample
14	Wien - Prater/ Lusthaus (Index 5) Am4	-	Tick DNA Sample
15	MM (Master-mixture) + dH_2O	-	Negative Control
16	Wien - Prater/ Lusthaus (Index 5) Aw1	-	Tick DNA Sample
17	Wien - Prater/ Lusthaus (Index 5) Aw2	-	Tick DNA Sample
18	Wien - Prater/ Lusthaus (Index 5) Aw3	-	Tick DNA Sample
19	Wien - Prater/ Lusthaus (Index 5) Aw4	-	Tick DNA Sample
20	Step Ladder	-	-

Tab. 29 PCR gel mixture table referring to Fig. 32

The following two gels show the final results of the tick screening taken together (Fig. 33-34 and Tab. 30-31). All PCRs have been performed with the external primer set p24 E and p24 E2 and the internal primer set N-p24 E and N-p24 E2. The bands, in the case that the tick sample is positive, should be at 390-391bp which represent the amplicon of the internal primer set on the *Bartonella* 16S rRNA gene. For a better visualization the 400bp bands of the step ladder has been marked with a red rectangle.

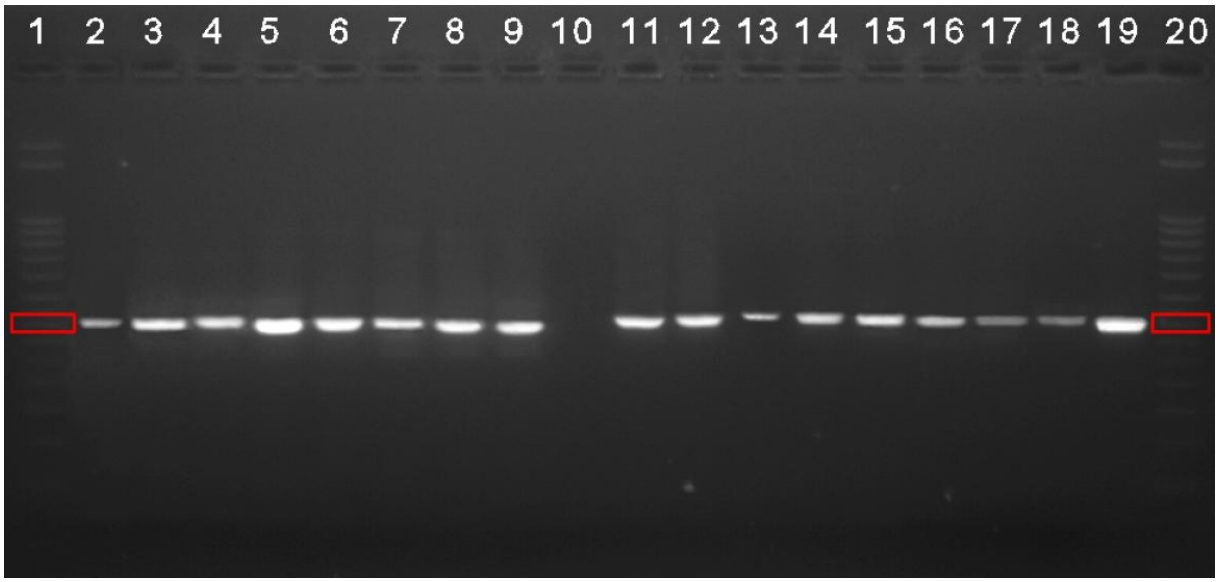


Fig. 33 Nested PCR tick screening final summarized results of all regions part I

Lanes	Mixture	Concentration [pg/µl]	Sample Type
1	Step Ladder	-	-
2	Niederösterreich - St. Pölten Aw6	-	Tick DNA Sample
3	Salzburg- Goldegg N4	-	Tick DNA Sample
4	Salzburg- Goldegg N6	-	Tick DNA Sample
5	Salzburg - Adnet bei Hallein Aw7	-	Tick DNA Sample
6	Salzburg- Goldegg N8	-	Tick DNA Sample
7	Oberösterreich - Niederottensheim bei Linz Aw5	-	Tick DNA Sample
8	Oberösterreich - Niederottensheim bei Linz Aw6	-	Tick DNA Sample
9	Oberösterreich - Niederottensheim bei Linz Am3	-	Tick DNA Sample
10	MM (Master-mixture) + dH_2O	-	Negative Control
11	Steiermark - Mürzzuschlag Am4	-	Tick DNA Sample
12	Steiermark - Mürzzuschlag Aw5	-	Tick DNA Sample
13	Steiermark - Mürzzuschlag Aw6	-	Tick DNA Sample
14	Steiermark - Mürzzuschlag Aw7	-	Tick DNA Sample
15	Steiermark - Mürzzuschlag Aw8	-	Tick DNA Sample
16	Wien - Prater/ Lusthaus (Index 5) L1	-	Tick DNA Sample
17	Wien - Prater/ Lusthaus (Index 5) L2	-	Tick DNA Sample
18	Wien - Prater/ Lusthaus (Index 5) N1	-	Tick DNA Sample
19	*B. henselae* spiked pos. Control	6,72	Standard
20	Step Ladder	-	-

Tab. 30 PCR gel mixture table referring to Fig. 33

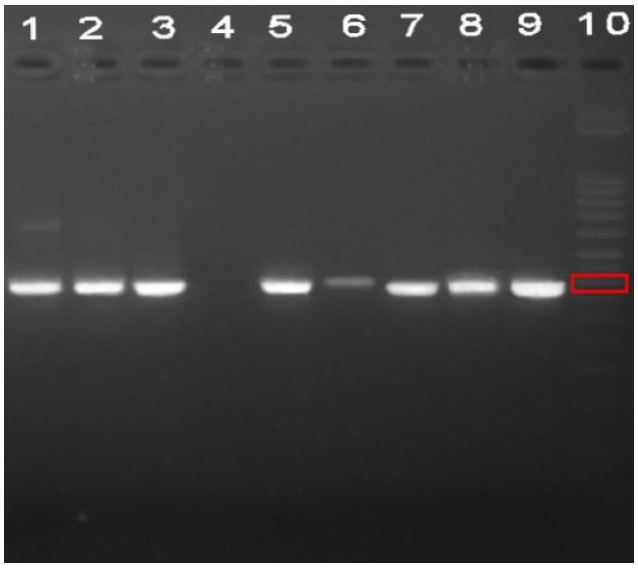


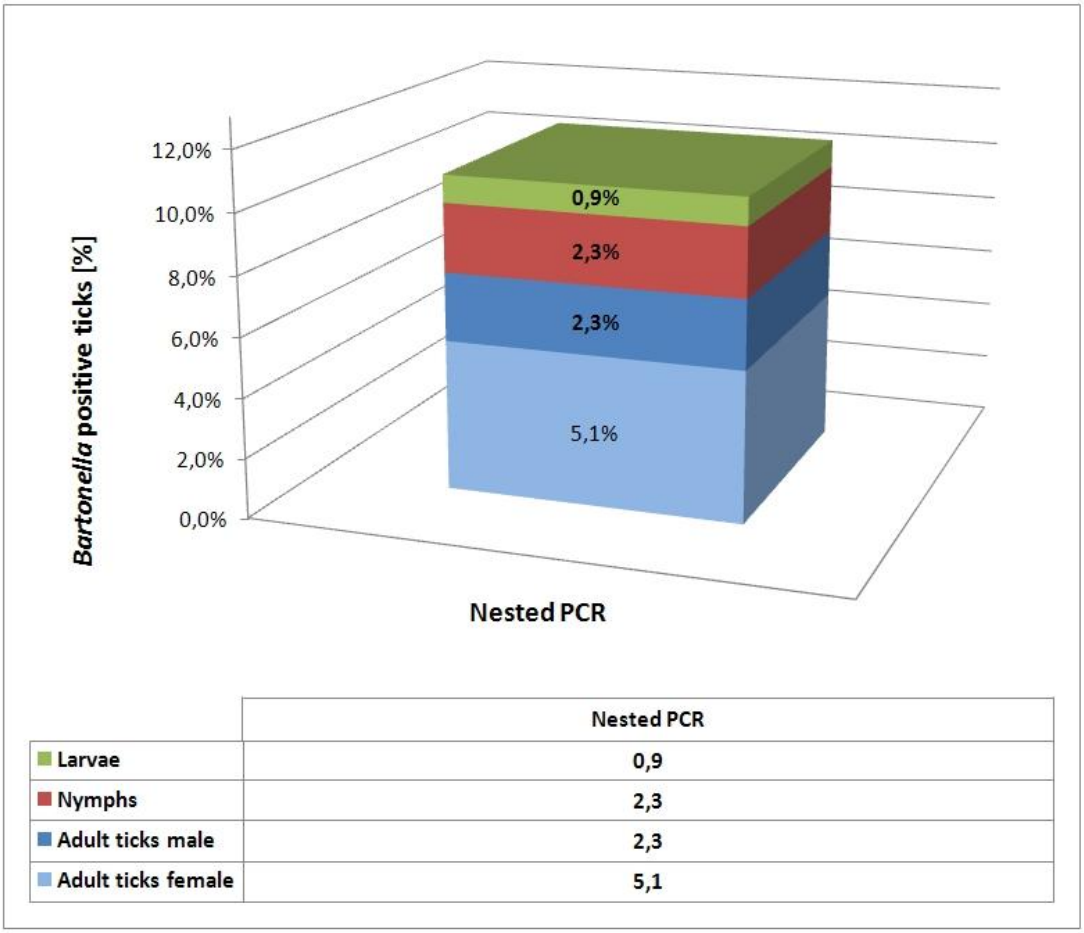
Fig. 34 Nested PCR tick screening final summarized results of all regions part II

Lanes	Mixture	Concentration [pg/µl]	Sample Type
1	Wien - Prater/ Lusthaus (Index 5) Aw6	-	Tick DNA Sample
2	Wien - Prater/ Lusthaus (Index 5) Aw7	-	Tick DNA Sample
3	Wien - Prater/ Lusthaus (Index 5) Aw8	-	Tick DNA Sample
4	MM (Master-mixture) + dH_2O	-	Negative Control
5	Wien - Prater/ Lusthaus (Index 5) Am1	-	Tick DNA Sample
6	Wien - Prater/ Lusthaus (Index 5) N2	-	Tick DNA Sample
7	Wien - Prater/ Lusthaus (Index 5) Am3	-	Tick DNA Sample
8	Wien - Prater/ Lusthaus (Index 5) Am4	-	Tick DNA Sample
9	*B. henselae* spiked pos. Control	6,72	Standard
10	Step Ladder	-	-

Tab. 31 PCR gel mixture table referring to Fig. 34

So in the end 23 ticks out of 216 could have been tested positive for *Bartonella* (Fig. 35). This corresponds to a percentage of infection of 10.6%. Especially the results of Vienna showed a very high rate of infection with 41.7% (Tab. 32). There are several possibilities that could cause such a high rate of infection. Because of the close proximity to the city and a high tick density in the area several transmission methods are benefitted. First the transstadial and transovarial transmission of the pathogens can result in a higher rate of infection. Then there is the possibility, even if it has a rare occurrence, of passive transmission of bacteria through feeding in close proximity to other ticks (cofeeding) [184, 193, 204]. This could be a possible explanation of a rate of infection close to 50%.

	Adult ticks		Nymphs	Larvae	Total amount
	male	female			
***Bartonella* positive ticks**	5	11	5	2	**23**
Tested tick developmental stages in total	72		72	72	**216**
Percentage [%]	2,3	5,1	2,3	0,9	**10,6**



	Nested PCR
Larvae	0,9
Nymphs	2,3
Adult ticks male	2,3
Adult ticks female	5,1

Fig. 35 Nested PCR tick screening results diagrammed

Province of Austria	Region	Positive tick sample and developmental stages	Regional percentage [%]
Niederösterreich	St. Pölten	Adult female 6	**4,2**
Salzburg	Goldegg	Nymphe 4	**16,7**
Salzburg	Goldegg	Nymphe 6	
Salzburg	Goldegg	Nymphe 8	
Salzburg	Adnet bei Hallein	Adult female 7	
Oberösterreich	Niederottensheim bei Linz	Adult male 3	**12,5**
Oberösterreich	Niederottensheim bei Linz	Adult female 5	
Oberösterreich	Niederottensheim bei Linz	Adult female 6	
Steiermark	Mürzzuschlag	Adult male 4	**20,8**
Steiermark	Mürzzuschlag	Adult female 5	
Steiermark	Mürzzuschlag	Adult female 6	
Steiermark	Mürzzuschlag	Adult female 7	
Steiermark	Mürzzuschlag	Adult female 8	
Wien	Prater/ Lusthaus (Index 5)	Larva 1	**41,7**
Wien	Prater/ Lusthaus (Index 5)	Larva 2	
Wien	Prater/ Lusthaus (Index 5)	Nymphe 1	
Wien	Prater/ Lusthaus (Index 5)	Nymphe 2	
Wien	Prater/ Lusthaus (Index 5)	Adult male 1	
Wien	Prater/ Lusthaus (Index 5)	Adult male 3	
Wien	Prater/ Lusthaus (Index 5)	Adult male 4	
Wien	Prater/ Lusthaus (Index 5)	Adult female 6	
Wien	Prater/ Lusthaus (Index 5)	Adult female 7	
Wien	Prater/ Lusthaus (Index 5)	Adult female 8	

Tab. 32 Regional distribution of the *Bartonella* positive ticks

5.1.4.2. Real-Time PCR

The first step was to test the Real-Time PCR with the primer set p24 E and E2 provided for it (Fig. 36-38 and Tab. 33). For the PCR standard curve at least 3 points are needed and every concentration should be run at least in duplicate. This results in a more accurate standard curve. The NCs provide a mechanism to control for external contamination or other factors that can result in a nonspecific increase in the fluorescence signal. Ideally, signal amplification should not be observed in the NC sample wells. If the NTCs do cross the threshold, their Cts should be at least five cycles, and preferably more than ten cycles, from the Cts of your least concentrated samples [220].

Furthermore a Dissociation (Melting) Curve Analysis was performed with SYBR Green. All products generated during the PCR amplification reaction are melted at 95°C, then annealed

at 55°C and subjected to gradual increases in temperature. During the incremental temperature increases, fluorescence data are collected until the reaction reaches 95°C. The result is a plot of raw fluorescence data units, R, versus temperature (Fig.38). As the temperature increases, the DNA melts and the fluorescence intensity decreases. The temperature at which a DNA molecule melts depends on its length and sequence; therefore, if the PCR products consist of molecules of homogeneous length and sequence, a single thermal transition will be detected. So even primer dimers or primer-probe binding can be detected [220].

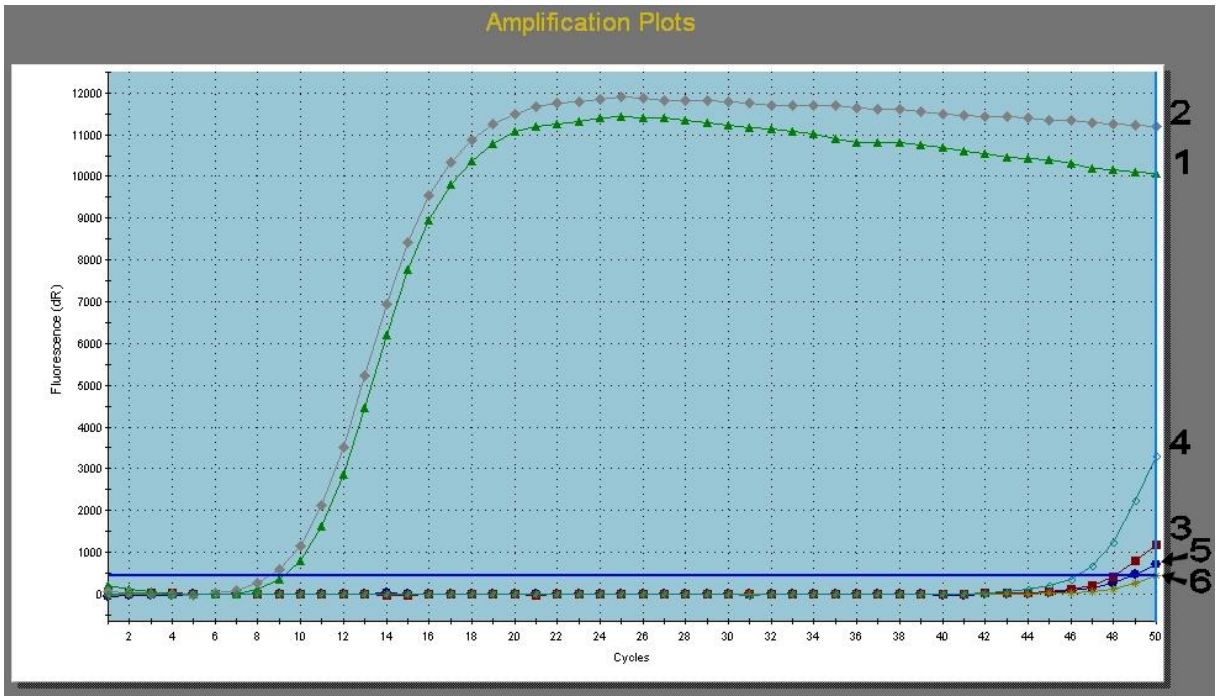


Fig. 36 Real-Time PCR adjustment – Three positive controls in duplicate

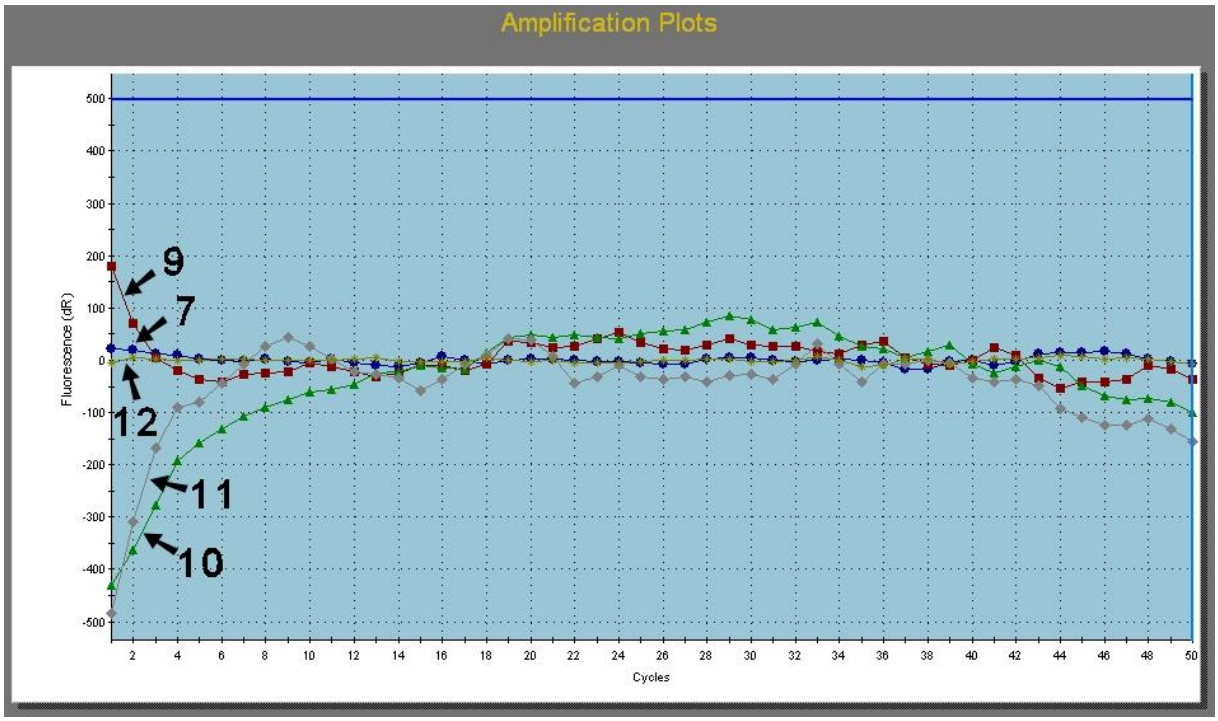


Fig. 37 Real-Time PCR adjustment – Negative controls

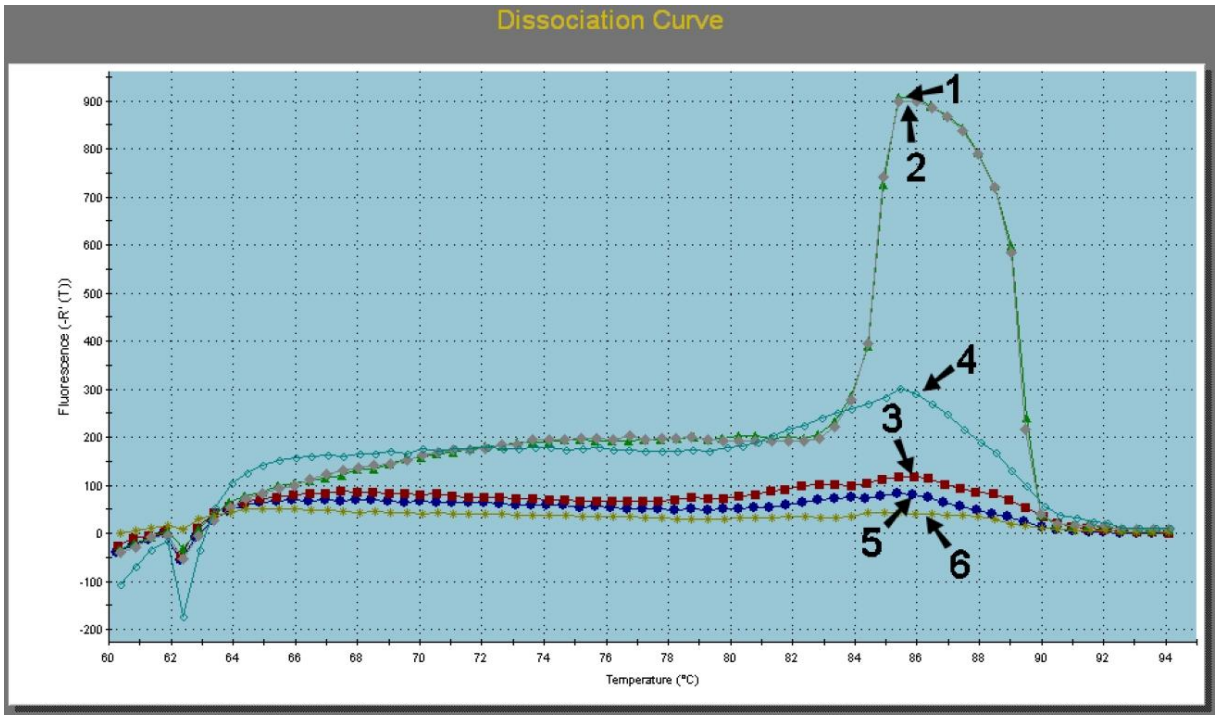


Fig. 38 Real-Time PCR adjustment – Dissociation Curve

No.	Well No.	Sample	Sample Type	Concentration [ng/µl]
1	A1	*B. henselae* spiked	Standard	43,92
2	B1	*B. henselae* spiked	Standard	43,92
3	C1	*B. henselae* spiked	Standard	12,56
4	D1	*B. henselae* spiked	Standard	12,56
5	E1	*B. henselae* spiked	Standard	6,88
6	F1	*B. henselae* spiked	Standard	6,88
7	G1	MM+dH_2O	NC	-
8	H1	blank	-	-
9	A2	Primer+Probe+dH_2O	NC	-
10	B2	Primer as+Probe+dH_2O	NC	-
11	C2	Primer s+Probe+dH_2O	NC	-
12	D2	dH_2O	NC	-

Tab. 33 Real-Time PCR mixture table referring to Fig. 36-38

The *B. henselae* spiked positive controls have been used in three different dilutions and in duplication (Fig. 36). The results of the Dissociation curve analysis showed only peaks at the same temperature level. This means that the PCR resulted in only one amplicon product and no primer dimers have been formed (Fig. 38). In the case of the NCs no fluorescence could have been detected (Fig. 37).

The following plots show the final results of the tick screening with Real-Time PCR and the p24 E and E2 primer set (Fig. 39-45 and Tab. 34-40). The increased fluorescence after 48 to 50 cycles is a result due to thermal, non-specific, degradation of the hydrolysis probe. So an increase of the fluorescence after these cycles shouldn't be rated as a positive result.
In plot 39 and 40 only the first positive control duplicate has been detected due to errors in the PCR Eppis which resulted in the vaporisation of the reaction mixture. But it had no further effects on the results of the plots.

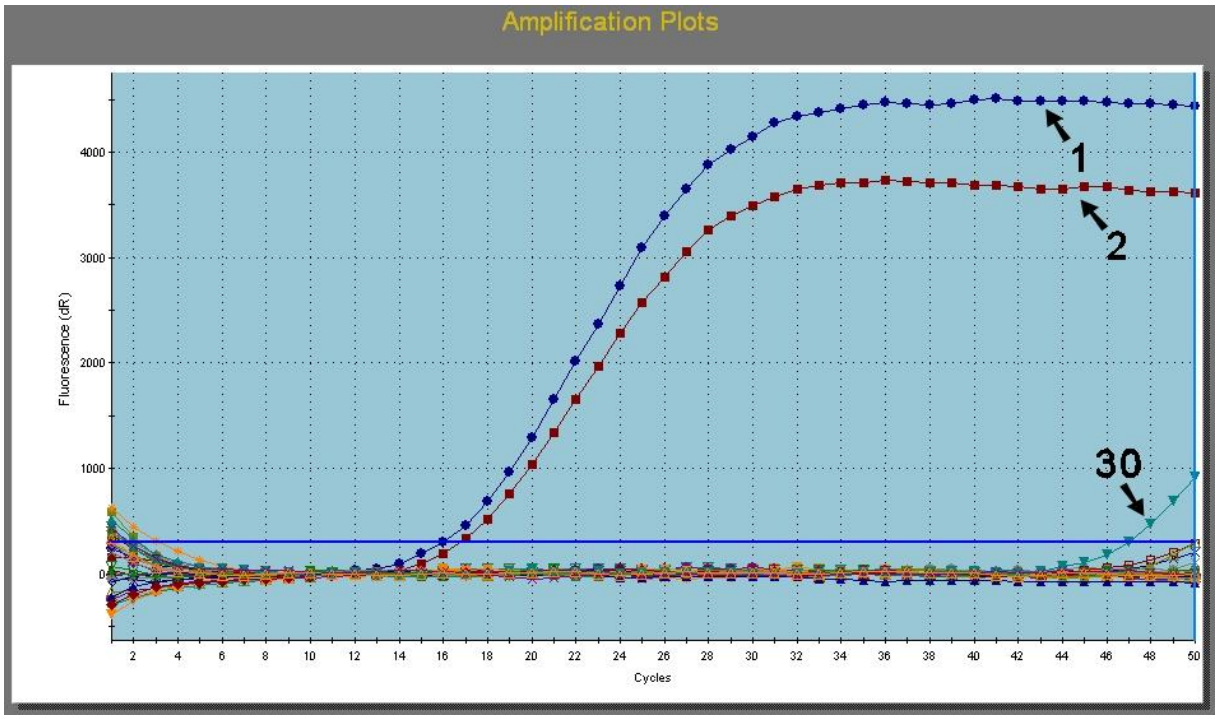


Fig. 39 Real-Time PCR Screening Niederösterreich

No.	Well No.	Sample	Sample Type	Concentration [ng/µl]
1	A1	*B. henselae* spiked	Standard	43,92
2	A2	*B. henselae* spiked	Standard	43,92
3	A3	*B. henselae* spiked	Standard	12,56
4	A4	*B. henselae* spiked	Standard	12,56
5	A5	*B. henselae* spiked	Standard	6,88
6	A6	*B. henselae* spiked	Standard	6,88
7	A7	-	-	-
8	A8	MM + dH_2O	NC	-
9	B1	NÖ - Irnfritz L1	Tick DNA Sample	-
10	B2	NÖ - Irnfritz L2	Tick DNA Sample	-
11	B3	NÖ - Irnfritz L3	Tick DNA Sample	-
12	B4	NÖ - Irnfritz L4	Tick DNA Sample	-
13	B5	NÖ - Irnfritz L5	Tick DNA Sample	-
14	B6	NÖ - Irnfritz L6	Tick DNA Sample	-
15	B7	NÖ - Irnfritz L7	Tick DNA Sample	-
16	B8	NÖ - Irnfritz L8	Tick DNA Sample	-
17	C1	NÖ - St.Pölten N1	Tick DNA Sample	-
18	C2	NÖ - St.Pölten N2	Tick DNA Sample	-
19	C3	NÖ - St.Pölten N3	Tick DNA Sample	-
20	C4	NÖ - St.Pölten N4	Tick DNA Sample	-
21	C5	NÖ - St.Pölten N5	Tick DNA Sample	-
22	C6	NÖ - St.Pölten N6	Tick DNA Sample	-
23	C7	NÖ - St.Pölten N7	Tick DNA Sample	-
24	C8	NÖ - St.Pölten N8	Tick DNA Sample	-
25	D1	NÖ - St.Pölten Am1	Tick DNA Sample	-
26	D2	NÖ - St.Pölten Am2	Tick DNA Sample	-
27	D3	NÖ - St.Pölten Am3	Tick DNA Sample	-
28	D4	NÖ - St.Pölten Am4	Tick DNA Sample	-
29	D5	NÖ - St.Pölten Aw5	Tick DNA Sample	-
30	**D6**	**NÖ - St.Pölten Aw6**	**Tick DNA Sample**	**POSITIVE**
31	D7	NÖ - St.Pölten Aw7	Tick DNA Sample	-
32	D8	NÖ - St.Pölten Aw8	Tick DNA Sample	-

Tab. 34 Real-Time PCR mixture table referring to Fig. 39

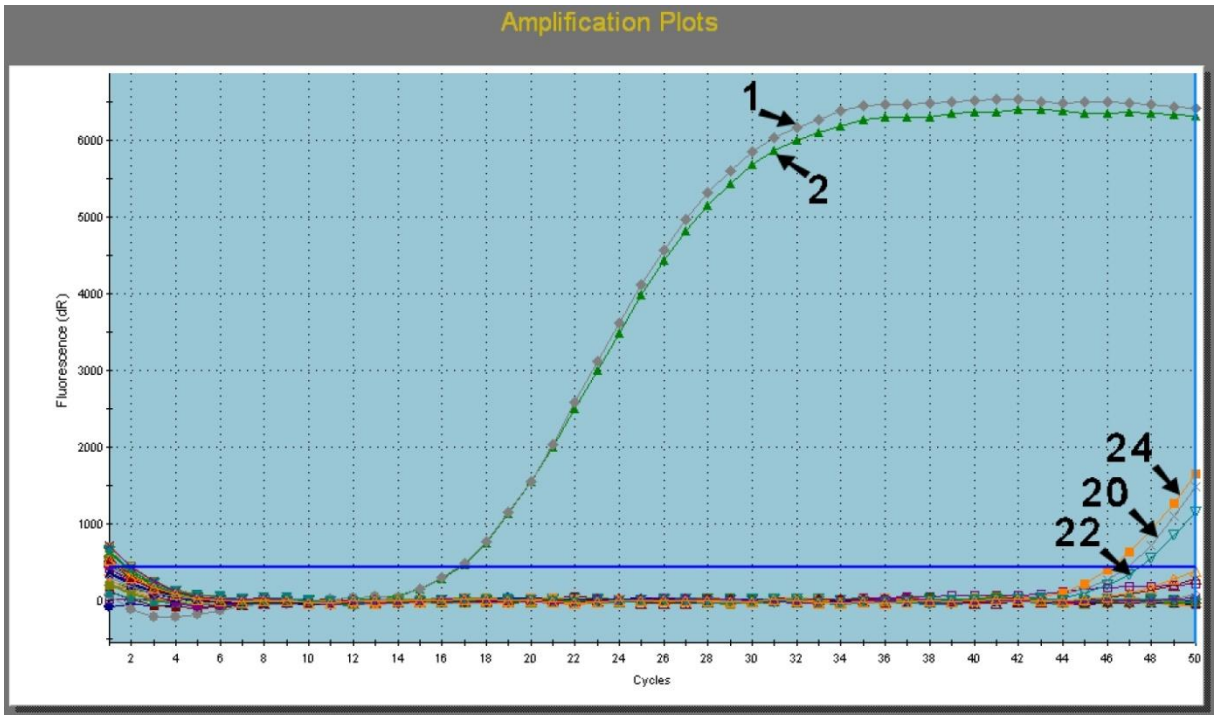


Fig. 40 Real-Time PCR Screening Salzburg

No.	Well No.	Sample	Sample Type	Concentration [ng/µl]
1	A1	*B. henselae* spiked	Standard	43,92
2	A2	*B. henselae* spiked	Standard	43,92
3	A3	*B. henselae* spiked	Standard	12,56
4	A4	*B. henselae* spiked	Standard	12,56
5	A5	*B. henselae* spiked	Standard	6,88
6	A6	*B. henselae* spiked	Standard	6,88
7	A7	-	-	-
8	A8	MM + dH_2O	NC	-
9	B1	S - Adnet bei Hallein L1	Tick DNA Sample	-
10	B2	S - Adnet bei Hallein L2	Tick DNA Sample	-
11	B3	S - Adnet bei Hallein L3	Tick DNA Sample	-
12	B4	S - Adnet bei Hallein L4	Tick DNA Sample	-
13	B5	S - Adnet bei Hallein L5	Tick DNA Sample	-
14	B6	S - Adnet bei Hallein L6	Tick DNA Sample	-
15	B7	S - Adnet bei Hallein L7	Tick DNA Sample	-
16	B8	S - Adnet bei Hallein L8	Tick DNA Sample	-
17	C1	S - Goldegg N1	Tick DNA Sample	-
18	C2	S - Goldegg N2	Tick DNA Sample	-
19	C3	S - Goldegg N3	Tick DNA Sample	-
20	**C4**	**S - Goldegg N4**	**Tick DNA Sample**	**Positive**
21	C5	S - Goldegg N5	Tick DNA Sample	-
22	**C6**	**S - Goldegg N6**	**Tick DNA Sample**	**Positive**
23	C7	S - Goldegg N7	Tick DNA Sample	-
24	**C8**	**S - Goldegg N8**	**Tick DNA Sample**	**Positive**
25	D1	S - Adnet bei Hallein Am1	Tick DNA Sample	-
26	D2	S - Adnet bei Hallein Am2	Tick DNA Sample	-
27	D3	S - Adnet bei Hallein Am3	Tick DNA Sample	-
28	D4	S - Adnet bei Hallein Am4	Tick DNA Sample	-
29	D5	S - Adnet bei Hallein Aw5	Tick DNA Sample	-
30	D6	S - Adnet bei Hallein Aw6	Tick DNA Sample	-
31	D7	S - Adnet bei Hallein Aw7	Tick DNA Sample	-
32	D8	S - Adnet bei Hallein Aw8	Tick DNA Sample	-

Tab. 35 Real-Time PCR mixture table referring to Fig. 40

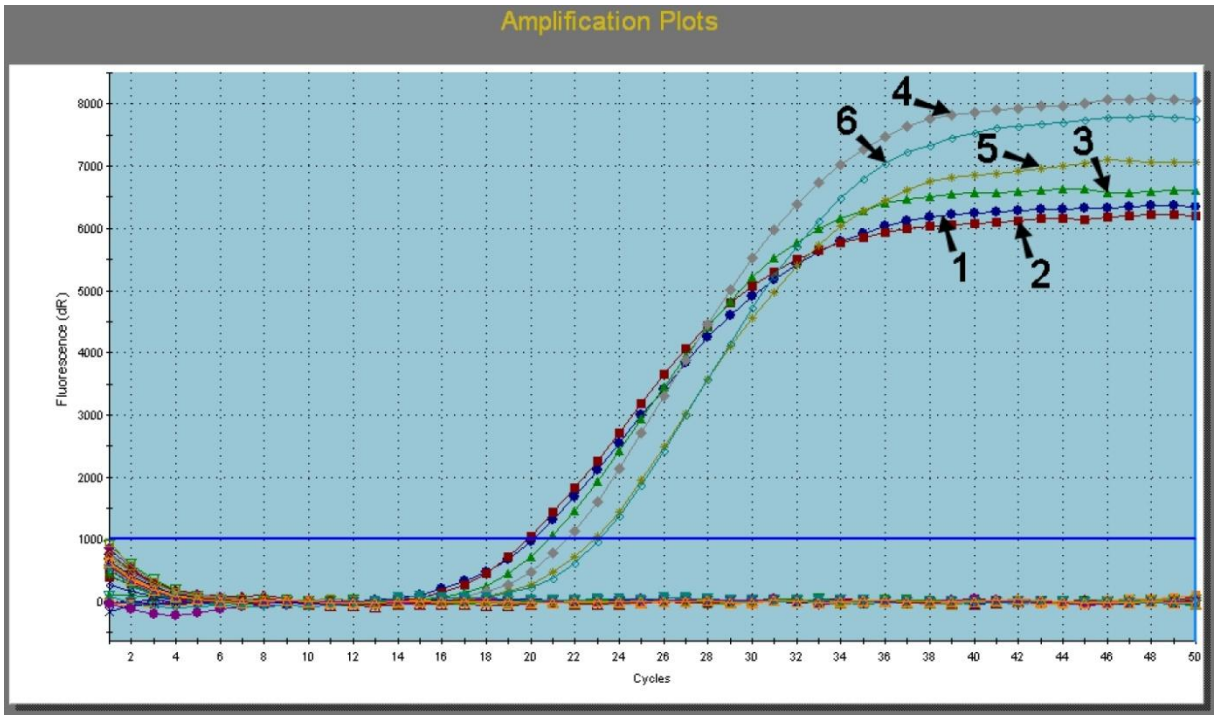


Fig. 41 Real-Time PCR Screening Kärnten

No.	Well No.	Sample	Sample Type	Concentration [ng/µl]
1	A1	*B. henselae* spiked	Standard	43,92
2	A2	*B. henselae* spiked	Standard	43,92
3	A3	*B. henselae* spiked	Standard	12,56
4	A4	*B. henselae* spiked	Standard	12,56
5	A5	*B. henselae* spiked	Standard	6,88
6	A6	*B. henselae* spiked	Standard	6,88
7	A7	-	-	-
8	A8	MM + dH_2O	NC	-
9	B1	K - Faaker See/ Drobolach L1	Tick DNA Sample	-
10	B2	K - Faaker See/ Drobolach L2	Tick DNA Sample	-
11	B3	K - Faaker See/ Drobolach L3	Tick DNA Sample	-
12	B4	K - Faaker See/ Drobolach L4	Tick DNA Sample	-
13	B5	K - Faaker See/ Drobolach L5	Tick DNA Sample	-
14	B6	K - Faaker See/ Drobolach L6	Tick DNA Sample	-
15	B7	K - Faaker See/ Drobolach L7	Tick DNA Sample	-
16	B8	K - Faaker See/ Drobolach L8	Tick DNA Sample	-
17	C1	K - Faaker See/ Drobolach N1	Tick DNA Sample	-
18	C2	K - Faaker See/ Drobolach N2	Tick DNA Sample	-
19	C3	K - Faaker See/ Drobolach N3	Tick DNA Sample	-
20	C4	K - Faaker See/ Drobolach N4	Tick DNA Sample	-
21	C5	K - Faaker See/ Drobolach N5	Tick DNA Sample	-
22	C6	K - Faaker See/ Drobolach N6	Tick DNA Sample	-
23	C7	K - Faaker See/ Drobolach N7	Tick DNA Sample	-
24	C8	K - Faaker See/ Drobolach N8	Tick DNA Sample	-
25	D1	K - Egelsee bei M. Am1	Tick DNA Sample	-
26	D2	K - Egelsee bei M. Am2	Tick DNA Sample	-
27	D3	K - Egelsee bei M. Am3	Tick DNA Sample	-
28	D4	K - Egelsee bei M. Am4	Tick DNA Sample	-
29	D5	K - Egelsee bei M. Aw5	Tick DNA Sample	-
30	D6	K - Egelsee bei M. Aw6	Tick DNA Sample	-
31	D7	K - Egelsee bei M. Aw7	Tick DNA Sample	-
32	D8	K - Egelsee bei M. Aw8	Tick DNA Sample	-

Tab. 36 Real-Time PCR mixture table referring to Fig. 41

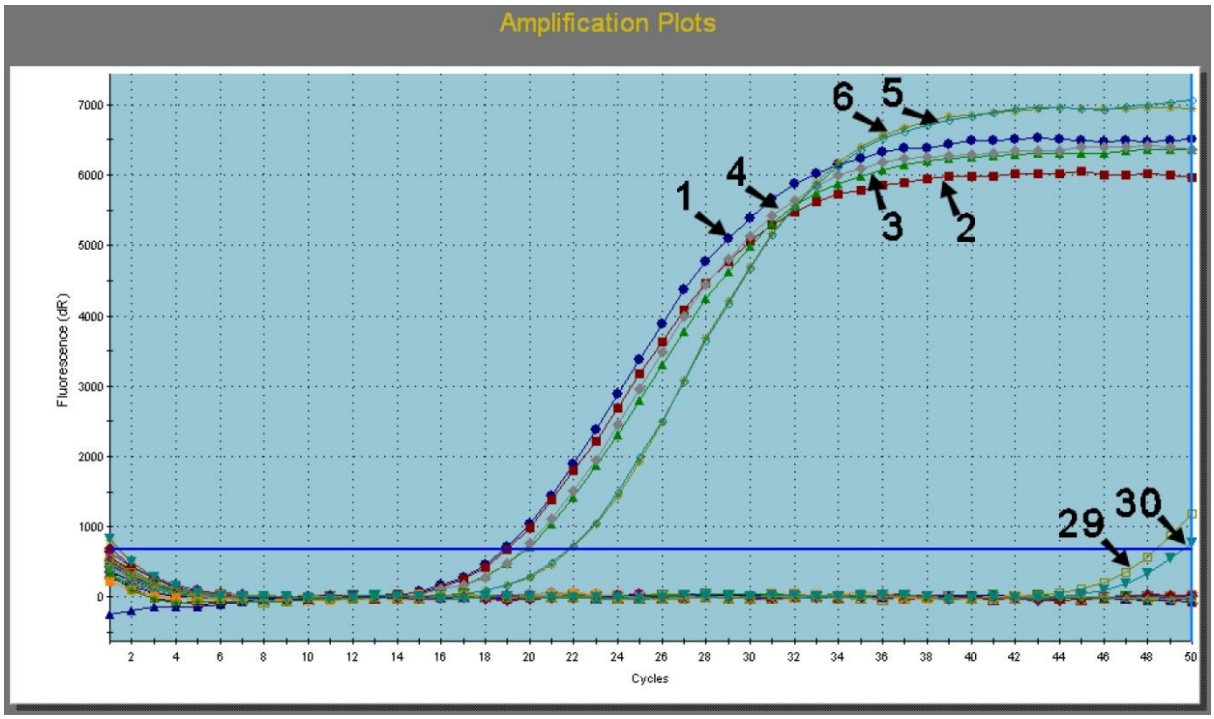


Fig. 42 Real-Time PCR Screening Oberösterreich

No.	Well No.	Sample	Sample Type	Concentration [ng/µl]
1	A1	*B. henselae* spiked	Standard	43,92
2	A2	*B. henselae* spiked	Standard	43,92
3	A3	*B. henselae* spiked	Standard	12,56
4	A4	*B. henselae* spiked	Standard	12,56
5	A5	*B. henselae* spiked	Standard	6,88
6	A6	*B. henselae* spiked	Standard	6,88
7	A7	-	-	-
8	A8	MM + dH_2O	NC	-
9	B1	OÖ - Niederottensheim bei Linz L1	Tick DNA Sample	-
10	B2	OÖ - Niederottensheim bei Linz L2	Tick DNA Sample	-
11	B3	OÖ - Niederottensheim bei Linz L3	Tick DNA Sample	-
12	B4	OÖ - Niederottensheim bei Linz L4	Tick DNA Sample	-
13	B5	OÖ - Niederottensheim bei Linz L5	Tick DNA Sample	-
14	B6	OÖ - Niederottensheim bei Linz L6	Tick DNA Sample	-
15	B7	OÖ - Niederottensheim bei Linz L7	Tick DNA Sample	-
16	B8	OÖ - Niederottensheim bei Linz L8	Tick DNA Sample	-
17	C1	OÖ - Niederottensheim bei Linz N1	Tick DNA Sample	-
18	C2	OÖ - Niederottensheim bei Linz N2	Tick DNA Sample	-
19	C3	OÖ - Niederottensheim bei Linz N3	Tick DNA Sample	-
20	C4	OÖ - Niederottensheim bei Linz N4	Tick DNA Sample	-
21	C5	OÖ - Niederottensheim bei Linz N5	Tick DNA Sample	-
22	C6	OÖ - Niederottensheim bei Linz N6	Tick DNA Sample	-
23	C7	OÖ - Niederottensheim bei Linz N7	Tick DNA Sample	-
24	C8	OÖ - Niederottensheim bei Linz N8	Tick DNA Sample	-
25	D1	OÖ - Niederottensheim bei Linz Am1	Tick DNA Sample	-
26	D2	OÖ - Niederottensheim bei Linz Am2	Tick DNA Sample	-
27	D3	OÖ - Niederottensheim bei Linz Am3	Tick DNA Sample	-
28	D4	OÖ - Niederottensheim bei Linz Am4	Tick DNA Sample	-
29	**D5**	**OÖ - Niederottensheim bei Linz Aw5**	**Tick DNA Sample**	**Positive**
30	**D6**	**OÖ - Niederottensheim bei Linz Aw6**	**Tick DNA Sample**	**Positive**
31	D7	OÖ - Niederottensheim bei Linz Aw7	Tick DNA Sample	-
32	D8	OÖ - Niederottensheim bei Linz Aw8	Tick DNA Sample	-

Tab. 37 Real-Time PCR mixture table referring to Fig. 42

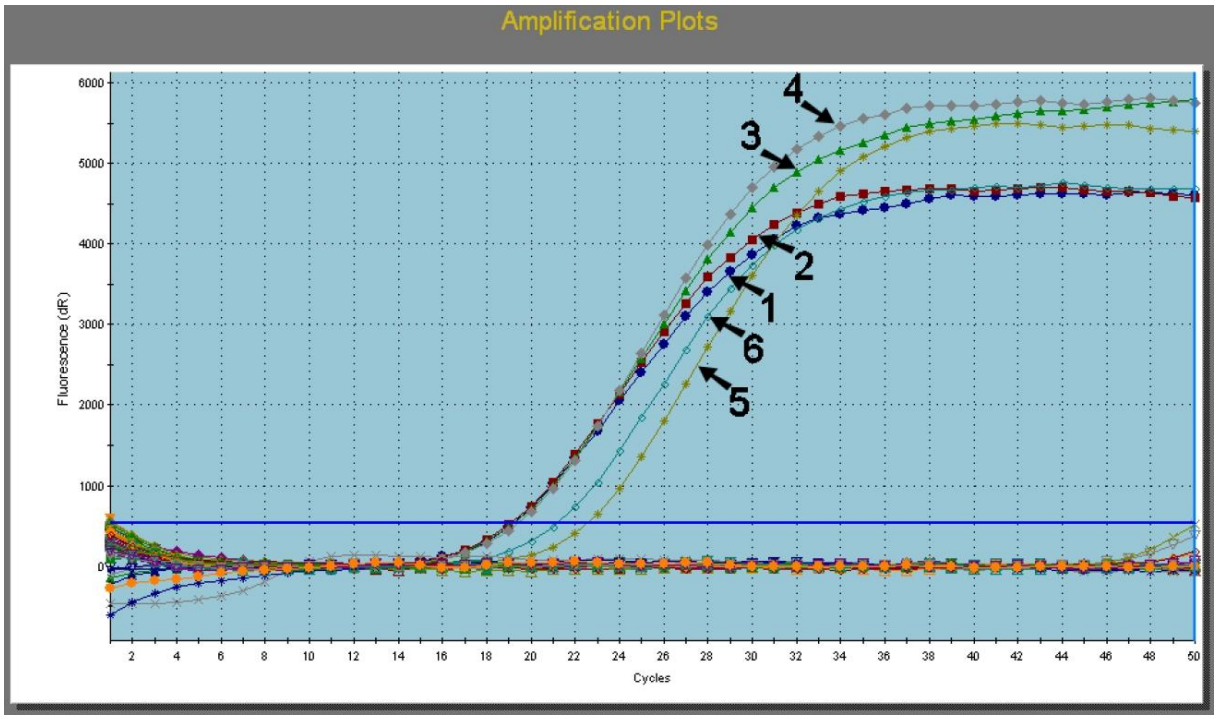


Fig. 43 Real-Time PCR Screening Tirol and Vorarlberg

No.	Well No.	Sample	Sample Type	Concentration [ng/µl]	No.	Well No.	Sample	Sample Type	Concentration [ng/µl]
1	A1	*B. henselae* spiked	Standard	43,92	33	E1	V - Thüringen L1	Tick DNA Sample	-
2	A2	*B. henselae* spiked	Standard	43,92	34	E2	V - Thüringen L2	Tick DNA Sample	-
3	A3	*B. henselae* spiked	Standard	12,56	35	E3	V - Thüringen L3	Tick DNA Sample	-
4	A4	*B. henselae* spiked	Standard	12,56	36	E4	V - Thüringen L4	Tick DNA Sample	-
5	A5	*B. henselae* spiked	Standard	6,88	37	E5	V - Thüringen L5	Tick DNA Sample	-
6	A6	*B. henselae* spiked	Standard	6,88	38	E6	V - Thüringen L6	Tick DNA Sample	-
7	A7	-	-	-	39	E7	V - Thüringen L7	Tick DNA Sample	-
8	A8	MM + dH_2O	NC	-	40	E8	V - Thüringen L8	Tick DNA Sample	-
9	B1	T - Mils L1	Tick DNA Sample	-	41	F1	V - Klaus N1	Tick DNA Sample	-
10	B2	T - Mils L2	Tick DNA Sample	-	42	F2	V - Klaus N2	Tick DNA Sample	-
11	B3	T - Mils L3	Tick DNA Sample	-	43	F3	V - Klaus N3	Tick DNA Sample	-
12	B4	T - Mils L4	Tick DNA Sample	-	44	F4	V - Klaus N4	Tick DNA Sample	-
13	B5	T - Mils L5	Tick DNA Sample	-	45	F5	V - Klaus N5	Tick DNA Sample	-
14	B6	T - Mils L6	Tick DNA Sample	-	46	F6	V - Klaus N6	Tick DNA Sample	-
15	B7	T - Mils L7	Tick DNA Sample	-	47	F7	V - Klaus N7	Tick DNA Sample	-
16	B8	T - Mils L8	Tick DNA Sample	-	48	F8	V - Klaus N8	Tick DNA Sample	-
17	C1	T - Imst N1	Tick DNA Sample	-	49	G1	V - Klaus Am1	Tick DNA Sample	-
18	C2	T - Imst N2	Tick DNA Sample	-	50	G2	V - Klaus Am2	Tick DNA Sample	-
19	C3	T - Imst N3	Tick DNA Sample	-	51	G3	V - Klaus Am3	Tick DNA Sample	-
20	C4	T - Imst N4	Tick DNA Sample	-	52	G4	V - Klaus Am4	Tick DNA Sample	-
21	C5	T - Imst N5	Tick DNA Sample	-	53	G5	V - Klaus Aw5	Tick DNA Sample	-
22	C6	T - Imst N6	Tick DNA Sample	-	54	G6	V - Klaus Aw6	Tick DNA Sample	-
23	C7	T - Imst N7	Tick DNA Sample	-	55	G7	V - Klaus Aw7	Tick DNA Sample	-
24	C8	T - Imst N8	Tick DNA Sample	-	56	G8	V - Klaus Aw8	Tick DNA Sample	-
25	D1	T - Imst Am1	Tick DNA Sample	-					
26	D2	T - Imst Am2	Tick DNA Sample	-					
27	D3	T - Imst Am3	Tick DNA Sample	-					
28	D4	T - Imst Am4	Tick DNA Sample	-					
29	D5	T - Imst Aw5	Tick DNA Sample	-					
30	D6	T - Imst Aw6	Tick DNA Sample	-					
31	D7	T - Imst Aw7	Tick DNA Sample	-					
32	D8	T - Imst Aw8	Tick DNA Sample	-					

Tab. 38 Real-Time PCR mixture table referring to Fig. 43

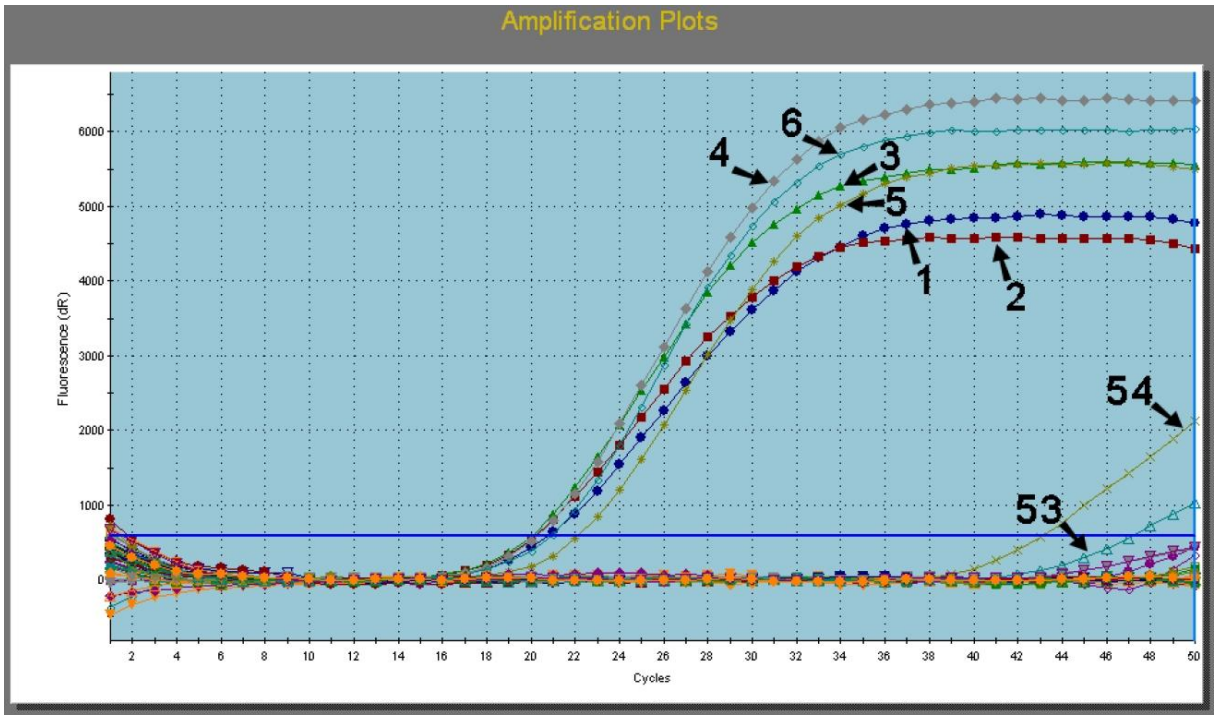


Fig. 44 Real-Time PCR Screening Burgenland and Steiermark

No.	Well No.	Sample	Sample Type	Concentration [ng/µl]	No.	Well No.	Sample	Sample Type	Concentration [ng/µl]
1	A1	*B. henselae* spiked	Standard	43,92	33	E1	St - Admont L1	Tick DNA Sample	-
2	A2	*B. henselae* spiked	Standard	43,92	34	E2	St - Admont L2	Tick DNA Sample	-
3	A3	*B. henselae* spiked	Standard	12,56	35	E3	St - Admont L3	Tick DNA Sample	-
4	A4	*B. henselae* spiked	Standard	12,56	36	E4	St - Admont L4	Tick DNA Sample	-
5	A5	*B. henselae* spiked	Standard	6,88	37	E5	St - Admont L5	Tick DNA Sample	-
6	A6	*B. henselae* spiked	Standard	6,88	38	E6	St - Admont L6	Tick DNA Sample	-
7	A7	-	-	-	39	E7	St - Admont L7	Tick DNA Sample	-
8	A8	MM + dH_2O	NC	-	40	E8	St - Admont L8	Tick DNA Sample	-
9	B1	B - Oberwart L1	Tick DNA Sample	-	41	F1	St - Mürzzuschlag N1	Tick DNA Sample	-
10	B2	B - Oberwart L2	Tick DNA Sample	-	42	F2	St - Mürzzuschlag N2	Tick DNA Sample	-
11	B3	B - Oberwart L3	Tick DNA Sample	-	43	F3	St - Mürzzuschlag N3	Tick DNA Sample	-
12	B4	B - Oberwart L4	Tick DNA Sample	-	44	F4	St - Mürzzuschlag N4	Tick DNA Sample	-
13	B5	B - Oberwart L5	Tick DNA Sample	-	45	F5	St - Mürzzuschlag N5	Tick DNA Sample	-
14	B6	B - Oberwart L6	Tick DNA Sample	-	46	F6	St - Mürzzuschlag N6	Tick DNA Sample	-
15	B7	B - Oberwart L7	Tick DNA Sample	-	47	F7	St - Mürzzuschlag N7	Tick DNA Sample	-
16	B8	B - Oberwart L8	Tick DNA Sample	-	48	F8	St - Mürzzuschlag N8	Tick DNA Sample	-
17	C1	B - Breitenbrunn N1	Tick DNA Sample	-	49	G1	St - Mürzzuschlag Am1	Tick DNA Sample	-
18	C2	B - Breitenbrunn N2	Tick DNA Sample	-	50	G2	St - Mürzzuschlag Am2	Tick DNA Sample	-
19	C3	B - Breitenbrunn N3	Tick DNA Sample	-	51	G3	St - Mürzzuschlag Am3	Tick DNA Sample	-
20	C4	B - Breitenbrunn N4	Tick DNA Sample	-	52	G4	St - Mürzzuschlag Am4	Tick DNA Sample	-
21	C5	B - Breitenbrunn N5	Tick DNA Sample	-	**53**	**G5**	**St - Mürzzuschlag Aw5**	**Tick DNA Sample**	**Positive**
22	C6	B - Breitenbrunn N6	Tick DNA Sample	-	**54**	**G6**	**St - Mürzzuschlag Aw6**	**Tick DNA Sample**	**Positive**
23	C7	B - Breitenbrunn N7	Tick DNA Sample	-	55	G7	St - Mürzzuschlag Aw7	Tick DNA Sample	-
24	C8	B - Breitenbrunn N8	Tick DNA Sample	-	56	G8	St - Mürzzuschlag Aw8	Tick DNA Sample	-
25	D1	B - Breitenbrunn Am1	Tick DNA Sample	-					
26	D2	B - Breitenbrunn Am2	Tick DNA Sample	-					
27	D3	B - Breitenbrunn Am3	Tick DNA Sample	-					
28	D4	B - Breitenbrunn Am4	Tick DNA Sample	-					
29	D5	B - Breitenbrunn Aw5	Tick DNA Sample	-					
30	D6	B - Breitenbrunn Aw6	Tick DNA Sample	-					
31	D7	B - Breitenbrunn Aw7	Tick DNA Sample	-					
32	D8	B - Breitenbrunn Aw8	Tick DNA Sample	-					

Tab. 39 Real-Time PCR mixture table referring to Fig. 44

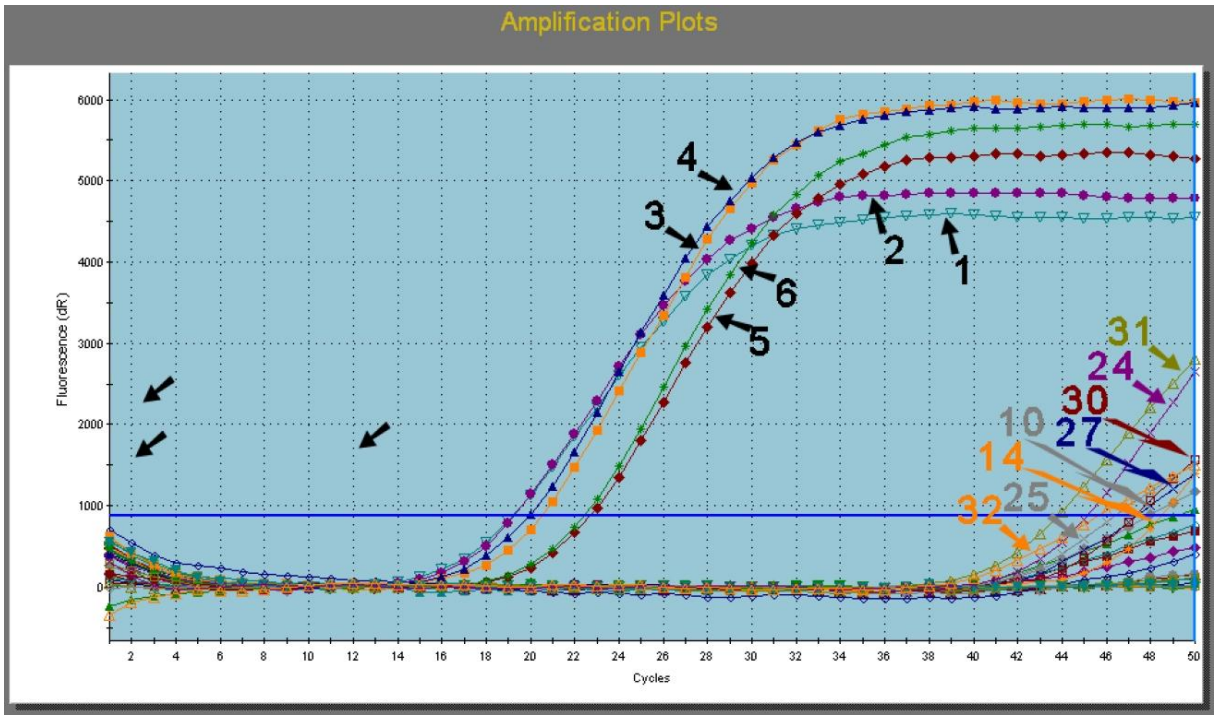


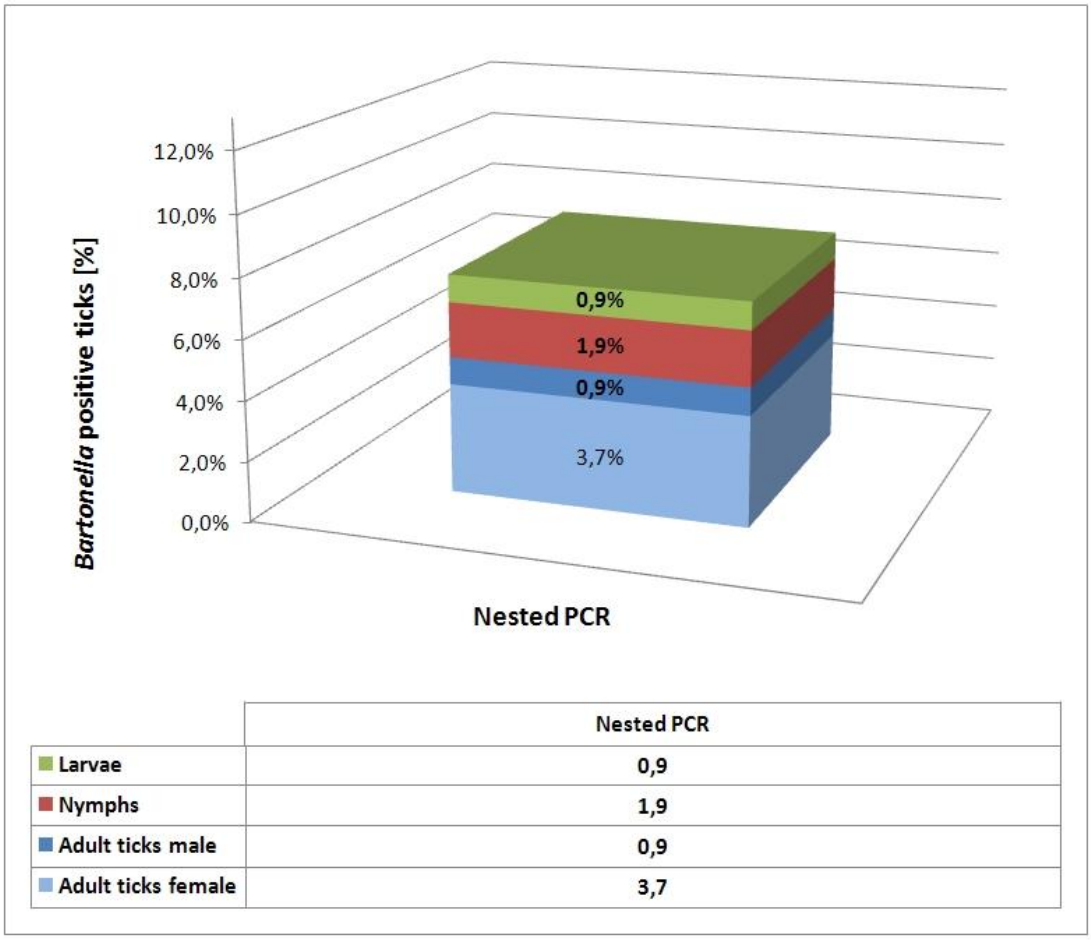
Fig. 45 Real-Time PCR Screening Wien

No.	Well No.	Sample	Sample Type	Concentration [ng/µl]
1	A1	*B. henselae* spiked	Standard	43,92
2	A2	*B. henselae* spiked	Standard	43,92
3	A3	*B. henselae* spiked	Standard	12,56
4	A4	*B. henselae* spiked	Standard	12,56
5	A5	*B. henselae* spiked	Standard	6,88
6	A6	*B. henselae* spiked	Standard	6,88
7	A7	-	-	-
8	A8	MM + dH_2O	NC	-
9	B1	W - Prater/Lusthaus L1	Tick DNA Sample	-
10	**B2**	**W - Prater/Lusthaus L2**	**Tick DNA Sample**	**Positive**
11	B3	W - Prater/Lusthaus L3	Tick DNA Sample	-
12	B4	W - Prater/Lusthaus L4	Tick DNA Sample	-
13	B5	W - Prater/Lusthaus L5	Tick DNA Sample	-
14	**B6**	**W - Prater/Lusthaus L6**	**Tick DNA Sample**	**Positive**
15	B7	W - Prater/Lusthaus L7	Tick DNA Sample	-
16	B8	W - Prater/Lusthaus L8	Tick DNA Sample	-
17	C1	W - Prater/Lusthaus N1	Tick DNA Sample	-
18	C2	W - Prater/Lusthaus N2	Tick DNA Sample	-
19	C3	W - Prater/Lusthaus N3	Tick DNA Sample	-
20	C4	W - Prater/Lusthaus N4	Tick DNA Sample	-
21	C5	W - Prater/Lusthaus N5	Tick DNA Sample	-
22	C6	W - Prater/Lusthaus N6	Tick DNA Sample	-
23	C7	W - Prater/Lusthaus N7	Tick DNA Sample	-
24	**C8**	**W - Prater/Lusthaus N8**	**Tick DNA Sample**	**Positive**
25	**D1**	**W - Prater/Lusthaus Am1**	**Tick DNA Sample**	**Positive**
26	D2	W - Prater/Lusthaus Am2	Tick DNA Sample	-
27	**D3**	**W - Prater/Lusthaus Am3**	**Tick DNA Sample**	**Positive**
28	D4	W - Prater/Lusthaus Am4	Tick DNA Sample	-
29	D5	W - Prater/Lusthaus Aw5	Tick DNA Sample	-
30	**D6**	**W - Prater/Lusthaus Aw6**	**Tick DNA Sample**	**Positive**
31	**D7**	**W - Prater/Lusthaus Aw7**	**Tick DNA Sample**	**Positive**
32	**D8**	**W - Prater/Lusthaus Aw8**	**Tick DNA Sample**	**Positive**

Tab. 40 Real-Time PCR mixture table referring to Fig. 45

With the Real Time PCR screening of the tick samples 16 ticks out of 216 could have been tested positive for *Bartonella* (Fig. 46). This corresponds to a percentage of infection of 7.4%. The regional distribution of Bartonella positive ticks had its highest level in Vienna with 33.3% (Tab. 41).

	Adult ticks		Nymphs	Larvae	Total amount
	male	female			
***Bartonella* positive ticks**	2	8	4	2	**16**
Tested tick developmental stages in total	72		72	72	**216**
Percentage [%]	0,9	3,7	1,9	0,9	**7,4**



	Nested PCR
Larvae	0,9
Nymphs	1,9
Adult ticks male	0,9
Adult ticks female	3,7

Fig. 46 Real Time PCR tick screening results diagrammed

Province of Austria	Region	Positive tick sample and developmental stages	Regional percentage [%]
Niederösterreich	St. Pölten	Adult female 6	**4,2**
Salzburg	Goldegg	Nymphe 4	**12,5**
Salzburg	Goldegg	Nymphe 6	
Salzburg	Goldegg	Nymphe 8	
Oberösterreich	Niederottensheim bei Linz	Adult female 5	**8,3**
Oberösterreich	Niederottensheim bei Linz	Adult female 6	
Steiermark	Mürzzuschlag	Adult female 5	**8,3**
Steiermark	Mürzzuschlag	Adult female 6	
Wien	Prater/ Lusthaus (Index 5)	Larva 1	**33,3**
Wien	Prater/ Lusthaus (Index 5)	Larva 2	
Wien	Prater/ Lusthaus (Index 5)	Nymphe 2	
Wien	Prater/ Lusthaus (Index 5)	Adult male 1	
Wien	Prater/ Lusthaus (Index 5)	Adult male 3	
Wien	Prater/ Lusthaus (Index 5)	Adult female 6	
Wien	Prater/ Lusthaus (Index 5)	Adult female 7	
Wien	Prater/ Lusthaus (Index 5)	Adult female 8	

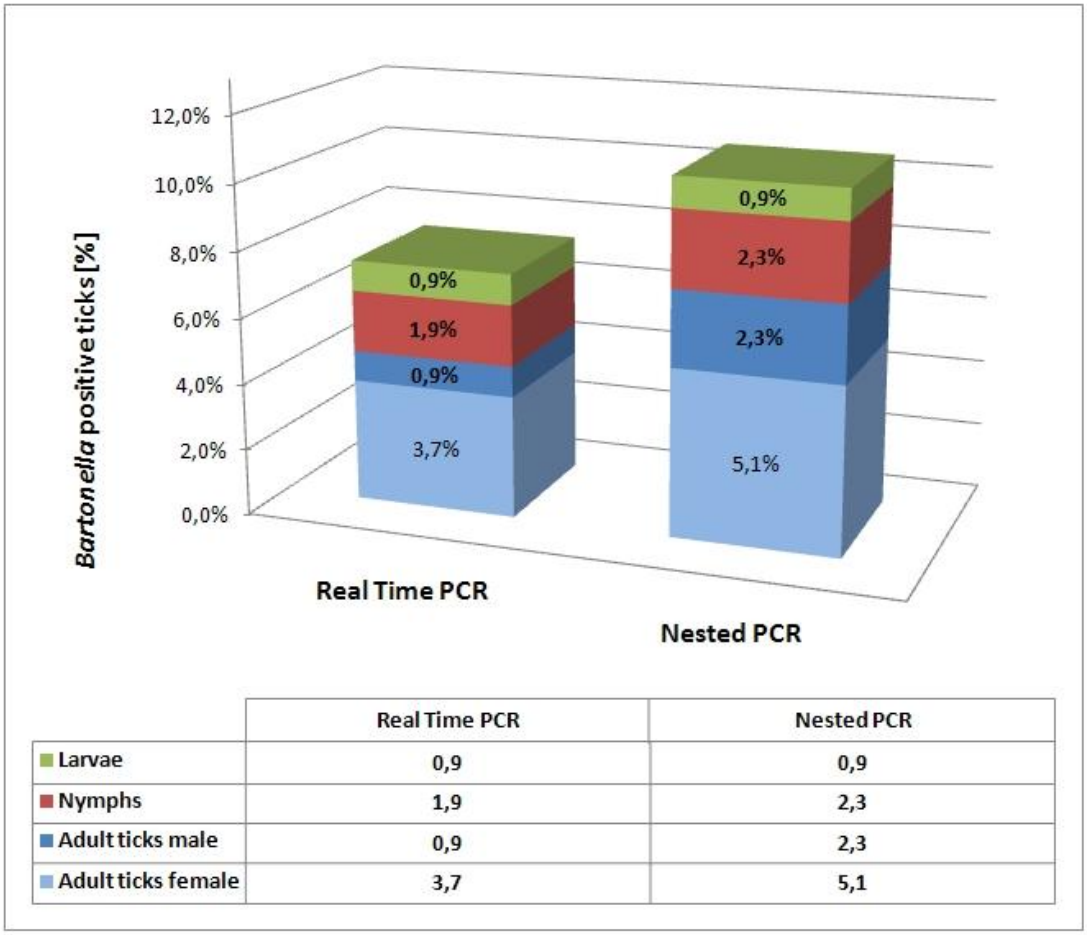
Tab. 41 Regional distribution of the *Bartonella* positive ticks

5.1.4.3. Comparison of the Results Real Time and Nested PCR

With both methods the same tick samples could have been detected as positives. However the Nested PCR resulted in 7 more positives (Fig. 47). This 7 positive samples had been tested a second time to verify these positive results. This raises the question why there was such a great difference between the two methods. I wouldn't say that a Nested PCR in general is more sensitive than a Real Time PCR. Much more accurate is that the Real Time PCR could have been adjusted not optimally. This could result in the decrease of the sensitivity.

As a disadvantage of the Real Time PCR has to be mentioned that the 16-23S Intergenic Spacer Region is not suitable to be used as a target sequence. The sequences between the primer binding sites are so differing among the *Bartonella* species that it is not possible to find a proper probe binding site (Fig. 23). So a species differentiation has to be performed with the Nested PCR.

	Adult ticks		Nymphs	Larvae	Total amount
	male	female			
	Real Time PCR				
Bartonella positive ticks	2	8	4	2	**16**
Tested tick developmental stages in total	72		72	72	**216**
Percentage [%]	0,9	3,7	1,9	0,9	**7,4**
	Nested PCR				
Bartonella positive ticks	5	11	5	2	**23**
Tested tick developmental stages in total	72		72	72	**216**
Percentage [%]	2,3	5,1	2,3	0,9	**10,6**



	Real Time PCR	Nested PCR
Larvae	0,9	0,9
Nymphs	1,9	2,3
Adult ticks male	0,9	2,3
Adult ticks female	3,7	5,1

Fig. 47 Comparison of the Real Time and Nested PCR tick screening results diagrammed

5.1.4.4. PCR for species differentiation

The PCR to differentiate between the *Bartonella* species was performed with the primer set 321s and 321as. The target sequence was the 16-23S Intergenic Spacer Region. As already mentioned above this target sequence was not suitable for the Real Time PCR so the

differentiation was only performed with the Standard PCR method. As a matter of fact, during the diploma theses it was not possible to receive proper results cause of some unknown problems with the PCR gel results (Fig. 48). The primer design for the primer set 321 has been repeated and the primer test results had been good (see Chapter 5.1.4 PCRs). So there was no explanation for the multiple bands in the gel results.

The gel in figure 48 shows one of these not interpretable results. In lane 1 and 19 the step ladder was loaded. Lane 6 shows the *B. grahamii* spiked positive control at about 300bp which correlates with the Clustal X value of 307bp (see Tab. 28). In lane 18 was the negative control. Only lane 2 shows a proper result which points to *B. grahamii* or *B. vinsonii sub berkhoffii*. But because of the remaining unclear results the one in lane 2 couldn't be assessed. Furthermore the sequencing of the amplicon in lane 2 didn't lead to any result.

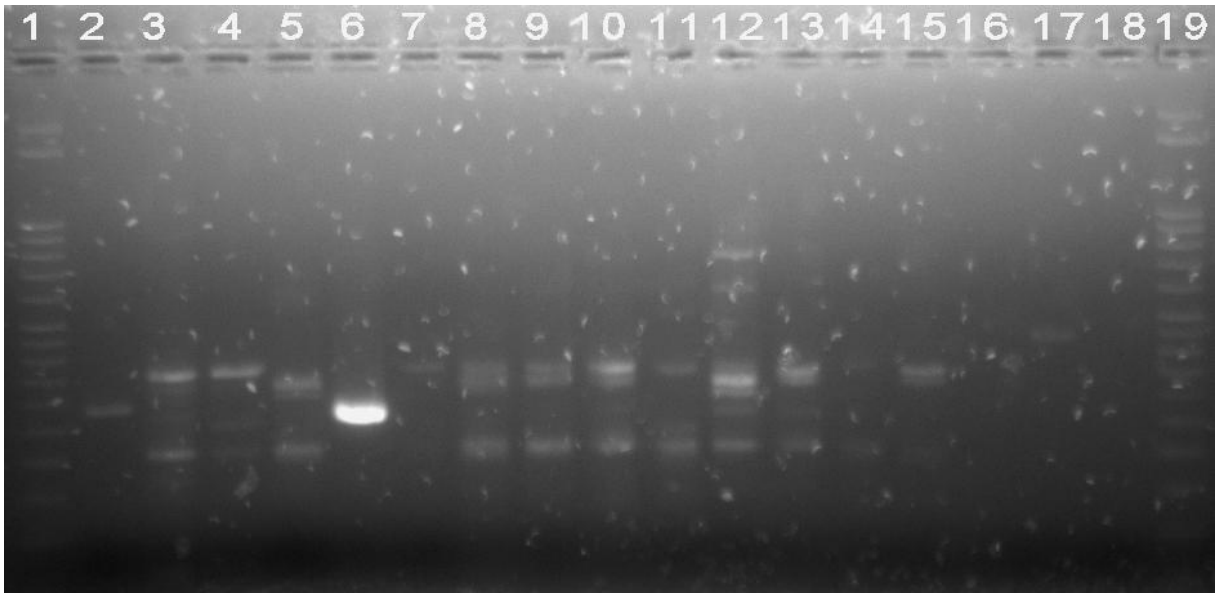


Fig. 48 Differentiation PCR result with primer set 321s and as

5.1.5. Sequencing PCR and Sequencing

The sequencing PCR was performed with the primer p24 E in the case of the cleaned up Real Time PCR positive products. For the Nested PCR the primer N-p24 E2 was used. The choosing of the primers has been made due to the primer design results. In this case the two primers p24 E and N-p24 E2 fulfilled the criteria for primer design at best. In both cases the

amplicons should be a partial sequence of the 16S rRNA gene of *Bartonella* and thus resulting only in a BLAST hit for *Bartonella* species.

The result for the Real Time PCR 16s rRNA partial sequence with 384bp (Fig. 49 and 50):

TGTCGCGGNNGTTGGGTTAACAAACGTAACGAGCGCAACCNCNCCCCTTAGTTGC
CAGCATTTAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGG
TGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTAC
AATGGTGGTGACAGTGGGCAGCGAGACCGCGAGGTCGAGCTAATCTCCAAAAGC
CATCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAG
TAATCGTGGATCAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGC
CCGTCACACCATGGGAGTTGGTTTTACCCGAAGGTGCTGTGCTACCGGCNAAGGA
A

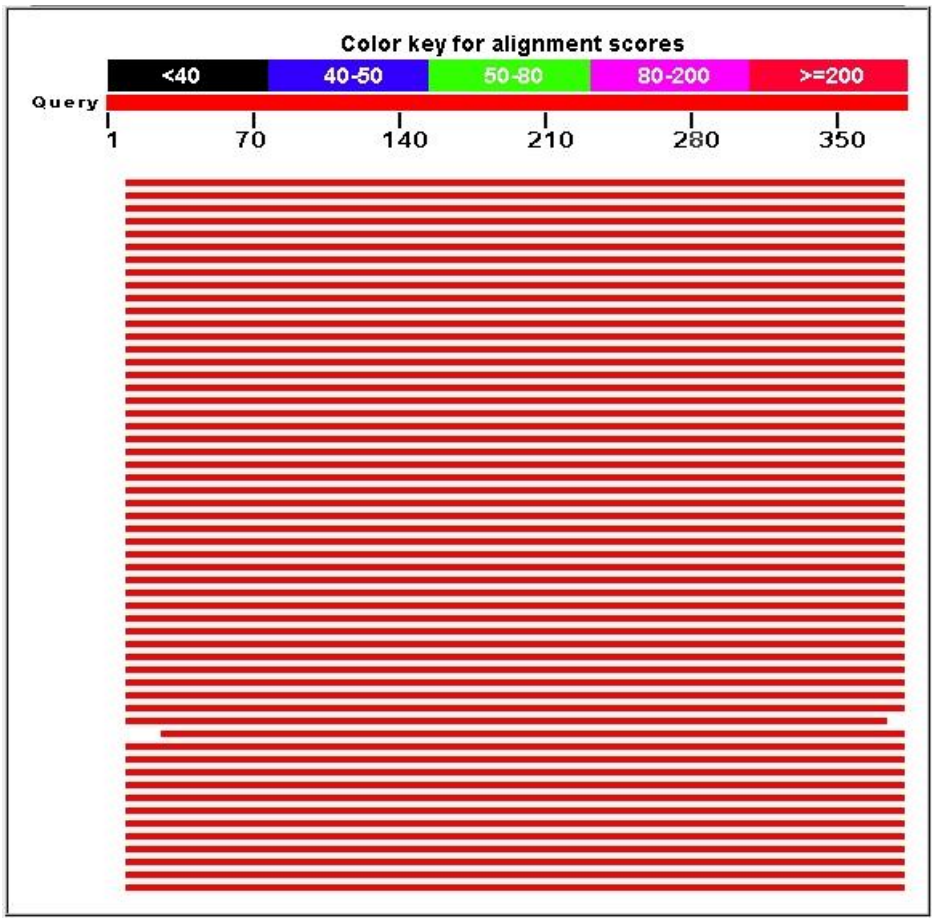


Fig. 49 Colour key for the alignment scores of the Real Time PCR positive results

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP001562.1	Bartonella grahamii as4aup, complete genome	628	1257	97%	5e-177	97%
EU111758.1	Bartonella queenslandensis strain AUST/NH15 16S ribosomal RNA gene	628	628	97%	5e-177	97%
EU111757.1	Bartonella queenslandensis strain AUST/NH11 16S ribosomal RNA gene	628	628	97%	5e-177	97%
EU111756.1	Bartonella queenslandensis strain AUST/NH8 16S ribosomal RNA gene,	628	628	97%	5e-177	97%
EU111755.1	Bartonella queenslandensis strain AUST/NH5 16S ribosomal RNA gene,	628	628	97%	5e-177	97%
EU111754.1	Bartonella queenslandensis strain AUST/NH12 16S ribosomal RNA gene	628	628	97%	5e-177	97%
AB426636.1	Bartonella grahamii gene for 16S rRNA, partial sequence, strain: Cg428	628	628	97%	5e-177	97%
AB426635.1	Bartonella grahamii gene for 16S rRNA, partial sequence, strain: Cg426	628	628	97%	5e-177	97%
AB426634.1	Bartonella grahamii gene for 16S rRNA, partial sequence, strain: PTZB	628	628	97%	5e-177	97%
AB426631.1	Bartonella grahamii gene for 16S rRNA, partial sequence, strain: Tokus	628	628	97%	5e-177	97%
AB426630.1	Bartonella grahamii gene for 16S rRNA, partial sequence, strain: Hokka	628	628	97%	5e-177	97%
AB426629.1	Bartonella grahamii gene for 16S rRNA, partial sequence, strain: Hokka	628	628	97%	5e-177	97%
AM260525.1	Bartonella tribocorum CIP 105476 complete genome, type strain CIP 1	628	1257	97%	5e-177	97%
AY993936.1	Bartonella sp. RF255YX 16S ribosomal RNA gene, partial sequence	628	628	97%	5e-177	97%
NR_025278.1	Bartonella tribocorum strain IBS 506 16S ribosomal RNA, partial seque	628	628	97%	5e-177	97%
Z70004.1	Bartonella sp. 16S rRNA gene (strain C7rat)	628	628	97%	5e-177	97%
Z70008.1	Bartonella sp. 16S rRNA gene (strain C5rat)	628	628	97%	5e-177	97%
AB529507.1	Bartonella grahamii gene for 16S rRNA, partial sequence, strain: cg147	623	623	97%	2e-175	96%
AB529498.1	Bartonella grahamii gene for 16S rRNA, partial sequence, strain: Korea	623	623	97%	2e-175	96%
AB529494.1	Bartonella sp. Shimane 84-1 gene for 16S rRNA, partial sequence	623	623	97%	2e-175	96%
AB529490.1	Bartonella sp. Okinawa 19-1 gene for 16S rRNA, partial sequence	623	623	97%	2e-175	96%
AB440632.1	Bartonella japonica gene for 16S rRNA, partial sequence	623	623	97%	2e-175	96%
EU111749.1	Bartonella rattaustraliani strain AUST/NH4 16S ribosomal RNA gene, pa	623	623	97%	2e-175	96%

Fig. 50 Scores for the BLAST of the Real Time PCR positive results

The result for the Nested PCR 16s rRNA partial sequence with 380bp (Fig. 51 and 52):

TACCCNGGNGGGGTGNNNGNTTNTACGNAANTGGATGAGCCCGCGTTGGATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCATAGCTGGTCTGAGAG
GATGATCAGCCACACTGGGTACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
GAGNGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGAG
TGATGAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAA
CCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGG
GGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCATGTAGGCGGATNATTTAA

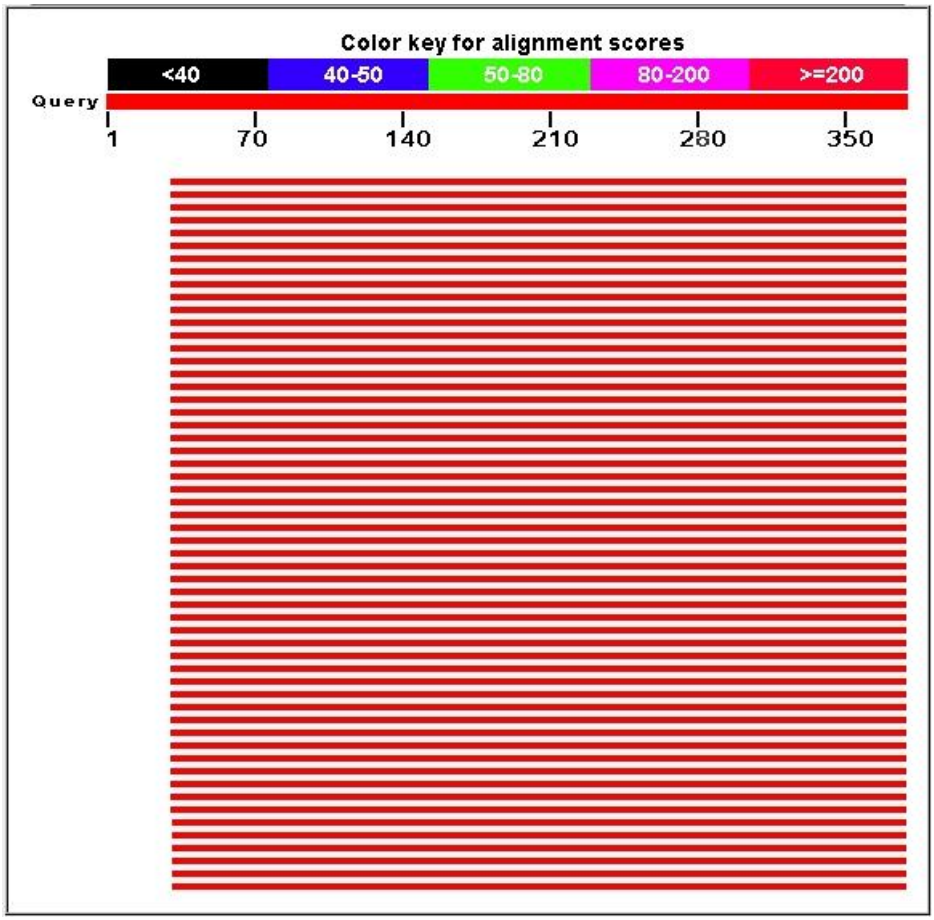


Fig. 51 Colour key for the alignment scores of the Nested PCR positive results

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ645426.1	Bartonella henselae strain M40SHD 16S ribosomal RNA gene, partial se	623	623	91%	2e-175	98%
AY513504.1	Bartonella henselae strain 882_ANT5 16S ribosomal RNA gene, partial	623	623	91%	2e-175	98%
BX897699.1	Bartonella henselae strain Houston-1, complete genome	623	1246	91%	2e-175	98%
AF214556.1	Bartonella henselae 16S ribosomal RNA, partial sequence	623	623	91%	2e-175	98%
AJ223780.1	Bartonella henselae 16S rRNA gene, isolate FR97/K7	623	623	91%	2e-175	98%
AJ223779.1	Bartonella henselae 16S rRNA gene, isolate FR96/BK38	623	623	91%	2e-175	98%
AJ223778.1	Bartonella henselae 16S rRNA gene, isolate FR96/BK3	623	623	91%	2e-175	98%
M73229.1	Rochalimaea henselae 16S ribosomal RNA, partial sequence	623	623	91%	2e-175	98%
Z11684.1	R.henselae 16S rRNA gene	619	619	91%	3e-174	98%
HM481198.1	Bartonella doshiae strain ZJ03/2009 16S ribosomal RNA gene, partial s	617	617	91%	1e-173	98%
AB529507.1	Bartonella grahamii gene for 16S rRNA, partial sequence, strain: cg147	617	617	91%	1e-173	98%
AB529498.1	Bartonella grahamii gene for 16S rRNA, partial sequence, strain: Korea	617	617	91%	1e-173	98%
AB529490.1	Bartonella sp. Okinawa 19-1 gene for 16S rRNA, partial sequence	617	617	91%	1e-173	98%
AB519066.1	Bartonella washoensis gene for 16S rRNA, partial sequence, strain: ER1	617	617	91%	1e-173	98%
CP001562.1	Bartonella grahamii as4aup, complete genome	617	1235	91%	1e-173	98%
FJ577650.1	Bartonella sp. Tel Aviv Rr 16S ribosomal RNA gene, partial sequence	617	617	91%	1e-173	98%
EU979532.1	Bartonella sp. Cr28647 16S ribosomal RNA gene, partial sequence	617	617	91%	1e-173	98%
EU111758.1	Bartonella queenslandensis strain AUST/NH15 16S ribosomal RNA gene	617	617	91%	1e-173	98%
EU111757.1	Bartonella queenslandensis strain AUST/NH11 16S ribosomal RNA gene	617	617	91%	1e-173	98%
EU111756.1	Bartonella queenslandensis strain AUST/NH8 16S ribosomal RNA gene,	617	617	91%	1e-173	98%

Fig. 52 Scores for the BLAST of the Nested PCR positive results

At the end of the sequencing, the BLAST results could confirm the outcomes of the PCR screenings.

5.1.6. Indirect Immunofluorescence-Test

For the IIF-Test 100 samples of each serum library (see Tab. 6) have been tested. This test was developed to detect in vitro human IgG – antibodies against *Bartonella henselae* and *quintana* in human serum or plasma. Two positive controls were used: one control with antibodies against *B. henselae* and the second with antibodies against *B. quintana* (Fig. 53). As a negative control an anti-*Bartonella*-negative solution was provided with the EUROIMMUN kit (Fig. 54).

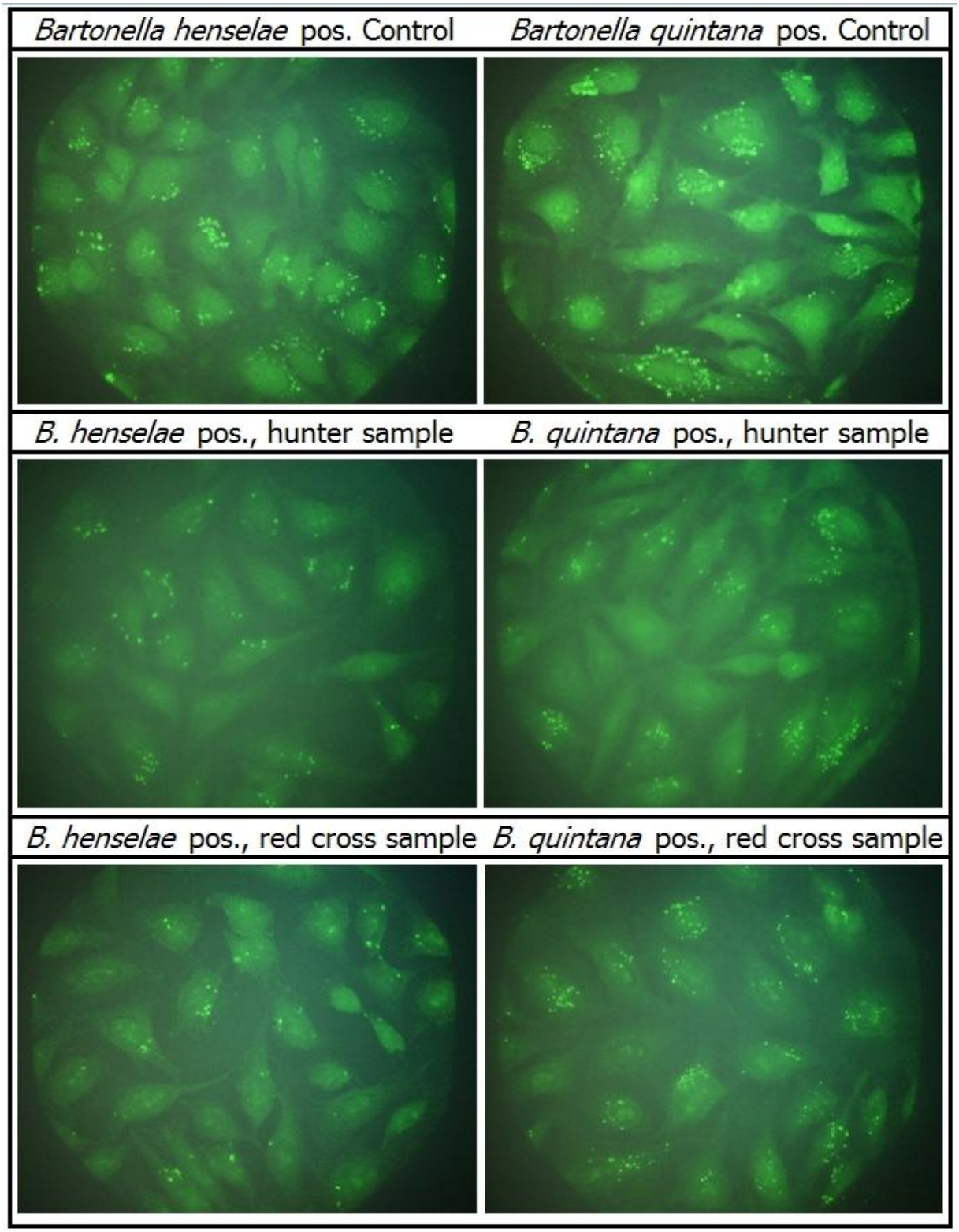


Fig. 53 IIF-Test positive controls and positive samples

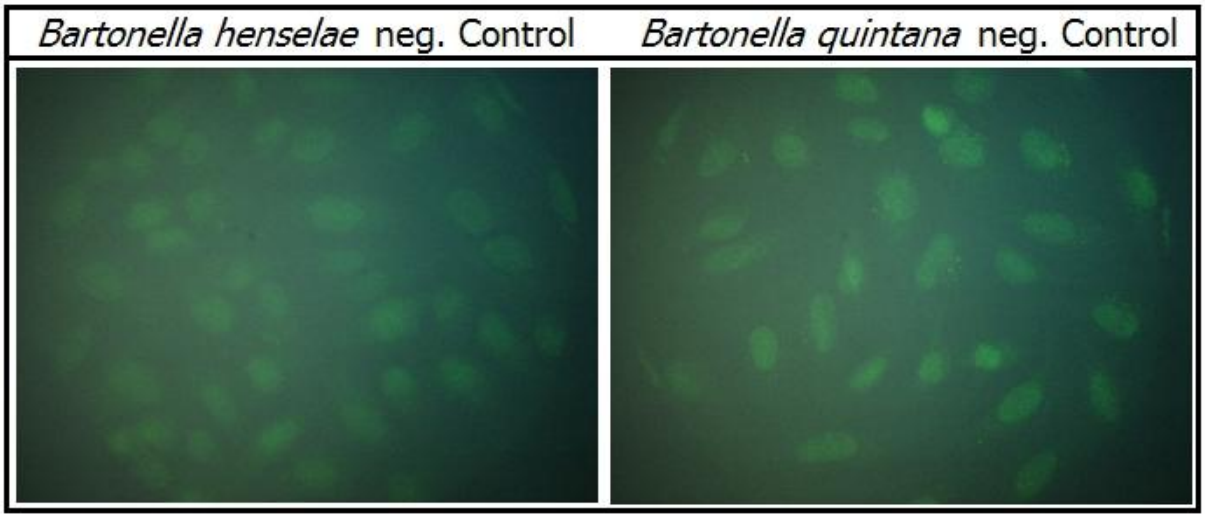


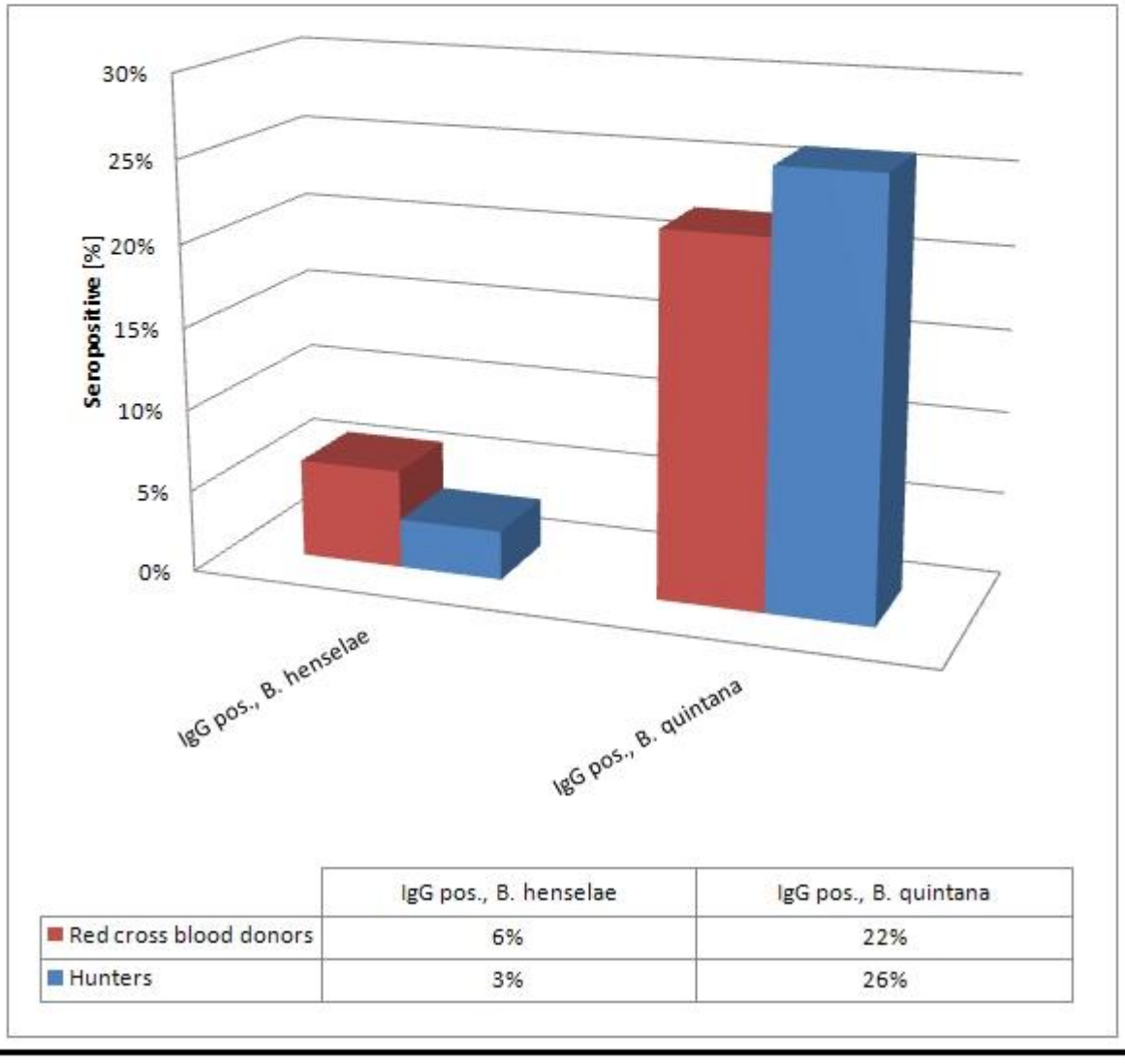
Fig. 54 IIF-Test negative controls

Of the 200 tested sera 57 were positive (Fig. 55). The antibody titer was measured at 1/320 and analysed with a UV-Microscope with 40x magnification. Of the reference serum library 50 men and 50 women of different age have been chosen to receive a representative sample. In the case of the hunters, due to the fact that this profession is mainly a male domain, it was only possible to have 25% women in the sample. In order to compare the results between the two serum libraries with the inclusion of the gender it was necessary to construct a separate table (Fig. 56). The age of the donors reaches from 20 to 89 years. In the last table the focus lies on the positives in comparison with the age of the donors (Tab. 42). The results showed that the mean age with the highest risk to get infected was 38 years in the case of the blood donors and 45 years for hunters. The influence of hunters on these results, considered as a high risk group, can be ignored due to the results of this study. There was no significance that the infection rate of hunters is higher due to their overexposure to ticks. But the infection rate with *B. quintana* was much higher than with *B. henselae*. In the case of the gender comparison there was a slightly higher infection rate of the male patients especially with *B. quintana*.

Evaluation of the IIF-Tests - Age Comparison		
	Amount	**Middle Age**
Positive samples total amount	57	42 years
Red cross sera	28	38 years
Hunter sera	29	45 years

Tab. 42 IIF-Test results with an age comparison

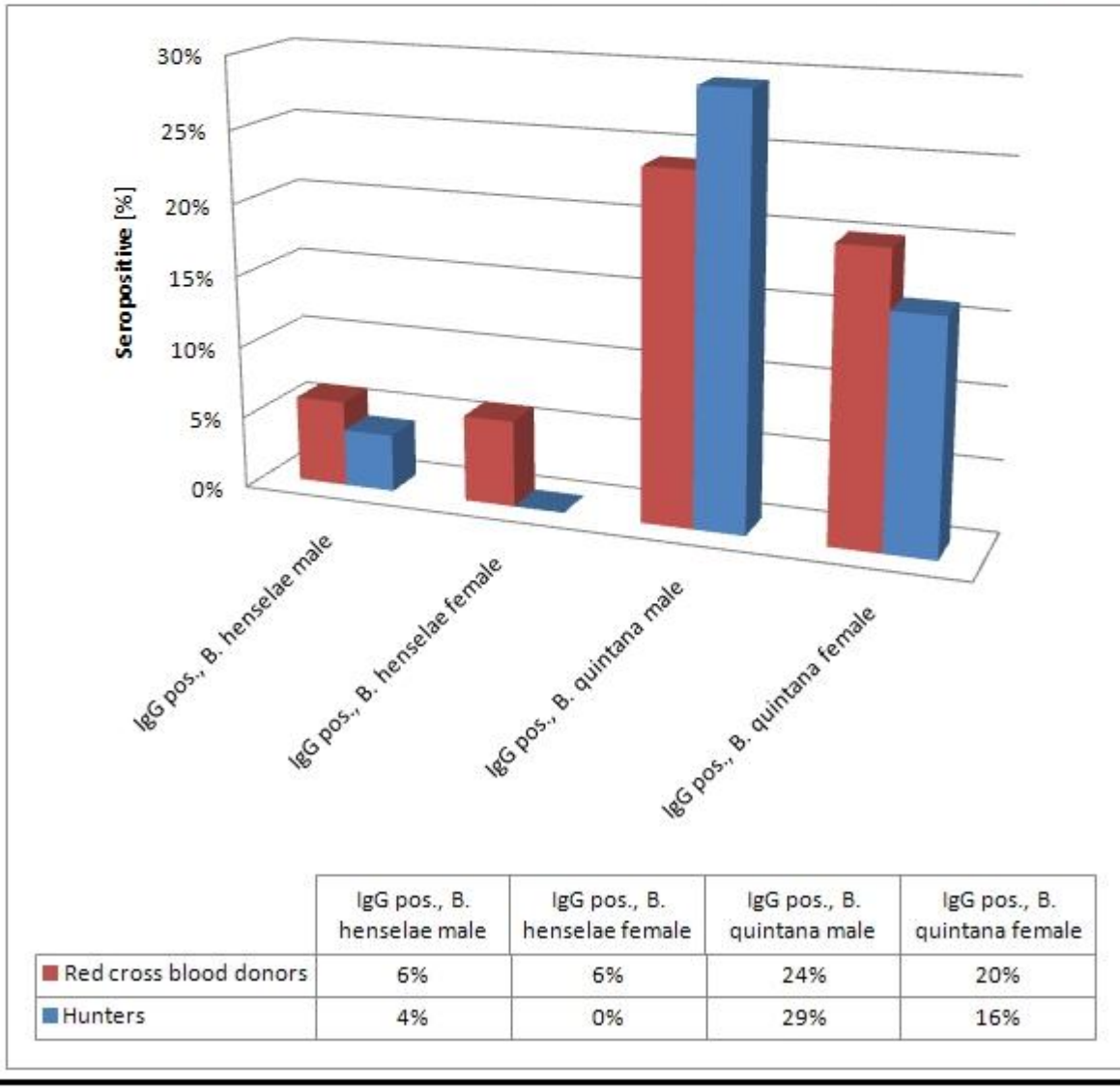
Evaluation of the IIF-Tests				
	Amount		Percentage	
Gender	male	female	male	female
Serum samples total amount	200		-	
Red cross blood donors	50	50	100%	
Hunters	75	25	100%	
Red cross sera	**Amount**		**Percentage**	
IgG Positive, *Bartonella henselae*	6		6%	
IgG Positive, *Bartonella quintana*	22		22%	
Hunter sera				
IgG Positive, *Bartonella henselae*	3		3%	
IgG Positive, *Bartonella quintana*	26		26%	



	IgG pos., B. henselae	IgG pos., B. quintana
Red cross blood donors	6%	22%
Hunters	3%	26%

Fig. 55 IIF-Test results I

Evaluation of the IIF-Tests - Gender comparison				
	Amount		Percentage	
Gender	male	female	male	female
Serum samples total amount	200		-	
Red cross blood donors	50	50	100%	100%
Hunters	75	25	100%	100%
Red cross sera	**Amount**		**Percentage**	
Gender	male	female	male	female
IgG Positive, *Bartonella henselae*	6		-	
	3	3	6%	6%
IgG Positive, *Bartonella quintana*	22		-	
	12	10	24%	20%
Hunter sera				
IgG Positive, *Bartonella henselae*	3		-	
	3	0	4%	0%
IgG Positive, *Bartonella quintana*	26		-	
	22	4	29%	16%



	IgG pos., B. henselae male	IgG pos., B. henselae female	IgG pos., B. quintana male	IgG pos., B. quintana female
Red cross blood donors	6%	6%	24%	20%
Hunters	4%	0%	29%	16%

Fig. 56 IIF-Test results II with a gender comparison

6. Discussion

The aim of this study was to found a proper screening method for *Bartonella* species in ticks. Furthermore the relevance of *Ixodes ricinus* as possible tick vector should be investigated.

A PCR screening method was successfully found in the present study. Due to the results of the IIF screening there was no evidence that *Ixodes ricinus* serves as vector for *Bartonella* species.

6.1. Tick DNA Extraction and *Bartonella* cultivation

The tick DNA extraction with the DNeasy Blood and Tissue Kit after the protocol of Beati and Keirans resulted in a high yield [221]. It is a fast and reliable method to extract tick DNA.

As stated of Heller and colleges *Bartonella* cultivation requires sensitive culture methods for the optimal recovery of these bacteria. They used blood agar plates for *Bartonella* cultivation but had problems to cultivate *B. clarridgeiae* and hence had to work with a second cultivation method, the BACTEC Peds Plus vial (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) [222].
In the present work even *B. clarridgeiae* could have been cultivated with blood agar plates. But there was a significant difference in the cultivation time. So if cultivating *B. clarridgeiae* it has to be considered that this species takes approximately 10 days longer for the first visible colonies.

6.2. PCRs

Because of their fastidious nature, *Bartonella* species are difficult to differentiate on the basis of phenotypic properties; for example, all are more or less inert in the standard biochemical tests used in bacterial identification. However, a number of PCR-based methods for the identification and delineation of *Bartonella* species have been described, targeting a range of

genetic loci including fragments of 16S rRNA gene, the 16-23S rRNA gene intergenic transcribed spacer region (ITS), the citrate synthase-encoding gene (gltA), the 60-kDa heatshock protein-encoding gene (groEL), a cell division protein-encoding gene (ftsZ), the RNA polymerase beta subunit-encoding gene (rpoB) and others [23, 52, 158, 196, 216, 217, 223, 224, 225]. So until now more than seven different target sequences and even more primer sets are in use. As a matter of fact, there is no standard procedure for the identification and delineation of *Bartonella* species.

The primer design of this work showed good results for the *Bartonella* screening. The primer p24 E was used in the study of Relman et al and Schouls et al [23, 196]. As a second primer they used p12B which produced an amplicon of 241bp length. The short amplicon length especially with the 16S rRNA gene as a target sequence resulted in similarities with other eubacteria; especially *Brucella abortus*. To prevent these high similarities the second primer for this study, p24 E2, was designed in a more distant and specific location on the 16s rRNA gene. The resulting amplicon sequence was 437 to 438bp long with much fewer similarities to other eubacteria.

Except from the primer set Bart 2 and 1R all primer pairs showed good results with a focus on p24 E and E2 (Tab. 43). But overall, after the results of this study, p24 E and E2 should be used for *Bartonella* screening in ticks. This would provide a good basis for a standard procedure for the identification and delineation of *Bartonella* species.

Target Sequence	Primer
1 - 16S rRNA Gen	p24 E [196]
	p24 E2
2 - 16S rRNA Gen	BTNi F [216]
	BTNi R
3 - 16S rRNA Gen	Bart 2
	Bart 1R
4 - 16-23S Intergenic Spacer Region	321 s [217]
	321 as
5 - Riboflavin Synthase Gen	Barton 1[158]
	Barton 2 [158]
6 - 16S rRNA Gen	N-p24 E
	N-p24 E2
7 - 16S rRNA Gen	p24 E [196]
	p24 E2-RT

Tab. 43 Overview primer sets with their target sequences

The ITS region, which lies between the 16S and 23S rRNA genes within the rRNA encoding operon, has been characterized in several *Bartonella* species and has been found to be somewhat larger than observed among other bacteria, ranging between 906 and 1529bp [217]. As most of the ITS is noncoding, it is prone to hyper-variability and thus interspecies and interstrain ITS sequence variation is markedly higher than that observed at other genetic loci [54]. Birtles and colleagues described the use of PCR-based amplification of ITS fragments to detect and identify *Bartonellae* in the blood of rodents. Direct detection was of particular use in the longitudinal survey of *Bartonella* bacteraemia that involved the field collection of very small amounts of blood from wild-living rodents [217, 223].

So the ITS region is a proper target sequence for *Bartonella* detection and differentiation. Results with the ITS region as target sequence couldn't be achieved in this study do to unknown interference factors. As already mentioned in chapter 5.1.4.4 - PCR for species differentiation, the results of the primer design did not correlate with the PCR results. There should not occur any unspecific primer binding and hence no unwanted amplicon products. Step per step the primers had been tested again with good results. All reagents of the master-mixture had been discarded and new ones have been used to eliminate the possibility of a contamination. Furthermore another laminar flow cabinet was used. But in the end the ITS - PCR screening results stayed non evaluable.

However the *Ixodes ricinus* tick screening for the presence of *Bartonella* DNA showed good results with the new established PCRs. In the case of the Nested PCR 23 out of 216 ticks could have been tested *Bartonella* DNA. This corresponds to a percentage of 10.6%. The rate of infection had its highest level in female adult ticks and decreased from male adult ticks to nymphs and larvae. This tendency correlates with other studies like the one of Hercík and colleagues [226]. It was an Ixodid tick screening study of 2003-2005. 327 ticks have been collected in different urban and suburban areas of Czechia. Half of the collected ticks had been male and female adults and the other half nymphs. They used Nested PCR techniques with the 16S rRNA gen and the ITS region as a target sequence. It resulted in a rate of infection of 1.2%, 2 males, one female and one nymph. The infection rate was much lower than in Austria but the tendency was the same. This decrease of the rate of infection from adult ticks over nymphs to larvae had been observed in many studies [196, 199, 200, 202, 216 226]. Especially the work of Halos and colleagues of France showed this significance. They had investigated 94 *Ixodes ricinus* ticks, 18 adults and 76 nymphs, in the Lille area in 2002. The screening was performed not only for *Bartonella* sp, but also for *Borrelia burgdorferi*

sensu lato and *Babesia* sp. For all three pathogens adult ticks showed a significantly higher infection rate with 67% compared to nymphs with 15% [202]. There are several hypotheses to explain this difference. One favoured explanation is the number of potentially infected meals which is higher for adults than for nymphs (two blood meals for adults versus one for nymphs). This could either be related to the existence of stage-specific factors, favouring the acquisition of the pathogen by the tick at a special stage as previously shown for other vector arthropods such as tsetse flies [202, 227].

The availability of improved molecular techniques for the characterization of *Bartonellae* led not only to changes in taxonomy but also promoted the discovery of several new species [217]. Today, an ever increasing number of *Bartonellae*, infecting a wide range of mammalian hosts, continue to be encountered [228, 229].

6.3. IIF-Screening

For the IIF screening 200 sera had been tested with a EUROIMMUN dual kit. 57 of them were positive for *Bartonella quintana* and / or *Bartonella henselae* IgG – antibodies. The antibody titer was measured at a ratio of 1/320. With a focus on the age of the positive patients, the mean age for the highest risk to get infected was 38 years for the blood donors and 45 years for the hunters (Tab. 44). In the case of the gender comparison there was a slightly higher infection rate of the male patients especially with *B. quintana* of the hunters but with no significance due to the differing gender values of the hunters.

	Evaluation of the IIF-Tests					
	Positive Samples		**Gender dependent**		**Age dependent**	
	Percentage		**Percentage**		**Percentage**	
Gender	male	female	male	female	male	female
Serum samples total amount			-		-	
Red cross blood donors	100%		100%	100%	100%	
Hunters	100%		100%	100%	100%	
Red cross sera	**Percentage**		**Percentage**		**Percentage**	
IgG Positive, *Bartonella henselae*	6%		6%	6%	28% with a mean age of 38	
IgG Positive, *Bartonella quintana*	22%		24%	20%		
Hunter sera						
IgG Positive, *Bartonella henselae*	3%		4%	0%	29% with a mean age of 45	
IgG Positive, *Bartonella quintana*	26%		29%	16%		

Tab. 44 Overview results of the IIF - Assay

In the work of Yilmaz and colleagues the aim was to investigate the seroprevalence of *B. henselae* in volunteer blood donors and the related risk factors. Seropositivity was detected in 6% (48/800) of the donors. This correlates with the findings of *B. henselae* positives in this study (see Tab. 44). But their study didn't find any significant statistical differences in terms of age, sex, chronic disorders, sport activities, outside behaviours, being injured by any wild or domestic animals, working outdoors, geographical properties of the area of inhabitancy, hunting and travelling [230].

Pons and colleagues examined serum samples of 218 patients in their study. 99 were female and 119 male, with a mean age of 34 to 36 years (range 0–91 years). Nineteen (8.7%) reacted with *B. henselae* antigens. Of all the factors concerning the seroprevalence rate being studied (age, sex, contact with animals, residential area), only age was statistically significant. Their results show a good compliance to this work; especially their findings regarding the statistically significant association between *B. henselae* seropositivity and age. Their observed *B. henselae* seropositivity was significantly more prevalent in subjects aged 30 – 44 years [231].

Further seroprevalence values for *B.* henselae reported by other investigators: 30% by Sander et al. [232] in Germany, 19.8% by Tea et al. [233] in Greece, 24.7% by Garcia-Garcia et al. [234] in Sevilla, Spain, 3.0% by McGill et al. [235] in Sweden and 5.88% by Blanco Ramos [236] in La Rioja, Spain. Two of these studies also screened for *B.* quintana IgG antibodies with the following results: Tea et al. 15% [233] and McGill et al. 0.2%. So if these values are compared to the results of this study in Austria the seroprevalence has a much lower level than in most of the other European countries. Whereas the value of the *Bartonella quintana* seropositivity is higher than in Greece and especially than in Sweden.

The influence of hunters on this works results, considered as a high risk group, can be ignored. There was no significance that the infection rate of hunters is higher due to their overexposure to ticks.

But vector biologists and epidemiologists have suggested that ticks may have a role in *Bartonella* transmission [237]. In 1996 Kruszewska et al. reported the preliminary finding of a *Bartonella* strain in *Ixodes ricinus* ticks from a park in Walz, Poland [238]. Through restriction fragment length polymorphism – PCR (RFLP-PCR) it was detected as *Bartonella bacilliformis*. Unfortunately, the strain has not been further characterized. More recently, different *Bartonella* species, including *B. quintana*, *B. henselae*, *B. washoensis*, and *B.*

vinsonii suspecies *berkhoffii*, have been detected in 19.2% of *Ixodes pacificus* ticks collected in California by amplification and sequencing of a fragment of the gltA gene [198]. In a study conducted by Lucey et al. in 1992 ticks had been suspected of being vectors of *B. henselae*. The tested patients recalled a tick bite but had no history of cat contacts. The authors reported a high *B.* henselae bacteremia level in these patients [239]. Ticks were also reported as possible source of infection in some human cases of concurrent infections of the central nervous system by *Borrelia burgdorferi* and *Bartonella henselae* [197]. So the evidence that ticks may serve as *Bartonella* vectors appears to be rapidly accumulating. As a consequence I would not proclaim that ticks cannot serve as *Bartonella* vectors due to the results of this study. It only means that there was no significance in this test series.

It will require much more work in the field of standardised *Bartonella* detection and differentiation methods as well as the construction of suitable test groups to verify if ticks serve as vector for *Bartonella* species.

7. Abstract in German

DETEKTION VON PATHOGENEN *BARTONELLA* SPEZIES IN ÖSTERREICHISCHEN *IXODES RICINUS* ZECKEN

Bartonellen sind Gram-negative, fakultativ-intrazelluläre aerobe Stäbchen-Bakterien und sind hauptsächlich als Erreger der Katzenkratzkrankheit bekannt, eine durch Bartonella henselae ausgelöste Erkrankung des Menschen, mit Lymphadenopathie und Fieber. Die Übertragung erfolgt entweder direkt über Katzen, welche für *Bartonella henselae* als Reservoirwirt dienen oder indirekt über den Katzenfloh. Mittlerweile hat man jedoch erkannt, dass viele weitere Bartonella-Spezies Bedeutung als Krankheitserreger des Menschen haben. Von den derzeit beschriebenen 24 *Bartonella* Arten sind zumindest 9 Arten humanpathogen. Bartonellen verursachen neben der Katzenkratzkrankheit auch die Carrión Krankheit, das Schützengrabenfieber (Trench fever), die Bazilläre Angiomatose, und Endokarditis. Auch Zecken kommen als Vektoren von Bartonellen in Betracht, wobei hier Risikogruppen wie Jäger und Forstarbeiter besonders hervorzuheben sind. Weitere *Bartonella* Vektoren neben der Zecke und dem Katzenfloh sind die Kleiderlaus und die Sandfliege.

Das Ziel der vorliegenden Studie war es, die Durchseuchungsrate österreichischer Zecken mit Bartonellen zu erheben. Außerdem ist die Prävalenz von Antikörpern gegen Bartonellen in Risikogruppen (beispielsweise Jäger) ermittelt worden. Zunächst wurden zwei PCR-Systeme etabliert, welche es ermöglicht haben Bartonellen in Zecken und deren Wirten nachzuweisen. Ein Nested PCR Verfahren und eine Real Time PCR wurden eingesetzt und verglichen. Für den Nachweis und auch die Differenzierung der *Bartonella* Spezies wurden verschiedene Zielgene (16S rRNA, 16S-23S intergenic spacer) verwendet und jeweils spezifische Primer entwickelt. Die nötige Kontroll-DNA für die Etablierung der PCR und die weiterführenden Untersuchungen wurde aus *Bartonella* Kulturen gewonnen. Hierzu wurden Plattenkulturen der Arten *Bartonella henselae, B. grahamii, B. doshiae* und *B. clarridgeiae* angelegt. Die Bakterien wurden in der exponentiellen Wachstumsphase von den Platten geerntet und deren DNA isoliert.

Aus allen 9 Bundesländern von Österreich gesammelte Adulte, Nymphen und Larven der Zecke Ixodes ricinus wurden auf Bartonella sp. untersucht. Von 216 getesteten Zecken Proben, bestehend aus 8 Adulten, 8 Nymphen und 8 Larven jedes Bundeslands, konnten 16 Positive bei der Real Time PCR und 23 Positive bei der Nested PCR detektiert werden. Dies

entspricht einer Durchseuchungsrate von 7,4% im Falle der Real Time PCR und 10,4% bei der Nested PCR. Des Weiteren konnte gezeigt werden, dass die Durchseuchungsrate von Larven über Nymphen zu Adulten Zecken markant angestiegen ist.

Im zweiten Teil der Arbeit wurden zwei Serotheken auf Antikörper gegen *Bartonella* getestet mithilfe eines Immunfluoreszenz Tests (IIF-Test für *B. henselae* und *B. quintana* IgG, Euroimmun Biochip Kit). Die erste Serothek bestand aus 891 Jäger Seren aus dem Jahr 2002 und die Zweite, welche als Referenz verwendet wurde, aus 624 Seren von Blutspendern des Roten Kreuzes aus dem Jahr 2006. Das Resultat war eine leicht höhere Durchseuchungsrate innerhalb der Jäger als Risikogruppe; 13,5% seropositve Jäger im Vergleich zu 11,5% der Blutspender.

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9. Curriculum vitae

1979.03.22	in Klagenfurt, Kärnten, Österreich
1985 bis 1989	Besuch der Volkschule Klagenfurt – Viktring
1989 bis 1997	Besuch des Bundesrealgymnasiums Klagenfurt – Viktring
1997	Matura im naturwissenschaftlichen Zweig
1997 bis 1998	Wehrdienst mit Ausbildung zum Rettungssanitäter
1998 bis 2010	Diplomstudium Biologie, Universität Wien
	Schwerpunkt Genetik / Mikrobiologie
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2000 bis 2006	Arbeit als Outdoor – Experience Trainer in Degi's Abenteuerschule
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2001/08 bis 2001/09	Labortätigkeit, Firma Clariant, Burgkirchen, Deutschland
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