

RESEARCH

Open Access

Absence of zoonotic *Bartonella* species in questing ticks: First detection of *Bartonella clarridgeiae* and *Rickettsia felis* in cat fleas in the Netherlands

Ellen Tijssse-Klasen¹, Manoj Fonville¹, Fedor Gassner³, Ard M Nijhof^{6,7}, Emil KE Hovius⁴, Frans Jongejan⁶, Willem Takken³, Johan R Reimerink², Paul AM Overgaauw^{5*} and Hein Sprong^{1*}

Abstract

Background: Awareness for flea- and tick-borne infections has grown in recent years and the range of microorganisms associated with these ectoparasites is rising. *Bartonella henselae*, the causative agent of Cat Scratch Disease, and other *Bartonella* species have been reported in fleas and ticks. The role of *Ixodes ricinus* ticks in the natural cycle of *Bartonella* spp. and the transmission of these bacteria to humans is unclear. *Rickettsia* spp. have also been reported from as well ticks as also from fleas. However, to date no flea-borne *Rickettsia* spp. were reported from the Netherlands. Here, the presence of *Bartonellaceae* and *Rickettsiae* in ectoparasites was investigated using molecular detection and identification on part of the *gltA*- and 16S rRNA-genes.

Results: The zoonotic *Bartonella clarridgeiae* and *Rickettsia felis* were detected for the first time in Dutch cat fleas. *B. henselae* was found in cat fleas and *B. schoenbuchensis* in ticks and keds feeding on deer. Two *Bartonella* species, previously identified in rodents, were found in wild mice and their fleas. However, none of these microorganisms were found in 1719 questing *Ixodes ricinus* ticks. Notably, the *gltA* gene amplified from DNA lysates of approximately 10% of the questing nymph and adult ticks was similar to that of an uncultured *Bartonella*-related species found in other hard tick species. The *gltA* gene of this *Bartonella*-related species was also detected in questing larvae for which a 16S rRNA gene PCR also tested positive for "*Candidatus* Midichloria mitochondrii". The *gltA*-gene of the *Bartonella*-related species found in *I. ricinus* may therefore be from this endosymbiont.

Conclusions: We conclude that the risk of acquiring Cat Scratch Disease or a related bartonellosis from questing ticks in the Netherlands is negligible. On the other hand fleas and deer keds are probable vectors for associated *Bartonella* species between animals and might also transmit *Bartonella* spp. to humans.

Background

Bartonella species are facultative intracellular Gram-negative bacteria which can infect humans and a wide range of animal species. Cat-scratch disease (CSD) is probably the most common *Bartonella* infection in the northern hemisphere [1,2]. The hallmark of CSD is enlargement and tenderness of lymph nodes draining

the site of inoculation of the microorganism [3]. Regional lymphadenopathy usually develops 2 to 3 weeks after exposure and normally resolves spontaneously after several months [4]. Thirty percent of the patients report low-grade fever and a skin or mucous membrane lesion may be observed at the site of inoculation for 25% to >90% of patients [3,5]. Extranodal clinical manifestations, such as encephalopathy, neuroretinitis, arthritis, and lytic bone lesions, occur in approximately 10% of patients [3,5-8]. Furthermore, *Bartonella* spp. are an important cause for blood-culture negative endocarditis [9-11]. Disease symptoms depend on the immune status of the host; in immuno-compromised hosts, the bacteria

* Correspondence: P.A.M.Overgaauw@uu.nl; HSprong@gmail.com

¹Laboratory for Zoonoses and Environmental Microbiology, National Institute for Public Health and Environment (RIVM), Bilthoven, The Netherlands

⁵Institute for Risk Assessment Sciences, Division of Veterinary Public Health, Utrecht University, Utrecht, The Netherlands

Full list of author information is available at the end of the article

are often present in blood and involved in angioproliferative disorders such as bacillary angiomatosis and peliosis hepatis [12]. The domestic cat is the major reservoir of *Bartonella henselae*, with a confirmed link to disease in humans [13,14]. Infected cats are usually asymptomatic and develop relapsing bacteraemia for long periods [15]. In a Dutch study, half of the cats were serological positive, and 22% were found to have a *B. henselae* bacteremia [16]. Bites or scratches from infected cats are associated with development of CSD. Cat fleas, *Ctenocephalides felis*, are involved in transmission between cats and may be also able to transmit *B. henselae* to humans: Approximately 30% of patients with CSD do not recall traumatic cat contact [17-19]. Solid evidence to support transmission via cat fleas is lacking. Other *Bartonella* species, including *Bartonella clarridgeiae* and *Bartonella grahamii*, have also been linked to human disease. However, there are only few reports of disease cases linked to these species [20-23].

The number of cases with tick-borne diseases in the Netherlands is on the rise [24]: This is illustrated by the fourfold increase in reported cases of erythema migrans since 1994, up to 22,000 patients in 2009. The most straightforward explanation is the reported increase in the incidence of tick bites [24]. The same tick species transmitting the etiologic agents of Lyme disease may also serve as vector of the causative agent of CSD and maybe also other *Bartonella* species [25-27]. Several PCR-based studies have demonstrated *B. henselae* DNA in various Ixodid tick species [26,28-32]. A recent study demonstrated that *B. henselae* can be transmitted across the developmental stages of *Ixodes ricinus* [33]. Altogether, these studies imply that CSD can be acquired from ticks, but studies contradicting this conclusion have also been published [34]. One of the aims of this study is to investigate whether *Bartonellaceae*, particularly *B. henselae*, are present in *I. ricinus* ticks in the Netherlands and form a risk to public health.

Another class of pathogens that can be transmitted by both Ixodid ticks and cat fleas is *Rickettsiae*. They are fastidious, mostly obligate intracellular alpha-proteobacteria. Hard ticks (Ixodidae) have been identified as vectors of the spotted fever syndrome in humans, which is caused by at least 15 different *Rickettsia* species [35]. Two infamous members of this group are *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever, and *Rickettsia conorii*, the causative agent of Mediterranean spotted fever [36]. *Rickettsia helvetica* is the most prevalent rickettsial species found in *I. ricinus* ticks in the Netherlands [37,38]. To date, the pathogenic potential of *R. helvetica* is unclear but infection with *R. helvetica* has been suspected in acute perimyocarditis, unexplained febrile illness, sarcoidosis and recently also meningitis [39-47]. Laboratory diagnosis of rickettsioses

is predominantly based on serology. Currently, micro-immunofluorescence is considered as a reference serological assay, but most commercially available tests offer a very limited set of antigens, mostly *R. rickettsii* and/or *R. conorii*, and serological cross-reaction with other rickettsial pathogens are common. Notably, some patients suspected to have (suffered from) a (mild) rickettsiosis do not recall a tick bite. In these cases, other potential sources of infection, for example cat fleas, might be involved. Cat fleas may maintain and transmit *Rickettsia felis* which is the causative agent of flea-borne spotted fever, also called cat flea typhus or summer flu [48]. Clinical signs are similar to those of murine typhus and other febrile illnesses [49]. Patients usually have fever, fatigue, headache, myalgia, rash and elevation of liver enzymes, although these clinical manifestations do not occur in all patients. They can also present abdominal pain, pleuric chest pain, diarrhea, nausea, vomiting, conjunctivitis, and neurological symptoms [50-54]. *R. felis* appears to have a global distribution [48], and it is not unlikely that *R. felis* is also present in the Netherlands. Nevertheless, evidence for the presence of *R. felis* in Dutch cat fleas is lacking and no autochthonous clinical cases of flea-borne rickettsiosis have been reported. In this study, we investigated whether *R. felis* is present in Dutch cat fleas.

Methods

Collection of ticks, fleas, deer keds and wildlife samples

Nymphs and adult ticks were collected for recent studies between 2006 and 2010 by flagging vegetation at 16 different locations in The Netherlands (Table 1). Additionally, questing ticks from all stages were collected from vegetation in Vrouwenpolder in October 2010.

Ticks from cats were collected by veterinarians between 2006 and 2009 as described [55]. More than 200 veterinarian clinics expressed interest in the study and were supplied with information packages containing posters, brochures and collection tubes. Participating clinics were asked to record host species, residence area of the pet owner, date of collection and whether the pets had travelled outside the Netherlands recently.

Deer keds (*Lipoptena cervi*) and attached ticks were collected from red deer by hunters.

All ticks used for this study were identified as *Ixodes ricinus* according to current keys, and life stage and sex of the ticks were recorded.

Tissue samples (ears) were collected from wood mice caught in Duin and Kruidberg area [37].

Fleas from bank voles (*Myodes glareolus*) and wood mouse (*Apodemus sylvaticus*) were derived from a previous study [37].

Fleas from 109 cats and 44 dogs were collected by 15 veterinary clinics and volunteers throughout the

Table 1 Geographic distribution of questing *I. ricinus* ticks (nymphs and adults)

Location	(Previous study)	Ticks (n)	<i>Bartonella</i> -related spp. (EF662054)
Apeldoorn	Gassner <i>et al.</i> , 2010 [74]	40	4
Bergherbos	Tijssse-Klasen <i>et al.</i> , 2010 [38]	17	0
Duin en Kruidberg	Sprong <i>et al.</i> , 2009 [37]	157	36
Ede	Gassner <i>et al.</i> , 2010 [74]	271	14
Eijsden	Gassner <i>et al.</i> , 2010 [74]	49	2
Gieten	Gassner <i>et al.</i> , 2010 [74]	22	1
Haaksbergen	Gassner <i>et al.</i> , 2010 [74]	15	0
Heumensoord	Tijssse-Klasen <i>et al.</i> , 2010 [38]	67	5
Hoog Baarlo	Gassner <i>et al.</i> , 2010[74]	221	12
Hullenberg	Tijssse-Klasen <i>et al.</i> , 2010 [38]	60	6
Kwade Hoek	Gassner <i>et al.</i> , 2010 [74]	122	15
Leusderheide	Tijssse-Klasen <i>et al.</i> , 2010 [38]	140	12
Twiske	Gassner <i>et al.</i> , 2010 [74]	167	25
Veldhoven	Gassner <i>et al.</i> , 2010 [74]	16	1
Wassenaar	Gassner <i>et al.</i> , 2010 [74]	67	3
Vrouwenpolder	This study	96	41
Total		1527	177

Tick lysates were tested for the presence of *Bartonella* DNA using the *gltA*-primers. Samples were analysed by gel electrophoresis. PCR products of 380 bp were sequenced. Only *Bartonella*-related DNA sequences <99% similar to EF662054 were detected.

Netherlands between October 2009 and October 2010. Fleas were identified according to current taxonomic keys and pools of fleas were formed per host animal and per flea species.

Ticks, fleas and deer keds were immersed in 70% ethanol immediately after collection and stored at -20°C until DNA extraction.

DNA extraction

DNA from vegetation ticks, deer keds and fleas collected from cats and dogs was extracted by alkaline lysis as described earlier [56]. DNA of engorged ticks was extracted using the Nucleospin Tissue kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol for the purification of genomic DNA from insects. DNA of fleas from a previous study was extracted by disruption in liquid nitrogen with pestles followed by homogenization in 600 µl buffer RLT using a Qiashredder homogenizer according to the manufacturer's instructions (RNeasy minikit, Qiagen). DNA was extracted from 300 µl homogenate using the QIAamp DNA mini kit (Qiagen). DNA was eluted in 50 µl elution buffer. DNA of tissue samples from mice was extracted with the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

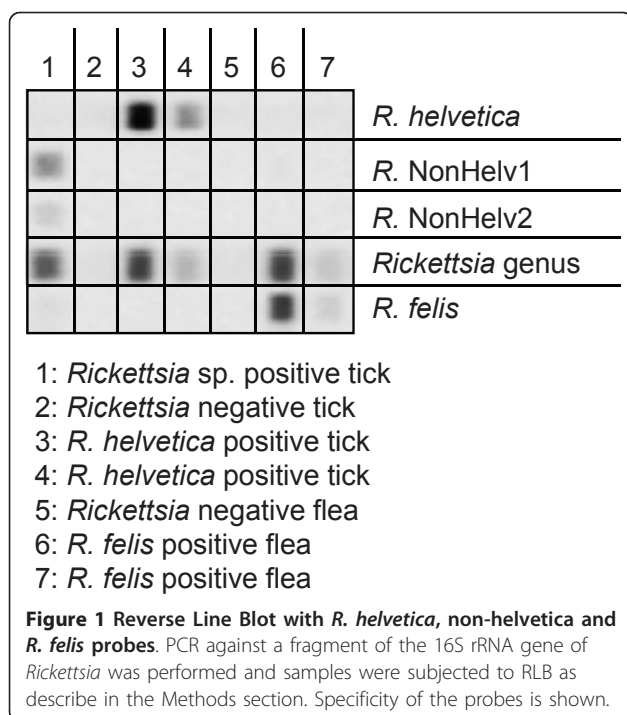
PCR-detection of *Bartonella* spp

Nymphal and adult vegetation ticks, larvae from Vrouwenpolder, ticks collected from cats and deer, deer keds, rodent tissue samples, fleas collected from rodents and

pet animals were tested for the presence of *Bartonella* spp.. *Bartonella* spp. DNA was detected by PCR followed by sequencing as previously described (59). Briefly, the *gltA* gene was amplified using 5'-GGGGAC-CAGCTCA_tGGTGG and 5'-AATGCAAAAAGAACAG-TAAACA as primers, yielding amplification products of approximately 380 base pairs. A serial dilution of the cultivated *B. henselae* ATCC 49882 strain was used as positive control [57]. The highest dilution was used as a positive control in PCR and to spike tick- and flea lysates to identify samples that contain PCR-inhibitory components. Inhibition was negligible in all tick and flea samples. PCR amplification of parts of the 16S rRNA gene was done exactly as described by García-Esteban and colleagues, using 16S-R and P24Emod as primer pairs [58].

PCR-detection of *Rickettsia* spp

Tickets collected from cats as well as fleas from dogs and cats were tested for the presence of *Rickettsia* spp. DNA by PCR followed by reverse line blotting (RLB) as previously described [38] but with minor modification (Figure 1). Briefly, the 16S rRNA gene was amplified using 5'-AACGCTATCGGTATGCTTAACA and 5'-Biotin-ACTCACTCGGTATTGCTGGA as primers. For RLB analysis the following amino labeled probes were used: 5'-TTTAGAAATAAAAAGCTAATACCG (catch all), 5'-CTTGCTCCAGTTAGTTAGT (*R. conorii*), 5'-GCTAATACCATATATTCTCTATG (*R. helvetica*), 5'-GTATATTCTCTACGGAAAAAAG (*Rickettsia* sp. IRS3), and 5'-TATATTCTCTACAGAGGAAAGATT (*R.*



felis). In order to detect potential double infections of ticks with *R. helvetica* and other rickettsial species, two RLB probes which were able to hybridize to DNA of most *Rickettsia* species except for *R. helvetica*: 5'-AATACCGTATATTCTCTACGGA (NonHelv1) and 5'-AATACCGTATATTCTCTGCGGA (NonHelv2). Plasmids containing a 16S rRNA sequence from *R. helvetica*, *R. conorii*, *R. typhi* or *R. prowazekii* were used as positive controls.

Amplification of parts of the citrate synthase gene was done as described by De Sousa and colleagues using cs409d/rp1258n and cs535d/rp1258n as primer pairs [59].

PCR detection of (Candidatus) *Midichloria mitochondrii* and phylogenetic analyses

Part of the 16S rRNA gene of “*Ca. Midichloria mitochondrii*” was amplified from tick lysates of larvae collected in Vrouwenpolder as described [60], using 5'-GCTACAGCTCTTGCCCGT (IrESF) and 5'-CAAAACCGACTCCCATGGC (IrESR) as primers. PCR amplicons were purified with the Qiaquick gel extraction kit (Qiagen Inc.) and sequenced using an ABI PRISM Big-Dye Terminator Cycle sequencing Ready Reaction kit (Perkin Elmer, Applied Biosystems). All sequences were confirmed by sequencing both strands. Sequences were compared with sequences in Genbank using BLAST. Phylogenetic analysis was performed using Bionumerics version 6.1 (Applied Maths, Gent, Belgium). Reference sequences were retrieved from Genbank. Sequences

were aligned using Clustal X and distance-based analyses were conducted using Kimura 2-parameters distance estimates and trees were constructed using the Neighbour-Joining (NJ) algorithm, implemented in the MEGA program version 4.0. Bootstrap proportions were calculated by the analysis of 1000 replicates for NJ trees.

With the exception of the 16S rRNA PCR done for *Rickettsia* sp., which were analyzed by RLB, all PCR products were separated and analyzed by TAE agarose gel-electrophoresis. To minimize cross contamination and false-positive results, positive and negative controls were included in each batch tested for *Bartonella* or *Rickettsia* by the PCR. In addition, DNA extraction, PCR mix preparation, sample addition, and PCR analysis were performed in separated, dedicated labs.

Results

Vegetation ticks

To investigate the presence of *Bartonella henselae* in the Dutch tick population 1527 *Ixodes ricinus* nymphal and adult ticks from 16 geographically different locations were collected from recent field studies (Table 1).

Vegetation types of most sampling areas have been described previously with the exception of Vrouwenpolder. Vrouwenpolder is a vegetation rich dune area with several species of deciduous trees and shrubs and approximately 70% of the soil was covered with vegetation litter. Only very scarce and incomplete information is available about the fauna of all locations. Nymphs (98%) and adults (2%) were tested for *Bartonella* spp. by PCR on the *gltA* gene. PCR products of the expected size of approximately 380 bp were observed in 177 tick lysates. Sequencing was successful on 98 of these samples. Except for five sequences with one or two point mutations, all these sequences were identical, and 99-100% homologous (372 bp) to an uncultured *Bartonella*-related sp. isolate from *I. scapularis* (Genbank accession number EF662054). Neither *B. henselae* nor any other *Bartonella* species were detected in any of the lysates of these questing ticks.

Next, lysates from 192 questing *I. ricinus* larvae from Vrouwenpolder were subjected to the same PCR against the *gltA*-gene. 41 larvae were positive and their sequences were identical to the *Bartonella*-related sp. from nymphs and adults. Under normal conditions, questing larvae have not yet had a blood meal. Transstadial but not transovarial (vertical) transmission of *Bartonella* has been described in ticks [33]. Therefore, it is unlikely that the *Bartonella*-related sp. in larvae is directly derived from a vertebrate host. The sequences of the *Bartonella*-related sp. might be derived from another microorganism present in ticks, which is capable of vertical transmission. This is supported by the fact that *gltA*-gene of this *Bartonella*-related sp. does

not cluster with other *Bartonella* species (Figure 2). Therefore, the presence of the endosymbiont “*Ca. Midichloria mitochondrii*” in the same larval tick lysates was tested by PCR and sequencing using primers specifically against part of the 16S rRNA gene of “*Ca. Midichloria mitochondrii*” [61]. 56 tick lysates yield a PCR product of the expected size (1250 bp), which successful sequences (n = 43) were all identical to sequences designated to “*Ca. Midichloria mitochondrii*” (AJ566640). An additional, independent PCR using generic primers on the 16S rRNA of *Bartonella* species was performed [58] on the same 192 samples. Five of these samples yielded a PCR product of the expected size (440 bp). Sequencing of these products yielded 5 identical sequences (372 bp) which were 99% similar to a plethora of uncultured bacteria in Genbank, varying from an uncultured *Bartonella* isolated from the gut of *Apis mellifera mellifera* (EU055544), *Phyllobacterium myrsinacearum* (HQ380017) to a presumed *Bartonella grahamii* isolated from *Apodemus agrarius* (AB529498). As so many different microorganisms had similar homology, we concluded that this part of 16S rRNA gene is not suitable for detection and identification of *Bartonella* species.

Ticks from cats

As domestic cats are the major reservoir host of *B. henselae*, adult ticks may acquire this pathogen while feeding on cats. 265 adult *I. ricinus* ticks removed from domestic cats were tested by PCR. *B. henselae* could not be detected in any of these tick lysates. In 56 lysates a fragment of approximately 380 bp was observed. Sequencing revealed that these samples contained the same *Bartonella*-related sp. as found in questing ticks (Table 2). The tick lysates from cats were also tested for *Rickettsiae* by PCR [62]. PCR products were analyzed by Reverse Line Blotting (RLB), which could differentiate several *Rickettsia* species, including *R. felis* (Figure 1). In 215 lysates *R. helvetica* was detected (Table 2). No other *Rickettsia* species were identified.

Deer keds and ticks from deer

The absence of *Bartonella* in questing ticks seemed contradictory to previous findings in the Netherlands [63], where more than 70% of the ticks collected from red deer were positive for *Bartonella* or closely related species. Therefore, 96 lysates from adult ticks fed on red deer were analysed by PCR on the *gltA* gene. Indeed,

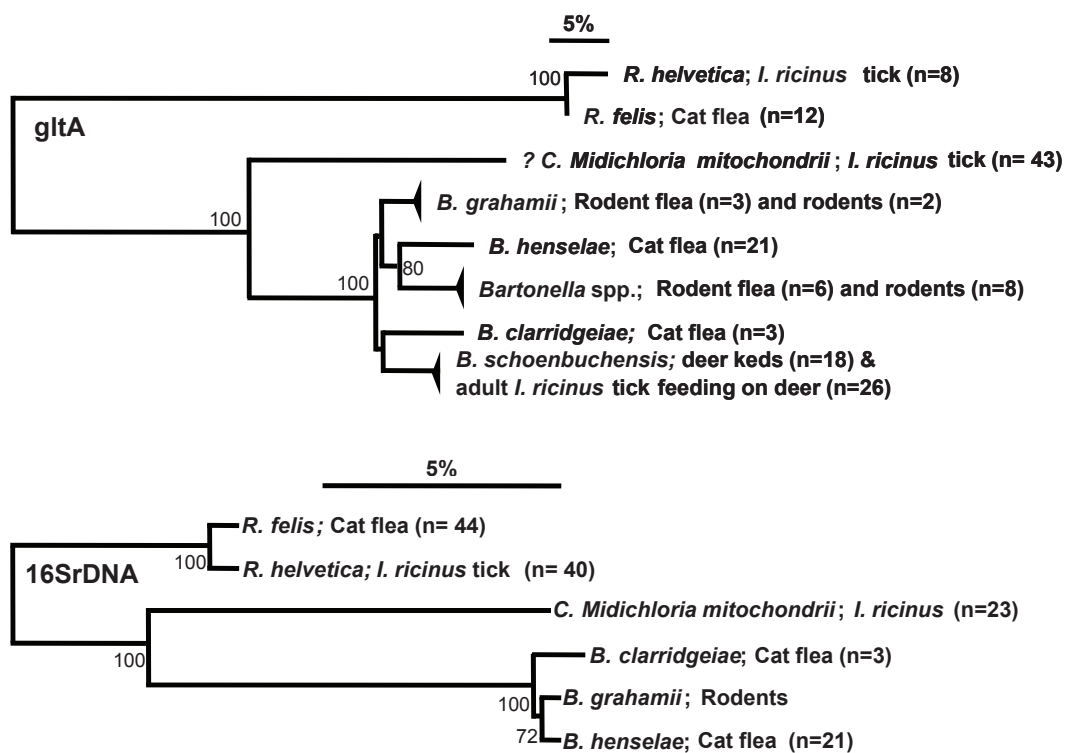


Figure 2 Phylogenetic analysis of the microorganisms found in ectoparasites. Neighbor-joining trees were based on the fragments of the *gltA* and 16S rRNA genes of the microorganisms found in the ectoparasites and rodents described in this study. Sequences were aligned and analysed as described in the Methods section. The number of sequenced isolates is between brackets. Small triangles indicate less than 1% variation. Only bootstrap values >75 are indicated. The *Bartonella*-related sp. found in ~ 10% of the questing ticks is indicated in the *gltA*-tree as “*Ca. Midichloria mitochondrii*”.

Table 2 *Bartonella* and *Rickettsia* in ticks feeding on cats

Microorganism (DNA)	Positive ticks (%)
<i>Bartonella henselae</i>	0 (0-1.4%)
<i>Bartonella</i> -related sp.	56 (16-27%)
<i>Rickettsia helvetica</i>	215 (76-86%)
<i>Rickettsia felis</i>	0 (0-1.4%)
Number of ticks tested	265

Adult *I. ricinus* ticks were removed from cats tested and tested for *Bartonella* and *Rickettsia* by PCR. *Rickettsia* species were identified by RLB. For *Bartonella*, the PCR products of 380 bp were sequenced and analysed. Six ticks were positive for both *R. helvetica* and *Bartonella*-related sp..

the PCR products of 72 lysates (75%) yielded a 380 bp on the agarose gels. Sequencing revealed that at least 26 ticks contained DNA identical to *B. schoenbuchensis* (AJ564632), and 13 ticks contained the *Bartonella*-related sp. Sequencing was not successful in all cases, probably also because of mixed sequences of both *Bartonella*-related sp and *B. schoenbuchensis* (not shown). As *B. schoenbuchensis* was never detected in questing ticks (Table 1), we wondered how *B. schoenbuchensis* was transmitted between red deer. In the literature, deer keds (*Lipoptena cervi*) have been suggested to transmit *B. schoenbuchensis* between red deer as they have shown to be infected with *B. schoenbuchensis* [64]. We tested whether this was also the case in the Netherlands. For this, 56 deer keds were collected, lysed and a PCR on part of the *gltA* gene was performed. 46 deer keds were found to be positive of *B. schoenbuchensis* (AJ564632).

Tissue samples from rodents

The origin of the *Bartonella*-related sp. from questing ticks was further investigated. One of the most prominent vertebrate hosts of *I. ricinus* are probably rodents. To determine whether mice are a potential reservoir of this *Bartonella*-related sp., 96 DNA samples from ear tissue of small rodents, *Myodes glareolus* and *A. sylvaticus*, from the Duin en Kruidberg area were tested by PCR and sequencing a 380 bp part of the *gltA* gene. Twelve rodent samples were positive, but sequencing revealed that these sequences (n = 5) were 98-99% similar to *Bartonella* isolates from *A. flavicollis* (yellow-necked mouse) from Slovenia (347 bp, DQ155393) and Greece (338 bp, AY435110). The *Bartonella* sequences from these rodents were only 70% similar to the *Bartonella*-related sp. from questing ticks (Figure 2).

Fleas from rodents

PCR of the *gltA*-gene on 24 flea lysates from wild rodents, *M. glareolus* and *A. sylvaticus*, caught in the Netherlands revealed that the *Bartonella* sp. found in *A. sylvaticus* was similar to those found in their fleas (Figure 2). Since the *Bartonella* sequences found in mouse were similar to those found in their fleas, but not similar

to the *Bartonella* sequences found in questing *I. ricinus* ticks, these data suggested that the *Bartonella* found in rodents are most likely transmitted via fleas, not ticks.

Fleas from pet animals

Although the DNA of a laboratory *B. henselae* strain was used as a positive control, we wondered whether the *gltA* PCR was specific and sensitive enough to detect *B. henselae* in questing ectoparasites. For this, fleas collected from pets were also tested under the same conditions as the tick lysates for both *Bartonella* and *Rickettsia*. 32 of 204 pools of cat fleas (*Ctenocephalides felis*) were positive for *B. henselae*. Three pools were positive for *B. clarridgeiae* and 43 pools for *R. felis* (Table 3). The identity of the latter two was confirmed by PCR and sequencing parts of the 16S rRNA and *gltA* genes, respectively (Figure 1). In one out of 17 pools of dog fleas (*Ctenocephalides canis*) DNA of *R. felis* was detected. No *Bartonella* sp. was detected in dog fleas. One third of the sampled dogs (n = 44) and cats (n = 109) carried fleas positive for *R. felis*, and one sixth of the animals had fleas positive for *B. henselae* (Table 4). Three cats had fleas positive for *B. clarridgeiae*.

Discussion

One of the major aims of this study was to investigate whether questing *I. ricinus* ticks transmit *Bartonella* species, particularly *B. henselae*, to humans [31,65,66]. In the 1719 questing ticks that were analysed, *B. henselae* was not found (Table 1). A *gltA* sequence, which was found in approximately 10% of the nymph and adult ticks, was closely related to a sequence found in *I. scapularis* ticks [67]. This sequence was designated previously as a *Bartonella* sp.. Phylogenetic analysis showed that the *gltA* sequence of this *Bartonella*-related species did not cluster with other *Bartonella* species (Figure 2). Furthermore, this *Bartonella*-related sequence was also found in lysates from 41 of 192 questing *I. ricinus* larvae, implying that the sequence is either from an environmental contamination or from a microorganism that is transmitted transovarially. To

Table 3 Pathogens found in fleas from pets

	<i>C. canis</i>	<i>C. felis</i>
Total (number)	48	528
Pools (size)	17 (1-7)	204 (1-17)
<i>B. henselae</i> -positive (pools)	0	32
<i>B. clarridgeiae</i> -positive (pools)	0	3
<i>R. felis</i> -positive (pools)	1	43

Ctenocephalides canis and *C. felis* fleas were separated based on morphological markers and were pooled per animal and flea species. In several cases more than one pool per animal was taken. Fleas were analysed by PCR and sequencing for the presence or absence of DNA from *Bartonella* and *Rickettsia* species.

Table 4 Prevalence of pets with fleas carrying zoonotic pathogens

	Dogs	Cats
Total	44	109
<i>R. felis</i>	13 (30%)	34 (31%)
<i>B. henselae</i>	7 (16%)	17 (16%)
<i>B. clarridgeiae</i>	0	3 (3%)
<i>R. felis</i> & <i>B. henselae</i>	2	3

Data are from Table 3, but now presented as prevalence in cats and dogs with fleas.

the best of our knowledge, transovarial transmission of *Bartonella* has never been demonstrated. Our data support this: *B. schoenbuchensis* was found in 75% of the adult ticks feeding on deer, but never in the questing larvae or nymphs tested in this study. We conclude that it is unlikely that the *Bartonella*-related sequence found in ticks is from a *Bartonella* species. Instead, we propose that the *gltA* sequence found in the questing *I. ricinus* ticks is from “*Ca. Midichloria mitochondrii*”, an endosymbiont found in various hard ticks, including *I. ricinus* [61]. Only a few genes, but not the *gltA* gene, of this microorganism have been amplified and sequenced [68]. The presence of “*Ca. Midichloria mitochondrii*” in Dutch *I. ricinus* ticks was shown here by PCR and sequencing part of its 16S rRNA gene (Figure 2). “*Ca. Midichloria mitochondrii*” is present in mitochondria of tick cells and cannot be isolated or cultured. However, it cannot be excluded that the *gltA* gene isolated from tick lysates is unrelated to “*Ca. Midichloria mitochondrii*”.

In this study, we have identified several *Bartonella* species: *B. henselae* and *B. clarridgeiae*, *B. grahamii* and another rodent-related *Bartonella* species and *B. schoenbuchensis*. As far as we know, the presence of these *Bartonella* species in the Netherlands is described here for the first time, except for *B. henselae* [69]. None of these were detected in this study in questing *I. ricinus* ticks. Statistically, less than 0.2% of the questing ticks might be infected with *B. henselae* or any other *Bartonella* species (95% exact binomial confidence interval). Therefore the risk of contracting bartonellosis from *I. ricinus* ticks in the Netherlands seems to be very low.

Questing *I. ricinus* larvae were subjected to PCR using generic primers on the 16S rRNA of *Bartonella* species [64]. Five of these samples were positive and yielded 5 identical sequences which were 99% similar to a plethora of uncultured bacteria in Genbank, varying from an uncultured *Bartonella* isolated from the gut of *Apis mellifera mellifera*, *Phyllobacterium myrsinacearum* to a presumed *Bartonella grahamii*. This high variety of hits indicates that the 16S rRNA gene has too little resolution power to positively identify a *Bartonella* species or to distinguish *Bartonella* from other, closely related

genera. Furthermore, results of a BLAST depend on the quality of sequences in the database. In an earlier publication we discussed the pitfalls of using a database with only verified sequence types which neglects large amounts of less well verified but valuable data [70]. Using a large and freely accessible database like that of NCBI, however, has its own pitfalls as the quality of submitted sequences is not always good and identities of sequenced species are not always verified. *Bartonella* sp., *Rickettsia* sp. and “*Ca. Midichloria mitochondrii*” are very difficult to culture and some species have not yet been cultured at all. For uncultured microorganisms often only a limited number of genes are known and based on the limited data available it can be difficult to identify a microorganism to genus level. This can lead to misnaming of database entries which in turn can lead to further misidentifications [70].

While ticks seem to play a negligible role in *Bartonella* transmission, other ectoparasites, especially fleas, might be involved in the enzootic cycle of *Bartonella* in animals. *B. henselae* and *B. clarridgeiae* were found in cat fleas, and *B. grahamii* and another rodent-related *Bartonella* species were found in fleas collected from rodents, and *B. schoenbuchensis* in deer keds. All these ectoparasites could transmit *Bartonella* between animals and maybe also from animals to humans. *B. henselae* is well known to be associated to human cases of bartonellosis in the form of cat scratch disease [7,8]. It is mainly transmitted directly from cats to humans but might also be transmitted by infected cat fleas [19]. To date, including the current study, the transmission of *B. henselae* to humans via ticks has not been proven and the risk of transmission by tick is probably negligible [71]. *B. clarridgeiae* has also been frequently reported from cats and their ectoparasites [17,72] but has so far only incidentally been reported from human cases [20,22]. Cat fleas could play a role in *B. clarridgeiae* transmission but the route of transmission has not yet been established.

B. schoenbuchensis has been hypothesized to be involved in the development of deer ked dermatitis, a sometimes long-lasting skin condition following bites of deer keds [73]. While the exposure of the general population to deer keds is rather low, some specific groups like forest workers and hunters are at high risk to be bitten by these arthropods. In this limited population, deer keds might play a role in transmission of *B. schoenbuchensis*. In the general population exposure to *B. henselae* is much more likely than exposure to any of the other discussed *Bartonella* species. It is therefore also likely to cause most bartonellosis cases. Further research is necessary to determine the risk of zoonotic transmission of different *Bartonella* species upon a bite of cat fleas and other arthropods.

Conclusions

Risk of human bartonellosis transmitted by *I. ricinus* ticks is negligible while other arthropods, including deer keds and fleas, can potentially transmit *Bartonella* sp. to humans. Earlier reports of high infection rates of questing ticks with a *Bartonella* species might in fact be due to the misidentification of "*Ca. Midichloria mitochondrii*" as *Bartonella* sp..

Acknowledgements

The authors are very grateful to the volunteers and co-workers of the Central Veterinary Institute (Cor Gaasenbeek, Fred Borgsteede and Kitty Maassen) of Wageningen University who have dedicated much time and effort to monthly collections of ticks. We thank Marieta Braks for initial flea identification, Annelies van Goor and her dedicated students, particularly Rudy Muts, for collecting fleas from pet animals and Marieke Mensink, Sabine van Dijk, Christa Drenth and Tineke Bolleman for their excellent technical assistance. We are very grateful to the veterinary practices who participated in collecting fleas and ticks from pets. We are indebted to Margriet Montizaan (Dutch Royal Hunting Society, Amersfoort) for collecting deer keds. This study was financially supported by the Dutch Food and Consumer Product Safety Authority (VWA) and by the Ministry of Health, Welfare and Sport (VWS).

Author details

¹Laboratory for Zoonoses and Environmental Microbiology, National Institute for Public Health and Environment (RIVM), Bilthoven, The Netherlands.

²Laboratory for Infectious Diseases and Screening, National Institute for Public Health and Environment (RIVM), Bilthoven, The Netherlands.

³Laboratory of Entomology, Wageningen University, Wageningen, The Netherlands.

⁴Companion Animal Hospital 't Heike, Veldhoven, The Netherlands.

⁵Institute for Risk Assessment Sciences, Division of Veterinary Public Health, Utrecht University, Utrecht, The Netherlands.

⁶Utrecht Centre for Tick-borne Diseases (UCTD), Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

⁷Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Berlin, Germany.

Authors' contributions

FJ, WT, JRR, HS and PAMO were involved in study design. FG, EKEH, AMN and PO collected ticks and fleas, contributed to laboratory analyses and analyzed data. ETK and MF developed new methodology, performed laboratory analyses and analyzed data. HS acquired funding, was involved in data analyses and wrote the initial draft. ETK performed phylogenetic analyses and wrote the final draft. All authors were involved in completing the manuscript and approved the final version.

Competing interests

The authors declare that they have no competing interests.

Received: 14 February 2011 Accepted: 18 April 2011

Published: 18 April 2011

References

- McElroy KM, Blagburn BL, Breitschwerdt EB, Mead PS, McQuiston JH: Flea-associated zoonotic diseases of cats in the USA: bartonellosis, flea-borne rickettsioses, and plague. *Trends Parasitol* 2010, **26**:197-204.
- Salzberger B, Franzen C, Fatkenheuer G: [Update in infectious diseases. Part I: epidemiology]. *Med Klin (Munich)* 2000, **95**:314-320.
- Carithers HA: Cat-scratch disease. An overview based on a study of 1,200 patients. *Am J Dis Child* 1985, **139**:1124-1133.
- Loutit JS: Bartonella infections. *Curr Clin Top Infect Dis* 1997, **17**:269-290.
- Margileth AM, Wear DJ, English CK: Systemic cat scratch disease: report of 23 patients with prolonged or recurrent severe bacterial infection. *J Infect Dis* 1987, **155**:390-402.
- Wheeler SW, Wolf SM, Steinberg EA: Cat-scratch encephalopathy. *Neurology* 1997, **49**:876-878.
- Maman E, Bickels J, Ephros M, Paran D, Comaneshter D, Metzkor-Cotter E, Avidor B, Varon-Graidy M, Wientroub S, Giladi M: Musculoskeletal manifestations of cat scratch disease. *Clin Infect Dis* 2007, **45**:1535-1540.
- Giladi M, Maman E, Paran D, Bickels J, Comaneshter D, Avidor B, Varon-Graidy M, Ephros M, Wientroub S: Cat-scratch disease-associated arthropathy. *Arthritis Rheum* 2005, **52**:3611-3617.
- Houpikian P, Raoult D: Blood culture-negative endocarditis in a reference center: etiologic diagnosis of 348 cases. *Medicine (Baltimore)* 2005, **84**:162-173.
- Lamas CC, Eykyn SJ: Blood culture negative endocarditis: analysis of 63 cases presenting over 25 years. *Heart* 2003, **89**:258-262.
- Fournier PE, Thuny F, Richet H, Lepidi H, Casalta JP, Arzouni JP, Maurin M, Celard M, Mainardi JL, Caus T, et al: Comprehensive diagnostic strategy for blood culture-negative endocarditis: a prospective study of 819 new cases. *Clin Infect Dis* 2010, **51**:131-140.
- Welch DF, Pickett DA, Slater LN, Steigerwalt AG, Brenner DJ: Rochalimaea henselae sp. nov., a cause of septicemia, bacillary angiomatosis, and parenchymal bacillary peliosis. *J Clin Microbiol* 1992, **30**:275-280.
- Koehler JE, Glaser CA, Tappero JW: Rochalimaea henselae infection. A new zoonosis with the domestic cat as reservoir. *Jama* 1994, **271**:531-535.
- Regnery R, Martin M, Olson J: Naturally occurring "Rochalimaea henselae" infection in domestic cat. *Lancet* 1992, **340**:557-558.
- Kordick DL, Wilson KH, Sexton DJ, Hadfield TL, Berkhoff HA, Breitschwerdt EB: Prolonged Bartonella bacteremia in cats associated with cat-scratch disease patients. *J Clin Microbiol* 1995, **33**:3245-3251.
- Bergmans AM, de Jong CM, van Amerongen G, Schot CS, Schouls LM: Prevalence of Bartonella species in domestic cats in The Netherlands. *J Clin Microbiol* 1997, **35**:2256-2261.
- Rolain JM, Franc M, Davoust B, Raoult D: Molecular detection of Bartonella quintana, B. koehlerae, B. henselae, B. clarridgeiae, Rickettsia felis, and Wolbachia pipientis in cat fleas, France. *Emerg Infect Dis* 2003, **9**:338-342.
- Shaw SE, Kenny MJ, Tasker S, Birtles RJ: Pathogen carriage by the cat flea Ctenocephalides felis (Bouche) in the United Kingdom. *Vet Microbiol* 2004, **102**:183-188.
- Zangwill KM, Hamilton DH, Perkins BA, Regnery RL, Plikaytis BD, Hadler JL, Cartter JM, Wenger JD: Cat scratch disease in Connecticut. Epidemiology, risk factors, and evaluation of a new diagnostic test. *N Engl J Med* 1993, **329**:8-13.
- Sander A, Zagrosek A, Brecht W, Schiltz E, Piemont Y, Lanz C, Dehio C: Characterization of Bartonella clarridgeiae flagellin (FlaA) and detection of anti-flagellin antibodies in patients with lymphadenopathy. *J Clin Microbiol* 2000, **38**:2943-2948.
- Eremeeva ME, Gerns HL, Lydy SL, Goo JS, Ryan ET, Mathew SS, Ferraro MJ, Holden JM, Nicholson WL, Dasch GA, Koehler JE: Bacteremia, fever, and splenomegaly caused by a newly recognized bartonella species. *N Engl J Med* 2007, **356**:2381-2387.
- Kordick DL, Hilyard EJ, Hadfield TL, Wilson KH, Steigerwalt AG, Brenner DJ, Breitschwerdt EB: Bartonella clarridgeiae, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch disease). *J Clin Microbiol* 1997, **35**:1813-1818.
- Kerkhoff FT, Bergmans AM, van Der Zee A, Rothova A: Demonstration of Bartonella grahamii DNA in ocular fluids of a patient with neuroretinitis. *J Clin Microbiol* 1999, **37**:4034-4038.
- Hofhuis A, van der Giessen JW, Borgsteede FH, Wielinga PR, Notermans DW, van Pelt W: Lyme borreliosis in the Netherlands: strong increase in GP consultations and hospital admissions in past 10 years. *Euro Surveill* 2006, **11**, E060622 060622.
- Vilcins IM, Kosoy M, Old JM, Deane EM: Bartonella-like DNA detected in Ixodes tasmani ticks (Acari: Ixodidae) infesting Koalas (Phascolarctos cinereus) in Victoria, Australia. *Vector Borne Zoonotic Dis* 2009, **9**:499-503.
- Chang CC, Chomel BB, Kasten RW, Romano V, Tietze N: Molecular evidence of Bartonella spp. in questing adult Ixodes pacificus ticks in California. *J Clin Microbiol* 2001, **39**:1221-1226.
- Angelakis E, Billeter SA, Breitschwerdt EB, Chomel BB, Raoult D: Potential for tick-borne bartonellosis. *Emerg Infect Dis* **16**:385-391.
- Reye AL, Hubschen JM, Sausy A, Muller CP: Prevalence and seasonality of tick-borne pathogens in questing Ixodes ricinus ticks from Luxembourg. *Appl Environ Microbiol* **76**:2923-2931.
- Dietrich F, Schmidgen T, Maggi RG, Richter D, Matuschka FR, Vonthein R, Breitschwerdt EB, Kempf VA: Prevalence of Bartonella henselae and

- Borrelia burgdorferi sensu lato DNA in ixodes ricinus ticks in Europe.** *Appl Environ Microbiol* 2006, **72**:1395-1398.
30. Podsiadly E, Chmielewski T, Sochon E, Tylewska-Wierzbanowska S: **Bartonella henselae in Ixodes ricinus ticks removed from dogs.** *Vector Borne Zoonotic Dis* 2007, **7**:189-192.
 31. Holden K, Boothby JT, Kasten RW, Chomel BB: **Co-detection of Bartonella henselae, Borrelia burgdorferi, and Anaplasma phagocytophilum in Ixodes pacificus ticks from California, USA.** *Vector Borne Zoonotic Dis* 2006, **6**:99-102.
 32. Morozova OV, Cabello FC, Dobrotvorskyy AK: **Semi-nested PCR detection of Bartonella henselae in Ixodes persulcatus ticks from Western Siberia, Russia.** *Vector Borne Zoonotic Dis* 2004, **4**:306-309.
 33. Cotte V, Bonnet S, Le Rhun D, Le Naour E, Chauvin A, Boulouis HJ, Lecuelle B, Lilin T, Vayssier-Taussat M: **Transmission of Bartonella henselae by Ixodes ricinus.** *Emerg Infect Dis* 2008, **14**:1074-1080.
 34. Telford SR, Wormser GP: **Bartonella spp. transmission by ticks not established.** *Emerg Infect Dis* 2006, **12**:379-384.
 35. Heyman P, Cochez C, Hofhuis A, van der Giessen J, Sprong H, Porter SR, Losson B, Saegerman C, Donoso-Mantke O, Niedrig M, Papa A: **A clear and present danger: tick-borne diseases in Europe.** *Expert Rev Anti Infect Ther* 2010, **8**:33-50.
 36. Parola P, Paddock CD, Raoult D: **Tick-borne rickettsioses around the world: emerging diseases challenging old concepts.** *Clin Microbiol Rev* 2005, **18**:719-756.
 37. Sprong H, Wielinga PR, Fonville M, Reusken C, Brandenburg AH, Borgsteede F, Gaasenbeek C, van der Giessen JW: **Ixodes ricinus ticks are reservoir hosts for Rickettsia helvetica and potentially carry flea-borne Rickettsia species.** *Parasit Vectors* 2009, **2**:41.
 38. Tijssse-Klasen E, Fonville M, Reimerink JH, Spitzen-van der Sluijs A, Sprong H: **Role of sand lizards in the ecology of Lyme and other tick-borne diseases in the Netherlands.** *Parasit Vectors* 2010, **3**:42.
 39. Nielsen H, Fournier PE, Pedersen IS, Krarup H, Ejlersten T, Raoult D: **Serological and molecular evidence of Rickettsia helvetica in Denmark.** *Scand J Infect Dis* 2004, **36**:559-563.
 40. Fournier PE, Allombert C, Supputamongkol Y, Caruso G, Brouqui P, Raoult D: **Aneruptive fever associated with antibodies to Rickettsia helvetica in Europe and Thailand.** *J Clin Microbiol* 2004, **42**:816-818.
 41. Nilsson K: **Septicaemia with Rickettsia helvetica in a patient with acute febrile illness, rash and myasthenia.** *J Infect* 2009, **58**:79-82.
 42. Nilsson K, Lindquist O, Pahlson C: **Association of Rickettsia helvetica with chronic perimyocarditis in sudden cardiac death.** *Lancet* 1999, **354**:1169-1173.
 43. Nilsson K, Liu A, Pahlson C, Lindquist O: **Demonstration of intracellular microorganisms (Rickettsia spp., Chlamydia pneumoniae, Bartonella spp.) in pathological human aortic valves by PCR.** *J Infect* 2005, **50**:46-52.
 44. Nilsson K, Pahlson C, Lukinius A, Eriksson L, Nilsson L, Lindquist O: **Presence of Rickettsia helvetica in granulomatous tissue from patients with sarcoidosis.** *J Infect Dis* 2002, **185**:1128-1138.
 45. Cinco M, Luzzati R, Mascioli M, Floris R, Brouqui P: **Serological evidence of Rickettsia infections in forestry rangers in north-eastern Italy.** *Clin Microbiol Infect* 2006, **12**:493-495.
 46. Fournier PE, Grunnenberger F, Jaulhac B, Gastinger G, Raoult D: **Evidence of Rickettsia helvetica infection in humans, eastern France.** *Emerg Infect Dis* 2000, **6**:389-392.
 47. Nilsson K, Elfving K, Pahlson C: **Rickettsia helvetica in patient with meningitis, Sweden, 2006.** *Emerg Infect Dis* 2010, **16**:490-492.
 48. Reif KE, Macaluso KR: **Ecology of Rickettsia felis: a review.** *J Med Entomol* 2009, **46**:723-736.
 49. Gillespie JJ, Ammerman NC, Beier-Sexton M, Sobral BS, Azad AF: **Louse- and flea-borne rickettsioses: biological and genomic analyses.** *Vet Res* 2009, **40**:12.
 50. Galvao MA, Mafra C, Chamone CB, Calic SB, Zavala-Velazquez JE, Walker DH: **Clinical and laboratory evidence of Rickettsia felis infections in Latin America.** *Rev Soc Bras Med Trop* 2004, **37**:238-240.
 51. Perez-Arellano JL, Fenollar F, Angel-Moreno A, Bolanos M, Hernandez M, Santana E, Hemmersbach-Miller M, Martin AM, Raoult D: **Human Rickettsia felis infection, Canary Islands, Spain.** *Emerg Infect Dis* 2005, **11**:1961-1964.
 52. Oteo JA, Portillo A, Santibanez S, Blanco JR, Perez-Martinez L, Ibarra V: **Cluster of cases of human Rickettsia felis infection from Southern Europe (Spain) diagnosed by PCR.** *J Clin Microbiol* 2006, **44**:2669-2671.
 53. Perez-Osorio CE, Zavala-Velazquez JE, Arias Leon JJ, Zavala-Castro JE: **Rickettsia felis as emergent global threat for humans.** *Emerg Infect Dis* 2008, **14**:1019-1023.
 54. Renvoise A, Joliet AY, Raoult D: **Rickettsia felis infection in man, France.** *Emerg Infect Dis* 2009, **15**:1126-1127.
 55. Black WC, Piesman J: **Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences.** *Proc Natl Acad Sci USA* 1994, **91**:10034-10038.
 56. Wielinga PR, Gaasenbeek C, Fonville M, de Boer A, de Vries A, Dimmers W, Akkerhuis Op Jagers G, Schouls LM, Borgsteede F, van der Giessen JW: **Longitudinal analysis of tick densities and Borrelia, Anaplasma, and Ehrlichia infections of Ixodes ricinus ticks in different habitat areas in The Netherlands.** *Appl Environ Microbiol* 2006, **72**:7594-7601.
 57. Bergmans AM, Peeters MF, Schellekens JF, Vos MC, Sabbe LJ, Ossewaarde JM, Verbakel H, Hooft HJ, Schouls LM: **Pitfalls and fallacies of cat scratch disease serology: evaluation of Bartonella henselae-based indirect fluorescence assay and enzyme-linked immunoassay.** *J Clin Microbiol* 1997, **35**:1931-1937.
 58. Garcia-Esteban C, Gil H, Rodriguez-Vargas M, Gerikagoitia X, Barandika J, Escudero R, Jado I, Garcia-Amil C, Barral M, Garcia-Perez AL, et al: **Molecular method for Bartonella species identification in clinical and environmental samples.** *J Clin Microbiol* 2008, **46**:776-779.
 59. De Sousa R, Edouard-Fournier P, Santos-Silva M, Amaro F, Bacellar F, Raoult D: **Molecular detection of Rickettsia felis, Rickettsia typhi and two genotypes closely related to Bartonella elizabethae.** *Am J Trop Med Hyg* 2006, **75**:727-731.
 60. Beninati T, Lo N, Sacchi L, Genchi C, Noda H, Bandi C: **A novel alpha-Proteobacterium resides in the mitochondria of ovarian cells of the tick Ixodes ricinus.** *Appl Environ Microbiol* 2004, **70**:2596-2602.
 61. Sasser D, Beninati T, Bandi C, Bouman EA, Sacchi L, Fabbri M, Lo N: **'Candidatus Midichloria mitochondrii', an endosymbiont of the tick Ixodes ricinus with a unique intramitochondrial lifestyle.** *Int J Syst Evol Microbiol* 2006, **56**:2535-2540.
 62. Christova I, Van De Pol J, Yazar S, Velo E, Schouls L: **Identification of Borrelia burgdorferi sensu lato, Anaplasma and Ehrlichia species, and spotted fever group Rickettsiae in ticks from Southeastern Europe.** *Eur J Clin Microbiol Infect Dis* 2003, **22**:535-542.
 63. Schouls LM, Van De Pol J, Rijpkema SG, Schot CS: **Detection and identification of Ehrlichia, Borrelia burgdorferi sensu lato, and Bartonella species in Dutch Ixodes ricinus ticks.** *J Clin Microbiol* 1999, **37**:2215-2222.
 64. Matsumoto K, Berrada ZL, Klinger E, Goethert HK, Telford SR: **Molecular detection of Bartonella schoenbuchensis from ectoparasites of deer in Massachusetts.** *Vector Borne Zoonotic Dis* 2008, **8**:549-554.
 65. Dietrich F, Schmidgen T, Maggi RG, Richter D, Matuschka FR, Vonthein R, Breitschwerdt EB, Kempf VA: **Prevalence of Bartonella henselae and Borrelia burgdorferi sensu lato DNA in ixodes ricinus ticks in Europe.** *Appl Environ Microbiol* 2010, **76**:1395-1398.
 66. Angelakis E, Pulcini C, Waton J, Imbert P, Socolovschi C, Edouard S, Dellamonica P, Raoult D: **Scalp eschar and neck lymphadenopathy caused by Bartonella henselae after Tick Bite.** *Clin Infect Dis* 2010, **50**:549-551.
 67. Swanson KI, Norris DE: **Co-circulating microorganisms in questing Ixodes scapularis nymphs in Maryland.** *J Vector Ecol* 2007, **32**:243-251.
 68. Epis S, Sasser D, Beninati T, Lo N, Beati L, Piesman J, Rinaldi L, McCoy KD, Torina A, Sacchi L, et al: **Midichloria mitochondrii is widespread in hard ticks (Ixodidae) and resides in the mitochondria of phylogenetically diverse species.** *Parasitology* 2008, **135**:485-494.
 69. Bergmans AM, Schellekens JF, van Embden JD, Schouls LM: **Predominance of two Bartonella henselae variants among cat-scratch disease patients in the Netherlands.** *J Clin Microbiol* 1996, **34**:254-260.
 70. Tijssse-Klasen E, Fonville M, van Overbeek L, Reimerink JH, Sprong H: **Exotic Rickettsiae in Ixodes ricinus: fact or artifact?** *Parasit Vectors* 2010, **3**:54.
 71. Telford SR, Wormser GP: **Bartonella spp. transmission by ticks not established.** *Emerg Infect Dis* 2010, **16**:379-384.
 72. Tsai YL, Lin CC, Chomel BB, Chuang ST, Tsai KH, Wu WJ, Huang CG, Yu JC, Sung MH, Kass PH, Chang CC: **Bartonella Infection in Shelter Cats and Dogs and Their Ectoparasites.** *Vector Borne Zoonotic Dis* 2010.
 73. Dehio C, Sauder U, Hiestand R: **Isolation of Bartonella schoenbuchensis from Lipoptena cervi, a blood-sucking arthropod causing deer ked dermatitis.** *J Clin Microbiol* 2004, **42**:5320-5323.

74. Gassner F, van Vliet AJ, Burgers SL, Jacobs F, Verbaarschot P, Hovius EK, Mulder S, Verhulst NO, van Overbeek LS, Takken W: **Geographic and Temporal Variations in Population Dynamics of *Ixodes ricinus* and Associated *Borrelia* Infections in The Netherlands.** *Vector Borne Zoonotic Dis* 2010.

doi:10.1186/1756-3305-4-61

Cite this article as: Tijssse-Klasen *et al.*: Absence of zoonotic *Bartonella* species in questing ticks: First detection of *Bartonella clarridgeiae* and *Rickettsia felis* in cat fleas in the Netherlands. *Parasites & Vectors* 2011 4:61.

**Submit your next manuscript to BioMed Central
and take full advantage of:**

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

