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Detection and Identification of *Ehrlichia*, *Borrelia burgdorferi* Sensu Lato, and *Bartonella* Species in Dutch *Ixodes ricinus* Ticks

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A sensitive and specific PCR hybridization assay was developed for the simultaneous detection and identification of *Ehrlichia* and *Borrelia burgdorferi* sensu lato. In separate assays the 16S rRNA gene of *Ehrlichia* species and the 23S-5S rRNA spacer region of *B. burgdorferi* sensu lato were amplified and labeled by PCR. These PCR products were used in a reverse line blot hybridization assay in which oligonucleotide probes are covalently linked to a membrane in parallel lines. Hybridization of the samples with the oligonucleotide probes on this membrane enabled the simultaneous detection and identification of *Ehrlichia*, *B. burgdorferi*, and *Bartonella* species in 40 different samples. The application of the assay to DNA extracts from 121 *Ixodes ricinus* ticks collected from roe deer demonstrated that 45% of these ticks carried *Ehrlichia* DNA. More than half of these positive ticks carried species with 16S rRNA gene sequences closely related to those of *E. phagocytophila* and the human granulocytic ehrlichiosis agent. The majority of the other positive ticks were infected with a newly identified *Ehrlichia*-like species. In addition, 13% of the ticks were infected with one or more *B. burgdorferi* genospecies. In more than 70% of the ticks 16S rRNA gene sequences for *Bartonella* species or other species closely related to *Bartonella* were found. In five of the ticks both *Ehrlichia* and *B. burgdorferi* species were detected.

The zoonotic vector-borne diseases form a large proportion of the emerging bacterial infectious diseases. The most prominent of these diseases are Lyme disease, ehrlichiosis, and bartonellosis. In The Netherlands 10 to 35% of the *Ixodes ricinus* ticks are infected with *Borrelia burgdorferi*, the causative agent of Lyme disease (25, 26). In 1994, general practitioners in The Netherlands reported seeing 33,000 patients who had sustained tick bites and approximately 6,500 patients with erythema migrans (7). These findings not only underline the importance of borreliosis but also suggest that other vector-borne diseases may occur in The Netherlands.

Presently, two tick-transmitted *Ehrlichia* species have been shown to cause human disease. The first is *Ehrlichia chaffeensis*, which causes human monocytic ehrlichiosis and which is transmitted by *Amblyomma americanum*, a tick species found only in the United States. Until now, very few cases of *E. chaffeensis* infection in Europe have been described (5, 15, 21). The second *Ehrlichia* species pathogenic for humans is the human granulocytic ehrlichiosis agent (HGE). The exact nature of this organism is still unclear, but on the basis of its 16S rRNA sequence it is shown to be closely related to *Ehrlichia phagocytophila* and *Ehrlichia equi*. The HGE agent is transmitted by *Ixodes scapularis*, but possibly also by other vectors like *I. ricinus*. Again, the initial reports of disease of human patients with HGE came from the United States. Remarkably, there have been very few reports of cases of disease caused by HGE in Europe. The major indication that ehrlichiosis may play a role in Europe comes from serosurveys performed in several European countries including Sweden, Norway, Switzerland, and the United Kingdom (5, 7, 28). However, von Stedingk et al. (28) have recently detected *Ehrlichia* species in Swedish *I. ricinus* ticks, and *Ehrlichia* was also detected in a French *I. ricinus* tick (19). These findings indicate that *Ehrlichia* species that are pathogenic for humans may be present in Western Europe as well.

The clinical manifestations of HGE infection can vary from a flu-like disease to severe life-threatening acute febrile disease with thrombocytopenia, leukopenia, and elevated liver transaminase levels. Because of the diffuse nonspecific symptoms of this disease, diagnosis relies heavily on laboratory tests. Serology, particularly immunofluorescence, is commonly used, but serology often does not detect antibodies in the acute phase of disease. Culture of HGE is possible, but it is very labor intensive and has not been validated as far as sensitivity is concerned. Microscopic examination of stained blood smears can be used to detect characteristic enclosures in infected leukocytes. However, this method is insensitive and requires special expertise. The sensitivity and specificity of PCR for the detection of the *Ehrlichia* probably exceed those of the other methods. Several PCRs for detection of *Ehrlichia* species have been described (1, 6, 9, 14); however, all of these assays enable the detection of just a single species. In this report we describe a PCR-hybridization assay that enables the simultaneous detection and species identification of a variety of *Ehrlichia*, *B. burgdorferi*, and *Bartonella* species in a single sample. This method allowed us to screen a large number of Dutch tick samples for the presence of these tick-borne pathogens.

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MATERIALS AND METHODS

Ticks and bacterial strains. *I. ricinus* ticks were collected from infested roe deer (*Capreolus capreolus*) shot in the Flevopolder in The Netherlands, an area where roe deer are abundant (26). Immediately after collection, the ticks were immersed in 70% ethanol and stored. The four genomic groups of *B. burgdorferi* sensu lato were represented by *B. burgdorferi* sensu stricto HB4, *Borrelia garinii*

AR-1, *Borrelia afzelii* A39S, and *Borrelia valaisiana* M19. Crude DNA extracts from the following *Ehrlichia* species were used: *E. phagocytophila* (kindly provided by F. Jongejan) and HGE, *Ehrlichia canis*, and *E. chaffeensis* (kindly provided by S. Dumler). The two *Bartonella* species used in this study were *Bartonella henselae* ATCC 49882 and *Bartonella quintana* 90-268 (3).

Preparation of DNA extracts from ticks. Ticks were processed as described before (10, 23). Briefly, the ticks were taken from the 70% ethanol solution, air dried, and boiled for 20 min in 100 μl of 0.7 M ammonium hydroxide to free the DNA. After cooling, the vial with the lysate was left open for 10 min at 90°C to evaporate the ammonia. The tick lysate either was used directly for PCR or was stored at −20°C until use.

PCR amplification. PCR amplifications were performed in an Omnigene thermal cycler (Hybaid Ltd., Teddington, United Kingdom). DNA amplification was done in 50-μl reaction volumes. For the amplification of *Ehrlichia* DNA, each reaction mixture contained 10 pmol of primer 16S8FE and B-GA1B, 1.25 U of SuperTaq DNA polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom), 0.275 μg of the TaqStart antibody (Clontech Laboratories, Palo Alto, Calif.), and standard amounts of amplification reagents (each deoxynucleoside triphosphate at a concentration of 200 μM, 10 mM Tris · HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100). A 25-μl overlay of paraffin oil was added to the tubes, followed by the addition of 5 μl of the tick DNA extract. To minimize nonspecific amplification a touchdown PCR program was used: 3 min at 94°C, two cycles of 20 s at 94°C, 30 s at 67°C, and 30 s at 72°C, and then two cycles with conditions identical to the previous cycles but with an annealing temperature of 65°C. During subsequent two cycle sets the annealing temperature was lowered by 2°C until it reached 57°C. Then, an additional 40 cycles each consisting of 20 s at 94°C, 30 s at 57°C, and 20 s at 72°C, followed the touchdown program, were performed. The PCR was ended by an extra incubation for 7 min at 72°C. For the amplification of *B. burgdorferi* sensu lato DNA, conditions similar to those described above were used, except that 40 pmol of the primers 23SN2 and 5SCB and double the amounts of SuperTaq and TaqStart were used. In addition, the touchdown PCR temperature ranged from 60 to 50°C. For the amplification of *Bartonella* DNA, the previously described PCR protocol of Bergmans et al. (4) was used.

To monitor for the occurrence of false-positive PCR results, negative controls were included during extraction of the tick samples: one control sample for each six tick samples, with a minimum of two controls. In addition, each time that the PCR was performed, negative and positive control samples were included. In order to minimize contamination, the reagent setup, the extraction and sample addition, and the PCR and sample analysis were performed in three separate rooms, of which the first two rooms were kept at positive pressure and had airlocks.

Reverse line blot hybridization. The reverse line blotting technique has been described before (11, 12, 25). Briefly, solutions with 5′ amino-linked oligonucleotide probes ranging from 10 to 800 pmol were coupled covalently to an activated Biodyne C membrane in a line pattern by using a miniblotter (Immunetics, Cambridge, Mass.). After binding of the oligonucleotide probes the membrane was taken from the miniblotter, washed in 2× SSPE (360 mM NaCl, 20 mM $Na_2HPO_4 \cdot H_2O$, 2 mM EDTA) with 0.1% sodium dodecyl sulfate (SDS) at 60°C, and again placed in the miniblotter with the oligonucleotide lines perpendicular to the slots. Ten microliters of the biotin-labeled PCR product was diluted in 150 μl of 2× SSPE–0.1% SDS, denatured for 10 min at 99°C, and cooled rapidly on ice. The slots of the miniblotter were filled with the denatured PCR product, and hybridization was performed for 1 h at 42°C. The membrane was removed from the miniblotter and was washed twice for 10 min each time in 2× SSPE–0.1% SDS at 51°C. Subsequently, the membrane was incubated for 30 min at 42°C with streptavidin-peroxidase (Boehringer Mannheim GmbH, Mannheim, Germany) diluted 1:4,000 in 2× SSPE–0.5% SDS and was washed twice for 10 min in 2× SSPE–0.5% SDS. Hybridization was visualized by incubating the membrane with enhanced chemiluminescence detection liquid (Amersham International plc, Den Bosch, The Netherlands) and exposing the membrane to X-ray film (Hyperfilm; Amersham). For species identification the biotinylated *Ehrlichia* PCR product was hybridized with seven different oligonucleotide probes in the reverse line blot assay. Similarly, the biotinylated spacer fragment of the *Borrelia* PCR was hybridized with five *B. burgdorferi* genospecies-specific oligonucleotide probes. For identification of *Bartonella* species a region between bases 964 and 1243 of the 16S rRNA gene was amplified and was used in a reverse line blot assay. All primers and probes are described in Table 1.

DNA sequencing and data analysis. The PCR products used for DNA sequencing were purified with Qiaquick PCR purification kits (Qiagen, Hilden, Germany). For DNA sequencing reactions, the fluorescence-labeled dideoxynucleotide technology was used (Perkin-Elmer, Applied Biosystems Division). The sequenced fragments were separated, and data were collected on an ABI 377 automated DNA sequencer (Perkin-Elmer, Applied Biosystems Division). The collected sequences were assembled, edited, and analyzed with the DNAStar package (DNAStar Inc., Madison, Wis.). The phylogenetic tree was constructed by using the Clustal analysis in the Megalign module of the DNAStar package.

Nucleotide sequence accession number. The 16S rRNA gene sequence of the *Ehrlichia*-like organism found in this study is available in the GenBank database under accession no. AF104680.

RESULTS

Sensitivity of the PCR for *Ehrlichia* and *Borrelia*. The sensitivity of the PCR for *Ehrlichia* and *Borrelia* was assessed by spiking the samples with known concentrations of previously produced PCR products. Extracts from ticks that were negative by previous PCRs were spiked with serial dilutions of *E. phagocytophila* or *B. burgdorferi* PCR products. All experiments were performed in duplicate. Repeatedly, the detection limit for both assays in which the PCR yielded a positive result was five copies of the target sequence (data not shown). The detection limit of the PCR for *Bartonella* has been determined in another study and was shown to correspond to one genome copy (4).

Specificity of the reverse line blot hybridization. A reverse line blot hybridization assay was designed to differentiate the various *Ehrlichia*, *B. burgdorferi*, and *Bartonella* species. Some of the *Ehrlichia* species differ by only one nucleotide in the target sequence (Fig. 1). The oligonucleotide probes were designed in such a way that the melting temperature of all oligonucleotides was approximately 55°C under the conditions used. As a result the oligonucleotide probes differ in length. In order to obtain specific and sensitive signals in the assay the optimal oligonucleotide probe concentrations and hybridization conditions were determined empirically. To assess the specificity of the assay, control samples were amplified and used in the reverse line blot hybridization assay under stringent hybridization conditions. Figure 2 shows the reactivities of the control samples for *Ehrlichia*, *B. burgdorferi*, and *Bartonella* species in the optimized reverse line blot assay. No cross-hybridization between the various species occurred, indicating that the system distinguished target sequences that differed by only a single base pair.

PCR detection of *Ehrlichia*, *Borrelia*, and *Bartonella* DNAs in ticks. The various PCRs were applied to DNA extracts from 121 *I. ricinus* ticks collected from 38 different roe deer. The majority of the ticks were adults, mainly females; and some were nonengorged, some were semiengorged, and some were fully engorged (Table 2). Regardless of whether these PCRs yielded a visible fragment on agarose gels, all samples were analyzed by the reverse line blot assay. Fifty-four of the 121 samples (45%) reacted with one or more of the *Ehrlichia*-specific probes (Table 2). Of these, 3 reacted with the HGE-specific probe, 3 reacted with the *E. phagocytophila*-specific probe, 11 reacted with the HGE variant-specific probe, 9 reacted with the *E. phagocytophila* variant-specific probe, and 7 reacted with both the HGE variant-specific and the *E. phagocytophila* variant-specific probes. In addition, 19 of the samples reacted solely with the *Ehrlichia* genus-specific probe. Sixteen of the same 121 tick samples (13%) reacted in the *Borrelia* PCR hybridization assay with the *B. burgdorferi* species-specific probes (Table 2). Coinfection with two different *B. burgdorferi* genospecies was detected in four tick samples. None of the ticks analyzed was infected solely with *B. garinii* or with *B. burgdorferi* sensu stricto. However, these genospecies were found in ticks coinfected with different genospecies.

The 121 ticks were collected from 38 roe deer, which implies that several ticks originated from the same animal. The distribution of the *Ehrlichia* and *Borrelia* species found in the ticks and their origins are displayed in Table 3. These results indicate that ticks collected from the same roe deer carried a variety of *Ehrlichia* and *Borrelia* species. This suggests that these bacterial species did not originate from the roe deer only but must have been taken up by the ticks during previous feeds on other animals.

Five of the tick samples carried various *Ehrlichia* and *B. burgdorferi* species, indicating the occurrence of coinfection

TABLE 1. Oligonucleotide primers and probes used in PCR and hybridization assays

Oligonucleotide name	Oligonucleotide sequence[a]	Target organism	Target gene	Nucleotide position	Reference
Primer					
5SCB	5′-biotin-GAGAGTAGGTTATTGCCAGGG	*B. burgdorferi* sensu lato	23S-5S spacer	243–263	25
23SN2	ACCATAGACTCTTATTACTTTGACCA	*B. burgdorferi* sensu lato	23S-5S spacer	469–444	25
Probes					
SL	5′-amino-CTTTGACCATATTTTTATCTTCCA	*B. burgdorferi* sensu lato	23S-5S spacer	453–430	25
SS	5′-amino-AACACCAATATTTAAAAAACATAA	*B. burgdorferi* sensu stricto	23S-5S spacer	322–299	25
GA	5′-amino-AACATGAACATCTAAAAACATAAA	*B. garinii*	23S-5S spacer	322–298	25
AF	5′-amino-AACATTTAAAAAATAAATTCAAGG	*B. afzelii*	23S-5S spacer	305–278	25
VS	5′-amino-CATTAAAAAAATATAAAAAATAAATTTAAGG	*B. valaisiana*	23S-5S spacer	303–278	25
Primers					
16S8FE	GGAATTCAGAGTTGGATCMTGGYTCAG	Eubacteria	16S rRNA gene	8–27	4
16S1523RM	CAGGAAACAGCTATGACCAAGGAGGTGATCCADCCVCA	Eubacteria	16S rRNA gene	1543–1523	This study
B-GA1B	5′-biotin-CGGGATCCCGAGTTTGCCGGGACTTCTTCT	*Ehrlichia* genus	16S rRNA gene	476–456	This study
GA1IC	AGAAGAAGTCCCGGCAAACTC	*Ehrlichia* genus	16S rRNA gene	456–476	This study
Probes					
A-EhrAll	5′-amino-TTATCGCTATTAGATGAGCC	*Ehrlichia* genus	16S rRNA gene	203–222	This study
A-HGE	5′-amino-GCTATAAAGAATAGTTAGTGG	HGE	16S rRNA gene	87–107	This study
A-Phago	5′-amino-TTGCTATAAAGAATAATTAGTGG	*E. phagocytophila*	16S rRNA gene	85–107	This study
A-D-HGE	5′-amino-GCTATGAAGAATAGTTAGTG	HGE variant	16S rRNA gene	87–106	This study
A-D-Phago	5′-amino-TTGCTATGAAGAATAATTAGTG	*E. phagocytophila* variant	16S rRNA gene	87–106	This study
A-ESchot	5′-amino-GCTGTAGTTTACTATGGGTA	*Ehrlichia*-like organism	16S rRNA gene	76–95	This study
A-ECan	5′-amino-TCTGGCTATAGGAAATTGTTA	*E. canis*	16S rRNA gene	85–105	This study
A-EChaf	5′-amino-ACCTTTTGGTTATAAATAATTGTTA	*E. chaffeensis*	16S rRNA gene	83–107	This study
Primers					
P24E	GGAATTCCCTCCTTCAGTTAGGCTGG	*Bartonella*	16S rRNA gene	966–982	22
P12B	CGGGATCCCGAGATGGCTTTTGGAGATTA	*Bartonella*	16S rRNA gene	1224–1243	22
Probes					
A-BartAll	5′-amino-GTTGGGCACTCTARGG	*Bartonella* genus	16S rRNA gene	1084–1099	This study
A-Hens	5′-amino-TGCCAGCATTTGGTTGG	*B. henselae*	16S rRNA gene	1072–1088	This study
A-Quin	5′-amino-TTGCCATCATTAAGTTGGG	*B. quintana*	16S rRNA gene	1071–1089	This study

[a] The underlined sequences represent the *Eco*RI and *Bam*HI restriction sites. The double underlined sequence denotes the M13 sequence which was used for DNA sequencing.

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CGGCAAGCTTAACACATGCAAGTCGAACGGATTATTCTTTATAGCTTGCTATAAAGAATAATTAGTGGCAGA  E. phagocytophila
.........C.................................................................  E. equi
...........................................................G..............  HGE
.....................................................G.....................  E. phagocytophila variant
.....................................................G.....G...............  HGE variant
          49                                          92    100
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FIG. 1. Multiple alignment of the variable part of the 16S rRNA gene sequences of the *E. phagocytophila* group. The region where differences were detected is shown for *E. phagocytophila*, *E. equi*, HGE, and the variants of *E. phagocytophila* and HGE. The positions of the residues that differ in the 16S rRNA gene are shown below the multiple alignment. Residues identical to those of the *E. phagocytophila* sequence are indicated by a dot. The sequence of *E. equi* was obtained from GenBank (accession no. M73223). The 500 bp of the 5′ end of the 16S rRNA gene sequences of *E. phagocytophila* and HGE were determined in this study and compared with the sequences in the GenBank and EMBL database and were found to be identical to published sequences (accession nos. M73320 and U02521, respectively). The 16S rRNA sequences of the *E. phagocytophila* variant and the HGE variant were determined in this study.

with these two pathogens (Table 4). Hybridization of the *Bartonella* PCR products yielded hybridization signals with the *Bartonella* genus-specific probe in 73 of the 121 samples (60%). However, none of these PCR products reacted with either the *B. henselae*-specific or the *B. quintana*-specific oligonucleotide probes.

DNA sequence analysis. In order to confirm the results obtained by the reverse line blot assay, 15 of the *Ehrlichia*-positive samples were also analyzed by DNA sequencing. Sequencing of the PCR product obtained by PCR for *Ehrlichia* revealed that we had correctly identified the various species by the reverse line blot hybridization assay. Furthermore, sequence analysis of the samples that reacted with both the HGE variant-specific and the *E. phagocytophila* variant-specific probes revealed an ambiguous nucleotide at position 100 in the 16S rRNA gene. Cloning of these PCR products and subse-

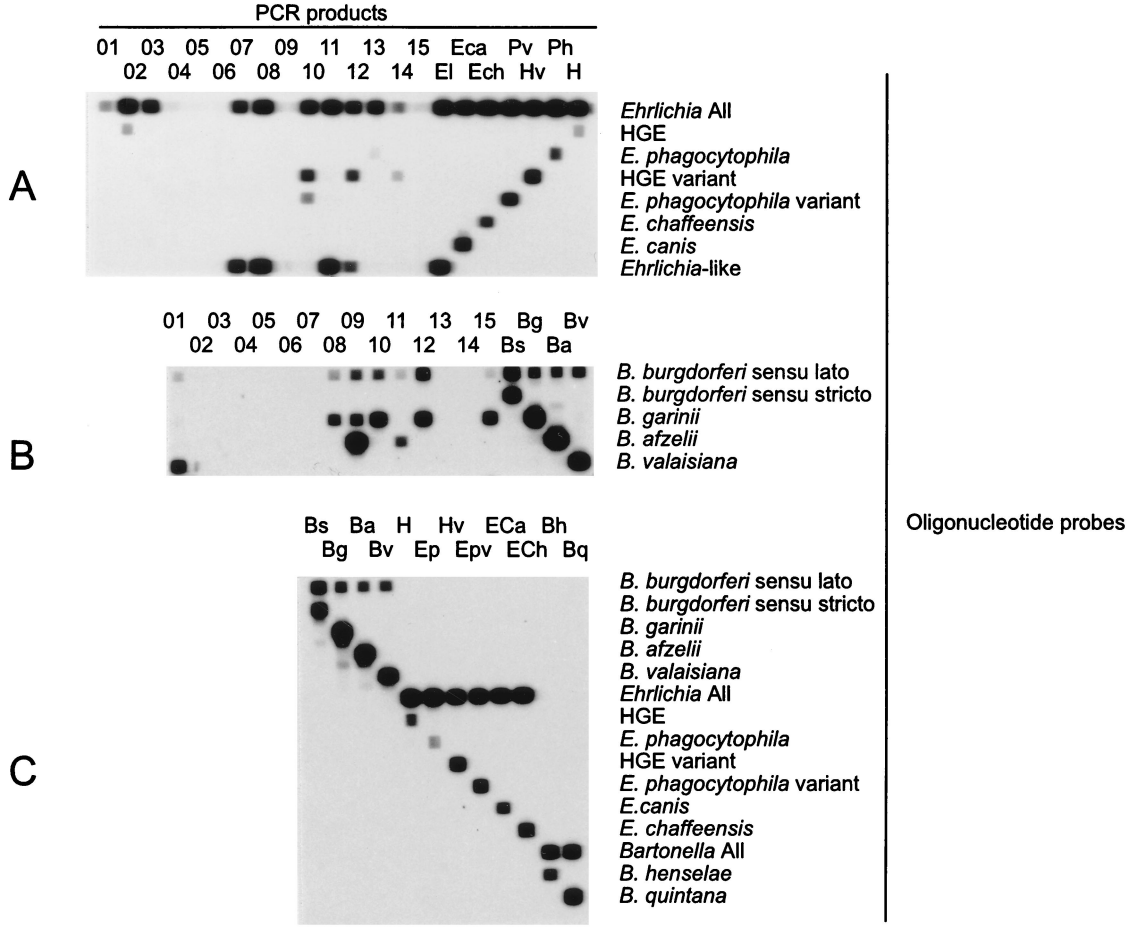

FIG. 2. Reverse line blot hybridization assay analyses for the detection and identification of *Ehrlichia*, *B. burgdorferi*, and *Bartonella* spp. in ticks. (A) Membrane carrying *Ehrlichia*-specific oligonucleotide probes; (B) membrane carrying *B. burgdorferi* genospecies-specific probes; (C) combined membrane carrying probes for *Ehrlichia*, *B. burgdorferi*, and *Bartonella* species. The oligonucleotide probes are attached to the membrane in the horizontal direction, and the PCR samples were applied perpendicularly in the vertical direction. The numbered lanes represent tick-derived PCR products, and the lanes marked with letters show the PCR products obtained from the positive control samples. El, *Ehrlichia*-like; Eca, *E. canis*; Ech, *E. chaffeensis*; Epv, *E. phagocytophila* variant; Hv, HGE variant; Ep, *E. phagocytophila*; H, HGE; Bs, *B. burgdorferi* sensu stricto; Bg, *B. garinii*; Ba, *B. afzelii*; Bv, *B. valaisiana*; Bh, *B. henselae*; Bq, *B. quintana*.

quent reverse line blot assay analysis and DNA sequencing of the clones revealed the presence of two types of cloned 16S sequences, indicating that these tick samples indeed carried a mixture of two different *Ehrlichia* sequences.

Nineteen of the PCR products obtained by the PCR for *Ehrlichia* reacted with the *Ehrlichia* genus-specific probe only. To determine the phylogenetic positions of these *Ehrlichia*-like organisms, the complete 16S rRNA gene sequences of three of these samples were determined. For this purpose two PCR fragments from each tick sample were generated and sequenced. The first PCR fragment covered bases 8 through 476 and was amplified with primer set 16S8FE and B-GA1B. The second fragment was generated by PCR with oligonucleotides A-EhrAll and 16S1523R and covered the region from positions 203 through 1543 of the 16S rRNA gene. The 16S sequences of these three samples were identical, and comparison with the DNA sequences in the GenBank data bank revealed that this 16S rRNA gene sequence differed markedly from all other published *Ehrlichia* sequences. The most closely related 16S rRNA gene sequences were those of *Cowdria ruminantium* (96% similarity) and those of members of the monocytic *Ehrlichia* group (Fig. 3). For this reason we designate this species *Ehrlichia*-like.

On the basis of the sequence analysis, a new probe (A-Eschot) specific for this *Ehrlichia*-like organism was designed for use in the reverse line blot assay and was used to screen the products obtained from the tick samples by PCR for *Ehrlichia*. Hybridization showed that of the 19 samples that initially reacted with the *Ehrlichia* genus-specific probe only, 8 reacted with the newly designed probe. Only one of the other *Ehrlichia*-positive samples reacted with this probe; this comprised a tick sample which reacted with both the *E. phagocytophila* variant-specific probe and the newly designed probe. As a result, the species infecting 11 of the samples that reacted with the *Ehrlichia* genus-specific probe remained undetermined.

Sequencing of 11 of the products obtained by PCR for *Bartonella* revealed that none represented *B. henselae* or *B. quintana* but closely resembled *Bartonella vinsonii*. However, the region of the 16S rRNA gene that was used for the PCR for *Bartonella* does not carry enough variation to reliably distinguish *B. vinsoni* from other closely related *Bartonella* and *Rhizobium* species.

DISCUSSION

We developed a PCR-based reverse line blot hybridization assay in which *Ehrlichia*, *B. burgdorferi*, and *Bartonella* species can be detected and differentiated. The assay was specific enough to detect single-base-pair changes with immobilized oligonucleotide probes and enabled us to differentiate *Ehrlichia* variants. The reverse line blot technique is a relatively easy and rapid method for the simultaneous detection and identification of microorganisms in field samples such as ticks. In its present form we can combine the hybridization of PCR products obtained in separate PCRs. We are now developing a multiplex PCR that will enable us to have an even more convenient method for the screening of samples. These samples could be tick lysates but could also be other material such as blood from patients suffering from a febrile disease with an unknown origin.

In the study presented here we used this method to detect and identify *Ehrlichia* and *B. burgdorferi* species in Dutch *I. ricinus* ticks. Analysis of the ticks showed an unexpected high rate of infection with *Ehrlichia* species (45%). The high infection rate may be partly due to the fact that the ticks originated from roe deer, which may serve as a reservoir for *Ehrlichia*.

TABLE 2. Results of reverse line blot assay analysis of PCR products obtained from 121 ticks with *Ehrlichia*-specific and *Borrelia*-specific oligonucleotide probes

Sex and stage	No. of ticks	No. of ticks containing the following[a]:															
		Ehrlichia species								*Borrelia* species							
		Ehrlichia	HGE	E.phago	HGEvar	E.phagovar	Ehr-like	HGE + E.phagovar	HGEvar + E.phagovar	HGEvar + Ehr-like	*B. burgdorferi* sensu lato	*B. afzelii*	*B. valaisiana*	*B. afzelii* + *B. valaisiana*	*B. afzelii* + *B. garinii*	*B. burgdorferi* sensu stricto + *B. garinii*
Nymph	11	0									1	1				
Male	26	13		1	3	4				1	1			1		
Female																
Nonengorged	21	10			5	1	1		1		4	1	2		1	
Semiengorged	24	8			1	1	3		2		4	2		1		1
Engorged	39	23	3	2	2	3	4	1	4		6	5	1			
Total	121	54	3	3	11	9	8	1	7	1	16	9	3	2	1	1

[a] E.phago, *E. phagocytophila*; E.phagovar, *E. phagocytophila* variant, HGEvar, HGE variant; Ehr-like, *Ehrlichia*-like organism.

TABLE 3. Origin of ticks and distribution of *Ehrlichia* and *Borrelia* species in the ticks

Deer no.	No. of ticks	No. of ticks containing the following *Ehrlichia* and *Borrelia* species[a]:																
		Ehrlichia	HGE	E.ph	HGEvar	E.phvar	Ehr-like	HGE + E.ph	HGEvar + E.phvar	HGEvar + Ehr-like	Ehr-Unspec	*B. burgdorferi*.sl	B.af	B.va	B.af + B.va	B.af + B.ga	B.ss + B.ga
758	18	11		1	2		5				3	3	2	1			
768	2	0										0					
770	1	0										0					
775	8	6	2			3		1				0					
778	3	2				2						3	1	1	1		
783	4	2			1	1						0					
784	2	0										0					
787	3	0										1	1				
790	2	0										0					
791	1	0										0					
792	2	0										0					
795	1	1					1					0					
796	3	0										0					
798	1	0										0					
799	3	1					1					1				1	
800	2	0										0					
801	4	2	1								1	1	1				
804	2	0										1	1				
805	1	1									1	0					
809	1	1					1					0					
810	2	0										0					
812	2	0										1	1				
813	3	0										0					
814	2	0										0					
815	1	0										0					
816	3	2		1							1	0					
817	7	6		1		2					3	0					
823	2	1			1							0					
824	2	1			1							0					
825	2	0										0					
827	1	1			1							0					
829	2	0										0					
831	5	2							1		1	1		1			
832	2	2			2							0					
837	8	6			3	1			1		1	1		1			
840	10	6							5	1		2	1	1			
865	1	0										1	1				
AA	2	0										0					
Total	121	54	3	3	11	9	8	1	7	1	11	16	9	3	2	1	1

[a] E.ph, *E. phagocytophila*; HGEvar, HGE variant; E.phvar, *E. phagocytophila* variant, Ehr-like, *Ehrlichia*-like organism; Ehr-Unspec, *Ehrlichia*-like organism but the species was not determined; sl, sensu lato; B.af, *B. afzelii*; B.va, *B. valaisiana*; B.ga, *B. garinii*; B.ss, *B. burgdorferi* sensu stricto.

However, there was no significant correlation between sex and engorgement of the ticks and infection with *Ehrlichia* species. In addition, ticks collected from the same roe deer carried a variety of *Ehrlichia* and *Borrelia* species. This suggests that the ticks may have been infected before feeding on the roe deer and that the *Ehrlichia* spp. originated from other reservoirs. In order to get a more accurate impression of the prevalence of *Ehrlichia* infection in Dutch ticks, we are now analyzing a large number of ticks collected from the vegetation. Whatever the reservoir may be, the results obtained in this survey suggest that Dutch ticks may pose a serious health threat to both humans and animals and should be used to warn clinicians to be aware of the possible presence of ehrlichiosis in The Netherlands.

TABLE 4. Combinations of *Ehrlichia* and *Borrelia* species found in ticks

Deer no.	*Ehrlichia* species	*Borrelia* species
778	*E. phagocytophila*-variant	*B. afzelii* + *B. garinii*
778	*E. phagocytophila*-variant	*B. afzelii* + *B. valaisiana*
837	HGE variant	*B. valaisiana*
840	*E. phagocytophila*-variant + HGE variant	*B. valaisiana*
840	*Ehrlichia*-like spp. without species determination	*B. afzelii*

The majority of the *Ehrlichia* species found in this study belong to the *E. phagocytophila* group. As expected, neither *E. canis* nor *E. chaffeensis* was found in any of the ticks. Analysis of PCR products revealed that the 16S rRNA gene sequences of the *E. phagocytophila* group showed slight variations. In total, four types of *E. phagocytophila*-like sequences were found: species with the *E. phagocytophila* or the HGE 16S rRNA gene sequences and two variants of these sequences that carried a substitution of a single base pair at position 92 of the 16S rRNA gene. This corroborates the findings of a Swedish group (28) and a group from the United States (2) that also found *Ehrlichia* species in which the A at position 92 of the 16S gene was substituted by a G. It remains to be determined

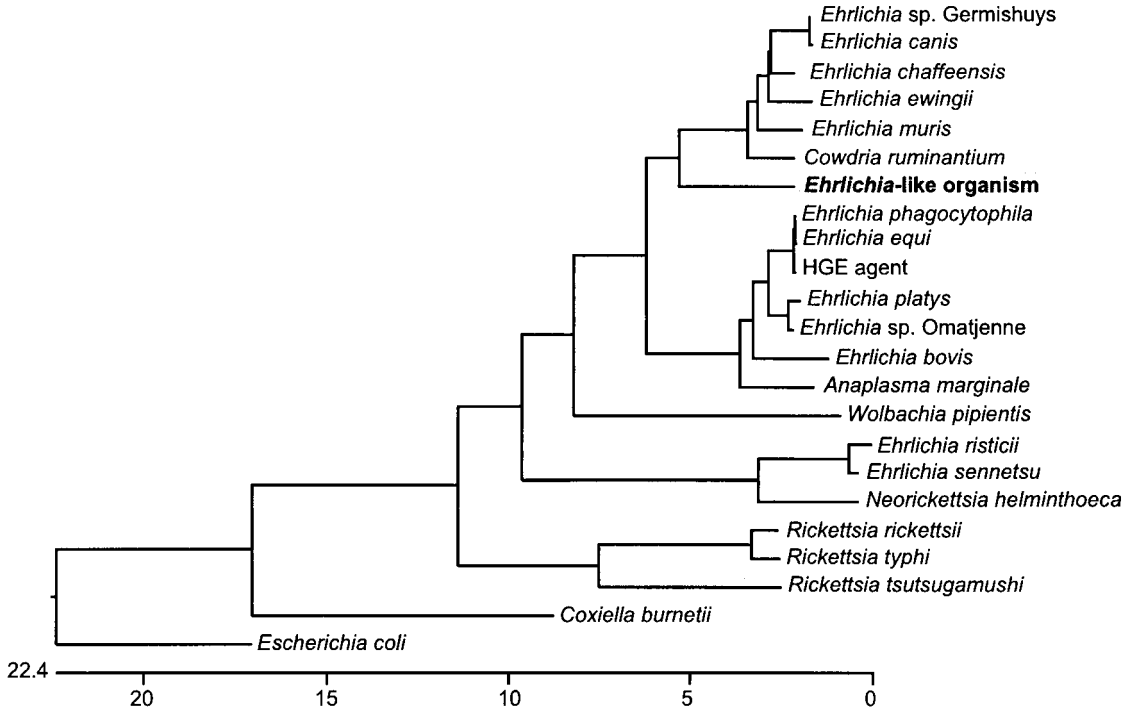

FIG. 3. Dendrogram showing the phylogenetic relationships of the 16S rRNA gene sequences of the newly identified *Ehrlichia*-like and those of other rickettsiae. The tree was constructed by comparing sequences of the segment of the 16S rRNA gene ranging from bases 40 to 1434 (*E. phagocytophila* coordinates). The scale beneath the tree measures the distance between sequences expressed as the number of substitution events. The sequences used for comparison were obtained from the GenBank and EMBL database.

whether the 16S rRNA variants represent different *Ehrlichia* species. It is possible that the HGE agent, *E. phagocytophila*, and the variants found in this study all belong to the same species and should be designated *E. phagocytophila* subspecies. Furthermore, it is unclear whether these variants can cause disease in humans or animals. It was remarkable that in none of the samples of the *E. phagocytophila* group from which the 16S rRNA gene sequences were determined was a C found at position 49 in the 16S rRNA gene. The presence of a C at this position may be characteristic for *E. equi*. This would corroborate earlier observations that *E. equi* was not found in Europe.

More than 6% of the ticks were infected with an *Ehrlichia*-like organism not described before. This organism is closely related to but clearly distinct from the monocytic group of *Ehrlichia* species and *C. ruminantium*. It is unclear whether this organism can cause disease in mammals, but experimental infection of animals may confirm its infectious nature. The newly identified organism may represent an endosymbiont. Examples of such endosymbionts in ticks are the *Francisella* and *Wolbachia* species, which are found at high rates in particular tick species (16–18). However, the relatively low frequency of infection of the ticks would argue against this hypothesis.

Analysis of the 121 ticks showed that 13% of the ticks carried *B. burgdorferi* species and confirmed earlier findings that 10 to 35% of the Dutch *I. ricinus* ticks are infected with *B. burgdorferi* genospecies (24). Interestingly, 5 of the 121 ticks were coinfected with *Ehrlichia* and two genospecies of *B. burgdorferi*. Due to its immunosuppressive nature, coinfection with *Ehrlichia* and *B. burgdorferi* may increase the severity of Lyme borreliosis.

Transmission of *Bartonella* species by ticks is speculative. However, at least one study reports on three patients with *B. henselae* bacteremia. These patients had no history of contact with cats but sustained tick bites prior to the bacteremia (13). From the study presented here it is clear that a large proportion of the ticks carry *Bartonella* species or species closely related to *Bartonella* but not the human pathogens *B. henselae* and *B. quintana*. The *Bartonella* species found might originate from small rodents on which the ticks may have been feeding. This could indicate that transmission of *Bartonella* species between rodents is, at least in some part, tick mediated. Further studies with other arthropods such as body lice and perhaps also blood from rodents such as rats may disclose the reservoirs and vectors for *B. quintana*.

Until now there have been no reports of ehrlichiosis in Dutch patients. Therefore, the high rate of infection of Dutch ticks with *Ehrlichia* species raises the question of whether human ehrlichiosis does occur in The Netherlands. It is known that *Ehrlichia* species cause infections in cattle, sheep, and dogs in Europe. However, until now there have been very few reports on human ehrlichiosis in Europe (15, 20, 27). In fact, only recently was the first case of granulocytic ehrlichiosis infection reported, and that was in Slovenia (20). Although the seroprevalence in several European serosurveys suggest that infections with *Ehrlichia* do occur in Europe, there seems to be a paucity of reported cases. There may be several explanations for this phenomenon. First, it is possible that there really are very few cases of human ehrlichiosis. Second, the majority of cases may go unnoted because they are caused by less virulent variants of HGE that result in a mild course of disease. Finally, cases of ehrlichiosis may remain unnoted because clinicians do not recognize the disease. Relatively few clinicians know that the disease exists and therefore cannot make the correct diagnosis. Furthermore, the tools used to diagnose ehrlichiosis are

usually lacking. Very few laboratories in The Netherlands are equipped to perform serology studies for *Ehrlichia*, and PCR is performed in none of these laboratories. Therefore, at least in The Netherlands, ehrlichiosis may have been overlooked. Recently, a Swedish group reported on three PCR-confirmed cases of HGE infection in humans (PROMED file 980418193622). Two of the three patients were seronegative, which forewarns us that serology may not suffice for the diagnosis of ehrlichiosis. The patients showed a variety of clinical symptoms, of which only fever and headache were seen in all three patients. Remarkably, the initial diagnosis for one of the patients was neuroborreliosis, and the patient was treated for this condition. These findings indicate that HGE infections do occur in Europe and suggest that there may indeed be an underdiagnosis of ehrlichiosis and that surveillance is required to determine the true extent of the problem.

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