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Quantitative Real-Time PCR for Detection of Members of the *Ehrlichia phagocytophila* Genogroup in Host Animals and *Ixodes ricinus* Ticks

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A TaqMan PCR was established for identification and quantitation of members of the *Ehrlichia phagocytophila* group in experimentally infected cows and in *Ixodes ricinus* ticks. The TaqMan PCR identified a 106-bp section of the 16S rRNA gene by use of a specific fluorogenic probe and two primers. This technique was specific for members of the *E. phagocytophila* group, which include *E. phagocytophila*, *Ehrlichia equi*, and the agent of human granulocytic ehrlichiosis. The TaqMan system identified 10 copies of a cloned section of the 16S rRNA gene of *E. phagocytophila*. The sensitivity and specificity of the TaqMan PCR were similar to those of conventional nested PCR. The numbers of ehrlichiae in leukocytes of the two cows experimentally infected with *E. phagocytophila* were measured daily by TaqMan PCR and had a course similar to that of the percentages of infected leukocytes determined daily by light microscopy. The prevalence of infected free-living ticks, which were collected from areas where bovine ehrlichiosis is endemic and from regions with sporadic occurrences of granulocytic ehrlichiosis in dogs and horses, was identical as determined by nested PCR and TaqMan PCR.

Ehrlichiosis is a rickettsial disease of animals and humans caused by various species of *Ehrlichia*. These obligate intracellular microorganisms have an affinity for eukaryotic cells and, as reviewed by Rikihisa (17), are usually transmitted by ticks. Based on the nucleotide sequence of the 16S rRNA gene, the members of this genus have been divided into the *E. phagocytophila*, *E. canis*, and *E. sennetsu* groups (1). The *E. phagocytophila* group includes *E. phagocytophila*, the cause of tick-borne fever in goats, sheep, and cattle in Europe; *E. equi*, the cause of equine ehrlichiosis in the United States; and the recently discovered agent of human granulocytic ehrlichiosis (HGE), which causes disease in humans, horses, and dogs in both the United States and Europe (6, 9, 10, 13).

The diagnosis of granulocytic ehrlichiosis in animals in the acute phase is based on clinical findings (fever, anorexia, apathy, limb oedema, and petechial hemorrhages), laboratory findings (leukopenia, aenemia, thrombocytopenia, and detection of intracytoplasmic inclusion bodies in leukocytes), tick infestation, season, and geographical location (17). In addition, a sensitive and specific PCR for detection of *Ehrlichia* DNA in host blood or in vectors has been recently described (2, 3, 8, 14). This report describes a quantitative real-time PCR with the TaqMan fluorogenic detection system for detection of members of the *E. phagocytophila* group. This new system uses a specific fluorogenic probe and two primers. The fluorescence released by the endogenous 5′-3′ nuclease activity of *Taq* DNA polymerase is proportional to the accumulated PCR products. Quantitation of *Ehrlichia* in an unknown sample is performed by comparison of the fluorescence signals of the sample to those of a standard curve. Cows with bovine ehrlichiosis and free-living ticks were used to evaluate this method for detection of members of the *E. phagocytophila* genogroup.

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MATERIALS AND METHODS

Oligonucleotide design of the probe and primers. The TaqMan probe and the primer sequences were designed with the software program Primer Express (Perkin-Elmer, Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. The sequence of the probe was selected based on previously described criteria (11): predicted cross-reactivity to *E. phagocytophila*, *E. equi*, and the agent of HGE; melting temperature of the probe of 69°C; lack of predicted dimer formation with corresponding primers and of self-annealing; a 10°C higher melting temperature of the probe than of the primers; and no stretches of identical nucleotides longer than four and no G at the 5′ end of probe. The fluorescent reporter dye at the 5′ end of the TaqMan probe (Ep.80p, CCTATGCATTACTCACCCGTCTGCCACT) was 6-carboxy-fluorescein (FAM); the quencher at the 3′ end was 6-carboxy-tetramethyl-rhodamine (TAMRA). Primers Ep.50r (5′-TCGAACGGATTATTCTTTATAGCTTG-3′) and Ep.145f (5′-CCATTTCTAGTGGCTATCCCATACTAC-3′) amplified a 106-bp fragment of the 16S rRNA gene.

Fluorogenic PCR. The 25-μl PCR mixtures contained 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 400 nM each primer, 20 nM fluorogenic probe, 0.125 U of *Taq* DNA polymerase per reaction, and 10 μl of diluted template or plasmid standard. After target denaturation for 3 min at 95°C, amplification conditions were five cycles of 30 s at 95°C and 20 s at 62°C, followed by 40 cycles of 40 s at 85°C and 60 s at 62°C. Amplification, data acquisition, and data analysis were carried out with an ABI 7700 Prism Sequence Detector (Perkin-Elmer, Applied Biosystems). Data were calculated with the Sequence Detector software.

Plasmid standard for absolute quantitation. A plasmid standard for absolute quantitation was prepared as follows. The 106-bp fragment obtained with the two primers Ep.145f and Ep.50r was cloned into pCR2.1-TOPO and transformed into *Escherichia coli* TOPO10 (Topo TA cloning kit; Invitrogen, NV Leek, The Netherlands). Purification of the plasmid DNA was carried out with a commercial plasmid kit (Qiagen, Basel, Switzerland). For bidirectional DNA sequencing of the insert, the M13 forward primer (5′-GTAAAACGACGGCCAG-3′) and M13 reverse primer (5′-CAGGAAACAGCTATGACC-3′) were used. The nucleotide sequence was detected with a fluorescence-based automated sequencing system (ABI 377A DNA sequencer) by Microsynth, Balgach, Switzerland. The plasmid insert was sequenced to confirm its identity with *E. phagocytophila*.

Specificity and sensitivity of the TaqMan PCR. Samples of seven rickettsial species were obtained to examine the specificity of the TaqMan PCR. The rickettsiae tested and their sources were as follows: *E. phagocytophila* (Swiss strain), *E. equi* (J. E. Madigan, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis), HGE agent (Protatek International, St. Paul, Minn.), *E. canis* (Protatek International), *E. risticii* (J. E. Madigan), *Anaplasma marginale* (Protatek International), and *Rickettsia rickettsii* (T. Glaus, Department of Veterinary Internal Medicine, University of Zurich, Zurich, Switzerland).

Dilutions of the linearized and purified plasmid standard were used to test the analytic sensitivity in the range of 1 to 10^9 copies of plasmid standard.

Experimental infection. Two clinically healthy Swiss Braunvieh cows aged 4 (cow 1) and 5 (cow 2) years were used for experimental infections. All cows originated from tick-free regions and had no antibodies to *E. phagocytophila*, as determined by indirect immunofluorescence. The cows were housed in a vector-proof facility at the University of Zurich and inoculated intravenously with 50 ml of thawed whole blood from an infected cow. The blood contained 460 leukocytes infected with *E. phagocytophila* (Swiss strain) per μl. The cows were monitored twice daily for clinical signs of bovine ehrlichiosis. Blood samples were drawn each morning for routine hematology, conventional PCR, and TaqMan PCR. From each blood sample, blood smears were prepared, stained with May-Grünwald Giemsa stain, and examined at a magnification of ×1,000. Five hundred leukocytes per blood smear were examined for *E. phagocytophila* organisms, and the percentage of positive cells was calculated. A washed leukocyte pellet was obtained from a sodium citrate-preserved blood sample. The DNA was prepared and the nested PCR was done as described previously (14). The number of *Ehrlichia* equivalents per μg of DNA extracted from the buffy coat was determined for the TaqMan PCR results.

Tick samples. A total of 2,320 free-living *Ixodes ricinus* ticks were collected from regions where bovine ehrlichiosis is endemic ($n = 653$) and from regions in Switzerland where there have been sporadic occurrences of granulocytic ehrlichiosis in dogs and horses ($n = 1,667$). The ticks were previously examined in two studies using the conventional nested PCR (15, 16). The prevalences of TaqMan PCR-positive ticks from the different sources were determined. In addition, the *Ehrlichia* load of infected ticks was expressed as *Ehrlichia* equivalents per μg of tick DNA.

RESULTS

Specificity and sensitivity of the TaqMan PCR. Of the rickettsial species examined, only *E. phagocytophila*, *E. equi*, and the HGE agent produced a TaqMan PCR specific fluorescence. All other rickettsial species were not able to generate a TaqMan fluorescence after 45 PCR cycles.

The analytical sensitivity of the TaqMan PCR was comparable to that of the previously described nested PCR (14). Both systems detected 10 copies of the standard plasmid. The samples containing fewer than 10 copies and the no-template controls gave a negative result. The amplification of a plasmid standard dilution over eight 10-fold dilutions showed linearity over the whole range.

Experimental infection study. The experimentally infected cows became ill with symptoms of bovine ehrlichiosis after incubation periods of 6 (cow 2) and 7 (cow 1) days. The most important clinical signs were fever (>39.5°C); decreased milk production; a mildly disturbed general condition; respiratory symptoms such as polypnea, nasal discharge, cough, and abnormal lung sounds; leukopenia; thrombocytopenia; and erythropenia. *Ehrlichia* organisms were first observed in the cytoplasm of neutrophils at days 5 (cow 2) and 6 (cow 1) and were seen for 10 days in both cows. During the phase of bacteremia, the percentages of infected leukocytes ranged from 1 to 18% in cow 1 and from 1 to 20% in cow 2. The nested PCR with blood buffy-coat cells became positive on day 4 in both cows and remained positive through day 20 (cow 1) or 22 (cow 2).

The course of the TaqMan PCR was identical to that of the nested PCR. A fluorescent signal was detected from the day 4 to 20 in cow 1 and from the day 4 to 22 in cow 2. Over the observation period, the numbers of *Ehrlichia* organisms quantified by the TaqMan PCR had a course that paralleled the percentages of infected leukocytes determined by light microscopy (Table 1).

Prevalence of granulocytic *Ehrlichia* in *I. ricinus*. Of the 653 free-living ticks collected from regions where bovine ehrlichiosis is endemic, 5 (0.8%) were positive in the TaqMan PCR (Table 2). Of the 1,667 ticks from regions with sporadic occurrences of granulocytic ehrlichiosis in dogs and horses, 21 (1.3%) had fluorescence specific for *Ehrlichia*. These results are in agreement with those of previous studies using nested PCR (15, 16). In those studies, nucleotide sequencing of the isolated products of the nested PCR characterized them as part of the 16S rRNA gene of *E. phagocytophila* and of a species of *Ehrlichia* with 100% homology to the agent of HGE in the United States. The *Ehrlichia* load of infected ticks was in the range of 73,000 to 170,000 *Ehrlichia* equivalents per μg of tick DNA, with a mean of 120,000 *Ehrlichia* equivalents per μg of tick DNA.

TABLE 1. Detection of *E. phagocytophila* by microscopy, nested PCR, and TaqMan PCR

Day	Cow 1			Cow 2		
	% Infected leukocytes	Result of:		% Infected leukocytes	Result of:	
		Nested PCR	TaqMan PCR (molecules/μg)		Nested PCR	TaqMan PCR (molecules/μg)
0	0	−	0	0	−	0
4	0	+	1.9×10^2	0	+	2.5×10^3
5	0	+	9.4×10^2	1	+	3.2×10^5
6	5	+	2.5×10^3	11	+	1.5×10^6
7	13.5	+	4.1×10^6	17	+	3.1×10^6
8	18	+	3.3×10^7	20	+	2.1×10^7
9	16.5	+	6.8×10^6	6	+	9.5×10^5
10	7	+	1.1×10^4	3	+	6.8×10^4
11	1	+	3.8×10^3	1	+	4.6×10^4
12	2	+	4.7×10^4	2	+	2.5×10^4
13	2	+	4.5×10^4	1.5	+	1.1×10^4
14	2	+	2.5×10^4	1	+	2.4×10^4
15	1	+	2.4×10^3	0	+	4.0×10^4
16	0	+	2.1×10^3	0	+	8.9×10^3
17	0	+	3.2×10^3	0	+	1.1×10^4
18	0	+	2.4×10^3	0	+	6.5×10^3
19	0	+	2.5×10^2	0	+	7.0×10^3
20	0	+	1.5×10^2	0	+	2.5×10^3
21	0	−	0	0	+	2.9×10^2
22	0	−	0	0	+	6.3×10^2
23	0	−	0	0	−	0

DISCUSSION

The *Ehrlichia*-specific TaqMan assay described here takes advantage of the endogenous 5′-3′ nuclease activity of *Taq* DNA polymerase to digest the TaqMan probe, which hybridizes to the amplicon during PCR. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence, primarily by Förster-type energy transfer. During PCR, forward and reverse primers hybridize to a specific sequence of the target DNA. The TaqMan probe hybridizes to a target sequence within the PCR product. The reporter dye and quencher dye are separated upon cleavage, resulting in increased fluorescence of the reporter. The fluorescence intensity is directly related to the amount of input target DNA and can be detected with an automated fluorometer. Quantitation is accomplished by comparison of the fluorescence signals obtained from samples with unknown *Ehrlichia* DNA loads with the fluorescence signals obtained from *Ehrlichia* plasmid standard dilutions. The TaqMan PCR has been used for quantification of bacteria such as *Listeria monocytogenes* (4), *Salmonella* spp. (5), *Mycobacterium tuberculosis* (7), and *Yersinia pestis* (12). However, to our knowledge, this technique has not been used for members of the genus *Ehrlichia*. The advantages of the TaqMan PCR, in comparison to conventional nested PCR, are a shorter working time (shorter cycling time and absence of gel electrophoresis), determination of larger sample numbers (up to 96 samples per round), and a lower risk of contamination. The sensitivities and specificities of the two methods are identical.

TABLE 2. Results of TaqMan PCR for the identification of granulocytic *Ehrlichia* in *I. ricinus* from different areas

Origin	No. of nested PCR-positive ticks/total (%)	No. of TaqMan PCR-positive ticks/total (%)	Isolated *Ehrlichia*
Areas where tick-borne fever is endemic	5/653 (0.8)[a]	5/653 (0.8)	*E. phagocytophila*
Areas where canine and equine granulocytic ehrlichioses are endemic	21/1,667 (1.3)[b]	21/1,667 (1.3)	HGE agent

[a] Data are from reference 15.
[b] Data are from reference 16.

Based on previous studies involving nested PCR, the probe and two primers used in this study were expected to be specific for *E. phagocytophila*, *E. equi*, and the agent of HGE. The specificity of the TaqMan PCR was confirmed via sequencing of the PCR products. There was no cross-reactivity between any of the *Rickettsia* species outside the *E. phagocytophila* genogroup. The analytical sensitivity of the TaqMan PCR was comparable to that of the previously described nested PCR (14). Both systems detected 10 copies of the standard plasmid.

Infection of two cows with *E. phagocytophila* resulted in characteristic clinical signs of bovine ehrlichiosis, and intracytoplasmic inclusions were seen on cytological examination of blood smears for 10 days. In contrast, the nested PCR was positive 1 or 2 days before and 5 to 8 days after the first and last microscopic inclusions were observed, respectively. In this same time period, there was TaqMan PCR specific fluorescence for *Ehrlichia*. Thus, there was 100% agreement between the nested PCR and the TaqMan PCR. Over the observation period, the number of *Ehrlichia* equivalents per μg of leukocyte DNA had a pattern similar to that for the percentage of infected leukocytes. In the infected cows, there was a rapid increase in the number of *Ehrlichia* equivalents to 10^7 molecules per μg of leukocyte DNA, followed by a slow decrease. The number of molecules is dependent on the leukocyte count, the percentage of infected leukocytes, and the differential leukocyte count. In addition to monitoring the course of an infection, the TaqMan PCR can also be used for quantification of material for experimental infection or for production of antigen.

The prevalence of infected ticks determined by the TaqMan PCR was identical to that determined by conventional nested PCR. To our knowledge, quantification of *Ehrlichia* agents in ticks has not been reported. Presumably, the amount of *Ehrlichia* DNA in ticks is dependent on a number of biological factors, such as the type and stage of tick, the time of year, the geographical region, and the tick's nutritional status. In the future, the TaqMan PCR can be used to study the biology of ticks infected with *Ehrlichia*.

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