Detection and quantitation of *Ehrlichia risticii* genomic DNA in infected horses and snails by real-time PCR

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Abstract

A real-time quantitative PCR using the TaqMan fluorogenic detection system (TaqMan PCR) was established for identification of *Ehrlichia risticii*, the agent of Potomac horse fever (PHF). The TaqMan PCR identified an 85 base pair section of the 16S rRNA gene by use of a specific fluorogenic probe and two primers. This technique was specific for eight tested *E. risticii* strains. The TaqMan system identified 10 copies of a cloned section of the 16S rRNA gene of *E. risticii*. The sensitivity and specificity of the TaqMan PCR were similar to those of conventional nested PCR. The TaqMan PCR was evaluated on horses with infectious colitis and on freshwater stream snails collected from regions with a history of PHF. *E. risticii* could be detected in 22 of 153 (14.4%) horses with infectious colitis and in 25 of 234 (10.7%) snails in the TaqMan PCR. The same results were obtained in the conventional nested PCR. The *Ehrlichia*-load was in the range of 10,000–9,000,000 and 35,000–680,000,000 *Ehrlichia* equivalents per μg leukocyte DNA and snail DNA, respectively. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Real-time PCR; *Ehrlichia risticii*; Horse; Snail

1. Introduction

Ehrlichia risticii is the causative agent of Potomac horse fever (PHF) and has been confirmed in various places throughout North America, Canada and Europe since its first detection in horses along the Potomac River (Knowles et al., 1983). Clinical signs are fever, anorexia, depression, mild colic, watery diarrhea, dehydration, edema, leukopenia

and in some cases laminitis or abortion. The case fatality rate varies from 5 to 30% (Palmer, 1993). The mode of transmission of *E. risticii* remained unidentified. There is no evidence for spread of the disease by arthropod vectors such as ticks (Schmidtmann et al., 1986; Hahn et al., 1990; Levine et al., 1990). Recent work has shown the close phylogenetic relationship between *E. risticii* and the helminth-associated *Neorickettsia helminthoeca*, the agent of salmon poisoning in dogs (Rikihisa, 1991; Pretzman et al., 1995). We have recently described the detection of *E. risticii* DNA in tissues of freshwater operculate snails (Pleuroceridae: *Juga* spp.) and their trematode cercariae collected from stream waters on a PHF enzootic pasture in northern California and from the blood of a horse with clinical signs of PHF residing within that pasture (Barlough et al., 1998; Reubel et al., 1998). Meanwhile, additional evidence to support association between *E. risticii* in equids and trematodes has been demonstrated through successful transmission of *E. risticii* to horses by subcutaneous inoculation with virgulate cercariae from *Juga yrekaensis* snails (Pusterla et al., 2000).

The diagnosis of PHF often is based on clinical signs and seasonal and geographical occurrence of illness. Diagnosis is hampered because of the high percentage of false-positive test results, when using the indirect immunofluorescence assay (Madigan et al., 1995). Isolation of the agent in cell culture is possible but time consuming and not routinely available in many laboratories (Ristic et al., 1986). Recent developments of nested PCR tests for DNA amplification have enhanced the diagnosis (Barlough et al., 1997; Mott et al., 1997). This report describes a real-time PCR using the TaqMan fluorogenic detection system to detect and quantify *E. risticii* (Holland et al., 1991). 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. During amplification the reporter and quencher dyes are separated upon cleavage, resulting in increased fluorescence of the reporter. 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 fluorescence signals obtained from samples with unknown *Ehrlichia* DNA load with the fluorescence signals obtained from *Ehrlichia* plasmid standard dilution. Horses with infectious colitis and freshwater stream snails collected from regions with a history of PHF were used to evaluate this method for detection of *E. risticii*.

2. Materials and methods

2.1. Oligonucleotide TaqMan probe and primers for real-time PCR

Primers and TaqMan probe design were based on a sequence alignment with known 16S rRNA gene sequences of *Ehrlichia* species deposited in the GenBank. Sequences were analyzed with the Genetics Computer Group (GCG, University of Wisconsin, Madison, WI, USA) sequence analysis package. TaqMan probe and primer sequences were designed with Primer Express software (Version 1, Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). The fluorescent reporter dye of the TaqMan probe (ER. 77p: ACGCACCCGTCTGCCACGGGA) at the 5′ end was FAM, the quencher at the 3′ end TAMRA. Primers ER. 133f (5′-GTTATTCCCTACTACCAGGCAAGTTC-3′) and ER. 54r

(5′-AACGGAATCAGGGCTGCTT-3′) amplified an 85 base pair fragment of the 16S rRNA gene.

2.2. Plasmid standard for absolute quantitation

To verify the specificity of the reaction, a TaqMan PCR product was cloned and sequenced as follows: the 85 base pair fragment obtained with the two primers ER. 133f and ER. 54r was cloned into PCR$^{®}$2.1-TOPO and transformed into *Escherichia coli* TOPO10 strain (Topo TA Cloning Kit, Invitrogen, NV Leek, The Netherlands). Purification of the plasmid DNA was carried out using a commercial plasmid kit (Qiagen, Basel, Switzerland). For bi-directional DNA sequencing of the insert, the following primers were used: M 13 forward primer (5′-GTAAAACGACGGCCAG-3′) and M 13 reverse primer (5′-CAGGAAACAGC TATGACC-3′). The nucleotide sequence was detected with a fluorescence-based automated sequencing system (ABI 377A DNA sequencer, Microsynth, Balgach, Switzerland). The plasmid insert was sequenced to confirm its identity with *E. risticii*. The cloned PCR product was used as a plasmid standard to test for analytical sensitivity in the range from 1 to 10^9 copies and for absolute quantitation of *Ehrlichia*-load in leukocyte and snail genomic DNA (gDNA). The number of *Ehrlichia* equivalents per µg leukocyte and snail gDNA was determined by adjusting the TaqMan PCR results to the volume of the aliquot used in the PCR assay and the gDNA concentration.

To test the influence of the carrier DNA on the recognition of the plasmid standard in the TaqMan PCR, we amplified the plasmid standard against a broad range of carrier DNA. Two types of carrier DNA were used: calf thymus DNA (Calf thymus DNA, Sigma, Buchs, Switzerland) and gDNA from an *E. risticii* negative horse and snail. Ten copies of plasmid standards were amplified with varying amounts of carrier DNA ranging from 6000 to 5 µg/ml in a 25 µl reaction volume.

2.3. Real-time TaqMan PCR

The 25 µl PCR mixtures contained 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM MgCl$_2$, 2.5 mM deoxynucleotide triphosphates, 400 nM of each primer, 80 nM of fluorogenic Taq-Man probe, 0.625 U of AmpliTaq Gold per reaction, and 10 µl of diluted template or plasmid standard. After AmpliTaq Gold activation for 10 min at 95°C, amplification conditions were: 45 cycles of 15 s at 95°C and 60 s at 60°C. Amplification, data acquisition and data analysis were carried out in a ABI 7700 Prism Sequence Detector (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). Data were calculated with the Sequence Detector Software.

2.4. Specificity of the TaqMan PCR

DNA samples of 17 strains of rickettsiae obtained from various sources (Table 1) were used to test the specificity of the TaqMan PCR.

2.5. Specimens from horses with infectious colitis and snails

From 1995 to 1998, blood specimens were obtained from 153 horses in California and Oregon with infectious colitis. The samples were collected from veterinary practitioners

Table 1
Specificity of the *Ehrlichia risticii* TaqMan PCR

Organism	Source	TaqMan PCR detection
Ehrlichia risticii — Illinois strain[a]	American Type Culture Collection	Yes
Ehrlichia risticii — Kentucky strain[a]	Y. Rikihisa, The Ohio State University	Yes
Ehrlichia risticii — Ohio-081 strain[a]	Y. Rikihisa, The Ohio State University	Yes
Ehrlichia risticii — SRC strain[a]	J.E. Madigan, University of California, Davis	Yes
Ehrlichia risticii — SHSN-1[b]	J.E. Madigan, University of California, Davis	Yes
Ehrlichia risticii — SHSN-2[b]	J.E. Madigan, University of California, Davis	Yes
Ehrlichia risticii — JUGA[b]	J.E. Madigan, University of California, Davis	Yes
Ehrlichia risticii — KLSN[b]	J.E. Madigan, University of California, Davis	Yes
Ehrlichia chaffeensis	Protatek International, St. Paul, MN	No
Ehrlichia phagocytophila	N. Pusterla, University of Zurich	No
Ehrlichia equi — MRK strain	J.E. Madigan, University of California, Davis	No
HGE agent — BDS strain	J.S. Bakken, Duluth Clinic, Duluth, MN	No
Ehrlichia canis	Protatek International, St. Paul, MN	No
Neorickettsia helminthoeca	Y. Rikihisa, The Ohio State University	No
Anaplasma marginale	Protatek International, St. Paul, MN	No
Rickettsia rickettsii	Hillcrest Biologicals, Cypress, CA	No
Rickettsia typhi	Hillcrest Biologicals, Cypress, CA	No

[a] Indicates a strain originated from a horse.
[b] Indicates a strain originated from a snail.

and from veterinarians at the Veterinary Medical Teaching Hospital at the University of California at Davis and submitted to our laboratory for the DNA-based diagnosis of PHF. The extraction of gDNA and the procedure of nested PCR were done as described previously (Barlough et al., 1997). The TaqMan PCR results were compared to the nested PCR results. In order to quantify the agent in the blood specimens, the number of *Ehrlichia* equivalents per μg gDNA extracted from the buffy-coat was determined for the TaqMan PCR results.

Collected snails (*n*=234 *Juga* spp.) were extracted from their shell using sterile scissors and tweezers and kept in phosphate-buffered saline. The mixture was shortly vortexed to allow the fragile snail tissue to disrupt and to release the trematode stages. Each mixture (50 μl) containing trematode stages and snail tissue was processed for DNA extraction using a described alkaline extraction protocol for gDNA (Rudbeck and Dissing, 1998). The prevalence of snails obtained by TaqMan PCR was compared to that obtained with nested PCR. In addition, the *Ehrlichia*-load of snails was determined and expressed as *Ehrlichia* equivalents per μg of snail gDNA.

3. Results

3.1. Specificity and sensitivity of the TaqMan PCR

TaqMan PCR specific fluorescence was obtained only with *E. risticii*, irrespective of the origin of the strain (Table 1). The TaqMan PCR was negative with the DNA from other rickettsial species after 45 cycles of amplification.

Sequencing of the cloned TaqMan PCR product showed 100% sequence identity to the 16S rRNA gene of *E. risticii* (GenBank Accession No. M21290). The TaqMan PCR was able to detect 10 copies of the standard plasmid suspended either in calf thymus DNA or in gDNA from a negative horse and snail as carrier DNA. The analytical sensitivity of the TaqMan PCR was comparable to the previously described nested PCR (Barlough et al., 1997). Negative results were obtained with samples that contained less than 10 copies of the plasmid and in the control reaction that did not have any template DNA. The amplification of a plasmid standard dilution over eight orders of magnitude showed linearity over the whole range.

3.2. Horse and snail specimens

Of the 153 horses with infectious colitis, 22 (14.4%) were positive in the TaqMan PCR. The same results were obtained by the nested PCR. The *Ehrlichia*-load of infected horses was in the range of 10,000–9,000,000 *Ehrlichia* equivalents per μg leukocyte DNA.

Of the 234 freshwater stream snails collected from regions with a history of PHF 25 (10.7%) were positive in the TaqMan PCR. These results are in agreement with those using nested PCR. The *Ehrlichia*-load of infected snails was in the range of 35,000–680,000,000 *Ehrlichia* equivalents per μg snail DNA with a mean of 96,000,000 *Ehrlichia* equivalents per μg snail DNA.

4. Discussion

To our knowledge, the TaqMan PCR has not been used previously for the detection of *E. risticii*. The advantages of the TaqMan PCR, in comparison to conventional nested PCR, are shorter working time (shorter cycling time, gel electrophoresis is not required), determination of larger sample numbers (up to 96 samples per round), lower risk of contamination and lower reagent costs.

Based on a sequence alignment with known 16S rRNA gene sequences of *Ehrlichia*, the TaqMan probe and two primers used in this study were only specific for the horse and snail strains of *E. risticii*. The analytical sensitivity of the TaqMan PCR was comparable to the previously described nested PCR (Barlough et al., 1997) with a detection limit of 10 copies of the standard plasmid.

The prevalence of PHF among the horses with infectious colitis appears to be low. A serological study carried out among horses with clinical signs of PHF from various regions of California (Madigan et al., 1995) showed a prevalence of 16.3%, which is comparable to that of our study. The low percentage of TaqMan PCR positive horses suggests that *E. risticii* was not the cause of the infectious colitis in most of the horses tested. Other causes of the clinical signs in these horses could have, included salmonellosis, *Clostridium difficile* infection, alterations of the gastrointestinal flora, colitis X and idiopathic colitis. There was 100% agreement between the nested PCR and the TaqMan PCR. The number of *Ehrlichia* equivalents per μg of leukocyte DNA demonstrated a large range of variation. The number of *E. risticii* molecules present in a blood sample is dependent on the leukocyte count, the percentage of infected leukocytes and the differential white blood cell count. This is

similar to the situation in cows experimentally infected with *E. phagocytophila* (Pusterla et al., 1999). The variation of these parameters during the course of the disease is probably responsible for the wide range of the *Ehrlichia*-load in horses infected with *E. risticii*. Quantitation by TaqMan PCR can be used to monitor the course of an infection, quantify material for experimental infection or antigen production.

The prevalence of infected snails determined by the TaqMan PCR was identical to that determined by conventional nested PCR. Barlough et al., (1998) have recently shown that pleurocerid stream snails may play a role in the life cycle of *E. risticii*. The authors found a minimum of 3.5% infected snails harboring the microorganism. Presumably, the percentage of infected snails and the amount of *Ehrlichia* DNA in snails is dependent on a number of biological factors correlated to the snails and the vectors such as the type and maturation status of snail or helminth vector, the time of year and the geographical region. In the future, the TaqMan PCR will be used to study the role of intermediate hosts and vector in the life cycle of *E. risticii*.

Acknowledgements

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