



Published in final edited form as:

Ticks Tick Borne Dis. 2016 February ; 7(1): 146–149. doi:10.1016/j.ttbdis.2015.10.004.

A real-time PCR assay for detection of the *Ehrlichia muris*-like agent, a newly recognized pathogen of humans in the upper Midwestern United States

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Abstract

The *Ehrlichia muris*-like agent (EMLA) is an emerging, tick-transmitted human pathogen that occurs in the upper Midwestern United States. Here, we describe the development and validation of a *p13*-based quantitative real-time PCR TaqMan assay to detect EMLA in blood or tissues of ticks, humans, and rodents. The primer and probe specificities of the assay were ascertained using a large panel of various *Ehrlichia* species and other members of Rickettsiales. In addition to control DNA, both non-infected and EMLA-infected human blood, *Mus musculus* blood, and *Mus musculus* tissue extracts were evaluated, as were non-infected and EMLA-infected *I. scapularis* and uninfected *D. variabilis* DNA lysates. The specificity of the probe was determined via real-time PCR. An EMLA *p13* control plasmid was constructed, and serial dilutions were used to determine the analytical sensitivity, which was found to be 1 copy per 4 μ l of template DNA. The sensitivity and specificity of this assay provides a powerful tool for ecological studies involving arthropod vectors and their mammalian hosts.

Keywords

Ehrlichia muris; *Ehrlichia muris*-like (EML) agent; Ehrlichiosis; Tick-borne Disease

Introduction

Ehrlichioses are tick-borne diseases caused by obligate intracellular bacteria in the genus *Ehrlichia* that affect various mammals including humans. In the United States, ehrlichiosis has historically been caused by infection with *Ehrlichia chaffeensis* or *Ehrlichia ewingii* (Paddock and Childs 2003). However, in 2009, a new ehrlichial pathogen, designated the *Ehrlichia muris*-like agent (EMLA), was identified in patients and *Ixodes scapularis* ticks in Minnesota and Wisconsin (Pritt et al. 2011). Since the initial report, this pathogen has been identified in more than 50 patients, as well as white-footed mice (*Peromyscus leucopus*) and

additional *I. scapularis* ticks in these states, though there have been no successful efforts to identify EMLA outside of Wisconsin and Minnesota to date (Pritt et al. 2011, Castillo et al. 2014, Wormser and Pritt 2015).

EMLA is genetically most closely related to *E. muris*, a species of *Ehrlichia* found in Asia and Japan, and may in fact represent a subspecies of *E. muris* (Wen et al. 1995, Rar et al. 2008, Rar et al. 2010, Karpathy et al. 2015). Because of this close genetic relationship between EMLA and *E. muris*, molecular detection in research labs has been primarily limited to broad range PCR with additional analysis via sequencing or melt curve analysis. While this is effective, it is time consuming and generally expensive. The availability of a real-time TaqMan PCR assay for specific detection of EMLA and exclusion of *E. muris* will provide a useful tool in both the continued search for EMLA outside of Minnesota and Wisconsin as well as ecological studies of EMLA, which are needed to more clearly elucidate the natural reservoir and other possible vectors of the pathogen.

Materials and Methods

Design of PCR primers and probe

All available EMLA gene sequences in GenBank were compared to the *E. muris* AS145^T sequences. The percent identity of outer membrane protein *p13* for the two bacteria is 91%, significantly less than that of other loci, and thus this locus was chosen as a good potential PCR target for differentiating the two. The EMLA *p13* gene sequence (KF523727) was aligned with the *E. muris* strain AS145^T *p13* sequence (KC595882) using MEGA version 5.1 software to identify any regions that differ substantially between the two species (Tamura et al. 2011). Primers P13-116-F and P13-196-R were designed to take advantage of a 12 nucleotide deletion at position 120 of the EMLA *p13* gene that is not found in the type strain of *E. muris* (Figure 1). The forward and reverse primers were designed using NCBI's Primer-BLAST and NetPrimer (www.premierbiosoft.com) to amplify a 99 bp fragment of the EMLA *p13* gene. The 25 bp probe P13-147-FAM was then designed to bind the amplicon and to have a melting temperature that was 10 °C higher than the primers (Table 1). Oligonucleotides were synthesized by the Biotechnology Core Facility Branch at CDC.

PCR optimization

DNA extracted from EMLA-infected DH82 cells was used throughout the optimization process as template DNA. Temperatures ranging from 55 to 65 °C were tested to determine the optimal annealing temperature. The PCR reactions were always conducted in a final volume of 25 µl, and the amplified PCR products were visualized by UV lamp in a 1.5% agarose gel containing 0.1 µg/ml ethidium bromide.

PCR amplification

The PCR assay was performed in a BioRad CFX 96 thermal cycler using the QuantiTect Multiplex PCR Kit (QIAGEN, Valencia, CA). Each 25 µl reaction consisted of 12.5 µl QuantiTect Master Mix, 7 µl PCR-grade water, 1 µl of the probe at 5 µM, 0.25 µl each of the forward and reverse primers at 20 µM, and 4 µl of template DNA. Cycling conditions were as follows: initial denaturation at 95 °C for 15 minutes followed by 45 cycles of 95 °C for 1

minute and 60 °C for 1 minute. Fluorescence data was acquired at the end of the annealing step of each cycle.

Cloning and sequencing

PCR was used to amplify the *p13* region from EMLA template DNA using primers P13-116-F and P13-196-R. The amplicon was purified using the Promega Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) and cloned into pCR2.1 using the TOPO TA cloning kit (Life Technologies, Grand Island, NY). The cloned products were sequenced in both directions and assembled using Sequencher 5.1 (Gene Codes, Ann Arbor, MI) to verify insertion of the *p13* amplicon.

Specificity and sensitivity

To characterize the specificity of the PCR reaction, the primers and probe were used to analyze positive controls from cell culture for other ehrlichial species, as well as DNA of cell culture samples of other members of the order Rickettsiales. In addition to control DNA, non-infected and EMLA-infected *Mus musculus* blood and tissue extracts were screened, as were non-infected *I. scapularis* and *D. variabilis* DNA lysates and EMLA-infected *I. scapularis* lysates, all of which were previously tested using established 16S primers ECHSYBR-F and ECHSYBR-R, which detect all species within the family Anaplasmataceae (Table 2) (Li et al. 2002).

All Rickettsiales in Table 2 are controls from cell culture that were verified using a hemi-nested *ompA* assay and subsequently sequenced for species confirmation (Regnery et al. 1991, Roux et al. 1996). *Anaplasmataceae* culture samples in Table 2 were verified using 16S primers and sequencing, as were *Neoehrlichia lotoris* (obtained from the blood of a raccoon), and *Neoehrlichia mikurensis* (obtained from the blood a patient in Europe) (Li et al. 2002). Human cells came from the American Type Culture Collection [ATCC] (Manassas, VA). All *Mus musculus* and *Ixodes scapularis* samples from Table 2 were generated at CDC during the course of mouse transmission and co-feeding experiments with EMLA in work that is not yet published.

Further evaluation was performed using a panel of 55 blinded specimens provided by the Division of Clinical Microbiology at Mayo Clinic in Minnesota, comprising DNA extracts from 41 questing adult *I. scapularis* ticks, 13 human patients (EDTA whole blood), and 1 *E. muris* cell culture isolate. Ticks were collected from Camp Phillips, WI in October 2013 and June 2014 and were extracted using either a BioLine (BioLine, London, UK) or MagNA Pure LC Total Nucleic Acid Isolation Kit (MagNA Pure Instrument, Roche Applied Science). All patient samples and the one cell culture sample were also extracted using the MagNA Pure kit. In this panel, 16 were positive for EMLA, 9 were positive for *A. phagocytophilum*, 3 were positive for *E. chaffeensis*, 1 was positive for *E. muris*, 1 was positive for *E. ewingii*, and 25 were negative for all *Ehrlichia* species using a previously reported assay at Mayo Clinic (Table 3) (Bell and Patel 2005, Castillo et al. 2014).

Included with all PCR reactions were negative controls without DNA template as well as positive control reactions containing plasmid DNA with the EMLA *p13* insert. Primer

specificity was further examined by resolving completed PCR reactions on 1.5% agarose gels to check for amplicons.

To determine the sensitivity of the assay, control plasmid DNA concentration was determined using a Qubit 2.0 fluorometer (Life Technologies, Grand Island, NY). Copy number was calculated based on concentration of DNA, and serial dilutions were prepared to attain copy numbers of 10^5 , 10^4 , 10^3 , 10^2 , 10, 5, 2.5, and 1 per 4 μ l of DNA. This dilution series was screened on the BioRad CFX96 Touch thermal cycler to create a standard curve for determining the limit of detection (Figure 2).

Results and Discussion

For all controls listed in Table 2, DNA amplification was detected only when EMLA-positive controls were used as template DNA. Of the 55 tick and patient samples sent from Mayo Clinic, all 39 EMLA-negative samples were also negative with the *p13* assay. 15 of the 16 EMLA-positive samples were detected with the *p13* assay. The one EMLA-positive tick sample that was negative using the *p13* assay was also screened and determined to be negative with a nested PCR assay targeting the *gltA* gene of *E. muris* and EMLA (Tamamoto et al. 2007, Telford III et al. 2011). Because this tick sample also tested negative with the nested *gltA* assay, it is possible that the DNA in the sample had degraded since the time it was tested at Mayo Clinic. Further examination of this sample by the Mayo Clinic established real-time assay may have resolved this question, but a lack of sample volume prevented further testing. The analytical sensitivity and limit of detection of the assay was determined to be 1 copy of plasmid DNA with an R^2 of the standard curve for the dilution series of 0.995. Because the assay detected 1 copy of plasmid DNA at a threshold cycle of 39, the cutoff for future use of the assay was determined to be 40 cycles.

Conclusions

As efforts continue to determine the geographic range, natural reservoir, and arthropod vector of EMLA, more efficient molecular detection methods for the pathogen are necessary. This report describes the successful development, optimization, and performance testing of a *p13* real-time PCR assay for the detection of EMLA in ticks, humans, and mammals. The sensitive and specific PCR developed here provides a powerful tool for use in ecological studies of EMLA as well as in the continued search to find the pathogen outside of the upper Midwest.

Acknowledgments

We would like to thank Susan M. Paskewitz, PhD and Xia Lee, MS and the Tick Surveillance Team from the University of Wisconsin–Madison, Madison, WI, and Diep K. Hoang Johnson, BS, from the Wisconsin Department of Health Services, Madison, WI, for collecting ticks used in this study.

The research reported here was supported in part by an appointment of M. Allerdice to the Research Participation Program at the Centers for Disease Control and Prevention administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and CDC. The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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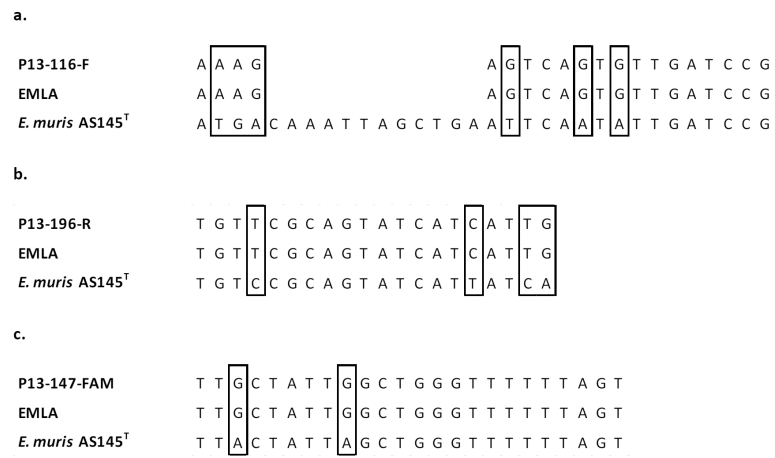


Figure 1. Primer and probe alignments for the **a.** forward primer, **b.** reverse primer, and **c.** probe. Boxes highlight areas of the primers or probe that are different between *E. muris* AS145^T and EMLA.

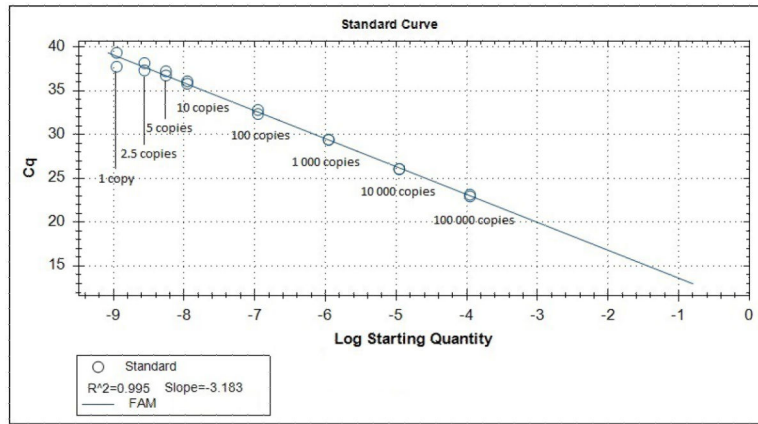


Figure 2. Standard curve from sensitivity testing of assay.

Table 1

Primer and probe sequences.

Primers/Probe	Sequences (5'-3')	Melting Temperature	Product Size
p13-116-F	AAA GAG TCA GTG TTG ATC CG	52.1 °C	99 bp
p13-196-R	CAA TGA TGA TAC TGC GAA CA	52.24 °C	
p13-147-FAM	TTG CTA TTG GCT GGG TTT TTT AGT	61.88 °C	

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Table 2

List of controls used for specificity testing.

Control Templates Tested	
<i>A. phagocytophilum</i> strain USG3	<i>R. parkeri</i> strain HMAC
<i>E. canis</i> strain Jake	<i>R. peacockii</i> strain Rustic
<i>E. chaffeensis</i> strain Arkansas	<i>R. sibirica</i> strain SE
<i>E. muris</i> strain AS145	<i>R. slovaca</i> strain D
<i>R. africae</i> strain Z9-Hu	<i>R. typhi</i> strain Willmington PP
<i>R. akari</i> strain HS CWPP	<i>Neoehrlichia lotoris</i>
<i>R. amblyommi</i> strain GAT8-2	<i>Neoehrlichia mikurensis</i>
<i>R. australis</i> strain JC-2	<i>O. tsutsugamushi</i> strain Karp PP
<i>R. canadensis</i> strain McKiel 24	<i>I. scapularis</i> -uninfected
<i>R. conorii</i> strain Malish 7	<i>I. scapularis</i> -EMLA infected*
<i>R. felis</i>	<i>D. variabilis</i> -uninfected
<i>R. helvetica</i> strain C9P9	Human Aorta Cells
<i>R. honei</i> strain FSF	Human Lung Endothelial Cells
<i>R. phillipii</i> strain 364D	Human Microvascular Endothelial Cells
<i>R. prowazekii</i> strain Breinl	Human Umbilical Endothelial Cells
<i>R. rhipicephali</i> strain 12T	<i>E. coli</i> strain INVαF ¹
<i>R. rickettsii</i> strain AZ-1	<i>B. elizabethae</i> strain F9251
<i>R. rickettsii</i> strain BSF Rab 1	<i>B. henselae</i> strain Houston-1
<i>R. rickettsii</i> strain Colombia	<i>B. vinsonii</i> strain Berkhoffii
<i>R. rickettsii</i> strain Costa Rica	EMLA-negative <i>Mus musculus</i> blood
<i>R. rickettsii</i> strain HLP #2	EMLA-infected <i>Mus musculus</i> blood*
<i>R. rickettsii</i> strain OSU 84-21c	EMLA-negative <i>Mus musculus</i> lung
<i>R. rickettsii</i> strain Sheila Smith	EMLA-infected <i>Mus musculus</i> lung*
<i>R. massiliae</i> strain Mtu1	EMLA-negative <i>Mus musculus</i> spleen
<i>R. montanensis</i> strain OSU 85-930	EMLA-infected <i>Mus musculus</i> spleen*

All samples were negative with the *p13* assay except those marked with an (*), which were controls positive for EMLA.

Table 3

Summary of Mayo Clinic blind samples results

Host / Pathogen (number of samples)	Number Positive by <i>p13</i> Assay	% Concordance with Conventional PCR
Human / EMLA (8)	8	100
Human / <i>E. chaffeensis</i> (3)	0	100
Human / <i>E. ewingii</i> (1)	0	100
Human / No agent (1)	0	100
<i>I. scapularis</i> / EMLA (8)	7	88
<i>I. scapularis</i> / <i>A. phagocytophilum</i> (9)	0	100
<i>I. scapularis</i> / No agent (24)	0	100
<i>E. muris</i> (1)	0	100

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