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Development and Validation of a Sensitive and Specific *sodB*-Based Quantitative PCR Assay for Molecular Detection of *Ehrlichia* Species

Barbara A. Qurollo,* Dana Riggins, Alaire Comyn, Merone T. Zewde, Edward B. Breitschwerdt

Intracellular Pathogens Research Laboratory and the Center for Comparative Medicine and Translational Research, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA

We developed a sensitive and specific *sodB*-based quantitative PCR assay to detect *Ehrlichia* spp. The assay's limit of detection was 5 copies/reaction, and it did not amplify nonspecific DNA. Compared with a 16S rRNA gene PCR target, the *sodB* target may offer an improved molecular diagnostic assay to detect *Ehrlichia* spp.

Ehrlichia spp. are intracellular bacteria that infect hematopoietic cells, resulting in disease in many mammals, including dogs and humans. The current diagnostic PCR methods designed to detect both *Anaplasma* and *Ehrlichia* spp. employ assays that target highly conserved regions on the 16S rRNA gene (1, 2) that can result in nonspecific amplification of environmental bacteria (3).

We aimed to develop a highly specific quantitative PCR (qPCR) assay using a target that would amplify existing as well as new *Ehrlichia* spp. The primers designed by our research group using *Ehrlichia* sp. sequences available in the NCBI nucleotide database amplified a conserved *sodB* region by conventional PCR (cPCR), confirming a Panola Mountain *Ehrlichia* sp. (PME) infection in a dog from a previous case report (4). In this paper, we evaluate *sodB* for use as a specific and sensitive target in qPCR to detect *Ehrlichia* spp. in diagnostic samples.

The positive *Ehrlichia* sp. samples consisted of EDTA-anticoagulated whole blood from naturally infected canine samples or infected canine DH82 histocytic cells (Table 1). Prior to amplifying and cloning *sodB* from diagnostic samples infected with *Ehrlichia ewingii* and PME, we confirmed the presence of these species by amplification and sequencing of 3 additional species-specific PCR targets (data not shown). DNA from 200 μl of EDTA-anticoagulated whole blood or DH82 cell suspensions was extracted on the QIAsymphony instrument using a MagAttract DNA mini M48 kit (catalog no. 953336; Qiagen, USA). DNA was quantified by spectrophotometry using a NanoDrop ND-1000^{n} spectrophotometer and stored at −20°C.

To amplify a 304-bp region of *sodB*, the primers sodbF (5′-TTTAATAATGCTGGTCAAGTATGGAATCAT) and sodbR (5′-AAGCGTGTTCCCATACATCCATAG) were designed manually after alignment of *E. canis*, *E. chaffeensis*, *E. muris*, and *E. ruminantium sodB* sequences (GenBank accession numbers CP000107, CP000236, CP006917, and CR925677, respectively) (4). The amplification was performed in a CFX96 real-time detection system combined with a C1000 thermal cycler (Bio-Rad, USA) using a 25-μl final volume reaction mixture containing 12.5 μl of SYBR Green Supermix (catalog no. 172-5271; Bio-Rad, USA), 0.2 μl of each primer at 50 μM (Sigma-Aldrich), 7 μl of molecular-grade water, and 5 μl of DNA template. The thermocycler conditions were 94°C for 2 min, followed by 40 cycles at 94°C for 10 s, 57°C for 15 s, and 72°C for 10 s, with melting temperature measurements between 65 and 88°C at 0.5-s intervals. All PCRs included a no-template control consisting of filter-sterilized, molecular-grade water.

TABLE 1 *Ehrlichia* species DNA source and GenBank accession numbers used for sequence comparisons in this study

Ehrlichia sp. (strain)	DNA source	*sodB* GenBank accession no.
E. canis (Jake)	DH82 cell culture	CP000107
E. chaffeensis (Arkansas)	DH82 cell culture	CP000236
E. ewingii	Dog-infected EDTA-whole blood	KC778986
E. muris	DH82 cell culture	CP006917
PME	Dog-infected EDTA-whole blood	KC702804

Plasmid clones, used as standards for the qPCR optimization and sensitivity analysis, were constructed using *sodB* amplicons from each *Ehrlichia* spp. examined with the pGEM-T easy vector system (Promega, Madison, WI), as recommended by the manufacturer. Sequencing was provided by Genewiz Inc. (Research Triangle Park, NC), and alignments were made with GenBank sequences using AlignX software (Vector NTI Suite 6.0; InforMax, Inc.). Plasmid copy numbers were calculated assuming an average base pair weight of 650 Da and Avogadro number (6.022×10^{23}) using the following equation: copy number = $(\text{DNA ng} \cdot 6.022 \times 10^{23})/(\text{length} \cdot 1 \times 10^{9} \cdot 650)$ (5). Duplicate, serial 10-fold dilutions in molecular-grade water resulted in 10 to 100,000 copies/reaction of plasmid DNA, and standard curves of quantification cycle (C_q) values were plotted against the logarithm of plasmid copy numbers/reaction. The PCR efficiency was estimated through linear regression of the dilution curve [$10^{(-1/\text{slope})-1) \cdot 100}$]. Coefficients ($R^2$) were calculated using Bio-Rad CFX Manager software. To determine the analytical sensitivity, plasmids were diluted in canine genomic DNA (gDNA) to 1 copy/μl and added to the reaction wells, resulting in 5 copies/reaction. The average C_q and standard deviation (SD) for the C_q variance using the limit of detection (LOD) (5 copies/reaction) was calculated with 20 inter-

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Address correspondence to Edward B. Breitschwerdt, ebbreits@ncsu.edou.
* Present address: Barbara A. Qurollo, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA.
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TABLE 2 Optimization of a quantitative PCR assay for *Ehrlichia* species (*E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris*, and Panola Mountain *Ehrlichia* spp.) partial *sodB* gene plasmids[a]

Plasmid *sodB* DNA for:	Melting temp (°C)	Standard curve				LOD (5 copies/reaction)[b]		
		Slope	*y* intercept	Efficiency (%)	R^2	% positive	Average C_q	SD for the C_q variance
E. canis	79.5	−3.544	43.74	92	0.99	100	37.17	0.83
E. chaffeensis	79	−3.578	43.45	90	0.99	100	37.03	0.694
E. ewingii	79	−3.585	42.92	90	0.99	100	35.63	0.969
E. muris	79	−3.479	40.44	94	0.99	85	37.58	1.061
PME	79	−3.486	43.47	94	0.99	95	37.79	1.884

[a] Standard curves were determined using the logarithm of plasmid copy numbers/reaction after 10-fold dilutions from 10 to 100,000 copies/reaction.
[b] The limit of detection (LOD) was determined to be 5 copies of plasmid/reaction. Percent positive and the SD of the C_q variance were determined based on positive results from 20 interassay technical replicates.

assay technical replicates. Specificity was determined using gDNA (10 to 30 ng/μl) from uninfected or infected dog and cat EDTA-whole blood samples. Infected samples included *Anaplasma platys*, *Anaplasma phagocytophilum*, *Rickettsia rickettsii*, *Cytauxzoon felis*, *Bartonella henselae*, "*Candidatus* Mycoplasma haematoparvum," and *Babesia gibsoni*.

The optimized *sodB*-based qPCR (*sodB*-qPCR) assay was compared to the *Anaplasma/Ehrlichia* 16S rRNA gene-based cPCR (16S-cPCR) assay utilized by the Vector-Borne Disease Diagnostic Laboratory (VBDDL)-North Carolina State University (NCSU). Dog DNA samples from EDTA-anticoagulated whole blood diagnostic specimens ($n = 203$) submitted to the VBDDL-NCSU for *Anaplasma/Ehrlichia* molecular diagnostics were assayed and consisted of 16S-cPCR amplicon-positive samples (*Ehrlichia* spp. [*E. canis* or *E. ewingii*; $n = 46$] or nonspecific DNA [$n = 39$]) and 16S-cPCR amplicon-negative samples ($n = 118$).

The *sodB*-qPCR assay produced a 304-bp product with all *Ehrlichia* sp. DNA samples examined. No amplicons were generated with the negative controls. The sequenced partial *sodB* clones for *E. canis*, *E. chaffeensis*, and *E. muris* showed 100% identity with 100% coverage to *sodB* sequences reported in GenBank (accession numbers CP000107, CP000236, and CP006917, respectively). Sequences obtained from *E. ewingii* and PME clones had been deposited in GenBank (accession numbers KC778986 and KC702804, respectively). For all 5 *Ehrlichia* spp. tested, the linear dynamic range extended to 5 $\log_{10}$ concentrations. The melting temperature, amplification efficiency, LOD, analytical sensitivity, interassay precision, and repeatability for each species-specific *sodB* plasmid are reported in Table 2.

The *sodB*-qPCR assay did not amplify DNA from the uninfected dog and cat gDNA (10 to 30 ng/μl) or the diagnostic samples positive for *A. platys*, *R. rickettsii*, *C. felis*, *B. henselae*, "*Candidatus* Mycoplasma haematoparvum," and *B. gibsoni*. One canine DNA sample previously identified as *A. platys* positive through 16S-cPCR and sequencing was identified as also being positive for *Ehrlichia* spp. in the *sodB*-qPCR. This DNA sample was further identified to the species level in additional qPCR assays to confirm a coinfection with *A. platys* and *E. canis* (data not shown). *A. phagocytophilum* DNA (0.5 ng/μl) extracted from DH82 cells and 2/9 *A. phagocytophilum*-positive dog samples generated an amplicon with a melting temperature of 83.5°C, higher than that of *Ehrlichia* spp. Based on standard curves with an *Anaplasma*-specific qPCR, *A. phagocytophilum* DNA copy numbers in the 2 positive dog samples were higher than those of all other positive dog samples. The *sodB*-qPCR amplified 45/46 (98%) samples positive for *Ehrlichia* spp. by 16S-cPCR, all of which agreed with 16S rDNA gene sequencing results (*E. canis* or *E. ewingii*) when identified to the species level in species-specific qPCRs. The 1 negative sample was also negative by additional PCRs. The *sodB*-qPCR did not amplify any of the 39 samples where nonspecific DNA was amplified by the 16S-cPCR and amplified *E. ewingii* DNA from 1/118 samples negative by 16S-cPCR, which was confirmed with additional PCRs.

This report describes the development and validation of a sensitive and specific *sodB*-qPCR assay for detection of at least 5 *Ehrlichia* spp. Amplification of *sodB*, which encodes Fe superoxide dismutase, was used to document PME infection in a dog (4) and has been used in loop-mediated isothermal amplification (LAMP) to detect *E. ruminantium* with high species specificity (6). The primers used in this study were not designed to identify species but instead to amplify all *Ehrlichia* spp; however, *sodB* orthologs contain unique regions of sequences, and primers highly specific for these regions are utilized in our research group to identify to the species level *E. ewingii*, *E. canis*, and PME (B. Qurollo, unpublished data).

The *Anaplasma*/*Ehrlichia* species 16S-cPCR assay amplifies other bacterial species due to primer degeneracy and high sequence identity between the prokaryotic 16S rRNA gene orthologs (3). Considering the nonspecificity, sequencing should be performed for all 16S rRNA gene amplicons to confirm species amplification. In the experience of the VBDDL, approximately 10% of 16S rRNA gene amplicons are nonspecific, ubiquitous bacterial DNA (B. Thomas and B. Hegarty, unpublished data), resulting in additional costs and delays to confirm results. Hence, an assay with increased specificity for *Ehrlichia* spp. would reduce the number of samples needing sequencing. This was demonstrated when the *sodB*-qPCR did not amplify nonspecific bacterial DNA that was amplified by the 16S-cPCR assay. Furthermore, the *sodB*-qPCR assay detected 5 copies of target DNA/reaction, an improvement in sensitivity compared to the reported 10 copies of target DNA/reaction detected with the 16S-cPCR assay (1). This was demonstrated when the *sodB*-qPCR detected *E. ewingii* in a sample, which was negative by 16S-cPCR. Moreover, the detection of *E. canis* in an *A. platys* sample positive by 16S-cPCR illustrates the challenge of amplification bias when using one universal target in coinfected animals. Specificities among other commonly diagnosed canine vector-borne diseases were demonstrated, with the exception of *A. phagocytophilum*, which was amplified from concentrated *A. phagocytophilum* gDNA. It is conceivable that samples with higher

concentrations of *Anaplasma* spp. may yield a qPCR product; however, this is not a robust assay for *Anaplasma* spp.

In summary, we have generated a sensitive and specific qPCR assay utilizing an orthologous gene target from five *Ehrlichia* spp., which if used in combination with an equally sensitive *Anaplasma*-specific qPCR, may prove more efficient and economical in *Anaplasma*/*Ehrlichia* diagnostic testing than use of a single, less specific PCR target.

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