Detection of Medically Important *Ehrlichia* by Quantitative Multicolor TaqMan Real-Time Polymerase Chain Reaction of the *dsb* Gene

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***Ehrlichia* species are the etiological agents of emerging and life-threatening tick-borne human zoonoses, in addition to causing serious and fatal infections in companion animals and livestock. We developed the first tricolor TaqMan real-time polymerase chain reaction assay capable of simultaneously detecting and discriminating medically important ehrlichiae in a single reaction. Analytical sensitivity of 50 copies per reaction was attained with templates from *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and *Ehrlichia canis* by amplifying the genus-specific disulfide bond formation protein gene (*dsb*). *Ehrlichia* genus-specific *dsb* primers amplified DNA from all known *Ehrlichia* species but not from other rickettsial organisms including *Anaplasma platys*, *Anaplasma phagocytophilum*, *Rickettsia conorii*, or *Rickettsia typhi*. High species specificity was attained as each species-specific TaqMan probe (*E. chaffeensis*, *E. ewingii*, and *E. canis*) identified homologous templates but did not cross-hybridize with heterologous *Ehrlichia* templates at concentrations as high as 10⁸ copies. Identification of *E. chaffeensis*, *E. ewingii*, and *E. canis* from natural and experimental infections, previously confirmed by polymerase chain reaction and serological or microscopic evidence, demonstrated the comparable specificity and sensitivity of the *dsb* real-time**

assay. This assay provides a powerful tool for prospective medical diagnosis for human and canine ehrlichioses and for ecologic and epidemiological studies involving arthropod and mammalian hosts. (J Mol Diagn 2005, 7:504–510)

The emergence of multiple *Ehrlichia* species as etiological agents of newly discovered human zoonoses and the previous recognition of these agents as causing serious disease in companion animals and livestock have intensified the need for rapid diagnostics for medically important ehrlichiae. Ehrlichiae are tick-transmitted obligate intracellular gram-negative bacteria that are maintained in nature by persistent infection of mammalian hosts. Humans are incidental hosts of these pathogens, and the recent emergence of ehrlichiosis in humans has been associated with changes in ecology, demographics, and host susceptibility.¹ *Ehrlichia chaffeensis*,^{2–4} *Ehrlichia ewingii*,^{5,6} and *Ehrlichia canis*^{7,8} cause ehrlichioses of medical and veterinary importance, with *E. chaffeensis* and *E. canis* being the primary agents of severe and sometimes fatal human monocytotropic ehrlichiosis (HME) and canine monocytic ehrlichiosis (CME), respectively. *E. chaffeensis* was first identified as an agent of human disease in 1987 in the United States,² whereas globally distributed *E. canis* was first identified in Africa as early as 1935⁹ and in North America in 1962.⁸

The potential for a severe life-threatening or fatal outcome of HME and CME emphasizes the need for early diagnosis. Currently, retrospective diagnosis of HME and CME is primarily determined serologically with paired sera using an indirect immunofluorescence assay (IFA). Accurate and reliable serological diagnosis of ehrlichiosis by IFA has inherent limitations related to the absence of antibodies early in the course of infection and in immunocompromised patients and lack of laboratory standardization of the procedure. At the time of presentation, a minority of patients will have developed antibodies to *E.*

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chaffeensis,^{10,11} and absence of antibodies at the time of therapeutic decisions when diagnosis is critical can contribute to a fatal outcome. Detection of *E. chaffeensis* in all culture-positive patients by polymerase chain reaction (PCR) during initial presentation of illness when only 44% were seropositive demonstrates its clinical utility for diagnosis and disease surveillance.¹¹

The development of molecular assays for ehrlichial agents has shown promise toward rapid, sensitive, and specific detection required for prospective diagnosis of the ehrlichioses. A limited number of gene targets have been available for genus- and species-specific detection of *Ehrlichia* infections because of the inability to culture some *Ehrlichia* species and the lack of genomic sequence information. PCR and reverse transcriptase PCR-based assays for *E. chaffeensis*, *E. ewingii*, and *E. canis* have most commonly targeted the 16S rRNA gene,^{3,12–15} but other genes including the variable-length PCR target, *groESL*, and *p28* have been used for detection of these ehrlichiae.^{11,16–18} Because of increased analytical sensitivity, nested PCR has been used routinely for detection of ehrlichemia,^{3,10,11,19,20} but it involves additional cost and labor and can be more susceptible to false positives as a result of exogenous contamination. A newly developed species-specific TaqMan real-time PCR assay targeting the 16S rRNA gene has detected *E. chaffeensis* with analytical sensitivity (10 copies) comparable with that provided by nested PCR.²¹ However, a PCR assay capable of simultaneous species-specific detection of the three medically important *Ehrlichia* species in the United States has not been reported.

We herein report the development of the first multicolor real-time PCR assay capable of detection and discrimination of medically important *Ehrlichia* species in a single reaction by amplification of a genus-specific disulfide bond formation protein gene (*dsb*). The analytical sensitivity of the assay is comparable with the most sensitive molecular assays reported and is able to specifically distinguish amplicons from *E. chaffeensis*, *E. ewingii*, and *E. canis* and detect co-infections within the same sample. This powerful closed-system assay will provide new capabilities that will be useful in the diagnosis and treatment of infected patients and companion animals and can also be used in epidemiological studies.

Materials and Methods

Real-Time (TaqMan) PCR Primer and Probe Design for the *dsb* Gene

Dsb proteins (thio-disulfide oxidoreductases) have conserved protein domains and are functionally conserved among bacteria, yet nucleic acid sequences encoding *dsb* genes among different bacterial genera are significantly divergent. Hence, they are excellent targets for genus-specific molecularly based assays. The *dsb* genes of *E. chaffeensis* and *E. canis* were previously identified and functionally characterized, and the sequences were determined to be unique to the genus *Ehrlichia*.²² The *dsb* genes of *Ehrlichia muris* and a yet to be named

Ehrlichia sp. (Anan strain, designated "IOE") isolated from *Ixodes ovatus* ticks were previously determined and are available in GenBank (accession nos. AY236484 and AY236485). The *dsb* gene of *Ehrlichia ruminantium* was identified by performing a BLASTn of the Entrez nucleotide databases at the National Center for Biotechnology Information with the *E. chaffeensis dsb* gene sequence and is available in GenBank (accession no. AF308669, clone 18hw). The *dsb* gene sequence (partial) of *E. ewingii* was determined by PCR amplification of infected tick DNA (kindly provided by Wesley Long, University of Texas Medical Branch) with *dsb* primers Dsb-330, 5'-GAT GAT GTC TGA AGA TAT GAA ACA AAT-3' (forward), and Dsb-728, 5'-CTG CTC GTC TAT TTT ACT TCT TAA AGT-3' (reverse). The amplicon was purified using a PCR purification kit (Qiagen, Valencia, CA) and sequenced directly with the same primers and the sequence deposited in GenBank (accession no. AY428950). *Dsb* gene sequences for all known *Ehrlichia* spp. were aligned using the CLUSTAL algorithm by the MegAlign program (DNASTar; Lasergene, Madison, WI). The *dsb* gene is conserved (69.5–91.5%) within the genus, with *E. ruminantium* and *E. ewingii* being the most divergent, and *E. muris* and IOE the most similar. Ehrlichial *dsb* genes have highly conserved and heterologous nucleic acid regions necessary to develop genus-specific primers and species-specific probes. Genus-specific *dsb* primers were manually designed complementary to highly conserved regions of *dsb* that flank heterogeneous regions used for species-specific probe design. Oligonucleotide primers (Sigma-Genosys, The Woodlands, TX) consisted of a single forward primer for all *Ehrlichia* species and two reverse primers: one reverse primer for *E. chaffeensis* and *E. canis* containing a single degenerate nucleotide position, and a completely complementary reverse primer for *E. ewingii* (Table 1). Amplification with these primers produces a product that is 378 bp from each of the ehrlichiae. A region of heterogeneity and higher G + C content (bases 411 to 441) in the *dsb* gene among the three *Ehrlichia* species was identified, and TaqMan probes with $T_m \sim 10^\circ\text{C}$ higher than the primer T_m complementary to this region were designed for species-specific detection. Dual-labeled fluorescent TaqMan probes for *E. chaffeensis*, *E. ewingii*, and *E. canis* were synthesized and labeled with 5'Quasar 670/3'Black Hole Quencher (BHQ) 2, 5' TAMRA/3' BHQ-2, and 5' FAM/3' BHQ-1, respectively (Biosearch Technologies, Navato, CA) (Table 2). Primers and probes were evaluated for potential sequence complementarity with the oligo analysis and plotting tool (Qiagen; <http://qiagen.com>).

Ehrlichia Multicolor Real-Time PCR

The *dsb* gene was amplified from *E. chaffeensis* (Arkansas strain) genomic DNA, *E. ewingii* from an infected tick, and *E. canis* (Jake strain) genomic DNA with primers Dsb-330 and Dsb-728 and cloned into a pCR 2.1 TOPO vector (Invitrogen, Carlsbad, CA). *Escherichia coli* was transformed with the *dsb*-containing plasmids and grown overnight, and plasmid was purified using a plasmid purification kit

Table 1. Multicolor Real-Time PCR *Ehrlichia* Genus-Specific *dsb* Primers and Homology to Each Species

<i>Ehrlichia</i> species (GenBank accession)	Primer Dsb-321* (forward, 5'–3') TTGCAAAATGATGTCTGAAGATATGAAACA T_m (°C) = 66.4	Primer Dsb-671 ^{†‡} (reverse, 5'–3') GCTGCTCCACCAATAAATGTATCYCCTA T_m (°C) = 66.1
<i>E. chaffeensis</i> (AF403711)		T....
<i>E. canis</i> (AF403710)		A....G.....C....
<i>E. ewingii</i> (AY428950)	ND	GCAGCTCCACCAATGAATGTATTTCCAA [§]

ND, not determined.

*The *Ehrlichia* forward primer sequence corresponds to nucleotides 321 to 350 from *dsb* in each species.

[†]All *Ehrlichia* reverse primer sequences correspond to the reverse complement of nucleotides 671 to 698 from *dsb* in each respective species.

[‡]Y = T or C.

[§]Dsb-671-ew reverse primer is 100% complementary to the *E. ewingii* *dsb* sequence.

(Qiagen). The plasmid DNA was quantified using NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Montchanin, DE). Plasmids containing *dsb* genes were used to optimize the PCR conditions. Optimal conditions for amplification of ehrlichiae were determined using an annealing temperature gradient (55–65°C) and by testing a range of concentrations to establish optimal concentrations of MgCl₂, primer, and TaqMan probe. The optimized real-time PCR assay used in all subsequent amplifications was performed using iQ Supermix (BioRad Laboratories, Hercules, CA) in a reaction volume of 50 μ L containing primers Dsb-321 and Dsb-671 (400 nmol/L each), Dsb-671-ew (200 nmol/L), and 25 μ mol/L of each TaqMan probe (three probes) with final concentration of 4 mmol/L MgCl₂. The thermal cycling protocol consisted of a single melting cycle of 95°C for 5 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The real-time PCR was performed in 96-well plates on an iCycler iQ multicolor real-time PCR detection system equipped with the appropriate filter sets and analyzed with iQ software version 3.1 (BioRad Laboratories). A sample was considered positive and threshold cycle (C_T) was determined after it crossed the minimum threshold level above background fluorescence after base-line subtraction.

Multicolor *dsb* Real-Time PCR Sensitivity and Specificity

The sensitivity of the real-time PCR assay was determined using 10-fold serial dilutions of the respective plasmid DNA in reactions that included all three TaqMan probes in the presence and absence of human DNA (250 ng; amount of DNA typically recovered from blood samples [100 μ L] is 20 to 35 ng/ μ L, and 5 to 10 μ L of template DNA is used per reaction) in five replicates. The number of *dsb*-containing plasmid copies was determined according to the formula (X g/ μ L DNA/[plasmid length in

bp $\times$ 660]) $\times$ 6.022 $\times$ 10²³ = plasmid copies/ μ L. Specificity was determined similarly, except *dsb* plasmids from *E. chaffeensis*, *E. ewingii*, *E. canis*, and *E. muris* were included separately as templates in reactions that each contained all of the species-specific probes. Intergenous specificity of the *dsb* primers was determined using genomic DNA from closely related organisms, *Anaplasma platys*, *Anaplasma phagocytophilum*, *Rickettsia conorii*, and *Rickettsia typhi*.

Real-Time PCR Assay Comparison

The analytical sensitivity of the *dsb* real-time assay was compared with another recently published real-time assay for detection of the *E. chaffeensis* 16S rRNA gene.²¹ The *E. chaffeensis* 16S rRNA real-time assay was extensively compared with other published PCR assays used for detection of *E. chaffeensis* and was found to be one of the most sensitive and specific. Because comparable sensitivity was obtained with plasmid dilutions of *E. chaffeensis*, *E. ewingii*, and *E. canis* in this study, *E. chaffeensis* was chosen to perform comparative analysis of the relative assay sensitivity. *E. chaffeensis* genomic DNA was quantified and diluted fivefold; the relative sensitivity of *E. chaffeensis* detection by the 16S rRNA gene was determined (five replicates) with Brilliant Quantitative-PCR core reagents (Stratagene, La Jolla, CA) as previously described;²¹ and the *dsb* real-time PCR on *E. chaffeensis* DNA was performed as described above.

Species-Specific Detection of *Ehrlichia* in Cases of Natural and Experimental Human and Canine Ehrlichiosis

The *dsb* real-time PCR was evaluated with human and canine clinical samples with detectable *E. chaffeensis* or

Table 2. *Ehrlichia* Species-Specific Dual-Labeled TaqMan Fluorogenic Probes for *dsb* Multicolor Real-Time PCR

<i>Ehrlichia</i> spp.	<i>dsb</i> TaqMan probe sequence*	T_m (°C)	Fluorophore/quencher	Excitation/emission wavelength (nm)
<i>E. chaffeensis</i>	5'–TGCTAGTGTCTGCTTGAACAGCTTTTCAGTGAT–3'	72.1	5' Quasar 670/BHQ-2 3'	649/670
<i>E. ewingii</i>	5'–AGCCAATGCTGCACGTACTGCTTTCAATGAT–3'	74.4	5' TAMRA/BHQ-2 3'	555/576
<i>E. canis</i>	5'–AGCTAGTGTCTGCTTGGGCAACTTTGAGTGAA–3'	74.0	5' FAM/BHQ-1 3'	494/534

*All TaqMan *Ehrlichia* species-specific probe sequences correspond to reverse complement of *dsb* nucleotides 411 to 441 from each respective species.

E. ewingii DNA using nested 16S rRNA PCR assays.^{15,19} *E. canis*-infected dogs were experimentally inoculated and monitored for ehrlichiae in peripheral blood by PCR amplification of the *p28* gene. PCR of *E. canis* *p28* was conducted by the Louisiana State University Veterinary Medical Diagnostic Laboratory using primers ECa282F239, 5'-CAA GCA GCA GCC ACA CAA T-3' (forward), and ECa28R701, 5'-GAT GCT TCT GGG CTT ATG GAG TA-3' (reverse), with thermal cycling protocol of 10 minutes at 95°C, 40 cycles of 95°C for 30 seconds, 50°C for 1 minute, and 72°C for 1 minute and finished with 10 minutes at 72°C as previously described.²³ DNA was extracted from 100 μ l of whole blood using a DNeasy kit (Qiagen) from naturally infected human samples (eight) and canine samples (nine) with detectable ehrlichial DNA by nested 16S rRNA PCR for ehrlichiosis.¹⁹ Total DNA template added to each reaction was between 100 and 200 ng contained in a maximum of 10 μ l. In addition, peripheral blood was collected from three dogs experimentally infected with *E. canis* and monitored weekly for 9 weeks after inoculation. DNA was extracted similarly and amplified by the *dsb* real-time PCR assay.

Results

Sensitivity of the Multicolor *dsb* Real-Time PCR Assay

The analytical sensitivity of the *dsb* real-time PCR for each ehrlichial species (*E. chaffeensis*, *E. ewingii*, and *E. canis*) independently was determined to be 50 copies per reaction using single species-specific *dsb* gene plasmids diluted 10-fold (data not shown). The effect of eukaryotic DNA that would routinely be found in clinical samples on assay sensitivity was evaluated by testing the efficiency of the assay with plasmid templates in the presence and absence of human DNA (250 ng). No reduction in the analytical sensitivity (C_T value) of the assay by the addition of host DNA was observed over the range of four plasmid dilutions (10^2 – 10^5 ; data not shown).

A comparison of analytical sensitivity of the *dsb* real-time PCR with another reported real-time PCR assay for detection of *E. chaffeensis* was performed. The 16S rRNA gene assay was previously reported to have a sensitivity of 10 copies for *E. chaffeensis*.²¹ Dilutions (fivefold) of genomic *E. chaffeensis* DNA were amplified by both PCR assays to compare the analytical sensitivity. There was a fivefold difference in analytical sensitivity between the 16S rRNA real-time PCR and *dsb* real-time PCR using dilutions of *E. chaffeensis* DNA (fivefold), but both assays were capable of detecting relatively low copy *E. chaffeensis* DNA (Table 3). Because *Ehrlichia* DNA is purified along with varying amounts of host cell DNA, the DNA per reaction is expressed as dilutions and not as concentrations of ehrlichial DNA. The reported analytical sensitivity of 16S rRNA using plasmid template (10 copies) and limits of detection using *E. chaffeensis* genomic DNA as template were similarly observed with the *dsb* real-time PCR assay (50 copies) (Table 3).

Table 3. Sensitivity Comparison of *dsb* and 16S rRNA Real-Time TaqMan PCR Assays for Detection of *E. chaffeensis* Genomic DNA

Genomic DNA dilution (5-fold)	<i>E. chaffeensis dsb</i> *	<i>E. chaffeensis</i> 16S rRNA†
1/50	+	+
1/250	+	+
1/1250	+	+
1/6250	+	+
1/31,250	+	+
1/156,250	+	+
1/781,250	–	+
1/3,906,250	–	–

*Sensitivity determined to be 50 copies of *dsb* gene plasmid.

†Sensitivity of 10 plasmid copies as reported by Loftis et al.²¹

Specificity of the Multicolor *dsb* Real-Time PCR Assay

Dilutions of plasmids containing the *dsb* genes from each species were tested in separate reactions containing all three species-specific probes. Amplicons were detected by the corresponding species-specific probe, but no signal was detected in fluorescent channels corresponding to either of the heterologous probes even with plasmid concentrations equivalent to 10^8 organisms (Table 4). A sample containing *dsb* plasmids from each of the three species was able to simultaneously detect each of the species. Amplicons for all *Ehrlichia* species tested were amplified by the *dsb* primers as detected by SYBR Green (data not shown). Intra- and intergenus specificity was determined using purified genomic DNA from other ehrlichiae and related rickettsial agents. DNA from *E. muris*, IOE, *A. platys*, *A. phagocytophilum*, *R. conorii*, and *R. typhi* were all negative by the multicolor TaqMan assay (Table 4). Amplicons from *E. muris* and IOE were detected by SYBR Green but were not observed with non-*Ehrlichia* genus templates. For comparison, the 16S rRNA real-

Table 4. Specificity of *Ehrlichia dsb* TaqMan Real-Time Multicolor PCR Assay

DNA	Specificity with templates from		
	<i>E. chaffeensis dsb</i>	<i>E. canis dsb</i>	<i>E. ewingii dsb</i>
	Quasar 670	FAM	TAMRA
<i>E. chaffeensis</i> *	+	–	–
<i>E. canis</i> *	–	+	–
<i>E. ewingii</i> *	–	–	+
<i>E. muris</i> †	–	–	–
<i>Ehrlichia</i> sp. (IOE)‡	–	–	–
<i>A. platys</i> §	–	–	–
<i>A. phagocytophilum</i> ¶	–	–	–
<i>R. conorii</i>	–	–	–
<i>R. typhi</i> **	–	–	–
Human††	–	–	–

*Results with *dsb* plasmid template copies of 10^8 to 10^1 .

†DNA from P388D₁-infected cells.

‡DNA from infected mouse spleen (250 ng).

§DNA from canine platelet-rich plasma (100 ng).

¶DNA from infected HL-60 cells (250 ng).

||DNA from purified *R. conorii* (100 ng).

**DNA from purified *R. typhi* (100 ng).

††DNA from THP-1 cells (250 ng).

Table 5. Comparison of PCR Results from Peripheral Blood Samples from Human Patients with Ehrlichiosis

Sample	dsb real-time PCR			16S rRNA nested PCR*		
	<i>E. cf</i>	<i>E. ca</i>	<i>E. ew</i>	<i>E. cf</i>	<i>E. ca</i>	<i>E. ew</i>
1	+	—	—	+	—	—
2	+	—	—	+	—	—
3	+	—	—	+	—	—
4	+	—	—	+	—	—
5	—	—	—	—	—	+†
6	+	—	—	+	—	—
7	—	—	+	—	—	+
8	+	—	—	+	—	—

DNA extractions: *E. cf*, *E. chaffeensis*; *E. ca*, *E. canis*; *E. ew*, *E. ewingii*.

*Assay described by Paddock et al.¹⁵

†Positive result in one of three separate extractions.

time PCR for *E. chaffeensis* was tested for specificity with the same rickettsial controls, and a positive signal was detected by the 16S rRNA assay in samples that contained the IOE template, but not by the *dsb* assay (data not shown).

Simultaneous Detection of Heterologous Templates with the Multicolor *dsb* Real-Time PCR Assay

Equal dilutions (in the range of 10² to 10⁵) of the *E. chaffeensis*, *E. canis*, and *E. ewingii* templates were detected simultaneously with the same efficiency (no effect on C_T) when compared with the C_T of each template run alone (data not shown). However, a decrease in sensitivity during simultaneous amplification of multiple templates was observed in the presence of 100-fold excess of any one of three templates. The sensitivity of simultaneous detection was not improved by additional primer, probe, Taq polymerase, MgCl₂, or dNTPs.

Detection of Ehrlichiae by Multicolor *dsb* Real-Time PCR with Clinical Samples

The specificity of the *dsb* multicolor TaqMan assay was evaluated blindly with human (eight) and canine (nine) samples confirmed as containing one of the three agents by laboratories using 16S rRNA PCR along with additional corroborative serology, microscopic, and hematological information. The *dsb* real-time assay detected six *E. chaffeensis*, one *E. ewingii*, and one negative sample from the eight human samples tested (Table 5). The specificity of the *dsb* real-time assay matched previous results obtained using a nested 16S rRNA assay.¹⁵ One sample was negative by *dsb* real-time PCR (three independent DNA extractions). These DNA templates (three) were subsequently retested by the laboratory of origin, and *E. ewingii* was detected in one of the samples. The *dsb* real-time PCR amplified *E. ewingii* from eight of nine canine samples that were previously reported as containing *E. ewingii* as determined by a nested 16S rRNA PCR assay (Table 6). Two co-infections were also detected, one *E. canis* and another *E. chaffeensis*, that were not

Table 6. Comparison of PCR Results from Peripheral Blood Samples of Canines with Ehrlichiosis

Sample	dsb real-time PCR			16S rRNA nested PCR*		
	<i>E. cf</i>	<i>E. ca</i>	<i>E. ew</i>	<i>E. cf</i>	<i>E. ca</i>	<i>E. ew</i>
1	—	—	+	—	—	+
2	—	—	+	—	—	+
3	—	—	+	—	—	+
4	—	—	+	—	—	+
5	—	—	+	—	—	+
6	+	—	+	—	—	+
7	—	+	+	—	—	+
8	—	—	+	—	+	+
9	—	—	—	—	—	+

E. cf, *E. chaffeensis*; *E. ca*, *E. canis*; *E. ew*, *E. ewingii*.

*Assay described by Goodman et al.¹⁹

detected by 16S rRNA nested PCR, and only *E. ewingii* was detected in one dog identified as co-infected with *E. ewingii* and *E. canis* by 16S rRNA nested PCR (Table 6).

The ability of the assay to detect *E. canis* was evaluated using samples from three dogs experimentally infected with *E. canis* by subcutaneous inoculation. Peripheral blood samples were collected weekly for 9 weeks, and ehrlichemia were monitored by PCR using primers targeting the *p28* gene. The *dsb* real-time assay correctly identified the infection as being that of *E. canis* and not *E. chaffeensis* or *E. ewingii* in three dogs that were experimentally infected with *E. canis*. *E. canis* was detected in peripheral blood samples (weeks 2 to 9) from two of the dogs, which was consistent with results obtained by *p28* PCR. In the third dog, *E. canis* was detected by both *dsb* real-time PCR and *p28* PCR (weeks 3, 5, 6, 7, and 9), but *dsb* also detected *E. canis* in four samples (weeks 1, 2, 4, and 8) in which *E. canis* DNA was not detected by *p28* PCR (data not shown).

Discussion

We describe herein the first multicolor real-time PCR assay that can detect and discriminate all of the known medically important ehrlichiae, including *E. chaffeensis*, *E. canis*, and *E. ewingii*. Difficulties in cultivating *Ehrlichia* species *in vitro*, distinguishing the specific agent serologically, and the recent emergence of two *Ehrlichia* species as agents of tick-borne zoonoses have emphasized the need for a simple and rapid molecular diagnostic assay for reliable and specific detection of all medically important ehrlichiae. The absence of antibody early in infection when patients usually seek medical attention highlights the importance and clinical utility of molecularly based assays such as PCR for prompt identification of the infecting agent.¹¹ Two studies have demonstrated the high clinical sensitivity of PCR in patients when the organism was concurrently isolated from the blood or in patients co-infected with human immunodeficiency virus.^{11,15,24} However, in another study, a substantial number of confirmed cases of HME had undetectable ehrlichial DNA by PCR.²⁵ Because of the limited data available and lack of a standard PCR protocol, current recommendations for

the diagnosis of HME suggest that PCR is a valuable adjunct to IFA.²⁶ Nevertheless, more studies are needed to unequivocally determine the clinical sensitivity of PCR during initial presentation in the primary care setting.

An important consideration in the development of any molecularly based assay is identification of an appropriate gene target that defines the sensitivity, reliability, and specificity of the assay. The availability of gene targets for development of molecularly based assays for *Ehrlichia* spp. has been limited because of the lack of genomic sequence information. The 16S rRNA gene is the most widely used molecular target to amplify ehrlichiae, but heterogeneous regions where species-specific probes can be targeted are very limited; thus designing probes to distinguish specific organisms in a particular genus can be difficult. This constraint can be an impediment to development of a new-generation molecularly based assay that can use multicolor probes for detection and distinction of multiple agents in a single-tube format. An example of this problem was revealed in this study in which the real-time assay previously reported as specific for *E. chaffeensis*²¹ detected IOE DNA. This is not particularly unexpected because the IOE and *E. chaffeensis* 16S rRNA genes are >98% conserved,²⁷ and the annealing sequence for the probe contained only four mismatches, the least among the *Ehrlichia* spp. The 16S rRNA gene sequences are highly conserved among bacteria, and nonspecific amplification of unintended targets does frequently occur. Although the *dsb* gene sequences among the *Ehrlichia* genus members are conserved, enough sequence heterogeneity exists to allow design of species-specific probes.

The objective of this investigation was not clinical validation of the *dsb* PCR assay, thus the predictive values of the test and its clinical specificity and sensitivity were not determined. However, the specificity of the assay was tested with confirmed clinical samples from natural human as well as natural and experimental canine infections to demonstrate its sensitivity and microbiological specificity as compared with commonly used 16S rRNA nested PCR. The *dsb* real-time PCR demonstrated similar specificity when compared with results obtained with nested 16S PCR with both clinical human and canine samples. Co-infections were identified in two of the canine *E. ewingii*-infected samples only by *dsb* real-time PCR. The co-infecting agents had low copy numbers compared with that of *E. ewingii*, and this could be an explanation for the discrepancies in the ability to detect co-infections by the nested 16S rRNA and *dsb* assays. Alternatively, a false-positive result is possible, but unlikely, considering the internal controls and extensive specificity testing performed in this investigation.

The 16S rRNA gene is the most frequently used molecular target for *Ehrlichia* detection, but a "gold standard" for molecular detection of ehrlichiae has not been established. The sensitivity of the *dsb* assay for detection of ehrlichiae is comparable with all reported PCR-based methods, as illustrated by the direct comparison with the 16S rRNA assay with *E. chaffeensis* genomic DNA, which has been thoroughly compared with other existing PCR assays.²¹ Although the analytical sensitivity of the *dsb*

assay using genomic and plasmid templates was comparable, our assay identified two clinical samples as negative that were positive for *E. ewingii* by nested 16S rRNA PCR (Tables 5 and 6). We attribute discrepancies to very low copy number of ehrlichiae as demonstrated by absence of detectable ehrlichiae in two of three DNA extractions (human sample 5) or in combination with unusually low concentration of template DNA (<10 ng/ μ l template in canine sample 9) and the potential loss of ehrlichial DNA after repeated freeze-thaw cycles (minimum of two) of the sample between the initial test performed with the nested 16S rRNA assay and the test performed for this study using the *dsb* assay. Although we have not determined the precise gene sequence of *E. ewingii dsb* forward primer region (Table 1), this region was highly conserved among all ehrlichiae. The analytical sensitivity of *E. ewingii* using plasmid dilutions was equal to that of *E. chaffeensis* and *E. canis*. Therefore, reduced sensitivity of the assay for *E. ewingii* potentially related to primer mismatches is not a likely explanation for the negative results obtained with these two samples.

The *dsb* multicolor real-time PCR assay is simple by design in having only three primers capable of amplifying all ehrlichial templates tested and species-specific probes to determine the pathogen identity in real time. An advantage of this multicolor assay over a typical multiplex PCR design is consistent and efficient amplification of all ehrlichial templates, which is often difficult to achieve in reactions containing multiple primer pairs. The real-time PCR format will increase the speed at which support for clinical decisions can be provided, reduce costs for performing the test, eliminate the need for gene sequencing to confirm the identity of PCR products, and assist in epidemiological case studies. An additional advantage of the single-step closed-system real-time based assays is the significantly lower risk of false positives due to exogenous contamination. Thus, some precautions taken to prevent contamination in open-system PCR diagnostics (nested protocols) would now be considered excessive for closed-system assays.

Molecularly based assays such as *dsb* real-time PCR will facilitate more comprehensive epidemiological studies and diagnostic identification of these difficult-to-culture and potentially lethal emerging infectious agents in the future. The lack of a molecularly based assay using a simple single reaction for tracking the geographical prevalence of each of the medically important ehrlichial species in patient and ticks has complicated epidemiological investigations and clinical diagnosis. The value of the *dsb* real-time assay will be in its ability to assist in rapid and specific identification of the agents responsible for human ehrlichioses and in the insight provided into which important ehrlichiae reside in or co-infect different tick species and mammalian reservoirs.

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