Use of Chimeric DNA-RNA Primers in Quantitative PCR for Detection of *Ehrlichia canis* and *Babesia canis*\(^\dagger\)

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To overcome the problem of nonspecific by-products in quantitative PCR (qPCR) assays, we constructed DNA-RNA chimeric primers and evaluated their use in the detection and quantification of the *Ehrlichia canis* 16S rRNA, *Babesia canis* Hsp70, and canine β-actin genes. Several RNA bases were incorporated into specific positions in the DNA primers, while no RNA stretches were allowed. qPCR reactions were carried out without preamplification steps. This resulted in decreased formation of undesirable by-products and a 10-fold increase in assay sensitivity.

Quantitative real-time PCR (qPCR) is a reliable technique for quantitative analysis of specific DNA and RNA sequences (10). The use of nonspecific dyes in qPCR has several advantages, including the ability to detect contamination and nonspecific products. Nonspecific dyes enable melting temperature (\(T_m\)) analysis and high-resolution melt analysis (13, 26). The generation of nonspecific amplification products resulted from inappropriate hybridization products designated primer dimers. These undesired by-products are formed due to weak complementarity between the 3′ ends of a primer and bases in nontarget oligonucleotide strands in the reaction mixture. The by-products enable the annealing of primers to nontarget strands, followed by the initiation and elongation of nonspecific dimers by thermostable DNA polymerase. This problem worsens in qPCR due to the appearance of nonspecific by-products that mask the detection of low concentrations of the target sequence after 30 cycles when complementarity of at least one nucleotide at the 3′ end is present and after cycle 40 when no 3′-end complementarity is present (2, 25). This problem is augmented in multiplex PCR amplification reactions due to the presence of several primer sets in the reaction mixture, leading to a decline in the sensitivity and quality of the reaction. Ferrie et al. (7) showed that under cold start conditions, every possible combination of two different primers in a multiplex reaction generates primer dimers, irrespective of primer complementarity. Nonspecific amplification products can be reduced by careful primer design and the use of stringent PCR protocols (6) and “hot start” enzymes (4, 5). “Hot start” enzymes and careful primer design are often insufficient to detect and quantify low copy numbers of target DNA.

Researchers are reluctant to use DNA-RNA chimeric primers for PCR or qPCR due to the incompatibility of RNA bases as a template for DNA-dependent DNA polymerases. However, DNA-RNA chimeras are frequently used for preamplification processes (24), isothermal linear nucleic acid amplification (14, 16, 23), site-specific mutagenesis (12, 19), and PCR assays using *rTth* DNA polymerase that functions as both DNA polymerase and reverse transcriptase (15).

We constructed chimeric DNA-RNA primers and evaluated their use in a singleplex qPCR assay for the detection of *Ehrlichia canis*, *Babesia canis* vogeli, and the canine β-actin gene. The chimeric primers enabled a highly sensitive qPCR reaction and reduced primer dimer formation.

**Primers.** The primers for target gene detection were designed using Genaphora’s Singleplexer software (Table 1). The primer sets amplified gene fragments consisting of 85 bp of the *B. canis* vogeli Hsp70 gene; 124 bp of the *E. canis* 16S rRNA gene; and 88 bp of the canine β-actin gene (*ACTB*), which served as a reference gene. qPCR detection was enabled by using SYBR green fluorescent dye. Highly complex primers were selected. DNA-RNA chimeric primers were constructed by incorporating RNA bases at the 5′ nearest neighbor to each suspected position for primer dimer initiation, while RNA base stretches were not permitted. In addition, the calculations did not predict either dimer formation with corresponding primers or self-anneling. The predicted annealing temperature of the primers was 60°C. All chimeric primers were synthesized and purified by Integrated DNA Technologies (IDT, Coralville, IA), and the DNA primers were synthesized by Sigma-Genosys (Rehovot, Israel).

**Plasmid construction.** Fragments containing the amplified sequences for qPCR of the genes of interest were cloned into pGEM-T Easy vector (Promega, Madison, Wisconsin). For the *E. canis* 16S rRNA gene, the pE. *canis*-16S plasmid was constructed by cloning a 1,501-base fragment, including 377 bases upstream of the qPCR amplicon and 1,000 bases downstream from the 3′ end of the qPCR amplicon region. For the *B. canis* Hsp70 gene, the pB. *canis*-Hsp70 plasmid was constructed by cloning a 1,622-base fragment beginning 310 bases upstream of the qPCR amplicon and ending 1,227 bases downstream from...
At the end of the cycles, incubation at cycles of 93°C for 10 s, 61°C for 30 s, data acquisition, 72°C for
mix (Finnzymes, Espoo, Finland): hold at 95°C for 7 min, 40
was used for nonspecific dye experiments with the Flash F-415
annealing temperatures of the Chromo4. Reactions were per-
annealing temperatures was carried out using the gradient
95°C with data acquisition every 1°C, hold for 1 s. SYBR green
61°C for 1 min was followed by a melting curve from 65°C to
5°C with data acquisition every 1°C, hold for 1 s. SYBR green
Results were recorded as the cycle in which fluorescence of
PCR product was detected above the cycle threshold (C\textsubscript{T})
or crossing points (CP) or quantification cycle (C\textsubscript{q}) as
determined by the MIQE guidelines (3). Calibration was carried out
using plasmid DNA templates consisting of pGEM-T Easy:
gene of interest. In regulated systems, \Delta C\textsubscript{q} is determined as
the delta between time zero and treated samples. As there is
no background level of expression in pathogen detection, \Delta C\textsubscript{q}
was defined as the difference between the C\textsubscript{q} values of the
tested sample and the negative control (naïve dog or non-
template control [NTC]). If neither of the negative controls
demonstrated an exponential fluorescence slope, \Delta C\textsubscript{q}
calculated as the difference between the last cycle and the
C\textsubscript{q} of the examined sample.

To evaluate the effect of low efficiency in chimeric-primer
assays on copy-number ratio, we calculated the copy-number
ratio (eff\textsubscript{[Target]}/eff\textsubscript{[Control]}, where eff is efficiency) for the
blood samples tested in the chimeric-primer assay and in the
DNA primer assay, as previously described (18).

Table 3 presents a comparison between the \Delta C\textsubscript{q} values of
DNA and chimeric primers for detection and quantification of
the E. canis 16S rRNA, B. canis 16S rRNA, and canine β-actin
genes. For all three genes and at all plasmid concentrations,
\Delta C\textsubscript{q} was higher when chimeric primers were used than when
DNA primers were used. The \Delta C\textsubscript{q} of the E. canis 16S rRNA
gene assay using DNA primers was 16 cycles, compared to 20
when chimeric primers were used (see Fig. S1A in the
supplemental material). The \Delta C\textsubscript{q} of the B. canis Hsp70 gene
using DNA primers was 10, compared to 14 when chimeric primers
were used (see Fig. S1B in the supplemental material). The
difference between DNA primers and chimeric primers was
demonstrated by qPCR fluorescence plots of serial decimal
dilutions of pE. canis-16S and pB. canis-Hsp70 (see Fig. S1B in the
supplemental material). In all DNA primer assay plots, low
plasmid concentrations (–10 copies/µl) merged with the NTC
sample plot, while in both chimeric-primer assays, low
concentrations were still distinguishable and different from the
NTC sample plots (see Fig. S2 in the supplemental material). The
efficiency of these reactions using DNA primers was 100%.

Table 1. Primer sequences used for amplification of target and reference genes

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’–3’)</th>
<th>Gene</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. canis 16S-fwd</td>
<td>TCGCTATTAGATGAGGCCCTACGT</td>
<td>E. canis 16S rRNA</td>
<td>911–931</td>
</tr>
<tr>
<td>E. canis 16S-rev</td>
<td>GAGTCTGGAGGCTATCTAGAT</td>
<td>E. canis 16S rRNA</td>
<td>1014–1034</td>
</tr>
<tr>
<td>B. canis Hsp70-fwd</td>
<td>GTCATAGCTTGCTGGTACGT</td>
<td>B. canis Hsp70</td>
<td>427–448</td>
</tr>
<tr>
<td>B. canis Hsp70-rev</td>
<td>GCAATGACGGTTAGACGCGCAAT</td>
<td>B. canis Hsp70</td>
<td>490–511</td>
</tr>
<tr>
<td>Canine β actin-fwd</td>
<td>CCAGAAGTACTCTGTGAT</td>
<td>Canine actin</td>
<td>1005–1025</td>
</tr>
<tr>
<td>Canine β actin-rev</td>
<td>CGGTGTGAGGGACGCTACGT</td>
<td>Canine actin</td>
<td>1072–1092</td>
</tr>
<tr>
<td>Chimera 16S-fwd</td>
<td>TCGCTATTAGATGAGCCTACGT</td>
<td>E. canis 16S rRNA</td>
<td>911–931</td>
</tr>
<tr>
<td>Chimera 16S-rev</td>
<td>GAGTCTGGAGGCTATCTAGAT</td>
<td>E. canis 16S rRNA</td>
<td>1014–1034</td>
</tr>
<tr>
<td>Chimera Hsp70-fwd</td>
<td>GTCATAGCTTGCTGGTACGT</td>
<td>B. canis Hsp70</td>
<td>427–448</td>
</tr>
<tr>
<td>Chimera Hsp70-rev</td>
<td>GCAATGACGGTTAGACGCGCAAT</td>
<td>B. canis Hsp70</td>
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</tr>
<tr>
<td>Chimera β actin-fwd</td>
<td>CCAGAAGTACTCTGTGAT</td>
<td>Canine actin</td>
<td>1005–1025</td>
</tr>
<tr>
<td>Chimera β actin-rev</td>
<td>GCGGTACTCTGTGAT</td>
<td>Canine actin</td>
<td>1072–1092</td>
</tr>
</tbody>
</table>

* The first six sequences are DNA primers, while the last six are DNA/RNA chimeric primers. The lowercase “r” designates an upstream RNA base. Those bases
are presented in bold font. fwd, forward; rev, reverse.

Table 2. List of clinically and molecularly diagnosed dogs naturally infected with Ehrlichia canis and/or Babesia canis

<table>
<thead>
<tr>
<th>Sample or dog</th>
<th>Result of previous conventional PCR</th>
<th>E. canis 16S rRNA</th>
<th>B. canis 16S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>37599</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>36799</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>6800</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Jar-Jar (naïve puppy)</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Candy</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

* DNA was extracted from the blood of naturally infected dogs. The diagnosis
was confirmed by using dual-labeled qPCR probes. The samples were detected
by nonspecific-dye PCR using DNA primers and chimeric primers.
whereas the use of DNA-RNA chimeric primers decreased the efficiency to ~84% (see Fig. S3 in the supplemental material). Although chimeric primers were less efficient, their sensitivity was higher (Table 3). The use of chimeric DNA-RNA primers for the detection of the *E. canis* 16S rRNA gene enabled the detection of ~10 copies/µl, while the use of DNA primers comprised of the same sequence allowed a minimal detection concentration of ~100 copies/µl. The detection threshold for the *B. canis* Hsp70 gene was ~10 molecules/µl using chimeric primers, while the detection threshold of the DNA primers was ~100 plasmid copies. When examining the detection threshold for the β-actin gene, the two primer sets (DNA primers and chimeric primers) showed high sensitivities, with a detection threshold of ~10 copies/µl, and yet, in higher concentrations, the ΔCq values of the chimeric primers were higher than those of the DNA primers.

Testing the sensitivity of the qPCR assay using chimeric primers on DNA extracted from naturally infected dogs showed that the ΔCq values of the *E. canis* and *B. canis* qPCR reactions were at least 2 to 3 cycles higher than the values acquired by using DNA primers (Fig. 1A and B). In these samples, the ΔCq values of the chimeric-primer reactions were higher than those of the DNA primers for both the *E. canis* 16S rRNA gene (Fig. 1A) and the *B. canis* Hsp70 gene (Fig. 1B). In samples 37599 (naturally infected with *E. canis*) and 6800 (naturally coinfected with *E. canis* and *B. canis*), the detection threshold of chimeric-primer assays was 10-fold higher than that of DNA primer assays. There was a delay of 2 to 3 cycles in Cq values in the chimeric-primer assay compared to the results for the DNA primer assay. Nevertheless, the sensitivity of chimeric primers was higher due to a delay of more than 7 cycles in the appearance of Cq values in NTC and the negative control compared to the results for the DNA primers. For DNA extracted from blood of healthy dogs, the sensitivities of both DNA and chimera assays for the detection of the canine β-actin gene were equal (Fig. 1C). In both assays, a dilution of 1/10,000 was detected, although in Jar-Jar’s sample, the standard deviation of the DNA assay of the 1/10,000 dilution was higher than the average ΔCq, as might occur at very low concentrations. The blood sample results are consistent with the plasmid results (Table 3), whereas the chimera assays for the *E. canis* 16S rRNA and *B. canis* Hsp70 genes detected 10-fold higher dilutions than the DNA primers, and the detection of the canine β-actin gene was maximal in both cases.

Utilization of chimeric primers demonstrated higher sensitivity than was found for DNA primers (Table 3). Calculation of the eff(ΔCq) ratios of Hsp70/ACTB and 16S rRNA/ACTB genes and comparison between the results of the DNA assay and chimeric-primer assay revealed that although the eff(ΔCq) ratio is significantly higher in the case of the chimeric-primer assay (see Fig. S4A and B in the supplemental material), the ratio between the results of the DNA primer assay and the chimeric-primer assay remained the same in each run for *B. canis* and *E. canis* (see Fig. S4C in the supplemental material). 

We initially hypothesized that the efficiency of the initiation of DNA synthesis in a DNA-dependent DNA polymerase amplification reaction mixture is hampered when RNA bases are incorporated in the initiation zone. Indeed, the amplification efficiency was reduced. Since the initiation zone of the RNA-

### Table 3. Comparison of ΔCq values of qPCR using DNA-RNA chimeric primers and DNA primers with SYBR green

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of plasmid copies/µl</th>
<th>ΔCq value found using:</th>
<th>DNA primers</th>
<th>Chimeric primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ehrlichia canis</em> 16S rRNA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.44</td>
<td>1.94</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.79</td>
<td>6.66</td>
<td>6.66</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>6.65</td>
<td>9.91</td>
<td>9.91</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>10.18</td>
<td>14.21</td>
<td>14.21</td>
</tr>
<tr>
<td><em>Babesia canis</em> Hsp70</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.85</td>
<td>4.97</td>
<td>4.97</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.23</td>
<td>8.45</td>
<td>8.45</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>6.44</td>
<td>12.79</td>
<td>12.79</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>9.94</td>
<td>16.58</td>
<td>16.58</td>
</tr>
<tr>
<td>Canine actin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.47</td>
<td>2.77</td>
<td>2.77</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5.32</td>
<td>10.59</td>
<td>10.59</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>8.85</td>
<td>14.59</td>
<td>14.59</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>11.9</td>
<td>17.07</td>
<td>17.07</td>
</tr>
</tbody>
</table>

* Each of the plasmids pE. canis-16S, pB. canis-Hsp70, and pACTB was diluted in decimal dilutions, and 3 µl of each dilution was added to the reaction mixture.
DNA chimeric primer dimers harbored more RNA bases (Fig. 2), the primer dimer artifacts in reaction mixtures with small amounts of template were reduced, resulting in increased detection sensitivity. The other possible explanation for the reduction of nonspecific by-products arose from the results obtained using dual-labeled probe together with chimeric primers, where the reaction annealing temperature was reduced by at least 2°C (data not shown). This effect was enhanced in the presence of two chimeric primers. Figure 2 demonstrates how two hypothetical chimeric primers anneal to the target sequence using six chimeric junctions, while primer dimers harbor about nine chimeric junctions. Moreover, the reduction in the expected $T_m$ for a hypothetical chimeric primer dimer ($\Delta T_m = -2.6°C$) was higher than the reduction of the expected target-and-chimeric-primer hybrid ($\Delta T_m = 0°C$ and $-1.2°C$). Irrespective of the mechanism proposed, this

FIG. 1. $\Delta C_q$ values from qPCR assays using chimeric primers and DNA primers. Results for four serial decimal dilutions of DNA samples extracted from blood of naturally infected and healthy dogs (Table 2) are presented, demonstrating the detection of the *Ehrlichia canis* 16S rRNA gene (A), the *Babesia canis* Hsp70 gene (B), and the canine $\beta$-actin gene (C). Bars: vertical lines, undiluted samples; horizontal lines, 10-fold dilutions; diagonal lines, 10^2-fold dilutions; dots, 10^3-fold dilutions; grids, 10^4-fold dilutions.
reduction in the appearance of reaction background increased the assay’s sensitivity. Nakano et al. (17) investigated the thermodynamics of base pairing chimeric junctions to DNA and showed that the nearest-neighbor \( T_m \) of chimeric junctions was different from that of DNA hybrids. It was not always increased but occurred more frequently. It is well known that annealing temperatures do not correlate absolutely with the \( T_m \) (9), and our results were probably influenced by the annealing temperature of the chimeric primers.

This approach is different from the locked nucleic acids (LNA) approach where the incorporation of LNA into oligonucleotide primers has been shown to increase template binding strength, allowing the primers to be shortened and increasing specificity (1). Contrary to the results with LNAs, the incorporation of RNA bases into DNA primers probably destabilized base pairing. However, destabilization of the primer dimers was probably more profound than the destabilization of the primer-target DNA hybrid. This method is different from all-chimera modification in which RNase activity is involved during isothermal amplification (14, 22) or the use of uracil-DNA N-glycosylase (22). It is also different from using RNA stretches in ordinary PCR (15). However, it should be worthwhile to examine whether using \( rTth \) improves reaction efficiency. Modification of the primer with artificial compounds at the 3’ end reduces the appearance of nonspecific products (24). However, using these modifications also requires primer cleavage. In world patent WO 01/64952 A2, Dean et al. (5a) described a method for reducing artifacts in nucleic acid amplification by performing modifications in the 5’ region of the primers, including the addition of RNA bases to the 5’ end. Dean’s method differs from our technique since it reduces the appearance of nonspecific products at the 5’ end of the primer, while our technique incorporates single RNA bases at the nearest neighbor of the suspected primer dimer initiation site even if (and preferably when) located at the 3’ end of the primer. Our method relies on the thermodynamics of primer dimers and kinetics of DNA-dependent DNA polymerase in the initiation of nonspecific products. The different base-pairing affinities of chimeric primers to DNA strands widen the number of possible combinations of base pairs for PCR and enable fine-tuning of the annealing temperature. In addition, this thermodynamic flexibility enables the construction of highly sensitive qPCR assays. Hence, the use of DNA-RNA chimeric primers appears to be a simple and efficient method for reducing the formation of by-products and increasing the sensitivity and specificity of qPCR assays.

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REFERENCES

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20. Reference deleted.
21. Reference deleted.