



A newly developed droplet digital PCR for *Ehrlichia canis* detection: comparisons to conventional PCR and blood smear techniques

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ABSTRACT. Canine monocytic ehrlichiosis caused by *Ehrlichia canis* infection is a life-threatening vector-borne disease in dogs worldwide. Routine blood smear has very low sensitivity and cannot accurately provide a quantitative result. Conventional PCR (cPCR) and real-time PCR (qPCR) are widely used as molecular methods for *E. canis* detection. qPCR is quantitative but relies on standard curves of known samples. To overcome this difficulty, this study developed a new *E. canis* quantitative detection method, using droplet digital polymerase chain reaction (ddPCR). ddPCR was evaluated against cPCR and blood smears. PCR amplicons and genomic DNA (gDNA) from 12 microscopic positive samples were used to identify the limits of detection (LODs) in ddPCR and cPCR. Our ddPCR was assessed in 92 field samples, it was compared with cPCR and blood smears. ddPCR showed LOD=1.6 copies/reaction, or 78 times more sensitive than cPCR (LOD=126 copies/reaction), using PCR amplicons as a template, whereas both ddPCR and cPCR had equal LODs at 0.02 ng gDNA/reaction. In addition, ddPCR had 100% sensitivity and 75% specificity for *E. canis* detection compared to cPCR and no cross-reaction with other blood pathogens was observed. ddPCR identified more positive samples than cPCR and blood smear. ddPCR improved the overall performance of *E. canis* detection, with a better LOD and comparable sensitivity and specificity to cPCR. The technique might be helpful for diagnosis of *E. canis* in light infection, evaluating the number of *E. canis* and follow-up after treatment.

KEYWORDS: blood smear, conventional polymerase chain reaction, detection, droplet digital polymerase chain reaction, *Ehrlichia canis*

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Ehrlichia canis is an obligate intra-cellular bacterium and causes canine monocytic ehrlichiosis (CME), a life-threatening tick-borne rickettsial disease. *Ehrlichia canis* is the most common species affecting dogs worldwide, especially in tropical areas and is probably the most widely distributed canine vector-borne diseases [10, 16, 22, 38, 51]. The prevalence of infection in dogs varies depending on the method of detection. In the United States, as many as 15% of dogs tested had antibodies to *Ehrlichia* spp. [4, 6, 34], while prevalence of *E. canis* in different European countries was in the range 0–50% [11, 13, 41, 56]. In Thailand, *E. canis* was found to be the most frequently detected dog blood pathogens, with a molecular prevalence ranging from 1.3–38.3%

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[32, 35, 48, 49, 55]. Similar *E. canis* infection distributions were identified in studies in Eastern and Southeast Asian countries [10, 16, 22]. Clinical signs of CME may vary ranging from mild to severe, with great morbidity and mortality in animals [8, 57]. *Rhipicephalus sanguineus* is considered the main vector for *E. canis* and this species is the predominant tick species in Thailand [44]. Co-infections with other blood pathogens occur frequently and worsen the disease conditions as well as complicating the diagnosis [17, 30, 54, 55]. Although *E. canis* typically infects dogs, this bacterium has potential to be transmitted to humans [36, 46]. Detection at the late stage may eventually develop to the chronic infection contribution to a life-threatening in dog as well as transmission to human. Therefore, early detection in animals would help early elimination, leading to the effective prevention and control of possible zoonosis.

Diagnosis of *E. canis* infection can be very challenging, because CME presents a variety of clinical signs, for example, fever, anemia or thrombocytopenia, which are similar to other blood pathogen infections [12, 17]. Currently, diagnosis of *E. canis* infection is primarily based on microscopic examination of intracytoplasmic morulae in dog monocytes and macrophages; however, this method has a limited sensitivity [42] and only a few morulae can be found [42, 56]. Furthermore, this technique needs experienced examiners to distinguish between *E. canis* infections from other cytoplasmic inclusions [14, 19] or related organisms [7, 25]. Serological diagnostic methods are also important in the cases with clinical or laboratory findings consistent with CME [5]. The indirect fluorescent-antibody assay (IFA) is the most widely used to diagnose *E. canis* infection [62]; however, the detection of antibodies may be problematic due to cross reactivity with closely related pathogens [61]. In addition, polymerase chain reaction (PCR)-based techniques are considered to be the most reliable methods to diagnose *E. canis* infection [19]. The conventional PCR (cPCR) is the simplest technique which has been demonstrated to be able to identify species [35, 55]. Recently, using the *16S* rDNA gene as an amplification target helped diagnose low pathogen infections [1, 25, 47]. However, the cPCR assay showed low sensitivity for detection in mild infection cases and does not provide any quantitative result [43].

Currently, droplet digital polymerase chain reaction (ddPCR) assay is a recent generation PCR test that allows absolute measurement of nucleic acids, by evenly distributing the PCR reactions into about 20,000 droplets. Although it measures the fluorescence intensity, calculation will class the signal as positive or negative, using a Poisson algorithm, which is no longer dependent on the signal intensity. Thus, the obstacle of faint band intensity measurement found in cPCR can be eliminated, allowing ddPCR to produce precise and accurate results without external standards, even compared to quantitative real-time PCR, as used in previous parasitic studies [24, 28, 53, 64]; it also led to better detection, when low amounts of DNA target were present [60]. The ddPCR technique has been used for the detection of medical important parasites, including *Echinococcus multilocularis* [39], *Plasmodium* spp. [37], *Leishmania* spp. [52], gastrointestinal parasites [3], *Trypanosoma cruzi* [53], *Wuchereria bancrofti* and *Brugia malayi* [24], *Babesia microti* and *B. duncani* [63] and *Cryptosporidium* spp. [64]. To improve *E. canis* diagnosis, we developed a new ddPCR assay, targeting the *16S* rDNA gene, for quantitative diagnosis in dog blood samples. The performance of the new developed ddPCR was compared to cPCR and routine blood smears on the same samples.

MATERIALS AND METHODS

Sample collection and DNA extraction

Blood samples were obtained from 101 dogs, including 92 field samples obtained from Buriram, Saraburi and Bangkok provinces, Northeastern and Central Thailand and samples positive for *B. canis* ($n=4$), *H. canis* ($n=2$) and *A. platys* ($n=3$), identified based on blood smears and confirmed using cPCR based on our published protocol [55]. In total, 101 samples were 43% male and 57% female. All blood samples were collected in an EDTA tube and immediately prepared for blood smear examination. Blood samples were kept in a 1.5-ml microcentrifuge tube, labeled, and stored at -20°C for later DNA extraction. Briefly, 250 μl of each EDTA blood sample was used for DNA extraction, using a E.Z.N.A. Blood DNA Mini Kit (Omega Bio-tek, Norcross, GA, USA), following the manufacturer's protocol. The total DNA concentration of all samples was measured using nanodrop spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -20°C until use. The research protocol of this study was approved by the Animal Ethics Committee of the Faculty of Veterinary Technology, Kasetsart University, Thailand (ACKU62-VTN-0011).

Primers and probe design

Primers and probes for ddPCR and cPCR were designed using the Primer3 program (<http://bioinfo.ut.ee/primer3-0.4.0/>) to check the flanks of the selected DNA region. *16S* rDNA was used as a target sequence for detection of *E. canis*, *16S* rDNA sequences of *E. canis* (GenBank accession no. U26740.1), *E. chaffeensis* (GenBank accession no. NR_074500.2), *E. ewingii* (GenBank accession no. U96436.1), *A. platys* (GenBank accession no. LC269822.1), *A. phagocytophilum* (GenBank accession no. M73224.1) and *Mycoplasma haemocanis* (GenBank accession no. MZ221174.1), retrieved from the NCBI nucleotide database. These were used as input for DNA alignment based on the Clustal Omega program. The polymorphic regions were used for target DNA priming sites, avoiding cross positive amplification among other pathogens. The primers and the probe used in this study were: forward 5'-AACTACAATAGGTTGCGAGAC-3', reverse 5'-ACGTATTCACCGTGGTGTGA-3' and probe FAM-5'-AGAGCATGAAGTCGGAATCGC-3'. The same primer sequences were used in ddPCR and cPCR. The expected amplicon size was 136 base pairs.

Alignment of six species revealed polymorphic regions among the same genes, as shown in Fig. 1. Selected regions showed a promising capacity for the primer design, using as a molecular marker separating the *Ehrlichia* sp. infection. In the *16S* rDNA primer design, the forward 3' end was specifically bound to an *Ehrlichia* spp. DNA template, since only the base pairing at the 3'

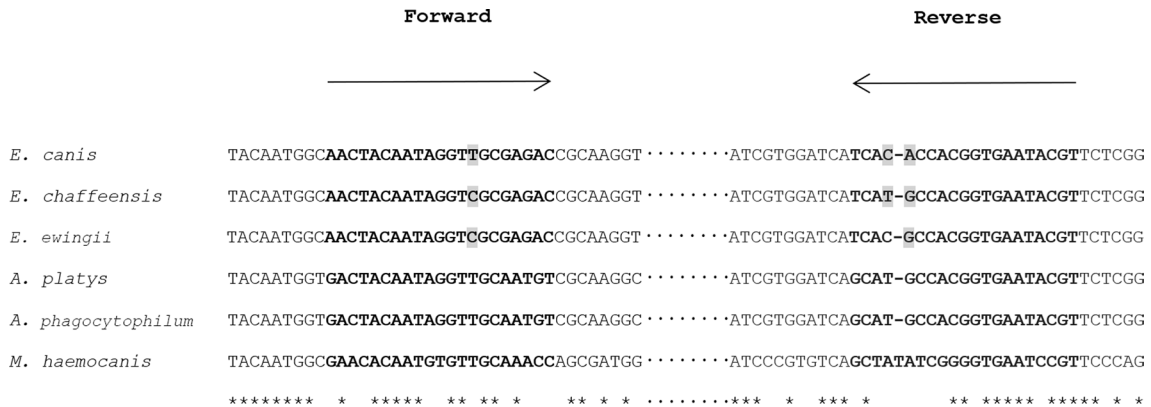


Fig. 1. Simplified DNA alignment showing selected polymorphic regions, targeted in primer design of *16S* rDNA. Dots indicate regions that were not involved in the interpretation and discussion. Shaded areas were the mismatch priming sites among *Ehrlichia* spp.

end of the primer would play an important role in the DNA extension. Likewise, the reverse 3' end also showed specific binding to the *Ehrlichia* spp. DNA template. Thus, the designed primers were genus-specific.

Conventional PCR amplification

Newly designed primers were used for cPCR. Genomic DNA (gDNA) samples were pretreated using EcoRI restriction enzyme digestion (Thermo Fisher Scientific) following the manufacturer's instructions for the best amplification result. Briefly, 1 µl EcoRI was mixed with a 1 or 10 ng/µl DNA sample in proper buffer and nuclease free water at 37°C for 1 hr. The reaction was inactivated using heat incubation (65°C, 20 min). cPCR reactions were set up in a total of 25 µl. Each reaction was composed of 1x DreamTaq Green buffer (Thermo Fisher Scientific), 0.2 mM dNTP each, 1 µM PCR primer, 2 µl EcoRI-digested gDNA template and 1.25 units DreamTaq DNA polymerase (Thermo Fisher Scientific). The PCR amplification cycle consisted of an initial denaturation step (95°C, 2 min), followed by 40 cycles of denaturation (95°C, 30 sec), with annealing temperature ranging from 46 to 60.7°C for 30 sec, and extension (72°C, 30 sec) and a final extension cycle (72°C, 5 min). The PCR products were subjected to electrophoresis in 1.5% agarose gel, containing SYBR Safe DNA gel stain (Thermo Fisher Scientific) to verify the amplicon size under UV light. Positive samples were directly sequenced from both ends using a 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, USA). Later, all DNA sequences were blasted against the NCBI database and the results confirmed 100% identity to *E. canis*.

Droplet digital polymerase chain reaction (ddPCR) assay

ddPCR used the QX200 Droplet Digital PCR system (Bio-Rad, Hercules, CA, USA). The same samples used in cPCR, were used for detection and quantification of the *E. canis 16S* rDNA gene in ddPCR. A total of 20 µl of each reaction contained 2 µl of EcoRI-digested gDNA template, 10 µl ddPCR Supermix (Bio-Rad), 7 µl distilled water and 1 µl primers and probe. Then, 20 µl PCR mix was added to the sample wells and 70 µl of droplet generation oil was added to each oil well of the cartridge. A 40 µl droplet partitioned mixture was obtained using a QX200 droplet generator (Bio-Rad). Then all samples were transferred to a 96-well PCR plate, sealed, and amplified using a 100TM Thermal Cycler (Bio-Rad) with the optimized PCR condition being: an initial step (95°C, 10 min), followed by 40 cycles of denaturation (94°C, 1 min) and annealing and extension at a temperature ranging from 46 to 60.7°C for 2 min, with a final cycle (98°C, 5 min). The 96-well PCR plate was transferred and read in the FAM channel using the QX200 reader (Bio-Rad). Droplet fluorescence data were analyzed using the QuantaSoft program (Bio-Rad).

Limit of detection evaluation for known copy number of *E. canis 16S* rDNA amplicons

LODs between ddPCR and cPCR were evaluated in sample with known template concentrations. DNA was extracted from an *E. canis* infected dog. The *16S* rDNA gene was amplified using cPCR with the newly designed primers. The known *16S* rDNA copy number sample was prepared using the *16S* rDNA amplicons. The amplicons were purified using a FavorPrep™ GEL/PCR Purification Kit (Favorgen, Ping-Tung, Taiwan), to obtain the known *16S* rDNA gene in mole equivalents. The molecular weight of double stranded *16S* rDNA amplicons was estimated based on the Sequence Manipulation Suite (https://www.bioinformatics.org/sms2/dna_mw.html), showing 83,901.74 g/mol. The amplicons concentration was initially adjusted to 1 ng/µl. This concentration was recalculated as described previously [59] using these steps:

$$1 \text{ ng}/\mu\text{l} = \frac{1 \times 10^{-9}}{83,901.74} \text{ mol}/\mu\text{l} = 1.19 \times 10^{-14} \times 6.02 \times 10^{23} = 7.175 \times 10^9 \text{ molecules}/\mu\text{l} \text{ (copies}/\mu\text{l)}$$

Then, the sample was adjusted to 10,000 copies/µl before being ten-fold diluted from 10,000 to 0.01 copies/µl. 2 µl of each diluted amplicon that was used as a template for ddPCR and cPCR assays. Since one *E. canis* genome had only one copy of the *16S* rDNA gene [65], the gene copy number can be directly converted into the number of *E. canis*.

Limit of detection for total gDNA extracted from 12 *E. canis* microscopic positive samples

The LODs in total gDNA for both ddPCR and cPCR were also evaluated in 12 *E. canis* microscopic positive samples. First, thin blood smears were made for all 12 samples and counted for % *E. canis* infected monocytes. Then the blood smear slides were fixed in methanol and stained for 45 min in 1:6-diluted Giemsa stain (pH 7.2) for *E. canis* identification. The slides were evaluated by an experienced examiner under oil immersion fields (1,000× magnification) in a minimum of 200 monocytes. The criterion for a positive detection was defined as the detection of the morula stage in the monocyte cytoplasm. All infected monocytes were counted, regardless of stages, as well as the number of *E. canis* per monocyte cell. Second, total gDNA in blood from each of the 12 *E. canis* infected dogs was extracted and adjusted to 1 or 10 ng/μl on a case-by-case basis. Each DNA sample was then ten-fold serially diluted to 0.00001 ng/μl and 2 μl of DNA template was used for ddPCR and cPCR assays.

Data and statistical analysis

Diagnostic performance of ddPCR was evaluated from 92 field samples. The sensitivity and specificity of the ddPCR assay for *E. canis* detection were calculated using cPCR as the reference. Data analysis used the SPSS version 22 for Windows software (SPSS Inc., Chicago, IL, USA). Correlations between the DNA copy number measured using the ddPCR software and the DNA copy number evaluated using spectrophotometry or the correlation between % infected monocytes and the DNA copy number were evaluated using Spearman's rho correlation coefficient. Agreement between two detection methods (ddPCR and cPCR) was estimated using Cohen's kappa coefficient, κ , where κ ranges from 0 to 0.20 indicated no agreement, from 0.21 to 0.39 indicated minimal agreement, from 0.40 to 0.59 indicated weak agreement, from 0.60 to 0.79 indicated moderate agreement, from 0.80 to 0.90 indicated strong agreement and above 0.90 indicated almost perfect agreement (McHugh, 2012). A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Optimization of ddPCR and cPCR assay

To obtain the optimal signal during amplification, the annealing temperature of each amplification was optimized against *E. canis* positive DNA. The cPCR annealing temperatures varied in the range 46–60.7°C, as shown in Fig. 2a. The most favored annealing temperature was 55°C as it produced higher relative band intensities than the other temperatures. To facilitate the comparison with ddPCR, Fig. 2b presents the same annealing temperatures. Clearly, the highest mean amplitude of positive droplets was for the 55°C annealing temperature (Fig. 2b); thus 55°C was selected for further experiments. Based on this optimization, the threshold for the positive detection level was set at 2,200 relative fluorescence units for *E. canis* using this set of primers.

Specificity evaluation

Although newly designed primers were used in the ddPCR and cPCR reactions, cross amplification to other pathogens had to be verified in both approaches. Based on the cPCR technique, the result clearly showed the specific band of 136 bp amplicon size as shown in Fig. 3a, when an *E. canis* infected sample was used as a positive control, while no cross-amplification was observed among other most common dog blood pathogens found in Thailand, consisting of *Babesia canis* (*n*=4), *Hepatozoon canis* (*n*=2) and *Anaplasma platys* (*n*=3). This 136-bp positive band was further confirmed to be 16S rDNA gene based on DNA sequencing.

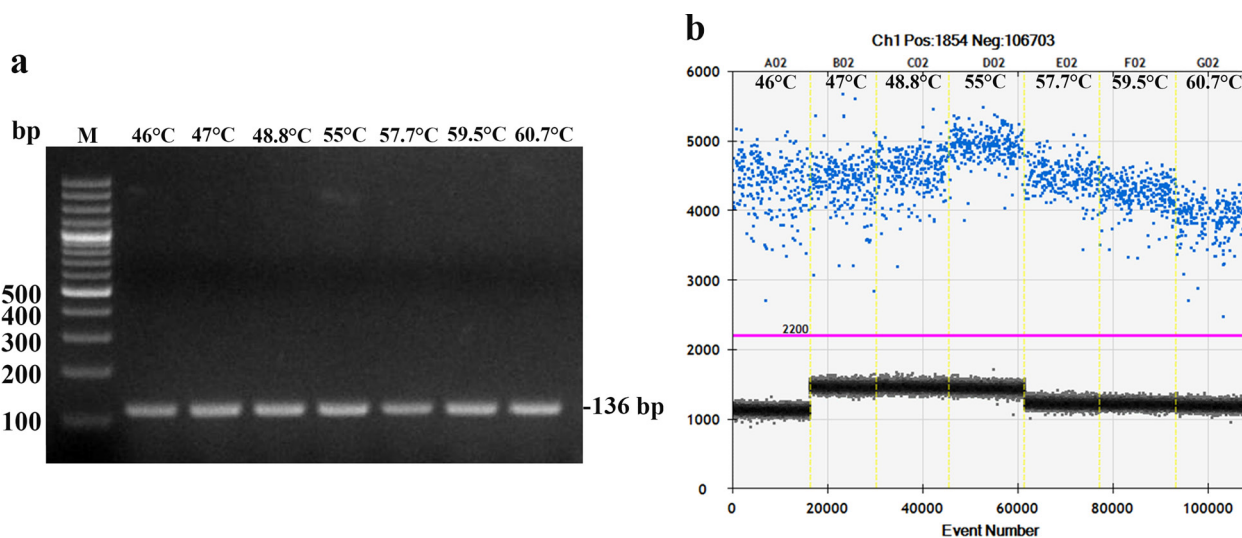


Fig. 2. (a) Conventional PCR shows positive bands at 136 bp products in 46–60.7°C annealing temperature optimization, (b) droplet digital PCR shows positive droplets (blue) and negative droplets (black) separations by manually adjustable threshold (pink line) in 46–60.7°C annealing temperature optimization.

ddPCR was also verified for no cross amplification using the same set of primers as applied in cPCR. The result clearly delivered no cross reaction with *B. canis*, *H. canis* or *A. platys*, as shown in Fig. 3b.

Limit of detection evaluation for known copy number of *E. canis* 16S rDNA amplicons

The limits of detection (LODs) of ddPCR and cPCR were evaluated using purified 136 bp amplicons of *E. canis* 16S rDNA as a template. The amplicon concentration was prepared ranging from 20,000 to 0.02 copies/reaction. When verified for LOD, cPCR still showed the 136-bp positive band when a template was lowered to 20 copies/reaction, measured using spectrophotometry (Fig. 4a). However, this LOD changed to 126 copies/reaction (6.3 copies/ μ l) when verified using ddPCR and the QuantaSoft software. In addition, ddPCR still produced a positive droplet when the template was lowered to 0.2 copies/reaction, measured using spectrophotometry (Fig. 4b, lane F09) and the QuantaSoft software calculated the copy number for 1.6 copies/reaction (0.08 copies/ μ l) (Fig. 4c). Interestingly, based on the copy number determined using the QuantaSoft software, ddPCR had a 78 times lower LOD than cPCR. Notably, the calculated gene copy number obtained using spectrophotometry and the QuantaSoft software were different, with the copy numbers from the QuantaSoft software being more reliable than that using spectrophotometry. However, Spearman's rho correlation coefficient was used to assess the relationship between DNA copy numbers evaluated using spectrophotometry with that using ddPCR. There was a strong positive correlation between the two ($r_s=0.986$, $P<0.01$).

Limit of detection-total gDNA extracted from 12 microscopic positive samples

The LODs of ddPCR and cPCR were further evaluated in 12 naturally infected *E. canis* samples, which had tested positive based on blood smear examination under various degree of infection in the range 1–30% infected monocytes (Table 1). Both techniques were consistent in confirming *E. canis* infections in all 12 samples. All gDNA samples were 10-fold serially diluted and used as a template for amplifications. In the cPCR reaction, the lowest concentration, producing the 136-bp positive band, was recorded for the LOD in each sample. The LODs were in the range 0.02–2 ng of gDNA/reaction, as shown in Table 1, with the lowest amount of cPCR detection at 0.02 ng/reaction of selected gDNA samples (Fig. 5a), whereas the ddPCR produced LODs in the same range of 0.02–2 ng of gDNA/reaction, with the lowest amount of ddPCR detection at 0.02 ng/reaction (Fig. 5b). Based on these results, we suggest using the gDNA template at 2 ng/reaction in either cPCR or ddPCR. However, ddPCR produced lower LODs than cPCR in 4 (case 5, 6, 7 and 9) out of 12 cases. In addition, Spearman correlation indicated that there was a weak positive correlation between the percentage of infected monocytes and the DNA copy number evaluated using ddPCR ($r_s=0.096$, $P=0.76$).

Diagnostic performance of ddPCR for *E. canis*

The diagnostic performance of ddPCR was applied in 92 field samples compared with cPCR. gDNA was extracted and the prepared DNA solution was directly used as a template in both amplification techniques. Tables 2 and 3 show that ddPCR had the highest number of positive samples (46/92) compared to cPCR (31/92) or blood smears (12/92). Furthermore, 15 cPCR negative samples were positive by ddPCR and no positive sample from cPCR was negative in ddPCR. From these results, the correlation indicated moderate agreement between ddPCR and cPCR ($\kappa=0.674$). In addition, samples that were cPCR-negative but ddPCR-positive had very low DNA copy numbers (mean: 0.16 copies/ μ l), whereas cPCR and ddPCR positive samples had significantly higher DNA copy numbers (mean: 9.32 copies/ μ l, $P<0.001$), as shown in Supplementary Table 1. This result implied that the cPCR false-negative cases were mainly due to very low DNA copy numbers. However, the sensitivity and specificity of a blood smear and ddPCR were calculated based on cPCR as reference. The results for *E. canis* detection were 39% sensitivity and 100% specificity for a blood smear and 100% sensitivity and 75% specificity for ddPCR.

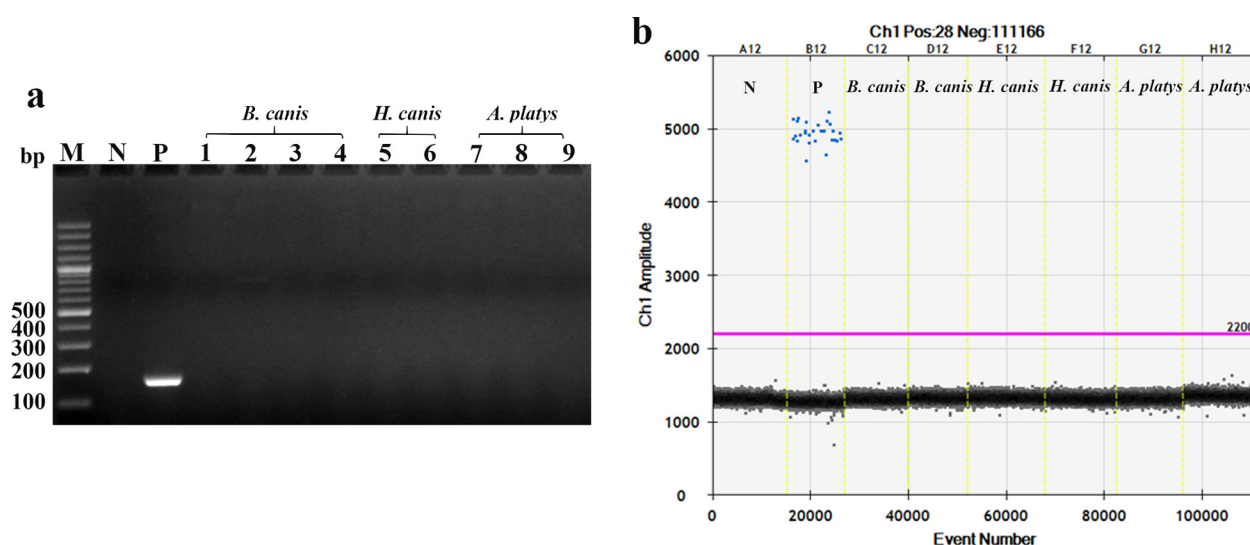


Fig. 3. Species-specific primer evaluation verified by (a) conventional PCR and (b) droplet digital PCR (P=*Ehrlichia canis* as a positive control, *B. canis*=*Babesia canis*, *H. canis*=*Hepatozoon canis*, *A. platys*=*Anaplasma platys* and N=No DNA template (negative control)).

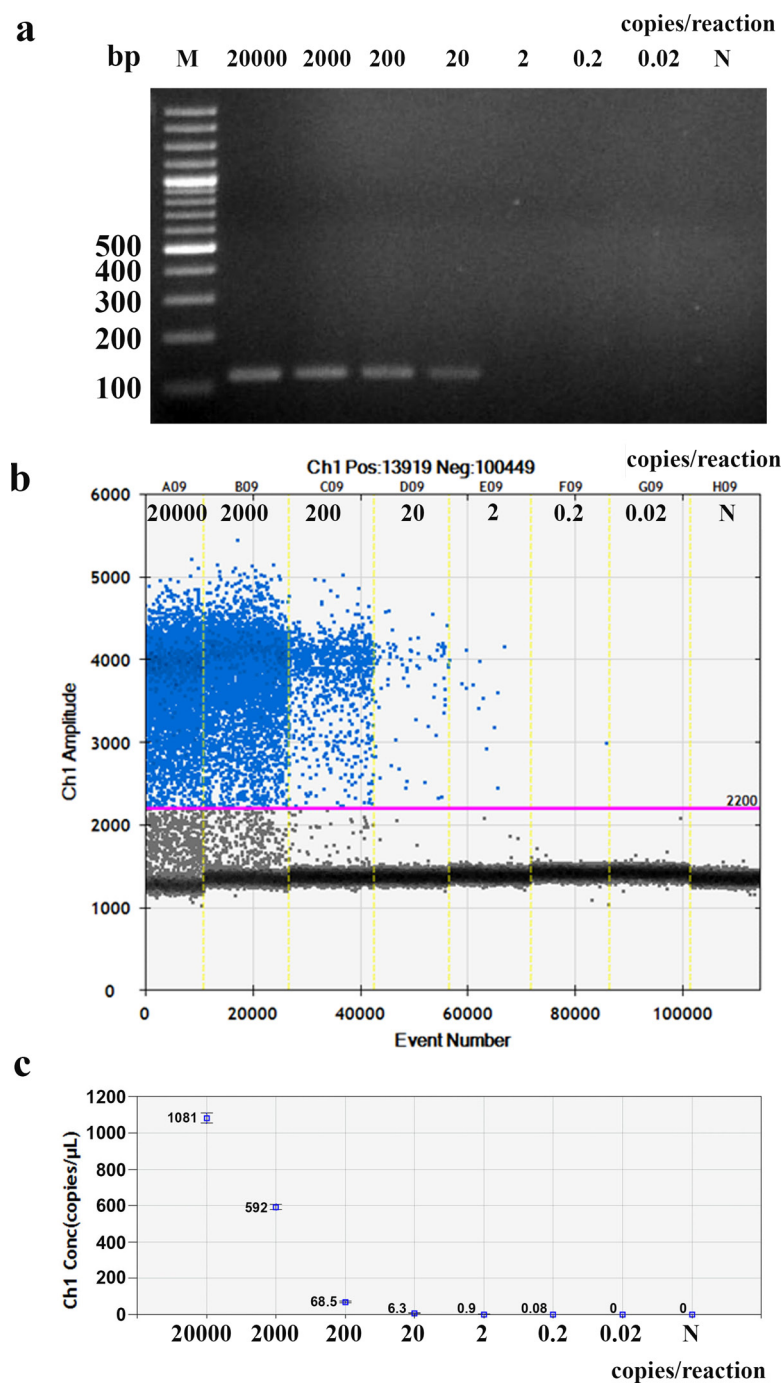


Fig. 4. Amplification of the 10-fold serial dilutions of *Ehrlichia canis* 16S rDNA amplicons starting at 20,000 copies/ μ L template using (a) conventional PCR, (b) droplet digital PCR and (c) calculated copy number from QuantaSoft software ((M=DNA marker, N=No DNA template (negative control))).

experiment. This difference could be explained by fluctuating DNA concentration measurements from absorption or pipetting errors [64]. The protocol demonstrated that ddPCR, targeting 16S rDNA, had a very low LOD compared to most PCR-based studies (see Table 4).

When the LOD validation of total gDNA was extended to 12 dogs showing positive based on blood smears, we found that ddPCR performed better than cPCR in 4 out of 12 cases (33%). This also indicated that even in a sample with various amounts of natural *E. canis* infections (1–30% infected monocytes), ddPCR and cPCR were able to detect *E. canis* DNA in at least 0.02–2 ng of total gDNA template/reaction, suggesting 2 ng/reaction was needed in clinical use to guarantee successful amplification. ddPCR could help in the early stage or chronic CME diagnosis, where it is difficult to find *E. canis* using microscopic examination.

DISCUSSION

Our study was the first to develop and assess a ddPCR assay for *E. canis* detection in Thailand. ddPCR was evaluated using known DNA concentrations, microscopic positive cases and field samples. The new developed assay was able to provide a quantitative result. 16S gene-specific primers were designed for priming in the sequence variable region, specifically at the 3' priming sites of both primers to avoid cross amplification by other blood pathogens found in Thailand, including *A. platys* and *M. haemocanis* [26, 35, 49, 55]. *Ehrlichia canis* itself is closely related to other *Ehrlichia* species, so that 16S rDNA gene alignment (Fig. 1) showed unimportant mismatch bases (shaded areas) that the applied primers could possibly use and be further applied to detect *E. muris*, *E. chaffeensis* and *E. ewingii*. However, *E. muris*, *E. chaffeensis* and *E. ewingii* have been reported in some countries [18, 31, 33, 58] and any *Ehrlichia* spp. infection can be treated in the same way. Although the tests with cPCR and ddPCR revealed that 55°C was the favored annealing temperature, which led to the highest relative band intensity from agarose gel (cPCR) and the highest relative fluorescence intensity from the Ch1 detector (ddPCR), any temperature between 46 and 60.7°C could be used.

To detect *E. canis* infection in any further samples, the LOD was evaluated using the known DNA concentrations from purified amplicons, following other studies [9, 52, 53, 64]. Based on the *E. canis* complete genome sequence annotation data [65], the 16S rDNA gene had only one copy per genome, making it possible to convert the 16S rDNA copy number to the number of *E. canis* cells directly. Our results demonstrated that the ddPCR QuantaSoft software was able to correctly measure concentrations of the 16S rDNA gene. When we calculated the template amount based on nanodrop spectrophotometry, the LOD of ddPCR was 0.2 copies/reaction. Theoretically, the success in reaction amplification should lead to at least 1 copy/reaction, thus a 0.2 copies/reaction result would be impossible by measurement of absorbances at 260 nm. However, with this low amount of DNA template, the QuantaSoft software indicated that the DNA template amount was 1.6 copies/reaction, that is it was more reliable than the spectrophotometric measurement, since at least one copy should be present. The absorbance measurement was 8 times less sensitive than the ddPCR measurement in this

Table 1. Blood smear examination data vs. limit of detection for droplet digital PCR and conventional PCR

Dog No.	Blood smear examination (% infected monocytes)	Limit of detection	
		Conventional PCR (ng/reaction)	Droplet digital PCR (ng/reaction)
1	5	0.02	0.02
2	3	2	2
3	7	0.02	0.02
4	1	0.2	0.2
5	5	2	0.2
6	17	2	0.2
7	3	2	0.2
8	21	0.02	0.02
9	7	2	0.2
10	5	0.2	0.2
11	30	0.2	0.2
12	7	2	2

Table 2. Diagnostic performance of blood smear for *Ehrlichia canis* detection in natural field samples (n=92)

Methods	Conventional PCR –	Conventional PCR +	Total
Blood smear –	61	19	80
Blood smear +	0	12	12
Total	61	31	92

* +=positive, -=negative.

Table 3. Diagnostic performance of droplet digital PCR for *Ehrlichia canis* detection in natural field samples (n=92)

Methods	Conventional PCR –	Conventional PCR +	Total
Droplet digital PCR –	46	0	46
Droplet digital PCR +	15	31	46
Total	61	31	92

* +=positive, -=negative.

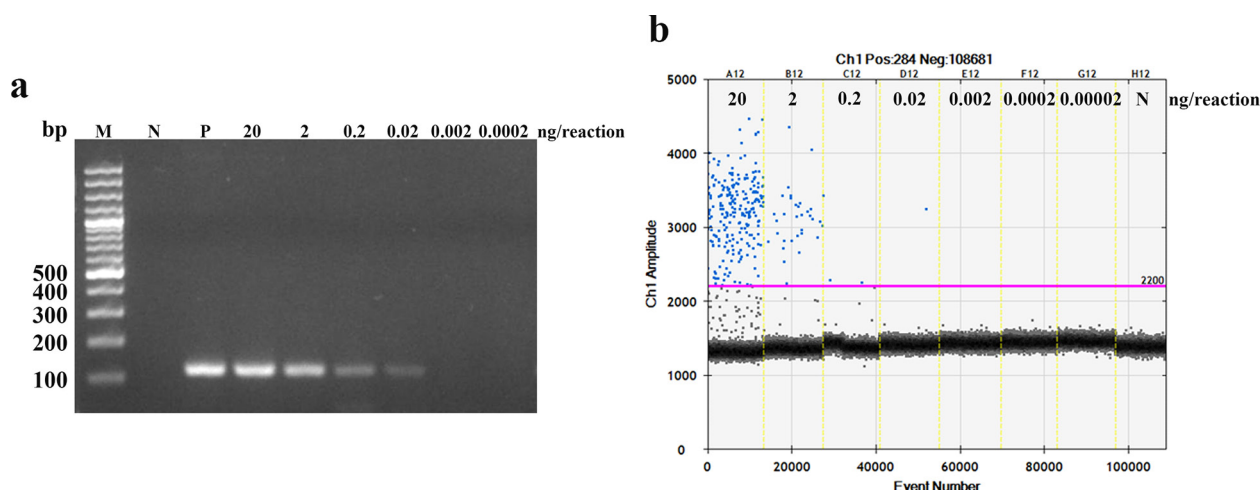


Fig. 5. Representative amplification of 10-fold serial dilutions of *Ehrlichia canis* infected blood samples. (a) Conventional PCR showing limit of detection=0.02 ng/reaction, (b) droplet digital PCR showing limit of detection=0.02 ng/reaction, ((M=DNA marker, P=positive control, N=No DNA template (negative control)).

Table 4. Limit of detection comparison between our optimized droplet digital PCR and other studies

Species	Gene target	Technique	Limit of detection	Ref.
<i>Ehrlichia canis</i>	16S rDNA	Droplet digital PCR	1.6 copies/reaction (or 0.02 ng/reaction)	This study
<i>E. canis</i>	Citrate synthase	Loop-mediated isothermal amplification	7.41×10^4 copies/reaction	[9]
<i>E. canis</i>	Virb9 gene	Multiplex PCR	0.01 ng/reaction	[27]
<i>E. canis</i>	16S rDNA	Conventional PCR	22 copies/ μ l	[8]
<i>E. canis</i>	Virb9 gene	Multiplex PCR	1.2×10^5 copies/reaction	[2]
<i>E. canis</i>	Virb9 gene	Real-time fluorescence resonance energy transfer	6.6×10^3 copies/reaction	[29]
<i>E. canis</i>	16S rDNA	Multiplex real-time PCR	1–10 copies/ μ l	[45]
<i>E. canis</i>	16S rDNA	Conventional PCR-biotinylated primers	6.4 ng/reaction	[40]

Furthermore, we found a weak positive correlation between the percentage of infected monocytes and the *16S* rDNA copy number ($r_s=0.084$, $P=0.76$). The weak correlation could be explained by the fact that a single infected white blood cell can be infected by variable numbers and stages of parasites [20, 50]. Therefore, evaluation of *E. canis* infection using ddPCR was more reliable than microscopy. The developed protocol could be further applied in clinical detections of *E. canis* using, for example, whole blood PCR.

ddPCR detection was then applied to 92 field samples. ddPCR produced higher positive cases (50%), whereas cPCR produced only 34% and blood smears only 13%. The higher sensitivity of ddPCR versus cPCR and blood smears agreed with an avian haemosporidian parasite study [21]. The higher positive rates from ddPCR were explained by the improved LOD, which was about 78 times better than for cPCR. In addition, we found that cPCR negative cases had significantly lower copy numbers than cPCR positive cases (Supplementary Table 1). Although ddPCR produced lower LODs compared to cPCR, the limitation of this method was the confirmation of positive droplets, as we found only one droplet in a very low concentration template. cPCR true positives can be confirmed based on DNA sequencing, while ddPCR cannot be confirmed in the same way, due to the extremely low number of amplicons in a 1 nl droplet. When non-specific amplification in ddPCR took place, this false-positive was included in the calculation, leading to incorrect identification. Any false-positive could result from species-specific primers, accidentally matched with a host DNA fragment, as has been reported in birds [21], since the dog genome (~2.4 Bb) [15] is about 3,700 times longer than the *E. canis* genome (~1.3 Mb) [65]. Theoretically, ddPCR is more reliable and specific than cPCR because it uses a TaqMan hydrolysis probe to improve specificity; droplets are independent, so non-specific amplification is unlikely to coincide with nano-litre reaction volume helps dilute inhibitors in the ddPCR system while increasing amplification efficiency [21].

Blood smear observations had low sensitivity (39%) in this study, which agreed with other studies, reporting many negative cases, for which PCR assay showed them to be positive [23, 32, 55]. The reported microscopic examination sensitivity of acute phase *E. canis* infection, using peripheral blood smears, was only 8% compared to four other approaches [42] and although several modified and time-consuming microscopic methods were applied, only a few *E. canis* morulae have been detected [42, 56]. It should be mentioned that in the current study used a variety of assays (microscopy, cPCR, and ddPCR) to determine whether a case was positive or negative based on the signal in each technique. qPCR was not used in this investigation because the technique used Cq value for analysis. The Cq value was relatively close to the Cq of the negative control due to the primer dimer band formed in the qPCR reaction at the very low copy number template used in the experiment.

The successful treatment, prevention, and control of *E. canis* in dogs can only achieved when accurate diagnosis is performed. The above findings provide valuable informations for selecting appropriate *E. canis* detection methods based on facilities and purposes. Although ddPCR detection has several advantages, the cost was 2–3 times higher than qPCR or approximately 1.5 USD per sample for reagents [21]. In addition, ddPCR is more instrument-dependent compared to cPCR and traditional blood smear examination. We also suggest further studies on larger sample sizes to confirm the clinical efficacy of ddPCR compared to qPCR for *E. canis* diagnosis.

In conclusion, this study is the first report of a ddPCR assay for *E. canis* detection under optimized conditions that comprehensively evaluated ddPCR performance. The study showed that the ddPCR technique was a powerful tool for accurately evaluating *E. canis* numbers, producing much lower LODs than cPCR. However, cPCR remains effective for routine diagnosis. The high performance of the PCR techniques can complement microscopic examinations because microscopic examination has lower sensitivity.

CONFLICT OF INTEREST. The authors declare no conflicts of interest.

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