

## Research article

## Detection of specific IgM and IgG antibodies in acute canine monocytic ehrlichiosis that recognize recombinant gp36 antigens

Sarawan Kaewmongkol<sup>a</sup>, Eukote Suwan<sup>a</sup>, Theerapol Sirinarumitr<sup>c</sup>, Sathaporn Jittapalapong<sup>a</sup>, Stanley G. Fenwick<sup>b</sup>, Gunn Kaewmongkol<sup>c,\*</sup><sup>a</sup> Faculty of Veterinary Technology, Kasetsart University, Bangkok, Thailand<sup>b</sup> Department of Infectious Disease and Global Health, Cummings School of Veterinary Medicine, Tufts University, Boston, USA<sup>c</sup> Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand

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## ABSTRACT

The efficacy of antibody detection tools for all stages of *Ehrlichia canis* infections and for various genotypes remains unclear. We produced recombinant gp36 (rgp36) antigens from different isolates of Thai *E. canis* to confirm the immunoreactivities to these recombinant proteins from naturally infected dogs. Sera and blood samples were taken from 21 dogs naturally infected with *E. canis* and in the clinical stages of acute phase ehrlichiosis. The expression vectors and competent *E. coli* produced two isolates of rgp36. These two major rgp36s were recognized by the dogs' sera in Western blotting, with both anti-dog IgM and IgG used as secondary antibodies. The two different genotypes of these local recombinant immunoreactive proteins were gp36 subgroup A (isolate 1055) and subgroup B (isolate 533). The Western blot analyses successfully identified both specific IgM and IgG from the dogs' sera. Of all 21 cases, five dogs presented specific IgM, twenty dogs presented specific IgG, and the commercial test used found fifteen seropositive dogs. There were four dogs that presented both specific IgM and IgG. Only one dog presented specific IgM only. This report is the first identification of a specific IgM in dogs in response to acute infections with *E. canis*. The recombinant gp36 isolates may be useful as potential antigenic material for subsequent serological tests that have a high possibility for differentiating between acute, chronic, primary, and nonprimary infections with *E. canis*.

## 1. Introduction

*Ehrlichia canis* is a gram-negative bacterium within the  $\alpha$ -Proteobacteria group in the order Rickettsiales. This organism is a major cause of canine monocytic ehrlichiosis (CME), which is one of the most important tick-borne diseases in dogs in the tropics, particularly in the region of Southeast Asia. Currently, serological diagnostic tests for canine monocytic ehrlichiosis include IFAT, ELISA, and in-house commercial test kits commonly used by routine veterinary medicine practices. Specific IgG against *E. canis* in the suspected dog's sera or whole blood is a primary target of these serological tests (Cárdenas et al., 2007; Baneth et al., 2009; Moroff et al., 2014). The experimental infections by Baneth et al. in 2009 demonstrated a comparison between ehrlichial DNA copy number and the kinetics of the antibody responses. The real-time PCR detected ehrlichial DNA in both blood and spleen at days 7–10 postinoculation. An ELISA (the ImmunoComb ELISA (Biogal, Kibbutz Gal'ed, Israel), containing plastic combs sensitized with *E. canis* antigen derived from mouse

J774A1-infected cells) detected specific IgG to *E. canis* at the cutoff level at day 12 postinoculation. The experimentally infected dogs presented clinical signs at days 9–12 postinoculation (Baneth et al., 2009). According to this information from the experimental infections, there is the possibility that the serological tests could show negative results in dogs with clinical signs in natural infections. Moroff et al. (2014), performed experimental infections in 8 dogs. A commercial test kit targeting specific IgG, the Snap 4Dx<sup>R</sup>, was used in this experiment. The Snap 4Dx<sup>R</sup> assay (IDEXX Laboratories Inc., USA) uses two specific *E. canis* recombinant proteins, p30 and p30-1, which are the major outer membrane proteins (OMPs), as antigens (Ohashi et al., 1998; Harrus et al., 2002; Harrus and Waner, 2011). This test kit provided positive results at day 17 postinfection in one out of eight dogs. Three dogs showed seropositive results at day 28 postinfection, and all eight dogs showed seropositivity at day 42 postinfection. Cárdenas et al. (2007), produced a novel ELISA for detecting specific IgG against the gp36 antigen of *E. canis*. However, in the experimental infections, this ELISA detected specific IgG as early as

\* Corresponding author.

E-mail address: [gunn\\_kaew@yahoo.com](mailto:gunn_kaew@yahoo.com) (G. Kaewmongkol).<https://doi.org/10.1016/j.heliyon.2020.e04409>

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day 14 postinfection (Cárdenas et al., 2007). Moreover, the antibody responses to five different recombinant antigens in three infected dogs was still at high levels up until day 42 postinfection, and these prolonged antibody responses in the infected dogs continued for 6–15 months (antibody titers remained increased for 31 months in some reports) (Perille and Matus, 1991; Waner et al., 1997).

According to several previous reports, high titers of specific IgG to *E. canis* can be observed in acute infections, recovery phases, chronic disease states, and persistent/subclinical infections, and these situations demonstrate the considerable disadvantage for the use of specific IgG detection for the diagnosis of canine ehrlichiosis. Although the serological tests for CME diagnosis are convenient, rapid, cost-effective and have a high specificity, there are some significant limitations.

*Ehrlichia chaffeensis*, an obligate intracellular bacterium, is the cause of human monocytic ehrlichiosis (HME). The major immunoreactive proteins of *E. chaffeensis* include P28 (OMP1), TRP32 (32-kDa tandem repeat-containing protein), TRP47, TRP120, and Ank200 (200-kDa ankyrin protein), and these proteins are strongly recognized by sera from both *E. chaffeensis*-infected humans and dogs (Luo et al., 2010). The TRP47 (gp47) and TRP36 (gp36), ortholog tandem repeat proteins of *E. chaffeensis* and *E. canis*, respectively, are immunoreactive proteins that contain protective linear antibody epitopes in the tandem repeat (TR) domains. Previous reports have demonstrated that TRP has various interactions with host proteins in the regulation of biological processes. This major immunoreactive protein of *E. chaffeensis* interacts with multiple host proteins involved in post-transcriptional modification processes (Lina et al., 2016; Kibler et al., 2018).

The diversity of *E. canis* genotypes, based on the *gp36* gene, has been reported worldwide. Studies have proposed that the geographical distribution of *E. canis* could be related to distinct strains, genotypes or serotype antigenic variabilities (Zhang et al., 2008; Huang et al., 2010; Hsieh et al., 2010; Kaewmongkol et al., 2017; Nambooppha et al., 2018). The *gp36* gene of *E. canis* consists of three regions: a 5' end pre-repeat region, a tandem repeat region with variable numbers of the repeat unit depending on the isolate, and a 3' end region. Phylogenetic trees of *E. canis*, based on the *gp36* protein, revealed two genetic clades in Thailand that included the US and Taiwanese genogroups (Nambooppha et al., 2018). Amino acid substitutions in these immunodominant antigens that were previously reported could play important roles in the different immunoreactivities of the protein with heterologous antisera compared with homologous antisera (Zhang et al., 2008; Huang et al., 2010).

The purpose of this study was to express recombinant *gp36* (rgp36) antigens from two different isolates of Thai *E. canis* and to confirm the immunoreactivities to these recombinant proteins in the sera from naturally infected dogs.

## 2. Materials and methods

### 2.1. Acute canine monocytic ehrlichiosis

Sera and blood samples were taken from 21 dogs naturally infected with *E. canis* in the clinical stages of acute phase ehrlichiosis on their initial visit to the veterinary hospitals. The cases had presented clinical signs within 4 weeks and had no previous history of the relevant clinical signs. The *E. canis* infections were also characterized by blood parameters, blood smear examinations, specific PCR from blood samples, and commercial test kits. The clinical signs and blood parameters were recorded (Tables 1 and 2). The DNA was extracted and purified from 200  $\mu$ l whole blood in EDTA using an E.Z.N.A.® Tissue DNA Kit according to the manufacturer's instructions (E.Z.N.A.® Tissue DNA Kit, Omega Bio-Tek, Inc., Norcross, GA). This animal use protocol has been approved by the Kasetsart University Institutional Animal Care and Use Committee and found to be in accordance to the guidelines under the Ethical Review Board of the Office of National

**Table 1.** Clinical signs in the studied dogs on their first visits at the veterinary hospital.

Parameter	Studied dogs N = 21 %
Fever (>102.5°F)	7 (36%)
Pale mucous membrane	6 (27%)
Jaundice	6 (27%)
Petechial hemorrhage	6 (27%)
Ecchymosis	4 (18%)
Bleeding tendency	2 (1%)
• Epistaxis	
• Gingival bleeding	
Multiple lymph node enlargement	12 (55%)
Multiple sites of muscle pain	6 (27%)
Hyperesthesia	2 (1%)
Vomiting	2 (1%)
Melena	2 (1%)
Productive cough	2 (1%)
Increased lung sound	2 (1%)
Incidental findings	4 (18%)

Research Council of Thailand (NRCT) (The Approval ID# ACKU61-VET-087).

### 2.2. Amplification of the *gp36* locus from *Ehrlichia canis* genomic DNA

PCR detection of *E. canis* was performed with PCR primers for the *gp36* gene (EC36-F1 (5'-GTATGTTTCTTTTATATCATGGC-3') and EC36-R1 (5'-GGTTATATTTTCAGTTATCAGAAG-3')) (Table 3). The PCR cycles and conditions used were based on previous studies (Hsieh et al., 2010; Kaewmongkol et al., 2016, 2017).

Another set of PCR primers for the *gp36* gene was modified for the process of cloning into the expression vectors. The forward primer EC36F1 (5'-GTATGTTTCTTTTATATCATGGC-3') was used to amplify the *gp36* gene isolate 533, and the forward primer EC36F2 (5'-ATGC-TACTTTTACTAATGGGTTATTG-3') was used to amplify the *gp36* isolate 1055. EC36R (5'-GTACAACATGTTAAGAATATCAG-3') was used as a reverse primer for both isolates. The amplification reaction included 1 pmol each of the forward and reverse primers, 0.2 mM dNTP, 2.5 mM MgCl<sub>2</sub>, 0.2 U *Taq* DNA polymerase with buffer (Invitrogen™, USA), and 2  $\mu$ l of *E. canis* genomic DNA. The amplification conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 53 °C for 45 s, 72 °C for 1 min, and 72 °C for 5 min. The PCR products were verified by 1.2% agarose gel electrophoresis. The PCR products with the expected band sizes were purified by UltraClean® 15 DNA Purification (MO BIO, USA), ligated into pBAD/TOPO® ThioFusion™ and transformed into *Escherichia coli* TOP10 (Invitrogen™, USA) according to the manufacturer's instructions. The obtained clones were further confirmed by colony PCR in the same manner. DNA sequencing was performed on the clones determined to be positive by the PCR.

**Table 2.** Average white blood cell count (Wbc), hematocrit (Hct), red blood cell count (Rbc), hemoglobin (Hb), and platelet count.

Blood parameters	Average	Reference ranges
Wbc	13.12 $\pm$ 4.89 $\times$ 10 <sup>3</sup> cell/ $\mu$ l	6–17
Hct	32.03 $\pm$ 8.45%	37–55
Rbc	4.966 $\pm$ 1.23 $\times$ 10 <sup>6</sup> cell/ $\mu$ l	5.5–8.5
Hb	11.11 $\pm$ 3.26 g/dL	12–18
Platelet count	154.6 $\pm$ 40.03 $\times$ 10 <sup>3</sup> cell/ $\mu$ l	180–500

**Table 3.** Results from the PCR, Western blotting with IgM and IgG, and a commercial test kit for IgG on the dogs presenting with the clinical signs and blood parameters of acute canine monocytic ehrlichiosis.

Dog patients	PCR for gp36	Western blotting IgM	Western blotting IgG	SNAP 4Dx <sup>R</sup>
Case 1	+	+	+	+
Case 2	+	+	+	+
Case 3	-	-	+	+
Case 4	-	-	+	-
Case 5	+	-	+	+
Case 6	+	+	+	-
Case 7	-	-	+	+
Case 8	-	-	+	+
Case 9	-	+	+	+
Case 10	+	-	+	+
Case 11	+	+	-	+
Case 12	-	-	+	-
Case 13	-	-	+	+
Case 14	-	-	+	+
Case 15	-	-	+	+
Case 16	-	-	+	-
Case 17	-	-	+	-
Case 18	-	-	+	-
Case 19	+	-	+	+
Case 20	-	-	+	+
Case 21	-	-	+	+
Total positive results	7/21 (33%)	5/21 (24%)	20/21 (95%)	15/21 (71%)

### 2.3. Protein expression of gp36

A single colony of a recombinant clone was inoculated in Luria-Bertani (LB) broth supplemented with 50 µg/mL ampicillin and grown at 37 °C at 200 rpm, overnight. The culture was added to fresh LB broth supplemented with 50 µg/mL ampicillin at a ratio of 1:100 and grown in the same conditions until the O.D.600 reached 0.4. The recombinant protein was induced by adding 0.1% L-(+)-arabinose and further incubating the culture for 5 h. The cells were harvested by centrifugation at 10,000 rpm for 10 min and resuspended in a solution

containing 50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride and 6 M urea. The suspension was lysed by sonication, and the soluble protein was separated by centrifugation at 10,000 rpm for 10 min at 4 °C. The protein solution was subjected to immobilized metal-ion affinity chromatography (GE Healthcare, USA). The protein was eluted with a stepwise gradient of 50–500 mM imidazole in a solution containing 50 mM sodium phosphate, pH 8.0 and 300 mM sodium chloride. The protein fractions were analyzed by 12% SDS-PAGE.

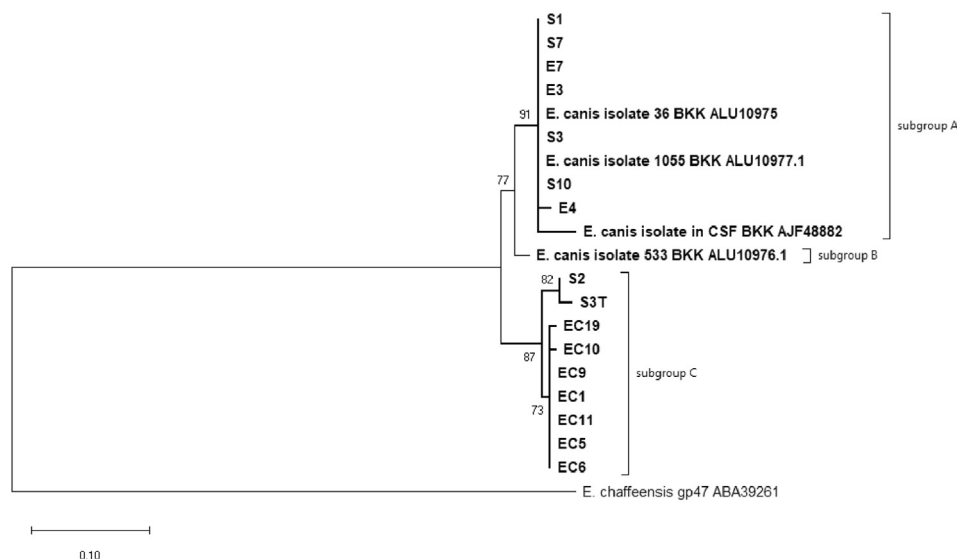
### 2.4. Western blot analysis

Western blot analysis was performed. Briefly, the protein was subjected to denaturing 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane. The membrane was blocked with blocking buffer (5% skim milk in 1% PBS buffer with 0.1% tween 20) at 4 °C overnight and incubated at room temperature for 1–2 h with either the sera of the naturally infected *E. canis* dogs (1:100) or a rabbit anti-polyhistidine immunoglobulin G (IgG) monoclonal antibody (MoAb) (1:3,000) (Invitrogen<sup>TM</sup>, USA). The membrane was triple washed with the same buffer as listed above. The membrane was then incubated with a goat anti-dog IgM conjugated to horseradish peroxidase, a goat anti-dog IgG conjugated to horseradish peroxidase (1:3,000) (KPL, USA) or goat anti-rabbit IgG/IgA/IgM (H+L) conjugated to horseradish peroxidase (Invitrogen<sup>TM</sup>, USA) for 1 h, washed and developed with a diaminobenzidine (DAB) Substrate Kit (Thermo Scientific, USA) for 3–5 min at room temperature. The specific protein band showed a brown color on the membrane.

## 3. Results

### 3.1. Phylogenetic analysis of the gp36 proteins from isolates in Thailand and reference sequences from GenBank

Specific PCR targeting of the *E. canis* gp36 gene was performed, which revealed the diversity of the local Thai genotypes of *E. canis* (Figure 1). Different numbers of the tandem repeats resulted in different lengths of the PCR products among the Thai genotypes. Nucleotide sequences were generated for all loci, and the amino acid sequences were analyzed using Chromas Lite version 4.0 (<http://www.technelysium.com.au>) and aligned with the reference sequences from GenBank using Clustal W (<http://www.clustalw.genome.jp>). The deduced protein sequences of



**Figure 1.** Maximum-likelihood phylogenetic tree of gp36 of *Ehrlichia canis* in this study and gp47 of the validated genotypes of *E. chaffeensis* retrieved from GenBank ABA39261. The percentage bootstrap support from 1,000 pseudoreplicates is indicated at the left of the supported node.

*E. canis* were assembled with BioEdit software (Version 5.0.9). The maximum-likelihood method was used for the phylogenetic analysis of the amino acid sequences. Phylogenetic analysis of the amino acid sequences was completed using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar et al., 2018) (Figure 1).

### 3.2. DNA sequencing and sequence alignment results of the two major subgroups of the *gp36* loci used in this study

Two *E. canis* genomic DNA samples, isolate 533 and isolate 1055, were used as templates to amplify *gp36* 533 and *gp36* 1055. Based on the *gp36* 533 sequence, this gene contained a stop codon in the coding sequence, and therefore, a new forward primer, EC36F2, was designed and used to amplify the 1055 *E. canis* genomic DNA sample. The PCR product sizes were 833 and 735 bp for the 533 and 1055 isolates, respectively. The sequencing results of the *gp36* isolate 533 showed 100% sequence identity with the *E. canis* isolate 533 (*gp36* protein gene, complete cds, GenBank accession number KT363876.1), and the *gp36* isolate 1055 showed 100% nucleotide sequence identity with the *E. canis* isolate 1055 (*gp36* protein gene, complete cds, GenBank accession number KT363877.1). These sequences shared 96.2% sequence identity and similarity with each other, but length of the *gp36*

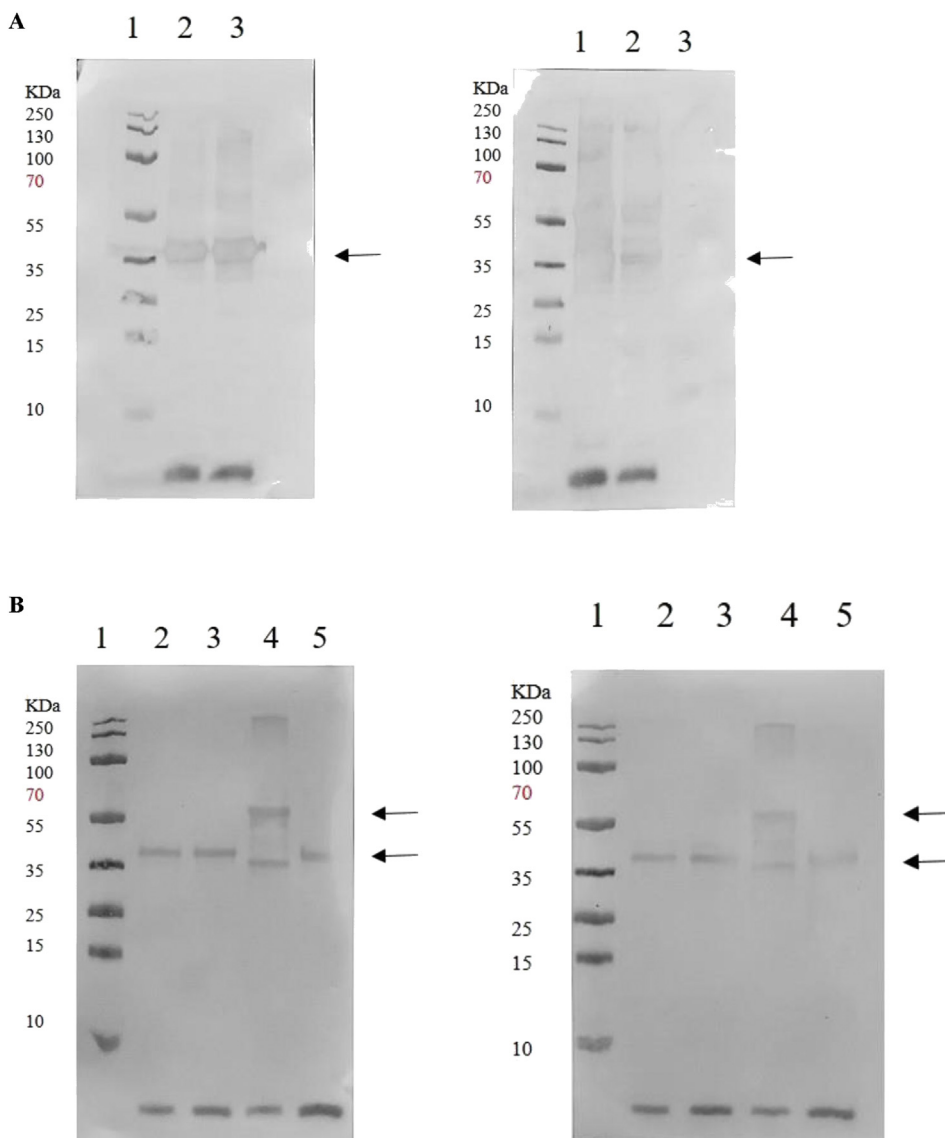
533 was longer than that of *gp36* 1055 due to the different number of tandem repeats.

### 3.3. Protein expression of *gp36*

The thioredoxin protein is approximately 12 kDa and is found in living organisms, including bacteria. This protein is used as a fusion protein to increase translation efficiency, and in some cases, the solubility of eukaryotic proteins expressed in *E. coli* (pBAD/TOPO® Thio-Fusion™ Expression Kit user manual, Invitrogen™, USA). Due to the thioredoxin fusion tag, the protein solubility and yield of *gp36* 1055 were higher than those of *gp36* 533. The *gp36* 1055 showed two bands, representing the protein with and without the thioredoxin fusion tag.

### 3.4. The acute phase of canine monocytic ehrlichiosis in infected dogs

Blood and serum were taken from the dogs that were in the early stage of acute disease. Clinical signs and blood parameters related to acute disease were recorded, including fever, jaundice, petechial hemorrhage, anemia, and thrombocytopenia (Tables 1 and 2). The average hematocrit (Hct) ( $32.03 \pm 8.45\%$ ) of the infected dogs in this study was lower than the normal range (35–55%). The averages of the red blood



**Figure 2.** The western blot results on the 6x His – tag protein (A) and the dog sera (B). (A) rgp36 533 (left) and rgp36 1055 (right); lane 1 is the protein ladder, and lanes 2–3 are the protein elution fractions. (B) The results of the anti-dog IgM (left) and anti-dog IgG (right) detection; lane 1 is the protein ladder, lanes 2 and 3 are the EC36 533 elution fractions and lanes 4 and 5 are the EC36 1055 elution fractions. The target protein bands are indicated with an arrow. Non-adjusted images of the western blot were presented as supplementary material.

cell count (Rbc) ( $4.966 \pm 1.23 \times 10^6$  cell/ $\mu$ l), hemoglobin (Hb) (11.11  $\pm$  3.26 g/dL) and platelet count ( $154.6 \pm 40.03 \times 10^3$  cell/ $\mu$ l) were also lower than the reference ranges (Rbc, 5.5–8.5  $\times 10^6$  cell/ $\mu$ l; Hb, 12–18 g/dL; platelet count, 180–500  $\times 10^3$  cell/ $\mu$ l) (Table 2). PCR targeting of the *gp36* locus was used to detect ehrlichial DNA in the whole blood samples. Serum samples were collected from the infected dogs on their first visit to the veterinary hospitals. Those sera were also tested by SDS-PAGE, Western blot analysis, and a commercial test kit, SNAP 4Dx<sup>R</sup> (Table 3). The Western blotting, using anti-dog IgM and IgG as the secondary antibodies, showed that the dogs' sera showed specific IgM and IgG that was reactive to the Thai rgp36s (Figure 2). Deduce amino acid sequences of the rgp36s and dot blot analysis results were presented as supplementary materials.

#### 4. Discussion

The studies in *E. chaffeensis* demonstrated a major epitope in the tandem repeat (TR) region of the TRP47, the ortholog *gp36* protein in *E. canis* (Doyle et al., 2006; Luo et al., 2010). The minor epitopes were identified in the TRP47-N and -C termini (Luo et al., 2010). The TR region of the TRP47 presented more solid immunoreactivity than the TRP47 -N and -C. However, some *E. chaffeensis* infected human patients developed specific immunity only to the minor epitopes and not to the TR epitopes (Luo et al., 2010). Three different genotypes based on amino acid sequences of the TR of *gp36* included *E. canis* US TRP36 (TEDSV-SAPATEDSVSAPA) (Doyle et al., 2006), Brazil (BR) TRP36 (ASVV-PEAEASVVPEAEASVVPEAE) (Aguiar et al., 2013) and Costa Rica (CR) TRP36 (EASVVPAEAPQPAQQTDEFFSDGIEA). These variations of the TR sequences serologically distinguished different *E. canis* genotypes and revealed coinfection or multiple sequential infections of these genotypes (Aguiar et al., 2016; Arroyave et al., 2020). In our Thailand study, the Maximum-likelihood phylogenetic analysis of *gp36* revealed three different genotypes, including subgroup A, B, and C (Figure 1). The TR region (region II) of *gp36* is identical to the US TRP36 (TEDSVSAPA). The number of repeats of the *E. canis* genotypes in our study (Figure 1) ranged from 6 to 12 repeats with genetic variations identified in region I (N-terminal) and region III (C-terminal). According to the previous studies in HME, the immunoreactivity of *E. canis* infected dogs in Thailand might mainly respond to the TR region (TEDSVSAPA). However, diversity of the minor epitopes in region I and III might be associated with antigenic variabilities in different subgroups of *E. canis*. The rgp36s from three subgroups should be produced and tested for variable immunoreactivities.

The specific IgG to *E. canis* rgp36s was detected by the Western blot in 20 of 21 dogs (95%) in this study. In comparison with the commercial test, seropositive dogs were found in 15 of 21 dogs (71%). The Western blot results presented a much higher positivity rate than the commercial test. The detection of this protein in the supernatant of the *E. canis* in vitro culture predicted the secretions of *gp36* in the tick salivary gland or in the mammalian host (Doyle et al., 2006). The early host immune response to this secreted *gp36* glycoprotein might explain the Western blot's higher positive rate. However, The Fisher exact test statistic value was at 0.093 (data not shown). In conclusion, the seropositive results of the Western blot compared with the commercial test were not statistically different at  $P < 0.05$ .

#### 5. Conclusion

Previous publications suggest the highly conserved properties of the major immunoreactive proteins of *E. canis*, including the *gp36* protein, has the potential to be used for serodiagnosis. Conversely, this study demonstrated the variation in *E. canis* *gp36* in the tandem repeats of this major immunoreactive protein isolated from naturally infected dogs in Thailand. Therefore, there are still some questions for the global value of this highly variable protein. Based on our knowledge and on previous literatures, this is the first identification of a specific dog IgM reactive to

recombinant *gp36* isolates obtained from *E. canis* local genotypes using Western blot analysis. Persistent increases in specific IgG have been observed to be prolonged until 15–30 months post infection. The characterization of the specific IgM, together with the IgG in the same infected dogs in this study, could explain the continuous re-exposure to the organism in the endemic area. We hypothesized that this reinfection cycle of the diverse genotypes of *Ehrlichia canis*, which is an intracellular bacterium that infects the monocyte, could trigger inappropriate immune responses in dogs. Here, we characterized the novel target proteins for detection of both specific IgM and IgG. The next step is to obtain these promising antigens with the great potential for differentiating between acute, chronic, primary and nonprimary infections of *E. canis* for studying the various pathogenicities of canine monocytic ehrlichiosis.

#### Declarations

##### Author contribution statement

Sarawan Kaewmongkol: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Eukote Suwan: Performed the experiments.

Theerapol Sirinarumit, Sathaporn Jittapalpong, Stanley G. Fenwick: Conceived and designed the experiments.

Gunn Kaewmongkol: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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##### Competing interest statement

The authors declare no conflict of interest.

##### Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2020.e04409>.

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