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Clinical Observations, Hematological Profile, Serological Testing, and Molecular Detection of *Erlichia Canis* in Veterinary Clinics in Bali, Indonesia

I Nyoman Suartha[1], Anak Agung Sagung Istri Pradnyantari[2], I Gusti Made Krisna Erawan[1] and I Gusti Ngurah Kade Mahardika[3]

[1]Department of Clinical Veterinary, Faculty of Veterinary Medicine, Udayana University, Denpasar Bali Indonesia
[2]Veterinary Clinic Kuta, Kedonganan Badung Bali Indonesia
[3]Laboratory of Molecular Biology, Faculty of Veterinary Medicine, Udayana University Denpasar Bali Indonesia
***Corresponding author:** nyoman_suartha@unud.ac.id

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ABSTRACT

Ticks of Rhipicephalus species can serve as a vector for transmitting the Zoonotic Ehrlichiosis disease from dogs to humans. In Indonesia, epidemiological data on the prevalence of *Ehrlichia canis* are very limited. The incidence of Ehrlichiosis is usually reported based on clinical symptoms and serological results. In contrast, accurate diagnosis mainly relies on microscopic examination of a stained blood smear and polymerase chain reaction (PCR). The present study was conducted to evaluate the correlations among clinical observations, hematological profile, serological testing, and molecular detection of *E. canis* in dogs examined at different animal clinics in Bali, Indonesia. We collected 109 samples from Ehrlichiosis suspected dogs. The disease in these dogs was confirmed through a hematological profile, serologically, and PCR test. The hematological examination was per-formed with ICUBIO iCell-800Vet. We did a serological examination with Rapid Test BioNote© E. canis. DNA extraction was carried out with DNeasy Blood and Tissue Kit product Qiagen. PCR amplification was performed with Personal Thermal Cycler MJ Mini BIO-RAD. The results revealed that clinical signs such as epistaxis, fever, and pale mucous membranes were strongly associated with serological detection of E. canis. In contrast, none of these signs was significantly correlated with PCR detection of E. canis. Total erythrocytes counts were significantly associated with serologic detection. The total erythrocytes, thrombocytes, and hemoglobin levels were significantly associated with PCR detection. The 16S-rDNA, collected from PCR and amplified, showed the *E. canis* gene, indicating that *E. canis* organisms were found in Bali. E canis Bali is one cluster with clusters from South America, Europe, and Asian countries like Thailand, Taiwan, Japan, and Israel. In conclusion, *E. canis* infection was confirmed through clinical, serological, and molecular approaches in dogs in Bali, Indonesia.

Key words: *E. canis*, clinical signs, haematology, serology, molecular detection.

INTRODUCTION

Ticks of Rhipicephalus species can serve as a vector for transmission of the Zoonotic Ehrlichiosis disease from dogs to humans (Nair et al. 2016; Obaidat and Alshehabat 2018). Dog Ehrlichiosis disease (*Canine Monocytic Ehrlichiosis*) is caused by *Ehrlichia canis spp.* (Harrus and Warner 2011; Harrus 2015; Cetinkaya et al. 2016). Incidences of Canine Monocytic Ehrlichiosis have been reported worldwide (Sainz et al. 2015; Kubo et al. 2015; Ybanez et al. 2018; Piratae et al. 2019). In Indonesia, epidemiological data on the prevalence of *E. canis* are very limited. The reports regarding the incidence of Erhlichiosis are usually based on clinical symptoms, and results of serological test kits (Hadi et al.2016; Erawan et al. 2017).

The most common clinical signs of Erhlichiosis include inappetence, lethargy, fever, epistaxis, and pale mucosa. Hematological profile is characterized by microcytic normochromic anemia, leucocytosis, and lymphocytosis. Hemolytic anemia and thrombocytopenia can also be seen (da Silva et al. 2012; Saeng-Chuto et al. 2016; Zhang et al. 2018; Piratae et al. 2019). The affected dogs have history or the presence of tick infestation (Ybanez et al. 2016; Erawan et al. 2017).

The diagnosis of dog blood parasites by private veterinary practisioners is mostly based on clinically signs, and use of serological diagnostic test kits

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(Mylonakis et al. 2014). In clinical veterinary practice with more tools, complete diagnosis based on microscopic examination of blood smears, complete blood count, and molecular approaches, is carried out at veterinary hospitals. Each of these diagnostic techniques has its own advantages and disadvantages. Clinical signs are very common and can be the result of various disease processes. Examination of blood smears has low sensitivity, particularly in cases with low parasitemia, and it also requires an experienced examiner (Sainz et al. 2015; Azhahianambi et al. 2018; Rucksaken et al. 2019). A serological diagnostic kit has been used for diagnosis of *Ehrlichia* spp. to detect antibodies against the parasite after onset of the infection (Kottadamane et al. 2017; Mitta et al. 2017; Piratae et al. 2019). Polymerase chain reaction (PCR) is a widely used molecular technique to confirm blood parasite infection due to its high sensitivity and specificity (Liu et al. 2016; Csokai et al. 2017; Yuasa et al. 2017; Konto et al. 2017; da Costa et al. 2019); Kovacevic et al. 2018; Rucksaken et al. 2019).

This study was conducted to investigate possible correlations among different diagnostic techniques like clinical signs, haematological profile, serological diagnostic kits, and molecular techniques. The main goal of this article was to provide a practical guideline for veterinary practitioners on the diagnosis, treatment, and prevention of Ehrlichiosis in dogs.

MATERIALS AND METHODS

Ethical Approval

The procedures performed in this study were guided by the principles of animal welfare, Animal Welfare Act of the Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia.

Research Design

The present study was a cross sectional project, which involved testing of suspected dogs from selected veterinary clinics for *Ehrlichia* spp. Health profile of dogs, presenting clinical signs, hematological parameters, serological data, and findings of molecular technique were recorded.

Research Subjects and Environment

A total of 109 dogs, regardless of sex, age, and breed, which had been suspected for Ehrlichiosis, were selected on the basis of health criteria including fever, pale mucosa, inappetence, epistaxis, and the presence or history of tick infestation. All dogs that were admitted to the clinics in 2019 that showed any clinical sign of Erhlichiosis such as fever, anemia, and epistaxis were sampled. The samples were taken in all seven private veterinary clinics in Bali, Indonesia, including Kedonganan Vet Clinic, Seminyak Vet Clinic, Nusa Dua Vet Clinic, Pecatu Vet Clinic, Denpasar Vet Clinic, Ubud Vet Clinic and Dangin Carik Tabanan Vet Clinic. They were subjected to routine examination of blood (hemoglobin concentration, red blood cell count, thrombocytes count, and white blood cell count), serologically test kit, and PCR testing. The hematological examination was performed at the Laboratory of Internal Medicine, Faculty of Veterinary Medicine, using ICUBIO iCell-800Vet. The serological examination was performed at the respective clinic with Rapid Test BioNote© *E. canis*. DNA extraction and PCR testing were performed at the Indonesian Biodiversity Research Centre (IBRC), and Biomedical Laboratory of the Faculty Veteinary Medicine, Udayana University, Bali, Indonesia.

Clinical Signs

Physical examination of experimental dogs was carried out to record all clinical signs suggestive of Erhlichiosis. Inclusion criteria for clinical signs included fever, pale mucosa, epistaxis, and the history or the presence of tick infestation.

Sample Collection and Processing

Blood samples were aseptically collected from the chepalic vein of experimental dog, and were divided into aliquots for DNA extraction and complete blood counts (in the sterile ethylene diamine tetra acetic acid tube) and serological tests by using commercical kit (Antigen Rapid, Bionote Inc., Hwaseong, South Korea). Blood samples for DNA extraction were stored at −20°C until further use.

DNA Extraction

DNA extraction was performed using DNeasy Blood & Tissue Kit (Qiagen), following the procedures recommended by the manufacturer. Briefly, 20µL protease K were mixed with 100µL whole blood with anti-coagulant. AL buffer (200µL) was added and vortexed, then incubated for 10s at 56ºC. Then absolute ethanol (200µL) was added to the mixture and vortexed. The mixture was than pipetted to DNA-easy minispin column provided and centrifuged at 8000rpm for one minute. The column was added with 500µL Buffer AW1 and recentrifuged for one minute at 8000rpm. The column was added further with 500µL Buffer AW2 and recentrifuged for three minutes at 14000rpm. Buffer AE of 200µL was added to the column for elusion in a new Eppendorf tube and incubated for one minute at room temperature and centrifuged for one minute at 8000rpm. The flow trough was collected and stored at −20°C until further use (Kubo et al. 2015).

PCR Profile

The primer for E. canis detection was designed based on database of 16S-rRNA downloaded from GenBank. The sequences were Canis_668F 5'-CTATCTGG TTCGATACTGACA-3' and Canis_1224R 5'-ATGRATT AGCTAAACCTTGCGGTC-3 for forward and backward primer, respectively. Primer was designed at Biomedical Laboratory, Faculty of Veterinary Medicine, Udayana University. The PCR was conducted with the mixture of Taq Plus PCR Master Mix 5mM (0.1U/µL *Taq* Plus Polymerase, 500µM dNTP, 20mM Tris-HCl (pH 8.3), 100mM KCl, 2mM $MgCl_2$), 3mM ddH2O, 1µL extracted DNA, 1.8µL of each primer in the concentration of 10µM. Amplification was performed with Personal Thermal Cycler MJ Mini BIO-RAD. After initial heating at 95ºC for 7s, 40 cycles were conducted with denaturation at 94ºC for 45s; annealing at 50ºC for 45s, and elongation at

72ºC for one second. PCR products were identified by 1% agarose gel stained with ethidium bromide and visualized under ultraviolet light. DNA product was subjected to sequencing at 1st Base, Malaysia using automatic Sanger's protocol.

Data Collection and Analysis

Hematological profile, clinical signs, serology kit values and PCR values of the experimental dogs were recorded and encoded in Microsoft Excel, using appropriate variable coding. Data were imported into the statistical software SPSS version 25 (IBM) and subjected to non- parametric statistics.

RESULTS

Clinical Signs of *Ehrlichiosis*

A total of 109 dogs showed clinical signs of infection with *Ehrlichia* spp. Among these, 58.7% (64/109) were positive for *E. canis* antibodies, while 15.63% (10/64) showed positive reaction with PCR. Clinical signs recorded were epistaxis, fever, pale mucosa, infection with tick Rhipicephalus spp. and a history of infection with tick Rhipicephalus spp. These clinical signs were seen either singly or in combination of two or more signs. Among dogs which were positive for *E. canis* antibodies, 6.4, 29.4, 17.69 and 34.38% had epistaxis, fever, pale mucous membranes, and were infected with ticks, respectively. Among samples that were positive for PCR, two showed clinical signs of epistaxis, six had fever, five exhibited pale mucous membranes, and four were infected with ticks (Table 1).

Hematological Profile

The following blood profile were recorded in dogs which were positive for *E. canis* antibodies, 1.83, 6.42, 26.61, 44.77, 2.75, and 22.94% had leukopenia, leukocytosis, anemia, thrombocytopenia, thrombocytosis, and hemoglobinemia, respectively. . Meanwhile, among the dogs that were positive in PCR, 3.33, 7.81, 1.56, 3.12, and 7.81% had leukocytosis, anemia, polycythemia, thrombocytopenia, and hemoglobinemia, respectively (Table 2).

PCR Amplifications

The length of the amplified *E. canis* PCR product was 556bp (Fig. 1), while the readable sequence was 536bp. The results of the Basic Local Alignment Search Tool (BLAST) showed that the resulting sequence was the 16S-rRNA gene, with 100% homology and 100% query cover after matching with Gen Bank data (Fig. 2).

Table 1: Association of clinical signs, serologic detection, and PCR detection of *E. canis* in dogs admitted to animal clinics in Denpasar, Bali, Indonesia

Parameter	Serological detection						Molecular detection					
Clinical Detect	(+)		(-)		Total	P Value	(+)		(-)		Total	P value
	N	%	n	%			N	%	N	%		
Epistaxis												
Yes	7	6.4	3	2.8	10	0.000*	2	1.83	6	5.50	8	0.227
No	57	52.3	42	38.5	99		8	7.34	48	44.03	56	
Temp(℃)												
38.0-39.2	32	29.4	23	21.1	55	0.221	4	3.67	26	23.85	30	0.118
>39.2	32	29.4	22	20.2	54		6	5.50	28	25.69	34	
Mucosa												
pink	44	40.7	31	28.7	75	0.000*	5	4.59	37	33.93	42	0.774
Pale	20	17.6	14	13.0	34		5	4.59	17	15.59	22	

*P<0.05

Table 2: Association of hematologic values, serologic detection, and PCR detection of *E. canis* in dogs admitted to animal clinics in Denpasar, Bali, Indonesia

Parameters	Serological detection						Molecular detection					
	(+)		(-)		Total	P Value	(+)		(-)		Total	P Value
	N	%	N	%			N	%	N	%		
Total Leukocytes												
Leukopenia	2	1.83	6	5.50	8	0.116	0	0.0	6	9.38	6	0.083
Leukocytes normal	55	50.46	38	34.86	93		8	12.5	41	64.06	49	
Leukocytosis	7	6.42	1	0.92	8		2	3.13	7	10.94	9	
Total Erythrocytes												
<Normal	29	26.61	15	13.76	44	0.027*	5	7.81	25	39.06	30	0.000*
Normal	31	28.44	30	27.52	61		4	6.25	26	40.63	30	
>Normal	4	3.67	0	0.00	4		1	1.56	3	5.56	4	
Total Thrombocytes												
Thrombocytopenia	27	24.77	14	12.84	41	0.194	2	3.12	26	40.63	28	0.022*
Thrombocyte normal	34	31.19	30	60.55	64		8	12.5	25	39.06	33	
Thrombocytosis	3	2.75	1	0.92	4		0	0.0	3	4.69	3	
Total Hemoglobin												
<Normal	25	22.94	15	13.76	40	0.242	5	7.81	21	32.81	26	0.004*
Normal	35	32.11	29	26.61	64		5	7.81	29	45.31	34	
>Normal	4	3.67	1	0.92	5		0	0.0	4	6.25	4	

*P<0.05.

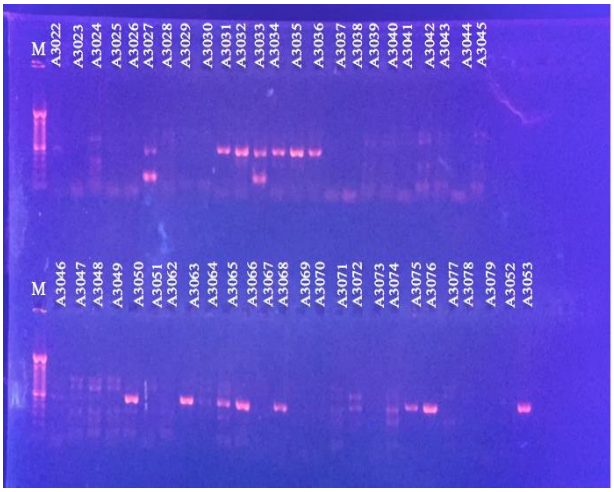

Fig. 1: The result of PCR of 16S-rDNA of suspected *E. canis* infection from dogs admitted to animal clinics in Bali Indonesia. M is a 100-bp DNA ladder (Invitrogen). PCR products were electrophoresed in 1% agarose gel and stained with etidium bromide. Only those with sharp bands at expected position were regarded as positive.

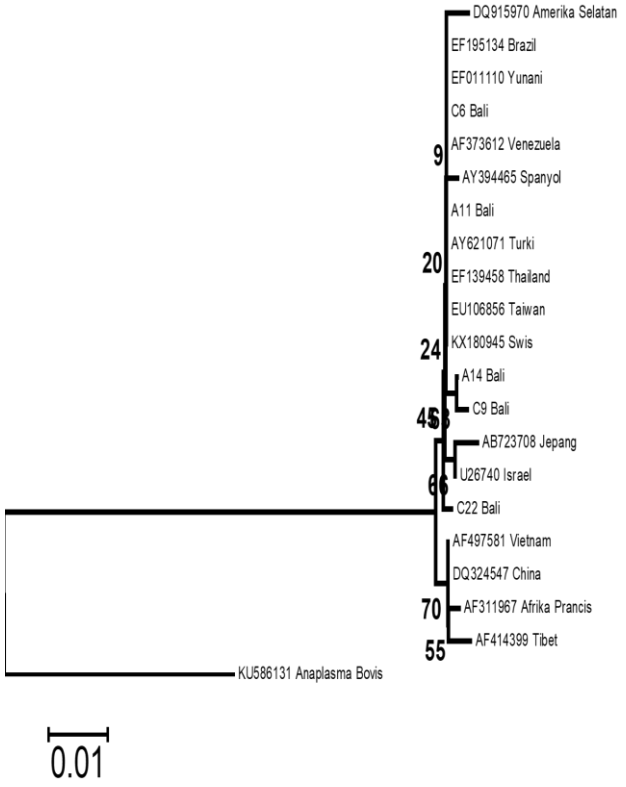

Fig. 2: The evolutionary relationship of species taxa Ehrlichia spp. in Bali with *E. canis* in the world. Sample code C6 Bali, A11 Bali, A14 Bali, C9 Bali, C22 Bali is a positive sample of *E. canis.*

DISCUSSION

This study shows that the presence of Ehrlichia sp. in dogs in Bali can be detected clinically, serologically, and molecularly. The prevalence of *Ehrlichia canis* infection was found to be 58.7% serologically and 30.43% molecularly. The prevalence of infection with Ehrlichia spp. has been reported to vary in different parts of the world, as determined by various techniques (Ahmet et al. 2001; Ansari-Mood et al. 2015; Csokai et al. 2017; Azhahianambi et al. 2018; Bunroddith et al. 2018; Piratae et al. 2019; ALhassan et al. 2021). In Indonesia, a study on Ehrlichia spp. was performed on dogs in the Jakarta, and west Java, which revealed a prevalence of 12% through serological technique (Upik et al. 2016). Differences in prevalence of *Ehrlichia canis* infection in different countries can be attributed to differences in detection techniques, and environmental conditions of each region (Ansari-Mood et al. 2015; Perez-Macchi et al. 2019). Based on this premise, we did not analyses veterinary clinic parameters as this study using PCR is the first to be conducted in Indonesia. We planned to achieving an overall picture how is the Erlichiosis burden in Bali.

Clinical signs including epistaxis, fever, and anemic mucus membranes were strongly associated with serological detection of *E. canis*; while none of these signs was significantly correlated with PCR detection (Tabel 1). Clinical signs of epistaxis occur in acute stage of *E. canis* infection (Ansari-Mood et al. 2015; Pat-Nah et al. 2015). Epistaxis leads to pale mucous membranes (Ybanez et al. 2016). In this study, 57 dogs showed no clinical signs of epitaxis, but were positive for *E. canis* on serologis test, while eight dogs without clinical signs of epistaxis showed positive result on molecular technique. These dogs were suspected for having the infection at a sub-clinical stage (Ansari-Mood et al. 2015).

Total erythrocytes count was significantly associated with serologic detection, while other hematological parameters including total leucocytes count, tolal thrombocytes and total hemoglobin were not significantly associated with serologic detection. However, the total erythrocytes, thrombocytes, and hemoglobin level, but not leucocytes, were significantly associated with PCR detection (Table 2). The diagnosis of Ehrlichiosis can be challenging because of different stages of infection with diverse clinical manifestations. *Canine Monocytic Ehrlichiosis* can be suspected in dogs with a history of having lived in an area where Ehrlichiosis was endemic, or in dogs infected with ticks, as well as showing characteristic clinical signs with hematological and biochemical abnormalities in the blood (Harrus and Waner 2011; Waner et al. 2014). The results of this study indicate that there is a relationship between total erythrocytes count and the prevalence of Erhlichiosis infection. These results are in accordance with the report that anemia and leukopenia could be seen in dogs infected with acute or chronic *E. canis* infection. The dogs infected with ticks and positive for Ehrlichiosis also show thrombocytopenia (Nakaghi et al. 2008; da Silva et al. 2012; Saeng-Chuto et al. 2016; Ybanez et al. 2016; Zhang et al. 2018; Chochlios et al. 2019; Piratae et al. 2019).

The PCR has been reported to be sensitive in detection of E. canis (Ansari-Mood et al. 2015). The PCR can detect the parasite prior to the onset of antibody production (Mojgan et al. 2013; Nasari et al. 2013). Phylogenetic analysis (Fig. 2) shows that 16S-RNA sequence found in Bali, is *E. canis*. The Bali E. canis was clustered with E. canis from South America, Europe, and from Asian countries like Thailand, Taiwan, Japan, and Israel. The sequence was separated with E. canis Vietnam, China, Africa, and Tibet.

Conclusion

Infections of *E. canis* were detected through clinical, serological and molecular approaches in dogs at Bali, Indonesia. Clinical signs of epistaxis, fever, and pale membrane were strongly associated with serological detection of *E. canis*; while none of these signs was significantly correlated with results of PCR. Total erythrocyte count was significantly associated with serologic detection. The total erythrocytes, thrombocytes, and hemoglobin were significantly associated with PCR. That sekuens 16S-RNA which found in Bali is *E. canis*. *E canis* Bali are one cluster with cluster from South America, Europe, and from Asian countries like Thailand, Taiwan, Japan, and Israel.

Authors' Contributions

IN Suartha and IGM Krisna Erawan conceptualized the study and wrote the manuscript. AA Istri Pradnyandari carried out sample collection and data analyses. IGNK Mahardika gave valuable insights and support in the conduct of the study. All authors finally read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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