International Journal of Livestock Research, Vol. 10 (4) APR' 2020
http://www.ijlr.org
eISSN: 2277-1964
NAAS Rating 2020 – 5.36

Original Research

Application of Nested PCR for Confirmative Diagnosis of Canine Ehrlichiosis

L. Narayana Rao*[1], B. Shobhamani[2], V. Vaikunta Rao[3] and K. V. Subramanyam[4]

[1]Assistant Professor (Contractual), Department of Veterinary Clinical Complex (Veterinary Medicine), NTR CVSc, Gannavaram, INDIA
[2]Professor& Head, Department of Veterinary Medicine, CVSc, Tirupati, INDIA
[3]Professor & Head, Department of Veterinary Clinical Complex, CVSc, Tirupati, INDIA
[4]Professor & Head Department of Veterinary Microbiology CVSc, Proddatur, INDIA

*Corresponding Author: lnrao402@gmail.com

How to cite this paper: Lenka, N., Bollam, S., Velamala, V., & Subramanyam, V. (2020). Application of Nested PCR for Confirmative Diagnosis of Canine ehrlichiosis. *International Journal of Livestock Research*, 10(4), 85-91. doi: http://dx.doi.org/10.5455/ijlr.20190827043651

Received : Aug 27, 2019
Accepted : Feb 29, 2020
Published : Apr 30, 2020

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Abstract

In a retrospective study, 50 Ehrlichia suspected dogs were tested with different diagnostic methods. Among them 8, 18 and 30 percent of samples were detected as positive for ehrlichiosis by peripheral blood smear, buffy coat smear examination and Anigen Rapid E.canis Ab test kit, respectively. The above test results were compared with nested PCR, of them 34 percent samples were positive for E.canis. First step PCR genus-specific primers amplified a 477 bp suggestive band of Ehrlichia and second step species-specific nested PCR amplified at 387 bp suggestive band of Ehrlichia canis. When qualitative results were compared based on Kappa statistical analysis revealed that nested PCR 72% agreement (k= 0.21) with peripheral blood smear examination, 84 % agreement (k=0.59) with buffy coat smear examination and 94% agreement (k=0.86) with Anigen Rapid E.canis Ab test kit. The sensitivity and specificity of the tests were found to be 0.17 (17%) and 1.0 (100%) for the peripheral blood smear, 0.52(52%) and 1.0 (100%) for buffy coat smear examination, and 0.87(87%) and 0.97(97%) for the Anigen Rapid E.canis Ab test kit. In conclusion, nested PCR is more sensitive and reliable diagnostic methods for diagnosis of early as well as subclinical form of canine ehrlichiosis.

Keywords: Anigen Rapid *E. canis* Ab test kit, Blood Smear Examination, *Ehrlichia canis*, Nested PCR

Introduction

Ehrlichiosis is a highly prevalent tick-borne infectious disease-affecting canines. *Ehrlichia* belongs to alpha-proteobacteria, which are Gram-negative obligate intracellular pathogens colonizing primarily in leukocytes. Donatien and Lestoquard in Algeria initially identified Ehrlichiosis disease in 1935 (Donatien and Lestoquard 1937). In India it was first reported in Chennai by Mudaliar in 1944 (Mudaliar S V, 1944). A handful of studies investigating the prevalence of canine ehrlichiosis in India using conventional examination of blood smears have reported prevalence between 0.35 percent in Punjab (Rani *et al.* 2010), one study reported using a species- specific nested PCR is 50 percent and 19 percent by microscopy (Lakshman *et al*. 2007). The overall serological prevalence of *E. canis* in India was found to be 57.5% percent by enzyme-linked immunosorbent assay (Kurkreti *et al.* 2018) and molecular prevalence is 20.6 percent (Rani *et al.* 2011) and overall prevalence based on Nested PCR is 30 percent (Bai laxmi *et al.* 2017) .

There are various species within the genus *Ehrlichia* viz., *E. canis*, *E. chaffeensis*, *E. ewingi, E. muris* and *E. ruminatiumetc* of this *Ehrlichia canis* is a predominant pathogen of dogs, causing monocyte ehrlichiosis, which is a deadly disease of dogs. *E. canis* is transracially transmitted by brown dog tick *Riphicephalus sanguineous* (Rotondano *et al*. 2015). The disease can be subclinical, acute or chronic and it affects all dogs regardless of their age and gender. Dogs with mild clinical signs of the acute stage of illness show dramatic improvement in clinical and hematologic parameters within 24 to 48 h after therapy is instituted and in chronic stage of ehrlichiosis have a poor prognosis. Chronic ehrlichiosis thus may result in either high mortality or the clinical recovery of the dogs, which then become carriers and may serve as a source of infection hence early stage diagnosis ehrlichiosis is extremely important, which can improve the prognosis of treated dogs and Identification of the most sensitive and reliable test will also help in screening dogs for their carrier status (Iqbal *et al* .1994).

The diagnosis of the disease can be challenging due to its different phases and multiple clinical manifestations. Ehrlichiosis should be suspected when a compatible history of previous tick exposure, typical clinical signs and characteristic hematological and biochemical abnormalities are present. For confirmatory laboratory investigations, conventional (blood/buffy coat smear examination, cell culture method), immunological (detection of antibodies by indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and western blot (WB) assay), and molecular (demonstration of genus and species specific DNA by polymerase chain reaction) methods are also available. Microscopic demonstration remains the 'gold' standard for the routine diagnosis of ehrlichia infections, but the demonstration of morulae is of limited value because of the low level of parasitaemia in peripheral blood (Lakshmanan *et al.* 2007). The cell culture reisolation method is reported to be very sensitive and definitive, but is not a convenient method, as it requires 14-34 days to give results and thus defeats the whole purpose of early diagnosis. Serological methods including IFAT, ELISA and western blot assay are effective for detection of antibodies to *E. canis* but failure of the techniques to differentiate current and past infections and early infections limit their reliability as a confirmative indicator of the disease. However, a definitive diagnosis of *E. canis* infection requires molecular techniques (Harrus and waner 2011). PCR is the most sensitive and specific diagnostic test whereas blood smear examination and serology can support to get a confirmative diagnosis of canine monocytic ehrlichiosis (Kottadamane *et al.* 2016).

In the present study, nested PCR protocol and comparison of Anigen rapid *E. canis* Ab Test kit, Peripheral and Buffy coat smear with nested PCR for diagnosis of canine ehrlichiosis were reported.

Materials and Methods

Clinical Cases

The retrospective study was carried out during the period from January 2018 to October 2018. 750 dogs were clinically examined from NTR College of Veterinary Science, Gannavaram, NTR Super Specialty Veterinary Hospital, Vijayawada, of them 126 infested with ticks out of the 126 dogs, 50 dogs suspected for ehrlichiosis, which were selected and screened for ehrlichiosis by different diagnostic techniques.

Clinical and Laboratory Materials

Whole blood samples was collected in EDTA vacutainer from suspected dogs for rapid diagnosis and PCR analysis.

The rapid diagnosis was carried out by using Anigen Rapid *E. canis* Ab test kit (BIO NOTE, republic of Korea) as per standard protocol recommended by the manufacturer. Thin peripheral and buffy coat smears were made from suspected dogs and subjected to direct microscopic examination (100X) using Giemsa stain (Fishers Scientifics) and slides were read carefully.

Genomic DNA Extraction

Isolation of DNA from whole blood was performed by using the QI Aamp DNA Mini kit (QIAGEN, Germany,), as per the Quick Start protocol recommended by the manufacturer. Extracted genomic DNA was quantified by Nanodrop spectrophotometer (Thermo Scientific Nanodrop 2000c, USA).

Primers

The below-described primer sequences were used in the study (Murphy *et al*. 1998; Banu *et al.* 2009).

Table 1: Primers used for Nested PCR

Ehrlichia canis	Primer Sequence-Nested PCR	Target Gene
Genus specific Primers	ECC (5' - AGA ACG AAC GCT GGC GGC AAG C-3') ECB (5'-CGT ATT ACC GCG GCT GCT GGC A-3').	16SrRNA
Species-specific Primers	ECAN5-(5' CAA TTA TTT ATA GCC TCT GGC TAT AGG A- 3' HE3 – (5' TAT AGG TAC CGT CAT TAT CTT CCC TAT-3')	16SrRNA

Nested PCR amplification. PCR assay was set up as 25 µl reaction cycle consisting of 7µl of Template DNA, 1.25µL of Forward primer (10 Pico mole), 1.25µL Reverse primer (10 Pico mole), 12.5 µL of Master Mix and 3µL of Nuclease free water used to make final volume.

Table 2: Nested PCR Protocol

PCR Protocol for *Ehrlichia spp.*					**Nested PCR Protocol for *Ehrlichia canis***	
S. No		Step	**Temperature**	**Time**	**Temperature**	**Time**
1		Initial Denaturation	95 ºC	2 min	95 ºC	3 min
2	A	Denaturation	94 ºC	1min	94 ºC	1 min (30 cycles)
	B	Annealing	67 ºC	2min	58ºC	1 min (30 cycles)
	C	Extension	72 ºC	1.30min	72 ºC	1 min (30 cycles)
3		Final extension	72 ºC	7 min	72 ºC	7 min

Nested PCR was performed in two rounds the first round of amplification used genus-specific primes. The amplicons obtained were subjected to nested PCR for confirmation of the species of Ehrlichia. Nested reactions were performed using five microlitres of this amplicon as a template with species-specific primers.

The PCR amplification was performed in a gradient type of PCR Thermal cycler (TaKaRa, JAPAN).PCR products electrophoresed on ethidium bromide-stained 1.5 % agarose gel, at 80 Volts for 54 minutes, visualized under U.V transilluminator or Gel imaging system (SYNGENE, UK)

Statistical Analysis

Kappa statistics were performed to assess the qualitative association between the nested PCR and other three tests (Peripheral blood smears examination, Buffy coat smear examination and Anigen rapid *E. canis* Ab Test kit), Sensitivities and specificities for each respective test were also calculated in relation to the nested PCR. Statistical analysis was carried out according to the principles of Landis and Koch, 1977.

Results and Discussion

Diagnosis

Peripheral Blood Smears Examination

Among the 50-suspected samples, four samples (8%) found positive for pink or purple colored cytoplasmic inclusions in monocytes suggestive of *Ehrlichia canis* (Plate 1). In comparison with nested PCR, sensitivity and specificity of this test was found to be 0.17(17%) and one (100%) respectively.

The presence of cytoplasmic inclusions or morula is a snapshot diagnostic method but the probability of detection of an organism is more during the acute or rickettsemic phase of the disease. Once the animal moves into the subclinical or chronic stage, diagnosis by this method is of low value. The present findings are agreed with the reports of earlier workers Lakshmanan *et al.,* 2007 and Banu *et al.,* 2009).

Buffy Coat Smear Examination

Fifty Ehrlichia suspected samples were examined by buffy coat smear examination, among them nine (18%) smears had pink or purple colored cytoplasmic inclusions bodies suggestive of *Ehrlichia canis* morula (Plate 2) and the test had 0.52(52%) sensitivity and one (100%) specificity.

In the buffy coat smear, the leukocytes are concentrated so better visibility of *E. canis* is achieved, this increases the probability of finding infected leukocytes with initial body or morulae. When compared to peripheral blood smear examination, a buffy coat smear examination was found to be highly sensitive in the diagnosis of CME and offering a quick and inexpensive diagnosis. The current findings are in agreement with previous findings (Mylonakis *et al.,*.2003; Kumar *et al.,* 2004).

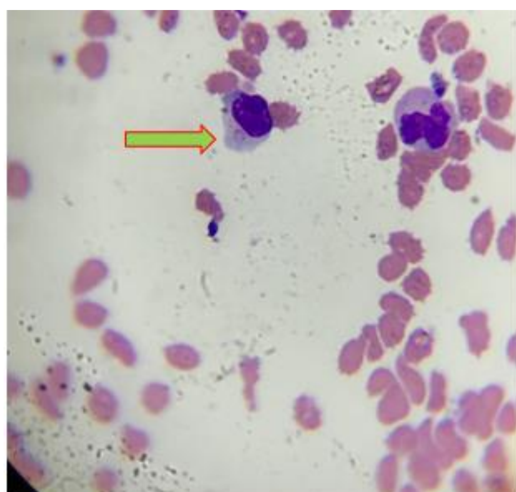

Figure 1: *E. canis* morula in Peripheral blood smears

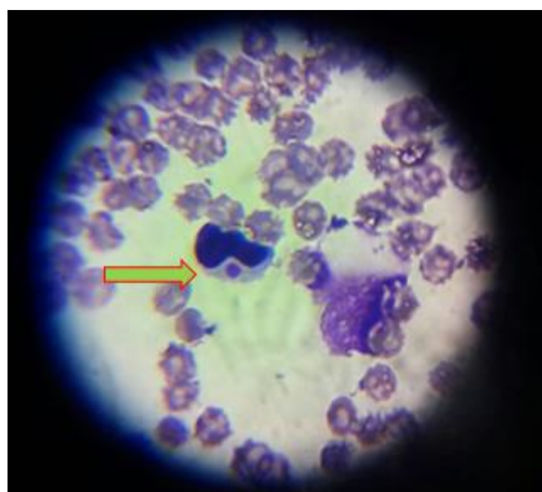

Figure 2: *E. canis* morula in buffy coat smears

(Purple colored inclusion bodies within the cytoplasm of monocyte suggestive of *E.canis* morula, Giemsa stain X 1000)

Anigen rapid *E. canis* Ab Test kit

Smear examined samples were subjected for Immuno chromatography based anigen Rapid *E. canis* Ab Test kit. Of them 15 (30%) samples were found to be positive for the *E.canis* antibodies (Plate 3).The test kit was shown 0.87(87%) sensitivity and 0.97(97%) specificity as compare with nested PCR.

Immuno-chromatography based 'Anigen Rapid *E. canis* Ab Test kit' is commercially available exclusively for the detection of *E.canis* antibodies. In present 30 percent, cases were positive for *Ehrlichia* antibodies. However, Tresamol *et al.,* (1998) had used the Immunoflorocent antibody test kit to study the seroprevalence of *E. canis* in Chennai city. Bhadesiya *et al.,* (2015) used a dot ELISA based immunocomb® antibody test kit for diagnosis of ehrlichiosis.

Polymerase Chain Reaction (PCR)

The DNA isolated from 50 blood samples which were examined by peripheral blood smears and buffy coat smear examination Anigen Rapid *E. canis* test kit were subjected to nested PCR, of them, 17 (34%) samples were positive for *E. canis*. The first step of amplification yielded amplicons of 477 bp suggestive of genus *Ehrlichia* from 17 samples (Plate 4). Second step amplification using five-microliter PCR product from the first step as template yielded amplicons of 387 bp suggestive of *E. canis* from 17 samples (Plate 5). The samples which were positive by Peripheral blood smears (n=3) and Buffy coat smear (n=9) and Anigen Rapid *E. canis* Ab test kit (n=14) were also found positive by nested PCR and one sample positive with Anigen Rapid *E. canis* Ab test kit shown negative for nested PCR method. Based on Kappa statistical analysis, nested PCR 72% agreement (k= 0.21) with peripheral blood smear examination, 84 % agreement (k=0.59) with buffy coat smear examination and 94% agreement (k=0.86) with Anigen Rapid *E. canis* Ab test kit.

Table 3: Comparison of nPCR with peripheral blood smear examination, Buffy coat smear examination and Anigen Rapid E. canis Ab test kit (n=50)

Method of diagnosis	Number positive	Positive Percent	Sensitivity	Specificity	k value
Peripheral blood smear examination	3	6%	17 %	100%	0.21
Buffy coat smear examination	9	18%	52%	100%	0.59
Anigen Rapid *E.canis* Ab test kit	15	30%	87%	94%	0.86

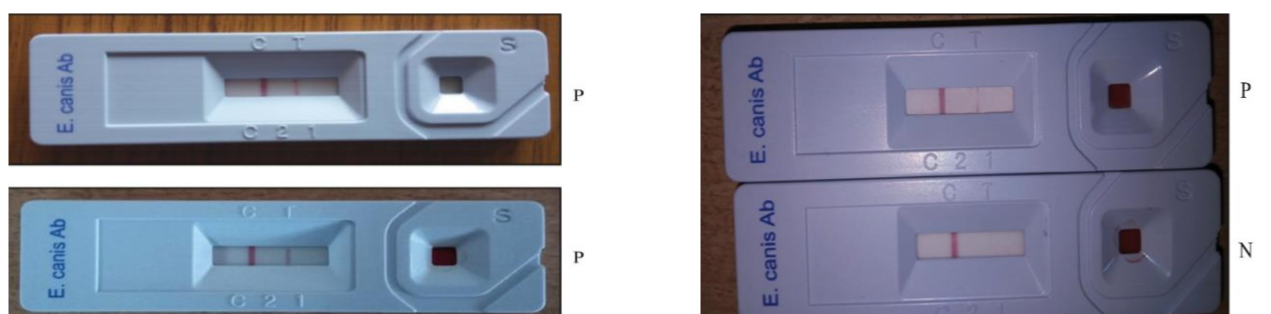

Figure 3: Results of Anigen Rapid *E. canis* Ab Test Kit
P – Positive for *E. canis* antibodies (Two bands are seen, one in the control region 'C' of the result window and others in the test region 'T'; **N** – Negative for *E. canis* antibodies (Single band was observed in the control region 'C' of the result window)

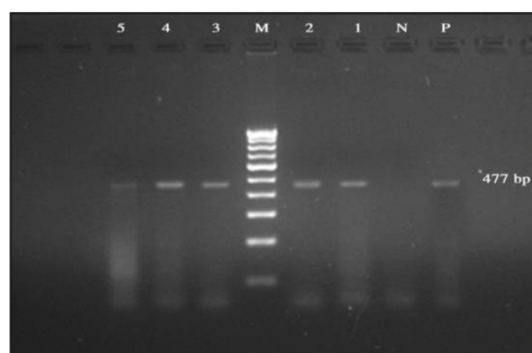

Figure 4: *Ehrlichia spp.* Genus specific Nested PCR (**M**-100 bp ladder; **P** – known control of *Ehrlichia spp.*; **N**- Negative control of *Ehrlichia spp*; **1-5** – positive samples of *Ehrlichia spp.*)

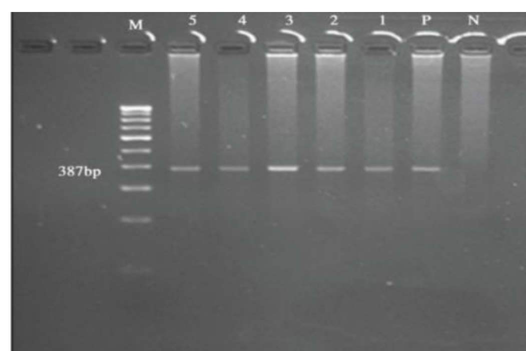

Figure 5: *Ehrlichia canis* species specific Nested PCR (**M**-100 bp ladder; **P** – known control of *Ehrlichia canis.*; **N**- Negative control of *Ehrlichia canis*; **1-5** –positive samples of *Ehrlichia canis.*)

In the present study, confirmatory diagnosis was performed by direct microscopic examination of *E. canis* morulae from peripheral blood smears and buffy coat smears, detection of antibodies against *E. canis* by commercially available immune-chromatography based Anigen Rapid *E. canis* Ab test kit and molecular detection by demonstration of genus and species specific DNA by using nested PCR. Presence of morula in blood smear

examination is snap shot approach for diagnosis of ehrlichiosis (Islam *et al.,* 2017) , in present study peripheral blood smear examination shown fair agreement (72%) and buffy coat smear examination shown moderate agreement (84%) with nested PCR, both the testes were found as high specific and low sensitive than the immunological and molecular diagnostic approaches, of them buffy coat smear examination had better sensitivity as compared to peripheral smear examination, these findings were corroborated with the reports of Harrus *et al* (1999) Lakshmanan *et al.* (2007) and Banu *et al*. (2009). Lakshmanan *et al.* (2007) recorded that 50 and 19.38 percentage of samples were positives with nested PCR and blood smear examination, respectively. Banu *et al.* (2009) documented that 1.25 and, 47.7 percent samples were positive with blood smear examination and nested PCR respectively. This might be due to the percentage of *E. canis* infected cells in the peripheral blood may be low and identification of *E. canis* morulae in blood smear examination is only possible in the acute stage of diseases as opined by Pat–nah *et al.* (2015). Detection of antibodies against *E. canis* could be done by using commercially available immune chromatography based Anigen Rapid *E. canis* Ab test kit and the results were close agreement (94%) with nested PCR and high sensitive than microscopic smear examination techniques. The antibody test kit may show serological cross-reactivity and fail to distinguish a current infection from neither previous infection nor exposure without establishment of infection, moreover in case with positive by nested PCR and negative with Anigen Rapid *E.canis* Ab test kit results can be considered to be in the early stage of infection before the development of detectable antibody titers from blood sample. This finding was supported with Neer *et al.* (2002), who reported that results could be negative in the acute phase of infection before seroconversion. In the present study more number (n=17) of cases were detected as positive by nested PCR, hence nested PCR has been considered as reliable technique and more sensitive as compared to conventional methods, further it could be employed for diagnosis of early and subclinical phase of infections, as opined by Banu *et al. (*2009) and Rajagopal e*t al*. (2009).

Conclusion

The present study suggested that parallel use different diagnostic methods like peripheral blood smear, buffy coat smear examination, Anigen Rapid *E. canis* Ab test kit and nested PCR assay will increase the chance of detection of ehrlichiosis infections. Among them nested PCR is more sensitive and reliable diagnostic methods for diagnosis of early as well as subclinical form of canine ehrlichiosis.

Conflict of Interests

The author expresses no conflict of interest with any other individual or organisation regarding the information discussed in the manuscript

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