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## Molecular identification and phylogenetic analysis of *Anaplasma platys* from naturally infected dogs of South India

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**Abstract**

Present study represents the first report on the molecular identification and phylogenetic analysis of *Anaplasma platys* infecting dogs of Kerala, South India. A total of 60 blood samples were collected from the dogs that presented to the University Veterinary Hospital and Teaching Veterinary Clinical Complex with clinical signs. Whole-blood samples submitted to molecular lab attached to the hospital for examination and blood smears were made from blood samples of all 60 dogs for the identification of *Anaplasma platys*. For each sample, polymerase chain reaction (PCR) was carried out after DNA extraction to amplify 16S rRNA gene for the detection of *Ehrlichia* genus, *Ehrlichia canis* and *Anaplasma platys*. Out of 60 animals treated, 7 (11.6%) were positive with *Ehrlichia* genus primer, which was later confirmed as *Ehrlichia canis* on species-specific PCR. Of the sixty dogs, five (8.3%) were positive for *Anaplasma platys* species specific PCR even though direct blood smear examination and *Ehrlichia* genus specific PCR results were negative.

**Keywords:** *Anaplasma platys*, *Ehrlichia canis*, polymerase chain reaction, 16S rRNA

**1. Introduction**

*Anaplasma platys* (*Ehrlichia platys*) is a gram-negative rickettsial, obligate intracellular organism, which specifically infects dog platelets [1]. Based on the genetic analyses of groESL, 16S rRNA gene and surface protein genes, genus *Ehrlichia* and other related genus like *Cowdria*, *Wolbachia* and *Neorickettsia* were re-organized and incorporated in family *Anaplasmataceae* in 2001 [2, 3]. Since then *Ehrlichia platys* was known as *Anaplasma platys*. *Anaplasma platys* causes a disease condition called Infectious Canine Cyclic Thrombocytopenia (ICCT) in dogs [4] and its occurrence was first reported from Florida [5]. *Rhipicephalus sanguineus*, commonly known as Brown dog tick, suspected to be the vector that transmitting this pathogen since the DNA of *Anaplasma platys* amplified from the tick [6]. In dogs, organism is present within platelets as morulae composed of variable number of inclusions that is difficult to detect by direct blood smear examination [5]. *Anaplasma platys* infection is generally considered as benign disease without any severe clinical signs, although clinical abnormalities like anorexia, pyrexia, uveitis and petechial hemorrhage have been reported earlier [7, 8, 9, 10]. However, more pathogenic strains of *Anaplasma platys* have been reported from several countries of Mediterranean basin and Brazil especially when co-infections are involved [3].

Characteristic hematological alteration noticed in *Anaplasma platys* infection is cyclic thrombocytopenia, which occurs in a cyclic pattern at 7-14 days intervals [11]. Even though the organism is worldwide in its distribution, the prevalence is more common in tropics and subtropical climates [12]. Diagnosis of *Anaplasma platys* infection is very difficult since the infection shows symptoms, which overlaps with *Ehrlichia canis* [13]. Detection of organism is usually made by the examination of Romanowsky-stained blood smears or buffy-coat smears, which lacks sensitivity and specificity because of cyclic thrombocytopenia [14]. The aim of the present study is to use PCR as a tool for the diagnosis of the infection. As far as we know, this is for the first time molecular characterization and sequencing have been carried out for the identification and phylogenetic analysis of *Anaplasma platys* from naturally infected dogs in South India.

## 2. Materials and methods

### 2.1. Sample collection

Sixty canine whole blood samples collected in EDTA from the dogs suspected for ehrlichiosis submitted to Molecular Lab attached to University Veterinary Hospital (UVH) and Teaching Veterinary Clinical Complex (TVCC), Mannuthy, Kerala from various parts of South India. All these dogs were having a history of pyrexia (104°F), anorexia, tick infestation and depression. The owners also reported that vaccination and deworming history were proper for each animal. On general examination, mucous membrane was pale with generalized enlargement of lymph nodes. Blood samples collected for laboratory examination were kept at 4°C, until it was processed within 12-24 hours for measuring hematological parameters using hematology analyzer (Orphee Mythic-18, Switzerland) and examination of blood smear. Thin blood smears were prepared and air-dried during the sampling process. Smears then fixed in methanol for 5 minutes and stained with Wright- Giemsa stain by trained laboratory technicians.

### 2.2. Genomic DNA extraction

Genomic DNA was isolated from 100 µl of blood using DNeasy® blood and tissue kit (Qiagen, Germany), as per manufacturers protocol with following minor modifications; incubated at 56°C for 2 hours. Isolated DNA was then eluted in 200 µl of Tris-EDTA buffer and kept at -20°C for further study. Concentration of the extracted DNA was measured using a NanoDrop® 2000C spectrophotometer (Thermo Scientific, USA).

### 2.3. Polymerase chain reaction

DNA isolated from all the 60 blood samples were first subjected to genus-specific PCR using set of primers EHR 16SD (5' – GGT ACC YAC AGA AGA AGT CC – 3') and EHR 16SR (5' – TAG CAC TCA TCG TTT ACA GC – 3') that amplify DNA fragment of 345 bp from the 5' half of the 16S rRNA gene. This primer set can amplify various species like, *Anaplasma marginale*, *Anaplasma centrale*, *Anaplasma platys*, *Anaplasma phagocytophilum*, *Ehrlichia ruminatum*, *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia muris*, *Ehrlichia equi*, *Ehrlichia sennetsu*, *Neorickettsia helminthoeca*, *Neorickettsia risticii* and *Wolbachia pipientis* [15]. To confirm the presence of *Anaplasma platys*, all the isolated DNA samples were screened using species- specific primers specific for *Ehrlichia canis* and *Anaplasma platys*. Screening for *Ehrlichia canis* was done using primers: - forward ECAF (5'-AAC ACA TGC AAG TCG AAC GGA-3') and reverse HE3R (5'-TAT AGG TAC CGT CAT TAT CTT CCC TAT-3') [16] resulting in an amplicon size of 412 bp. Thermal cycler condition and PCR mixture composition were same as that of earlier report [16]. Screening for the presence of *Anaplasma platys* DNA was carried out using species-specific:- forward primer PLATYS (5'- GAT TTT TGT CGT AGC TTG CTA TG-3') and reverse primer EHR 16SR to produce an amplified product of approximately 678 bp in size [17]. Amplification of *Ehrlichia* genus and *Anaplasma platys* species-specific fragments was carried out with a 50 µl reaction mixture containing 5 µl template DNA, 25 pmol of each primer, 1 U Taq DNA polymerase, 3 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleoside triphosphates, 20 mM Tris-HCl, and 100 mM KCl. The amplification was performed in an automated thermal cycler (BIO-RAD T100™, USA) with following cycling conditions: an initial denaturation at 95°C

for 2 minute, 35 cycles of 95°C for 1 minute, 51°C or 54°C for 1 minute (depending on the primers used), 72°C for 1 minute and final extension at 72°C for 5 minute.

### 2.4. Sequencing and phylogenetic analysis of data

Products obtained after polymerase chain reaction were gel extracted and purified using QIAquick gel extraction kit (Qiagen, Germany) according to manufacturer's instructions. Samples were sequenced in both directions using available sequencing facility at Agri Genome Labs Private Ltd., Cochin, and Kerala. Obtained nucleotide sequences were analysed for homology with other published sequences available in GenBank using BLAST programme of NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>) and submitted to GenBank database. Nucleotide sequence specific to *Anaplasma platys* were aligned with sequences of several *Anaplasma platys* sequences obtained from different countries using Clustal W software [18]. Aligned sequences were trimmed to equal length (including gaps) and from which phylogenetic tree was constructed with maximum likelihood method using MEGA 7.0 software [19]. Evolutionary history was calculated based on Kimura 2-parameter (K2P) model and reliability of the topology was tested using bootstrap value of 1000 replicates.

## 3. Results and Discussion

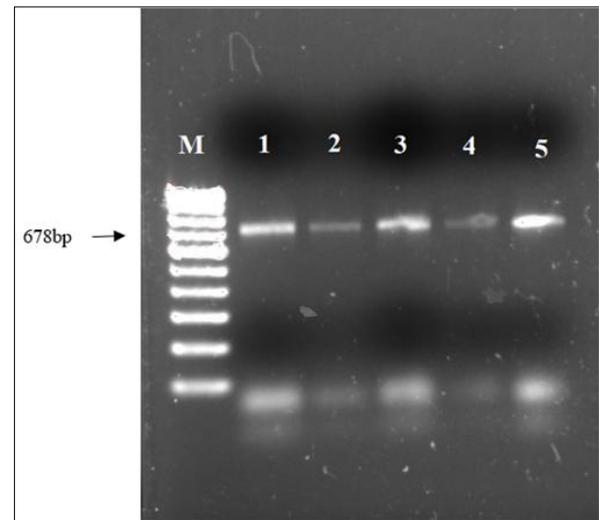
Complete blood count analysis of all the sixty samples revealed anisocytosis, moderate to severe normocytic normochromic anaemia and mild to severe thrombocytopenia. All the animals tested were having anemia and thrombocytopenia. In some samples, the platelets were bigger in size in accordance with the earlier reports [20, 1, 21]. All the selected samples were reported with infestation of tick *Rhipicephalus sanguineus*. Anaemia, a non-specific finding in *Anaplasma platys* infection was noticed in the positive samples. This may be due to the chronic inflammation in *Anaplasma platys* infection as explained earlier [1]. Of the sixty samples that were subjected to blood smear examination, none of them were positive for any of the *Ehrlichia* species. Out of sixty, five samples had abnormal sized platelets. On direct examination of the blood smears, intracytoplasmic basophilic inclusion bodies could not be detected in any of the blood samples. Diagnosis of *Anaplasma platys* infection based on the presence of inclusion bodies in platelets by direct blood smear examination is not sensitive and time consuming [9, 22]. The reason for this could be due to the cyclic appearance of parasitised platelets [23] and these parasitised platelets are rare or even absent when the platelet count is at its lowest [20]. Abnormal sized platelets prompted us to carry out a PCR- based assay using genus specific primer and species specific primers coupled with sequencing for the identification of ehrlichial organisms.

DNA samples from all 60 dogs were screened for the presence of *Ehrlichia/Anaplasma* species using genus and species specific primers. Gel analysis revealed that among 60 samples, 7 samples (11.6%) were positive for *Ehrlichia* genus specific PCR, which were later confirmed as *Ehrlichia canis* by species specific PCR, and 5 samples (8.3%) were positive for *Anaplasma platys* specific PCR. As expected, 345 bp band size were obtained for amplification with *Ehrlichia* genus specific primer and 412 bp for amplification with *Ehrlichia canis* specific PCR. NCBI-BLAST analysis of the sequences showed 98% homology with published 16S rRNA sequences of *Ehrlichia canis*. Polymerase chain reaction of 5 samples,

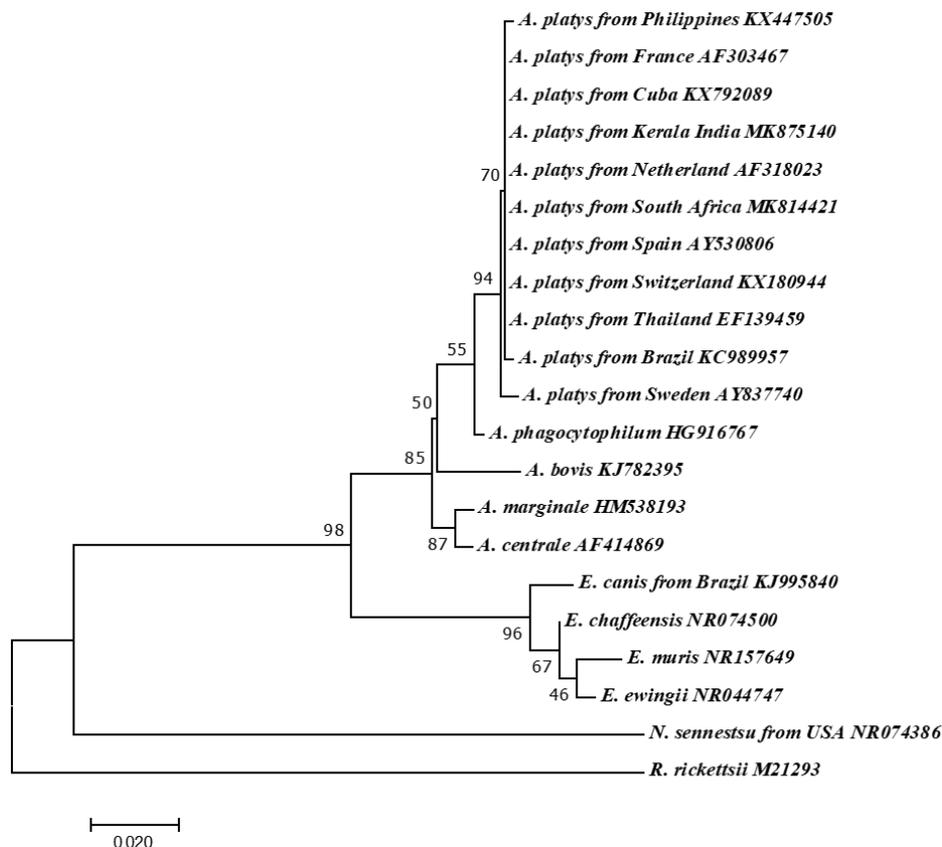
which were PCR negative with *Ehrlichia* genus specific PCR, having abnormal platelets on blood smear examination were found to be positive with *Anaplasma platys* species-specific primer with an expected amplicon length of 675 bp as shown in the figure 1. Unlike other reports [3, 17], samples which were positive on *Anaplasma platys* species specific PCR found to be negative with *Ehrlichia* genus specific PCR. This may be due to low parasitemia as reported earlier [24]. This finding strongly suggest that a combination of blood smear with abnormal platelets, clinical signs and direct PCR using species specific primers could be used as a diagnostic tool for the routine testing for *Anaplasma platys* in clinical labs. The NCBI-BLAST and phylogenetic analysis of *Anaplasma platys* 16S rRNA sequence after excluding the primer sequences showed 99.41% similarity with published sequences. Phylogenetic relationship based on 16SrRNA sequences of *Anaplasma platys* isolated from Kerala, India and other species of the *Ehrlichia*, *Anaplasma*, *Rickettsia* and *Neorickettsia* genera as shown in the figure 2. All *Anaplasma platys* isolated from different parts of the world clustered together with a nodal support of 93 percentage. As proposed earlier [25, 26, 27, 28, 29], clinical signs in *Anaplasma platys* infection depends on geographical location, immune status, breed and stress condition of the animal.

Many studies have reported the presence of human and animal *Anaplasma platys* infection from different parts of the world [30, 31]. Eventhough the presence of *Anaplasma platys* infection in bovines is speculated by 16S rRNA analysis [32],

there is no such conclusive evidence for the presence of *Anaplasma platys* in carnivores or herbivores. However, till now there is no such clinical cases have been reported in dogs from Kerala, India. In respect to the above data, the current study affirms the existence of natural infection of *Anaplasma platys* in dogs of Kerala, India for the first time.



**Fig 1:** PCR amplified product of 16S rRNA gene using *Anaplasma platys* specific primers. M- Molecular marker, lane 1 to 5- positive samples with a band size of 678bp



**Fig 2:** Phylogenetic tree constructed using 16S rRNA gene sequences of *Anaplasma platys* and other closely related genera. Evolutionary history was calculated by using the Maximum Likelihood method based on the Kimura 2-parameter (K2P) model.

#### 4. Conclusion

In contrary to the previous reports, samples that were positive on *Anaplasma platys* species-specific PCR found to be negative with *Ehrlichia* genus-specific PCR. Hence, a

combination of clinical signs, direct blood smear examination for platelets having an abnormal size and a single-step direct PCR with *Anaplasma platys* species-specific primer could use as a routine diagnostic tool.

## 5. Acknowledgements

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## 6. References

- Baker DC, Simpson M, Gaunt SD, Corstvet RE. Acute *Ehrlichia platys* infection in the dog. *Veterinary Pathology*. 1987; 24:449-453.
- Dumler JS, Barbet AF, Bekker CPJ, Dasch GH, Ray SC, Rikihisa Y *et al.* Reorganization of genera in the Families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species combinations and designation of *Ehrlichia equi* and 'HE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *The International Journal of Systematic and Evolutionary Microbiology*. 2001; 51:2145-2165.
- Aguirre E, Tesouro MA, Ruiz L, Amusatogui I, Sainz A. Genetic Characterization of *Anaplasma (Ehrlichia) platys* in Dogs in Spain. *Journal of Veterinary Medicine Series B*. 2006; 53(4):197-200.
- Cardoso L, Tuna J, Vieira L, Yisaschar-Mekuzas Y, Baneth G. Molecular detection of *Anaplasma platys* and *Ehrlichia canis* in dogs from the North of Portugal. *Veterinary Journal*. 2010; 183:232-23.
- Harvey JW, Simpson CF, Gaskin JM. Cyclic Thrombocytopenia Induced by a Rickettsia-Like Agent in Dogs. *The Journal of Infectious Diseases*. 1978; 137(2):182-188.
- Sparagano OAE, de Vos AP, Paoletti B, Cammà C, de Santis P, Otranto D *et al.* Molecular Detection of *Anaplasma platys* in Dogs Using Polymerase Chain Reaction and Reverse Line Blot Hybridization. *Journal of Veterinary Diagnostic Investigation*. 2003; 15(6):527-534.
- Hoskins JD. *Ehrlichial* diseases of dogs: diagnosis and treatment. *Canine Practice*. 1991; 16:13-21.
- Baker DC, Gaunt SD, Babin SS. Anemia of Inflammation in Dogs Infected with *Ehrlichia platys*. *American Journal of Veterinary Research*. 1988; 49:1014-6.
- Bradfield JF, Vore SJ, Pryor WH. *Ehrlichia platys* infection in dogs. *Laboratory Animal Science*. 1996; 46:565-568.
- Glaze MB, Gaunt SD. Uveitis associated with *Ehrlichia platys* infection in a dog. *Journal of the American Veterinary Medical Association*. 1986; 188:916-917.
- Eddlestone SM, Gaunt SD, Neer TM, Boudreaux CM, Gill A, Haschke E *et al.* PCR detection of *Anaplasma platys* in blood and tissue of dogs during acute phase of experimental infection. *Experimental Parasitology*. 2007; 115(2):205-210.
- Abarca K, López J, Perret C, Guerrero J, Godoy P, Veloz A *et al.* *Anaplasma platys* in Dogs, Chile. *Emerging Infectious Diseases*. 2007; 13(9):1392-1395.
- Dhivya B, Latha BR, Azhahianambi P. Molecular detection of *Anaplasma platys* infection in dogs in Chennai, Tamil Nadu, India- A pioneer report. *Journal of Entomology and Zoology Studies*. 2017; 5(3):1608-1610.
- Soares R, Ramo CA, Pedroso T, Babo-Terra V, Cleveland H, Araújo FD. Molecular survey of *Anaplasma platys* and *Ehrlichia canis* in dogs from Campo Grande, Mato Grosso do Sul, Brazil. *Anais da Academia Brasileira de Ciências*. 2017; 89(1):301-306.
- Inokuma H, Raoult D, Brouqui P. Detection of *Ehrlichia platys* DNA in Brown Dog Ticks (*Rhipicephalus sanguineus*) in Okinawa Island, Japan. *Journal of Clinical Microbiology*. 2000; 38:4219-21.
- Gal A, Loeb E, Yisaschar-Mekuzas Y, Baneth G. Detection of *Ehrlichia canis* by PCR in different tissues obtained during necropsy from dogs surveyed for naturally occurring canine monocytic ehrlichiosis. *Veterinary Journal*. 2008; 175(2):212-217.
- Brown GK, Martin AR, Roberts TK, Aitken RJ. Detection of *Ehrlichia platys* in dogs in Australia. *Australian Veterinary Journal*. 2001; 79:554-558.
- Thompson JD, Higgins DG, Gibson TJ. Clustal W. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic acids Research*. 1994; 22:4673-4680.
- Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*. 2016; 33(7):1870-1874.
- French TW, Harvey JW. Serologic diagnosis of infectious cyclic thrombocytopenia in dogs using an indirect fluorescent antibody test. *American Journal of Veterinary Research*. 1983; 44:2407-2411.
- Sainz A, Amusatogui I, Tesouro MA. *Ehrlichia platys* infection and disease in dogs in Spain. *Journal of Veterinary Diagnostic Investigation*. 1999; 11:382-4.
- Harrus S, Aroch I, Lavy E, Bark H. Clinical manifestations of infectious canine cyclic thrombocytopenia. *Veterinary Record*. 1997; 141(10):247-250.
- Woody BJ, Hoskins JD. Ehrlichial diseases of dogs. *Veterinary Clinics of North America: Small Animal Practice*. 1991; 21(1):75-98.
- Martin AR, Brown GK, Hugh Dunstan R, Roberts TK. *Anaplasma platys*: an improved PCR for its detection in dogs. *Experimental Parasitology*. 2005; 109(3):176-180.
- Inokuma H, Ohno K, Onishi T, Raoult D, Brouqui P. Detection of ehrlichial infection by PCR in dogs from Yamaguchi and Okinawa Prefectures, Japan. *The Journal of Veterinary Medical Science*. 2001; 63(7):815-817.
- Suksawat J, Pitulle C, Arraga-Alvarado C, Madrigal K, Hancock SI, Breitschwerdt EB. Coinfection with three *Ehrlichia* species in dogs from Thailand and Venezuela with emphasis on consideration of 16S ribosomal DNA secondary structure. *Journal of Clinical Microbiology*. 2001; 39(1):90-93.
- Inokuma H, Beppu T, Okuda M, Shimada Y, Sakata Y. Epidemiological survey of *Anaplasma platys* and *Ehrlichia canis* using ticks collected from dogs in Japan. *Veterinary Parasitology*. 2003; 115(4):343-348.
- Sanogo Y, Davoust B, Inokuma H, Camicas JL, Parola P, Brouqui P. First evidence of *Anaplasma platys* in

- Rhipicephalus sanguineus* (Acari: Ixodida) collected from dogs in Africa. Onderstepoort Journal of Veterinary Research. 2003; 70:205-212.
29. Beaufils JP, Inokuma H, Martin-Granel J, Jumelle P, Barbault-Jumelle M, Brouqui P. *Anaplasma platys* (*Ehrlichia platys*) infection in a dog in France: description of the case and characterization of the agent. Revue de Medecine Veterinaire. 2002; 153:85-90.
  30. Maggi RG, Mascarelli PE, Havenga LN, NaidooV, Breitschwerdt EB. Co-infection with *Anaplasma platys*, *Bartonella henselae* and *Candidatus Mycoplasma haematoparvum* in a veterinarian. Parasite and Vectors. 2013; 6(1):103.
  31. Breitschwerdt EB, Hegarty BC, Quorollo BA, Saito TB, Maggi RG, Blanton LS *et al.* Intravascular persistence of *Anaplasma platys*, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii* DNA in the blood of a dog and two family members. Parasites and Vectors. 2014; 7(1):298.
  32. Pradeep RK, Nimisha M, Sruthi MK, Vidya P, Amrutha BM, Kurbet PS *et al.* Molecular characterization of South Indian field isolates of bovine *Babesia* spp. and *Anaplasma* spp. Parasitology Research. 2018; 118(2):617-630.