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Molecular detection of *Anaplasma platys* infection in dogs in Chennai, Tamil Nadu, India- A pioneer report

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Abstract
Diagnosis of blood parasites has been based on the morphological identification in a blood smear. However, in recent times with the advent of molecular diagnostic techniques it has been proved that morphological diagnosis alone does not lead to confirmatory results. Taking these criteria into account the current study on canine anaplasmosis was designed. *Anaplasma platys* and *A. phagocytophilum* are the etiological agents of Canine anaplasmosis. *A. platys* appear as inclusions within the thrombocytes and often go unnoticed in a blood smear examination. Clinical signs in *A. platys* infection are similar to canine ehrlichiosis and hence diagnosis based on clinical signs and blood smear examination seldom lead to confirmatory results. In the current study, a nested-PCR assay was utilized for confirmatory diagnosis of *A. platys* infection among dogs in Chennai. About 141 blood samples from suspected dogs were collected of which 23 (16.31%) were positive for *A. platys*. This is the first confirmed molecular evidence on occurrence of *A. platys* infection in the canine pet population in Chennai.

Keywords: Dogs, *Anaplasma platys*, Chennai, Nested-PCR

1. Introduction
Indian sub-continent has a sub-tropical climate which is conducive for the existence of not only human and animals but also the pathogens. There is a relative paucity on the reports of tick-borne infectious disease [1] among pet population compared to the livestock species. Metropolitan cities in India, viz., New Delhi, Kolkata, Mumbai and Chennai have large canine populations which include both native and exotic breeds. With the introduction of new dog species due to importation, we are indirectly introducing new pathogens which were not known to us earlier. *Anaplasma platys* recorded for the first time in Chennai in this study is one such example.
Canine anaplasmosis is a tick-borne rickettsial disease caused by *A. platys* and *A. phagocytophilum. A .platys* is an intracellular rickettsial agent found in the platelets. *A. platys* can be detected as basophilic inclusions in the platelets of thrombocytopenic dogs [2]. The incubation period for the parasite is usually 1 to 2 weeks and is characterized by fever, lethargy, weight loss, pale mucous membranes, petechiae, nasal discharge, and lymphadenomegaly [3]. The detection of inclusion bodies in blood platelets is possible in earlier stage of infection only and is non-specific later as there are non-parasitic inclusions within these figured elements [4]. As the clinical signs of *E. canis* and *A. platys* overlap and since detection of *A. platys* in routine blood smear examination is difficult the reports on this rickettsial parasite is scarce. Hence, the present study of molecular detection of *A. platys* infection is present among dogs in Chennai was undertaken.

2. Materials and Methods
2.1 Collection of blood samples
Whole blood samples in an anticoagulant were collected from dogs presented at the Small Animal Clinics of Madras Veterinary College Teaching Hospital and from cases presented in private clinics in Chennai for a period of one year from January'2014 to December' 2014. Blood samples were collected from 141 dogs with clinical signs such as pyrexia with lymphadenitis, tick infestation, epistaxis and history of recurrent pyrexia. Ticks from infested dogs were collected for morphological identification.
Hematological parameters were also analysed for all the samples collected. Blood smears from the suspected animals were stained using Giemsa stain to detect the presence of haemoprotozoan parasites.

2.2 Isolation of DNA
DNA was isolated using QIAMP DNA mini kit (Qiagen, Germany). The step by step protocol was followed as given by the manufacturer. The isolated genomic DNA was the subjected to quantification and purity assessment by nanodrop technique.

2.3 DNA amplification and Nested Polymerase Chain Reaction
DNA amplification and Nested Polymerase chain reaction was carried out [5]. The primer sequences utilized for amplification were as follows.

Genus specific primers
8F: 5'-AGTTTGATCATGGCTCAG- 3'
1448R:5'-TGGCGTGACGGGCAGTG- 3'
Thermalcycler conditions: Initial denaturation at 94ºC for 2 minutes, followed by 40 cycles of denaturation at 94ºC for 1 minute, annealing at 45ºC for 1 minute and extension at 72ºC for 1 minute. Final extension for 5 minutes at 72ºC. Expected band size- 1445 bp.

Species specific primers
PLATYS: 5' - GAT TTT TGT CGT AGC TTG CTA TG – 3' combined with reverse primer
EHR16SR: 5' - TAG CAC TCA TCG TTT ACA GC – 3', which amplifies a 678- bp fragment from 16S ribosomal RNA.
Cycling conditions were: Initial denaturation at 94ºC for 1 minute, followed by 40 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds and extension at 72ºC for 30 seconds. Final extension at 72ºC for 5 minutes. Expected band size-678 bp.

2.4 DNA sequencing
Nested PCR reaction was carried out with a positive sample and the PCR product was gel electrophoresed. The 678bp PCR product in the gel was cut, eluted using QIAMP gel elution kit and the product was sent for sequencing to Eurofins laboratory, Bangalore.

3. Results and Discussion
The haematological parameters revealed severe thrombocytopenia in 94 (66.6%) out of the 141 cases. In Giemsa stained blood smears, identification of inclusion bodies inside the blood platelets was difficult. The reason for this could be attributed to low level of parasitemia [6]] and also cyclical nature of the disease wherein there is decreased platelet count during infection and consequently decrease in circulating micro-organisms [7]. Hence for the diagnosis of chronic, sub clinical infections and in prevalence studies, molecular detection has been found to play a vital role for accurate results as evident in the current study. Therefore, all the samples were subjected to nested-PCR analysis. Out of the 94 cases with thrombocytopenia, 36 were positive for *Ehrlichia canis* in the prelimimary screening of blood smears. Of the 141 suspected samples that were subjected to nested PCR assay for detection of *Anaplasma platys*, 23 (16.31%) samples were positive with a band evident at 678 bp (nested PCR) as shown in the figures 1 and 2. This is the first record of prevalence of the pathogen in dogs in Chennai.

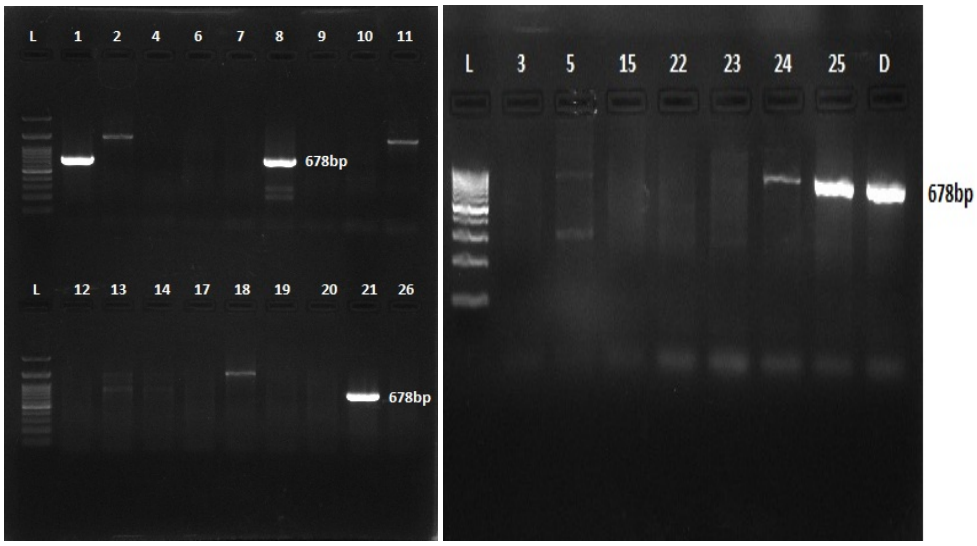

Fig 1, 2: L-1000bp ladder, positive samples with a band at 678bp with *Anaplasma platys* specific primers

To further confirm the finding, DNA sequencing was carried out and it has been found that the sequence has 98 percent homology with the Bareilly strain of *A. platys*. Among the confirmed cases of *A. platys* by nested PCR, 2 of the animals had co-infection with *E. canis* that was evident by blood smear examination. The molecular detection of *A. platys* in naturally infected dogs in Chennai in this study is the first confirmatory record of the prevalence of infection in Tamil Nadu though *A. platys* has been reported among dogs in North India earlier [8].
Data collected pertaining to tick infestation showed that 98 (69.5%) out of 141 cases were infested with ticks. Ticks collected were identified as *Rhipicephalus sanguineus* based on the morphological features. *A. platys* infection was detected in *R. sanguineus* infested dogs which are imperative to conclude that it serves as the vector for *A. platys* in Tamil Nadu similar to the previous studies [8, 9].
Co-infections are common in canine tick-borne diseases [10]. In this study, co-infection with *Ehrlichia canis* was detected in 2 (12.5%) of *A. platys* positive cases. Co-infections of *A. platys* and *E. canis* have been reported earlier [11]. However, the percentage of co-infected animals is slightly higher to those observed in Delhi (7%) and Mumbai (4.5%) [8]. It has been reported that co-infections of *E. canis* and *A. platys* occur frequently as they share the same arthropod vector [3].

4. Conclusion

This is the first record of *A. platys* infection in dogs in Chennai. The results obtained in the present study suggest that molecular technique is more specific and sensitive in diagnosis of *A. platys* infections compared to conventional blood smear examinations, as none of the samples were concluded positive in the initial screening of blood smears. *A. platys* is of zoonotic importance and hence this finding proves to be the stepping stone for further research on the public health significance of this parasite.

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