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Taqman based real time polymerase chain reaction (qPCR) for detection of granulocytic anaplasmosis in dogs in Thrissur district, Kerala state

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Abstract
Anaplasma phagocytophilum formerly *Ehrlichia phagocytophilum*, the causative agent of human, equine and canine granulocytic anaplasmosis, is a small obligate intracellular bacterium which has infective stage inside granulocytes. Even though infective stage of bacterium is evident inside neutrophils, in the peripheral blood smears prepared from many of the canine patients, it is very difficult to arrive at an accurate and confirmatory diagnosis without a molecular diagnostic technique. Hence the present study was undertaken to standardize a Taqman based real time PCR targeting specific segment of 16SrRNA gene for the qualitative detection of *A. phagocytophilum* from suspected canine peripheral blood samples and also in ticks recovered from them. The results of the present study show that Taqman based Real Time PCR can be reliably used as an aid in accurate and confirmatory diagnosis based on the qualitative detection of *A.phagocytophilum* 16SrRNA gene. To authors knowledge this is the first report of molecular detection of *A.phagocytophilum* among canine population and in ticks recovered from them in Thrissur district, Kerala State.

Keywords: Anaplasma phagocytophilum, dogs, real time PCR, 16SrRNA gene

1. Introduction
Anaplasma phagocytophilum the etiological agent of human, equine and canine granulocytic anaplasmosis (CGA) is an obligate intracellular pathogen that parasitizes the granulocytes-primarily neutrophils of wild/domestic animals and man [1]. The bacterium is reported to be transmitted by *Ixodes* species ticks. Dogs naturally infected with *A. phagocytophilum* generally remain healthy and become asymptomatic carriers, but clinical manifestations such as lethargy, anorexia, depression, fever and splenomegaly are described [2]. The clinical manifestation of *A. phagocytophilum* infection in dogs had been defined most commonly as granulocytic ehrlichiosis, anaplasmosis and granulocytic anaplasmosis [3, 4, 5, 6, 7].
The clinical signs in dogs suffering from CGA is not specific, but rise in temperature, in appetence, lethargy, vomiting, polyarthritis, splenomegaly, lymphadenopathy and anemia had been documented earlier [8, 9, 10, 11, 12] and cases of co infections with *Borrelia burgdorferi*, *Babesia* species and tick borne encephalitis virus also had been described [13, 14, 15].
The infective stage of the bacterium is elementary bodies or morules in cytoplasm of neutrophils and sometimes eosinophil's which is seen 4 to 18 days after infection and will be visible only for a short period of time usually 4 to 8 days [3]. Even though the presence of infective stage of this bacterium has been evident in neutrophils- in peripheral blood smears prepared from many of the canine patients-suspected for tick transmitted diseases; it is very difficult to arrive at a confirmatory and accurate diagnosis. The detection of infective stage of *A. phagocytophilum* morulae in granulocytes can give a clue to identify the disease [3]. But for a more reliable diagnosis additional diagnostic test like, indirect immunofluorescence, PCR and isolation need to be conducted [16].
Confirmatory and early diagnosis based on accurate and sensitive molecular methods for tick borne diseases like granulocytic anaplasmosis is not currently available for canine patients in Kerala state. Tentative diagnosis of tick borne diseases affecting companion animals in veterinary practice is based on clinical signs, complete blood count, serum biochemical profile, peripheral blood smear examination, antibody detection tests and history of exposure to ticks. But it is very difficult to arrive at a final diagnosis from the above said methods because clinical signs overlap in many of the diseases.

Antibody detection methods based on immune chromatography (lateral flow tests) usually gives negative results when dogs are presented in acute stage of clinical illness. It is very difficult to assess the time when detectable levels of antibodies are present in blood and do sampling at that exact time.

Conventional diagnostic methods based on microscopy will not be sufficient to differentiate between pathogens having similar infective stages; like *A.phagocytophilum* and *Ehrlichia ewingii*-which have similar infective stages inside neutrophils. Moreover a single tick bite might be transmitting multiple tick borne infections like granulocytic anaplasmosis due to *A.phagocytophilum*, granulocytic ehrlichiosis due to *Ehrlichia ewingii*, babesiosis due to *Babesia canis*/*gibsoni* and lyme disease due to *Borrelia burgdorferi*. In most of these diseases, clinical signs over lap and clinicians will be relying on one treatment protocol without actually knowing whether single or multiple pathogen is responsible for the disease. Dogs infected with *A. phagocytophilum* can also remain as asymptomatic carriers, at the same time transmitting infection between human and animal population. Even though lot of research had been conducted on canine equine as well as human granulocytic anaplasmosis abroad, not much study had been carried out in Kerala state. Moreover veterinarians and dog owners of Kerala are unaware of the zoonotic risk of this tick transmitted bacterium.

Hence this study was aimed to use Taqman based qualitative Real Time PCR (qPCR) as an aid in early, accurate and confirmatory diagnosis of granulocytic anaplasmosis among canine patients. Confirmatory diagnosis for the presence of *Anaplasma phagocytophilum* based on qPCR in dogs and in the ticks recovered from them will be the first report of its kind in the state of Kerala. This will also give a clear evidence for the carrier status of the bacterium among dog population and also the presence of infective stage of bacterium among ticks recovered from dogs in Thrissur district. This also gives an insight into the possibility of zoonosis from canine to human as well as to bovine or caprine population.

2. Materials and Methods

2.1 Samples

A total of one hundred and eighty seven (187) peripheral blood samples collected from dogs presented to the district veterinary center clinics, under department of Animal Husbandry, Thrissur District, Kerala State, with any one of the following clinical signs, *viz*; fever, lethargy, anorexia, polyarthritis, dragging of hind limbs, recurrent dermatological problems and infertility brought for treatment purpose during the period from 2014 to 2018 were included in the study. Out of 187 dogs, 48 were police dogs who had undergone training at State Dog Training School (SDTS), Kerala Police Academy (KEPA), Thrissur district during the period (2014-15 & 2015-16), 6 were police dogs maintained with District Police Chief, Thrissur City (2014-18), 6 were police dogs maintained at Central Prison, Viyyur (2014-18), Thrissur district, 6 were pups belonging to Indian breeds selected to Mounted Police Unit, Thiruvananthapuram during 2018, and remaining 94 were domesticated or owned dogs presented for treatment during the four year study period from 2014 to 2018. About twenty five (25) tick specimens collected randomly from dogs with severe tick infestation at the time of presentation, were also processed as templates for the study. From each dog the following samples were collected, peripheral blood in EDTA from ear tip for preparation of template DNA and blood smear, whole blood for hematology and serum for serum biochemistry.

Out of the 48 Police Dogs from SDTS KEPA, two dogs were sampled five times, one dog was sampled four times, three dogs were sampled three times and rest of 42 dogs were sampled two times during study period. The sampling was not done at regular intervals but whenever they were presented with any one of the clinical signs described previously. Police dogs maintained with District Police Chief, Thrissur City, with Central Prison, Indian breed pups selected to Mounted Police Unit, during 2018 and all the domesticated dogs presented were sampled only once for the purpose, as and when they are presented with any one of the clinical signs described above. Hematological and serum biochemistry parameters were also studied for all the 187 dogs on the same day when they are presented with active clinical signs.

2.2 DNA extraction

Peripheral blood in EDTA was stored at-80°C until usage and processed for preparation of template DNA as per the protocols described in DNA isolation kit for mammalian blood (Version 7) and High Pure PCR template preparation kit (Version 21-Roche Diagnostics). Ticks collected from infected dogs were stored at -80°C until usage and processed for preparation of template DNA as per protocols described in DNA isolation kit for cells and tissues (Version7&21-Roche Diagnostics). The DNA templates prepared from whole blood and ticks were stored at -80°C until usage.

2.3 Estimation of concentration of Template DNA

The DNA templates prepared from whole blood were processed for estimation of DNA concentration as per protocols described for (Invitrogen-Qbit dsDNA High Sensitivity Assay Kit) and estimated in Qbit.3.0 Fluorometer (Thermo Fischer Scientific, USA).

2.4 Primers and Probes for PCR

The pair of forward and reverse primers and probe sequences specific for a specific fragment *A. phagocytophilum* 16SrRNA gene (Accession number KM349220) described previously [1] and designed from (Tibmolbiol, Roche) was used in the study. Forward and reverse primers specific for *Anaplasma phagocytophilum*16SrRNA gene-Aph 16S-f &Aph 16S-r which produces a 97 bp amplicon with a Taqman probe Aph 16Sp labeled at the 5' and 3' ends with dye FAM (5'Caboxy Fluorescein) and BBQ respectively were used. Name and sequences of Real Time PCR primers and probes are given in table.1.

Table 1: Details of primers and probes used for qPCR

Oligo Name	Primer Sequence (5'-3')	Product Size	Target Genome	References
Aph 16S-f	cgggagaggatagcggaattc	97 bp	*Anaplasma phagocytophilum* 16SrRNA gene Accession Number KM349220	[1]
Aph16S-r	cgtcagtaccggaccagatag			
Aph 16S-p	FAM-cgccttcgccactggtgttcctcc-Q			

2.5 Optimized conditions for Real Time PCR

Format for Real Time PCR followed –with total reaction volume of 20 µl, using Fast Start Essential DNA Probes Master 2X concentration -10µl (Roche Diagnostic's, Version 6), 1 µl each of forward and reverse primer (10µM), 2 µl of probe (10µM), 1 µl of water and 5 µl of template DNA extracted from whole blood and ticks. Negative and positive controls were included in all the runs. *A.phagocytophilum* positive control was prepared by custom synthesizing entire sequence of *A.phagocytophilum* 16SrRNA gene with accession number (KM349220) and cloned into *pUGM* Plasmid (SciGenom Labs PVT Ltd, Kakkanad, Cochin). Five micro liter of clone (initial concentration-95ng/ µl) was diluted to serial one in tenths concentration and five micro liters from each 1:10 dilution was used as positive control in reaction mixture. Similarly templates prepared from whole blood samples which were found positive in the initial probe based experiment- were also used as positive controls in the subsequent experiments for qualitative detection.

Primer efficiency testing was conducted by plotting a standard curve. Custom synthesized positive clone in *pUG*M plasmid, with initial concentration 95ng/ µl was used for primer efficiency testing and standardization of cycling conditions, optimal for detection of 16S rRNA gene from suspected samples. The positive clone was diluted in serial 1:10 dilutions in PCR grade water (Roche) so as to contain one genomic equivalent (GE) per 5 µl in last dilution. Six serial dilutions in duplicate were prepared for the experiment and all the dilutions were tested in RT PCR assay to find out the limit of detection and efficiency of the primers designed.

Cycling conditions optimized for primer efficiency testing and for testing of samples after standardization with positive control, included an initial activation (preincubation) at 95° C for 10 min followed by 50 cycles of 15s denaturation at 95° C followed by a 1 min annealing -extension step at 60° C.

3. Results

3.1 Peripheral blood smear

Peripheral blood smears prepared from the dogs, stained with overnight Giemsa stain revealed the presence of inclusions or vacuoles inside the neutrophils (Fig.1&2) suggestive of morulae of *A.phagocytophilum.* Three police dogs had concurrent infection with *Babesia gibsoni* detected in blood smear as signet ring shaped merozoites inside RBC's and treated accordingly.

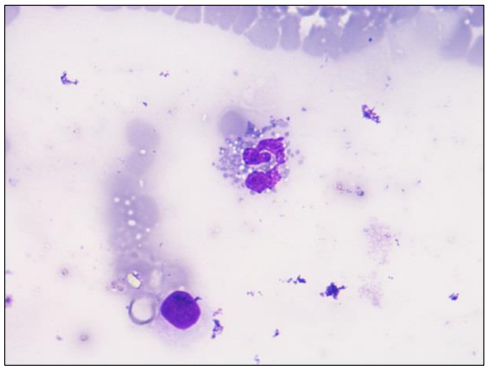

Fig 1: Giemsa stained peripheral blood smears-Morulae in neutrophil

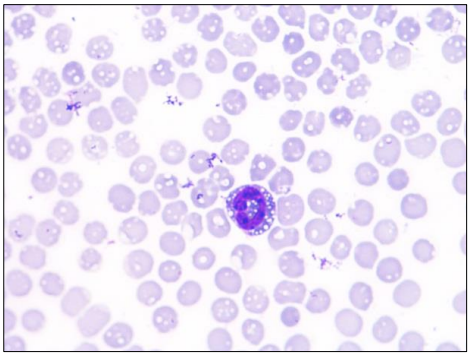

Fig 2: Giemsa stained peripheral blood smears-Intracytoplasmic vacoulations-neutrophil

3.2 Estimation of concentration of Template DNA

DNA concentrations were estimated for all the template DNA samples prepared from whole blood prior to Real Time PCR experiments.

3.3 Primers and Probes and Optimized conditions for Real Time PCR

Real Time PCR for amplifying specific fragments of the 16SrRNA gene of *A.phagocytophilum*, was used for qualitative detection of target genome in blood samples collected from suspected canine cases and in 25 tick templates collected randomly from infected dogs.The cycling conditions followed was optimal and successfully amplified *A.phagocytophilum* 16SrRNA gene with specific primers Aph16S-f &Aph16S-r along with a Taqman probe Aph 16Sp (Fig. 3).

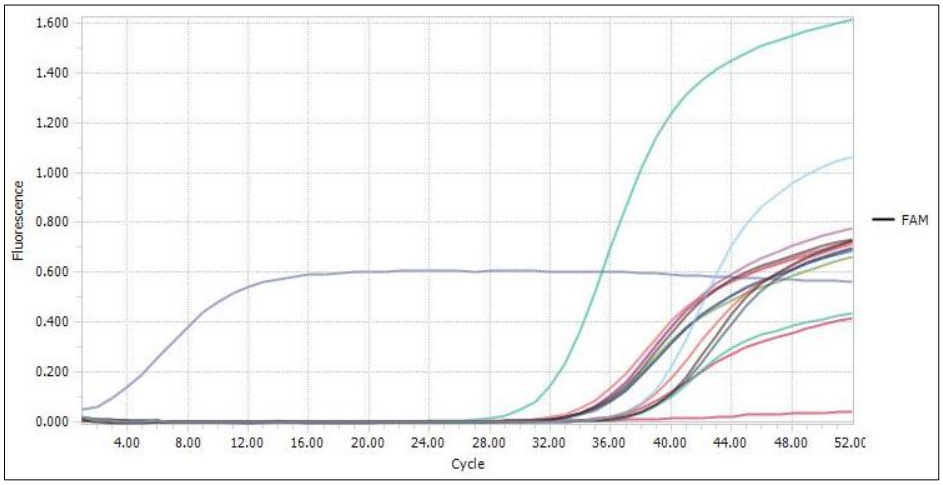

Fig.3: Amplification curve of positive test samples

3.4 Primer efficiency testing

Primer efficiency testing was conducted by plotting a standard curve, custom synthesized positive clone in *pUGM* plasmid, with initial concentration 95ng/ µl was used as template for the plotting of standard curve. Six tubes in duplicate with serial 1:10 dilutions prepared for the experiment showed, change of approximately 3.3 cycles between 10 fold dilutions of the template. The slope of the standard curve generated after primer efficiency testing showed an efficiency of 2.16 and R value 0.99 (Fig.4).

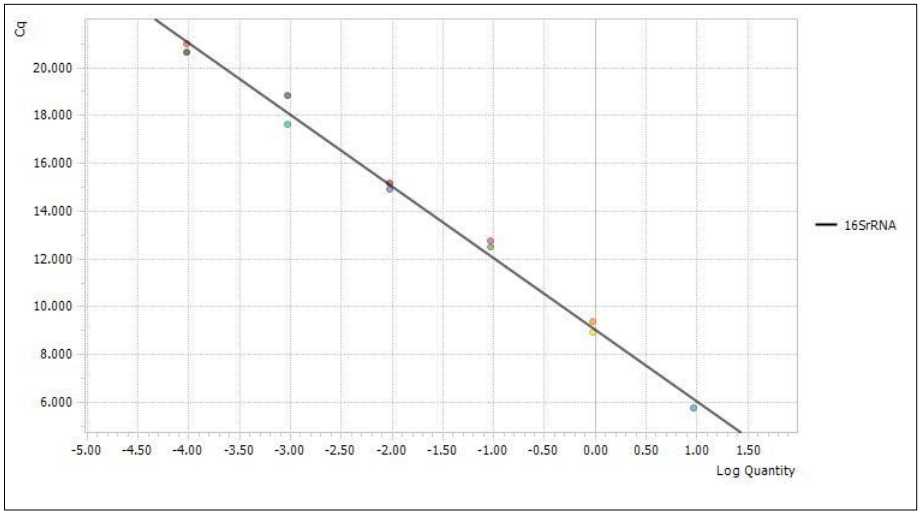

Fig 4: Slope of standard curve for primer efficiency testing- Primers used Aph16S-f & Aph16S-r along with Taqman probe Aph 16Sp

3.5 Results of the test samples

The samples were considered positive with threshold cycle (Ct) levels less than 45 (Fig.3). The average Ct value observed for 125 positive samples was 28.64+0.5 cycles ranging from 17.07 to 45.46 cycles (Fig.3).

Out of the 48 Police Dogs from State Dog Training School, Kerala Police Academy, 42 dogs (87.5%) were found to harbor *A.phagocytophilum* 16SrRNA gene, All 6 police dogs (100 %), maintained with District Police Chief, Thrissur City were found to be positive, Out of 6 dogs maintained at Central Prison, Viyyur, Thrissur, Kerala State, 4 dogs were found to be positive (66%), all 6 Indian breed pups selected for police force during 2018 turned out to be positive (100%) and out of 94 domestic dogs screened 70 dogs were found to be positive (74%). *A.phagocytophilum* 16SrRNA gene could be detected in all repeat samples attempted from positive police dogs as and when they are presented with active clinical signs.

All together 125 (67%) out of total 187 dogs and 19 tick templates (76%) out of 25 tick specimens collected randomly-from dogs with severe tick infestation screened were found to harbor *A.phagocytophilum* indicating a very high prevalence for the presence of bacterium among canine population studied.

Out of the 48 positive dogs of SDTS KEPA, two dogs which were sampled five times, one dog sampled four times, three dogs sampled three times and rest 42 dogs sampled two times whenever they were presented with active clinical signs, *A.phagocytophilum* 16SrRNA gene could be detected in all the repeat samples during a sampling period of 9 months. This indicates a carrier status for the bacterium with occasional presence in the blood stream even though there is remission of clinical signs after the treatment period. Repeat sampling could not be carried out at regular intervals because, the protocol followed was to take peripheral and whole blood sample as when the animals were presented with active clinical signs.

3.6 Hematological and serum biochemical parameters

There were not many abnormalities in hematological and serum biochemical parameters studied, except for three police dogs belonging to SDTS KEPA, which were affected with acute canine babesiosis due to *Babesia gibsoni* as concurrent infection with *A.phagocytophilum* during their training period. These dogs had significantly reduced hemoglobin values (2-4g %), low PCV (10 %) and reduced platelet count (50-100 X 10^9/L), but responded very well to the treatment protocol followed. Generally for police or owned dogs studied, platelet counts were in the range of 50-250X10^9/L, which was the only hematological parameter noticed slightly deviating from normal value.

There was not much abnormality in serum biochemical parameters (liver function tests-AST, ALT, ALP, total bilirubin, total protein and Albumin Globulin ratio) studied because most of the dogs were presented with acute clinical signs and were treated immediately for the cause with oral doxycycline@10mg/kg for 30 days along with liver correctives (LIV 52 suspension–HIMALAYA), hematinics (aRBC PET-Vetoquinol, India) and suspensions containing all essential amino acids /vitamins orally. All the animals responded very well to the treatment protocol followed and recovered fully, except that they had recurrent infection with *A.phagocytophilum.* Recurrence could not be associated with any specific periodicity, but varies in individual dogs depending upon the environmental or managemental stress they are exposed to. But once accurate diagnosis was made, treatment protocol followed was successful with 100 % remission of clinical signs for the prescribed time period.

The clinical signs described previously viz; fever, lethargy, anorexia, polyarthritis, dragging of hind limbs and recurrent dermatological problems in canines can be suggestive of CGA and the results of present study indicate that the Taqman based qualitative Real Time PCR (qPCR) can be used as an accurate and confirmatory diagnostic method to detect the

presence of *A.phagocytophilum* in the peripheral blood samples very well before the changes in hematological and serum biochemistry values are evident.

4. Discussion

Ehrlichia and *Anaplasma* bacteria are transmitted by ticks which become affected after feeding on infected animals. Humans, cats and other domestic animals can occasionally become infected, but dogs are the main hosts [17]. The predilection site for *A.phagocytophilum*, the neutrophil, indicates that the pathogen has unique adaptations and pathogenesis mechanisms. As per descriptions by earlier workers interactions with host-cell signal transduction and possibly eukaryotic transcription had been documented. This interaction leads to permutations of neutrophil function and could permit immunopathologic changes, severe disease, and opportunistic infections [18].

In the present study the clinical signs exhibited by dogs with CGA and co infections with *B. gibsoni* is in concurrence with the studies documented earlier [8, 9, 10, 13, 14, 15]. Three police dogs were showing acute clinical signs of babesiosis due to *Babesia gibsoni* as concurrent infection with *A.phagocytophilum*.

Peripheral blood smears prepared from the dogs, stained with overnight Giemsa stain revealed the presence of inclusions or vacuoles inside the neutrophils suggestive of morulae of *A.phagocytophilum* which is sufficient to identify the disease [3]. Earlier workers had described presence of between 5% and 37% infected granulocytes in dogs with clinical signs of *A. phagocytophilum* infection [16, 19, 20, 22]. As per ref [3] the elementary bodies or morules in cytoplasm of neutrophils and eosinophil's is seen 4 to 18 days after infection and will be visible only for a short period of time usually for 4 to 8 days.

Detection and quantification of *A.phagocytphilum* and *Babesia spp* in *Ixodes ricinus* ticks by real time PCR was reported earlier [1] and primers specific, Aph 16S-f and Aph-16S-r, targeting 16SrRNA gene, which is a highly conserved sequence for *A.phagocytophilum*, produced a 97 bp amplicon with a Taqman probe Aph 16Sp. Among 1875 ticks (1084 adults and 791 nymphs) collected, 0.9% was found to be infected with *A.phagocytophilum* and 2.5% with *Babesia spp.*

In the present study also the set of primer pairs and probes described [1] could successfully amplify 16SrRNA gene of *A.phagocytophilum* from clinical samples. Molecular detection of *A. phagocytophilum* targeting full length 16SrRNA gene using fD1 and Rp2 primers from a clinical case of canine granulocytic anaplasmosis also had been described [23].

Molecular detection of *A.platys* and *A.phagocytophilum* using primers targeting 16SrRNA and *groESL* gene using nested primers had been documented [24] in dogs in Colombia. One (1.1%) of the 91 sampled dogs showed inclusions suggestive of *Anaplasmataceae.* Nine hundred and seventy-four dogs with clinical signs suspicious for canine granulocytic anaplasmosis were tested for *A. phagocytophilum* DNA by modified real-time PCR out of which 72 dogs (7.4%) turned out to be positive [27]. Detection of *Ixodid* tick transmitted multiple pathogens including *Anaplasma phagocytophilum* targeting 16SrRNA gene by a Multiplex Real-Time Reverse Transcription-PCR Assay was also reported earlier [28].

A multiplex real-time quantitative PCR assay for human infection by *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* was documented [29] and they reported clinical sensitivity and specificity of 100% (39/39) and 100% (143/143), respectively for *A. phagocytophilum* and 95% (20/21) and 99% (159/161) for *E. chaffeensis*. They concluded that these assays support early diagnosis, treatment and high through put testing required for large epidemiologic studies. The use of sensitive multiplex PCR assays to differentiate Lyme spirochetes and emerging tick borne pathogens like *A. phagocytophilum* and *B. microti* were reported [33] Anaplasma citrate synthase gene gltA were also targeted for detection of *A. phagocytophilum* in *Ixodes ricinus* ticks [34].

The standard curves in absolute qPCR can be generated using circular plasmids, and a linear relationship can be established between C_t and $\log_{10}$ values for primer efficiency testing and also to estimate the abundance of microorganisms in tick specimens [1, 2]. In the present study also circular *pUGM* plasmid inserted with target sequence was used as positive control for primer efficiency testing, to generate slope of the standard curve.

All together 125 (67%) out of total 187 dogs and 19 tick templates (76%) out of 25 tick specimens were found to harbor *A.phagocytophilum* showing a very high prevalence of the bacterium when compared to earlier studies [1, 24, 27, 28] among canines and in ticks recovered from them in Thrissur district. The presence of pathogen in ticks also indicates that a significant relationship exists between demonstration of pathogen in ticks and expression of disease in dogs warranting a detailed study, including more number of tick specimens in concurrence with the number of clinical cases taken for the study. Here only few tick samples could be collected even though a total of 187canine clinical cases were included. This is because ticks were not always visible in their body at the time of presentation, even though previous exposures to ticks were confirmed by their owners; well before the clinical signs were manifested.

The result of this study *i.e.* high prevalence of *A.phagocytophilum* infection among dogs and ticks is very relevant because climatic change and human pressure on environment may result in increased prevalence of ticks and tick borne infections [25]. Moreover climatic conditions in Kerala, high temperature and humidity is ideal for survival and breeding of ticks [26] and risk of acquiring infection to dogs is closely related to prevalence of pathogens in active ticks and thereby raising a potential zoonotic risk to dog owners and handlers. Prevalence studies of *A.phagocytophilum* infection among ticks targeting *Msp2* gene using real time PCR in an urban and natural habitat in South-Western Slovakia had been studied earlier [31] and confirmed that *Ixodes ricinus* populations are harboring the pathogen at a higher rate than in natural habitat.

The results of study by earlier workers [30] showed that *A. phagocytophilum* MSP4 and HSP70 are involved in host-pathogen interactions, with a role for HSP70 during pathogen infection. All the positive templates obtained in the present study must be subjected further to Real Time PCR targeting genes coding for MSP2 and MSP4 surface proteins as part of pathogenicity studies.

In conclusion the present study indicates that besides being supportive in early, accurate and confirmatory diagnosis of CGA in dogs with acute clinical signs, Taqman based qPCR also can be recommended for identifying the carrier status among clinically normal dogs, so that, occurrence of the disease can be anticipated by the owner at any time during the lifespan of the dog and necessary precautions can be taken by the owner to prevent disease transmission by accidental tick

bite. To our knowledge this is the first report of molecular diagnosis of CGA due to *A. phagocytophilum* and the presence of *A. phagocytophilum* in ticks recovered from infested dogs in the state of Kerala.

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