SHORT COMMUNICATION

Clinico-pathological and Molecular Findings in Canine *Anaplasma phagocytophilum* Infection and its Therapeutic Management

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Abstract

A 4-year-old female Golden Retriever presented to the Veterinary Clinical Complex of the College revealed the clinico-pathological findings such as fever, tachycardia, tachypnea, pale mucous membranes, dehydration, haemorrhage, hind limb pain; anaemia, leukocytosis, lymphocytosis, monocytopenia, eosinopenia; elevated ALT, ALP, total bilirubin, indirect bilirubin, BUN, and CRP; with proteinuria, bilirubinuria, and haematuria. The oxidative stress indices revealed decreased TAOA and SOD, and increased LPO levels. Blood smear examination revealed the presence of intracytoplasmatic inclusions in neutrophils associated with high positive serology for *Anaplasma phagocytophilum*. Species-specific PCR analysis confirmed serological diagnosis of A. *phagocytophilum*. Data indicates that *A. phagocytophilum* circulates in natural environments of the northeast region of India and its prevalence in dogs could be underestimated because the clinical signs are frequently nonspecific and a certain diagnosis requires the combination of clinico-pathological and molecular assays. Pets living in this area should be regularly monitored and treated for ectoparasites to minimize health risks for humans and pets.

Key words: *A. phagocytophilum*, Anaemia, Doxycycline, Morulae, PCR, Rapid antibody detection, Thrombocytopenia.

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Introduction

Anaplasmosis is a tick-borne disease caused by Gram-negative, obligate, intracellular bacteria belonging to the genus *Anaplasma* under the family *Anaplasmataceae* (Dumler *et al*., 2001). In dogs, *Anaplasma* organisms mainly infect neutrophils and platelets and characterized by basophilic inclusions in the cells, so-called morulae, which contain one or more subunits (Arraga-Alvarado *et al*., 2003). The disease is characterized by thrombocytopenia. However, immune-mediated mechanisms of thrombocytopenia become more important in subsequent thrombocytopenic episodes (Thakur *et al*., 2019). In general, the infection is accompanied by unspecific and mild clinical manifestations including anorexia, depression, generalized lymph node enlargement, pale mucous membranes, elevated rectal temperatures, lameness, vomiting, and epistaxis (Kohn *et al*., 2008; Eberts *et al*., 2011). It is transmitted by *Ixodes* spp., *Dermacentor* spp., and *Haemaphysalis* spp. (Lee *et al*., 2016). Additional diagnostic procedures in clinical practice are frequently necessary to reach a correct diagnosis of canine anaplasmosis. In this study, a case of canine granulocytic anaplasmosis has been documented by complete clinical and clinico-pathological description and by molecular investigation of the etiological agent with therapeutic approach.

Case History and Observations

A 4-year-old, female Golden Retriever dog was presented to Teaching Veterinary Clinical Complex, CAU, Selesih, Mizoram, India in April, 2023 with a history of inappetence, lethargy, bloody diarrhoea, haematemesis and lameness. Owner also reported the history of ticks. The dog was regularly vaccinated and dewormed.

The dog examined clinically, revealed fever (103.2° F), tachycardia (130 beats/min), tachypnoea (60 breaths/min), lethargy, pale mucous membranes, dehydration, cutaneous petechial haemorrhages and hind limb pain. No abnormalities were noticed on thoracic auscultation. Ultrasonographic examination revealed heterogenecity of both liver and spleen (Fig. 1A & 1B), and cystitis where thickness of the urinary bladder wall was 0.43 cm (Fig. 1C).

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Haematology and serum biochemistry were performed at days 0, 14, and 28 for therapeutic evaluation using an automated haematology system (MS4s® haematology analyzer, France) and a chemistry analyzer (Fujifilm dry chem. NX700, Japan). CRP and oxidative stress indices (TAOA, LPO, and SOD) were also done with the help of commercial kits. The haemato-biochemical findings of the laboratory variables observed are presented in Table 1. At the blood smear examination, a mean of 10% of neutrophils presented cytoplasmic inclusions that were characterized by blue-violet aggregates of punctiform bodies, coherent with morulae of *A. phagocytophilum* (Fig. 2A & 2B). Final interpretation of the blood report was leukocytosis, lymphocytosis, monocytopenia, eosinopenia, and thrombocytopenia. Serum biochemistry indicated elevated liver enzymes (ALT, ALP), total bilirubin, indirect bilirubin, BUN. CRP and oxidative stress indices, *viz.*, LPO were increased with decreased levels of TAOA and SOD in *Anaplasma phagocytophilum* infected dog on day of hospitalization. Urine analysis done with help of urine analyser (Urit 50, Biogeny Pvt. Ltd. India) revealed proteinuria, bilirubinuria, and haematuria.

Thin blood smear was prepared and stained with Giemsa stain for cytological examination. A serological study conducted using rapid antibody test kit (PetX, England) for detection of vector-borne infections - *Ehrlichia canis*, *Anaplasma spp.*, *Babesia gibsoni*, and *B. Canis*, revealed it positive for *Anaplasma* spp. and negative for *E. canis, B. gibsoni, B. canis* (Fig. 3). It was also confirmed by species-specific primer of *A. phagocytophilum* in PCR assay (Fig. 4).

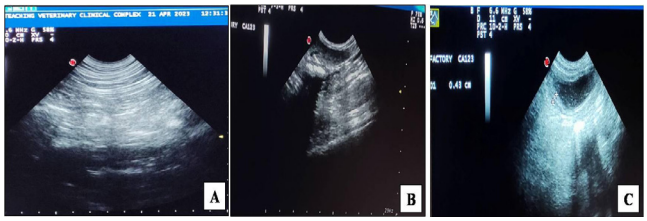

Fig.1: USG examination of infected dog revealed: A) Heterogenicity of liver, B) Heterogenecity of spleen, and C) Cystitis (urinary bladder wall thickness - 0.43 cm)

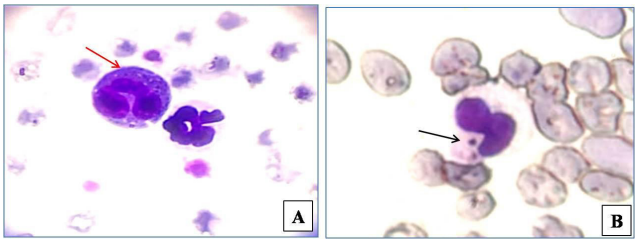

Fig. 2: Blood smear (Giemsa staining) showing *A. phagocytophilum* inclusion bodies (A: red arrow) and morulae (B: black arrows) in the cytoplasm of neutrophil granulocytes of infected dog.

Table 1: Haemato-biochemical parameters in dog with granulocytic anaplasmosis

Parameters	Day hospitalized	14th day post-therapy	28th day post-therapy	Reference range *
Hb (g/dL)	6.2	8.4	9.8	11.9-18.9
PCV (%)	17.7	25.2	30.6	35-57
TEC (millions/mm^3)	2.7	3.8	5.28	4.95-7.87
TLC (x 10^3 cells/mm^3)	22.93	13.5	10.6	5-14.1
Neutrophils (%)	60	73.6	65.8	58-85
Lymphocytes (%)	34.7	16.4	19.3	8-21
Monocytes (%)	1.8	5	6.4	2-10
Eosinophils (%)	1.7	9	7.9	0-9
Basophils (%)	0.9	1	0.6	0-1
Platelets (lakhs/mm^3)	0.93	2.4	2.85	2.11-6.21
Total protein (g/dL)	5.3	5.8	5.9	5.4-7.5
Albumin (g/dL)	2.2	2.5	2.4	2.3-3.1
Total bilirubin (mg/dL)	0.4	0.2	0.2	0-0.3
ALT (U/L)	117	87	91	10-109
ALP (U/L)	148	54	46	1-114
BUN (mg/dL)	29	21	14	8-28
Creatinine (mg/dL)	1.1	0.9	1.2	0.5-1.7
C-reactive protein (µg/mL)	48	12	0	2-9
TAOA	0.47	0.68	1.31	-
LPO	2.01	1.35	1.02	-
SOD	0.64	0.96	1.27	-

*The Merck Veterinary Manual (2016), 11th edn.

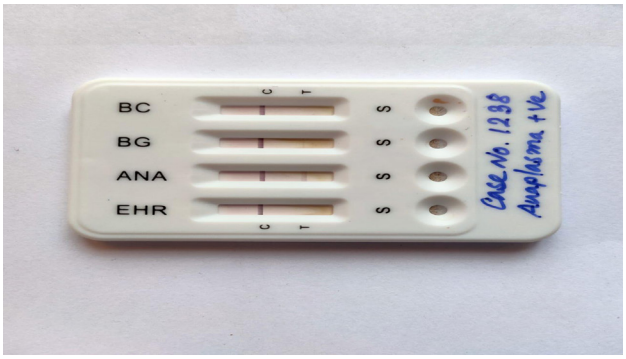

Fig. 3: Rapid diagnostic kit showed *Anaplasma* positive

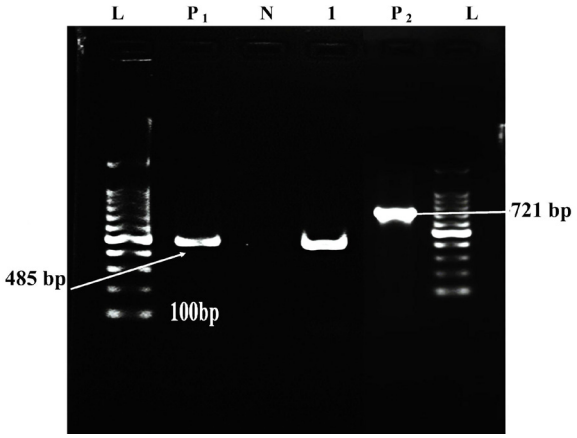

Fig. 4: Amplification of 16S rDNA gene of *A. phagocytophilum:* L denotes 100 bp DNA ladder, P_1 positive control for *A. phagocytophilum* (485 bp), and P2 positive control for *A. platys*

N negative control (no template), and 1 positive sample for *A. phagocytophilum* (485 bp)

For molecular diagnosis, DNA extraction was carried out from 200 µL of whole blood using the QIAamp DNA Blood Mini Kit (Qiagen) as per manufacturer's instruction and PCR assay was performed for confirmation of both *Anaplasma platys* and *A. phagocytophilum* using species-specific primers. A nested PCR approach was employed for the identification of *A. platys*. In the first round, primers 8F (5'-AGT TTG ATC ATG GCT CAG-3') and 1448R (5'-CCA TGG CGT GAC GGG CAG TGT G-3') were used to amplify 16S rRNA genes. This was followed by a second round of PCR utilizing an *A. platys*-specific primer, PLATYS (5'-GAT TTT TGT CGT AGC TTG TG-3'), and an *Ehrlichia* genus-specific primer, EHR16SR (5'- TAGCACTCATCGTTTACAGC-3'), resulting in a 678 bp amplicon as outlined by Martin *et al.* (2005). The first round of amplification involved initial denaturation at 94°C for 5 minutes followed by 40 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min 40 sec at 72°C with an additional 5 min extension at 72°C. For the second round of amplification, the cycle profile included an initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, with a final extension for 5 min at 72°C. And for *A. phagocytophilum* a semi-nested PCR approach was used which amplified 16S rDNA species-specific gene fragment of approximately 485 bp. In the first round, primers A480F and A900R were employed. For the second round of amplification, primer A520F and A900R were used as described by Werszko *et al.* (2019). Amplification conditions included an initial denaturation for 5 min at 94°C followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min, and final elongation for 5 min at 72°C in thermocycler. The reactions were conducted in 25 µL reaction mixture containing 12.5 µL of commercially available PCR master mix (Takara EmeraldAmp® GT PCR Master Mix), forward and reverse primers at a concentration of 10 pmol each, 2 µL of the extracted DNA template, and the remaining volume was adjusted with nuclease-free water. The second round of amplification involved 1 µL of first round PCR amplified product used as template DNA within another 25 µL reaction.

Treatment and Discussion

The dog was hospitalized and immediately treated with fluid therapy, pantoprazole, ondansetron, prednisolone, ethamsylate for symptomatic relief, and a 28-day course of doxycycline @ 10 mg/kg B.W orally daily along with liver support and haematinic. By the 14th day, the dog exhibited improved activity, normal appetite, and resolution of clinical signs. Haematological parameters returned to normal, although a mild decrease in haemoglobin and PCV persisted. Proteinuria also observed in urine analysis (Table 1). Blood smear examination was negative, but PCR remained positive. Subsequent evaluations revealed no clinical or laboratory abnormalities on 28 days confirming the successful management of *Anaplasma phagocytophilum* infection in the Golden Retriever.

Clinical pathology of canine anaplasmosis caused by *A. phagocytophilum* varies from a subclinical infection to an acute febrile condition. The majority of naturally infected dogs with *A. Phagocytophilum* likely remain healthy (Beall *et al.,* 2008). Clinical manifestations of anaplasmosis can vary, but the disease typically manifests as an acute febrile syndrome characterized by elevated body temperature, lethargy, anorexia, and reluctance to move (Harrus *et al.,* 2005). Occasionally, less common clinical signs in dogs infected with *A. phagocytophilum* include polydipsia, pale mucous membranes, vomiting, diarrhoea, as well as haemorrhagic manifestations like mucosal petechiae, melena, or epistaxis (Poitout *et al.,* 2005) which were also observed in this case probably due to coagulation abnormalities (Rikihisa, 2011). According to Carrade *et al.* (2009) anaemia is frequently associated with canine granulocytic anaplasmosis (CGA) that was consistent with our findings.

Haematological abnormalities with CGA included left-shift regeneration of neutrophils, lymphocytosis, eosinopenia, monocytosis, monocytopenia, neutropenia, and neutrophilia and thrombocytopenia as the most noticeable haematological alterations (Ravnik *et al.,* 2011). Thrombocytopenia was also reported as the most common finding by Chirek *et al.* (2018). Thrombocytopenia may result

from immune-mediated platelet destruction, disseminated intravascular coagulation, sequestration in the spleen, or the production of inhibitory factors (Almizraq and Branch, 2021). Elevated liver enzyme levels and hyperbilirubinemia were also documented by Chirek *et al*. (2018) which indicated the pathological effect of *A*. *phagocytophilum* on hepatic dysfunction. The rise in BUN level suggests possible indirect damage to renal tissue, along with the release of globulin catabolites from erythrocyte breakdown via erythrophagocytosis (Alsaad, 2009).

Elevated CRP values observed in our study align with findings reported by Pantchev *et al*. (2009). CRP serves as an unbiased indicator of inflammatory activity of *A*. *Phagocytophilum*, providing clinically relevant information that goes beyond what is offered by a complete blood count (CBC) and clinical observations. Increased LPO is significantly influenced by *Anaplasma* spp. infection, leading to an excess of free radicals and a decline in antioxidant levels. This suggests a direct correlation between anaplasmosis severity, oxidative stress, and the reduction of antioxidant reserves. The study indicates that LPO plays a significant role in anaemia development, while antioxidants help protect erythrocytes by mitigating oxidative stress. The observed negative association between LPO and anaemia, along with the positive correlation between SOD, catalase, and anaemia, supports these findings.

Diagnostic testing has variable utility in acute presentations. The gold standard diagnosis is IgG serologies which carry a sensitivity > 95% but are often negative in the first ten days of illness (Bakken *et al.,* 2002; CDC, 2016). PCR has a high sensitivity during the first week of infection especially within four days of symptom onset (Schotthoefer *et al.,* 2013). But, in most areas of the Northeast region of India, PCR testing is not widely available and impacting treatment decisions (CDC, 2016). Initiation of doxycycline administration can act as a substitute marker for diagnosis as patients often respond rapidly to treatment within 2-3 days (CDC, 2016). Blood smears provide a widely available option and can show morulae or inclusion bodies during acute presentations; however they have a variable sensitivity around 50-60% (Schotthoefer *et al.,* 2013).

This immune-mediated pathogenesis has important clinical implications and suggests that immunosuppressive therapy when combined with antibiotics may be beneficial in severe anaplasmosis. The combination of doxycycline and dexamethasone treatment improved the clinical signs, haemato-biochemical parameters, and oxidative stress indices after 14 days onwards of post-therapy, as was also reported by Divya (2023) and Melter *et al.* (2007). Therefore, it can be concluded that immunosuppressive agents may be safe and beneficial when combined with doxycycline in severe anaplasmosis.

In areas where the prevalence of the disease is unclear, it is advisable to consider anaplasmosis in dogs as well as in other animals that have a history of tick exposure. The disease is important because it threatens animals as well as humans because of its zoonotic potential.

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References

Almizraq, R.J., & Branch, D.R. (2020). Efficacy and mechanism of intravenous immunoglobulin treatment for immune thrombocytopenia in adults. *Annals of Blood*, *6*, 1-20.

Alsaad, K.M. (2009). Clinical, haematological and biochemical studies of anaplasmosis in Arabian one-humped camels (*Camelus dromedaries*). *Journal of Animal and Veterinary Advances, 8*(11), 2106-2109.

Arraga-Alvarado, C., Palmar, M., Parra, O., & Salas, P. (2003). *Ehrlichia platy* (*Anaplasma platy*) in dogs from Maracaibo, Venezuela: An ultrastructural study of experimental and natural infections. *Veterinay Pathology*, *40,* 149-156.

Bakken, J.S., Haller, I., Riddell, D., Walls, J.J., & Dumler, J.S. (2002). The serological response of patients infected with the agent of human granulocytic ehrlichiosis. *Clinical Infectious Diseases, 34,* 22-27.

Beall, M.J., Chandrashekar, R., Eberts, M.D., Cyr, K.E., Diniz, P.P.V., Mainville, C., & Breitschwerdt, E.B. (2008). Serological and molecular prevalence of *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Ehrlichia* species in dogs from Minnesota. *Vector Borne Zoonotic Diseases*, *8*(4), 455-464.

Carrade, D.D., Foley, J.E., Borjesson, D.L., & Sykes, J. E. (2009). Canine granulocytic anaplasmosis: A review. *Journal of Veterinary. Internal Medicine*, *23*(6), 1129-1141.

CDC (2016). Diagnosis and Management of Tickborne Rickettsial Diseases: Rocky Mountain Spotted Fever and Other Spotted Fever Group Rickettsioses, Ehrlichioses, and Anaplasmosis - United States. Center for Disease Control and Prevention MMWR, 2016; pp. 65. https://doi.org/10.15585/mmwr.rr6502a1

Chirek, A., Silaghi, C., Pfister, K., & Kohn, B. (2018). Granulocytic anaplasmosis in 63 dogs: clinical signs, laboratory results, therapy and course of disease. *Journal of Small Animal Practitioner, 59*(2), 112-120.

Divya, V. (2023). Diagnosis of *Anaplasma platys* infection in a dog from Palakkad district, Kerala by direct microscopy and real time PCR: A case report. *Pharma Innovation, 12*(4), 5-7.

Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A. , Palmer, G.H., Ray, S.C., Rikihisa, Y., & Rurangirwa, F.R. (2001). 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*, descriptions of six new species combinations and designation of *Ehrlichiaequi* and ‹HGEagent› as subjective synonyms of *Ehrlichia phagocytophila*. *International Journal of Systematic and Evolutionary Microbiology, 51*(6), 2145-2165.

Eberts, M.D., Vissotto de Paiva Diniz, P.P., Beall, M.J., Stillman, B.A., Chandrashekar, R., & Breitschwerdt, E.B. (2011). Typical and atypical manifestations of *Anaplasma phagocytophilum* infection in dogs. *Journal of American Animal Hospital Association*, *47*(6), e86-e94.

Harrus, S., Waner, T., Bjoersdorff, A., & Shaw, S.E. (2005). Ehrlichiosis and anaplasmosis. In: Shaw, S.E., Day, M.J. (Eds.), *Arthropod-*

borne Infectious Diseases of the Dog and Cat. Manson Publishing, London, pp. 120-133.

Kohn, B., Galke, D., Beelitz, P., & Pfister, K. (2008). Clinical features of canine granulocytic anaplasmosis in 18 naturally infected dogs. *Journal of Veterinary Internal Medicine, 22*(6), 1289-1295.

Lee, S., Lee, S.H., VanBik, D., Kim, N.H., Kim, K.T., Goo, Y.K., & Kwak, D. (2016). First molecular detection and phylogenetic analysis of *Anaplasma phagocytophilum* in shelter dogs in Seoul, Korea. *Ticks and Tick Borne Diseases*, *7*(5), 945-950.

Martin, A.R., Brown, G.K., Dunstan, R.H., & Roberts, T.K. (2005*). Anaplasma platys:* An improved PCR for its detection in dogs. *Experimental Parasitology, 109*(3), 176-180.

Melter, O., Stehlik, I., Kinska, H., Volfova, I., Ticha, V., & Hulínska, D. (2007). Infection with *Anaplasma phagocytophilum* in a young dog: A case report. *Veterinarni Medicina*, *52,* 10.17221/2001-VETMED.

Pantchev, N., Schaper, R., Limousin, S., Norden, N., Weise, M., & Lorentzen, L. (2009). Occurrence of *Dirofilaria immitis* and tick-borne infections caused by *Anaplasma phagocytophilum, Borrelia burgdorferi sensulato* and *Ehrlichia canis* in domestic dogs in France: Results of a countrywide serologic survey. *Parasitology Research, 105,* 101-114.

Poitout, F.M., Shinozaki, J.K., Stockwell, P.J., Holland, C.J., & Shukla, S.K. (2005). Genetic variants of *Anaplasma phagocytophilum* infecting dogs in Western Washington State. *Journal of Clinical Microbiology*, *43*(2), 796-801.

Ravnik, U., Tozon, N., Smrdel, K.S., & Zupanc, T.A. (2011). Anaplasmosis in dogs: The relation of haematological, biochemical and clinical alterations to antibody titre and PCR confirmed infection. *Veterinary Microbiology*, *149*(1-2), 172-176.

Rikihisa, Y. (2011). Mechanisms of obligatory intracellular infection with *Anaplasma phagocytophilum*. *Clinical Microbiology Reviews, 24*(3), 469-489.

Schotthoefer, A.M., Meece, J.K., Ivacic, L.C., Bertz, P.D., Zhang, K., Weiler, T., Uphoff, T.S., & Fritsche, T.R. (2013). Comparison of a real-time PCR method with serology and blood smear analysis for diagnosis of human anaplasmosis: Importance of infection time course for optimal test utilization. *Journal of Clinical Microbiology, 51,* 2147-2153.

Thakur, N., Chethan, G.E., Kumar, A., Gaykwad C., Reena K.K., Kumar A., Rajesh J.B., De, U.K., Mahendran K., & Banerjee P.S. (2019). Concurrent infection of *Leptospira icterohaemorrhagiae*, *Hepatozoon canis* and *Anaplasma phagocytophilum* in a Labrador retriever dog and its therapeutic management. *Comparative Clinical Pathology, 28*, 1845-1850

Waner, T., Harrus, S., Weiss, D.J., Bark, H., & Keysary, A.(1995). Demonstration of serum antiplatelet antibodies in experimental acute canine ehrlichiosis. *Veterinary Immunology and Immunopathology, 48*(1-2), 177-182.

Werszko, J., Szewczyk, T., Steiner-Bogdaszewska, Z., Laskowski, Z., & Karbowiak, G. (2019). Molecular detection of *Anaplasma phagocytophilum* in blood-sucking flies (Diptera: Tabanidae) in Poland. *Journal of Medical Entomology*, *56*(3), 822-827.