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Original Article

Molecular Detection of *Cytauxzoon* Spp. Infection and Haemato-Biochemical Alterations Associated with It in Domestic Cats

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Abstract

Background: Cytauxzoonosis is a tick borne hemoprotozoal disease of wild and domestic felids. Its epidemiology and clinical importance is less appreciated in India. The aim of this study was to diagnose the disease in domestic cats and identify the associated clinico-haemato-biochemical changes.

Methods: Three hundred ninety domestic cats, presented at Veterinary Clinical Complex, Bihar Veterinary College and three private clinics in Bihar, India were screened, out of which 90 cats showing symptoms of cytauxzoonosis were included in microscopic and molecular examination. The clinical, haematological and biochemical findings of the positive cats were recorded.

Results: The microscopic and molecular prevalence was 26.66% (24/90) and 37.77% (34/90) respectively. Lethargy, fever, anorexia, tachypnea and tachycardia were the most common clinical findings. The significant haematological changes recorded were decreased haemoglobin, haematocrit, TEC and thrombocytopenia. The significant biochemical changes were increased ALT and AST activities, hyperbilirubinemia, hypoproteinaemia with hypoalbuminemia and increased BUN concentration.

Conclusion: The infection of *Cytauxzoon* sp. has reached the domestic cats of Bihar, India which was earlier thought to be endemic in North America. The clinical manifestations and hemato-biochemical changes are somewhat similar to other hemoprotozoan diseases. Molecular diagnosis is inevitable for confirmation of infection. Further studies are required to reveal the vector responsible for transmission of the parasite in India.

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Introduction

Cytauxzoonosis is an emerging disease, initially considered as 100% fatal, has also manifested as subclinical chronic infection in domestic cats. It is caused by an intracellular blood parasite *Cytauxzoon* spp. which firstly infects leukocytes and then enters erythrocytes in subsequent stage. *Cytauxzoon* spp. is an apicomplexan protozoon of the order Piroplasmida and family Theileriidae (1). It infects both domestic and wild felids and first time reported in domestic cats from the United States of America in the year 1976 by Wagner (2). *Cytauxzoon* organism has two host life cycle, a tick and a felid for sexual and asexual reproductive processes, respectively (3). In vertebrate hosts, it has a commencing tissue phase where schizonts are present within macrophages of blood vessels' lining followed by an erythrocytic phase (4). Generally, the schizogenous phase causes vascular occlusion in vital organs such as the lungs, liver, kidney, and spleen and thus is responsible for the occurrence of a rapidly-progressive systemic disease and death within 3 week post-infection (5). The most common clinical findings in affected cats are anorexia, depression, anemia, vomiting, icterus, and high fever. Non-regenerative anemia, thrombocytopenia, neutrophilic leukocytosis, hyperproteinemia, hypoalbuminemia, and hyperbilirubinemia are the major haemato-biochemical changes identified in the clinically infected cats.

Cytauxzoonosis in domestic cats was only reported in North America and South America initially, but in recent years the disease has also been registered in Europe and Asia. *Cytauxzoon* spp. of Europe appear to be less virulent than the *C. felis* in domestic cats in the USA based on physical examination and laboratory findings. Cats infected with the European *Cytauxzoon* spp. are usually presented with subclinical infections and seldom associated with mild anemia (6). There is limited information on cytauxzoonosis in domestic cats from Asia and very less study has been done on this disease in India.

Hence, the present study was undertaken with an aim to describe the clinical, haematological and biochemical manifestations of cytauxzoonosis affected cats in Patna, Bihar, India.

Materials and Methods

Area and animal of study

A total of 390 cats presented at Veterinary Clinical Complex, Bihar Veterinary College, Patna and at three private clinics in Patna were screened for the previously defined symptoms of cytauxzoonosis namely anorexia, vomition, fever, icterus, dyspnea, tachycardia, lethargy, dehydration, vocalization and diarrhoea from August, 2023 to January, 2024 (six months). Among them 90 cats showing two or more of these symptoms, irrespective of their age, sex, or breed were suspected for cytauxzoonosis and their blood sample were sent for blood smear examination and PCR detection. The work was done in an ethical manner complying with all relevant codes of experimentations and legislations.

The experimental protocol was approved by institutional animal ethical committee, Bihar Animal Science University, India (vide letter no- F.26-1/2022-23/DR). The owner's consent was taken for every case included in this study.

Sample collection

Total 3 ml blood was collected from the lateral or medial saphenous vein of all the cats suspected for cytauxzoonosis. Out of that 1 ml was taken in EDTA containing vial for smear examination, haematology, and DNA extraction and 2 ml in the plain vial for biochemical tests.

Analytical methods

Haematological estimation for Haemoglobin (Hb), Haematocrit, Total Erythrocyte

Count (TEC), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin Concentration (MCHC), Total Leukocyte Count (TLC), Absolute Neutrophil count (ANC), Absolute Lymphocyte count (ALC), Platelets Count, was done using automated hematology analyser (Nihon Kohden Celltac). Biochemical estimation for Alanine Transaminase (ALT), Aspartate Aminotransferase (AST), total protein, albumin and globulin, total bilirubin, direct and indirect bilirubin, Blood Urea Nitrogen (BUN) and creatinine were estimated using standard kits manufactured by coral clinical systems and semi-automated clinical chemistry analyser (AGD 2020) as per the manufacturer's protocol.

Ultrasonographic examination

Ultrasonographic evaluation of abdomen in the affected cats was performed as per the method described by Penninck and D'Anjou (7). Assessment for hepatomegaly, splenomegaly, or any textural changes in the liver and spleen was made. Linear probe set at the frequency of 8 MHz was used in the procedure.

Molecular study

Genomic DNA from clinically ill cats was isolated using 300 µL whole blood sample and HiPurA® SPP blood DNA isolation kit (HIMEDIA) following the manufacturer's instructions. The isolated DNA sample was stored at -20 °C for further use. Molecular detection of piroplasms was carried out using a conventional PCR assay using the sense primer 5'CCAGCAGCCGCGGTAATT3' and the antisense primer 5'CTTTCGCAGTAGTTYGTCTTAACAAA TCT3' that amplify a fragment of ~373 bp of the 18S rRNA gene as reported by Diaz-Reganon et al. (8). PCR reactions were carried out in a final volume of 25 µL containing 12.5 µl of Taq PCR Master Mix (2×Premix) containing Taq DNA Polymerase, optimized PCR buffer and dNTPs, 1.0 µl of each primer (Forward and Reverse), 2 µl of the DNA template and 8.5 µl nuclease free water to make the total volume of 25 µl.

The thermal cycling conditions consisted of an initial denaturation: 94 °C for 3 min 35 cycles of 94 °C for 30 s 64 °C for 45 s 72°C for 30 s. Final extension: 72 °C for 7 min. All reactions were performed using an automated thermal cycler (BIO-RAD, T100). The amplicons were visualized under UV illumination in gel documentation system after electrophoresis of 10 µL of the reaction solution in a 2% agarose Gel.

Statistical analysis

The data obtained were statistically analysed as per the methods described by Snecdecor and Cochran (9) using the IBM SPSS 29.0 (IBM Corp., Armonk, NY, USA) standard version for Windows (IBM Corporation). The independent t-test for equality of means with unequal variances was carried out. Variables with $P > 0.05$ were considered as statistically "non-significant." variables with $P < 0.05$ were considered as statistically "significant."

Results

Clinical observations

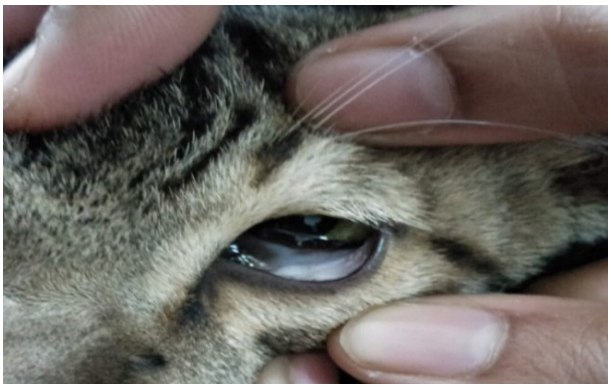


Fig. 1: Pale conjunctival mucous membrane in a cytauxzoonosis positive cat



Fig. 2: Icteric gum in a cytauxzoonosis positive cat

The clinical signs and symptoms observed in confirmed cases of cytauxzoonosis in cats were lethargy (17 / 18, 94.4%), fever and hyporexia/anorexia (16/18, 88.19%), tachypnea (13/18, 72.22%) and tachycardia (12/34, 66.67%), pale/whitish mucus membrane (Fig. 1; 8 /18, 44.44%), icterus (Fig. 2; 3/18, 16.67%), vomition (4/18, 22.22%), diarrhoea and dehydration (2/18, 11.11%), vocalization (1/18, 5%), stomatitis (1/18, 5%), abortion (1/18, 5%) and dermatitis (1/18, 5%). Anamnesis revealed the presence of ecto-parasites (tick, lice or fleas) and outdoor exposure in cats (16/18, 88.89%) although none of the cats had ticks or other ecto-parasite on their body at the time of presentation.

Ultrasonographic examination

In this study, ultrasonography showed splenomegaly (Fig. 3; 6/18, 33.33%) and rounded liver margins indicating hepatomegaly (2/18, 11.11%).

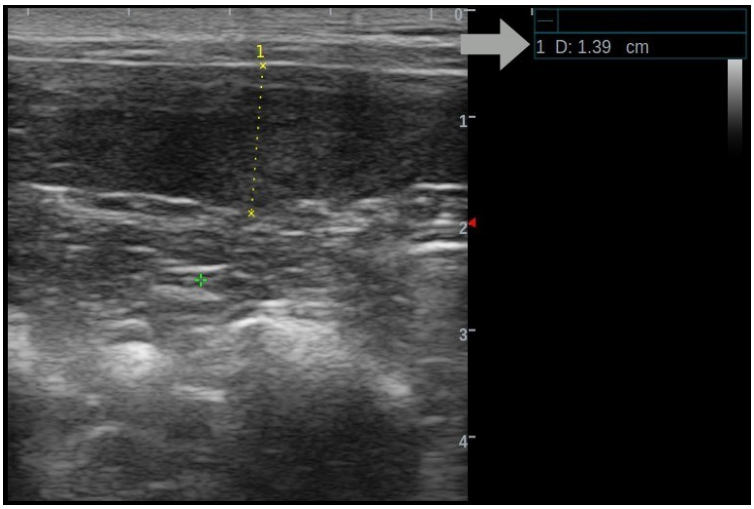


Fig. 3: Splenic height of 1.39 cm (>1cm) indicating splenomegaly

Haematological examination

Mean haemoglobin, haematocrit and TEC levels showed significantly ($P < 0.05$) lower values in all the treatment groups on 0-day, indicating anaemia to be a common finding in cytauxzoonosis affected cats.

The mean values of MCV and MCHC did not show any significant difference from the healthy group. Thus the anaemia found in infected cats were normocytic normochromic in nature. The mean of platelets count was significantly ($P < 0.05$) lower from the control group level. Hematological analysis showed no significant decrease or increase in mean TLC, ANC and ALC (Table 1).

Table 1: Haematological changes in cytauxzoonosis positive cats

Parameter	Healthy cats (n=6)	Cytauxzoonosis Positive cats (n=18)	*P* value
Haemoglobin (g/dL)	13.20±0.46	8.28±0.42*	0.000
TEC (×10⁶/µL)	7.93±0.17	5.18±0.33*	0.000
PCV (%)	38.62±1.48	25.17±1.24*	0.000
MCV (fL)	48.71±0.87	50.28±1.56^{NS}	0.389
MCHC (g/dL)	36.94±2.64	32.87±0.30^{NS}	0.185
Platelets Count (×10³/µL)	265.33±31.00	137.72±12.07*	0.007
TLC (×10⁶/µL)	10.65±1.24	12.07±1.42^{NS}	0.468
ANC (×10⁶/µL)	6.10±0.80	8.73±1.10^{NS}	0.055
ALC (×10⁶/µL)	3.17±0.56	2.28±0.29^{NS}	0.173

*significant, ^{NS} Non significant

Biochemical examination

The mean activities of serum enzymes ALT and AST were significantly higher in diseased from the healthy cats. There was significant ($P < 0.05$) increase in diseased cat's total, direct and indirect bilirubin level when compared to healthy control. Total serum protein and albumin level was found to be significantly lower ($P < 0.05$) from the healthy control while mean globulin level had no significant alteration. Mean value of serum urea nitrogen concentration in the present study showed significant ($P < 0.05$) increase in infected cats. However, serum creatinine concentration did not differ significantly (Table 2).

Table 2: Biochemical changes in cytauxzoonosis positive cats

Parameter	Healthy cats (n=6)	Cytauxzoonosis Positive cats (n=18)	*P* value
ALT (U/L)	59.00±1.15	90.05±3.43*	0.000
AST (U/L)	35.50±3.19	42.78±1.79*	0.040
Total Bilirubin (mg/dL)	0.25±0.03	1.68±0.36*	0.001
Direct Bilirubin (mg/dL)	0.14±0.02	0.78±0.18*	0.003
Indirect Bilirubin (mg/dL)	0.13±0.02	0.75±0.18*	0.003
Total Protein (g/dL)	7.18±0.07	6.87±0.06*	0.005
Albumin (g/dL)	3.28±0.08	2.76±0.08*	0.000
Globulin (g/dL)	3.90±0.04	4.11±0.11^{NS}	0.088
BUN (mg/dL)	22.60±1.01	27.97±1.42*	0.006
Creatinine (mg/dL)	1.06±0.06	1.04±0.06^{NS}	0.823

*significant, ^{NS} Non significant

Blood smear examination

0ut of 90 suspected samples for cytauxzoonosis blood smear examination revealed signet-ring shaped intra-erythrocytic piroplasm in 24 cases (Fig. 4) revealing 26.66% positivity of *Cytauxzoon* spp. in blood smear examination. Concentration of parasitemia was graded mild to moderate in all of them.

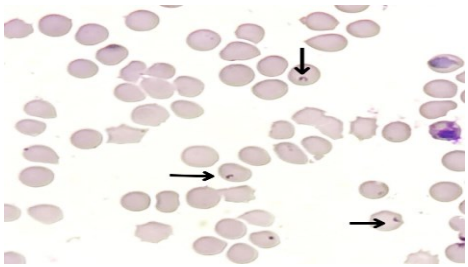


Fig. 4: Microscopic view of signet-ring shaped intra-erythrocytic piroplasm of *Cytauxzoon* spp.

Molecular detection

The molecular prevalence of cytauxzoonosis in the present study found to 37.77 %. The PCR assay of blood samples from suspected 90 animals gave a bright band of targeted amplicon 373 bp (lying in between 350 and 400 bp) in 34 cases (Fig. 5).

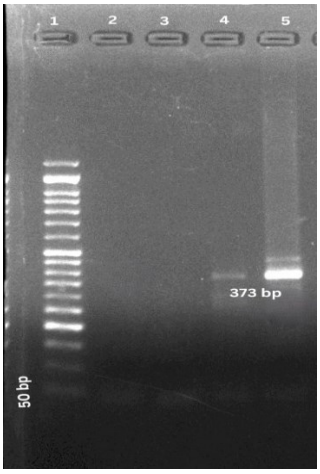


Fig. 5: Gel electrophoresis image showing an amplicon of 373 bp of 18S rRNA gene of *Cytauxzoon* sp., where lane 1 shows ladder of 50 bp, lane 2 shows negative control, lane 3 shows negative sample and lane 4 and 5 shows positive samples

Discussion

The clinical signs in cytauxzonoosis affected cats in our study were in concurrence with reports of other workers who also showed similar trends in the occurrence of clinical signs (10-12). Weisman et al. (13) first time reported abortion in cytauxzoonosis positive cat. But they did not find any piroplasms or schizont-laden macrophages in the placenta, fetal muscle, or bone marrow. Birkenheuer et al. (14) found tick on cat's body only on 2 of the 34 cases while Moghaddam et al. (11) did not find any ticks at the time of presentation analogous to this study. They assumed this to be a failure to examine the cats carefully for tick infestation, quick removal of ticks by the cats due to their grooming behaviour, use of ecto-parasiticides as reported by some owners herein, or an alternative mode of disease transmission.

In the present study, 26.66% positivity of *Cytauxzoon* spp. in blood smear examination was found with the concentration of parasitaemia graded mild to moderate in all of them. Similar mild parasitaemia was reported by Carli et al. (15). Detection of erythrocytic piroplasm in cytauxzoonosis affected cats was previously done (11, 16). It is to be noted that on the day of presentation sensitivity of erythrocytic piroplasms identification can be as low as 50%. This is because acute cytauxzoonosis is the result of schizogenous stage of the parasite (in mononuclear cells) and therefore ill-

ness can occur before piroplasms are identifiable within the RBCs (17, 18). The stain precipitant, Howell-Jolly bodies or artifacts can be mistaken for piroplasms and sometimes too low parasitaemia can be overlooked in micorscopic examination thus decreasing the sensitivity of this method of diagnosis.

The molecular prevalence of cytauxzoonosis in the present study was found to be 37.77 %. Molecular detection of *Cytauxzoon* spp. using different targets in 18S rRNA (19), ribosomal internal transcribed spacer regions 1 and 2 (ITS1, ITS2) (20) and mitochondrial gene cox3 (21) had been performed earlier. We targeted a 373 bp region of 18S rRNA using the primer and protocol described by Diaz-Reganon et al. (8). Further, sequencing and phylogenetic analysis in this study is expected for species identification and taxonomic classification.

Mean haemoglobin, haematocrit and TEC levels showed significantly ($P < 0.05$) lower values in all the treatment groups on 0-day in concurrence with earlier workers (22-24). Comparison between infected and non-infected cats revealed haematocrit in the infected cats were significantly lower than non-infected cats (11). Carli et al. (25) found anaemia in 14/39 (35.9%) infected cats however, it was not statistically associated with cytauxzoonosis when compared to anaemia in non-infected cats. Anaemia probably results, due to phagocytosis of red blood cells, because immune mediated erythrophagocytosis is an important finding in many organs (26).

Thrombocytopenia was a common finding in Cytauxzoon infected cases in previous reports (14, 19, 22, 23, 25, 27) contrastingly found higher platelets level in infected cats. Platelet aggregation earlier noted in felines with cytauxzoonosis in the USA (26) may be the reason behind thrombocytopenia. Conner et al. (28) reasoned increased consumption of platelets due to disseminated intravascular coagulation (DIC) behind thrombocytopenia. According to Levi et al. (29) systemic inflammation leads to activation of coagulation mechanism through tissue factor mediated thrombin synthesis, down regulation of anti-coagulant mechanisms and fibrinolysis inhibition. However, an accurate platelet counts in cats often is difficult to get as their platelets are susceptible to clumping (30). Carli et al. (25) in their case-control study did not find any significant changes in total leucocyte, neutrophil, and lymphocyte counts in concurrence to our findings.

Significantly higher mean activities of serum enzymes ALT and AST in cytauxzonoon infected cats during this study may be due to increased activity of liver enzymes (14). Maia et al. (31) described increased ALT and AST activities in a domestic cat with coinfection of Cytauxzoon and Mycoplasma. Similar findings also have been described in some case reports of domestic cat infected with *C. felis* (19, 22, 23, 27). Increased hepatic enzymes may indicate damage due to occlusion of blood vessels supplying the liver, by parasite-distended mononuclear cells or result of hypoxia due to anaemia (10, 32). Moghaddam et al. (11) found higher total protein and higher globulin concentration in infected as compared to non-infected cats. Legraux et al. (27) also encountered hyperproteinaemia with hypergammaglobulinemia in concurrence to our report. Hypoalbuminemia alike the present study was reported by Birkenheuer et al. (14) and Moghaddam et al. (11). Cohn et al. (10) reported a lowered total serum protein (< 6 g/dL) in 12/51 of infected cases. Albumin is a negative acute-phase protein, so during acute phase response its production is impeded by pro-inflammatory cytokines (33). However, various other processes such as vasculitis, liver dysfunction, and even protein losing nephropathy and enteropathy, can contribute in reduced serum level of albumin in cytauxzoonosis (34).

Peixoto et al. (35) revealed an elevated urea nitrogen concentration and normal creatinine in a lioness died of cytauxzoonosis. Maia et al.

(31) in a case report of cytauxzoonosis affected cat found increased BUN concentration similar to this report. Alho et al. (23) also came across azotaemia in cytauxzoonosis affected cat. However, in many instances like with Moghaddam et al. (11) and Carli et al. (25) the serum urea nitrogen in infected cats was normal unlike our findings. Increase in urea concentration might be accredited to dehydration. Increased urea level associated with raised creatinine concentration could be directly related to renal disorder.

Carli et al. (25) found the total bilirubin level within the normal range in the infected cats while Carli et al. (15), Cohn et al. (5) and Moghaddam et al. (11) reported hyperbilirubinemia. Hyperbilirubinemia is a common biochemical abnormality in cytauxzoonosis resulting from both extravascular haemolysis and liver damage because of infiltration of schizont-laden macrophages (32, 36).

Ultrasonographic splenomegaly and rounded liver margins indicating hepatomegaly in our study are similar to finding of Nentwig et al. (19). Hepatomegaly and splenomegaly are common imaging findings due to parasitic vascular occlusion as per Aschenbroich et al. (37) and Cohn and Birkenheuer (10).

In the present study we could not spot ticks on any of the infected cats. Thus, the tick specie or any other vector responsible for transmission of the disease remains ambiguous. As the study period was of six months only, the seasonal trend in epidemiology of disease could not be ascertained. Further, longer study period would have increased the sample size, increasing the representativeness of the population.

Conclusion

The Cytauxzoon organism is emerging as the most important haemoprotozoan parasites of domestic cats with variable clinical symptoms. Seroprevalence study of 90 suspected cases out of 390 cases of cats presented to Veterinary Clinical Complex hospital, Bihar Veterinary College, Patna in a duration of 6 months periods reveals 26.66% (24/90) and 37.77% (34/90) positive for *Cytauxzoon* spp. in blood smear and PCR based on piroplasmid 18S rRNA gene, respectively. The most common clinical signs noted in present study were lethargy, fever, anorexia, tachypnoea and tachycardia. The haematological changes recorded in the cytauxzoonosis affected cats in present study were normocytic normochromic anaemia (decreased haemoglobin, haematocrit, TEC and normal MCV and MCHC) and thrombocytopenia. The biochemical changes recorded in the cytauxzoonosis affected cats in present study were increased ALT and AST activities, hyperbilirubinemia, hypoproteinaemia with hypoalbuminemia and increased BUN concentration.

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Conflict of interest

Authors declare that there is no conflict of interest.

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