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Molecular detection of combined infection of anaplasmosis and Theileriosis in a goat

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Abstract
A three years old nondescript female goat was presented to Veterinary Clinical Complex, Veterinary College and Research Institute, Namakkal with the complaint of intermittent cough, anorexia and fever for four days. Clinical examination revealed fever (41 °C), tachycardia, tachypnoea, anaemia, tick infestation with rough hair coat, enlargement of prescapular lymph node and submandibular oedema. Haemato-biochemical examination revealed macrocytic hypochromic anaemia, hypoproteinaemia with hypoalbuminemia. *Theileria spp.* and *Anaplasma spp.* were detected at 298 bp and 139 bp, respectively in duplex PCR however, neither blood smear nor lymph node aspiration biopsy smear showed the presence of any haemoparasites. Animal was treated with Buparvaquone, Oxytetracycline, Haematinics, B complex vitamins, protein supplements and showed uneventful recovery.

Keywords: goat, anaplasmosis, Theileirosis, duplex PCR

Introduction
Theileriosis and anaplasmosis are the most prevalent vector borne parasitic diseases of ruminants in tropical and subtropical countries (Uilenberg, 1997) [1]. Out of six *Theileria spp.* affecting small ruminants, three species (*T. lestoquardi, T. luwenshuni*, and *T. uilenbergi)* are considered under the pathogenic group whereas *T. seperata, T. ovis*, and *T. recondita* are generally considered to be mildly pathogenic (Schnittger *et al*., 2000) [2]. Taha and El Huseein (2010) [3] found that *Hyalomma anatolicum* and *Rhipicephalus turanicus* were the transmitting agents of *T. lestoquardi* which causes malignant ovine theileriosis in sheep and goats. Anaplasmosis is a widely distributed infection caused by *Anaplasma ovis* which is transmitted by ixodid ticks, mechanical and iatrogenic transmission. High fever, anaemia, tachycardia, enlargement of superficial lymph nodes, intermittent cough and corneal opacity were the consistent clinical signs of goats with haemoparasite infection (Minnat and Abdulwadood, 2012) [4]. Milking goats might experience a significant drop in milk production that could last for several weeks. In the study, combined infection of anaplasmosis and theileriosis is documented using Duplex Polymerase Chain Reaction (Duplex PCR) in a goat.

Materials and Methods
A three years old nondescript female goat was presented to Veterinary Clinical Complex, Veterinary College and Research Institute, Namakkal with the history of intermittent cough, anorexia and fever for four days. Detailed clinical examination was done. Peripheral blood smear collected from the ear vein was stained with Giemsa stain and was examined under oil immersion (100x magnification) for intraerythrocytic forms of blood parasites (Sahoo *et al*., 2017) [5]. Two 2ml of blood sample in vacutainers with EDTA for haematology and PCR analysis and 3ml blood sample in a vacutainer with clot activator for serum biochemical analysis were collected aseptically from jugular vein. A complete blood count including haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), total leukocyte count (TLC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count and serum biochemical values of total protein, albumin, aspartate aminotransferase (AST), creatinine, blood urea nitrogen (BUN) were analysed using autoanalyzer. Fine needle aspiration biopsy (FNAB) smear from enlarged prescapular lymph nodes was collected and stained with Giemsa stain to identify intra lymphocytic schizonts (Koch's blue bodies).

DNA extraction
DNA was extracted from whole blood samples using DNA extraction kit (QiaAmp DNA kit-

QIAGEN) as per the manufacture's instructions. Two hundred micro litres of blood samples were lysed in 200 µl lysis buffer and then proteins were degraded with 20 µl Protease enzyme and kept in water bath for 10 min at 56 ºC. 200 µl of ethanol was added to the solution and the entire volume was transferred to the mini spin columns after thorough vortexing. The column was first centrifuged at 8000 rpm for one minute and then washed twice with 500µl washing buffer. Finally, the DNA was eluted using 200 µl elution buffer from the column.

PCR amplification

The duplex PCR method was employed for the simultaneous detection of *Theileria spp*. and *Anaplasma spp*. Two pairs of primers targeting 18S rRNA gene sequence of *Theileria spp.* and 16S rRNA gene sequence of *Anaplasma spp.* were utilized for the amplification. The primers used for *Theileria spp.* and *Anaplasma spp*. were, forward strand primer 5´-ATTCCCGCATCCTATTTAGCAG-3´ and reverse strand primer 5´-CGACTCCTTCAGCACCTT-3´ and forward strand primer 5´-ACACGGTCCAGACTCCTACG-3´ and reverse strand primer 5´-AGGTACCGTCATTATCTTCCCTACT-3´ respectively. The PCR was carried out in a total reaction volume of 25 µl including 12.5 µl master mix (Amplicon), 1µl of each primer (both forward and reverse), 3 µl of template DNA and 5.5 µl nuclease free water. The amplification was carried out in a thermocycler with cyclic program as follows; an initial denaturation step of 95 ºC for 5 minutes, 36 cycles of denaturation step of 95 ºC for 30s, annealing step at 56 ºC for 45s, extension at 72 ºC for 50s and a final extension at 72 ºC for 10 min. The amplified products (8 µl) were subjected to agarose gel electrophoresis in a 2 % agarose gel containing ethidium bromide at 80V for 40 minutes (Cui *et al*., 2017) [6].

Results and Discussion

The goat was dull and depressed and had fever (41 °C), tachycardia (84/min), tachypnoea (46/min), pale conjunctival mucous membrane (Fig 1), enlargement of prescapular lymph nodes and submandibular oedema (Fig 2). The goat had rough hairy skin and coat with tick infestations. Earlier researchers observed similar clinical findings in goats with Theileriosis (Hassan *et al*., 2015; Nazi *et al*., 2012) [7, 8] and Anaplasmosis (Smith and Sherman, 2009) [9]. In the present study, the peripheral blood smear and lymph node smear did not detect either *Theileria spp.* or *Anaplasma spp*. Theileria was tiny pleomorphic piroplasm in the blood smear and it needed skill for identification (Li *et al*., 2014) [10] however, Anaplasma would appeared compact, round intraerythrocytic masses on or near the edge of red blood cells (OIE, 2008) [11]. Minnat and Abdulwadood (2012) [4] identified Koch blue bodies in the white blood cells of goats with Theileriosis. Haematological evaluation revealed decreased haemoglobin, PCV, RBC, MCHC and increased MCV, MCH indicating macrocytic hypochromic anaemia in combined infection with Theileriosis and anaplasmosis in the present study (Table 1). Macrocytic hypochromic anaemia (Meenu *et al*., 2021) [12], macrocytic normochromic anaemia (Jayalakshmi *et al*., 2019) [13] in Theileriosis and microcytic hypochromic anaemia in Anaplasmosis (Nadiq *et al*., 2017) [14] in goats were reported. The activity of macrophages in eliminating piroplasms from infected erythrocytes might be responsible for anaemia in Theileria infected animals (Al-Amery and Hassso, 2002) [15]. According to Thrall (2012) [16], the presence of a greater number of reticulocytes that were unable to achieve the same haemoglobin concentration as mature erythrocytes might be the cause of the decrease in MCHC in haemoprotozoan affected animals. Excessive erythrocyte destruction or an increase in the number of immature erythrocytes could lead to an increase in the average mass of haemoglobin (MCH) per RBC (Schalm *et al*., 2010) [17]. In the present study, there was leucocytosis however normal leucocyte count was reported by Jayalakshmi *et al*. (2019) [13]. Serum biochemical evaluation showed hypoproteinaemia with hypoalbuminemia and all other serum biochemical parameters were within normal range (Table 1). Al-fetly (2012) [18] recorded that sheep with haemoprotozoan illnesses had considerably reduced total protein and albumin levels. According to Al-Amery and Hasso (2002) [14] hypoproteinaemia and hypoalbuminemia were the sole reason for the intermandibular oedema in clinically affected goat.

Theileria spp. and *Anaplasma spp.* were detected at 298 bp targeting 18S rRNA and 139 bp targeting 16S rRNA, respectively in duplex PCR (Fig 3) (Cui *et al.*, 2017) [6] however traditional microscopic blood smear examination did not detect the blood parasites. It showed that the PCR technique could be the best diagnostic tool for the identification of subclinical or stay blood parasitic infection in goats. Bami *et al*. (2009) [19] employed nested PCR to differentiate *Theileria spp.* and concluded that the microscopic examination of blood smears could not detect subclinical illness. Molecular diagnosis of anaplasmosis by PCR using major surface protein 4 was sensitive and specific (Hamedani *et al*., 2009) [20]. Affected goat was treated with single shot of buparvaquone @ 2.5mg/kg bwt intramuscularly and Ivermectin @ 0.2 mg/kg subcutaneously and oxytetracycline @ 20 mg/kg bwt intravenously after diluting with normal saline for five days with supportive therapy with haematinics and B complex vitamins. The owner of the animal was advised to provide protein supplements. The animal showed improvement in the feeding and also clinically improved from anaemia and fever. In the present study, molecular detection of combined blood parasitic infestation by duplex PCR technique was more sensitive and helped to provide appropriate treatment to save the animal.

Table 1: Haemato-biochemical parameters of a goat with combined Theileriosis and Anaplasmosis

Parameter	Goat with Theileriosis & Anaplasmosis	Reference Value
Haemoglobin (g/dl)	2.1	8-12
Packed cell volume (%)	13.1	22-38
Total red blood cell count (x 10^6/µl)	4.26	8-18
Total leucocyte count (x 10^3/µl)	17	4-13
Platelet count (x10^5/µl)	5.0	3-6
Neutrophil (%)	33	30-48
Lymphocyte (%)	61	50-70
Eosinophils (%)	3	3-8

Monocyte (%)	3	1-4
MCV (fl)	24	16-25
MCH (pg)	4.8	5.2
MCHC (g/dl)	21	30-36
Total protein (g/dl)	3.6	6.4-7.0
Albumin (g/dl)	1.2	2.7-3.9
Creatinine (mg/dl)	1.1	1.0-1.8
BUN (mg/dl)	18	10-20
AST (U/L)	139	167-513

Fig 1: Submandibular oedema in the affected goat

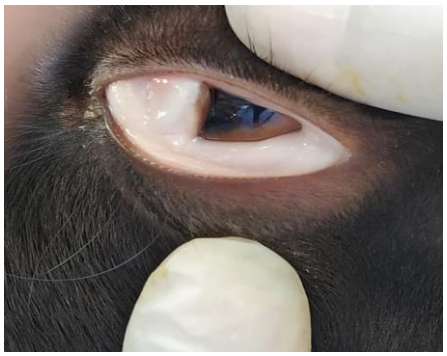

Fig 2: Pale conjunctival mucous membrane

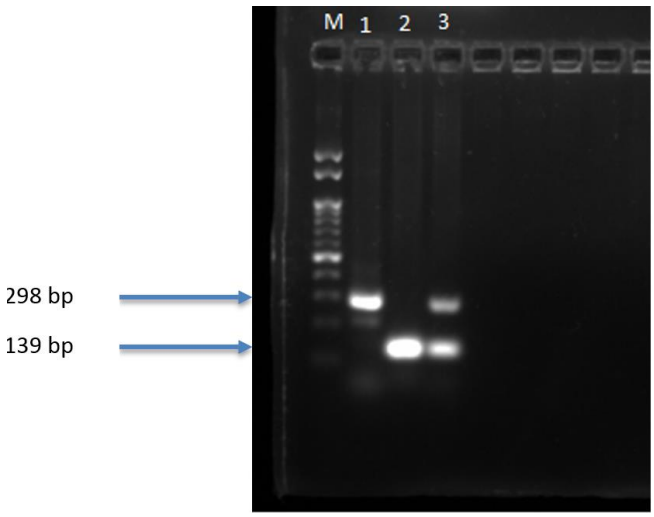

Fig 3: Agarose gel electrophoresis of amplification products. Lanes: M : molecular weight standards (100bp) Lane 1: Positive control of *Theileria spp.*, Lane 2: Positive control of *Anaplasma spp.*, Lane 3: *Theileria spp.* and *Anaplasma spp.* specific band at 298bp and 139bp, respectively from the affected goat blood sample

Conclusion

Caprine theileriosis and anaplasmosis are the major haemoparasitic diseases which cause huge impact on economy of goat farming. Mixed blood parasitic infection is rare in occurrence in small ruminants. A goat with combined T*heileria spp.* and *Anapalsma spp.* infection was diagnosed by duplex PCR and the major clinical findings were marked macrocytic normochromic anaemia, hypoproteinaemia and hypoalbuminemia. Duplex PCR was found to be effective in identifying the low parasitaemia from blood when microscopic examination of peripheral blood smear failed to detect.

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