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Molecular Detection of Haemoprotozoan Diseases in Anaemic Goats of Southern India: A Pilot Study

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ABSTRACT

Background: Anaemia is a common and important clinical presentation in goats due to infectious, traumatic, nutritional, haemoprotozoal, endo and ecto parasitic causes. Haemoprotozoan are obligate intracellular pathogens transmitted by ticks with an impact on animal health. *Anaplasma ovis*, *Babesia ovis*, *Babesia motasi* and *Theileria luwenshuni* that infects goats can be diagnosed by different methods like giemsa staining, PCR or competitive ELISA.

Methods: Goats presented to Large Animal Clinics of Madras Veterinary College Teaching Hospital, Chennai, India, were screened for the signs of anaemia. In this study, PCR method was used for the detection of *Anaplasma*, *Babesia* and *Theileria* infection.

Result: Among 25 blood samples collected from anaemic goats, 48 per cent were found positive for haemoprotozoan disease. Out of which 32 per cent were *Anaplasma ovis* followed by 4 per cent of *Babesia motasi* and *Babesia ovis* each whereas remaining 8 per cent showed mixed infection. This indicated a high incidence of haemoprotozoan infection as a major cause of anaemia in goats reared under field conditions.

Key words: Anaemia, *Anaplasma ovis*, *Babesia ovis*, *Babesia motasi, Therileria luwenshuni.*

INTRODUCTION

India's livestock sector is one of the largest in the world with a holding of 11.6% of world livestock population. Among different domestic animals, goats being multipurpose animal provide meat, milk and fibre (Bhardwaj *et al*., 2018). Anaemia is an important clinical presentation in goats manifested by pale or white mucous membranes, exercise intolerance, tachypnea, tachycardia, possible systolic murmurs, weakness, jaundice, haemoglobinuria, melena, submandibular oedema, ascites, weight loss, prolonged capillary refill time and even collapse of the animal in extreme cases (Balcomb and Foster, 2014).

According to Smith and Sherman (2011), anaemia is classified as haemolytic anemia, blood loss anemia and due to impaired erythropoiesis. Causes of haemolytic anaemia in goats include the haemoparasitic diseases such as anaplasmosis, babesiosis and theileriosis. Anaplasmosis is caused by intra-erythrocytic rickettsial pathogen *Anaplasma ovis*, which is mostly found in sheep, goats, and wild ruminants. According to Torina *et al.* (2012), *Anaplasma ovis* infection in sheep and goats often has no symptoms. Co-infection with other pathogens and stress brought on by numerous conditions, such as a hot environment and transportation, frequently predispose to the disease (Aravind *et al.,* 2020). Srivastava *et al*. (2022) reported 11.7% of pre-weaning mortality in goat kids was due to debility and anemia.

Babesiosis is one of the most common tick borne infections in ruminants. In acute condition it produces haemolytic anaemia, fever, inappetence, jaundice, and hemoglobinuria, all of which can be deadly in extreme instances (Hurtado *et al.,* 2019). *Babesia motasi* is more prevalent in goats but may be less virulent in sheep. Haemaphysalis and Rhipicephalus ticks can spread *B.motasi* (Smith and Sherman 2011). In India clinical disease in goats with mixed infection of *B. ovis* and *B. motasi* has been reported by Tufani *et al.* (2018).

Theileriosis encompasses a group of tick-borne protozoal diseases of ruminants affecting primarily the hemic-lymphatic system. It leads to anemia and lymphopenia in affected goats. Koch's Blue Bodies may be identified in Giemsa-stained smears of lymph nodes or characteristic piroplasms seen in circulating RBCs (Smith and Sherman (2011). According to Yin *et al.* (2008) *T. lestoquardi, T. uilenbergi* and *T. luwenshuni* were characterized as highly pathogenic. Nagaraj *et al.* (2019)

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stated that goats affected with theileriosis appear to be apparently healthy. This study investigated the incidence of haemoprotozoan diseases in anaemic goats.

MATERIALS AND METHODS

Goats presented to Large Animal Clinics of Madras Veterinary College Teaching Hospital, Chennai India, between March and December, 2023 was screened for the signs of anaemia. Twenty five anaemic goats were included in this study.

Screening of *Anaplasma* spp., *Theileria* spp. and *Babesia* spp. in blood smear

Blood smears were collected and fixed in methanol and stained with Leishman Giemsa (L-G) cocktail stain for 30 minutes and examined under oil immersion objective of the microscope (Gajendra *et al.,* 2015). About 10 microscopic fields per smear of RBCs were screened for *Theileria* spp., *Anaplasma* spp. and *Babesia* spp.The sample was considered to be positive even if single piroplasm was seen

Molecular detection of *Anaplasma* spp. *Theileria* spp. and *Babesia* spp

Blood samples were collected from the jugular vein into a tube containing anticoagulant (EDTA) from 25 goats affected of anaemia. Genomic DNA was extracted successfully from 150 µl of blood using method adopted by Surzycki (2000) (Addition of 0.25% Sarcosyl was the modification done which aids in the lysis of cells) and DNA was stored at -20 °C until further use.

Polymerase chain reaction (PCR)

The DNA samples of goats were subjected to PCR amplification with the genus specific primers and later confirmed by species specific primers of *Theileria, Anaplasma* and *Babesia*. The primers details and cycling conditions are listed in Table 1 and 2. PCR assay was carried out in thermal cycler. Each PCR reaction was added with 10 ρmole of genus specific primers and species specific primers encoding the 18S rRNA of *Theileria spp.,* 16S RNA *of Anaplasma spp.* and 16S RNA of *Babesia spp.* and made up to the final volume of 20 µl with nuclease free water. Red dye master mix (2x, Ampliqon III) was used to prepare PCR reaction mix. Each 20 µl reaction mixture comprised 5 µl of template DNA, 10 µl Red dye PCR Master Mix (2X, Ampliqon), 1 µl of each primers at 10 pmol/µl (forward and reverse primer) and 3 µl nuclease free water. In addition to this test DNA samples, a negative control (DNA extracted from known negative healthy goat blood sample) were included in each of the PCR amplification. 10 µl of amplified PCR product was loaded for electrophoresis in 1.5% Agarose gel along with 100 bp DNA ladder (Lee *et al.,* 2012). The images were captured and documented using gel documentation system (Bio Rad., USA).

RESULTS AND DISCUSSION

Among 25 blood samples and blood smears 12 samples (48 per cent), were found positive for haemoprotozoan disease.

Microscopic examination

The Leishman Giemsa (L-G) stained blood smears were screened for *Anaplasma* spp., *Theileria* spp. and *Babesia* spp. Among 25 blood smears from goats, 6 cases (24 per cent) were found to be positive for *Anaplasma* spp. and1case (4 per cent) was positive for *Theileria* spp. showing characteristic piroplasms stages in the RBCs (Fig 1a,1b).

Molecular detection

The amplicon of genus specific *Anaplasma ovis*, *Babesia ovis*,*Babesia motasi* and *Theileria luwenshuni* was observed as a single band of 347 bp, 636 bp, 642 bp and 389 bp, respectively (Fig 2-5). Out of 25 blood samples, 12 (48 per cent) cases were found positive for haemoprotozoan.

Table 1: Primer sequence for the identification of *Theileria* spp., *Anaplasma* spp., *Babesia* spp. in anaemic goat.

Gene	PCR Targeted for	Primer sequence Forward and Reverse (5'-3')	Product length (bp)	Reference
18SrRNA	*Theileria* genus specific PCR	F-AGTTTCTGACCTATCAG	1098bp	Allsopp *et al.*,1993
		R-TTGCCTTAAACTTCCTTG		
	Theileria luwenshuni	F-GGTAGGGTATTGGCCTACTGA	389bp	Yin *et al.*,2008
		R-TCATCCGGATAATACAAGT		
16S rRNA	*Anaplasma* genus specific PCR	F-GCCACACTGGAACTGAGATAC	796bp	KC470064.1
		R-GGCAACTAAGGATGAGGGTTAC		
	Anaplasma ovis	F-TGAAGGGAGCGGGGTCATGGG	347bp	Torina *et al.,* 2012
		R-GAGTAATTGCAGCCAGGCACTCT		
16S rRNA	*Babesia* genus specific PCR	F-CAGCTTGACGGTAGGGTATTG	646bp	U09834.1
		R-AGATACCGTCGTAGTCCTAACC		
	Babesia motasi	F-CAGCTTGACGGTAGGGTATTG	642bp	AY260178.1
		R-GGTTAGGACTACGACGGTATCT		
	Babesia ovis	F-CAGCTTGACGGTAGGGTATTG	636bp	AY260179.1
		R-GGTTAGGACTACGACGGTATCT		

Table 2: PCR cycling conditions for the identification of different *Theileria* spp., *Anaplasma* spp. and *Babesia* spp. in anemic goat.

PCR Targeted for	Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Referance
Theileria genus specific PCR	18s rRNA	94°C for 3 min	94°C for 30s for 35 cycles	48°C for 1min for 30 cycles	72°C for 1 min for 30 cycles	72°C for 5 min	Allsopp *et al.*, 1993,
Theileria luwenshuni		94°C for 3 min	94°C for 30s for 35 cycles	56°C for 1min for 35 cycles	72°C for 2 min for 35 cycles	72°C for 7 min	Yin *et al.*, 2008
Anaplasma genus specific PCR	16s rRNA	94°C for 3 min	94°C for 30s for 35 cycles	60°C for 1min for 35 cycles	72°C for 1 min for 35 cycles	72°C for 5 min	
Anaplasmaovis		94°C for 3 min	94°C for 30s for 35cycles	64°C for 1min for 35 cycles	72°C for 1 min for 35 cycles	72°C for 5 min	
Babesia genus specific PCR	16s rRNA	94°C for 3 min	94°C for 30s for 35cycles	60°C for 1 min for 35 cycles	72°C for 1 min for 35 cycles	72°C for 5 min	
Babesiamotasi		94°C for 3 min	94°C for 30s for 35cycles	58.6°C for 1 min for 35 cycles	72°C for 1 min for 35 cycles	72°C for 5 min	
Babesia ovis		94°C for 3 min	94°C for 30s for 35cycles	60°C for 1 min for 35 cycles	72°C for 1 min for 35 cycles	72°C for 5 min	

*-Annealing temperature determined by the gradient PCR.

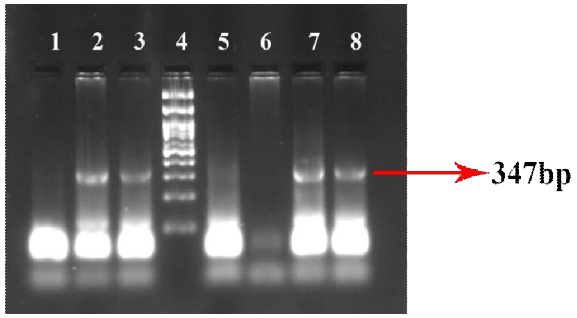
In which 8 cases were found positive for *Anaplasma ovis* and one case found positive for *Babesia motasi*, *Babesia ovis* each and two cases were found positive for mixed infection (*Anaplasma ovis* with *Babesia ovis* and *Theileria luwenshuni*; *Anaplasma ovis*–with *Babesia ovis*) by PCR.

In the present study, anaemic goats had highest incidence of *Anaplasma ovis* (8 cases; 32 per cent) which is in agreement with findings of Aravind *et al.* (2020). Due to co-infection with other pathogens and stress brought on by numerous conditions, such as a hot environment and transportation, frequently predispose goats to the diseases (Aravind *et al.,* 2020).

In this study, one animal was found positive for *Theileria luwenshuni, Anaplasma ovis* and *Babesia ovis* mixed infection. Similarly a study conducted by Nagaraj *et al.,* (2019), observed that the prevalence of *T.luwenshuni* were 32.58 percent in apparently healthy goats.

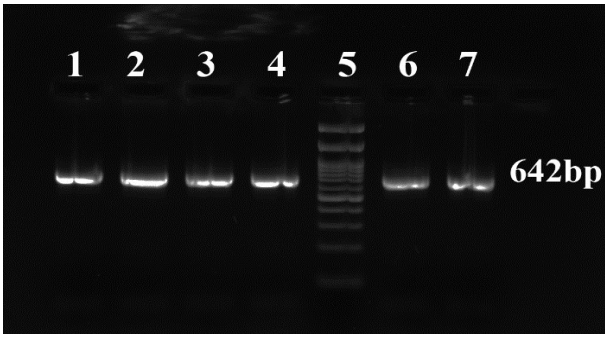
Theileriosis was confirmed by detection of Koch's Blue Bodies (KBB) using FNAC from swollen pre-scapular lymph nodes (Banka *et al.,* 2020). According to Yin *et al.* (2008), blood smear examination is reliable for detection of acute cases; it has a limited value for detection of infection in chronic cases where only a few number of piroplasms exist. A study conducted by Jayalakshmi and Premalatha (2020), blood smear examination revealed 18.54% goats were positive for haemoparasites like *Anaplasma* spp and

Fig 1: Blood smear positive for *Anaplasma* spp and *Theileria* spp piroplasms.



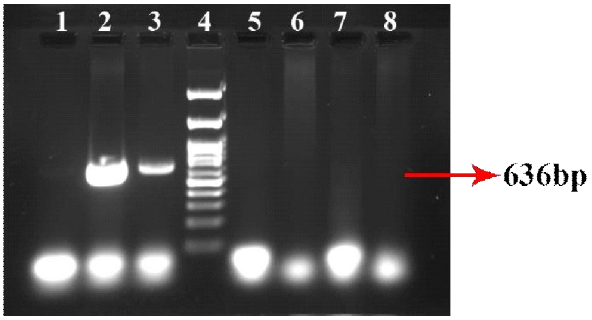
Lane 4: 100 bp ladder (Thermo scientific). Lane 1: Negative control
Lane 2 to 8: Field DNA samples

Fig 2: PCR for *Anaplasma ovis.*



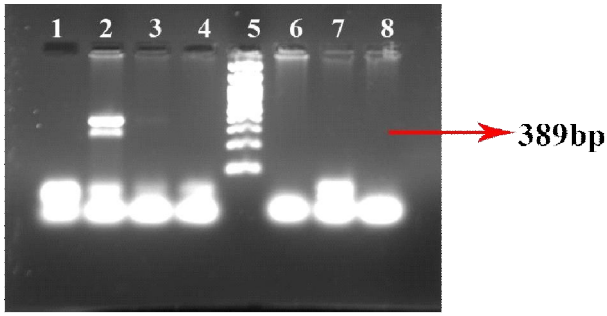
Lane 5: 100 bp ladder (Thermoscientific). Lane 1: Positive control.
Lane 2 to 7: Field DNA samples.

Fig 4: PCR fo r*Babesia motasi.*



Lane 4: 100 bp ladder (Thermoscientific). Lane 1: Negative control.
Lane 2 to 8: Field DNA samples.

Fig 3: PCR for *Babesia ovis.*



Lane 5: 100 bp ladder (Thermoscientific). Lane 1: Negative control.
Lane 2 to 8: Field DNA samples.

Fig 5: PCR for *Theileria luwenshuni.*

Theileria spp. of which 15.32% was *Anaplasma* spp and 3.22% animals showed mixed infection of *Theileria* spp and *Anaplasma* spp.

In this study, one case (4 per cent) was found positive for *Babesia motasi* and *Babesia ovis* each. Remaining two cases (8 per cent) were found positive for mixed infection (*Babesia ovis, Anaplasma ovis* and *Theileria luwenshuni*; *Babesia ovis* and *Anaplasma ovis*). *Babesia ovis* and *Babesia motasi* also reported by others in goats (Uilenberg, 2001; Smith and Sherman, 2009; Haq *et al.,* 2017) and *Theileria luwenshuni* in sheep (Dhaygude, 2021). According to Rather *et al.* (2016), among ruminants the goats had resistance against babesiosis so prevalence of the babesiosis in goats was reported to be 0.75% per cent compared to other ruminants. But climatic changes are expected to increase the risks of vector borne diseases due to rise of environmental temperature and change in rainfall pattern, it leads to an increase in the vector population (Sharma, 2011). Ingle (2019) reported an overall prevalence of 29 per cent for *Babesia* species using PCR where 11% were positive for *B. ovis*. According to Ingle (2019) the use of PCR in the surveillance of babesiosis will enable the detection of asymptomatic carrier animals that could not be detected using conventional methods.

CONCLUSION

The present study revealed that anemia is common in goats and almost 50% of anemia is due to haemoprotozoan diseases. Anaplasma ovis is the common infection followed by mixed infection of Babesia and Theileria. Polymerase chain reaction was found useful molecular technique for diagnosis and surveillance of haemoprotozoan diseases in goats.

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Conflict of interest

on behalf of all authors I declare that there is no conflict of interest.

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