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ORIGINAL ARTICLE

Detection of incidence of *Babesia* spp. in sheep and goats by parasitological diagnostic techniques

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Abstract The present study was undertaken on epidemiology and diagnosis of babesiosis in sheep and goats in Bengaluru Urban and Rural districts of Karnataka state from November 2017 to May 2018. Out of 343 (225 sheep and 118 goats) blood smears examined by Giemsa and acridine orange (AO) fluorescent dye staining methods, 3.55 and 4.0 per cent of sheep and 0.84 and 1.69 per cent of goat samples were found positive for *Babesia* organisms, respectively. The sensitivity and specificity was found to be higher in AO fluorescent dye staining method. In agewise susceptibility, the percent positivity was found to be higher in animals > 6 months old. In genderwise susceptibility, the percent positivity was found to be higher in females than males. Hence, AO fluorescent dye staining method is found to be very rapid and cost effective diagnostic method for treatment and control of babesiosis.

Keywords *Babesia* · Diagnosis · Goats · Morphometrics · Sheep

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Introduction

Among tick borne haemoprotozoan diseases, babesiosis is an economically important disease of small ruminants in tropical and subtropical countries. In India, the estimated loss due to tick borne diseases is around US$ 498.7 million per annum (Minjauw and McLeod 2003) and in the world it is roughly US$ 7000 million (FAO 1984). Among *Babesia* spp., *Babesia motasi* and *Babesia ovis* are considered to be pathogenic; *Babesia crassa*, *Babesia foliata* and *Babesia taylori* are mild to non-pathogenic in sheep and goats (Levine 1985; Kaufmann 1996).

Based on morphology, many authors have described pleomorphic forms of *Babesia* spp. throughout the world. Lestoquard (1925) reported a parasite ($2.5 - 4.0 \times 1.2$ µm) in goat blood from Algeria. Thomson and Hall (1933) described *B. motasi* ($2.5 - 3.5 \times 1.2 - 1.5$ µm) from sheep in northern Nigeria. Ried et al. (1976) described the parasite as a small *Babesia* (2 µm in length). Christensson and Thunegard (1981) reported *B. motasi* (3.1×1.9 µm) as large and small forms (2.2×1.8 µm). Bai et al. (2002) reported two *Babesia* spp. *viz.*, *B. ovis* and large polymorphic form of *Babesia* ($1.8 - 2.5 \times 0.9 - 1.8$ µm) from sheep in eastern part of Gansu province, China. Shayan et al. (2008) reported large polymorphic form of *B. ovis* (2.7×0.4 µm). In India, the first observation on ovine babesiosis was recorded in Mysore (Achar and Sreekantaiah 1934) and designated as *B. motasi.* Subsequently, the disease has been reported by Sarwar (1935), Ray and Raghavachari (1941), Madhav (1966), Jagannath et al. (1974), Prabhakar (1976), Muraleedharan et al. (1994), Vidya et al. (2011), Muthuramalingam et al. (2014) and Haq et al. (2017).

Though Giemsa staining continues to be the "gold standard method" for diagnosis of babesiosis in most of the

countries with the sensitivity ranging from 10^{-5} to 10^{-6}. However, considerable progress has been made with application of fluorescent dyes in diagnosis of blood parasites particularly with acridine orange (AO). The advantage of AO is that results are readily available within 3 to 10 min and has shown higher sensitivity compared to Giemsa staining in the detection of *Babesia* species (Bose et al. 1995). Hence, the present literature describes an application of Giemsa and AO fluorescent dye staining methods in epidemiological studies and quick detection of babesiosis.

Materials and methods

Study area and collection of samples

A total of 343 (225 sheep, 118 goats) blood samples were collected aseptically from both clinically suspected (10 sheep, 8 goats) and apparently healthy animals (215 sheep, 110 goats) without any symptoms but infested with ticks in and around Bengaluru Urban and Rural districts of Karnataka during the study period from November 2017 to May 2018 (Table 1). Out of 343 blood samples, 18 were collected from Veterinary Hospital, Veterinary College, Hebbal, Bengaluru from sheep and goats which were showing clinical signs *viz.,* pyrexia (104–107°F), reduced appetite, watery discharge from oral and nasal cavity, respiratory distress, haemoglobinuria and pale mucous membrane. The details on age and gender were also recorded.

Microscopic examination of blood smears

Two set of blood smears were made immediately after the blood collection. One set of blood smears stained with Giemsa stain were examined under a light microscope for the presence of the intra-erythrocytic piroplasms (Soulsby 1982). Second set of blood smears were stained with 0.01% AO fluorescent stain. The AO (0.01%) stain was prepared by adding 20 mg of AO powder (Himedia Laboratories) to 190 ml acetate buffer (pH 5.6). The blood smears were stained with AO stain as per the method described by Ravindran et al. (2007) with slight modification and were screened for the presence of *Babesia* organisms under 400 × magnification of Inverted fluorescent microscope at 460 nm (ZEISS, Germany). The morphological characterization was carried out by micrometric studies.

Statistical analysis

The statistical analysis of data was carried out by Chi square test using graphpad prism software, version 5.01.

Results and discussion

Many alternative molecular approaches to Giemsa staining have been developed but none of these methods have been able to replace Giemsa staining due to their high costs in the diagnosis of babesiosis. Several other methods have been described with use of fluorescent dye but the most extensively studied dye is AO. In the present study, out of 343 (225 sheep and 118 goats) blood samples examined by Giemsa and AO fluorescent dye staining methods, 3.55 and

Table 1 Number of blood samples found positive for babesiosis in sheep and goats by Giemsa and AO fluorescent dye staining method

Sl. no.	Districts	Taluks	No. of samples collected		No. positive (Giemsa staining method)		No. positive (AO staining method)	
			Sheep	Goats	Sheep	Goats	Sheep	Goats
1	Bengaluru Urban	Bengaluru North	39	30	2 (5.12%)	0	2 (5.55%)	0
		Bengaluru South	20	12	1 (5.0%)	0	2 (10.0%)	0
		Bengaluru East	28	11	0	0	0	0
		Anekal	22	13	0	0	0	0
2	Bengaluru Rural	Devanahalli	14	10	0	0	0	0
		Hosakote	28	12	1 (3.57%)	1 (8.33%)	2 (7.14%)	2 (16.66%)
		Doddaballapura	38	17	1 (2.63%)	0	0	0
		Nelamangala	36	13	3 (7.69%)	0	3 (7.69%)	0
Total			225	118	8 (3.55%)	1 (0.84%)	9 (4.0%)	2 (1.69%)

The statistical differences between the groups and methods was found to be non-significant ($P<0.05$)

4.0 per cent of sheep and 0.84 and 1.69 per cent of goats were found positive for *Babesia* organisms, respectively. In AO, the nucleus of the *Babesia* organisms fluoresced apple green color, whereas light orange colored fluorescence was observed from the cytoplasm (Fig. 1). Among eight taluks, Nelamangala revealed 7.69 per cent of positive cases (Table 1). The sensitivity of Giemsa and AO fluorescent dye staining methods was found to be 56.25 and 68.75 per cent, respectively. The specificity was found to be 99.70 and 100.0 per cent, respectively.

These findings are in accordance with Razmi et al. (2003) from Iran who recorded 23.5 (92/391) and 0.5 (2/385) per cent of *B. ovis* in sheep and goats, respectively. In contrast to the present findings, Naderi et al. (2017) from Iran reported higher percentage of babesiosis in goats (17.0%) than compared to sheep (12.41%). In the present study, fluorescent dye staining method showed a better visual contrast for detection of *Babesia* organisms due to differential fluorescence of organisms with less chance of missing the protozoan organisms and fewer artifacts, which helped in detecting subclinical cases of *Babesia* infection which could not be detected in Giemsa stained blood smear indicating higher sensitivity of fluorescent dye staining when compared to Giemsa staining technique. Similar observations were also reported by Hansen et al. (1970); Bose et al. (1995); Ravindran et al. (2007) and Nair et al. (2011).

Examination of Giemsa stained blood smears by microscopy revealed the presence of highly pleomorphic organisms with predominant of single (31%) and paired pyriforms (28%) followed by amoeboid (17%), oval (11%), elongated (4%), ring (4%) and other forms (5%) (Fig. 2). The micrometric studies revealed two forms of *B. ovis* organisms in both sheep and goats which was later confirmed by PCR (Figs. 3 & 4). Small form of *Babesia* was observed in three sheep samples and measured about 1.0 to 1.31 µm in length and 0.5 to 2.5 µm in breadth. A large form of *Babesia* organism was observed in three sheep and two goat samples with 1.8 to 2.7 µm in length and 0.9 to 1.5 µm in breadth. This is the first report from India on occurrence of two forms of organisms in *B. ovis*. The parasitaemia percentage ranged from 1.0 to 1.8 in clinically and microscopically confirmed cases.

Different morphological forms of piroplasms observed in the present study were in agreement with Shayan et al. (2008) from Iran; Zangana and Naqid (2011) from Iraq and Sevinc et al. (2013) from Turkey and Spain who have observed different morphological forms of piroplasms *viz*.,

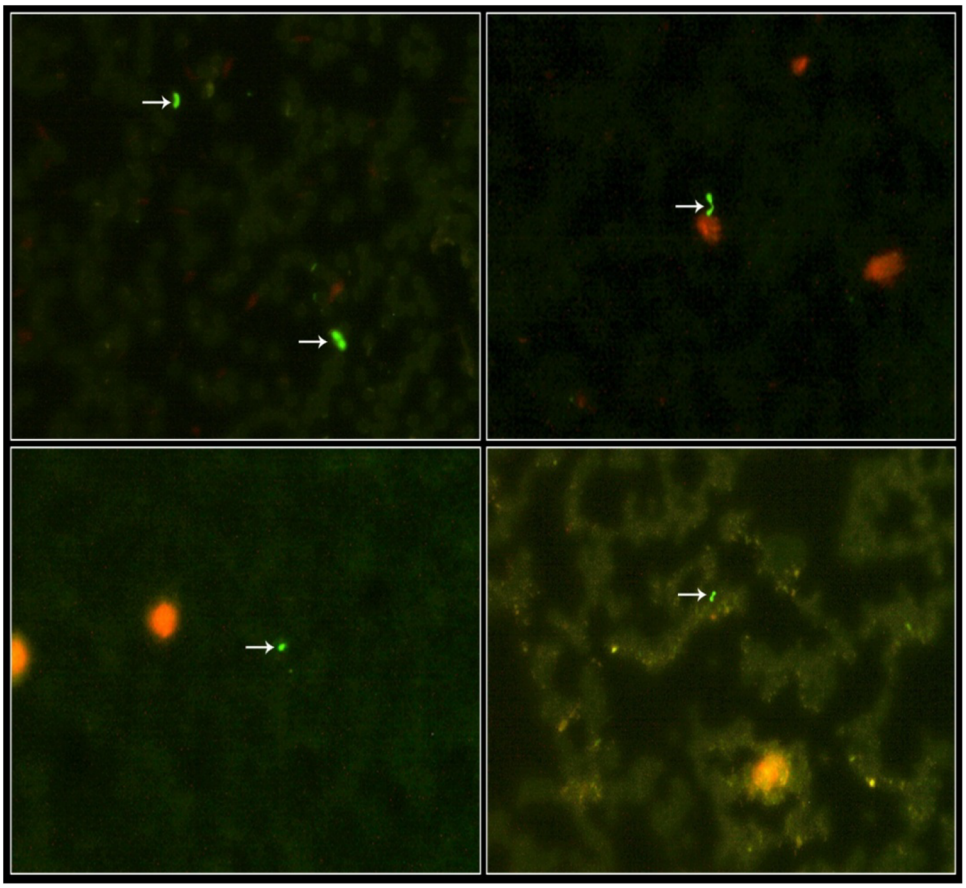

Fig. 1 Acridine orange stained blood smears showing *Babesia* organisms (400 ×)

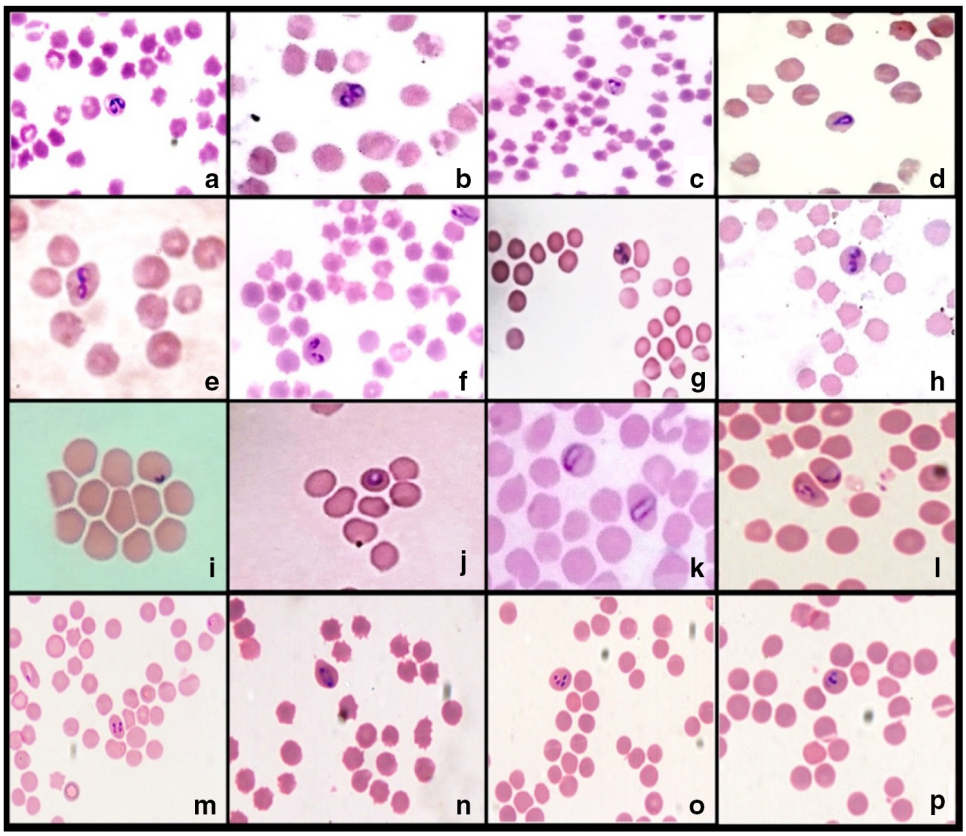

Fig. 2 Pleomorphic forms of *Babesia* organisms in Giemsa stained blood smears (1000 ×). **a–c** Paired pyriforms with acute angle, **d** single pyriform, **e–h** paired forms with obtuse angle, **i** ring form, **j** Amoeboid form, **k** elongated form, **l–p** Other forms

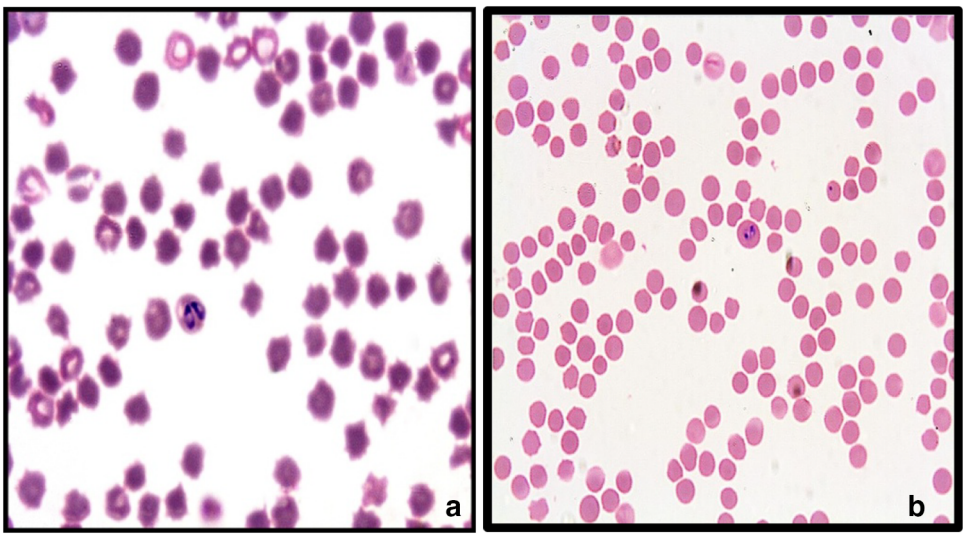

Fig. 3 Large (**a**) and small form of *Babesia* (**b**) organisms (1000 ×)

single and paired pyriforms, amoeboid forms, oval forms, elongated forms, ring forms, three leaf shaped single subspherical and other forms. Similar to the level of parasitaemia recorded during this study, Altay et al. (2008) reported 0.01 to one per cent of parasitemia in sheep from Turkey, Papadopoulos et al. (1996) recorded one per cent of parasitemia in *Babesia* infected sheep and goats from Macedonia region of Greece. However, Yeruham et al. (1996) from Israel recorded higher parasitemia of 5.3 and 7.04 per cent in ewes and hoggets, respectively. These variations in the percentage of parasitemia could be attributed to the stage of the disease at which the blood smears were made from the animals because, high parasitemia will be observed during acute/clinical stage of the disease whereas, low parasitemia is a characteristic feature of carrier or chronic stage of the disease (Yin et al. 2007).

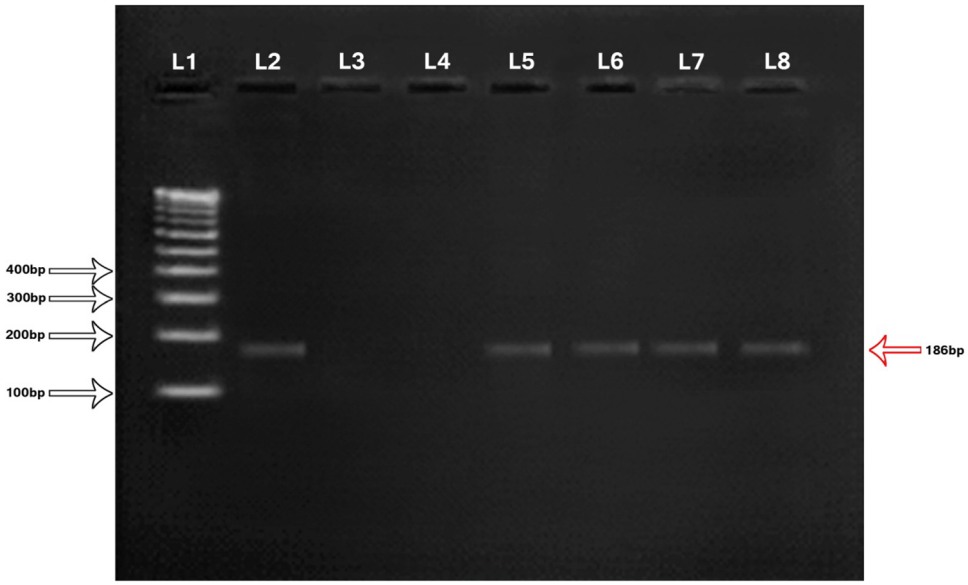

Fig. 4 PCR gel showing amplification of 186 bp DNA fragment specific for *B. ovis*. Lane 1: 100 bp DNA Ladder, Lane 3: Negative control, Lane 4: No template control, Lane 2, 5, 6: Field samples of sheep, Lane 7, 8: Field samples of goats

Fall in blood haemoglobin values of infected animals was the significant haematological change recorded during this study and is in accordance with Haq et al. (2017) from Jammu and Kashmir (India); Zangana and Naqid (2011) from Iraq and Vidya et al. (2011) from Karnataka (India).

Agewise susceptibility revealed that in below six months of age group, out of 68 sheep and 30 goats examined, 4.41 and zero per cent were found to be positive for *Babesia* spp. respectively. In above 6 months of age, out of 157 sheep and 88 goats, 5.73 and 4.54 per cent were found to be positive for *Babesia* spp. respectively. However, the lower infection was observed in young animals less than 6 months of age.

In genderwise susceptibility, out of 95 blood smears examined from males, 4.10 and zero per cent of sheep and goats were found positive for babesiosis, respectively. In 248 females, 5.92 per cent of sheep and 4.16 per cent of goats were found positive for babesiosis. The present study showed that infection is higher in females than males. During this study, there was no statistically significant differences between gender (males and females) and age groups in both sheep and goats ($P < 0.05$) which correlates with the findings of Reijbei et al. (2014) from Tunisia; Ali shah et al. (2017) from Pakistan; Aktaş et al. (2007) from Bazmani et al. (2018) from Iran.

Conclusion

In conclusion, babesiosis in sheep and goats caused by *B. ovis* can be considered as an emerging disease in Karnataka state. AO fluorescent dye staining method was found to be very sensitive, specific and cost effective diagnostic parasitological technique for epidemiological studies and quick detection of disease. However, it needs further research work on prevalence of *B. ovis* and ticks in other regions of Karnataka with their probable role in transmission of the disease.

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Authors' contribution SK, GSM and PED conceived of or designed study. SK performed research. SK, JNL and SS analyzed data. SK and GSM contributed new methods or models. SK and GSM wrote the paper.

Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

Ethical approval The work was carried out in a Teaching Veterinary Clinical Hospital and Research Institute, Veterinary College, Bengaluru, Karnataka Veterinary, Animal and Fisheries Sciences University (KVAFSU), Bidar, India. The KVAFSU 2004 Act legislates collection of clinical materials for the diagnosis and treatment of animals.

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