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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(11): 2106-2109

www.thepharmajournal.com
Received: 25-09-2022
Accepted: 28-10-2022

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Incidence and diagnosis of anaplasmosis in cattle

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Abstract
Bovine anaplasmosis, formerly known as gall sickness, is an infectious, non-contagious, tick-borne disease of domesticated and wild ruminants caused by Anaplasma species. It is considered as one of the economically important rickettsial diseases affecting ruminants and is principally transmitted by a tick of *Rhiphicephalus* spp. Present study on incidence and diagnosis of anaplasmosis in cattle was conducted at the Department of Veterinary Medicine, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand during October 2021 to March 2022. Total 66 suspected cattle were screened for anaplasmosis. They were subjected to blood smear examination and molecular test (PCR). Out of them, 22 cattle were found positive for anaplasmosis by microscopic examination and 34 cattle were found positive by PCR. Overall incidence of anaplasmosis by blood smear examination was 33.34 percent and by PCR 51.52 percent.

Keywords: Anaplasmosis, incidence, blood smear examination, PCR

Introduction
Anaplasma is one of the most important parasites transmitted by at least 20 hard ticks species and mechanically by some biting arthropods There are many Anaplasma species parasites, but *Anaplasma marginale* (*A. marginale*) and *Anaplasma centrale* (*A. centrale*) are the most important species. Bovine anaplasmosis is usually caused by *Anaplasma marginale* (Wahba *et al.* 2017) [1]. Anaplasma invades and multiplies within erythrocytes, causing clinical signs include anaemia, jaundice, fever, weight loss, abortion, decreased milk production, hyperexcitability and sudden death (Kocan *et al.*, 2003) [2]. Diagnosis of anaplasmosis is performed routinely by Giemsa-stained blood smears which can indeed be used as a suitable method to detect Anaplasma in animals clinically suspected for acute diseases, but it is not applicable to determine pre-symptomatic and carrier animals (Carelli *et al.*, 2007) [3]. Nucleic-acid-based tests polymerase chain reaction (PCR) have also been developed that are capable of detecting the presence of low-level infection in carrier cattle and tick vectors. (Aubry and Geale, 2011) [4] So present study was undertaken to rule out Incidence and the diagnostic efficacy of blood smear examination and PCR of anaplasmosis in cattle.

Materials and Methods
The blood samples from 66 cattle suspected of having anaplasmosis were collected from animals from the field area nearby Anand and Nadiad. Blood samples were collected from the jugular vein in sterile plastic K_3-Ethylenediamine tetra acetic acid (K_3EDTA) vials for microscopic as well as for molecular diagnosis.

Blood Smear Examination
Total 66 animals were screened by thin blood smear examinations. All the collected blood samples were processed within 24 hours following collection. Blood was collected from the peripheral circulation such as ear tip for making smears. A small drop of blood was placed on the pre-cleaned and grease free slide. Another slide was used as spreader for making thin blood smears. The edge of spreader slide was touched with the blood drop by keeping at 30-45˚ angle on first glass slide and a thin smear was made. The smears were labelled and allowed to air-dry for 10-15 minutes.

Giemsa's Staining Technique
The dried blood smears were flooded with methyl alcohol for 10 minutes, left to air-dry and stained with Giemsa's stain (Himedia Laboratories Pvt. Ltd, Mumbai, India).

The blood smears were immersed in staining fluid containing 30 drops (0.67 ml) Giemsa's stain in 30 ml distilled water and kept in stain for 45 minutes. They were washed with distilled water and allowed to dry. The blood smears were examined under the oil immersion lenses (x100) of microscope.

Molecular Diagnosis

The whole genomic DNA was extracted from the blood samples of 66 cattle by using commercial DNA Extraction kit (QIAamp-DNA extraction blood mini kit, Quigen, USA) according to the manufacturer's instructions. For molecular detection, Anaplasma oligo-primers specifically amplified at 429 bp, 16s rRNA gene of Anaplasma genus (Seong *et al*., 2015) [13] were used. The information of oligo-primers used is given in Table 1

Table 1: *Anaplasma* oligonucleotide Primer sequence used for PCR

Sr. No.	Primer	Primer sequence (5' to 3')	Amplicon Size
1	Forward Primer	TACCTCTGTGTTGTAGCTAACGC	429 bp
2	Reverse Primer	CTTGCGACATTGCAACCTATTGT	

Seong *et al.*, 2015 [13]; Park *et al.*, 2018 [11].

To optimize the PCR reaction, PCR was done at different annealing temperatures and concentration of DNA samples. The PCR amplification reaction was carried out in a programmable thermal cycler. The PCR procedure was run using appropriate PCR protocol as given in Table 2

Table 2: PCR protocol followed for DNA amplification of *Anaplasma* spp.

Sr. No.	Steps	Temperature & Time	Cycle
1	Initial denaturation	94 °C for 3 minutes	1
2	Denaturation	94 °C for 30 seconds	30
3	Annealing	56 °C for 1 minute	
4	Extension	72 °C for 1 minute	
5	Final extension	72 °C for 5 minutes	1

Results and Discussion

Incidence of anaplasmosis in cattle

In present study, a total 66 number of cattle suspected for anaplasmosis were screened on the basis of clinical signs, blood smear examination (Giemsa's stain), and by PCR test. The findings are shown in Table 3.

Table 3: Incidence of anaplasmosis in cattle

No. of animals screened	No. of animals positive by blood smear examination	No. of animals positive by PCR
66	22 (33.34%)	34 (51.52%)

Out of 66 cattle, 22 cattle were found positive for anaplasmosis by blood smear examination with overall incidence of 33.34 percent. The present finding agreed with Talulkar *et al.* (2001) [16]. who reported 33.00 percent occurrence of anaplasmosis in cattle. Rajput *et al.* (2005) [12] and Bhatnagar *et al.* (2015) [3] reported 41.00 and 42.07 percent occurrence of anaplasmosis in cattle, while Chowdhury *et al.* (2006) [5] reported very high 70.00 percent occurrence and Murleedharan *et al.* (2005) [10] reported very low incidence (1.33%) of anaplasmosis in cattle.

By PCR test, 34 out of 66 cattle were found positive for anaplasmosis contributing 51.52 percent incidence. This is nearer to the observations of 45.33 percent prevalence of anaplasmosis reported by Sharma *et al.* (2013) [14]. However, comparatively low incidence of 8.6 to 24.7 percent anaplasmosis has been documented through PCR by other researchers (Torioni De Echaide *et al.*, 1998; M'ghirbi *et al.*, 2016; Ganguly *et al.*, 2018; Shaukat *et al.*, 2019; Ashraf *et al.*, 2021) [17, 9, 6, 15, 1].

Diagnosis of anaplasmosis in cattle

Based on blood smear examination

A total number of 66 anaplasmosis suspected animals were selected for screening. All the animals were examined by Giemsa-stained blood smears under oil immersion (100x) microscope. Out of them, 22 (33.34%) animals were found positive by observing spherical, compact dot-like organisms in the vacuoles on or around the margin of erythrocytes.

In present study, shape of Anaplasma organism was observed in thin blood smear examination by Giemsa's staining technique. On microscopic examination, *Anaplasma* spp. were seen as compact spherical masses, inside the red blood cells near the periphery of RBCs wall (Figure 1). These observations are in accordance with Kumar *et al.* (2015) [8].

Based on molecular test

PCR amplification was used to investigate 66 blood samples from cattle, including those that were positive for anaplasmosis based on clinical signs and blood smear examination.

All the isolated DNA samples from naturally infected 18 cattle were amplified using different forward and reverse primers specific for anaplasma, which produced in a particular band on 429 bp, and the PCR results were analysed using a 2% agarose gel electrophoresis. Out of 66 samples 34 (51.52%) samples were found positive for *Anaplasma* species.

Comparison of diagnostic methods for anaplasmosis in cattle

Sensitivity and specificity tests were performed to determine the efficacy of various diagnostic techniques, with PCR serving as the standard diagnostic tool for confirmation of anaplasmosis in cattle. The difference was found when the results of diagnostic tools were compared.

Table 4: Sensitivity and specificity of blood smear examination

Screening test results (Blood smear examination)	Disease Status		Total
	Diseased	Non-diseased	
Positive	22	0	22
Negative	12	32	44
Total	34	32	66

In the present study, the sensitivity and specificity of thin blood smear examination was 64.71 percent and 100 percent as compared to that of the gold standard test PCR with 100 percent sensitivity and specificity. The 100 percent sensitivity and specificity of PCR proved the superiority of molecular diagnostic test over conventional thin blood smear examination (Table 4).

The results of present investigation are in line with reports of El-Ashker *et al.* (2015) in cattle affected with anaplasmosis. However, thin blood smear examination was less sensitive (64.71%), but 100 percent specificity suggested that it can be used as primary laboratory test for diagnosis of anaplasmosis under field conditions.

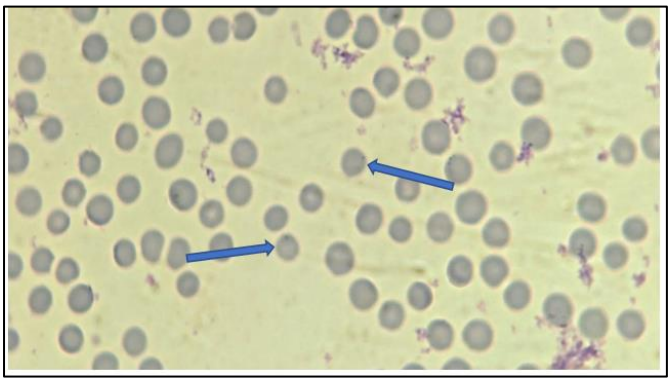

Fig 1: Dot shaped *Anaplasma marginale* on margin of erythrocyte

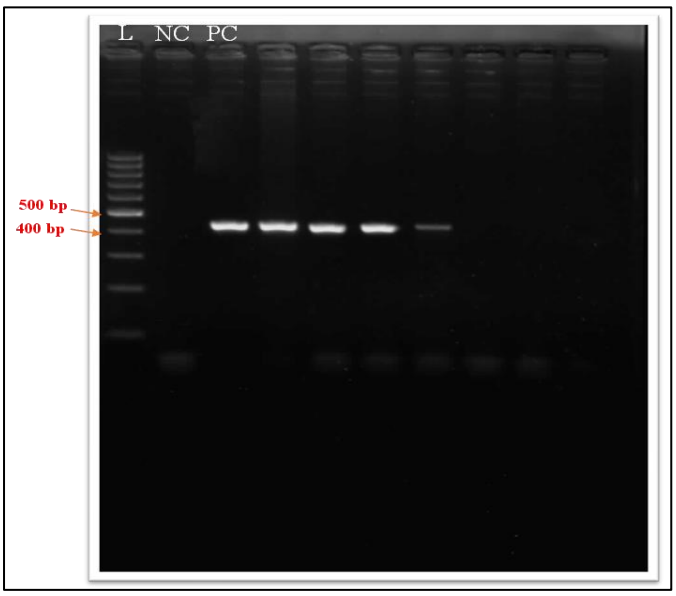

Fig 2: Gel image of *Anaplasma* spp. 429bp L-Leader, NC-Negative Healthy, PC-Positive Healthy

Conclusion

The overall incidence of anaplasmosis in cattle by blood smear examination was 33.34% (22/66) and by PCR 51.52% (34/66). The blood smear examination can be used for primary diagnosis of anaplasmosis in field condition, but it has 100% specificity and less sensitivity (64.71%). However, PCR having 100% sensitivity as well as specificity and it will also help in the detection of asymptomatic carrier.

Acknowledgement

The authors are grateful to the Dean of veterinary college and authorities of KU, Anand for the facilities and encouragement provided for this work.

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