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Molecular studies on carrier status of bovine Anaplasmosis in and around Chennai

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Abstract
A total of 34 blood samples were collected from different breeds of cattle came to large animal outpatient unit, Madras veterinary College in Chennai. The samples were collected with details of sample number, age, place and date of collection. Blood smear were taken from 34 animals and stained with cocktail preparation of Giemsa-leishman stain. Among 34 samples only one smear showed positivity for Anaplasma marginale and remaining 33 samples were negative. From the collected blood samples DNA was extracted and DNA purity was checked by Nano-drop method and PCR was performed. The PCR products were loaded in 1.5 percent agarose gel and view under gel-doc UV illuminator showed the presence of 427 bp. product of *Anaplasma marginale* in three samples. In the present study, the presences of *Anaplasma marginale* in healthy carrier's bovines were confirmed using microscopical and molecular techniques. Microscopy could detect only one positivity among 34 samples for *Anaplasma marginale.* No *Anaplasma bovis* could be detected by microscopy as well as by PCR. The present study clearly indicated that higher sensitivity and specificity for PCR to detect the *Anaplasma marginale* carrier in healthy animals.

Keywords: Anaplasmosis, subclinical anaplasmosis, carrier state of anaplasmosis, PCR

Introduction
Bovine anaplasmosis is an infectious disease of cattle caused by the obligate inter cellular bacterium. *Anaplasma marginale* which is of the order Rickettsiales (Hanzlicek). Anaplasmosis is one of the most prevalent and economically important rickettsial diseases through the country. Carrier animals can act as the source of infection for naïve hosts. Detection of *A.marginale* using a nucleic acid approach offers an alternative diagnostic tool, studies have been undertaken using this approach for its detection from carrier cattle (Noaman and Shayan, 2010) [8].
The disease primarily occurs in tropical and subtropical regions and can provide significant issues regarding beef and dairy production potential if untreated. (Kocan).
Signs and symptoms include fever, weight loss, abortion, and potentially death (for cattle older than 2 years), although juvenile cattlelessthan9monthsoldareusuallyasymptomatic.Cattle who survive exposure to Anaplasma become immune to the disease; however, they carry the disease for life, which is a concern formative portions of the population (Hairgrove)

Materials and Methods
This data was conducted from 2021 November at large Animal Medicine unit, madras veterinary teaching hospital and consist of 34 blood samples collected from crossbred cattle cows which were brought to veterinary hospital. A total of 34 blood samples were collected from different breeds of cattle came to large animal outpatient unit, Madras veterinary College in Chennai. The samples were labelled properly with details of sample number, age, place and date of collection.
The blood samples were collected randomly from the cattle in heparinised EDTA vial as well as in serum tubes with peripheral blood smears. All the collected samples were brought to the laboratory, where the heparinised samples were kept in deep freezer i.e.-20 C till processing.
LG (Leishman and Giemsa) cocktail was prepared by (Garbyal RS and Agarwal N, Kumar P, 2006) [5].
The unit volume of Giemsa stock was filtered and mixed with an equal unit volume of distilled water to prepare a Giemsa working solution (1:1 dilution from stock). (The dilutions can be changed according to one's preference, up to 1:7.).

An equal volume of Leishman's stain was filtered and mixed with an equal volume of the above Giemsa working solution (1:1). (The dilutions can be changed according to one's preference, up to 1:7.)

Isolation of Dna-Qiagen Dneasy Blood & Tissue Kit method of DNA

Extraction

1. Add 300 µl of EDTA blood and add 100 µl of phosphate Buffer saline, vertex add 20 µl of Proteinase K into 2ml micro centrifuge tube and incubate at 56 ºC for 10 minutes.
2. Vertex it. Add 200 µl of AL buffer, vertex it and incubate at 56 ºC for 10 minutes, vertex.
3. Add 200 µl of Chilled absolute ethanol, vertex it and mix into spin column maximum capacity 600 µl.
4. Centrifuge at 8000 rpm for 3 minutes and discard, keep the spin column into another collection tube, add 500 µl of AW1 (Alkaline wash) buffer, centrifuge at 8000 rpm for 2 minutes at room temperature. Discard the collection content.
5. Keep spin column into new collection tube, add 500 µl of AW2 buffer, centrifuge at 8000 rpm at 2 minutes at room temperature and discard/empty the collection tube content.
6. Centrifuge the tubes at 14000 rpm for 3 min at room temperature.
7. Keep the spin column into new 1.5 ml centrifuge tube.
8. Add 30 µl of pre-treated Nuclease free water into spin column. Wait for 2minutes.
9. Centrifuge the tube contain 30 µl of DNA stored at -20 ºC.
10. This final pellet was used as template for PCR. Crude DNA isolated from the blood of an adult infected cattle of Chennai, Tamil Nadu formed the Positive control.

DNA Concentration

DNA concentration of 34 samples detected by Nano drop method using Thermo scientific Nano drop one- equipment.

Fig 1: Nano-drop equipment

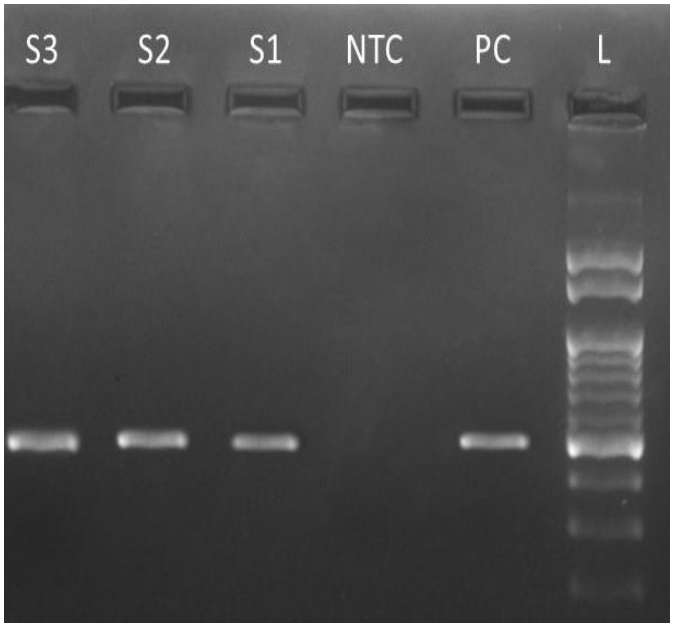

Fig 2: 1.5% Agarose gel PCR product of *Anaplasma marginale*

L – Ladder, PC- Positive control, NTC- Negative Control, S1 – Positive sample 1, S2- Positive sample 2, S3-Positive sample 3 (457 bp).

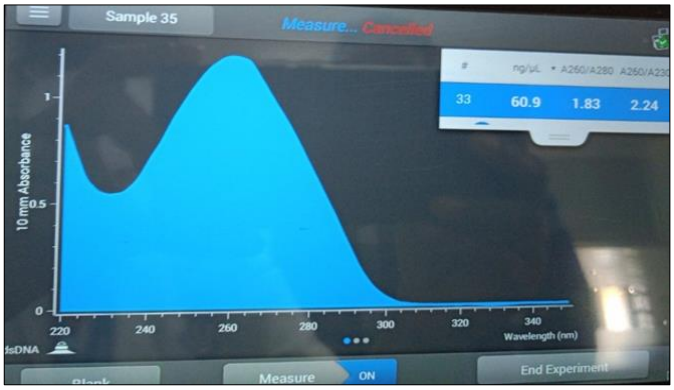

Fig 3: DNA Concentration curve – Good concentration value -1.8

PCR for the detection of A. marginale using primers amplifying msp5 gene with a product size of 457 bp was performed.

Table 1: PCR for 25 µl reaction

Master mix	12.5 µl
Forward primer	1 µl
Reverse Primer	1 µl
DNA	2 µl
NFW	8.5 µl

Table 2: Negative control for primers

Master mix	12.5 µl
Forward primer	1 µl
Reverse Primer	1 µl
NFW	10.5 µl

Cycling Conditions for detection of *A. marginale* using primers amplifying msp5 gene

The cycling conditions were initial denaturation of 5 min at 95 ºC, 35 cycles each consisting of denaturation at 9 5ºC for 1

min, annealing 65 ºC for 2 min and extension 72 ºC for 1 min with a final extension 72 ºC for 10 min followed by cooling to 4 °C. The reactions were performed using T100 thermal cycler.

After amplification/digestion reaction 5 µl of the product was electrophoresed in an ethidium bromide-stained 1.5 percent agarose gel and visualised in a transilluminator under UV light. A 100 bp (Bangalore genei, India) ladder was used as molecular standard.

Results and Discussion

Only one *A. marginale* could be detected by microscopy. *A. marginale* was detected in one out of 34 LG (Leishman and Giemsa) cocktail-stained blood smear. Only one sample positivity was confirmed by Microscopy that was confirmed by PCR also. Two samples of *A. marginale* carrier was detected out of 34 samples that two carrier samples was not detected by Microscopy. Totally 3 samples were detected in PCR as positive.

The most important haemorickettsial organism affecting cattle of the study area was *A. marginale*. PCR showed the maximum sensitivity in detecting these organisms than microscopy.

In the present study, the presences of A. marginale and A. bovis in healthy Carriers bovines were confirmed using microscopical and molecular techniques. Microscopy could detect only one positivity among 34 samples for *Anaplasma marginale.* No *Anaplasma bovis* could be detected by microscopy as well as by PCR. Sreekumar *et al.* (2000) [10] could not detect A. bovis in a blood smear of infected animals. Microscopy is widely accepted technique for the diagnosis of haemoprotozoan and haemorickettsial organisms, but this technique lack high sensitivity. The present study clearly indicated that higher sensitivity and specificity for PCR. PCR assay showed high density and specificity than microscopy in detecting tick-borne parasites.

Anaplasmosis is one of the most prevalent and economically important rickettsial diseases through the country. Carrier animals can act as the source of infection for naïve hosts. Detection of *A. marginale* using a nucleic acid approach offers an alternative diagnostic tool, studies have been undertaken using this approach for its detection from carrier cattle (Noaman and Shayan, 2010) [8]. In the present study, though the carrier animals did not exhibit any symptoms they remain patent to the vectors and remain silent source of infection to other susceptible animals (Kieser *et al.,* 1990). The higher prevalence of Anaplasmosis in suspected cross-bred animal indicates the presence of sub-clinical infection or carrier status of this disease. (B.) *microplus* was reported as the commonest tick species in Tamil Nadu (Koshy *et al.,* 1982) [7]. The presence of biting flies (Tabenus spp. And Stomoxys spp.) due to hot and humid climatic conditions prevailing in the state may augument the mechanical transmission to the naïve animals.

The present study concluded that there age, breed, sex, season, acaricide application and prevalence. Losses due to this disease can be prevented by application of management practices and control over theses predisposing factors

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