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# Bovine carriers of *Anaplasma marginale* and *Anaplasma bovis* in South India

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**Abstract.** Carriers of bovine anaplasmosis in Northern Kerala, South India were detected using conventional microscopical and molecular techniques. PCR-RFLP and nested PCR techniques were used for detection of *Anaplasma marginale and Anaplasma bovis* respectively and the PCR products were confirmed by sequencing. Out of 150 samples tested, 25 were detected positive for *A. marginale* and five for *A. bovis* based on molecular tests. The inclusion bodies of *A. marginale* could be detected by microscopy in two blood smears after staining by giemsa while acridine orange staining detected three smears positive. The data clearly suggest the higher sensitivity of molecular techniques for diagnosis of these diseases.

## INTRODUCTION

Bovine anaplasmosis or gall sickness caused by the intraerythrocytic rickettsia, *Anaplasma marginale* (Rickettsiales: Anaplasmatacea) is transmitted biologically by infected ticks or mechanically by biting flies and contaminated fomites (Ristic, 1981). Acute anaplasmosis is characterized by fever, progressive haemolytic anemia, weight loss, abortion and even death resulting in significant loss to meat and milk production (Alderink & Dietrich, 1981). The economic loss due to infections caused by *Babesia* and *Anaplasma* infections in India was estimated to be $57 million (Tick Cost Version, 1.0, 1999).

The genus *Anaplasma* also includes *Anaplasma bovis*, formerly referred as *Ehrlichia bovis* (Dumler *et al.*, 2001). *Anaplasma bovis* is transmitted by *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and *Hyalomma truncatum* and occur in India and Africa (Radostits *et al.*, 2000). The symptoms of bovine ehrlichiosis include fever, enlargement of peripheral lymphnodes, apathy, anorexia, loss of weight, shaking of head and in most of the cases one or both ears held over the parotid region with tilting of head towards that side (Woldehiwet & Ristic, 1993).

Animals that recover from primary attack remain as life long carriers (Piercy, 1956). During the carrier stage, rickettsaemia occurs, which may permit vector transmission to take place and hence diagnosis of carrier status is important. Routine laboratory diagnosis in acutely infected cattle is based on the microscopic examination of peripheral blood smears. Serological tests even though developed, lack the required specificity and sensitivity for a

reliable diagnosis (Singh, 1997; Aubry & Geale, 2011). Molecular DNA based methods can address some of these problems and are widely used nowadays.

The present communication deals with the detection of these haemorickettsials in carrier bovines using conventional and molecular techniques from blood samples collected from five northern districts of Kerala, South India.

## MATERIALS AND METHODS

### Animals and samples

A cross-sectional study (convenient sampling) was conducted using a total of 150 blood samples collected in heparinised eppendorf tubes from apparently normal / healthy crossbred cattle (crossbreds of Jersey, Holstein Fresian or Swiss Brown with the indigenous cattle) of Northern Kerala, 30 each from five districts *viz*., Wayanad, Malappuram, Kozhikode, Palakkad and Kannur during June to December, 2007. The bovine populations (Quinquennial Livestock Census, 2003) in these districts are as follows; Wayanad (159858), Malappuram (134703), Kozhikode (163404) Palakkad (263763) and Kannur (103694). Six random samples from 5 different localities contributed 30 samples from each district (Fig. 1). Samples were collected from animals kept by individual households and not from farms. Two peripheral blood smears were also prepared from each animal and were fixed with methanol.

### Staining techniques

#### Giemsa staining

One smear from each animal was stained with giemsa stain at 1 in 10 dilutions for 30 minutes and examined under oil immersion (100X) objective of the microscope. A minimum of 5000 RBCs were screened before declaring negative for presence of any rickettsial organisms.

#### Acridine orange staining

Acridine orange staining was performed as per the method of Lauer *et al.* (1981) with slight modifications (Ravindran *et al.*, 2007). Briefly, blood smears were flooded with 0.01 per cent acridine orange stain, allowed to act for two minutes and then washed slowly in tap water. The smears were mounted using coverslip and examined when moist, under

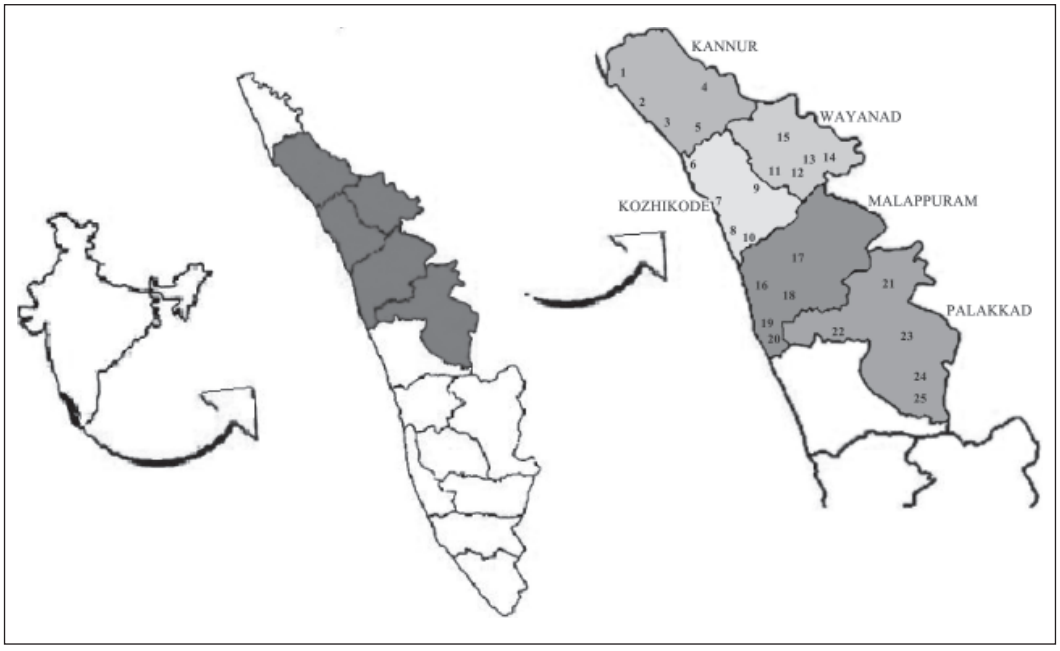


Figure 1. Map of India and Kerala showing the area of collection of blood samples

the 100X objective of fluorescent microscope (Olympus). A minimum of 50 peripheral fields were screened before declaring negative for the presence of any rickettsial organisms.

**Isolation of DNA**

The blood samples were processed for extraction of crude DNA based on the method described for the detection of *Plasmodium* sp. from human blood (Tirasophon *et al.*, 1991) with slight modifications. Heparinized blood samples (150 µl) were treated by 1300 µl dehaemoglobinization buffer (0.015 per cent saponin, 3.5 mM NaCl and 1 mM EDTA) and centrifuged at 12,000 rpm for 2 min. The supernatant was discarded. The pellet was washed once with 750 µl of reaction mixture buffer (10 mM Tris HCl, 50 mM $MgCl_2$ and 0.01 per cent gelatin) and centrifuged at 6000 rpm for one minute. Again, the supernatant was discarded carefully. The final pellet was resuspended in 75 µl of distilled water, boiled for 10 min and 10 µl from this was used as template for PCR. The blood sample of a day old calf processed by the similar technique was used as negative control in all PCR reactions. Crude DNA isolated from the blood of an adult infected cattle of Chennai, Tamil Nadu formed the positive control.

**Molecular detection**

PCR for the detection of *A. marginale* using primers (20 pmol each) amplifying msp5 gene (Forward 5'-GCA TAG CCT CCG CGT CTT TC-3'; reverse 5'-TCC TCG CCT TGG CCC TCA GA-3') with a product size of 457 bp was performed as per the protocol described by Shimada *et al.* (2004). The cycling conditions were: initial denaturation of 5 min at 95°C, 35 cycles each consisting of denaturation at 95°C for 1 min, annealing 65°C for 2 min and extension 72°C for 1 min with a final extension at 72°C for 10 min followed by cooling to 4°C. To determine the specificity, all the amplicons were digested with *Eco* RI restriction endonuclease (Bangalore Genei, India) according to the manufacturer's instructions.

A nested PCR was standardized for the detection of *A. bovis.* Universal eubacterial primers (PC5 and PO mod) which recognize conserved sequences of 3' end of 16S r DNA (Wilson *et al.*, 1990; Chen *et al.*, 1994) and amplify 1500 bp product were used for the initial reaction. The forward (5'-TAC CTT GTT ACG ACT T 3') and reverse (5'-AGA GTT TGA TCM TGG 3') primers (25 pmol each) were used. The cycling conditions were: initial denaturation of 3 min at 95°C, 25 cycles each consisting of denaturation at 95°C for 30s, annealing 52°C for 1 min and extension 72°C for 2 min with a final extension at 72°C for 5 minutes followed by cooling to 4°C. One microliter of the product from the first amplification was used as the template DNA for amplifying *A. bovis* 16S r RNA gene specific 551 bp product using 25 pmol each of forward (5'-CTC GTA GCT TGC TAT GAG AAC-3') and reverse (5'-TCT CCC GGA CTC CAG TCT G-3') primers. The cycling conditions were: initial denaturation of 3 min at 95°C, 40 cycles each consisting of denaturation at 94°C for 1min, annealing 55°C for 1 min and extension 72°C for 1 min with a final extension at 72°C for 5 min followed by cooling to 4°C.

All amplification reactions were performed in a final volume of 25 µl containing 10 µl of DNA, 0.2 mM of dNTPs, 5ml of buffer (20mM Tris- HCl, 50 mM KCl, 1.5 mM $MgCl_2$) and 1.25 U *Taq* DNA polymerase (Bangalore Genei, India). The reactions were performed using a thermal cycler with heated lid (Eppendorf, Germany).

After amplification / digestion reaction, 15 µl of the product was electrophoresed on a 1.5 per cent agarose gel. A 100 bp ladder (Bangalore Genei, India) was used as molecular size standard. Results were statistically analysed using Chi square test.

The PCR products (one each of *A. marginale* and *A. bovis*) were directly sequenced using an automated DNA sequencing service of Bangalore Genei Pvt. Ltd, Bangalore. Database homology search with newly obtained sequence information was conducted using BLAST (<http://www.ncbi.nih.gov/BLAST/>) programme for confirmation.

Statistical analysis (SPSS, IBM, USA) was performed based on Fisher's Exact (Chi square) test.

RESULTS

**Microscopical examination of blood smears**

Only *A. marginale* could be detected by microscopy. *A. marginale* was detected in two out of 150 giemsa stained blood smears while acridine orange staining (Fig. 2) revealed the organism in three smears.

**Polymerase Chain Reaction**

The *A. marginale* specific primers amplified a 457 bp product without any spurious products from the crude DNA isolated from the blood of infected cattle from Chennai, Tamil Nadu which was used as positive control. The primers did not amplify any product when bovine leucocyte DNA was used as template.

*Anaplasma marginale* specific PCR-RFLP was performed on samples which yielded the desired 457 bp product. On digestion with *Eco* RI, products of size 265 bp and 192 bp were observed. Out of 150 samples, 25 (16.67 per cent) were positive for *A. marginale*. *Anaplasma marginale* was detected from 5 samples of Wayanad, 4 samples from Malappuram, 5 samples of Kozhikode, 9 samples from Palakkad and 2 samples from Kannur.

Nested PCR was performed for detection of *A. bovis*. A product of 1500 bp fragment was observed after amplification with universal eubacterial primers. An amplicon of size 551 bp detected the presence of *A. bovis*. Seven out of 150 samples revealed the 1500 bp product, out of which only one (from Menangady, Wayanad) was identified as *A. bovis*. However, four samples which did not produce 1500 bp product, revealed *A. bovis* specific 551 bp fragment. Out of 150 samples, one sample showed mixed infection due to both organisms. The most important haemorickettsial organism affecting cattle of the study area was *A. marginale*.

Sequencing of the PCR products amplified by *A. marginale* Chennai isolate (Accession number: JQ706074), Malappuram isolate (Accession number: JQ706075) and *A. bovis* (Accession number: JQ706076) confirmed the presence of the organisms. NCBI-BLAST revealed maximum identity of 99 per cent of Chennai and Malappuram isolates of *A. marginale* with Macheng, China (DQ317449),

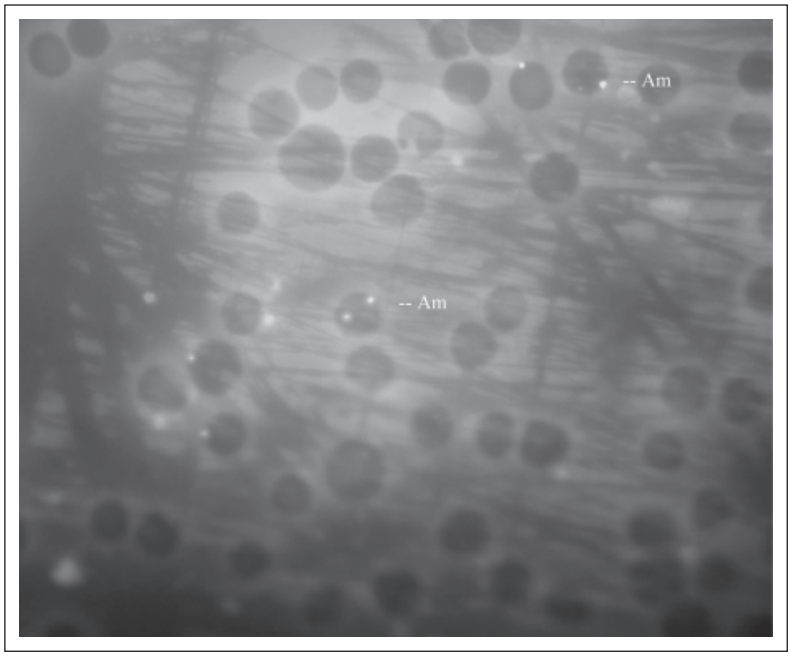


Figure 2. Acridine orange stained blood smear showing *Anaplasma marginale* inclusions
Am : *Anaplasma marginale* inclusion

Hogan, China (DQ317448), Cebu, Phillipines (AB704328), Liangdang, China (EF546443), Brazil (AY714547) and Pernambuco-Zona da Mata, Brazil (AY245428) strains of *A. marginale*. *Anaplasma bovis* isolate from Wayanad revealed 98 per cent identity with many isolates *viz.*, caprine isolates from China (JN558829, JN558828, JN558825, JN558825, JN558819, HQ913645, HQ913644, HM595732, FJ389577, FJ389573, FJ169956), bovine isolate from China (FJ169957) sheep isolate from Iran (JF514513), *Boophilus annulatus* isolate from Iran (JF514508) South Korean water deer isolates (GU556627, GU556626, EU682764) South Korean canine isolate (EU409558) South Korean *Haemaphysalis longicornis* isolates (GU064902, GU064901, EU181143) deer isolate from Japan (AB454073) *H. longicornis* isolate from China (EU179238, EU179237) *etc*.

**Comparison of giemsa, acridine orange and PCR techniques**

Acridine orange staining was equally / more sensitive to giemsa staining in detecting haemorickettsials. PCR showed the maximum sensitivity in detecting these organisms. The comparison of results of the two tests is shown in Table 1. Statistical analysis (SPSS, IBM, USA) using Fisher's Exact (Chi square) test revealed that there is an association between PCR and staining results ($p=0.007<0.05$, Pearson chi square value =12.773).

Table 1. Results of staining (Acridine orange and PCR) on samples for *A. marginale*

| | Staining +ve | Staining -ve | Total |
|---|---|---|---|
| PCR +ve | 3 | 26 | 29 |
| PCR -ve | 0 | 121 | 121 |
| Total | 3 | 147 | 150 |

## DISCUSSION

In the present study, the presence of *A. marginale* and *A. bovis* in healthy carrier bovines were confirmed using microscopical and molecular techniques. Microscopy could detect a maximum of three samples positive for *A. marginale*. No *A. bovis* could be detected by microscopy. Molecular techniques detected 25 samples for *A. marginale* and one sample for *A. bovis*. Sequencing of the products confirmed the presence of the organisms in these animals.

Although microscopy is widely accepted and cost effective technique for the diagnosis of haemoprotozoans and haemorickettsial organisms, the technique lacks the higher sensitivity. The low prevalence observed in the present study may be due to the fact that carrier cattle usually do not reveal inclusions in their blood films (Ristic, 1981). The present study also clearly indicated the higher sensitivity and specificity of the molecular methods (Figueroa *et al*., 1993; Gale *et al*., 1996) compared to the conventional methods especially in detecting low level rickettsaemia seen in carrier animals.

Microscopy could not detect a single case of *A. bovis* in the present study even though Sounderrajan & Rajavelu (2006) could detect 0.53 per cent out of 150 blood smears collected from Chennai. Sreekumar *et al.* (2000) couldn't detect *A. bovis* in a blood smear of an infected buffalo and later identified the etiology by culturing the organism in blood mononuclear cells.

Previously, Naranjo *et al*. (2006) demonstrated that *A. marginale* infections in Southern Europe are maintained in cattle and deer with ticks and tabanids serving as vectors. In Thailand, Parola *et al.* (2003) identified three different types of *Anaplasma* as well as *Ehrlichia* from ticks based on molecular studies. Various other studies from different parts of the world include, Italy (Torina & Caracappa, 2007), Mexico (Rodríguez *et al.*, 2009), Hungary (Hornok *et al*., 2007), South Africa (Mtshali *et al.*, 2007). Molecular characterization of MSP1a repeat sequences (de la Fuente *et al.*, 2007) using 131 strains of *A. marginale* from countries in North and South America, Europe, Asia, Africa and Australia revealed genetic heterogeneity of organism.

Anaplasmosis is considered as one of the top ten economically important rickettsial diseases affecting ruminants in India (PD-ADMAS Annual Report, 2005-06). The higher

prevalence of the anaplasmosis in clinically normal crossbreds of Northern Kerala of South India indicates subclinical infections or carrier status of these vector borne diseases. Though the carrier animals didn't exhibit any symptoms; they remain patent to the vectors and remain silent source of infection to other susceptible animals (Kieser *et al.*, 1990). *Rhipicephalus (B.) annulatus* is reported as the commonest tick species in southern region of the country (Jagannath *et al.*, 1979; Rajamohanan, 1982; Koshy *et al.*, 1982). The abundance of biting flies (*Tabanus* spp.and *Stomoxys* spp.) due to the hot and humid tropical climatic conditions prevailing in the state; augment the mechanical transmission of the disease to naïve animals. Hence, the results of the study is of great importance on the backdrop of the changing tick epidemiology of the country by replacement of multiple host ticks with one host ticks (Khan, 1990,1994; Sangwan *et al.*, 2000).

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