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## Diagnosis and confirmation of *Theileria annulata* infection in cattle in Odisha, India

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### Abstract

The present research was undertaken in and around Bhubaneswar, Odisha for a period of three consecutive years from March 2012 to February 2015 to study the incidence of bovine tropical theileriosis in cattle through examination of Giemsa stained blood smears and confirm theileria annulata infection by Polymerase Chain Reaction (PCR) method. Out of the 5237 suspected cattle blood samples, 3876(74%) were found positive by blood smear examination and 1361(26%) were found negative. Fifty fresh blood samples including 25 positive and 25 negative cases, declared after blood smear examination, were subjected to PCR test and 34 were found positive. The PCR product was sequenced and submitted to National Center for Biotechnology Information (NCBI). It was assigned a new accession number KT222946 and named as OMSA-1 by NCBI. This is also the first report on PCR diagnosis of theileriosis in the state. As PCR improved the diagnosis by blood smear examination, it can be used for diagnosis to avoid false positive and false negative results.

**Keywords:** Cattle, PCR, *Theileria annulata*, infection

### 1. Introduction

Theileriosis (*Theileria annulata* infection) has emerged as one of the fatal disease of crossbred animals in last two decades. The disease is present in the entire Indian subcontinent and it is endemic in India [1]. It is transmitted by the tick of genus Hyalomma. Indigenous cattle and buffaloes have an inherent resistance to the disease and harbour theilerial piroplasm in their erythrocytes as symptomless carriers. In general, calves are more susceptible to theileriosis as compared to aged animals. The indigenous cattle on recovery from the disease, in endemic areas, become resistant to re-infection but develop a persistent carrier state [2, 3]. These carriers are potentially dangerous to uninfected and newly introduced crossbred animals, by acting as reservoirs of infection in the presence of ticks. There is need for study on the clinicopathological aspects of the disease and suitable molecular diagnostic methods for early and effective detection of the parasite in both clinical and subclinical cases of the disease. Hence, the present work was conducted to study the incidence of the disease in cattle in areas in and around Bhubaneswar over three years time period from 2012 to 2015. Also the molecular test was done to confirm the causative agent i.e. *Theileria annulata* parasite from suspected blood sample.

### 2. Materials and methods

#### 2.1 Collection of clinical sample and data

The blood samples received at Teaching Veterinary Clinical Complex (TVCC), College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar, Odisha with complain of anorexia, non-remitting pyrexia, drop in milk yield, enlarged superficial lymph nodes, pale conjunctival mucous membrane, haemoglobinuria, nasal discharge, coughing etc. during the period from March 2012 to February 2015 were included in this study. Patient data regarding age, sex, stage of lactation, parturition history, clinical signs etc. were also recorded. Screening of the blood samples for theileriosis was primarily done by Giemsa stained smear examination. A total of 5237 number of blood samples were collected from suspected cases of theileriosis in cattle. The blood samples were tested for hematological parameters. Thin blood smear was prepared from all the samples, stained with Giemsa stain and examined under microscope by oil emersion lens for demonstration of theileria piroplasm inside erythrocytes.

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## 2.2 Polymerase Chain Reaction (PCR) and nucleotide sequencing

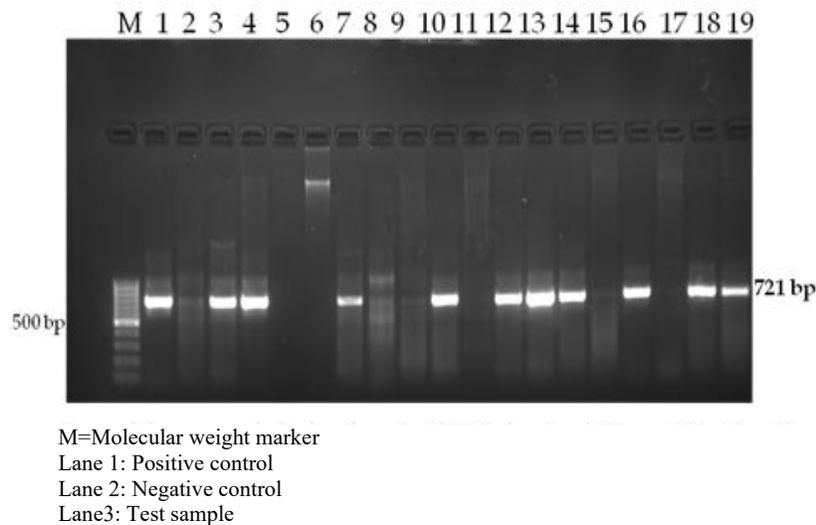
The deoxyribonucleic acid (DNA) was extracted from 50 fresh blood samples including twenty five positive cases and twenty five clinically suspected cases but found negative in blood smear examination using commercial kit from Merck India. The forward and reverse primer sequences as per previous report GTAACCTTTAAAAACGT and GTTACGAACATGGGTTT respectively were used for PCR of extracted parasitic DNA [4]. The thermal profile such as initial denaturation at 94 °C for 3min, denaturation at 94 °C for 45sec, annealing at 55 °C for 30sec, extension at 72 °C for 50sec, step2 to step4 were repeated for 35 cycles and final extension was done at 72 °C for 5min was followed. The PCR product was subjected to 1.2% gel electrophoresis. The results were visualised in molecular imager (Biorad). The positive gel band was cut, extracted by gel extraction kit and sent for sequencing.

## 2.3 Analysis of result

The nucleotide sequences obtained were subjected to BLAST analysis. The similar sequences from public database were retrieved and phylogenetic tree was constructed using MEGA 7 tool. The obtained nucleotide sequence showed variation from public database and thus submitted to GenBank for record.

## 3. Results

A total of 5237 blood samples from clinically suspected cases of bovine theileriosis were examined by blood smear examination and 3876 were found positive. Fifty fresh blood samples including twenty five positive cases and twenty five clinically suspected cases but found negative in blood smear examination were subjected to molecular diagnosis using PCR technique. An expected amplified product of 721bp (fig-1) was obtained from 34 out of 50 blood samples indicating the presence of *Theileria* parasite (as confirmed later in sequencing) and 16 samples were found negative.



**Fig 1:** Agarose gel electrophoresis (1.2%) showing PCR amplification of 721bp fragment of *Theileria annulata* major merozoite surface antigen.

The nucleotide sequence obtained after sequencing was submitted to GenBank with accession number KT222946. The strain was identified as *Theileria annulata* based on previous reports [5, 6]. The sequence analysis also revealed that the obtained nucleotide sequence was having homology with other Indian strains with Accession No. KP235484, KP235485 from Andhra Pradesh, EF618728 from Tamil Nadu and JX648210.1 from Kerala with respect to that particular amplified portion.

Neighbour-joining tree based on the partial gene sequence of OMSA-1 obtained in this study and reference sequences obtained from the NCBI GenBank database. The tree was constructed using the maximum composite likelihood based

substitution method with 1000 bootstrap replicate for test of phylogeny in the MEGA 7 software (fig 2). For phylogenetic analysis, six closely related sequences from five countries (Japan, China, Netherland, Egypt and Tunisia,) and four closely related sequences from three states of India (Andhra Pradesh, Tamilnadu and Kerala) were retrieved from GenBank. The sequence of Tams-1 gene isolated from Andhra Pradesh and Tamil Nadu were having high sequence similarity occupying a single cluster. The sequence isolated from Odisha is having high homology with sequence isolated from Andhra Pradesh. The sequence isolated from China was having high homology with sequence isolated from Japan.

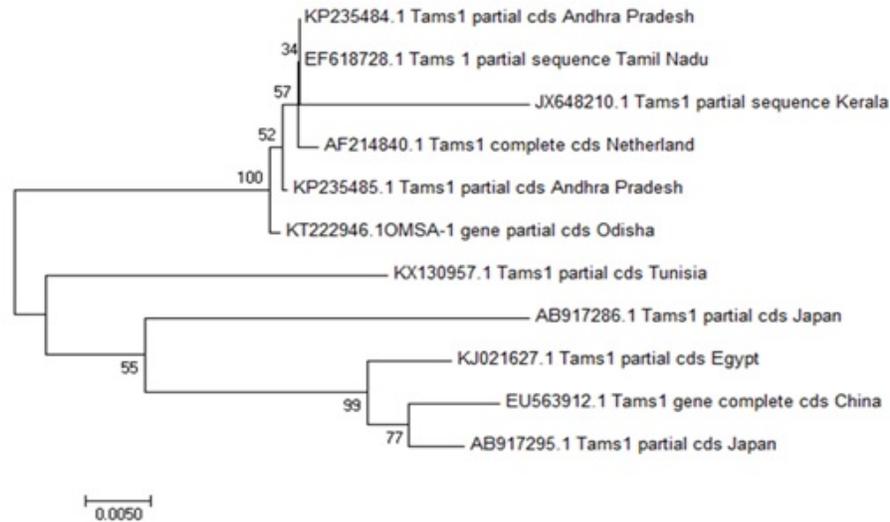


Fig 2: Phylogenetic tree of OMSA-1 gene sequence

#### 4. Discussion

A total of 5237 blood samples from suspected cases of theileriosis were screened by blood smear examination. Piroplasms indicating *Theileria annulata* were detected inside RBCs in blood smears examination in 3876 (74%) out of 5237 cases. Many previous workers have reported prevalence of theileriosis by blood smears examination in various states of India. In Haryana state, Galhotra and Chandiramani [7] reported 35.53% prevalence in cattle and buffaloes. Shastri *et al.* (1985) reported 48.0% prevalence among 553 buffaloes in Marathwada, Madhya Pradesh and Uttar Pradesh. Singh [8] examined 5454 blood smear from apparently normal crossbred cattle during 1989 and found 14.94% prevalence. Chengalva *et al.* [9] reported 10.50% prevalence in cattle and buffaloes from Andhra Pradesh. Shinde *et al.* [10] reported 4.33% prevalence in cattle from Nagpur region. Roy *et al.* [11] reported 8.17% prevalence of theileriosis in Durg, Rajhandgaon, Raipur and Bastar districts of Chhattisgarh from April 2000 to March 2002. Raina *et al.* [12] examined 116 blood smears and revealed the prevalence of theileriosis as 3.44%.

In the present study, PCR was done to confirm the presence of the parasite in bovine blood samples. Fifty fresh blood samples including twenty five positive cases and twenty five clinically suspected cases but found negative in blood smear examination were subjected to molecular diagnosis using PCR technique. Total 34 cases were found positive for theileriosis by PCR method in comparison to 25 positive cases in blood smear examination which improved the diagnosis by 14%. Many workers have also similar reports on the efficacy of molecular diagnosis of theileria parasite over blood smear examination [4, 6, 13, 14, 15, 16, 17]. It was found that PCR is a more sensitive technique in comparison to microscopic examination for diagnosis of theileriosis. There is high pleomorphism among the piroplasms and ambiguity between Koch's Blue Bodies and azurophilic granules. So the microscopic examination of blood smear is more likely to give false positive diagnosis of theileriosis. In the chronic recurrent cases of theileriosis treated by high doses of tetracycline for several days, the KBB or piroplasms are not visible by microscopic examination. It may lead to false negative diagnosis of theileriosis [18, 19, 20]. To overcome the limitations of blood smear examination and to study the

presence of parasite in large cattle population, PCR based molecular diagnosis using the above primers can be adopted.

#### 5. Conclusion

In the present study it was concluded that occurrence of theileriosis is very high in cattle (74% in samples suspected for blood protozoa) in the districts in and around Bhubaneswar. The disease is highly prevalent in crossbred cattle particularly during rainy season due to high tick population. The piroplasms are seen in different forms and may confuse with other intra-cytoplasmic inclusions giving false results. Molecular diagnosis (PCR) due to its higher specificity and sensitivity can overcome this difficulty. The strain obtained was confirmed as *Theileria annulata* by sequence analysis. It was the first report on molecular diagnosis of the parasite in Odisha and the strain was assigned an accession No KT222946 and name "*Theileria annulata* isolate Odisha major merozoite surface antigen (OMSA-1) gene, partial cds". Further work is required to cover more areas under the study and to know presence of other strains of the parasite in affected cattle.

#### 6. Acknowledgements

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