A PCR BASED DETECTION OF *Theileria orientalis* IN CATTLE IN GALLE AND RATHGAMA VETERINARY RANGES

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Introduction

Tick-transmitted *Theileria* parasites of cattle cause a great negative impact on the livestock industry worldwide. Pathogenisity of *Theileria spp* differs as pathogenic and benign. Base d on this classification, *Theileria parva* and *Theileria annulata* are considered as the most pathogenic *spp* whereas, *Theileria orientalis* is considered to be less pathogenic. However, in recent years, *Theileria orientalis* infection has been reported as a clinically significant condition in cattle in the Asia Pacific region.

According to the epidemiological data of the Department of Animal Production and Health, Sri Lanka, in the year 2014, suspected Theileriosis cases of cattle were reported with a significant increase from the Southern Province. In the field situation, diagnosis is based on the clinical signs, but they are not specific for Theileriosis. Traditionally, the parasite identification is done by the microscopic examination of Giemsa stained blood smears. However, the Identification of different *Theileria* spp is very important for the definitive diagnosis since the pathogenicity differs among different species as well as different genotypes. Therefore, this study was carried out for the confirmatory diagnosis and the detection of *Theileria spp* present in previously Theileriosis suspected farms in Galle and Rathgama veterinary ranges by PCR analysis targeting the Major Piroplasmic Surface Protein (MPSP) gene of *Theileria orientalis* and the Merozoite Surface Antigen (Tams 1) gene of *Theileria annulata.*

Methodology

The study was done using routinely collected samples for diagnostic purpose at the Veterinary Research Institute, Gannoruwa. Thus, the ethical approval was not necessary. Five milliliters of blood were collected from the Jugular vein into EDTA tubes from 16 healthy cattle randomly selected from previously Theileriosis suspected farms and stored at - 4 °C in the laboratory. Blood smears were prepared with the blood obtained from tail vein stained with Leishman stain. Stained smears were examined under the light microscope (10×100) to detect the intra erythrocytic pleomorphic *Theileria* piroplasms including rod, comma and cygnet shapes. DNA was extracted from blood using QIAGEN® DNeasy Blood and Tissue kit following the manufacturer's protocol and stored at -20 ^{0}C. Concentration of the extracted was measured with the JENWAYGENOVA NANO spectrophotometer prior to the PCR.

PCR was performed to identify the presence of *Theileria orientalis* and *Theileria annulata.* For *Theileria orientalis*, 776 bp fragment of the MPSP gene was targeted by the forward primer, MPSP-F (5'-CTTTGCCTAGGATACTTCCT-3') and the reverse primer, MPSP-R (5'-ACGGCAAGTGGTGAGAACT-3') whereas for *Theileria annulata,* 785 bp fragment of

the Tams 1 gene was targeted by the forward primer, Tams1-F (5'-ATGCTGCAAATGAGGAT-3') and the reverse primer, Tams1-R (5'-GGACTGATGAGAAGACGATGAG-3'). All PCR reactions consisted of 25 µl total reaction volume including 5 µl of 5 Colorless GoTaq® Flexi buffer, 2 µl of $MgCl_2$, 0.5 µl of Invitrogen PCR Nucleotide mix (10mM), 1.25 µl of Forward primer (10 µM), 1.25 µl of Reverse primer (10 µM), 0.125 µl of Go Taq® DNA Polymerase (5U/µl), 13.375 µl of DNA/RNA free water (Sigma) and 1.5 µl (40ng) of the DNA template. PCR was performed by the BIORAD - C1000 Touch ™ thermal cycler under the conditions of initial denaturation at 94 ^{0}C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 59 ^{0}C for 1 minute, extension at 72 ^{0}C for 1 minute and final extension at 72 ^{0}C for 10 minutes repeating for 35 cycles. PCR products were subjected to 1 % Agarose gel electrophoresis, stained with Ethidium Bromide (Promega, U. S. A.) at 110 V for 40 minutes and visualized under UV light by Gene flash gel documentation system.

Results and Discussion

Microscopic examination of blood smears revealed that 68.75% (11/16) of the samples were positive for intra erythrocytic *Theileria* piroplasms which were pleomorphic including rod, comma and cygnet shapes. Rod shape was the most common (Plate 1). According to the results of the PCR assay, 90.9% (10/11) of the smear positive samples were positive for *Theileria orientalis* forming a 776 bp band comparable to the positive control for *Theileria orientalis* in the Agarose gel electrophoresis (Plate 2). All the samples were negative for *Theileria annulata.*

According to the previous studies carried out in Nuwara Eliya, Jaffna, Ampara and Polonnaruwa districts, the predominant *spp* was *Theileria orientalis. Theileria orientalis* was highly prevalent in the wet zone (Nuwara Eliya) whereas *Theileria annulata* was predominant in the dry zone (Ampara). This is similar to the finding of the present study, the presence of *Theileria orientalis* in Galle and Rathgama veterinary ranges belonged to the wet zone. This suggests that the climatic conditions may be favoring the existence of *Theileria orientalis* in the wet zone. Recent studies in the Asia-Pacific region revealed that *Theileria orientalis* is no more a benign heamoprotozoan parasite uniformly due to the identification of several genotypes of the same species in association with the clinical disease including fever, anaemia, weakness, jaundice, abortion and / or mortality as well as a significant loss in milk production in cattle.

Eleven genotypes of *Theileria orientalis* were identified by molecular techniques as Type 1, 2, 3, 4, 5, 6, 7, 8, N1, N2 and N3. Only Type 1 and 2 are considered to be pathogenic. Other than that, Type 7 had been associated with a fatal infection in cattle in South India recently. Type 1, 3, 5 and 7 were identified in Nuwara Eliya, Jaffna, Ampara and Polonnaruwa districts. Therefore, there is a possibility that the identified *Theileria orientalis* in the risk group of Galle and Rathgama veterinary ranges to be consisted of pathogenic genotypes such as Type 1 or 7 which may be the reason for the clinical disease. However, for the confirmation it is suggested to perform a genotype specific PCR assay.

Microscopic examination of the Geimsa stained blood smear is the recommended test of confirming clinical cases of Theileriosis. In the present study, 1/11 smear positive samples were negative for the PCR. Since *Theileria* piroplasms are pleomorphic, that

smear finding could be false positive and the other possibility is, *Theileria* parasites present in that sample could be another *Theileria spp* targeted by the PCR. To confirm the smear finding, a *Theileria* genus specific PCR can be performed. As a whole, blood smear examination is less sensitive than molecular techniques in the diagnosis of *Theileria* infection and generally incapable of detecting the infection at the carrier stage due to low parasitaemia.

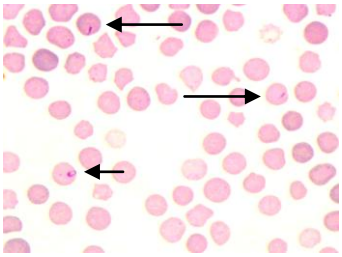

Figure 1. *Theileria spp* seen in a Leishman stained blood smear (10×100). Close up of cygnet (short arrow) and rod-shaped (long arrows) *Theileria* piroplasms in RBC s taken by the Trinocular light microscope with imaging system

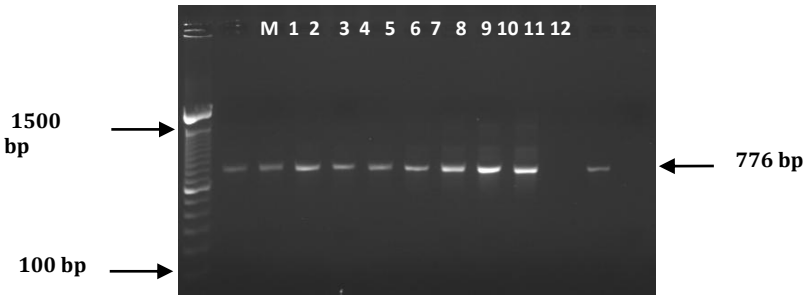

Figure 2. Verification of the PCR products by Agarose gel electrophoresis. *Molecular size marker. Lane 1 – Lane 9: Positive samples for Theileria orientalis. Lane 10: Negative sample for Theileria orientalis. Lane 11: Positive control. Lane 12: Negative control.*

Conclusions and Recommendations

The identified *Theileria spp* in cattle of previously Theileriosis suspected farms in Rathgama and Galle veterinary ranges was *Theileria orientalis*. A genotype specific PCR assay will help to further identify the pathogenic genotypes of *Theileria orientalis*.

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References

Ariyarathne, MESAD Gothami, WS & Rajapakse, RVPJ (2014), Application of PCR Technique on Confirming *Theileria* infection in Cattle and Buffaloes with Determining the Relationship between Animals' PCV and WBC Count with the infection, International Journal of Scientific and Research Publications 4.

OIE Terrestrial Manual, 2014.

Perera, KP Gasser, RB Firestone, SM Anderson, GA Malmo, J Davis, G Beggs, DS & Jabbar, A (2014), Oriental Theileriosis in dairy cows causes a significant milk production loss*, Parasites and Vectors* 7:73.

Sivakumar, T Yoshinaria, T Igarashia, I Kothalawala, Abeyratne, SAE Vimalakumar, SC Meewewa, AS Kuleswarakumare, K Chandrasiri, AND & Yokoyama, N, (2012a), Genetic diversity within *Theileria orientalis* parasites detected in Sri Lankan cattle*, Ticks and Tick-borne Diseases* 4, 235-241.

Sivakumar, T Kothalawala, H Abeyratne, AS Vimalakumar, SC Meewawe, AS Hadirampela, DT Puvirajan, T Sukumar, S Kuleswarakumar, K Chandrasiri, ADN Igarashi, I. & okoyama, N, (2012b), A PCR-based survey of selected *Babesia* and *Theileria* parasites in cattle in Sri Lanka, *Veterinary Parasitology* 190, 263–267.