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

M.E.S.A.De S. Ariyaratne[*], W.S. Gothami[*], R.V.P.J. Rajapakse[**]

* National Zoological Garden, Dehiwala,
** Division of Parasitology, Department of Veterinary Pathobiology, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya

Abstract- Theileriosis is a bovine haemoprotozoal infection caused by *Theileria* species. It is a tick transmitted haemoparasite and the disease can develop as clinical or subclinical infections. This study was carried out in order to determine *Theileria* infection by Polymerase Chain Reaction in cattle and buffaloes in a National Livestock Development Board farm at Polonnaruwa, Sri Lanka.   Forty-one blood samples were collected from the herd of diseased and non diseased cattle and buffaloes. Packed cell volume of each blood sample was analyzed using microcenrifugation which revealed that 85.7% (18/21) cattle and 20% (4/20) buffaloes had PCV values below the average. White Blood Cell count was calculated using haemocytometer method and 33.3% (7/21) cattle and 15% (3/20) buffaloes had WBC count above average value. Light microscopic examination of thin blood smears was used to detect *Theileria* infection primarily where 7.31% (3/41) samples became positive and Polymerase Chain Reaction was carried out in order to confirm the infection and to identify the *Theileria* species. For the amplification of *Theileria* genus, 989F and 990R primers were used to amplify a 1098-bp fragment of the ssurRNA gene. *T. annulata* species amplification was done using N516(F) and N517(R) primers derived from the 721-bp fragment of the gene encoding the 30-kDa major merozoite surface antigen for *T. annulata.* Amplification of *T. parva* was done using TPF and TPR primers which used to amplify a 277-bp internal fragment located between bases 2784 and 3061 of the p104 gene. Three samples which became positive for *Theileria* piroplasms were highly specific for the primers used for the *Theileria* genus. PCR reaction of species specific primers used for *T. parva* and *T. annulata* became negative. DNA sequencing was proposed for the positive samples to identify the *Theileria* species since it is not either of *T. annulata* or *T.parva.*

Index Terms- *Theileria*, Sri Lanka, PCR, WBC, PCV

I. INTRODUCTION

Theileriosis is a disease caused by several species of *Theileria,* causing livestock production losses in Africa, Asia and Middle East (Urquhart *et al.*, 1996). In Sri Lanka *Theileria orientalis* is predominant in Nuwara Eliya while *Theileria annulata* is more common in dry zone (Sivakumar *et al.*, 2012). This is associated with infections which range from clinically in-

apparent to fatal. The two species of major veterinary importance in cattle are *Theileria annulata* and *Theileria parva.* Mildly pathogenic species infecting cattle has also been identified (Urquhart *et al.*, 1996). Intraerythrocytic *Theileria* piroplasms are commonly rod-shaped and can also occur in round, oval and ring shapes. There are three phases in an acute *Theileria* infection and each phase last for about a week. First phase is the incubation period in which parasite and lesions are not yet evident. This is followed by the second phase in which the hyperpalasia and expansion of the lymphoblasts occur. In the third phase lymphoid depletion, disorganization associated with lymphocytolysis and reduction in lymphopoesis will occur (Urquhart *et al.*, 1996). Theileriosis is characterized by a marked anaemia and fever (Durrani, *et al.*, 2008). In *Theileria parva* infections the lymph node draining the area of tick bite which is usually the parotid becomes enlarged and later swelling of the superficial lymph nodes will occur. In addition loss of body condition, dyspnoea, terminal diarrhoea and petechial haemorrhages may occur under the tongue and on the vulva (Urquhart *et al.*). Clinical signs developed in *Theileria annulata* infections are similar but in later stages there is haemolytic anaemia an icterus (Urquhart *et al.*, 1996). Diagnosis of clinical theileriosis in cattle is usually based on Giemsa stained blood smear examination and detection of piroplasms in red cells, but the detection of the infective agent by this method is not reliable in carrier stages (Durrani, *et al.*, 2008). Also in sick animals, macroschizonts can be demonstrated in biopsy smears of lymph nodes and dead animals, in impression smears of lymph nodes and spleen (Urquhart *et al.*, 1996). PCR is a relatively non invasive technique, which is fairly rapid and affordable.

II. METHODOLOGY

Forty-one blood samples were collected haphazardly from cattle and buffaloes in a NLDB farm at Polonnaruwa. Volume of 5ml was collected in to EDTA coated blood collecting tubes with 18 gauge needles and 10ml syringes. Blood smears from each sample was made and stained with Leishmann stain according to method described by Silva, (1996). Also micro capillary tubes were used to detect Packed Cell Volume and the readings were obtained using the microhaematocrit reader according to the method described by Silva, (1996). Each blood sample was mixed well by repeated inversions. Blood was diluted with WBC

diluting fluid in 1:20 ratio and chambers of the haemocytometer was filled with the mixture and allowed 3-5 minutes for the WBCs to settle down. WBC count for each sample was determined using this method by examining under the light microscope. DNA was extracted using Promega Wizard Genomic DNA purification kit (Promega, Madison WI, USA) according to manufacturer's instructions. Extracted DNA was stored at 4 ^{0}C in a refrigerator. Primer sets used in this study are listed in Table 1. For the amplification of *Theileria* genus, 989F and 990R primers were used to amplify a 1098-bp fragment of the ssurRNA gene. *Theilera annulata* species amplification was done using N516 (F) and N517(R) primers derived from the gene encoding the 30-kDa major merozoite surface antigen for *T. annulata.* Amplification of *Theileria parva* was done using TPF and TPR primers which used to amplify a 277-bp internal fragment located between bases 2784 and 3061 of the p104 gene. PCR was performed for 30μl of total reaction volume containing 13.5 µl of Promega DEPC water, 1.5 µl of $MgCl_2$ (25mM/ml), 2.0 µl of Promega dNTP, 2.5 µl of 10×Taq buffer, 0.5 µl of 50u/ µl Promega Taq polymerase and 1.5 µl of each primer and 2 µl of template DNA. The reaction for *Theileria* genus was repeated for 30 cycles under the following conditions: 5 minutes at 94 ^{0}C, 30 seconds at 94 ^{0}C, 30 seconds at 55 ^{0}C, 45 seconds at 72 ^{0}C and 7 minutes at 72 ^{0}C. The reaction for *Theileria annulata* and *Theileria parva* was repeated for 35 cycles under the following conditions: 3 minutes at 94 ^{0}C, 1 minutes at 94 ^{0}C, 1 minute at 57 ^{0}C, 1 minute at 72 ^{0}C and 7 minutes at 72 ^{0}C.

Table1: *Theileria* genus specific and species specific primers used in this study.

Primer	Primer sequence	Predicted amplification size	Gene name	Reference
989(F) 990(R)	*Theileria* specific AGTTTC TGACCT ATCAG TTGCCT TAAACT TCCTTG	1098 bp	Small subunit rRNA gene	Durrani, *et al.*, 2008
N516(F) N517(R)	*Therileria annulata* specific GTAACC TTTAAA AACGT GTTACG AACATG GGTTT	721bp	Gene encoding the 30-KDa major merozoite surface antigen	Durrani, *et al.*, 2008

TPF TPR	*Theileria parva* specific GGCCAA GGTCTC CTTCAG ATTACG TGGGTG TGTTTC CTCGTC ATCTGC	277bp	Protein 104-KDa gene	Odongo, *et al.*, 2009

Table 2: Master mixture of constitutes for the PCR

Constitute	Volume
10× Promega Taq buffer	2.5 µl
$MgCl_2$ (25mM/ml)	1.5 µl
Promega dntp	2.0 µl
Primer (F)	1.5 µl
Primer (R)	1.5 µl
DNA template	2.0 µl
50u/ µl Promega Taq polymerase	0.5 µl
DEPC water	13.5 µl

Each of the above values are for a one sample and these values should be multiplied from the sample number and additional one (eg: sample number 3 + 1 = 4) to minimize the possible pipetting error.

Agarose gel electrophoresis was used for the analysis of amplified DNA and a 0.8% gel was prepared. Gel electrophoresis was performed under 50v for 30 minutes and results were visualized using UV transilluminator.
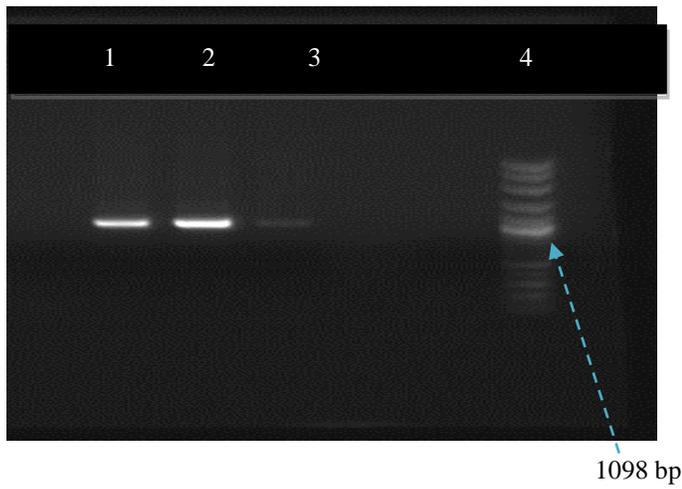
III.   RESULTS AND DISCUSSION

Theileria piroplams are commonly comma shaped intra erythrocytic organisms. Structural abnlormalities of erythrocytes were also examined in *Theileria* positive blood smears which included anisocytosis, poiklocytosis and presence of reticulocytes. Blood smear examination revealed 7.31% (3/41) samples to be positive for intraerythrocytic piroplasms of *Theileria.* Detection of PCV values showed that 85.7 % (18/21) cattle were having PCV below the average value of 35±1 (Mahima, 2013) where as 20% (4/20) of the buffaloes were having PCV values below the average value of 27 (Naveen Chandra *et al.*, 2008). Statistical analysis of the data using a chi-square with MINITAB software revealed that there is a considerable relationship between being infected by *Theileria* and reduced Packed Cell Volume. (P value which is < than 0.05 and the null hypothesis which is there is a relationship between two variables was ruled in). According to the study 33.3% (7/21) cattle were having high WBC counts than normal value (8.59 – 14.81 × 10^3/μl of blood) (Mahima, 2013) where as 15% (3/20) of buffaloes were having high WBC count than normal (3.6 – 20 × 10^3/μl of blood) (Silva, 2010).

Figure 1: Agarose gel electrophoresis for PCR products of *Theileria* positive samples DNA extracts with primers specific for *Theileria* genus. A band size of 1098 is visible from three samples.

1098 bp

Molecular size marker and samples as indicated above, 1,2,3 – Three samples idendified as *Theileria* positive from blood smear examination.(sample 1 and 2 are cattle blood samples, sample 3 was a buffalo sample) 4 – 100 bp DNA ladder.

Genus *Theileria* is composed of many species which can infect cattle and buffaloes. *Theileria annulata* and *Theileria parva* are the two main species responsible for infections in cattle. These species have different vectors that transmit the merozoites to cattle. Three host tick *Rhipicephalus* transmits *T. parva* while *Hyalomma* transmits *T. annulata.* Therefore it is essential for the tick to be present in the particular region for an infection to occur (Urquhart *et al.*, 1996). Theleriosis is a disease that can lead to major economic losses in animal production (Urquhart *et al.*, 1996). Clinical sings developed in infected animals may be slightly different due to each *Theileria* species. Diagnosis of the disease based on clinical signs is very non-specific because there are other infections such as Babesiosis and Trypanasomiosis which may lead to similar clinical manifestations. Examination of Geimsa stained blood smears and detection of piroplasms is the conventional method of identification of *Theileria.* But the detection of infective agent by this method is not reliable and is almost impossible in carrier stages (Durrani, *et al.*, 2008). Also this method cannot differentiate between each species of *Theileria* genus which can infect cattle and buffaloes. There are different methods to differentiate between *Theileria* species but in most cases it is necessary to use a combination of these methods to identify a species definitively. Geographic distribution, vector specificity, morphology, host immunity, pathogenicity, cross immunity, serology, DNA probes and restriction fragment length polymorphism are some of those methods used for the species identification (Morzaria, 1987). The geographical distribution of two most important *Theileria* species for cattle, *T. parva* and *T. annulata,* correlates well with the distribution of their vector. *Theileria parva* is transmitted predominantly by the brown ear tick, *Rhipicephalus appendiculatus,* which is restricted to eastern, central and southern Africa; *T. annulata* is transmitted by several *Hyalomma* species, which are distributed widely in North Africa, southern Europe, the Middle East, India, southern Russia and China (Morzaria, 1987). Even though it is not very accurate, geographic distribution of the vector and vector specificity of each *Theileria* species can be used in species identification. It is difficult to differentiate *Theileria* species by examining the morphology of the parasites because parasites of different species look alike in most piroplasm and schizont stages (Morzaria, 1987). Pathogenecity of each *Theileria* species can also be used in species differentiation. *Theileria parva* replicates mainly in lymphocytes and it causes destruction of the lymphocytes where as Erythrocytic merogony is limited and haemolytic anaemia is not present. *Theileria annulata* replicates in both lymphocytes and erythrocytes, thus causing disease with severe lymphocytopaenia, anaemia and occasionally jaundice (Morzaria, 1987). One of the serological tests used to diagnose *Theileria* species is the Indirect Fluorescent Antibody Test (IFAT). Cross-reactions have been observed among *T. parva, T. mutans* and *T. annulata* species when IFAT is used. Under experimental conditions and using appropriate controls, the test can be useful in identifying *Theileria* species. But its usefulness in the field reduces the usage. DNA probes are also a powerful and sensitive technique in identifying mixed theilerial infections, in cattle exposed to natural tick challenge where differentiation of *Theileria* species is needed (Morzaria, 1987). But use of DNA probes is expensive compared to other methods. Polymerase Chain Reaction was used to identify *Theileria* species in the current study. PCR was an accurate, easy to perform, less time consuming and a less expensive method compared to the other methods. DNA extraction was carried out prior to PCR to the samples which were identified as positive for the infection from conventional stained blood smear examination method. The DNA extraction procedure used in this study was able to extract genomic DNA successfully for amplification. It was less expensive but was time consuming compared to rapid, ready to use, one step commercial DNA extraction solutions. And this reduced the value of PCR as a rapid test to detect *Theileria* infections. PCR with *Theileria* genus specific 989F and 990R primers which used to amplify 1098-bp fragment of the ssurRNA gene (Durrani, et *al.*, 2008) was positive for all the three samples which was identified as positive from blood smear examination. *Theilera annulata* species amplification was done using N516(F) and N517(R) primers derived from the gene encoding the 30-kDa major merozoite surface antigen for *T. annulata* (Durrani, *et al.*, 2008) which the samples became negative. For the amplification of *Theileria parva* TPF and TPR primers were used to amplify a 277-bp internal fragment located between bases 2784 and 3061 of the p104 gene (Odongo, *et al.*, 2009) and correct size fragment was not amplified. Since negative results can occur due to the mechanical errors during sample handling, pippeting etc. and also due to failure of annealing temperature optimization, PCR was carried out for the second time to the DNA extracts together with primers specific for genus and both the species. It was proposed to perform DNA sequencing on the genus positive samples because it suggested that this could be another *Theileria* species other than *Theileria annulata* or *Theileria parva.* Cattle and buffaloes PCV and WBC count were also measured in this study. All the animals infected with *Theileria* were having Packed Cell Volume below average where as only one out of three infected animals had White Blood

Cell count higher than the average. This suggests that there is a relationship between drop in Packed Cell Volume and *Theileria* infections (Durrani, *et al*., 2008). This hypothesis was proved by the statistical data analysis.

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AUTHORS

First Author – M. E. S. A. De S. Ariyaratne, BVSc, Central Veterinary Investigation Centre, Veterinary Research Institute. anupama.ariyaratne@gmail.com

Second Author – W.S. Gothami, BVSc, Central Veterinary Investigation Centre, Veterinary Research Institute. sugandhikagothami@yahoo.com

Third Author – R. V. P. J. Rajapakse, BVSc, PhD, Division of Parasitology, Department of Veterinary Pathobiology, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya., jayanthar@pdn.ac.lk

Correspondence Author – M. E. S. A. De S. Ariyaratne, anupama.ariyaratne@gmail.com, 0094772347770