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Comparison of primer sets for amplification of major piroplasm surface protein (MPSP) antigen of *Theileria orientalis*

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Abstract
Theileria orientalis is a tick-born haemoprotozoan parasite distributed throughout the world. It causes significant economic losses to livestock industry, because no commercially effective medicines or vaccines are currently available for its control. Among the different molecular markers are used to study and characterise this parasite, major piroplasm surface protein (MPSP) gene is most commonly used and reliable marker to characterise this parasite. We compared the efficiency of two primer sets for diagnosis of *Theileria orientalis* in 32 cattle blood samples that were showing clinical signs suggestive of theileriosis. Light microscopic examination revealed twenty two cattle were positive for theileriosis. In PCR we used two primer sets that amplify the 32kDa major piroplasm surface protein (MPSP) antigen gene of *T. orientalis*. Polymerase chain reaction (PCR) based methods showed higher sensitivity in identifying positive cases than detected by light microscopy. Primer set 1 identified 23 cattle as positive, whereas primer set 2 identified all the 32 cattle examined as positive for *T. orientalis*. Our preliminary analysis concludes that PCR based method was more efficient in detecting oriental theileriosis in cattle and primer set 2 is the first choice for epidemiological studies of *T. orientalis.*

Keywords: Cattle, major piroplasm surface protein gene, *Theileria orientalis*

Introduction
Oriental theileriosis is a tick born haemoprotozoan disease of cattle caused by *Theileria orientalis* (also known historically as *T. sergenti* and *T. buffeli*) [1]. *Theileria orientalis* proliferates inside the erythrocytes as piroplasm and during intra- erythrocytic stage causes haemolysis and subsequent anaemia, which is the primary clinical finding in the affected cattle [2]. The major piroplasm surface protein (MPSP) is an immunodominant antigen expressed during intraerythrocytic piroplasm stage and conserved among *Theileria* spp. [3]. Based on the sequence variations in the MPSP gene, currently 11 distinct genotypes of *T. orientalis* were identified globally and they are designated as Chitose or type 1, Ikeda or type 2, Buffeli or type 3, types 4 to 8, N1 to N3 [4].

In recent years increased number of clinical cases of oriental theileriosis have been reported from Asia-Pacific region [5, 6, 7, 8]. Clinical outbreaks of disease have been reported mainly with Chitose and Ikeda genotypes of *T. orientalis* in dairy and beef cattle [6, 9]. The affected cattle show a combination of clinical signs such as anorexia, anaemia, lethargy, ill-thrift, diarrhoea, decreased milk production, late term abortions and mortality in severe cases [1]. The severity of the disease depends on degree of anaemia in affected animals [10]. Transtadial transmission of *T. orientalis* occurs through *Haemaphysalis longicornis* ticks [11] and infection of cattle with *T. orientalis* is considered to last life-long [12]. Light microscopic examination of the Giemsa stained blood smears is routinely practised for identification of *Theileria* spp. and it remains as fast method to detect theileriosis in clinical cases [13]. But the major disadvantage with light microscopy is the difficulty in detection of carrier animals with low level of parasitaemia which may go unnoticed regularly and the difficulty to differentiate different *Theileria* spp. [14]. The diagnosis of oriental theileriosis now relies on polymerase chain reaction (PCR) assays by amplification of major piroplasm surface protein gene [15, 16]. Polymerase chain reaction could be used for early diagnosis of theileriosis in cattle, two weeks ahead of *Theileria* infected erythrocytes detected by light microscopy [16].

Tanaka *et al*. 1993 [17] and *Ota et al*. 2009 [16] designed different sets of oligonucleotide primers for diagnosis of *T. orientalis* by PCR. These primers have been used by different researchers in the epidemiological studies of *T. orientalis* separately but there are no reports

comparing the efficiency of the two primer sets in detection of *T. orientalis* infection in cattle. The aim of this study was to compare the efficiency of the two primer sets for molecular diagnosis of *T. orientalis*.

Materials and Methods

A total of 32 cattle showing the symptoms of anorexia, anemia and decreased milk production suggestive of theileriosis were included in this study.

Sample collection

Thin blood smears were prepared using a drop of blood collected from ear vein and immediately fixed in methanol for three minutes. The blood smears were stained by Giemsa staining technique and observed under the oil immersion objective of the microscope for the presence of *Theileria* piroplasms. Whole blood samples were collected in EDTA coated vials and stored at -20ºC till DNA extraction.

Polymerase chain reaction (PCR)

DNA was extracted from whole blood samples using commercial kit (DNeasy® Blood and Tissue kit, Qiagen, Germany). All the DNA samples were tested first for *Theileria* infection by using *Theileria* genus specific primers targeting the small subunit (SSU) rRNA gene of *Theileria*. For species specific PCR two primers sets (Table 1) were used to amplify the major piroplasm surface protein gene of *T. orientalis*. PCR was performed in 25µl reaction mixture containing 12.5µl of master mix, 10 pmol of each forward and reverse primer, 5µl of DNA as template and 5.5µl of nuclease free water. Thermal cycling conditions used for amplification reactions were mentioned in Table 2. The DNA extracted from *Theileria* infected cattle that was positive by blood smear examination and by using two primers sets was used as positive control. Negative control with known negative DNA was also run in each PCR. The PCR reactions were performed in thermal cycler (Bio-Rad, USA) with 35 cycles for each primer set. The amplified PCR products were subjected to electrophoresis in 1.2% agarose gel stained with ethidium bromide and then gel documentation was done.

Table 1: Oligonucleotide primers used to amplify MPSP gene of *T. orientalis*

Primers	Sequence	Amplicon size	Reference
Primer set 1			
MPSP-F	5'-CACGCTATGTI'GTCCAAGAG-3'	875 bp	Tanaka *et al*. 1993 [17].
MPSP-R	5'-TGTGAGACTCAATGCGCCTA-3'		
Primer set 2			
MPSP-F	5'-CTTTGCCTAGGATACTTCCT-3	776 bp	Ota *et al*. 2009 [16].
MPSP-R	5'-ACGGCAAGTGGTGAGAACT-3'		

Table 2: Thermal cycling conditions used in PCR

S. No.	PCR programme	Primer set 1 (Tanaka *et al*., 1993) [17].		Primer set 2 (Ota *et al*., 2009) [16].	
		Temperature	Time	Temperature	Time
1	Initial denaturation	94 ºC	3 min	94ºC	10 min
2	Denaturation	94 ºC	1 min	94ºC	1 min
3	Annealing	57 ºC	1 min	58ºC	1 min
4	Extension	72 ºC	3 min	72ºC	1 min
5	Final extension	72 ºC	4 min	72ºC	4 min
6	Hold	4 ºC	Till retrieval	4ºC	Till retrieval

Results and Discussion

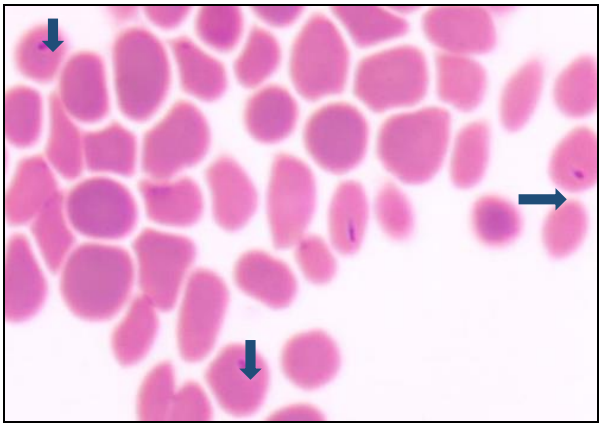

Fig 1: *Theileria* piroplasms in blood smear

Out of the 32 animals examined, 22 cattle (68.6%) were found positive by blood smear examination. The theilerial piroplasms were seen as thin or thick rods with trailing cytoplasm inside the erythrocytes (Fig 1.). By genus specific PCR, all the 32 animals were found positive for *Theileria*, but in species specific PCR using two different primer sets the results varied. Primer set 1 detected *T. orientalis* infection in 23 cattle (71.9%), whereas all the animals were found positive for *T. orientalis* by primer set 2.

The aim of the present study was to compare the efficiency of two primer sets for detection of MPSP gene of *T. orientalis* by PCR. Conventional light microscopic examination of blood smears was routinely practised under field conditions for the detection of haemoprotozaons.

But it is difficult to identify animals with low level of parasitaemia by light microscopy and these animals will act as potential carriers for transmission of disease.

Table 3: Comparison of results of conventional method and PCR assays for diagnosis of *T. orientalis*

S. No.	Conventional method (blood film examination)	Polymerase chain reaction	
		Primer set 1	Primer set 2
1	+	+	+
2	+	+	+
3	+	+	+
4	-	+	+
5	+	-	+
6	-	+	+
7	-	-	+
8	+	+	+
9	-	+	+
10	+	+	+
11	-	-	+
12	-	-	+
13	-	-	+
14	+	+	+
15	-	-	+
16	+	-	+
17	-	+	+
18	-	-	+
19	+	+	+
20	+	+	+
21	+	+	+
22	+	-	+
23	+	+	+
24	+	+	+
25	+	+	+
26	+	+	+
27	+	+	+
28	+	+	+
29	+	+	+
30	+	+	+
31	+	+	+
32	+	+	+

Table 4: Number and percent of cattle infected with *T. orientalis*

Conventional method (blood film examination)	PCR	
	Primer set 1	Primer set 2
Positive (No.)%	Positive (No.)%	Positive (No.)%
22 68.6	23 71.9	32 100

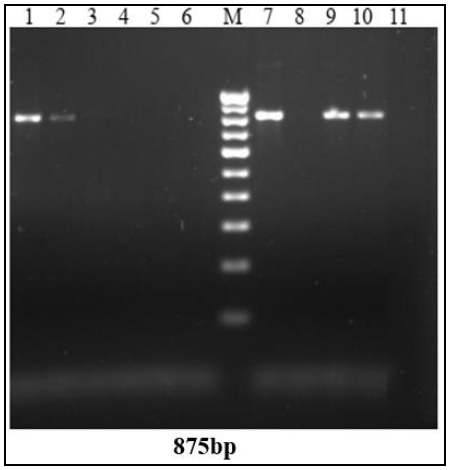

Fig 2: PCR gel doc image showingamplification of 875bp product of T. orientalis using primer set 1

Lane 1,2,7 and 9 samples positive for *T. orientalis*
Lane 3,4,5,6 and 8 samples negative
Lane M- 100bp ladder
Lane 10: Positive control
Lane 11: Negative control

PCR is highly sensitive and specific compared to conventional diagnostic methods and can be used to detect infection in clinical as well as carrier animals. The number of positive cases detected by primer set 2 was higher compared to the number detected by primer set 1. Some animals which were negative in both blood smear examination and in PCR using primer set 1 were also diagnosed as positive by primer set 2. The primer set 2 designed by Ota *et al*. (2009) [16] were able to detect parasite DNA in greater number of cattle blood samples and could be used to detect hidden infections in cattle. Detection of these hidden infections will help in reducing the economic losses to livestock producers caused by oriental theileriosis.

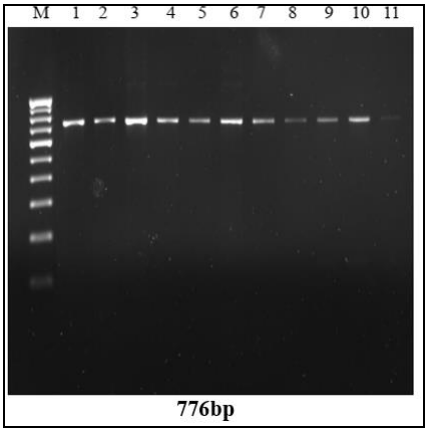

Fig 3: PCR gel doc image showing Amplification of 776bp product of *T. orientalis* using primer set 2

Lane M- 100bp ladder
Lane 1 to 9 samples positive for *T. orientalis*
Lane 10: Positive control
Lane 11: Negative control

Conclusion

Oriental theileriosis caused by *T. orientalis* was considered as benign for many years, but in recent years clinical outbreaks of oriental theileriosis are recorded from different countries in Asia-pacific region. The diagnosis of *T. orientalis* can be made by clinical signs, detection of piroplasms in blood smears and by molecular methods. Traditional method of blood smear examination by light microscopy routinely used in field conditions was not sensitive enough to detect animals with low level of parasitaemia. Hence molecular methods using different molecular markers are used to study and characterise the *T. orientalis*. Major piroplasm surface protein (MPSP) is the most commonly used and reliable marker widely used to characterise the *T. orientalis*. In the present study comparison of two primer sets targeting the MPSP gene of *T. orientalis* revealed that, primers amplifying the 776bp fragment (primer set 2) of MPSP gene of *T. orientalis* were able to detect more number of infected animals as positives compared to the primers that amplify the 875bp fragment (primer set 1) of the same. Hence, it could be concluded that primer set 2 is the first choice in epidemiological studies of oriental theileriosis for detection of clinical as well as carrier animals.

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References

1. Watts JG, Playford MC, Hickey KL. *Theileria orientalis*: a review. New Zealand Veterinary Journal 2015;64(1):3-9.
2. Jenkins C, Micallef M, Alex SM, Collins D, Djordjevic SP, Bogema DR, *et al*. Temporal dynamics and subpopulation analysis of *Theileria orientalis* genotypes in cattle. Infection, Genetics and Evolution 2015;32:199-207.
3. Kawazu SI, Sugimoto C, Kamio T, Fujisaki K. Molecular cloning and immunological analysis of immunodominant piroplasm surface proteins of *Theileria sergenti* and *T. buffeli*. Journal of Veterinary Medical Science 1992;54(2):305-311.
4. Sivakumar T, Hayashida K, Sugimoto C, Yokoyama N. Evolution and genetic diversity of Theileria. Infection, Genetics and Evolution 2014;27:250-263.
5. Aparna M, Ravindran R, Vimalkumar MB, Lakshmanan B, Rameshkumar P, Kumar KA, *et al*. Molecular characterization of *Theileria orientalis* causing fatal infection in crossbred adult bovines of South India. Parasitology International 2011;60(4):524-529.
6. McFadden AMJ, Rawdon TG, Meyer J, Makin J, Morley CM, Clough RR, *et al*. An outbreak of haemolytic anaemia associated with infection of *Theileria orientalis* in naive cattle. New Zealand Veterinary Journal 2011;59(2):79-85.
7. Vinodkumar K, Shyma V, Justin DK, Ashok S, Anu JP, Mini K, *et al*. Fatal *Theileria orientalis* N2 genotype infection among Asian water buffaloes (*Bubalus bubalis*) in a commercial dairy farm in Kerala, India. Parasitology 2016;143(1):69-74.
8. Jackson BJ, Tresamol PV, Vijayakumar K, Justin Davis K. Diagnosis and molecular confirmation of oriental theileriosis in a cow: A case report. The Pharma Innovation 2018;7(8):398- 400.
9. Kamau J, de Vos AJ, Playford M, Salim B, Kinyanjui P, Sugimoto C, *et al*. Emergence of new types of *Theileria orientalis* in Australian cattle and possible cause of theileriosis outbreaks. Parasites &Vectors 2011;4(1):22-31.
10. Kim S, Yu DH, Kang SW, Chae JB, Choi KS, Kim HC, *et al*. Hematological changes associated with *Theileria orientalis* infection in Korean indigenous cattle. Korean Journal of Parasitology 2017;55(5):481-490.
11. Hammer JF, Emery D, Bogema DR, Jenkins C. Detection of *Theileria orientalis* genotypes in *Haemaphysalis longicornis* ticks from southern Australia. Parasites & Vectors 2015;8(1):229-237.
12. Kubota S, Sugimoto C, Onuma M. Population dynamics of *Theileria sergenti* in persistently infected cattle and vector ticks analysed by a polymerase chain reaction. Parasitology 1996;112(5):437-442.
13. Izzo MM, Poe I, Horadagoda N, De Vos AJ, House JK. Haemolytic anaemia in cattle in NSW associated with *Theileria* infections. Australian Veterinary Journal 2010;88(1-2):45-51.
14. Mans BJ, Pienaar R, Latif AA. A review of Theileria diagnostics and epidemiology. International Journal for Parasitology: Parasites and Wildlife 2015;4(1):104-118.
15. Kim SJ, Tsujia M, Kubota S, Wei Q, Lee JM, Ishihara C, *et al*. Sequence analysis of the major piroplasm surface protein gene of benign bovine *Theileria* parasites in East Asia. International Journal of Parasitology 1998;28(8):1219-1227.
16. Ota N, Mizuno D, Kuboki N, Igarashi I, Nakamura Y, Yamashina H, *et al*. Epidemiological survey of *Theileria orientalis* infection in grazing cattle in the eastern part of Hokkaido, Japan Journal of Veterinary Medical Science 2009;71(7):937-944.
17. Tanaka M, Onoe S, Matsuba T, Katayama S, Yamanaka M, Yonemichi H, *et al*. Detection of *Theileria sergenti* infection in cattle by polymerase chain reaction amplification of parasite-specific DNA. Journal of Clinical Microbiology 1993;31(10):2565-2569.