Rapid detection, discrimination and quantification of *Theileria orientalis* types using ribosomal DNA internal transcribed spacers

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ABSTRACT

We report the population structure analysis of *Theileria orientalis* types (Ikeda, Buffeli and Chitose), the causative agent of theileriosis in cattle and its cohorts, using ITS1 and ITS2 spacers by fragment genotyping. We utilized primers flanking the two ribosomal RNA internal transcribed spacers (ITS1 and ITS2). Due to varying degrees of sequence polymorphism in the ITS regions found within and between species, we exploited the insertions and or deletions in these regions which resulted in different fragment sizes. On the basis of fragment size polymorphism, we were able to discriminate the three commonly found types of *Theileria orientalis.* ITS1 was capable of discriminating all three types (Ikeda-251bp, Chitose-274bp and Buffeli-269bp) in one single reaction by fragment genotyping. However, using ITS2, Ikeda (133-bp) a more pathogenic type was distinguishable from Buffeli/Chitose (139-bp). In addition, we quantified parasite load in experimental animals using ITS1. When compared with previous PCR detection method, ITS1 and ITS2 genotyping were found to be more sensitive methods with high specificity in population analysis and can be deployed in molecular epidemiology studies.

Keywords: Ribosomal RNA intergenic spacer; *Theileria orientalis* types; genotyping, MPSP

1.0 INTRODUCTION

Asia-Pacific theileriosis is caused by a parasite known variously as *T. sergenti* or *T. orientalis*; although the name *T. sergenti* is now considered redundant (C. Sugimoto, pers. Comm.). Theileriosis due to *T. orientalis* is prevalent throughout Japan, Korea and parts of Russia and China (Chae et al., 1999; Fujisaki et al., 1991; Kawazu et al., 1992a, b; Onuma et al., 1998) and has been reported to occur in Australia (Callow, 1984; Kamau et al., 2010). The distribution of *T. orientalis*-buffeli appears to be worldwide, but is less frequently associated with clinical disease although some fatal cases have been reported to occur in America (Chae et al., 1999; Stockham et al., 2000; Cossio-Buyagur et al., 2002). Its transmitted by the three-hosts tick vector *Haemaphysalis longicornis*, and characterized clinically by anemia and weakness, leading to death, especially in animals experiencing metabolic stress such as parturition. There are eight genetic types (genotypes) currently comprising the *T. orientalis* complex. These types include type1 (Chitose), type2 (Ikeda), type3 (Buffeli) and type-4-8 yet to be classified with taxonomically (Kim et al., 1998; Ota et al., 2009). Mixed infection with different types of *T. orientalis* in cattle are common in Japan (Kim et al., 2004; Kubota et al., 1995), Korea (Baek et al., 2002) and Thailand (Sarataphan et al., 2003) but their clinical significance remain unclear. According to Shimizu et al., (1992; 2000), at least one to two billion yen (one to two million US$) per year is spent to fight theileria infections in Japan.

Diagnosis of a variety of disease syndromes caused by the *T. orientalis* parasites is principally based on clinical signs, knowledge of the disease, tick-vector distribution, and identification of parasites in Giemsa-stained blood smears. The most widely used

diagnostic test for *Theileria* species is the indirect fluorescent antibody (IFA) test (Burridge et al., 1974; Kiltz et al., 1986). However, the test is hindered by cross-reactivity among some *Theileria* species and hence it is limited for large-scale surveys in areas where species distribution overlaps. Moreover, it is commonly not possible to discriminate the pathogenic Ikeda type from the less pathogenic *Theileria orientalis* types (Chitose and Buffeli) in cases of mixed infections.

Molecular diagnostic assays, particularly those based on the PCR (D'Oliveira et al., 1995) and reverse line blot hybridisation are already established as powerful tools for characterizing parasite polymorphisms, defining parasite population genetics and generating epidemiological data (Gubbels et al., 1999). The small subunit (SSU) rRNA gene is widely used as a taxonomic marker in many organisms, but it is highly conserved and may not always reliably discriminate between closely related species. The rRNA internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2), which are separated by the 5.8S gene and flanked by the SSU and large subunit rRNA genes in most eukaryotes, are not subject to the same functional constraints as the rRNA genes. As a result, the spacer regions are subject to higher evolutionary rates leading to greater variability in both nucleotide sequence and length (Hillis and Dixon, 1991). The ITS1 and ITS2 spacers have been used for strain differentiation and species identification in different organisms (Som et al., 2000). They have been found to be reliably valuable in more discrete phylogenetic separation of closely related species, recognition of new species, determination of conspecificity between isolates, discrimination within a species,

and differentiation between piroplasm species and subspecies (Fazaeli et al., 2000; Zahler et al., 1998; Collins and Allsopp, 1999) compared to SSU rRNA.

Herein, we report the development and application of a method on ITS1 and ITS2 with enhanced sensitivity and specificity in typing of *T. orientalis* types from field samples from suspected, infected or carrier cattle by fragment capillary electrophoresis. We further show the application of this method in quantifying (monitoring) parasites load and parasitemia in cattle experimentally infected with *T. orientalis* types. For easy of reference, capillary electrophoresis will be referred as genotyping.

2.0 MATERIALS AND METHODS

2.1 Theileria isolates:

The cattle examined in the present study were grazed on 4 public farms in the eastern part of Hokkaido (Taiki, Otofuke, Shintoku, and Shin- Hidaka districts) (Fig. 1). A total of 325 blood samples were collected from grazing cattle in these districts. In Taiki district, Holstein cattle aged 12–20 months were firstly allowed to graze from 4th June, 2007, and 91 blood samples collected from the cattle. Thirty seven and 83 blood samples were collected from cattle in the Shintoku (age approximately 9 months) and Shin-Hidaka districts (different age groups) on 17th July and 25th July, 2007, respectively. While the rest 20 samples, were collected from cattle in the Otofuke district (different age group) on 14th July, 2008.

***2.2 Experimentally infected animals**:*

Four calves approximately 1 year of age were screened and selected for being theileria-free by PCR method, then maintained in experimental tick-proof pens. The animals were experimentally infected with three different types of *T. orientalis* (Ikeda, Chitose and Buffeli) stock. Parasitemia and anemia were monitored through collecting 2ml of blood from the tail veins which was added to ethylenediaminetetraacetic acid, to a final concentration of 0.15–0.22%. Thin blood smears were prepared using 10% Giemsa stain for classical microscopic examination. Parasitemia was classified by counting the number of infected cells per field by Ishihara method (Ishihara, 1971).–; no detection, +; 1 parasite in 10 fields (<0.05%), ++; >1 parasite in 10 fields (0.05–0.5%), +++; more than 1 parasite in 1 field (0.5–5%), ++++; more than 10 parasites in 1 field (> 5%), (Table 1).

2.3 DNA extraction and plasma preparation:

Genomic DNA was extracted from 100 µl of blood sample using a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 200 µl of eluted DNA solutions (approximately 6 µ g/ µl) were obtained and stored at –30 ^{0}C until use.

2.4 Detection of T. orientalis by conventional PCR

Two primers sets were used Table1, one targeting the Major Piroplasm Surface Protein (MPSP) gene (Ota et al., 2009) and our newly designed primer pair flanking both ribosomal DNA internal transcribed spacers (ITS1 and ITS2) including the 5.8S gene (Fig. 2). PCR was performed using 10µl of GoTaq 2X Master mix, 50ng of DNA, 1µl of 10 mM Primer with a final total volume of 20µl. The thermal cycling conditions consisted of an initial denaturation of 95 ^{0}C for 2 min followed by 30 cycles at 95 ^{0}C for 30 s, 57 ^{0}C for 30 s, and 72^{0}C for 30 s, with a final extension at 72 ^{0}C for 5 min and hold at 4 ^{0}C. The amplified PCR products were electrophoresed in 1.2% agarose gel and stained with GelRed (Biotium Inc. Hayward, CA, USA) and visualized under UV light.

2.5 Genotyping by capillary electrophoresis:

Twenty sequences of *T. orientalis* spp were retrieved from Genbank and aligned using Genetyx software version 9 (http://www.sdc.co.jp/genetyx/updates/genetyx_9/). Two sets of primers targeting ribosomal DNA internal transcribed spacers (ITS1 and ITS2) were designed (Fig. 2). Oligonucleotides and their fragment sizes used in this study are described in (Table 2). PCR was performed using AmpliTaq Gold Master mix, 50ng of DNA, 1.25µl of 10 mM Primer with a final total volume of 25µl. The thermal cycling included initial denaturation of 95 ^{0}C for 5 min followed by 40 cycles at 95 ^{0}C for 15 s, 57 ^{0}C for 15 s, and 72^{0}C for 30 s, with a final extension at 72 ^{0}C for 7 min and hold at 4 ^{0}C. The amplified PCR products were electrophoresed in 1.2% agarose gel, stained with GelRed (Biotium Inc. Hayward, CA, USA) and visualized under UV

light. Depending on band intensity, the samples were diluted accordingly. The loading premix for capillary electrophoresis (genotyping) was prepared using 1µl of diluted PCR product, 0.5µl of internal marker (GeneScan™-600 Liz size standard-Applied Biosystem) and 9µl of Hi-Di. The genotyped peaks were analyzed using GeneMapper® Software version 4.1.

2.6 Quantification of parasitemia by genotyping

To optimize and subsequent quantify parasitemia load by ITS genotyping method, DNA extracted from piroplasm of individual *T. orientalis* types (Ikeda, Chitose, Buffeli) was amplified targeting ITS1 and ITS2. The PCR products were cloned into pGEM-T easy Vector according to manufacturer's instructions for the TA cloning kit (Invitrogen, Carlsbad, CA, USA). Recombinant plasmid clones were standardized into concentrations of 50ng, 100ng, 150ng and 200ng per µl which served as DNA template. PCR was performed using AmpliTaq Gold Master mix, Primer 1.25µl of 10mM with a final total volume of 25µl. The thermal cycling included hot stat at 95 ^{0}C for 5 min followed by 40 cycles at 95 ^{0}C for 15 s, 57 ^{0}C for 15 s, and 72 ^{0}C for 30 s, with a final extension at 72 ^{0}C for 7 min and hold at 4 ^{0}C. The amplified PCR products were electrophoresed in 1.2% agarose gel, stained with GelRed (Biotium) and visualized under UV light. PCR products were not diluted but directly capillary electrophoresed. The loading premix for genotyping was prepared as described previously. The genotyped peaks were analyzed using GeneMapper® Software version 4.1.

3.0 RESULTS:

3.1 PCR amplification and capillary electrophoresis of rRNA ITS1 and ITS2

The ITS1 PCR amplification products were separated by agarose gel electrophoresis revealing specific single bands under UV light. The PCR products from ITS1 were genotyped resulting into three peaks corresponding to Ikeda-251bp, Buffeli-269bp and Chitose-274bp. Most samples had mixed infections with two or three *T. orientalis* type infections as reflected by multiple peaks respectively (Fig 4). To validate the ITS1 genotyping method, field samples were genotyped and the summary results are as indicated (Table 3). Individual animals showed single or mixed infection with two or three *T. orientalis* types. In case of ITS2, PCR amplification products were gel electrophoresed and capillary electrophoresed as above. The results of ITS2 genotyping indicated Ikeda-133-bp and Buffeli/Chitose 139-bp (Fig 5). ITS2 genotyping was not tested against field isolates.

3.2 Specificity of the capillary electrophoresis (genotyping) method:

The specificity of the method was determined by comparing percentage discrimination between PCR amplification of MPSP gene and ITS1 genotyping. PCR products generated using species-specific MPSP primer targeting MPSP gene were gel electrophoresed and fragment size classified belonging to Ikeda, Chitose, or Buffeli (Table 4). ITS1 genotyping was specific, capable of picking positive samples and discriminating parasite population into the various types (Ikeda, Chitose, and Buffeli) in contrast with MPSP species-specific primer that did not discriminate certain individual samples.

3.3 Sensitivity of the capillary electrophoresis genotyping in comparison to conventional PCR detection:

To determine and test the detection limit of ITS1 genotyping, products of PCR amplification targeting MPSP and an additional newly designed primer targeting combined region of ITS1 and ITS2 including the 5.8S were gel electrophoresed and results compared. Out of 322 total samples examined, MPSP detected 50.8%, ITS1-5.8S-ITS2 primer 88.6% while ITS genotyping detected 80.6% (Table 5). ITS1-5.8S- ITS2 PCR amplification and ITS1 genotyping were found to have high detection rate even when the template DNA concentration was 2ng/µl.

3.4 Correlation of parasitemia with parasite DNA detected by capillary electrophoresis within a host:

To correlate the parasitemia level to peak heights in capillary electrophoresis genotyping, three naive animals experimentally infected with inoculums containing Ikeda, Chitose, and Buffeli types were microscopically examined for their parasitemia levels for a period of 3 months and parasite counts recorded (Table 1). In the first seven days no parasites were observed via giemsa staining in cows number 5690 and 5694 with cow number 5692 having zero parasitemia until 27th Aug. Parasitemia started being recorded from 24th August in cow's number 5690 and 5694 with cow number 5692 starting on 29th August. Levels of parasitemia started to increase with highest parasitemia being recorded between 9th Sep and 13th Oct (Table 1). Thereafter the levels started to fall with the exception of cow number 5694 which the parasitemia started to decrease starting 6th November. The

experiment was terminated on 16th November and animals treated. ITS1 genotyping was able to detect the parasite and discriminate the *T. orientalis* types present when the detection by microscopic was not possible. Using ITS1 peak heights, we correlated the parasitemia levels detected microscopically with peak heights. The peaks were shorter when parasitemia was low and increased as the level of parasitemia increased (Table 1).

DISCUSSION

Eight types (Type1-8) of *Theileria orientalis* are known to infect cattle in the Asia-pacific region (Jeong et al., 2010: Ota et al., 2009); five of which have been confirmed to occur in Japan (Type1-5). Ikeda (Type 2) is the most virulent of the eight types accounting for most of the reported cases in recent Theileriosis outbreak in Australia outbreak (kamau et al., 2010). Various criteria and methods have been used to detect and identify these parasites (types) (Jeong et al., 2003; Kawazu et al., 1992; Sugimoto et al., 1991; Tanaka et al., 1993). It's difficult to differentiate between those genotypes solely on the basis of the morphology of the piroplasm and or sero-diagnosis due to cross-reactivity (Kim et al., 1998; Choi et al., 1997).

In this study we describe a novel method for detection, discrimination and quantification of *T. orientalis* types by capillary electrophoresis genotyping all in a single reaction. The method consists of PCR amplification, gel electrophoresis and genotyping based on ITS1 or ITS2 rRNA spacer regions, followed by scoring the different fragment sizes as peaks representing different types. In addition, PCR amplification targeting MPSP and rRNA ITS1-5.8S-ITS2 regions were used. The whole procedure can be completed within hours

with accuracy of identification being 80.3%. This method provides an accurate alternative for detection and identification of *T. orientalis* types specifically type1 (Chitose), type2 (Ikeda) and type3 (Buffeli), which are difficult to differentiate by conventional PCR methods.

The sensitivity of detection methods targeting MPSP gene and rRNA ITS1-5.8S-ITS2 region when compared to that of ITS1 capillary electrophoresis genotyping were 50.8%, 88.6%, and 80.3% respectively. Experimentally infected cows that were either negative or positive microscopically tested positive with ITS1 genotyping; yet it took two weeks for cow number 5090 and 5094, and five weeks for cow number 5092 to be microscopically positive. This result demonstrates subclinical cases of *T. orientalis* infection may exist even when the piroplasms of *T. orientalis* cannot be detected by microscopic examination. It also indicates that our newly designed ITS1 capillary electrophoresis genotyping and rRNA ITS1-5.8S-ITS2 PCR detection methods provide useful means for detecting hidden infections before the appearance of symptoms in the cattle and the method could be applied for prophylactic purpose, to quantify of theileria for prognosis or after treatment (Jeong et al., 2003). The clinical application of these methods could help livestock producers to avoid economic damage caused by bovine theileriosis.

Specificity of the capillary electrophoresis genotyping method was determined based on percentage of *T. orientalis* types the method could detect and differentiate, compared to those detected and discriminated by PCR amplification of species-specific MPSP gene.

The results indicate ITS1 genotyping method is capable of detecting more than one type compared to MPSP. These findings indicates that the overall sensitivity and specificity by ITS1 genotyping can be increased up to 2-fold, compared with conventional MPSP diagnostic methods.

Theileriosis is one of the chronic diseases that results in invisible economic losses in feed efficiency and growth retardation through cycling of infections in endemic areas. Currently, the control of theileriosis largely relies on the application of acaricides to reduce tick infestation, thus necessitating proper monitoring (Dolan, 1986). However, the exact assessment of theileriosis is limited mainly by its complicated pathogenesis. Parasitemia is a factor used to institute therapeutic interventions and it helps determine the initial dose of treatment. To quantify the parasites DNA and relate that to level of parasitemia, correlation between parasites load infecting the host animal was compared to capillary electrophoresis peak height. The assumption made was that, the starting DNA template would be influenced by the parasite load that will be reflected in the DNA amount isolated. The peak height was taken to mean the level of the starting DNA concentration with higher DNA concentration resulted to higher peaks. To confirm the hypothesis, three cows experimentally challenged were microscopically examined at different time points over a span of three months and parasitemia level recorded. Each time-point blood was taken and DNA isolated. The results of genotyping peak heights correlated well with the level of parasitemia. Peak heights were low when the parasitemia level was low and increased with increase in parasitemia. When parasitemia levels started falling, the genotyping peak heights were maintained over time before eventually starting

to reduce. Presence of parasite DNA is fundamentally constant throughout its life-cycle and is not degraded due to short-term environmental stress factors that can alter transcriptional and post-transcriptional events. Thus, molecular based identification methods of the parasite genome are generally not limited to any particular developmental stage (Zarlenga et al., 2001). The genotyping heights started decreasing towards the end of three months conceivably the time at which the host immune system had eliminated most of the parasites. This indicated ITS1 genotyping was capable of detecting carrier state indicating that cattle with less than 0.01% parasitemia may require careful monitoring, especially in the absence of clinical signs (Lee and Kim, 1987). Although the standard PCR is essentially a non-quantitative method, the peak height fluctuations with parasitemia probably reflect variations in the number of parasites in the host blood (Skilton et al., 2002). This method of ITS1 genotyping may be a useful tool in screening recent outbreaks, naturally infected animals or where carrier animals are suspected to occur (Dolan, 1986, Bishop et al., 1992, 1995).

In experimentally challenged cows, presence of type3 (buffeli) was detected by ITS1 genotyping only in the first two weeks and thereafter the test became negative. We suspected that pressure may occur when an animal or the ticks are co-infected with Ikeda, Chitose and Buffeli; such an environment may result to interaction, synergy and or interference between different parasites types resulting to mutation that may lead to loss of one or more of the parasite types. This phenomenon may cause the remaining parasite type(s) to be more virulent and or adaptable to different vectors transmission ability (Tindih et al., 2010).

The ITS1, ITS2 and rRNA ITS1-5.8-ITS2 detection methods constitute molecular diagnostic tools valuable addition to the repertoire of parasite detection, discrimination and quantification tools that are currently available for molecular epidemiological studies of tick-borne pathogens in field conditions. The adoption of the technique which is fast, easy to use, cost-effective, highly sensitive and specific may contribute to enhanced understanding of the epidemiology of theileriosis. In addition, the method can be applied in monitoring, evaluation of preventive measures; and as an epidemiological tool to understanding complicated pathogenesis of theileriosis in endemic regions.

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LEGENDS TO TABLES:

Table1: Summary of experimental challenge, fragment heights and parasitemia. Type1, type2 and type3 represents Chitose, Ikeda and Buffeli respectively. The ID indicates the identification number for the challenge animal, while date indicates the dates at which the experimental animal was tested microscopically for parasitemia and blood collected for DNA isolation and typing. The plus (+) and negative (-) signs indicates the sample typed positive or negative for a particular *T. orientalis* type respectively. The numbers in bracket indicates the peak height which corresponds to the parasitemia scores. Minus (-) indicates negative.

Table2: Newly designed Oligonucleotide (Kamau et al., 2010) used in the study. Expected fragment sizes ranges from Ikeda-251bp, Buffeli-269bp, and Chitose-274-bp for ITS1.

Table3: Distribution of *Theileria orientalis* types based on rRNA ITS1 capillary electrophoresis. Abbreviations used are I-Ikeda, C-Chitose and B-Buffeli. Numbers in the columns indicate positive samples with certain T. orientalis type infection in different farms. Samples showing mixed infection are abbreviated as I+C+B, I+C, C+B or I+B respectively.

Table4: Comparison of ITS1 capillary electrophoresis genotyping versus conventional PCR amplification of species-specific MPSP gene. Numbers in bracket indicates animals sampled. The figures in column indicate number of *Theileria orientalis* types detected and discriminated in each farm.

Table5: The sensitivity of ITS1 genotyping when compared to detection by PCR amplification of species-specific MPSP gene and rRNA ITS1-5.8S-ITS2. The figures in column indicate number of animal's detected positive by farm while numbers in bracket indicates total animals sampled.

LEGENDS TO FIGURES:

Fig 1: Geographical map of Hokkaido, Japan. Stars indicate the locations of the four districts (Otofuke, Shintoku, Taiki, and Shin-Hidaka) where the blood samples were collected.

Fig 2: rRNA internal transcribed spacer regions and the primer design targets used in this study.

Fig 3a: Primer position for the rRNA ITS1 region after alignment of twenty published sequences of *T. orientalis* Ikeda, Chitose and Buffeli types. The highlighted red box indicates the forward and reverse primer locations. Multiple alignments were done using Genetyx software.

Fig 3b: Primer design for the rRNA ITS2 after alignment of twenty retrieved database sequences of *T. orientalis* Ikeda, Chitose and Buffeli types. Sequences with deletions represent the Ikeda sequences. The highlighted red box indicates the forward and reverse primer locations.

Fig 4: rRNA ITS1 Genescan-derived electropherogram traces of capillary electrophoresis genotyping which represents three types of *T. orientalis* spp. The scale represents fragment size (in base pairs), and three different *T. orientalis* genotypes are represented as Ikeda (251bp), Buffeli (269bp), Chitose (274bp) while

that of mixed infection, different genotypes are indicated as three different genotypes by their fragment size differences.

Fig 5: rRNA ITS2 Genescan-derived electropherogram traces of capillary electrophoresis genotyping which represents three *T. orientalis* spp. The scale represents fragment size (in base pairs), and different *T. orientalis* genotypes are represented as Ikeda (133bp), Buffeli/Chitose (139bp) while that of mixed infection, two distinct peaks indicating Ikeda as 133bp, and Buffeli/Chitose as 139bp according to their fragment size differences.

Table 1: Summary of experimental challenged animals, parasitemia, fragment height and ITS Genotyping

Animal ID	Date	Parasitemia	Chitose/Height	Ikeda/Height	Buffeli/Height
5690	10-Aug	0	+ (86)	+ (172)	+ (94)
5690	17-Aug	0	+ (525)	+ (160)	+ (651)
5690	24-Aug	0.03	+ (7532)	+ (725)	-
5690	31-Aug	0.57	+ (8190)	+ (1346)	-
5690	**7-Sep**	1.1	+ (8664)	+ (2092)	-
5690	**14-Sep**	1.38	+ (8410)	+ (8190)	-
5690	**21-Sep**	1.19	+ (7796)	+ (8507)	-
5690	**28-Sep**	1.63	+ (1115)	+ (8695)	-
5690	**5-Oct**	1.43	+ (347)	+ (8572)	-
5690	**13-Oct**	1.1	+ (629)	+ (8619)	-
5690	**19-Oct**	0.77	+ (1321)	+ (8223)	-
5690	26-Oct	0.62	+ (1753)	+ (8093)	-
5690	2-Nov	0.96	+ (339)	+ (674)	-
5690	9-Nov	0.3	+ (7637)	+ (8023)	-
5690	16-Nov	0.056	+ (1333)	+ (4541)	-
5692	10-Aug	0	+ (55)	+ (88)	+ (92)
5692	17-Aug	0	+ (2615)	+ (3616)	+ (204)
5692	24-Aug	0	+ (6505)	+ (1581)	-
5692	31-Aug	0.05	+ (5756)	+ (8187)	-
5692	**7-Sep**	0.36	+ (644)	+ (8513)	-
5692	**14-Sep**	1	+ (641)	+ (7987)	-
5692	**21-Sep**	2.17	+ (343)	+ (8251)	-
5692	**28-Sep**	1.47	+ (3439)	+ (8027)	+ (55)
5692	**5-Oct**	0.77	+ (8339)	+ (8643)	-
5692	13-Oct	0.49	+ (7714)	+ (8231)	-
5692	19-Oct	0.34	+ (7067)	+ (8492)	-
5692	26-Oct	0.4	+ (6226)	+ (8643)	+ (137)
5692	2-Nov	0.3	+ (4506)	+ (8460)	-
5692	9-Nov	0.074	+ (1019)	+ (6525)	-
5692	16-Nov	0.093	+ (1571)	+ (7197)	-
5694	10-Aug	0	+ (44)	+ (75)	+ (306)
5694	17-Aug	0	+ (2805)	+ (4775)	+ (593)
5694	24-Aug	0.01	+ (8201)	+ (7980)	+ (171)
5694	31-Aug	0.37	+ (6068)	+ (6277)	-
5694	7-Sep	0.23	+ (1622)	+ (8021)	-
5694	**14-Sep**	1.8	+ (1063)	+ (8488)	-
5694	**21-Sep**	5.59	+ (199)	+ (8656)	-
5694	**28-Sep**	4.34	+ (3344)	+ (8512)	-
5694	**5-Oct**	4.23	+ (5889)	+ (8419)	-
5694	**13-Oct**	2.52	+ (4908)	+ (8386)	-
5694	**19-Oct**	4.77	+ (8480)	+ (8426)	-
5694	**26-Oct**	3.44	+ (8221)	+ (8643)	-
5694	**2-Nov**	1.34	+ (7762)	+ (8375)	-
5694	9-Nov	0.68	+ (5621)	+ (6251)	-
5694	16-Nov	0.28	+ (5077)	+ (6203)	-

Table 2: Oligonucleotide primers used and expected fragment sizes

Primer	Sequences	Expected Size
MPSP type specific Forward Primer		
Ts-Ikeda	5'-AAGGATCCGTCTCTGCTACCGCCGC-3'	826**bp** (Kubota et al., 1996)
Ts-Chitose	5'-GCGGATCCTCATCGTCTCTGCAACT-3'	831**bp** "
Ts-Buffeli	5'GCGGATCCGCTCTGCAACCGCAGAG-3'	825**bp** "
MPSP type specific Universal Reverse Primer		
Ts-R	5'-TGTGAGACTCAATGCGCCTA-3'	
rRNA ITS1 Forward	5'-CGCTTGTTGAGAGGATGCCT-3'	Ikeda 251**bp**, Chitose 274**bp**, Buffeli 269**bp**
rRNA ITS1 Reverse	5'- CCTTCATCGTTGTGTGAGCCAA -3'	(Kamau et al., 2010)
rRNA ITS2 Forward	5'-TGGCTCACACAACGATGAAG-3'	Ikeda 133**bp**, Chitose/Buffeli 139**bp**
rRNA ITS2 Reverse	5'-GGTTCACTGAAATGGGAGTAC-3'	(Kamau et al., 2010)
rRNA ITS1-5.8-ITS2 Forward	5'-CGCTTGTTGAGAGGATGCCT-3'	400**bp**
rRNA ITS1-5.8-ITS2 Reverse	5'-GGTTCACTGAAATGGGAGTAC-3'	(Kamau et al., 2010)

Table 3: Different Genotypes of *Theileria orientalis* detected by rRNA ITS1 per individual cow

Farms	I+C+B	I+C	C+B	I+B	I	C	B	None
Otofuke	8	0	3	1	2	0	6	0
Shintoku	13	4	8	5	1	0	5	1
Shinhidaka-A	2	20	1	3	35	2	0	31
Shinhidaka-B	7	26	0	3	43	0	1	3
Taiki	8	0	0	7	46	0	1	29
Total	**38**	**50**	**12**	**19**	**127**	**2**	**13**	**64**

Table 4: Comparisons of ITS1 genotyping versus PCR amplification of conventional species-specific MPSP to determine the specificity of the two detections methods

	Species-specific MPSP			**ITS1 Genotyping**		
Farm & Number of samples	Ikeda	Chitose	Buffeli	Ikeda	Chitose	Buffeli
Otofuke (20)	2	0	0	11	11	18
Shintoku (37)	6	6	0	26	28	32
Shinhidaka -A- (94)	35	13	0	25	60	6
Shinhidaka -B- (83)	44	23	0	79	33	11
Taiki (91)	40	0	0	61	8	16
Total (325)	127	42	0	202	140	83

Table 5: Comparing parasite detection by ITS1 capillary electrophoresis genotyping with PCR amplification of rRNA ITS1-5.8-ITS2 and species-specific MPSP gene

Farm & Number of examined cows	MPSP	rRNA ITS1-5.8S-ITS2	ITS1
Otofuke (20)	2	12	20
Shintoku (37)	9	26	36
Shinhidaka -A- (94)	47	84	63
Shinhidaka -B- (83)	48	82	80
Taiki (91)	59	84	62
Total Examined cows (325)	50.8%	88.6%	80.3%

Fig 1: **Geographical map of Hokkaido, Japan. Stars indicate the locations of the four districts (Otofuke, Shintoku, Taiki, and Shin-Hidaka) where the blood samples were collected**.

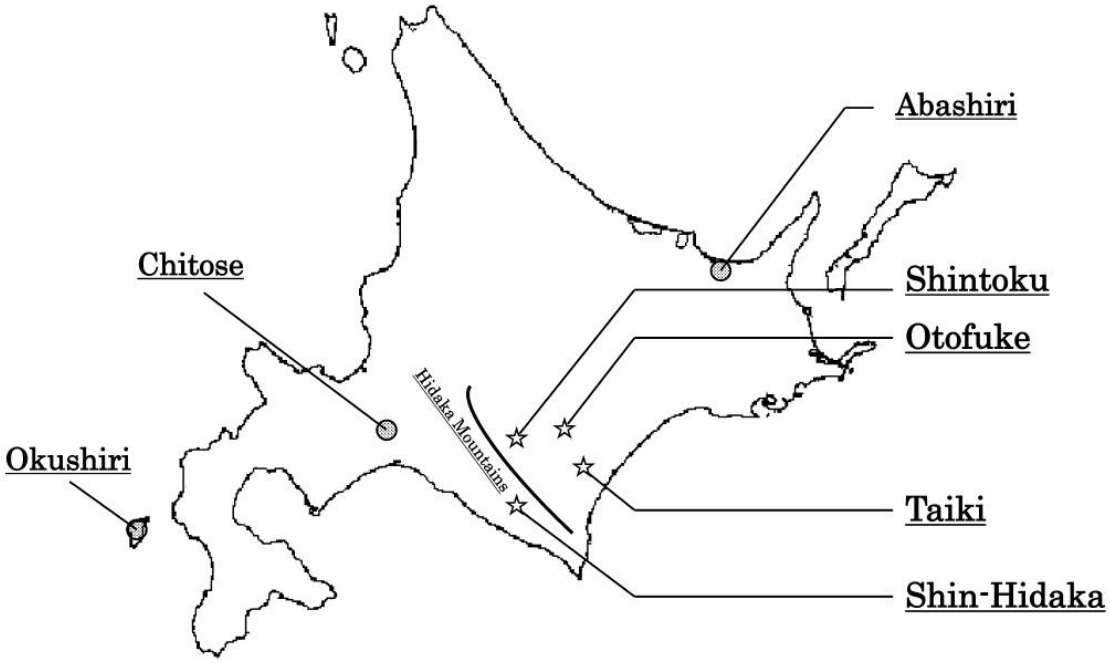

Fig 2: rRNA Internal transcribed spacer region and the Primer design target regions

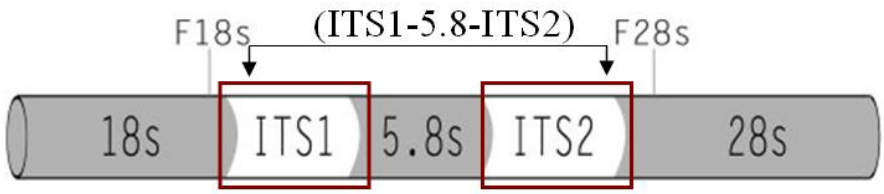

Fig 3a: Alignment of retrieved *Theileria orientalis* types and ITS1 primer location

Fig 3b: Alignment of retrieved *Theileria orientalis* types ITS2 primer location

Fig 4: the three *Theileria orientalis* types on capillary electrophoresis genotyping plot by ITS1

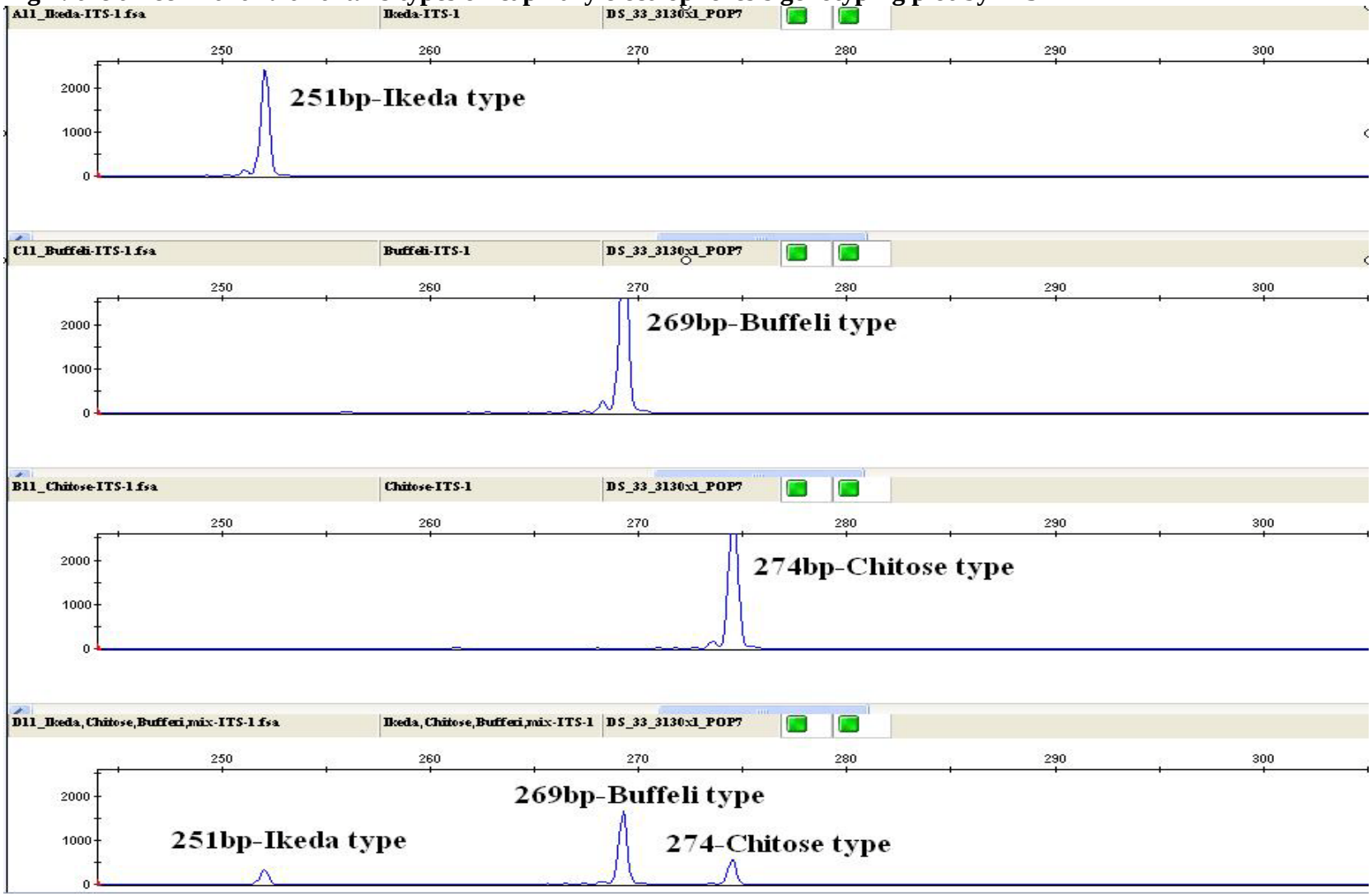

Fig 5: the three *Theileria orientalis* types on capillary electrophoresis genotyping plot by ITS2