Development and Validation of a Quantitative PCR Assay Using Multiplexed Hydrolysis Probes for Detection and Quantification of *Theileria orientalis* Isolates and Differentiation of Clinically Relevant Subtypes

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Theileria orientalis is an emerging pathogen of cattle in Asia, Australia, and New Zealand. This organism is a vector-borne hemoparasite that causes clinical disease characterized by anemia, abortion, and death, as well as persistent subclinical infections. Molecular methods of diagnosis are preferred due to their sensitivity and utility in differentiating between pathogenic and apathogenic genotypes. Conventional PCR (cPCR) assays for *T. orientalis* detection and typing are laborious and do not provide an estimate of parasite load. Current real-time PCR assays cannot differentiate between clinically relevant and benign genotypes or are only semiquantitative without a defined clinical threshold. Here, we developed and validated a hydrolysis probe quantitative PCR (qPCR) assay which universally detects and quantifies *T. orientalis* and identifies the clinically associated Ikeda and Chitose genotypes (UIC assay). Comparison of the UIC assay results with previously validated universal and genotype-specific cPCR results demonstrated that qPCR detects and differentiates *T. orientalis* with high sensitivity and specificity. Comparison of quantitative results based on percent parasitemia, determined via blood film analysis and packed cell volume (PCV) revealed significant positive and negative correlations, respectively. One-way analysis of variance (ANOVA) indicated that blood samples from animals with clinical signs of disease contained statistically higher concentrations of *T. orientalis* DNA than animals with subclinical infections. We propose clinical thresholds to assist in classifying high-, moderate-, and low-level infections and describe how parasite load and the presence of the Ikeda and Chitose genotypes relate to disease.

Theileria orientalis is a vector-borne hemoparasite that infects cattle and buffalo and is generally spread by ticks of the *Haemaphysalis* genus (1–3). Historically, this organism has been referred to as *Theileria sergenti*, *Theileria buffeli*, or the *T. orientalis*/*T. sergenti*/*T. buffeli* complex; however, the name *T. sergenti* is now considered invalid (4), and *T. orientalis* is commonly used to refer to all (5). From a clinical perspective, *T. orientalis* can cause anemia, lethargy, jaundice, fever, abortion, and mortality in cattle (6). Clinical infection can also result in decreased milk production in dairy cattle (7). *T. orientalis* is a conditional pathogen, and while pathogenic forms are largely limited to Eastern Asia and Australasia (5, 6, 8–10), it is frequently detected in asymptomatic animals; these benign forms are globally spread (5, 11–14). Analysis of the most common genotyping locus (p32), encoding the variable major piroplasm surface protein (MPSP), currently identifies 11 distinct *T. orientalis* genotypes (13, 15, 16). Of these genotypes, type 2 (Ikeda) and to a lesser extent type 1 (Chitose) are typically found in association with clinical disease (6, 9, 10, 17–22). The presence of pathogenic and benign forms of *T. orientalis* greatly complicates its clinical diagnosis, with standard blood film analysis unable to identify the pathogenic genotypes.

Multiple conventional PCR (cPCR) assays have been published for the identification of *T. orientalis* in blood samples (9, 21, 23, 24). The most commonly cited assays detect and genotype *T. orientalis* by amplifying unique regions of the MPSP gene (21). These assays use two universal primers to detect *T. orientalis* infection and specific forward primers to identify the Ikeda, Chitose, and Buffeli genotypes. While this method is highly sensitive and has been validated in prior studies (19, 21), it is not multiplexed

and is highly susceptible to PCR inhibitors (6), which are often found in nucleotide extractions obtained from blood (25, 26). To overcome inhibition, undiluted and diluted nucleotide extracts can be examined in parallel to prevent false-negative results (6, 19, 27). However, if multiple reactions per sample are required to determine the presence of infection, the procedure becomes both expensive and time-consuming. Furthermore, cPCR does not give an accurate representation of parasite load and therefore cannot provide an indication of the severity of *T. orientalis* infection. A recently developed multiplexed tandem PCR (28) is able to discriminate between four genotypes of *T. orientalis*, but it is only semiquantitative and therefore cannot be used to define a clinical threshold.

Hydrolysis probe quantitative PCR (qPCR) assays employ sequence-specific, fluorescently labeled probes attached to duplex-

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TABLE 1 Oligonucleotides used in this study

Oligonucleotide	Sequence (5'–3')	Target	Final concn	Reference
MPSP-F	GCAACAAGGATTTGCACGC	MPSP gene, all genotypes	300 nM	This study
MPSP-R	TGTGAGACTCAATGCGCTAGA	MPSP gene, all genotypes	300 nM	This study
Pr-U	FAM-TCGACAAGTTCTACCAC-MGB-NFQ	MPSP gene, all genotypes	100 nM	This study
Pr-I	VIC-CATGAACAGTGCTTGCG-MGB-NFQ	Ikeda MPSP gene	250 nM	This study
Pr-Ca	NED-TCCTCAGCGCTGTTCT-MGB-NFQ	Chitose MPSP gene	100 nM	This study
Pr-Cb	NED-TCCTCGGCGCTGTTCT-MGB-NFQ	Chitose MPSP gene	150 nM	This study
TsI	CACCATCGTCTGCTACCGCCGC	Ikeda MPSP gene (cloning primer)	500 nM	Modified from 21
TsC	CACCTTCCTCATGCTCTCTGCAACT	Chitose (cloning primer)	500 nM	Modified from 21
TsB	CACCTTCCTCATGCTCTCTGCAACT	Buffeli (cloning primer)	500 nM	Modified from 21
TsR	CACCTGCTCTGCAACCGCAGAG	<i>T. orientalis</i> (cloning primer)	500 nM	Modified from 21
Chb mutF	CTATGTGAGACTCAATGCGCCTA	Chitose plasmid mutagenesis forward primer	500 nM	This study
Chb mutR	GATGAGAACAGCGCCGAGGACGGCAAGTG	Chitose plasmid mutagenesis reverse primer	500 nM	This study
T7F	CACTTGCCGCTCTCGGCGCTGTTCTCATC	pET100/GW/D-TOPO vector forward primer	800 nM	This study
T7R	TAATACGACTCACTATAGGG	pET100/GW/D-TOPO vector reverse primer	800 nM	This study
RTF-I	ATTGGTAGACGGAATGGAAGAAGG	Ikeda (sequencing primer)	800 nM	This study
RTR-I	GAGACTCAATGCGCTAGAGATAATAGA	Ikeda (sequencing primer)	800 nM	This study
RTF-C	CCGGTGATGAGAGATTCAAGGAAGTA	Chitose (sequencing primer)	800 nM	This study
RTR-C	GACTCAATGCGCTAGARATAGTAGG	Chitose (sequencing primer)	800 nM	This study

stabilizing molecules and are frequently used to accurately identify and quantify genetic sequences within clinical samples (29–32). These assays can be multiplexed by using multiple sequence-specific probes attached to fluorescent molecules with unique and distinguishable emission spectra (33). While one hydrolysis probe qPCR assay was previously developed for *T. orientalis* detection, that assay did not identify genotypes associated with clinical disease (34). In this study, we describe the development and validation of a multiplex hydrolysis probe qPCR which can detect *T. orientalis* infection, quantify parasite load, and identify the two genotypes associated with clinical disease.

MATERIALS AND METHODS

Samples. Conventional and quantitative PCR analyses were performed on 318 blood samples that were separated into sensitivity and specificity panels. The sensitivity panel comprised 237 samples that were positive for *T. orientalis* in any of the four cPCR assays used for detection and typing of *T. orientalis* (19, 21). These samples were either derived from animals and/or herds that had clinical cases consistent with theileriosis or from herds that were screened as part of a *Theileria* surveillance program. Samples were derived from diverse geographic regions of Australia, including the states of New South Wales, Victoria, Queensland, and Western Australia. Clinical signs considered consistent with theileriosis were a combination of some or all of the following: lethargy, ataxia, increased respiratory rate, fever, pale and/or jaundiced mucous membranes, and abortion in pregnant animals. Hematological measures were also considered and comprised anemia, as determined by packed cell volume (PCV) and a blood film positive for *Theileria* piroplasms and for erythrocytic changes consistent with regenerative anemia (e.g., nucleated erythrocytes, poikilocytosis, polychromasia, and Howell-Jolly bodies).

The specificity panel was composed of 81 samples that were identified as *T. orientalis* negative by previous cPCR analysis. Of these samples, 50 were sourced from areas with no history of *T. orientalis* outbreaks at the time of sampling (South Australia) and from cattle without any clinical symptoms of *T. orientalis* infection. A further 31 samples, used for analytical specificity testing, were also free of *T. orientalis* (as confirmed by cPCR); however, 25 of these samples were sourced from cattle known to be infected with *Babesia bigemina* or *Babesia bovis*, causative agents of the clinically similar disease tick fever. The remaining 6 samples were derived from cattle immunized with a live vaccine strain of *Anaplasma centrale*, which is used for controlling anaplasmosis. All samples were collected in

vacuum blood tubes containing either EDTA (309 samples) or lithium-heparin (9 samples). Blood was decanted into sterile sample tubes and stored at -20°C for later extraction.

DNA extraction. Purification of sample DNA was performed using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) with a 100- μl starting volume of blood and a 100- μl elution volume. Ten-fold dilutions of template DNA were made in molecular-grade water (Sigma-Aldrich, St. Louis, MO, USA). Negative extraction controls were included at a ratio of at least 1 per 20 DNA extractions as a quality control measure.

Primer and TaqMan probe design. Dual-labeled TaqMan hydrolysis probes were used for the qPCR assay. A triplex qPCR (UIC triplex) was designed that consisted of a quantitative universal (U) assay for the detection of all *T. orientalis* types and two type-specific semiquantitative assays aimed at differentiating the Ikeda (I) and Chitose (C) genotypes. A single primer set was used for amplification, with the hydrolysis probes conferring specificity in each channel of the assay. This assay design was chosen to avoid interactions between primer sets and allow for accurate quantification in the universal component of the assay. The forward primer was designed to target a highly conserved sequence within the 3' end of the MPSP gene, while the reverse primer was similar to the TsR primer designed and validated in prior studies (19, 21). Each probe was 5' labeled with a fluorophore (6-carboxyfluorescein [FAM], VIC, and NED for the U, I, and C components, respectively) and 3' labeled with a nonfluorescent quencher (NFQ). All probes contained an MGB moiety to confer additional probe specificity and were purchased from Life Technologies (Carlsbad, CA, USA). All primer and probe sequences are listed in Table 1. An *in silico* analysis was performed on all primers and probes via comparison with existing sequence data in GenBank. The *T. orientalis* U probe was designed as a 100% match for >95% of all available *T. orientalis* MPSP gene sequences. Similarly, the I and C probes were designed to maximize detection of their respective subtypes. In the case of the Chitose probe, a degenerate design was used to account for a polymorphism present in some of the Chitose sequences (Table 1).

cPCR. cPCR analysis was performed on all samples used in the study to provide a benchmark for comparison of the qPCR assay. cPCR is considered a gold standard for *T. orientalis* detection and differentiation. The cPCR assay used in this study detected >50% of blood smear-negative animals (19) and has been validated in prior studies (21, 27). The specificity of the cPCR has also been independently verified in our laboratory via DNA sequencing of PCR products from each genotype assay (unpublished data). The cPCR was performed as 6 separate assays (one each for universal [p32] and the Ikeda and Chitose genotypes on both undiluted

and 10-fold dilutions of DNA extracts). A Buffeli-specific cPCR was also performed to account for samples that were positive in the p32 assay but negative in the Ikeda and Chitose assays. All cPCRs were performed as previously described (27), except that GelRed (Biotium, Hayward, CA, USA) was used as a DNA stain in place of ethidium bromide due to its improved biosafety and sensitivity characteristics.

Generation of plasmid standards. Plasmid DNA standards were generated by PCR from blood samples sourced from New South Wales and Queensland known to contain the Ikeda, Chitose, and Buffeli genotypes (GenBank accession numbers [KM624619](#) to [KM624621](#)). Type-specific forward primers and a universal reverse primer targeting the MPSP genes of each genotype (Table 1) (21) were modified to facilitate cloning into the pET100/GW/D-TOPO vector (Life Technologies). MPSP gene PCR products were cloned, as described previously, into *Escherichia coli* TOP10 cells (35). To account for the Chitose polymorphism mentioned above, a plasmid standard variant (Chitose B) was generated by site-directed mutagenesis of the original Chitose plasmid (Chitose A), as described in reference 36 and using mutagenic primers (Table 1). Plasmids were extracted from transformant colonies with the QIAprep Spin miniprep kit (Qiagen). All plasmids were examined with conventional dye terminator sequencing to confirm correct sequences using T7 forward and reverse primers (Table 1). Plasmid DNA was quantified by spectrophotometry at 260 and 280 nm. Plasmid standards were prepared as serial 10-fold dilutions in (1:250) tRNA from *Saccharomyces cerevisiae* (Sigma-Aldrich).

qPCRs. All qPCRs were performed in parallel with cPCR analyses using the same nucleotide extracts. UIC multiplex qPCRs were prepared by combining TaqMan Environmental MasterMix 2.0 (final concentration, 1×; Life Technologies) with primers and probes described in Table 1. Reagent mixes were aliquoted into MicroAmp Fast Optical 96-well reaction plates (catalog number 4346906; Life Technologies), and 2 µl of extracted DNA sample was added, to yield a total volume of 20 µl. Plates were subsequently sealed with optical adhesive covers (catalog number 4360954; Life Technologies). Thermal cycling was performed in a 7500 Fast real-time PCR system (Life Technologies) with an initial holding stage of 95°C for 10 min followed by a cycling stage (95°C for 15 s and then 60°C for 1 min) for 45 cycles.

Probe specificity was tested using purified single and pairwise combinations of the different plasmid standards (Ikeda, Chitose A and B, and Buffeli plasmids). Buffeli plasmid was included in the standards to ensure that the universal component of the assay efficiently detected this prevalent (albeit benign) genotype. To test for equivalent quantification of all targets in the U component of the assay, single and pairwise combinations of 10-fold serial dilutions of the different plasmid standards ranging from 3×10^1 to 3×10^7 MPSP gene copies/µl (GC/µl) were tested.

Equimolar and unequal pairwise combinations (10- to 1,000-fold differences in concentration) of the plasmids were used to determine whether the I and C assays were efficient at detecting the dominant genotype in a given template mix. These assays were performed in triplicate.

The limit of detection (LOD) was defined as the limit where 95% of qPCRs were successful (37), and it was experimentally estimated by testing 8 replicates of an equimolar mixture of Ikeda, Chitose (A and B), and Buffeli plasmid DNA at dilutions of 15,000, 1,500, 150, 50, 15, 5, 1.5, and 0.5 MPSP GC/µl against each probe in uniplex and multiplex, followed by probit analysis.

Potential PCR inhibition from blood components was tested during UIC assay development (using a cocktail containing 900 nM primers and 250 nM U probe) by testing both undiluted and 10-fold-diluted DNA extracts from the samples in the specificity panel ($n = 237$). In addition, PCR inhibition in the final UIC assay was tested in a 10-fold dilution series of Ikeda, Chitose (A and B), and Buffeli plasmids (1.5×10^1 to 1.5×10^7 MPSP GC/µl) spiked into a pool of 10 *Theileria*-negative DNA extracts. The negative extracts were derived from EDTA blood samples from cattle located in areas where *Theileria* was not enzootic (i.e., negative samples from the specificity panel) and that were further confirmed to be *T. orientalis* negative by both cPCR and qPCR.

Data analysis. Data were collected and analyzed using the Life Technologies 7500 software v2.3. Threshold cycle (C_T) values were determined by calculating the cycle number when fluorescence reached a ΔR_n (i.e., fluorescence of the reporter dye relative to that of a passive reference dye) of 0.1. The universal component of the UIC assay was quantified by comparison to a standard curve generated from a 10-fold dilution series ranging from 1.5×10^1 to 1.5×10^7 MPSP GC/µl of an equimolar mix of Ikeda, Chitose (50% Chitose A and 50% Chitose B), and Buffeli plasmids. Quality acceptance parameters for each of the standard curves were an R^2 value of >0.98 and an amplification efficiency between 90 and 110%. Nucleic acid concentrations used for analytical and diagnostic statistics (MPSP GC/µl) were obtained using plasmid molecular masses, calculated from plasmid sequences as previously described (38). McNemar's test was used to determine whether there was a significant difference in the proportion of positives between the two tests between pairs of treatments (cPCR versus qPCR). Discrepant results were resolved via Sanger sequencing at the Australian Genome Research Facility (AGRF) by using the relevant sequencing primers detailed in Table 1. Confidence intervals (CIs) for the sensitivity and specificity were calculated using the Clopper-Pearson method (39). The values for GC/µl and GC/µl packed erythrocytes (PE) were used for correlations with parasitemia and the PCV. GC/µl PE values were calculated to account for variable erythrocyte concentrations (i.e., calculated from the GC/µl by multiplying it by the nucleotide extraction volume [100 µl]/PCV). Correlations were examined using Spearman's method; two-tailed P values are listed in the Results section.

Comparison with clinical data. To determine whether there was a significantly higher concentration of parasite DNA in clinically affected animals, samples were divided into four groups based on their individual case histories and herd status: (i) subclinical animals from herds without clinical cases; (ii) clinically affected individuals; (iii) recovering animals; (iv) in-contact animals. Animals were considered subclinical ($n = 55$) if they were positive via cPCR but belonged to herds that did not display any cases consistent with clinical theileriosis at the time of sampling (e.g., surveillance samples). Animals were considered clinically affected ($n = 110$) if they displayed a combination of symptoms consistent with theileriosis at the time of sampling (anemia, jaundice, depression, ataxia, lethargy tachypnoea, tachycardia, pyrexia, or late-term abortion) and tested positive for *T. orientalis* in cPCR. Cases with potentially confounding diagnoses were excluded from this group. Recovering animals ($n = 25$) were those that had symptoms consistent with theileriosis in the preceding weeks but had recovered at the time of sampling and were positive in cPCR. Many of these animals also had evidence of regenerative anemia on blood smears, indicating prior illness. In-contact animals ($n = 24$) were cohorts of clinically affected animals at the time of sampling that were not displaying obvious symptoms of disease themselves. MPSP allele concentrations (GC/µl) for each group were compared using one-way analysis of variance (ANOVA), a Kruskal-Wallis test, and Dunn's multiple comparison posttest within Prism 4.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Analytical sensitivity and specificity. The linear dynamic range and analytical sensitivity statistics were determined in multiplex and uniplex for each probe used in the UIC qPCR assay. The linearity of each standard curve from reactions performed in uniplex and multiplex is demonstrated in Fig. 1A. For the U probe, a linear range is observed between 3.0×10^1 and 9.0×10^7 MPSP GC/µl in both uniplex and multiplex. Similarly, the I and C components of the UIC assay exhibited a linear range between 1.5×10^1 and 1.5×10^7 MPSP GC/µl in both uniplex and multiplex. All linear ranges were observed with a coefficient of determination greater than 99.5% (Table 2). Detection of single and pairwise combinations of the Ikeda, Chitose (A and B), and Buffeli plasmids within the universal (quantitative) component of the UIC

FIG 1 The U component of the UIC assay is quantitative. (A) Linear dynamic range for the hydrolysis probe qPCR, produced from four replicate assays using U (top), I (middle), and C (bottom) probes. All qPCRs were performed using an equimolar mixture of Ikeda, Chitose (A and B), and Buffeli plasmids. Data from qPCRs performed in multiplex (×) and in uniplex (△) are compared. Lines of best fit for multiplex and uniplex data are shown as solid and broken lines, respectively. Error bars represent standard deviations from

assay was linear over the previously defined linear range (R^2 for each combination ranged from 0.997 to 0.999) (Fig. 1B). Furthermore, the means of each plasmid combination at each serial dilution were detected within a coefficient of variation (CV) of <20%, which is consistent with existing PCR assays that are considered quantitative (40, 41). The specificity of each probe for its target was confirmed using plasmids containing Ikeda, Chitose (A and B), and Buffeli MPSP gene sequences (data not shown). In addition, 31 samples collected from cattle cPCR negative for *T. orientalis* that were infected with either *Anaplasma centrale*, *Babesia bigemina*, or *Babesia bovis* were all negative in the UIC assay (Table 3).

The LOD for each assay was defined as the point where 95% of replicate assays were positive, and the LODs were determined for each probe in uniplex and when run in the UIC multiplex format. LODs were shown to be of the same order of magnitude when the assay was run in uniplex or multiplex (Table 2).

Diagnostic sensitivity and specificity. The use of qPCR for the detection of infection requires the identification of a diagnostic threshold for the delineation of animals which are positive or negative for the presence of the organism. To identify this threshold, the sensitivity and specificity of the U component of the UIC qPCR assay was examined against potential diagnostic thresholds. A diagnostic threshold above the calculated LOD (Table 2) was selected that maximized both sensitivity and specificity of the U probe. From this analysis, a diagnostic threshold of 30 MPSP GC/ μ L was selected.

Next, 237 samples determined via cPCR to be *T. orientalis* infected were analyzed, and of these, 233 returned a positive result with the U component of the UIC qPCR assay (Table 3). Samples were considered qPCR positive if the concentration calculated from the U component was above the diagnostic threshold of 30 MPSP GC/ μ L. To rigorously test the detection limits of the qPCR assays used in this study, we examined samples that were weakly positive in the cPCR. Twenty-nine samples with very faint bands in cPCR were also identified as positive in qPCR (<1,000 MPSP GC/ μ L), demonstrating that the UIC qPCR compares very well with the corresponding cPCR assays at low concentrations. An overall sensitivity of 98.3% was observed for the detection of *T. orientalis* infection, and the specificity was 100.0%. No significant difference in the proportion of positives between the two tests was identified between the UIC qPCR assay and *T. orientalis* infection status as determined by cPCR using McNemar's test (Table 3). While the sensitivity of the U probe was very high, a small number of *T. orientalis*-infected samples ($n = 4$) did not test positive in the UIC qPCR assay (Table 3) but were weakly positive (faint bands observed) in the p32 cPCR. Of these four samples, three demonstrated detectable amplification in the UIC qPCR but fell below the diagnostic threshold. DNA sequencing indicated that two of these three samples were of the Buffeli genotype and the remaining sample was of the Chitose genotype. The fourth sample was negative upon retest in the p32 cPCR. Conversely, two samples included in the *T. orientalis*-infected group, which were detected as cPCR positive for Chitose and Buffeli but were negative when

means. (B) Means and ranges of the U component from triplicate UIC assays performed on serial dilutions of all possible combinations of Ikeda, Chitose, and Buffeli plasmids (ICB, IC, IB, CB, I, C, and B). R^2 values for each combination ranged from 0.997 to 0.999.

TABLE 2 Analytical sensitivity of U, I, and C assays run in multiplex and uniplex

Parameter	U		I		C	
	Multiplex	Uniplex	Multiplex	Uniplex	Multiplex	Uniplex
R ²	0.999	0.999	0.998	0.995	0.998	0.998
% efficiency (95% CI)	95.0 (93.7–96.4)	95.4 (94.1–96.8)	95.2 (92.8–97.8)	96.7 (93.0–100.7)	100.0 (97.5–102.7)	97.1 (95.5–99.5)
LOD (GC/μl) (95% CI)	17 (8–45)	28 (14–60)	27 (8–90)	16 (6–46)	20 (6–65)	16 (7–38)

tested with the p32 cPCR, were positive when tested with the U component of the UIC qPCR assay. Both of these samples subsequently yielded positive results in the p32 cPCR assay upon retesting, and both samples were confirmed to be genuine Chitose and Buffeli positives upon DNA sequencing.

Subtype differentiation. To detect the presence of *T. orientalis* genotypes associated with clinical disease, two probes were included in the UIC assay to detect the Ikeda and Chitose genotypes. A diagnostic threshold of 30 GC/μl was similarly selected for the I and C components of the UIC assay, as this was above the limits of detection (Table 2) and maximized the sensitivity and specificity of these components. In preliminary experiments with plasmid, detection of the I and C genotypes was found to be nearly identical for equimolar mixtures of template. In the case of unequal concentrations of template, the dominant genotype was detected in all cases (data not shown). The minor (nondominant) genotype was detected in the majority of reaction mixtures in which the dominant type was up to 3 orders of magnitude higher in concentration than the minor genotype (data not shown). The abilities of the I and C assays to detect each genotype were then assessed against the genotype-specific cPCRs by using the 237 *T. orientalis*-infected blood samples from the sensitivity and specificity panels. Of the 181 samples that tested positive for the Ikeda type in cPCR, 180 were Ikeda positive in qPCR. The remaining sample yielded an amplicon in the I component of the UIC assay but fell below the diagnostic threshold and was therefore scored as negative. The UIC assay detected a further two samples that tested Ikeda negative in the cPCR. These samples were later confirmed as genuine positives by DNA sequencing. The overall sensitivity of the I component of the UIC assay was 99.4%, and the specificity was 96.4% (Table 4). A total of 144/237 samples were positive when tested with the Chitose type-specific cPCR, and of these 129 were positive with the C component of the UIC qPCR assay, giving a sensitivity of 89.6%. The samples testing Chitose negative in the qPCR but positive in the cPCR were all from samples with mixed infections containing extremely high levels of the Ikeda genotype, suggesting that competition for reagents was a factor in the lower sensitivity of the C component of the UIC assay. Despite this, the C component of the UIC assay detected a further 7 positives that tested negative in the Chitose-specific cPCR, yielding a specificity

of 92.6% (Table 4). These 7 samples were later confirmed to be genuine positives via DNA sequencing. Although the number of Chitose-positive samples detected by the UIC was lower overall, there was no statistical difference in the number of samples testing positive in cPCR ($n = 144$) versus qPCR ($n = 136$) ($P < 0.05$).

Investigation of potential PCR inhibition. Previous studies identified that neat DNA extracts and 10-fold dilutions of sample must be run in parallel when performing the *T. orientalis* cPCR assays to account for PCR-inhibiting contaminants present within nucleic acid extracts prepared from blood (6, 19, 27). To overcome this, we used a DNA polymerase master mix that has been demonstrated to be effective at reducing contaminant-based inhibition (TaqMan environmental master mix) (42). We assessed the efficacy of this master mix in reducing or eliminating the effects of PCR inhibition during development of the UIC assay. No additional positive reactors were detected when 10-fold-diluted extracts of the 237 samples from the sensitivity panel were examined in the UIC assay. Evidence of inhibition in the cPCR assays (i.e., no or significantly less PCR product observed in undiluted extracts compared to levels in the 10-fold dilutions) was observed in 10 of the 237 extracts from the specificity panel. All 10 cPCR-inhibited samples were positive in both undiluted and 10-fold-diluted extracts when tested with the UIC assay. Furthermore, a higher MPSP allele concentration was observed in the quantitative U component of the UIC assay for all undiluted extracts compared to their respective 10-fold dilutions. To further examine whether PCR inhibition had an effect on assay quantification, we prepared serially diluted plasmid DNA containing the Ikeda, Chitose, and Buffeli alleles and spiked these into nucleic acid extracts of samples from regions where *T. orientalis* was not enzootic and that were also confirmed negative by both cPCR and qPCR. Plasmids were serially diluted 10-fold to concentrations spanning 1.5×10^1 to 1.5×10^7 MPSP GC/μl. In all experiments, the qPCR-predicted concentrations were comparable to those determined by spectrophotometry (data not shown). Together, these data indicate that PCR inhibition is not significant for this assay. Nonetheless, it is recommended that a positive control, or a negative control spiked with plasmid DNA, be included in each DNA extraction run, along with a negative-control sample as a batch control measure.

TABLE 3 Contingency tables and descriptive statistics comparing detection of *T. orientalis* infection using the UIC qPCR assay with infection status determined by the 6 cPCR assays (Universal, Ikeda, and Chitose at two dilutions)

qPCR result	cPCR detection of <i>T. orientalis</i>			% relative sensitivity (95% CI) ^a	% relative specificity (95% CI) ^a
	Present	Absent	Total		
Positive	233	0	233	98.3 (95.7–99.5); NS	100 (95.6–100.0)
Negative	4	81	85		
Total	237	81	318		

^a The statistical significance (at a P level of <0.05) is also reported. NS, not significant.

TABLE 4 Comparison of UIC multiplex qPCR and type-specific cPCR for the detection of *T. orientalis* subtype

Type	No. with qPCR/cPCR results				% relative sensitivity (95% CI)	% relative specificity (95% CI)	Statistical difference ^a
	+/+	+/-	-/+	-/-			
Ikeda	180	2	1	54	99.4 (96.6–100.0)	96.4 (87.9–99.6)	NS
Chitose	129	7	15	86	89.6 (83.4–94.1)	92.6 (85.3–97.0)	NS

^a The statistical significance (at a *P* level of <0.05) is reported. NS, not significant.

Correlation with parasitemia and PCV. Of the 237 samples from the specificity panel, 135 had corresponding data for the PCV and parasitemia, and these were used for comparative analyses with the total MPSP allele concentration determined by the U component of the UIC qPCR (Fig. 2). Because blood films were scored as the percentage of erythrocytes containing piroplasms (percent parasitemia), a correction factor (described in Materials and Methods) was also applied to the qPCR data to account for variable erythrocyte levels, yielding an erythrocyte-independent measure, the number of gene copies per μl of packed erythrocytes (GC/ μl PE). Strong and significant positive correlations ($P < 0.0001$) were observed between MPSP allele concentrations (in GC/ μl and GC/ μl PE) and the percent parasitemia (Fig. 2A and B). A moderate negative correlation ($r = -0.482$, $P < 0.0001$) was observed when MPSP allele concentrations expressed in the erythrocyte concentration-dependent measure (GC/ μl) were compared with PCV values (Fig. 2C). This correlation was stronger when MPSP allele concentrations were corrected for erythrocyte concentration ($r = -0.640$, $P < 0.0001$) (Fig. 2D).

Determination of a clinical threshold. As total MPSP allele concentration strongly correlates with parasitemia and PCV, we investigated whether the UIC qPCR could be used to define a clinical threshold for *T. orientalis* infection. Samples from the sensitivity panel were divided into four groups based on the overall clinical presentation of the donor animals as well as herd status (Fig. 3A). One-way ANOVA demonstrated that animals that were clinically affected at the time of sampling ($n = 110$) had significantly higher mean MPSP allele concentrations (GC/ μl) than recovering ($n = 25$) or in-contact ($n = 24$) animals from herds with clinical cases, as well as subclinical (but *Theileria*-positive) animals from herds without clinical cases ($n = 55$) ($P < 0.001$). In addition, recovering and in-contact animals displayed significantly higher MPSP allele concentrations than the subclinical animals from the herds without clinical cases ($P < 0.001$). Recovering and in-contact animals from herds with clinical cases did not have statistically different MPSP allele concentrations ($P > 0.05$). Based on these data, we propose three groups of *T. orientalis* infection severity, as determined in the UIC qPCR. The first group contains high-level *T. orientalis* infection strongly associated with

FIG 2 MPSP allele concentrations determined by the UIC assay correlate with blood film analysis and PCV. (A and B) Correlation of the MPSP allele concentration (calculated from the U component of the UIC qPCR multiplex) expressed in measures dependent on (A) or independent of (B) erythrocyte concentration with parasitemia, as determined by blood film examination. (C and D) Correlation of MPSP allele concentration (calculated from the U component of the UIC qPCR multiplex), expressed in measures dependent on (C) or independent of (D) erythrocyte concentration with PCV. Anemia corresponds to a PCV of <24% and is represented by the vertical dotted lines.

FIG 3 (A) Association of MPSP allele concentration with clinical data allows the delineation of clinical thresholds. MPSP allele concentrations (calculated from the U component of the UIC qPCR multiplex) were separated into 4 clinical groups: subclinical animals from herds without clinical cases (i.e., herds with no clinical cases of theileriosis but situated in areas where *T. orientalis* is enzootic), animals with clinical symptoms consistent with theileriosis at time of sampling (clinical), animals that had exhibited clinical symptoms prior to sampling (recovering), and animals in contact with theileriosis-affected animals but not exhibiting clinical symptoms at time of sampling (in contact). Clinical thresholds are shown as dotted lines. (B) Association of MPSP allele concentration with clinical genotype detected. MPSP allele concentrations (calculated from the U component of the UIC qPCR multiplex) were separated into 4 groups based on genotype, detected by the I and C components. Clinical thresholds are shown as dotted lines.

clinical signs of disease and corresponding to GC/μl levels of $>3.0 \times 10^5$ (Fig. 3). The second group consists of samples from animals with a moderate level *T. orientalis* infection corresponding to a GC/μl of $>1.5 \times 10^4$ but $\leq 3.0 \times 10^5$. Samples from the third group were defined by a low level of *T. orientalis* DNA corresponding to GC/μl levels of $\leq 1.5 \times 10^4$. Using these thresholds, 100% of animals ($n = 55$) from herds without clinical cases were classified into the low-level infection group. In contrast, 91% (100/110) of animals that displayed clinical symptoms at the time of sampling were within the moderate- to high-level ranges. Above the upper threshold of 3×10^5 MPSP GC/μl (high-level infection), 95% of animals (42/44) were clinically affected. In addition, the majority of animals (23/25) that were recovering from clinical theileriosis fell within the low and moderate ranges, while all in-contact animals ($n = 24$) fell within the low and moderate ranges.

Associations of Ikeda and Chitose genotypes with parasite load. The relationships between the presence of the Ikeda and Chitose genotypes and MPSP allele concentration were examined by scoring each sample positive in each of the three channels of the UIC assay (Fig. 3B). The samples that tested positive in the

U assay only ($n = 24$) were all identified as Buffeli positives in the cPCR assay. Animals harboring the Ikeda type only ($n = 69$) or the Ikeda type in combination with the Chitose type ($n = 103$) had significantly higher MPSP allele concentrations than animals that were Chitose-only positive or samples in which no pathogenic genotype was detected ($P < 0.001$). The mean MPSP allele concentrations of the C-only and U-only positive samples were not significantly different ($P > 0.05$). Similarly, the mean allele concentrations of the I- and IC-positive samples were not statistically different ($P > 0.05$). These data suggest that the Ikeda genotype in particular is associated with higher overall parasite loads. Indeed, 100% of samples collected from clinically affected and recovering animals examined in this study were found to be I-only positive or both I and C positive. Semiquantitative data from the I and C assays indicated that *T. orientalis* Ikeda was the dominant genotype in 58% of mixed Ikeda-Chitose infections. Of animals that tested C positive only ($n = 18$), 16 were from subclinical herds, while the remaining 2 samples were derived from in-contact animals within clinically affected herds.

DISCUSSION

Bovine theileriosis caused by *T. orientalis* is a serious problem for cattle producers in Eastern Asia and is an emerging disease in Australia and New Zealand, causing significant losses to meat and milk production (6, 7). Disease outbreaks have been closely associated with the Ikeda type of *T. orientalis* and to a lesser extent the Chitose type (6, 10, 18). The ubiquity of benign *T. orientalis* genotypes, which are morphologically and serologically indistinguishable from the clinically associated types (19, 27), necessitates the use of molecular tests for clinical diagnosis of bovine theileriosis. Furthermore, due to the high prevalence of subclinical carriers of *T. orientalis* Ikeda and Chitose, a quantitative test that can provide an accurate estimate of parasite load is essential.

The multiplex qPCR assay developed and validated in this study represents the first quantitative assay for *T. orientalis* detection that incorporates a clinical genotyping component. While real-time assays that detect *T. orientalis* have been developed, most have focused on species-level detection (not discrimination of genotypes) (34), discrimination of different *Theileria* spp. (43, 44), or are only semiquantitative (28). The UIC multiplex qPCR assay developed here was both sensitive and specific for *T. orientalis* detection compared to cPCR and reliably identified the clinically relevant Ikeda and Chitose genotypes. Furthermore, the UIC assay was more specific and had near-identical sensitivity to that reported for a recently published multiplexed tandem PCR assay (28). The U component of the UIC multiplex targets highly conserved regions of the MPSP gene in order to account for the genotypic diversity within *T. orientalis* (11 types observed currently) (13, 15, 16). Therefore, the U component allows for additional flexibility in detecting any genotypes not typically associated with disease or for which the clinical relevance has not been fully established (45).

Quantitative PCR has previously been used to determine parasite load within clinical blood samples (34, 46). In this study, we observed a strong and significant correlation between MPSP allele concentration and parasitemia (as determined by blood film), demonstrating the utility of the U component of the UIC assay in quantifying parasite load. While the correlation between total MPSP allele concentration and PCV was also significant, it was not

as strong as the correlation observed between total MPSP allele concentration and parasitemia. This observation could be due to the cyclical nature of apicomplexan infections, which can recrudescence periodically (46, 47). Furthermore, a lag period between peak parasitemia and minimum PCV has been observed in prior studies of both *Babesia* and *Theileria* species (46, 47). In field-based studies of *T. orientalis* infection, we have observed that parasitemia can peak up to 8 days before cattle become anemic and up to 15 days before minimum PCV is reached (unpublished observations).

In this study, we present the first comprehensive comparison of MPSP allele concentration and genotype with clinical data from *T. orientalis*-positive animals. Indeed, the quantitative data derived from the UIC assay were found to be strongly associated with individual clinical presentation and herd status, enabling the establishment of clinical thresholds for the assay. MPSP allele concentrations of $>1.5 \times 10^4$ GC/ μ l (moderate infection) were strongly associated with clinical theileriosis at the herd level and within individuals. Furthermore, 95% of animals with MPSP allele concentrations in excess of 3×10^5 (high-level infection) displayed symptoms of clinical theileriosis. In contrast, 100% of subclinical animals from herds without clinical cases had MPSP allele concentrations below 1.5×10^4 (low-level infection). These thresholds can be applied as a guide in the differential diagnosis of theileriosis caused by *T. orientalis*, in addition to information derived from the genotyping components of the UIC assay. Based on the samples tested, there was strong evidence for the association of the Ikeda genotype, alone or in combination with the Chitose genotype, with clinical disease (100% of clinical cases examined). Where the Chitose genotype occurred as a sole infection, it was most often associated with herds having only subclinical cases. These findings are consistent with prior cPCR studies (6, 18) investigating the association of MPSP genotypes with clinical cases in New South Wales, Australia. Nonetheless, other studies conducted elsewhere have implicated the Chitose genotype as a direct cause of disease (10, 22); therefore, the role of this genotype in clinical cases, in the presence or absence of the Ikeda genotype, warrants further investigation.

Due to the persistent nature of infection, *T. orientalis* Ikeda or Ikeda/Chitose genotypes are frequently detected at low or moderate levels in convalescent animals. Similarly, *T. orientalis* can be detected at low or moderate levels in clinically normal animals that have recently become infected through contact with clinically diseased animals. The UIC assay accurately quantifies parasite load and also detects clinically associated genotypes, which allows for the detection of potential early stage clinical infections. In these instances, knowledge of herd infection status and history is critical, and monitoring of these animals and their cohorts (including follow-up qPCR testing) is warranted in the event that clinical signs develop. Previously, repeat cPCR testing of herds where *T. orientalis* had already been detected was considered unrewarding due to the long-term nature of infection (19); however, the additional information provided by qPCR may be useful for monitoring parasite loads within at-risk animals, particularly given that stress is a known factor in disease recrudescence.

In summary, the UIC qPCR assay presented here exploits hydrolysis probe (TaqMan) chemistry, enabling detection of multiple products in a single well, thereby circumventing laborious processes involved in running multiple screening and genotyping PCR assays. In addition, the assay can be adapted to

any platform allowing multiple fluorophore-based detection. As a genuine multiplex assay, the UIC qPCR can be applied to high-throughput clinical testing, enabling hundreds of UIC assays to be performed in a single day. The UIC qPCR also offers a considerable advantage over other available assays which require dilution of DNA extracts to overcome PCR inhibition (6, 9, 27, 28). Indeed, the number of assays required per sample to achieve a result is reduced from six, via cPCR detection, to just a single assay with the UIC qPCR. This represents a considerable (approximately 75%) reduction in turnaround time. The UIC assay is also cost-effective, at an estimated A\$10 to A\$15 per sample, including standards and batch controls, depending on the number of samples per run (i.e., within a range of 10 to 80 samples). Inclusion of a non-MGB probe-based internal control in the red spectrum may be feasible with additional optimization; however, the inclusion of a positive extraction control is a cost-effective alternative in batches of ≥ 4 samples.

Finally, the quantitative data from the U assay, combined with those from the IC genotyping assays, provide veterinarians with a powerful tool for clinical diagnosis of bovine theileriosis caused by *T. orientalis* that can inform herd management.

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