Semiquantitative Multiplexed Tandem PCR for Detection and Differentiation of Four *Theileria orientalis* Genotypes in Cattle

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Oriental theileriosis is an emerging, tick-borne disease of bovines in the Asia-Pacific region and is caused by one or more genotypes of the *Theileria orientalis* complex. This study aimed to establish and validate a multiplexed tandem PCR (MT-PCR) assay using three distinct markers (major piroplasm surface protein, 23-kDa piroplasm membrane protein, and the first internal transcribed spacer of nuclear DNA), for the simultaneous detection and semiquantification of four genotypes (Buffeli, Chitose, Ikeda, and type 5) of the *T. orientalis* complex. Analytical specificity, analytical sensitivity, and repeatability of the established MT-PCR assay were assessed in a series of experiments. Subsequently, the assay was evaluated using 200 genomic DNA samples collected from cattle from farms on which oriental theileriosis outbreaks had occurred, and 110 samples from a region where no outbreaks had been reported. The results showed the MT-PCR assay specifically and reproducibly detected the expected genotypes (i.e., genotypes Buffeli, Chitose, Ikeda, and type 5) of the *T. orientalis* complex, reliably differentiated them, and was able to detect as little as 1 fg of genomic DNA from each genotype. The diagnostic specificity and sensitivity of the MT-PCR were estimated at 94.0% and 98.8%, respectively. The MT-PCR assay established here is a practical and effective diagnostic tool for the four main genotypes of *T. orientalis* complex in Australia and should assist studies of the epidemiology and pathophysiology of oriental theileriosis in the Asia-Pacific region.

Tick-borne diseases (TBDs) pose a major threat to livestock production worldwide and can have a significant impact on farming communities due to economic losses (1). Theileriosis is one of the important TBDs of cattle, sheep, and/or other ruminants, mainly in tropical and subtropical regions of the world (2). In cattle, East Coast fever (ECF) and Mediterranean/tropical theileriosis are due to *Theileria parva* and *Theileria annulata*, respectively, whereas oriental theileriosis is caused by *Theileria orientalis*. The prevalence of various forms of theileriosis in different parts of the world is dependent on the occurrence of suitable tick vectors for their transmission (3).

Oriental theileriosis is caused by one or more genotypes of the *T. orientalis* complex and is transmitted by ixodid ticks, primarily *Haemaphysalis* spp. (4–6). Presently, 11 genotypes of *T. orientalis* complex (designated Chitose or type 1, Ikeda or type 2, Buffeli or type 3, types 4 to 8, and N-1 to N-3) have been identified using a number of molecular markers, including major piroplasm surface protein (MPSP) (7, 8), 23-kDa piroplasm membrane protein (p23) (9–11, 60), small-subunit (SSU) rRNA gene (8, 12, 13), and/or the first and second internal transcribed spacers of nuclear ribosomal DNA (ITS-1 and ITS-2, respectively) (12, 14). Of these genotypes, Ikeda and Chitose are recognized to be associated with clinical outbreaks of oriental theileriosis, mainly in the Asia-Pacific region (15–21). The major clinical signs of this disease include fever, anemia, jaundice, lethargy, weakness, abortion, and/or mortality (16–18), with significant production losses in dairy cattle (22). Thus far, four genotypes (Buffeli, Chitose, Ikeda, and type 5) of *T. orientalis* have been reported in Australia (13, 18, 20–23).

Currently, the diagnosis of oriental theileriosis is usually based on the observation of clinical signs, the detection of piroplasms of *T. orientalis* in blood smears (19, 24, 25), and/or the use of serological (26) or conventional molecular techniques (7, 27, 28). Each of these approaches has limitations. For example, clinical

diagnosis is subjective and usually requires further laboratory investigations to confirm the presence of infection/disease. Microscopy is commonly used and involves the detection of *T. orientalis* piroplasms in blood smears. Although microscopy might be used to quantify the level of parasitemia (28), it is relatively time-consuming and inaccurate and does not provide any genetic information on the parasite. Serological tests can detect anti-*T. orientalis* antibodies early in an infection (29), but there are issues with immunological cross-reactivity among genotypes of *T. orientalis* (G. J. Eamens, personal communication), and it is not possible to unequivocally differentiate among exposure, current infection, and past infection by *Theileria* spp. (30). Conventional PCR techniques can be more sensitive than the aforementioned methods; however, their diagnostic performance can be affected by blood constituents (e.g., hemoglobin and lactoferrin) that are inhibitory to PCR, and they do not allow the quantitation of parasites (21, 31–33). Some of these issues can be overcome using real-time PCR assays, which allow the relative or absolute quantification of the parasites present in blood (34). Such assays have been developed

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TABLE 1 Demographic and characteristics of cattle farms selected for this study from various locations in Victoria

Farm condition and no.	Location	Geographical coordinates	Farm enterprise			Cattle breed(s)	Sample collection date (day/mo/yr)	No. of individuals tested
			Beef	Dairy	Mixed			
With oriental theileriosis outbreaks								
1	Bairnsdale	37°82′ S, 147°62′ E	—	—	+	Mixed beef and dairy breeds	3/14/2012	11
2	Balmattum	36°65′ S, 145°64′ E	+	—	—	Mixed beef breeds, including Brangus	3/26/2012	8
3	Bena farm 1	38° 41′ S, 145° 76′ E	—	+	—	Friesian	3/14/2012	9
4	Bena farm 2	38 41′ S, 145°76′ E	—	+	—	Friesian	3/14/2012	18
5	Bena farm 3	38° 41′ S, 145° 76′ E	—	+	—	Friesian	3/4/2012	4
6	Benalla ^b	36°55′ S, 145°98′ E	—	—	—	Angus	7/1/2012	3
7	Bete Bolong	37°69′ S, 148°39′ E	—	+	—	Friesian	3/6/2012	21
8	Bethanga	36°12′ S, 147°09′ E	+	—	—	Angus	3/8/2012	10
9	Bunyip	38°09′ S, 145°72′ E	+	—	—	Angus × Belgian blue	3/20/2012	16
10	Corryong	36°19′ S, 147°91′ E	+	—	—	Angus	4/3/2012	10
11	East Gippsland	37° 45′ S, 148° 18′ E	+	—	—	Angus	3/8/2012	9
12	Freeburgh	36°76′ S, 147°03′ E	+	—	—	Angus	3/20/2012	3
13	Girgarre	36°40′ S, 144°98′ E	—	+	—	Illawarra Shorthorn	3/28/2012	3
14	Katandra	36°24′ S, 145°63′ E	—	+	—	Holstein	4/10/2012	12
15	Orbost	37° 71′ S, 148° 45′ E	—	—	+	Angus	3/6/2012	9
16	Pranjip	36°76′ S, 145°39′ E	+	—	—	Hereford	3/28/2012	20
17	Staghorn	36°24′ S, 146°93′ E	+	—	—	Hereford	3/5/2012	7
18	Tallangatta	36°28′ S, 147°43′ E	+	—	—	Simmental	5/3/2012	18
19	Warragul	38°16′ S, 145°93′ E	+	—	—	Angus	6/3/2012	9
With no history of oriental theileriosis ^a								
20	Curdievale	38°51′ S, 142°88′ E	—	+	—	Friesian and Holstein	11/21/2013	21
21	Jancourt East	38°41′ S, 143°13′ E	—	+	—	Friesian and Holstein	2/4/2014	27
22	Prinetown	38° 64′ S, 143° 21′ E	—	+	—	Holstein	2/4/2014	29
23	Timboon West	38°56′ S, 142°92′ E	—	+	—	Holstein and Jersey	11/21/2013	33

^a Western District of Victoria.^b Epidemiological data for this farm could not be collected.

for *Theileria sergenti* (35), *T. parva* (36, 37), and *T. equi* (38, 39) but have not yet been established for members of the *T. orientalis* complex.

A real-time PCR method that shows major promise is multiplexed tandem PCR (MT-PCR) (40). This technique can use multiple primer pairs for the detection of multiple pathogens. It consists of two amplifications: (i) multiplexed amplification (primary target enrichment), which involves a small number of PCR cycles and multiplexed or outer primer sets, and (ii) a subsequent quantification amplification which utilizes a diluted product from the primary amplification as a template and specific, nested, or inner primers (40). Although MT-PCR was originally developed to quantify gene transcription (40), MT-PCR has been applied to the sensitive and simultaneous detection of some fungi, such as *Candida* spp. (41, 42), enteric pathogens of humans (43, 44), gastrointestinal nematodes of sheep (45), and toxigenic cyanobacteria (46). As two genotypes of the *T. orientalis* complex (i.e., Chitose and Ikeda) are presently recognized to relate to clinical disease, there is a need to identify and differentiate each of them from nonpathogenic genotypes (i.e., Buffeli and type 5) of *T. orientalis* known to occur in southeast Australia (21). MT-PCR could offer a useful means of achieving such differential diagnosis as well as estimating the infection intensities of individual *T. orientalis* genotypes in bovines.

The aim of the present study was to establish and evaluate an MT-PCR assay for the simultaneous detection and differentiation of the four distinct genotypes, Buffeli, Chitose, Ikeda, and type 5, representing the *T. orientalis* complex known to occur in Australasia as well as for the semiquantitation of DNA of each of these genotypes in blood samples from cattle.

MATERIALS AND METHODS

Blood and genomic DNA samples. Blood samples were available from 200 cattle (group 1; symptomatic or asymptomatic animals) from a previous study from 19 farms on which clinical outbreaks of oriental theileriosis were recorded (Table 1) (21). These blood samples had already been characterized using a conventional PCR-based approach (i.e., 170 test-positive samples, 20 samples showing PCR inhibition [using 2 µl of template], and 10 previously test-negative samples). In addition, blood samples were collected from 110 cattle (group 2) from the coccygeal vein (using an 18-gauge needle) into EDTA tubes by registered, practicing veterinarians (see Acknowledgments) from the Western District in Victoria, a region in which no outbreaks of oriental theileriosis and/or *T. orientalis* infections have been reported to date (Table 1). Genomic DNAs were extracted from individual blood samples (200 µl) using a DNeasy blood and tissue kit (catalog no. 69506; Qiagen, USA), according to the manufacturer's protocol, and eluted in 100 µl. In addition, genomic DNAs of other common blood parasites of cattle, including *T. parva*, *T.*

annulata, *Babesia bovis*, and *Anaplasma centrale*, were available from colleagues (see Acknowledgments).

MT-PCR. The Easy-Plex platform (AusDiagnostics Pty., Ltd., Australia) was used, which includes a Rotor-Gene 6000 real-time PCR thermocycler (Qiagen, Germany) and a Gene-Plex CAS1212 liquid handling robot (AusDiagnostics). The primary amplification (target enrichment) was conducted using primer pairs designed to the sequences of the ITS-1 and the p23 gene of *T. orientalis* for genotypes Ikeda and Buffeli, respectively, and to the MPSP gene for both Chitose and type 5. Current information on the *T. orientalis* genome (Japanese Ikeda strain) indicates that it has two copies of ITS-1 and one copy each of the p23 and MPSP genes (47). The secondary amplification for semiquantification used nested primer pairs to internal regions of these loci (catalog no. 4023; AusDiagnostics); these internal primer pairs amplify a region of 107 bp from ITS-1 (genotype Ikeda), a region of 115 bp from the p23 gene, and regions of 70 to 112 bp from the MPSP gene (Chitose and type 5). In addition, an independent primer pair is included in each reaction mixture as a reference for quantitation and to assess the efficiency of amplification from 10,000 copies of a synthetic oligonucleotide template (internal spike control).

The final protocol was as follows: for primary amplification (15 cycles of 10 s at 95°C, 20 s at 60°C, and 20 s at 72°C), 5 µl of genomic DNA representing each test sample or 5 µl of water (negative control) was dispensed into 0.2-ml PCR strips and placed into a 24-well thermocycling block within the Gene-Plex robotic platform. Following the dispensing of each sample and the initiation of the assay, the following setup process and analysis were executed by the program Easy-Plex Assay Setup (AusDiagnostics), with the secondary amplification in MT-PCR and the melting curve analysis being semiautomated (44, 48). A sample was recorded as test positive using the auto-call function of the Easy-Plex software (AusDiagnostics) if the amplicon produced a single melting curve which was within 1.5°C of the expected melting temperature, the height of the peak was higher than 0.2 dF/dT (where dF/dT is the derivative of fluorescence over temperature), and the peak width was $\leq 3.5^\circ\text{C}$. Cycle threshold (C_T) values were recorded for each test-positive sample, and the DNA copy number for each genotype in each sample was determined by comparison with C_T data determined for an internal spike control (40) for each sample tested. In instances ($n = 9$) where the internal spike control did not reach the expected DNA copy number of 10,000, the genomic DNA sample was diluted to 1:10 or 1:100 and retested, and the DNA copy number was calculated for the undiluted sample. Using this protocol, a minimum of 2.5 DNA copies (1 fg) could be detected. Finally, genotypes Buffeli, Chitose, Ikeda, and type 5 were assigned according to their mean ($\pm$ standard deviation) expected peak melting temperatures of $83.6 \pm 1.5^\circ\text{C}$, $82.1 \pm 1.5^\circ\text{C}$, $87.4 \pm 1.5^\circ\text{C}$, and $81.6 \pm 1.5^\circ\text{C}$, respectively. The DNA copy number determined can be used as a measurement of the intensity of infection for each genotype. The relative intensities of infection by genotypes Buffeli, Chitose, and type 5 were estimated as the DNA copy number recorded for individual genotypes, while relative intensity of infection by genotype Ikeda was estimated by dividing the DNA copy number recorded by 2. In order to verify the specificity of MT-PCR as well as to assess nucleotide variation among amplicons in relation to peak melting temperature, selected samples ($n = 100$) were subjected to single-strand conformation polymorphism analysis (SSCP) and sequenced ($n = 10$) using an established cloning-based protocol (49).

Statistical analyses. To assess repeatability of the MT-PCR assay, the coefficient of variation (CV) was estimated using the program Microsoft Excel (2010). Owing to a positive skew, copy number data were log transformed and are presented as medians and geometric means (the back-transformed mean of the log-transformed copy number estimates) according to the following equation:

$$\text{geometric mean} = \exp\left[\frac{1}{n} \sum \ln(x_j)\right]$$

where n is the sample size, j is 1, . . . , n , and x_j is the j th data value.

Pairwise comparisons of the relative intensity of each genotype in

mixed genotypic infections were conducted (using Ikeda as the reference). For samples in which two genotypes were present (e.g., Buffeli and Ikeda), pairwise comparisons were conducted with paired-sample t tests of the geometric mean copy numbers, whereas for infections of more than two genotypes (e.g., Buffeli, Chitose, and Ikeda), linear mixed models were used to estimate the difference in geometric means using the program Stata (release 13; StataCorp LP, College Station, TX) by incorporating a random effect term to account for nonindependent observations (i.e., multiple genotypes in each individual). Models were of the following form: $\log_{10}(\text{gene copy number}) = \beta_0 + \beta_1 \cdot \text{Chitose} + \beta_2 \cdot \text{Buffeli} + \beta_3 \cdot \text{type 5} + \text{Individual}_j + \epsilon$, where β_0 is an intercept which can be interpreted as the expected geometric mean copy number for the reference category (Ikeda), and β_1 , β_2 , and β_3 are regression coefficients for categorical variables and may be interpreted as the difference in geometric mean copy number between genotype Ikeda and the genotypes Buffeli, Chitose, and type 5, respectively. We assumed that Individual_j , which is the random effect term for the j th of N individual cows (where N is the total sample size of the study), was normally distributed, along with the residual error (ϵ), with a standard deviation of $S_{\text{individual}}$ according to the following: $\text{Individual}_j \sim \text{Normal}(0, S_{\text{individual}})$, for $j = 1, \dots, N$.

The diagnostic specificity and sensitivity of the MT-PCR were estimated following the recommended Bayesian latent class modeling approach (50, 51) for two conditionally dependent tests on two populations (groups 1 and 2) in the absence of a gold standard (i.e., reference samples of known disease status). Conventional PCR cannot be considered a gold standard because it has been shown that the analytical sensitivity of the MT-PCR assay was 1,000 times higher than that of conventional PCR. Conventional PCR is a suitable diagnostic technique to detect *T. orientalis*. However, in MT-PCR, depending on the selected cutoff DNA copy number, a higher diagnostic sensitivity or higher diagnostic specificity than that of conventional PCR can be achieved. The Bayesian latent class modeling approach makes no assumptions about the status of animals from the two populations (groups 1 and 2). Prevalence was assumed to be distinct in each population, and diagnostic specificity and sensitivity were assumed to be constant across the two populations. The tests were assumed to be dependent (conditional on infection status) because they had the same biological basis, that is, the detection of nucleic acids of genotypes of *T. orientalis*. Prior information about the diagnostic specificity and sensitivity of the MT-PCR assay was modeled using independent and informative beta distributions elicited from a technical expert (R. B. Gasser) with knowledge of the populations and test performance yet not involved in the sample collection or testing (52). The most likely (modal) value and the $(\alpha - 100)$ th percentile of the corresponding beta distribution were elicited by asking the expert to specify that he was $(100 - \alpha)\%$ sure that the diagnostic sensitivity of the MT-PCR was $> X$, and the most likely value for this parameter was Y (51). Prior information was similarly elicited for the prevalence in each population, while diagnostic specificity and sensitivity of the conventional PCR assay were specified as diffuse priors based on elicited modal values only, following Branscum et al. (51). Dependence parameters were specified as uninformed independent uniform distributions, and Bayesian inferences were based on the joint posterior distribution, numerically approximated using the program WinBUGS (53), running 110,000 model iterations, discarding the first 10,000 iterations as burn-in, and thinning by 10 to minimize auto-correlation. Agreement statistics (prevalence-adjusted bias-adjusted kappa [PABAK]) (54) were directly calculated as model outputs. Final inferences were presented as the 50%, 2.5%, and 97.5% quantiles of the marginal posterior distributions for each of the parameters, corresponding to a posterior median point estimate and 95% probability interval (PI), respectively.

Analyses were repeated by applying different DNA copy number cutoff values for dichotomizing the MT-PCR results as test positive, which enabled estimations of the two-way receiver-operator-characteristic (ROC) curve and optimal cutoff. Sensitivity analyses were performed as recommended previously (50, 52) to test for the influence of elicited pri-

FIG 1 Detection of various genotypes of the *Theileria orientalis* complex using the MT-PCR assay. Cycling (A) and melting (B) curves for the genotypes Buffeli, Chitose, Ikeda, and type 5 of *T. orientalis* are shown. (C) Cycling curves of a blood DNA sample showing partial inhibition/delayed amplification of the spike control. (D and E) Cycling curves using undiluted template and showing no inhibition when the template was diluted at 1:10 (D) and 1:100 (E).

ors on the final results, inputting vague (flat) priors, and comparing all model outputs.

RESULTS

Establishment of the MT-PCR assay. In setting up the MT-PCR assay, a series of experiments was conducted to establish the optimum cycling protocol, the specificity and sensitivity of the MT-PCR, and the repeatability of results. The analytical specificities of individual primer sets (genotypes Buffeli, Chitose, Ikeda, and type 5 of the *T. orientalis* complex) were assessed using well-defined genomic DNA samples representing each of the four genotypes (positive controls; $n = 4$) (from Perera et al. [21]) as well as from *T. annulata*, *T. parva*, *A. centrale*, and *B. bovis* (negative controls; $n = 4$). Each of the four primer sets designed and tested amplified products exclusively from the expected genotypes (Fig. 1A and B). The identity of individual products was confirmed by SSCP analysis and sequencing, and no products were amplified from *T. annulata*, *T. parva*, *A. centrale*, or *B. bovis* DNA. Using the same, well-defined samples, repeatability of the copy number was greater within a run (CV of 12%) than among runs (CV of 26%), and genotypes were always correctly assigned (CV of 0%) for samples with ≥ 30 DNA copies.

Validation of the MT-PCR assay. Two hundred DNA samples representing cattle from 19 farms on which oriental theileriosis outbreaks had occurred (group 1) and 110 samples representing cattle farms where no outbreaks had occurred in Victoria (group 2) were tested in MT-PCR. Of the 200 samples from cattle in group 1, all genomic DNA samples that tested positive in a previous conventional PCR study (21) also tested positive (>0 DNA copies) by MT-PCR ($n = 170$). In addition, 17 of the samples that showed PCR inhibition (using 2 μ l of template) in conventional

PCR ($n = 20$) (21) did not inhibit MT-PCR. Of 10 samples that were previously negative by conventional PCR (21), eight samples tested positive by MT-PCR, with all eight positive samples containing 4 to 15 DNA copies. In addition, of 110 samples from group 2, 2 tested positive for *T. orientalis* by both MT-PCR and conventional PCR, and a further 6 samples tested positive by MT-PCR only. Of all 200 samples from group 1, 9 samples showed inhibition using 5 μ l of template but did not when the original template was diluted to 1:10 or 1:100 and retested (Fig. 1C to E). SSCP analysis of 100 amplicons representing all four genotypes (Buffeli, $n = 30$; Chitose, $n = 25$; Ikeda, $n = 30$; type 5, $n = 15$) of *T. orientalis* revealed four main profiles (see Fig. S1 in the supplemental material); minor SSCP profile variation was repeatedly observed within genotypes Buffeli and Chitose, which was reflected in differences in the peak melting temperatures (0.9 to 1.0°C). DNA sequencing of amplicons revealed that nucleotide variation of 1.4 to 1.7% was associated with these differences (data not shown).

Of 200 blood samples collected from group 1, 198 tested positive in MT-PCR (applying a cutoff >0 DNA copy number detected). In this group, the prevalences of individual genotypes (i.e., Buffeli, Chitose, Ikeda, and type 5) of the *T. orientalis* complex in cattle included in these outbreaks were 92.9% (184/198), 57.1% (113/198), 95.5% (189/198), and 32.3% (64/198), respectively. The prevalence of *T. orientalis* infections with single or mixed genotypes detected is shown in Fig. 2. The number of infections with mixed genotypes was higher (93.43%; 185/198) than those with single genotypes (6.57%; 13/198). There was a high prevalence (38.9%) of mixed infections with genotypes Buffeli and Ikeda, followed by infection with all four genotypes (31.3%)

FIG 2 Prevalence of genotypes of the *Theileria orientalis* complex detected by the MT-PCR assay. Letters C, B, I, and T denote single infections by genotypes Chitose, Buffeli, Ikeda, and type 5, respectively. Various combinations of letters with the plus sign denote mixed infections with two or more genotypes.

and with genotypes Buffeli, Chitose, and Ikeda (21.2%) (Fig. 2). Most of the oriental theileriosis outbreaks (31.6%; 6/19) had a high prevalence of genotype Ikeda, followed by infection with genotype Buffeli (Table 2). Ten of 19 farms had a prevalence of 100% for genotype Ikeda. Type 5 showed the lowest prevalence among the four genotypes (Table 2). Compared with other regions, a

comparatively high average relative intensity of infection by genotype Ikeda was recorded in Bairnsdale, Balmattum, Benalla, Bena farm 1, Bethanga, Bunyip, Corryong, Katandra, and Tallangatta, where deaths and/or abortions were reported (Table 2).

Although all four genotypes were detected in cattle experiencing clinical oriental theileriosis, the relative intensity of infection

TABLE 2 Numbers of cows that died or aborted and average relative intensity of infection by genotypes of *Theileria orientalis* in each outbreak at each location (farm)

Farm no.	Location (n) ^a	No. of cows that died due to theileriosis	No. of cows that aborted due to theileriosis	Prevalence of genotype (%)				Avg intensity of infection by genotype (DNA copies)			
				Ikeda	Chitose	Buffeli	Type 5	Ikeda	Chitose	Buffeli	Type 5
1	Bairnsdale (11)	1	0	100	36.4	100	9.1	165,636	14,797	110,165	9
2	Balmattum (8)	1	2	87.5	37.5	100	37.5	120,177	107,017	102,661	4,355
3	Bena farm 1 (9)	16	6	77.8	0	88.9	11.1	65,023	0	21,800	1,000
4	Bena farm 2 (18)	1	0	94.4	88.9	94.4	55.6	238,319	270,6797	106,535	16
5	Bena farm 3 (4)	0	1	100	0	75	0	38,514	0	15,145	0
6	Benalla (3) ^b			33.3	100	33.3	0	20,689	9	10,750	0
7	Bete Bolong (21)	7	0	100	90.5	100	42.9	59,959	22,843	137,234	33,42
8	Bethanga (10)	4	4	100	10	100	0	195,658	15	72,115	0
9	Bunyip (16)	5	0	100	6.3	100	12.5	150,092	6,033	106,745	1,602
10	Corryong (10)	4	0	100	20	90	10	50,331	6,305	16,673	9
11	East Gippsland (9)	22	12	88.9	88.9	88.9	55.6	166,249	221,099	505,150	2,163
12	Freeburgh (3)	0	1	66.7	33.3	0	0	8	16	0	0
13	Girgarre (3)	0	3	66.7	66.7	66.7	0	84,918	108,368	302,627	0
14	Katandra (12)	6	0	100	58.3	83.3	16.7	110,907	97,482	47,436	20
15	Orbost (9)	1	0	100	100	100	100	24,857	156,553	338,766	44,072
16	Pranjip (20)	0	2	100	100	100	95	111,315	186,715	115,938	8,403
17	Staghorn (7)	2	1	100	14.3	85.7	14.3	3,268	108,368	40,302	16,847
18	Tallangatta (18)	1	1	94.4	50	94.4	5.6	191,611	147,184	120,811	201
19	Warragul (9)	0	0	88.9	77.8	88.9	0	24,587	770	27,561	0

^a n, number of cows.

^b Epidemiological data for this farm could not be collected.

TABLE 3 Relative intensity of infection (DNA copies) by genotypes of *Theileria orientalis* in regions where outbreaks occurred

Type of mixed infection (n) ^a	Genotype	Category	Median DNA copy no. (min, max) ^b	Geometric mean DNA copy no.	Difference of geometric mean DNA copy no. (95% CI) ^c	P value ^d
Ikeda + Chitose (3)	Ikeda	0	8 (8, 2,500)			
	Chitose	1	19 (8, 9,000)			
Ikeda + Buffeli (77)	Ikeda	0	22,061 (3, 706,132)	11,599	−6,744 (−8,063, −4,933)	<0.001
	Buffeli	1	10,000 (4, 792,570)	4,855		
Ikeda + Chitose + Buffeli (42)	Ikeda	0	28,577 (6, 621,062)	16,604	0	
	Chitose	1	207 (10, 266,168)	460	−16,144 (−16,450, −15,233)	<0.001
	Buffeli	2	24,812 (11, 1,324,465)	21,510	4,906 (−9,389, 47,519)	0.642
Ikeda + Buffeli + type 5 (1)	Ikeda	0	8,424			
	Buffeli	1	21,778			
	Type 5	2	3,175			
Ikeda + Chitose + Buffeli + type 5 (62)	Ikeda	0	40,958 (6, 706,132)	38,004	0	
	Chitose	1	76,866 (8, 1,412,263)	38,815	811 (−19,553, 43,651)	0.97
	Buffeli	2	89,387 (9, 1,412,263)	67,702	29,698 (−5,821, 104,419)	0.13
	Type 5	3	35 (5, 249,621)	217	−37,787 (−37,900, −37,548)	<0.001

^a n, number of animals.^b min, minimum; max, maximum.^c CI, confidence interval.^d P values were obtained by comparing each category with the reference group (i.e., category 0). For three mixed infections, data were analyzed by pair-wise comparisons using genotype Ikeda as the reference category. Statistical analysis for two mixed infections was not performed as the sample size was low. Results were estimated using linear mixed models, adjusting for variability among samples. Significant values are in boldface.

by each of these genotypes showed that genotypes Ikeda and Buffeli dominated the other two genotypes (Chitose and type 5) (Table 3). For the most prevalent, mixed infections (i.e., with genotypes Buffeli and Ikeda), the genotype Ikeda showed a significantly higher relative intensity of infection than the genotype Buffeli ($P < 0.001$) (Table 3; Fig. 3). Genotype Ikeda was significantly more dominant ($P < 0.001$) than genotype Chitose in mixed infections with genotypes Buffeli, Chitose, and Ikeda. Of 110 DNA samples from group 2, eight samples tested positive in MT-PCR; four had single infections with genotype Buffeli (copy number range, 11 to 26), and four had mixed infections with genotypes Buffeli (copy number range, 5 to 30,019) and Ikeda (copy number range, 3 to 90,547).

The diagnostic specificity of the MT-PCR (94.0%; 95% PI, 90.1 to 96.8%) was lower than that of the conventional PCR (96.8%; 95% PI, 93.0 to 98.8%); the diagnostic sensitivity of the MT-PCR was 98.8% (95% PI, 96.7 to 99.7%) if test positivity was defined based on a cutoff of >0 DNA copies, compared with 95.1% (95% PI, 91.6 to 97.5%) for the conventional PCR. When the MT-PCR

was interpreted using the test-positive cutoff of >20 DNA copies (Fig. 4), diagnostic performance was equivalent to that of the conventional PCR (see Fig. S2 and Table S1 in the supplemental material). There was excellent agreement between the two diagnostic tests in both groups of samples (posterior median PABAK of >0.864 in all iterations), and the prevalence estimates in the two populations were relatively stable (group 1, $>95.1\%$; group 2, 1.3%) even as the MT-PCR cutoff was altered. Changes in inference were negligible when the Bayesian latent class model was populated with flat (uninformative) priors.

DISCUSSION

The present study established and validated an MT-PCR assay for the detection, differentiation, and semiquantitation of four genotypes (i.e., Buffeli, Chitose, Ikeda, and type 5) of the *T. orientalis* complex in Australia in blood samples from cattle. Bayesian latent class analysis estimated that 95% of 200 cattle specifically selected from infected farms and only 1.3% of 110 cattle from farms from an area in Victoria (Western District) where theileriosis cases have

FIG 3 Box plot diagrams showing the number of DNA copies of genotypes in mixed infections as indicated on each panel. The DNA copy number recorded for genotype Ikeda was divided by 2 to determine the DNA copy numbers shown in the figure.

FIG 4 Diagnostic sensitivity and specificity of the MT-PCR at different cutoff points.

not been reported tested positive for one or more of the four genotypes. Moreover, the levels of parasite DNA in blood were substantially higher (30 times) in most cattle in the region of endemicity (group 1) than in the eight cattle from the Western District of Victoria (group 2) that tested positive in the MT-PCR (see Table S2 in the supplemental material). It is possible that these test-positive cattle were recently introduced into this district as cattle transport from regions of endemicity to regions where the parasite is not endemic within Victoria as well as from New South Wales is common (Department of Primary Industries, New South Wales Government [www.dpi.nsw.gov.au]). Conventional PCR (21) detected DNA of *T. orientalis* in only two of the eight cattle with the highest intensity of infection inferred from the MT-PCR.

Electrophoretic mutation scanning analysis and targeted sequencing demonstrated specificity for all four sets of primers, all of the amplicons produced, and the conditions of MT-PCR. In addition, the DNA samples from four heterologous blood pathogens (*T. annulata*, *T. parva*, *A. centrale*, and *B. bovis*) tested were, as expected, all negative. Nonetheless, future studies should re-evaluate the specificity of the MT-PCR assay in regions where other blood-borne bovine pathogens are endemic (including viruses and bacteria). Although intended for genotypic detection/differentiation and semiquantitation, the present MT-PCR assay might also be useful as a mutation scanning tool to detect genetic variability within individual genotypes of *T. orientalis* because SSCP-coupled sequencing was able to show that subtle variation ($\sim 0.9^\circ\text{C}$) in peak melting temperature linked to sequence difference of 1.4 to 1.7% (one or two nucleotide alterations) in loci for the Buffeli and Chitose types was readily detectable.

The minimum amount of DNA detectable (i.e., 1 fg or 2.5 DNA copies) by MT-PCR was comparable to that of previous studies using the same platform (44, 46), and the test was approximately 1,000 times more sensitive than conventional PCR (21).

Given the ability of the MT-PCR to detect ≥ 1 fg of *T. orientalis* DNA, the present study has shown that most infections are multigenotypic, in contrast to previous results achieved by conventional (one-step) PCRs. The performance of MT-PCR is comparable to or better than that reported for some real-time TaqMan PCRs established for *T. equi* and *T. sergenti* (35–39). In our MT-PCR, the DNA copy number estimate for each genotype and sample relative to the internal spike control (i.e., 10,000 DNA copies of a synthetic oligonucleotide template amplified by specific primers) is likely to be more accurate and repeatable than for other assays used previously. The DNA copy number determined can be used as a measurement of the intensity of infection for each genotype. Given that high DNA copy numbers of pathogenic genotypes (Chitose and Ikeda) of *T. orientalis* in cattle might relate to disease (our unpublished data), the ability to estimate intensity could be useful to predict the risk of an outbreak, but this proposal warrants testing.

Coinfections with multiple *T. orientalis* genotypes were commonly detected by MT-PCR, consistent with previous studies in Australia (13, 20, 21, 23). However, here, Ikeda was the most common genotype, followed by the Buffeli, Chitose, and type 5 genotypes, in contrast to previous evidence showing that Chitose was the second most prevalent genotype (21–23). This difference in prevalence is likely due to the ability of the present MT-PCR to detect tiny amounts (≥ 1 fg) of parasite DNA compared with conventional PCRs (21–23). The sensitivity of the MT-PCR assay also explains why the prevalence of the Buffeli type was higher than recorded in previous studies (20, 21) and also provides additional support for the proposal that the Buffeli genotype is endemic in Australia (55, 56); however, a large-scale nation-wide survey would be needed to establish the geographical distribution of different genotypes of the *T. orientalis* complex. Currently, the MT-PCR assay has been designed for the four genotypes of *T. orientalis* known to occur in Australia (20, 21, 23). The assay could be readily modified to include loci or gene regions for genotypes not included in the present assay, provided that the markers to be used have been prevalidated for specificity to detect additional genotypes prior to their inclusion in the assay.

In conclusion, the semiautomated MT-PCR assay established here is a cost-effective, time-efficient, and practical diagnostic tool. It provides a major advance because it allows a qualitative and quantitative evaluation of four distinct genotypes of *T. orientalis* at once. Currently, the estimated cost per sample is Aus\$19, which is approximately half that of our conventional PCR-based testing (21, 23), and the time required from sample preparation to test result is about one-fifth (about 1 day) of that using the conventional approach. In our opinion, the MT-PCR assay has broad applicability and can now be utilized to support investigations into the epidemiology, pathophysiology, and transmission of oriental theileriosis. For example, the assay could be readily used to explore the temporal changes in genotypes that occur within individual cattle (proposed by Perera et al. [22]), population dynamics suggested to occur during transmission from cattle to ticks and vice versa (57), and/or to test the hypothesis that definitive and intermediate hosts other than cattle and *Haemaphysalis*, respectively, are involved in disease spread (58, 59). For instance, it would be interesting to explore whether water buffaloes or deer might act as reservoir hosts. Importantly, the present MT-PCR assay will be useful for the surveillance and monitoring of oriental theileriosis in Australasia and should be readily applicable in other

countries in the Asia-Pacific region where this disease significantly impacts livestock health, welfare, and production.

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REFERENCES

- Minjauw B, McLeod A. 2003. Tick-borne diseases and poverty: the impact of ticks and tick-borne diseases on the livelihoods of small-scale and marginal livestock owners in India and eastern and southern Africa. Department for International Development, Animal Health Programme, Centre of Tropical Veterinary Medicine, University of Edinburgh, Midlothian, United Kingdom. http://r4d.dfid.gov.uk/PDF/Outputs/RLAHTickBorn_Book.pdf.
- Uilenberg G. 1995. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Vet Parasitol* 57:19–41. [http://dx.doi.org/10.1016/0304-4017\(94\)03107-8](http://dx.doi.org/10.1016/0304-4017(94)03107-8).
- Dobbelaere DAE, McKeever DJ. 2002. *Theileria*. Kluwer Academic Publishers, Boston, MA.
- Uilenberg G, Mpangala C, McGregor W, Callow LL. 1977. Biological differences between African *Theileria mutans* (Theiler 1906) and two benign species of *Theileria* of cattle in Australia and Britain. *Aust Vet J* 53: 271–273. <http://dx.doi.org/10.1111/j.1751-0813.1977.tb00214.x>.
- Uilenberg G. 1981. Theilerial species of domestic livestock, p 4–37. In Irvin AD, Cunningham MP, Young AS (ed), *Advances in the control of theileriosis*. Springer, Dordrecht, The Netherlands.
- Jongejan F, Musisi FL, Moorhouse PD, Snacken M, Uilenberg G. 1986. *Theileria taurotragi* in Zambia. *Vet Q* 8:261–263. <http://dx.doi.org/10.1080/01652176.1986.9694051>.
- Kakuda T, Shiki M, Kubota S, Sugimoto C, Brown WC, Kosum C, Nopporn S, Onuma M. 1998. Phylogeny of benign *Theileria* species from cattle in Thailand, China and the U.S.A. based on the major piroplasm surface protein and small subunit ribosomal RNA genes. *Int J Parasitol* 28:1261–1267. [http://dx.doi.org/10.1016/S0020-7519\(98\)00113-1](http://dx.doi.org/10.1016/S0020-7519(98)00113-1).
- Gubbels MJ, Hong Y, van der Weide M, Qi B, Nijman IJ, Guangyuan L, Jongejan F. 2000. Molecular characterisation of the *Theileria buffeli/orientalis* group. *Int J Parasitol* 30:943–952. [http://dx.doi.org/10.1016/S0020-7519\(00\)00074-6](http://dx.doi.org/10.1016/S0020-7519(00)00074-6).
- Sako Y, Asada M, Kubota S, Sugimoto C, Onuma M. 1999. Molecular cloning and characterisation of 23-kDa piroplasm surface proteins of *Theileria sergenti* and *Theileria buffeli*. *Int J Parasitol* 29:593–599. [http://dx.doi.org/10.1016/S0020-7519\(99\)00004-1](http://dx.doi.org/10.1016/S0020-7519(99)00004-1).
- Yokoyama N, Ueno A, Mizuno D, Kuboki N, Khukhuu A, Igarashi I, Miyahara T, Shiraishi T, Kudo R, Oshiro M, Zakimi S, Sugimoto C, Matsumoto K, Inokuma H. 2011. Genotype diversity of *Theileria orientalis* detected from cattle grazing in Kumamoto and Okinawa prefectures of Japan. *J Vet Med Sci* 73:305–312. <http://dx.doi.org/10.1292/jvms.10-0263>.
- Ota N, Mizuno D, Kuboki N, Igarashi I, Nakamura Y, Yamashina H, Hanzaike T, Fujii K, Onoe S, Hata H, Kondo S, Matsui S, Koga M, Matsumoto K, Inokuma H, Yokoyama N. 2009. Epidemiological survey of *Theileria orientalis* infection in grazing cattle in the eastern part of Hokkaido, Japan. *J Vet Med Sci* 71:937–944. <http://dx.doi.org/10.1292/jvms.71.937>.
- Aktas M, Altay K, Dumanli N. 2006. A molecular survey of bovine *Theileria* parasites among apparently healthy cattle and with a note on the distribution of ticks in eastern Turkey. *Vet Parasitol* 138:179–185. <http://dx.doi.org/10.1016/j.vetpar.2006.01.052>.
- Kamau J, de Vos AJ, Playford M, Salim B, Kinyanjui P, Sugimoto C. 2011. Emergence of new types of *Theileria orientalis* in Australian cattle and possible cause of theileriosis outbreaks. *Parasit Vectors* 4:22. <http://dx.doi.org/10.1186/1756-3305-4-22>.
- Kamau J, Salim B, Yokoyama N, Kinyanjui P, Sugimoto C. 2011. Rapid discrimination and quantification of *Theileria orientalis* types using ribosomal DNA internal transcribed spacers. *Infect Genet Evol* 11:407–414. <http://dx.doi.org/10.1016/j.meegid.2010.11.016>.
- Sugimoto C, Fujisaki K. 2002. Non-transforming *Theileria* parasites of ruminants, p 93–106. In Dobbelaere DAE, McKeever DJ (ed), *Theileria*. Kluwer Academic Publishers, Boston, MA.
- Izzo MM, Poe I, Horadagoda N, De Vos AJ, House JK. 2010. Haemolytic anaemia in cattle in NSW associated with *Theileria* infections. *Aust Vet J* 88:45–51. <http://dx.doi.org/10.1111/j.1751-0813.2009.00540.x>.
- Aparna M, Ravindran R, Vimalkumar MB, Lakshmanan B, Rameshkumar P, Kumar KG, Promod K, Ajithkumar S, Ravishankar C, Devada K, Subramanian H, George AJ, Ghosh S. 2011. Molecular characterization of *Theileria orientalis* causing fatal infection in crossbred adult bovines of South India. *Parasitol Int* 60:524–529. <http://dx.doi.org/10.1016/j.parint.2011.08.002>.
- Islam MK, Jabbar A, Campbell BE, Cantacessi C, Gasser RB. 2011. Bovine theileriosis—an emerging problem in south-eastern Australia? *Infect Genet. Evol* 11:2095–2097. <http://dx.doi.org/10.1016/j.meegid.2011.08.012>.
- McFadden AM, Rawdon TG, Meyer J, Makin J, Morley CM, Clough RR, Tham K, Mullner P, Geysen D. 2011. An outbreak of haemolytic anaemia associated with infection of *Theileria orientalis* in naive cattle. *N Z Vet J* 59:79–85. <http://dx.doi.org/10.1080/00480169.2011.552857>.
- Eamens GJ, Bailey G, Jenkins C, Gonsalves JR. 2013. Significance of *Theileria orientalis* types in individual affected beef herds in New South Wales based on clinical, smear and PCR findings. *Vet Parasitol* 196:96–105. <http://dx.doi.org/10.1016/j.vetpar.2012.12.059>.
- Perera PK, Gasser RB, Anderson GA, Jeffers M, Bell CM, Jabbar A. 2013. Epidemiological survey following oriental theileriosis outbreaks in Victoria, Australia, on selected cattle farms. *Vet Parasitol* 197:509–521. <http://dx.doi.org/10.1016/j.vetpar.2013.06.023>.
- Perera PK, Gasser RB, Firestone SM, Anderson GA, Malmo J, Davis G, Beggs DS, Jabbar A. 2014. Oriental theileriosis in dairy cows causes a significant milk production loss. *Parasit Vectors* 7:73. <http://dx.doi.org/10.1186/1756-3305-7-73>.
- Cufos N, Jabbar A, de Carvalho LM, Gasser RB. 2012. Mutation scanning-based analysis of *Theileria orientalis* populations in cattle following an outbreak. *Electrophoresis* 33:2036–2040. <http://dx.doi.org/10.1002/elps.201200082>.
- Becerra VM, Eggen AAS, Rooy RC, Uilenberg G. 1983. *Theileria orientalis* in cattle in Ethiopia. *Res Vet Sci* 34:362–364.
- Altay K, Aydin MF, Dumanli N, Aktas M. 2008. Molecular detection of *Theileria* and *Babesia* infections in cattle. *Vet Parasitol* 158:295–301. <http://dx.doi.org/10.1016/j.vetpar.2008.09.025>.
- Jeong W, Kweon CH, Kim JM, Jang H, Paik SG. 2005. Serological investigation of *Theileria sergenti* using latex agglutination test in South Korea. *J Parasitol* 91:164–169. <http://dx.doi.org/10.1645/GE-3294>.
- Kawazu S, Sugimoto C, Kamio T, Fujisaki K. 1992. Antigenic differences between Japanese *Theileria sergenti* and other benign *Theileria* species of cattle from Australia (*T. buffeli*) and Britain (*T. orientalis*). *Parasitol Res* 78:130–135. <http://dx.doi.org/10.1007/BF00931654>.
- Tanaka M, Onoe S, Matsuba T, Katayama S, Yamanaka M, Yonemichi H, Hiramatsu K, Baek BK, Sugimoto C, Onuma M. 1993. Detection of *Theileria sergenti* infection in cattle by polymerase chain reaction amplification of parasite-specific DNA. *J Clin Microbiol* 31:2565–2569.
- Kajiwaru N, Kirisawa R, Onuma M, Kawakami Y. 1990. Specific DNA probe for the detection of *Theileria sergenti* infection in cattle. *Jpn J Vet Sci* 52:1199–1204. <http://dx.doi.org/10.1292/jvms.1993.52.1199>.
- Bishop R, Sohanpal B, Kariuki DP, Young AS, Nene V, Baylis H, Allsopp BA, Spooner PR, Dolan TT, Morzaria SP. 1992. Detection of a carrier state in *Theileria parva*-infected cattle by the polymerase chain reaction. *Parasitology* 104:215–232. <http://dx.doi.org/10.1017/S0031182000061655>.

31. Panaccio M, Lew A. 1991. PCR based diagnosis in the presence of 8% (v/v) blood. *Nucleic Acids Res* 19:1151. <http://dx.doi.org/10.1093/nar/19.5.1151>.
32. Wilson IG. 1997. Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol* 63:3741–3751.
33. Hoofar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P. 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. *J Clin Microbiol* 42:1863–1868. <http://dx.doi.org/10.1128/JCM.42.5.1863-1868.2004>.
34. Bell AS, Ranford-Cartwright LC. 2002. Real-time quantitative PCR in parasitology. *Trends Parasitol* 18:337–342. [http://dx.doi.org/10.1016/S1471-4922\(02\)02331-0](http://dx.doi.org/10.1016/S1471-4922(02)02331-0).
35. Jeong W, Kweon CH, Kang SW, Paik SG. 2003. Diagnosis and quantification of *Theileria sergenti* using TaqMan PCR. *Vet Parasitol* 111:287–295. [http://dx.doi.org/10.1016/S0304-4017\(02\)00388-6](http://dx.doi.org/10.1016/S0304-4017(02)00388-6).
36. Sibeko KP, Oosthuizen MC, Collins NE, Geysen D, Rambritch NE, Latif AA, Groeneveld HT, Potgieter FT, Coetzer JAW. 2008. Development and evaluation of a real-time polymerase chain reaction test for the detection of *Theileria parva* infections in Cape buffalo (*Syncerus caffer*) and cattle. *Vet Parasitol* 155:37–48. <http://dx.doi.org/10.1016/j.vetpar.2008.03.033>.
37. Papli N, Landt O, Fleischer C, Koekemoer JO, Mans BJ, Pienaar R, Josemans A, Zweygarth E, Potgieter F, Latif AA. 2011. Evaluation of a TaqMan real-time PCR for the detection of *Theileria parva* in buffalo and cattle. *Vet Parasitol* 175:356–359. <http://dx.doi.org/10.1016/j.vetpar.2010.10.038>.
38. Kim C, Blanco LBC, Alhassan A, Iseki H, Yokoyama N, Xuan X, Igarashi I. 2008. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. *Vet Parasitol* 151:158–163. <http://dx.doi.org/10.1016/j.vetpar.2007.10.023>.
39. Bhoora R, Quan M, Franssen L, Butler CM, Van der Kolk JH, Guthrie AJ, Zweygarth E, Jongejan F, Collins NE. 2010. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. *Vet Parasitol* 168:201–211. <http://dx.doi.org/10.1016/j.vetpar.2009.11.011>.
40. Stanley KK, Szewczuk E. 2005. Multiplexed tandem PCR: gene profiling from small amounts of RNA using SYBR Green detection. *Nucleic Acids Res* 33:e180. <http://dx.doi.org/10.1093/nar/gni182>.
41. Lau A, Sorrell TC, Chen S, Stanley K, Iredell J, Halliday C. 2008. Multiplex tandem PCR: a novel platform for rapid detection and identification of fungal pathogens from blood culture specimens. *J Clin Microbiol* 46:3021–3027. <http://dx.doi.org/10.1128/JCM.00689-08>.
42. Lau A, Halliday C, Chen SCA, Playford EG, Stanley K, Sorrell TC. 2010. Comparison of whole blood, serum, and plasma for early detection of candidemia by multiplex-tandem PCR. *J Clin Microbiol* 48:811–816. <http://dx.doi.org/10.1128/JCM.01650-09>.
43. Stark D, Al-Qussab SE, Barratt JLN, Stanley K, Roberts T, Marriott D, Harkness J, Ellis JT. 2011. Evaluation of multiplex tandem real-time PCR for detection of *Cryptosporidium* spp., *Dientamoeba fragilis*, *Entamoeba histolytica*, and *Giardia intestinalis* in clinical stool samples. *J Clin Microbiol* 49:257–262. <http://dx.doi.org/10.1128/JCM.01796-10>.
44. Jex AR, Stanley KK, Lo W, Littman R, Verweij JV, Campbell BE, Nolan MJ, Pangasa A, Stevens MA, Haydon S, Gasser RB. 2012. Detection of diarrhoeal pathogens in human faeces using an automated, robotic platform. *Mol Cell Probes* 26:11–15. <http://dx.doi.org/10.1016/j.mcp.2011.10.004>.
45. Roeber F, Jex AR, Campbell AJD, Nielsen R, Anderson GA, Stanley KK, Gasser RB. 2012. Establishment of a robotic, high-throughput platform for the specific diagnosis of gastrointestinal nematode infections in sheep. *Int J Parasitol* 42:1151–1158. <http://dx.doi.org/10.1016/j.ijpara.2012.10.005>.
46. Baker L, Sendall BC, Gasser RB, Menjivar T, Neilan BA, Jex AR. 2013. Rapid, multiplex-tandem PCR assay for automated detection and differentiation of toxigenic cyanobacterial blooms. *Mol Cell Probes* 27:208–214. <http://dx.doi.org/10.1016/j.mcp.2013.07.001>.
47. Hayashida K, Hara Y, Abe T, Yamasaki C, Toyoda A, Kosuge T, Suzuki Y, Sato Y, Kawashima S, Katayama T, Wakaguri H, Inoue N, Homma K, Tada-Umezaki M, Yagi Y, Fujii Y, Habara T, Kanehisa M, Watanabe H, Ito K, Gojonori T, Sugawara H, Imanishi T, Weir W, Gardner M, Pain A, Shiels B, Hattori M, Nene V, Sugimoto C. 2012. Comparative genome analysis of three eukaryotic parasites with differing abilities to transform leukocytes reveals key mediators of *Theileria*-induced leukocyte transformation. *mBio* 3(5):e00204–12. <http://dx.doi.org/10.1128/mBio.00204-12>.
48. Szewczuk E, Thapa K, Anninos T, McPhie K, Higgins G, Dwyer DE, Stanley KK, Iredell JR. 2010. Rapid semi-automated quantitative multiplex tandem PCR (MT-PCR) assays for the differential diagnosis of influenza-like illness. *BMC Infect Dis* 10:113. <http://dx.doi.org/10.1186/1471-2334-10-113>.
49. Abeywardena H, Jex AR, Firestone SM, McPhee S, Driessen N, Koehler AV, Haydon SR, von Samson-Himmelstjerna G, Stevens MA, Gasser RB. 2013. Assessing calves as carriers of *Cryptosporidium* and *Giardia* with zoonotic potential on dairy and beef farms within a water catchment area by mutation scanning. *Electrophoresis* 34:2259–2267. <http://dx.doi.org/10.1002/elps.201300146>.
50. World Organisation for Animal Health (OIE). 2014. Principles and methods of validation of diagnostic assays for infectious diseases, chapter 1.1.5. Manual of diagnostic tests and vaccines for terrestrial animals, 7th ed. World Organisation for Animal Health (OIE), Paris, France. <http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/>.
51. Branscum A, Gardner I, Johnson W. 2005. Estimation of diagnostic-test sensitivity and specificity through Bayesian modeling. *Prev Vet Med* 68:145–163. <http://dx.doi.org/10.1016/j.prevetmed.2004.12.005>.
52. Christensen R, Johnson WO, Branscum AJ, Hanson TE. 2011. Bayesian ideas and data analysis: an introduction for scientists and statisticians. CRC Press, Boca Raton, FL.
53. Lunn DJ, Thomas A, Best N, Spiegelhalter D. 2000. WinBUGS—a Bayesian modelling framework: concepts, structure, and extensibility. *Stat Comput* 10:325–337. <http://dx.doi.org/10.1023/A:1008929526011>.
54. Byrt T, Bishop J, Carlin JB. 1993. Bias, prevalence and kappa. *J Clin Epidemiol* 46:423–429. [http://dx.doi.org/10.1016/0895-4356\(93\)90018-V](http://dx.doi.org/10.1016/0895-4356(93)90018-V).
55. Callow LL. 1984. Protozoal and rickettsial diseases, p 264. Animal health in Australia, vol 5. Australian Bureau of Animal Health/Australian Government Publishing Service, Canberra, Australia.
56. Stewart NP, Uilenberg G, de Vos AJ. 1996. Review of Australian species of *Theileria*, with special reference to *Theileria buffeli* of cattle. *Trop Anim Health Prod* 28:81–90. <http://dx.doi.org/10.1007/BF02250731>.
57. Kubota S, Sugimoto C, Kakuda T, Onuma M. 1996. Analysis of immunodominant piroplasm surface antigen alleles in mixed populations of *Theileria sergenti* and *T. buffeli*. *Int J Parasitol* 26:741–747. [http://dx.doi.org/10.1016/0020-7519\(96\)00047-1](http://dx.doi.org/10.1016/0020-7519(96)00047-1).
58. Altangerel K, Battsetseg B, Battur B, Sivakumar T, Batmagnai E, Javkhlan G, Tuvshintulga B, Igarashi I, Matsumoto K, Inokuma H, Yokoyama N. 2011. The first survey of *Theileria orientalis* infection in Mongolian cattle. *Vet Parasitol* 182:343–348. <http://dx.doi.org/10.1016/j.vetpar.2011.05.040>.
59. Sivakumar T, Tattiyapong M, Fukushi S, Hayashida K, Kothalawala H, Silva SSP, Vimalakumar SC, Kanagaratnam R, Meewewa AS, Suthakaran K, Puvirajan T, de Silva WK, Igarashi I, Yokoyama N. 2014. Genetic characterization of *Babesia* and *Theileria* parasites in water buffaloes in Sri Lanka. *Vet Parasitol* 200:24–30. <http://dx.doi.org/10.1016/j.vetpar.2013.11.029>.
60. Perera PK, Gasser RB, Jabbar A. Assessment of sequence variability in a p23 gene region within and among genotypes of the *Theileria orientalis* complex from Australia. *Ticks Tick Borne Dis*, in press. <http://dx.doi.org/10.1016/j.ttbdis.2014.10.006>.