Original article

Assessing the performance of multiplexed tandem PCR for the diagnosis of pathogenic genotypes of *Theileria orientalis* using pooled blood samples from cattle

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ABSTRACT

Oriental theileriosis caused by multiple genotypes of *Theileria orientalis* is an important tick-borne disease of bovines. Here, we assessed the performance of an established multiplexed tandem PCR (MT-PCR) for the diagnosis of the two recognized, pathogenic genotypes (*chitose* and *ikeda*) of *T. orientalis* in cattle using pooled blood samples. We used a total of 265 cattle blood samples, which were divided into two groups according to previous MT-PCR results for individual samples. Samples in group 1 (*n* = 155) were from a herd with a relatively high prevalence of *T. orientalis* infection; and those in group 2 (*n* = 110) were from four herds with a low prevalence. For group 1, 31 and 15 batches of five- and ten-pooled samples (selected at random), respectively, were formed. For group 2, 22 and 11 batches of five- and ten-pooled samples (selected at random), respectively, were formed. DNAs from individual pooled samples in each batch and group were then tested by MT-PCR. For group 1, the apparent prevalences estimated using the 31 batches of five-pooled samples (97%) and 15 batches of ten-pooled samples (100%) were significantly higher compared with individual samples (75%). For group 2, higher apparent prevalences (9% and 36%) were also recorded for the 22 and 11 batches of pooled samples, respectively, compared with individual samples (7%). In addition, an estimate of true prevalence Overall, the average infection intensity recorded for the genotypes of *chitose* and *ikeda* were considerably lower in pooled compared with individual samples. The diagnostic specificities of MT-PCR were estimated at 95% and 94%, respectively, when batches of five- and ten-pooled samples were tested, and 94% for individual samples. The diagnostic sensitivity of this assay was estimated at 98% same for all individual, five- and ten-pooled samples. This study shows that screening batches of five- and ten-pooled blood samples from cattle herds are similar to those obtained for individual samples, and, importantly, that the reduced cost for the testing of pooled samples represents a considerable saving to herd managers.
Keywords: *Theileria orientalis*, Pooled blood sample, Diagnosis, Cattle, Multiplexed-tandem PCR

1. Introduction

*Theileria orientalis* (Apicomplexa: Piroplasmida; Theileriidae) is a group of intracellular haemoprotozoan parasites that causes oriental theileriosis in bovines, and is distributed worldwide [1]. These parasites are transmitted by ixodid ticks of the genus *Haemaphysalis*, although mechanical transmission by biting insects (e.g., mosquitoes) can also occur [2]. Previously, oriental theileriosis was considered to be a benign disease of cattle globally; however, recently, hundreds of outbreaks of oriental theileriosis have occurred in Australia [3-5] and New Zealand [6]. Oriental theileriosis is manifested by pyrexia, haemolytic anaemia, productivity losses, abortions and/or mortality in dairy and beef cattle [3-7].

Based on the sequence of the major piroplasm surface protein (*MPSP*) gene, to date, 11 *T. orientalis* genotypes (designated *chitose* or *type* 1, *ikeda* or *type* 2, *buffeli* or *type* 3, *types* 4 to 8, and *N1* to *N3*) have been recorded worldwide [1]. Of particular note are the genotypes *chitose* and *ikeda*, which are consistently found to be associated with severe disease in cattle in the Asia-Pacific region [3-10].

For the diagnosis of oriental theileriosis, conventional techniques such as the detection of piroplasms of *T. orientalis* in blood smears, and/or the use of serological or conventional polymerase chain reaction [11-16] have been used, although each of these have limitations, including labor, cost, and low diagnostic sensitivity and/or specificity. To overcome these limitations, Perera et al. [17] developed a multiplexed-tandem PCR (MT-PCR) assay for the
simultaneous detection and differentiation of the four commonest genotypes (buffeli, chitose, ikeda and type 5) of the T. orientalis complex in Australasia and their quantitation in bovine blood samples. This assay proved to be an invaluable tool for the routine diagnosis, epidemiological studies and for the exploration of theileriosis outbreaks [10, 18-20].

Although very useful, a possible limitation of this MT-PCR assay (e.g., in outbreak situations) can be the cost to the herd manager, when relatively large numbers of samples need to be tested. Recently, we calculated that the cost of the test for pathogenic (i.e., chitose and ikeda) as well as non-pathogenic (i.e., buffeli and type 5) genotypes of T. orientalis per individual sample is ~ AUD 19.00. One way of reducing costs would be to pool blood samples from numbers of individual cattle, and test for pathogenic genotypes (chitose and ikeda) only. Although the analytical specificity and sensitivity (ability to specifically detect ≥ 2.5 DNA copies or 1 fg of genomic DNA) of MT-PCR are very high [17], one cannot assume that this assay performs the same for pooled samples as it does for individual blood samples. Indeed, diagnostic specificity and sensitivity depend on a number of factors, including inhibitory factors in the sample matrix, pool size and robustness of the assay [21-22], and each of these aspects need to be critically assessed. Therefore, in the present study, we evaluated the performance of the MT-PCR assay for the diagnosis of the two pathogenic genotypes (chitose and ikeda) of T. orientalis in cattle using individual and pooled blood samples. We also estimated and compared the cost to the farmer for the testing of pooled versus individual samples.

2. Materials and methods

2.1. Blood samples and DNA extraction
Blood samples \((n = 265)\) from cattle were available from previous studies conducted in Australia [17-18]. Extracted DNA of all of these samples had been individually tested for *T. orientalis* infection by MT-PCR. Based on the prevalence of *T. orientalis* infection, these blood samples were divided into two groups. Group 1 \((n = 155)\) were from beef cattle from South Australia (Furner) with a relatively high prevalence \((75\%)\) of *T. orientalis* infection [18]. Group 2 \((n = 110)\) were from four dairy herds from Victoria (Western Districts) with a low prevalence of *T. orientalis* \((7\%)\) [17].

For group 1, 31 and 15 batches of five- and ten-pooled samples (selected at random), respectively, were formed. For group 2, 22 and 11 batches of five- and ten-pooled samples (selected at random), respectively, were formed. A volume of 200 µL of each blood sample was taken and introduced into each pool (Fig. 1). Genomic DNA was extracted from individual pooled blood samples (in each batch and group) using mini-columns (DNeasy Blood and Tissue Kit; cat. no. 69506, Qiagen, USA) and eluted in 100 µL buffer employing an established approach [18].

2.2. MT-PCR assay

Each DNA sample was tested using MT-PCR. Initially, this assay was developed and validated by Perera et al. [17] for the detection, differentiation and quantitation of pathogenic (i.e., *chitose* and *ikeda*) and non-pathogenic (i.e., *buffeli* and *type 5*) genotypes of *T. orientalis* which commonly infect cattle in Australasia. However, in this study, we used this assay only for the detection, differentiation and quantitation of *ikeda* and *chitose* genotypes of *T. orientalis*. The assay was conducted in the Easy-Plex platform (AusDiagnostics Pty Ltd, Australia) as described previously by Perera et al. [17] using primers (Cat. no. 38170R; AusDiagnostics) designed specifically to the major piroplasm surface protein (*mpsp*) gene (genotype *chitose*) and the first internal transcribed spacer (ITS-1) of nuclear ribosomal DNA.
(genotype *ikeda*). Following primary and secondary amplifications, the peak high resolution melting (HRM) temperature of each amplicon was compared with those of pre-determined reference temperatures representing each genotype: *chitose* (82.1 ± 1.5 °C) and *ikeda* (87.4 ± 1.5 °C) [17]. The relative intensity of infection of each of these two pathogenic genotypes was expressed as a DNA copy number [17]. All amplicons had the peak melting temperatures within respective reference values; randomly selected amplicons representing each genotype were subjected to single-strand conformation polymorphism (SSCP) analysis and targeted sequencing [5, 23].

2.3. Data analyses

Data were entered into Microsoft Excel 2013 spreadsheet, and simple descriptive statistics were performed. Cost of testing individual, five- and ten-pooled samples for *T. orientalis* by MT-PCR was estimated.

The diagnostic sensitivity and specificity of the *Theileria* MT-PCR assay were determined using Bayesian latent class modelling [24-25] as previously described [17] using the posterior estimates of the parameters defining the beta distribution for diagnostic sensitivity and diagnostic specificity of the MT-PCR for individual samples [17]. We used a Bayesian approach to estimate true prevalence as well as the diagnostic sensitivity and diagnostic specificity of the MT-PCR assay for five- and ten-pooled samples. Here, pooled sensitivity (*Se*<sub>p</sub>) was a function of the sensitivity of the MT-PCR for individual samples (*Se*), the size of the pools (*k*) and the true prevalence of infection (*Pt*):

\[
Se_p = 1 - [(1 - Se)^{kp} \times Sp^k (1-Pt)]
\]

Pooled specificity (*Sp*<sub>p</sub>) was a function of the specificity of the MT-PCR for individual samples (*Sp*) and pool size (*k*):

\[
Sp_p = Sp^k
\]
The expression for apparent prevalence ($P_A$) was then:

$$P_A = P_T \times Se_p + (1 - P_T)(1 - Sp_p)$$

Pooled sensitivity, pooled specificity and true prevalence estimates were calculated using Markov chain Monte Carlo sampling. We used an uninformed (‘flat’) prior distribution for true prevalence and Bayesian inferences based on the joint posterior distribution were numerically approximated using WinBUGS [26] running 110,000 model iterations and discarding the first 10,000 iterations as burn-in.

DNA copies (i.e., intensity of infection) of each genotype were log-transformed ($\log_{10}$). One-way analysis of variance (ANOVA) was used to compare the mean $\log_{10}$ DNA copy number of each genotype between each blood group: individual, five- and ten-pooled samples. Pairwise comparisons following one-way ANOVA were conducted using Tukey’s Honestly Significant Difference test (HSD). Bonferroni’s correction to the $P$-value was used to account for multiple comparisons. The association between genotype prevalence within and between blood groups was analyzed using Pearson’s chi-squared test and Fisher’s exact test. The SPSS Statistics 22 package (IBM) was used for statistical analyses, and a $P$-value of $< 0.05$ was considered as statistically significant.

3. Results

3.1. T. orientalis infection prevalence estimates

For group 1, the prevalence estimates using all 31 five- (97%) and 15 ten-pooled samples (100%) were significantly higher (individual vs pool of five: $P < 0.008$; individual vs pool of ten: $P < 0.02$) than that of recorded (75%) for individual samples (Table 1). For group 2, higher apparent prevalences (9% and 36%) were also recorded for the 22 five- and 11 ten-pooled samples, respectively, than that of (7%) recorded using individual samples (Table 1),
but this difference was not statistically significant. In group 1, the estimated true prevalence of *T. orientalis* was 77%, 48% and 62% in individual, five-pooled and ten-pooled blood samples, respectively (Table 1). Similarly, in group 2, the estimated true prevalence of *T. orientalis* was low, 7%, 2% and 2% in individual, five-pooled and ten-pooled blood samples, respectively (Table 1).

### 3.2. Prevalences and intensities of genotypes

Both pathogenic genotypes (i.e., *ikeda* and *chitose*) were detected by MT-PCR, irrespective of whether individual or pools of samples were tested. In group 1, when samples were originally tested individually [18], the prevalence of genotype *ikeda* (91%) was found significantly higher compared to that of genotype *chitose* (19%) \((P < 0.0001)\). When five- and ten-pooled samples were tested, the prevalence estimate of genotype *ikeda* was 97% and 100%, respectively, being significantly higher \((P < 0.03)\) than for *chitose* 43% and 53%, respectively (Table 1). In addition, for the pooled samples, the prevalence estimates for *ikeda* and *chitose* were relatively higher than for the individual samples (Table 1).

Overall, in group 1, genotype *ikeda* dominated, and its relative intensity of infection was significantly lower (*ikeda* in individual vs pool of five: \(P < 0.0001\); *ikeda* in individual vs pool of ten: \(P < 0.002\)) in five-pooled (318,505) and ten-pooled (190,406) samples than in individual (329,775) samples. The DNA copy numbers of genotype *ikeda* was significantly higher \((P < 0.05)\) than for genotype *chitose* in all individual, five- and ten-pooled samples (Table 2).

In group 1, for individual, five- and ten-pooled samples, the average DNA copies of genotype *ikeda* (329,775, 318,505 and 190,406, respectively) was significantly higher \((P < 0.05)\) than for *chitose* (125,462, 87,815 and 10,889, respectively) (Table 2). The average DNA copies of genotype *ikeda* in individual samples (329,775) was found significantly
higher than those recorded in five- and ten-pooled samples ($P < 0.033$), whereas there was no significant differences among average DNA copies recorded in individual, five- and ten-pooled samples for genotype *chitose* (Table 2). Overall, the average DNA copy numbers for genotype *ikeda* had a narrower distribution for pooled samples than when samples were tested individually. By contrast, the average DNA copy numbers for *chitose* was similar, irrespective of whether pooled or individual samples were tested (Fig. 2).

In group 2, only genotype *ikeda* was detected (Table 1), and the average DNA copy numbers was low (range: 14,714 to 47,229), irrespective of whether pooled or individual samples were tested (Table 2).

### 3.3. Diagnostic sensitivity and specificity

The estimated diagnostic specificity and sensitivity of MT-PCR were negatively correlated, irrespective of whether pooled or individual samples were tested. The diagnostic sensitivity of the assay was 98% (95% credible interval (CrI), 97%, 99%), 98% (95% CrI, 97%, 99%) and 97% (95% CrI, 97%, 99%) in individual, five- and ten-pooled blood samples, respectively. Conversely, the diagnostic specificity of the assay was 94% (95% CrI, 91%, 96%), 95% (95% CrI, 92%, 97%) and 94% (95% CrI, 92%, 97%) in individual, five- and ten-pooled blood samples, respectively.

### 4. Discussion

In this study, we evaluated the performance of the MT-PCR assay for the diagnosis of the two pathogenic genotypes (*chitose* and *ikeda*) of *T. orientalis* infections in cattle using pooled blood samples from cattle, and compared test results with those for individual samples. Overall, the apparent prevalences of *T. orientalis* in five- (97%) and ten-pooled (100%) blood
samples were significantly higher ($P < 0.05$) than individual samples (75%). In addition, the infection intensity of the genotypes *chitose* and *ikeda* estimated was lower when pooled samples were tested compared with individual samples. These prevalence differences possibly relate to the random pooling of blood samples, such that one *T. orientalis*-positive blood sample with either four or nine other *T. orientalis*-negative samples would render a pooled sample test-positive. Additionally, in a herd with high prevalence, the apparent (75%) and true prevalences (77%) of individual samples were almost similar. However, in five- and ten-pooled samples, apparent prevalence of 97% and 100%, respectively, was quite high when compared to true prevalence which was 48% and 62% for five- and ten-pooled samples, respectively. A similar trend was observed in low prevalence herds (see Table 1). These results indicate that apparent prevalence in pooled samples were overestimated due to false-positive samples; whereas, the true prevalence seems to be more reliable estimate which is closer to the individual animal level prevalence of *T. orientalis*.

To date, there is no report on the testing of piroplasms (*Babesia* and *Theileria* spp.) using pooled blood samples from bovines or other animals. However, some PCR-based studies, for instance [27-29] have used pooling strategies for the detection of human haemoprotezoan parasites, *Plasmodium* spp., and found this approach to be sensitive and efficient for malaria surveillance in low-transmission settings, thereby enabling them to detect asymptomatic, occult infections with significant savings in cost and labor. The practicality and cost-effectiveness of testing pooled samples have also been demonstrated in other studies using either blood or faecal samples for the specific detection of bacteria (e.g., *Salmonella enteritidis*, *S. typhimurium*, *S. agona*; [30-31], protozoa (e.g., *Plasmodium* spp., *Tritrichomonas* sp.; [28-29, 32-33], helminths (*Ascaris lumbricoides*, *Muellerius capillaris* and *Trichuris trichiura*; [34-35] and viruses (bovine leukemia and viral diarrhoea; [36-37]).
The relative intensities of *T. orientalis* infections (average DNA copies) estimated herein for the two pathogenic genotypes were higher in individual than in pooled blood samples. For instance, individual blood samples had higher average DNA copies of *ikeda* (329,775) and *chitose* (125,462) than five- (318,505 and 87,815, respectively) and ten-pooled samples (190,406 and 10,889, respectively) (see Table 2). The relatively low average DNA copy numbers for the two pathogenic genotypes in pooled samples were likely due to the dilution effect when mixing samples prior to DNA extraction (see Fig. 1). Previously, [21] had also showed that the dilution of serum (pools of three, four, five or seven) for the detection of avian pneumovirus antibodies using an enzyme-linked immunosorbent assay led to false-negative results. Similarly, Chatterjee et al. [38] investigated the detection of hepatitis B virus in human blood donors using nucleic acid testing (NAT), and found that the pooling of blood samples led to an increase in false-negative results, particularly for samples with a low virus load.

In the present study, the estimated diagnostic sensitivity and specificity of MT-PCR was negatively correlated in all blood samples (individual: sensitivity 98%, specificity 94%; five-pooled: 98%, 95%; and ten-pooled: 98%, 94%, respectively) tested. Higher sensitivity of the MT-PCR for the testing of five- and ten-pooled means that the probability of a false-negative result is low. The findings of the present study shows that the testing of either five- or ten-pooled blood samples are most suitable and economical approach for estimating the herd-level prevalence of *T. orientalis* infections compared with testing numerous individual samples in cattle without considering the chances of getting false-positive and/or false-negative results due to a dilution effect, particularly when parasitaemia is low.

Previously, Perera et al. [17] estimated that the cost to test one blood sample (for all four genotypes) using MT-PCR assay was approximately AUD 19. However, in the present study, we used the MT-PCR assay only for the detection of the two pathogenic genotypes (*chitose*
and *ikeda*) of *T. orientalis*, which reduced the cost of individual sample testing from AUD 19 to AUD 14 per sample. Therefore, we used this new figure (AUD 14 per sample) to estimate the cost for testing of pooled samples. In a herd with higher prevalence of *T. orientalis*, the cost of estimating the herd-level prevalence using ten-pooled samples was the lowest (AUD 210), followed by five-pooled (AUD 434) and individual (AUD 2,170) samples (Table 3). However, the cost of re-testing individual blood samples following pooling was the lowest (AUD 2,170) for individual blood samples, followed by ten- (AUD 2,310) and five-pooled (AUD 2,534) samples (Table 3). In a region with low prevalence of *T. orientalis* infection, a similar trend was observed for the herd-level testing (ten-pooled: AUD 154; five-pooled: AUD 308; individual: AUD 1,540); however, the cost of re-testing individual blood samples following a pooling was the lowest for ten-pooled samples (AUD 434) followed by five-pooled (AUD 448) and individual (AUD 1,540) samples (Table 3). These comparisons show that the testing of pooled blood samples using the MT-PCR is cost-effective; however, if animal-level prevalence were to be estimated in a herd by re-testing all samples in test-positive pools, the testing of individual blood samples would be less expensive than a pooling method in a region with a high prevalence of infection (75% in this study). By contrast, in a region with a low prevalence (7% in this study), the pooling strategy would be less expensive than testing individual bloods. In addition, the pooling of blood samples prior to DNA extraction considerably reduces labor associated with DNA extraction, without compromising the diagnostic sensitivity, similar to findings from a previous study aimed at detecting *Plasmodium* spp. [28].

In conclusion, this study, for the first time, highlights that pooling blood samples and then testing by MT-PCR is a practical and cost-effective approach for the rapid detection, differentiation and quantitation of the two pathogenic genotypes (*chitose* and *ikeda*) of *T. orientalis*, also in case of oriental theileriosis outbreaks, in Australasia. In future studies, this
approach should be used to monitor the prevalence of *T. orientalis* in endemic and non-endemic regions, and could possibly be extended to include other haemoproteozoa of livestock.

**Conflict of interest**

The authors declare no conflict of interest.

**Acknowledgements**

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Table 1
Prevalences and intensities of *Theileria orientalis* genotypes detected using MT- PCR in cattle.

<table>
<thead>
<tr>
<th>Group (prevalence of <em>T. orientalis</em>; number of cattle)</th>
<th>Location</th>
<th>Type of blood sample tested</th>
<th>AP (95% CI)</th>
<th>TP (95% Cr I)</th>
<th>No. of test positive samples by genotypes % (proportion)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>ikeda</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (high; 155)</td>
<td>Furner, South Australia</td>
<td>Individual</td>
<td>75 (68 to 82) (^a)</td>
<td>77 (69 to 83)</td>
<td>91 (106/117) (^a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pools of five samples</td>
<td>97 (84 to 99) (^b)</td>
<td>48 (30 to 70)</td>
<td>97 (29/30) (^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pools of ten samples</td>
<td>100 (80 to 100) (^b)</td>
<td>62 (23 to 100)</td>
<td>100 (15/15) (^b)</td>
</tr>
<tr>
<td>Group 2 (low; 110)</td>
<td>Western districts, Victoria</td>
<td>Individual</td>
<td>7 (4 to 14) (^a)</td>
<td>7 (0 to 16)</td>
<td>50 (4/8) (^a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pools of five samples</td>
<td>9 (2 to 28) (^a)</td>
<td>2 (0 to 5)</td>
<td>100 (2/2) (^a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pools of ten samples</td>
<td>36 (15 to 65) (^a)</td>
<td>2 (0 to 7)</td>
<td>50 (2/4) (^a)</td>
</tr>
</tbody>
</table>

*AP*: Apparent prevalence.

*TP*: True prevalence.

*CI*: Confidence interval.

*Cr I*: Credible interval.

Different superscript letters (a, b, c) within columns and rows denote proportions that are significantly different (*P* < 0.05).

Comparisons were made within rows for test positive samples by genotypes, whereas within columns for total test positive samples and each genotype among individual, and pools of five and ten samples.

Comparisons were made separately for groups 1 and 2.
Table 2
Average DNA copies of the two pathogenic genotypes of *Theileria orientalis* estimated using MT-PCR in cattle.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Average DNA copies</th>
<th>Median DNA copies (min, max)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type of blood sample tested</td>
<td>Individual</td>
</tr>
<tr>
<td></td>
<td>Group 1</td>
<td>ikeda</td>
</tr>
<tr>
<td></td>
<td></td>
<td>chitose</td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>ikeda</td>
</tr>
</tbody>
</table>

Different superscript letters (a, b, c) within columns and rows denote significantly different (*P* < 0.05) DNA copies. Comparisons were made within rows for DNA copies of each genotype among and within individual, pools of 5 and 10. Comparisons were made separately for groups 1 (a herd with a history of theileriosis outbreak (number of cattle = 155) and 2 (herds with no history of theileriosis outbreak (n = 110)).
Table 3
Cost of testing individual and pooled blood samples of cattle for *Theileria orientalis* infection using MT-PCR.

<table>
<thead>
<tr>
<th>Group (prevalence of <em>T. orientalis</em>; number of cattle)</th>
<th>Type of blood sample tested</th>
<th>Total samples</th>
<th>No. of test positives</th>
<th>No. of test negatives</th>
<th>Cost per sample (AUD, US$)</th>
<th>Sub-total cost (AUD, US$)</th>
<th>Cost of re-testing (AUD, US$)**</th>
<th>Total cost (AUD, US$)***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (high; 155)</td>
<td>Individual</td>
<td>155</td>
<td>117</td>
<td>38</td>
<td>14, 10</td>
<td>2,170, 1,611</td>
<td>0, 0</td>
<td>2,170, 1,611</td>
</tr>
<tr>
<td></td>
<td>Five-pooled</td>
<td>31</td>
<td>30</td>
<td>1</td>
<td>14, 10</td>
<td>434, 322</td>
<td>2,100, 1,560</td>
<td>2,534, 1,882</td>
</tr>
<tr>
<td></td>
<td>Ten-pooled</td>
<td>15</td>
<td>15</td>
<td>0</td>
<td>14, 10</td>
<td>210, 156</td>
<td>2,100, 1,560</td>
<td>2,310, 1,715</td>
</tr>
<tr>
<td>Group 2 (low; 110)</td>
<td>Individual</td>
<td>110</td>
<td>8</td>
<td>102</td>
<td>14, 10</td>
<td>1,540, 1,143</td>
<td>0, 0</td>
<td>1,540, 1,144</td>
</tr>
<tr>
<td></td>
<td>Five-pooled</td>
<td>22</td>
<td>2</td>
<td>20</td>
<td>14, 10</td>
<td>308, 229</td>
<td>140, 104</td>
<td>448, 333</td>
</tr>
<tr>
<td></td>
<td>Ten-pooled</td>
<td>11</td>
<td>2</td>
<td>9</td>
<td>14, 10</td>
<td>154, 114</td>
<td>280, 208</td>
<td>434, 322</td>
</tr>
</tbody>
</table>

*Total number of samples tested × AUD14 or US$10

**The cost needed for re-testing of individual samples from the positive five- or ten-pooled blood samples

***The sum of sub-total and re-testing costs
Figure legends

**Fig. 1.** Schematic diagram showing different groups of cattle blood samples tested for *Theileria orientalis*.

**Fig. 2.** Box plot diagrams showing the number of DNA copies (log10-transformed) of the two pathogenic genotypes, *ikeda* (A) and *chitose* (B) detected in individual, five- and ten-pooled blood samples.