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Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR

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Abstract

A TaqMan-based real-time PCR assay was developed for the diagnosis of *Anaplasma marginale* infection of cattle. The established assay was proven to be highly specific, since no cross-reactions were observed with other *Anaplasma* species of ruminants, including the closely related *Anaplasma centrale*, or other haemoparasites of ruminants (*Anaplasma bovis*, *Anaplasma ovis*, *Anaplasma phagocytophilum*, *Babesia bovis*, *Babesia bigemina*, *Theileria annulata*, *Theileria buffeli*). The detection limit was equal to that of nested (n)PCR (10^1 copies of standard DNA and 3×10^1 infected erythrocytes ml^{-1} of blood). The assay was also reproducible, as shown by satisfactory low intra-assay and interassay coefficients of variation. Fifty-four blood samples of ruminants (cattle, $n = 51$; sheep, $n = 2$; goats, $n = 1$), that had been tested previously by reverse line blot (RLB) hybridisation, were subjected to a nPCR assay and the newly established real-time PCR assay. By using real-time PCR, *A. marginale* DNA was detected in 40/51 bovine samples, with DNA titres ranging from 3.60×10^3 to 5.70×10^8 copies ml^{-1} of blood, whereas sheep and goat samples tested negative. The concordance with nPCR was 100%, whereas a unique sample that had tested negative by RLB gave positive results by nPCR and real-time PCR. The established assay could overcome the limitations of existing diagnostic methods, allowing for simultaneous detection and quantification of the *A. marginale* DNA in bovine blood, that is essential to support the clinical diagnosis, to assess the carrier status of the animals and to evaluate the efficacy of vaccines and antirickettsial drugs.

Keywords: *Anaplasma marginale*; real-time PCR; tick-borne diseases; cattle.

1 1. Introduction

2 Among tick-borne diseases, bovine anaplasmosis represents one of the most important diseases of
 3 ruminants worldwide, causing significant economic losses in tropical and subtropical areas (Losos,
 4 1986; Palmer, 1989; Kocan et al., 2000). The main causative agent of bovine anaplasmosis is
 5 *Anaplasma marginale* (Theiler, 1910), an intraerythrocytic pathogen belonging to the genus
 6 *Anaplasma* (Rickettsiales: Anaplasmataceae) (Dumler et al., 2001), that is transmitted biologically
 7 by infected ticks or mechanically by biting flies or blood-contaminated fomites. The closely related
 8 species or subspecies *Anaplasma centrale* (Theiler, 1911) causes only mild forms of disease and it
 9 is used for extensive vaccination of cattle in endemic areas in order to provide a certain degree of
 10 protection against *A. marginale* (Callow and Dalglish, 1980). A third *Anaplasma* species,
 11 *Anaplasma ovis*, causes disease in sheep and goats (Lestoquard, 1924). Acute anaplasmosis, caused
 12 by *A. marginale*, is characterised by a progressive haemolytic anaemia associated with fever, weight
 13 loss, abortion, decreased milk production and in some cases death of the infected cattle (Palmer,
 14 1989).

15 Diagnosis of bovine anaplasmosis is made by finding *Anaplasma* in Giemsa-stained blood smears
 16 in animals clinically suspected, during the acute disease, whereas it is not useful to detect pre-
 17 symptomatic and carrier animals. Several serological tests have employed extensively for
 18 epidemiological studies, although they do not discriminate between different *Anaplasma* species
 19 because of antigenic similarity (Palmer, 1989; Dreher et al., 2005). Molecular methods, with a high
 20 degree of sensitivity and specificity, have been developed to identify *A. marginale* DNA (Eriks et
 21 al., 1989; Ge et al., 1995; Gale et al., 1996; French et al., 1998; Bekker et al., 2002; Molad et al.,
 22 2006). However, the conventional PCR assays do not permit an accurate estimation of the initial
 23 concentration of target DNA as they can provide only semi-quantitative results. Therefore, a
 24 quantitative assay with higher reproducibility is needed to determine the degree of infection with *A.*
 25 *marginale*. Recently, real-time PCR assays have been developed for quantification of *A. marginale*
 26 *msh5* gene copies and *msh2* RNA transcripts in infected ticks (Löhr et al., 2002; Futse et al., 2003).

Those methods, albeit sensitive, are based on detection of amplified DNA using SYBR Green intercalating dye and lack determination of diagnostic specificity with particular regard to the related species *A. centrale*.

In the present study, the development of a real-time PCR assay based on TaqMan technology is described for rapid detection and accurate quantification of *A. marginale* DNA in blood samples of cattle naturally infected.

2. Materials and methods

2.1. Blood samples and DNA extraction

Fifty-four blood samples collected in 2000 from 51 cattle belonging to 18 different herds of three regions of southern Italy (Apulia, Calabria, Basilicata) were used throughout the study.

Additionally, 2 sheep and 1 goat blood samples were tested. The samples were recruited from an epidemiological cross-sectional study (Carelli et al., 2001) carried out by reverse line blot (RLB) assay for simultaneous detection and identification of tick-borne haemoparasites (Gubbels et al., 1999; Bekker et al., 2002).

DNA was extracted from 200 µl of blood (stored at -20°C) with the QIAamp DNA Blood Mini Kit (QIAGEN S.p.A., Milan, Italy), following the manufacturer's instructions. DNA of each sample was eluted in 200 µl of AE buffer and diluted 1:10 in distilled water prior to molecular analysis in order to reduce residual inhibitors of DNA polymerase activity to an ineffective concentration (Decaro et al., 2005, 2006a, b, c).

2.2. Design of primers and TaqMan probe

In order to establish a real-time PCR assay specific for *A. marginale*, the sequences of the *msp1b* gene of two *A. marginale* strains (accession numbers AY841153 and M59845) were retrieved from GenBank and aligned using the Bioedit software package (www.mbio.ncsu.edu/BioEdit/bioedit.html). Real-time PCR target region was identified by visual

inspection of sequence alignment and then primer and probe sequences were chosen by using a primer design software (Beacon Designer, Bio-Rad Laboratories Srl, Milan, Italy), in order to amplify a 95-bp fragment within the gene region amplified by a nested PCR assay specific for *A. marginale* (Molad et al., 2006). Primers and probe were synthesised by MWG Biotech AG (Ebersberg, Germany), with the TaqMan probe labeled with the fluorescent reporter dye 6-carboxyfluorescein (6-FAM) at the 5' end and with blackhole quencher 1 (BHQ1) at the 3' end. The position and sequence of the primers and probe used for real-time PCR amplification are reported in Table 1.

2.3. Plasmid construction

For construction of *A. marginale* standard DNA, a PCR assay, using primers AM456 and AM1164 (Table 1) targeting the *msp1b* gene (Molad et al., 2006), generated a 729-bp fragment that was cloned into pCR®4-TOPO (Invitrogen srl, Milan, Italy) and propagated in competent *Escherichia coli* TOP10F' cells, following the manufacturer's instructions. Plasmid DNA was purified from transformed cells using Wizard Plus Midiprep (Promega Italia, Milan, Italy) and quantified by spectrophotometrical analysis at 260 nm on the basis of plasmid size and the corresponding DNA mass. Ten-fold dilutions of the plasmid, representing 10^0 - 10^8 copies of DNA/10 µl of template, were carried out in a blood sample collected from a calf that had been tested *Anaplasma*-negative by gel-based PCR. Aliquots of each dilution were frozen at -70°C and used only once.

2.4. Real-time PCR

Real-time PCR for simultaneous detection and quantification of *A. marginale* DNA was performed on a 7500 Real-time PCR System (Applied Biosystems, Foster City CA) with iTaq™ Supermix added with ROX (Bio-Rad Laboratories Srl, Milan, Italy). The reaction mixture (25 µl) contained 12,5 µl of iTaq™ Supermix, each primer at a concentration of 600 nmol l^{-1} and the probe at a concentration of 200 nmol l^{-1} , and 10 µl of template or plasmid DNA. The thermal cycling

consisted of activation of iTaq DNA polymerase at 95° C for 10 min and 45 cycles of denaturation at 95° C for 45 s and annealing-extension at 60 °C for 1 minute. The TaqMan assay was carried out in duplicate for each unknown and standard sample and a no-template control was included in each assay. The increase in fluorescent signal was registered during the extension step of reaction and the data were analysed with the appropriate sequence detector software (7500 System Software v.1.3.1). The growth of PCR product is proportional to an exponential increase in fluorescence (ΔRn). The application software produces an amplification curve resulting from a plot of ΔRn versus cycle number. The threshold cycle number (C_T) for each analysed sample was regarded as the cycle number at which the amplification curve crossed the threshold which is usually automatically selected from the average of the ΔRn of the samples between cycles 3 and 15. Lower C_T values corresponded to a greater amount of initial template and a negative result was considered to have a C_T value of 40 or more cycles.

2.5. Internal control

In order to verify the absence of DNA losses during the extraction step and of PCR inhibitors in the DNA templates, an internal control (IC), consisting of canine parvovirus type 2 (CPV-2) DNA (Desario et al., 2005), was added to the lysis buffer (AL buffer, QIAGEN S.p.A.) at the concentration of 10,000 DNA copies ml⁻¹ of buffer prior to blood digestion. The fixed amount of the IC added to each sample had been calculated to give a mean C_T value in a real-time PCR assay (Decaro et al., 2005) of 36.26 with a S.D. of 0.81 as calculated by 50 separate runs. Real-time PCR for IC detection was carried out in a separate run, using primers CPV-For (5'-AAACAGGAATTAACATACTAATAATATTTA-3') and CPV-Rev (5'-AAATTTGACCATTGATGATAAACT-3') and probe CPV-Pb (5'-6-FAM-TGGTCCTTTAACTGCATTAAATAATGTACC-TAMRA-3'). Samples in which the C_T value for the IC was > 37.88 (average plus 2 S.D.) were excluded from the analysis.

2.6. Evaluation of specificity, sensitivity, dynamic range and reproducibility

To exclude cross-reactivities between *A. marginale* and other pathogens responsible for tick-borne diseases of ruminants, including the closely related *A. centrale*, the assay was evaluated for specificity by testing DNA extracts from blood samples positive to the following species: *A. marginale*, *A. centrale*, *A. bovis*, *A. ovis*, *A. phagocytophilum*, *Babesia bovis*, *Babesia bigemina*, *Theileria annulata*, *Theileria buffeli*. Blood samples collected from uninfected calves as well as sterile water were also included in the analysis as negative controls.

To evaluate the detection limits of the real-time PCR assay, 10-fold dilutions of the plasmid DNA, ranging from 10^8 to 10^0 copies, were made in a blood sample collected from an *Anaplasma*-negative calf and subsequently tested. In addition, 10-fold dilutions of an *A. marginale*-positive blood sample, containing 3×10^8 *Anaplasma*-infected erythrocytes ml^{-1} , were made in blood that contained uninfected erythrocytes and subsequently processed.

Serial 10-fold dilutions of plasmids containing from 10^1 to 10^8 copies of standard DNA and corresponding C_T values were used to plot the standard curve for *A. marginale* DNA absolute quantification.

Reproducibility of the assay was evaluated by testing repeatedly blood samples containing amounts of *A. marginale* DNA spanning the whole range covered by real-time PCR, as previously described (Decaro et al., 2005, 2006a, b, c; Elia et al., 2006).

2.7. Gel-based PCR

A nested (n)PCR assay, targeting the *msp1b* gene and based on ethidium-bromide staining, was performed for specific detection of *A. marginale* DNA in blood samples as previously described by Molad et al. (2006), with minor modifications. LA PCR Kit Ver. 2.1 (TaKaRa Bio Inc., Shiga, Japan) was used for both PCR and nested amplifications. First amplification was conducted in a 50- μl reaction containing $1 \mu\text{mol l}^{-1}$ of external primers AM456 and AM1164, LA PCR Buffer (Mg^{2+} plus) 1x, 8 μl of dNTP mixture (corresponding to $400 \mu\text{mol l}^{-1}$ of each dNTP), 2.5 units of TaKaRa

LA TaqTM and 10 µl of 1:10 diluted template DNA. The cycling protocol consisted of preheating of 94°C for 3 min following by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, with a final extension of 72°C for 10 min. The 50-µl reaction for the second round amplification (nPCR) consisted of 1 µl of the primary PCR product, 1 µmol l⁻¹ of internal primers AM100 and AM219, LA PCR Buffer (Mg²⁺ plus) 1x, 8 µl of dNTP mixture (corresponding to 400 µmol l⁻¹ of each dNTP) and 2.5 units of TaKaRa LA TaqTM. The thermal conditions were as for the first amplification, except that the annealing temperature were 54°C. The PCR products were detected by electrophoresis through a 1.5% agarose gel and visualisation under UV light after bromide ethidium staining.

3. Results

3.1. Performances of real-time PCR for *A. marginale*

No template controls, negative blood samples and other haemoparasites of ruminants, including *A. centrale*, did not produce any detectable fluorescence signal, confirming that real-time PCR was highly specific for the detection of *A. marginale* DNA.

The detection limit of the assay was assessed as 10¹ DNA copies 10 µl⁻¹ of plasmid template and 3 x 10¹ *Anaplasma*-infected erythrocytes ml⁻¹ (corresponding to dilution 10⁻⁷) for standard DNA and *A. marginale*-positive blood sample, respectively.

Ten-fold dilutions of standard DNA were tested and used to construct the standard curve by plotting the plasmid copy number logarithm against the measured C_T values. The generated standard curve covered a linear range of eight orders of magnitude (from 10¹ to 10⁸ copies of standard DNA) and showed linearity over the entire quantification range (slope = -3.482), providing an accurate measurement over a very large variety of starting target amounts.

To determine the reproducibility of the assay, intra-assay and inter-assay studies were undertaken (Fig. 1). Intra-assay CVs ranged from 2.92% (samples containing 10³ DNA copies) to 16.82% (10¹

DNA copies), while the inter-assay CVs were comprised between 9.00% (10^4 DNA copies) and 15.71 (10^1 DNA copies).

3.2. Internal control detection

The IC was detected in all the examined samples, with C_T values below the threshold value of 37.88. Therefore, significant DNA losses did not occur during nucleic acid extraction, as well as no DNA polymerase inhibition was observed during real-time PCR amplification.

3.3. Analysis of blood samples by real-time PCR and nPCR

The 54 blood samples were tested in parallel by nPCR and real-time PCR for detection of *A. marginale* DNA. The results of the molecular assays are reported in Table 2. By the established real-time PCR, *A. marginale* was detected in 40/51 bovine blood samples, with DNA titres ranging from 3.60×10^3 to 5.70×10^8 copies mL^{-1} of blood.

A 100% concordance was demonstrated between real-time PCR and nPCR, whereas 1 sample (no. 18) that tested positive by real-time and nPCR had given negative results for *A. marginale* by RLB hybridisation, testing positive for *A. centrale* and *B. bovis*. Sequence analysis of the nPCR product obtained from this sample confirmed the presence of *A. marginale* (100% nucleotide identity to *A. marginale* reference strains).

By RLB, single infections caused by *A. marginale* and *A. centrale* had been detected only in 7 and 3 bovine blood samples, respectively. Multiple parasitosis had been observed in 36 out of 51 tested samples of bovine origin (Table 2).

The blood samples collected from sheep ($n = 2$) and goats ($n = 1$) tested negative for *A. marginale* by real-time PCR and nPCR, whereas by RLB they had been found to contain *A. ovis* alone (sheep samples no. 52 and 53) or in association with *A. bovis* (goat sample no. 54).

4. Discussion

In this study a real-time PCR assay was developed for detection and quantification of *A. marginale* DNA in blood of cattle naturally infected. The assay was highly sensitive proving to be able to detect as few as 10^1 DNA copies $10 \mu\text{l}^{-1}$ of standard DNA and 3×10^1 *Anaplasma*-infected erythrocytes ml^{-1} . This sensitivity is higher with respect to previously described gel-based PCR, RNA probe, PCR-ELISA and hybridisation assays (Eriks et al., 1989; Aboytes-Torres and Buening, 1990; Figueroa et al., 1993; Ge et al., 1995; Gale et al., 1996; French et al., 1998; Molad et al., 2006). The intra-assay and interassay CVs were satisfactory low, although the results obtained for reproducibility within run were less linear in comparison to reproducibility between runs, with optimal intra-assay CVs for samples containing 10^3 and 10^6 copies of *Anaplasma* DNA (2.92% and 3.81%, respectively). No reactivity was obtained with DNA from other pathogens present in many blood samples as resulted by RLB assay (Table 2), showing the high specificity of the established real-time PCR assay. Therefore, the co-presence in the same blood samples of other bovine haemoparasites, including those antigenically related to *A. marginale*, does not affect the performances of the assay. This finding represents a major advantage of the real-time PCR assay as cattle are frequently affected by multiple infections (Table 2). A 100% concordance was demonstrated between real-time PCR and nPCR and of 97.5% between these techniques and RLB. Apart from the high sensitivity of RLB which was approximately equal to that of nPCR (Molad et al., 2006), the major advantage of this technique is the possibility to test multiple samples against multiple probes at the same time. Moreover, nPCR determines an increased risk of carry-over contamination and RLB is time consuming particularly in preparation of membranes with species-specific probes. In addition, neither nPCR nor RLB are able to give a precise estimation of the rickettsemia levels, which is useful for a definitive diagnosis of disease induced by *A. marginale*. In fact, the presence of low levels of *A. marginale* is suggestive of the carrier status of the infected cattle, whereas the acute phase of infection, during which there is the occurrence of clinical signs, is characterised by high levels of rickettsemia (Kieser et al., 1990). Compared with molecular methods

1 established previously, the real-time PCR assay described in the present paper displayed several
2 advantages, increasing the laboratory throughput and allowing for simultaneous processing of
3 several samples. With respect to classical PCR protocols, the processing time required by real-time
4 PCR is shorter, the contamination risks are lower because of the lack of post-PCR steps and the
5 specificity is increased by the probe hybridisation. These advantages make real-time PCR an
6 attractive tool for the laboratory diagnosis of *A. marginale* infection and for a precise evaluation of
7 the extent and duration of rickettsemia in animals infected naturally. The only limit of this study is
8 represented by the limited geographical region where samples were collected (southern Italy).
9 Considering that the *A. marginale* strains tested are not representative of the diversity of strain types
10 encountered on a more global scale, the applicability of the assay with strains from other countries
11 should be evaluated.

12 In conclusion, the established *A. marginale* real-time PCR assay could be employed for several
13 aims: i) detection and quantification of rickettsemia in carrier, pre-symptomatic and symptomatic
14 cattle; ii) assessment of the precise correlation between levels of rickettsemia and occurrence of
15 clinical signs; iii) evaluation of the efficacy of vaccines; iv) evaluation of the follow-up after
16 treatment with antirickettsial drugs.

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20

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Figure legend

Fig. 1. Coefficients of variation intra-assay and interassay over the dynamic range of the *A. marginale* real-time PCR assay.

FIG. 1

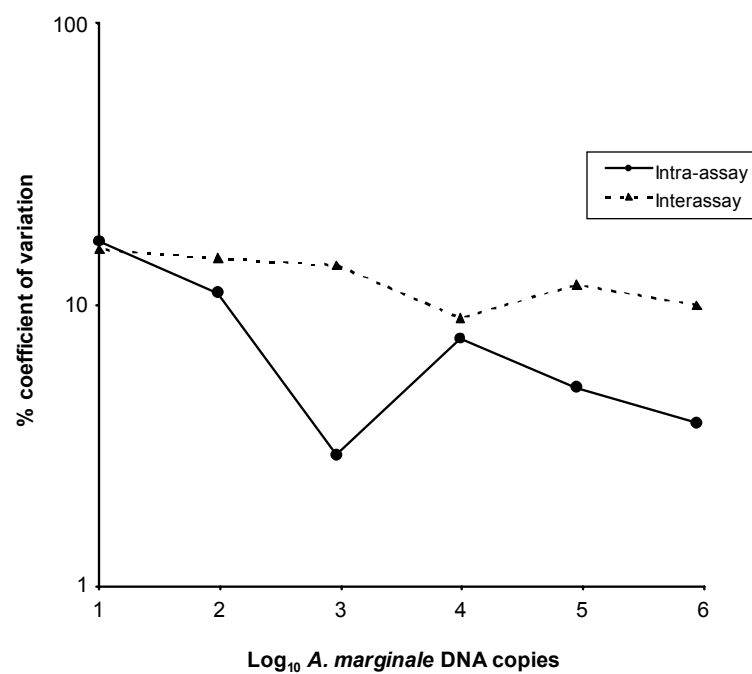


Table 1. Sequence, position and specificity of the oligonucleotides used in the study.

Assay	Reference	Primer/probe	Sequence 5' to 3'	Polarity	Position ^a	Amplicon size
Real-time PCR	This study	AM-For	TTGGCAAGGCAGCAGCTT	+	825-842	74 bp
		AM-Rev	TTCCGCGAGCATGTGCAT	-	902-919	
		AM-Pb	6FAM-TCGGTCTAACATCTCCAGGCTTTCAT-BHQ1	-	873-898	
n-PCR	Molad et al., 2006	AM456	CCATCTCGGCCGTATTCCAGCGCA	+	456-479	732 bp
		AM1164	CTGCCTTCGCGTCGATTGCTGTGC	-	1164-1187	
		AM100	CAGAGCATTGACGCACTACC	+	740-759	246 bp
		AM101	TTCCAGACCTTCCCTAACTA	-	966-985	

^a Oligonucleotide positions are referred to the sequence of *msp1b* gene of *A. marginale*, accession number M59845.

Table 2. Results of real-time PCR and nested-PCR for *A. marginale* detection carried out on blood samples of ruminants tested with reverse line blot hybridisation.

Sample No.	Species	RLB	Real-time PCR*	n-PCR
1	Cattle	<i>A. centrale</i>	-	-
2	Cattle	<i>A. bovis</i> , <i>T. buffeli</i>	-	-
3	Cattle	<i>A. marginale</i>	2.07×10^6	+
4	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	2.35×10^7	+
5	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	4.32×10^6	+
6	Cattle	<i>A. marginale</i> , <i>A. centrale</i> , <i>T. buffeli</i>	3.01×10^5	+
7	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	2.00×10^4	+
8	Cattle	<i>A. centrale</i>	-	-
9	Cattle	<i>A. marginale</i>	1.67×10^4	+
10	Cattle	<i>A. centrale</i> , <i>B. bovis</i>	-	-
11	Cattle	<i>A. marginale</i>	5.70×10^8	+
12	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	2.42×10^6	+
13	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	7.23×10^3	+
14	Cattle	<i>A. marginale</i>	2.33×10^5	+
15	Cattle	<i>A. marginale</i> , <i>B. bovis</i>	9.87×10^5	+
16	Cattle	<i>A. centrale</i>	-	-
17	Cattle	<i>A. phagocytophilum</i> , <i>T. buffeli</i>	-	-
18	Cattle	<i>A. centrale</i> , <i>B. bovis</i>	1.16×10^5	+
19	Cattle	<i>A. marginale</i>	2.31×10^6	+
20	Cattle	<i>T. buffeli</i>	-	-
21	Cattle	<i>A. marginale</i> , <i>A. centrale</i> , <i>B. bigemina</i>	3.71×10^5	+
22	Cattle	<i>A. marginale</i> , <i>A. centrale</i> , <i>B. bigemina</i> , <i>B. bovis</i>	5.55×10^5	+
23	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	3.07×10^6	+
24	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	1.40×10^4	+
25	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	3.60×10^3	+
26	Cattle	-	-	-
27	Cattle	-	-	-
28	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	2.08×10^6	+
29	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	2.47×10^6	+
30	Cattle	<i>A. marginale</i>	1.18×10^6	+
31	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	5.25×10^5	+
32	Cattle	<i>A. marginale</i> , <i>A. centrale</i> , <i>T. buffeli</i>	3.16×10^6	+
33	Cattle	<i>A. marginale</i> , <i>A. centrale</i> , <i>T. buffeli</i>	1.03×10^6	+
34	Cattle	<i>A. marginale</i>	4.33×10^6	+
35	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	6.39×10^4	+
36	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	6.08×10^5	+
37	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	1.28×10^6	+
38	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	6.71×10^4	+
39	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	2.20×10^5	+
40	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	3.18×10^5	+
41	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	2.45×10^5	+
42	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	6.82×10^5	+
43	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	1.33×10^5	+
44	Cattle	-	-	-
45	Cattle	-	-	-
46	Cattle	<i>A. marginale</i> , <i>A. centrale</i> , <i>T. buffeli</i>	5.86×10^4	+
47	Cattle	<i>A. marginale</i> , <i>T. buffeli</i> , <i>B. bigemina</i>	3.15×10^5	+
48	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	4.85×10^5	+
49	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	1.61×10^5	+
50	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	6.81×10^6	+
51	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	4.19×10^5	+
52	Sheep	<i>A. ovis</i>	-	-
53	Sheep	<i>A. ovis</i>	-	-
54	Goat	<i>A. ovis</i> , <i>A. bovis</i>	-	-

*Titres of *A. marginale* are expressed as DNA copy numbers ml⁻¹ of blood.
+, positive; -, negative.

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