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

3

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1 **Abstract**

2 A TaqMan-based real-time PCR assay was developed for the diagnosis of *Anaplasma marginale*  
3 infection of cattle. The established assay was proven to be highly specific, since no cross-reactions  
4 were observed with other *Anaplasma* species of ruminants, including the closely related *Anaplasma*  
5 *centrale*, or other haemoparasites of ruminants (*Anaplasma bovis*, *Anaplasma ovis*, *Anaplasma*  
6 *phagocytophilum*, *Babesia bovis*, *Babesia bigemina*, *Theileria annulata*, *Theileria buffeli*). The  
7 detection limit was equal to that of nested (n)PCR ( $10^1$  copies of standard DNA and  $3 \times 10^1$  infected  
8 erythrocytes  $\text{ml}^{-1}$  of blood). The assay was also reproducible, as shown by satisfactory low intra-  
9 assay and interassay coefficients of variation. Fifty-four blood samples of ruminants (cattle,  $n = 51$ ;  
10 sheep,  $n = 2$ ; goats,  $n = 1$ ), that had been tested previously by reverse line blot (RLB) hybridisation,  
11 were subjected to a nPCR assay and the newly established real-time PCR assay. By using real-time  
12 PCR, *A. marginale* DNA was detected in 40/51 bovine samples, with DNA titres ranging from  
13  $3.60 \times 10^3$  to  $5.70 \times 10^8$  copies  $\text{ml}^{-1}$  of blood, whereas sheep and goat samples tested negative. The  
14 concordance with nPCR was 100%, whereas a unique sample that had tested negative by RLB gave  
15 positive results by nPCR and real-time PCR. The established assay could overcome the limitations  
16 of existing diagnostic methods, allowing for simultaneous detection and quantification of the *A.*  
17 *marginale* DNA in bovine blood, that is essential to support the clinical diagnosis, to assess the  
18 carrier status of the animals and to evaluate the efficacy of vaccines and antirickettsial drugs.

19

20 **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 *msp5* gene copies and *msp2* RNA transcripts in infected ticks (Löhr et al., 2002; Futse et al., 2003).

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

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

7

## 8 **2. Materials and methods**

### 9 **2.1. Blood samples and DNA extraction**

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

12 Additionally, 2 sheep and 1 goat blood samples were tested. The samples were recruited from an  
13 epidemiological cross-sectional study (Carelli et al., 2001) carried out by reverse line blot (RLB)  
14 assay for simultaneous detection and identification of tick-borne haemoparasites (Gubbels et al.,  
15 1999; Bekker et al., 2002).

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

21

### 22 **2.2. Design of primers and TaqMan probe**

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

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

### 10 **2.3. Plasmid construction**

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

### 21 **2.4. Real-time PCR**

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

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

13

#### 14 **2.5. Internal control**

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

26



## 1 **2.6. Evaluation of specificity, sensitivity, dynamic range and reproducibility**

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

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

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

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

19

## 20 **2.7. Gel-based PCR**

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

1 LA Taq<sup>TM</sup> and 10 µl of 1:10 diluted template DNA. The cycling protocol consisted of preheating of  
2 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  
3 final extension of 72°C for 10 min. The 50-µl reaction for the second round amplification (nPCR)  
4 consisted of 1 µl of the primary PCR product, 1 µmol l<sup>-1</sup> of internal primers AM100 and AM219,  
5 LA PCR Buffer (Mg<sup>2+</sup> plus) 1x, 8 µl of dNTP mixture (corresponding to 400 µmol l<sup>-1</sup> of each  
6 dNTP) and 2.5 units of TaKaRa LA Taq<sup>TM</sup>. The thermal conditions were as for the first  
7 amplification, except that the annealing temperature were 54°C. The PCR products were detected  
8 by electrophoresis through a 1.5% agarose gel and visualisation under UV light after bromide  
9 ethidium staining.

10

### 11 **3. Results**

#### 12 **3.1. Performances of real-time PCR for *A. marginale***

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

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

19 Ten-fold dilutions of standard DNA were tested and used to construct the standard curve by plotting  
20 the plasmid copy number logarithm against the measured C<sub>T</sub> values. The generated standard curve  
21 covered a linear range of eight orders of magnitude (from 10<sup>1</sup> to 10<sup>8</sup> copies of standard DNA) and  
22 showed linearity over the entire quantification range (slope = -3.482), providing an accurate  
23 measurement over a very large variety of starting target amounts.

24 To determine the reproducibility of the assay, intra-assay and inter-assay studies were undertaken  
25 (Fig. 1). Intra-assay CVs ranged from 2.92% (samples containing 10<sup>3</sup> DNA copies) to 16.82% (10<sup>1</sup>

1 DNA copies), while the inter-assay CVs were comprised between 9.00% ( $10^4$  DNA copies) and  
2 15.71 ( $10^1$  DNA copies).

3

### 4 **3.2. Internal control detection**

5 The IC was detected in all the examined samples, with  $C_T$  values below the threshold value of  
6 37.88. Therefore, significant DNA losses did not occur during nucleic acid extraction, as well as no  
7 DNA polymerase inhibition was observed during real-time PCR amplification.

8

### 9 **3.3. Analysis of blood samples by real-time PCR and nPCR**

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

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

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

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

25

#### 1 4. Discussion

2 In this study a real-time PCR assay was developed for detection and quantification of *A. marginale*  
3 DNA in blood of cattle naturally infected. The assay was highly sensitive proving to be able to  
4 detect as few as  $10^1$  DNA copies  $10 \mu\text{l}^{-1}$  of standard DNA and  $3 \times 10^1$  *Anaplasma*-infected  
5 erythrocytes  $\text{ml}^{-1}$ . This sensitivity is higher with respect to previously described gel-based PCR,  
6 RNA probe, PCR-ELISA and hybridisation assays (Eriks et al., 1989; Aboytes-Torres and Buening,  
7 1990; Figueroa et al., 1993; Ge et al., 1995; Gale et al., 1996; French et al., 1998; Molad et al.,  
8 2006). The intra-assay and interassay CVs were satisfactory low, although the results obtained for  
9 reproducibility within run were less linear in comparison to reproducibility between runs, with  
10 optimal intra-assay CVs for samples containing  $10^3$  and  $10^6$  copies of *Anaplasma* DNA (2.92% and  
11 3.81%, respectively). No reactivity was obtained with DNA from other pathogens present in many  
12 blood samples as resulted by RLB assay (Table 2), showing the high specificity of the established  
13 real-time PCR assay. Therefore, the co-presence in the same blood samples of other bovine  
14 haemoparasites, including those antigenically related to *A. marginale*, does not affect the  
15 performances of the assay. This finding represents a major advantage of the real-time PCR assay as  
16 cattle are frequently affected by multiple infections (Table 2). A 100% concordance was  
17 demonstrated between real-time PCR and nPCR and of 97.5% between these techniques and RLB.  
18 Apart from the high sensitivity of RLB which was approximately equal to that of nPCR (Molad et  
19 al., 2006), the major advantage of this technique is the possibility to test multiple samples against  
20 multiple probes at the same time. Moreover, nPCR determines an increased risk of carry-over  
21 contamination and RLB is time consuming particularly in preparation of membranes with species-  
22 specific probes. In addition, neither nPCR nor RLB are able to give a precise estimation of the  
23 rickettsemia levels, which is useful for a definitive diagnosis of disease induced by *A. marginale*. In  
24 fact, the presence of low levels of *A. marginale* is suggestive of the carrier status of the infected  
25 cattle, whereas the acute phase of infection, during which there is the occurrence of clinical signs, is  
26 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.

17

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1 **Figure legend**

2

3 **Fig. 1.** Coefficients of variation intra-assay and interassay over the dynamic range of the *A.*

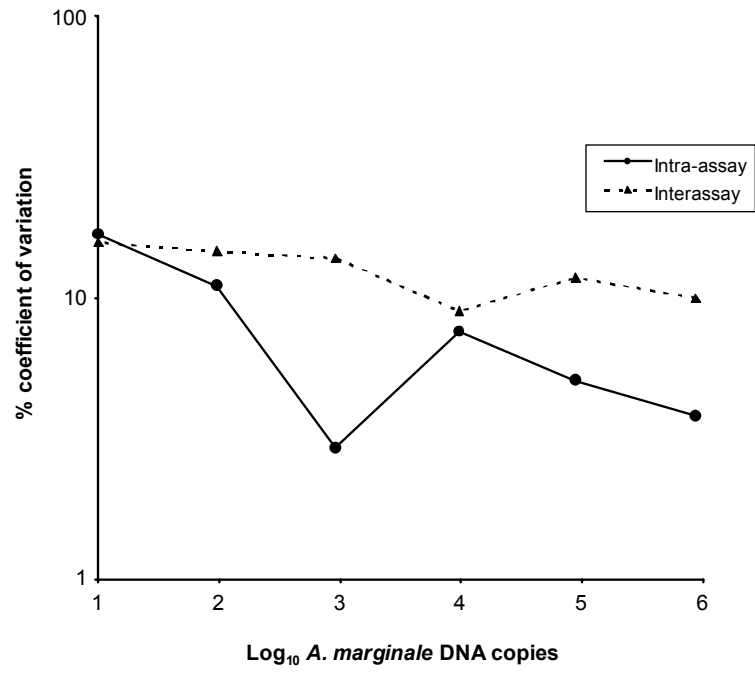
4 *marginale* real-time PCR assay.

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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 <sup>a</sup>	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	TTCCAGACCTCCCTAACTA	-	966-985	

<sup>a</sup> 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 x 10 <sup>6</sup>	+
4	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	2.35 x 10 <sup>7</sup>	+
5	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	4.32 x 10 <sup>6</sup>	+
6	Cattle	<i>A. marginale</i> , <i>A. centrale</i> , <i>T. buffeli</i>	3.01 x 10 <sup>5</sup>	+
7	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	2.00 x 10 <sup>4</sup>	+
8	Cattle	<i>A. centrale</i>	-	-
9	Cattle	<i>A. marginale</i>	1.67 x 10 <sup>4</sup>	+
10	Cattle	<i>A. centrale</i> , <i>B. bovis</i>	-	-
11	Cattle	<i>A. marginale</i>	5.70 x 10 <sup>8</sup>	+
12	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	2.42 x 10 <sup>6</sup>	+
13	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	7.23 x 10 <sup>3</sup>	+
14	Cattle	<i>A. marginale</i>	2.33 x 10 <sup>5</sup>	+
15	Cattle	<i>A. marginale</i> , <i>B. bovis</i>	9.87 x 10 <sup>5</sup>	+
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 x 10 <sup>5</sup>	+
19	Cattle	<i>A. marginale</i>	2.31 x 10 <sup>6</sup>	+
20	Cattle	<i>T. buffeli</i>	-	-
21	Cattle	<i>A. marginale</i> , <i>A. centrale</i> , <i>B. bigemina</i>	3.71 x 10 <sup>5</sup>	+
22	Cattle	<i>A. marginale</i> , <i>A. centrale</i> , <i>B. bigemina</i> , <i>B. bovis</i>	5.55 x 10 <sup>5</sup>	+
23	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	3.07 x 10 <sup>6</sup>	+
24	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	1.40 x 10 <sup>4</sup>	+
25	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	3.60 x 10 <sup>3</sup>	+
26	Cattle	-	-	-
27	Cattle	-	-	-
28	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	2.08 x 10 <sup>6</sup>	+
29	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	2.47 x 10 <sup>6</sup>	+
30	Cattle	<i>A. marginale</i>	1.18 x 10 <sup>6</sup>	+
31	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	5.25 x 10 <sup>5</sup>	+
32	Cattle	<i>A. marginale</i> , <i>A. centrale</i> , <i>T. buffeli</i>	3.16 x 10 <sup>6</sup>	+
33	Cattle	<i>A. marginale</i> , <i>A. centrale</i> , <i>T. buffeli</i>	1.03 x 10 <sup>6</sup>	+
34	Cattle	<i>A. marginale</i>	4.33 x 10 <sup>6</sup>	+
35	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	6.39 x 10 <sup>4</sup>	+
36	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	6.08 x 10 <sup>5</sup>	+
37	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	1.28 x 10 <sup>6</sup>	+
38	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	6.71 x 10 <sup>4</sup>	+
39	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	2.20 x 10 <sup>5</sup>	+
40	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	3.18 x 10 <sup>5</sup>	+
41	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	2.45 x 10 <sup>5</sup>	+
42	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	6.82 x 10 <sup>5</sup>	+
43	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	1.33 x 10 <sup>5</sup>	+
44	Cattle	-	-	-
45	Cattle	-	-	-
46	Cattle	<i>A. marginale</i> , <i>A. centrale</i> , <i>T. buffeli</i>	5.86 x 10 <sup>4</sup>	+
47	Cattle	<i>A. marginale</i> , <i>T. buffeli</i> , <i>B. bigemina</i>	3.15 x 10 <sup>5</sup>	+
48	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	4.85 x 10 <sup>5</sup>	+
49	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	1.61 x 10 <sup>5</sup>	+
50	Cattle	<i>A. marginale</i> , <i>A. centrale</i>	6.81 x 10 <sup>6</sup>	+
51	Cattle	<i>A. marginale</i> , <i>T. buffeli</i>	4.19 x 10 <sup>5</sup>	+
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<sup>-1</sup> of blood.  
+, positive; -, negative.

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