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Development of fluorogenic probe-based PCR assays for the detection and quantification of bovine piroplasmids

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ABSTRACT

This paper reports two new quantitative PCR (qPCR) assays, developed in an attempt to improve the detection of bovine piroplasmids. The first of these techniques is a duplex TaqMan assay for the simultaneous diagnosis of *Babesia bovis* and *B. bigemina*. This technique is ideal for use in South America where bovids harbour no theilerids. The second technique, which is suitable for the diagnosis of both babesiosis and theileriosis worldwide, involves fluorescence resonance energy transfer (FRET) probes. In FRET assays, *Babesia bovis*, *B. divergens*, *Babesia* sp. (*B. major* or *B. bigemina*), *Theileria annae* and *Theileria* sp. were all identifiable based on the melting temperatures of their amplified fragments. Both techniques provided linear calibration curves over the 0.1 fg/μl to 0.01 ng/μl DNA range. The assays showed good sensitivity and specificity. To assess their performance, both procedures were compared in two separate studies: the first was intended to monitor the experimental infection of calves with *B. bovis* and the second was a survey where 200 bovid/equine DNA samples from different countries were screened for piroplasmids. Comparative studies showed that duplex TaqMan qPCR was more sensitive than FRET qPCR in the detection of babesids.

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1. Introduction

Piroplasmid protozoa of the genera *Babesia* and *Theileria* cause serious infections in cattle and other mammals. The blood cells are invaded by the parasites and infection can lead to death. Bovine babesiosis is endemic in tropical areas but more occasional in temperate regions. Bovine theileriosis is not present in South America, but it is widespread in other regions of the world, including the Mediterranean Basin. Early detection of the pathogen improves the prognosis. However, the classical detection method (microscopy) is of low sensitivity and usually fails to diagnose carrier animals. The advantages offered by real-time PCR techniques (reduced contamination risks,

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precise quantification of parasite DNA and high sensitivity) have recently led to attempts to use them for diagnosing bovine piroplasmids (Jeong et al., 2003; Buling et al., 2007; Kim et al., 2007; Iseki et al., 2007). However, all these techniques have shown shortcomings, such as the inability to detect several species at the same time or the production of an excess of primer-dimers, or involve cumbersome procedures. Many researchers therefore prefer to use reverse line blotting (RLB), which does allow for the simultaneous detection of multiple piroplasmid species (Gubbels et al., 1999).

The present work reports two qPCR methods for the detection of piroplasmids: (1) a duplex TaqMan qPCR assay for the simultaneous detection of *B. bovis* and *B. bigemina* (this test ought to be useful in South America where no theilerids are present in cattle); and (2) a qPCR assay employing FRET probes for the identification of bovine piroplasmids at the genus or species level (this test ought to be of use anywhere in the world where babesids and theilerids infect cattle). The new diagnostic methods were tested by: (i) monitoring calves experimentally infected with *B. bovis*, and (ii) screening for piroplasmid infections in 200 bovid and equine blood samples from six countries. The results returned by these methods were compared against those obtained with alternative molecular procedures.

2. Materials and methods

2.1. Duplex TaqMan qPCR assay

Table 1 shows the primers and TaqMan minor groove binder (MGB) probes used in the duplex TaqMan procedure. The cytochrome b gene was chosen as the target owing to its greater number of copies compared to the small ribosomal subunit gene (approximately 100:1, according to Criado-Fornelio, 2007). All primers/probes were designed using Primer Express software. Three commercial kits were used to perform the assay, allowing their comparison: the TaqMan Universal PCR Master Mix Kit (Applied Biosystems), the Fast Start TaqMan Probe Master Kit with ROX (Roche Diagnostics, Mannheim Germany) and the Quantitect Multiplex Kit (Qiagen, Valencia, California, USA). All assays were performed following the manufacturers' instructions and employing an ABI 7500 Fast thermocycler (Applied Biosystems). To test the versatility of the assay, attempts were also made to use the Lightcycler 1.5 thermocycler (Roche Diagnostics).

2.2. FRET probe hybridisation assay

Table 1 shows the primers and anchor and sensor probes employed in this assay. Due to lack of information on cytochrome b gene sequences in many bovine piroplasmids, the target gene was the small ribosomal subunit. The UPFR primers are universal for piroplasmids. The TAFA/TAFS probes are based on the *T. annulata* 18S rRNA sequence (which is shared with other theilerids; see Fig. 1). The primers and probes were designed using LCPDS software v.1.0 (Roche, Basel, Switzerland). All assays were performed using the FastStart DNA MasterPlus Hybprobe Kit (Roche) and employing the Lightcycler 1.5 thermocycler (Roche Diagnostics), following the manufacturer's recommendations.

2.3. Specificity, sensitivity and reproducibility of the proposed qPCR assays

The specificity of the duplex TaqMan assay was tested by attempting to amplify the DNA of pathogens other than *B. bovis* and *B. bigemina* (see Section 2.4 for the species involved). The specificity of the FRET assay (which should detect all piroplasmids) was tested by attempting to amplify the DNA of *Toxoplasma gondii* (strain RH-type I), kindly provided by the Parasitology Unit of the *Instituto de Salud Carlos III*, Majadahonda (Madrid), Spain. In addition, the DNA of *Sarcocystis* sp. and *Neospora caninum* were tested. *Sarcocystis* sp. DNA was extracted from tissue macrocysts recovered from infected bovine muscle. Two cattle DNA samples, diagnosed as *Neospora*-positive by nested PCR (Buxton et al., 1998), were also employed in specificity tests. The sensitivity of the duplex TaqMan and FRET assays was tested as previously published for the SYBR green assay of Buling et al. (2007). The reproducibility of both assays was tested as described by Buling et al. (2007), employing samples reflecting mild (low DNA content) and severe infection (moderate DNA content).

2.4. Biological samples used in the development of the duplex TaqMan and FRET assays

DNA from *Babesia* spp.- and *Theileria* spp.-positive blood samples, previously identified as such (Criado-Fornelio et al., 2003b, 2006, 2009) by standard PCR and sequencing techniques (see Section 2.7), was used as reference material for assessing the performance of the proposed qPCR methods. Parasite DNA samples came from

Table 1
Oligonucleotides (primers and probes) employed in the present study.

Assay type	Primers	Probes
Duplex TaqMan-MGB	BIT-F = 5′-ttatgttccaggagatgttga-3′ BIT-R = 5′-cccaacccatattaacctcagt-3′	Probe BIP (*B. bigemina*) 5′ FAM-cgaatgtgttatcagagtat-3′ TAMRA Label = 5′ FAM (5-carboxyfluorescein, succinimidyl ester); probe includes a MGB molecule.
	BOT-F = 5′-tgttcctggaagcgttgattc-3′ BOT-R = 5′-ccaacccatattgacttcagc-3′	Probe BOP (*B. bovis*) 5′ VIC-tga atgtgtaattagagtgc-3′ TAMRA Label = 5′ VIC (proprietary name of Applied Biosystems); probe includes a MGB molecule.
FRET probes	UPFR-F = 5′-ttgatcctgccagtagt-3′ UPFR-R = 5′-agctgatrggtcagaaac-3′	(Anchor) TAFA = 5′-actgttgtaatgagccattcgcagtttc-3′ Fluorescein (Sensor) TAFS = 5′ LCRED 705-tataaaagcttatacttagacatgcatggc-3′

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Acc. No. / Species     /-----ANCHOR PROBE-------    -----SENSOR PROBE------- / No. differences
AY150056 – T. annulata--TATGCTTGTCTTAAAGATTAAGCC-AT-GCATGTCTAAGTATAAGCTTTTAT = probe
DQ287959 – T. buffeli---TATGCTTGTCTTAAAGATTAAGCC-AT-GCATGTCTAAGTATAAGCTTTTAT = probe
AF013148 – T. parva-----TATGCTTGTCTTAAAGATTAAGCC-AT-GCATGTCTAAGTATAAGCTTTTAT = probe
AY150069 – T. annae-----TATGCTTGTCTTAAAGATTAAGCC-AT-GCATGTCTTAGTATAAGCTTTTAT   1
EU622907 – B. major-----TATGCTTGTCTTAAAGATTAAGCC-AT-GCATGTCTAAGTACAAGCTTTTTA   3
DQ785311 – B. bigemina--TATGCTTGTCTTAAAGATTAAGCC-AT-GCATGTCTAAGTACAAGCTTTTTA   3
AJ439713 – B. divergens-TATGCTTGTCTTAAAGATTAAGCC-AT-GCATGTCTAAGTACAAACTTTTTA   4
AY150059 – B. bovis-----TATGCTTGTCTTAAAGACTAAGCC-AT-GCATGTCTAAGTACCAGCTTGTTA   5 (+1 anchor)

AY150062 – T. equi------TATGCTTGTCTTAAAGATTAAGCC-AT-GCATGTCTAAGTATAAGCTTTTAT = probe
AY346370 – B. caballi---TATGCTTGTCTTAAAGATTAAGCC-AT-GCATGTCTAAGTACAAGCTTTTTA   3
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Fig. 1. Alignment of piroplasmid 18S rRNA gene sequences targeted by the FRET probes. The GenBank® accession numbers for piroplasmid sequences, FRET probe binding regions (both anchor and sensor), and the number of base differences (compared to FRET probes) are indicated. Conserved positions in piroplasmid sequences are shown by asterisks. Variable positions in sequences are underlined. FRET probes are based on *T. annulata*/*buffeli* sequences.

the following sources: eight cows positive for either *B. bovis* (2 samples), *B. bigemina* (2 samples), *B. divergens*, *B. major*, *T. annulata* or *T. buffeli*, two dogs (one positive for *T. annae* and the other for *B. canis canis*), two goats (both infected with *B. ovis*), and four horses (two positive for *B. caballi* and two for *T. equi*).

2.5. Testing the performance of the proposed qPCR assays by monitoring the progress of infection in calves experimentally infected with B. bovis

The performance of the proposed qPCR assays was assessed by using them to monitor the change in protozoan DNA loads in blood from calves experimentally infected with *B. bovis*. Three Hereford calves (8 months old) were inoculated with 3 ml of infected blood containing 10^7 *B. bovis*-parasitized erythrocytes per ml at the INTA-Corrientes facilities in Argentina. The blood inoculum was obtained from a naturally-infected cow. Every week for 11 weeks, 10 ml of blood were obtained from the calves for DNA extraction (for details see Section 2.6). A single dose of imidocarb dipropionate (3 mg/kg live weight) was administered to the animals in the second week post-infection to ensure their survival over the experimental period. Purified parasite DNA samples were stored at −20 °C for shipping to Spain for quantitative analysis using the proposed qPCR assays. The performance of the two novel methods was compared to that of previously described qPCR tests: the SYBR-green assay of Buling et al. (2007) and the TaqMan assay of Kim et al. (2007). All assays were performed in duplicate on two different occasions (both methods).

2.6. Screening survey to detect piroplasmids in blood samples from around the world using the proposed qPCR assays

The proposed qPCR assays, once optimised, were used to screen for piroplasmid DNA in 200 blood samples taken from different animal species in six countries: 40 cows from central Spain (with no sign of disease), 40 cows from central Portugal (with no sign of disease), 24 buffaloes from northern Argentina (*Bubalus bubalis*) positive for *B. bovis* by nested PCR (Figueroa et al., 1992), 45 horses positive to *T. equi* by ELISA (Asenzo et al., 2008) from northern Argentina, 5 cows (symptomatic) and 10 horses (with no sign of disease) from southern Italy, 10 cows from central Brazil (symptomatic), and finally 26 cows with no sign of disease from southern France (a non-endemic area). The horses were included in the above list in an attempt to determine whether they can act as a significant reservoir of *B. bovis* or *B. bigemina*. In all cases, blood samples were aseptically drawn (using EDTA as an anticoagulant) and kept at 4 °C until use. DNA was extracted from these blood samples using several methods depending on the country where this step was performed: (i) the Blood-spin Kit (Mobio) was used in Spain and France; (ii) the Qiagen Flexi Gene Kit (Qiagen) in Argentina; (iii) the Blood Archive Pure Kit (5 Prime, Hamburg, Germany) was used in Portugal; (iv) the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma–Aldrich, St Louis, Missouri, USA) in Italy; and (v) the Genomic DNA extraction Kit (Real Biotech, ChungHo, Taiwan) in Brazil.

2.7. Conventional PCR and sequencing methods used to confirm the identity of piroplasmids in survey samples

Preliminary identification of piroplasmid DNA by conventional PCR and sequencing was performed as previously described (Criado-Fornelio et al., 2003a,b). However, since the sensitivity of this assay is relatively low, further primers (Buling et al., 2007) were used for the reliable identification of *B. bovis* and *B. bigemina*.

2.8. Reverse line blotting

Samples from Portugal were screened for piroplasmid infection by RLB (routine in the corresponding laboratory), as described by Brígido et al. (2004).

2.9. Statistical analysis

The Student *t*-test was used to compare the melting temperatures of the amplification products and the DNA concentrations obtained with the different qPCR methods. Statgraphics software (Manugistics, Rockville, Maryland, USA) was used for all calculations. Significance was set at $p < 0.05$.

3. Results

3.1. Optimisation of the duplex TaqMan assay

Of the three commercial kits tested, the Quantitect Multiplex Kit (Qiagen) showed the best amplification

Table 2
Performance of different qPCR assays for monitoring calves experimentally infected with *B. bovis*.

Comparative parameters	Type of qPCR assay–target gene		
	Duplex TaqMan–cyt. b	SYBR G–cyt. b	FRET (18S rRNA)
DNA yield (DNA concentration in 1 sample[a]—average ± S.D.)	269.5 ± 13.9[b]	123.6 ± 10.4	89.6 ± 7.1
Total DNA quantified in 31 samples (fg/μl)[c]	956	527	564
Total negative samples (out of 31)[d]	0	10	14

[a] The mean DNA concentrations and standard deviations were obtained by measuring (in duplicate) the DNA content of the sample with the greatest amount.
[b] Student *t*-test analysis showed that the duplex TaqMan assay yielded higher amounts of DNA than the other tests ($p < 0.05$).
[c] Total DNA represents the cumulative DNA concentrations obtained in the 31 samples analysed.
[d] Total negative samples refers to how many times no *B. bovis* DNA was detected in the 31 samples.

performance. The optimised duplex TaqMan reaction (final volume = 20 μl) finally contained 10 μl of Quantitect Multiplex mix, 250 nM of each probe (see Table 1), 0.5 μM of each primer (see Table 1), 3–8 μl of sterile, distilled water, and 2–5 μl of template DNA (the volumes were adjusted as needed to include at least 5 ng of DNA per assay). The optimal thermocycling conditions were 15 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, and an annealing/extension step at 60 °C for 1 min.

3.2. Optimisation of the FRET assay

The optimised FRET reaction (final volume = 20 μl) contained 4 μl of FastStart DNA MasterPlus Hybprobe (Roche), 0.5 μM of each primer (see Table 1), 2 μl each of sensor and anchor probe (20 μM each), 11–8 μl of sterile, distilled water, and 2-5 μl of template DNA (the volumes were adjusted as needed to include at least 5 ng of DNA per assay). The optimal thermocycling conditions were an initial denaturation cycle at 95 °C for 10 min, followed by 40 amplification cycles (with a temperature transition rate of 20 °C/s in all steps) consisting of 95 °C for 5 s, annealing at 55 °C for 15 s, and an extension step at 72 °C for 15 s. Fluorescence acquisition was set at "single" in the annealing step.

3.3. Specificity, sensitivity and reproducibility of the duplex TaqMan and FRET assays

The duplex TaqMan assay produced no amplification products with non-*B. bigemina* DNA, non-*B. bovis* DNA or any other non-piroplasmid DNA. No cross-reactivity between *B. bovis* and *B. bigemina* probes was observed (data not shown). In the FRET assay, no amplification products were obtained with non-piroplasmid DNA. The FRET assay accurately distinguished between many bovine piroplasmids (Fig. 2A and B); the Student *t*-test showed significant differences between their amplicon melting temperatures ($p < 0.001$ or lower, data not shown). The test also detected *Theileria* sp. and *Babesia* sp. in horses. The melting temperatures of the amplification products of *T. equi* and *B. caballi* DNA (positive controls) were comparable to those of *Theileria* sp. and *B. bigemina*/*B. major*, respectively. The sensitivities of the proposed techniques were similar. Both provided linear response curves over the 10^3–10^8 copies range (0.1 fg of the target DNA). The *B. bovis* and *B. bigemina* standard curves, determined using either the packaged ABI FAST 7500 or Lightcycler software, showed correlation coefficients of close to 0.99 and slope values of close to −3.2 (curves not shown); this indicates that the qPCR assays were well optimised. Reproducibility varied between 3% and 34%. Primers BIT-F/BIT-R and the TaqMan-MGB probe (labelled with FAM) were found usable for amplification purposes in both the ABI 7500 and Lightcycler instruments.

3.4. Performance: comparison of the proposed techniques with other qPCR methods

3.4.1. Detection of B. bovis DNA in experimentally infected calves

All three calves challenged with *B. bovis* survived the experiment. However, there was a problem in DNA extraction with one animal and only nine samples (weeks

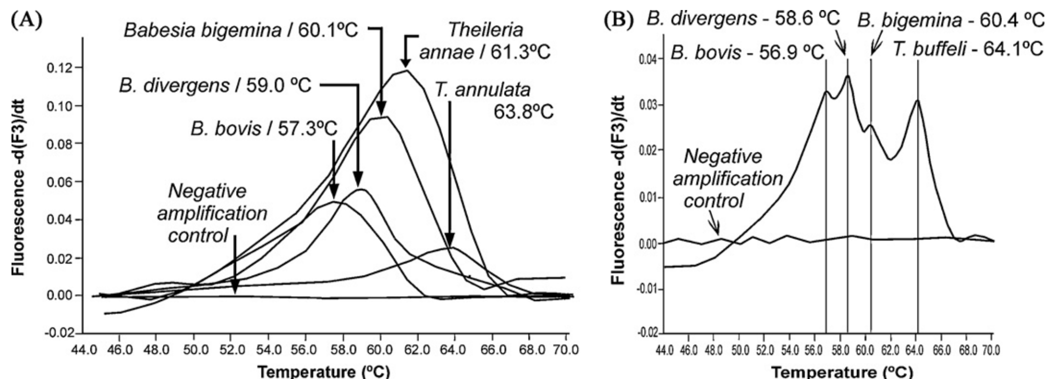

Fig. 2. Melting curve analysis obtained in the FRET probe method. (A) Curves obtained with control samples (*B. bovis*, *B. divergens*, *B. bigemina*, *T. annae* and *T. annulata*). Peaks show differences in melting temperature (comparing species in pairs) of ≥1 °C. Such differences were always statistically significant at $p < 0.001$ or lower. (B) Melting curves obtained with a negative control and in a capillary spiked with parasite DNA from three different bovines (1 positive for *B. bovis*, 1 for *B. bigemina* and the last for both *B. divergens* and *T. buffeli*). Approximately 2 μl of each sample were included in the assay. Four peaks (in agreement with the expected melting temperature) are easily observed.

1 to 9) could be obtained. In total, 31 DNA samples were available at the end of the experiment. The monitoring of their condition with the different qPCR methods showed parasitaemia to be irregular and to vary greatly between the calves (data not shown). The treatment with imidocarb in week 2 post-infection did not eliminate *B. bovis* from the bloodstream, although it did secure the survival of the calves. The individual performance of each qPCR method is shown in Table 2. The duplex TaqMan assay was superior to other methods in the three comparative variables studied (DNA yield, total DNA, and assay negativity). For comparative purposes, an attempt was made to quantify *B. bovis* DNA in calves using the TaqMan assay of Kim et al. (2007). However, problems caused by genetic polymorphism in the probe-binding region prevented this, both in the experimentally infected calves and survey samples.

3.4.2. Results of the screening survey performed with different diagnostic methods

Different babesid prevalences were found depending on the diagnostic technique employed (Table 3). The most sensitive method was the duplex TaqMan assay (41% of the samples showed the presence of babesid DNA). Lower prevalence figures were obtained with the FRET (18%) and conventional PCR plus sequencing (8%) methods. The FRET assay was again better than standard PCR plus sequencing for the diagnosis of theilerids (Table 3). The prevalence values obtained with these methods were 30% and 19%, respectively. Both the FRET and duplex TaqMan assays were able to diagnose simultaneous infections with two piroplasmid species in 2–3% of samples (all from Portugal and Argentina). Conventional PCR, however, was unable to detect these mixed infections. The new qPCR tests showed good specificity. Samples from healthy cows from France (where babesiosis is not endemic) were all negative for *B. bovis* and *B. bigemina*. Samples from South American bovids (where theileriosis is not endemic) were all negative for theilerids according to the FRET assay. The results obtained with conventional PCR and sequencing generally agreed with those obtained with the proposed qPCR methods in examinations of bovid and equine samples, with only one exception. The duplex TaqMan test (but not the other diagnostic methods used) returned different results at different times for one bovine sample from Italy. However, the dilution of this sample (at 1/25 and 1/50) yielded positive results. In the Italian horses the FRET assay was able to diagnose both *Babesia* sp. and *Theileria* sp. The duplex TaqMan assay was less sensitive than the nested PCR technique of Figueroa et al. (1992) in the detection of *B. bovis* as shown by the results obtained for the Argentine buffaloes (these tests returned 75% and 100% positive results, respectively). The same was true for the FRET assay and RLB in Portugal (prevalences obtained were 28% and 33%, respectively). Interestingly, 77% of the Argentine horses were found to harbour a babesid infection (in most cases *B. bovis*) when samples were tested with the new duplex TaqMan assay. Quantification of bovine piroplasmid DNA by the TaqMan technique

Table 3
Results of the survey for *Babesia*/*Theileria* sp. in 200 bovid/equine DNA samples using different methods.

Country	Number of samples	Stand. PCR/sequencing	FRET qPCR[a]	Duplex TaqMan
France	26 cows	Negative	Negative	Negative
Spain	40 cows	Negative for *Babesia* 4 *Theileria annulata*	1 *Babesia* sp. 8 *Theileria* sp.	5 *B. bigemina*
Portugal[b,c]	40 cows	 1 *B. bovis* 19 *Theileria* sp.[d]	1 *Babesia* sp. 5 *B. bovis* 1 *B. divergens* 25 *Theileria* sp. 3 *Theileria* + *Babesia* sp.	5 *B. bigemina* 2 double infection
Italy	5 cows	3 *B. bigemina* 2 *T. annulata*	3 *Babesia* sp. 2 *Theileria* sp.	2 *B. bigemina*
	10 horses	Negative for *Babesia* 2 *T. equi*	1 *Babesia* sp. 3 *Theileria* sp.	1 *B. bovis*
Argentina[b]	24 buffaloes	1 *B. bovis*	7 *B. bovis*	16 *B. bovis* 2 double infection
	45 horses	1 *B. bovis* 1 *B. bigemina* 11 *Theileria* sp.[e]	9 *B. bovis* 1 *Babesia* sp. 16 *Theileria* sp. 2 mixed infection[f]	33 *B. bovis* 2 double infection
Brazil	10 cows	5 *B. bovis* 5 *B. bigemina*	5 *B. bovis* 5 *Babesia* sp.	5 *B. bovis* 5 *B. bigemina*
Babesia sp./*Theileria* sp. prevalence (%)		8%/19%	17%/30%	41%/not applicable
Double infection prevalence (%)		0%	2%	3%

[a] All isolates of *Babesia* sp. in this column were identified by sequencing as *B. bigemina*, except that from an Italian horse, identified as *B. caballi*.
[b] Due to the high number of infected animals, only 30% of positives were sequenced in both countries (12 samples from Portugal and 21 from Argentina).
[c] All positive samples diagnosed by qPCR in Portugal were confirmed by RLB.
[d] Five isolates were sequenced and identified as either *T. annulata* or *T. buffeli*.
[e] Six isolates were sequenced and identified as *T. equi*.
[f] One horse was infected with *Babesia* sp. + *Theileria* sp. and the other with *B. bovis* + *Theileria* sp.

Table 4
List of isolates sequenced in the present work.

Country	Host	Isolate/Accession number (if applicable)	Closest GenBank entry/% identity (by BLASTn®)
Spain	Cow	*B. bigemina* (cyt. b gene)	AF109354, *B. bigemina* Spain 1/100%
		B. bigemina FJ426362 **(cyt. b gene SPC2)**[a]	AF109354, *B. bigemina*/>99%
		B. bigemina FJ426363 **(cyt. b gene SPC3)**	AF109354, *B. bigemina*/>99%
Portugal	Cow	**B. bovis** FJ426367 **(cyt. b gene)**	DQ785310, *B. bovis*/>99%
		B. bigemina (cyt. b gene)	AF109354, *B. bigemina*/100%
		T. annulata (18S rRNA gene)	AY150156, *T. annulata*/100%
		T. buffeli (18S rRNA gene)	DQ287959, *T. buffeli*/100%
		B. divergens (18S rRNA gene)	AJ439713, *B. divergens*/100%
Italy	Cow	**T. annulata** FJ426369 **(18S rRNA gene)**	DQ287944, *T. annulata*/>99%
		T. buffeli FJ426360 **(18S rRNA gene)**	DQ287959, *T. buffeli*/>98%
		B. bigemina (cyt. b gene)	AF109354, *B. bigemina*/100%
	Horse	**B. bovis** FJ426366 **(cyt. b gene)**	DQ785310, *B. bovis*/98%
		T. equi (18S rRNA gene)	DQ287951, *T. equi*/100%
		B. caballi (18S rRNA gene)	AY309955, *B. caballi*/100%
Argentina	Horse	**T. equi** FJ426368 **(18S rRNA gene)**	AY150062, *T. equi*/99%
		B. bovis (18S rRNA, cyt. b gene)	DQ785313, DQ785308, *B. bovis*/100%
		B. bigemina (18S rRNA, cyt. b gene)	DQ785311, DQ785312, *B. bigemina*/100%
Brazil	Cow	**B. bovis** FJ426364 **(18S rRNA gene)**	DQ785913, *B. bovis*/>99%
		B. bovis FJ426365 **(cyt. b gene)**	DQ785310, *B. bovis*/>99%
		B. bigemina FJ426361 **(18S rRNA gene)**	X59607, *B. bigemina*/>99%

[a] New sequences are indicated in boldface. Those sequences identical to entries previously deposited in GenBank® were not introduced in this database.

showed the symptomatic cattle from Italy and Brazil to have the highest quantities of babesid DNA, ranging from 1.24×10^{-1} to 1.68×10^{-4} ng/μl. According to the FRET tests, the cattle/horses infected with *Theileria* sp. in Argentina, Spain, Portugal and Italy showed moderate levels of parasite DNA (ranging from 3.5×10^{-4} to 8.7×10^{-6} ng/μl). Many asymptomatic animals in different geographic locations showed lower DNA concentrations with either qPCR assay, and were probably parasite carriers.

3.5. New sequences obtained in the present work

Generally, the piroplasmid genes sequenced in the present work were identical or very similar to those already held in the GenBank database (Table 4). Two new genetic variants of the *B. bigemina* cytochrome b gene were found in Spain, and a new *B. bovis* cytochrome b gene sequence was found in an Italian horse.

4. Discussion

The present results show that the proposed qPCR tests can be used for diagnosing infection with bovine piroplasmids. The sensitivity of the duplex TaqMan assay seems to be slightly lower than that of RLB or nested PCR. Similarly, nested PCR was also found to be more sensitive than the SYBR-green assay of Buling et al. (2007) (which also targets the cytochrome b gene) (Olegario et al., 2007). However, the duplex TaqMan technique has important advantages over that of Kim et al. (2007), who actually pointed out that their system had possible flaws since the target region for the probe is polymorphic in *B. bovis* (this was observed in the present work as well). In contrast, MGB technology involves the use of shorter probes (Kutyavin et al., 2000), which minimizes the chances of probe hybridisation failure due to mutations in the target sequence. The proposed duplex TaqMan assay is also more sensitive than the SYBR-green assay of Buling et al. (2007), which is limited to 35 amplification cycles. It is also less labour-intensive than the assay of Iseki et al. (2007), which is also prone to contamination problems due to the need to conduct RFLP analysis for definitive babesid identification. Finally, it should be remembered that the new duplex TaqMan method is versatile. This assay (at least for *B. bigemina*) was performed easily with two different qPCR instruments.

Since sensitivity determinations using serial dilutions of infected blood with known levels of parasitaemia were not performed, the minimum parasite load detectable with the new assays could not be ascertained. Based on previous estimations of cytochrome gene-based (Criado-Fornelio, 2007) and small ribosomal subunit based qPCR assays (Kim et al., 2007), the new tests may have sensitivities of between 0.75 and 2.5 parasites/μl of infected blood. Some problems with the reproducibility of the duplex TaqMan assay were encountered with one Italian sample. Since the amplification results were clearly positive when DNA dilutions were employed, it is likely that inhibitors were present in the sample that returned a negative result. However, standard PCR and FRET assays were not affected by these possible inhibitors, probably because fewer primers (or primers and probes) are employed in these methods (Wei et al., 2008). The fact that identification at the genus level only could be obtained for *Theileria* and some *Babesia* might be seen as a shortcoming of the FRET method. However, this may be of little importance in clinical practice. Current treatments for babesiosis and theileriosis are not species-dependent (Vial and Gorenflot, 2006; Wilkie et al., 1998).

T. annae has been has been reported in cattle and horses in Italy (Pietrobelli et al., 2007). Accordingly, it should be taken into account when designing any molecular test for

these herbivores. Although the diagnosis of piroplasmosis in horses was not a primary aim of this work, the FRET assay proved to be a good alternative to conventional PCR plus sequencing. Infections caused by bovine piroplasmids have been described in horses in Spain (Criado-Fornelio et al., 2006; Buling et al., 2007). The finding that horses in Argentina and Italy were infected by bovine babesids is therefore not surprising. Since the Argentine horses were positive for *T. equi* antibodies, it is likely that most of them had been exposed to ticks. Thus, the prevalence of bovine babesids in South American equines cannot be accurately estimated in such a biased sample. However, this is a clear indication that any babesiosis control campaign needs to take into account the role played by non-bovine hosts as disease carriers.

5. Conclusions

In the present study, two new qPCR tests were found to be valuable tools for diagnosing piroplasmid infection in experimental and survey animals. The duplex TaqMan assay is highly sensitive and particularly suitable for South American countries where only *B. bovis* and *B. bigemina* are found in bovids. The FRET test, albeit less sensitive than the former, has the advantage that it can be used worldwide to detect either babesids or theilerids, both in bovids and horses.

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References

Asenzo, G., Wilkowsky, S., Barrandeguy, M., Mesplet, M., Benitez, D., Florin-Christensen, M., 2008. Development of an indirect ELISA for the diagnosis of equine piroplasmosis. Ann. N. Y. Acad. Sci. 1149, 235–238.

Brígido, C., Pereira da Fonseca, I., Parreira, R., Fazendeiro, I., do Rosário, V.E., Centeno-Lima, S., 2004. Molecular and phylogenetic characterization of *Theileria* spp. parasites in autochthonous bovines (Mirandesa breed) in Portugal. Vet. Parasitol. 123, 17–23.

Buling, A., Criado-Fornelio, A., Asenzo, G., Benitez, D., Barba-Carretero, J.C., Florin-Christensen, M., 2007. A quantitative PCR assay for the detection and quantification of *Babesia bovis* and *B. bigemina*. Vet. Parasitol. 147, 16–25.

Buxton, D., Maley, S.W., Wright, S., Thomson, K.M., Rae, A.G., Innes, E.A., 1998. The pathogenesis of experimental neosporosis in pregnant sheep. J. Comp. Pathol. 118, 267–279.

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Saraña, A., Barba-Carretero, J.C., 2003a. Presence of *Mycoplasma haemofelis, Mycoplasma haemominutum* and piroplasmids in cats from southern Europe: a molecular study. Vet. Microbiol. 93, 307–317.

Criado-Fornelio, A., Martínez-Marcos, A., Buling-Saraña, A., Barba-Carretero, J.C., 2003b. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in Southern Europe—Part I: Epizootiological aspects. Vet. Parasitol. 113, 189–201.

Criado-Fornelio, A., Martinez, J., Buling, A., Barba, J.C., Merino, S., Jefferies, R., Irwin, P.J., 2006. New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome b genes. Vet. Parasitol. 142, 238–247.

Criado-Fornelio, A., 2007. A review of nucleic acid-based diagnostic tests for *Babesia* and *Theileria* with emphasis on bovine piroplasmida. Parassitologia 49S, 39–44.

Criado-Fornelio, A., Buling, A., Pingret, J.L., Etievant, M., Boucraut-Baralon, C., Alongi, A., Agnone, A., Torina, A., 2009. Prevalence and molecular characterization of haemoprotozoa of domestic animals in France. Vet. Parasitol. 159, 73–76.

Figueroa, J.V., Chieves, L.P., Johnson, G.S., Buening, G.M., 1992. Detection of *Babesia bigemina*-infected carriers by polymerase chain reaction amplification. J. Clin. Microbiol. 30, 2576–2582.

Gubbels, J., de Vos, A., van der Weide, M., Viseras, J., Schouls, L.M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J. Clin. Microbiol. 7, 1782–1789.

Iseki, H., Alhassan, A., Ohta, N., Thekisoe, O.M., Yokoyama, N., Inoue, N., Nambota, A., Yasuda, J., Igarashi, I., 2007. Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine *Babesia* parasites. J. Microbiol. Methods 71, 281–287.

Jeong, W., Kweon, C.H., Kang, S.W., Paik, S.G., 2003. Diagnosis and quantification of *Theileria sergenti* using TaqMan PCR. Vet. Parasitol. 111, 287–295.

Kim, C., Iseki, H., Herbas, M.S., Yokoyama, N., Suzuki, H., Xuan, X., Fujisaki, K., Igarashi, I., 2007. Development of TaqMan-based real-time PCR assays for diagnostic detection of *Babesia bovis* and *Babesia bigemina*. Am. J. Trop. Med. Hyg. 77, 837–841.

Kutyavin, I.V., Afonina, I.A., Mills, A., Gorn, V.V., Lukhtanov, E.A., Belousov, E.S., Singer, M.J., Walburger, D.K., Lokhov, S.G., Gall, A.A., Dempcy, R., Reed, M.W., Meyer, R.B., Hedgpeth, J., 2000. 3′-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res. 28, 655–661.

Olegario, L., Lima, R., Picoloto, G., Freitas, A., Madruga, C., Kessler, R., Araujo, F., Rosinha, G., Criado-Fornelio, A., Florín-Christensen, M., 2007. Comparison of semi-nested PCR and real time PCR for detection of *Babesia bovis* and *Babesia bigemina* in cattle. Parassitologia 49S1, 99.

Pietrobelli, M., Cancrini, G., Moretti, A., Tampieri, M.P., 2007. Animal babesiosis: an emerging zoonosis also in Italy? Parassitologia 49S1, 33–38.

Vial, H.J., Gorenflot, A., 2006. Chemotherapy against babesiosis. Vet. Parasitol. 138, 147–160.

Wei, T., Lu, G., Clover, G., 2008. Novel approaches to mitigate primer interaction and eliminate inhibitors in multiplex PCR, demonstrated using an assay for detection of three strawberry viruses. J. Virol. Meth. 151, 132–139.

Wilkie, G.M., Brown, C.G., Kirvar, B.E., Thomas, M., Williamson, S.M., Bell-Sakyi, L.J., Sparagano, O., 1998. Chemoprophylaxis of *Theileria annulata* and *Theileria parva* infections of calves with buparvaquone. Vet. Parasitol. 78, 1–12.