Title:

Equine Piroplasmosis status in the United Kingdom: an assessment of laboratory diagnostic submissions and techniques

Authors:

Robert M Coultous[a], Paul Phipps[b], Charlie Dalley[b], Jane Lewis[b], Toni-Ann Hammond[c], Brian R Shiels[d], William Weir[a] and David G M Sutton[a]

Affiliations:

[a] School of Veterinary Medicine
College of Medical, Veterinary and Life Sciences
University of Glasgow
Bearsden Road
Glasgow
Scotland
G61 1QH
United Kingdom

[b] Animal and Plant Health Agency
Woodham Lane
New Haw
Surrey
England
KT15 3NB
United Kingdom

[c] Diagnostic Laboratory Services

Animal Health Trust
Lanwades Park
Kentford
Newmarket
England
CB8 7UU
United Kingdom

[d] Institute of Biodiversity Animal Health and Comparative Medicine
College of Medical, Veterinary and Life Sciences
University of Glasgow
Bearsden Road
Glasgow
Scotland
G61 1QH
United Kingdom

Corresponding author:
Robert Coultous
r.coultous.1@research.gla.ac.uk
Telephone +44 (0)141 330 7516
Fax +44 (0)141 330 2271
School of Veterinary Medicine
College of Medical, Veterinary and Life Sciences
Room 217, Henry Wellcome Building
University of Glasgow

Bearsden Road
Glasgow
Scotland
G61 1QH
United Kingdom

Abstract

Equine piroplasmosis (EP) has historically been of minor concern to UK equine practitioners, primarily due to a lack of competent tick vectors. However, increased detection of EP tick vector species in the UK has been reported recently. EP screening is not currently required for equine importation, and when combined with recent relaxations in movement regulations, there is an increased risk regarding disease incursion and establishment into the UK.

This study evaluated the prevalence of EP by both serology and polymerase chain reaction (PCR) among 1,242 UK equine samples submitted for EP screening between February and December 2016 to the Animal and Plant Health Agency and the Animal Health Trust. Where information was available, 81.5 % of submissions were for the purpose of UK export testing, and less than 0.1 % for UK importation. Serological prevalence of EP was 8.0 %, and parasite DNA was found in 0.8 % of samples.

A subsequent analysis of PCR sensitivity in archived clinical samples indicated that the proportion of PCR-positive animals is likely to be considerably higher. We conclude the current threat imposed by UK carrier horses is not adequately monitored and further measures are required to improve national biosecurity and prevent endemic disease.

Introduction

The UK has historically remained free from endemic equine piroplasmosis (EP), despite a near ubiquitous global presence (1). Consequently, the disease has been of minimal concern to the UK equine practitioner and diagnostic testing has not been undertaken routinely, even in horses presenting with classical clinical signs such as haemolytic anaemia.

The basic pathology of EP together with the life-cycle of its causative pathogens, *Theileria equi* and *Babesia caballi*, are well described in the literature (1, 2, 3). Following inoculation by an infected tick vector, the protozoan parasite invades host erythrocytes, with additional invasion of host leukocytes in the case of *T. equi*. The parasite replicates in the equine erythrocytes leading to rupture of the infected cell. This releases parasite merozoites into the circulation, which further invade and replicate within erythrocytes, perpetuating the infection. Within the tick host, transmission of *T. equi* is through the transtadial route, while for *B. caballi* transtadial and transovarian transmission both occur (3). The clinical presentation of infection with one or both of these parasites is similar. Acute cases typically present with anaemia, pyrexia, lethargy, dehydration and anorexia with death occurring in severe or neglected cases (1, 2, 3). In chronic disease, clinical signs are less severe, with animals displaying variable anaemia, malaise, anorexia, weight loss and reduced performance (1, 2, 3). Infection with *T. equi* has been detrimentally associated with athletic performance (4) and has a significant impact on the racing industry of endemic areas (5). An association also has been claimed between EP and reduced fertility and abortion, with a reported 11 % of South African thoroughbred abortions being attributed to *T. equi* infection (6).

Importantly, the insidious nature of chronic and subclinical forms of the disease can lead to the creation of a latent carrier state that is particularly common in endemic regions. This has important implications for biosecurity. It is reported that *B. caballi* carrier status is self-limiting with clearance achieved four years post-infection (7), but this may be due to infection entering a latent stage (1). Clearance of *B. caballi* infection can been achieved through treatment with imidocarb dipropionate (8). *Theileria equi* carrier status is thought to be life-long and can be maintained despite medical treatment (9). The unmonitored importation of these carrier animals to different regions of the UK, compounded by a lack of tick control and prolonged co-grazing and mixing with naïve individuals, presents a potential means by which the infection could become established in the UK.

Although EP seropositive equids have been imported and present in the UK for many years, the lack of endemic EP in the British Isles has historically been attributed to a small and geographically limited vector tick population (10). Up to 33 tick species have been identified as known or potential vectors for EP (11), but *Dermacentor reticulatus* is the only confirmed EP vector species currently established in the UK. *Dermacentor reticulatus* populations were thought to be limited to areas in western Wales and Devon, however recent studies have documented geographical expansion of the species, with recognised populations now present in Essex (12). The epidemiological importance of these new *D. reticulatus* vector populations in the transmission of tick-borne disease was highlighted in a recent canine piroplasmosis outbreak in the Essex area (13).

EP has also been moving geographically closer to the UK in recent years, with an isolated *T. equi* outbreak in Ireland in 2009 (14), autochthonous cases of both *T. equi* and *B. caballi* reported in Holland in 2011 (15) and evidence of both parasites being well established in the Camargue of France (16). When combined with current policies mitigating restrictions of certain equine movements, such as the Tripartite Agreement of 2014 (17) and the proposed High Health High Performance (HHP) scheme (18), the threat of EP to the resident UK horse population is becoming of increasing concern.

The latest World Organisation for Animal Health (OIE) status of EP in the UK (July - December 2017) is ‘infection/infestation in domestic animals’, and ‘disease absent in wild animals’ (19). This reflects the presence of imported EP seropositive equids, with the absence of any autochthonous cases of endemic disease.

Currently EP is not reportable or notifiable in the UK and imported animals are not tested routinely, despite the fact that seropositive chronic carrier horses are known to act as reservoirs of parasite infection for suitable sympatric tick species if present (20). Serological testing in the UK is largely restricted to animals being exported to disease-free countries with compulsory import screening, such as USA, Australia and Japan, where the disease is notifiable and controlled.

It is useful to consider the diagnostic tests presently available for EP screening. Current OIE guidelines recommend the indirect fluorescent antibody test (IFAT) and the competitive enzyme-linked immunosorbent assay (cELISA) as the screening tests for international trade (21), and the older complement fixation test (CFT) is still available and utilised commercially. Although sensitive, serological testing such as the cELISA does not reflect level of parasitaemia or provide information on the likelihood of onward transmission to feeding ticks, since antibodies persist for many months after apparent clearance of infection (22). Polymerase chain reaction (PCR) methods and, specifically, nested PCR are considered to be the best means of establishing parasite burden in equids (3). Despite the description of many PCR protocols in the literature, a commercial PCR screening assay for EP is not readily available to UK practitioners.

The main aim of this pilot study was to investigate the potential risk posed by seropositive horses resident in the UK, using follow-up nested PCR to determine animals with a parasite burden. A nested PCR protocol was developed and validated in-house using known positive field specimens. Results from UK diagnostic submissions for EP serology were also collated to facilitate estimation of the proportion of this sampled population that was serologically and PCR positive, therefore presenting a potential transmission risk to feeding tick species.

Materials and methods

This prospective study utilised routine samples submitted by UK practitioners for EP serology testing at the Animal and Plant Health Agency (APHA) and the Animal Health Trust (AHT), between February and December 2016. Serological testing performed comprised CFT, IFAT and cELISA either singularly or in combination as requested by the submitting veterinary surgeon. The CFT, which was only available at the APHA, was performed in accordance with OIE standards using an in-house protocol. The APHA also performed IFAT assays using an in-house protocol in accordance with OIE standards; titres ≥ 1/80 were reported as positive. IFATs requested on AHT submitted samples were performed at the APHA, although the results have been associated with the AHT for data consistency (Table 1). For cELISA testing, both the AHT and APHA used commercially available kits (*Babesia caballi* 273-2 and *Babesia equi* 274-2, VMRD, USA), with a result of ≥ 40 % reported as positive.

Following EP serological screening, all samples from both institutes were then forwarded to the University of Glasgow as anonymised clotted equine blood samples. They were then subjected to nested PCR, allowing subsequent comparison to the serological test results supplied by each laboratory. As the samples were submitted for the primary purpose of serology testing, only clotted blood was available for PCR screening.

For DNA extraction, 200 µl of clotted blood was mechanically agitated then enzymatically digested with proteinase K prior to extraction with the QIAamp DNA Mini Kit (Qiagen), using the manufacturer's recommended protocol. A total of 1,211 samples were screened by nested PCR with a modified *Babesia/Theileria* 18S SSU rRNA catch-all primer set, with outer primers (23) and inner primers (24) as described previously. These primers were reported to effectively detect a range of *Theileria/Babesia* spp., including *T. equi* and *B. caballi* (23). Prior to sample screening, the reaction conditions were optimised in-house with known EP positive samples from Morocco, Gambia and Oman. Reaction conditions were an initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of 94 °C for 45s, with annealing at 67 °C (external primers) or 57 °C (internal primers) for 60s, elongation at 72 °C for 60s, and with a final extension at 72 °C for 5 minutes. A 1:10 dilution of the primary reaction product was used as a template for the secondary reaction. The final product was visualised on a 1 % agarose electrophoresis gel. The PCR product was purified (QIAquick PCR purification kit, Qiagen) prior to Sanger DNA sequencing (Eurofins Genomics, Germany).

Sequences were subject to BLAST comparison (https://blast.ncbi.nlm.nih.gov/) with the non-redundant NCBI database to achieve species identification.

In each case, the result of the nested PCR was then compared to the EP serological test result as supplied by the original laboratory. Although all data were anonymised, and information about sampled animals was unavailable, the reason for EP serological test submission was known for the majority of specimens. Additionally, an acute case of piroplasmosis was confirmed during the study period, seen in a horse previously imported but now resident in the UK. Samples from this horse were used to compare the effect of coagulated and anti-coagulated blood samples on nested PCR performance.

Results

Serological test results and nested PCR results from the full 1, 242 UK laboratory EP submissions are presented in Table 1. In summary, 5.9 % of samples submitted during the study period were serologically positive for *T. equi* (n = 70), and 4.4 %

serologically positive for *B. caballi* (n = 52). Overall EP seroprevalence was 8.0 % (n = 96), with 27.1 % of these (n = 26) being seropositive for both parasites. *Theileria equi* parasite DNA was detected in 0.8 % (n = 10) of the samples from these laboratory submissions. Sanger sequencing revealed that all nucleotide sequences detected had 97-100 % identity to the relevant section of the 18S SSU rRNA gene of *T. equi*. *Babesia caballi* DNA was not detected in any sample.

The purpose of EP serology as stated on the submission form, and where permitted without breach of data confidentiality, is summarised in Table 2. Testing prior to potential export is highlighted as the predominant reason (81.5 % of submissions), with only a single animal for UK importation being tested. It is unknown what proportion of seropositive horses in the present dataset had previously been imported to the UK. Specific data regarding the testing purposes for the 'other' category were not available.

In order to evaluate the sensitivity of EP serology, a comparison was made between those animals positive on nested PCR and serological status (see Table 3). Only four of the ten samples identified to have parasite DNA present were found to be seropositive, with variations between cELISA, CFT and IFAT test results. It was not possible to infer statistical agreement between the different test types, as not all samples were subjected to each test.

The effect of sample submission type (coagulated versus anti-coagulated EDTA blood) on PCR test results is demonstrated in Figure 1, with samples from a confirmed UK case of EP submitted to the study. The affected horse (L1) in this case was imported several months previously and had developed clinical signs of anaemia and pyrexia, consistent with acute piroplasmosis. After positive cELISA and IFAT serology for EP from AHT, a blood sample was collected for PCR analysis. Importantly, both a clotted and an anti-coagulated (EDTA) jugular blood sample were collected at the same time and stored identically before submission. DNA extraction and nested PCR EP testing were performed concurrently and in triplicate on the submitted samples, and the results compared. The coagulated sample produced negative results in each case, whilst all three of the anti-coagulated sample replicates produced a strong band that was subsequently sequenced and confirmed to be *T. equi* in origin.

Discussion

Within the 1,242 samples submitted to the UK diagnostic services during the period February to December 2016 from horses resident in the UK, the overall seroprevalence of EP was 8.0 %. Although there is sparse information regarding EP seroprevalence in northern Europe, this is in line with similar datasets from Holland (15) and Switzerland (25) with 4 % and 7.3 % seroprevalence reported in these countries, respectively. Additional PCR-RLB performed by Butler *et al* (15) on EDTA blood detected *T. equi* DNA in 1.6 % of samples and did not detect any *B. caballi* DNA. However, this is not directly comparable to the current study's *T. equi* DNA detection rate of 0.8 % and absence of detectable *B. caballi* DNA, as the use of EDTA samples by Butler *et al.* (15) may have provided greater sensitivity. Additionally, the sampled equine populations are not directly comparable between these and the current study. Butler *et al* (15) performed a cross-sectional study of 300 horses known to have been resident in the same location within Holland for at least one year. Sigg *et al* (25) reported that of their 689 sampled animals, 459 (66.6 %) were imported (having been brought to Switzerland up to five years prior to testing) and all of those had arrived from a European country. Seroprevalence was 8.5 % in these imported horses versus 4.8 % in indigenous horses (25). In both studies the previous movement history was limited or absent, making the geographical source of infection unclear. No geographical data or previous travel history was available for the current study samples due to data confidentiality.

Within the set of seropositive samples identified in this study, 27.1 % were found to be positive for both *T. equi* and *B. caballi*. This may be representative of exposure or infection by both parasites or serological false-positives (26); cross-reactivity with B. caballi has been noted at low titres with CFT and IFAT using serum from experimental *T. equi* infections (27). Due to a lack of further sampling and the absence of *B. caballi* identification by PCR, further investigation of this finding is beyond the scope of this study.

Discrepancies between IFAT, cELISA and nested PCR results have been reported in experimental infection (9), and this was noted in the present study. The discrepancies encountered were:

i) Serologically negative, PCR positive samples. It is shown in Table 3 that 6 of the 10 samples where *T. equi* DNA was detected had negative serology results. Conventional logic would suggest that a detectable level of parasite DNA should promote a detectable immune response. The absence of seroconversion in the presence of parasite DNA could either be due to an early stage of infection or a fluctuating parasitaemia, where samples were taken at a time of parasite proliferation but before the rise of a detectable antibody titre. This anomaly has been noted in the early course of experimental infection (9), and there is indication that CFT may be more sensitive than other serological methods in these early stages of infection (28). Disease recrudescence in EP has been noted to occur at times of increased stress and immunosuppression, such as may occur with increased handling, transport, co-infection and even lactation (29). This phenomenon results in parasitic multiplication and the development of clinical signs in previously disease-free carrier animals. Whilst recent movement may have resulted in parasite recrudescence in a proportion of the animals in this study, it is unlikely that all of them would have been free from detectable levels of antibodies because once established as carriers, animals seroconvert to EP (9).

The discrepancy between test modalities may have resulted also from the intrinsic limitations of the serological testing. Serological tests can give false-negative results (26) and this incongruity has been observed in previous studies. One example is a recent Venezuelan study which found *T. equi* to have a much higher PCR prevalence (61.8 %) than seroprevalence (14.0 %) (30). Additionally, Bhoora *et al* (31) postulated that genetic variation of the EMA-1 antigen, on which the cELISA used by APHA and AHT is based, may have prevented the detection of some South African strains of *T. equi* using this diagnostic technique.

ii) High-titre serologically positive, PCR negative results. It was anticipated that a high serological titre would be associated with the presence of circulating parasite DNA and a positive PCR result. However, this was not seen in 15 high-titre (≥ 1/640) IFAT positive samples that were evaluated (data not shown). Titre values for the cELISA were not available. A potential reason for this became evident following a private sample submission to the project from an imported horse (L1). This horse was undergoing veterinary evaluation following presentation with acute anaemia and pyrexia. Tested in triplicate, Figure 1 shows that template DNA derived from EDTA blood samples provided clear positive bands, while the clotted blood samples were consistently negative. The reasons for this may include the degradation or reduction of available parasite DNA within the clotted samples and transfer of inhibitors during DNA extraction. Regardless of the exact cause, this clearly demonstrates a significant reduction in PCR sensitivity using clotted blood samples, although the full extent of this requires validation in additional cases.

All PCR screening in this study was performed on clotted blood samples, using the residual sample following serological evaluation. These were the only diagnostic specimens available to the group in this instance. Given the evidence presented in Figure 1, if clotted blood samples cannot provide a repeatable PCR positive result for EP from a horse with active disease and acute clinical signs, then this has important implications for reported negative PCR results. Despite the screening data initially appearing consistent with results from comparable studies in other countries, the availability of primarily clotted blood samples in this study is likely to have significantly underestimated the number of *T. equi* PCR positive carrier animals in the sample set. This may also explain the complete absence of *B. caballi* detection by PCR despite serological detection among the samples. Consequently, we recommend avoiding the use of clotted blood samples for PCR screening.

iii) Low-titre serologically positive, PCR negative samples. Typically these may simply represent previous disease exposure, though in the case of EP it could signify a latent carrier state that lacks sufficient circulating parasite for DNA detection. Alternatively, these could be serological false-positive results, an issue inherent with serological testing (26). However, given the apparent reduction of PCR sensitivity in this study, no further interpretation can be made on these samples.

Another conspicuous finding of this study is the apparently low uptake of EP testing in horses in the UK following importation (Table 2). Strikingly, only a single sample of 1,097 submitted to APHA was for the purpose of determining EP status at time of importation to the UK, strongly suggesting that there is widespread lack of awareness or indifference to EP biosecurity within the UK veterinary and equine industries. The most common purpose cited for sample submission was pre-export testing. This implies that the main driver for EP screening is to meet mandatory requirements for foreign export and not clinical investigation, and highlights the more stringent EP biosecurity controls imposed by other non-endemic countries such as USA, Australia, New Zealand and Japan.

In summary, this study shows that a small but important proportion of equids residing in the UK are seropositive for EP, and that parasite DNA is detectable in a further proportion of these. Given the diagnostic limitations imposed in this study, namely the use of remnant clotted material following serological testing, it is likely that piroplasmosis DNA is present in a higher proportion of UK equids than reported here. As it is known that carriers of EP may undergo disease recrudescence at times of co-infection, stress and immunosuppression, UK veterinary practitioners should be aware that EP should be a differential diagnosis for horses presenting with characteristic clinical signs in this country, which may include pyrexia, lethargy and evidence of haemolysis.

Although a detailed distribution of EP vector tick species within the UK is not fully known, the presence of equids positive for parasite DNA in tick-infested pasture should be considered a potential risk for disease transmission to co-grazing equids, and this requires assessment. The authors note that the factors of reduced restrictions on international equine movement and an absence of any UK formal import screening for EP, coupled with the limitations of current testing methods, present a continued risk to the UK equine population and industry. This study suggests that a combined approach of serology and parasite DNA detection is required to provide the most efficacious EP screening protocol. It is also suggested that in the event of positive animals being identified in the UK, follow-up screening of co-grazing animals and ticks could be considered as a means of local and national disease surveillance. The authors believe that a change in attitude towards the disease and national EP biosecurity is required before endemic disease establishment creates a complex problem that is more difficult to resolve.

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	No. of samples	*T. equi* serology (No. of positives/total no. of tests)					*T. equi* PCR	*B. caballi* serology (No. of positives/total no. of tests)					*B. caballi* PCR
		CFT	IFAT	cELISA	**Total unique seropositives**			CFT	IFAT	cELISA	**Total unique seropositives**		
APHA	1097	31/482	39/502	9/562	66/1050	**6.3 %**	7/1066	17/479	33/504	2/563	49/1049	**4.7 %**	0/1066
AHT	145	NA	4/9	4/145	4/145	**2.8 %**	3/145	NA	1/9	2/145	3/145	**2.1 %**	0/145
Total	1242	**6.4 %**	**8.4 %**	**1.8 %**	**5.9 %**		**0.8 %**	**3.5 %**	**6.6 %**	**0.6 %**	**4.4 %**		**0 %**

Table 1. Breakdown by test type of EP positive results from samples screened between February and December 2016. The results are listed by submitting organisation and test type. As some samples were found to be positive by multiple serological methods; the 'Total unique seropositives' columns show the number of discrete positive samples for each species.

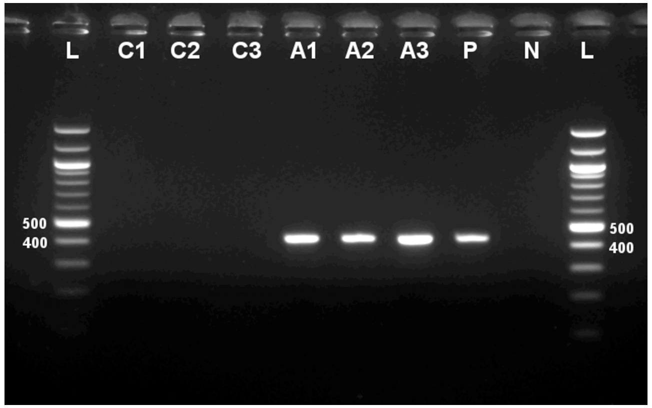
	Reason for EP testing			
	Import	Export	Other	Unknown
APHA	1/1097	894/1097	189/1097	13/1097
AHT	NA	NA	NA	145/145

Table 2. Reason for sample submission as noted by the submitting veterinary surgeon. Most samples were submitted prior to intended export, highlighting that some countries require EP serology status to be determined prior to granting an importation licence. Notably only one sample was specifically submitted to determine EP serological status at time of importation to the UK.

Samples positive by nested PCR							
ID	Organisation	CFT (*T. equi*)	IFAT (*T. equi*)	cELISA (*T. equi*)	CFT (*B. caballi*)	IFAT (*B. caballi*)	cELISA (*B. caballi*)
VLA12	APHA	NA	Negative	NA	NA	Negative	NA
VLA14	APHA	NA	Negative	NA	NA	Negative	NA
VLA15	APHA	NA	Negative	NA	NA	Negative	NA
VLA255	APHA	NA	***Positive***	NA	NA	***Positive***	NA
VLA265	APHA	***Positive***	***Positive***	***Positive***	Negative	NA	Negative
VLA269	APHA	NA	NA	Negative	NA	NA	Negative
VLA761	APHA	***Positive***	Negative	***Positive***	***Positive***	Negative	Negative
AHT18	AHT	NA	Negative	Negative	NA	Negative	Negative
AHT21	AHT	NA	Negative	Negative	NA	Negative	Negative
L1	AHT	NA	***Positive***	***Positive***	NA	Negative	Negative

Table 3. Serological data for samples found to be positive by nested PCR during the study. These samples were all positive for T. equi and negative for B. caballi on sequencing of the PCR product.

Figure 1. An electrophoresis gel showing the final PCR product from sample L1. The expected fragment length for T. equi was 433 bp. Template DNA was extracted from clotted blood samples (C1-3) and from EDTA samples (A1-3). Controls using DNA extracted from known EP positive (P) and EP negative (N) horse blood are shown together with a 100 bp ladder (L).



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