Title

Investigating equine piroplasmosis presence in Ireland

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Abstract (200)

Background

Equine piroplasmosis (EP) is a notifiable disease in Ireland and a significant concern to domestic and international equine industries. Information regarding EP presence in Ireland is currently limited. This retrospective surveillance study

describes a serological and molecular analysis of blood samples submitted to the Irish Equine Centre (IEC) for EP testing between January 2013 and April 2016.

Methods

Following serological testing, seropositive samples were screened using a polymerase chain reaction (PCR) targeting the 18S ribosomal RNA gene. Amplicon sequences were bioinformatically analysed to identify the parasite species and to assess genetic diversity.

Results

From 2,099 screened equine blood samples, 2.5 % and 1 % were seropositive for *Theileria equi* and *Babesia caballi* respectively. *T. equi* DNA was detected in 9 % of the seropositive samples while *B. caballi* DNA was not detected in any sample. The *T. equi* DNA sequences displayed no genetic diversity at this locus, in contrast to samples from the UK and from endemic areas.

Conclusion

Detection of EP seropositive and parasitaemic horses in Ireland indicates a clear and present health risk to the equine population. It is recommended that owners adopt appropriate biosecurity measures and clinicians are mindful of this disease as a differential diagnosis.

Introduction

Equine piroplasmosis (EP) is a disease of global economic and welfare significance for horses and is caused by single or co-infection with the apicomplexan haemoparasites *Theileria equi* and *Babesia caballi (1)*. Natural transmission occurs via a number of identified tick vector species *(2)*, but several

74 outbreaks in non-endemic areas have been attributed to iatrogenic transmission
75 from the use of contaminated hypodermic needles and other equipment *(3,4)*.
76 Although EP can cause severe disease including death, recent outbreaks have
77 shown that less than 1 % of infected equines may exhibit clinical signs of acute
78 disease *(5)*. A major concern is the chronic form of the disease, which is much
79 more common in affected populations than acute manifestation of infection.
80 Chronic EP can have a negative impact on athletic performance *(6,7)* and
81 increase the incidence of abortion *(8)*. Importantly, chronically infected animals
82 may act as a reservoir of infection, with parasites potentially persisting
83 indefinitely and acting as a source for onward transmission of EP *(9)*. These
84 carrier animals may exhibit mild or no clinical signs, potentially leading to the
85 insidious establishment of disease in naïve equine populations, as documented in
86 EP outbreaks in Florida in 2008 *(4)* and Texas in 2009 *(5)*.
87 It is not just the direct effects of clinical disease, abortion and reduced
88 performance that have an impact on the equine industry. A number of countries
89 prominent in the international equine community currently hold EP-free status,
90 including Ireland *(10)*. Several of these countries, such as the USA, Australia and
91 Japan, prohibit the entry of EP-positive horses in order to maintain national EP
92 biosecurity. Since EP carrier status can be life-long *(11)*, these restrictions have
93 the potential to permanently exclude EP-positive competition horses from
94 international travel. Such disruption to competition and trade at a multi-national
95 level has been a significant logistical and financial burden to the global equine
96 industry for many decades *(12)*.
97 EP is a notifiable disease in Ireland *(13)*, however there is currently no
98 compulsory screening programme for imported horses and no active EP

surveillance is undertaken in the country. Ireland is understood to be free of tick species capable of acting as vectors for the parasite, although no comprehensive national tick survey has been conducted to date *(14)*. Despite this, an EP outbreak was reported in 2009 on an equine establishment in County Meath, where 28 of the 60 horses on the premises were found to be EP positive at the time of investigation (15).

This study presents the results of a retrospective analysis of EP-positive samples submitted to the Irish Equine Centre (IEC) between January 2013 and April 2016 and the relevance and implications of these findings are considered.

Materials and methods

This retrospective study utilised available remnant serum samples from horses screened as serologically positive for EP by the IEC between January 2013 and April 2016. Permission for sample use in research studies was obtained by the IEC at the point of submission.

Samples were provided to the University of Glasgow in an anonymised fashion, with the only metadata available being the date of sample submission and the parasite species to which they were recorded as seropositive. Although the IEC offers complement fixation test (CFT), indirect fluorescent antibody test (IFAT) and competitive ELISA (cELISA) testing, due to data protection legislation and a requirement to maintain full anonymity, the diagnostic method that resulted in the samples being deemed seropositive was not recorded in the present study.

DNA was extracted from serum samples using the Qiamp DNA Mini Kit (Qiagen, Germany) following the manufacturer's recommended protocol.

Extracted DNA samples were screened with a *Babesia/Theileria* 18S SSU rRNA catch-all nested PCR, using published primers and reaction conditions previously used to detect EP parasite DNA in a similar survey of samples submitted to UK diagnostic laboratories (16). PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) following the manufacturer's recommended protocol prior to commercial Sanger DNA sequencing (Eurofins Genomics, Germany).

The sequences generated were initially subject to Basic Local Alignment Search Tool (BLAST) comparison (https://blast.ncbi. nlm.nih.gov/), using the non-redundant National Center for Biotechnology Information (NCBI) database, to allow species identification.

The sequences were then subject to genetic analysis, which included comparison to sequences from UK laboratory submissions (16) and field samples from the EP-endemic countries of Morocco, Oman and The Gambia (17) generated using the same nPCR method. Sequence alignments were performed using the MUSCLE method (18) within the AliView software package (19), and nucleotide diversity statistics, including expected heterozygosity (H_e), nucleotide diversity (π) and haplotype multiplicity, were generated using DnaSP (20). A neighbour-joining phylogenetic tree was created and visualised using MEGA7 (21) to establish the phylogenetic positioning of the detected sequences. Additionally, heat maps were generated in Microsoft Excel to illustrate genetic similarity between sequences.

Results

147 A total of 2,099 blood samples were submitted to the IEC for EP serology
148 screening in the period between January 2013 and April 2016. Overall, 74
149 (3.5 %) of these submissions were EP-seropositive, with 53 samples (2.5 %)
150 seropositive for *T. equi* and 21 samples (1 %) seropositive for *B. caballi*. No
151 samples were found to be seropositive for both parasites.
152 Of the 74 EP-seropositive serum samples from this period, 67 were available for
153 further screening with the *Babesia/Theileria* catch-all nested PCR (nPCR) assay.
154 Amplicons were generated for 7 of the 67 serum samples, however Sanger
155 sequencing was only successful for six of the seven samples, with one sample
156 producing a poor-quality sequence which was excluded from further analysis.
157 BLAST analysis revealed that all sequences were *T. equi.* Thus, *Theileria equi* DNA
158 was detected in 9 % of the 67 serum samples while *Babesia caballi* DNA was not
159 detected in any sample. All samples where *T. equi* DNA was detected were
160 seropositive for *T. equi* only.
161 All sequences obtained from isolates in this study were submitted to GenBank
162 (NCBI), available with accession numbers MN934822-MN934827 for the Irish
163 isolates, and accession numbers MT613662-MT613682 for isolates from the UK
164 and endemic countries.
165 The metadata and laboratory results associated with the six sequenced *T. equi*
166 PCR positive samples are shown in Table 1.
167 To compare the level of genetic diversity in Irish samples to those from another
168 non-endemic area and endemic areas, a further collection of field isolates was
169 typed using the same method. These represented samples from the UK (n = 9),
170 Morocco (n = 6), Oman (n = 1) and The Gambia (n = 5). Multiple sequence
171 alignment was undertaken using these sequences together with sequences

generated from published studies, downloaded from GenBank (NCBI). A phylogenetic tree was constructed and annotated with the previously described *T. equi* clades (A-E) (22,23) (Figure 1).

The analysis confirmed all novel sequences as *T. equi*. The Irish samples were restricted to clade E and displayed no genetic diversity at this locus, appearing completely identical to one another. In order to test whether the observed lack of clade-level genetic diversity was meaningful, the distribution of genotypes from Irish samples was compared to those from the UK, where EP is also not endemic. These were found to be statistically significantly different, ($P = 0.0278$, Fisher's exact test with Freeman-Halton extension). To investigate the variation in diversity between the samples from different regions at the sequence level, within-country expected heterozygosity (H_e) was calculated using the allelic sequences derived from the Irish, UK, Moroccan and Gambian samples (Table 2). Relatively high heterozygosity ($H_e \geqslant 0.75$) was evident in all sample sets, except those from Ireland. To further investigate this issue, for each country, the nucleotide diversity (π) and average number of differences between samples (k) was calculated. This analysis further highlighted the lack of diversity among the Irish samples, which contrasted with that of other countries which showed substantial variation at the nucleotide level (Table 2). Finally, pair-wise similarity was calculated between the sequences used in this study and illustrated in the form of a triangular matrix heat map (Figure 2). Three of the nine sequences derived from UK horses were found to be identical to the sequence detected in the Irish samples.

Discussion

This report highlights that, despite EP being a notifiable disease under current regulations, a small but important proportion of the Irish equine population is EP seropositive. When compared to our previous study of UK laboratory EP screening submissions, approximately half as many samples were found to be seropositive in the Irish dataset (3.5 %) compared to the UK (8.0 %). However, among EP-seropositive samples, both countries displayed a similar proportion of *T. equi* seropositives (UK 72.9 %; Ireland 71.4 %). In contrast, a higher proportion of EP-seropositives was recorded as *B. caballi* in the UK samples (54.2 %) versus the Irish samples (28.6 %). Interestingly, whilst 27.1 % of the UK EP-seropositive samples were positive for both parasites, co-infection was not noted in any of the Irish samples.

The *B. caballi* prevalence and co-infection disparity between the two studies is challenging to explain. While this could be representative of the differing geographical origins of horses imported into each country, the necessary metadata to investigate this issue was not available in either study. EP serological detection can have variable sensitivity depending on the species and genotype being tested; issues have been described with CFT (24), IFAT (25) and cELISA (26,27). Again, as the required information on the diagnostic methods used to screen each sample was not available this issue could not be explored further.

Among the seropositive Irish samples, nine percent of these had detectable circulating parasite DNA within the serum, confirming their status as EP-carrier animals with a potential for onward transmission. Detecting piroplasm DNA in such a high proportion of seropositive serum samples was surprising, as the EP parasites spend the majority of their lifecycle within erythrocytes of the equine

host, which are extremely scant within serum samples. Therefore, it is likely that detected parasite DNA was derived either from the parasite's transient extracellular merozoite stage or from piroplasms released from erythrocytes damaged during the original centrifugation of the blood sample. Consequently, it is justifiable to speculate that the true number of carrier animals with circulating parasite DNA in this sample population was considerably higher.

Theileria equi positive samples from this Irish survey were unusual as, despite their temporal spread, being collected from animals over a period of 14 months, all belonged to the same clade, namely clade E (Figure 1). This finding was somewhat unexpected, as *T. equi* is known to have an unusually high level of intra-species genetic polymorphism evident at the 18S SSU rRNA target locus (28), including considerable within-clade diversity. Up to five *T. equi* clades (A-E) have been proposed (22), however some contemporary authors have maintained a four clade classification system (A-D) based on the variation at the 18S rRNA locus (17,23,29), considering clade E to be a part of clade B. Indeed, the presence of novel species within the *T. equi* umbrella has been suggested, such as the recently described *Theileria haneyi* (30). Unlike the Irish samples, the *T. equi* samples derived from endemic countries were found to be distributed across more than one clade. This was expected, based on the observations of other authors in well-established, endemic *T. equi* areas *(23,28,31)*. UK positive samples were also dispersed across the tree, with samples representing clades A, D and E detected. Given these UK cases are likely to be epidemiologically unrelated and represent imported carrier animals originating from a variety of geographical origins, it is not unexpected they contain a variety of *T. equi* genotypes. Furthermore, the genetic diversity of the UK samples, relative to the

247 marked lack of clade-level diversity in the Irish samples, was found to be
248 statistically significant (P = 0.0278). This was further supported by a low
249 expected heterozygosity and nucleotide diversity within the Irish samples (Table
250 2) and is visually demonstrated on the heat map (Figure 2).
251 This finding raised two potential hypotheses:
252 i) A reason for the single clade grouping of Irish samples may simply be that EP-
253 positive animals were imported from similar, or even different, geographical
254 locations, with all samples co-incidentally belonging to the same 18S SSU rRNA
255 clade. A study of 100 Swiss equine samples discovered that five of the seven *T.*
256 *equi* samples detected by PCR were confirmed to be derived from clade E, and of
257 those, three were known to have originated from different countries (Italy,
258 France and Hungary) (32). In the previous UK study (16), three of the ten
259 detected samples were also derived from clade E, further indicating the
260 prevalence of this clade. Unfortunately, the travel history or even domicile of the
261 sampled Irish equines was unavailable, due to data confidentiality, and in the
262 absence of such data this theory cannot be verified.
263 ii) An alternative and more concerning hypothesis also needed to be considered.
264 The statistically significant lack of genetic diversity in the Irish equine samples
265 would be consistent with some or all parasite isolates being isogenic clones from
266 a point source of infection, thereby suggesting possible local transmission
267 events. Indeed, four of the DNA positive samples were submitted within 48 hours
268 of each other, with three also possessing sequential identifiers suggesting they
269 may be epidemiologically linked, either as submissions from a single premises or
270 as a cohort of imported animals. Although Ireland has been described as free
271 from *T. equi* vector tick species, the lack of a national survey or active

surveillance programme does not rule out the possibility of undocumented tick populations with the potential to transmit the parasite (14). Spread through mechanical means via contaminated hypodermic needles and equipment is also another potential mode of transmission, which has been implicated in outbreaks in other EP-free countries (4). Unfortunately, the low quantity of DNA in the source serum samples prevented investigation of diversity at other genetic loci, precluding further bioinformatic investigation of the point-source infection hypothesis.

Following the completion of this study, the IEC was able to help address the hypotheses generated by providing specific metadata on the seropositive samples, whilst maintaining confidentiality. The authors can report that the majority of EP seropositive samples detected (64 %) came from a single premises holding a substantial population of horses imported from France, temporarily resident in Ireland for the thoroughbred breeding season. This premises pursues a policy of monitoring known EP-positive horses during their residency in the country and six of these animals were repeat sampled during their stay. The second largest contributor of EP seropositive animals (15 %) was found to be an equine rescue premises, which included imported *B. caballi* seropositive donkeys. All other seropositive samples came from animals with a history of foreign importation, and no signs of clinical piroplasmosis had been reported in any of the submitted samples. Additionally, the PCR positive samples identified in this study were confirmed to be from unique individuals and not the product of repeat sampling. These observations strongly support the first hypothesis in explaining the lack of clade-level diversity among the study's samples.

It should be noted that the sample collection for Ireland analysed in this study was limited in scale and timespan, and not necessarily representative of the ongoing situation in the country as a whole. Our findings do indicate, though, that to detect and investigate the origin of putative outbreaks in future, such as the outbreak reported in 2009 (15), high-resolution genotyping would be a useful addition to the epidemiological and serological approaches currently used.

This report highlights the presence of a small but important proportion of EP-seropositive animals present in the Irish equine population. Significantly, a subset of these was shown to have detectable circulating parasitaemia, posing a risk for forward transmission of the disease. Horse owners should be cognisant of the potential presence of equine piroplasmosis in Ireland and adopt suitable biosecurity measures. Similarly, veterinary clinicians should consider this condition as a differential diagnosis when confronted with a case displaying appropriate clinical signs, and be aware of the possibility of clinical disease occurring within horses resident within the country. It is advised that positive cases should be investigated further with the aim of determining the origin of infection, especially in those cases without a history of foreign travel.

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Competing interests

The authors declare they have no competing interests.

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ID	GenBank accession number	Sampling date	*T. equi* serology	*B. caballi* serology	*Babesia/Theileria* nPCR catch-all	Sequencing identification
13C06470	MN934822	28/08/13	Positive	Negative	Positive	*T. equi*
13C06503	MN934824	29/08/13	Positive	Negative	Positive	*T. equi*
13C06505	MN934827	29/08/13	Positive	Negative	Positive	*T. equi*
13C06679	MN934826	05/09/13	Positive	Negative	Positive	*T. equi*
14C05489	MN934825	13/06/14	Positive	Negative	Positive	*T. equi*
14C10368	MN934823	14/11/14	Positive	Negative	Positive	*T. equi*

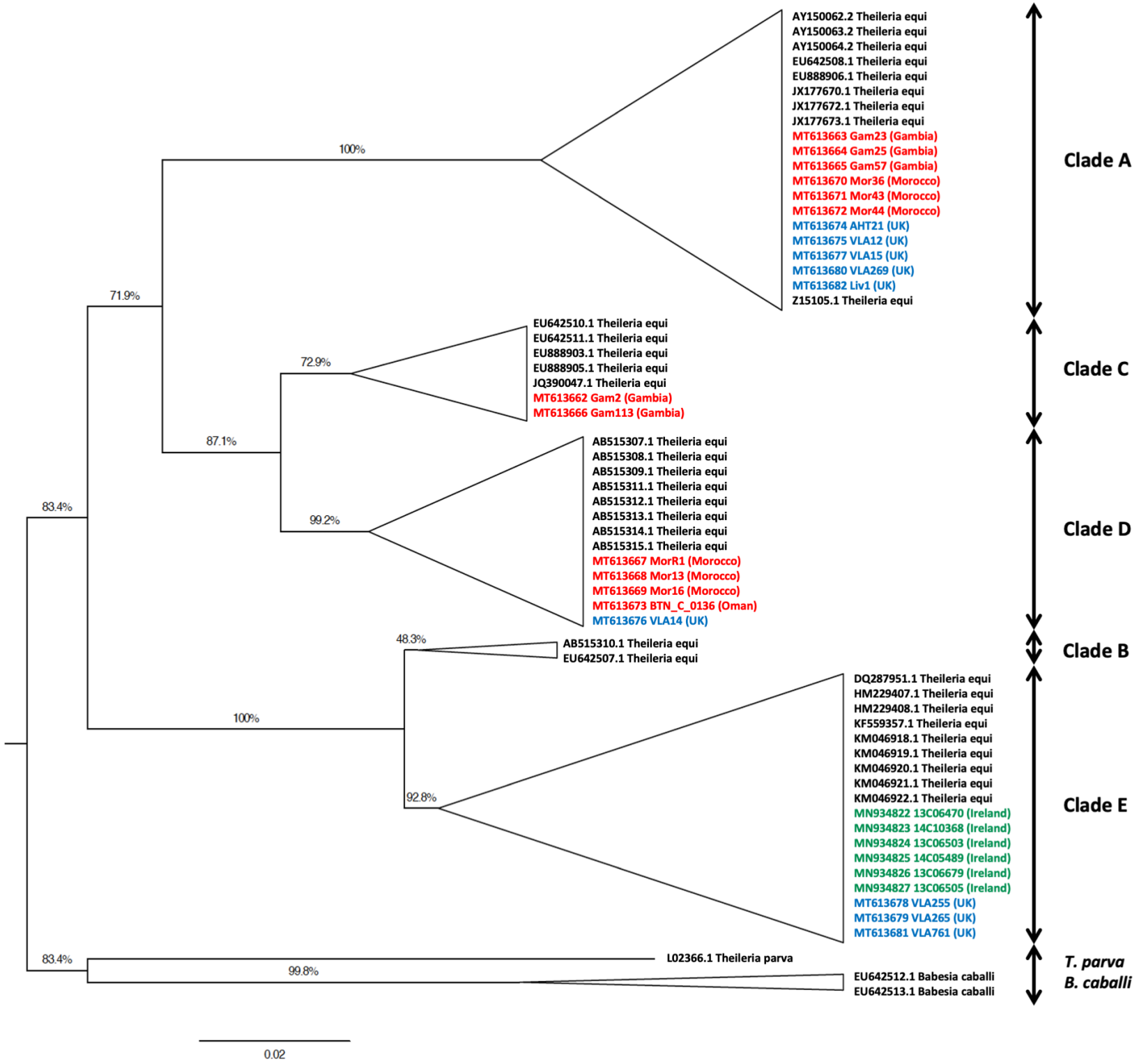
Table 1 - Summary of the metadata and laboratory results for the samples in which T. equi DNA was detected. For reasons of confidentiality, the exact method of serological testing was unavailable.

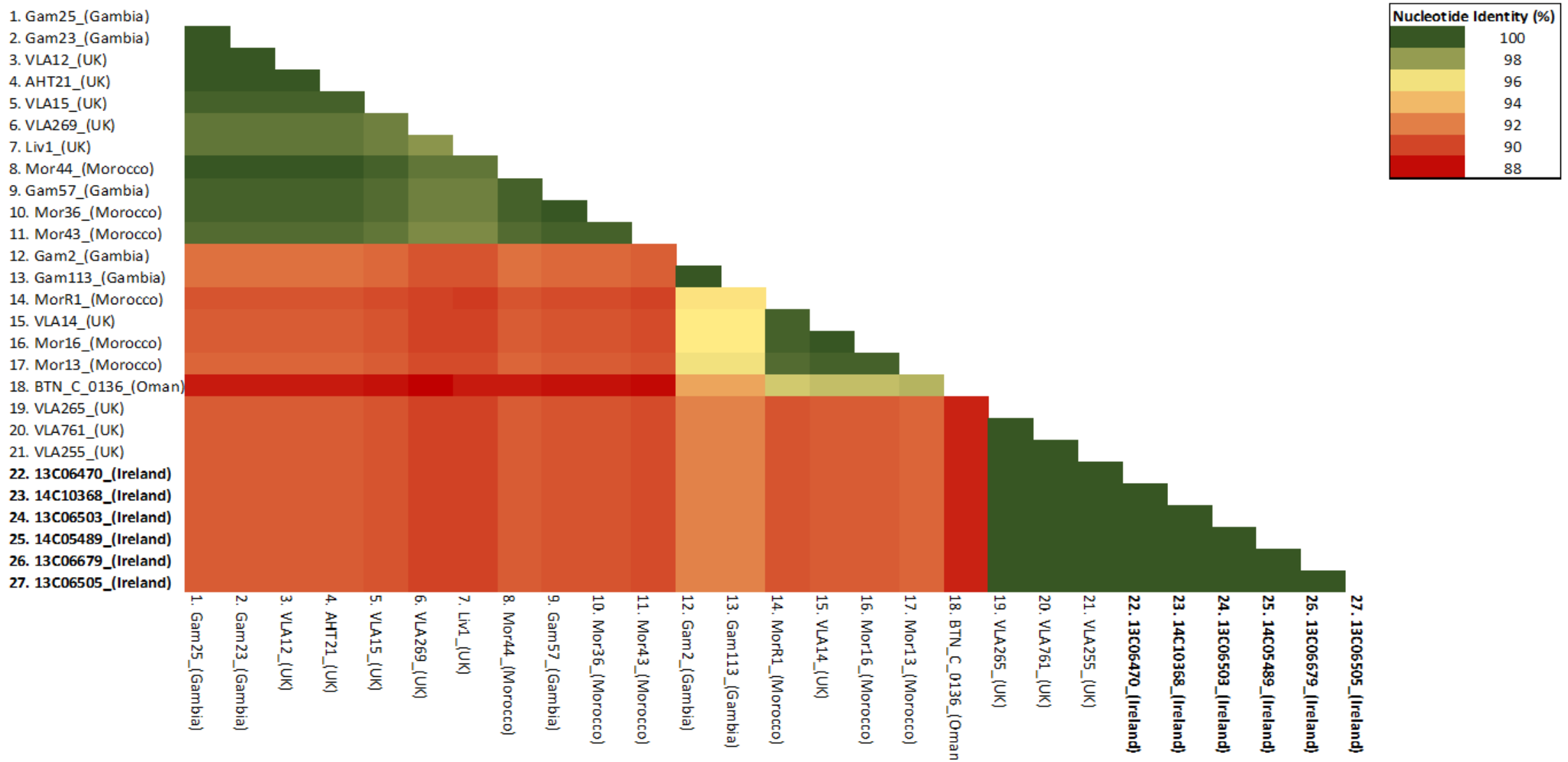
Country of origin	Number of sequences	Number of haplotypes	Expected heterozygosity (H_e)	Nucleotide diversity (π)	Average number of pair-wise differences between samples (k)
UK	9	4	0.750	0.056	17.778
Gambia	5	3	0.800	0.037	11.800
Morocco	6	6	1.000	0.052	16.733
Ireland	6	1	0.000	0.000	0.000

Table 2 – An analysis of genetic diversity at the 18S SSU rRNA locus present within samples from different geographical origins.

Figure 1 - A neighbour-joining tree inferring the evolutionary relationship of the 18S SSU rRNA gene segments from the T. equi samples identified in this survey. Included are all detected T. equi sequences from Ireland (green), the UK (blue) and endemic countries (red). Other representative sequences of T. equi, T. parva and B. caballi (to which the tree is rooted) are included with GenBank accession numbers. Bootstrap values are shown as a percentage, based on 1,000 replications. The previously described T. equi clades, A-E, have been annotated, and external branches have been collapsed to the clade level.

Figure 2 – A heat map demonstrating the nucleotide identity of Theileria equi samples from a number of geographical regions. 100% nucleotide identity was noted between all detected Irish samples (highlighted in bold).


AY150062.2 Theileria equi
AY150063.2 Theileria equi
AY150064.2 Theileria equi
EU642508.1 Theileria equi
EU888906.1 Theileria equi
JX177670.1 Theileria equi
JX177672.1 Theileria equi
JX177673.1 Theileria equi
MT613663 Gam23 (Gambia)
MT613664 Gam25 (Gambia)
MT613665 Gam57 (Gambia)
MT613670 Mor36 (Morocco)
MT613671 Mor43 (Morocco)
MT613672 Mor44 (Morocco)
MT613674 AHT21 (UK)
MT613675 VLA12 (UK)
MT613677 VLA15 (UK)
MT613680 VLA269 (UK)
MT613682 Liv1 (UK)
Z15105.1 Theileria equi
Clade A
100%
71.9%
EU642510.1 Theileria equi
EU642511.1 Theileria equi
EU888903.1 Theileria equi
EU888905.1 Theileria equi
JQ390047.1 Theileria equi
MT613662 Gam2 (Gambia)
MT613666 Gam113 (Gambia)
72.9%
Clade C
87.1%
AB515307.1 Theileria equi
AB515308.1 Theileria equi
AB515309.1 Theileria equi
AB515311.1 Theileria equi
AB515312.1 Theileria equi
AB515313.1 Theileria equi
AB515314.1 Theileria equi
AB515315.1 Theileria equi
MT613667 MorR1 (Morocco)
MT613668 Mor13 (Morocco)
MT613669 Mor16 (Morocco)
MT613673 BTN_C_0136 (Oman)
MT613676 VLA14 (UK)
83.4%
99.2%
Clade D
48.3%
AB515310.1 Theileria equi
EU642507.1 Theileria equi
Clade B
DQ287951.1 Theileria equi
HM229407.1 Theileria equi
HM229408.1 Theileria equi
KF559357.1 Theileria equi
KM046918.1 Theileria equi
KM046919.1 Theileria equi
KM046920.1 Theileria equi
KM046921.1 Theileria equi
KM046922.1 Theileria equi
MN934822 13C06470 (Ireland)
MN934823 14C10368 (Ireland)
MN934824 13C06503 (Ireland)
MN934825 14C05489 (Ireland)
MN934826 13C06679 (Ireland)
MN934827 13C06505 (Ireland)
MT613678 VLA255 (UK)
MT613679 VLA265 (UK)
MT613681 VLA761 (UK)
100%
92.8%
Clade E
83.4%
L02366.1 Theileria parva
99.8%
EU642512.1 Babesia caballi
EU642513.1 Babesia caballi
T. parva
B. caballi
0.02


1. Gam25_(Gambia)
2. Gam23_(Gambia)
3. VLA12_(UK)
4. AHT21_(UK)
5. VLA15_(UK)
6. VLA269_(UK)
7. Liv1_(UK)
8. Mor44_(Morocco)
9. Gam57_(Gambia)
10. Mor36_(Morocco)
11. Mor43_(Morocco)
12. Gam2_(Gambia)
13. Gam113_(Gambia)
14. MorR1_(Morocco)
15. VLA14_(UK)
16. Mor16_(Morocco)
17. Mor13_(Morocco)
18. BTN_C_0136_(Oman)
19. VLA265_(UK)
20. VLA761_(UK)
21. VLA255_(UK)
22. 13C06470_(Ireland)
23. 14C10368_(Ireland)
24. 13C06503_(Ireland)
25. 14C05489_(Ireland)
26. 13C06679_(Ireland)
27. 13C06505_(Ireland)
Nucleotide Identity (%)
100
98
96
94
92
90
88