Title:

Molecular confirmation of *Sarcocystis fayeri* in a donkey

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Abstract:

Sarcocystis fayeri is a canine protozoan parasite with an equine intermediate host. Historically classified as an incidental pathogen, recent literature has described the toxic effects of *Sarcocystis fayeri* in human food poisoning, and highlighted potential involvement in equine neuromuscular disease. Until now, horses were believed to be the exclusive intermediate host. This study reports the first molecular confirmation of *S. fayeri* in a donkey, and gives rise to the consideration of donkeys being a potential reservoir for the parasite. This finding is of particular importance in understanding the epidemiology of this disease.

Keywords:

Sarcocystis fayeri; sarcosporidiosis; donkey; Gambia

1. Introduction

The protozoan parasite *Sarcocystis fayeri* was first described in the United States following post-mortem identification in horses at slaughter (Dubey et al., 1977). These naturally infected horses were used to experimentally demonstrate the parasite's intermediate equine 'prey host' and final canine 'predator host' life-cycle, that is typical of the genus. Bradyzoite infected equine tissue was fed to naïve domestic dogs which produced sporocysts in their faeces from twelve days following ingestion of infected meat (Dubey et al., 1977). The canine hosts remained free of clinical signs throughout the study. Sporocysts obtained from experimentally infected dogs were subsequently used to inoculate naïve ponies, with schizonts observed histologically in cardiac capillaries from ten days post-inoculation. Intramuscular cysts in the tongue, oesophagus, diaphragm and skeletal muscle of the ponies were noted from 50 days post-inoculation, and these cysts were infective to naïve canine hosts from day 77 (Fayer and Dubey, 1982).

Pyrexia and mild anaemia were documented in the infected ponies of these experimental studies (Fayer and Dubey, 1982). Other authors studying naturally infected animals also described signs of muscular weakness, ataxia and weight loss (Cawthorn et al., 1990). Granulomatous and eosinophilic myositis has been documented histologically in cases where the pathogen has been confirmed by nested polymerase chain reaction (PCR) (Herd et al., 2015). Despite these descriptions of clinical disease, *Sarcocystis fayeri* has largely been considered an incidental finding in equids (Valentine, 2008). However, a recent study of sarcocyst involvement in the skeletal muscle of horses with neuromuscular disease has suggested that it may not be incidental in all cases. In this study, DNA was extracted from muscle samples of 15 horses showing signs of neuromuscular disease and in which encysted sarcocysts had been detected in muscle biopsies. Following nested PCR, sequence analysis demonstrated *S. fayeri* in six of these 15 samples, suggesting that this species may be of greater pathogenic significance than previously thought (Aleman et al., 2016).

Pathogenic potential for humans has also been highlighted in Japan recently, where reports of food poisoning from the consumption of horsemeat has lead to the issue of public notifications regarding the safe preparation of raw horsemeat for human consumption (Harada et al., 2013). Further research has indicated a protein fraction of *S. fayeri* sarcocysts as a causal toxic agent (Kamata et al., 2014).

Sarcocystis spp. infection within horses has a reported histological prevalence of up to 93 % in some areas (Fukuyo et al., 2002). Sarcosporidiosis has also been reported in mules and donkeys (Kirmse, 1986) but this has been attributed to *S. bertrami* (*S. equicanis*), with *S. fayeri* undocumented in any equid other than the horse (Dubey et al., 2016). Here the authors report the first identification of *S. fayeri* in a donkey, which may have implications for epidemiology of disease.

2. Study methodology and results

As part of a study into haemoparasitic disease prevalence, 114 horse and donkey blood samples were collected from equids in villages in the Central River District of The Gambia between 2012 and 2013. Ethical approval for this study was granted by the University of Glasgow Ethics Committee.

Blood samples were taken by jugular venipuncture into EDTA tubes and these were frozen at -20 °C, heat treated at 56 °C for 30 minutes for UK importation and then frozen at -20 °C until DNA extraction. Each sample had a dedicated form recording the animal's packed cell volume, total plasma protein concentration and the findings of a detailed clinical examination and body condition score (Carrol and Huntington,

1988) as assessed by an experienced veterinary surgeon (AGR). The presence of neurological signs or other concurrent debilitating disease led to the exclusion of the animal from the study due to avoid any confounding effect on outcome.

The samples were screened for piroplasmosis by nested PCR with a modified *Babesia/Theileria* 18S SSU rRNA catch-all primer set (Criado-Fornelio et al., 2003). Reaction conditions were an initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of 94 °C for 45s, annealing at 67°C (external primers) or 57 °C (internal primers) for 60s, elongation at 72 °C for 60s, 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.

One sample, 'Gam56', produced a larger PCR product (~400 bp) than expected for *Babesia* or *Theileria* genera parasites (~385 bp). The PCR product was purified (QIAquick PCR purification kit, Qiagen®) and sent for sequencing (Eurofins Genomics®, Germany). Following BLAST analysis, the nucleotide sequence was found to have a high level of identity (up to 97 %) with *Sarcocystis fayeri* 18S SSU rRNA gene sequences deposited in the non-redundant NCBI database.

To investigate this finding, and assess for *Sarcocystis* presence in cohort animals, Gam56 and 20 other donkey blood samples taken from the same village were subjected to a *Sarcocystis* 18S-specific nested PCR utilising previously published primers, S5/ S4 (external) and S7/S2 (internal) (Fischer and Odening, 1998), and reaction conditions (Aleman et al., 2016). Again, Gam56 generated an amplicon, which following direct sequencing, displayed a high level of identity to the *S. fayeri* sequences in the NCBI database (up to 97%). All other tested samples did not generate a detectable amplicon.

The target areas of the 'catch-all' and *Sarcocystis*-specific nested reactions overlapped. Using the forward and reverse sequences representing the amplicons from both primer sets, a consensus sequence was generated and submitted to GenBank™, under the accession number **KY039162**. A phylogenetic tree was generated using this consensus sequence together with sequences from sets of species closely related to and including *S. fayeri,* namely *S. neurona*, *S. hominis* and *S. cruzi*; a *Babesia caballi* sequence was included as an outlier in order to root the tree. An alignment was generated using MUSCLE (Edgar, 2004) and a boot-strapped phylogenetic tree constructed with MEGA7 (Kumar et al., 2016), using a neighbour-joining method. A neighbour-joining tree was deemed more suitable than a maximum-likelihood tree due to the presence of indel areas within the nucleotide alignment. The tree was visualised within MEGA7 and is illustrated in Figure 1, along with the relevant GenBank™ accession numbers. The novel sequence derived from the donkey was clearly placed within the clade representing *S. fayeri*. An alignment of the Gam56 consensus with the three closest identified GenBank™ sequences is shown in supplementary data.

The samples were also screened for trypanosomosis by PCR using previously described primers (Masiga et al., 1992). Gam56 was found to be PCR positive for *Trypanosoma congolense* only. The clinical examination and blood analysis findings are summerised in Table 1. Using PCR primers specific for the donkey ND2 gene (Kesmen et al., 2007), the animal that provided the Gam56 sample was verified as being a donkey. This donkey was re-sampled weekly for two weeks after the initial sampling. The samples were subjected to the same *Sarcocystis*-specific nested PCR, which proved negative (Table 1).

3. Conclusion

Sarcocystis fayeri is gaining recent interest as both a pathogen of horses and as an inducer of toxic food poisoning.

The animal identified in this report demonstrated anaemia (reduced packed cell volume and pale mucous membranes), depression, intermittent pyrexia and increased pulse and respiration rate (likely secondary to the anaemia and pyrexia). There was an increase in plasma total protein, although probably due to an inflammatory response following the trypanocidal treatment. These are common clinical signs of trypanosomosis, so due to the co-infection with *T. congolense*, the clinical significance of the *S. fayeri* infection could not be determined. Also, the potential association of *S. fayeri* and neuromuscular disease suggested by other authors (Aleman et al., 2016) was not evident in this case, although follow-up only took place over a two-week period.

The discovery of *Sarcocystis*-DNA in a blood sample is unusual considering the typical cyst location for these parasites within tissues, principally muscle of chronically infected hosts. One hypothesis to explain the results is that the detected DNA was derived from merozoites during their haematogenous dissemination following endodyogeny in endothelial cells of blood vessels. This blood-borne stage has been observed in other *Sarcocystis* spp. and utilised for experimental infection via blood transfusion (Fayer and Leek, 1979) and its transient nature may explain the negative findings in subsequent weeks.

Another hypothesis, although less likely, is that during sampling the needle passed through a schizont in the endothelium of the jugular vein (Dubey et al., 2001), thus contaminating the sample with sufficient DNA to detect at PCR. This theory would also be in keeping with the later negative results, as the sample would have been taken from a different part of the jugular.

In the instance of this case, it was not possible to confirm cyst formation within the subject. However, with presence of circulating parasite DNA it is not unreasonable to assume their establishment, raising the possibility of the donkey as an intermediate host.

The subject also received a treatment of diminazene diaceturate (3.5 mg/kg i.m.) at week 1. While this drug is known for its activity against piroplasmosis and trypanosomosis (Peregrin and Mamman, 1993), it is not a recognised treatment for sarcosporidiosis in equines (Dubey et al., 2001). Therefore, it is considered unlikely that this treatment is related to the subsequent negative results.

The confirmation of *S. fayeri* in a donkey host has not been previously reported. The NCBI database currently contains 15 reference sequences annotated as *S. fayeri*. Whilst there is a relatively large degree of polymorphism between these sequences, the isolate sequenced within this study falls firmly within the *S. fayeri* clade.

This finding raises the possibility of the donkey acting as an alternative reservoir for the parasite. Such a scenario may be of particular importance given the huge size of the working donkey population present in many areas of the world. In light of recent work into the possible under-diagnosis of *S. fayeri* disease in both equine (Aleman et al., 2016) and human health (Harada et al., 2013), the potential role of the donkey in the epidemiology of this parasite should not be over-looked.

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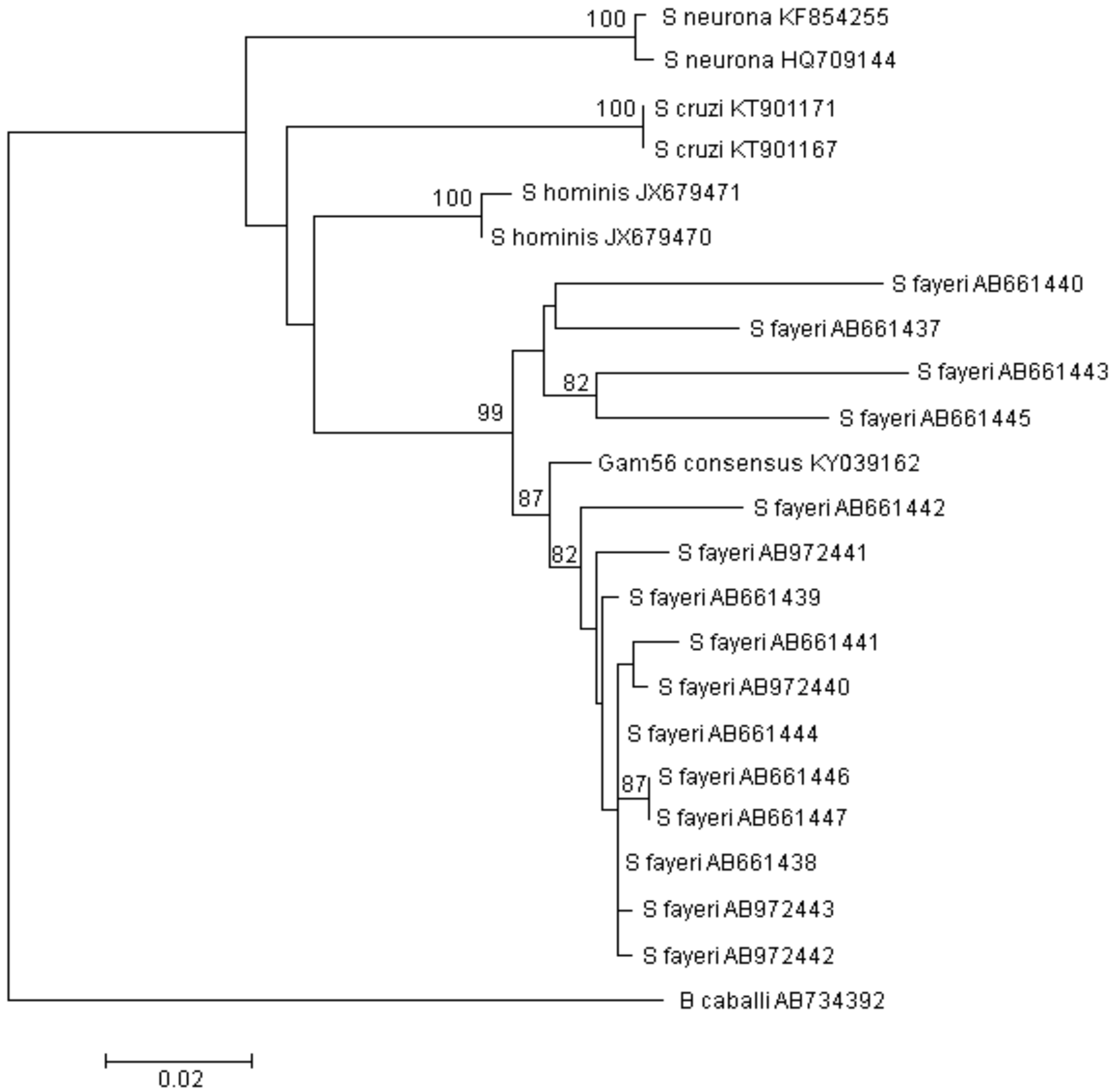
Table 1. Clinical and diagnostic data pertaining to sample 'Gam56' described in this study. Units of measurement and reference ranges are shown in parentheses where applicable.

Identity	**Estimated Age**	**Sex**	**Estimated Weight**
Gam56	6 years	Male	135kg
	Week 1	**Week 2**	**Week 3**
Attitude	Dull	Bright	Bright
Temperature (36.2 – 37.8°C)	38.2°C	39.1°C	37.6°C
Pulse Rate (beats per minute) (40-53)	72	80	72
Respiratory Rate (breaths per minute) (18-24)	64	40	24
Mucous Membranes	Pale	Pale	Pale
Body Condition Score (1-5)	2.5	2	2
Packed Cell Volume (%) (≥24)	12	26	27
Total Protein (g/L) (60-80)	74	94	90
Treatment Given	Diminazene diaceturate	None	None
***T. congolense* PCR**	+	+	+
***T. vivax* PCR**	-	-	-
***T. brucei* PCR**	-	-	-
***Babesia/Theileria* PCR**	+ (larger size amplicon)	-	-
***Sarcocystis* PCR**	+	-	-

Figure 1. A neighbour-joining tree inferring the evolutionary relationship of the 18S SSU rRNA gene fragment from the *Sarcocystis fayeri* sample (KY039162) described in this study. Included are all current comparable *S. fayeri* sequences available through GenBank™, and representative sequences of *S. neurona*, *S. cruzi*, *S. hominis*, and *B. caballi* (to which the tree is rooted) with accession numbers noted. Bootstrap values >70 % are shown, as generated from 1,000 replications. Up to 95 % alignment gaps were allowed at any position (5 % site coverage). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (sum of branch lengths = 0.53). The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The tree was created using the MEGA7 package (Kumar et al., 2016). The GenBank™ accession numbers are included.


100
S neurona KF854255
S neurona HQ709144
100
S cruzi KT901171
S cruzi KT901167
100
S hominis JX679471
S hominis JX679470
S fayeri AB661440
S fayeri AB661437
82
S fayeri AB661443
99
S fayeri AB661445
Gam56 consensus KY039162
87
S fayeri AB661442
82
S fayeri AB972441
S fayeri AB661439
S fayeri AB661441
S fayeri AB972440
S fayeri AB661444
87
S fayeri AB661446
S fayeri AB661447
S fayeri AB661438
S fayeri AB972443
S fayeri AB972442
B caballi AB734392
0.02

Supplementary Figure 1. An alignment of the Gam56 consensus (KY039162) with the three top-scoring BLAST ‘hits’ from the GenBank™ database. The alignment was performed with MUSCLE 3.8 (Edgar, 2004).

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CLUSTAL multiple sequence alignment by MUSCLE (3.8)

KY039162.1      CCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATATCTGCTGGGA
AB661447.1      CCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATATCTGCTGGGA
AB661446.1      CCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATATCTGCTGGGA
AB661439.1      CCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATATCTGCTGGGA
                ************************************************************

KY039162.1      GCAATCAGTCCGCCCTTTTTATAGGGGTGTGCACTTGATGAATTCTGGCATGTTTTTATC
AB661447.1      GCAATCGGTCCGCCC-TTTTACAGGGGTGTGCACTTGATGAATTCTGGCATGTTTTTATC
AB661446.1      GCAATCGGTCCGCCC-TTTTACAGGGGTGTGCACTTGATGAATTCTGGCATGTTTTTATC
AB661439.1      GCAATCGGTCCGCCC-TTTCATAGGGGTGTGCACTTGATGAATTCTGGCATGTTTTTATC
                ****** ******** *** * **************************************

KY039162.1      --TTTCC------TAATGATTATTATTAGGTTAATTCCTAGTAATAATTAGTATTGGGAT
AB661447.1      TATTTCCTAATGATAATGATTATTATTAGGTTAATTCCTAATAATAATTATTATTGGGTT
AB661446.1      TATTTCCTAATGATAATGATTATTATTAGGTTAATTCCTAATAATAATTATTATTGGGTT
AB661439.1      --TTTCCTAATGATAATGATTATTATTAGGTTAATTCCTAATAATAATTATTATTGGGTT
                  *****      *************************** ********* ******* *

KY039162.1      AGATAGACCGTTACTTTGAGAAAATTAGAGTGTTTAATGCAGGCTGTATTATGCCTTGAA
AB661447.1      AGATAAACCGTTACTTTGAGAAAATTAGAGTGTTTAATGCAGGCTGTTTTATGCCTTGAA
AB661446.1      AGATAAACCGTTACTTTGAGAAAATTAGAGTGTTTAATGCAGGCTGTTTTATGCCTTGAA
AB661439.1      AGATAAACCGTTAC-TTGAGAAAATTAGAGTGTTTAATGCAGGCTGTTTTATGCCTTGAA
                ***** ******** ******************************** ************

KY039162.1      TACTGCAGCATGGAATAACAATATAGGATTTCGGTTCTATTTTGTTGGTTTTTAGGACTG
AB661447.1      TACTGCAGCATGGAATAACAATATAGGATTTCGGTTCTATTTTGTTGGTTTTTAGGACTG
AB661446.1      TACTGCAGCATGGAATAACAATATAGGATTTCGGTTCTATTTTGTTGGTTTTTAGGACTG
AB661439.1      TACTGCAGCATGGAATAACAATATAGGATTTCGGTTCTATTTTGTTGGTTTTTAGGACTG
                ************************************************************

KY039162.1      GAATAATGGTTAATAGGGACAGTTGGGGGCATTCGTATTTAACTGTCAGAGGTGAAATTC
AB661447.1      GAATAATGGTTAATAGGGACAGTTGGGGGCATTCGTATTTAACTGTCAGAGGTGAAATTC
AB661446.1      GAATAATGGTTAATAGGGACAGTTGGGGGCATTCGTATTTAACTGTCAGAGGTGAAATTC
AB661439.1      GAATAATGGTTAATAGGGACAGTTGGGGGCATTCGTATTTAACTGTCAGAGGTGAAATTC
                ************************************************************

KY039162.1      TTAGATTTGTTAAAGACGAACTAATGCGAAAGCATTTGCCAAAGATGTTTTCATTAATCA
AB661447.1      TTAGATTTGTTAAAGACGAACTAATGCGAAAGCATTTGCCAAAGATGTTTTCATTAATCA
AB661446.1      TTAGATTTGTTAAAGACGAACTAATGCGAAAGCATTTGCCAAAGATGTTTTCATTAATCA
AB661439.1      TTAGATTTGTTAAAGACGAACTAATGCGAAAGCATTTGCCAAAGATGTTTTCATTAATCA
                ************************************************************

KY039162.1      AGAACGAAAGTTAGGGGCTCG
AB661447.1      AGAACGAAAGTTAGGGGCTCG
AB661446.1      AGAACGAAAGTTAGGGGCTCG
AB661439.1      AGAACGAAAGTTAGGGGCTCG
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