Veterinary Microbiology 276 (2023) 109604

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

First report and molecular characterization of cases of natural *Taylorella asinigenitalis* infection in three donkey breeds in Spain

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ARTICLE INFO

Keywords:
Taylorella asinigenitalis
Contagious Equine Metritis
Donkeys
Spain
MLST

ABSTRACT

Taylorella asinigenitalis is a non-pathogenic bacteria isolated from the genital tract of donkeys but also a cause of metritis and vaginal discharge in mares. It is closely related to *Taylorella equigenitalis*, the cause of Contagious Equine Metritis (CEM) in horses, and has been present in different countries in Europe since 1995. Up to date, there are no studies on the prevalence of *T. asinigenitalis* in the equine or asinine populations in Spain; this is the first report of the presence of *T. asinigenitalis* in donkeys (*Equus asinus*) from different breeds in three regions of Spain. A total of 106 healthy animals of three different Spanish donkey breeds: Andaluza (26), Majorera (12) and Zamorano-Leonés (68) were sampled between June and July 2017 and a real-time PCR was used to detect *T. asinigenitalis* in all samples. A total of 39/221 (17,65 %) samples from 22/106 (20,75 %) animals yielded a positive result and were further characterized by MLST; an allelic profile and Sequence Type (ST) could be assigned to 11 of the 39 positive samples, resulting in four novel STs and no clonal complexes within the PubMLST database. There were statistically significant differences in the percentage of positive animals by breed and sex, and also in the variability of STs between farms. Breeding management would have an influence on the percentage of positives in a farm; artificial insemination and separating jacks from jennies should be implemented. Further studies to detect and characterize *T. asinigenitalis* in donkeys and horses from Spain would be required to obtain a broader epidemiological picture in this country.

1. Introduction

Contagious equine metritis (CEM), a venereal disease affecting equids and causing mucopurulent vaginal discharge, endometritis, cervicitis, vaginitis and temporary infertility in mares (Timoney, 1996), was first described in United Kingdom in 1977 (Crowhurst, 1977), and its causative agent first designated as *Haemophilus equigenitalis* (Taylor et al., 1978). Due to its phenotypic characteristics, *Haemophilus equigenitalis* was proposed to be reclassified and included in the new genus *Taylorella*, renamed as *Taylorella equigenitalis* (Sugimoto, 1985).

Taylorella equigenitalis was reported in the United States in 1978 (Bryans and Hendricks, 1979), where screening and controls measures led to the isolation of a very similar organism from the semen of a donkey jack in California in October 1997 and the genital tract of asymptomatic animals (several mares, one stallion and two donkeys) in Kentucky in January 1998 (Jang et al., 2001). Considering the genetic (97.6 % 16 S rRNA similarity with *T. equigenitalis*) and phenotypical (lower growth rate, poor IFI reaction against *T. equigenitalis* antibodies) differences found in those isolates, a new species was proposed and designated as *Taylorella asinigenitalis* (Jang et al., 2001).

T. asinigenitalis was first reported in 2004 in Europe (Sweden) after sequencing the 16S rRNA gene of a strain from a stallion with natural infection that had tested positive for CEM (Baverud et al., 2006). Later, in 2008, the bacteria was isolated from two donkeys in Italy (Franco et al., 2009). A retrospective study demonstrated the presence of *T. asinigenitalis* in France since 1995, even before its first description in

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https://doi.org/10.1016/j.vetmic.2022.109604
Received 19 January 2022; Received in revised form 23 August 2022; Accepted 18 November 2022
Available online 24 November 2022

1998 (Breuil et al., 2011). Recently, in 2018, a case report also described the isolation of the bacteria from an onager in a zoo in UK (Mawhinney et al., 2018).

Despite being considered a non-pathogenic bacteria, an experimental study revealed that two mares intra-uterine exposed to the Kentucky strain developed vaginal and cervical discharge, but less severe than those exposed to *T. equigenitalis* (Katz et al., 2000). Recently, a new strain of *T. asinigenitalis* showed different pathogenicity in mares and Jenny donkeys, with the first ones developing severe, purulent endometritis while the others were asymptomatic (Wilsher et al., 2021).

The whole genome of *T. equigenitalis* and *T. asinigenitalis* were first sequenced in 2011 (Hebert et al., 2011) and 2012 (Hebert et al., 2012), respectively, and allowed the development of a single multi-locus sequence typing scheme (MLST) (Duquesne et al., 2013), which can be used for molecular epidemiological studies. While other techniques such as pulsed field gel electrophoresis (PFGE) and clustered regularly interspaced short palindromic repeat (CRISPR) have also been used to characterize the genetic diversity of isolates from *Taylorella* genus, MLST is probably the most useful to carry out large-scale or international epidemiological studies (Duquesne et al., 2013).

Up to date, there are no studies on the presence of *T. asinigenitalis* in the asinine populations in Spain; therefore, the aim of the study was to determinate the presence/absence of *T. asinigenitalis* in donkeys (*Equus asinus*) from different breeds in three regions of Spain and to characterize isolates by MLST.

2. Material and methods

2.1. Animals sampled

A total of 106 animals of three different Spanish donkey breeds: Andaluza (26), Majorera (12) and Zamorano-Leonés (68), residing in three provinces of Spain (Badajoz, Ávila and Zamora, respectively) were included in the study.

A convenience sampling was carried out by a veterinary surgeon in four different premises between June and July 2017 as part of their annual CEM screening programme;.

Samples consisted in genital swabs from clitoris and clitoral fossa in females, and distal urethra, urethral fossa including urethral sinus, and glans surface/prepuce (depending on voluntary exteriorization of the penis) in males. In addition, semen samples from two Jacks were collected with an artificial vagina and were included in the study. A total of 46 samples were collected from intact males (n = 16), while 5 samples were taken from castrated males (n = 5) and 170 samples were recovered from females (n = 85).

Swabs were shipped refrigerated to the laboratory in Amies charcoal medium (Deltalab®, Barcelona, Spain) within 48 h, where they were stored at − 80 °C until DNA extraction.

2.2. Identification at species level

2.2.1. DNA extraction

Total genomic DNA was extracted individually from genital swabs and semen using QIAamp DNA mini kit (Qiagen, Spain), according to the manufacturer's protocol and was preserved at − 40 °C until multiplex real-time PCR was carried out.

2.2.2. Real-time PCR

A previously described multiplex real-time PCR was used to detect the presence of 16S rDNA fragments from *T. equigenitalis* and *T. asinigenitalis* (Wakeley et al., 2006).

Briefly, the PCR mix contained a total reaction volume of 25 μl comprising: 8,5 μl of ultrapure sterilized water, 5 μl of 5x Quantifast Pathogen PCR Master mix, 0,5 μl of each forward and reverse primer Tay377 F [20 μM] and Tay488 R [20 μM], 0,5 μl of the *T. asinigenitalis* specific Taqman probe labeled with 5′-TexasRed and 3′-BHQ2 [10 μM], 2,5 μl of an internal DNA control template and 2,5 μl of internal control primer/probe set (Qiagen, Spain) and 5 μl of target DNA. A positive control (strain ATCC 700933), a negative extraction control and a negative PCR control (ultrapure sterilized water) were included in each PCR assay. Amplifications at Cq < 40 were considered as positive.

Real-time PCR reactions were performed in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Cycling conditions were as follows: initial denaturation cycle at 95 °C for 5 min, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The fluorescence data obtained was analyzed using Bio-Rad CFX Manager (Version 3.1; Bio Rad Laboratories, Hercules, CA, USA).

2.2.3. MLST analysis

The positive samples by real-time PCR were further characterized by MLST, using ATCC strain 700933 as a positive control and the primer pairs for the amplification of each of the seven housekeeping genes (*gltA, gyrB, fh, shmt, tyrB, adk, txn*) proposed by Duquesne et al. (2013). Each 50 μl PCR reaction was carried out in a BioRad T100 Thermal Cycler and C1000 Thermal Cycler using 3 μl of the DNA extraction, 0,5 μl of each primer (20 μM), 25 μl of Taq PCR Master Mix and 21 μl of Nuclease-Free Water (Sigma Aldrich, Missouri, USA). The amplification protocol for each housekeeping gene was the following: initial denaturation at 94 °C for 3 min and 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Electrophoresis in 2 % agarose gel (Biotools) using 1x TAE buffer and 10 μl of SYBR Safe™ DNA gel stain (Invitrogen) was carried out in amplified products to confirm the presence of specific bands for each gene (Duquesne et al., 2013). The bands were visualized using BioRad Molecular Imager® Gel Doc XR+ Imaging System transilluminator.

Amplicons showing a clear, specific band were purified using Illustra ExoProStar™ 1-step enzyme according to the manufacturer and sequenced using the Sanger method. Chromatograms for forward and reverse sequences were visualized using the Chromas Lite version 2.01 software. Non-determined nucleotides (N) were eliminated by aligning both sequences with the software MEGA X (https://www.megasoftware.net/). Each sequence was then analysed in the NCBI BLAST database (https://blast.ncbi.nlm.nih.gov/) and compared to the allelic sequences for *Taylorella sp*. of PubMLST database (Jolley et al., 2018). Exactly matching sequences were assigned an existing allele number whereas unique sequences were assigned new alleles. The combination of the alleles for the seven loci (*gltA, gyrB, fh, shmt, tyrB, adk, txn*) was then used for designation of allelic profiles or ST (sequence typing) by the database curator.

Using software goeBURST (PHYLOViZ) (Feil et al., 2004), STs were classified as single-locus variants (SLVs), double-locus variants (DLVs) or individual unlinked STs, and the presence of clonal complexes was evaluated (Maiden, 2006). Finally, a phylogenetic analysis using the concatenated sequences of the seven housekeeping genes (3521 bp) was carried out by the Maximum Likelihood method using MEGA X (https://www.megasoftware.net/) including all *T. asinigenitalis* and *T. equigenitalis* strains published in the PubMLST database (Jolley et al., 2018).

2.3. Statistical analysis

Positivity rates by real time PCR for males and females were analyzed using Chi square test (X2). The differences between *T. asinigenitalis* real-time PCR Cq value and copy number in males and females, were assessed using Mann-Whitney U test.

3. Results

3.1. Real-time PCR results

A total of 39/221 (17,65 %) samples from 22/106 (20,75 %)

different animals yielded a positive result for *T. asinigenitalis* (Table 1), while there were no positive results for *T. equigenitalis*. Considering breed, 91,67 % (11/12) of the Majorera breed animals residing in Ávila were positive, whereas the percentage of positives was lower for the Andaluza breed animals located in Badajoz [3,85 % (1/26)] and the Zamorano-Leonés breed animals residing in Zamora [14,71 % (10/68)].

Regarding sex, there were significant differences between the percentages of positives of females [8,23 % (7/85)] and males [71,43 % (15/21)] ($p < 0.001$). Within the positive males, 10 were jacks and the rest were geldings. Within the positive jennies located in Ávila (4), 3 were pregnant at the time of sampling and the other one had foaled recently. The positive jenny detected in Badajoz was 10 years-old and had previously foaled 4 times but wasn't pregnant when samples were taken. From the positive jennies in Zamora (2), one was pregnant and the other one had a foal; both had been mated with a positive jack the previous year.

3.2. Characterization by MLST

Samples previously identified to species level as *Taylorella asinigenitalis* by real-time PCR were further characterized using MLST. This technique allowed PCR amplification of the seven housekeeping genes (*gltA, gyrB, fh, shmt, tyrB, adk and txn*) in 11 of the 39 positive samples (Table 2). Fig. 1 shows the amplification of *gltA* gene in some of the samples in our study.

The allele numbers assigned to each sequence are shown in Table 2. The combination of the 7 loci from each strain resulted in four novel STs. No clonal complexes (CC), using the SLV criteria, were assigned by PubMLST for STs 75, 76, 77 and 78. STs 76 and 78 were classified as DLV, and individual STs as unlinked STs by eBURST. No STs were shared between *T. equigenitalis* and *T. asinigenitalis*.

The phylogenetic tree based on the 3521-bp concatenated sequences of the seven MLST housekeeping genes, constructed from the sequences of the 78 STs available in the *Taylorella spp*. database in PubMLST showed that there was a common ancestor for *T. equigenitalis* and *T. asinigenitalis* (Fig. 2). The STs assigned in the population of this study were included together with all the *T. asinigenitalis* STs in PubMLST, and in the same branch with isolates from France.

Table 1
Number of positive *T. asinigenitalis* real-time PCR samples (n = 39) by province, breed, sex and sample site.

Province	Breed	Sex	Sample site	Number of positive samples
Ávila	Majorera	Male	Distal urethra	1
			Urethral fossa	1
			Glans surface	1
			Prepuce	1
		Castrated male	Prepuce	5
		Female	Clitoris	4
			Clitoral fossa	3
Badajoz	Andaluza	Male	Distal urethra	0
			Urethral fossa	0
			Glans surface	0
			Semen	0
		Female	Clitoris	1
			Clitoral fossa	0
Zamora	Zamorano-Leonés	Male	Distal urethra	7
			Urethral fossa	6
			Glans surface	7
		Female	Clitoris	0
			Clitoral fossa	2

Table 2
Allelic profile assigned by PubMLST to each sequence characterized in this study.

Strains	*gltA*	*gyrB*	*fh*	*shmt*	*tyrB*	*adk*	*txn*	ST
VSE17/01095-1	6	8	34	19	26	8	16	75
VSE17/01095-2	6	8	34	19	26	8	16	75
VSE17/01095-3	6	8	34	19	26	8	16	75
VSE17/01100	6	8	34	19	26	8	16	75
VSE17/01104	6	8	–	19	26	–	16	
VSE17/01111	6	8	34	19	26	8	16	75
VSE17/01432-1	22	9	35	4	–	–	28	
VSE17/01432-2	22	9	35	4	25	–	28	
VSE17/01432-3	22	9	35	4	25	15	28	78
VSE17/01435-1	23	6	35	4	25	–	28	
VSE17/01435-2	23	6	35	4	25	15	28	76
VSE17/01435-3	23	6	35	4	25	15	28	76
VSE17/01440-1	22	6	36	4	25	15	25	77
VSE17/01440-2	22	6	36	4	25	15	25	77
VSE17/01440-3	22	6	36	4	25	15	25	77

4. Discussion

This is the first study describing the presence of *T. asinigenitalis* in Spain. In agreement with other publications detecting *T. asinigenitalis* in donkeys (Carleton et al., 2015; Donahue et al., 2012; Franco et al., 2009; Jang et al., 2001), all the positive animals were asymptomatic carriers since no clinical signs were observed.

While there are no data published about prevalence of *T. asinigenitalis* in different breeds, and the design of the present study did not allow to determine the prevalence by breed, the different percentage of positive animals between breeds could be related with differences in the breeding protocols and general management from each premises. In the premises in Ávila, with the highest percentage of positives, females were naturally mated and remained on a single group together with males and their foals, whereas in Zamora, where a high percentage of positive males was found, males and females remained in two different premises and were naturally mated. Finally, in Badajoz, where the lowest percentage of positive animals was observed, females and their foals were physically separated from males, which were individually hosted in boxes and breeding was carried out by using artificial insemination.

A higher percentage of positive animals was observed in males compared to females. However, a higher positive rate recovery has been reported from swabs collected from the proximal reproductive tract (Meade et al., 2010) in female donkeys, and neither clitoral sinuses nor endometrial or cervical swabs were sampled in the female donkeys included in the present study. Therefore, some positive female animals could have been misidentified and the difference between males and females in our study could be expected to be lower.

The positive gelded males had never been used for breeding so, as suggested before there must be a different way of transmission other than venereal transmission, such as transmission from the Jenny to the fetus, at birth or by direct contact (Baverud et al., 2006; Donahue et al., 2012).

In our study, MLST characterization was performed on DNA from field samples, without prior isolation of *T. asinigenitalis* by culture; this involved that the DNA load was insufficient to do the complete MLST analysis in all our positive real-time PCR samples. Considering that the samples were gathered and analysed by real-time PCR in 2017, while the characterization by MSLT was carried out in 2021, these samples could also have been subjected to a number of manipulations, including thawing, which could affect the amount and quality of DNA (Brunstein,

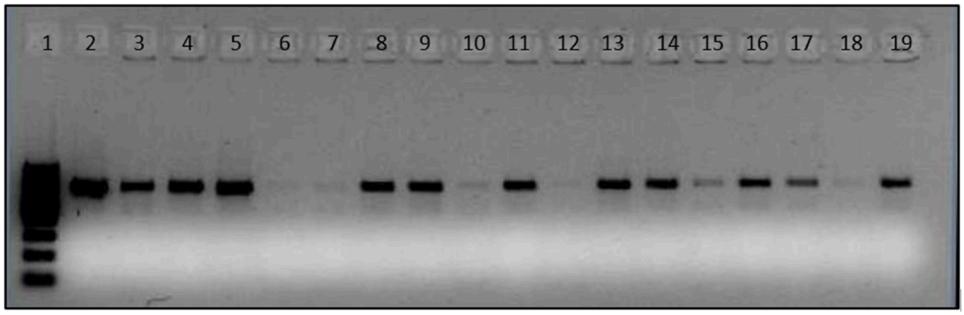

Fig. 1. Amplification of the *gltA* gene. Lane 1: MW 100 bp ladder; Lane 2: VSE17/01095–1; Lane 3: VSE17/01095-2; Lane 4: VSE17/01095-3; Lane 5: VSE17/01100; Lane 6: VSE17/01101; Lane 7: VSE17/01103; Lane 8: VSE17/01104; Lane 9: VSE17/01111; Lane 10: VSE17/01432-1; Lane 11: VSE17/01432-2; Lane 12: VSE17/01432-3. Lane 13: VSE17/01435-1; Lane 14: VSE17/01435-2; Lane 15: VSE17/01435-3; Lane 16: VSE17/01439-1; Lane 17: VSE17/01439-2; Lane 18: VSE17/01439-3; Lane 19: VSE17/01440-1.

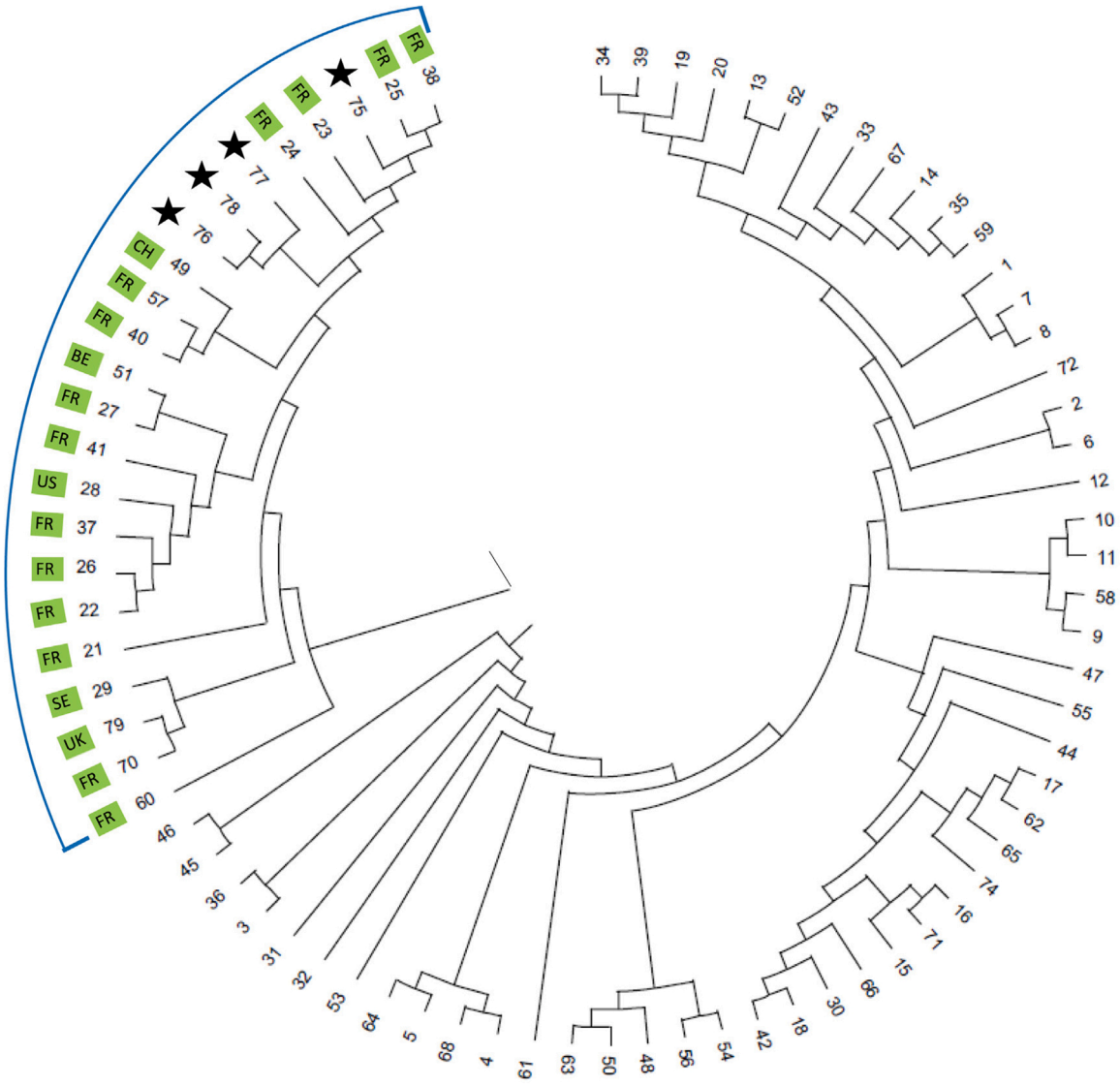

Fig. 2. Phylogenetic tree based on 3521-bp concatenated sequences of seven MLST housekeeping genes constructed from the sequences of the 78 STs available in the *Taylorella spp*. database in PubMLST. Each number refers to a different ST. *Taylorella asinigenitalis* STs are shown inside the blue bracket whereas the rest of STs belong to *Taylorella equigenitalis*. STs identified for the first time in this study in Spain are marked with a star; the rest of STs were found in the following countries: France (FR), Switzerland (CH), Belgium (BE), United States (US), Sweden (SE), and United Kingdom (UK).

2015). Furthermore, the use of swabs in Amies medium with charcoal could explain why it was only possible to carry out characterization in 11 out of the 39 real-time PCR samples considering possible inhibition caused by agar (Gibb and Wong, 1998). Therefore, the procedure carried out in this study could be an option for samples yielding a negative result by culture but a positive result by real-time PCR, but also for clinical samples avoiding a prior culture step, as already carried out by other authors with *T. equigenitalis* (May et al., 2019).

Regarding the STs assigned to amplicons in our study, in the premises in Ávila, all amplicons belonged to the same ST (75), except for sample VSE17/01104 in which only 5 of the 7 loci were amplified. The fact that these five loci were the same as the rest of the sequenced samples from

the same farm could suggest the spread of a single ST in this premises. However, in Zamora the characterization showed a greater variability of STs (76, 77, 78). This could be explained by the fact that Zamora had the highest number of resident donkeys probably coming from different sources.

The phylogenetic tree showed that the STs from Spain would be close to STs isolated in France (Fig. 2); however, up to our knowledge there are no data on importation of donkeys from this country to Spain. The STs from our study greatly differ from the first ST from *T. asinigenitalis*, isolated in France in 1995 (ST 22) and are related to STs isolated in France between 1998 and 2006; this could date the presence of *T. asinigenitalis* in Spain in the past 15–25 years, although the phylogenetic tree also shows relationships between STs isolated in very distant years.

This study is the first report of the presence of *Taylorella asinigenitalis* in Spain. According to our results, the breeding management could have an influence on the percentage of positives in a farm; artificial insemination and separating jacks from jennies would be recommended. However, further studies to detect and characterize *T. asinigenitalis* in donkeys and horses from Spain would be required to corroborate these findings.

Funding

Herranz, C. was supported by PEJD-2019-PRE/BIO-16734 Grant.

Authors' declaration of interests

No competing interests have been declared.

Acknowledgements

We are grateful to the owners from the farms included in this study for agreeing to use samples for this study. We are also grateful to Kelly Alejandra Carvajal and Almudena Casamayor (technicians at VISAVET Health Surveillance Centre) for their help with the analysis of samples.

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