Received: 12 November 2023 | Accepted: 5 June 2024

DOI: 10.1111/evj.14134

ORIGINAL ARTICLE

Comparison of morphological and molecular *Strongylus* spp. identification in equine larval cultures and first report of a patent *Strongylus asini* infection in a horse

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Funding information

Deutsche Forschungsgemeinschaft, Grant/Award Number: 251133687/GRP2046

Abstract

Background: Surveillance of *Strongylus vulgaris* and other *Strongylus* spp. in equids is important for targeted intervention in parasite control, requiring reliable routine diagnostic methods.

Objectives: Comparing morphological examination and PCR analyses of larval cultures to identify *Strongylus* spp. species based on German diagnostic samples from 2018.

Study design: Method comparison.

Methods: During the routine diagnostic investigations, in total 712 strongyle-egg positive equine faecal samples were cultured. Third-stage larvae (L3) were morphologically differentiated. For molecular validation, samples were examined using *S. vulgaris* real-time PCR and *Strongylus edentatus*/*Strongylus equinus*/*Strongylus asini* high-resolution melting PCRs.

Results: Based on 28S rRNA PCR, 594 samples positive for nematode DNA were included in the study. The inter-rater reliability to compare morphological and molecular species identification was fair for *Strongylus* spp. without species identification and for *S. edentatus*, slight for *S. equinus* and poor for *S. vulgaris*. The frequency based on morphological and molecular data in this study were for *S. vulgaris* 0% and 0.8%, respectively, for *S. edentatus* 0.3% and 1.5%, respectively, and for *S. equinus* 2.0% and 0.2%, respectively. Based on molecular analyses, one sample obtained from a domestic horse contained *S. asini* DNA, which was confirmed by sequencing.

Main limitations: For many samples, no or only incomplete data regarding clinical history, the exact geographical location and whether samples were obtained on individual or farm level, were available.

Conclusions: Results of morphological and molecular examination methods of strongyle L3 from equine samples can differ substantially. Further evaluation of these

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methods is required to provide reliable and cost-effective methods of screening equine parasites. Further studies using approaches suitable to detect *S. asini* are needed to evaluate its clinical and epidemiological relevance.

KEYWORDS
Germany, horse, large strongyle, larval culture, *Strongylus* spp.

1 | INTRODUCTION

Parasites of the family Strongylidae represent the most prevalent and abundant parasites of equines worldwide. *Strongylus vulgaris* is considered to be the most pathogenic gastrointestinal parasite within the Strongylidae.[1]

Coproculture, with subsequent morphological identification and differentiation of larvae, is still a widely applied method to detect patent infections with *Strongylus* spp. at individual horse and farm levels.[2–6] In previous studies based on larval culture and subsequent morphological differentiation of the third-stage larvae (L3), the prevalence of *S. vulgaris* in Germany was estimated to be 0%–1.3% at the individual horse level.[7–10] Patent infection with *S. vulgaris* can also be detected by applying various polymerase chain reaction (PCR) methods.[11–14] High-resolution melt (HRM) PCR was introduced to detect and discriminate *Strongylus edentatus*, *Strongylus equinus* and *Strongylus asini*.[15] Using qPCR, a *S. vulgaris* prevalence of 1.9% at individual horse level was observed.[9] A recent study comparing serology and qPCR on the same horses reported *S. vulgaris* seroprevalence as high as 21.2%, while qPCR detected *S. vulgaris* in only 1.3% of all faecal samples from which nematode DNA was amplifiable.[15] However, potential cross-reactivity with orthologous proteins from other species of Strongylidae cannot be excluded. In some Scandinavian countries, an increase in the *S. vulgaris* prevalence (based on larval culture or PCR) was detected on the farm level during the past decades, with up to 64% of farms positive for *S. vulgaris*.[16,17] This re-emergence was considered to be associated with selective treatment without prior examination regarding presence of *S. vulgaris*. To avoid clinical disease and re-emergence of *S. vulgaris* infections, selective anthelmintic treatment approaches need to be combined with routine surveillance for the presence of *S. vulgaris*.[2,18]

Although it is impossible to reliably distinguish eggs of Cyathostominae and Strongylinae, it is possible to discriminate and identify the L3 of Strongylidae to a certain extent using morphological features such as body length and width and, in particular, the number of intestinal cells (IC).[4–6] Since most cyathostomin L3 have eight distinct triangular shaped IC, species identification is not possible for most species. Only species of the genus *Poteriostomum* with 16 IC and *Gyalocephalus capitatus* with 12 IC can be identified to the genus or species level, respectively.[4] *S. vulgaris* L3 have 28–32 rectangular-shaped IC, while those of *S. edentatus* have a thinner body with 18–20 poorly defined IC.[6] Most *Triodontophorus* spp. larvae also have 18–20 rectangular-shaped IC but the body is wider compared with *S. edentatus*.[6] The only exception is *Triodontophorus serratus*, which has in total 16 IC with rectangular elongated shape in the anterior and pentagonal shaped in the posterior intestine.[5] In addition, 16 IC have also been described for *S. equinus*, *Craterostomum acuticaudatum* and *Oesophagodontus robustus*. Therefore, the sizes of the larvae must also be considered in addition to the number of IC to unequivocally identify the nematode species.[4–6] The increasing demand for selective treatment approaches also increases demands for diagnostic methods to determine yards where highly pathogenic parasitic nematodes are endemic and selective treatment might pose an unacceptable risk.

The current study aimed to compare morphological and molecular methods to investigate the occurrence of *Strongylus* spp. in larval cultures prepared from diagnostic samples using an explorative study design.

2 | MATERIALS AND METHODS

2.1 | Study population

Surplus material of 712 strongyle egg-positive equine faecal samples, which were examined between January and December 2018 as part of routine diagnostics at the Institute for Parasitology, University of Veterinary Medicine Hannover, Germany, with the combined sedimentation–flotation or McMaster technique, was available for the current study. No specific sampling was conducted during this study, but samples sent in for routine diagnosis were used for convenience. Recorded metadata included the examination date as well as animal species, age and sex of the individual and the submitter's zip code as provided in the sample submission form by the animal owner or veterinarian submitting the samples (Data S1). Most samples were obtained from individual horses but for some samples, the owner did not provide any information if the sample was taken from a single horse or pooled with other horses.

2.2 | Larval culture and morphological examination

2.2.1 | Larval culture

If possible, coprocultures were prepared on the day of arrival, otherwise the faecal samples were stored at 4°C until further processing. A minimum of 20 g faeces were mixed with wood chips and supplemented with tap water. The mixture was transferred into a glass jar (500 mL) with a loosely fitted screw cap and cultured at 26°C, 84%

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relative humidity for 7–10 days in a dark climate chamber. To obtain developed L3, the jar was carefully filled with tap water to the brim, a glass Petri dish was pressed onto the jar, and the culture was turned upside down. Then, the Petri dish was half filled with tap water and the larvae were allowed to migrate into the water of the Petri dish for at least 12 h. The water in the Petri dish containing the larvae was collected and examined under a microscope.

2.2.2 | Morphological examination of strongyle L3

Morphological strongyle L3 identification was based on the number of IC, and cuticular sheath with tail shape and larvae size were also considered. If possible, larvae were extracted for photography of morphology including size measurement. If a sample was too crowded with larvae, it was split into aliquots, which in turn were diluted with tap water to facilitate screening. If possible, 250 L3 per coproculture were identified morphologically. If less than 250 L3 were obtained from a faecal sample, all available larvae were examined. All examinations were carried out by one and the same examiner.

After microscopic examination, the larvae were flushed into vessels (one per faecal sample) with tap water and stored at 4°C until shipping to the Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Germany. To facilitate shipment, larvae were sedimented in conical tubes, the supernatant removed, and transferred to 2 mL tubes. To blind further procedures, only the sample IDs were provided. After arrival in March 2020, the supernatant from all samples was removed and the samples were frozen at −20°C until further processing.

2.3 | Genomic DNA isolation

All genomic DNA extractions from the larval pools per sample were performed using the NucleoSpin 8 Soil kit (Macherey-Nagel) following the manufacturer's instructions using SL1 and SX buffer. The SpeedMill P12 (Analytik Jena AG) was used to homogenise the samples in the bead tubes delivered with the DNA isolation kit. The DNA was eluted with 100 μL elution buffer.

2.4 | Molecular detection of *Strongylus* spp. infections

2.4.1 | Detection of nematode DNA

The presence of amplifiable nematode DNA in the isolated DNA was verified using a pan-nematode PCR targeting the 28S rRNA gene as described by Demeler et al.[19] PCR reactions contained 200 nmol/L of each primer in 15 μL 1X GoTaq® qPCR Master mix and 5 μL template DNA. As positive control, plasmid DNA (500, 50 and 5 copies per reaction) containing the corresponding 28S rRNA fragment of *Haemonchus contortus* cloned in the pCR®2.1-TOPO vector (TOPO® TA Cloning® Kit; Thermo Fisher Scientific) was used. After an initial denaturation at 94°C for 2 min, 40 cycles of 94°C for 10 s, 65°C for 30 s, and 72°C for 30 s were performed. During the elongation phase, fluorescence was measured. A conventional melting analysis was performed after amplification by raising the temperature from 65°C to 95°C in 0.5°C increments with each step lasting for 5 s each and fluorescence read at each step to confirm the identity of the PCR fragment and exclude primer dimers. Negative samples were diluted 1:5 with DEPC-treated water due to the possibility of PCR inhibitors and the PCR was repeated. In further analyses, only DNA samples positive in the pan-nematode PCR were included.

2.4.2 | Detection of *S. vulgaris* DNA

The *S. vulgaris* specific hydrolysation-probe-based real-time PCR was initially published by Nielsen et al.[12] The PCR targets a partial ITS-2 region of *S. vulgaris*. Plasmid DNA containing the ITS-2 region of *S. vulgaris* was used as positive control. Three different dilutions of the positive control (500, 50 and 5 copies per reaction) were analysed in duplicate in parallel with DNA from the larval culture samples. The PCR of the samples was conducted as singleplex, but PCRs were repeated to confirm the results. PCR reactions contained 200 nmol/L of each primer and probe in 20 μL Maxima Probe/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific) and 5 μL template DNA. After an initial denaturation at 95°C for 15 min, 50 cycles of denaturation at 95°C for 15 s, annealing at 61°C for 30 s and elongation at 72°C for 30 s were run. PCR products were purified using the DNA Clean & Concentrator®-5 purification kit (Zymo Research) and cloned into the pCR®2.1-TOPO vector (TOPO® TA Cloning® Kit; Thermo Fisher Scientific). Plasmid products were sent to LGC Genomics (Berlin, Germany) for Sanger sequencing. The determined DNA sequences were compared with the NCBI database using Blastn.[20]

2.4.3 | Detection of *S. edentatus*, *S. equinus* and *S. asini* DNA

A real-time PCR followed by HRM curve analysis was conducted for the identification of samples positive for *S. edentatus*, *S. equinus* and *S. asini* targeting the partial ITS-2 region. The different target species are differentiated based on HRM data and cross-reactivity with *S. vulgaris* DNA was excluded according to previously published data.[15] Since no genomic DNA for *S. equinus* and *S. asini* was available, plasmids were ordered from Shanghai ShineGene Molecular Bio-Technologies, Inc. containing the ITS-2 according to the GenBank accession number X77808 for *S. equinus* and X99345 for *S. asini*. In each PCR run, plasmid DNA samples containing the corresponding amplicons from *S. edentatus*, *S. equinus* and *S. asini* as template at concentrations of 500, 50 and 5 copies per reaction were used in duplicates as positive controls and references for melting curve analyses.

All PCRs were conducted twice with each reaction containing 500 nmol/L of each primer and 5 μL template DNA in 20 μL

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1X GoTaq® qPCR Master Mix using a Bio-Rad CFX-96 cycler. The raw relative fluorescence units (RFU) data were obtained in BioRad CFX Maestro 2.0. Normalisation was conducted using BioRad Precision Melt Analysis™ Software V1.0534.0511. After an initial denaturation at 94°C for 2 min, 50 cycles of denaturation at 94°C for 10 s, annealing at 63°C for 30 s and elongation at 72°C for 30 s were performed. Fluorescence was measured throughout the elongation phase. Subsequently, an HRM analysis was performed by raising the temperature from 65°C to 98°C in 0.1°C increments with each step lasting for 10 s and fluorescence was read at each step. The melting curves of the PCR products were analysed using the Precision Melt Analysis Software 1.0534.0511 (Bio-Rad). Of all positive samples, the PCR products were purified, cloned as described above and validated by Sanger sequencing at LGC Genomics (Berlin).

2.5 | Data analyses

For statistical analyses, only 28S rRNA pan-nematode PCR-positive samples were considered. The frequency of positive samples with 95% confidence intervals (CIs) was calculated using the binom.wilson() function from the epitools 0.5-10,1 package in R 4.1.1. In total 594 samples were included for downstream statistical analysis to determine the inter-rater reliability. To compare the morphological examination with the molecular methods, the inter-rater agreement was estimated by calculating the Cohen's kappa κ coefficients with 95% CIs using the cohen.kappa() function from the package DescTools 0.99.45 in R.

3 | RESULTS

3.1 | Morphological identification and differentiation of *Strongylus* spp. of L3 larvae

Using morphological examination of 712 samples, 12 samples (1.7%) were considered to be *Strongylus* spp. positive. None of the examined larvae had more than 20 IC, therefore the presence of *S. vulgaris* was excluded using this method. Only in two samples (0.3%) larvae were assigned to *S. edentatus*, and in both samples, *S. equinus* larvae were additionally observed. In total, *S. equinus* L3 were observed in 12 samples (1.7%).

3.2 | Detection of nematode DNA

By conducting a 28S rRNA pan-nematode PCR on all 712 larval culture samples, the presence of amplifiable nematode DNA was confirmed in 594 samples. After a fivefold dilution of the remaining 118 samples, the PCR was repeated, but no 28S rRNA DNA could be detected. Thus, a total of 594 pan-nematode positive samples were further subjected to *Strongylus* spp. specific real-time PCRs. Among the 118 samples that were excluded, none was positive for *Strongylus* spp. in the morphological examination.

3.3 | Detection of *Strongylus* spp. DNA in third-stage larvae

Frequency of different *Strongylus* spp. species as detected in the morphological and molecular analyses for the 594 samples is shown in Table 1. *S. vulgaris* DNA was detected in 5 out of 594 samples (0.8%) using real-time PCR. The results of all five samples were verified by Sanger sequencing. Based on peaks in the first derivative of the melt curve (Figure 1A) and the normalised HRM curve data (Figure 1B) and confirmed by sequencing, nine samples were *S. edentatus* positive (1.5%). One melting profile indicated a co-infection with *S. edentatus* and *S. equinus* (Figure 1). The PCR product of this sample was cloned. Due to a low number of colonies obtained even after repeated transformation, only four different colonies could be sequenced. None contained *S. equinus* DNA, which was, according to the first derivative of the melting curve, the rarer amplification product. This sample taken from an 8-year-old horse mare from the German federal state of North Rhine-Westphalia showed a co-infection with *S. edentatus*, *S. equinus* (both detected by HRM PCR) and *S. vulgaris* (detected in the *S. vulgaris* real-time PCR). One sample obtained from a horse from the federal state of Schleswig-Holstein reproducibly showed a melting curve profile with the same pattern as the positive control plasmid of *S. asini* (frequency 0.2%) (Figure 1). The PCR was repeated, the product was cloned, and two clones were sequenced and compared with the sequence of the reference plasmid. The two obtained sequences were identical (deposited in GenBank with the accession number OR419839) and had an identity of only 99% (117/118 positions, 100% query coverage) and it was therefore concluded that it was highly unlikely that the positive PCR was caused by contamination of the template or the PCR reaction with the reference plasmid.

3.4 | Monthly differences in detection of *Strongylus* spp. infections

Strongylus spp. infection as detected based on morphological data were found between July and December 2018 (Table 1). PCRs detected *Strongylus* spp. in February and between June and November 2018. For three samples information about the month of the taken sample was not available (Table 1). Due to the small number of positive samples and missing metadata for some samples, no statistical analysis of seasonality was conducted, although there was a tendency that observations clustered in summer/autumn. However, the total number of samples in these seasons was also higher than in winter and spring.

3.5 | Comparison of morphological and molecular identification of *Strongylus* spp.

The agreement between the morphological identification method and real-time PCR/HRM was calculated using Cohen's kappa κ coefficients (Table 2). Cohen's κ values were interpreted according to the

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TABLE 1 Monthly detection of *Strongylus* spp. based on morphological (Mo) and molecular tests (PCR) in 2018.

		Strongylus vulgaris		*Strongylus edentatus*		*Strongylus equinus*		*Strongylus asini*	
Month	*N*	Mo	PCR	Mo	PCR	Mo	PCR	Mo	PCR
January	4	0	0	0	0	0	0	0	0
February	17	0	1	0	2	0	0	0	0
March	47	0	0	0	0	0	0	0	0
April	61	0	0	0	0	0	0	0	0
May	22	0	0	0	0	0	0	0	0
June	48	0	0	0	1	0	0	0	0
July	85	0	1	1	0	1	0	0	0
August	30	0	0	0	1	0	0	0	0
September	92	0	0	0	1	4	0	0	1
October	71	0	1	0	1	3	0	0	0
November	44	0	1	1	1	2	1	0	0
December	18	0	0	0	0	2	0	0	0
n.a.	55	0	1	0	2	0	0	0	0
Total	594	0	5	2	9	12	1	0	1
Frequency (%)		0	0.8	0.3	1.5	2.0	0.2	0	0.2
95% CI (%)		0–0.6	0.4–2.0	0.1–1.2	0.8–2.9	1.2–3.5	0.02–0.9	0–0.6	0.02–0.9

Abbreviations: CI, confidence interval; *N*, sample number; n.a., no data on month available.

guidelines outlined by Landis and Koch.[21] The inter-rater agreement for detecting any *Strongylus* spp. irrespective of the particular species was only fair (Cohen's κ 0.31). The inter-rater agreement for *S. edentatus* and *S. equinus* was slight (Cohen's κ 0.18 and 0.15, respectively) and for *S. vulgaris* and *S. asini* only poor (Cohen's κ 0 for both).

4 | DISCUSSION

For diagnostic examinations of faecal samples for detection of strongyle infections, reliability, robustness and reproducibility of the method are of high relevance, but also economic aspects (examination time and material cost) need to be considered, as owners often refrain from examinations due to comparatively high costs. Following the coproscopical identification of strongyle eggs, the morphological examination of the L3 stage after larval cultivation is a commonly used diagnostic method for the discriminatory identification of Strongylinae and Cyathostominae. However, in the absence of knowledge regarding potentially relevant species-specific differences in the optima of culture conditions, such as humidity and temperature between equine strongyle species, larval cultures might introduce some bias. Accordingly, this approach has limitations regarding quantitative as well as qualitative species differentiation since some species might develop better than others under the chosen conditions. Furthermore, the host species, breed, nutritional status and geographic origin, as well as strongyle species diversity or egg shedding intensity, might also have an impact on the size of the larvae,[4,22–24] which may also affect morphological diagnosis.

The current study demonstrates that the agreement between morphological and molecular (real-time PCR and HRM PCR) methods was poor to, at best, fair. Regarding *S. equinus*, only 1 of the 12 morphologically positive samples could be confirmed by PCR. Moreover, only one of the two morphologically *S. edentatus*-positive samples was identified by molecular analysis, but the latter detected an additional eight samples as *S. edentatus*-positive. Similarly, *S. vulgaris* and *S. asini* were identified molecularly in five and one samples, respectively, while they went undetected in the morphological examination.

These discrepancies might be for different reasons. Regarding *S. equinus*, the most probable cause appears to be morphological misidentification, since L3 of *T. serratus*, *C. acuticaudatum*, *Oesophagodontus* spp. and *Poteriostomum* spp. have also 16 IC, and other morphological characteristics are often not uniquely assignable as well.[4–6] Since it is assumed, that *Triodontophorus* spp., *Oesophagodontus* spp. and *C. acuticaudatum* do not perform a body migration and that the pathogenicity resembles that of Cyathostominae, misidentification with more pathogenic *Strongylus* species should be excluded. Conversely, a number of PCR-positive *Strongylus* spp. samples remained morphologically negative. These larvae could have been overlooked, or alternatively, this is attributable to the maximum number of 250 larvae examined morphologically. Especially in the case of high larval numbers, which usually result from Cyathostominae, *Strongylus* spp. larvae may simply not have been present in the aliquot of the sample that was analysed. However, the molecular analysis could also have produced false positive results in some samples, for example, due to contamination between samples and/or with the positive control plasmids. However, this can be ruled out, at least for the one *S. asini*-positive sample, as sequencing revealed a nucleotide

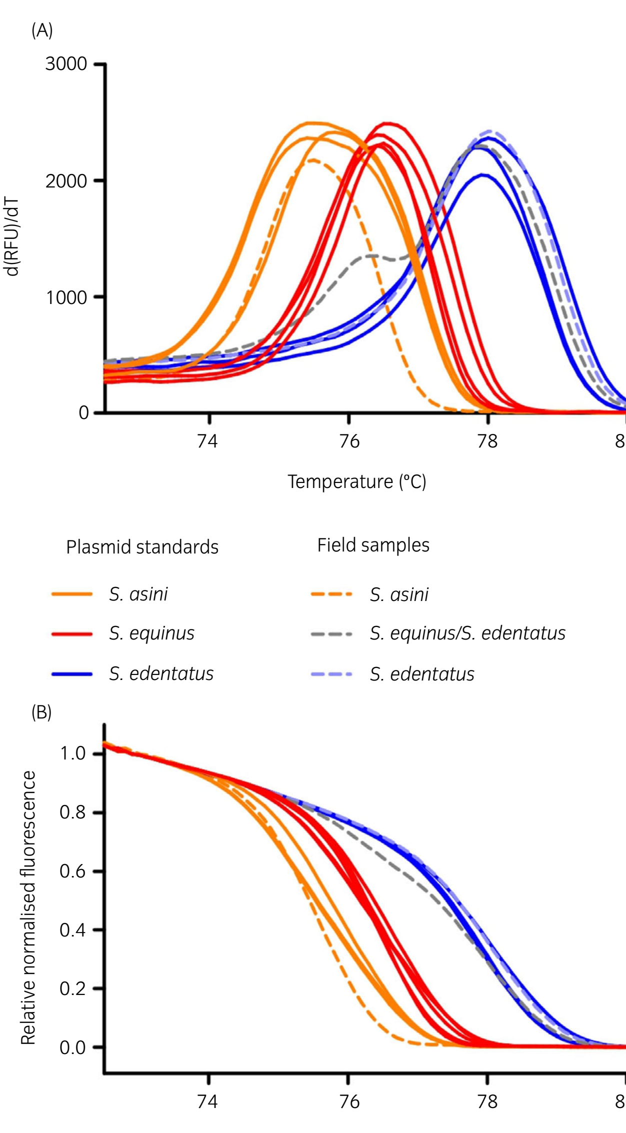

FIGURE 1 High-resolution melting curve analysis of representative samples positive for *Strongylus* spp. data are viewed as the first derivative of the melting curve (A) or as the normalised melting curve (B). Plasmids containing the PCR product for one species were used as standard template.

polymorphism to the reference plasmid. Moreover, polymorphisms in the target DNA sequences might prevent amplification or detection of *Strongylus* spp. in morphologically positive but molecularly negative samples due to imperfect hybridisation of primers or probes. In theory, a very low concentration of target sequences could be a conceivable reason for a false negative molecular analysis, especially in samples with high numbers of Cyathostominae DNA. Thus, by chance, a single *Strongylus* spp. larvae might have been present in the morphologically analysed subsample although the frequency of these larvae was too low to be detected by PCR. In the case of *Strongylus* spp. coinfections, it also needs to be considered that the amplification reaction, due to its exponential function, can sometimes favour frequent target sequences and suppress very rare ones. Nevertheless, the discrepancies observed in the current study are most probably attributable to the limitations of the morphological analysis as demonstrated before already for *S. vulgaris* but not for the other *Strongylus* spp.[9,11,12]

Avoiding false negative results is particularly crucial with regard to *S. vulgaris* infections. Since this parasite is associated with high pathogenicity, false negative results might lead to problematic treatment decisions, as twice-yearly treatments required to interrupt the parasite's life cycle on a particular farm would not be applied. The current study indicates that the *S. vulgaris* PCR is more sensitive than morphological evaluation since all PCR-positive samples were scored negative using morphology. The frequency of 0.8% for *S. vulgaris* among strongyle egg-positive samples based on PCR is comparable with previously conducted studies in Germany that showed prevalences in the range of 0%–1.3% on individual horse level based on morphological examination and 1.9% based on a qPCR approach.[7–10] Morphological and molecular methods were further compared by calculating Cohen's κ values for inter-rater-agreement. Due to the small number of positive samples, Cohen's kappa, supported by the low 95% CI values, should be considered carefully. Additional data, including more positive samples, are required to come to robust conclusions about the agreement of the methods. Currently, it is impossible to obtain such data from field samples in Central Europe since prevalences of *Strongylus* spp. are simply too low to obtain such data with a reasonable effort. This is particularly true for rarely found *Strongylus* species. Applying both approaches to wild equine populations might offer a solution to this problem.

To the authors' knowledge, this is the first report of a naturally infected horse with *S. asini*. Regarding the metadata of this sample, the only information provided by the owner was that the sample originated from a horse and was sent from the federal state of Schleswig-Holstein, Germany. No information could be obtained if the horse was grazing on a pasture shared with donkeys or zebras, the natural hosts of *S. asini*. The only report of an experimental *S. asini* infection in a single horse suggests that only a few larvae undergo migration and that this is associated with less pathology than infections with other *Strongylus* spp.[25] This study was terminated by euthanising the study animals, followed by necropsies 4 months after the infection. Only L4 larvae could be obtained during necropsy. Therefore, no information is available on some of the larvae that would have developed into gravid adult worms in the gut. To the best of the authors' knowledge, data regarding the prepatent period are missing. Since only one other study systematically searched for the presence of *Strongylus* spp. including *S. asini* in horses using PCR,[15] it might well be that rare patent infections of horses with this parasite have been overlooked so far. Most probably, co-grazing with donkeys and zebras, the competent hosts of *S. asini*, would increase the chance to find *S. asini*-positive horses, but this needs to be further investigated in the future.

In conclusion, the current study showed that results of PCR and morphological diagnosis of L3 from equine samples can differ considerably, most probably due to limitations of the morphological

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TABLE 2 Inter-rater reliability between morphological (Mo) and molecular tests (PCR).

Comparison	Mo^+/PCR^+	Mo^-/PCR^+	Mo^+/PCR^-	Mo^-/PCR^-	Cohen's kappa	95% CI
Strongylus spp.	4	9	8	573	0.31	0.06–0.55
Strongylus vulgaris	0	5	0	589	0	0–0
Strongylus edentatus	1	8	1[a]	584	0.18	−0.13 to 0.48
Strongylus equinus	1[b]	0	11[c]	582	0.15	−0.11 to 0.41
Strongylus asini	0	1	0	593	0	n.d.

Abbreviations: CI, confidence interval; n.d., not defined.
[a]Tested positive for *S. vulgaris* and *S. equinus* by PCR.
[b]Sample was also positive for *S. vulgaris* and *S. edentatus* by PCR.
[c]Three of 11 tested positive for *S. edentatus*.

evaluation. Particularly, measurement of all individual larvae in length and width is usually too time consuming during routine diagnostic processes. This situation makes morphological species identification of equine strongyle larvae a challenging task that should be critically evaluated. In addition to the deployment of experienced laboratory personnel, an initial classification into Cyathostominae with 7–12 IC and Strongylinae ≥16 IC could be implemented. If Strongylinae are present, further species differentiation might be achieved using molecular approaches as part of a quality assurance strategy. Due to further improvements, developments and the associated cost reductions in molecular examination methods it will probably be possible to offer this examination method cost-effectively in the near future. The finding of *S. asini* DNA in a sample from a domestic horse will need further investigation to determine its epidemiological relevance.

FUNDING INFORMATION

This research was funded by Deutsche Forschungsgemeinschaft (German Research Foundation, DFG) through the Research Training Group GRK 2046 “Parasite Infections: From experimental models to natural systems” (project number 251133687/GRP2046).

ACKNOWLEDGEMENTS

The authors thank the research training group GRK 2046 (Graduierten-Kolleg 2046) for stimulating discussions that substantially improved the manuscript. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Irina Diekmann: Conceptualization; data curation; writing – original draft; investigation; validation; formal analysis. **Katrin Blazejak:** Investigation; validation; formal analysis; data curation; writing – review and editing. **Jürgen Krücken:** Conceptualization; methodology; data curation; formal analysis; supervision; visualization; writing – original draft; writing – review and editing. **Christina Strube:** Conceptualization; supervision; funding acquisition; writing – review and editing. **Georg von Samson-Himmelstjerna:** Conceptualization; methodology; funding acquisition; project administration; resources; writing – review and editing.

DATA INTEGRITY STATEMENT

Georg von Samson-Himmelstjerna had full access to all data and takes responsibility for the integrity of the data and accuracy of the data analysis.

ETHICAL ANIMAL RESEARCH

Ethics permission was not required for this study as surplus material from diagnostic samples (noninvasively collected faecal samples) was used.

INFORMED CONSENT

Written consent was obtained from the owners or veterinarians who sent samples for diagnostics that the samples may be further used for research in general.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request: Open sharing exemption granted by editor.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Diekmann I, Blazejak K, Krücken J, Strube C, von Samson-Himmelstjerna G. Comparison of morphological and molecular *Strongylus* spp. identification in equine larval cultures and first report of a patent *Strongylus asini* infection in a horse. Equine Vet J. 2025;57(2):522–9. https://doi.org/10.1111/evj.14134

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