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PRELIMINARY DATA ON OCCURRENCE OF *Strongylus vulgaris* IN HORSES, ROMANIA: LARVAL CULTURES AND REAL-TIME PCR

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Abstract

Strongylus vulgaris (Nematoda: Strongylidae) is the most pathogenic helminth parasite of horses. Despite of the increasingly reports on reducing its prevalence in well managed horse farms, surveys of horse populations across the world still document the presence of this parasite. Therefore, this study aimed to investigate the occurrence of S. vulgaris in different horse establishments in Romania. For this purpose, a total of 439 horses, originated from 20 premises and nine counties of Romania, including horses (n = 228) from three stud farms, four sport / recreational units (n = 43), and working horses (n = 168), which tested positive for strongyle infection, were included in the study. Individual fresh faecal samples with the strongyle egg per gram (EPG) count > 100 were subjected for larval culturing; subsequently third stage larvae (L3), harvested using a Baermann technique, were further subjected for morphological analysis to identify S. vulgaris species. Additionally, 64 pooled samples (obtained from 5-10 individual coprocultures) of mixed strongyle-type L3 larvae, including 27, 4, and 33 samples from stud, sport, and working-horses, respectively) were molecularly screened for S. vulgaris by Real-Time PCR. On microscopic examination of individual coprocultures, S. vulgaris L3 larvae were identified only in working horses (15.5% at individual level). Additionally, the RT-PCR confirmed S. vulgaris-DNA in 8 of screened pooled-samples, of which 7 were from working horses and 1 from stud farm horses. Altogether, the results revealed the occurrence of S. vulgaris in Romanian horses and particularly emphasized the higher sensitivity of PCR-based methods for its monitoring in horse populations. Moreover, these preliminary findings are the base for on-going molecular investigations of horses for S. vulgaris at individual level to provide useful information for a sustainable control of equine strongyles in Romania.

Key words*: equine, Strongylus vulgaris, coprocultures, Real-Time PCR.*

INTRODUCTION

Strongyles (Nematoda: Strongylidae) are the most common helminth parasites (Reinemeyer et al., 1984; Mitrea, 2011). Equine strongyles, members of two subfamilies, Strongylinae and Cyathostominae, are of significant veterinary interest because of their high pathogenicity due to the species-specific visceral migration, and their development of anthelmintic resistence, respectively (Kaplan, 2004; Lyons et al., 2008a; 2008b; 2011a). Of them, particularly, the large strongyle *Strongylus vulgaris* (subfamily Strongylinae) is regarded as the most pathogenic parasite of horses (Drudge and Lyons, 1986; Mitrea, 2011). The adult worms parasitize in the cecum and the right ventral colon, while the larvae (the fourth-L4 and fifth-L5 stages) migrate in the circulatory system, the cranial mesenteric arterial tree, causing fibrinous endarteritis, trombosis, and severe aneurysms (Duncan and Pirie, 1985).

Despite of the increasingly reports on reducing its prevalence in well managed horse farms, surveys of horse populations across the world still document the presence of this parasite (Herd, 1990; Kaspar et al., 2017).

Due to the inability of the classical and widely applied copro-parasitological techniques, which are used for detecting strongyle-type eggs, to differentiate the strongyle species that inhabit the digestive tract of horses, currently it is a large interest for developing and applying complementary techniques, such as larval culturing and molecular techniques (Andersen et al., 2013). Moreover, larval cultures (copro-cultures) is a practice used in many countries (i.e. Denmark, Germany), where, in order to reduce the intensity of antiparasitic treatments and to minimize the development of chemoresistance a targeted selective therapy regimen is applied, being part of the surveillance program implemented to monitor

the occurrence of *S. vulgaris* (Pfister and van Doorn, 2018).
Identification of the strongyle L3 it is a very important tool for epidemiological studies of equine strongyles, as it is known that the most prevalent larval types are produced by the most resistant species (Madeira de Carvalho et al., 2008, Matthews et al., 2004).
Accordingly, based on morphological keys (number, shape, and arrangement of intestinal cells, type of oesophagus, length of tail), there have been described different L3 morphotypes, for the both, cyathostomins and strongylins (L3 with different arrangemments of the 6 to 16 and 16 to 32 intestinal cells, respectively) (Kornas et al., 2009).
Currently, larval culture and subsequent morphological identification of third larvae (L3) (the standard method) and Real-Time PCR are useful tools for differentiation of cyathostomins and strongylins (Nielsen et al., 2012). Therefore, this study aimed to preliminary assess the occurrence of *S. vulgaris* in horses from different horse establishments in Romania by using larval cultures and, for the first time in Romania, by Real-Time PCR.

MATERIALS AND METHODS

Animals and samples
A total of 439 horses, originated from 20 premises and nine counties of Romania, including horses (n = 228) from three stud farms, four sport/recreational units (n = 43), and working horses (n = 168), were enrolled in a coprological study. From them, individual fresh faecal samples were collected and analyzed using a modified McMaster method for strongyle egg per gram (EPG) counting (with a limit of detection of 25 epg). All individual fecal samples with the strongyle EPG count ≥100 were subjected for larval culturing.

Larval cultures
Larval cultures were performed as described by Henriksen and Korsholm (1983). Briefly, 10 grams of faeces were incubated at 22-24ºC for 14 days. Regularly, the cultures were checked for dessication and moistened. Third stage larvae (L3) were harvested after 24 h sedimentation using a Baermann technique. For each sample, 1 ml of sediment was collected, centrifuged (at 3000 rpm, 5 min), and was subjected for morphological analysis of the strongyle L3, as described before (Bellaw and Nielsen, 2015; Anutescu et al., 2016). Briefly described, of each 1 ml sediment, harvested larvae morphological identification was performed from an aliquot of 100 µl, to characterize the structure of strongyle-populations and the remaining sediment (900 µl) was examined for the the presence of *S. vulgaris* larvae (Buzatu et al., 2017).
Identification and differentiation of harvested larvae into different strongyle L3 morphotypes were performed using morphological (number, shape, and arrangement of intestinal cells, type of oesophagus, length of tail) keys (Kornas et al., 2009).
A total number of 64 pooled samples of mixed strongyle-type L3 larvae, including 27, 4, and 33 samples from stud, sport, and working-horses, respectively) were constituted using collected larvae from 5 to 10 individual coprocultures and subjected for molecular screening for *S. vulgaris* by using Real-Time PCR.

DNA extraction and Real-Time PCR
DNA-extraction from harvested larvae was carried out following the manufacturer's recommendations (PureLink® Genomic DNA Kit, Invitrogen) according to the blood or body fluid spin protocol (Qiagen, Hilden, Germany). DNA samples were stored at –20°C until used for RT-PCR analysis.
Samples were screened for *S. vulgaris* by real-time PCR using the StepOnePlus Real Time PCR System (Applied Biosystems, Darmstadt, Germany), targeting rDNA sequences of the ITS-2 (the second internal transcribed spacer).
The amplification RT-PCR protocol was adapted after Nielsen et al. (2008) and Kaspar et al. (2017).

RESULTS AND DISCUSSIONS

Larval cultures
The number of larvae morphologically identified (in 100 µl of the sediment) from larval cultures of stud, sport, and working horses varied, depending on the initial EPG values, from 5 to 118 (average 69), 1 to 77

(average 56), and from 1 to 88 (average of 49), respectively.

The analysis of the larval coprocultures, respectively the differentiation and identification of L3 larvae revealed the presence of mixed larvae populations, with the both cyathostomine and strongyline species.

However, coprocultures revealed the presence of *S. vulgaris* L3 only in working horses, with a mean prevalence of 15.50%.

Synthesized data about the occurrence of different strongyle larval morphotypes/species in the investigated horses are presented in Table 1.

Table 1. Sintetized data on number and percentage of positive horses for different strongyle L3 morphotypes/species, horses from different breeding systems (data on larval cultures)

Breeding system		Third stage strongyle larval morphotypes/species of cyathostomines and strongylines (from individual aliquot of 1000 µl sediment)															
		Cyathostominae										*Strongylinae*					
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	***Gy***	***Po***	***Sv***	***Sed***	***Seq***	***Oe***	***Cr***	***Tr***
Stud farms																	
L.C.	**n=228**	228	7	166	77	9	26	10	19	4	6	0	0	0	0	0	2
	%	*100*	*3.07*	*72.80*	*33.80*	*3.95*	*11.40*	*4.40*	*8.33*	*1.75*	*2.60*	*0*	*0*	*0*	*0*	*0*	*0.88*
Sport units																	
L.C.	**n=43**	43	0	24	4	0	1	1	0	0	0	0	0	0	0	2	0
	%	*100*	*0*	*55.80*	*9.30*	*0*	*2.33*	*2.33*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*4.65*	*0*
Working horses																	
L.C.	**n=168**	168	1	82	36	2	6	1	9	10	9	26	1	2	8	10	3
	%	*100*	*0.60*	*48.80*	*21.40*	*1.20*	*3.50*	*0.60*	*5.30*	*5.95*	*5.36*	*15.50*	*0.60*	*1.20*	*4.70*	*5.60*	*1.80*

L.C.= larval cultures; n= number of horses; %: percentage: A-H: Cyathostominae morphotypes; Gy: *Gyalocephalus capitatus;*
Po: *Poteriostomum* spp.; Strongylinae (subfamily): S.v: *Strongylus vulgaris*; S.ed: *Strongylus edentatus*; S. eq: *Strongylus equinus;*
Oe: *Oesophagodontus robustus;* Cr: *Craterostomum acuticaudatum*; Tr: *Triodontophorus* spp.

Real-Time PCR

A total number of 64 pooled samples, including 33, 27, and 4 samples from working, stud farms, and sport horses, respectively, containing mixed strongyle-type L3 larvae, were tested for *S. vulgaris* DNA. Each sample contained, between 37 and 200 larvae.

Subsequently to the molecular analysis, the RT-PCR confirmed *S. vulgaris* DNA in working horses (7/33 pooled samples, including all individual samples positive at larval cultures) but also in one pooled samples from horses originating from stud farm (1/27 pooled samples), while none of the pooled samples from sport horses were positive for *S. vulgaris* DNA.

A detection threshold of 500 fluorescence units (dR) was used and results were recorded as the mean PCR cycle number at which the fluorescence detection threshold had been exceeded (Ct) (Table 2).

Table 2. Data on the *Strongylus vulgaris* positive samples by RT-PCR compared with the larval cultures

ID	*Orig. county	Type establishment	*Strongylus vulgaris*		RT-PCR
			LC	RT-PCR	*Ct
P_1	TL	working	positive	positive	29.6337
P_2	TL	working	positive	positive	30.1878
P_3	TL	working	positive	positive	30.4090
P_4	BV	working	negative	positive	35.9485
P_5	BV	working	positive	positive	30.4395
P_6	IL	working	negative	positive	33.7626
P_7	IL	working	positive	positive	34.4597
P_8	BV	stud farm	negative	positive	30.8645
Positive control [PC]				**positive**	**20.8894**

P1-P8: pooled samples of mixed strongyle third stage larvae from larval cultures; *horse originating county; TL: Tulcea; BV: Brasov: IL: Ialomita; **Ct: mean PCR cycle number threshold; LC: larval culture

Discussion

Performing larval culture for identification of infesting larval stages (L3) of strongyles plays a very important role for studying the epidemiology of equine strongyles; previous reports showed that the most prevalent strongyle larval morpoypes are produced by the most resistant species (Madeira de Carvalho et al., 2008; Matthews et al., 2004).

In this respect, the results of the present study are particularly relevant, showing the morphotypes A with highest frequency; in previous studies the morphotype A is reported to be produced by the most prevalent and chemoresistant cyathostomin species (Madeira de Carvalho et al., 2008). Moreover, it has been shown that regular deworming impacts the structure of strongyle populations in horses. For this reason, investigations on natural equine strongyle infections and the species-composition of strongyle populations are essential for a better understanding of the anthelmintic chemo-resistance mechanisms developed by small strongyle species in horses (Lyons et al., 2011b).

In Romania, strongyle infections are very common in horses, with high prevalence and frequency in different equine breeding systems, throughout all the country. Recent studies have been reported prevalence values for strongyle infection from 44.0%, 79.3% up to 88.4% for sport, working and stud farm horses, respectively (Ionita et al., 2013; Buzatu et al., 2014, 2016). Moreover, 100% extensivity for strongyle infection was reported by both coproparasitologic examination and necropsy, in horses from eastern Romania (Covasa and Miron, 2011). Other similar studies, conducted in western, centre, or eastern areas of in horses from Romania, investigating the strongyle-species population composition, have reported also the cyathostomine morphotype A as the most common. Additionally, among the cyathostomine and strongyline species, *Cyathostomum catinatum, C. pateratum*, *Cylicostephanus goldi, C. longibursatus*, and *Strongylus vulgaris, S. edentatus, Oe. robustus, Triodontophorus* spp., respectivelly, have been reported (Cernea et al., 2003, 2015; Badea et al., 2015: Anutescu et al., 2016; Buzatu et al., 2017).

Knowledge of the biodiversity of larval morphotypes among strongylid populations in Romania will therefore allow an indirect assessment of the susceptibility and/or resistance status of different subpopulations of cyathostomins (Cernea et al., 2015), as a basis for further studies on the epidemiology of strongyle infections in horses in Romania.

A comparative study by Madeira et al. (2008) in dewormed and non-dewormed horses and donkeys, from Romania and Portugal, revealed that morphotype A cyatostomins had the highest prevalence in dewormed animals, followed by type C and D. Therefore, a good identification of cyatostomines will provide knowledge about epidemiology, biology or even monitoring their antiparasitic resistance.

Regarding the presence of *S. vulgaris* species, it is rarely reported in horses in farms where rigorous long-term parasitological control programs are applied (for the last 50 years), based on frequent prophylactic anthelmintic treatments (Kaplan, 2004). However, there are also studies that report the presence of this species in such farms, even if at lower prevalence values, which could be due to also the investigation methods used (Mitrea, 2002; Schneider et al., 2014).

Also, there are currently studies showing that in horse farms where parasitological control programs based on selective therapy are already applied, based on the less frequent application of anthelmintic treatments, there is a risk of re-emergence of *S. vulgaris*, as reported in Denmark (Nielsen et al., 2006; 2012).

In contrast, the species *S. vulgaris* is more frequently reported in working horses or horses on farms where prophylactic treatments are performed with low frequency, an aspect confirmed also in our studies, with a prevalence of 15%. These results are in agreement with previous studies carried out in Romania in slaughtered horses: thus, Cernea (2006) reports a prevalence of 10.38% for *S. vulgaris* and 2.12% for *S. edentatus* and lower prevalence values (5.08-1.58%) for other strongyline species, such as *Oe. robustus*, *Triodonthophorus* spp., *Cr. acuticaudatum*.

More recently, a post-mortem examination study of 47 working horses from Romania revealed 100% positive for small strongyles (Cyathostominae) infections and 24 species were identified; of them, *Cyathostomum catinatum, Cylicocyclus insigne*, and *C. nassatus*

had the highest prevalence (100%) (Morariu et al., 2016).
In Poland, in slaughtered horses, *S. vulgaris* was the dominant species among nematodes (22.8%), followed by *S. edentatus* (18.3%) and *S. equinus* (1.7%) (Studzińska et al., 2012).
Thus, *S. vulgaris* is still a threat to horse health and therefore monitoring studies are required. Additional to the larval cultures, which are the standard method of monitoring the presence of this parasite in many European countries, such as Denmark, Germany, Italy, currently more sensitive methods, based on PCR, have been developed and applied (Ioniță et al., 2010; Nielsen et al., 2012; Kaspar et al., 2017).
In this study, RT-PCR confirmed the *S. vulgaris* DNA in all the samples which comprises the individual positive samples at larval cultures but also in samples which were negative at larval examinations, including one pooled sample from stud farm horses. This fact falls within the limits of the larval cultivation method and can be explained by the fact that the development of certain species of strongylides requires different conditions of temperature and humidity and thus it is possible the incomplete larval development (with indefinite appearance and/or indistinguishable morphological elements) (Roeber and Kahn, 2014).
PCR-based molecular biology techniques represent alternative or complementary tools with superior specificity and sensitivity that can validate the results (Kaspar et al., 2017).
Therefore, the findings of the present study confirmed the higher sensitivity of RT-PCR technique for investigating the occurrence of *S. vulgaris* in horse populations.

CONCLUSIONS

Altogether, the results revealed by larval culture and for the first time molecularly, by RT-PCR, the occurrence of *S. vulgaris* in Romanian horses.
Moreover, these preliminary findings are the base for on-going molecular investigations of horses for *S. vulgaris* at individual level to provide useful information for a sustainable control of this important parasite of horses, in Romania.

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