Investigating the Strongyle Populations, with Emphasis on *Strongylus vulgaris* (Nematoda: Strongylidae) in Romanian Horses, Based on Larval Cultures

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ABSTRACT

This study aimed to investigate the presence of *Strongylus vulgaris* on different horse establishments in Romania. A total number of 380 horses were enrolled in the study, representing: three stud farms (n=156); four sport/recreational establishments (n=75); and working horses (n=149). Overall, 106 premises/yards from 17 localities in 9 counties from North-Eastern, Center and Southern Romania were visited. Fresh fecal sample were collected from individual horses and were analyzed by a modified McMaster method for fecal strongyle egg per gram counting. Further, larval cultures were performed for morphological identification of *S. vulgaris* third stage larvae. Overall, of the investigated horses from studs, sport/recreational units, and working horses, 84.60%, 44.0%, and 79.20%, respectively were positive for strongyle. On coprocultures, cyathostomin-larvae were the most prevalent in all horse-establishment types, while *S. vulgaris* larvae were found only in working horses (12.1%; n=18). Other strongylin species have been identified, namely *S. equinus*, *S. edentatus*, *Oesophagodontus robustus*, and *Triodontophorus* spp. all in working horses, and *Craterostomum acuticaudatum*, in both working horses and horses for sport units. However, further studies are planned by using more sensitive technique for monitoring of *S. vulgaris* in Romanian horse farms.

Keywords: horses, larval cultures, Romania, *Strongylus vulgaris*

INTRODUCTION

The large strongyle *Strongylus vulgaris* (Nematoda: Strongylinae) is the most pathogenic strongyle species and the most important parasite in horses (Lyons *et al.*, 2011; Mitrea, 2011). Adult worms live in the cecum and right ventral colon, while its migrating fourth (L4) and fifth (L5) stage larvae are responsible for severe verminous endarteritis, tromboembolism and infarction of the cranial mesenteric artery and its branches characterized by painful colic syndrome, which can be fatal (Duncan and Pirie, 1975; Lyons *et al.*, 2011; Mitrea, 2011). Despite of the fact that the intensive anthelmintic treatment strategies designed to control *S. vulgaris* have been successful in reducing its prevalence, morbidity

and mortality due to this parasite (Kaplan, 2002), surveys of horse populations across the world still document its occurrence (Nielsen, 2012). Therefore, *S. vulgaris* still represents a potential threat to equine health and it is a need to monitor horse farms for its presence. In this context, field studies to investigate the occurrence and prevalence of different equine strongyle species in horse populations from different geographical areas and under different breeding systems are very important to develop sustainable control programs.

However, for living animals, the microscopic examination of fecal strongyle eggs does not allow species specific differentiation of mixed natural strongyle infections, due to the egg morphology

similarities (Lichtenfels *et al.*, 2008). Therefore, alternative methods have been developed, such as larval cultures. Larval culturing is the most practical method to differentiate and identify different equine strongyle species or genera (up to 17), based on the morphological characteristics of the third larval stage (L3) (Cernea *et al.*, 2008; Andersen *et al.*, 2013).

By this study was aimed to investigate the presence of *S. vulgaris* on different horse establishments in Romania, based on larval cultivation.

MATERIALS AND METHODS

Animals

A total number of 380 horses were enrolled in the study, representing: three horse farms (n=156); four recreational/sport establishments (n=75); and working horses (n=148) aging from 1.2 to 28 years. Overall, 106 yards from 17 localities in 9 counties from North-Eastern, Center and Southern Romania were visited (Tab. 1).

Fecal analyses, Larval culturing, and identification of the third larval stage

Fresh fecal samples were collected from individual horses and were firstly analyzed by a modified McMaster method for fecal strongyle egg per gram (EPG) counting. Further, individual larval cultures were performed on positive samples, for the morphological identification of *S. vulgaris* larvae (third stage L3), as described before (Anutescu *et al.*, 2016). Briefly, for individual larval cultures (LC), 10 grams of faeces were suspended in humidity chambers which were incubated for 14 days at 22-24°C: LC were daily ventilated and moistened if necessary. After culturing, L3 were harvested after 24 h sedimentation, using a modified Baermann method (Bellaw and Nielsen, 2015). For each larval culture, 1 ml of sediment was collected, centrifuged at 3000 rpm for 5 minutes, and analyzed according to the protocol described by Schneider *et al.* (2014), as follows: 100 µl aliquot was used for counting and morphological identification and differentiation of larvae (larvae were immobilized with Lugol's iodine); the remaining sediment (900 µl) was analyzed for identification of *S. vulgaris* larvae. The strongyle larvae were identified using morphological keys, based on: number, shape, and arrangement of the intestinal cells, type of esophagus, tail length (Cernea *et al.*, 2008; Kornas *et al.*, 2009).

RESULTS AND DISCUSSIONS

Overall, of the investigated horses from stud farms, sport/recreational units, and working horses, 84.60%, 44.0%, and 79.20%, respectively were positive for strongyle eggs (Tab. 1), with the individual of EPG counts varying from 0 to 6675, 5250, and 3700, respectively.

For morphological identification of *S. vulgaris* larvae, a total of 283 individual larval cultures were performed. The presence and frequency of different cyathostomin and strongylin L3 morphotypes / species was established based on the examination of larvae in 1 ml of sediment / each individual cultures and the prevalence based on the identified L3 from an aliquote of 100µl / each LC.

The morphological identification of L3 was made based on the following criteria: type of esophagus, intestinal cells (number, shape, and arrangement), and length of tail (Kornas *et al.*, 2009). Accordingly, there has been described different types of arrangements: of 8 intestinal cells encountered in cyathostomins, called A-D, as well as other morphotypes, namely type E (6 cells), type F (7 cells) and type G (8 nonspecific cells), H (9 cells), *Gyalocephalus capitatus* (12 cells), and *Poteriostomum* spp. (16 cells). Similar morphological keys are also described for the large strongyle species, which have higher number of intestinal cells (16 - 32) (Madeira de Carvalho *et al.*, 2008; Cernea *et al.*, 2008).

S. vulgaris larvae (Fig. 1) were identified based on the following characteristics: long dimension (900 - 1110 µm), short esophagus, long tail, and 28 - 32 distinct intestinal cells (Cernea *et al.*, 2008).

Overall, on coprocultures, larval differentiation and identification showed mixed strongyle infections, with small strongyle species (cyathostomins) the most prevalent in all horses investigated, represented by cyathostomin morphotype A, C, D as the most frequent, being identified in the majority of horses from all breeding systems, followed by the morphotype B, E, F, G, H, *Gyalocephalus capitatus*, and *Poteriostomum* spp., which were identified with a lower frequency (Tab. 2).

S. vulgaris larvae were found only in working horses (n=18; 12.1%); the positive animals aged between 2 and 20 years. Other strongylinae were identified, as follows: *Strongylus equinus*, *S. edentatus*, *Oesophagodontus robustus*, and *Triodontophorus* spp. all in working horses, and *Craterostomum acuticaudatum*, in both working horses and horses for sport (Tab. 2).

Tab 1. Prevalence of strongyle-egg-type infection and *Strongylus vulgaris* (L3) in horses, stratified by their geographical originating area and horse breeding systems, Romania

Breeding system/ origin area		Premises/yards		Horses: number; percentage (%)		
region	County*	Premises (L)	yards (number)	investigated	positive for strongyle egg	positive for <i>Strongylus</i> <i>vulgaris</i> L3
<i>Working horses (n=148)</i>						
North-Eastern	SV	L.1	8	10	8 (80.0)	1 (10.0)
		L.2	10	11	5 (45.4)	0
	BT	L.3	8	17	11 (64.7)	2 (11.7)
		L.4	6	13	8 (61.5)	4 (30.7)
Center	BV	L.5	19	32	25 (78.1)	0
		L.6	28	33	31 (93.9)	7 (21.2)
South/ South-Eastern	BZ	L.7	6	12	12 (100)	1 (8.3)
	IL	L.8	5	6	3 (50)	2 (33.3)
	IF	L.9	1	5	5 (100)	0
	TL	L.10	8	10	10 (100)	1 (10.0%)
Total (%)		10	99	149	118 (79.2)	18 (12.1)
<i>Sport/recreational horse establishments (n= 75)</i>						
South/ South-Eastern	TL	L11	1	10	7 (70.0)	0
	PH	L.12	1	12	11 (91.66)	0
	IF	L.13	2	53	15 (28.30)	0
Total (%)		3	4	75	33 (44.0)	0
<i>Stud farms (n= 156)</i>						
Center	BV	L.14	1	40	34 (85.0)	0
South-Eastern	BZ	L.15	1	56	49 (87.5)	0
	CT	L.16	1	60	49 (81.66)	0
Total (%)		3	3	156	132 (84.60)	0
TOTAL		16	106	380		

Legend: *BZ-Buzau; BV-Brasov; BT-Botosani; CT-Constanta; IF-Ilfov; PH-Prahova; SV: Suceava; TL-Tulcea

Tab. 2. Structure of strongyle populations, based on larval cultures, in horses from different Romanian breeding systems

Horses' Category	Number of horses positive for different cyathostominae ^c and strongyline ^s third stage larvae (L3)															
	A ^c	B ^c	C ^c	D ^c	E ^c	F ^c	G ^c	H ^c	Gy ^s	Po ^s	Sv ^s	Se ^s	Seq ^s	Oe ^s	Cr ^s	Tr ^s
Working horses (n=118)	118	0	88	35	0	0	0	4	11	3	18	1	2	4	5	1
Sport horses (n=33)	33	0	31	9	0	2	2	0	0	0	0	0	0	4	0	0
Stud farms (n=132)	132	7	101	68	9	26	10	4	3	2	0	0	0	0	0	0

Note: ^c: Cyathostominae: A – H: *cyathostomins* morphotype A to H; Gy: *Gyalocephalus capitatus*; Po: *Poterios-tomum* spp.; ^s: Strongylineae: S.v: *Strongylus vulgaris*; S.ed: *Strongylus edentatus*; S.eq: *Strongylus equinus*; Oe: *Oesophagodontus robustus*; Cr: *Craterostomum acuticaudatum*; Tr: *Triodontophorus* spp.

Fig. 1. Third stage of *Strongylus vulgaris* (recovered from larval cultures (100x))

Subsequently of examination of an aliquote of 100 µl in each larval culture (LC) a total number of 10430 (from 118 LC), 3630 (from 33 LC), and 13740 (from 149 LC) larvae were counted on working horses, horses from sport units, and stud farms, respectively. The cyathostomin morphotype A was the most prevalent (varying from 65.8 to 70.2%), followed by the morphotype C (prevalence

between 9.4 and 18.7%), and D (2.8 – 6%). Other small strongyle species were also identified, but in lower prevalence. Of the large strongyles, *S. vulgaris*, *S. edentatus*, *Triodontophorus* spp. larvae were identified all LC from working horses (0.4%), while *Craterostomum acuticaudatum* was found in those from the both working (0.4%) and sport horses (1.1%) (Tab. 3).

Tab. 3. Prevalence of cyathostomin and strongylin third larvae morphotypes (% of L3 identified in 100 µl) in horses, stratified by different breeding systems

Horses' category / L3 identified	L3 morphotypes / species of cyathostominae ^c and strongylinae ^s (identified in the aliquot of 100 µl of each LC)																
	A ^c	B ^c	C ^c	D ^c	E ^c	F ^c	G ^c	H ^c	Gy ^c	Po ^c	N	Sv ^s	Sed ^s	Seq ^s	Oe ^s	Cr ^s	Tr ^s
Working horses																	
L3 (n=10430)	65.8	0	16.9	2.8	0	0	0	0	0	0.4	12.7	0.4	0.4	0	0	0.4	0.4
Sport horses																	
L3 (n=3630)	70.2	0	18.7	3.0	0	0.6	0	0	0	0	6.3	0	0	0	0	1.1	0
Stud farms																	
L3 (n=13740)	67.1	0.7	9.4	6.8	0.5	3.1	0.3	0.3	0.3	0.15	11.6	0	0	0	0	0	0

Note: ^c: Cyathostominae: A-H: *cyathostomins* morphotype A to H; Gy: *Gyallocephalus capitatus*; Po: *Poteriostomum* spp.; ^s: Strongylinae: Sv: *Strongylus vulgaris*; Sed: *Strongylus edentatus*; Seq: *Strongylus equinus*; Oe: *Oesophagodontus robustus*; Cr: *Craterostomum acuticaudatum*; Tr: *Triodontophorus* spp.

Overall, this study emphasizes that strongyle infections are highly prevalent in all horse breeding systems. These findings are in line with previously reported data of epidemiological studies performed in different areas of Romania (Covasa and Miron, 2011; Morariu *et al.*, 2012; Buzatu *et al.*, 2013, 2014, 2016, Ionita *et al.*, 2013, Cernea *et al.*, 2008, 2015). However, data about prevalence of different strongyle species in Romanian horses, and particular about the most pathogenic large strongyle *S. vulgaris* are still scarce. Through larval culturing, this study revealed mixed strongyle populations, comprising a complex of cyathostomin species, with the morphotype A as predominant, but also strongylin species, including *S. vulgaris*, *S. edentatus*, and *S. equinus* in working horses. These results are consistent also with previous studies carried out in Romania reporting a prevalence of 10.3% for *S. vulgaris* (Cernea *et al.*, 2015). Different surveys showed that the *S. vulgaris* species is more commonly found in working horses, as it was in the present study, or in horses from farms where anthelmintic treatments are irregularly administered or with a lower intensity (Kyvsgaard *et al.*, 2011). Therefore, due to the intensive anthelmintic treatment regime in the last decades, *S. vulgaris* is reported now in lower

prevalence in well managed farms (Kaplan, 2002) as it is in Switzerland, Germany (Kaspar *et al.*, 2017). In Poland, in slaughtered horses was of *S. vulgaris* was the most dominant nematode (22.8%), followed by *S. edentatus* (18.3%) and *S. equinus* (1.7%) (Studzińska *et al.*, 2012).

The knowledge on the biodiversity of larval morphotypes within different strongyle populations might be an useful tool for indirect assessment of the susceptibility or chemoresistance of the cyathostomins populations. These findings are of relevance, as the morphotype A is reported as being produced by the most prevalent and resistant cyathostomins (Madeira de Carvalho *et al.*, 2008).

Nonetheless, *S. vulgaris* has become a rare parasite in well managed horse farms during the past 50 years (Kaplan, 2002) due to the frequent prophylactic anthelmintic treatments. Other studies, show occurrence of *S. vulgaris*, with low prevalence (Scheinder *et al.*, 2014), but it might be also depending on the method used for monitoring. However, currently, there are reports showing that stud farms using selective therapy, based on less frequency of anthelmintic treatments, might be at risk for re-emerging of *S. vulgaris* as reported in Denmark (Nielsen *et al.*,

2012). Therefore, this parasite is still a threat for the horse health and need to be monitored; larval cultures near by other more sensitive methods based on PCR are useful tools and standard method in some European countries (Nielsen *et al.*, 2012; Kaspar *et al.*, 2017). Recently, Kaspar *et al.* (2017) demonstrated that real-time PCR is more sensitive and detected a significantly higher proportion of positives of *S. vulgaris* than larval culture. Therefore, further studies are planned by using more sensitive technique PCR-based for monitoring of *S. vulgaris* in Romanian horse farms.

CONCLUSION

In this study we report the occurrence of *S. vulgaris* in working horse populations. This study represents the base for further investigations, including by using more sensitive tools, to monitor for *S. vulgaris* on Romanian horse farms.

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