USE OF LARVAL CULTURES TO INVESTIGATE THE STRUCTURE OF STRONGYLE POPULATIONS IN WORKING HORSES, ROMANIA: PRELIMINARY DATA

Sabrina Maria ANUȚESCU1, Marius Cătălin BUZATU1, Alexandra GRUIANU1, Jennifer BELLAW2, Ioan Liviu MITREA1, Mariana IONIȚĂ1

1University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine of Bucharest, 105 Splaiul Independentei, 050097, Romania
2M.H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY, USA

Corresponding author email: liviumitrea@yahoo.com

Abstract

Strongyle parasites infecting grazing horses have different pathogenity potentials. For this reason, strongyle eggs with horse feces need to be differentiated into small and large strongyle species, usually by means of larval cultivation and subsequent microscopic identification of third-stage larvae. The present study aims to provide an analysis of the strongyle populations composition based on different morphotypes of third stage strongyle larvae in naturally infected horses. In this regard, a coprological study was carried out to investigate the diversity of strongyle species in naturally infected working horses, in Northeastern Romania. For this, individual faecal samples were collected and examined qualitatively for parasitic infection, using a sodium chloride flotation method, and quantitatively for faecal strongyle egg count (eggs per gram - EPG), by a modified McMaster technique. Further larval cultures were performed from pooled positive samples for the identification of third-stage larvae (L3) of strongyle nematodes. All samples were positive for strongyle eggs, with an intensity rate varying from 30 to 2450 EPG. Larval identification showed mixed strongyle populations, with the following structure: small strongyles (92.9%), represented by the cyathostomin larvae type A (62%), type C (18.6%), type F (8.5%), and Gyalocephalus capitatus (3.8%), and large strongyles (4.5%) species, such as Oesophagodontus robustus (2.6%) and Craterostomum acuticaudatum (1.9%). Out of the total number of counted larvae, 2.6% could not be identified. The present study emphasizes that use of larval cultures allows a proper assessment of mixed strongyle populations in horses and it might represent an useful tool for further investigations into the epidemiology of equine strongyle infections in Romania.

Key words: strongyles, horses, larval cultures, cyathostomins.

INTRODUCTION

Equine gastro-intestinal parasites are ubiquitous and clinically important across the world (Andersen et al., 2013). Usually, mixed populations of multiple species are present in individual animals (Corning, 2009; Matthews, 2011). About 60 different species of strongyles (Nematoda: Strongylidae) have been described infecting equids. Of them, the cyathostomin (Cyathostominae) group, known also as small strongyles, consists of more than 50 species (Lichtenfels et al., 2008). Small strongyles are the most prevalent parasites in equine populations worldwide, regardless of climatic or management differences (Lyons et al., 1999).

Of the total worm burden of equids, cyathostomins often comprise 95-100%, followed by species such as Parascaris equorum, Oxyuris equi, Strongyloides westeri, Anoplocephala perfoliata and large strongyle (Strongylinae) species (S. vulgaris, S. edentatus, S. equinus, Triodontophorus spp.) (Nielsen, 2012).

The similar morphology of strongyle eggs does not allow species differentiation of mixed natural infections by faecal microscopy, not even to the subfamily level (Lichtenfels, 2008). For this reason, larval cultivation is the most practical and readily available method to differentiate between large and small strongyles, based on the morphology of the third-larval (L3) stage (Andersen et al., 2013).

Moreover, performance of coprocultures is now an established practice in many countries, such as Denmark, where, in order to reduce treatment intensities and to delay further development of resistance surveillance based parasite control programs are implemented (Nielsen, 2012).
The identification of horse strongyle infective larvae is extremely important for biological and epidemiological studies, since the most prevalent and abundant larval types are produced by the most prevalent and resistant species (Madeira de Carvalho et al., 2008). The present study aims to provide an analysis of the strongyle populations composition based on different morphotypes of third stage strongyle larvae in naturally infected horses, as basis for further evaluation of the status of susceptible and/or resistant cyathostomin subpopulations in Romanian horses.

MATERIALS AND METHODS

A total number of ten working horses aged between two and nine years originating from different households in Northeastern Romania (Suceava county), were included in the study. All animals were naturally infected and had not been treated with any anthelmintic for at least 3 months prior to sampling. Individual fresh faecal samples were collected in November, 2015.

The parasite burden for each horse was qualitatively analysed, using a sodium chloride flotation method. Additionally, strongyle infections were quantitatively investigated by a modified McMaster technique with a sensitivity of 25 eggs per gram.

All strongyle egg positive samples were further used for larval cultures, using humidity chambers as described by Henriksen and Korsholm (1983).

Four larval cultures were performed on pooled faeces, using one gram of faeces from each strongyle egg positive sample. Briefly, pooled faeces were suspended in humidity chambers created from disposable plastic cups and double-layered squares of cheesecloth (Bellaw and Nielsen, 2015). The humidity chambers were kept for 15 days at 22-24°C and regularly checked for dessication and moistened. The third stage larvae were harvested after sedimentation for 24 h using a modified Baermann analysis, as described by Bellaw and Nielsen (2015). 1 ml of sediment from each sample was collected and centrifuged at 3000 rpm for 5 min. Larvae were immobilized with Lugol's iodine for a better identification. Harvested larvae counting and identification were performed from an aliquot of 100 µl from each 1 ml sediment as described by Schneider et al. (2014). The remaining sediment (900 µl) was analysed for the presence of large strongyle larvae.

Finally, the mean number of counted larvae and percentages of identified larval types were calculated.

The morphological identification and differentiation of harvested larvae into free-living nematodes and different morphotypes of third stage small and large strongyle larvae were performed according to the identification keys described by Kornas et al. (2009) and Cernea et al. (2008), based on the following differentiation criteria: number, shape, and arrangement of intestinal cells, type of oesophagus, and length of tail.

RESULTS AND DISCUSSIONS

Overall, all fecal samples (10/10) collected from working horses and copro-parasitologically analyzed were positive for parasite eggs and/or oocysts, as follows: strongyles 100% (10/10), Parascaris equorum 10% (1/10) and Eimeria leuckarti 10% (1/10).

The overall mean output of strongylid eggs was 417.5 EPG (SD=721.2114), with individual intensity variation from 50 to 2450 EPG.

The faecal cultures from horses showed both free-living nematodes and infective strongyle larvae.

A total number of 156 third-stage strongyle larvae (L3) were counted from all the four analysed aliquots. On average, 39 infective larvae per sample were recovered.

Details on the structure of strongyle populations in investigated horses are presented in Table 1.

Small strongyle larvae were predominantly identified (92.9%). According to the identification keys (Kornas et al., 2009; Cernea et al., 2008), mixed populations of cyathostomins were classified in:
- type A (62.2%): with 8 rectangular intestinal cells, the first two parallel, the remaining six in a single row (Figure 1);
- type C (18.6%): with 8 intestinal cells, first 4 in a double row, rectangular shaped, the remaining in a single row, trapezoidal shaped (Figure 2);
Table 1. The structure of strongyle populations in working horses, Romania, using larval cultures

<table>
<thead>
<tr>
<th>L3 morphotype / species</th>
<th>Third stage strongyle larvae identified (number; %)</th>
<th>Average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Larval culture 1</td>
<td>Larval culture 2</td>
</tr>
<tr>
<td>Cyathostomininae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyathostomins Type A</td>
<td>18 (47.4%)</td>
<td>28 (59.6%)</td>
</tr>
<tr>
<td>Cyathostomins Type C</td>
<td>4 (10.5%)</td>
<td>13 (27.6%)</td>
</tr>
<tr>
<td>Cyathostomins Type F</td>
<td>9 (23.4%)</td>
<td>3 (6.4%)</td>
</tr>
<tr>
<td>Gyalocephalus capitatus</td>
<td>2 (5.3%)</td>
<td>0 (2.5%)</td>
</tr>
<tr>
<td>Strongylinae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oesophagodontus robustus</td>
<td>1 (2.6%)</td>
<td>2 (4.3%)</td>
</tr>
<tr>
<td>Craterostomum acuticaudatum</td>
<td>2 (5.3%)</td>
<td>0 (2.5%)</td>
</tr>
<tr>
<td>Unidentified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unidentified</td>
<td>2 (5.3%)</td>
<td>1 (2.1%)</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>47</td>
</tr>
</tbody>
</table>

- type F (8.3%): with 7 clearly defined elongated intestinal cells (Figure 3), and
- *Gyalocephalus capitatus* (3.8%) - with 12 intestinal cells, the first 6-10 elongated cells disposed in a double row, the remaining cells in a single row, trapezoidal shaped (Figure 4).

Additionally, larvae belonging to large strongyle species (4.5%) were detected, as follows:
- *Oesophagodontus robustus* (2.6%): with 16 elongated intestinal cells disposed in a double row (Figure 5), and
- *Craterostomum acuticaudatum* (1.9%): with 16 rectangular intestinal cells arranged in a double row at the anterior end, and a single row at the posterior end (Figure 6).

Out of the total number of counted infective larvae, there were four (2.6%) unidentified larval types, due to the less defined structure and arrangement of the intestinal cells.
respectively, for strongyle infections (intensity rates up to 3800 and 2775 EPG, of 87.97% and 70.3% respectively, and working horses also showed high prevalence, of 87.97% and 70.3% respectively, and intensity rates up to 3800 and 2775 EPG, respectively, for strongyle infections (Ioniță et al., 2013; Buzatu et al., 2013, 2014). In addition to EPG profiling, examination of cultivated third-stage strongyle larvae (L3) can determine whether eggs are produced by small or large strongyles. Although samples were collected in November, the cultivating temperature was according to the optimal values. Similar cultivation conditions were successfully used for the recovery of strongyle larvae in coprocultures as described before (Arias et al., 2012; Postoli et al., 2010; Denwood et al., 2012; Schneider et al., 2014). Moreover, for a maximum yield of strongylid larvae, fresh faeces from horses of stall/pasture-keeping conditions were collected, as it is recommended by Sengupta et al. (2016). The amount of harvested larvae was sufficient to analyse to what extent they belong to either the cyathostomins or strongylins. However, the specific diagnosis of equid strongyle infections based on larval culture technique and subsequent morphological identification of harvested L3 has its limitations. According to Roebel and Kahn (2014), the development of some strongylid species requires different temperature and humidity conditions. Thus, one cultivation technique is likely to favor the development of certain species over the others, which would provide incomplete results of the composition of a mixed strongyle infection. The technique may also suffer to some degree from bias, which may lead to incorrect results. Moreover, larval identification is inherently difficult because of indistinctive morphological features of some larvae. This situation was encountered during the present study, when 2.6% of larvae could not be identified due to unclearly defined number and arrangement of the intestinal cells.

In Romania, equid cyathostomin infections are highly prevalent as described by most of the studies related to strongyle parasitism in horses (Morariu et al., 2012; Badea et al., 2014; Cernea et al., 2015; Madeira de Carvalho et al., 2008). In the present study, cyathostomins were predominant (92.9%) in the strongyloid community of the investigated horses likewise. Third-stage larvae belonging to different morphotypes of cyathostomins were observed in all the examined faecal cultures. The predominant morphological type identified was type A (62.2%), followed by type C (18.6%) and F (8.5%). A very small percentage (4.5%) of L3 belonging to the large strongyle group was identified, respectively two species: O. robustus and C. acuticaudatum. No larvae belonging to Strongylus spp. were recovered. Similar studies in Romania showed a complex structure of strongyle populations, with also the
type A subpopulation being predominant (Cernea et al., 2015). The comparative study conducted by Madeira de Carvalho et al. (2008) in Portugal and Romania on cyathostomin infection in feral and domestic horses and donkeys, both dewormed and not dewormed, also revealed type A as the most prevalent, followed by type C, D and B. Most frequently, type A was recovered from dewormed horses, which strengthens the authors’ statement that this larval type is produced by the most prevalent and resistant cyathostomins. The A type cyathostomes larvae originated by highly prevalent species (i.e. *Cylicocyclus nassatus*, *Cylcocyclus insignie*, *Cyathostomum catinatum*, *Cylicostephanus longibursatus*) (Villa-Vicosa et al., 1997; Madeira de Carvalho et al., 2008). Thus, the knowledge on the biodiversity level of larval morphotypes within strongyle populations will allow an indirect evaluation of the status of susceptible or resistant cyathostomin subpopulations, as base for further studies for assessing the anthelmintic resistance phenomenon in Romanian horses.

**CONCLUSIONS**

The present study showed mixed third stage strongyle larvae (L3) subpopulations in working horses, and the morphotype A was dominant. However, further studies are planned in order to assess the biodiversity of larval types in strongyle populations while assessing the anthelmintic resistance in different equine strongyle populations in Romania. Subsequently, this study represents a base for further investigations into the epidemiology of strongyle infections in Romanian horses.

**ACKNOWLEDGEMENTS**

This work was supported by a grant of the Romanian National Authority for Scientific Research and Innovation, CNCS - UEFISCDI, project number PN-II-RU-TE-2014-4-1432.

**REFERENCES**


