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Original Article

Helminth infections and hybridization between *Haemonchus contortus* and *Haemonchus placei* in sheep from Santana do Livramento, Brazil

Infecções helmínticas e hibridização entre *Haemonchus contortus* e *Haemonchus placei* em ovinos criados em Santana do Livramento, Brasil

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Abstract

The occurrence and intensity of helminth infections were evaluated in sheep from pastures shared with cattle. In 2015 and 2016, young male sheep acquired in Santana do Livramento, Rio Grande do Sul, Brazil, were finished in integrated crop-livestock system. We selected the 12 sheep that showed the highest number of nematode eggs per gram of faeces to search for worms in the gastrointestinal tract. *Haemonchus contortus* and *Trichostrongylus colubriformis* were the major parasites. *H. contortus* presented mean intensities of 1,159 and 257 worms in 2015 and 2016, respectively. *T. colubriformis* displayed mean intensities of 4,149 and 2,427 worms in 2015 and 2016, respectively. Of the 127 male specimens of *Haemonchus* spp. analysed by Polymerase Chain Reaction (PCR), 125 were *H. contortus,* one *Haemonchus placei* and one hybrid. Other species detected were *Cooperia punctata*, *Cooperia pectinata*, *Cooperia spatulata*, *Cooperia curticei, Ostertagia ostertagi*, *Teladorsagia circumcincta*, *Trichostrongylus axei*, *Nematodirus spathiger*, and *Trichuris ovis.* Twenty lambs presented cysts of *Taenia hydatigena* in the liver and mesentery. One lamb presented *Coenurus cerebralis*, the larval stage of *Taenia multiceps*, in the brain. In conclusion, sheep from pasture shared with cattle presented a high diversity of nematode species. *H. contortus* and *H. placei* co-infection occur with consequent hybridization.

Keywords: *Cooperia*, *Nematodirus*, *Ostertagia*, *Taenia hydatigena*, *Teladorsagia*, *Trichostrongylus*.

Resumo

A ocorrência de infecções helmínticas foi avaliada em ovinos que compartilhavam pastagem com bovinos. Em 2015 e em 2016, cordeiros machos foram adquiridos em Santana do Livramento, Rio Grande do Sul, para serem terminados em sistema de lavoura – pecuária (ILP). Em cada ano, 12 cordeiros que tinham maior contagem de ovos nas fezes foram abatidos para recuperação dos vermes. *Haemonchus contortus* e *Trichostrongylus colubriformis* foram as principais espécies registradas. *H. contortus* apresentou intensidade média de 1159 e 257 vermes em 2015 e 2016, respectivamente. *T. colubriformis* apresentou intensidade média de 4149 e 2427 parasitas em 2015 e 2016, respectivamente. De 127 machos de *Haemonchus* spp. analisados por "Polymerase Chain Reaction" (PCR), 125 foram identificados como *H. contortus*, um como *Haemonchus placei* e um como híbrido. *Cooperia punctata*, *Cooperia pectinata*, *Cooperia spatulata*, *Cooperia curticei, Ostertagia ostertagi*, *Teladorsagia circumcincta*, *Trichostrongylus axei*, *Nematodirus spathiger* e *Trichuris ovis* foram as outras espécies de nematódeos registradas. Vinte cordeiros apresentaram cistos de *Taenia hydatigena* no mesentério e no fígado. Um cordeiro apresentou no cérebro *Coenurus cerebralis*, o estágio larval de *Taenia multiceps*. Em conclusão, ovinos criados com bovinos apresentam grande diversidade de nematódeos. A co-infecção de *H. contortus* e *H. placei* favorece a produção de híbridos.

Palavras-chave: *Cooperia*, *Nematodirus*, *Ostertagia*, *Taenia hydatigena*, *Teladorsagia*, *Trichostrongylus*.

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Introduction

Rio Grande do Sul State (RS) has the largest sheep population in Brazil, with an estimated 3,957,275 head (IBGE, 2015). In this State, most sheep are raised by continuous grazing on grassland vegetation (native pasture) that is usually shared with cattle in the biome of the Pampa, located in the southern half of RS. Southern Brazil occupies a transitional zone between tropical and temperate climates, with hot summers, cool winters, and no dry season. The southern half of RS and adjacent areas of Uruguay and Argentina have annual precipitation in the range of 1200-1600 mm and mean annual temperatures of 13-17 °C. Grass-dominated vegetation types prevail, with many herb, shrub, and tree let species co-occurring within the grass matrix (OVERBECK et al., 2007). In this environment, sheep production faces gastrointestinal nematode (GIN) infections, which are a significant cause of productivity losses and mortality. This problem has become worse due to the widespread emergence of nematode populations with resistance to anthelmintics (ECHEVARRIA et al., 1996).

Environmental conditions greatly influence the distribution of different nematode species and the risk of massive infections. Santiago et al. (1975) published the first report on the prevalence of helminths in sheep and cattle sharing the same pasture in RS, reporting that *Trichostrongylus colubriformis*, *Teladorsagia circumcincta*, *Oesophagostomum columbianum*, *Oesophagostomum venulosum* and *Nematodirus filicollis* were specifically found in sheep, while *Cooperia* spp. infected both sheep and cattle. Later, in a detailed study of GIN epizootiology in sheep in Itaqui, RS, Santiago et al. (1976) reported nine species of GINs. Among them, *Haemonchus contortus*, *Trichostrongylus axei*, *Trichostrongylus colubriformis*, and *Oesophagostomum columbianum* were the main causes of economic losses in sheep in that region. Regarding *Haemonchus* species, Santiago (1968) postulated that natural cross-infection of cattle and sheep with *Haemonchus* spp. in RS was negligible. Studies in São Paulo State demonstrated that cattle were the preferential host of *Haemonchus similis* and *Haemonchus placei* and that sheep were the preferential host of *Haemonchus contortus* (AMARANTE et al., 1997; SILVA et al., 2015).

Most studies on GIN epidemiology were performed decades ago, before the development of molecular techniques that can be used to distinguish nematode species. In studies that aim to evaluate the prevalence and intensity of infection, correct identification of parasite species is essential. The use of molecular tools is particularly important in *Haemonchus* spp. diagnosis because morphological measurements of spicules may overlap, raising issues about the precise differentiation among species (SANTOS et al., 2014, 2017). In addition, co-infection with *H. contortus* and *H. placei* can result in hybrids (HUSSAIN et al., 2014; AMARANTE et al., 2017). For this reason, the accurate identification of both species and hybrids is imperative in epidemiological studies where the species are sympatric, especially when small ruminants and cattle share the same pastures (AMARANTE, 2011). Therefore, the aim of this study was to evaluate the occurrence and intensity of helminth infection in sheep from pastures shared with cattle in RS, with an emphasis on the identification of *Haemonchus* spp.

Materials and Methods

This study was conducted in accordance with the Ethics Committee on Animal Use (CEUA) of São Paulo State University (UNESP), Instituto de Biociências, Botucatu, São Paulo, Brazil (protocol number 716-CEUA).

In 2015 and 2016, young sheep were purchased in Santana do Livramento, which is located in the southern half of RS State, to be finished in an integrated crop-livestock system (ICL) in Botucatu, São Paulo State, Brazil, where they rotationally grazed paddocks for a total of 72 days. At the farm of origin lambs grazed simultaneously with cattle (*Bos taurus*) of different ages.

They were allocated in a clean pasture that had been used in an ICL system in previous years. Before introduction of the experimental sheep, the area was left without animals for 300 days, for a period of crop production followed by pasture seeding and growth. This resulted in a clean pasture that was free of contamination by the free-living stages of GIN parasites. Decontamination of the area was confirmed by the use of worm-free tracer lambs that grazed the pasture and remained free of helminth infection (data not publish). We then analysed the worm population acquired by the sheep on their farm of origin in Santana do Livramento.

Experimental animals

In the first year (2015), we used 64 Poll Dorset x Corriedale (crossbred) uncastrated male sheep of 8 months of age on average and with a mean body weight of 24.4 ± 3.4 kg. In the second year (2016), we used 48 Texel x Corriedale (crossbred) male young sheep of the same age as the animals from the previous year and with a body weight of 26.4 ± 3.5 kg.

At the end of 72 days of grazing, we selected in each year the 12 sheep with the highest numbers of eggs per gram of faeces (EPG) based on the average of six EPG counts of faecal samples taken every two weeks. The selected lambs were slaughtered to count and characterize the worms in the gastrointestinal tract. At necropsy, the abomasum and small and large intestines were removed and opened and the contents were placed in graduated buckets. A 10% aliquot was frozen at -18 °C for subsequent enumeration and identification of the helminths (UENO & GONÇALVES, 1998). The small intestine was digested in saline solution (0.85% NaCl) for 4 h at 37 °C, and a 10% sample of the digested material was collected and frozen.

All internal organs were examined to search for cysts of *Taenia hydatigena* and *Echinococcus granulosus*, and the brain was examined to search for cysts of *Taenia multiceps*.

Nematodes were counted and sexed, and 20 male specimens of each genus were examined per lamb (or all males when fewer than 20 specimens were recovered) for the identification of species. Identification down to the species level of *Cooperia, Trichostrongylus*, *Nematodirus*, and *Trichuris* was based on the morphology of the spicules of male specimens (LEVINE, 1978; LICHTENFELS & PILITT, 1983; VICENTE et al., 1997; UENO & GONÇALVES, 1998). Identification of *Ostertagia* spp. and *Teladorsagia* spp. was conducted according to Lichtenfels & Hoberg (1993).

Ostertagia ostertagi and *Ostertagia lyrata* were considered as two morphotypes of a single species (LICHTENFELS et al., 1988; WYROBISZ et al., 2016).

Measurements of the spicules and barbs of *Haemonchus* spp. were used to calculate a discriminate function (DF) using the formula (DF = 0.0016TL + 0.128THr + 0.152THl – 9.97) described by Jacquiet et al. (1996) and Achi et al. (2003), where TL is the total length of the spicule, THr is the distance from the tip to the barb of the right spicule and THl is the distance from the tip to the barb of the left spicule. Species identification was established as follows:

- DF < 0.63: *H. contortus*;
- 0.63 < DF < 3: *H. placei*.

The number of females of each species was calculated based on the percentage of male species identified for each genus.

Molecular evaluation of Haemonchus spp. by Polymerase Chain Reaction (PCR)

In the second year (2016), we collected 127 *Haemonchus* adult male worms from 12 lambs. The spicule measurement and a DNA sample were obtained from each specimen according to the protocol described by Santos et al. (2014).

Genomic DNA was obtained from cattle and sheep blood samples (hosts) and from previously identified specimens of *H. contortus* and *H. placei* (based on morphological analysis) and these were used as positive and negative PCR controls.

DNA samples were extracted from single specimens of *Haemonchus* spp. with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Molecular identification of *Haemonchus* spp. was performed by PCR using the species-specific primer pairs HcBotuF1/R2 and HpBotuF/R (AMARANTE et al., 2017). Each primer pair amplified a single distinct band for each species. The primer pair HcBotuF1/R2 amplified an approximately 260 base pair (260 bp) band from *H. contortus* samples and HpBotuF/R amplified a PCR product of approximately 459 bp from *H. placei* samples. The PCR conditions were previously described by Amarante et al. (2017).

The PCR products were electrophoresed on a 2% agarose gel in 1% TAE buffer containing ethidium bromide and photographed under a UV light using a Sony Cyber-shot DSC-HX1 camera (Sony Electronics, San Diego, CA, USA). All PCR reactions were performed at least in duplicate.

Data analysis

Descriptive statistical analyses were used to summarise the data, as proposed by Bush et al. (1997), using the following terms:

- Prevalence: the number of hosts infected with each nematode species divided by the number of hosts examined;
- Intensity (of infection): the number of each nematode species in a single infected host;
- Mean intensity: the total number of parasites of a particular species divided by the number of hosts infected with that parasite.

Results

The selected lambs presented a mean of 4,242 EPG in the first year and 1,781 EPG in the second year. The following species of nematodes were recovered from their gastrointestinal tracts: *H. contortus*, *H. placei*, *T. axei*, *T. circumcincta*, *Ostertagia ostertagi*, *T. colubriformis*, *Cooperia curticei*, *Cooperia pectinata*, *Cooperia punctata*, *Cooperia spatulata*, *Nematodirus spathiger*, *Strongyloides papillosus* and *Trichuris ovis*.

Haemonchus spp.

In 2015, we performed a morphological evaluation of 178 male *Haemonchus* specimens. All of them showed measurements consistent with *H. contortus*: a spicule length of 418 ± 3 µm, a right spicule barb length of 41 ± 3 µm, and a left spicule barb length of 21 ± 2 µm (Figure 1A). In 2016, in addition to the spicule measurements, we performed a molecular identification by PCR (Table 1). We found 125 *H. contortus*, one *H. placei* (Figure 1C) and one hybrid specimen by PCR (Figure 2; Figure 1E; Table 1). Four specimens showed morphometrics consistent with *H. placei*; however, only one of these worms proved to be *H. placei* in the PCR analysis. The other three specimens were *H. contortus* with longer spicules and hooks. The prevalence of *H. contortus* was 100% in 2015 (mean intensity of 1,159 worms) and 92% in 2016 (mean intensity of 257 worms) (Table 2).

Ostertagia spp. and Teladorsagia circumcincta

Ostertagia spp. was found in 2015 and 2016, with a low prevalence and intensity of infection. We found 14 male specimens with the *O. ostertagi* morphotype (Figure 3A) and 8 with the

Table 1. Number of *Haemonchus* male specimens identified by PCR with its respective morphometrics (means followed by size range in parentheses).

Species	PCR	Morphometrics – Length in µm		
		Spicule	Right spicule barb	Left spicule barb
Haemonchus contortus	125	417 (375-463)	43 (38-53)	22 (19-28)
Haemonchus placei	1	450	53	25
Hybrid	1	438	47	25

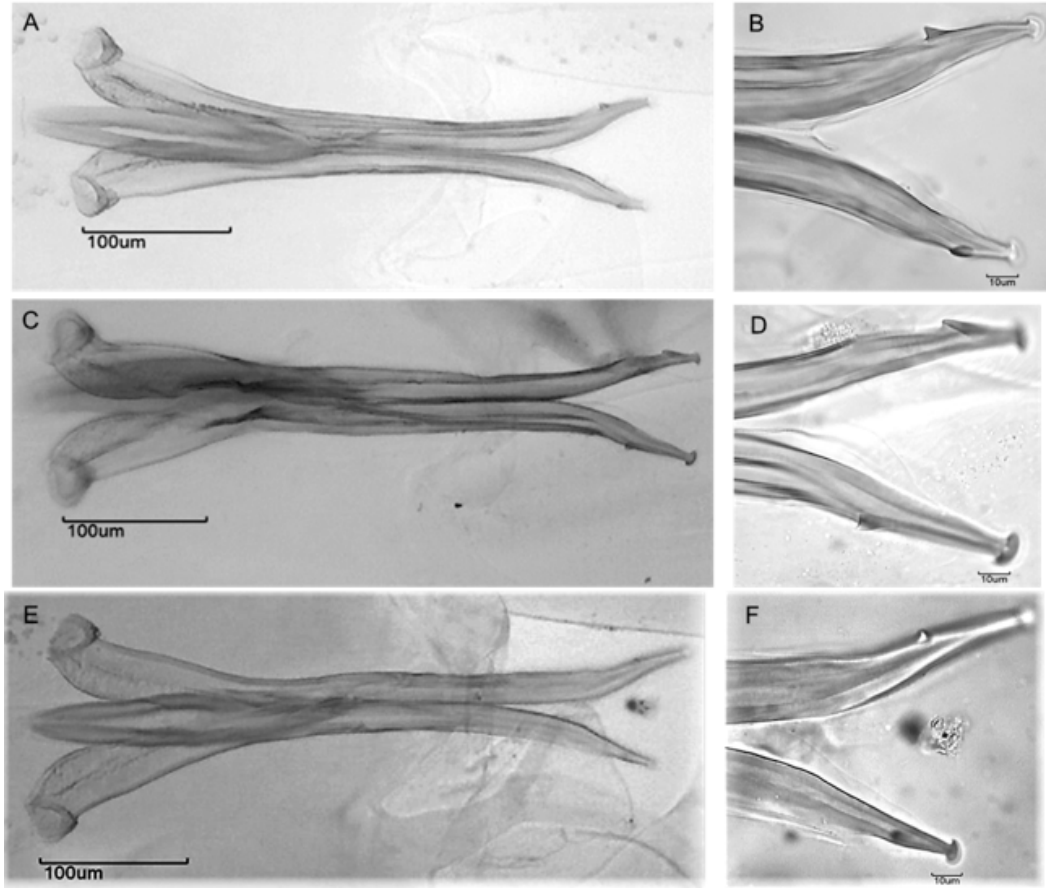

Figure 1. (A) Spicules and (B) spicule tips of *Haemonchus contortus*; (C) spicules and (D) spicule tips of *Haemonchus placei*; and (E) spicules and (F) spicule tips of a hybrid.

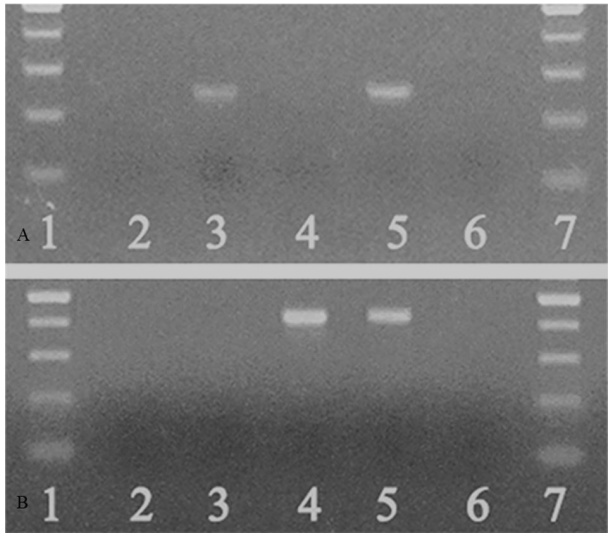

Figure 2. PCR with genomic DNA samples to identify *Haemonchus contortus, H. placei* and their hybrid. (A) Species-specific primer pair *HcBotuF1/R2* employed to amplify *H. contortus* (lane 3) and the hybrid (lane 5); (B) Species-specific primer pair *HpBotuF/R* employed to amplify *H. placei* (lane 4) and the hybrid (lane 5). Each photograph also shows reactions with: the control sample from ovine host (lane 2) and reagents without DNA in lane 6. Molecular marker (100 bp; GE Healthcare) were run in lanes 1 and 7 (A and B).

Ostertagia lyrata morphotype (Figure 3B). Both were considered to be a single species, *O. ostertagi,* with different morphotypes. *Teladorsagia circumcincta* (Figure 3C) was only recovered in 2016, with a prevalence of 25% and a mean intensity of 27 worms (Table 2).

Trichostrongylus spp.

Among the two species of *Trichostrongylus* found, *T. colubriformis* (Figure 4A) was by far the predominant species, with prevalence rates of 100% and 83% and mean intensities of 4,149 and 2,427 worms in 2015 and 2016, respectively (Table 2). *Trichostrongylus axei* (Figure 4B) was only recorded in 2016, with a prevalence of 17% and a mean intensity of 750 worms.

Cooperia spp.

Four species of *Cooperia* were recovered (Table 2). In 2015, *C. punctata* (Figure 5A) was the predominant species (prevalence, 92%; mean intensity, 810 worms) followed by *C. pectinata* (prevalence, 58%; mean intensity, 178; Figure 5B) and *C. spatulata* (prevalence, 17%; mean intensity, 191; Figure 5C). In 2016, *Cooperia* spp. was recovered in lower numbers than in 2015. The predominant species was *C. curticei* (prevalence, 67%; mean intensity, 260; Figure 5D)

followed by *C. punctata* (prevalence, 17%; mean intensity, 375) and *C. pectinata* (prevalence, 17%; mean intensity, 65).

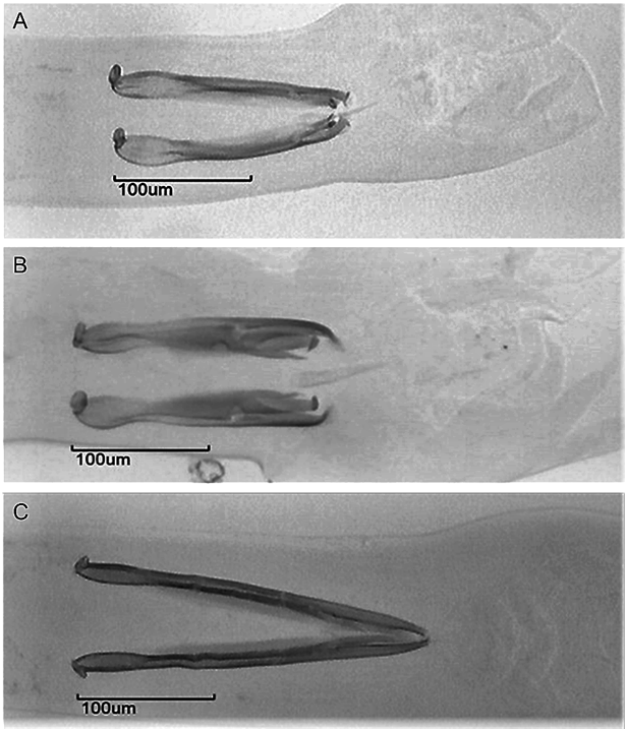

Figure 3. Spicules of *Ostertagia ostertagi* morphotype (A), *Ostertagia lyrata* morphotype (B) and *Teladorsagia circumcincta* (C).

Other helminths

In both 2015 and 2016, *N. spathiger* (Figure 6) was recovered, with prevalence rates of 83% in 2015 and 58% in 2016, but with a low mean intensity of infection (Table 2). In 2016, *S. papillosus* was detected in the small intestine, with a high prevalence (92%) and mean intensity (1033 worms; Table 2). Few specimens of *Trichuris* were recovered from the large intestine. In 2015, only three adult females were recovered; therefore, it was not possible to identify the species. In 2016, two adult *T. ovis* males were recovered. In 2015, the prevalence was 25% and the mean intensity was 10 worms, whereas in 2016, the prevalence was 8% and the mean intensity was 50 worms (Table 2).

In addition to the nematodes, 20 lambs (83.3%) presented cysts of *Taenia hydatigena* in the liver and mesentery. In 2015, one lamb presented *Coenurus cerebralis*, the larval stage of *Taenia multiceps*, in the brain. This animal did not show any clinical sign of a nervous disorder.

Discussion

In the present study, *H. contortus* and *T. colubriformis* were the major gastrointestinal parasites in sheep. These were also the main species, with the highest prevalence rates and infection intensities, in sheep from other Brazilian States, such as Santa Catarina (RAMOS et al., 2004) and São Paulo (WILMSEN et al., 2014). In other important sheep-producing areas of South America, such as Uruguay, *H. contortus* is also the most important parasite in

Table 2. Prevalence and mean intensity of infection of gastrointestinal nematodes in lambs from Santana do Livramento – RS, Brazil, in 2015 and 2016 (12 lambs in each year).

Year	Species	Prevalence (%)	Mean intensity*
2015	*Haemonchus contortus*	100	1159 (70-6530)
	*Ostertagia ostertagi***	25	127 (0-140)
	Trichostrongylus colubriformis	100	4149 (180-21290)
	Cooperia punctata	92	810 (0-4996)
	Cooperia pectinata	58	178 (0-655)
	Cooperia spatulata	17	191 (0-229)
	Nematodirus spathiger	83	83 (0-350)
	Strongyloides papillosus	67	123 (0-820)
	Trichuris spp.	25	10 (0-20)
2016	*Haemonchus contortus*	92	257 (0-1400)
	Haemonchus placei	8	30 (0-30)
	*Ostertagia ostertagi***	8	20 (0-20)
	Teladorsagia circumcincta	25	27 (0-40)
	Trichostrongylus axei	17	750 (0-800)
	Trichostrongylus colubriformis	83	2427 (0-12220)
	Cooperia punctata	17	375 (0-440)
	Cooperia pectinata	17	65 (0-80)
	Cooperia curticei	67	260 (0-1270)
	Nematodirus spathiger	58	26 (0-100)
	Strongyloides papillosus	92	1033 (0-9040)
	Trichuris ovis	8	50 (0-50)

*The mean intensity was calculated based on the examination of 10% of gastrointestinal contents from each animal. Minimum and maximum values are in parenthesis;
**It was found *Ostertagia ostertagi* and *Ostertagia lyrata,* which were considered as two different morphotypes of a single species (*O. ostertagi*).

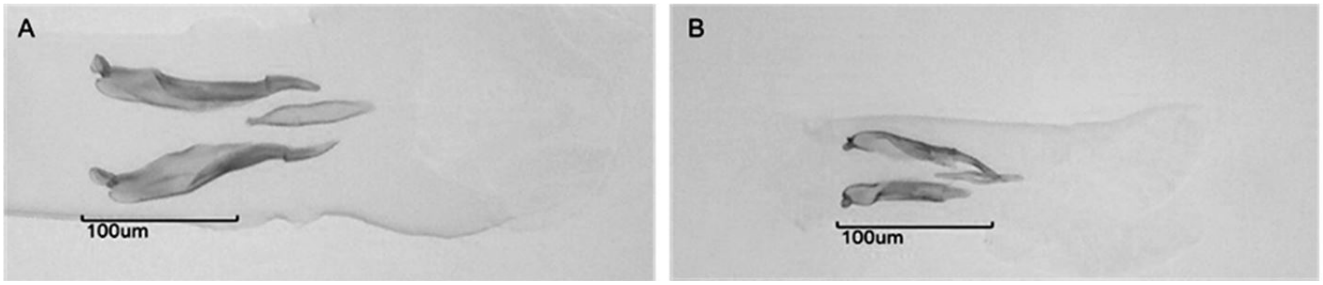

Figure 4. Spicules of *Trichostrongylus colubriformis* (A) and *Trichostrongylus axei* (B).

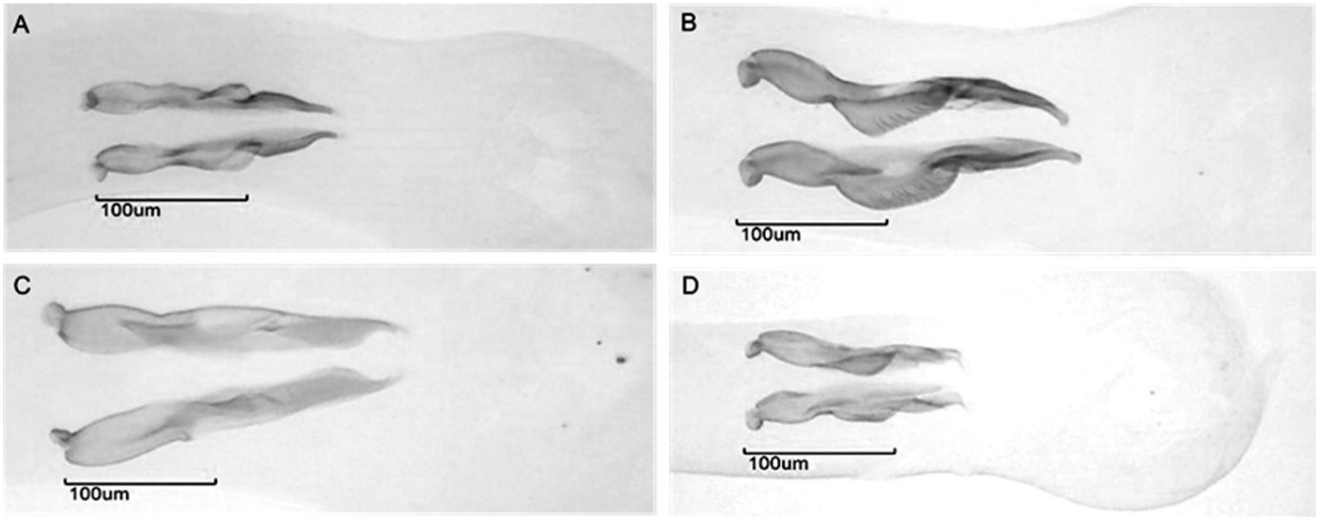

Figure 5. Spicules of *Cooperia punctata* (A), *Cooperia pectinata* (B), *Cooperia spatulata* (C) and *Cooperia curticei* (D).

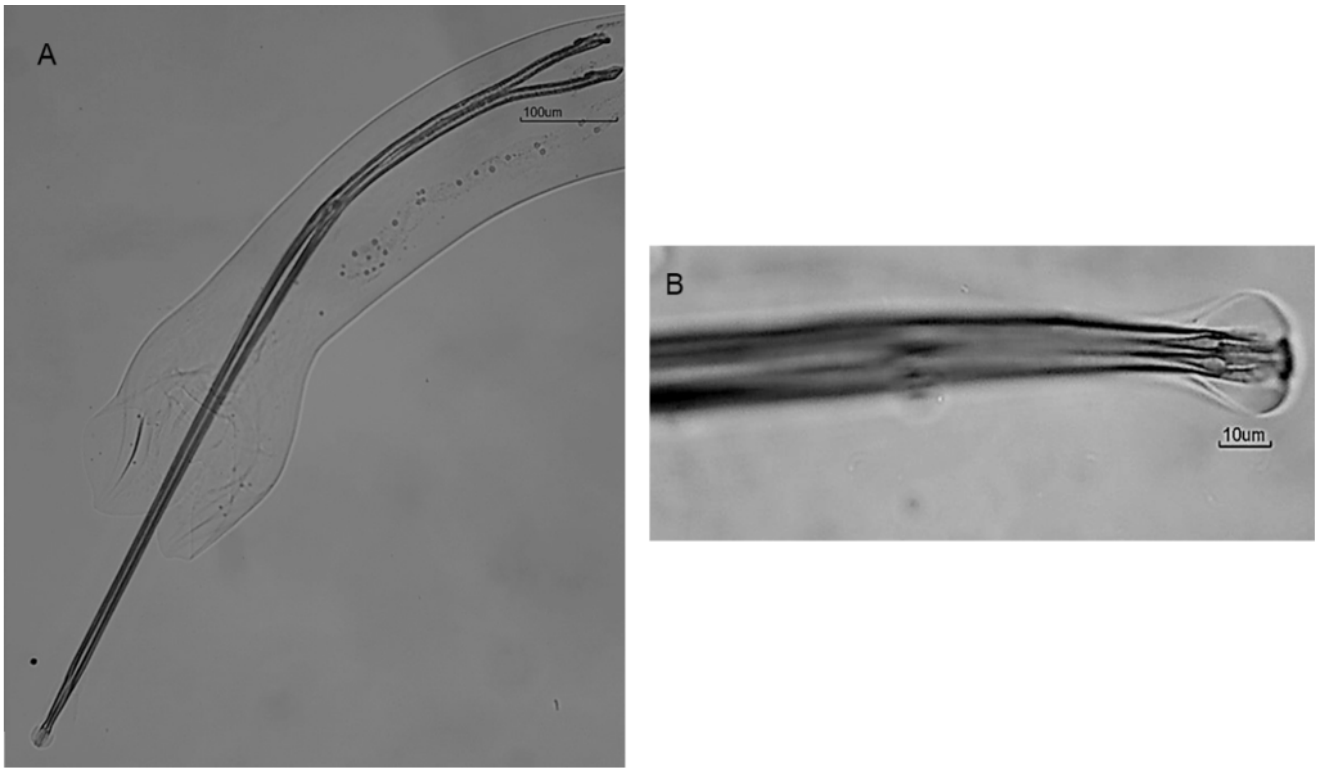

Figure 6. (A) Spicules and (B) spicule tips of *Nematodirus sphatiger*.

almost all seasons except winter, when *T. colubriformis*, and to a lesser extent, *T. axei*, *T. circumcincta* and *Cooperia* spp. are more important (NARI & CARDOZO, 1987). In Argentina, *H. contortus* is also the primary nematode in summer and autumn, while other parasites (i.e., *Nematodirus, Teladorsagia* and *Trichostrongylus*) are dominant in winter and spring (SUAREZ & BUSETTI, 1995; EDDI et al., 1996).

In our study, *Nematodirus* and *Teladorsagia*, which are typical parasites in temperate areas, occurred in small numbers. These nematodes have been reported at low infection intensities in sheep in RS (SANTIAGO et al., 1975, 1976) and Santa Catarina (RAMOS et al., 2004), especially during the winter. The free-living stages of *Nematodirus* and *Teladorsagia* are adapted to develop and survive in cold climates (MEDEROS et al., 2010).

We found a relatively high number of nematode species (13 total species) infecting the sheep. In contrast, Amarante et al. (2004) and Wilmsen et al. (2014) reported only five nematode species in sheep that grazed alone in São Paulo State. When sheep share pasture with cattle, which was the case in the present study, the helminth species diversity is higher. A similar situation was observed by Giudici et al. (1999) in the French West Indies.

Among the four species of *Cooperia* recorded in the present study, only *Cooperia curticei* was reported in sheep raised in the absence of cattle in São Paulo State (AMARANTE et al., 2004, 2009; WILMSEN et al., 2014). The other three species detected, *C. punctata*, *C. pectinata* and *C. spatulata*, are commonly found in cattle in Brazil (LIMA, 1998; BRICARELLO et al., 2007; SANTOS et al., 2010; BASSETTO et al., 2014). In the case of these last three species, bovine appears to be the source for sheep infection. A similar situation may have occurred with *Ostertagia ostertagi* and *H. placei*, two typical parasites of cattle (WILLIAMS et al., 1987; CARDOSO et al., 2013) that were also detected in the present study. Taken together, these findings demonstrate that the high diversity of nematode species results from cross infections in sheep-cattle mixed grazing systems. What is the economical consequence of such cross infections? Apparently, they are not relevant. Santiago et al. (1975), Amarante et al. (1997) and Rocha et al. (2008) considered nematode cross infections to be of low epidemiological importance when sheep and cattle share pastures, because of the low infection intensities, suggesting that integrated grazing systems using cattle and sheep could be used for pasture decontamination. However, the *H. contortus* and *H. placei* co-infections detected in the present study and others in which sheep and cattle share the same pasture (AMARANTE et al., 1997; ACHI et al., 2003) raise the possibility of interspecies hybridization (CHAUDHRY et al., 2015). By PCR, we detected one hybrid specimen among 127 *Haemonchus* individuals. Brasil et al. (2012) also reported the presence of 1.28% hybrids in a population of 156 *H. contortus* and *H. placei* recovered from sheep, cattle, goat, and buffalo. This hybridization could lead to interspecies introgression of genes and provide a mechanism for the transmission of anthelmintic resistance between parasite species (CHAUDHRY et al., 2015). This would be a major concern given the high prevalence of anthelmintic resistance in *H. contortus*. Molecular tools are very useful for elucidating the dynamics of *H. contortus* and *H. placei* infections when the two species are sympatric, because identification based on morphological analysis has limitations for differentiating species and detecting hybrids within a population (SANTOS et al., 2014; AMARANTE et al., 2017).

Twenty of the 24 lambs (83.3%) presented *Cysticercus tenuicollis*, the larval stage of *Taenia hydatigena*, and one lamb presented *Coenurus* larvae (*Taenia multiceps*) in the brain. Sheep acting as an intermediate host of dog cestodes is a relatively common problem in Brazil (RISSI et al., 2008; DE LA RUE et al., 2011; MORAIS et al., 2017). Our results demonstrate that dogs are still being fed raw sheep offal after home slaughtering and/or have access to dead sheep carcasses on farms. The high occurrence of these cestodes, reported in the present study, demonstrates the need for immediate action by veterinary authorities, due to the serious implications of dog cestodes on animal and public health.

In conclusion, sheep from pastures shared with cattle presented a high diversity of nematode species. Among them, *H. contortus* and *T. colubriformis* showed the highest prevalence and infection intensities. Co-infection with *H. contortus* and *H. placei* was also observed, with consequent hybridization.

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