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DOI 10.2478/s11687-014-0231-x

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HELMINTHOLOGIA, 51, 3: 210 – 214, 2014

Effectiveness of coproscopic concentration techniques

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Summary

The aim of the present study was to compare the effectiveness of the concentration techniques of flotation-centrifugation with zinc chloride (FZn) (δ = 1.45) with the spontaneous sedimentation (SSed) and the sucrose flotation-centrifugation (FSuc) (δ = 1.2) to recuperate gastrointestinal parasites from camelid fecal samples. The technique with more positive results for the detection of *Nematodirus* sp., *Trichuris* sp., strongyle-type eggs and *Eimeria macusaniensis* oocysts was the FZn. For *Trichuris* sp. and *Eimeria macusaniensis*, the higher coverglass counts were detected by FZn procedure. No significant differences were registered among centrifugation flotation techniques for *Nematodirus* spp. Coverglass count for strongyle-type eggs was significantly higher for FSuc than FZn ($p = 0.0005$) or SSed ($p = 0.0005$), being also significantly higher for FZn than for SSed ($p = 0.008$). FZn is a sensitive technique that allows the recovery of parasite elements with high density and it exerts low osmotic pressure avoiding parasite deformation.

Keywords: Concentration techniques; zinc chloride; parasite; camelid

Introduction

Stool examination must have high sensitivity, particularly when the parasitic material is present in low densities. Concentrations of parasites from herbivore faeces, including camelids, are often influenced by several factors that may contribute to the underestimation of gastrointestinal parasites; i.e. can be cited the amount of faeces processed for parasitological analysis, standardized at 2 – 5 g (Kaminsky, 2003) independently of the zoological origin of the faeces or the animal defaecation rate. Daily ungulate defecation rates are strongly more important than those of omnivores or of predators (Sánchez-Rojas & Gallina, 2000; Orr *et al*., 2012), being the volume of ungulate faeces processed for parasitology examination often not representative. During sampling, abundant vegetal fibers are retained on meshes in the course of the screening, when some parasites could be also retained.

Common nematode parasites of domestic ruminants also infect South American camelids (SAC). However, they are frequently parasitized by several specific gastrointestinal parasites, as *Lamanema chavezi*, *Eimeria macusaniensis*, *Nematodirus lamae* and *Trichuris tenuis*, among others (Leguía 1991, 1999; Richard & Bishop, 1991; Cafrune *et al*., 2001, 2009; Cebra & Stang, 2008). Eggs of *L. chavezi* and oocysts of *E. macusaniensis* also exhibit particular features such as their large sizes and high specific gravity (Jarvinen, 1999; Cafrune *et al*., 2009). Cebra and Stang (2008) suggested that eggs of *Trichuris* sp. collected from camelids have high densities, and proper techniques must be performed for their diagnosis.

During the selection of coproparasitological concentration techniques, some factors must be taken into account. For flotation procedures, specific gravity of the flotation solution, viscosity, the volume of flotation solution employed, application of additional centrifugation step, duration and speed of centrifugation and the elapsed time, among others, must be considered (O'Grady & Slocomb, 1980; Cringoli *et al*., 2004, 2010). Additionally important consideration is the selection of those procedures that causes the minimal egg distortion or destruction (O'Grady & Slocomb, 1980; Quinn *et al*., 1980; Cringoli *et al*., 2004).

Parasitological research on SAC usually utilizes flotation procedures with low specific gravities solutions (δ < 1.3) (Leguía & Casas, 1998; Jarvinen, 1999; Cafrune *et al*., 2001; Beldoménico *et al*., 2003; Cebra & Stang, 2008) being probable that some parasites with high density are underscore, as it was suggested for *E. macusaniensis* or *L. chavezi*, among others (Jarvinen, 1999; Cafrune *et al*., 2001, 2009; Cebra & Stang, 2008). Furthermore, reports evaluating the performance of common fecal diagnostic

techniques on camelid feces are scarce (Jarvinen, 1999; Cebra & Stang, 2008; Cafrune *et al*., 2009).
Zinc chloride flotation (δ = 1.9) is also a technique widely used in palynological studies (Gray, 1965) and it was recently demonstrated its effectiveness to recover gastrointestinal parasites from human faeces (Taglioretti *et al*. 2012).
The aim of the present study was to compare the effectiveness of the concentration techniques by flotation with zinc chloride (δ = 1.45) with two commonly used techniques in parasitology, the spontaneous sedimentation and the sucrose flotation (δ = 1.2), to recuperate gastrointestinal parasites in current camelid fecal samples.

Materials and methods

Studied materials
A total of 20 fecal samples of South American camelids were examined for parasites. Four corresponded to *Lama guanicoe* collected from National Park Perito Moreno (NPPM), Santa Cruz; 11 to *Lama lama* collected from "Estancia La Reserva", Buenos Aires and the other 5 camelid faecal samples, 2 of *Vicugna vicugna* and 3 of *L. lama*, were sent from the laboratory from Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria, Salta (National Institute of Agricultural Technology, Agricultural Experiment Station, Salta). Fecal samples proceeding from NPPM and "Estancia La Reserva" were collected from soil after animal defecation.

Methodological protocol
Each fecal sample was examined by 3 techniques: flotation-centrifugation with zinc chloride (δ = 1.45) (FZn), sucrose flotation-centrifugation (δ = 1.2) (FSuc) and spontaneous sedimentation (Lutz, 1919) (SSed). In order to achieve the volume enough to apply the techniques, 4 pellets were rehydrated in 50 ml tubes with distilled water from each sample. The pellets were homogenized and the homogenate was screened through 300 µm plastic mesh. The filtrate was first concentrated by gravitational sedimentation overnight in the refrigerator, and after, the supernatant was discarded.
With the aim to apply the different parasitological procedures, the concentrated filtrate obtained from the 4 pellets was homogenized and subsamples of 1 ml of the homogenate were transferred to 3 centrifuge tubes of 15 ml. In the flotation-centrifugation procedures, the specific gravity of the solutions, the duration of the period of flotation and the possible distortion of parasites remains, were taken into account.
Flotation techniques were conducted as follows: the flotation medium was added to the centrifuge tube, leaving 1 cm below the rim of the tube. For FZn, a few drops of hydrochloric acid 10 % were added to the sediment before the additions of the zinc chloride solution. The tube was centrifuged at 1000 r.p.m. by 5 min. Then, the tube was completely filled with the flotation solution, and a coverglass was placed on the top of the tube, in contact with the fluid during 5 min. After this period, the coverglass was removed and placed on a microscope slide for its examination. Once the first coverglass was removed, another one was placed on the top of the tube during 15 min more and the procedure was repeated for 30, 60 and 80 min (5 slides for each technique were observed). For spontaneous sedimentation procedure, 5 slides were also observed.
Each slide was then examined by light microscopy (Zeiss Primo Star) under 10X magnifications and parasite identifications and measurements were done under 40X magnifications. Images were registered by digital camera and edited by Image J 1.44p. It was also noted whether parasites remains were deformed, collapsed or distorted. Number of eggs and cysts which floated and adhered to a coverglass were counted and the coverglass counts were also registered for spontaneous sedimentation procedure.

Statistical analysis
The proportions of samples that yielded positive results for each parasite were compared among the 3 procedures by use of the Cochrane Q test. The percent of agreement (PA) among methods was also calculated according to Gordis (2000).
To investigate differences in eggs and cysts counts, positive results for each parasite were compared among methods using the Friedman repeated-measures on ranks. The Wilcoxon signed rank test with Bonferroni correction was used for pairwise comparisons. All comparisons were considered significant at values of $p < 0.05$. In cases where significant differences were detected by pairwise comparisons, Wilcoxon signed rank tests (alternative hypothesis: less) were also applied to detected which technique recovered more eggs at a significant level of $p = 0.05$.
Coverglass counts were done in different flotation periods for both flotation-centrifugation techniques, to assess whether the floating period could influence on the number of eggs or cysts recovered, and to estimate the optimal floating period in which most parasites can be found. Furthermore, total coverglass count was computed as the sum of coverglass counts for each flotation period of all positive samples for each procedure.

Results

Ability to detect positives samples among procedures
Of the 20 fecal samples examined, strongyle- type eggs were found in 12 samples, *Nematodirus* spp. in 13 samples, *Trichuris* spp. in 9 samples and *E. macusaniensis* in 4 samples for at least one of the three procedures.
The proportion of positive samples according to the method used was significantly different for *Nematodirus* sp., *Trichuris* sp. and strongyle-type eggs and also for *E. macusaniensis* oocysts (Table 1). In all cases, the technique with more positive results for the detection of parasites studied was the flotation-centrifugation with zinc clorhidric solution (FZn), being this the most sensitive procedure (Table 1).

Table 1. Number (N°) and proportion (%) of positive samples resulting from the 3 techniques

Parasite	Technique FSuc N° (%)	FZn N° (%)	SSed N° (%)	Total
E. macusaniensis [a]	1 (25)	4 (100)	0 (0)	4
***Trichuris* sp.** [a]	4 (44.4)	8 (88.8)	5 (55.5)	9
***Nematodirus* sp.** [a]	10 (76.92)	11 (84.6)	2 (15.4)	13
***Strongylus*-type** [a]	11 (91.66)	12 (100)	6 (50)	12

[a] Significant differences at $p < 0.05$ between the proportions of the samples that yielded positive results for each parasite; Total: No. total of positive samples for each parasite

Of the 12 samples positive for strongyle-type eggs, 6 were positive by the all 3 methods (PA = 50 %), 4 were positive by the 2 flotation techniques (PA = 33.3 %), 2 only by FZn and 1 only by FSuc. No positive samples by the SSed and negative by the other techniques were found. The percentage of agreement for *Nematodirus* sp. eggs by the 3 techniques was around 15 % (2/13), 1 sample was positive only by FSuc and 3 only by FZn, for both flotation techniques the PA was around 69 % (9/13). Positive samples for sedimentation were in all cases positive by the other 2 techniques. For *Trichuris* sp. eggs, the PA for the 3 procedures was around 44 % (4/9), 4 positive samples were detected by FZn only, 1 by SSed and all samples positive by FSuc (4) were also positive by the other procedures. Of the 4 samples positive for *E. macusaniensis* oocysts, none was positive for SSed, and the PA for both flotation procedures was around 25 % (1/4).

Coverglass counts among procedures

There were significant differences among the three procedures in the total coverglass count for *Nematodirus* sp. (Friedman chi-squared = 14.7568, p = 0.0006246), *Trichuris* sp. (Friedman chi-squared = 14, p = 0.0009119), strongyle-type eggs (Friedman chi-squared = 19.4634, df = 2, p-value = 5.937e-05) and *E. macusaniensis* oocysts (p = 0.02). Pairwise comparisons are shown in Table 2. For *Trichuris* sp. eggs and *E. macusaniensis* oocysts, the higher coverglass count was detected by FZn procedure, being for *Trichuris* sp. eggs significantly higher than from the other procedures ($p < 0.05$ in both cases). The FSuc was the technique that more strongyle-type and *Nematodirus* sp. eggs recovered. Nevertheless, no significant differences were detected among centrifugation flotation techniques for *Nematodirus* spp. eggs (Table 2). Coverglass count for strongyle-type eggs was significantly higher for FSuc than FZn (p = 0.0005) or SSed (p = 0.0005), being also significantly higher for FZn than for SSed (p = 0.008).

Coverglass counts for each flotation period

Total coverglass counts for different flotation periods for both flotation-centrifugation procedures are shown in Table 3; 60 % of the eggs of the parasites studied were recovered between 5 and 30 minutes of flotation, independently of the centrifugation-flotation technique employed; 100 % of the oocysts of *E. macusaniensis* were also found at this flotation period by FZn procedure, but the only oocyst registered by FSuc technique was at 80 min of the flotation period. Neither the number of eggs/oocysts nor the parasitic diversity increased with the increase of the flotation period before 30 min (Table 3).

Neither distortion nor alteration of morphology of organisms was observed by the application of the flotation-centrifugation techniques.

Discussion

Spontaneous sedimentation was the less sensitive technique to detect SAC gastrointestinal parasites, except for eggs of *Trichuris* sp. The centrifugation zinc chloride flotation technique yielded the highest proportion of positive results for all parasites and also the highest egg/oocyst counts for *Trichuris* sp. and for *E. macusaniensis*. This is what it was expected, since *Trichuris* sp. eggs, found in camelids, and *E. macusaniensis* oocysts are high density structures (Jarvinen, 1999; Cebra & Stang, 2008; Cafrune *et al*., 2009).

Table 2. Pairwise comparisons of coverglass counts among procedures (only positive samples were taken into account)

Technique	Parasite *E. macusaniensis* FSuc	*E. macusaniensis* FZn	*Trichuris* sp. FSuc	*Trichuris* sp. FZn	*Nematodirus* sp. FSuc	*Nematodirus* sp. FZn	Strongyle-type FSuc	Strongyle-type FZn
FSuc	-	NS	-	SD	-	NS	-	SD
FZn	NS	-	SD	-	NS	-	SD	-
SSed	NS	NS	NS	SD	SD	SD	SD	SD

Table 3. Total coverglass counts for different flotation period among flotation-centrifugation procedures

Parasite	Total coverglass counts by time period									
	FSuc (min)					FZn (min)				
	5	15	30	60	80	5	15	30	60	80
E. macusaniensis	-	-	-	-	1	6	3	3	-	-
***Trichuris* sp.**	28	5	9	4	14	13	37	39	22	30
***Nematodirus* sp.**	49	15	-	-	-	29	5	2	3	2
Strongyle-type	473	214	631	254	48	129	36	27	28	40

Studies carried out by Cebra and Stang (2008) suggested that a high specific gravity solution may improve *Nematodirus* spp. egg counts. Nevertheless, the highest counts of *Nematodirus* spp. in the present study were reported by the centrifugation-sucrose flotation technique, although differences with zinc chloride solution were no significant. This is argued with Grady and Slocomb (1979) who verified that *Nematodirus* spp. eggs floated equally well in nitrate solutions with specific gravity ranging from 1.22 to1.38.

The implementation of the centrifugation step improves the recovery of strongyle type eggs as it was reported for other flotation techniques with different media (Zajac *et al*., 2002; Cebra & Stang, 2008). In the present study, a centrifugation step was added to both flotation procedures, so differences in strongyle egg counts among flotation techniques are not solely dependent on specific gravities of solutions as it was proposed by other authors (Quinn *et al*., 1980; Cringoli *et al*., 2004), being probably that differences in the solution characteristics, like viscosity and/or osmotic pressure may be influencing eggs floating capacity.

Additionally, since strongyle type eggs have thin shells, the addition of drops of hydrochloric acid to the tube before the addition of the zinc chloride solution, in the case of the centrifugation in the zinc chloride flotation, could also destroy or modify egg shells, inhibiting their suspensions. Osmotic pressures increase as the density of the solution increases, and it is possible that osmotic pressure that exerts zinc chloride on these eggs is higher than those of the sucrose solution, altering the eggshells. Despite this, neither egg distortions nor alterations were observed at any elapsed time.

The elapsed time was taken into account, since a too early count can affect those slow-rising parasites, and a too late one may allow parasites to become distorted, particularly those with thin walls, like strongyle type eggs (Bowman *et al*., 2003, Cebra & Stang, 2008). However, additional elapsed time (up to 30 min.) did not increase egg or cyst counts as it was reported for *Trichuris* sp., *Capillaria* sp., strongyle type eggs and *E. macusaniensis* oocysts by the application of centrifugation sucrose flotation procedure (Cebra & Stang, 2008). *E. macusaniensis* was the exception, since the only oocyst detected by the centrifugation-sucrose flotation method was at 80 min.

Although during the performance of the centrifugation zinc chloride flotation there appeared increased amounts of fecal debris floating than in the centrifugation sucrose flotation technique, the amount of debris was less than that observed in slides processed by spontaneous sedimentation, and the recognition of eggs and cysts under the coverglass could be done successfully being the reading time significantly less.

The present study highlights the centrifugation zinc chloride flotation technique as a sensitive method to recover and to identify SAC gastrointestinal parasites, allowing the recovery of parasite elements of high density and exerting low osmotic pressure avoiding parasite deformation.

However, here it is proposed that techniques may be used in series, since although FZn had better diagnostic sensitivity compared with spontaneous sedimentation and centrifugation sucrose flotation, it was less effective in the recovery rate of strongyle-type eggs compared with sucrose solutions.

Efforts to improve egg/cyst recovery rates by the implementation of a high density solution and likely the additions of a centrifugation step in the flotation procedures were successful, except for strongyle-type eggs.

Acknowledgements

We thank to the National Parks Administration, Patagonia Regional Delegation; to Lic. Mercedes Cafrune, from the National Institute of Agricultural Technology, Agricultural Experiment Station, Salta and to Sandra Escobar from "Estancia La Reserva".

The present study was funded by grants from CONICET (PIP 090/11), UNMDP (EXA590/12), and FONCyT (PICT 1576/07).

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RECEIVED SEPTEMBER 30, 2013

ACCEPTED JULY 7, 2014