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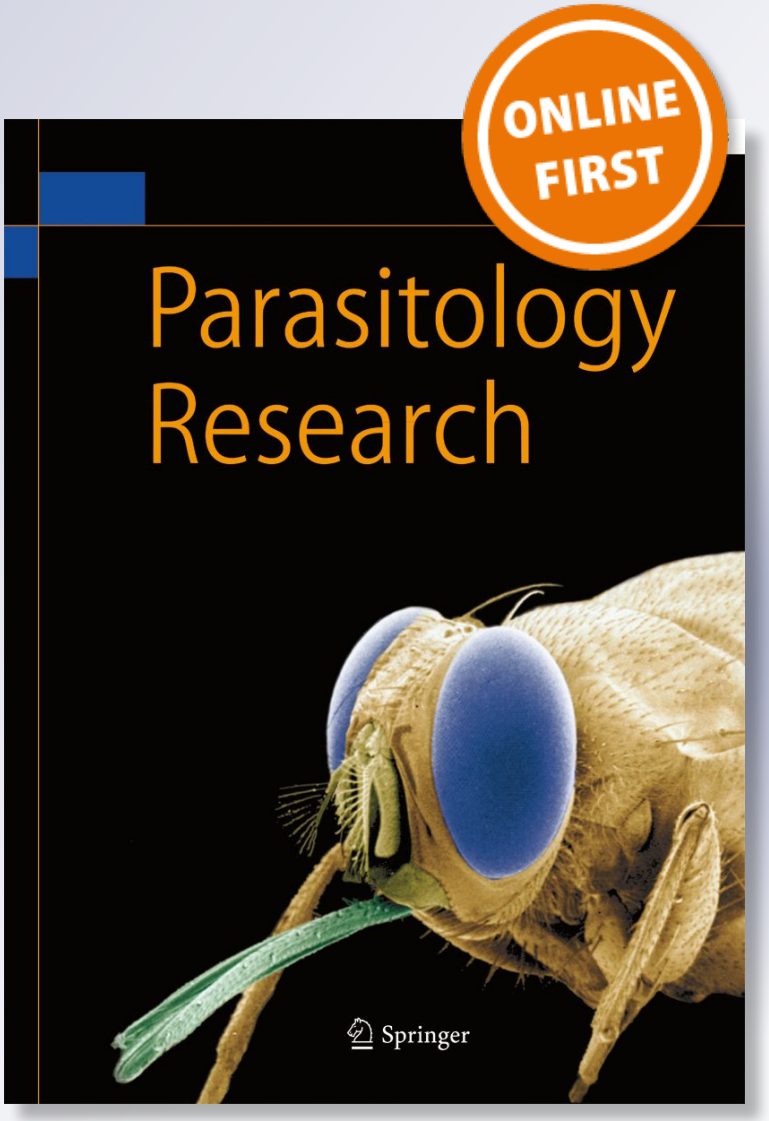

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ORIGINAL PAPER

Formalin-ethyl acetate concentration, FLOTAC Pellet and anal swab techniques for the diagnosis of intestinal parasites

Paola Cociancic[1] · Laura Rinaldi[2] · María Lorena Zonta[1] · Graciela Teresa Navone[1]

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Abstract

The aim of this study was to compare formalin-ethyl acetate concentration and FLOTAC Pellet techniques for the diagnosis of intestinal parasites in human stool samples. The anal swab method was used for the specific detection of *Enterobius vermicularis*. The study was performed in children and youth from Puerto Madryn (South Argentina). A total of 174 individuals were examined using the formalin-ethyl acetate concentration technique (FECT), the FLOTAC Pellet technique with saturated sodium chloride and zinc sulphate flotation solutions and anal swabs. The performance of copromicroscopic techniques was evaluated according to sensitivity, negative predictive value and Kappa index. Overall, 39.1% of the individuals were parasitised. The most prevalent species was *Blastocystis* sp. (19%) followed by *E. vermicularis* (17.8%), *Giardia lamblia* (6.3%), *Entamoeba coli* (5.7%), *Hymenolepis nana* and *Endolimax nana* (1.1%). The FECT was the most sensitive technique for *Blastocystis* sp., *G. lamblia* and *E. coli* infections, whereas FLOTAC Pellet techniques were the most sensitive for *H. nana* diagnosis. Anal swabs detected the highest percentage of *E. vermicularis* infection. This was the first time that the FLOTAC Pellet technique was used to detect intestinal parasites in humans. The FECT continues to be a reliable method for detecting protozoa and the FLOTAC Pellet technique gains importance in the diagnosis of helminths. Anal swab test remains the method of choice for the detection of *E. vermicularis*. However, when comparing techniques, key factors as preservation methods, preservation times and flotation solutions should be taken into account.

Keywords Intestinal parasite · Diagnosis · Formalin-ethyl acetate concentration technique · FLOTAC technique · Anal swabs

Introduction

Intestinal parasitoses caused by protozoa (e.g. *Blastocystis* sp., *Giardia lamblia, Entamoeba* spp.) and helminths (e.g. *Enterobius vermicularis*, hookworms, *Ascaris lumbricoides*, *Trichuris trichiura*) represent a public health problem and affect millions of people worldwide, especially children of developing countries (Osman et al. 2016; Pan American Health Organization 2016). These infections are mainly associated with faecal contamination, lack of safe drinking water and sewage services as well as with an inadequate hygiene (WASH). Likewise, parasitoses are favoured by people having limited access to health education and to the diagnosis and treatment of infections (Freeman et al. 2013; Pan American Health Organization 2016).

In Argentina, the prevalence of intestinal parasitoses varies among regions: it is high in the north (Menghi et al. 2007; Milano et al. 2007; Zonta et al. 2014; Rivero et al. 2017) and in the centre (Basualdo et al. 2007; Gamboa et al. 2009, 2011, 2014; Garraza et al. 2014; Zonta et al. 2016; Cociancic et al. 2017), and low in the south (Socías et al. 2014; Navone et al. 2017).

The selection of a diagnostic method for the detection of intestinal parasites depends on each parasitic species, considering the biological and morphological variability of the microorganism under examination (Anécimo et al. 2012). Since some of the main used methods have limitations as regards parasitological diagnosis, it often becomes necessary to evaluate the sensitivity, accuracy, precision and costs of the method to be adopted (Barda et al. 2014; Cringoli et al. 2017).

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✉ Paola Cociancic
paolacociancic@cepave.edu.ar

[1] Centro de Estudios Parasitológicos y de Vectores (CEPAVE-CONICET-UNLP), La Plata, Argentina

[2] Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, CREMOPAR, Naples, Italy

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Among the coproparasitological methods, sedimentation techniques use solutions that have lower specific gravity than the parasitic organisms, thus concentrating the latter in the pellet. The formalin-ethyl acetate concentration technique is the sedimentation technique used at Centers for Disease Control and Prevention. It is characterised for being a diphasic sedimentation method that allows the diagnosis of parasites from fixed samples (Centers for Disease Control and Prevention 2018). This qualitative technique allows the detection of protozoan cysts/oocysts and helminth eggs and larvae. In addition, it is easy to use and has a low probability of technical errors and a high sensitivity mainly for protozoa diagnosis (Navone et al. 2005; Kaminsky 2014; Cringoli et al. 2017).

FLOTAC techniques (Cringoli et al. 2010) were demonstrated to be powerful for diagnosing parasitic infections of human and veterinary importance (Steinmann et al. 2012; Barda et al. 2014; Maurelli et al. 2014). These techniques are quantitative and allow the diagnosis of protozoa and helminths from fresh or fixed samples, combining high sensitivity and accuracy. They involve a centrifugation of stool samples in a flotation solution (FS) followed by a translation of the apical portion of the suspension containing the parasites (Cringoli et al. 2010).

More specific diagnostic methods, such as Graham technique and anal swabs, are recommended for the detection of *E. vermicularis* since, due to this species biological cycle, it is not common to find its eggs in faeces (Cazorla-Perfetti 2014). Although this species is one of the most frequent helminths in children and youth, in some cases, its prevalence is underestimated because only copromicroscopic techniques are applied to diagnose it (Cazorla et al. 2006). In particular, anal swab technique is easy to collect and it is generally accepted by the population (Pezzani et al. 2004; Menghi et al. 2007; Navone et al. 2017).

Considering the above-mentioned information, the aim of this study was to compare formalin-ethyl acetate concentration and FLOTAC Pellet techniques for the diagnosis of intestinal parasites in stool samples of children and youth of a population in the South of Argentina. Likewise, the anal swab method was used for the specific detection of *E. vermicularis.*

Materials and methods

Study area

The study was carried out in Puerto Madryn (province of Chubut, South Argentina). Puerto Madryn (42° 46' S; 65° 02' W) is a city located in the northeast of the Patagonian region on the coast of the Argentine Sea. The climate is temperate-arid with an average annual temperature of 13 °C and annual rainfall between 100 and 200 mm. Soils are stony, with clayey silt texture and with low organic matter in general (Burkart et al. 1999). Due to its important industrial, fishing and tourist activity, Puerto Madryn is permanently visited by families from other cities and neighbouring countries (mainly from Bolivia and Paraguay), who stay and inhabit the peripheral area establishing informal settlements (Ferrari and Bozzano 2016; National Institute of Statistics and Censuses 2018).

Study population

A cross-sectional survey was conducted in May 2017 in the 5 municipal Child Development Centers (CDC): N° 1, N° 2, N° 3, N° 4 and N° 8. All individuals of both sexes under 14 years old attending the municipal CDC and their siblings were invited to participate voluntarily in the study. Children and youth whose parents and legal guardians had given written and oral consent were included and those who had been given some antiparasitic treatment by the time of the research were excluded.

The sample size calculated was 166 individuals, assuming a prevalence of about 38% from a previous study conducted in province of Chubut (Navone et al. 2017), in order to have at least 100 positive individuals, to allow a power of 80% and a difference of 20% between the diagnostic techniques with a 95% confidence level.

Sample collection and parasitological analysis

Meetings with parents and legal guardians were held to inform them about the biology of intestinal parasites, their means of transmission and strategies to prevent intestinal parasitosis. Free parasitological tests were offered and every consenting family was provided with 2 vials per participant for stool samples and anal swabs to diagnose intestinal parasites. Samples were collected by parents and legal guardians during 5 days, prior to verbal and written instructions. They were asked to fill the vial with a nut-sized stool sample each day. Anal swabs were specifically obtained each morning before getting up by rubbing the perianal margins with sterile gauze and the samples were placed in the vial with formalin immediately after (Pezzani et al. 2004; Cazorla-Perfetti 2014).

Samples were preserved in formalin 5% and stored at room temperature for 20 days before processing.

All stool samples were homogenised, filtered and processed using formalin-ethyl acetate concentration and FLOTAC techniques.

The formalin-ethyl acetate concentration technique (FECT) was employed as the standard procedure: 10 ml filtered suspension was centrifuged and 7 ml formalin and 3 ml ethyl acetate were added to the resulting pellet. The tubes were shaken vigorously and centrifuged. The plug was carefully removed and the pellet was examined under an optical microscope. This technique is recommended by the World Health

Organization with an initial centrifugation to obtain a clear pellet (World Health Organization 1991; Kaminsky 2014).

The FLOTAC technique was applied using the Pellet technique since the samples were fixed and the weight was unknown. In these circumstances, the weight of the faecal material to be analysed can be inferred by weighing the pellet in the tube after filtration and centrifugation (Cringoli et al. 2010; Rinaldi et al. 2012). Thus, 12 ml of filtered suspension was centrifuged and the pellet was weighed. Twelve millilitres of water was added and the volume contained in 0.3 g of the pellet was transferred to 2 tubes. The tubes were centrifuged and supernatants were discarded. The tubes were filled up to 6 ml with FS2 (saturated sodium chloride; s.g. = 1.2) and FS3 (zinc sulfate; s.g. = 1.2). Each suspension was homogenised and poured into the flotation chambers of the FLOTAC apparatus. The apparatus were closed and centrifuged. After centrifugation, the top parts of flotation chambers were translated and each chamber was examined under the microscope.

The anal swab vials were agitated vigorously and all suspension was placed in 15 ml tubes and then centrifuged for 10 min at 400*g* for the specific diagnosis of *E. vermicularis* (World Health Organization 1991).

Temporary staining with Lugol was used when necessary. Identification of parasitic elements (eggs/larvae/cysts/oocysts) was based on their morphological characteristics (Ash and Orihel 2013). Eggs of helminths found in stools samples were detected and counted. Eggs per gram of faeces were obtained by multiplying the total number of eggs by 4 (Cringoli et al. 2010). Eggs of *E. vermicularis* and cyst of protozoa were identified as well. The techniques were compared using the qualitative diagnosis for FECT and anal swabs are not quantitative methods.

All samples were blinded examined by the same experienced parasitologist (PC among the authors) to avoid interpersonal discrepancy.

Statistical analysis

Data were analysed by software R (R Core Team 2015). Prevalence was calculated as proportion of parasitised individuals over the total of analysed individuals and expressed in terms of percentage. A sample was considered positive if defined positive by any technique, and it was considered negative if all diagnostic techniques were negative. Therefore, the combined results of the techniques employed served as the gold standard.

Sensitivity, the negative predictive value (NPV) and the Kappa index (KI) were estimated to evaluate the performance of FECT, FLOTAC Pellet FS2 (FS2) and FLOTAC Pellet FS3 (FS3). Sensitivity (proportion of true-positives among the parasitised) and NPV (proportion of negative results among the non-parasitised) were expressed in terms of percentage with 95% confidence intervals (Agresti and Coul 1998; Ott and Longnecker 2010). The value of the KI was classified as a concordance: poor (KI ≤ 0), slight (KI = 0.01–0.20), fair (KI = 0.21–0.40), moderate (KI = 0.41–0.60), substantial (KI = 0.61–0.80) and almost perfect (KI = 0.81–1.00) (Sim and Wright 2005).

Results

One hundred seventy-four children and youth of both sexes were examined (48.9% boys and 51.1% girls); ages ranged from 1 to 14 years though the majority were 3 (31%), 4 (28.2%) and 5 years old (11.5%).

The results of the study are shown in Fig. 1, Table 1 and Table 2. Overall, 39.1% (68/174) of the individuals were positive for at least one parasite, by any diagnostic method. Six species were found and the most prevalent were *Blastocystis* sp. (19%), *E. vermicularis* (17.8%) and *G. lamblia* (6.3%). Two samples were positive for *Hymenolepis nana* (1.1%). Additionally, *E. coli* (5.7%) and *Endolimax nana* (1.1%) were also detected.

Eggs per gram of faeces (EPG) of *H. nana* were calculated with FLOTAC Pellet techniques. In this regard, the EPG detected by FS2 was higher than that obtained by FS3 in a sample (956 and 396, respectively). However, in a different sample, 12 EPG were detected with FS3 and 4 EPG with FS2.

The FECT revealed the greatest percentage of infection for *Blastocystis* sp., *G. lamblia*, *E. coli* and *E. nana*. FS2 and FS3 showed the highest number of *H. nana* infection. Anal swabs detected the highest percentage of *E. vermicularis* infection followed by FS3 and FS2 (Fig. 1). Likewise, the FECT was the most sensitive technique for *Blastocystis* sp., *G. lamblia* and *E. coli* followed by FS3 and FS2. However, FS2 and FS3 had the highest sensitivity for *H. nana*. Furthermore, NVP were higher than 92% with all techniques (Table 1).

Kappa indexes for agreement between techniques are shown in Table 2. KI was substantial for *Blastocystis* sp. between FECT and FS2 (0.73) and between FS2 and FS3 (0.73); it was almost perfect between FECT and FS3 (0.81). For *G. lamblia* infection, the agreement was moderate between FECT and FS2 (0.41), between FECT and FS3 (0.52) and between FS2 and FS3 (0.56). KI was almost perfect for *E. coli* between FECT and FS3 (0.81) and it was fair between FS2 and FS3 (0.38) and between FECT and FS2 (0.35). For *H. nana* infection, the agreement was substantial between FECT and FLOTAC Pellet techniques (0.66) and it was 1 between FS2 and FS3. Since *E. nana* and *E. vermicularis* were not found with all techniques, they were not included in the analysis of sensitivity and agreement so as to produce a meaningful statistic. In this respect, *E. nana* was only found in 2 samples analysed with the FECT. Moreover, anal swabs allowed the diagnosis of *E. vermicularis* in 26 samples (14.9%) (Fig. 1). However, 5 stool samples were positive with

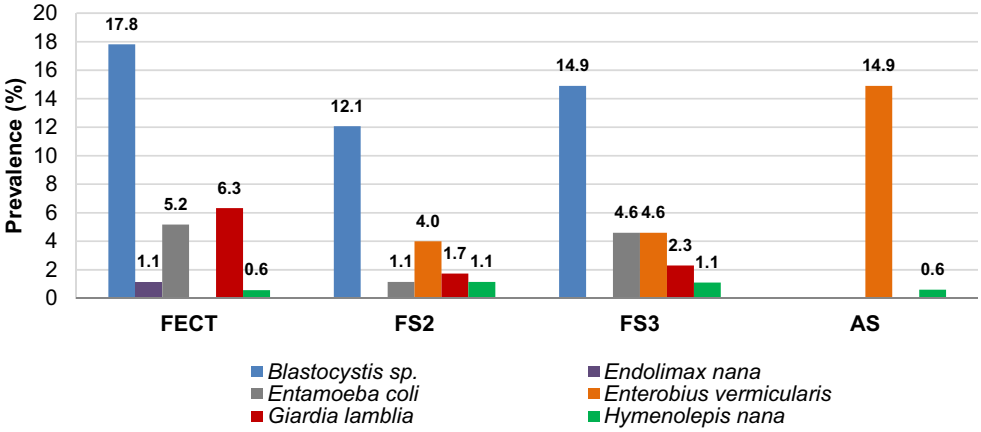

Fig. 1 Prevalence of intestinal parasites with the formalin-ethyl acetate concentration technique (FECT), the FLOTAC Pellet technique with FS2 (FS2) and FS3 (FS3), and anal swabs (AS)

FLOTAC Pellet techniques and these had not been diagnosed by anal swabs (5 samples for FS3 and 4 for FS2).

Discussion

Our findings provide information about the performance of diagnostic methods of intestinal parasites in people from an Argentine region where prevalence is low compared to other regions within the country. Moreover, this was the first time that the FLOTAC Pellet technique was used for detecting intestinal parasites in humans.

This study showed that 39.1% of children and youth analysed were parasitised by at least one of the 6 parasites identified. The most prevalent pathogenic species were *Blastocystis* sp., *E. vermicularis*, *G. lamblia* and *H. nana* and the most frequent non-pathogenic species were *E. coli* and *E. nana*. Several studies showed similar results in other populations of Argentina (Basualdo et al. 2007; Barda et al. 2014; Cociancic et al. 2017; Gamboa et al. 2014; Garraza et al. 2014; Menghi et al. 2007; Navone et al. 2017; Rivero et al. 2017; Zonta et al. 2014).

Soil-transmitted helminths were not found. This result shows a resemblance to that recorded in different populations from other Patagonian provinces, specifically in Chubut (Navone et al. 2017) and Neuquén (Soriano et al. 2005). The last mentioned study states that the soil-transmitted helminthiasis detected in Neuquén belonged to children who had resided in an endemic area.

In this study, all copromicroscopic techniques applied revealed the same intestinal parasites, except for *E. nana* that was detected only with the FECT and *E. vermicularis* that was found with anal swabs and FLOTAC. When comparing the sensitivity of the different techniques for the diagnosis of *Blastocystis* sp., *G. lamblia* and *E. coli*, the FECT showed the highest value. A previous study showed that the FECT was more effective than Willis and Charles-Barthelemy techniques for the diagnosis of these species (Navone et al. 2005). Likewise, Barda et al. (2013) also showed that the FECT was more sensitive for intestinal protozoa infections than direct faecal smear and Mini-FLOTAC. However, our results differed from those of Becker et al. (2011) and Gualdieri et al. (2011), who reported that the FLOTAC technique with FS4 (sodium nitrate; s.g. = 1.2) and FS7 (zinc sulfate; s.g. = 1.35) was more sensitive than the FECT for the detection of these protozoa. Various factors might explain this difference. For techniques based on flotation, the FS used might affect the visibility of intestinal parasites (Cringoli et al. 2010). Hence, the choice of FS is an important step for more accurate differential diagnosis. Secondly, the fixative used for faecal

Table 1 Sensitivity and negative predictive values (NPV) of the formalin-ethyl acetate concentration technique (FECT) and the FLOTAC Pellet technique with FS2 (FS2) and FS3 (FS3)

Parasite	*N*; sensitivity (95% CI)			NPV (95% CI)		
	FECT	FS2	FS3	FECT	FS2	FS3
Blastocystis sp.	31; 93.9 (79.4–99.3)	29; 63.6 (46.6–77.9)	21; 78.8 (61.9–89.6)	98.6 (94.7–99.9)	92.2 (86.7–95.6)	95.3 (90.4–97.9)
G. lamblia	11; 100.0 (71.5–100.0)	3; 27.3 (9.2–57.1)	4; 36.4 (14.9–64.8)	100.0 (97.8–100.0)	95.3 (90.9–97.8)	95.9 (91.6–98.1)
E. coli	9; 90.0 (55.5–99.7)	2; 20.0 (4.6–52.1)	8; 80.0 (47.9–95.4)	99.4 (96.7–99.9)	95.3 (90.9–97.8)	98.8 (95.4–99.9)
H. nana	1; 50.0 (9.4–90.5)	2; 100.0 (15.8–100.0)	2; 100.0 (15.8–100.0)	99.4 (96.8–99.9)	100.0 (97.8–100.0)	100.0 (97.8–100.0)

Table 2 Kappa index for agreement between the formalin-ethyl acetate concentration technique (FECT) and the FLOTAC Pellet technique with FS2 (FS2) and FS3 (FS3)

Blastocystis sp.	FECT	FS2	FS3
FECT		0.73	0.81
FS2	0.73		0.73
FS3	0.81	0.73	
G. lamblia	FECT	FS2	FS3
FECT		0.41	0.52
FS2	0.41		0.56
FS3	0.52	0.56	
E. coli	FECT	FS2	FS3
FECT		0.35	0.81
FS2	0.35		0.38
FS3	0.81	0.38	
H. nana	FECT	FS2	FS3
FECT		0.66	0.66
FS2	0.66		1.00
FS3	0.66	1.00	

preservation and the preservation time can affect the performance of techniques. The most commonly used fixatives are formalin, sodium acetate-acetic acid-formalin and merthiolate-iodine formalin (Utzinger et al. 2010; Becker et al. 2011). However, formalin 5% produces the most accurate results (Cringoli et al. 2010).

Moreover, FLOTAC Pellet techniques revealed a sensitivity of 100% for *H. nana* infection and showed a minimum of 4 EPG. In a different research, the FLOTAC with FS3 was the most sensitive technique for detecting this species in humans, followed by FS4, FS1 (Sheather's sugar solution; s.g. = 1.2) and FS2 (Steinmann et al. 2012). Based on the available evidence of their high sensitivity for detecting nematodes and trematodes, FLOTAC techniques have gained in importance as the optimal method for the diagnosis of intestinal parasites in humans (Utzinger et al. 2008; Knopp et al. 2011; Steinmann et al. 2012; Gerardi et al. 2018).

The agreement between the methods was variable (IK values ranged between 0.35 and 1.0), but generally substantial, mainly for FECT and FS3. The results suggested that the techniques applied in this study could be used for the diagnosis of parasitic diseases in areas where the prevalence of intestinal parasites is less than 40%. At this point, we considered that evaluating the disadvantages of each technique could be of help. The FECT uses formalin and ethyl acetate, which are hazardous to humans and the environment, it is expensive in general, its processing time takes longer compared to other methods and it is not quantitative. FLOTAC techniques, on the other hand, require centrifugation with different rotors and so an appropriately equipped laboratory is needed. As for flotation solutions, zinc sulfate is harmful to the environment; it is expensive and relatively difficult to find in some laboratories. Moreover, since flotation solutions can often alter the external membrane of some parasites (e.g. *E. coli*), well-trained laboratory technicians become necessary for reading FLOTAC disks (Becker et al. 2011; Cringoli et al. 2017).

Regarding *E. vermicularis*, this study demonstrated that copromicroscopic techniques are not sufficient and, therefore, that a more specific method such as the anal swab is needed. An observation we made is that FLOTAC Pellet techniques found eggs of *E. vermicularis* in stool samples. Although the eggs are not usually eliminated with faeces, they could be found in the first portion of stool passed after night (Jeandron et al. 2010). Nevertheless, this led us to confirm that the FLOTAC technique is a highly sensitive method for detecting helminths in general.

Limitations in the survey methods used in the present study should be borne in mind when interpreting the data. Most importantly, the study used a voluntary recruitment procedure that can affect the selection bias. In addition, it must be noted that in the absent of an independent diagnostic technique (e.g. PCR, Graham test), the combined results had to be considered as "true positive" in order to render a calculation of prevalence, sensitivity and NPV. Finally, FLOTAC Pellet has been tested only using the FS2 and FS3, but other FS could be tested to improve the accuracy of the technique.

The results of this study allow us to conclude that FECT and FLOTAC Pellet techniques should be recommended as complementary copromicroscopic methods for the diagnosis of intestinal parasites in humans. The FECT continues to be a reliable method for detecting protozoa and the FLOTAC Pellet technique gains importance in the diagnosis of helminths in populations where the prevalence is low. However, additional studies elucidating the performance of different fixatives and flotation solutions will help to improve the FLOTAC Pellet technique, particularly for concurrent diagnosis of both helminths and protozoa in fixed human stools. Likewise, the anal swab test remains the method of choice for the detection of *E. vermicularis*. The information presented can be useful to contribute to the improvement of parasitological diagnoses and to the monitoring of intestinal parasites to control and prevent these infections.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical aspects The study was carried out without affecting the physical, psychic and moral integrity of the participants and securing their identity. The present research was evaluated and approved by the Comité de Ética de la Escuela Latinoamericana de Bioética (CELABE) under Resolution No. 003, Record No. 73. The study was conducted attending the principles proclaimed in the Universal Declaration of Human Rights (1948), the ethical standards established by the Nüremberg Code (1947), the Declaration of Helsinki (1964) and its successive amendments. Special attention was also paid to Article 5 of the Regulation Decree of National Law 25.326.

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