Influence of Refrigeration and Formalin on the Floatability of *Giardia duodenalis* Cysts

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Giardia duodenalis *cysts obtained from fresh fecal samples, fecal samples kept under refrigeration and fecal samples treated with formalin were studied as to their floatability on sucrose solutions with the following specific gravities: 1,040 kg/m^3; 1,050 kg/m^3; 1,060 kg/m^3; 1,070 kg/m^3; 1,080 kg/m^3; 1,090 kg/m^3; 1,100 kgm^3; 1,150 kg/m^3; 1,200 kg/m^3; and 1,250 kg/m^3, contained within counting-chambers 0.17 mm high. Cysts that floated on and those settled down as sediments were counted, and had their percentages estimated. Sucrose solutions of 1,200 kg/m^3 specific gravity (the average specific gravity of diluting liquids employed in floatation techniques) caused to float 77.7%, 78.4% and 6.6% of the* G. duodenalis *cysts obtained, respectively, from fresh fecal samples, fecal samples kept under refrigeration, and fecal samples treated with formalin. Cysts obtained both from fresh fecal samples and fecal samples kept under refrigeration presented similar results concerning floatability. It was observed, however, that the treatment of feces with formalin diminished the cysts floatability under the various specific gravities studied. This results should influence, the recommendations for transport and storage of fecal samples used for parasitological coproscopy.*

Key words: parasitological coproscopy - floatability - *Giardia duodenalis* - floatation techniques

The efficiency of the concentration techniques used in parasitological coproscopy depends on the knowledge of physical properties of the fractions that compose the material to be examined (Ferreira 1972). Among these properties, the specific gravity is highlighted.

The specific gravity of parasitic elements has been studied through gradients of density (Ferreira & Carvalho 1971, Moitinho 1980, David & Lindquist 1982, Moitinho & Ferreira 1984, Moitinho et al. 1986).

A less hard-working technique - and therefore less liable to present inaccuracies - is the one that employs counting-chambers. Adopted by Silva (1984) and Moitinho and Ferreira (1992) in researches concerning specific gravities of samples of, respectively, helminth eggs and cysts of *Giardia duodenalis* and *Entamoeba coli*, this technique is based on the work of Sawitz et al. (1939) who used analogous methodology to investigate the floatability of *Ancylostomidae* eggs.

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However, the majority of studies concerning investigation on the floatability of parasitic elements are associated with the use of fecal samples lacking any kind of preservation procedures. The employment of both physical and chemical agents, such as refrigeration and formalin treatment for preserving fecal samples, is a common practice in clinical analysis laboratories. Although questions concerning possible side-effects of these agents on the floatability of parasitic elements do occur, they are not so frequent as it would be desired. As a result, in some laboratories, all kinds of fecal samples are equally submitted to the same diagnosing techniques, regardless of their being preserved or not.

Though being rare, a bibliographical survey disclosed some researches focusing on this issue, such as the ones carried out by Scholten and Yang (1974), reporting having found no concentration of parasites in feces after their being stored in formalin; Moitinho and Ferreira (1984), comprising studies on the effect of physical and chemical agents on the floatability of *Ancylostomidae* eggs; and, Amato Neto et al. (1992), providing data on the impact of formalin on the floatability of *Cryptosporidium* oocysts.

The purpose of the present paper was to disclose the impact of both procedures, refrigeration

and treatment with formalin, on the specific gravity of *G. duodenalis* cysts.

MATERIALS AND METHODS

Cyst suspensions - Were obtained from human fecal samples containing *G. duodenalis* cysts collected not more than 24 hr before. Each fecal sample was divided into three parts: one was let aside without any kind of treatment; another part was kept under refrigeration at 4ºC for three to seven days; and, a third part was kept in 10% formalin (one part of feces to two parts of formalin) also for a three to seven day period.

All the three different fecal portions - those not preserved, the ones preserved under refrigeration and those treated with formalin - were diluted into water, being then screened for taking macroscopic particles off. The resultant suspensions were centrifuged for 5 min at 800 x g. The process of centrifugation in water was repeated on till there remained only a limpid supernatant. Each sediment obtained was diluted into water for obtaining fecal suspensions at 5% concentration.

Sucrose solutions - Were obtained by diluting commercial refined sugarcane (União Company, São Paulo, SP, Brazil) into distilled water in order to get solutions of specific gravities equal to 1,312 kg/m^3; 1,300 kg/m^3; 1,267 kg/m^3; 1,200 kg/m^3; 1,180 kg/m^3; 1,160 kg/m^3; 1,140 kg/m^3; 1,120 kg/m^3; 1,100 kg/m^3; and 1,080 kg/m^3. They were checked on by means of an densimeter (Arba Glass Industry, Porto Alegre, RS, Brazil), in a 1,000 kg/m^3-1,500 kg/m^3 scale model. Each one of the solutions had its specific gravity estimated in order to obtain solutions that, after added with cysts suspensions, could yield solutions of specific gravities equal to 1,250 kg/m^3; 1,200 kg/m^3; 1,150 kg/m^3; 1,100 kg/m^3; 1,090 kg/m^3; 1,080 kg/m^3; 1,070 kg/m^3; 1,060 kg/m^3; 1,050 kg/m^3; and 1,040 kg/m^3, foreseen for the experiments.

Counting-chambers - Were made of glass slides to which rectangular supports, obtained from coverslips (0.17 mm high), were added providing boundary chambers of 16 mm x 18 mm x 0.17 mm (Moitinho & Ferreira 1992).

Floatability - In order to achieve the floatability determinations, fecal suspensions containing *G. duodenalis* cysts were blended by mixing them with sucrose solutions to get the foreseen specific gravity values. In order to obtain solutions of 1,250 kg/m^3 and 1,200 kg/m^3 specific gravities, cysts suspensions were mixed with sucrose solutions of, respectively, 1,312 kg/m^3 (in the proportion of 1:4) and 1,267 kg/m^3 (in the proportion of 1:3) specific gravities. Solutions of specific gravities equal to 1,150 kg/m^3; 1,100 kg/m^3; 1,090 kg/m^3; 1,080 kg/m^3; 1,070 kg/m^3; 1,060 kg/m^3; 1,050 kg/m^3; and 1,040 kg/m^3 were obtained after mixing equal volumes of cysts suspensions with sucrose solutions of specific gravities of 1,300 kg/m^3; 1,200 kg/m^3; 1,180 kg/m^3; 1,160 kg/m^3; 1,140 kg/m^3; 1,120 kg/m^3; 1,100 kg/m^3; and 1,080 kg/m^3, respectively. Employing Pasteur's pipettes, aliquots from the resultant mixtures were successively transferred to the counting-chambers. Both, floating cysts that adhered to the higher wall of the chamber and those whose sediments rested on the lower wall of the chamber were counted with the aid of a 400 x magnification light microscope (Olympus Tokio, Japan). *G. duodenalis* cysts, either those derived from fresh fecal samples (non preserved), or from fecal samples kept under refrigeration, or from fecal samples treated with formalin were analyzed.

Statistical analysis - Probit analysis was used to compare the results concerning floatability of *G. duodenalis* cysts obtained either from fresh fecal samples, or from those kept under refrigeration, or from the ones treated with formalin with the purpose of disclosing which, and in what extent, stool treatment or treatments did influence the *G. duodenalis* cysts floatability. Probit analysis transforms proportions of cysts into empirical probits. The equation $Y = 5 + (x - m)/s$ describes the relation between probits and log of specific gravity, in which *m* is the mean of the distribution of specific gravity and *s*, its standard deviation. A complete explanation on probit analysis and its application can be found elsewhere (Finney 1980).

A Basic programme, developed by Cláudio Santos Ferreira, was used for calculating the weighted linear regression, coefficients of determination (r^2) and, the percentage of floating cysts in a solution of an estimated specific gravity of 1,180 kg/m^3.

RESULTS

Table I shows, in percentages, the results of floating *G. duodenalis* cysts obtained either from fresh fecal samples, from fecal samples kept under refrigeration, or from those treated with formalin, all of them being immersed in sucrose solutions of different specific gravities.

Table II presents data on the median specific gravity for the floatability of *G. duodenalis* cysts obtained either from fresh fecal samples, from those kept under refrigeration, or from the ones treated with formalin, as well as, data on the weighted linear regressions, coefficients of determination (r^2) and, fiducial limits (FL).

Using the equation of probits, for a specific gravity equal to 1,180 kg/m^3, the proportions (%) of *G. duodenalis* cysts were estimated to be the following: 72.1% (95% FL, 38.1% - 93%), when obtained from fresh fecal samples; 69% (95% FL,

TABLE I

Floatability of *Giardia duodenalis* cysts obtained from fresh fecal samples, from those kept under refrigeration and from the ones treated with formalim

Especific gravity	Fecal samples					
	Fresh		Preserved			
			Refrigeration		Formalin	
(kg/m^3)	Cysts no.	Floatation %	Cysts no.	Floatation %	Cysts no.	Floatation %
1,040	4,326	0.2	4,069	0.9	3,229	0.0
1,050	4,054	1.8	4,094	1.2	3,107	0.7
1,060	3,410	7.4	3,673	5.6	3,717	0.9
1,070	3,576	17.4	3,661	13.9	3,225	1.3
1,080	3,522	31.1	3,341	28.3	3,418	1.4
1,090	3,633	39.5	3,778	38.5	3,209	0.4
1,100	3,932	50.3	3,409	44.4	3,087	1.6
1,150	3,827	67.5	3,326	62.1	3,214	3.5
1,200	1,770	77.7	1,650	78.4	1,484	6.6
1,250	948	85.9	870	84.5	758	33.0

TABLE II

Regression equations, coefficients of determination (r^2), median specific gravity and fiducial limits for the floatability of *Giardia duodenalis* cysts obtained from fresh fecal samples, from those kept under refrigeration and from the ones treated with formalim

Estimated values	Fecal samples		
	Fresh	Preserved	
		Refrigeration	Formalin
Regression equations	Y=2.555 + 42.145X	Y=2.576 + 40.839X	Y=1.761 + 24.413X
Coefficients of determination (r^2)	77.3%	82.4%	75%
Median specific gravity (kg/m^3)	1,117	1,122	1,299
Fiducial limits (95%)	(1,089; 1,165)	(1,098; 1,165)	(1,239; 1,468)

38.1% – 90.3%), when derived from fecal samples kept under refrigeration; and, 6.2% (95% FL, 2.9% - 11.8%), if derived from fecal samples previously treated with formalin.

Differences in floatation observed among cysts from fecal samples previously kept under refrigeration and those from fresh samples were found to be not significant when probit analysis was employed. However, cysts obtained from fecal samples treated with formalin presented strikingly and significantly different results concerning floatation when compared with those obtained with cysts from fresh fecal material.

DISCUSSION

The counting-chamber technique - simpler than that of gradients of density employed in researches on the floatability of parasitic elements - allows one to calculate the accumulated percentages of cysts that either float on or settle down as sediments when immersed in solutions of which the specific gravities are already known. In addition, data on the literature surveyed indicate that results of floatability obtained by means of gradients of density (Moitinho 1980, Moitinho et al. 1986) can be actually compared with those obtained by employing counting-chambers (Silva 1984, Moitinho & Ferreira 1992).

The investigation for ascertaining floatability using counting-chambers, adopted in the present paper, disclosed that solutions of specific gravity equal to 1,200 kg/m^3 - the average specific gravity of diluting liquids employed in techniques of floatation - caused 77.7% of the *G. duodenalis* cysts from non-preserved fecal samples to float (Table I). Analagous works carried out with this species showed indices of floatability equal to 74.6% and 88.5%, for this same specific gravity, when em-

ploying, respectively, sucrose gradients (Moitinho et al. 1986) and counting-chambers (Moitinho & Ferreira 1992).

By using counting-chambers, it was also investigated the impact of both variables - refrigeration and formalin treatment - on the floatability of *G. duodenalis* cysts. Reports on the influence of formalin on the yield of parasites concentration (Scholten & Yang 1974) and on the reduction of *Cryptosporidium* oocysts floatability (Amato Neto et al. 1992) can be found in the literature.

Furthermore, recommendations concerning the use of sedimentation procedures, specially the formalin-ether sedimentation, are reported for fecal samples treated with formalin (Ivey 1970, Garcia 1992a).

Though the above mentioned studies call attention either to the effects of formalin on the concentration and floatability of parasites or recommend sedimentation processes for fecal samples treated with formalin, there are others that, on the contrary, advocate mixing the stools with formalin as a previous procedure to the use of floatation techniques (Garcia 1992b).

The results of this paper show, unmistakably, the reduction of the floatability of *G. duodenalis* cysts after their being treated with formalin. No significant changes on the cysts floatability were observed after the stool samples were preserved by keeping them under refrigeration. Moitinho and Ferreira (1984), carrying out similar work, did not find out any reduction on the floatability of *Ancylostomidae* eggs by using refrigeration. These data are opposed to those of Sawitz et al. (1939), according to whom the permanence of stools in refrigerator reduces the floatability of eggs of this helminth.

Solutions of specific gravity equal to 1,200 kg/m^3 caused the floatation of respectively 77.7%, 78.4% and 6.6% of *G. duodenalis* cysts obtained from fresh fecal samples, from those kept under refrigeration and from the ones treated with formalin (Table I). Similar proportions of floating *G. duodenalis* cysts were estimated by the equation of probits for a specific gravity equal to 1,180 kg/m^3, which is the specific gravity of zinc sulfate solution employed by Faust et al. (1938). These results indicate that the percentage of non-recovered cysts is not high when one uses, for instance, the Faust et al.'s technique in the research of *G. duodenalis* cysts obtained from fecal material, either fresh or kept under refrigeration. Obviously, similar observations do not apply for fecal material previously treated with formalin.

Results presented in this paper shall influence, in the practice, the recommendations concerning transport and storage of fecal material destined for parasitological coproscopy.

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