ORIGINAL ARTICLE

WILLIS METHOD MODIFICATION WITH A VIEW TO IMPROVING ROUTINE COPROPARASITOLOGICAL DIAGNOSIS

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ABSTRACT

Intestinal parasites still cause high morbidity and mortality, due to poor hygiene and sanitary conditions, and therefore indiscriminate treatment has been routine practice advocated by the Public Health staff. Although there is a consensus regarding the need to diagnose such diseases, this is not performed with the necessary care, due to great demand and the lack of a wide ranged and highly sensitive technique. In this sense, most clinical laboratories use routine methods for fecal examination such as the Lutz sedimentation or modified Ritchie methods, which are complete and easy to execute, but do not have adequate sensitivity to detect low density eggs and protozoan cysts, especially when there is a predominance of low parasite burdens. In contrast, there are methods that are based on the flotation of low density developmental forms, namely, the Willis method (NaCl flotation d=1.120), which is rapid, easy to perform and allows high density egg flotation but with low sensitivity for protozoan cysts; and the Faust method, which is based on centrifugal flotation of developmental forms in a 33% $ZnSO_4$ (d=1.200) solution, but with the disadvantage of being lengthy and requiring a centrifuge. In this study, we verified the applicability of introducing an alteration in the Willis method, which consisted in the substitution of NaCl by $ZnSO_4$ in order to combine the advantages of this method with the Faust method. 208 samples were assessed by the Willis and Ritchie methods and by the proposed method (modified Willis). The latter proved superior to the other two ($\rho < 0,0001 - X^2$) regarding the detection of protozoan cysts, but similar to the Ritchie method in regard to other diagnosed parasites, therefore demonstrating the high potential for the introduction of this modified method in the routine of fecal diagnosis.

KEY WORDS: Coproparasitological diagnosis; Willis method; Modification.

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INTRODUCTION

In Brazil, despite regional differences, environmental contamination is still high, with a moderate prevalence of intestinal parasitoses often associating several parasites (Rivero et al., 2018). Intestinal parasitoses are still high morbidity and mortality diseases, being related to poor hygiene and sanitary conditions, and therefore indiscriminate treatment has been routine practice recommended by Public Health teams (Fernandez, 2006; Santos et al., 2017).

It is known, however, that the excessive use of antiparasitic drugs can trigger problems, among them the selection of resistant strains (Moller, 2004). Thus, there is a consensus regarding the need to carry out prior diagnosis of these diseases which, however, is not often performed with due care, in view of the high demand and the lack of a wide ranged and highly sensitive technique (Machado et al., 2001; Pereira et al., 2007).

Numerous methods, both quantitative and qualitative, have been suggested for fecal examination, which are often criticized for their complexity and low sensitivity, large number of samples and high execution cost in the routine of coproparasitological diagnosis, thus restricting their use in clinical analysis laboratories (Menezes et al., 2013).

In this sense, most laboratories adopt sedimentation methods, such as the Lutz method or the modified Ritchie method, which are accessible and easy to perform but do not present adequate sensitivity for the research of low density eggs and protozoan cysts, especially when there is a predominance of low parasitic loads (Mendes et al., 2005).

In contrast, there are methods that are based on the flotation of low density developmental forms, among which the Willis method (flotation in NaCl d=1,120), which is a fast, easy-to-execute method that prioritizes the flotation of low density eggs but also allows the flotation of high density eggs, but with low sensitivity for protozoan cysts; and the Faust method, which is based on the centrifugal flotation of developmental forms in a $ZnSO_4$ (d=1.200) solution, but with the disadvantage of requiring a centrifuge and being a time-consuming process (Willis, 1921; Faust et al., 1938; Garcia et al., 2006; Menezes et al., 2013).

The aim of the present work was to verify the applicability of a modification in the Willis method, which consisted in the substitution of NaCl by $ZnSO_4$, to combine the advantages of this method with those of the Faust method to standardize techniques with a view to providing better accuracy in routine clinical analyzes to facilitate laboratory procedures.

MATERIAL AND METHODS

To outline the present study, 208 unknown randomized human fecal samples were examined, all of them supplied by the "*Dr. Paulo Emilio D'Alessandro*" Municipal Clinical Analyzes Laboratory in Pindamonhangaba. It should be noted that the samples were from different patients, and there was no test repetition or more than one sample from the same patient.

The study was directed in a way that the researchers did not have access to the patient's identity. Each sample was examined concomitantly, as described below, by the Willis, Modified Willis and Ritchie methods. For each method, in order to reproduce the routine of a large number of clinical analyses laboratories, only one slide of each fecal material was examined. The Willis and Ritchie methods were performed according to techniques already described in the literature (Willis, 1921; Ritchie, 1948).

The modified procedure was similar to that used in the Willis method, with the difference of replacing the saturated NaCl solution by a saturated Zinc Sulphate (33% $ZnSO_4$, d=1.200) solution, as described below.

Modified Willis method: The basis of the method is the spontaneous flotation in a 33% $ZnSO_4$ saturated solution, d=1.120 (Zinc Sulphate), so that the developmental forms of the parasites can float on the surface of the tube containing the solution. The use of $ZnSO_4$ is part of the Faust method (Faust, 1938), but also applying a centrifugation step, which was not used in the method proposed in the present study.

About 2 grams of feces were diluted in 10 mL of saturated $ZnSO_4$ solution. The material was filtered through sterile gauze and transferred to a narrow-mouthed tube. Next, the volume was completed to the top of the tube, where a slide was placed.

The slide was kept in contact with the meniscus for 5 minutes. No air bubbles should form between the slide and the surface of the liquid. The drop containing the eggs adheres to the underside of the slide, which must be carefully removed by inversion.

It should be noted that only one slide was made per sample, in order to simulate a clinical analyzes laboratory routine with a large number of coproparasitological exams. According to Azevedo et al. (2017), the time, cost and complexity of coproparasitological examination methods are important factors in the choice of methods applied in routine laboratory examination.

Subsequently, a drop of lugol is added to the material on the slide which is then examined under a microscope.

This modification is based on the hypothesis that a precipitate will form without the presence of artifacts that would hinder the visualization of protozoan cysts, as well as inducing minor distortions, when observed with the use of saturated NaCl solutions.

The results were submitted to statistical analysis to verify concordance between the evaluated techniques, using the Kappa test, according to which, the closer the result of K is to 0.5, the greater the agreement between the methods compared, as reported by Rezende et al (2015).

In addition, the Chi-Square test was applied to verify whether the proportions of positivity differ from the standard test, at the significance level of 0.001 ($p<0.001$). For the statistical method, BioEstat 5.0 and GraphPad Prism 6 software were used.

Finally, the Sensitivity (S) and Specificity (SP) of the evaluated methods and the Negative Predictive Value (NPV) were also evaluated (Kawamura, 2002).

RESULTS AND DISCUSSION

After the coproparasitological examination of 208 samples, 49 (23.6%) were positive, with a higher number of protozoa such *Entamoeba coli* (15.4%) and *Giardia intestinalis* (6.3%) (Table 1).

Table 1. Frequency of diagnosed parasites in fecal samples using three different coproparasitological examination methods.

Parasites	Willis Method	Modified Willis Method	Ritchie Method
Ascaris lumbricoides	0	2 (1.0%)	1 (0.5%)
Hookworms	1 (0.5%)	1 (0.5%)	1 (0.5%)
Entamoeba coli	0[b]	32 (15.4%)[a]	14 (6.7%)[a]
Entamoeba histolytica/dispar	0	1 (0.5%)	1 (0.5%)
Enterobius vermicularis	2 (1.0%)	4 (1.9%)	1 (0.5%)
Giardia intestinalis	0[b]	13 (6.3%)[a]	5 (2.4%)[a]
Hymenolepis nana	0	1 (0.5%)	1 (0.5%)
Taenia spp.	0	3 (1.4%)	3 (1.4%)
Total positive samples	3 (1.4%)[b]	49 (27.4%)[a]	25 (12.0%)[a]

*a/*b: similar letters imply similar results in statistical terms, that is, with no significant difference.

Although the samples used were randomly chosen, the highest prevalence of *E. coli* agrees with results obtained by other authors, including Assis et al. (2013) who observed 40.8% of positive samples for this protozoan. Similarly, Machado et al. (2001) and Macedo (2005) diagnosed, respectively, 21.2% and 50% positivity for *E. coli*.

As previously mentioned, among the parasites, the most frequent was *G. intestinalis*, agreeing with the results presented by other researchers, among them Pardo et al. (2010), who diagnosed 30.7% positive for this protozoan among children from 2 to 9 years of age; and Assis et al. (2013) with 32% of the amostral space, in the Maxakali ethnic group in Minas Gerais.

It is known that the purpose of parasitological examination of feces is to diagnose intestinal parasites, through investigation of the different parasitic forms that are eliminated in the feces. These have particularities related to size, density and membrane permeability. So far, there is no accessible method capable of diagnosing effectively and concomitantly all parasitic forms.

To mitigate this problem, some solutions seem plausible, including the association of techniques and an increase in the number of samples per patient, but these alternatives are not practical considering the quantity of samples frequently present in the routine of a clinical analyzes laboratory. Therefore, the choice of technique for gold standard for the routine of the coproparasitological diagnosis is fundamental.

When comparing the positivity obtained in the evaluated methods using the Kappa method, greater agreement was noted between the Modified Willis method and the Ritchie method (K=0.4423, regular agreement) than for the Willis method (K=0,3894) (Kraeme & Bloch, 1988).

It is noteworthy that for the evaluation of the kappa index, the Ritchie technique was used as the gold standard, since it is a concentration technique that allows the detection of most parasitic forms and is also easy to handle, similar to the Hoffman, Pons and Janner methods, but with the advantage of allowing faster sample processing and obtainment of a precipitate with less amount of debris.

Regarding the efficacy of the compared methods, in quantitative terms the superiority of the proposed method is evident when compared to the Willis method, since it detected positive samples in a significantly higher quantity ($p<0.001 - X^2$). On the other hand, although a larger number of positive samples were detected by the Modified Willis method than that observed with the Ritchie method, this difference was not statistically significant, as shown in the figure.
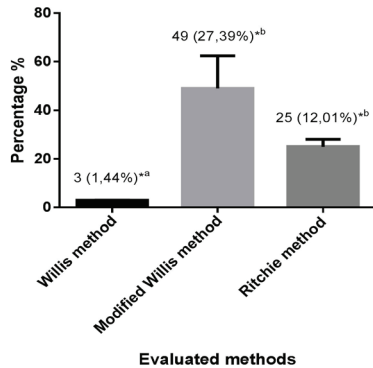
Figure. Efficacy (not of positive samples) of the evaluated methods for coproparasitological diagnosis.

It is known that clinical analysis laboratories use the Hoffman method or adaptations of the Ritchie method in routine coproparasitological diagnosis, particularly due to the large number of samples processed daily (Almeida et al., 2007). This study prioritized the comparative analysis of the proposed Modified Willis method over the Modified Willis and Ritchie methods. The Hoffman method was not included in the experiments, particularly due to some of its disadvantages, among which the time required to read the results and the large amount of debris, which may make it difficult to read the obtained results, thereby increasing the possibility of false negative results (Bica et al., 2011).

Despite the absence of a significant difference between Ritchie's method and the Modified Willis method, according to the results obtained in the present study, it is clear that the Ritchie method can compromise the reliability of routine reports, since the results obtained after outlining this method, presented a large number of false negative (lower NVP values) results, mainly for nonpathogenic (*Entamoeba coli*) and pathogenic protozoa (*Giardia intestinalis*). This did not occur after using the Modified Willis method (Table 2).

Besides, through the modified Willis method, a larger number of positive samples were detected, indicating significant sensitivity for detecting all species of helminths and protozoa diagnosed in the present study, as demonstrated in figure, highlighting that this method can be an important alternative for the test mentioned above regarding better diagnosis of such protozoa species. The NVP, sensitivity and specificity values of each method are shown in table 2.

Table 2. NPV (negative predictive value), sensitivity (S) and specificity (SP) of the evaluated coproparasitological methods, by species of detected parasite.

Parasites	Willis Method			Modified Willis Method			Ritchie Method		
	NPV	S	SP	NPV	S	SP	NPV	S	SP
A. lumbricoides	99%	0%	100%	100%	100%	100%	99%	50%	100%
Hookworms	100%	100%	100%	100%	100%	100%	100%	100%	100%
E. coli	85%	0%	100%	100%	100%	100%	91%	43%	100%
E.histolytica/dispar	100%	0%	100%	100%	100%	100%	100%	100%	100%
E. vermicularis	99%	50%	100%	100%	100%	100%	98%	25%	100%
G. intestinalis	94%	0%	100%	100%	100%	100%	96%	38%	100%
H. nana	100%	0%	100%	100%	100%	100%	100%	100%	100%
Taenia spp.	99%	0%	100%	100%	100%	100%	100%	100%	100%

The risk of releasing unreliable reports can be excluded on presenting a conciliatory and efficient assessment method. The present work proposes a modification to the Willis method by removing centrifugation, which is one of the steps in the Ritchie method, and at the same time eliminating the bias in the Willis method, which uses a saturated NaCl solution tending to induce deformation in protozoan cysts, possibly due to the rapid loss of water by osmosis or rupture (plasmolysis). According to Schossler et al. (2012), the effects of excess soluble salts are more striking especially in the presence of Na and Cl. NaCl limits the amount of water available, and may even lead to dehydration of microorganisms, consequently promoting plasmolysis (Rezende et al., 2011).

The superiority of the $ZnSO_4$ solution over NaCl solution to induce flotation of developmental forms of parasites has already been demonstrated by Santarém et al. (2009) who observed a significant difference in the recovery of *Toxocara canis* eggs from soil samples. According to these authors, physico-chemical properties of the solutions, such as viscosity, may be as important as density.

Therefore, rapid crystallization of the NaCl solution during the slide reading period may be a limiting factor for the diagnosis of protozoan cysts, since it leads to rapid loss of water and dryness, consequently impairing visualization

Regarding helminth diagnosis, the low number of positive samples did not show statistically significant differences between the results obtained after use of the various coproparasitological diagnostic techniques evaluated in the present study.

The efficacy in diagnosing Ancylostomidae eggs was similar in the three techniques studied. However, despite the absence of a significant difference, it can be infered that the Modified Willis method led to a greater detection of *Ascaris lumbricoides* and *Enterobius vermicularis* eggs, when compared to the results obtained with the other tested methods, as shown in Table 1. Ritchie's method results contrast with what would theoretically be observed, since in this method, which uses sedimentation by centrifugation, greater positivity would be expected for dense developmental forms (Mendes et al., 2005).

The superiority of the proposed flotation method disagrees with results obtained by other researchers, such as Barbosa et al. (2016). These authors compared five different methods for the detection of *Balantidium coli* cysts from swine feces and found a statistically significant difference ($p < 0.05$) in the frequency of cysts between swine and nonhuman primate samples which could only be observed through direct examination and the Lutz method.

It should be noted that these authors evaluated two methods of flotation by centrifugation, comparing them to two methods of sedimentation, one by centrifugation and the other without the use of a centrifuge (spontaneous). It is possible that the centrifugation process favors the sedimentation of dense developmental forms more, and negatively influences the flotation of these, justifying the results obtained by such authors.

In the experiments outlined in the present study, we attempted to evaluate a modification of the Willis method, which consists of spontaneous flotation, through the introduction of a saturated Zinc Sulphate solution, taking advantage of two different methods, since, as explained above, this solution, which is used in the Faust method routine, allows better visualization of developmental forms of parasites, without causing rapid loss of cytoplasmic liquid; and spontaneous sedimentation, which is related to the Willis method, allowing the flotation of denser developmental forms, without any decrease in sensitivity resulting from the centrifugation process, which is recommended in the original Faust method.

Based on the results obtained, it can be concluded that the coproparasitological examination method proposed in the present work, which consists in a modification of the Willis method, by substituting the saturated NaCl solution for a saturated $ZnSO_4$ (33%) solution, proved superior to the Willis method ($\rho < 0.001 - X^2$), being closer to the Ritchie method, concerning the study of protozoan cysts, and similar to the Willis method regarding helminth eggs, thus highlighting the advisability of introducing it in diagnostic routines especially in situations where the large number of daily samples makes it impossible to perform more than one coproparasitological examination.

As a suggestion for future research, we can highlight the design of experiments aimed at comparing the effectiveness of this methodology against the Faust method, which also uses a saturated Zinc Sulfate solution, but requires the use of a centrifuge and sample washing which makes it more laborious and expensive.

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