Comparison of Three Parasitological Stool Examination Methods with the Formalin-Ethyl Acetate Procedure for the Diagnosis of Intestinal Parasites in Humans


1Department of Medical Sciences and Life, University Center of the Ciénega, University of Guadalajara, Ocotlán, Jalisco, Mexico.
2Department of Physiology, Health Sciences University Center, University of Guadalajara, Jalisco, México.

Authors’ contributions

This work was carried out in collaboration among all authors. Authors MLGR and ALME conceptualized the study. Authors DADLOC, AREM and JJRR did the methodology. Authors MLGR and MACL did the software and formal analysis. Authors ALME and MLGR wrote the original preparation of the manuscript. Authors MLGR and ALME wrote the original draft preparation of this manuscript. Authors ALME and MLGR wrote, reviewed and edited the manuscript. Authors ALME carried out the visualization. Author MLGR supervised the study, did the project administration and funding acquisition. All authors read and agreed to the published version of the manuscript.

Article Information

DOI: 10.9734/IJTDH/2020/v41i43027

Editor(s):
(1) Dr. Nasser Mousa, Mansoura University, Egypt.

Reviewers:
(1) Si Thu Aung, University of Medicine Mandalay Myanmar and Khon Kaen University of Thailand, Thailand.
(2) Marta Cecilia Minvielle, Universidad Nacional de La Plata, Argentina.
Complete Peer review History: http://www.sdiarticle4.com/review-history/56558

Received 25 February 2020
Accepted 02 May 2020
Published 15 May 2020

ABSTRACT

Aims: The objective was to compare the sedimentation spontaneous in tube technique (SSTT), the direct smear, the zinc sulfate (surface film and sediment analyzed) methods with the formalin-ethyl acetate method as the reference standard.

Study Design: This a cross-sectional study performed in two populations around Chapala lake, Jalisco, Mexico.

Place and Duration of Study: Sample Department of Medical Sciences and Life, University Center of the Ciénega, University of Guadalajara, Ocotlán, Jalisco, Mexico.

*Corresponding author: Email: mlgalvanr@gmail.com;
1. INTRODUCCIÓN

Los parásitos intestinales son importantes porque están relacionados con la morbilidad y mortalidad en niños y adultos en todo el mundo. Los protozoos, como Blastocystis spp, Entamoeba histolytica (E. histolytica), Giardia lamblia (G. lamblia) y Cryptosporidium spp, son los más prevalentes en el mundo [1]. El transporte de estos parásitos en el mundo es mayor que las veces en el pasado. En México, la población general de San Juan Cosalá parece estar infectada con estos parásitos en un 77%. Los parásitos intestinales más prevalentes son: Blastocystis spp (49%), Entamoeba histolytica/E. dispar/E. moshkovskii/E. bangladeshi (Entamoeba complex) (37.5%) y G. intestinalis (11.5%). Algunos helmintes: Enterobius vermicularis (E. vermicularis) (18.3%) y A. lumbricoides (5.8%) [3]. En una investigación en Sonora se detectó una prevalencia de parásitos intestinales en niños de 60%. (Cryptosporidium spp 27%, G. lamblia 23%) [4].

La microscopía convencional todavía se utiliza para el diagnóstico de cistos, trofozoitos y oocistos de parásitos intestinales. Consiste en examinar directamente los muestras de heces. Con esta técnica se puede detectar la mayoría de los parásitos, como Ascaris lumbricoides (A. lumbricoides), Necator americanus y Ancylostoma duodenale (A. duodenale). Enfatizamos que este análisis es esencial para determinar si se requieren medidas de control y prevención de infecciones intestinales. En México, la población general de San Juan Cosalá parece estar infectada con estos parásitos en un 77%. Los parásitos intestinales more prevalentes son: Blastocystis spp (49%), Entamoeba histolytica/E. dispar/E. moshkovskii/E. bangladeshi (Entamoeba complex) (37.5%) y G. intestinalis (11.5%). Algunos helmintes: Enterobius vermicularis (E. vermicularis) (18.3%) y A. lumbricoides (5.8%) [3]. En una investigación en Sonora se detectó una prevalencia de parásitos intestinales en niños de 60%. (Cryptosporidium spp 27%, G. lamblia 23%) [4].

La microscopía convencional se utiliza para el diagnóstico de cistos, trofozoitos y oocistos de parásitos intestinales, y es esencial para determinar si se requieren medidas de control y prevención de infecciones intestinales. En México, la población general de San Juan Cosalá parece estar infectada con estos parásitos en un 77%. Los parásitos intestinales más prevalentes son: Blastocystis spp (49%), Entamoeba histolytica/E. dispar/E. moshkovskii/E. bangladeshi (Entamoeba complex) (37.5%) y G. intestinalis (11.5%). Algunos helmintes: Enterobius vermicularis (E. vermicularis) (18.3%) y A. lumbricoides (5.8%) [3]. En una investigación en Sonora se detectó una prevalencia de parásitos intestinales en niños de 60%. (Cryptosporidium spp 27%, G. lamblia 23%) [4].

Con la microscopía convencional se utilizan diferentes métodos para detectar parásitos intestinales, como el método de sedimentación espontánea en tubo (SSTT), que es un método sencillo y no requiere de reactivos tóxicos. Otra opción es el método de concentración con sulfato de zinc (ZnSO₄), que es más sensible que el método de concentración con acetato de etilo (ethyl acetate). Sin embargo, estos métodos no son comparables con métodos de concentración como el método de Faust con sulfato de zinc, que es un método limitado por su sensibilidad y especificidad. Otros métodos utilizados para el diagnóstico de parásitos intestinales son el método de concentración con formalina-aceato, que es más sensible que el método de concentración con sulfato de zinc, pero requiere de reactivos tóxicos. En México, la población general de San Juan Cosalá parece estar infectada con estos parásitos en un 77%. Los parásitos intestinales más prevalentes son: Blastocystis spp (49%), Entamoeba histolytica/E. dispar/E. moshkovskii/E. bangladeshi (Entamoeba complex) (37.5%) y G. intestinalis (11.5%). Algunos helmintes: Enterobius vermicularis (E. vermicularis) (18.3%) y A. lumbricoides (5.8%) [3]. En una investigación en Sonora se detectó una prevalencia de parásitos intestinales en niños de 60%. (Cryptosporidium spp 27%, G. lamblia 23%) [4].

Con la microscopía convencional se utilizan diferentes métodos para detectar parásitos intestinales, como el método de sedimentación espontánea en tubo (SSTT), que es un método sencillo y no requiere de reactivos tóxicos. Otra opción es el método de concentración con sulfato de zinc (ZnSO₄), que es más sensible que el método de concentración con acetato de etilo (ethyl acetate). Sin embargo, estos métodos no son comparables con métodos de concentración como el método de Faust con sulfato de zinc, que es un método limitado por su sensibilidad y especificidad. Otros métodos utilizados para el diagnóstico de parásitos intestinales son el método de concentración con formalina-aceato, que es más sensible que el método de concentración con sulfato de zinc, pero requiere de reactivos tóxicos.
2. MATERIALS AND METHODS

2.1 Type of Study and Area Participants

This is a cross-sectional study performed in two populations of around Chapala lake, Jalisco Mexico.

2.2 Pre-Analytical and Analytical Phase of Quality Control

The participants should not have used antibiotics or antiparasitic for at least one month or consumed antacids, antidiarrhea products at least 10 days before stool collection [6]. This information was obtained through the application of a questionnaire approved by the Institutional Research, Ethics, and Biosafety Committee registration number CI 053-2014 of University Centre of Health Sciences, University of Guadalajara.

The fecal samples were obtained by spontaneous evacuation, not contaminated with urine, water and soil and were previously identified with a serial number and the date and time of sample collection. The containers were then placed in a plastic bag and transported in a coolant gel to keep the cold chain to the laboratory and were stored between 4°C to 8°C for 24-72 h until analysis. On the other hand, Lugol, formalin, sodium chloride, and zinc sulphate solutions used for the tests were checked for any type of visible contamination prior to being used. In addition, the specific gravity of the zinc sulphate solution 1.18 g/dL (ZnSO₄) was verified using a hydrometer.

The ZnSO₄ method was applied and sediment of 0.5 to 1 mL for an effective concentration was obtained. Therefore, in order to detect all possible organisms and prevent their deformation we examined both surface film and sediment within 20 min following the last centrifugation with the ZnSO₄ solution. Each sample was processed and analyzed by two parasitologists and they observed the entire area coverslip [6].

2.3 Parasitological Methods

Each stool sample was homogenized for 60 s before starting the procedure direct or concentration.

2.3.1 Direct smear

This method is simple, fast, and economic. This allows the observation of internal parasite structures. Approximately 2 mg of stool sample was mixing and then thoroughly immersed in one drop the Lugol’s iodine solution. A coverslip (22x24mm) was placed on top of the suspension and microscopic examination was performed with 10X and 40 X objectives [6].

2.3.2 Flotation with zinc sulphate

This method is based on the flotation of protozoan cysts and oocysts, helminthes eggs and larvae, which occurs at a lower density than that of the ZnSO₄ solution. However, a few denser eggs tend to sediment [7].

Four g of fresh stool was transferred into 10 mL of 10% formalin and was mixed and fixed for 30 min. The mixture was filtered through gauze. Then, 15 mL 0.85% NaCl solution was added to each tube and centrifuged for 10 min at 500 x g. The supernatant was decanted, and the sediment (approximately 0.5 to 1 mL) then was suspended in 1 to 2 mL of ZnSO₄ solution. After the volume was completed to 15 mL with the same solution and centrifuged for 1 min at 500 x g. Carefully, the tubes were removing to a rack and it to stand for one minute. One drop of the surface film and 1 drop of sediment were remove with a wire loop and placed them onto a slide, and a drop of iodine solution was added and a coverslip (22x24 mm) was placed on top of each preparation and systematically observed using the 10X and 40 X objective [6,7].

2.3.3 Formalin-ethyl acetate

This method allows concentrating cysts, oocysts, and eggs and larvae, independently of their density [6]. The sample was fixed in 10% formalin and the fecal fat and mucoid substances were extracted with ethyl acetate. However, these two chemicals are toxic to humans and the environment [5]. Four g of fresh stool was mixed into 10 mL 10% formalin and fixed for 30 min. The suspension was filtered through gauze and transferred to a 15 mL conical centrifuge tube. A 0.85% NaCl solution was added to make the volume up to 15 mL and centrifuged for 10 min at 500 x g. The supernatant was decanted, and the sediment was suspended in 10 mL of 10% formalin. The tube was left to rest for 10 min, then, 4 mL of ethyl acetate was added, the tube was capped and the mixture was shaken vigorously for 30 s and the plug was removed after 15 to 30 s and centrifuged for 10 min at 500 x g. Four layers were observed, the parasites in the sediment. After loosening the debris plug, the top three layers were decanted, the sediment
was suspended in residual water and homogenized with gentle stirring, after with a Pasteur pipet a drop of iodine solution was added and coverslip (22x24 mm) was placed on top of each preparation and systematically observed using the 10X and 40 X objective [6,7].

2.3.4 Spontaneous sedimentation in tube technique

5 g of stools was dissolved in 10 mL of saline isotonic solution for approximately 30–60 s until a homogenized the sample. The solution was then filtered through surgical gauze into sterile conical tubes of 50 mL. Then the gauze was discarded. The tube was completely filled with normal saline solution and plug was placed. It was vigorously mixed for 30 s and then left in a vertical position at room temperature for at least 45 min. Then, a drop of sediment was taken using a Pasteur pipet and placed them onto a slide, and a drop of iodine solution was added and a coverslip (22x24 mm) was placed on top of each preparation and systematically observed using the 10X and 40 X objectives [8-10] Fig. 1.

2.4 Statistical Analysis

The results were statistically analyzed with the IBM-SPSS (Statistical Program for the Social Sciences) software package, version 20.0. The results were presented as the average of three samples per patient, in a number and percentage format. The different methods were compared, in terms of sensitivity and specificity, against the formalin-ethyl acetate method (reference standard) by using 2 × 2 contingency tables and chi-square test was performed.

The kappa index (k) was calculated through Tau-b of Kendall, to assess the agreement among the results obtained with all diagnostic techniques and the standard method. Interpretation of k value was as follows: k ≤ 0.19 (no agreement); k = 0.20–0.390 (P=poor agreement); k = 0.40–0.59 (G= good agreement); k = 0.60–0.79, (VG=very good agreement); k = 0.80–1.00 (E=excellent agreement). Statistical significance was considered for P values < 0.05 [13].

3. RESULTS

3.1 Comparison of Three Parasitological Methods Vs to Formalin-Ethyl Acetate Method for Detection of Pathogenic Protozoa

Our results showed that, for Entamoeba complex the SSTT and the direct smear methods showed a very good (VG) agreement (accuracy = 86.09%, k=0.697; accuracy=76.52%, k=0.662 respectively). The SSTT was the least accurate (accuracy = 73.91% and k=0.161) for the recovery of Blastocystis spp. Howe- ver, with direct smear and ZnSO₄ (surface film and sediment analyzed) methods also showed poor (P) agreement for this parasite. The SSTT was the most accurate for G. lamblia

![Fig. 1. Flow chart, shows the methods used in the diagnosis of intestinal parasites](image-url)
3.2 Comparison of Three Parasitological Methods Vs to Formalin-Ethyl Acetate Method for Detection of Commensal Protozoans

When the SSTT and direct smear method for the detection of commensal protozoa like Endolimax nana, were compared good (G) agreement was obtained (accuracy=82.81%, k = 0.567 and 80.00%, k= 0.487 respectively). The ZnSO₄ method was not effective (P agreement) for Endolimax nana (E. nana) and Entamoeba coli (E. coli), even when the surface was analyzed as a sediment. In the case of other non-pathogenic protozoa, such as Iodamoeba bütschlii (I. bütschlii) and Chilomastix mesnili (C. mesnili), there was no agreement at all. But nevertheless, the SSTT showed a VG agreement for the recovery of E. coli (accuracy= 91.30%, k = 0.791) (Table 2).

3.3 Comparison of Three Parasitological Methods vs to Formalin-Ethyl Acetate Method for Helminthes Detection

The present study showed that the direct smear and SSTT methods had VG agreement (k =0.755 and k=0.696 respectively) for the identification of A. lumbricoides while the ZnSO₄ (surface film and sediment) method had G concordance (k=0.473 and 0.524 respectively). For E. vermicularis there was no agreement at all (Table 3).

3.4 Comparison of Three Parasitological Methods vs to Formalin-Ethyl Acetate Method for Detection of Polyparasitism

The comparative study of these diagnostic methods determined that the sensitivity and specificity for detection of multiple parasites was the best when using the SSTT method (k=0.649) (Table 4).

4. DISCUSSION

In the present study, the direct smear method showed VG agreement for the recovery of A. lumbricoides eggs, Entamoeba complex cysts, and G. lamblia cysts. There are studies in which this method has been applied as a screening for the diagnosis of enteroparasites and for the confirmation of the results they used a concentration method like the Ritchie, obtaining better results with the concentration method [14-16]. The direct smear and the concentration methods by flotation and sedimentation such as SSTT applied were for the recovery of Blastocystis spp (P agreement). These results reflect the limitations of these methods to detect low levels of infection of this parasite. Contrasting results with respect to other authors [8,10]. The analysis of multiple stool samples is recommended to improve the detection of low levels not only of Blastocystis but also other parasites [17]. On the other hand, our results are similar of others authors [18].

There are reports that distilled water affects the vacular phase of Blastocystis and therefore it has been recommended to use isotonic saline solution in flotation and sedimentation concentration procedures such as Ritchie [7]. However, the SSTT method, it is suggested that it could be the most appropriate [17]. In our work in concentration methods with zinc sulfate and formaldehyde-ethyl acetate, isotonic saline solution was used precisely to avoid lysis of the parasite [6,7,17]. In 1938, the original concentration by flotation with ZnSO₄ solution was described [19]. Since then, several modifications have been introduced to it [19-22]. In the present study, we applied the recommendations from CLSI (2005) [6]. For example 0.85% NaCl was added for the washes to prevent the lysis of several stages of Blastocystis spp. The centrifugation speed was 500 x g, when the flotation concentration method with ZnSO₄ was applied, the surface film and the sediment were revised; to ensure detection of all possible organisms and however, the poor agreement was for Blastocystis spp.

Nevertheless, the flotation method was not effective (P agreement) during the recovery of intestinal protozoa such as E. nana and E. coli, even when the surface was analyzed as a sediment. In the case of other non-pathogenic protozoa, such as I. bütschlii and and C. mesnili, there was no agreement at all. The smaller protozoans are easily missed with the direct smear and concentration methods, thus the permanent stain is recommended [23]. It enables the detection of scanty organisms that might have been missed by employing only a direct smear [7,24].
Table 1. Performance of different diagnostic methods compared to formalin-ethyl acetate method for detection of pathogenic protozoa

<table>
<thead>
<tr>
<th>Parasites</th>
<th>Method</th>
<th>N+ (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
<th>NPV (%)</th>
<th>Kappa index</th>
<th>95%CI</th>
<th>P</th>
<th>Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entamoeba complex</td>
<td>Direct smear</td>
<td>31(27.0)</td>
<td>66.70</td>
<td>95.90</td>
<td>76.52</td>
<td>73.00</td>
<td>0.662</td>
<td>67.21-83.92</td>
<td>P&lt;.05</td>
<td>VG</td>
</tr>
<tr>
<td></td>
<td>SSTT</td>
<td>40(34.8)</td>
<td>78.60</td>
<td>90.40</td>
<td>86.09</td>
<td>88.80</td>
<td>0.697</td>
<td>78.39-91.83</td>
<td>P&lt;.05</td>
<td>VG</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(surface film)</td>
<td>15(13.0)</td>
<td>35.70</td>
<td>100.0</td>
<td>76.52</td>
<td>73.00</td>
<td>0.414</td>
<td>67.71-83.92</td>
<td>P&lt;.05</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(sediment)</td>
<td>9(7.80)</td>
<td>16.70</td>
<td>97.30</td>
<td>67.83</td>
<td>66.98</td>
<td>0.167</td>
<td>58.47-76.23</td>
<td>P&lt;.05</td>
<td>P</td>
</tr>
<tr>
<td>Blastocystis spp</td>
<td>Direct smear</td>
<td>29(25.2)</td>
<td>63.20</td>
<td>82.30</td>
<td>79.13</td>
<td>91.86</td>
<td>0.375</td>
<td>70.56-86.15</td>
<td>P&lt;.05</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>SSTT</td>
<td>25(21.7)</td>
<td>36.80</td>
<td>81.30</td>
<td>73.91</td>
<td>86.67</td>
<td>0.161</td>
<td>64.90-81.66</td>
<td>P&lt;.05</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(surface film)</td>
<td>31(27.0)</td>
<td>57.90</td>
<td>79.20</td>
<td>75.65</td>
<td>90.48</td>
<td>0.296</td>
<td>66.77-83.17</td>
<td>P&lt;.05</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(sediment)</td>
<td>25(21.7)</td>
<td>52.60</td>
<td>84.40</td>
<td>79.13</td>
<td>90.00</td>
<td>0.328</td>
<td>70.56-86.15</td>
<td>P&lt;.05</td>
<td>P</td>
</tr>
<tr>
<td>G. lamblia</td>
<td>Direct smear</td>
<td>14(12.2)</td>
<td>100.0</td>
<td>95.30</td>
<td>96.50</td>
<td>100.0</td>
<td>0.760</td>
<td>90.15-98.57</td>
<td>P&lt;.05</td>
<td>VG</td>
</tr>
<tr>
<td></td>
<td>SSTT</td>
<td>12(10.4)</td>
<td>100.0</td>
<td>97.20</td>
<td>93.39</td>
<td>100.0</td>
<td>0.843</td>
<td>92.57-99.40</td>
<td>P&lt;.05</td>
<td>E</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(surface film)</td>
<td>7(6.10)</td>
<td>66.70</td>
<td>99.00</td>
<td>96.49</td>
<td>97.20</td>
<td>0.731</td>
<td>91.26-98.04</td>
<td>P&lt;.05</td>
<td>VG</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(sediment)</td>
<td>10(8.70)</td>
<td>77.80</td>
<td>97.20</td>
<td>95.65</td>
<td>98.10</td>
<td>0.713</td>
<td>90.15-98.57</td>
<td>P&lt;.05</td>
<td>VG</td>
</tr>
</tbody>
</table>

Entamoeba complex: Entamoeba histolytica / E. dispar/E. moshkovskii/E. bangladeshi; SSTT: Spontaneous Sedimentation in Tube Technique; ZnSO₄(surface film): Concentration by flotation (surface film examined); ZnSO₄(sediment): Concentration by flotation (sediment examined); N+: Number of positive cases with the indicated parasite; NPV: Negative predictive value; 95%CI: Confident interval. Ranges of Agreement: Excellent (E) 0.80–1.00, Very good (VG) 0.60–0.79, Good (G) 0.40–0.59, Poor (P) 0.20–0.39.
Table 2. Performance of different diagnostic techniques compared to formalin-ethyl acetate method for detection of commensal protozoans

<table>
<thead>
<tr>
<th>Parasites</th>
<th>Method</th>
<th>N+ (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
<th>VPN (%)</th>
<th>Kappa Index</th>
<th>95% CI</th>
<th>P</th>
<th>Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. nana</td>
<td>Direct smear</td>
<td>30(26.1)</td>
<td>61.30</td>
<td>86.90</td>
<td>80.00</td>
<td>85.88</td>
<td>0.487</td>
<td>71.52-86.88</td>
<td>&lt;.05</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>SSTT</td>
<td>33(28.7)</td>
<td>71.00</td>
<td>86.90</td>
<td>82.61</td>
<td>89.09</td>
<td>0.567</td>
<td>74.43-89.04</td>
<td>&lt;.05</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(surface film)</td>
<td>13(11.3)</td>
<td>29.00</td>
<td>95.20</td>
<td>77.39</td>
<td>78.43</td>
<td>0.297</td>
<td>68.65-84.67</td>
<td>&lt;.05</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(sediment)</td>
<td>13(11.3)</td>
<td>25.80</td>
<td>94.00</td>
<td>75.45</td>
<td>77.45</td>
<td>0.243</td>
<td>66.77-83.17</td>
<td>&lt;.05</td>
<td>P</td>
</tr>
<tr>
<td>E. coli</td>
<td>Direct smear</td>
<td>19(16.5)</td>
<td>51.40</td>
<td>98.80</td>
<td>84.48</td>
<td>82.29</td>
<td>0.576</td>
<td>76.59-90.54</td>
<td>&lt;.05</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>SSTT</td>
<td>33(28.7)</td>
<td>82.90</td>
<td>95.00</td>
<td>91.30</td>
<td>92.68</td>
<td>0.791</td>
<td>84.59-95.75</td>
<td>&lt;.05</td>
<td>VG</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(surface film)</td>
<td>7(6.10)</td>
<td>20.00</td>
<td>100.00</td>
<td>75.65</td>
<td>71.07</td>
<td>0.258</td>
<td>66.77-83.17</td>
<td>&lt;.05</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(sediment)</td>
<td>5(4.30)</td>
<td>11.40</td>
<td>98.80</td>
<td>72.17</td>
<td>71.82</td>
<td>0.134</td>
<td>63.05-80.13</td>
<td>&lt;.05</td>
<td>P</td>
</tr>
<tr>
<td>I. bütschlii</td>
<td>Direct smear</td>
<td>1(0.90)</td>
<td>0.000</td>
<td>99.10</td>
<td>98.26</td>
<td>99.12</td>
<td>0.009</td>
<td>93.86-99.79</td>
<td>&lt;.05</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SSTT</td>
<td>3(2.60)</td>
<td>0.000</td>
<td>97.40</td>
<td>96.52</td>
<td>99.11</td>
<td>0.164</td>
<td>91.33-99.04</td>
<td>&lt;.05</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(surface film)</td>
<td>0(0.00)</td>
<td>0.000</td>
<td>100.00</td>
<td>47.90</td>
<td>50.00</td>
<td>0.000</td>
<td>41.40-51.45</td>
<td>&lt;.05</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(sediment)</td>
<td>0(0.00)</td>
<td>0.000</td>
<td>100.00</td>
<td>0.87</td>
<td>NA</td>
<td>0.000</td>
<td>0.02-4.750</td>
<td>&lt;.05</td>
<td>NA</td>
</tr>
<tr>
<td>C. mesnili</td>
<td>Direct smear</td>
<td>7(6.10)</td>
<td>0.000</td>
<td>93.80</td>
<td>91.30</td>
<td>97.22</td>
<td>0.038</td>
<td>84.59-95.75</td>
<td>&lt;.05</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SSTT</td>
<td>5(4.30)</td>
<td>33.30</td>
<td>96.40</td>
<td>94.78</td>
<td>98.18</td>
<td>0.225</td>
<td>88.99-98.06</td>
<td>&lt;.05</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(surface film)</td>
<td>0(0.00)</td>
<td>0.000</td>
<td>100.00</td>
<td>89.57</td>
<td>100.00</td>
<td>0.000</td>
<td>82.48-94.49</td>
<td>&lt;.05</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(sediment)</td>
<td>0(0.00)</td>
<td>0.000</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>0.000</td>
<td>96.84-100.0</td>
<td>&lt;.05</td>
<td>NA</td>
</tr>
</tbody>
</table>

SSTT: Spontaneous Sedimentation Technique in Tube; ZnSO₄(surface film): Concentration by flotation (surface film examined); ZnSO₄(sediment): Concentration by flotation (sediment examined); N+: Number of positive cases with the indicated parasite; VPN: Negative predictive value; 95%CI: 95% Confidence Interval. Ranges of Agreement: Excellent (E) 0.80–1.00, Very Good (VG) 0.60–0.79, Good (G) 0.40–0.59, Poor (P) 0.20–0.39, No agreement (NA) 0.00–0.19
### Table 3. Performance of different diagnostic methods compared to formalin-ethyl acetate method for helminthes detection

<table>
<thead>
<tr>
<th>Parasites</th>
<th>Method</th>
<th>N+(% )</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
<th>NPV (%)</th>
<th>Kappa index</th>
<th>95%CI</th>
<th>P</th>
<th>Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. lumbricoides</em></td>
<td>Direct smear</td>
<td>6(5.20)</td>
<td>71.40</td>
<td>99.10</td>
<td>97.39</td>
<td>98.17</td>
<td>0.755</td>
<td>92.57-99.46</td>
<td>P&lt;.05</td>
<td>VG</td>
</tr>
<tr>
<td></td>
<td>SSTT</td>
<td>7(6.10)</td>
<td>71.40</td>
<td>98.10</td>
<td>96.52</td>
<td>95.15</td>
<td>0.696</td>
<td>91.33-99.04</td>
<td>P&lt;.05</td>
<td>VG</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(surface film)</td>
<td>5(4.30)</td>
<td>42.90</td>
<td>98.10</td>
<td>94.78</td>
<td>96.36</td>
<td>0.473</td>
<td>88.99-98.06</td>
<td>P&lt;.05</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(sediment)</td>
<td>4(3.50)</td>
<td>42.90</td>
<td>99.10</td>
<td>95.65</td>
<td>96.40</td>
<td>0.524</td>
<td>90.15-98.57</td>
<td>P&lt;.05</td>
<td>G</td>
</tr>
<tr>
<td><em>E. vermicularis</em></td>
<td>Direct smear</td>
<td>2(1.70)</td>
<td>0.00</td>
<td>98.30</td>
<td>50.00</td>
<td>50.00</td>
<td>0.000</td>
<td>43.36-56.64</td>
<td>P&lt;.05</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SSTT</td>
<td>3(2.60)</td>
<td>0.00</td>
<td>98.30</td>
<td>50.00</td>
<td>50.00</td>
<td>0.000</td>
<td>43.36-56.64</td>
<td>P&lt;.05</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(surface film)</td>
<td>0(0.00)</td>
<td>0.00</td>
<td>100.0</td>
<td>50.00</td>
<td>50.00</td>
<td>0.000</td>
<td>43.36-56.64</td>
<td>P&lt;.05</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄(sediment)</td>
<td>0(0.00)</td>
<td>0.87</td>
<td>99.10</td>
<td>50.00</td>
<td>50.00</td>
<td>0.000</td>
<td>43.36-56.64</td>
<td>P&lt;.05</td>
<td>NA</td>
</tr>
</tbody>
</table>

SSTT: Spontaneous Sedimentation in Tube Technique; ZnSO₄(surface film): Concentration by flotation (surface film examined); ZnSO₄(sediment): Concentration by flotation (sediment film examined); N+: Number of positive cases with indicated parasite; NPV: Negative predictive value; 95%CI: Confidence interval. Ranges of Agreement: Excellent (E) 0.80–1.00, Very good (VG) 0.60–0.79, Good (G) 0.40–0.59, Poor (P) 0.20–0.39, No agreement (NA) 0–0.19

### Table 4. Performance of different diagnostic methods compared to formalin-ethyl acetate method for detection of multiple parasites

<table>
<thead>
<tr>
<th>Method</th>
<th>N+(% )</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy</th>
<th>NPV</th>
<th>Kappa index</th>
<th>95%CI</th>
<th>P</th>
<th>Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct smear</td>
<td>36(31.3)</td>
<td>58.50</td>
<td>83.80</td>
<td>74.78</td>
<td>78.46</td>
<td>0.43</td>
<td>65.83-82.42</td>
<td>0.000</td>
<td>G</td>
</tr>
<tr>
<td>SSTT</td>
<td>46(40.0)</td>
<td>82.90</td>
<td>83.80</td>
<td>83.48</td>
<td>89.86</td>
<td>0.64</td>
<td>75.41-89.75</td>
<td>0.000</td>
<td>VG</td>
</tr>
<tr>
<td>ZnSO₄(surface film)</td>
<td>16(13.9)</td>
<td>28.80</td>
<td>93.20</td>
<td>65.57</td>
<td>69.70</td>
<td>0.23</td>
<td>60.29-77.80</td>
<td>0.003</td>
<td>P</td>
</tr>
<tr>
<td>ZnSO₄(sediment)</td>
<td>12(10.4)</td>
<td>19.50</td>
<td>94.60</td>
<td>67.83</td>
<td>67.96</td>
<td>0.16</td>
<td>58.47-76.23</td>
<td>0.018</td>
<td>P</td>
</tr>
</tbody>
</table>

SSTT: Spontaneous Sedimentation Technique in Tube; ZnSO₄(surface film): Concentration by flotation (Surface film examined); ZnSO₄(sediment): Concentration by flotation (sediment examined); N+: Number of positive cases with the indicated parasite; NPV: Negative predictive value; 95%CI: Confidence interval. Ranges of Agreement: Excellent (E) 0.80–1.00, Very good (VG) 0.60–0.79, Good (G) 0.40–0.59, Poor (P) 0.20–0.39
On the other hand, no method recovered *E. vermicularis* the non agreement, could be attributed to the particular life cycle of this helminthes, as the female deposits the eggs in the perianal region, and the eggs can only be recovered with more sensitive techniques, such as the Graham method [7].

An additional advantage of the SSTT method is the absence of centrifugation to conserve the morphology of the stages parasite, in contrast with other methods of concentration [7,23,25]. Important difference when compared to the concentration method with zinc sulphate where the reading time should not exceed 20 min after centrifugation with the ZnSO₄ solution because the structures begin to distort and disintegrate [6]. When we perform this method of concentration had to analyze the samples in sections to avoid losing the parasitic stages. The time is an important factor especially for laboratories that process a large number of samples or even in epidemiological studies [9, 24,26].

In this work, the SSTT method yielded the highest number of positive samples (40%). Other authors have also recommended this method for the clinical and epidemiological diagnosis of enteroparasites [9]. The SSTT method revealed a VG agreement for *A. lumbricoides*. If we consider the densities of the fertile eggs (1.11-1.13 g/dL) and the infertile eggs (1.20 g/dL) of this helminthes [25] both types of eggs sediment with SSTT method regardless of its density. However, with the ZnSO₄ method a lower sensitivity was obtained, this may be influenced by the density of the ZnSO₄ solution used (1.18 g/dL) in such a way that the infertile eggs do not float. Therefore, we also analyze the sediment. In this study, however, better results were obtained with the SSTT, this can also be influenced because this method use greater amount of sample, thus increasing the possibility of recovery of both parasitic stages of *A. lumbricoides*.

The SSTT method has shown that the viability of parasites is conserved and do not experience distortions in parasite stages. This advantage could be used to visualize the motility of amoebas, flagellates, ciliates and larvae such as *Strongyloides stercoralis*. In this study, no parasite motility was observed due at the samples were conserved at 4°C by 72 h before analysis. However, epidemiological studies where liquid samples were processed and analyzed within the same study site have been reported. Therefore, it is essential to access to fresh stools to observe parasite mobility [15].

Even with the availability of a variety of methods, there is still a high risk of exposure of laboratory personnel and environment to toxic substances [27]. In our study we used ethyl acetate, which is less toxic than ether [27]. Still, this standard method has disadvantages when compared to the SSTT method. Therefore, the selection of methodologies that reduce occupational hazards is still a challenge. Regarding toxicity, several studies have shown the advantages of the SSTT method over ZnSO₄ and formalin-ethyl acetate methods [26].

Even though it is known that parasitological stool examination methods have lower sensitivity and specificity than molecular methods [28], they remain a valuable tool to diagnose enteroparasites. In Western Europe, real time PCR is rapidly replacing conventional microscopy. However, in developed countries, where most parasites are not endemic, detection and identification of protozoa and helminthes are challenge [23].

However, microscopy is still the first diagnostic method in most of the parasitological laboratories, and this procedure is sufficient to detect the most predominant parasite species in areas of high incidence and intensity of parasites [5,28,29]. Thus, the SSTT method has been proposed in countries where diagnostic resources are limited and where parasites are endemic [9].

5. CONCLUSION

The spontaneous sedimentation in tube technique (SSTT) constitutes a viable alternative in the diagnostic detection of intestinal parasites. Compared with the formalin-ethyl acetate technique, SSTT is a simple, economic, and fast method that recovers protozoa and helminthes in different phases.

SSTT method is until in low-resource settings where intestinal parasites are endemic. Moreover, the SSTT does not pose a risk of toxicity to personnel or environment, as it uses only isotonic saline solution rather than hazardous substances, as used in the formalin-ethyl acetate method.
CONSENT
As per international standard or university standard written participant consent has been collected and preserved by the authors.

ETHICAL APPROVAL
This information was obtained through the application of a questionnaire approved by the Institutional Research, Ethics, and Biosafety Committee registration number CI 053-2014 of University Centre of Health Sciences, University of Guadalajara.

ACKNOWLEDGEMENTS
All the authors thank the facilities granted by the staff of the clinic of Municipality of Jocotepec, the secretary of health.

COMPETING INTERESTS
Authors have declared that no competing interests exist.

REFERENCES


Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/56558