COMPARISON OF A POLYMERASE CHAIN REACTION AND THE KATO-KATZ TECHNIQUE FOR DIAGNOSING INFECTION WITH SCHISTOSOMA MANSONI

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Abstract. Fecal samples from 194 individuals living in an area of Brazil endemic for Schistosoma mansoni were analyzed by a polymerase chain reaction (PCR) and the Kato-Katz parasitologic examination. Statistical analysis of the results showed a kappa index of 0.8 between the two methods. The prevalence of infection was 30.9% in three fecal samples examined by the Kato-Katz method, but 38.1% in one fecal sample examined by the PCR technique. Repeated survey of discordant results showed that five (41.6%) of 12 parasitologically negative cases for which PCR gave positive results were misdiagnosed by Kato-Katz examinations. The PCR technique showed a sensitivity of 96.7% and a specificity of 88% when the parasitologic examination was used as the reference test. The efficacy of cure with praziquantel was 87.8% in three parasitologic stool examinations and 75.6% in one PCR survey. These results demonstrate that the PCR assay might be a valuable alternative for diagnosing Schistosoma infections.

INTRODUCTION

Schistosomiasis is a severe snail-borne disease endemic in many developing countries that affects more than 200 million people worldwide.1 Approximately 120 million individuals are symptomatically infected and 20 million are severely affected by the different species of the genus Schistosoma known to infect humans.1 Among these species, Schistosoma mansoni is endemic in 54 countries and territories in South America, Africa, and the Caribbean and the eastern Mediterranean regions. In Africa, where ≥80% of the global cases are concentrated, S. mansoni is the major species that is transmitted.2

Despite efforts to control this disease, which are based on treatment of infected people and elimination of snails, schistosomiasis remains a major health problem that results in significant financial burdens.2 The Kato-Katz parasitologic technique3 is currently the recommended method for diagnosing this disease1 because it is quantitative, relatively inexpensive, and simple. However, the sensitivity of this technique decreases when the prevalence and intensity of infection are low.4,6 Thus, this method is less efficient in areas of low endemicity, in post-treatment situations, and in the control of transmission.

Other diagnostic alternatives include immunologic methods, such as detection of parasite-specific antibodies and circulating antigens. Antibody detection assays have been shown to be more sensitive than the parasitologic examination,7 but generally lack specificity.8 The detection of circulating antigens is a highly specific assay7,9 but has not been shown to be more sensitive than the detection of eggs in areas of low endemicity.7,10,11

Despite remarkable advances achieved over the last decade in this field, including the development of a polymerase chain reaction (PCR),12 molecular techniques have not been extensively used in the diagnosis of human infections with Schistosoma. We have recently published preliminary results demonstrating that the PCR is a useful technique for the detection of S. mansoni in human feces and serum.13 The amplification reaction is capable of detecting as little as 1 fg of a highly repetitive Schistosoma mansoni DNA sequence, showed no cross-reactivity with other related helminths, and was 10 times more sensitive than the Kato-Katz technique.

This report presents the results of a more extensive comparison between the PCR assay and the parasitologic Kato-Katz technique, in which fecal samples of individuals from a area of Brazilian endemic for S. mansoni infection were analyzed by both procedures.

MATERIALS AND METHODS

Study population. One hundred ninety-four persons from a small village located in Comercinho, Minas Gerais, Brazil, an area endemic for infection with S. mansoni, participated in this work. All residents were invited to participate in the study. The community was selected based on the previous known low prevalence of the locality. The study group was composed of 49% females and 51% males with an age range of 5–76 years (mean age = 36 years). Informed consent was obtained from all adult participants and from the parents or legal guardians of minors. This project was reviewed and approved by the FIOCRUZ Ethics Review Board.

Fecal samples. Samples were collected and stored at −70°C until use, except during transportation to the research laboratory, through which they were kept on ice. The collections were done on three consecutive days for all patients. Fecal samples were analyzed by both a PCR (one sample) and Kato-Katz stool examination (three samples). The collection of new fecal samples, specifically conducted for the investigation of discordant results between the two methods, was performed on at least four different days and the samples were reanalyzed by both procedures.

Forty-one patients with a positive stool examination result by the Kato-Katz diagnostic assay were treated with a single oral dose of praziquantel (50 or 60 mg/kg for adults and children, respectively). New fecal collections from these patients were done on three consecutive days two months after treatment. These samples were then analyzed for cure evaluation by the PCR technique (one sample) and the Kato-Katz stool examination method (three samples).

Twenty fecal samples of non-exposed persons from Belo Horizonte, Minas Gerais, Brazil with negative Kato-Katz stool examination results were used to evaluate the specificity of the PCR assay.

Stool examination by the Kato-Katz method. Fecal samples were evaluated for the presence of S. mansoni eggs by the
quantitative Kato-Katz parasitologic method. Two glass slides (43 mg of feces per slide) were prepared for analyzing each sample, and the arithmetical mean number of eggs per gram of feces was the final result.

**Stool examination by the PCR method. Sample preparation.** Prior to extraction, approximately 0.5 g of feces were mixed with 1.0 mL of distilled water in a 1.5-mL microcentrifuge tube, vigorously agitated, and centrifuged at 100,000 × g for two minutes. The supernatant was discarded and the pellet was washed by the same procedure, except that the sample was gently agitated for 10 minutes to favor the rupture of S. mansoni eggs. The final pellet was used for isolation of parasite DNA.

**Isolation of DNA.** The DNA from fecal samples was extracted following a modification of the rapid one-step extraction (ROSE) method. Briefly, 0.7 mL of ROSE buffer (10 mM Tris, pH 8.0, 300 mM EDTA, pH 8.0, 1% sodium lauryl sulfate, 1% polyvinylpyrrolidone) were mixed with the washed fecal pellet and heated to 95°C for 20 minutes, with the sample being agitated vigorously briefly after the first 10 minutes of heating. The sample was then centrifuged at 14,000 × g for 10 minutes at room temperature to precipitate proteins and fecal debris. Five hundred microliters of the supernatant was then collected in a separate tube, mixed with 50 µL of cold (−20°C) 3 M sodium acetate, pH 5.3, and 1 mL of cold (−20°C) ethanol, and immediately centrifuged at 14,000 × g for 15 minutes. The supernatant was then discarded, and the pellet was washed once with 500 µL of 70% ethanol (−20°C) and centrifuged at 14,000 × g for 10 minutes. The supernatant was discarded, and the pellet was dried at 37°C for 15 minutes and resuspended in 100 µL of cold (4°C) buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0).

**Polymerase chain reaction assay.** The PCR primers were designed to amplify a highly repeated 121-basepair sequence of S. mansoni reported by Hamburger and others that comprises about 10% of the parasite genome (600,000 copies per cell). For the amplification of DNA extracted from stool specimens, samples were diluted 1:100 and 1 µL was used as template in a 10-µL final volume mixture containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.5 µM of each primer, 200 µM of the four dNTPs, and 0.75 units of Taq DNA polymerase. The amplification reaction was carried out for 35 cycles, with each cycle consisting of a denaturation step at 95°C for 40 seconds and an annealing step at 65°C for 30 seconds. The first cycle had an extended denaturation step for five minutes and the reaction was ended with an extension step at 72°C for five minutes.

To prevent carryover contamination, extraction and amplification of DNA and the preparation of the PCR mixture were conducted in separate facilities with total separation of materials. Aerosol barrier tips were used in all steps, including sample collection. Contamination was monitored by the inclusion of four negative controls containing water instead of extracted DNA in the amplification reaction, two of which came from the DNA extraction step. The PCR products were subjected to electrophoresis on 6% polyacrylamide gels and analyzed after silver staining.

**RESULTS**

The prevalence of S. mansoni infection, calculated with one, two, or three Kato-Katz examinations or by one PCR survey, is shown in Figure 1. The prevalence observed using the PCR technique was approximately 13% higher (χ² = 126, P < 0.0001) than that determined with the Kato-Katz stool examination.

The geometric mean number of eggs per gram of feces estimated by the Kato-Katz method in a positive stool sample was 33 eggs per gram of feces, which indicates a low intensity of infection. Comparison of results obtained by one PCR and three stool examinations by the Kato-Katz method is shown in Table 1. Statistical analysis showed a kappar index of 0.8, indicating good agreement between the two methods. Analysis of discordant results showed that 16 samples were positive only by the PCR and two positive samples were positive only by the Kato-Katz method. These two patients had very low egg outputs, with 12 eggs per gram of feces in the first sample and no eggs in the other two samples.

The samples of patients whose results differed in the PCR and Kato-Katz methods were re-analyzed by both methods using a greater number of fecal samples (at least four on consecutive days). Among 12 patients whose samples were negative by three Kato-Katz examinations, but positive by one PCR survey, five (41%) were positive by the Kato-Katz method after repeated examinations.

Diagnostic parameters were calculated by two different approaches: 1) taking the Kato-Katz results alone as the reference for comparison or 2) considering the specificity of both parasitologic examinations and one PCR survey. The prevalence of infection detected by one PCR survey was almost 13% higher (χ² = 126, P < 0.0001) than that detected by the three parasitologic examinations.

**TABLE 1**

Comparative evaluation of the polymerase chain reaction (PCR) and the Kato-Katz stool parasitologic examination (three samples) for the diagnosis of *Schistosoma mansoni* infection

<table>
<thead>
<tr>
<th>Kato-Katz stool examination</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Positive</td>
<td>58</td>
<td>16</td>
<td>74</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>118</td>
<td>120</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>134</td>
<td>194</td>
</tr>
</tbody>
</table>

* Kappa index = 0.8.
the Kato-Katz and PCR methods as being 100%, and assuming as infected person anyone with positive result by either method. The values estimated by each approach are shown in Table 2.

The sensitivity values of the PCR were high and approximately the same irrespective of the reference considered. Specificity values changes significantly depending on the reference used, but were still high (88%) even when the Kato-Katz method was taken as the reference. The specificity of the DNA amplification assay was found to be 100% by survey of 20 samples from individuals never exposed to *Schistosoma* infection and with negative Kato-Katz stool examination results.

Parasitic-positive patients were treated and new fecal samples were collected two months after treatment and analyzed by both methods for cure evaluation. Table 3 shows that the PCR technique detected more positive cases than the Kato-Katz method. The parasitologic results showed that two months after treatment, 87.8% of the infected individuals had been cured, a value that decreased to 75.6% ($\chi^2 = 17.6, P < 0.0001$) when the PCR results were used.

**DISCUSSION**

One of the main requirements for diagnosing schistosomiasis is the development of a more sensitive assay. Since the PCR is an extremely sensitive and specific technique with widespread use in the diagnosis of infectious diseases, it was evaluated as a diagnostic tool for detecting infection of humans with *S. mansoni*. As shown in Figure 1 and Table 1, a single PCR survey detected more cases of infection with *S. mansoni* than three Kato-Katz stool examinations. There are two possible explanations for this result: 1) the PCR could be more sensitive than the Kato-Katz technique, in which case those results would actually correspond to *S. mansoni*-infected samples, or 2) those results could, in reality, be false-positive results.

To better discern between these two possibilities, we conducted a re-investigation among 12 of the 16 patients with positive PCR and negative stool examinations results by performing at least four additional parasitologic examinations on each fecal sample. The other four discordant results could not be further scrutinized since their donors could not be located. The re-investigation of these discordant results showed that five (41.6%) of the 12 patients were infected with *S. mansoni*, demonstrating the higher sensitivity of the PCR. Similar results were obtained in a preliminary experimental comparison between the two methods.14 Seven samples remained negative even after more than four additional Kato-Katz examinations.

**Table 2**

Sensitivity, specificity, and predictive values of the Kato-Katz and the polymerase chain reaction (PCR) methods for the diagnosis of *Schistosoma mansoni* infection

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a (S) b (%)</td>
<td>a (S) b (%)</td>
<td>a (S) b</td>
<td>a (S) b</td>
</tr>
<tr>
<td>Kato-Katz</td>
<td>100</td>
<td>78.9</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PCR</td>
<td>96.7</td>
<td>97.4</td>
<td>88</td>
<td>100</td>
</tr>
</tbody>
</table>

*PPV = positive predictive value; NPV = negative predictive value; a = values calculated considering Kato-Katz results as the reference gold standard; b = values calculated assuming a specificity of 100% for both methods, and defining as infected all persons with a positive result by either method.

Predictive cure rates of *Schistosoma mansoni* infection two months after treatment with praziquantel evaluated by the polymerase chain reaction (PCR) technique and the Kato-Katz method

<table>
<thead>
<tr>
<th></th>
<th>Kato-Katz stool examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>5</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
</tr>
</tbody>
</table>

* Cure rates = 31/41 = 75.6% and 36/41 = 87.8%, as evaluated by one PCR survey and three Kato-Katz stool examinations, respectively ($\chi^2 = 17.6, P < 0.0001$).

**Table 3**

It is possible that having passed through such extensive survey these samples represent truly negative samples, a fact that would reduce the overall efficiency of the PCR technique. However, it is also possible that those individuals had egg outputs less than the 20 eggs/gram detection limit of the Kato-Katz examination, in which case the infection would have been detected only by the PCR survey. This possibility is supported by the higher sensitivity of the PCR, and is consistent with a previously reported underestimation of the prevalence by the Kato-Katz method, even after four additional investigations.19 False-positive results could have been due to either a lack of specificity by the PCR assay or carry-over contamination. Cross-reactivity with other helminth infections can be ruled out since only two of these patients were infected with other helminths and the DNA from these worms is not amplified by the *S. mansoni*-specific primers.13 Carryover contamination is often difficult to exclude as a cause of false-positive results in PCR-based diagnostic assays due to the exquisite sensitivity of the technique. However, strict measures to avoid contamination were taken during the entire handling and processing steps of the assay (see Materials and methods).

The PCR failed to detect two samples positive by the Kato-Katz technique. There is no reason to believe that these cases correspond to false-positive parasitologic results since this method has a specificity of 100%. They were certainly misdiagnosed by the DNA amplification assay, a fact that could be due to many factors, such as inhibition of the amplification reaction by feces compounds and/or DNA degradation during transportation from the field. The fecal DNA dilution was shown to be sufficient in earlier tests to overcome inhibition of the PCR inhibition. However, such inhibition cannot be ruled out for each individual sample unless internal controls are used. In this case, controls were not used to decrease costs. Another possibility that cannot be excluded is the effect that a variation in egg output and uneven distribution in feces could have on PCR detection of *S. mansoni* DNA, especially for these two PCR-negative, Kato-Katz positive samples, which contained only 24 eggs per gram of feces.

The diagnostic value of the PCR technique was estimated by two different approaches. In a traditional approach, the Kato-Katz assay was taken as the gold standard and all parameters were calculated using it as the reference. In another approach, both methods were considered to have specificities of 100%; thus, a true positive result was considered as one detected by either method. The validity of this approach would depend solely on the true efficiency of the PCR since the quality of the Kato-Katz technique has been established.
Several facts support the assumption of this efficiency. With respect to specificity, no cross-reactivity has been observed when DNA from related helminths was used as template, and the PCR showed a specificity of 100% when used with 20 fecal samples of non-infected persons. With regard to sensitivity, retesting with the parasitologic method showed that 41% of the discordant results analyzed had been correctly diagnosed by the DNA amplification technique. In addition, a previous report has shown greater sensitivity for the PCR assay. With respect to the overall efficiency of the PCR technique, the statistical agreement found between the two methods was high (kappa value = 0.8).

Sensitivity values of the PCR technique were high by both approaches (>96%). A specificity value calculated by the first approach was lower (88% versus 100%), but still satisfactory if one considers that it was based on results obtained from an endemic area, and that the high sensitivity of the technique would lead to higher detection rates. The same reasoning is valid for positive predictive values obtained with the PCR technique (78.4% versus 100%). Negative predictive values were high and identical (98.3%) irrespective of the adopted gold-standard. Since all of these parameters are highly dependent on the infection prevalence among the population, test performance under other conditions could be different.

Cure evaluation by both methods also showed a higher detection capacity of the DNA amplification reaction. Among the 60 patients positive by the stool examination, 41 were treated and provided three fecal samples two months after treatment. The other 19 patients could not be treated, either because they could no longer be located or they had a restrictive medical condition. Since it detected more positive cases, the PCR provides a lower estimation of the cure rate than the parasitologic technique (75.6% versus 87.8%). The cure rate for *S. mansoni* infection in Brazil, as estimated by the Kato-Katz method, ranges from 80% to 90% after two months of treatment. If one considers the high negative predictive value found for the PCR technique (98%), the real cure rate is unlikely to be very different from what was calculated from the data obtained with both methods.

The data presented in this study show the results of a stringent evaluation of the PCR technique as a diagnostic tool for schistosomiasis in patients in an area of medium intensity of infection. In the analysis of this specific population, a single PCR survey has shown a high statistical correlation with three parasitologic examinations (kappa value = 0.8). However, it also had a higher sensitivity (97% versus 79%). Thus, this technique might prove to be especially useful in circumstances of lower intensity or prevalence of infection, a condition for which the parasitologic examination shows a well-documented limitation of its sensitivity.

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