Evaluation of an Indirect Immunofluorescence Assay for Strongyloidiasis as a Tool for Diagnosis and Follow-Up

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The diagnostic accuracy of an indirect immunofluorescence antibody test (IFAT) for Strongyloides stercoralis at different serum antibody titers was evaluated. To assess diagnostic sensitivity, sera from 156 patients with known strongyloidiasis were collected. Negative control sera were obtained from a composite group of 427 subjects (blood donors and hospitalized patients). With an area under the receiver-operating characteristic plot of 0.98, the IFAT showed a high level of diagnostic accuracy for strongyloidiasis. An antibody titer of ≥1:20, with 97% sensitivity and 98% specificity, was identified as the diagnostic threshold with the best overall performance. Cross-reactions were evaluated with 41 additional samples from patients with other known helmint infections, and the IFAT detected low-titer positivity in only one subject with filariasis. A positive IFAT result at an antibody dilution of ≥1:80 was virtually 100% specific, with 71% sensitivity. To test the usefulness of the IFAT as a monitoring tool, the changes in specific-antibody titers after treatment in a group of 155 patients were evaluated. Seroreversion or a decrease in antibody titer of twofold or more was observed in 60% of the patients. Response to treatment was directly correlated to the initial antibody titer, and a baseline antibody titer of ≥1:80 was identified as the best predictor of response. In conclusion, a positive IFAT result at an antibody dilution of ≥1:20 is the optimal cutoff for screening. A titer of ≥1:80, with virtually no false-positive result, is a reliable cutoff for a serological assessment of treatment efficacy and for inclusion in clinical trials.

Strongyloidiasis is a chronic, soil-transmitted helminthiasis with a worldwide distribution. Current estimates indicate that it affects 30 to 100 million people, but accurate prevalence data are lacking. Although strongyloidiasis is endemic in many tropical and subtropical countries (sub-Saharan Africa, Latin America, Southeast Asia, and Northern Australia), foci of low endemicity are also reported in temperate climates, such as the Mediterranean coast, Eastern Europe, and the southeastern United States (1, 3, 8, 18, 20, 23, 26, 38, 39, 42, 47, 48, 50, 52, 53). Due to its unique autoinfective cycle, Strongyloides stercoralis may persist in the host for indefinite periods (21). Most infected individuals are asymptomatic or may present aspecific and intermittent clinical symptoms and unexplained eosinophilia (14, 38, 43). However, in cases of immunosuppression, strongyloidiasis may become a disseminated, life-threatening disease (7, 16, 29, 30, 54). Unfortunately, its diagnosis remains a challenge. Given the irregular and often low larval output in uncomplicated infections, the sensitivity of direct stool examination is very poor (12, 27, 59). Furthermore, currently available antiparasitic drugs do not always eradicate the infection (27). Consequently, the risk of failing to diagnose the disease is compounded by the difficult determination of treatment efficacy. Thus, an enzyme-linked immunosorbent assay (ELISA) and an indirect immunofluorescence antibody test (IFAT) have been developed, with a wide range of reported sensitivities and specificities (19, 40, 55, 56, 57, 58, 60). Assessing the accuracies of serological assays has been limited primarily by the absence of a gold standard. Therefore, the utility of immunoadsasses both for diagnosis and for follow-up remains controversial (38, 43, 57).

The aim of the present study was to assess the sensitivity and specificity of an IFAT for S. stercoralis at different antibody titers in order to determine the best cutoff both for a clinical decision and for enrollment in clinical trials. In addition, to test the usefulness of serology as a monitoring tool, changes in specific-antibody titers were measured after treatment in a group of 155 patients over a 4-month follow-up period.

MATERIALS AND METHODS

Samples used to assess diagnostic sensitivity. Sera were collected from 156 patients in whose stool specimens S. stercoralis larvae had been detected.

Samples used to assess diagnostic specificity. Negative control sera were obtained from a group of 427 subjects, comprising 229 healthy Italian blood donors and 198 patients admitted to the hospital in medical and pediatric departments for any reason. None of these subjects had a history of travel to high-risk areas or any other known epidemiologic risk factor for S. stercoralis infection. None of them had an eosinophil count of more than 300/µl or complained of any cutaneous, gastrointestinal, or respiratory symptom indicative of strongyloidiasis.

Samples used to assess cross-reactivity. The sera used to evaluate cross-reactions were obtained from 41 patients with the following other parasitologically proven helmint infections: filariasis (11 cases), schistosomiasis (9 cases), hookworm infections (7 cases), toxocariasis (5 cases), ascariasis (5 cases), trichuriasis (3 cases), and Strongyloides fuelleborni infection (1 case). All of them had multiple (at least five) stool specimens negative for S. stercoralis upon direct examination.

IFAT for S. stercoralis. Immunoglobulin G antibodies against S. stercoralis were detected by an IFAT developed and validated at the Centre for Tropical Diseases, Sacro Cuore Hospital, Negrar, Verona, Italy. Briefly, for antigen prepa-
ration, intact S. stercoralis filariform larvae were obtained from a positive charcoal fecal culture (15). By using the Baermann technique (5), all the liquid containing the vital larvae was collected and centrifuged at 4°C. The sediment was washed five times with phosphate-buffered saline (PBS), pH 7.2, at 4°C. It was then exposed to acetone for 30 min at 4°C and washed again five times with PBS. Ten reaction fields of 8 mm in diameter, with waterproof surroundings, were prepared on a microscope glass coverslip (thickness, 0.13 to 0.16 mm; 24 by 60 mm; Menzel-Glaser). The material containing the intact filariform larvae was then applied to the reaction fields, with at least 10 larvae/field. Each reaction field was covered with a 12-μm-pore-size filter of the same diameter. Twenty-five microliters of diluted serum sample was applied as an overlay onto the filter of each field. Negative and positive reference control serum samples of 25 μl at a 1/20 dilution were added. The coverslip was then incubated in a moist chamber at 37°C for 30 min. After the coverslips were rinsed initially for 15 min and then for 10 min with PBS, 25 μl of 50-times-diluted fluorescein isothiocyanate-conjugated anti-human immunoglobulin G (bioMérieux Italia SpA, Rome, Italy) containing 0.2% Evans blue (bioMérieux Italia SpA) was added to each field. The coverslip was again incubated at 37°C for 30 min. After being washed twice in PBS for 10 min, the specimens were embedded in Fluoroprep (bioMérieux Italia SpA) and applied upside-down onto a microscope slide. The light from the bound, fluorescein-conjugated antibodies was detected by an experienced microbiologist, blinded to clinical data, through a Leitz Laborlux S fluorescence microscope with a 20× objective. A positive reaction was indicated by the defined and complete fluorescence of larvae on the slide. Results were expressed as the greatest dilution in serum that gave a positive result.

Patients. Patients with a diagnosis of strongyloidiasis from October 1993 to March 2005 were identified from the database of the Centre for Tropical Diseases. They were included in the analysis if they met at least one of the following criteria: a positive microscopic stool examination, a positive fecal culture, and/or a positive S. stercoralis IFAT result with an antibody dilution of ≥1/20. Demographic and clinical data, including history of travel or immigration, geographical areas of long- or short-term residence, and presence or absence of eosinophilia (defined as an eosinophil count of ≥300/μl), and clinical signs and symptoms, were reviewed.

Treatment regimens. All patients received one of the two alternative treatment regimens considered to be the most effective for strongyloidiasis at the time of their admission: thiabendazole at 25 mg/kg of body weight twice a day orally for 2 days (74 patients) or ivermectin at 200 μg/kg orally in a single dose (81 patients).

Assessment of therapeutic efficacy. Patients were considered to be responsive to treatment if their positive baseline IFAT result reverted to negative and/or showed a decrease in antibody titer of twofold or more within 4 months of the initial treatment. For subjects with positive direct stool examinations (and/or cultures) upon recruitment, negative results after treatment were also mandatory as a criterion for cure.

Statistical analysis. Diagnostic accuracy was assessed as the area under the receiver-operating characteristic (ROC) plot. Diagnosis obtained by direct stool examination was the external gold standard. Sensitivity, specificity, and positive and negative likelihood ratios were calculated for the 1:20, 1:40, 1:80, and 1:160 serum antibody titer (31, 63).

For the population of patients treated for strongyloidiasis, baseline variables potentially associated with the serological response to treatment were evaluated. The distribution of continuous variables was reported as the median and the interquartile range (25th to 75th percentiles). Categorical variables were presented as numbers and percentages. The comparison between the responsive and nonresponsive groups was carried out using Student’s t test or the Mann-Whitney U test for continuous variables. Qualitative data were compared by using the chi-square test with Yates’ corrections and the Fisher exact test when necessary. The binary logistic regression model was used to assess the predictors of serological response to pharmacological treatment. A value of P of less than 0.05 was considered significant.

RESULTS

Decision cutoff. The complete spectrum of sensitivity and specificity combinations for the different serum antibody titers in the IFAT for S. stercoralis was represented by the ROC plot (Fig. 1). The area under the ROC plot of 0.98 (95% confidence interval, 0.97 to 0.99; P = 0.000001) showed the high diagnostic accuracy of this immunoassay. An antibody titer of ≥1/20, which represents the serum antibody titer closest to the upper left corner of the ROC plot, maximized the sensitivity-specificity index (Fig. 1). The estimated diagnostic accuracy of IFAT at different serum antibody titer is reported in Table 1. The best overall performance of IFAT was found at a titer of ≥1/20, with 97% sensitivity and 98% specificity. At a titer of ≥1/80, the test showed the maximum level of specificity, 99.8%, still with an acceptable level of sensitivity (71%). Among the group of 41 patients with other known helminth infections, only one positive IFAT result was observed, at an
TABLE 1. Estimated diagnostic accuracy of Strongyloides IFAT at different serum antibody titer

| Titer | Sensitivity | Specificity | Positive likelihood ratio | Negative likelihood ratio | No. of false-positive results | No. of false-negative results |
|-------|-------------|-------------|---------------------------|---------------------------|------------------------------|------------------------------|
| ≥1:20 | 97.4        | 97.9        | 46.2                      | 0.03                      | 9                            | 4                            |
| ≥1:40 | 88.5        | 98.8        | 75.6                      | 0.12                      | 5                            | 18                           |
| ≥1:80 | 70.5        | 99.8        | 301.1                     | 0.30                      | 1                            | 46                           |
| ≥1:160 | 48.1       | 100.0        | 0.52                      | 0                         | 8                            | 8                            |

TABLE 2. Main characteristics of the study population (155 patients)

| Variable            | No. (%) of patients |
|---------------------|---------------------|
| History of travel   |                     |
| Yes                 | 99 (63.9)           |
| No                  | 56 (36.1)           |
| Area of travel      |                     |
| Africa              | 60 (60.9)           |
| Asia                | 20 (20.0)           |
| South America       | 16 (16.4)           |
| Europe              | 3 (2.7)             |
| Clinical manifestation|                      |
| Asymptomatic        | 29 (18.7)           |
| Symptomatic         | 126 (81.3)          |
| Pruritus            | 74 (58.7)           |
| Abdominal pain      | 61 (48.4)           |
| Skin rash           | 31 (24.6)           |
| Respiratory symptoms| 18 (14.3)           |
| Diarrhea            | 12 (9.5)            |
| Weight loss         | 8 (6.3)             |
| Styphilis           | 7 (5.6)             |
| Nausea              | 6 (4.8)             |
| Vomiting            | 4 (3.2)             |

a The percentages were calculated for the 99 patients with a history of travel.
b The percentages were calculated for the 126 patients with symptoms.

tive to treatment, with either seroreversion (36%) or a decrease in antibody titers of twofold or more (24%). Among the nonresponsive patients, 23% of the subjects had unvaried or increased titers and 17% had a reduction by one titer (i.e., a reduction by one consecutive serum dilution).

Response to treatment was directly correlated to the baseline antibody titer (Spearman correlation, 0.25; \( P = 0.002 \)). Among the group of patients with an initial titer of ≥1:80, 54/74 (73%) patients met the criterion for cure. In contrast, among the group of patients with an initial titer of <1:80, the eradication rate was 39/82 (48%; \( P = 0.002 \)).

The binary logistic regression model identified a baseline titer of ≥1:80 as the best predictor of the response to treatment (\( P = 0.001 \)) (Table 3).

**DISCUSSION**

Recent reports of several cases of fatal disseminated strongyloidiasis in developed countries highlight the problem of a disease which is underestimated due both to the low level of clinical suspicion and to the insensitivity of direct diagnostic tools (4, 6, 11, 12, 24, 34, 35, 44, 45, 49, 51, 59).

The low sensitivity of conventional stool examination may well explain the underestimation of the disease prevalence on the one hand and the wide range of reported drug efficacies on the other.

Therefore, more-sensitive immunodiagnostic techniques have been analyzed. Most studies have evaluated the accuracy of ELISA, with reported sensitivities ranging from 80% to 97% and specificities ranging from 29% to 99% (9, 13, 17, 26, 33, 36, 55, 58), while little data are available on the IFAT (22, 33). The IFAT is technically more complex than ELISA and requires skillful personnel both for antigen preparation and for reading.

Compared to ELISA, the IFAT has the advantage of giving a quantitative result through a precise determination of the specific-antibody titer. This is particularly useful for follow-up. Recent studies reported a decrease in the antibody titer after successful treatment (28, 32, 38, 43, 46). As suggested by several authors, the optical density from an ELISA may be used as a proxy for the antibody titer (32, 38, 43), but the IFAT gives an objective determination of the titer through real serum antibody dilutions.

Our results show that both the sensitivity and the specificity
of the IFAT are very high and that the cross-reactivity rate, in subjects with other nematode infections, is negligible.

The ROC plot (Fig. 1) supported evidence of the high accuracy of the IFAT. The best overall performance of the IFAT was found at a titer of $\geq 1:20$. At a titer of $\geq 1:80$, the test showed the maximum level of specificity, still with an acceptable level of sensitivity.

In agreement with those from previous studies (38, 56), these results show that the immunoassay has much higher sensitivity, even at higher titers, than direct smear or agar plate culture.

Because in clinical practice the optimal serological cutoff for screening for a potentially fatal disease should correspond to the highest sensitivity, a positive titer of $\geq 1:20$ was found to be the best cutoff for screening for this infection. All subjects with a positive IFAT result at this dilution should be treated, regardless of the results of direct tests.

In contrast, the case definition for enrollment in a clinical trial should correspond to the highest specificity, as false positives could give rise to a misleading interpretation of the results, with underestimation of the drug efficacy (12). A positive IFAT result at a dilution of $\geq 1:80$, virtually 100% specific, with acceptable sensitivity, was identified as the optimal cutoff for enrollment in clinical trials.

A limited number of studies have evaluated the use of serology as a tool for monitoring therapeutic efficacy. Most (28, 32, 37, 38, 43, 46) reported a decline in antibody titer within 6 to 12 months. However, results are heterogeneous and difficult to compare due to variable and nonstandardized laboratory techniques and follow-up timings (26).

In the present study, after a $\geq 4$-month follow-up, seroreversion or a decrease in antibody titer of twofold or more was observed in 60% of patients. These results provide further evidence that serology is a useful tool to assess treatment efficacy (28, 32, 36, 43, 46).

However, this response rate was lower than expected. Previous studies with the same drug regimens (2, 10, 14, 25, 41, 43, 49–52) reported eradication rates ranging from 85% to 100%. Given the absence of a reference diagnostic strategy, these studies employed a variety of follow-up protocols. Moreover, all randomized trials used insensitive stool examination techniques to assess therapeutic efficacy, with a consequent overestimation of the anthelmintic effects of the drugs (2, 10, 14, 41, 61, 62).

A direct correlation between the diagnostic titer and the response to treatment as defined above was found. The eradication rate among the group of patients with baseline titers of $\geq 1:80$ was significantly higher than that achieved among the group of patients with diagnostic titers of $<1:80$ (73% versus 48%; $P = 0.002$). This difference may be explained by the suboptimal specificity of serology at lower titers: the inclusion of patients with false-positive results upon recruitment could account for an underestimation of therapeutic efficacy. A baseline serum antibody titer of $\geq 1:80$ was the best predictor of response to pharmacological treatment.

A limitation of this study is the scarce availability of the *Strongyloides* IFAT, currently used only by research laboratories. Our promising results suggest the need for a wider dissemination of this serological assay, both as a diagnostic and as a monitoring tool.

However, more studies are needed in order to identify the most appropriate follow-up timing. In conclusion, the IFAT is a sensible and specific diagnostic tool for strongyloidiasis. A titer of $\geq 1:20$ is the optimal cutoff for diagnosis. A titer of $\geq 1:80$ is a reliable cutoff for a serological assessment of treatment efficacy and for inclusion in clinical trials.

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