

DIAGNOSIS OF HUMAN STRONGYLOIDIASIS BY IMMUNOFLUORESCENCE, USING *STRONGYLOIDES RATTI* AND *S. STERCORALIS* LARVAE

DAVID I. GROVE AND A. JANE BLAIR

Department of Medicine, University of Western Australia, and the Repatriation General Hospital,
Nedlands, Western Australia 6009

Abstract. The sensitivity and specificity of an indirect immunofluorescent antibody assay for the diagnosis of human strongyloidiasis has been investigated. Sera were obtained from 160 Australian ex-servicemen who had been prisoners-of-war in Southeast Asia during World War II, 44 of whom were proven parasitologically to have strongyloidiasis; these men did not have concurrent infections with other helminths. In addition, sera were collected from 44 age- and sex-matched Australians who were not thought to have been exposed to *S. stercoralis*, and from 44 Filipino subjects. Antibodies were measured by using living filariform *S. ratti* larvae as the source of antigen. The assay was highly sensitive; antibodies were found at a titer of 1:4 or greater in 98% of men with strongyloidiasis and in 2% of Australian control subjects. Fifteen percent of exposed ex-servicemen in whom parasites had not been found had antibody titers of 1:4 or more, and it is thought that they had cryptic infections. Incubation of pooled positive sera with soluble *S. ratti* antigen produced a marked fall in antibody titer, but no changes were seen after incubation with soluble *Ascaris suum* or *Dirofilaria immitis* antigens. It is thought that this indicates that the test is specific and that the 84% of Filipinos with antibody titers of 1:4 or greater had unsuspected strongyloidiasis. When antibody titers against *S. ratti* were compared with those obtained using living filariform *S. stercoralis* larvae, a high correlation was found ($r = 0.89$, $P < 0.001$). It is concluded that this assay provides a simple, safe, and specific method for the diagnosis of strongyloidiasis.

A diagnosis of strongyloidiasis is made definitively when larvae are found in the stools, in duodenal fluid, or occasionally, in other tissues or fluids of infected persons. Unfortunately, parasitological confirmation of the presence of a suspected infection may be extremely difficult as worms are frequently absent from such specimens or are present only in very small numbers.¹⁻⁴ In these circumstances, repeated examination of the feces by a well-trained and motivated technician appears to be the best diagnostic procedure,⁴ but even so, these attempts may still fail to detect the parasite. The development of a reliable diagnostic test would be of considerable advantage as it would obviate the necessity for unpleasant and time-consuming fecal examinations. This study describes the sensitivity and specificity of an immunofluorescent assay for *Strongyloides* antibodies, using *S. ratti* and *S. stercoralis* antigens, in parasitologically-defined persons. Finally, the prevalence of antibodies in a tropical population in which strongyloidiasis was not suspected is reported.

MATERIALS AND METHODS

Patients

Sera were collected from 160 Australian ex-servicemen who had been prisoners-of-war in Southeast Asia during the Second World War. The clinical characteristics and parasitological aspects of these men have been described previously;⁴ a diagnosis of strongyloidiasis was confirmed in 44 persons and suspected, but not proven despite repeated testing, in a small proportion of the remainder. Sera were obtained from 44 age-matched Australian veterans who had not served overseas and who had not lived in the northern parts of Australia or elsewhere in the tropics. Finally, sera from 44 Filipino subjects were tested; they were not age-matched but all were adults, and 91% were males. These persons have been described elsewhere;⁵ 61% had filariasis. Furthermore, an earlier study had shown that 68% of similar persons living in the same area had *Ascaris lumbricoides* infections and 56% were infected with *Trichuris trichiura*.⁶ Hookworm eggs were seen in the feces of approximately 15% of subjects, but this is probably a gross underestimate as the Kato technique was employed; this is

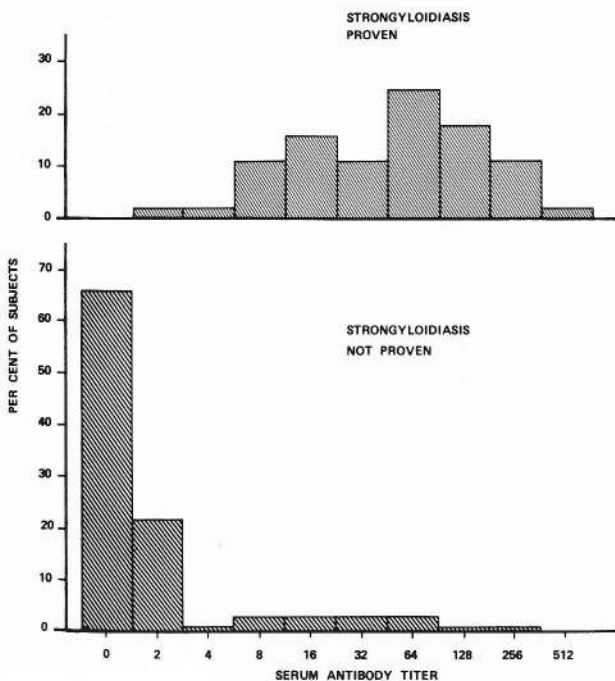


FIGURE 1. Serum fluorescent antibody titers in 44 Australian ex-prisoners-of-war in whom strongyloidiasis was proven parasitologically, and in 116 persons in whom worms were not found. The percentage of subjects is plotted against the reciprocal of the final serum dilution giving a positive reaction. A value of 0 represents no reaction with serum diluted 1:2, while a value of 2 represents a positive reaction with serum diluted 1:2.

an insensitive method for detecting this parasite. *S. stercoralis* was not specifically looked for and was not seen.

Strongyloides ratti

A homogenic strain of *S. ratti* was maintained by serial passage in Sprague-Dawley rats. Techniques for the isolation of filariform larvae have been described earlier.⁷

Strongyloides stercoralis

The parasite was obtained from the feces of one of the patients described above. Moistened feces were kept for 2 weeks at room temperature on a watch glass in a petri dish containing water. Filariform larvae were recovered from the water, washed several times in phosphate buffered saline (PBS) then a 7-week-old dog was infected percutaneously with 2,000 larvae. Worms appeared in the stools 3 weeks after infection and persisted thereafter. Larvae used in the antibody assay were obtained from feces of this animal.

Antibody assays

Preliminary studies compared fluorescence using heat-killed, formalin-killed, and living infective larvae of *S. ratti*. The clearest and most consistent fluorescence was seen with living worms, so fresh preparations of these larvae were employed routinely. Sera were diluted serially with PBS in 3-ml plastic tubes (Disposable Products, Ridleyton, South Australia). Approximately 50 filariform larvae in 200 μ l of PBS were added to each 200 μ l of serum dilution, then the tubes were incubated at 37°C for 1 hour. Larvae were washed three times by suspension in PBS and centrifugation at 200 \times g for 5 min at room temperature. Fluoresceinated sheep-anti-human globulin (Wellcome Laboratories, Beckenham, England) was diluted 20 times in PBS and 50 μ l was added to each tube. The suspension was incubated for a further hour at 37°C, then the worms were washed three times in PBS. The tubes were placed on an ice bath in order to inhibit movement of larvae, then the worms were transferred to glass slides and coverslips were added. A Zeiss fluores-

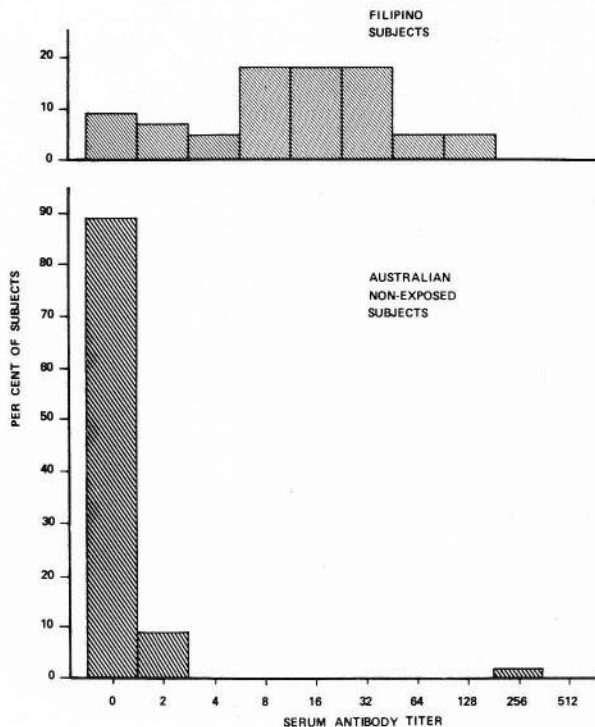


FIGURE 2. Serum fluorescent antibody titers in 44 Australian control subjects who had not been exposed to *S. stercoralis*, and in 44 Filipino subjects.

cent microscope with fluorescein-selective filter system 488710 was used. A larva was considered to show positive fluorescence when the cuticle fluoresced distinctly and completely on $400\times$ magnification. A minimum of six larvae were observed at each serum dilution; a positive result was recorded when at least two-thirds of larvae demonstrated such fluorescence. Negative and positive control sera were included in each test.

The same protocol was used initially for infective larvae of *S. stercoralis*, but very little fluorescence was seen. These larvae were noticed to be excessively motile when incubated at 37°C in comparison with those of *S. ratti*. It was thought that enhanced mechanical and metabolic activity may inhibit binding or wash off bound immunoglobulin. When worms were incubated at 8°C , fluorescence similar to that seen with *S. ratti* larvae was observed; this incubation temperature was used thereafter. Control tubes with *S. ratti* larvae and known positive serum were included on each occasion, and gave expected and consistent results.

Soluble antigens

Soluble antigens were prepared from infective larvae of *S. ratti*, adult *Dirofilaria immitis* (kindly supplied by Dr. J. Dunsmore, School of Veterinary Studies, Murdoch University, W.A.), and adult *Ascaris suum* (obtained from the local abattoir). The worms were ground in Pyrex tissue grinders, incubated at 4°C overnight, then centrifuged for 1 hour at $30,000 \times g$. The supernatant was collected and the protein content determined, then adjusted to a concentration of 1 mg/ml.

Antibody titers of pooled positive serum were measured before and after incubation at 37°C for 1 hour with equal volumes of *S. ratti*, *D. immitis* and *A. suum* antigen solutions.

RESULTS

Fluorescent antibody titers against infective larvae of *S. ratti* are shown in Figures 1 and 2. No antibody was detected in 89% of the Australian control subjects, and only one person had a titer

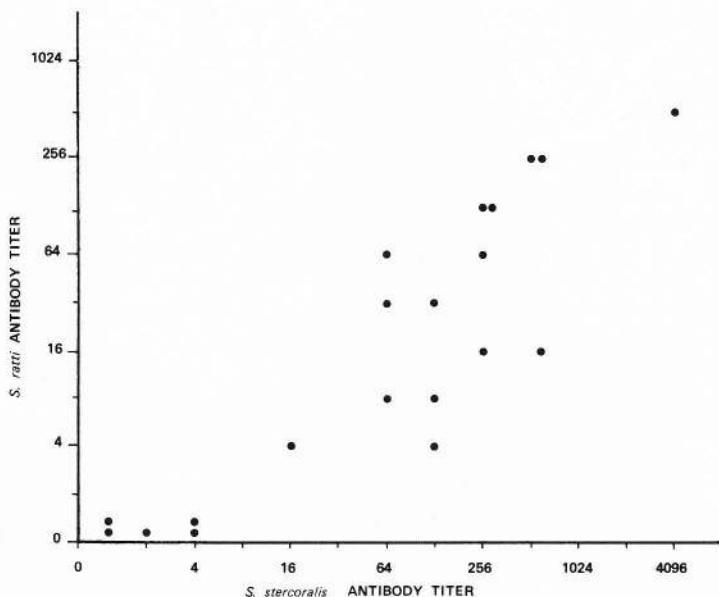


FIGURE 3. Comparison of serum fluorescent antibody titers of 20 selected sera with *S. ratti* or *S. stercoralis* as the source of antigen.

greater than 1:2. Antibody titers in Australian patients with proven strongyloidiasis followed an approximately normal distribution with titers ranging from 1:2 to 1:512. There was minimal overlap between patients and control subjects ($P < 0.001$, Wilcoxon's sum of ranks test).

Fifteen percent of veterans who had been prisoners-of-war, but in whom strongyloidiasis had not been proven parasitologically, had serum antibody titers $\geq 1:4$.

The majority of Filipino subjects tested had significant antibody titers against *S. ratti*. Again, there was an approximately normal distribution of serum antibody levels, but the mean titer (1:15)

was less than that in Australians with proven strongyloidiasis (1:45).

In order to determine whether there may be cross-reactivity between *Strongyloides* antigen and *Ascaris* and filarial antigens, serum was preincubated with antigen extracts of these parasites, then *Strongyloides* antibody titers were determined. There were no changes in levels after incubation with *Ascaris* and filarial antigens, but a dramatic 64-fold fall in titer occurred after incubation with *S. ratti* antigen (Table 1).

When infective larvae of *S. stercoralis* became available, antibody titers of 20 selected sera were measured. There was a highly significant correlation ($r = 0.89$, $P < 0.001$) between antibody levels obtained with *S. stercoralis* and *S. ratti* larvae, although they tended to be slightly higher with the former parasite (Fig. 3).

TABLE 1

Serum antibody titers of pooled immune serum before and after incubation with soluble antigen extracts of *Strongyloides ratti*, *Ascaris suum*, and *Dirofilaria immitis*

Antigen	Serum antibody titer	
	Pre-incubation	Post-incubation
<i>S. ratti</i>	128	2
<i>A. suum</i>	128	128
<i>D. immitis</i>	128	128

DISCUSSION

One of the major limitations in the immunodiagnosis of helminth infections is that the demonstration of antibodies indicates only the presence of infection but provides no information concerning the intensity of that infection. This is important in many helminthiases, as the severity of dis-

ease appears to be proportional to the worm burden. This difficulty does not apply in strongyloidiasis, however, as *S. stercoralis* has the capacity, unusual among worms, to replicate within the human host. This ability accounts not only for the long duration of infection in many persons, but also for the explosive illness which may supervene in immunosuppressed patients.⁹ A reliable serological assay would assist in confirming the diagnosis in symptomatic people, should aid in the identification of asymptomatic, infected persons who are at risk of developing overwhelming strongyloidiasis, and may be a useful parameter for assessment of the response to treatment.

A number of attempts have been made to develop an immunological test for the diagnosis of strongyloidiasis; these have included both serological assays¹⁰⁻¹⁴ and skin tests. There have been two major problems in the development of such assays. The first has resulted from the limited availability of suitable antigens. Indeed, some workers have relied on supposed cross-reactivity with other worms, e.g., filariae.¹³ We have maintained *S. ratti* in rats and this has provided a constant and safe source of antigens which seemed likely to be relevant. Secondly, many patients with strongyloidiasis have concurrent infections with other gastrointestinal helminths and the possibility of cross-reactions has placed doubts on the specificity of the assays. The ex-servicemen we investigated have provided a rare opportunity to assess serological responses in isolated *Strongyloides* infections. Many of these veterans were infected with hookworm, *Ascaris lumbricoides* and *Trichuris trichiura* during the war, but since these helminths cannot multiply, those infections have subsided spontaneously. In contrast, infection with *S. stercoralis* has persisted for at least 35 years.

The present study has clearly shown that when living filariform larvae of *S. ratti* are used, antibodies can be detected with a high degree of sensitivity and specificity using an immunofluorescent technique. Strongyloidiasis is not endemic in southern Australia, and only one of the control persons from this area had a serum antibody titer of $\geq 1:4$. His antibody titer was very high, suggesting that he may truly have been infected with *S. stercoralis*. Unfortunately, we were not able to investigate him intensively by parasitological means. However, he gave a history of having visited Fiji during a cruise in the South Pacific; he may have acquired the infection there as it is not

uncommon for Australian tourists to become infected with this worm after walking barefoot along the beaches in Fiji (W. B. Hennessy; St. Vincent's Hospital, Sydney, personal communication).

In contrast, only one veteran with parasitologically proven strongyloidiasis had a serum antibody titer of $< 1:4$. The reason for this low titer is uncertain, but it is possible that a laboratory error may have led to a false diagnosis of strongyloidiasis, particularly as this individual was asymptomatic. There is no doubt, however, that the veteran with a serum titer of $< 1:4$ was infected, as parasites were found both in 1946 and again recently. Nevertheless, there was very little overlap in the distribution of serum antibody titers in patients with proven strongyloidiasis and in control subjects without strongyloidiasis. In any case, the occasional false negative result is far less frequent than the number of false negatives given by parasitological examinations; 32% of persons with ultimately proven strongyloidiasis were negative on the first occasion, despite a combination of fecal microscopy, fecal culture and microscopy of duodenal fluid.⁴

Fifteen percent of veterans who were prisoners-of-war but in whom parasites were not found had a serum antibody titer of $\geq 1:4$. Since we have shown that the chances of finding larvae are proportional to the number of specimens examined,⁴ it seems likely that these persons have cryptic infections with *S. stercoralis*; indeed one of these men had pathognomonic larva currens.

When this study had been completed, a dog was successfully infected with *S. stercoralis* obtained from one of the veterans. This provided an opportunity to correlate antibody titers measured using this parasite with those found when the closely-related *S. ratti* was employed. There was a highly significant correlation between the two species, thus validating the usefulness of *S. ratti* as a source of antigen in the immunodiagnosis of strongyloidiasis. The antibody titers obtained with *S. stercoralis* were slightly higher than those found when *S. ratti* was employed; this may indicate greater sensitivity when the homologous parasite is used, or may reflect the difference in temperature at which the larvae were incubated.

These studies indicated the sensitivity of the assay but provided no information concerning its specificity. For these reasons, antibodies were sought in the sera of Filipino patients, many of whom were known to have filariasis. An earlier

study had shown that many similar people living in the same region were infected with *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm, but the technique used was inappropriate for estimating the prevalence of strongyloidiasis. When serum antibodies against *Strongyloides* were measured, the distribution was very similar to that seen in patients with known strongyloidiasis, with 84% of Filipinos having titers of $\geq 1:4$. This indicates either that strongyloidiasis is endemic among these people, or that there is cross-reactivity with other gastrointestinal helminths or with filariae.¹³ In order to differentiate between these two possibilities, pooled *Strongyloides* positive sera were preincubated with soluble antigens prepared from *S. ratti*, *A. suum* and *D. immitis*, the latter two being the worms most similar to the human parasites that were available. There was a marked fall in titer after incubation with *Strongyloides* antigen but no change in titer after incubation with *Ascaris* or *Dirofilaria* antigens, thus suggesting that there is no significant cross-reactivity between these parasites in this system. It is possible that humans may recognize metabolic larval antigens not obtained by extraction of adult *A. suum* and *D. immitis*, but it seems unlikely that this would produce false positive reactions since a similar somatic preparation of *S. ratti*, which did not include these products, produced a marked fall in antibody titer to *Strongyloides*.

In conclusion, this study has shown that an immunofluorescent antibody assay done with living filariform larvae of *S. ratti* is sensitive and specific for the diagnosis of human strongyloidiasis. *S. ratti* is easily available, safe to use, and the results correlate well with those found when *S. stercoralis* is employed. This test is simple, does not require the collection of feces or duodenal fluid, and is much less unpleasant than fecal microscopy, a procedure which sometimes needs to be repeated many times.

ACKNOWLEDGMENTS

This study was supported by grants from the Australian Department of Veterans' Affairs and the Rockefeller Foundation.

REFERENCES

- Napier, L. E., 1949. *Strongyloides stercoralis* infection. *J. Trop. Med. Hyg.*, 52: 25-30.
- Beal, C. B., Viens, P., and Grant, R. G., 1970. A new technique for sampling duodenal contents: Demonstration of upper small bowel pathogens. *Am. J. Trop. Med. Hyg.*, 19: 349-352.
- Gill, G. V., and Bell, D. R., 1979. *Strongyloides stercoralis* infection in former Far East prisoners of war. *Br. Med. J.*, 2: 572-574.
- Grove, D. I., 1980. Strongyloidiasis in Allied ex-prisoners of war in Southeast Asia. *Br. Med. J.*, 280: 598-601.
- Grove, D. I., and Forbes, I. J., 1979. Immunosuppression in bancroftian filariasis. *Trans. R. Soc. Trop. Med. Hyg.*, 73: 23-26.
- Grove, D. I., Cabrera, B. D., Valeza, F. S., Guinto, R. S., Ash, L. R., and Warren, K. S., 1977. Sensitivity and specificity of skin reactivity to *Brugia malayi* and *Dirofilaria immitis* antigens in bancroftian and malayan filariasis in the Philippines. *Am. J. Trop. Med. Hyg.*, 26: 220-229.
- Dawkins, H. J. S., Grove, D. I., Dunsmore, J. D., and Mitchell, G. F., 1980. *Strongyloides ratti*: Susceptibility to infection and resistance to reinfection in inbred strains of mice as assessed by excretion of larvae. *Int. J. Parasitol.*, 10: 125-129.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.
- Scowden, E. B., Schaffner, W., and Stone, W. J., 1978. Overwhelming strongyloidiasis; an unappreciated opportunistic infection. *Medicine (Baltimore)*, 57: 527-644.
- Sato, S., 1933. Immunologische studien ueber die *Strongyloides*-Infektion. *Fukuoka Ikwadaigaku Zasshi*, 26: 1526-1585 (in Japanese); 88-89 (German summary).
- Brannon, M. J. C., and Faust, E. C., 1949. Preparation and testing of a specific antigen for diagnosis of human strongyloidiasis. *Am. J. Trop. Med.*, 29: 229-239.
- Coudert, J., Ambroise-Thomas, P., Kien, T. T., and Pothier, M. A., 1968. Diagnostic serologique de l'anguillule humaine par immunofluorescence (resultats preliminaires). *Bull. Soc. Pathol. Exot.*, 61: 74-80.
- Kanani, S. R., and Rees, P. H., 1970. The diagnosis of strongyloidiasis with special reference to the value of the filarial complement fixation test as a screening test. *Trans. R. Soc. Trop. Med. Hyg.*, 64: 246-251.
- Dafalla, A. A., 1972. The indirect fluorescent antibody test for the serodiagnosis of strongyloidiasis. *J. Trop. Med. Hyg.*, 75: 109-111.