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Enzyme-linked Immunosorbent Assay (ELISA) in the Detection of IgG Antibodies in Onchocerciasis Using Blood Collected on Filter Paper

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Introduction

Various types of the serodiagnosis or immunodiagnosis of onchocerciasis and lymphatic filariasis have been developed so far (Ambroise-Thomas, 1980; Tada and Aoki, 1981). The enzyme-linked immunosorbent assay (ELISA) has been found to be simple, sensitive and suitable for the mass screening of most parasitic infections (Voller *et al.*, 1976). The ELISA has been also applied to detect antibodies against *Onchocerca volvulus* (Bartlett *et al.*, 1975; Marcoullis *et al.*, 1978; Speiser and Weiss, 1979; Speiser, 1980; Ambroise-Thomas *et al.*, 1980). The indirect hemagglutination (IHA) test for onchocerciasis has been performed with blood collected on filter paper (Ikeda *et al.*, 1978, 1979). Blood samples for mass examinations in an endemic area are easier to collect on filter paper than by venipuncture. Long *et al.* (1981) reported that seroactivity in blood samples on filter paper was kept even under the field condition by storage using a silica-gel desiccator. Thus, it is important to combine and to develop both methods which are useful epidemiologically.

In the present study we tried to measure IgG antibodies in human blood samples obtained by the filter paper technique with the ELISA using both pathogenic parasite *O. volvulus* and a parasite closely related *O. gutturosa* from cattle. Further, we discussed the usefulness of the ELISA for seroepidemiological analysis.

Materials and Methods

Blood samples and sera

Blood samples were taken on filter paper (Type I, Toyo Roshi, Ltd., Japan) from the ear lobe. A method of storage and extraction of the samples was of Ikeda *et al.* (1978).

Two paired blood samples, one obtained

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on filter paper and the other, sera, by venipuncture, were collected from 130 inhabitants of 2 coffee plantations (fincas), El Regalo and Santa Ines, in an endemic area of Chicaco, Guatemala. Blood samples of the other 245 inhabitants from these two fincas and 82 from Shamatari and Parima in an endemic area of Amazonas, the upper Orinoco, Venezuela, were taken by filter paper only. Clinical data from examinations by skin snip and palpation for nodules by "brigadas" were recorded in Guatemala. Blood samples from 54 inhabitants of a finca in an area nonendemic for onchocerciasis, Coatepeque, Guatemala, also were served as controls in order to evaluate nonspecific cross-reactions against *Onchocerca volvulus* antigens. Control sera were also taken from 9 co-workers of the laboratory in Guatemala City and 3 Japanese, who had not lived in any endemic area and were not suffering from any parasitic diseases.

Standard positive and negative pooled sera were prepared ten selected serum samples obtained from an endemic and a nonendemic area of Guatemala, respectively. The positive sera were selected for their high titers of IHA test against *O. volvulus* antigens as well as microfilaria (mf) positive case in skin snips.

Antigens

Adult *O. volvulus* was enzymatically recovered from the onchocercal nodules of Guatemalan patients using the method of Schulz-Key *et al.* (1977). Adult *O. gutturosa* was dissected out from the connective tissue of the cervical ligaments of the cattle gained in a slaughter house in Escuintla, Guatemala.

Whole worms were homogenized with polytron® (Kinematica, Switzerland) in 0.05 M carbonate bicarbonate buffer (CBB), pH 9.6, containing 0.02% sodium azide. After several times of freezing and thawing, the homogenates were kept in a refrigerator overnight and were then centrifuged at 10,000 *g* for 30 min at 4 C. The supernatant was used as a crude antigen. The concentration of protein was determined by the method of Lowry *et al.* (1951).

ELISA procedures

The procedure was slightly modified from that described by Nakao *et al.* (1981). The flat bottom wells in EIA microtitration plate (Flow Laboratories, Inc., U.S.A.) were sensitized overnight at 4 C with 100 μl of the antigen solution diluted in CBB and used within 2 weeks. Unbound antigen was removed by washing three times the wells with 0.05% (v/v) Tween 20 in saline. 100 μl of blood samples diluted in 0.05% Tween 20 in 0.01 M phosphate buffered saline, pH 7.0 (PBS-T) was added in each well and the plates were incubated at 37 C for 30 min. After washings, 100 μl of the solution of goat antibody to human IgG heavy and light chains bound to horseradish peroxidase (anti IgG-conjugate) (Miles Laboratories, Inc., U.S.A.) diluted in 0.05 M PBS-T was added to each well and the plates were incubated at 37 C for 30 min. Substrate solution containing 0.1 mg of orthophenylendiamine (OPD) per ml and 0.03% of H_2O_2 in 0.05 M acetate acetic acid buffer, pH 4.5, was applied in the amount of 150 μl per well and the plate was incubated at room temperature in the dark. The enzyme reaction was stopped by addition of 50 μl 4 N H_2SO_4. The absorbance was read by a spectrophotometer (Corona MTP-12, Nissey Sangyo, Japan) at 500 nm.

Fecal examination

Fecal samples from 146 persons who lived in endemic areas of Guatemala, and from 43 who lived in a nonendemic area, were examined parasitologically to determine the prevalence of intestinal parasites.

Results

For the determination of antigen concentration, the microplate wells were coated with serial dilutions of antigen solution. They were reacted with serial dilutions of a standard positive serum and of a standard negative serum. Anti human IgG peroxidase conjugate at 1:500 dilution was used in the test and the OD was recorded as shown in Fig. 1. Antigen concentration and the dilution of serum were optimal at 2.5 μg/ml and at 1:200, respectively.

For the determination of conjugate concentration, the microplate wells were coated with the antigen solution at 2.5 μg/ml and were reacted with several dilutions of the standard positive and negative sera. The plate was then reacted with serial dilutions of the conjugate. In Fig. 2, the results showed that the dilution of the conjugate at 1:500 was proper.

For the determination of the peroxidase reaction time, the microplate wells were sensitized with antigen at 2.5 μg/ml and were reacted with the standard positive and negative sera at 1:200 and 1:1000 followed by the conjugate at 1:500. The reaction was stopped at various time intervals after OPD-substrate was added. The reaction preceded rapidly in the initial 5 min and then gradually until 30 min at room temperature (Fig. 3). The peroxidase reaction time of 30 min was considered appropriate in the following experiments.

The standard condition for the detection of IgG antibodies against *O. gutturosa* was determined as above. The results of the checkerboard titration showed that the concentration of the antigens and the test serum were optimal at 2.5 μg/ml and 1:200, respectively.

Paired blood samples taken on filter paper and by venipuncture from inhabit-

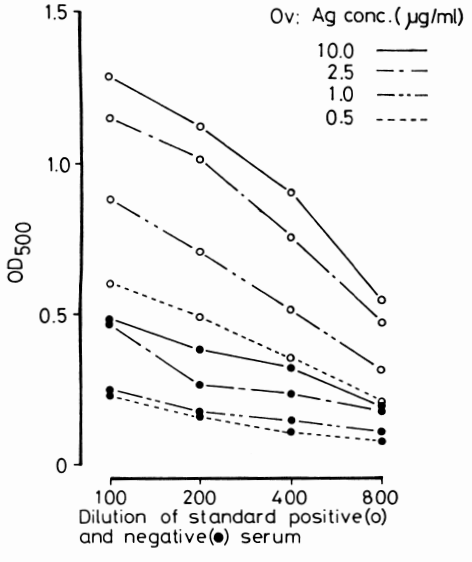

Fig. 1 Effect of antigen concentration on the ELISA values developed by serial dilutions of the standard positive and the standard negative serum.

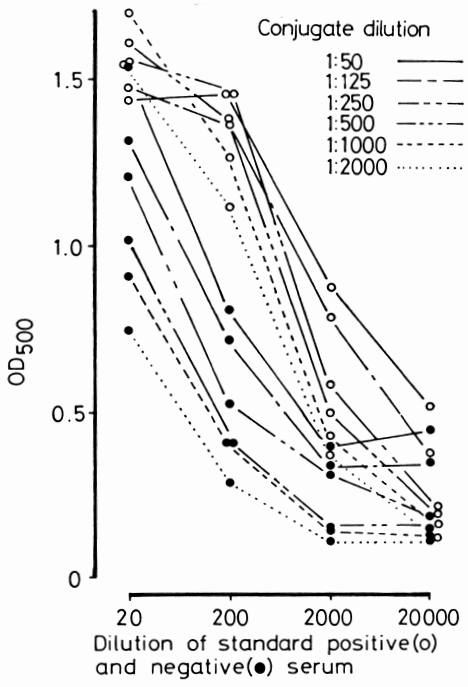

Fig. 2 Effect of conjugate concentration on the ELISA values developed by serial dilutions of the standard positive and the negative serum.

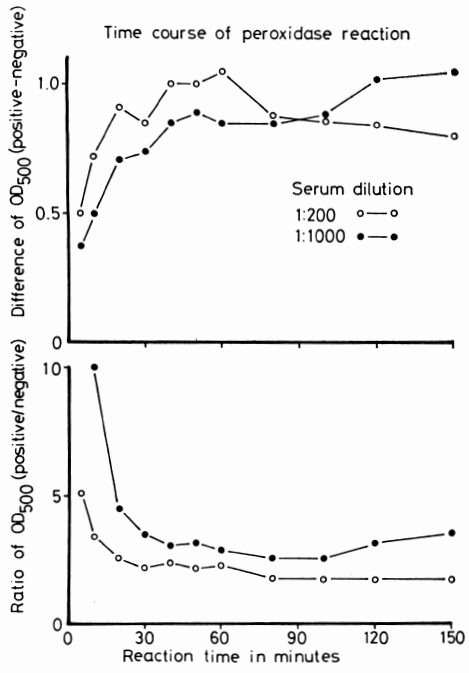

Fig. 3 Time course of the peroxidase reaction in the ELISA.

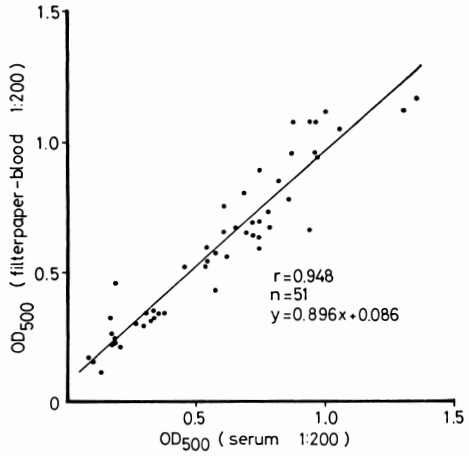

Fig. 4 Comparison of the ELISA values obtained with serum collected by venipuncture and blood collected on filter paper.

ants of the endemic areas were examined using *O. volvulus* antigens. The results shown in Fig. 4 reveal a close correlation

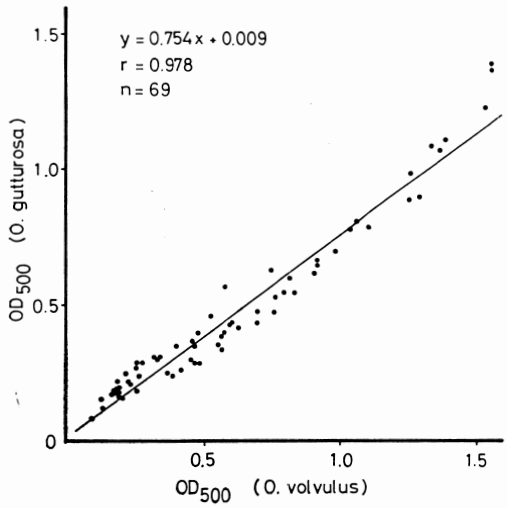

Fig. 5 Comparison of the ELISA values obtained with *O. volvulus* and *O. gutturosa* antigens using blood collected on filter paper.

(r=0.948, n=51) between 2 paired samples from identical subjects.

Blood samples taken on filter paper from 69 inhabitants of the endemic areas were tested by ELISA with *O. volvulus* or *O. gutturosa* as antigen in order to compare the antigenic similarity between two. The results are shown in Fig. 5. The data reveal a high correlation between the results from two different antigens, homologous and heterologous, in the detection of IgG antibodies (r=0.978).

Blood samples taken on filter paper from inhabitants of the endemic or nonendemic area in Guatemala and Venezuela were tested by ELISA with *O. volvulus* antigens. The distribution of the ELISA values of the five surveyed areas was plotted (Fig. 6). The mean ELISA value of a population closely correlates the corresponding mf rate.

To evaluate cross reactivity against other helminthic parasites, we examined fecal samples from persons in each locality in the endemic and the nonendemic areas. As shown in Table 1, no significant statistical difference in the prevalence of

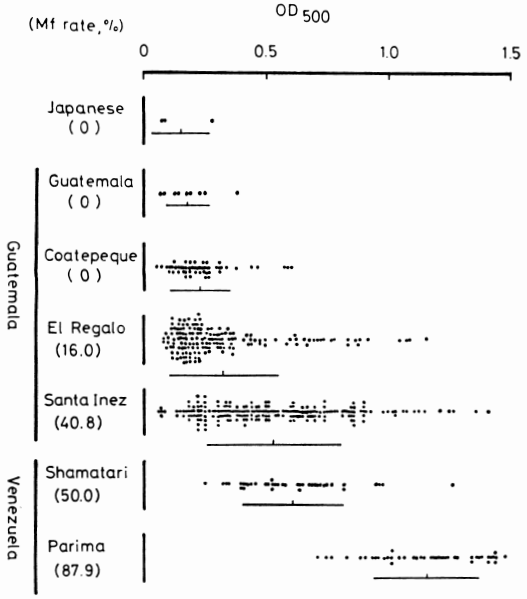

Fig. 6 The distribution of the ELISA values of blood samples taken on filter paper from inhabitants of the endemic and the nonendemic areas in Guatemala and Venezuela. Horizontal line represents mean ± SD.

several intestinal parasites was observed between individuals whose ELISA values were $\geqq 0.48$ (mean ELISA value of blood samples from nonendemic area+2 SD) or <0.48 in the endemic area, nor between the two area ($P>0.75$, except for *T. trichiura* infection between the two areas). This result suggests that the specificity of the ELISA for onchocerciasis is satisfactory.

The ELISA values of males who lived in either moderate or low endemic area are higher than those of females, and the mean ELISA value in males and females appeared to increase with age (Figs. 7 and 8). In comparison with El Regalo, a low endemic area, many of younger generation (0–19 years old) in Santa Ines, a moderate endemic area, had the high ELISA value. The mean ELISA values of females after 30 years old in Santa Ines showed as high as those of males of the same generation.

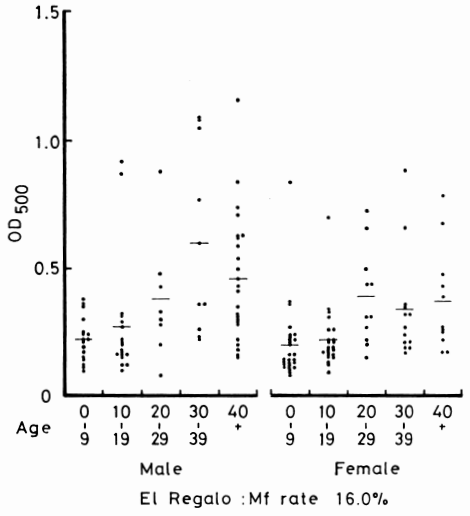

Fig. 7 The distribution of the ELISA values by age and sex of the inhabitants in low endemic area, El Regalo, Guatemala. Horizontal line represents mean.

Table 1 Prevalence of intestinal parasites among inhabitants of fincas in endemic and nonendemic areas in Guatemala

Parasites	Endemic area			Nonendemic area		
	POSITIVE*	NEGATIVE	Total	POSITIVE	NEGATIVE	Toatl
Ascaris lumbricoides	47 (68.1%) †	55 (71.4%)	102 (69.9%)	3 (100%)	20 (50%)	23 (53.5%)
Trichuris trichiura	49 (71.0%)	48 (62.3%)	97 (66.4%)	2 (66.7%)	15 (37.5%)	17 (39.5%)
Hookworm	34 (49.3%)	31 (40.3%)	65 (44.5%)	2 (66.7)	17 (42.5%)	19 (44.2%)
Negative	7 (10.1%)	5 (6.5%)	12 (8.2%)	0 (0%)	5 (12.5%)	5 (11.6%)
No. examined	69	77	146	3	40	43

* Case in which ELISA value was $\geqq 0.48$

† Prevalence rate

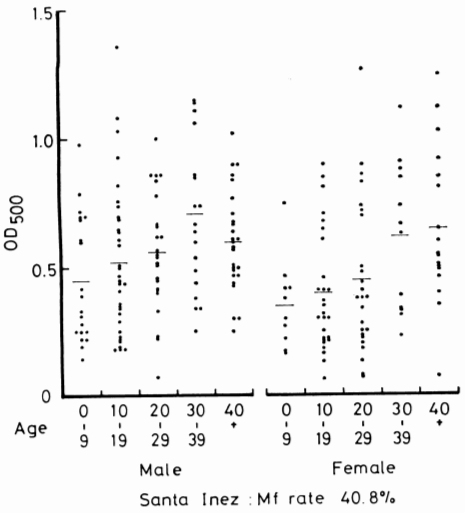

Fig. 8 The distribution of the ELISA values by age and sex of the inhabitants in medium endemic area, Santa Ines, Guatemala. Horizontal line represents mean.

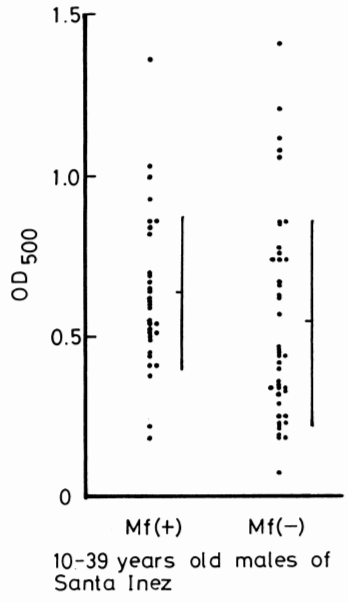

Fig. 9 The distribution of the ELISA values in 10–39 years old male subjects with or without microfilaria by skin biopsy in endemic area, Santa Ines, Guatemala. Vertical line represents mean ± SD.

To compare the distribution of the ELISA values between mf positive group and mf negative group under the same condition, 75 male inhabitants with 10–39 years of age of Santa Ines were chosen. The mean ELISA value (±SD) was 0.63 (±0.24) in mf positive group, and 0.54 (±0.32) in mf negative group. In 18 cases out of 43 in mf negative group, the ELISA values were higher than the mean ELISA value of blood samples from nonendemic area +2 SD (0.48) (Fig. 9).

Discussion

Collecting blood by filter paper is convenient and safe for epidemiological surveys (Ikeda *et al.*, 1978). In the present study, we evaluated the usefulness of the filter paper method in collecting blood for the serodiagnosis of human onchocerciasis by ELISA. The results showed the presence of higher correlation between blood taken on filter paper and serum in ELISA values. It has become difficult to obtain *O. volvulus* as antigen from human onchocercal nodules. The ELISA requires only a smaller amount of the antigen than other immunologic procedures such as IHA test in spite of its specificity in seroepidemiology (Ikeda *et al.*, 1979). The ELISA should be recommended for this point of view.

Various homologous and/or heterologous worm antigens have been used for serodiagnosis and seroepidemiology of onchocerciasis (Tada and Aoki, 1981). Our results also reveal that *O. gutturosa* antigens can be utilized for the ELISA using blood samples taken on filter paper instead of *O. volvulus* antigens. This coincides with the results reported previously (Bartlett *et al.*, 1975; Kamiya *et al.*, 1982). Soluble extract of an other filarial worm, *Dipetalonema viteae,* was found to be potent antigens in the ELISA for the detection of IgG antibodies (Speiser, 1980). We did not observe that *O. volvulus* antigens gave high ELISA values both with the negative control sera and with the diluent (Bartlett

et al., 1975). This might be due to difference between their and our preparation methods of the worm antigens from the onchocercal nodules.

We found that all of the examined inhabitants of Parima, tribe Yanomami, Venezuela, had markedly high ELISA values and mf rate. In Shamatari, mf rate was medium and the mean ELISA value ± SD was 0.61±0.21. Many inhabitants of Parima had high mf density in skin snip. It is difficult to analyze them epidemiologically on a level with surveys done in Guatemala, because of their unknown age. However, it was assumed from the pattern of the distribution of the ELISA values that active transmission occurred near the housing area.

The mean ELISA value of Coatepeque, nonendemic area, was slightly higher than those of laboratory control and Japanese control. This suggests that *O. volvulus* antigen(s) used in the ELISA might be slightly cross-reactive to the other helminths. Actually, intestinal parasitic infections were endemic there. Although history of three inhabitants who had higher ELISA values (>0.48) is unknown, it is likely that in the past they had been exposed to *O. volvulus* infections in endemic areas. When a positive reaction was chosen as in which the OD was >2 SD above the mean OD of nonendemic control sera, the false positive rate was 3.7%. This and the result shown in Table I indicate that the ELISA is as specific as the IHA test reported by Ikeda *et al.* (1978, 1979).

The parallel correlation of the mean ELISA value of a community with mf positive rate is analogous to the relation between the IHA positive rates and the mf positive rates (Ikeda *et al.*, 1979). The level of antibodies had a tendency to increase with the age. These suggest that the increase of the level of antibodies might be due to that in an exposure to the infection. There are differences in the age where positives appear and in the pattern of the distribution in the ELISA values by age according to the endemicity of both areas (Figs. 8 and 9). These findings are coincident with those of Ikeda *et al.* (1979).

In spite of mf negative cases, there were many positive ones by the ELISA in the endemic area. Yoshimura *et al.* (1982) calculated that 55% of patients with mean mf densities between 0.1 and 4.9/10 mm^2 would be diagnosed as negative, and 33% of those with mean mf densities of 5.0 to 9.9 would also be diagnosed as negative. Furthermore, Ikeda *et al.* (1979) reported considerable reversion from snip-negative to snip-positive by a follow-up study made 6–7 months after the first examination among IHA positives. Thus, those who were positive in the ELISA and negative for mf might be already sensitized with developing parasites but still in prepatency or had only a few mf at the time of the examination. On the other hand, there were the cases which were negative in the ELISA and positive for mf. In our system we could not detect antibodies besides IgG class. It is possible that only other classes of antibodies were produced in such cases. Since the role of individual Ig class in a infection with *O. volvulus* has been unclear so far, anti-human immunoglobulin conjugate which has wide spectrum might be used in the first step of the epidemiological survey.

Summary

The enzyme-linked immunosorbent assay (ELISA) for onchocerciasis was performed with a crude saline extract of adult *Onchocerca volvulus* and 2 types of blood samples from inhabitants in an endemic and a nonendemic areas in Guatemala and Venezuela. The ELISA values of blood samples taken on filter paper and of sera

obtained by venipuncture showed a high correlation (r=0.948). IgG antibodies in these human blood samples were measured with the ELISA using *O. volvulus* and *O. gutturosa* antigens. It was shown that *O. gutturosa* antigens were as potent with the ELISA as *O. volvulus* antigens. The results from five surveyed areas revealed that the mean ELISA value of a community closely correlated the corresponding microfilarial (mf) rate. A sex-related difference was evident in the ELISA values in the subjects from moderate and low endemic areas. The mean ELISA values of a population were age-dependent. Many subjects in mf negative subjects from endemic areas had high ELISA values. These false positive cases were thought to be due mainly to low sensitivity of skin biopsy and/or to early infection. These results suggest that the specificity and sensitivity of the ELISA for onchocerciasis are satisfactory.

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濾紙法によって採血したオンコセルカ症患者血液中 IgG 抗体の ELISA 法による検出

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Onchocerca volvulus 成虫生食水抽出抗原を用いた ELISA 法でグァテマラ及びベネズエラの流行地，非流行地住民の抗体価を測定した．濾紙採血法による血液材料と静脈採血によって得た血清の ELISA 価との間には非常に高い相関があった（r=0.948）．濾紙採血法による血液材料中の IgG 抗体価が *O. volvulus* と *O. gutturosa* を抗原として ELISA 法で測定された．その結果，*O. gutturosa* で得た ELISA 価と *O. volvulus* で得た価との間に強い相関が認められた．調査対象となった5つの部落の各々の平均 ELISA 価とそのマイクロフィラリア（mf）陽性率との間には関連性があった．中程度以下の浸淫地において ELISA 価には男女差があり，男性の ELISA 価の方が女性のそれよりも高い傾向があった．また年齢ごとの集団でその平均 ELISA 価を調べると，年齢が増すほど平均 ELISA 価は大きくなった．流行地の男性住民 10～39 歳のうち，mf 陰性にもかかわらず，高い ELISA 価を示す者が多数いた．この原因として，検皮法の mf 検出感度の低さ，あるいはこれら偽陽性患者がまだ感染初期にあることなどが考えられた．本研究の結果から，*O. volvulus* 成虫抗原と濾紙採血した血液材料を用いた ELISA 法の特異性と感度は十分に高いということが示唆された．