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Title	ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) AS A DIAGNOSTIC TOOL FOR GUATEMALAN ONCHOCERCIASIS USING A BOVINE FILARIA (ONCHOCERCA GUTTUROSA) ANTIGEN AND BLOOD SAMPLES COLLECTED ON FILTER PAPER
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ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)  
AS A DIAGNOSTIC TOOL FOR GUATEMALAN  
ONCHOCERCIASIS USING  
A BOVINE FILARIA (*ONCHOCERCA GUTTUROSA*)  
ANTIGEN AND BLOOD SAMPLES  
COLLECTED ON FILTER PAPER

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An ELISA for human onchocerciasis performed in Guatemala with a crude PBS extract of *Onchocerca gutturosa* and blood samples collected on filter paper showed a high sensitivity and specificity. A correlation between ELISA and IHA was obtained, and the former proved to be more sensitive than the latter. The ELISA values of blood samples collected on filter paper and of sera obtained from the same patient showed a high correlation. The linear correlation was also observed in ELISA values using two types of antigens, i. e., *O. gutturosa* and *O. volvulus*. 388 inhabitants of five coffee plantations were examined by the ELISA using *O. gutturosa* antigen. The results showed a positive reaction in 40(97.6%) of the 41 inhabitants with microfilariae(Mf)-positive/nodule-positive, and in 76(91.6%) out of 83 with Mf-positive/nodule-negative, but in only 17(21.8%) out of 78 with Mf-negative/nodule-negative in an endemic area, and 1(1.1%) out of 90 in a non-endemic area. Using a combination of *O. gutturosa* obtained from cattle and blood samples collected on filter paper, ELISA was found to be applicable for the immunodiagnosis of human onchocerciasis in Guatemala.

Key words: ELISA, *Onchocerca gutturosa*, *Onchocerca volvulus*, filaria, Guatemala

INTRODUCTION

At present, routine parasitological examinations by skin biopsy and nodule palpation are carried out as diagnostic tools for epidemiological survey under a programme authorized by the Onchocerciasis Control Project in Guatemala. However, these

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methods often fail to detect microfilarae (Mf) or adult worms of *Onchocerca volvulus* in patients because of their insufficient sensitivity.

A number of immunoserological tests has been developed to ensure more reliable results, as reviewed by AMBROISE-THOMAS (1980), i. e., the gel diffusion test by BIGUET et al. (1964), the indirect fluorescent antibody test (IFA) by LUCASSE (1962) and the indirect hemagglutination test (IHA) by IKEDA et al. (1978).

Recently, the enzyme-linked immunosorbent assay (ELISA) for the detection of serum antibodies has found wide application in parasitology.<sup>13,14</sup> ELISA is a potentially useful tool since it may be applied as a sensitive and quantitative tool for an immunodiagnosis of parasitic infection when the results are read accurately on a spectrophotometer.

The objective of our present study was to evaluate the reliability of the ELISA, comparing it with other diagnostic tests which are used to detect antibody in blood samples collected on filter paper, and employing *O. gutturosa* antigen for the diagnosis of Guatemalan human onchocerciasis.

#### MATERIALS AND METHODS

##### *Antigen*

Adult worms of *O. volvulus* were obtained from the onchocercal nodules by enzymatic digestion as reported by SCHULZ-KEY et al. (1977). The adult worms were isolated from the nodules, which were digested with 0.1% collagenase (Sigma, USA) in TC-199 (Nissui Seiyaku, Japan) containing 0.2mg gentamycin per ml for 6–48 h at 37°C. Adult worms of *O. gutturosa* were obtained surgically from the ligaments of cattle slaughtered at an abattoir in Guatemala.

The crude antigens from these adult worms were prepared in the manner reported by IKEDA et al. (1978). In brief, the adult worms were ground with cold acetone, and after centrifugation at 7,700 G for 30 min the sediment was mixed with 0.15 M phosphate buffered saline at pH 7.2 (PBS). The suspension was then stirred slowly for two days, centrifuged at 27,000 G for 30 min, and the supernatant was used as crude antigen.

##### *Blood samples and sera*

Blood samples were taken on filter paper (Type I, Toyo Roshi, Tokyo) from the ear lobes of 388 inhabitants of five coffee plantations in Guatemala: in the endemic areas, 91 inhabitants of Buena Vista, 72 of San José Guachipilín, 58 of Las Parasitas, 77 of San Fernando and in a non-endemic area, 90 of San Francisco Miramar as a control.

The sera from 72 inhabitants of San José Guachipilín were obtained using capillary tubes for blood collection.

*Indirect hemagglutination test (IHA)*

IHA was performed using formalin fixed tanned sheep blood cells which had been sensitized with the crude PBS extract of adult worms of *O. volvulus*.<sup>4,6,8)</sup> The antigen concentration was determined by checkerboard titration using immunized rabbit serum and sera from the patients. A reciprocal serum titer of 60 or more was considered as positive.

*Skin biopsy and nodule palpation*

Skin biopsy was performed by taking two snips of skin from the left scapular and left iliac region in the males, and from the left and right scapulars in the females. The snips were kept in saline solution for 1 h at room temperature, and then the Mf which emerged from the snips were examined microscopically. Palpation was carried out to determine the presence of onchocercal nodules.

*ELISA*

The test was based on the method of VOLLER et al. (1976). Crude PBS extracts of *O. volvulus* and *O. gutturosa* diluted properly to a protein concentration of 0.5–1.5  $\mu$ g/ml with 0.05 M carbonate buffer, pH 9.8, were used. The wells of a microplate (Nunc, Denmark) were sensitized with 0.25 ml of the respective antigens at 4°C overnight. The wells were washed four times with 0.15 M phosphate buffered saline with 0.05 M Tween-20 (PBS-Tween) for each step. 0.25 ml of the test sera and blood samples diluted with PBS-Tween to 1 : 500 on a serum volume basis were added to the wells and incubated for 2 h at room temperature. 0.25 ml of alkaline phosphatase conjugated goat anti-human IgG (Miles-Yeda, Israel) was added and incubated for 2 h at room temperature. Next, 0.25 ml of the substrate, 0.1% p-nitrophenylphosphate, was added and kept for 30 min at room temperature. Finally, the enzyme reaction was stopped by 0.05 ml of 2 M sodium hydroxide. The optimal concentration of antigens was determined by checkerboard titration before the tests. The results were expressed as an absorbance at 400 nm (ELISA value) in a spectrophotometer. An ELISA value of 0.50 or more was considered to represent a positive reaction.

*Fecal examination*

Fecal examinations were done parasitologically to determine the prevalences of intestinal helminth-parasites between two groups ; 30 inhabitants with onchocerciasis of an endemic area of Buena Vista, and 33 inhabitants of a non-endemic area of San Luis Buena Vista, who were confirmed to be Mf-negative by skin biopsy.

## RESULTS

*A comparison of O. volvulus and O. gutturosa antigens*

The correlation between the crude PBS extracts of *O. volvulus* and *O. gutturosa* as

an antigen in ELISA was investigated using 72 blood samples from the inhabitants in San José Guachipilín. The result is shown in figure 1. The ELISA value showed a close correlation between *O. volvulus* and *O. gutturosa* antigens ( $r=0.96$ ).

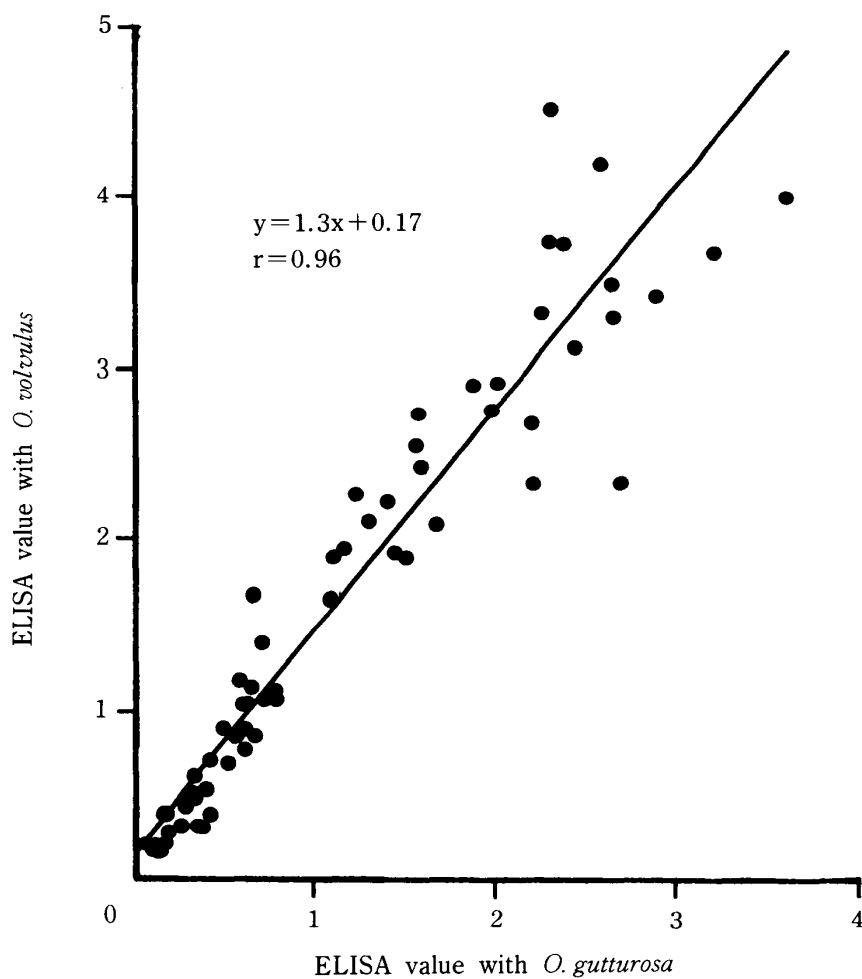


FIGURE 1 Correlation between two ELISA values with PBS extracts of *O. gutturosa* and *O. volvulus* using blood samples on filter paper obtained from 72 inhabitants of San José Guachipilín.

ELISA value means the absorbance at 400nm with a spectrophotometer.

*A comparison of sera and blood samples on filter paper*

Sixty-four sera in capillary tube and blood samples on filter paper collected parallelly from the inhabitants of San José Guachipilín, were also tested by ELISA employing the PBS extract of *O. gutturosa*. The ELISA value gave a close correlation between them ( $r=0.97$ ) as shown in figure 2. Based on these data, further tests were performed using blood samples collected on filter paper and PBS extract of *O. gutturosa* as an antigen.

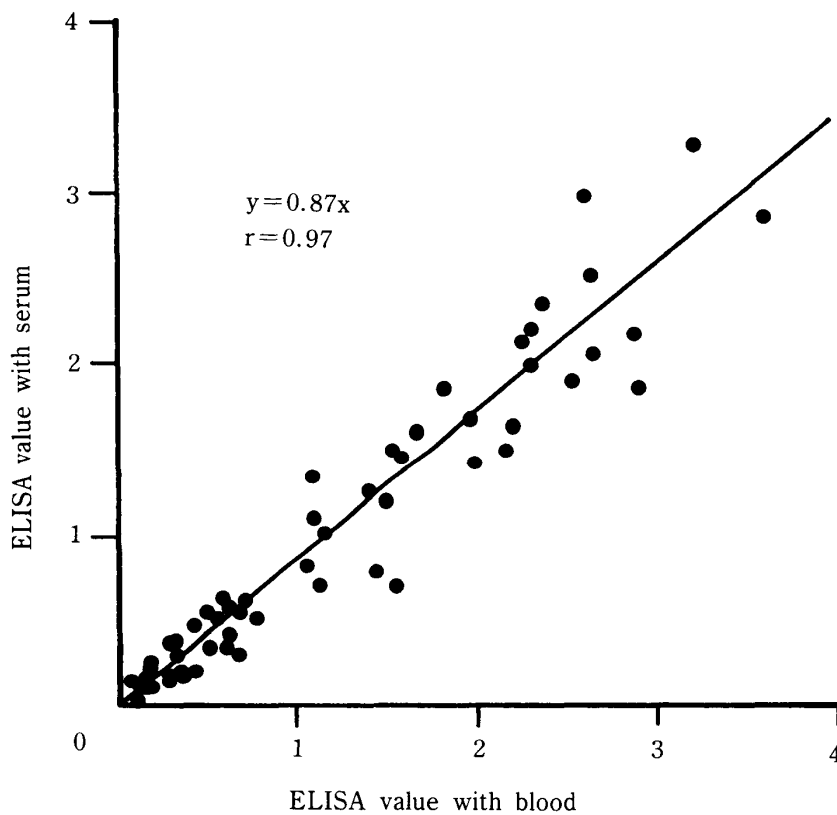


FIGURE 2 Correlation between two ELISA values with blood samples obtained on filter paper and sera from 64 inhabitants of San José Guachipilín, employing PBS extract of *O. gutturosa*.

*Influence of other helminthic parasites on ELISA*

To evaluate the cross reactivity with other helminthic parasites in ELISA, blood samples of 30 onchocercal patients living in Buena Vista and 33 inhabitants of a non-endemic area of San Luis Buena Vista, all of whom had been infected with common intestinal parasites, were examined by ELISA. The inhabitants were classified into three groups, that is, those with *Ascaris lumbricoides*, *Trichuris trichiura* and

hookworms, according to the results of fecal examinations. A significant difference of ELISA values was observed between the inhabitants of the two coffee plantations represented by the respective groups. This difference indicated that the infection with common intestinal parasites had little influence on the ELISA value (table 1).

TABLE 1 *Influence of other helminthic infections on ELISA value*

PARASITES <sup>a</sup>	ELISA VALUE (MEAN ± SD) <sup>b</sup>			
	Buena Vista (endemic area)		San Luis Buena Vista (nonendemic area)	
<i>A. lumbricoides</i>	21	1.63±0.90	23	0.23±0.33
<i>T. trichiura</i>	20	1.57±0.96	18	0.20±0.33
Hookworms	6	1.34±0.89	12	0.32±0.41
No. examined	30 (onchocerciasis)		33 (non-onchocerciasis)	

a. Parasites were determined by fecal examination.

b. Significant statistical difference was observed between ELISA values for workers in two coffee plantations by Student t-test ( $P < 0.05$ )

#### *Relations between ELISA and the other tests*

388 inhabitants of five coffee plantations, including 90 inhabitants of a non-endemic area used as a control, were examined by ELISA, skin biopsy, nodule palpation and IHA. These data are summarized in table 2.

#### *Relation between ELISA, skin biopsy and nodule palpation*

To investigate more detailed relationships among the ELISA, skin biopsy and nodule palpation tests, 221 inhabitants of Buena Vista, San José Guachipilín and Las Parasitas, an endemic area, were classified into four groups based on the result of skin biopsy and nodule palpation as shown in table 3. The following number of patients were positive in ELISA: 40(97.6%) out of 41 inhabitants with Mf-positive/nodule-positive, 76(91.6%) out of 83 with Mf-positive/nodule-negative, 11(57.9%) out of 19 with Mf-negative/nodule-positive, 17(21.8%) out of 78 with Mf-negative/nodule-negative in the endemic area. Whereas only 1(1.1%) out of 90 inhabitants of the non-endemic area was positive. The ELISA values were also given as  $1.63 \pm 0.68$  in the group of Mf-positive/nodule-positive, and  $1.38 \pm 0.75$  in the group of Mf-positive/nodule-negative, which were statistically higher than the figure  $0.46 \pm 0.48$  for the

TABLE 2 Examinations by ELISA and the other tests

LOCALITY	NO. TESTED	NO. POSITIVE (%)			
		Skin biopsy	Nodule	IHA	ELISA
Buena Vista	91	79(86.8)	25(27.5)	81(89.0)	83(91.2)
Sn. José Guachipilín	72	35(48.6)	27(37.5)	36(50.0)	47(65.3)
Las Parasitas	58	10(17.2)	8(13.8)	11(19.0)	15(25.9)
Sn. Fernando	77	2( 2.6)	0	5( 6.5)	5( 6.5)
Sn. Francisco Miramar <sup>a</sup>	90	0	0	2( 2.2)	1( 1.1)

a. This coffee plantation is located in a non-endemic area, and used as a control.

TABLE 3 Relations between ELISA, skin biopsy and nodule palpation

GROUP <sup>a</sup>	NO. TESTED	ELISA		
		No. positive	Positive rate (%)	Mean ELISA±SD
Mf(+), Nodule(+)	41	40	97.6	1.63±0.68
Mf(+), Nodule(-)	83	76	91.6	1.38±0.75
Mf(-), Nodule(+)	19	11	57.9	0.86±0.92
Mf(-), Nodule(-)	78	17	21.8	0.48±0.48
Mf(-), Nodule(-) <sup>b</sup>	90	1	1.1	0.23±0.11

a. 221 inhabitants of endemic areas in Buena Vista, San José Guachipilín and Las Parasitas were classified into four groups by skin biopsy and nodule palpation.

b. This group, which inhabited a non-endemic area of San Francisco Miramar, was used as a control.

group of Mf-negative/nodule-negative of the endemic area and that of  $0.23 \pm 0.11$  for the group of the non-endemic area ( $P < 0.05$ ).

*Correlation between ELISA and IHA*

According to the ELISA value and the IHA titer, four and five groups were made, respectively, as shown in table 4. The ELISA was confirmed to have a good correlation with IHA and to be more sensitive than IHA.

TABLE 4 *Correlation between ELISA value and IHA titer<sup>a</sup>*

IHA TITER	NO. EXAMINED	ELISA VALUE			
		$< 0.5$	$0.5 \leq < 1.0$	$1.0 \leq < 2.5$	$2.5 \leq$
30	37	25	9	3	—
60	17	—	5	12	—
240	14	—	1	7	6
960	2	—	—	1	1
3840	2	—	—	—	2

a. Reciprocal IHA titers of 72 inhabitants in San José Guachipilín.

#### DISCUSSION

BARTLETT et al. (1975) reported that *O. volvulus* antigen was not useful as it gave high values for both the negative control sera and the PBS in ELISA. In the present study, no significant difference was found in the ELISA values between PBS extracts of *O. volvulus* and *O. gutturosa*. This discrepancy may be due to the use of *O. volvulus* adult worms by collagenase digestion of the onchocercal nodule, which cause less damage to the worm and less contamination with human components. However, the use of *O. gutturosa* adult worms as a source of antigen seems to be more practical because of the limited supply of nodules containing *O. volvulus* and the availability of *O. gutturosa* from Guatemalan cattle, which has a high infection rate as reported by HASHIGUCHI et al. (1981).

IKEDA et al. (1978) reported the successful use of dried blood on the filter paper instead of serum in IHA for Guatemalan onchocerciasis. A similar result was obtained in the present ELISA. The use of filter paper to collect blood samples is advantageous to the epidemiological study because it helps to reduce apprehension of the examinee and to ease transportation of the samples to the laboratory.

ROFFI et al. (1982) reported that the ELISA with *O. volvulus* adult antigen gave cross reactions in bancroftian filariasis, schistosomiasis and dipetalonemiasis in Africa.

In Guatemala, the inhabitants of coffee plantations are usually infected with common intestinal parasites such as *A. lumbricoides* and *T. trichiura*, etc., but not with schistosome and another type of filaria parasite. To evaluate the influence of the former parasitic infections on the ELISA, we examined two comparative groups of workers who lived in endemic and in non-endemic areas, all of whom had been infected by general intestinal parasites. The result showed only a slight influence of these infection on ELISA values and confirmed that the present ELISA was a reliable diagnostic tool for Guatemalan onchocerciasis.

The results of examinations in five coffee plantations by ELISA, skin biopsy, nodule palpation and IHA showed that the infective rates determined by each diagnosis were relatively consistent and that ELISA was more sensitive than the other tests. The low sensitivity by ELISA observed in the group of Mf-negative/nodule-positive, may be attributed to the presence of non-onchocercal nodule as reported by IKEDA et al. (1978, 1979).

The ELISA using a combination of *O. gutturosa* obtained from cattle and dried blood samples collected on filter paper proved to be applicable for the immunodiagnosis of human onchocerciasis in Guatemala.

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