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Evaluation of the Double Diffusion Test for the Serodiagnosis of Onchocerciasis in Guatemala

MASATOSHI TAKAOKA*, ARACELI LUJAN T.†, YOSHIHISA HASHIGUCHI‡, MASATO KAWABATA§, YOICHI ITO♯, SIGEO HAYASHI§

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Introduction

A number of immunological studies on the diagnosis of filarial diseases have been carried out (Kagan, 1963; Lucasse and Hoeppli, 1963; Ciferri *et al.*, 1965; WHO Report, 1976; MacRae *et al.*, 1977). In most of these studies, heterologous antigens prepared from different filarial species have been used and some of them are found to be unsatisfactory for immunodiagnosis of disease because of the low specificity (Kagan, 1963; Takaoka *et al.*, 1973; WHO Report, 1979).

In onchocerciasis, however, it is easy to obtain the adult worm of *Onchocerca volvulus* from the onchocercoma located subcutaneously, thus homologous antigen can be used for the immunological test. Previously, various immunological methods including fluorescent antibody test (Lucasse, 1962; Woodruff and Wiseman, 1968), indirect haemagglutination test (Kagan *et al.*, 1963; Rose *et al.*, 1966; Ikeda *et al.*, 1978), complement fixation test (Ridley, 1956; MacRae, 1977), immunodiffusion test and the enzyme-linked immunosorbent assay (Bartlett *et al.*, 1975) have been attempted for the diagnosis of onchocerciasis. Of these tests the immunodiffusion test may be most effective one for qualitative analysis of filarial diseases (Capron *et al.*, 1968; Ulrich *et al.*, 1970; Neppert, 1975, 1979).

The purpose of the present study was to show whether immuno-diffusion test can be used as a diagnostic tool especially in the estimation of the effect of control measures for onchocerciasis in Guatemala.

Materials and Methods

Antigen

Fresh nodules were collected from the patients during the national denodulization campaign in Guatemala. Adults *Onchocerca volvulus* were teased out from these nodules, washed three times with physiological saline solution (0.15 M), homogenized with cold acetone in a glass homogenizer for delipidization and centrifuged at 3,000 rpm for 30 micutes. The sediment was added with 0.15 M phosphate buffered saline, pH 7.2 at 100 volumes of dry weight of worns. The suspension was

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* *Institute of Public Health in Saitama, Urawa, Japan.*
† *Servicio National de Erradicacion de la Malaria, Ministerio de Salud Publica, Guatemala.*
‡ *Department of Parasitology, Kochi Medical School, Kochi, Japan.*
§ *Department of Parasitology, National Institute of Health, Tokyo, Japan.*
♯ *Department of Parasitology, Kitasato University, School of Medicine, Kanagawa Japan.*

homogenized for 10 minutes in a glass homogenizer at 0 C, and allowed to rotate slowly with magnetic stirrer at 4 C for 2 days, centrifuged at 8,000 rpm for 30 minutes (4 C), and the supernatant obtained was then concentrated with the collodion bag (Sartorius Co., EMS KIKI, Japan). Antigen was kept at −20 C until used. Protein concentration of antigen was determined according to the method of Oyama and Eagle (1956). Protein concentration of antigen was standaridized at 3 mg/ml. For comparing cross-reactivity among different species of nematodes, extracts of *Dirofilaria immitis* (4.3 mg protein content/ml) was also prepared by the method of Ikeda *et al.* (1978).

Serum for assay

Two sources of sera were used for assay, namely hyper-immunized rabbit serum and sera obtained from inhabitants of endemic areas.

Sera from inhabitants: The blood was collected from the ear lobe of inhabitants in five endemic areas of onchocerciasis (Sibaja, Guachipilin, Hamburgo, Medio Monte, and la Cruz) in Guatemala in 1979 by capillary tubes for hematocrit (70 mm in length and 1 mm in diameter, Dramond Co., Japan). The tube fulfilled with blood was placed vertically positioned at room temperature for 2 to 3 hours in order to separate serum. Negative control serum was also obtained from school-children in Guatemala City, a non-endemic area of onchocerciasis.

Immunized rabbits sera: Antigen solution with an equal volume of Freund's complete adjuvant was injected subcutaneously into foot pads of two conventional rabbits, three times biweekly. On the seventh day after the last injection, they were bled to collect sera. These sera from rabbits immunized with antigens of *O. volvulus* or *D. immitis* were used to determine the specific precipitin bands by double diffusion test.

Procedure of double diffusion test

The method of Ouchterlony, as modified by Takaoka *et al.* (1973) was used for double diffusion test. A 1.0% agar (Difco Agar Noble) was prepared in 0.05 M veronal buffer, pH 8.6 and ionic strength of 0.05. Four ml of agar solution was poured to cover a slide glass. After the agar solidified, 7 wells with 3 mm diameter were punctured by an agar cutter, 5 mm apart from each other. Immunodiffusion was carried out for 3days at 4 C in a wet chamber, and the plates were washed with saline solution for 3 days changing solution frequently. Finally, the plate was stained with Coomassie brilliant blue R 250 (ICI) for 12 to 24 hours to read and recorded precipitin bands.

Examination of microfilarial density in the skin

Skin snips were removed from man's left scapula and left pelvis and from woman's right and left scapulas by Holth type corneoscleral punch (Hashiguchi *et al.* 1979). After the samples were immersed in saline solution (0.85%) for one hour at room temperature, emerged microfilariae were counted under a disecting microscope at the magnification of ×40.

Results

Reliability of double diffusion test

The skin biopsy for the examination of microfilariae and double diffusion test were performed on 572 inhabitants from five endemic areas of onchocerciasis (Sibaja, Guachipilin, Hambrugo, Medio Monte, and la Cruz) and 223 inhabitants or school-children from three non-endemic areas (el Faro, el Carrizal, Guatemala city) as controls in Guatemala. Results were summarized in Table 1. In endemic areas, 38.1% of the inhabitants were found positive by skin biopsy and 46.1% were found

Table 1 Results of examination with double diffusion test and skin biopsy for microfilariae to inhabitants from endemic and non-endemic areas of onchocerciasis in Guatemala, 1979

Area	Assay	No. of inhabitants examined	No. of positive	Percent of poditive
Endemic	Skin biopsy	572	218	38.1
	Double diffusion test	560	258	46.1
Non-endemic	Skin biopsy	223	0	
	Double diffusion test	223	3	1.3

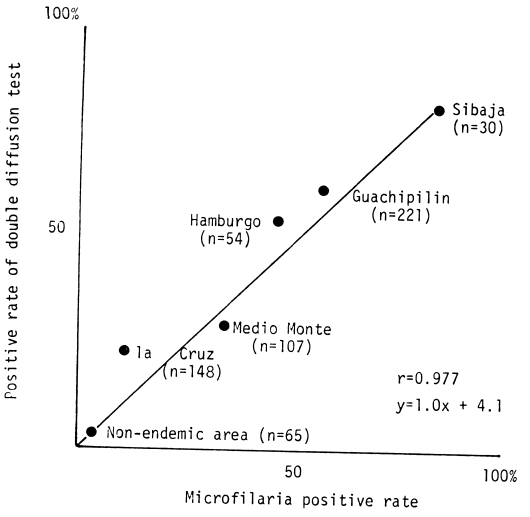


Fig. 1 Relationship between the results of double diffusion test and skin biopsy on the inhabitants of five endemic areas of onchocerciasis in Guatemala.

positive by double diffusion test showing 1–6 precipitin bands. Whereas, in non-endemic areas, none of 223 habitants was positive by skin biopsy and only 3 out of 223 reacted positive in double diffusion test, indicating a low false positive reaction of the double diffusion test. Within positive cases by double diffusion test, any relation was not observed between the number of precipitin bands and microfilarial density or nodule positiveness.

The positive rates in double diffusion test and skin biopsy for microfilariae on the inhabitants of endemic areas were shown in Fig. 1. As shown in figure, double diffusion positive rates were closely correlated with microfilarial positive rates (r=0.98), indicating reliability of double

Table 2 The results of skin biopsy and double diffusion test in 560 inhabitants in five endemic areas of onchocerciasis in Guatemala

Double diffusion test \ Skin biopsy	Positive (%)	Negative (%)	Total (%)
Positive	202 (36.1%)	56 (10.0%)	258 (46.1%)
Negative	15 (2.7%)	287 (51.3%)	302 (53.9%)
Total	217 (38.8%)	343 (61.3%)	560

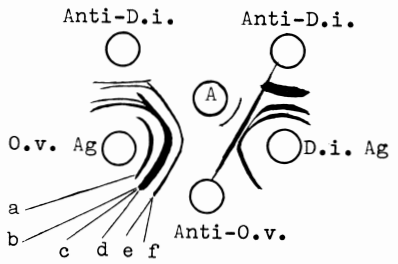
* Statistical analyses were performed by Mcnemar's test (1947).

diffusion test for diagnosing onchocerciasis.

The results of double diffusion test were compared with skin biopsy to determine its reliability (Table 2). Five hundred sixty inhabitants were examined and fifty six out of 343 microfilariae negative cases (16.3%) were positive in double diffusion test. Whereas, only 15 out of 217 microfilariae positive patients (6.9%) were negative in double diffusion test. This results indicated that low false negative reaction have been obtained by double diffusion test. On the other hand, a coincidence rate of the reaction of the reaction (positive or negative) between skin biopsy and double diffusion test was very high (87.3%). A significant coincidence was shown between these 2 examinations by Mcnemar's test ($p<0.001$).

Cross reaction with other human parasites

Studies were undertaken to determine the specific and common precipitin bands by using *O. volvulus* and *D. immitis* antigens



A : Serum of onchocercal patient
O. v.: *Onchocerca volvulus*
D. i. : *Dirofilaria immitis*
Ag : antigen

Fig. 2 Analysis of antibody in an onchocercal patient against *Onchocerca volvulus* and *Dirofilaria immitis* antigens.

and various serum samples. Sera used were obtained from rabbits immunized separately with *O. volvulus* adults and *D. immitis* adults and from onchocerciasis patients. Five or six bands were observed between *O. volvulus* antigen and *O. volvulus* immune rabbit serum, as well as between *O. volvulus* antigen and serum from onchocerciasis patients. Whereas, four bands were seen between *D. immitis* antigen and serum from onchocerciasis patients. Among the precipitin bands, specific one for *O. volvulus* could not be distinguished, but at least three bands (a, d and f) were seen in *O. volvulus* antigen-patients serum combi-

Table 3 Results of double diffusion test using antigens of *O. volvulus* and *D. immitis* and sera of inhabitants in endemic and non-endemic areas of onchocerciasis in Guatemala

Area	No. examined	Results of examination		Antigen			
		Double diffusion test	Skin biopsy	*O. volvulus*		*D. immitis*	
				No. persons	%	No. persons	%
Endemic	133	Positive	Positive	50	36.5	31	22.6
		Positive	Negative	11	8.0	12	8.8
		Negative	Positive	4	2.9	23	16.8
		Negative	Negative	72	52.6	71	51.8
Non-endemic	223	Negative	Negative	220	98.7	90	89.1
		Positive	Negative	3	1.3	11	10.9

Table 4 Results of fecal examination and double diffusion test using *O. volvulus* antigen among inhabitants in endemic and non-endemic areas of onchocerciasis in Guatemala

Area	No. examination	Results of double diffusion test		Results of fecal examination		
		No. positive	(%)	Parasite	No. positive	(%)
Endemic	125	60	48.0	*A. lumbricoides*	86	68.8
				*T. trichiura**	99	79.2
				Hookworm†	49	39.2
				Protozoa	85	68.0
Non-endemic	60	0		*A. lumbricoides*	33	55.0
				T. trichiura	38	63.3
				Hookworm	6	10.0
				Protozoa	43	71.7

, † Significant differences were observed between the infection rate of parasite in persons from endemic and non-endemic areas ($0.05>P>0.01$, †$P<0.01$).

nation which possessed characteristic in their mobilities and shapes in comparison with the bands in *D. immitis* antigen-patient serum combination (Fig. 2).

The cross reaction in double diffusion test using antiegns derived from *O. volvulus* and *D. immitis* and the sera of inhabitants in endemic areas (Sibaja and Medio Monte) and non-endemic areas (el Faro and Guatemala city) of onchocerciasis were examined. As shown in Table 3, when the antigen from *O. volvulus* adults was used, much higher coincidence rate (89.1%) was obtained between skin biopsy and double diffusion test, and a low false-negative rate (2.9%) by double diffusion test was demonstrated than the results using antigen from *D. immitis* (coincidence rate=74.4%; false-negative rate=16.8%). Furthermore, when sera from inhabitants in non-endemic areas was examined by double diffusion test, lower false-positive rate (1.3%) was observed on the reaction using *O. volvulus* antigen than that using *D. immitis* antigen (10.9%).

Common bands that could interfere with the specific diagnosis of onchocerciasis may arise from interaction between *O. volvulus* antigen and serum from patients with intestinal parasites. To clarify this point, 125 residents from onchocerciasis-endemic (Sibaja, Hamburgo and Medio Monte) and 60 school-children from non-endemic area (Guatemala City) were checked for intestinal parasites by fecal examination and their sera by double diffusion test using *O. volvulus* antigen (Table 4). None of 60 children in non-endemic area was positive by double diffusion test, whereas many persons in endemic and non-endemic areas havoured with one or more intestinal parasites, although a significant differences in infection rates with some species of parasites were observed between both areas. These results indicated that intestinal parasites infection may not affect to the diagnosis of onchocerciasis by double diffusion test using *O. volvulus* antigen.

Discussion

Double diffusion test is a relatively simple method and is often used for the diagnosis of parasitic infections. The present study was designed to evaluate if double diffusion test could be used as immunodiagnostic tool of *Onchocerca volvulus* infection in Guatemala.

Neppert (1975, 1979) reported that double diffusion test was less sensitive than both skin test and IHA test. Capron *et al.* (1968), Petithory *et al.* (1973) and Neppert (1974) investigated cross-reactivity between several nematodes antigens and sera from patients with these nematodes infection or from rabbits hyperimmunized against these nematodes extracts by using double diffusion and immunoelectrophoresis. They reported that strong cross reaction had been shown. Ulrich *et al.* (1970) in Venezuela showed that precipitin antibodies by double diffusion test against *O. volvulus* antigen were only positive in 64% of microfilarial positives.

Our investigation showed that two-hundred and two (93.1%) out of 217 microfilarial positives in endemic area were positive by double diffusion test, whereas only 3 (1.3%) out of 223 inhabitants from non-endemic areas reacted positively in double diffusion test. Furthermore, double diffusion positive rates from five endemic areas in Guatemala were closely correlated with their microfilarial positive rates. These results indicated a high specificity and reliability of the test for the diagnosis of onchocerciasis.

Cross reactivities were also studied using antigens from *O. volvulus* or *D. immitis* adults extracts and sera from *O. volvulus* infected patients or hyperimmunized rabbits against these antigens. Common precipitin bands were observed between different antigen-antibody combination. To clarify

if the cross reaction between *O. volvulus* antigen and antibody from patients with other parasite infection interfere with the diagnosis, fecal examination and double diffusion test using *O. volvulus* antigen were performed on the inhabitants from endemic and non-endemic areas of onchocerciasis. Results showed that intestinal parasite infection may not affect to the reaction of double diffusion test because of none of children harboured one or more intestinal parasites showing positive by double diffusion test.

Summary

A study was carried out for the diagnosis of onchocerciasis in Guatemala by double diffusion test using antigen derived from *Onchocerca volvulus* adult. Of 572 inhabitants from five endemic areas of onchocerciasis examined, 38.1% were found positive by skin biopsy and 46.1% by double diffusion test. On the other hand, only 1.3% were positive by double diffusion test in 223 sera from non-endemic areas and 2.7% were negative in 217 sera from microfilarial positive patients indicating low proportion of false-positive and false-negative reactions by double diffusion test. The positive rates in skin biopsy of the inhabitants of five endemic areas were closely correlated with the positive rate in double diffusion test (r=0.98).

Comparison of the results by double diffusion test using antigens derived from *O. volvulus* and *D. immitis* were performed on the serum samples from the inhabitants in endemic areas of onchocerciasis. *O. volvulus* antigen was more reliable for the diagnosis. The reactions between *O. volvulus* antigen and sera from patients were not interfered by intestinal parasitic infection.

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グァテマラ共和国におけるオンコセルカ症の二重拡散法による診断法の検討

高岡正敏
(埼玉県衛生研究所環境衛生部)
LUJAN A. T.
(グァテマラ共和国マラリア研究所)
橋口義久
(高知医科大学寄生虫学教室)
川端直人　林　滋生
(国立予防衛生研究所寄生虫研究部)
伊藤洋一
(北里大学医学部寄生虫学教室)

我々はグァテマラ共和国において，*Onchocerca volvulus* 成虫抗原を使った二重拡散法 (DD) のオンコセルカ症に対する診断の有効性について検討した．

オンコセルカ浸淫地区に居住する住民560名の血清において，DD test の陽性率は46.1%を示した．これに対し，同住民のミクロフィラリアの陽性率は38.1%であった．また，非感染地区の住民223名についてはミクロフィラリアは認めなかったが DD test では1.3の陽性を示した．ミクロフィラリア陽性者217名のうち，DD test 陽性を示したものは202 (93.1%) で，DD test と Skin Biopsy 法の結果との間には有意な一致が認められた ($P<0.001$)．

また，本診断法における他種寄生虫による交叉反応の影響はみられなかった．

以上の結果から，我々が行った DD test はオンコセルカ症の診断法として特異性及び感受性共に高く，有効な検査法であることがわかった．