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Stick enzyme-linked immunosorbent assay using the avidin–biotin system for detection of circulating antigen in bancroftian filariasis

K. A. PARKHE, P. RAMAPRASAD and B. C. HARINATH*
Mahatma Gandhi Institute of Medical Sciences, Sevagram 442 102, India

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Abstract. Detection of filarial antigen in different groups of sera was carried out by sandwich as well as inhibition enzyme-linked immunosorbent assays using antibody-coated sticks. Both systems were found to be equally sensitive in detecting antigen in 90% of microfilariae carriers. Incorporation of avidin-biotin in the sandwich assay system increased the sensitivity of antigen detection from 10^{-6} to 10^{-16} pg. A 67% decrease in the number of false negative results was observed when the sensitive avidin-biotin inhibition enzyme-linked immunosorbent assay system was used for analysis of filaria blood samples.

Keywords. *Wuchereria bancrofti;* filarial serum immunoglobulin G; enzyme-linked immunosorbent assay; biotinylated FSIgG; biotinylated ES antigen; avidin penicillinase.

Introduction

Diagnostic methods based on detection of parasite antigens are more useful in detecting active infection. Filarial antigens have been detected in blood, urine and hydrocoele fluid samples of filarial patients and animals (Kaliraj *et al.,* 1979; Dissanayake *et al.,* 1982, 1984; Hamilton *et al.,* 1984; Reddy *et al.,* 1984, 1986; Malhotra *et al.,* 1985a, b).

Cellulose acetate membranes (CAM) attached to plastic strips have been used in enzyme-linked immunosorbent assays (ELISA) for the detection of filarial antibody (Parkhe *et al.,* 1986). This paper reports the use of CAM attached to plastic strip (stick ELISA) for detection of antigen in sandwich as well as inhibition ELISA.

The strong interaction between avidin and biotin has been utilized in various systems such as specific staining of biological membranes in electron microscopy (Heitzmann and Richards, 1974), selective absorption of cells (Jasiewicz *et al.,* 1976), immunoenzymatic techniques (Guesdon *et al.,* 1979), and competitive inhibition assay (Wilson *et al.,* 1986) in the detection of circulating antigen levels in mice infected with *Toxocara canis* using direct ELISA (Bowman *et al.,* 1987). This communication reports the adaptation of stick ELISA to the detection of circulating antigen in filaria blood samples, and the increased sensitivity achieved by using the avidin–biotin system.

*To whom all the correspondence should be addressed.

Abbreviations used: CAM, Cellulose acetate membranes; ELISA, enzyme-linked immunosorbent assay; *Wb* mf ES Ag, *Wuchereria bancrofti* microfilariae excretory–secretory antigens; FSIgG, filarial serum immunoglobulin G; SPB, sodium phosphate buffer; PBS/T, phosphate buffered-saline with Tween 20; PVC, polyvinyl chloride.

Materials and methods

Sera

Human sera (30 samples), belonging to different groups, from normal subjects (non-endemic and endemic normal) and filarial patients (microfilaraemic and clinical filariasis) were screened. Serum was separated and stored at – 20°C after addition of sodium azide (0·1%) as preservative.

Filter paper blood samples were collected as described previously (Malhotra *et al.*, 1982).

Wuchereria bancrofti microfilariae excretory–secretory antigens

Wuchereria bancrofti microfilariae excretory-secretory antigen (*Wb* mf ES Ag) was prepared as described previously (Kharat *et al.,* 1982). The culture fluid was centrifuged at 13,000 *g* for 15 min. The supernatant (25 ml aliquots) was dialysed and lyophilized. The lyophilized powder was reconstituted in 2 ml of 0·05 M sodium phosphate buffer (SPB), pH 7·2 and protein was determined according to the method of Lowry *et al.* (1951).

Immunoglobulin-G fraction of human filarial serum immunoglobulins

The immunoglobulin G fraction of human filarial serum immunoglobulins (FSIgG) was prepared as described by Reddy *et al.* (1984).

Sandwich and inhibition ELISA

Twenty mg of FSIgG were conjugated to 1000 units of penicillinase (Sigma Chemical Co., USA) by the method of Avrameas (1969).

Conjugation of *Wb* mf ES Ag and penicillinase was achieved as described for the FSIgG–penicillinase conjugate, except that 125 μg of ES Ag protein was used instead of 20 mg. The substrate consisted of soluble starch (150 mg) in 27·5 ml of SPB (pH 7·2, 0·25 M) containing 10·6 mg of penicillin 'V' and 100 μl of 0·08 M iodine in 3·2 M potassium iodide solution. The substrate was prepared fresh before use.

Stick ELISA was carried out in small plastic vials (9 × 55 mm). Optimum amounts of FSIgG (in 5 μl SPB, pH 7·2, 0·05 M) containing 100 ng of protein, sera (1:600 dilution in PBS/T), FSIgG penicillinase conjugate (1:200 dilution in PBS/T), ES antigen penicillinase conjugate (1:100 dilution in PBS/T) were determined by Chequer Board titration.

Sandwich ELISA was carried out as described by Reddy *et al.* (1984) with some modifications. After applying 5 μl of FSIgG on CAM, the sticks were dried at room temperature and incubated at 37°C for 2 h with 3% gelatin (diluted in SPB, pH 7·2, 0·05 M). After washing 5 times at 5 min intervals with phosphate buffered saline (pH 7·2, 0·01 M) containing 0·05% Tween-20 (PBS/T), the sticks were incubated at 37°C for 2 h with 0·5 ml of 1:600 diluted sera or different dilutions of *Wb* mf ES Ag, *viz.,* 120, 12, 1·2, 0·12, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} pg/ml; PBS/T was used for

the control. After washing, the sticks were incubated at 37°C with 0·5 ml of FSIgG-penicillinase conjugate. After a thorough wash (10 times), 0·5 ml of substrate was added and the sticks were incubated for a further 30 min at 37°C. Disappearance of blue colour denoted positive reaction, while persistence of blue colour indicated a negative result.

Inhibition ELISA was carried out as described by Malhotra *et al.* (1984) and Ramaprasad *et al.* (1985). The procedure is the same as described above for sandwich ELISA except that 0·5 ml of ES Ag-penicillinase conjugate was added instead of 0·5 ml of FSIgG–penicillinase conjugate. A positive reaction in inhibition ELISA was indicated by the persistence of blue colour.

Biotin labelling of FSIgG and mf ES Ag

N-Hydroxysuccinimidobiotin (Sigma Chemical Co., USA) was conjugated to FSIgG as described by Guesdon *et al.* (1979) and Heitzmann and Richards (1974) with some modifications. A solution of 10 mg FSIgG in 1 ml of 0·1 M $NaHCO_3$ was mixed with 0·1 ml of N-hydroxysuccinimidobiotin solution (12 mg in 1 ml of dimethyl formamide). The mixture was kept at 25°C for 1 h and then dialysed at 4°C against 5 changes of SPB (pH 7·2, 0·01 M). After dialysis, the conjugate was removed and kept at –20°C with 0·02% sodium azide as a preservative until used.

Biotin labelling of *Wb* mf ES Ag was carried out in the same way except that 125 μg of *Wb* mf ES Ag protein was used instead of 10 mg FSIgG.

Coupling of avidin to penicillinase

Avidin was coupled to penicillinase as described by Guesdon *et al.* (1979). To 1 ml of SPB (pH 7·2, 0·25 M) containing 100 units of penicillinase and 2 mg of avidin (Sigma Chemical Co., USA) was added 40 μl of a 1% aqueous solution of glutaraldehyde, with stirring. After 3 h at 25°C, the preparation was dialysed for 48 h against 7 changes of SPB (pH 7·2, 0·01 M) at 4°C. It was then centrifuged at 4°C (30 min at 3000 g) and kept at –20°C after addition of 0·02% sodium azide as a preservative until used.

Sandwich and inhibition ELISA with avidin-biotin

The optimum dilutions of FSIgG (5 μl containing 100 ng protein), filter paper blood samples (1:600,000 dilution in PBS/T), biotinylated FSIgG (1:1000 dilution in PBS/T), biotinylated ES Ag (1:1000 dilution in PBS/T) and avidin–penicillinase conjugate (1:2000 dilution in PBS/T) were determined by Chequer Board titration.

Sandwich ELISA with avidin-biotin was carried out as follows. After applying 5 μl of FSIgG on CAM, sticks were dried and incubated at 37°C for 2 h with 3% gelatin. After washing, sticks were incubated at 37°C for 2 h with different dilutions of *Wb* mf ES Ag *viz*., 120, 12, 1·2, 0·12, 10^{-3}, 10^{-6}, 10^{-7}, 10^{-10}, 10^{-13}, 10^{-16}, 10^{-17}, 10^{-18}, and 10^{-19}pg/ml; PBS/T was used for the control. The sticks were washed and incubated at 37°C for 2 h with 0·5 ml of biotinylated FSIgG. After washing, the sticks were incubated at 37°C for 2 h with 0·5 ml of avidin-penicillinase conjugate. After a thorough wash (10 times) with PBS/T, 0·5 ml of freshly prepared substrate was

added and the sticks kept at 37°C for a further 30 min. Disappearance of blue colour denoted positive reaction. The procedure for inhibition ELISA with avidin–biotin was the same except that 0·5 ml of biotinylated ES Ag–conjugate was used instead of biotinylated FSIgG and filter paper blood eluates were used for antigen detection instead of *Wb* mf ES Ag. The persistence of blue colour denoted positive reaction.

Results

A total number of 30 sera belonging to different groups were screened by the stick ELISA method. Nine out of 10 microfilaraemic sera, 8 out of 10 clinical Filariasis sera and none of the 5 non-endemic and 5 endemic normal sera showed the presence of antigen by sandwich ELISA. In inhibition ELISA, 9 out of 10 microfilarial sera, 6 out of 10 clinical filariasis sera and none of the non-endemic and endemic normal sera showed the presence of antigen.

To determine the detectable limits of antigen by sandwich ELISA with and without the avidin–biotin system, different dilutions of *Wb* mf ES antigen were used, from 120 pg/ml up to 10^{-19} pg/ml. Antigen at concentration as low as 10^{-6} pg/ml was detected by sandwich ELISA; with incorporation of avidin–biotin the assay could detect as little as 10^{-16} pg/ml antigen. As inhibition ELISA detects specific antigen, further studies were carried out by inhibition ELISA. Filter paper blood eluates were used in place of sera, as described earlier (Malhotra and Harinath, 1984).

Twenty-two out of 191 microfilaraemia samples in the form of filter paper blood eluates were antigen-negative while the remaining were antigen-positive by inhibition ELISA in PVC plate assay (Ramaprasad, P., Bharati, M. S. and Harinath, B. C., unpublished results). Fifteen of these 22 antigen-negative filter paper blood eluates and 5 out of the remaining 169 antigen-positive samples were rescreened in inhibition ELISA incorporating the avidin–biotin system. Ten of these 15 "antigen-negative" microfilaraemia samples and all the 5 antigen-positive samples showed the presence of antigen.

Discussion

The diagnosis of filariasis based on the detection of antifilarial antibody has been extensively explored. However, detection of parasite antigen in body fluids may be more informative than antibody detection in the confirmation of active infection (microfilaraemia). The presence of circulating antigen has been reported in 77 and 82% of microfilaraemia carriers using counter-immunoelectrophoresis and sandwich ELISA respectively in bancroftian filariasis (Kaliraj *et al.,* 1981; Reddy *et al.,* 1984). Microfilariae ES antigen has also been determined in 70–75% of microfilaraemics by inhibition ELISA (Malhotra and Harinath, 1984; Ramaprasad *et al.,* 1985). Rabbit antiserum raised against bovine serum albumin was used in dilutions ranging from 1:4000 to 1:512,000 in the avidin-biotin technique (Guesdon *et al.,* 1979).

Stick ELISA (indirect), which was developed earlier (Parkhe *et al.,* 1986) for detection of antibody, has been adapted for detecting antigen. Further, incorporation of avidin-biotin in stick ELISA was attempted with a view to increase the sensitivity of detection of circulating antigen by sandwich as well as inhibition ELISA. The use of avidin-biotin in sandwich ELISA enhanced the sensitivity of the assay: the method could detect a concentration of antigen as low as 10^{-16} pg/ml. Inhibition

ELISA with avidin–biotin requires more dilute sera or filter paper eluates (1:600,000 dilution) compared to inhibition ELISA without avidin-biotin (1:600 dilution). Further, 10 out of 15 samples which were negative for antigen by inhibition ELISA showed the presence of antigen in inhibition ELISA incorporating avidin–biotin. Incorporation of avidin-biotin thus decreased the number of false negative results by 67%. The method has potential for being developed into a more sensitive test for detecting circulating antigen.

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