ASSESSMENT OF VALIDITY OF COUNTERIMMUNOELECTROPHORESIS AND ELISA IN THE ROUTINE DIAGNOSIS OF AMOEBIASIS

PHAKAKRONG SAMREJRONGROJ* and SAVANAT THARAVANIJ

Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok. *Department of Microbiology, Phramongkutklao Medical College, Bangkok, Thailand.

INTRODUCTION

Amoebiasis is still an important disease of international concern. The diagnosis of intestinal amoebiasis is in general not difficult because of the relative ease in the demonstration of haematophagous amoebae in the stool. The diagnosis of extra-intestinal amoebiasis especially amoebic liver abscess is more difficult, but several serological tests have been developed and proved to be of value in its diagnosis. Included among these tests are complement fixation (Craig, 1928), indirect haemagglutination (Kessel *et al.*, 1965; Thompson *et al.*, 1968), immunoelectrophoresis (Savanat and Chaicumpa, 1969), counter immunoelectrophoresis (CIE) (Sepulveda *et al.*, 1972; Krupp, 1974), immunofluorescence (Jeanes, 1966; Boonpucknavig *et al.*, 1967), latex agglutination test (Morris *et al.*, 1974; Tharavanij *et al.*, 1974), enzyme-linked immunosorbent assay (Bos *et al.*, 1976; Yang and Kennedy, 1979; Mohapatra, 1983) and cellulose acetate membrane precipitin test (Thammapalerd *et al.*, 1981). Among these tests, CIE is one of the rapid tests and has been used in our laboratory for routine serodiagnosis of amoebiasis since 1974, but its sensitivity and specificity have not been systematically analysed. The objectives of this study are:- (1) To assess the validity of the CIE test in the diagnosis of amoebiasis in sera routinely sent to our laboratory and to compare the result with that of ELISA, the highly sensitive test. (2) To compare the CIE results using antigens from two strains of *Entamoeba histolytica* in order to select the most suitable strain for future use.

MATERIALS AND METHODS

Antigen preparation: *E. histolytica* strains HK-9 and HT-12 were used. The HK-9 strain was originally obtained from Dr. L.S. Diamond in 1968 and the HT-12 isolated in 1970 from a Chinese patient with amoebic liver abscess (Wang *et al.*, 1974) was obtained from Dr. John Cross, then at NAMRU-2, Taipei, Taiwan in 1978. The amoebae were grown in TPS-1 with 10% horse serum according to the technique of Diamond (1968). Antigens were prepared from these 2 strains of *E. histolytica* using the technique previously described (Savanat *et al.*, 1973).

Sera: Sera were obtained from patients admitted to following hospitals:- The Hospital for Tropical Diseases, Siriraj Hospital, Chulalongkorn Hospital, Rajvithi Hospital and Phramongkutklao Hospital. All sera were sent to our laboratory during 1981-1982 for routine serodiagnosis of amoebiasis. They were inactivated at 56°C for 30 min and kept at -20°C until tested. The test was performed blind, and the diagnosis of the diseases was obtained thereafter by retrospective search of hospital records. By this approach, sera tested were classified into 4 groups as follows:-

(1) 46 patients with amoebic liver abscess the diagnosis of which was based on clinical criteria of fever and pain in the right hypo-

chondrium, aerobic bacteria-free anchovy sauce pus, and favorable response to anti-amoebic therapy.

(2) 99 patients suspected of amoebic liver abscess, but liver aspirate had not been performed or not available or search of hospital records could not be made.

(3) 22 patients with other parasitic infections comprising hookworms (8), opisthorchiasis (6), *Trichomonas hominis* (2), *Entamoeba coli* (2), *Endolimax nana* (2), *Trichuris trichiura* (1) and *Strongyloides stercolaris* (1).

(4) 25 patients with other liver diseases comprising viral hepatitis (22) and carcinoma of the liver with positive alpha-1 fetoprotein (3).

In addition, sera from 50 blood donors obtained from the Red Cross Institute were used as negative controls.

Serological tests: Counter-immunoelectrophoresis (CIE) test and ELISA were used. The CIE test was based essentially on the technique used by Gocke and Howe (1970) for the detection of hepatitis B antigen. Briefly, 10 ml of warm 0.85 % agarose (Indubiose A37) in 0.05M veronal buffer, pH 8.2 was poured on a 8 × 10 cm agarose coated glass plate. Nine pairs of 3 mm diameter wells were made at 3 mm apart. Ten µl of the antigen with the protein content of 20 mg/ml determined by Lowry's method and patient's serum were filled on the cathode and anode-side wells respectively. Positive and negative control sera were always included in each plate. Electrophoresis was carried out at room temperature using constant voltage of 6 volts/cm for 1 hour. Thereafter the plate was washed, dried and stained with Coomassie blue.

ELISA was performed using the technique of Engvall and Perlmann (1972) modified by Ambrois-Thomas and Desgeorges (1978). Briefly, polystyrene plates (Dynatech,M129-A) were coated with appropriately diluted antigen (11.7 µg/ml) in 0.1 M carbonate-bicarbonate buffer pH 9.6 and kept in a humid box at 4°C overnight. The plates were washed three times in physiological saline and the serum serially diluted in phosphate buffered saline (PBS) pH 7.2 with the initial dilution of 1 : 40 was added to each well and the plates incubated at 25°C for 30 minutes. Thereafter the plates were washed and peroxidase-conjugated rabbit anti-human immunoglobulins G-A-M (Institut Pasteur, Paris) was added to each well followed by incubation at 25°C for another 30 minutes. A solution of ortho-tolidine (Sigma) was then added and the plates were further incubated for 15-30 minutes, and the result read with-naked eyes.

For statistical analysis, Chi square analysis (with Yate correction) was used.

RESULTS

Comparison of CIE results using antigens from two *E. histolytica* strains was made. Ninety-nine sera from patients suspected of having amoebic liver abscesses were tested using antigens from HK-9 and HT-12 strains Thirty five and 39 sera were positive against HK-9 and HT-12 antigens with percent positivity of 35.4 and 39.4 respectively. This difference was not statistically significant ($p > 0.05$). In the subsequent study, only the antigen from the HK-9 strain was used.

The validity of CIE and ELISA in the routine diagnosis of amoebiasis is shown in Table 1. Forty-three of 46 patients (93.5%) with amoebic liver abscesses were positive with CIE but all 27 patients (100%) were positive with ELISA (titer > 1 : 320), whereas 25 patients with other liver diseases, 22 patients with other parasitic infections and 50 blood donors were all negative with both

Table 1

Comparison between CIE test and ELISA in patients with amoebic liver abscesses, other parasitic infections, and other liver diseases, and blood donors.

Clinical Entity	CIE			ELISA			
	No. tested	No. pos.	% pos.	No. tested	No. pos.	% pos.	*GMRT (range)
Amoebic liver abscess	46	43	93.5	28	28	100	7231 (320-81920)
Other parasitic Infections	22	0	0	22	0	0	< 80
Other liver diseases	25	0	0	25	0	0	< 80
Blood donors	50	0	0	50	0	0	< 80

* GMRT = Geometric mean reciprocal titer.

Table 2

Relationship between period after onset of clinical illness and seropositive rates with CIE test and ELISA.

Duration of illness (days)	CIE		ELISA		
	No. pos. No. tested	% pos.	No. pos. No. tested	% pos.	*GMRT (range)
0 - 5	4/6	66.7	3/3	100	507 (320-640)
6 - 10	14/15	93.3	10/10	100	12,600 (5,120-81,920)
11 - 20	14/14	100	8/8	100	11,500 (1,280-81,920)
> 20	11/11	100	7/7	100	6,231 (640-20,480)

* GMRT = Geometric mean reciprocal titer.

tests and individual ELISA titers were below 1 : 80.

Relationship between the duration of clinical illnesses (the day after the clinical onset of amoebic liver abscess and the time of serum collection) and the percent positivity is shown in Table 2. Four of six (66.7%) patients within 5 days of clinical illnesses, 14 of 15 patients (93.3%) with clinical illnesses of 6-10 days and all remaining 25 patients

tested on day 11 or more after the onset of clinical illnesses were CIE positive. In contrast, all 28 sera tested with ELISA were positive, but with low geometric mean reciprocal titer in patients tested within 5 days after the onset of clinical illnesses and with higher titers in patients tested thereafter (Table 2). Two CIE negative patients tested within 5 days after the onset of clinical symptoms were ELISA positive.

DISCUSSION

Antigenic analysis of different strains of *E. histolytica* has been reported using immunoelectrophoresis (Krupp, 1966) or two-dimensional immunoelectrophoresis (Chang *et al.*, 1979) showing some differences in precipitating band profiles when the antigens from a given strain of *E. histolytica* reacted with homologous and heterologous sera. In the present study, it was found that such difference (if exists) does not have any significant influence on the result of the routine CIE test currently used in our diagnostic laboratory using the HK-9 antigen, since no significant difference in seropositive rates was evident when 99 sera from amoebiasis suspected patients were tested with antigens from the HK-9 and HT-12 strains. It follows then that antigens from these two *E. histolytica* strains are equally suitable for use in the routine diagnostic service for amoebiasis.

Sensitivity of the CIE test in patients with amoebic liver abscesses were 93.5% which was slightly less than that of ELISA (100% sensitivity). Furthermore, ELISA can detect antibodies against *E. histolytica* in the situation where CIE cannot, since two patients, who were negative with the CIE test, were still positive with ELISA. In addition, ELISA can be used to detect antibody against *E. histolytica* much earlier than the CIE test. Only four of six (66.7%) patients tested within 5 days after the onset of clinical symptoms were postive with the CIE test, whereas all 3 patients tested within the same period were positive with ELISA. In term of specificity, both tests were equally specific (100%). With respect to practicability for use in the routine test, the CIE test is more practical especially in developing countries than ELISA for 3 reasons:- First, it is easy to read the result with naked eyes whereas with ELISA it is sometimes difficult to read the end point of titration, and in this case a more expensive ELISA reader is required. Second, CIE because of its simplicity can be performed even when a small number of serum samples is requested for testing. ELISA requires several steps of manipulation and thus would be impractical when a few serum samples are to be tested. Third, in the laboratory which is capable of producing its own antigen, the cost of setting up the CIE test is much less than that of ELISA.

SUMMARY

Counterimmunoelectrophoresis (CIE) was used to detect antibodies against *Entamoeba histolytica* in 99 sera from amoebiasis suspected patients using antigens from the HK-9 and HT-12 strains of axenically cultivated *E. histolytica*. There was no significant difference of seropositive rates using antigens from these two strains suggesting the both strains were equally suitable for use in the routine CIE test for the diagnosis of amoebiasis. In comparison with ELISA, the CIE test was a little less senstiive than ELISA with per cent sensitivity of 93.5 and 100 respectively. Two amoebic liver abscess patients who were CIE negative were ELISA positive. The CIE seropositive rate was related to the period after onset of clinical symptoms with per cent positivity of 66.7, 93.3 and 100 when the sera were tested on $\leq$ 5, 6-10 and $\geq$ 11 days of illnesses respectively.

REFERENCES

AMBROIS-THOMAS, P. and DESGEORGES, P.T., (1978). Diagnostic immunoenzymologique (ELISA) des maladies parasitaires par une micromethode modifiee. 1. Modalites techniques. *Bull. W.H.O., 56* : 609.

BOONPUCKNAVIG, S. and NAIRN, R.C., (1967), Serological diagnosis of amoebiasis by immunofluorescence. *J. Clin. Pathol., 20* : 875.

BOS, H.J. and VAN DEN EIJK, A.A., (1976). Enzyme-linked immunosorbent assay (ELISA) in the serodiagnosis of amoebiasis. *In*: Proceedings of the International Conference on Amoebiasis. Controle Estudios sombre Amebiasis, Mexico City, pp. 721-727, Supulveda, B. and Diamond, L.S. (ed).

CHANG, S.M., LIN, C.M., DUSANIC, D.G., and CROSS, J.H., (1979). Antigenic analyses of two axenized strains of *Entamoeba histolytica* by two-dimensional immunoelectrophoresis. *Amer. J. Trop. Med. Hyg., 28* : 845.

CRAIG, C.F., (1928). Complement fixation in the diagnosis of infection with *Entamoeba histolytica. Amer. J. Trop. Med., 8* : 29.

DIAMOND, L.S., (1968). Techniques of axenic cultivation of *Entamoeba histolytica* Schaudinn, 1903 and *E. histolytica*-like amoebae. *J. Parasitol., 54* : 1047.

ENGVALL, E. and PERLMANN, P., (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry, 8* : 871.

GOCKE, D.J. and HOWE, C., (1970). Rapid detection of Australia antigen by counterimmunoelectrophoresis. *J. Immunol., 104* : 1031.

JEANES, A.L., (1966). Indirect fluorescent antibody test in the diagnosis of hepatic amoebiasis. *Brit. Med. J., 1* : 1464.

KESSEL, J.F., LEWIS, W.P., PASQUEL, C.M. and TURNER, J.A., (1965). Indirect hemagglutination and complement fixation tests in amoebiasis. *Amer. J. Trop. Med. Hyg., 14* : 540.

KNOBLOCH, J. and MANNWEILER, E., (1983). Development and persistence of antibodies to *Entamoeba histolytica* in patients with amoebic liver abscess. Analysis of 216 cases. *Amer. J. Trop. Med. Hyg., 32* : 727.

KRUPP, I.M., (1966). Immunoelectrophoretic analysis of several strains of *Entamoeba histolytica. Amer. J. Trop. Med. Hyg., 15* : 849.

KRUPP, I.M., (1974). Comparison of counterimmunoelectrophoresis with other serologic tests in the diagnosis of amoebiasis. *Amer. J. Trop. Med. Hyg., 23* : 27.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J., (1951). Protein measurements with the Folin phenol reagent. *J. Biol. Chem., 193* : 265.

MOHAPATRA, T.M. and SEN, P.C., (1983). Evaluation of the micro-ELISA technique in the serodiagnosis of invasive amoebiasis. *Ann. Trop. Med. Parasitol., 77* : 101.

MORRIS, M.N., POWELL, S.J. and ELSDON-DEW, R., (1970). A rapid latex agglutination test for invasive amoebiasis. *S. Afr. Med. J., 44* : 594.

SAVANAT, T. and CHAICUMPA, W., (1969). Immunoelectrophoresis test for amoebiasis *Bull. W.H.O., 40* : 343.

SAVANAT, T., VIRIYANOND, P. and NIMITMONGKOL, N., (1973). Blast transformation of lymphocytes in amebiasis. *Amer. J. Trop. Med. Hyg., 22* : 705.

SEPULVEDA, B., LEE, E., TORRE, M. DE LA and LANDA, L., (1971). El diagnostico serlogico de la amebiasis invasora con la techica de inmunoelectroforesis cruzada. *Arch. Invest Med., 2 (suppl.)* : 263.

THAMMAPALERD, N., THARAVANIJ, S. and SAMREJRONGROJ, P., (1981). Cellulose acetate membrane precipitin test in amoebiasis using antigens from four different strains of *Entamoeba histolytica*. *Southeast Asian J. Trop. Med. Pub. Hlth., 12* : 353.

THARAVANIJ, S., TANTIVANICH, S. and KLONGKAMNUANKARN, K., (1974). Latex agglutination test for amoebiasis. *J. Med. Assoc. Thailand, 57* : 294.

THOMPSON, P.E., GRAEDEL, S.K., SCHNEIDER, C.R., STUCKI, W.P. and GORDON, R.M., (1968). Preparation and evaluation of standardised amoeba antigen from axenic cultures of *Entamoeba histolytica*. *Bull. W.H.O., 39* : 349.

WANG, L.T., JEN, G. and CROSS, J.H., (1974). Axenic cultivation of four strains of *Entamoeba histolytica* from liver abscess. *Southeast Asian J. Trop. Med. Pub. Hlth., 5* : 365.

YANG, J. and KENNEDY, M.T.,(1979). Evaluation of enzyme-linked immunosorbent assay for the serodiagnosis of amoebiasis. *J. Clin. Microbiol., 10* : 778.