APPLICATION OF LOCAL PRODUCTS R-PHYCOERYTHRIN AND MONOCLONAL ANTIBODY AS A FLUORESCENT ANTIBODY PROBE TO DETECT *ENTAMOEBA HISTOLYTICA* TROPHOZOITES

Nitaya Thammapalerd[1], Thararat Supasiri[2], Somchai Awakairt[2] and Suwalee Chandrkrachang[2]

[1]Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400; [2]Department of Chemistry, Faculty of Science, Srinakharinwirot University Prasarnmitr, Bangkok 10110, Thailand

Abstract. R-phycoerythrin (R-PE) was extracted from red algae, *Gracilaria fisheri* from Pattani Province, Thailand, with 50 mM sodium phosphate buffer, pH 7.0, followed by precipitation with 30-50% final concentration of saturated ammonium sulphate solution at O°C. The precipitate was further purified by DEAE-cellulose (DE-52) column chromatography. The purified R-PE showed a single band of Mr 240 kDa by polyacrylamide gel electrophoresis with the maximum absorption and maximum fluorescence emission at 565 nm and at 573 nm respectively, and the OD ratio of 565 to 280 nm was 6.7. The IgG fraction of a murine monoclonal antibody (Eh208C2-2 MIgG) raised against trophozoites of HM-1: IMSS strain of pathogenic *E. histolytica* was conjugated with purified R-PE by using the heterobifunctional compound N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). The conjugate was shown by direct immunofluorescent antibody (DIFA) assay to stain specifically both the culture-derived and stool-derived *E. histolytica* trophozoites.

INTRODUCTION

Phycoerythrin (PE) is one of 3 classes of phycobiliproteins containing multiple bilin chromophores which act as efficient light-harvesting systems in the photosynthetic apparatus of cyanobacteria (blue-green algae), and of two groups of eukaryotic algae, red algae and cryptomonads (Glazer, 1981). It cantains highly fluorescent proteins of 34 chromophores making it uniquely suited to function as components of specific fluorescent reagents in fluoro-immunoassay for analyses of molecules and cells (Oi *et al*, 1982). In Thailand, red algae, *Gracilaria* spp and *Polycavernosa* spp are distributed on both sides of the Gulf of Thailand and in the Indian Ocean and in brackish water lakes opening to the Andaman Sea. They are found in many provinces ranging from Rayong, Chanthaburi, Trat to Pattani, Songkhla, Surat Thani, and Ranong in the south (Fig 1). The local people eat them raw or after they have been cooked. Value-added products derived from seaweed processing, such as agar, agarose and a pigment, R-phycoerythrin (R-PE) from a local red algae, *Gracilaria fisheri*, from Pattani Province have been isolated at the Department of Chemistry, Faculty of Science, Srinakharinwirot University Prasarnmitr, and at the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, mouse monoclonal antibodies (MAbs) have been raised against whole cell lysate of the pathogenic HM-1 : IMSS strain of *E. histolytica*. One MAb, Eh208 C2-2, has been used successfully in an enzyme-linked immunosorbent assay (ELISA) for the detection of *E. histolytica* antigens in fecal specimens with positive rates of 100% and 66.7% in trophozoite-positive and cyst-positive patients, respectively (Thammapalerd and Tharavanij, 1991; Wonsit *et al*, 1992), as well as in immunohistochemical staining of *E. histolytica* trophozoite antigens in liver tissue sections of experimentally infected hamsters (Sherchand *et al*, 1994), and in the detection of circulating antigens of *E. histolytica* in hamsters (Thammapalerd *et al*, 1996). Together with extremely high absorptivities, high quantum efficiencies and wide excitation and emission bands across the visible spectrum, phycobiliprotein is potentially suitable for protein conjugation for use in various immunoassay techniques (Oi *et al*, 1982; Kronic, 1986; Kronic and Grossman, 1982). Therefore, attempts were made to prepare a conjugate between Eh208C2-2 MAb and the purified R-PE using the heterobifunctional cross-link-

Correspondence: Nitaya Thammapalerd.
Fax: 662 246-8340; Email: tmntm@mahidol.ac.th

ing reagent N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and to test its potential to be used as a fluorescent antibody probe to detect *E. histolytica*. In this study, we report the development of methods to prepare the conjugate between the purified R-PE isolated from *Gracilaria fisheri* and the IgG fraction of murine Eh208C2-2 MAb (MIgG) and to apply such conjugate as a fluorescent antibody probe for the detection of *E. histolytica* trophozoite antigens either derived from axenic cultures or stool samples by using the direct immunofluorescent antibody assay (DIFA).

MATERIALS AND METHODS

Amebae and culture conditions

HM-1 : IMSS and HTH-56 : MUTM strains of *E. histolytica* were grown axenically in screw-capped tubes in TPS-1 medium in the presence of 10% heat-inactivated bovine serum according to the technique described previously by Diamond (1968). The HM-1 : IMSS strain was kindly provided since 1986 by Prof Gordon B Bailey, Morehouse School of Medicine, Atlanta, Georgia, USA, while HTH-56 : MUTM strain of *E. histolytica* was locally axenized at the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (Thammapalerd *et al*, 1993).

Anti-*E. histolytica* MAb

Murine MAbs were raised against the pathogenic HM-1 : IMSS train of *E. histolytica* in SP2/0 myeloma cells according to the method of Galfre and Milstein as described in Thammapalerd and Tharavanij (1991). Several antibody-secreting hybridoma clones were obtained, among which Eh208C2-2 MAb was used throughout in the detection of *E. histolytica* trophozoites in this study. Ascites fluid from pristane-primed BALB/c mice was collected and centrifuged at 900g for 5 minutes and the supernatant was kept at -20°C. The IgG fraction of the ascites fluid (MIgG) was purified by Protein A Sepharose CL-4B^{R} affinity chromatography as described earlier (Wonsit *et al*, 1992).

Purification of R-phycoerythrin (R-PE)

R-Phycoerythrin (R-PE) was isolated and purified (Supasiri *et al*, unpublished). Briefly, it was extracted from red algae, *Gracilaria fisheri* from Pattani Province, Thailand, with 50 mM sodium phosphate buffer, pH 7.0, followed by precipitation with 30-50% final concentration of saturated ammonium sulphate solution at 0°C. The precipitate was further purified by DEAE-cellulose (DE-52) column chromatography.

Preparation of R-Phycoerythrin-labeled MIgG conjugate

SPDP conjugation of R-phycoerythrin: To 1.0 ml (10 mg) of *Gracilaria fisheri* R-phycoerythrin in a coupling buffer (0.1 M sodium phosphate, 0.1 M NaCl, pH 7.4) was added á fresh solution of 10 µl (0.13 mg) N-succinimidyl 3 - (2 - pyridyldithio) propionate (SPDP) in anhydrous methanol. After 60 minutes at room temperature, the reaction mixture was applied to a column of Sephadex G-25 (2.0 × 9.0 cm), equilibrated, and eluted with coupling buffer. The frontally eluted R-phycoerythrin-S-S-pyridyl derivative was kept at room temperature or stored lyophilized at 4°C if not being used immediately.

SPDP conjugation of IgG fraction of Eh 208C2-2 MAb: To 1 ml (1 mg) of MIgG in the above coupling buffer, was added 10 µl (0.13 mg) of SPDP in anhydrous methanol. After incubation of the reaction mixture for 60 minutes at room temperature, the mixture was applied to a column of Sephadex G-25 (2.0 × 9.0 cm), equilibrated and eluted with coupling buffer. The frontal eluates which contained MIgG-PDP were reduced by the addition of 1 M dithiothreitol (DDT) solution to a final concentration of 0.05 M of DTT in sodium phosphate buffer, pH 7.4. After a further 20 minutes, the reaction mixture was applied to a column of Sephadex G-25 (2.0 × 9.0 cm) and eluted with coupling buffer, fractions collected and then lyophilized.

SPDP disulfide crosslinking procedures: The SPDP disulfide crosslinking procedure used was modified from those of Kronick and Grossman (1982) and Oi *et al*, (1982). Lyophilized thiolated MIgG was mixed together with the lyophilized R-phycoerthythrin-S-S-pyridyl derivative and dissolved in the total volume of 5 ml of coupling buffer. After 18 hours incubation at room temperature, the iodoacetamide was added into the

reaction mixture to a final concentration of 0.03 M for 15 minutes. The reaction mixture was passing through a Sephadex G-25 column, fractions collected with the addition of bovine serum albumin (BSA, Cohn Fraction V, Sigma Chemical Company, St Louis, USA) to a final concentration of 1%, lyophilized and stored in the deep freezer (-78°C). When used it was reconstituted with PBS buffer containing 0.05% Tween 20.

Purification of R-PE-labeled MIgG conjugate

The conjugate was dissolved in PBS, pH 7.4 and 0.05 ml applied to an FPLC Superose® HR 10/30 column (Pharmacia) connected to a Pharmacia Biopump. The samples were eluted with 0.1 M NaCl, PBS buffer, pH 7.4 at a flow rate of 0.2 ml/minute (12 ml hr^{-1}). The absorption and fluorescence emission spectra were recorded using UV/visible spectrophotometer (LKB Bromma 2141, variable at 200-700 nm).

Preparation of *E. histolytica* antigen slides

Antigen coated slides were prepared according to the method described by Garcia *et al* (1982) with slight modification. Trophozoites from the HM-1 : IMSS and HTH-56 : MUTM strains as well as stool samples from patients with amebic dysentery were used as antigens for coating slides. Amebae from a 48-72 hour culture tube were chilled in an ice bath for 10 minutes to dislodge the parasites, followed by washing 3 times with 0.15 M NaCl solution (NSS) by centrifugation at 120*g* at room temperature for 5 minutes each. Thereafter the cell sediment was adjusted in NSS to give approximately 2×10^6 cells/ml. Two µl aliquots of the cell suspension were dropped on a clean and dry microscopic slide (25 mm × 75 mm) to form 16 spots. Smear slides were dried on a warm plate at 37°C for 30 minutes and then fixed in cold absolute ethanol for 10 minutes. Fresh stool samples were spotted or smeared directly onto microscopic slide, dried and fixed the same way as those cultured amebae. Prepared slides were wrapped with tissue paper and was then put in polyethylene bags containing a dehydrating agent (Silica-gel) and sealed. They were stored at -20°C until used.

Direct IFA test

To perform the IFA test, stored slides were removed from the freezer and left dry for 10 minutes at 37°C. Ten µl of R-PE-labeled Eh208C2-2MIgG conjugate, purified R-PE alone and PBS were applied to the antigen spots of one slide for the direct IFA test, whereas culture supernatant from EH208C2-2 clone as well as appropriately diluted immunized mouse sera (IMS) against those two pathogenic *E. histolytica*, normal mouse serum (NMS) and PBS were applied to the antigen spots of another slide for indirect IFA test, serving as three positive and two negative controls respectively. The two sets of slides were incubated in a humidified box at 37°C for two hours. The slides were then blotted individually with tissue paper in order to minimize the risk of mixing between adjacent supernatants and immediately washed by immersion four times for five minutes each in the Coplin jar containing cold PBS, and rinsed again with cold PBS. The slides were finally air dried. For the indirect IFA test, an 8 µl amount of R-PE labeled IgG rabbit anti-mouse IgG conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) in a 1 : 10 dilution in PBS containing 1% BSA was placed on every spot of tested area on the slide and covered with a cover glass (22 mm × 50 mm). This slide was reincubated at 37°C for another one hour in a humidified chamber, washed and dried as above. For examination, each slide was mounted under a cover glass (22 mm × 50 mm) in 70 µl of freshly prepared mounting solution comprising 5% n-propyl gallate in glycerol plus 20% 2M Tris base (Gilo and Sedat, 1982) and examined with a Zeiss fluorescence microscope (Zeiss, West Germany) equipped with a phase contrast and epi-illumination system at 250 and 400 times magnification. The fluorescence intensity was recorded as negative (-) or positive (+) ranging from one to four plus. Photographs were taken with Kodak color VR 400 film with 2.3 minute exposure time.

RESULTS

Characteristics of the purified R-PE isolated from local red algae, *Gracilaria fisheri* from Pattani province, Thailand

A map of Thailand (Fig 1 A) locates major provinces including Pattani at southern part of Thailand where the red algae, *Gracilaria fisheri* (Fig 1B) was obtained. The purified R-PE showed a single band of Mr 240 kDa by polyacrylamide gel electrophoresis (Supasiri *et al*, unpublished) and

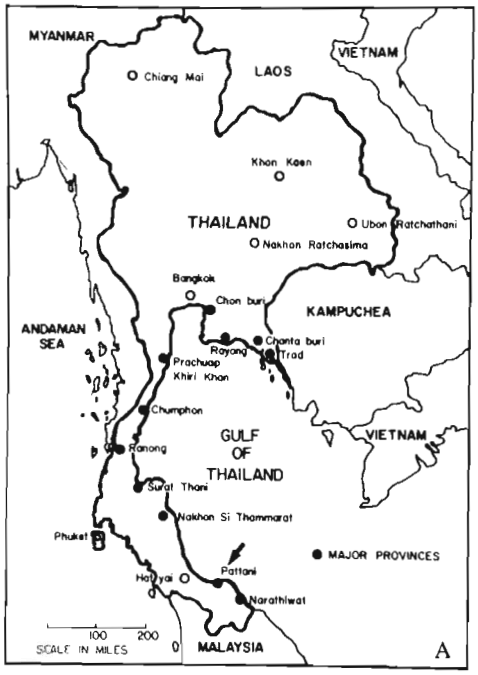

Fig 1–Map of Thailand (A) showing the distribution of red algae, *Gracilaria fisheri* (B) in various provinces (●) including Pattani (arrow head) where red algae, *Gracilaria fisheri* was obtained.

has absorption maxima at 3 wavelengths, 495,545 and 565 nm, of which 565 nm gives the maximum absorption, but only one single maximum fluorescence emission at 573 nm, respectively (Fig 2). The OD ratio of 565 to 280 nm was 6.7.

SPDP conjugation reactions of R-PE and Eh 208C2-2MIgG

Purified R-PE was successfully conjugated to the IgG fraction of mouse Eh208C2-2 MAb by

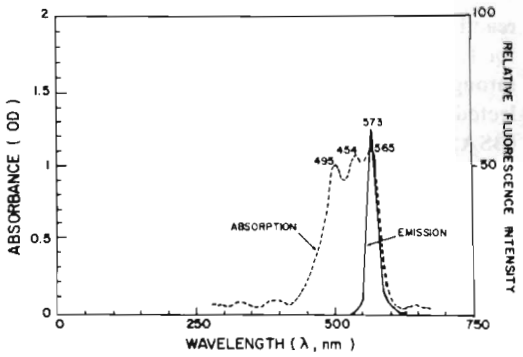

Fig 2–The maximum absorption (----) and maximum fluorescence emission (——)at 565 nm and at 576 nm respectively of purified R-PE isolated from *Gra-cilaria fisheri.*

using the heterobifunctional cross-linking reagent, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). The SPDP reagent can be coupled separately to both R-PE and MAb through free amino groups on the phycobiliprotein and MAb proteins. The resultant antibody-pyridyldithio groups can then be reduced by DTT to thiol groups and disulfide-linked to the R-PE-pyridyldithio groups with the release of 2-pyridinethione. A summary of SPDP labeling reactions for producing MAb-R-PE conjugates is shown in Fig 3.

Purification of conjugate

Purification of conjugate away from any unconjugated label or specific binding molecule that may remain was done by passing the conjugation (cross-linking) reaction mixtures through gel filtra-

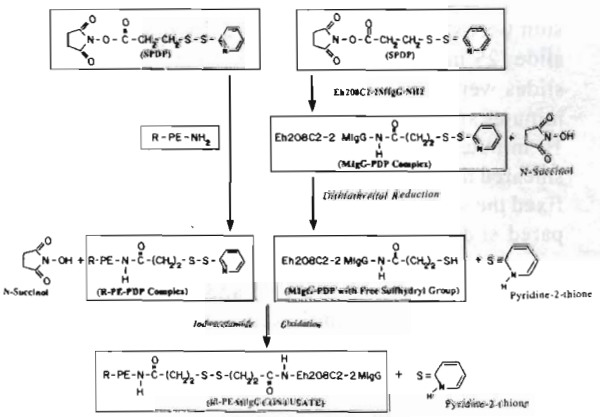

Fig 3–Summary of SPDP labeling reactions for producing R-PE-labeled Eh208C2-2MAb conjugates.

tion chromatography, for example, Sephacryl 300 or the FPLC-250 Superose™ 12 column, which have proven to be the best approach to conjugate purification. The fractions of purified conjugates were pooled on the basis of the retention time and corresponding molecular weight of the particular fractions. Fig 4 shows the elution profile of Eh208C2-2 MIgG conjugated with R-PE after passing through Superose[12] column chromatography using FPLC-250. Absorbance at both 565 nm and 280 nm is plotted to show content of both label (at 565 nm) and protein (at 280 nm) in each fraction. The characteristic two peaks of conjugated and unconjugated material are seen and that unconjugated R-phycoerythrin, is clearly separated from the conjugates.

Direct IFA test

Both culture-derived (Fig 5A) and stool-derived (Fig 5B) *E. histolytica* trophozoites displayed a bright yellow-orange fluorescence after staining with the R-PE-labeled Eh208C2-2-MIgG conjugate by DIFA assay. The staining pattern was similar to group I staining pattern previously described (Thammapalerd and Tharavanij, 1991). It stained brightly all trophozoites of *E. histolytica* both from axenic cultures and stool samples.

DISCUSSION

Very effective conjugates between purified R-PE and Eh208C2-2 MIgG could be produced using the heterobifunctional cross-linking reagent, SPDP. At 23°C it dissolves in absolute ethanol to give at least a 50 mM solution and must be used freshly.

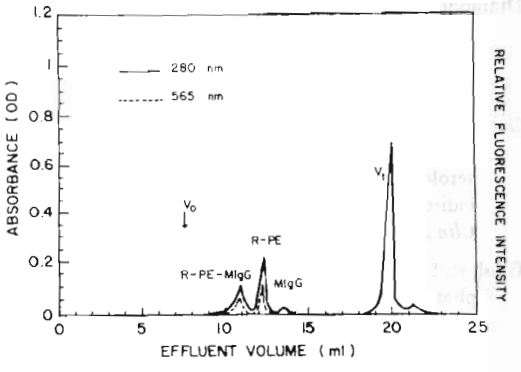

Fig 4–Elution profile of Eh208C2-2 MIgG conjugated with purified R-PE after passing through FPLC Superose 12 column chromatography and eluted with 0.1 M NaCl, PBS buffer, pH 7.4.

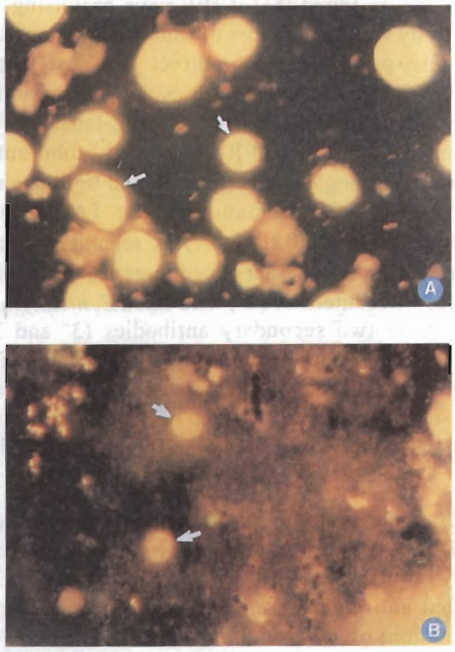

Fig 5–*E. histolytica* trophozoites either derived from cultures (A) or stool samples (B) displayed a bright yellow - orange fluorescence after staining with the R-PE-labeled Eh208C2-2-MIgG conjugate by direct immunofluorescent antibody assay (DIFA). The photograph was taken with Kodak color VR 400 film after 2.30 minute exposure (x25).

SPDP results a disulfide linkage between the coupled molecules. Thus, some workers who were concerned about the possible reduction of the disulfide bonds have preferred to use maleide-containing heterobifunctional reagents such as succinimidyl-4-(N-maleimidometyl) cyclohexane-1-carboxylate (SMCC) or succinimidyl-4-(p-maleimidophenyl) butyrate (SMPB) to produce thioether linkages (Hardy, 1986). They are commercially available from several sources and allow the separate activation of the two molecules of interest, the phycobiliprotein and the specific-binding molecule, using reagents under mild aqueous conditions at near neutral pH (Hardy, 1986).

The conjugate was stabilized by the addition of 1% BSA, aliquoted, wrapped with tin foil and kept in the deep freezer (-78°C) for 4, 8, 12, 24 and 36 months and retested again for its effectiveness to stain *E. histolytica* trophozoites by direct IFA

test. It was found that it still gave promising 4^+ reactivity without any loss of fluorescent intensity. We also performed the indirect IFA test using Eh208C2-2 MAb supernatant as the primary antibody and commercial available R-PE-labeled rabbit anti-mouse IgG and R-PE-labeled rabbit anti-mouse immunoglobulins (gamma and light chains specific) (TAGO Immunologicals, TAGO Inc, Burlingame, CA94011, USA) as secondary antibodies. It turned out that our conjugate gave higher fluorescence intensity (4^+) with fixed *E. histolytica* than those two secondary antibodies (3^+ and 3^+ respectively) (data were not shown). The conjugate could be used directly without any further purification since in each reaction step of IFA, any nonspecific reactants would be washed away during the test.

One interesting observation about the result of the IFA test using our conjugate is that it stained every trophozoite of *E. histolytica* whereas FITC - labeled anti-mouse conjugate stained only some populations of cultured *E. histolytica*. The latter is well observed among amebiasis researchers. The phycobiliproteins exhibit an extinction coefficient as much as thirty times that of a dye like fluorescence with no sacrifice in quantum yield. Our purified R-PE exhibited a 565 to 280 nm ratio as high as 6.7 which was above the recommended ratio of at least 4.0 for being suitable as a fluorescent label (Kronick, 1986). The concept of phyco-biliproteins as valuable labels was thus established. The phycobiliproteins were typically coupled through the ϵ-amino group of their many lysine side chains: B-phycoerythrin has an estimated 85 lysines per molecule and allophycocyanin has approximately 36 (Glazer and Stryer, 1984). To the best of our knowledge, there is no record of estimated numbers of ϵ-amino groups of lysine per molecule in R-PE. In general, phycobiliproteins are acidic and carry a negative charge at neutral pH. This negative charge and their generally hydrophilic character minimize non-specific binding that is often associated with the use of fluorescence as a label. The purified R-phycoerythrin should therefore be applicable for use in conjugation with avidin and other anti-immunoglobulin isotypes of various species of animals or humans for use as probes in fluoro-immunoassays and in fluorescence activated cell sorting.

The Eh208C2-2MAb, raised against ultrasonically disrupted *E. histolytica* trophozoites of the HM-1 : IMSS strain reacted with an epitope on *Entamoeba* pyruvate : ferredoxin oxidoreductase (Thammapalerd *et al*, 1996). The IFA staining pattern in this study together with previous IFA data (Thammapalerd and Tharavanij, 1991; Sosa *et al*, 1995) suggested that PFOR is an abundant, highly conserved and immunospecific protein.

ACKNOWLEDGEMENTS

The Eh208C2-2 MAb used in this study was produced with the support from the USAID/PSTC program, Grant No. 936-5542-G-00-6029-00. We thank Prof Gordon B Bailey, for the HM-1 : IMSS strain of *E. histolytica*, Dr Khanjanapaj Lewmanomont for the identification of *Gracilaria fisheri* from red algae from Pattani Province and Dr Sathit Pichyangkul, for commercially available R-PE-labeled rabbit anti-mouse IgG and R-PE-labeled rabbit anti-mouse immunoglobulins (gamma and light chains) as secondary antibodies used in the indirect IFA test. We are grateful to the late Prof Savanat Tharavanij for his kind interest. Thanks are also extended to Miss Jatuporn Chaiyawon for typing manuscript.

REFERENCES

Carlsson J, Drevin H, Axen R. Protein thiolation and reversible protein-protein conjugation, N-succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent, *Biochem J* 1978; 173 : 723-37.

Diamond LS. Technique of axenic cultivation of *Entamoeba histolytica* Schaudinn, 1903 and *E. histolytica*-like amoeba. *J Parasitol* 1968; 54 : 1047-56.

Garcia LS, Bruckner DA, Brewer TC, Shimizu RY. Comparison of indirect fluorescent-antibody amoebic serology with counterimmuno-electrophoresis and indirect hemagglutination amoebic serologies. *J Clin Microbiol* 1982; 15 : 603-5.

Giloh H, Sedat JW. Fluorescence microscopy: Reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. *Science* 1982; 217 : 1252-5.

Glazer AN. Photosynthetic accessory proteins with bilin prosthetic groups. In: Hatch MD and Boardman NK, eds. Biochemistry of Plants. New York: Academic Press, 1981; 8 : 51-96.

Grazer AN, Stryer L. Phycofluor probes. *Tends Biochem Sciences* 1984; 9 : 423-7.

Hardy RR. Purification and coupling of fluorescent proteins for use in flow cytometry. In: Wier DM, Herenberg LA, Blackwell CC, Herzenberg LA, eds. Handbook of Experimental Immunology. Edinburgh: Blackwell Scientific Publications, 1986; 31.1-31.12.

Kronick MN. The use of phycobiliproteins as fluorescent lebels in immunoassay. *J Immunol Methods* 1986; 92 : 1-13.

Kronick MN, Grossman PD. Immunoassay techniques with fluorescent phycobiliprotein conjugates. *Clin Chem* 1982; 29 : 1582-6.

Oi VT, Glazer AN, Stryer L. Fluorescent phycobiliprotein conjugates for analyses of cells and molecules. *J Cell Biol* 1982; 93 : 981-6.

Sherchand JB, Thammapalerd N, Riganti M, Tharavanij S, Punpoowong B. Monoclonal antibody-based immunohistochemical demonstration of *Entamoeba histolytica* in liver tissues of experimentally infected hamster *(Mesocricetus auretus). Int J Parasitol* 1994; 24 : 909-16.

Sosa RJ, Thammapalerd N, Awakairt S, Tharavanij S. Immunotoxin against *E. histolytica*: an *in vitro* study. In: Svasti J, *et al.* eds. Biopolymers and Bioproducts: Structure, Function and Applications. Bangkok: Samakkhisan (Dokya) 1995; 506-8.

Thammapalerd N, Kotimanusvanij D, Duchene M, *et al.* Pyruvate: ferredoxin oxidoreductase from *E. histolytica* recognized by a monoclonal antibody. *Southeast Asian J Trop Med Public Health* 1996; 27 : 63-70.

Thammapalerd N, Sherchand JB, Kotimanusvanij D, Chintana T. Monoclonal antibody-based ELISA for the detection of circulating *E. histolytica* antigens in hepatic amoebiasis in hamsters. *(Mesocricetus auretus)* (Submitted for publication in Southeast Asian J Trop Med Public Health).

Thammapalerd N, Tharavanij S. Biomedical applications of monoclonal antibodies (McAb) against *Entamoeba histolytica*. In: Maramorosch K, ed. Biotechnology for Biological Control of Pests and Vectors. Boca Raton, Florida: CRC PRess, Inc, 1991; 169-178.

Thammapalerd N, Tharavanij S, Wattanagoon Y. Axenic cultivation of *E. histolytica* from liver abscess and its zymodeme. *Southeast Asian J Trop Med Public Health.* 1993; 24 : 480-3.

Wonsit R, Thammapalerd N, Tharavanij S, Radomyos P, Bunnag D. Enzyme-linked immunosorbent assay based on monoclonal and polyclonal antibodies for the detection of *Entamoeba histolytica* antigens in faecal specimens. *Trans R Soc Trop Med Hyg* 1992; 86 : 166-9.