PYRUVATE : FERREDOXIN OXIDOREDUCTASE FROM *ENTAMOEBA HISTOLYTICA* RECOGNIZED BY A MONOCLONAL ANTIBODY

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Abstract. A mouse monoclonal antibody, Eh208C2-2 MAb, raised against whole cell antigens of *Entamoeba histolytica* trophozoites of the pathogenic strain HM-1 : IMSS and polyclonal antisera (PAb) against membrane antigens of *E. histolytica* trophozoites of strain HTH-56 : MUTM were screened against a cDNA library of the pathogenic strain, SFL3. The monoconal antibody detected many phage plaques expressing an *E. histolytica* protein. The DNA sequence encoding the protein was approximately 55% identical, over 1,100bp, to *Trichomonas vaginalis* pyruvate : ferredoxin oxidoreductase (PFOR) and pyruvate : flavodoxin oxidoreductase from *Klebsiella pneumoniae, Anabaena variabilis* and *Enterobacter agglomerans*. Two of seven clones detected by mouse polyclonal antisera also encoded this protein. Two others encoded *Entamoeba* Hsp70, another encoded *Entamoeba* alkyl-hydroperoxide reductase and the remaining two were unidentified sequences. *Entamoeba* PFOR is an abundant, antigenic protein which may be a useful target for the development of protective host immune responses against invasive amebiasis.

INTRODUCTION

Amebiasis is the third leading parasitic cause of death world-wide after malaria and schistosomiasis (WHO, 1992). The causative agent, *Entamoeba histolytica*, occurs throughout the world especially in tropical and subtropical climates. *E. histolytica* is a member of the group of amitochondrial, microaerophilic anaerobic protozoa which rely heavily on fermentative metabolism for ATP production. In these organism, a critical step in the energy production pathway is the decarboxylation of pyruvate and the enzyme responsible for this reaction is pyruvate : ferredoxin oxidoreductase (PFOR) (Kerscher and Oesterhelt, 1982). PFOR is also involved in the activation of metronidazole, a 5-nitroimidazole, and the most effective drug used to treat the parasitic members of the anaerobic protozoa (Townson *et al*, 1994a). Decarboxylation of pyruvate occurs concomitantly with reduction of the electron carrier, ferredoxin, which has a sufficiently low redox potential to reduce the 5-nitro group of the nitroimidazole drug family to toxic radicals (Townson *et al*, 1994b). The 5-nitroimidazoles are the only recommended drugs to treat amebiasis, the only effective drugs available to treat trichomoniasis and the drugs of choice for giardiasis (Upcroft and Upcroft, 1993).

PFOR has been implicated in the resistance of organisms to the 5-nitroimidazoles and in the case of *Trichomonas foetus* a complete absence of PFOR is correlated with resistance to high levels of drug (Johnson, 1993). In *Giardia* PFOR activity is decreased in lines resistant to the drug (Upcroft and Upcroft, 1993) and there is evidence that a decrease in PFOR is accompanied by alternative energy producing pathways (Townson, unpublished data). Since PFOR is so intimately involved in metronidazole activation and is central to the parasite's metabolism, this enzyme is an ideal host immune response or chemotherapeutic target.

Diagnostic and therapeutic applications of monoclonal antibodies (MAbs), one in particular, designated Eh208C2-2 MAb, have been clearly shown(Thammapalerd and Tharavanij, 1991;

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Wonsit *et al*, 1992; Sherchand *et al*, 1994; Sosa *et al*, 1994; Thammapalerd, 1994; Thammapalerd *et al*, unpublished). Here we report on the sequence of the gene encoding the antigen recognized by Eh208C2-2 MAb.

MATERIALS AND METHODS

Parasite culture

The axenic Thai strain of *E. histolytica*, HTH-56 : MUTM was used and grown as previously described (Thammapalerd *et al*, 1993). The techniques of maintenance and cultivation of stock cultures were based on that of Diamond *et al* (1978).

Monoclonal and polyclonal antibodies against pathogenic *E. histolytica*

The monoclonal antibody, Eh208C2-2 MAb, raised against HM-1 : IMSS, the Mexican strain of *E. histolytica*, has been described by Thammapalerd and Tharavanij (1991). Polyclonal antisera were raised in Quackenbusch mice against crude membrane preparations of parasites of the Thai strain (HTH-56 : MUTM). Parasites were frozen/thawed several times, washed in phosphate buffer saline (PBS) and the precipitate solubilized in 50 mM Tris/HCl, pH 8.0, in the presence of 1% Triton X-100 and 0.4% sodium deoxycholate (SDC). Mice were immunized on three successive occasions with parasite extracts in Freund's complete adjuvant, initially, and in incomplete adjuvant in subsequent immunizations.

cDNA library screening

A cDNA library was constructed from the *E. histolytica* strain SFL-3 using the Lambda ZAPII system (Stratagene) (Ortner *et al*, 1992). The amplified library was stored and plated as described by Plaimauer *et al* (1993). Plaque lifts were reacted with Eh 208C2-2 MAb and 500 fold dilutions of polyclonal sera absorbed against sonicated *E. coli* XL1-Blue cells (Stratagene). Specific clones were identified with the biotinylated second antibody-streptavidin linked horse-radish peroxidase system (Amersham) and diaminobenzidine (Sigma) for detection. Positive plaques were picked, replated and rescreened. Phagemids were rescued by *in vivo* excision as described in the manufacturer's instructions and DNA purified (Berghammer and Auer, 1993), cleaved with restriction enzymes EcoRI, HaeII and HinfI (New England Biolabs) and separated electrophoretically.

PCR amplification of phage DNA

The DNA of freshly picked phage was amplified using BlueScript (M13) forward and reverse primers. Assays contained 2 mM dNTP, 2.5 mM $MgCl_2$, primers and buffer in 25 µl. Amplification was over 30 cycles under standard conditions (Pharmacia LKB Biotechnology).

DNA sequencing

PCR amplified DNA or phagemid DNA was further purified by phenol/chloroform extraction and ethanol precipitation. DNA was sequenced using the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions and an automated sequencer (Applied Biosystems). Sequences were analyzed and compared using the program BESTFIT and PILEUP (Smith and Waterman, 1981) in the GCG suite of programs (Devereux *et al*, 1984) on a Sun Sparcstation 10 at the Queensland Institute of Medical Research. The program PRETTYPLOT (EMBL) was used to depict similarities between protein sequences.

RESULTS

Screening of the cDNA library of pathogenic *E. histolytica* strain SFL-3 with Eh 208C2-2 MAb

The library was plated at a density of 700 plaques per plate and 8 plaques reacted positively on each plate with the Eh208C2-2 MAb. All 8 positive plaques, D23-D30 were picked, processed and the size of the PCR product estimated after agarose gel electrophoresis (Table 1). All insert sizes were greater than 1,200bp and the largest was approximately 3,000bp (Table 1). The two largest phage inserts (D25 and D27) required several attempts to be rescued by *in vivo* excision, apparently as a result of the instability of the 5' region of the gene

Table 1

Clones derived from the cDNA library which react with Eh208C2-2 monoclonal and polyclonal antibodies.

Clone[a]	Size[b] (kb)	Encoded protein
D23	2	PFOR
D24	1.5	PFOR
D25	3	PFOR
D26	1.6	PFOR
D27	2.7	PFOR
D28	1.2	PFOR
D29	1.8	PFOR
D30	1.9	PFOR
X1	1.5	?
X2	1.1	Hsp70
X3	2	PFOR
X4	1.8	PFOR
X7	1.5	?
X13	2.3	Hsp70
X17	0.9	Alkyl-hydroperoxide reductase

[a] The D series clones were detected by the Eh208C2-2 monoclonal antibody. The X series were detected with polyclonal antisera.
[b] Size refers to the DNA insert.
PFOR = pyruvate : ferredoxin oxidoreductase.
Hsp = heat shock protein.

in *E. coli*. Those *E. coli* carrying the phagemid with the 2.7kb insert grew very slowly and only deletion products resulted from excision of the 3kb insert.

Screening of the cDNA library of pathogenic SFL-3 strain of *E. histolytica* with polyclonal antibody (PAb)

Screening of the library with polyclonal antisera, which had an IFA titer of 3,200 (data not shown), resulted in numerous positive clones. Seven were picked and designated X1, X2, X3, X4, X7, X13 and X17, and the sizes of these were estimated to range from 900bp to 2.3kb (Table 1). The 5' region of the insert of each clone was sequenced.

Mapping of cloned DNA

Restriction enzyme cleavages of *Entamoeba* DNA from the D23-D30 series of clones indicated that all encoded the same gene for PFOR (Fig 1) and

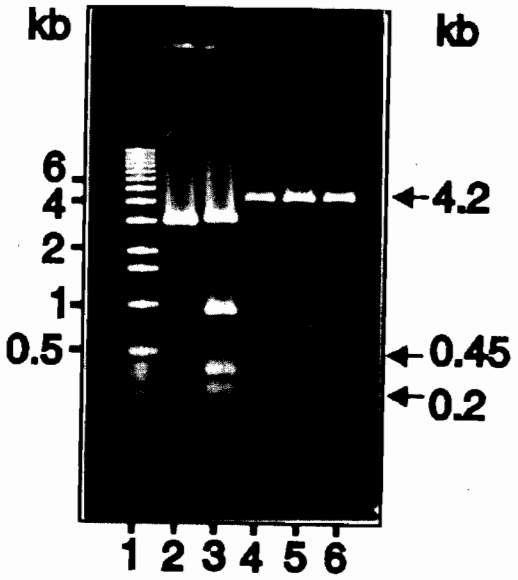

Fig 1–EcoRI cleavage of phagemids encoding *E. histolytica* PFOR. Arrows indicate 4.2kb of vector plus insert from the 3' terminus of the gene to the first upstream EcoRI site. Other EcoRI segments from D23 are 0.45 and 0.2kb and are consistent with the EcoRI sites at position 432 and 635 in the 1,100bp sequence indicated in Fig 2. The shorter insert in D24 does not have the 0.45kb 5' region of the PFOR gene and the phagemid (with more than 1kb deleted from the 5' end of the original phage insert) recovered after excision form D25 apparently has two segments of approximately 0.2kb. Lane 1, 1kb ladder (Gibco BRL); 2, phagemid vector; 3, a phagemid carrying an unidentified insert; 4, D23; 5, D24; 6, D25.

these were used to construct a gene map (Fig 2).

Comparison of sequences with those in the GenBank database

Sequences of the 3' region of the D series inserts, which included a polyT stretch and 3' untranslated region of the gene, confirmed that all clones belonged to the same gene and sequencing of the 5' region allowed a 1,100bp segment of the gene sequence to be compiled as result of the overlapping clones obtained (Fig 2). The sequence of the 1,100 bp contiguous *E. histolytica* gene from the D series clones identified by Eh208C2-2 MAb was compared with those in GenBank. *Trichomo-*

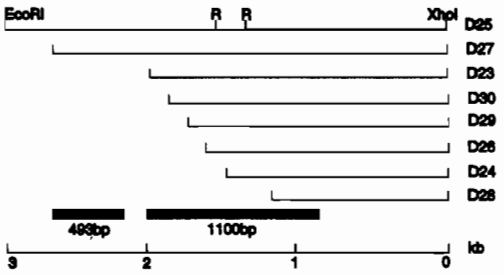

Fig 2–Restriction map of the *E. histolytica* partial PFOR gene. The map represents the insert of clone D25, the longest insert obtained from the cDNA library. Other clones (D27, 23, 30, 29, 26, 24 and 28) with shorter inserts (Table 1) are shown. The 5' region of each insert was sequenced and 1,100bp of contiguous sequence and 493bp from clone D27 was compiled (solid bars) and the sequence deposited in GenBank. Clone D25 did not produce sufficient PCR product for sequencing and was not rescued by *in vivo* excision. XhoI and EcoRI indicate restriction sites in the polylinker of the vector and constitute the 5' and 3' termini of each insert. Cloning was unindirectional so that the XhoI site represents the 3' terminal region of the gene in each case. The start codon of the gene was not apparent in the clones obtained. R, EcoRI Hae III restriction sites within the *Entamoeba* PFOR gene.

nas vaginalis subunits A and B PFOR (Hrdy and Müller, 1995) had 56% similarity with the *Entamoeba* sequence over approximately 800 bp and 55% and 60% identity with the *NifJ* gene encoding pyruvate: flavodoxin oxidoreductase in *Enterobacter agglo-merans* (Kreutzer *et al*, 1993) and *A. variabilis* (Bauer *et al*, 1993), over 870 and 360 bp, respectively (GenBank accession numbers X78558 and Q06879). Comparison of the translated protein sequence with sequences in GenBank showed 34% and 41.8% identity with *Klebsiella pneumoniae* (Cannon *et al*, 1988) and *A. variabilis* PFOR (GenBank accession numbers P03833 and L14925) over 375 and 287 amino acids respectively. Additionally, when the data were compiled by PRETTYPLOT both subunits of *T. vaginalis* PFOR and PFOR from *E. agglomerans* and *Rhodospirillum rubrum* (accession number X77515) showed significant homology with the *Entamoeba* protein (Fig 3). *Giardia duodenalis* PFOR (accession number L27221) shared some similarity with these proteins (data not shown).

The protein sequence encoded by the 5' terminal region of the longest 2.7kb *E. histolytica* DNA insert of D27 was homologous to PFOR from other organisms at the same level as the above mentioned proteins but there was no significant homology at the DNA level.

Of seven clones identified by polyclonal antisera raised against membrane antigens of HTH-56 : MUTM, two (X3 and X4) encoded the *Entamoeba* PFOR gene, two (X2 and X13) encoded Hsp70 (Ortner *et al*, 1992), the fifth (X17) encoded alkyl-hydroperoxide reductase (Bruchhaus and Tannich, 1993) and two others (X1 and X7) had no significant homology with either DNA or protein sequences in the GenBank data base.

DISCUSSION

The monoclonal antibody Eh208C2-2 MAb, raised against ultrasonically disrupted *E. histolytica* trophozoites of the HM-1 : IMSS strain reacted with an epitope on *Entamoeba* PFOR. Approximately 1% of cDNA clones derived from the SFL-3 strain in the Lambda ZAP library expressed this epitope and were detected in plaque assays by the monoclonal antibody. Polyclonal antisera raised against crude membrane preparations from the strain HTH-56 : MUTM, established in Thailand, also detected a predominance of clones expressing *Entamoeba* PFOR. These data indicate that in *E. histolytica*, PFOR is an abundant, highly conserved and immunogenic protein. Hsp70, alkyl hydroperoxide reductase as well as PFOR and two unidentified proteins were encoded by other clones selected with the polyclonal antisera.

Small clones (0.9kb) were detected in the cDNA library but the D series clones carrying the *E. histolytica* PFOR gene sequences were all larger than 1.2Kb. The epitope of Eh208C2-2 MAb is encoded by the 3' region of the gene since D28 encodes approximately one third of the gene at the 3' terminus. It is also seems likely that the epitope is a linear epitope which may prove very useful in constructing immunogenic peptides.

E. histolytica has an extremely AT rich genome with rDNA genes and flanking regions up to 85% AT rich (Sehgal *et al*, 1994) and *E. histolytica* PFOR gene is approximately 60% AT rich. The *Trichomonas* PFOR gene in comparison is 50% AT rich over an aligned region. The *A. variabilis* PFOR gene is also AT rich; over an alternative, aligned region, the *E. agglomerans* PFOR gene was approximately 50% AT rich in comparison to 60% for

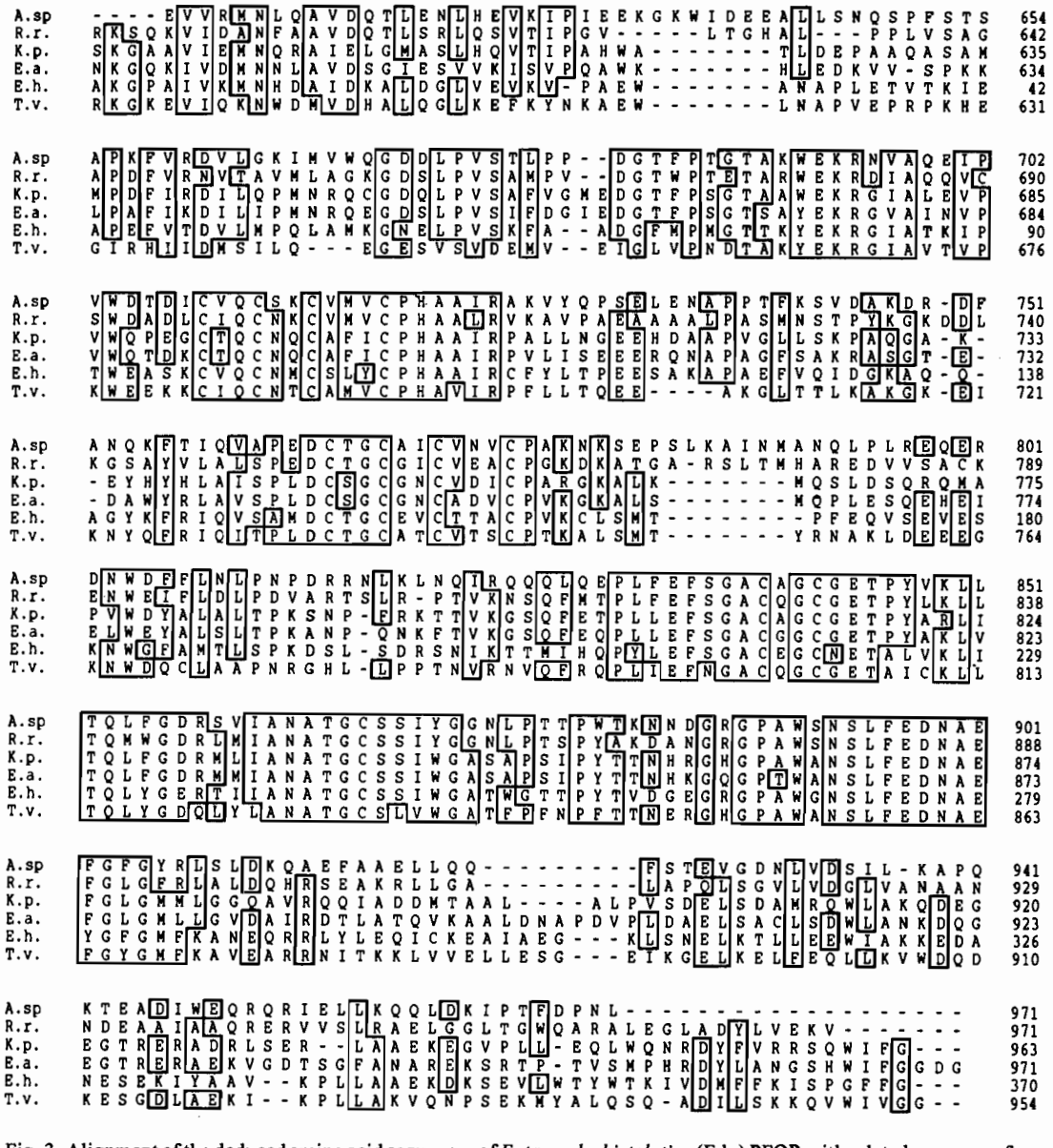

Fig 3–Alignment of the deduced amino acid sequences of *Entamoeba histolytica* (E.h.) PFOR with related sequences from *Trichomonas vaginalis* (T.v.), *Klebsiella pneumoniae* (K.p.), *Enterobacter agglomerans* (E.a.), *Anabaena variabilis* (A.v.) and *Rhodospirillum rubrum* (R.r.). The alignment was obtained with the PRETTYPLOT program in the extended GCG suite of programs (EMBL). The *E. histolytica* sequence (amino acid 1-370) reported here is equivalent to the GenBank sequence Z50193 from amino acid 590-959 with some significant differences.

Entamoeba. The *Giardia* PFOR gene, which is GC rich, was not homologous to the *Entamoeba* gene at the DNA level but the two protein sequences were similar (data not shown). Most of the variation in GC content of the PFOR genes occurs in the third base position of codons.

An immunotoxin (IT) comprising Eh208C2-2 MAb and the deglycosylated toxic moiety of ricin A (RA) chain, was prepared using the heterobifunctional cross-linker, N-succinimidyl 3-(2-pyridyldithiol) propionate (SPDP), and *in vitro* and *in vivo* tests suggest, both directly and indirectly, that the

IT produced was effective against *E. histolytica* (Sosa *et al*, 1994; Thammapalerd, 1994; Thammapalerd *et al*, unpublished). The purified IT when indirectly used in hamsters (*Mesocricetus auretus*), could protect the animals 100% (3 out of 3) from amebic liver abscess formation (Thammapalerd *et al*, unpublished). These data together with previous IFA data (Thammapalerd and Tharavanij, 1991) suggest that PFOR is membrane associated and at some stage probably appears on the parasite's surface. PFOR in the protozoa *Giardia* and *Trichomonas* is also membrane associated (Hrdy and Müller, 1995). Other successful immunizations against *E. histolytica* have involved gerbils and scid mice immunized with antibodies to serine rich *E. histolytica* protein (SREHP), native and highly conserved recombinant 29kDa cysteine-rich protein antigens, and recombinant 170 kDa surface adhesin (Zhang, *et al*, 1994a, b, c; Soong *et al*, 1995). Immunogenicity of the recombinant serine rich *E. histolytica* protein (rSREHP) vaccine in the African green monkey has also been demonstrated (Stanley *et al*, 1995).

The protection of parasite hosts by immunizations of parasite house keeping genes is not a novel phenomenon and mice, can be protected against schistosomiasis by immunization with parasite derived glyceraldehyde-3P-dehydrogenase (Goudot-Crouzel *et al*, 1989) and glutathione-S-transferase (Mitchell, 1989; Lui *et al*, 1995). Glutathione S-transferase vaccine cloned from *S. mansoni* could also cross protect patas monkey experimentally infected with *S. haematobium* (Boulanger *et al*, 1995). The advantage of using an essential parasite enzyme to raise a protective response is that variation in the target enzyme is minimal and likely to be highly conserved among all strains. Additionally, an enzyme such as PFOR, which is highly conserved among the anaerobic protozoa and bacteria, and which raises a protective response, is not present in the host so autoimmune responses to immunization are unlikely.

The prospect for immunotherapy of invasive amebiasis is now encouraging in animal models, including gerbils, African green monkey, scid mice and hamsters (Zhang *et al*, 1994, Soong *et al*, 1995; Stanley *et al*, 1995; Sosa *et al*, 1994; Thammapalerd, 1994; Thammapalerd *et al*, unpublished). The identification of a highly conserved essential *E. histolytica* enzyme susceptible to immunotherapy gives us new scope for drug and vaccine design.

Sequences reported in this paper have been submitted to the GenBank data base under the accession number L46793. Since we prepared this manuscript two sequences for *Entamoeba histolytica* PFOR from different strains have appeared in the data base and compare well with our sequence (accession numbers U30149 and Z50193), altnough there are significant differences, probably due to different parent strains used or subunit variation.

ACKNOWLEDGEMENTS

We wish to thank Dr Tan Chongsuphajaisiddhi, Dean, Faculty of Tropical Medicine, and Director, SEAMEO-TROPMED Regional Center for Tropical Medicine, Mahidol University, and Dr Donald McManus, Tropical Health Program, Queensland Institute of Medical Research, for their kind help in support of this study. Thanks are also extended to the Austrian Ministry of Sciences and Research, Thai-Austrian Cooperation in Science and Technology, Ministry of University Affairs, Mahidol University support (Trop Med) for the senior author to visit Austria and Australia and to the Tropical Health Program, Khon Kaen University for support for DK in Australia. Financial support by the National Research Council of Thailand and Faculty of Tropical Medicine, Mahidol University is gratefully acknowledged.

REFERENCES

Bauer CC, Scappino L, Haselkorn R. Growth of the cyanobacterium *Anabaena* on molecular nitrogen: *NifJ* is required when iron is limited. *Proc Natl Acad Sci USA* 1993; 90 : 8812-6.

Berghammer H, Auer B. "Easypreps": Fast and easy plasmid minipreparation for analysis of recombinant clones in *E. coli*. *BioTechniques* 1993; 14 : 527-58.

Boulanger D, Warter A, Trottein F, *et al*. Vaccination of patas monkeys experimentally infected with *Schistosoma haematobium* using a recombinant glutathione S-transferase cloned from *S. mansoni*. *Parasite Immunol* 1995; 17 : 361-9.

Bruchhaus I, Tannich E. Analysis of the genomic sequence encoding the 29kDa cysteine-rich protein of *Entamoeba histolytica*. *Trop Med Parasitol* 1993; 44 : 116-8.

Cannon M, Cannon F, Buchanan-Wollaston V, Ally D,

Ally A, Beynon J. The nucleotide sequence of the *nifJ* gene of *Klebsiella pneumoniae*. *Nucleic Acids Res* 1988; 16 : 11379.

Diamond LS, Harlow DR, Cunnick CC. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Trans R Soc Trop Med Hyg* 1978; 72 : 431-2.

Devereux J, Haeberli P, Smithies O. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* 1984; 12 : 387-95.

Goudot-Crozel V, Caillol D, Djabali M, Dessein AJ. The major parasite surface antigen associated with human resistance to schistosomiasis is a 37kD glyceraldehyde-3P-dehydrogenase. *J Exp Med* 1989; 170 : 2065-80.

Hrdy I, Müller M. Primary structure and eubacterial relationships of the pyruvate : ferredoxin oxidoreductase of the amitochondriate eukaryote *Trichomonas vaginalis*. *J Mol Evol* 1995; 41 : 388-96.

Johnson P. Metronidazole and drug resistance. *Parasitol Today* 1993; 9 : 183-6.

Kerscher L, Oesterhelt D. Pyruvate : ferredoxin oxidoreductase-new findings on an ancient enzyme. *TIBS* 1982; 7 : 371-8.

Kreutzer R, Dayananda S, Klingmuller W. Cotranscription of the electron transport protein genes *nifJ* and *nifF* in *Enterobacter agglomerans* 333. *J Bacteriol* 1991; 173 : 3252-6.

Liu SX, Song GC, Xu YX, Yang W, McManus DP. Anti-fecundity immunity induced in pigs vaccinated with recombinant *Schistosoma japonicum* 26kDa glutathione-S-transferase. *Parasite Immunol* 1995; 17 : 335-40.

Mitchell GF. Glutathione-S-transferase- potential components of anti-schistosome vaccines? *Parasitol Today* 1989; 5 : 34-5.

Ortner S, Plaimauer B, Binder M, Wiedermann G, Scheiner, O, Duchêne M. Humoral immune response against a 70-kilodalton heat shock protein of *Entamoeba histolytica* in a group of patients with invasive amoebiasis. *Mol Biochem Parasitol* 1992; 54 : 175-84.

Pharmacia LKB Biotechnology, Pharmacia. T^7 Sequencing™ Kit Instructions 1995; pp. 1-31.

Plaimauer B, Ortner S, Wiedermann G, Scheiner O, Duchene M. Molecular characterization of the cDNA coding for translation elongation factor-2 (EF-2) of pathogenic *Entamoeba histolytica*. *DNA Cell Biol* 1993; 12 : 89-96.

Sehgal D, Mittal V, Ramachandran S, Dhar SK, Bhattacharya A, Bhattacharya S. Nucleotide sequence organization and analysis of the nuclear ribosomal DNA circle of the protozoan parasite *Entamoeba histolytica*. *Mol Biochem Parasitol* 1994; 67 : 205-14.

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.

Smith TF, Waterman MS. Comparison of biosequences. *Adv Applied Math* 1981; 2 : 482-9.

Soong CJG, Torian BE, Abd-Alla MD, Jackson TFHG, Gatharim V, Ravdin JI. Protection of gerbils from amebic liver abscess by immunization with recombinant *Entamoeba histolytica* 29-kilodalton antigen. *Infect Immun* 1995; 63 : 472-7.

Sosa RJ, Thammapalerd N, Awaikairt S, Tharavanij S. Immunotoxin against *Entamoeba histolytica* : An *in vitro* study. 11th FAOBMB Symposium, Biopolymers and Bioproducts: Structure, Function and Applications, Marriott Royal Garden Riverside Hotel, Bangkok, Thailand, 15 - 18 November 1994. Abstract No. PO-D-07.

Stanley SL Jr, Blanchard JL, Johnson N, *et al*. Immunogenicity of the recombinant serine rich *Entamoeba histolytica* protein (SREHP) amebiasis vaccine in the African green monkey. *Vaccine* 1995; 13 : 947-51.

Thammapalerd N. Progress in amebiasis research in Thailand. Program Update Late-Breaker Submission. 43rd ASTMH Annual Meeting, Hyatt Regency Hotel Cincinnati, Ohio, USA. 12-19 November 1994, Abstract No. 213A.

Thammapalerd N, Tharavanij S. Biomedical applications of monoclonal antibodies (McAb) against *Entamoeba histolytica*. In: Biotechnology for Biological Control of Pests and Vectors. Maramorosch K. ed. Florida: CRC Press 1991: pp. 163-78.

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

Townson SM, Boreham PFL, Upcroft P, Upcroft JA. Resistance to the nitroheterocyclic drugs. *Acta Tropica* 1994a; 56 : 173-94.

Townson SM, Hanson G, Upcroft JA, Upcroft P. A purified ferredoxin from *Giardia duodenalis*. *Eur J Biochem* 1994b; 220 : 439-46.

Upcroft JA, Upcroft P. Drug resistance and *Giardia*. *Parasitol Today* 1993; 9 : 187-90.

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.

World Health Organization. Report on World Health, WHO, Geneva, Press Release WHO/28 May 1, 1992 : 1-7.

Zhang T, Cieslak PR, Foster L, Kunz-Jenkins C, Stanley SL Jr. Antibodies to the serine rich *Entamoeba histolytica* protein (SREHP) prevent amebic liver abscess in severe combined immunodeficient (SCID) mice. *Parasite Immunol* 1994a; 16 : 225-30.

Zhang T, Cieslak PR, Stanley SL Jr. Protection of gerbils from amebic liver abscess by immunization with recombinant *Entamoeba histolytica* antigen. *Infect Immun* 1994b; 62 : 1166-70.

Zhang T, Stanley SL Jr. Protection of gerbils from amebic liver abscess by immunization with a recombinant protein derived from the 170-kilodalton surface adhesin of *Entamoeba histolytica*. *Infect Immun* 1994c; 62 : 2605-8.