Biotechnological Approaches to Diagnosis, Prevention and Treatment of Infectious Diseases

Proceedings of a Workshop jointly organized by

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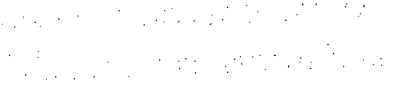

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TP 248.14

Edited by

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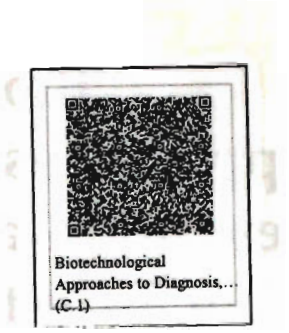

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CONTENTS

APPENDICES

DIAGNOSIS, PREVENTION AND TREATMENT OF INFECTIOUS DISEASES: MAIN ISSUES FOR POSSIBLE ASEAN-JAPAN CO-OPERATION

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INTRODUCTION

Throughout the ASEAN region, as throughout the rest of the developing world, infectious diseases constitute a main health problem along with malnutrition and other problems related with poor standards of living. Although these problems are related with poverty and social conditions, and their effective solutions would depend on socio-economic development in the affected countries, many dimensions of the problem are scientific and technological in nature. This is true especially for tropical infectious diseases, for which diagnosis, prevention and treatment are still largely subject to further improvement. This is so because these diseases affect mainly the people in developing countries, who can ill afford the cost of their prevention and treatment, and therefore do not provide the market for the major pharmaceutical companies of the world.

For the developing countries, the burden arising from these tropical infectious diseases are indeed heavy, not only in terms of deaths, but also in terms of economic and social costs due to illnesses and their effects thereafter. Although the ASEAN countries can be considered to be relatively "advanced" developing countries, the tolls from these diseases are still very high, especially in the case of malaria which is by far the major disease and is posing increasingly greater danger due to resistance of the parasites to various drugs and reduced effectiveness of insecticidal spray. It is compulsory therefore for the ASEAN countries to pay more attention to these problems for their own interest, and for the interest of the rest of the developing world.

In addition to the necessity to solve the current problems on tropical diseases of their own, there is another powerful reason for the ASEAN countries to pay special interest in these problems. Being advanced developing countries, the ASEAN countries are now in a unique position where not only the diseases are present, but the potential tools for solving the problems from these diseases are also available. Hence, unlike in other less developed countries, relatively advanced research can be done in the endemic ASEAN countries, ranging from new therapeutic and immunization trials to basic molecular biology and the search for new tools in biotechnology. An ideal opportunity is also open for other more advanced countries like

Japan to collaborate with the ASEAN countries in the search for new knowledge and tools against tropical infectious diseases, which comprise one of the last remaining frontiers in biomedical science in this century. The present situation of lack of effective immunization and therapy against these diseases is reminiscent of the situation a century ago, before Shibasaburo Kitasato, with Robert Koch and Emil von Behring, established immunization against tetanus and diphtheria. The difference is that in the present case the discoveries and development need not be in Berlin, but can be in Tokyo, Bangkok or Manila.

THE PROBLEMS

The problems of tropical infectious diseases are mainly due to parasites, mainly protozoa and helminths, but may also be defined broadly to include viruses and bacteria as well. The estimate of global incidence of major parasitic infections is given in Table 1

Most of the diseases listed in this Table occur and are major problems in Southeast Asia. The status of these problems in Southeast Asia has been recently reviewed[2]. In specific terms, Southeast Asia was the focus for the spread of chloroquine resistance of falciparum malaria in the early sixties, of Fansidar resistance in the early eighties and of mefloquine resistance at present. Some diseases are specific to the region such as liver fluke (*Opisthorchis viverrini*), elephantiasis due to *Brugia malayi*, and schistosomiasis due to *S. mekhongi*. Although some of these diseases can be effectively treated, for examples schistosomiasis and liver fluke disease by the drug praziquantel, they remain major public health problems due to various factors, including reinfection due to poor living conditions, eating habits, etc. and other socio-economic conditions. Despite the socio-economic dimension of the problem, technology is an important part of the sclution, since it can lead to effective prevention and treatment of these diseases.

Table 1 Incidence of major tropical infectious diseases.

Type	*Number of infections (millions)*
Protozoa	
Malaria	800
Amoebiasis	400
African trypanosomiasis	1
Chagas' disease	18
Helminths	
Ascariasis	1000
Hookworm	900
Lymphatic filariasis	90
Schistosomiasis	250
Liver fluke	
Viruses and bacterial	
Diarrhoeas	5000
Dengue	4
Japanese encephalitis	
Tuberculosis	1000

Based on Warren[1] and other estimates.

THE APPROACHES

In the light of the magnitude of the problems, and the fact that the ASEAN countries, like other developing countries, cannot afford large financial resources nor have enough trained manpower to provide adequate services, the most suitable approaches should be development of operationally simple and practical methods for monitoring and control of the diseases at the public health level, and inexpensive yet effective prevention and treatment measures at the individual level. These require the use of new advances in biomedical science, such as the use of nucleic acid hybridization for new diagnostic procedures, the use of subunit vaccines from genetic engineering or of synthetic peptide vaccines, and the use of new drugs designed from detailed knowledge of the target enzymes from the causative agents of the diseases. Table 2 indicates the new approaches which can be developed in greater detail.

Table 2 Approaches based on molecular biology and new biotechnology to diagnosis, prevention and treatment of tropical infectious diseases.

Problems	Approaches
Diagnosis	DNA probes, PCR
	Immunodiagnosis
	Fluorescence microscopy-based
Prevention	Subunit Vaccines
	Peptide vaccines
	Chemical prophylaxis
Treatment	Rational drug design
	Screening from natural products
Related problems (vector control, etc.)	Applied DNA technology for field uses

The search for these new tools and their development for practical use require research and development, ideally of collaborative nature between the institutions in developed and developing countries, and the applications of the new procedures developed require sufficient training of personnel both at the laboratory and the field levels.

RESEARCH ACCOMPLISHMENTS AND EXPERTISE OF THAI INSTITUTIONS

Table 3 gives information on the research interests and expertise of some institutions working on biomedical/biotechnological aspects of tropical infectious diseases.

Table 3 List of some institutes in Thailand with expertise and research accomplishment in biomedical/biotechnological aspects of tropical infectious diseases.

Institution	Expertise/Accomplishment
Mahidol University	
Faculty of Science	Molecular biology of malaria, cloning of parasite folate enzymes, antimalarial drug mechanisms, DNA probes for parasite/vector detection and classification, immunopathology of liver flukes, microanatomy of schistosomes/snails.
Faculty of Tropical Medicine	Diagnosis and treatment of various tropical diseases, clinical and field research on parasitic diseases and vectors, centre of SEAMEO tropical medicine programmes.
Faculty of Medicine, Ramathibodi	Malaria-thalassaemia interaction.
Chulalongkorn University	
Faculty of Science	Collection and classification of malaria parasites by antigenic diversity and drug resistance.
Faculty of Medicine	Metabolic pathways and enzymes of malaria parasites.

Institution	Expertise / Accomplishment
Armed Forces Research Institute	Various collaborative research of medical science at field and laboratory levels with Walter Reed Army Institute of Research, eg. malaria vaccines, new drug regimes, diagnosis of diarrhoeal diseases, vector studies.
Ministry of Public Health	Field works including research on monitoring and control of diseases.

Further details concerning the research interest and expertise of the author's laboratory in Mahidol University are given in Appendix 1.

POSSIBLE TOPICS AND MODES OF COLLABORATION

In order to establish successful collaboration between Japan and ASEAN countries, it is necessary to identify the leading institutions working on the applications of molecular biology and biotechnology to the problems of parasitic diseases. Some of these institutions and researchers have already been identified by the ASEAN Subcommittee on Biotechnology and are represented in this seminar, while others need to be identified further, possibly through their previous involvement with such programmes as the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Unlike the Special Programme which is global in scope and covers research and training in diverse areas, the possible ASEAN-Japan collaboration should be well defined to cover the biomedical/biotechnological aspects of the problems relevant to the Southeast Asia region.

It is proposed that selected institutions/laboratories from the ASEAN countries and Japan with interests and expertise in biomedical/biotechnological aspects of tropical infectious diseases join together to form a Network on Biotechnology for Tropical Diseases, with activities in joint research and training in diagnosis, prevention and treatment of tropical diseases through application of biotechnology. The objectives for such network are

- to undertake joint research on tropical infectious diseases with emphasis on new biotechnology and molecular biology

- to transfer technology between Japan and the ASEAN countries on appropriate aspects concerning diagnosis, prevention and treatment of tropial infectious diseases

- to organise regional training and workshop activities, with the aim of diffusion of new technologies to appropriate health and scientific personnel.

These objectives are sharply defined and are complementary to other related existing programmes such as UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and the SEAMEO (Southeast Asian Ministers of Education Organization) TROPMED Programme. The network should obtain funding from relevant agencies in Japan and the ASEAN countries, through joint proposals to be developed by the ASEAN Subcommittee on Biotechnology.

From these objectives, research and training agenda can be proposed as follows:

Diagnosis: Development of suitable DNA probes and primers for specific and sensitive detection of parasites and vectors common in the Southeast Asian region, and for their classification; rapid non-radioactive methods for detection procedures, suitable for use in field studies and field stations; development of suitable immunodiagnostic and other new diagnostic procedures; improvement in current procedures for diagnosis, eg. microscopic procedures improved through *in situ* hybridization techniques; diagnostic procedures for parasite drug resistance.

Prevention: Assessment of genetic basis of immune status to parasitic diseases; natural immunity and its basis; immune response to candidate vaccines; antigenic diversity and variation in parasites; mechanisms of immunity; role of host immune response in disease severity; chemical prophylaxis.

Treatment: Mechanisms of anti-parasitic drug action and drug resistance; metabolism of the parasites and their enzymes as potential targets for chemotherapy; cloning and expression of target enzymes; molecular structures and structures of the catalytic sites; design and synthesis of potential inhibitors; drug screening from natural products and other sources; drug combinations, especially with reversers of drug resistance.

The proposed network, once formed, should develop workplans which have appropriate research and training components to be implemented in the units of the network in various ASEAN countries and in Japan. The network should have two main types of fund, common fund and fund for each unit of the network. The common fund should be used for collaborative

research and training activities to be shared by all countries, while unit funding should be for exclusive use of the respective countries, with counterpart funding from sources within the countries. The network members should meet at least annually to review past achievements and plan further activities. It is proposed that the period for initial phase of the network should be 3-5 years, after which a review of performance should be undertaken.

It is hoped that Japan will take the leading role in the initiation and running of this network, which will be a unique contribution to ASEAN-Japan co-operation.

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APPENDIX 1

Unit of Parasite Biochemistry, Department of Biochemistry, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand.

Personnel : 3 Ph.D. staff, 1 M.Sc. staff, 3 Ph.D. students, 4 M.Sc. students, 4 research assistants.

This Unit, which started as a research group in 1974, and began to receive funding from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) in 1978, was officially created in 1980 with support from the Thai Government, the Rockefeller Foundation, TDR and other sources. The aims of the Unit are to study the biochemistry of parasites and mechanisms of chemotherapy of tropical diseases. Most of the work is concentrated on malaria, including studies of folate metabolism in the malarial parasites, mechanisms of action of antimalarials, changes in red cell membrane structure and function induced by malarial infection, and mechanisms of protection from *Plasmodium falciparum* infection of genetically variant red cells commonly found in Southeast Asia. Some of the achievements are:

1979 - First report of changes in phosphorylation of membrane proteins of malaria-infected red cells.

1984 - Kinetic and structural changes in malarial dihydrofolate reductase was shown as the basis for pyrimethamine resistance.

1985 and subsequent work - GTP cyclohydrolase, the first enzyme in malarial folate *de novo* synthesis pathway, was identified and characterized; folate salvage pathway was demonstrated; methionine synthesis pathway was characterized.

1986 and subsequent work - Enhanced phagocytosis of some genetically variant red cells infected by *P. falciparum* was demonstrated.

1987 - Demonstration of oxidative action of artemisinin, the new antimalarial first developed in China.

1990 - Cloning and expression of *P. falciparum* dihydrofolate reductase-thymidylate synthase in *E. coli*.

1992 - Design, cloning and expression of synthetic gene for *P. falciparum* dihydrofolate reductase-thymidylate synthase in *E. coli*.

Since its establishment, the Unit has published 90 papers in parasite biochemistry and related areas, and has produced a manual on "Application of Genetic Engineering to Research on Tropical Disease Pathogens with Special Reference to Plasmodia" (TDR, Geneva, pp. 554, 1986). It has trained 6 Ph.D. graduates and 31 M.Sc. graduates, most of whom are now staff members of various universities in Thailand. Since 1986, the Unit has been collaborating extensively with the research group of Prof. Dan V. Santi, University of California, San Francisco. Other collaborations include those with Dr. G. Jaureguiberry (INSERM, Paris) and Dr. S. Meshnick (University of Michigan, Ann Arbor).

A PARASITE CYSTEINE PROTEASE: PURIFICATION, MONOCLONAL ANTIBODIES, cDNA CLONING, AND POSSIBLE APPLICATION

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Previous works in our and other laboratories have demonstrated that a schistosome cysteine protease hydrolyzes host hemoglobin and is probably responsible for the parasite's nutritional requirement (Sauer and Senft, 1972; Aoki, 1980). In addition to this physiological significance, the enzyme released from the blood-dwelling parasite was thought to elicit host immune response, yielding the production of antibodies. This idea was proven experimentally that the partially purified parasite protease entailed substantial species specificity in immunodiagnostic tests such as skin test and RAST (Senft and Maddison, 1975; Aoki, 1980). Thereafter, we have been conducting two lines, basic and applied, of investigations on the parasite cysteine protease, using *Fasciola* sp. as a model material.

PURIFICATION (Yamasaki, Aoki and Oya, 1989)

A proteolytic enzyme was purified 576-fold from the homogenate of liver fluke *Fasciola* sp. by ammonium sulfate fractionation, Sephadex G-75 gel filtration, and activated thiol-Sepharose covalent chromatography. The purified enzyme migrated as a single band with a molecular weight of 27 kDa on SDS-PAGE, and consisted of about 20% acidic, 10% basic, and 40% hydrophobic amino acids. Marked inhibition of the enzyme activity by specific inhibitors, such as leupeptin, antipain, TLCK, and stimulation by sulfhydryl reagent, dithiothreitol (DTT), indicate that the enzyme is categorized as a cysteine protease and does not belong to the class of serine, aspartic acid or metal protease.

MONOCLONAL ANTIBODIES (Yamasaki, Aoki and Oya, 1989)

To further characterize this protease, we attempted to produce monoclonal antibodies specific for the enzyme. For the immunization, the mouse received a subcutaneous injection of partially purified enzyme (Sephadex G-75 step). After the two more booster injections of the antigen, mouse spleen cells and myeloma cells were subjected to the fusion in the presence of polyethyleneglycol. HAT selection and cloning of hybridomas resulted in the cells secreting monoclonal antibodies.

Specificity of these monoclonal antibodies was verified by two ways. (1) Upon immunoblotting analysis, these monoclonal antibodies recognized a single protein band of 27 kDa, corresponding to the molecular weight of the purified enzyme. (2) The binding of the enzyme with monoclonal antibodies and separation by centrifugation of the immune complex yielded the precipitated activity. The precipitate was composed of only 27 kDa protein and IgG molecules.

INTRACELLULAR LOCALIZATION
(Yamasaki, Kominami and Aoki, 1992)

The intracellular localization of the parasite cysteine protease was examined by light and electron microscopic immunocytochemistry using a monoclonal antibody specific to the enzyme. Immunoperoxidase staining was predominantly restricted to large numbers of granules in the parasite intestinal epithelial cells and to host erythrocytes present in the intestinal lumens.

In immunogold electron microscopy, the gold particles were consistently deposited on the electron-dense secretory granules in intestinal epithelial cells and on the intestinal contents. These findings strongly suggest that the *Fasciola* protease in the secretory granules is secreted as a digestive enzyme into the intestinal lumen, where it may play an important role in the extracellular degradation of host proteins, including hemoglobin, for the parasite nutrition.

cDNA CLONING (Yamasaki and Aoki, manuscript in preparation)

For more basic understanding of physiology and molecular structure of this particular cysteine protease, we have started to clone a gene encoding this protein. For this purpose, first of all, a *Fasciola* cDNA library has been constructed in the λgt11 cloning vector. Plaques were screened by two different probes, i.e., mouse monoclonal or polyclonal antibodies and nonRI (digoxigenin)-labeled cDNA fragment (amplified by PCR, 498 bp), the latter probe giving satisfactory results.

The digoxigenin-labeled probe was prepared as described previously (Sakanari *et al.*, 1989). For the design of primers and amplification by PCR of cDNA fragment, we noticed two highly conserved sequences of amino acids (QGQCGSCW---------YWIVKNSW) which contain the active site Cys and Asn residues of cysteine proteases. According to these sequences, two oligonucleotide primers, 5'-*Eco* RI site-CAR GGI CAR TGY GGI TCI TYG TGG-3' and 3' -ATR ACC TAR CAI TTY TTR AGI ACC-*Hind* III site, were synthesized. These primers and template *Fasciola* cDNA gave a PCR product, which was purified by agarose-gel electrophoresis, determined for nucleotide sequence and found to be composed of 498 bp, and labeled with digoxigenin.

Four positive clones were isolated through the screening of about 20,000 plaques. Among them, the clone B7-2 had a 1.1 kb fragment which was sub-cloned in a plasmid vector pUC18 for nucleotide sequencing. The sequence was determined by dideoxy method in an automated DNA sequencer of Applied Biosystems (model 373A).

An open reading frame of cDNA contained 978 bp which correspond to 326 amino acids. The deduced amino acid sequence is divided into 3 parts, namely, pre-region, pro-region, and mature enzyme, consisting of a precursor protein for cysteine protease. The N-terminal pre-region has 15 amino acid residues starting with Met, the majority of which are hydrophobic amino acids, and thus is regarded as signal sequence for the targeting of precursor protein to endoplasmic reticulum; the pro-region contains about 90 amino acid residues; the C-terminal part comprising about 220 amino acid residues is the mature enzyme. Alignment of the amino acid sequence of our mature enzyme with those of other cysteine proteases indicates that Cys (25), His (162), and Asn (182) are believed to be the active site residues of the enzyme, and conserved in all cysteine proteases examined.

The percentage of homologies were calculated for amino acid sequences between our protease and other various cysteine proteases. Pre- and pro-regions showed a low homology ranging from 27 to 30%. The mature enzyme had high homologies (46 to 50%) when compared with mammalian cathepsins H and L, and lower homologies with mammalian cathepsin B (27-29%). These results suggest that the primary structure of our mature enzyme resembles mammalian cathepsin L and H; these cathepsins are known to occur in the cytosolic organelle, the lysosome. Interestingly, the *Fasciola* cysteine protease resembling the lysosomal cathepsin L and H appears to occur in the secretory granules in the intestinal epithelial cells.

(POSSIBLE) APPLICATION

We had a preliminary result of application of the partially purified *Fasciola* cysteine protease (Sephadex G-75 step) as antigen for ELISA in the immunodiagnosis of human fascioliasis. This method was very sensitive without any false negative or false positive reactions, and highly specific, giving little detectable cross-reactivity with several other human parasitic diseases; this is an example of actual and useful application.

Theoretically, we now have several possible applications of our tools, i.e., homogeneously purified cysteine protease, monoclonal antibodies, and cDNA encoding the enzyme.

The purified enzyme could be used for the N-terminal amino acid sequencing in automated sequencer and for the peptide sequences after fragmentation by proteases and separation by HPLC. The information of

these sequences could lead to the design of oligonucleotide primers for PCR. These techniques, results, and informations, in general, are all applicable for the cloning of particular gene(s).

Monoclonal and mono-specific antibodies could be used as probes for the particular protein to determine intracellular localization, as reported in this paper. In Western blotting analysis or immuno-precipitation, the specific monoclonal antibodies only recognized the protease, and thus these antibodies could be used for the detection of particular antigens in biological materials such as human sera (antigen detection in immunodiagnosis)

Since we now know the deduced amino acid sequence of our protease precursor, we could synthesize polypeptides corresponding to partial sequences found in pre-region, pro-region, and mature enzyme to produce monospecific antibodies. These antibodies could be used as probes in immunocytochemistry of cysteine protease which might indicate loci of processing of the pre- and pro-regions, i.e., intracellular transport or targeting of the particular protein.

We could also produce a large amount of precursor protein and mature cysteine protease in *E. coli* and other host cells, and then could purify these proteins easily. The purified proteins could be available for immunodiagnosis for human and cattle parasitic diseases. Considering the essential nature of particular proteins in parasites, they could be tested as candidates for vaccination antigens.

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REVIEW : INFECTIOUS DISEASE PROJECT OF JICA IN THAI-NIH, WITH SPECIAL EMPHASIS ON THE MOLECULAR AND BIOTECHNOLOGICAL APPROACHES

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SUMMARY

Since 1985, an infectious disease project has been developing in Thai National Institute of Health (Thai-NIH) by Cooperation between Thai and Japanese scientists under the sponsorship of Japan International Cooperation Agency (JICA). This project is to strengthen the reference and research activities of Department of Medical Sciences, Ministry of Public Health, in the area of infectious disease control.

The main items for cooperation were concerned with laboratory diagnosis of microbial diseases, pilot study of vaccine production and quality control, molecular epidemiology of viral diseases, ecology of vector insects and rats, breeding of SPF mice and quails, and establishment of P3 and RI laboratories. Special emphasis was placed on the application of molecular and biotechnological approaches which may serve as a simple and rapid means for diagnosis and surveillance of infectious diseases. The project activities are reviewed briefly in this text.

INTRODUCTION - Background of Project

Much improvements in public health conditions have been made for the past three decades in Thailand under successive Five-Year National Economic and Social Development Plans. This is evidenced by the decrease of mortality, extension of the average life expectancy, and the enlargement of population. However, the improvement in this way does not always mean that infectious and parasitic diseases have been brought under the complete control in this country.

Though the mortality became lower than before, the morbidity of those diseases is still high as in other developing countries, and they were prevalent as an epidemic, endemic or sometimes pandemic disease.

In view of this situation, the Ministry of Public Health had an idea to establish a new institute which may function as the technological and reference center in the national network of public health laboratory services, especially such as that equipped with advanced facilities and modern laboratory apparatus. At the same time, the introduction of molecular biology and biotechnology was actively discussed and planned for the control of infectious

diseases. However, it must be noted that the ultimate target of the Ministry's plan was to upgrade health services in rural areas through the above-stated national network.

INITIATION AND DEVELOPMENT OF THE RESEARCH PROMOTION PROJECT IN THAI-NIH

The plan to establish a new central institute by the Ministry of Public Health as stated above was then shown to Japanese Government asking the financial assistance (Grant-Aid for the building and equipment) together with technological cooperation in the area of infectious disease control. This request was accepted by Japanese side. Building constraction was started in December 1984 and completed 2 years later.

Meanwhile, a project with the title The Research Promotion Project of the National Institute of Health started on April 28, 1985 under the Record of Discussion (R/D) signed by the authorities of the Thai Government and the Japanese Implementation Survey Team, JICA, on that day. The proposed period of cooperation was five years. When the project period came to the end with many successful achievements, a new agreement was made that the project should be extended two more years expecting the further development of cooperation and the reinforcement of still unsatisfactory items. This extension period was terminated in July, 1992, but immediately succeded by the follow-up period of two years under the agreement between two countries to bring this project into a successful end.

MECHANISM AND STRATEGY IN THE PROJECT (Fig. 1-3)

The mechanism of our JICA technological cooperation consists of four components; dispatch of Japanese experts, training of Thai scientists (fellows) in Japan, provisions of machinery and equipments, and budgetary assistance for the middle level staff training course. Up to this time, 135 Japanese experts of various speciality came to NIH and 47 Thai fellows stayed in Japan to learn laboratory technologies or to make observation trip (Fig. 1).

The objective of our project is rather clear, namely the transfer of technologies which are available for the diagnosis and prevention of infectious diseases and the introduction of modern approaches to the research activity in this area. To work along this line, we set up a strategy as follows.

First of all, efforts were made to promote the technical capability of Thai staffs in diagnostic microbiology and immunology. In some instances, the development of reagents and kits was considered to serve reference and

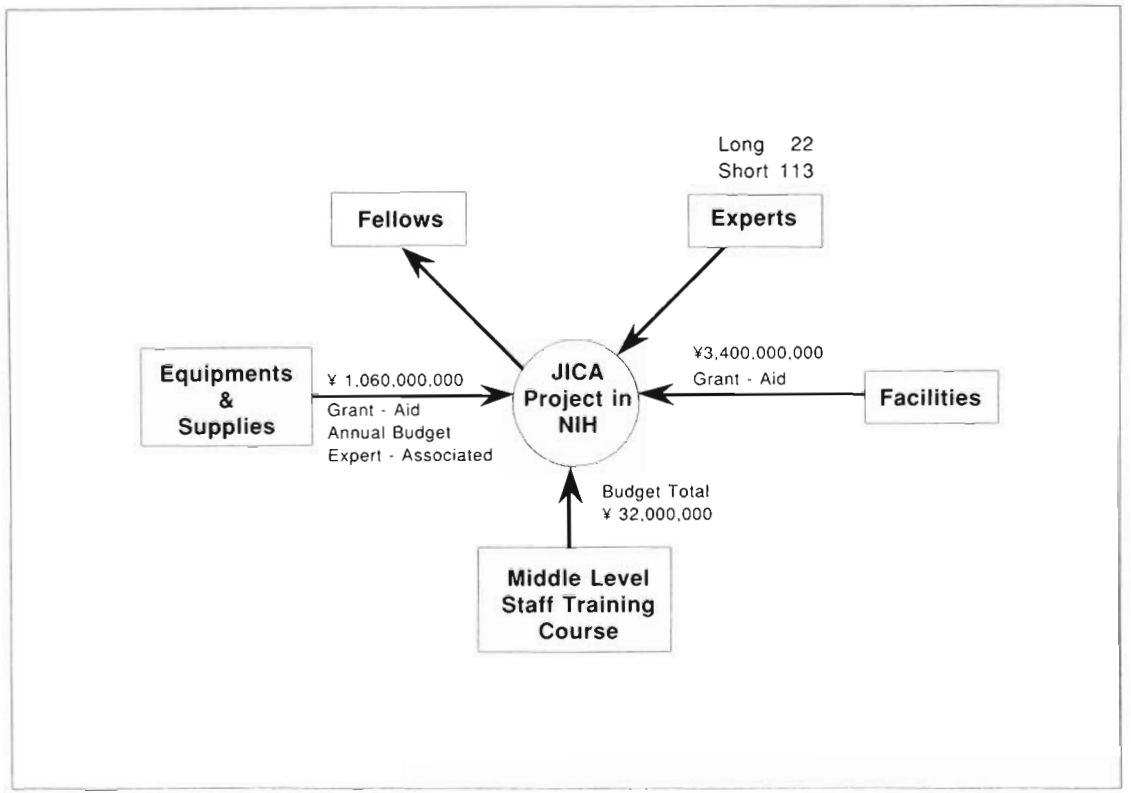


Fig. 1. Mechanisms of Cooperation.

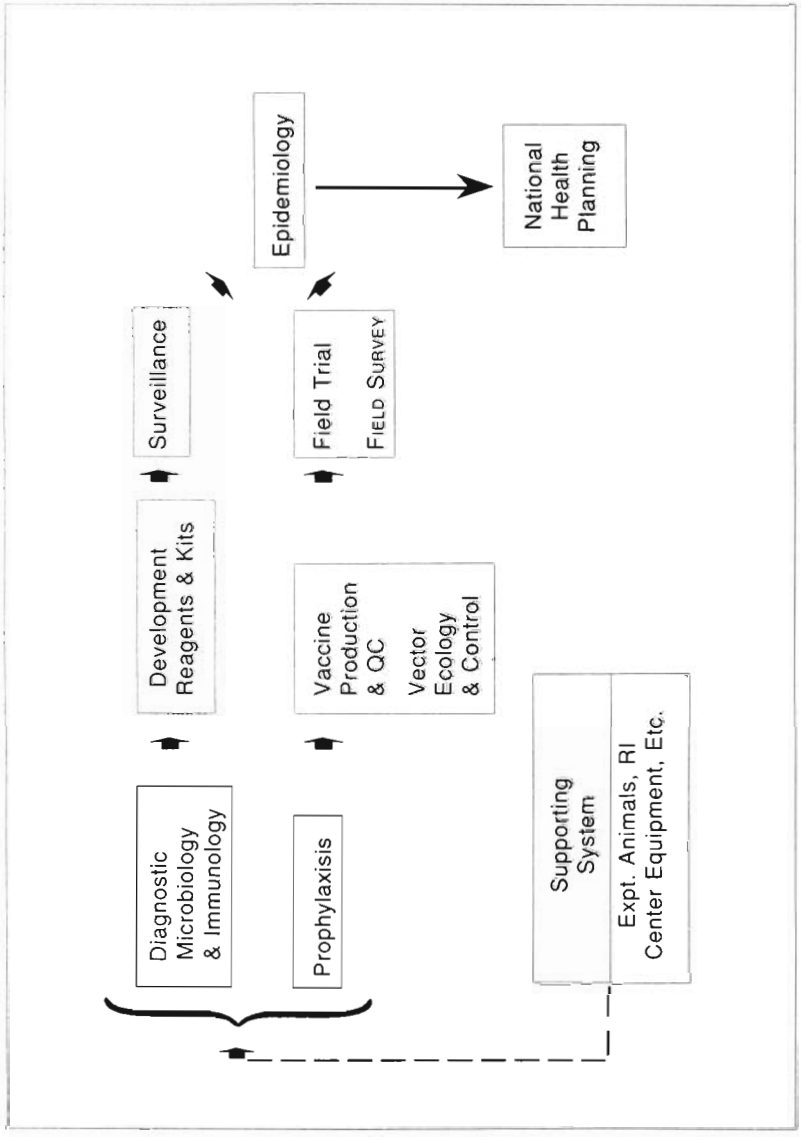


Fig. 2. Strategy in Infectious Disease Control Project: Basic Ideas Defined in RD.

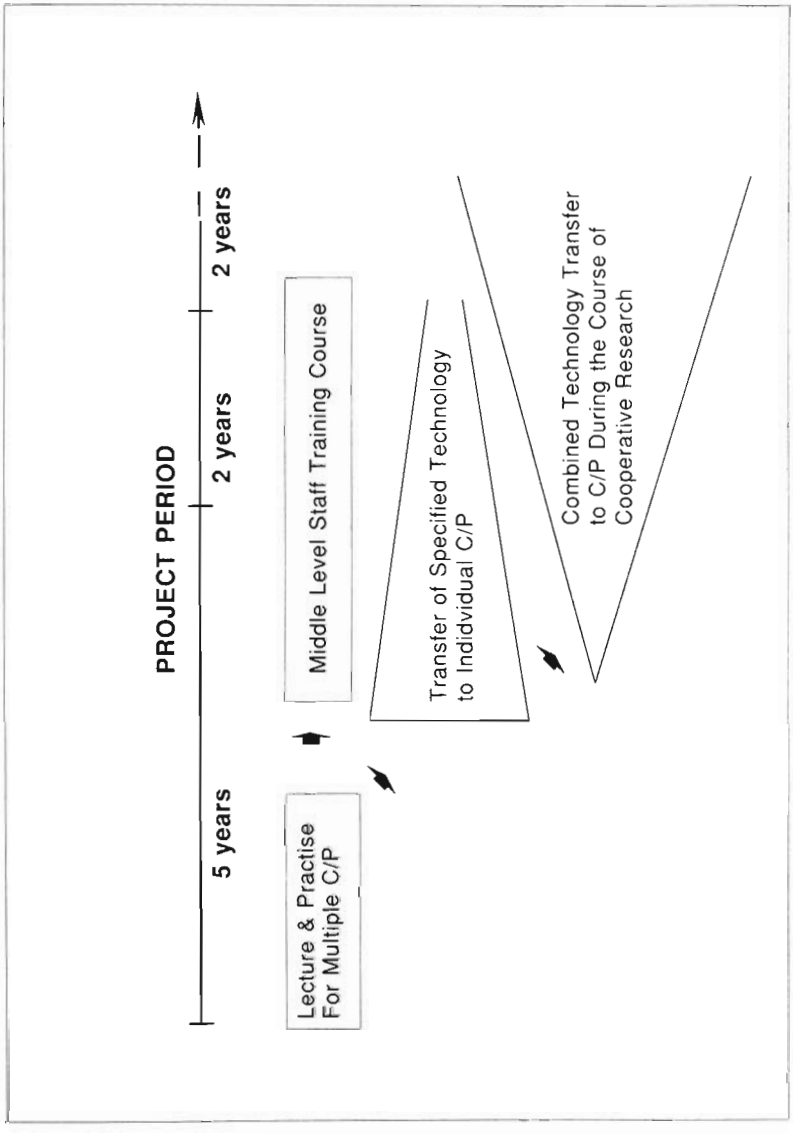


Fig. 3. The Change in the Strategy of Technological Cooperation.

surveillance activities of the institute. Meanwhile, another effort was directed to the prophylactic aspect such as vaccine development including quality control and vector ecology aiming at the device of control measure against insects.

These two lines of technical cooperation developed into various field trials and epidemiology, and the results were expected to serve public health administration of the Ministry (Fig. 2).

As for the style of technology transfer, it has been changing according to the stage of project. In the initial stage, "lecture and practise" as in universities was employed mainly for the training in the general or common laboratory techniques. Then, it was replaced by the transfer of specific technology to the individuals who need it frequently. In the later stage, technology transfer was made during the cooperative research activity so that Thai counterparts can acquire variour laboratory techniques and procedures by using them repeatedly from their own purpose and interest (Fig. 3).

REQUESTED ITEMS FOR CO-OPERATION

In the field of diagnostic bacteriology, the following items were requested as the subject of technology transfer.

..... General laboratory techniques

..... Numerical classification

..... Special identification methods

1) Analysis of fatty acid pattern by GLC
2) DNA hybridization
3) Plasmid pattern analysis
4) Phage typing
5) MIC (drug sensitivity test)

..... Identification of individuals bacterial species

1) Mycoplasma
2) *Legionella*
3) *Clostridium defficile*
4) Gram-negative non-fermentative rod bacteria
5) Cholera toxins

In addition, the next three subjects were employed as the co-operative research projects.

..... Melioidosis and *Pseudomonas pseudomallei*

..... Molecular approach for the detection of *Salmonella* contamination in foods

..... Introduction of laboratory-originated information system for enteropathogenic bacteria

In the area of virology, the following items were requested as the subject of technology transfer.

..... Introduction of molecular and genetical technologies in the study on rotavirus, influenza virus, HIV, dengue virus, HCV, and AHCV

1) RNA-PAGE
2) Oligonucleotide mapping
3) Cloning
4) Seguencing
5) PCR

..... Introduction of monoclonal antibodies for the virology of rotavirus, rabies virus, JE virus and dengue virus

..... Preparation of diagnostic (IFA) kits for HIV

..... Molecular epidemiology of virus infection; rotavirus, HIV, influenza

In the area of vaccine development and quality control, the following 4 items were requested as the subjects of technology transfer.

..... Tissue culture rabies vaccine

..... Japanese encephalitis vaccine from infected mouse brains

..... Rubella vaccine

..... Acellular pertussis vaccine

In the case of entomology, the main items of cooperation were as follows.

..... Development of museum

..... Vector ecology

..... Biological and chemical control of vector insects and rats

In the area of experimental animals, the technical cooperation was made in the following items.

..... The general method of breeding

..... Microbial monitoring

..... Genetic monitoring

..... Introduction of 4 colonies of SPF mice, SPF quail colony, hypertension rats

Other area of cooperation was as below.

..... Establishment of P3 laboratory

..... Establishment of RI laboratory

..... Publication of the achievements in international journals

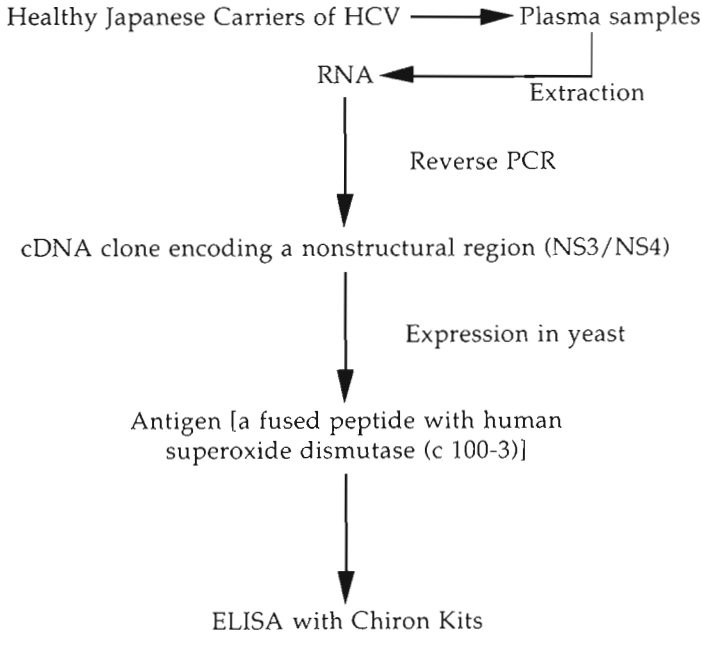
TRANSFER OF BIOTECHNOLOGY

Transfer of biotechnology in our project consists of two aspects; one is the introduction of biotechnological techniques together with necessary apparatus and reagents, another is the application of biotechnological approaches to each research subjects. Biotechnology in our case is simply one of the methodology and not the purpose.

Without the conventional techniques and procedures in the laboratory, biotechnology does not work efficiently and does not bring any benefit to our research activities. However, the recent development of computer-equipped apparatus and reagents has made the biotechnological procedure very simple and rapid. Without the deep knowledge of molecular biology, we can obtain extensive information about the structure of gene. Therefore, what is the most important is how to apply biotechnological approaches to our research activities. In the following chapter, I would like to show some examples which were made in our project.

COOPERATIVE RESEARCH ACTIVITIES BY BIOTECHNOLOGICAL APPROACHES

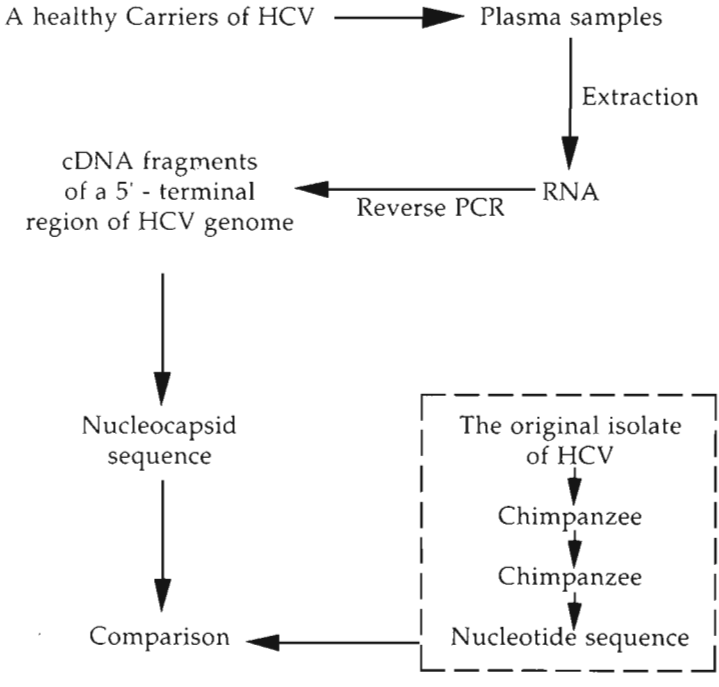
Example 1. Serosurveillance of HCV infection in Thai people



Antibody-positive rate 2.6% in healthy blood donors >
2.8% in healthy pregnant women > USA
5.7% in acute NANB hepatitis < Japan
15.4% in chronic NANB hepatitis < Europe

Ref. No	Participants	
	Thai side	Japanese side
1	Boonmar, S. Pojanagaroon, B.	Watanabe, Y. Tanaka, Y. Saito, I. Miyamura, T.

Example 2. Demonstration of the putative nucleocapsid (core) and envelop protein genes of HCV



Genome region	Identity rate between the 2 isolates		
	Nucleotide sequences	Polypeptide sequences	Function
5' untranslated	99%		Regulation
putative nucleocapsid	91%	97%	of life cycle
the region immediate	74%	75%	Adaptation to
downstream			environment

Ref. No.	Participants	
	Thai side	Japanese side
2-5	Boonmar, S.	Takeuchi, K. Saito, I. Miyamura, T. *et al.*

Example 3. Molecular epidemiology of Rotavirus infection in Thailand

Technology transfer

1987-1988 { ELISA, IAHA,
SDS-PAGE (RNA analysis)

1989-1990 { Monoclonal antibodies (serotype-specific)

1990-1991 { Homology by nucleotide sequencing of virus genome
RNA-RNA hybridization
PCR

Epidemiology

..... Subgroup and serotype distribution of human, bovine, and porcine rota virus in Thailand and confirmation by PCR technique

..... Characterization of bovine rotavirus prevalent in Thailand

1) Serology by ELISA and RNA-RNA hybridization
2) Nucleotide sequence of the VP7 gene

Ref. No.	Participants	
	Thai side	Japanese side
6-10	Pongsuwanna, Y.	Taniguchi, K. Urasawa, S. *et al.*

Example 4. Molecular epidemiology of influenza

Technology transfer

1987-1988	Tissue culture technique, Quantitative determination of virus growth
1988-1989	Serology with monoclonal antibodies
1988-1991	RNA extraction, Oligonucleotide mapping of RNA Sequencing, Advises to set up the surveillance system

Molecular epidemiology

Taking advantage of the above techniques, a survey of influenza epidemic was made in Thailand. In this survey, swine HINI influenza viruses were first isolated in this country, which showed a high serological and genetical relationship with A/NJ/8/76.

In Thailand, the human influenza virus is prevalent in the summer season (May to July), meanwhile swine influenza virus is prevalent in the winter season (November to January).

Ref. No.	Participants	
	Thai side	Japanese side
11-12	Kupradinun, S. Peanpijit. P. Phodhikosoom, P.	Yoshida, Y. Endo, A. Nerome, K.

Example 5. Demonstration of the complete nucleotide sequence of a variant of coxsackievirus A 24

This work was done when a Thai fellow (Mrs. Kasama) was staying in NIH-Tokyo for training.

The overall structure of the genome of CA24 was found to be 7.461 nucleotides long with poly (A) tail at the 3' end. Following a 750 nucleotides 5' non-coding region, there is long open reading frame of 7.392 nucleotides, which serve to encode a viral polyprotein consisting of 2,214 amino acids.

Since CA 24V emerged from a single focus as a new human pathogen, the above findings led us to the evolutionary and phylogenetic study.

Ref. No.	Participants	
	Thai side	Japanese side
13	Supanaranond, K. Warahit, P.	Takeda, N. Yamazaki, S.

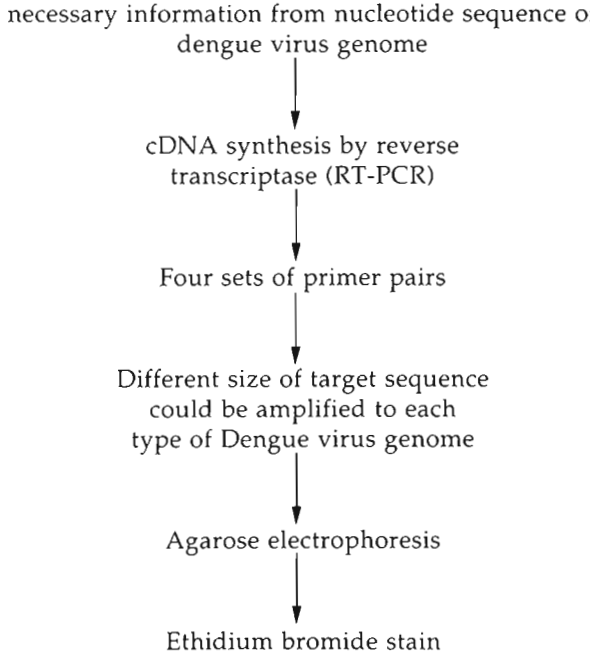
Example 6. Molecular epidemiology of Dengue in Thailand

Tachnology transfer

1991-1992 Development of reverse transcriptase polymerase chain reaction (PCR) for rapid identification of dengue virus serotypes by Japanese experts.

Molecular epidemiology

The above PCR method was employed in the field of dengue epidemic in Thailand. It was found that the dengue virus primer here employed is applicable to any types of dengue virus prevalent in Thailand. The primers were prepared as below.



Ref. No.	Participants	
	Thai side	Japanese side
14-15	Maneekarn, N. Warachit, P. *et al.*	Morita, K. Tanaka, M. Igarashi, A.

Example 7. Preparation of monoclonal antibodies against nucleocapsid proteins of rabies virus

Technology transfer

1988	Introduction of EIA and FIA to titrate neutralizing antibodies
1989	Labeling of antibodies with peroxidase
1991	Donation of hybridomas to produce monoclonal antibodies against nucleocapsid protein of rabies virus
1992	Promotion of mouse ascites and separation and purification of IgG

Application to laboratory diagnosis and fild survey

	Participants	
	Thai side	Japanese side
	Samuthananon, P.	Tokiyoshi, S.

Example 8. Introduction of biotechnological approaches to the laboratory diagnosis of HIV infection

Technology transfer

1987-1988	Set-up of P3 laboratory
1988-1989	Introduction of T-4 cell line which release HIV into culture medium persistently
1989-1990	Proparation of IFA-kits
1990-1991	PCR technology and HIV isolation system

Reference and surveillance activities

AIDS laboratory in NIH is functioning as a reference laboratory in the national network of AIDS surveillance.

Ref. No.	Participants	
	Thai side	Japanese side
16-17	Auwanit, W. Isarangkul Na	Yamanishi, K. Kurata, T. Ikuta, K. Takebe, Y.

Example 9 Application of molecular and biotechnological methods to rapid detection of the contamination of foods with pathogenic enterobacteria

Technology transfer

1989	Analysis of plasmide profile
1990	Colony hybridization technique using enterotoxin probe conjugated with peroxidase
1991	Use of commercially available kits for the repid detection of *Salmonella*
1992	Review of ever introduced techniques

Food contamination test

..... Laboratory diagnosis of enterotoxigenic *E. coli* by hybridization and PCR

..... Rapid and sensitive detection of *Salmonella* by the reverse dot blot hybridization method using 16 S ribosomal RNA gene as probe

Ref. No.	Participants	
	Thai side	Japanese side
18-21	Bangtrakulnonda, A. *et al.*	Danbara, H. *et al.*

Example 10. Simple and rapid genetic identification of *Legionella* species with photobiotin-labeled DNA

Technology transfer

1977 — A dot hybridization technique with photobiotic-labeled DNA was developed to identify *Legionella* species. The techniques was rapid and reliable not only for clinical isolates but also for environmental isolates.

Ref. No.	Participants	
	Thai side	Japanese side
22	Dejsirilert, S. *et al.*	Ezaki, T. *et al.*

DISCUSSION

The main objective of the present infectious disease project is to promote the research activities in NIH by introducing modern technologies, facilities, and equipments. The promotion of the research potential is necessary to strengthen the function of NIH as the central reference laboratory in the national network of public health laboratory services.

Our cooperation with Thai staffs of the institute has already been made for 7 years and a half and is expected to continue until the end of July 1994. Though not satisfactory enough, it is evident that the morale, motive and activity in research has been increasingly great during the part years.

The JICA policy in foreign aid is usually called "request base", which means that the assistance and cooperation will be made in response to the requests from counter-countries, not positively from Japanese side.

This is also the case in our project. As described in this review, many requests were presented as the desired technologies to be transferred. Most of them are concerned with molecular biology and biotechnologies. There was no reason to reject these proposals. However, when some world-famous Japanese expert of bacterial taxonomy emphasized the importance

of traditional techniques of routine use, his opinion was not welcomed by some Thai staffs. Certainly, it is ridiculous that some one is enthusiastic in molecular biology without capability to prepare a buffer solution of given molar concentration.

Nevertheless, even during the short period of our project, the progress in biotechnology was so rapid. We first used oligonucleotide mapping in the molecular epidemiology, but it was soon replaced by sequencing and PCR. Anyway, what is important is to introduce the modern advanced technologies in a good harmony with the existing conventional techniques (Fig. 4).

The requests, diagnostic kits and necessary machinery in biotechnology are rather expensive to be used routinely. In such a case, we must employ the more economical methods from due reasons.

Finally, it must be emphasized that the ultimate purpose of the project such as this is to enlarge the human resources in the concerned field, not the transfer of simple knowledges, materials, and technologies which may not be used positively (Fig. 5).

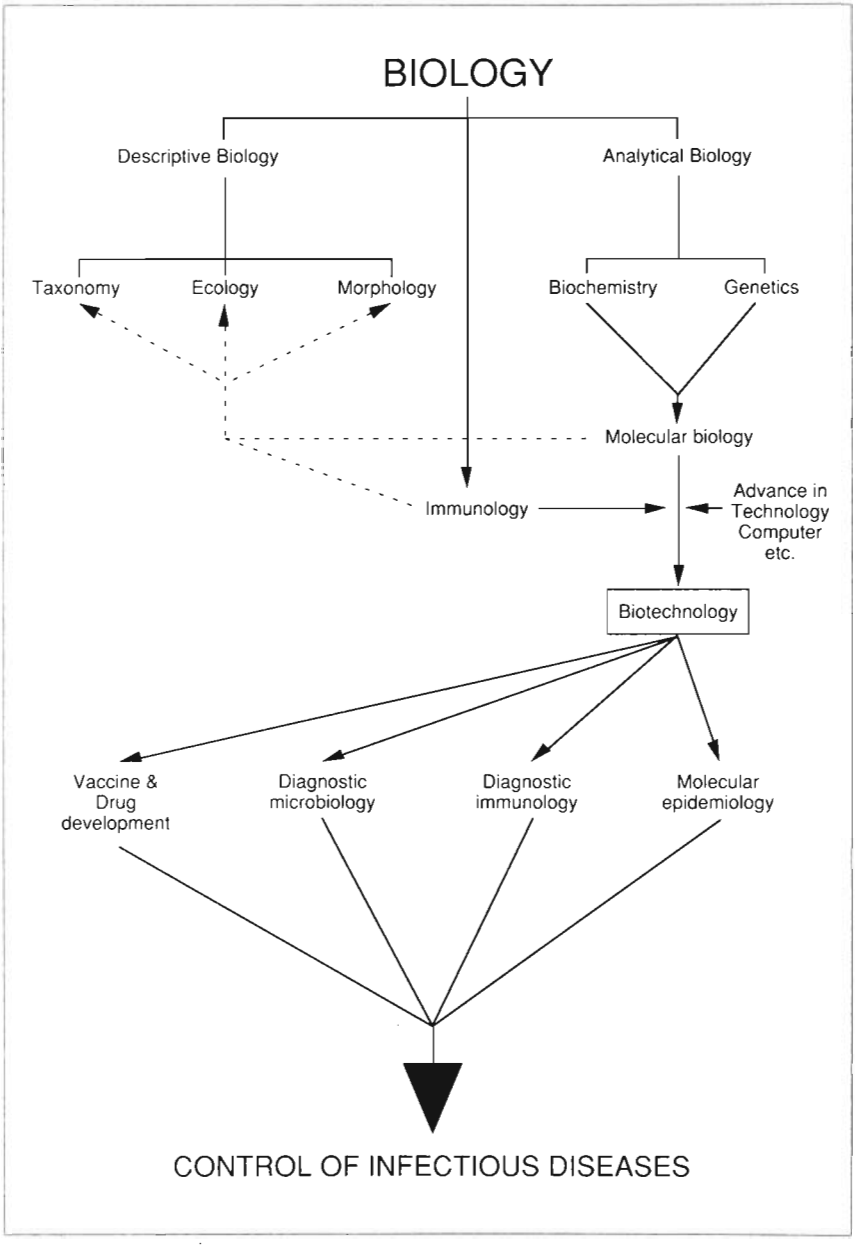


Fig. 4. Development of biotechnology and its application to infectious disease control.

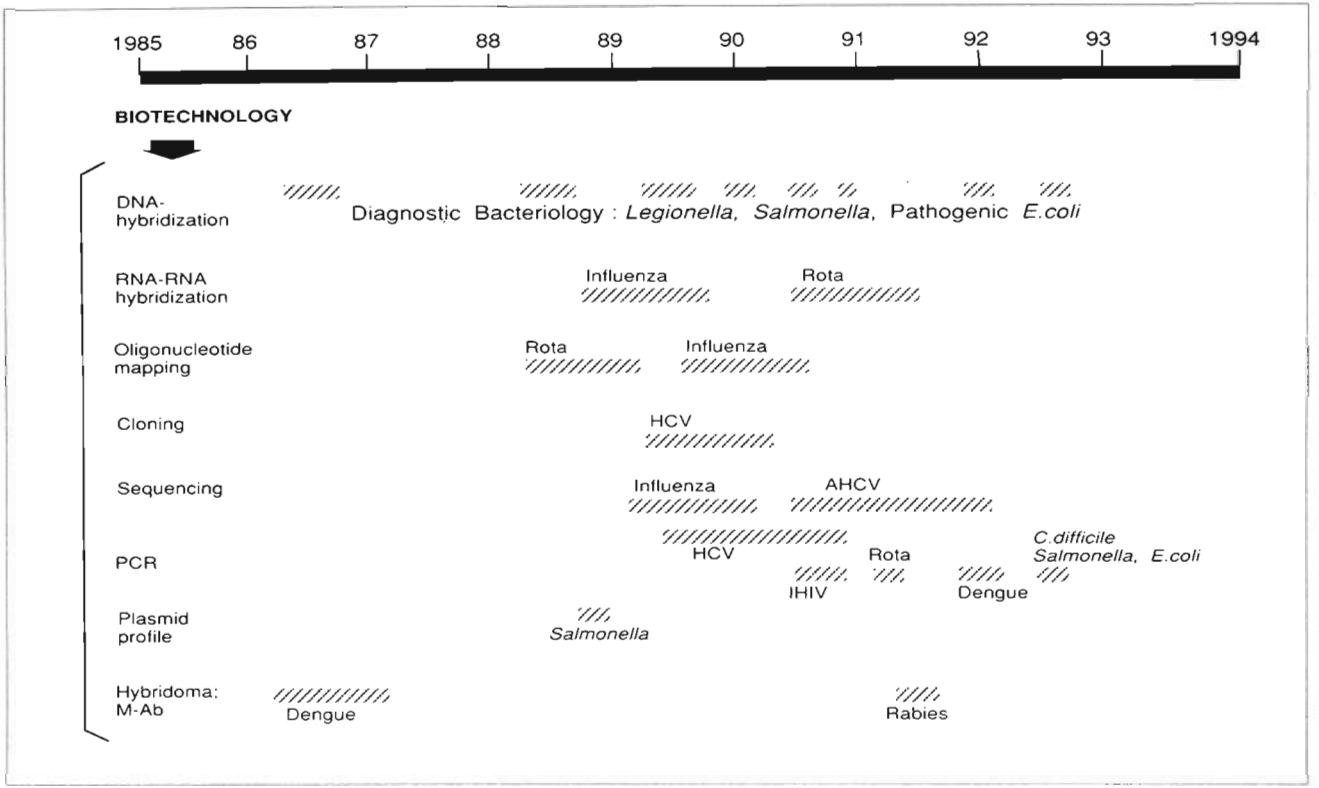


Fig. 5. Transferred biotechnologies in NIH and their application to.

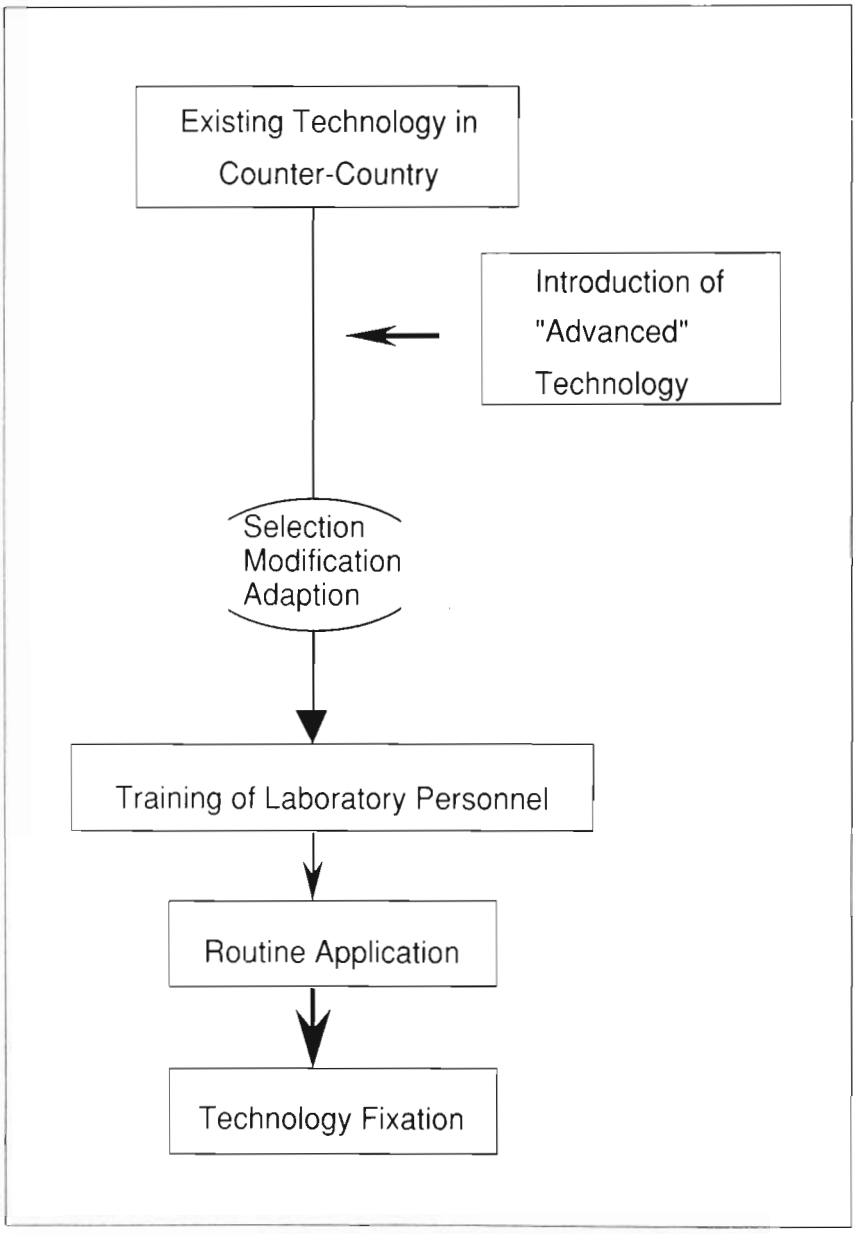


Fig. 6. Technology transfer.

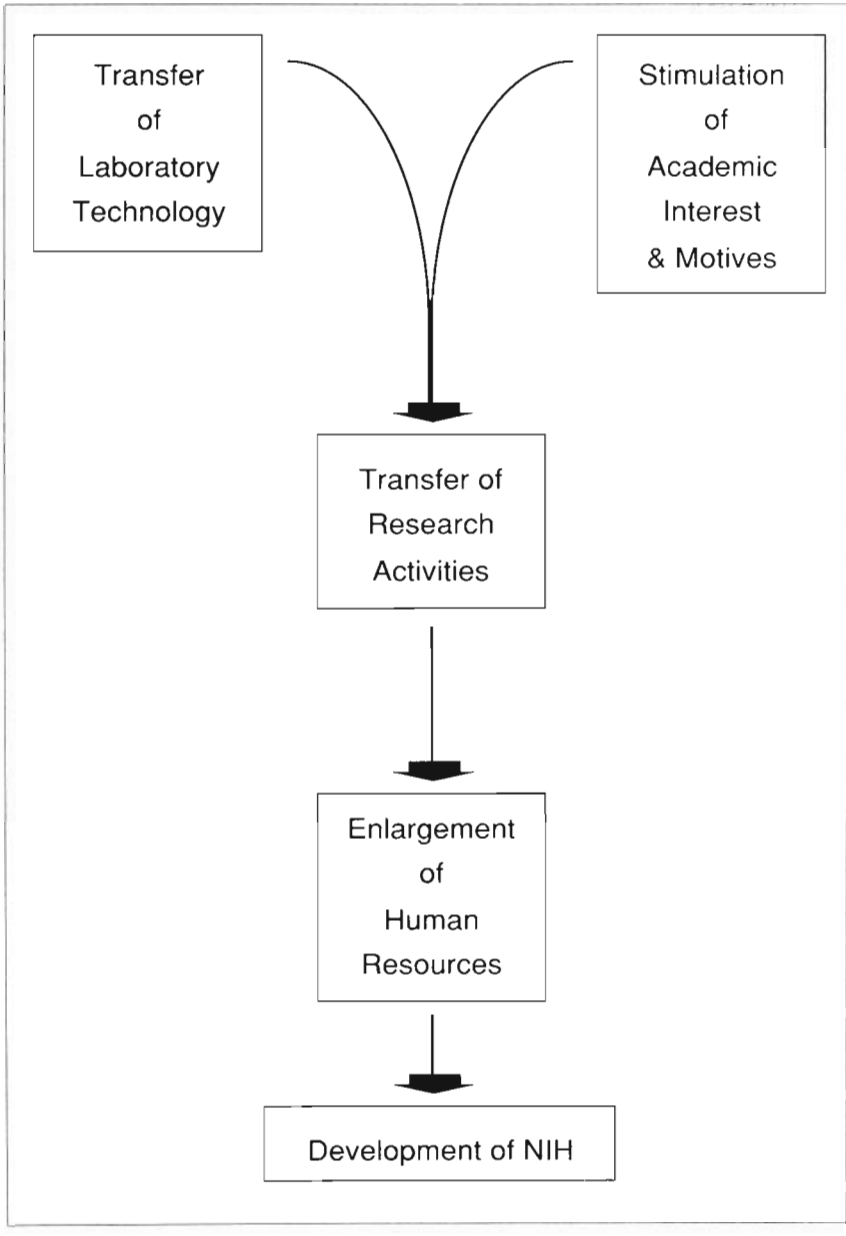


Fig. 7. Research Promotin Project.

ACKNOWLEDGEMENT

The project reported here was supported by Japan International Cooperation Agency. The experts who worked for technology transfer and cooperative research came from 9 universities, 5 research institutes, 5 prefectural laboratories and 4 private sectors. I would like to express my deepest thanks to all of them for their cooperation. Some of their names are found as the authors of publications shown in Reference.

Finally, I thank Suwanna Navacharoen for her cooperation in the preparation of this manuscript.

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HEPATITIS VIRUS IN INDONESIA: SEQUENCE VARIATION OF HEPATITIS B VIRUS

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The prevalence of Hepatitis B virus (HBV) infection marker is high in South Asia; for Hepatitis B surface antigen (HBsAg) it is 7-20%. In Mataram, a city in Lombok island, Indonesia, 25-50% of the population has been infected with this virus. Carrier rates among voluntary blood donors in big cities of this country range from 2.1% to 17.5%. Infection could manifest as acute or chronic hepatitis, asymptomatic carrier, cirrhosis or carcinoma of the liver.

Molecular approaches have been used in research and diagnostic of this infection that gave us a better understanding of the virus and its disease.

This communication reveals a review of several molecular researches on HBV from Indonesia that have been published as a result of joint programme between The Department of Microbiology, Medical Faculty, University of Indonesia and Immunology Division, Jichi Medical School, Japan and highlights some steps of the methods used which applicable in biotechnology approaches for diagnosis, prevention and treatment of infectious diseases.

HEPATITIS B VIRUS GENOME

The genome of HBV is a circular double-stranded DNA molecule that is not covalently closed. It consists of an L(-) strand which has four open reading frames (genes S,C,P,X) and a short S(+) strand of variable length. The length of HBV genome is around 3200 basepairs. Within the S gene laid the determinants for subtypes of HBsAg (hepatitis B surface antigen). The determinant *d* is specified by the codon 122 when it encodes for amino acid lysine. If this codon encodes for arginine, the determinant will be *y*. Similarly, the determinant *w* is specified by codon 160 encodes for lysine and *r* by that of arginine. A point mutation of A to G at nucleotide 365 or 497, which converts codon 122 or 160 from lysine (AAg/AAA) to arginine (AGG/AGA) could change the subtype of HBsAg.

METHODS TO OBTAIN THE SEQUENCES OF HBV

The methods used to obtain sequences could be devided in two pathways (Fig.1). Both pathways utilized plasma of blood donors as sample. After enzyme immunoassay to determine the subtype of HBsAg and dot hybridization test to detect the presence of HBV DNA, the HBV genome

was liberated and subjected to subsequent steps. The first path (I) was cloning the whole genome of HBV-DNA into a plasmid vector pSP65 followed by transformation of the recombinant into bacteria *E. coli* DH1. The plasmid containing HBV DNA was then isolated from the bacteria and its HBV DNA was subcloned into bacteriophage M13 for sequencing. The second path (II) was through PCR (polymerase chain reaction) to amplify the target (a part of S gene) to be sequenced. As we could see in Fig. 1, the use of PCR technique eliminated the step of cloning HBV DNA in plasmid vector pSP65 and transformation into *E. coli.*

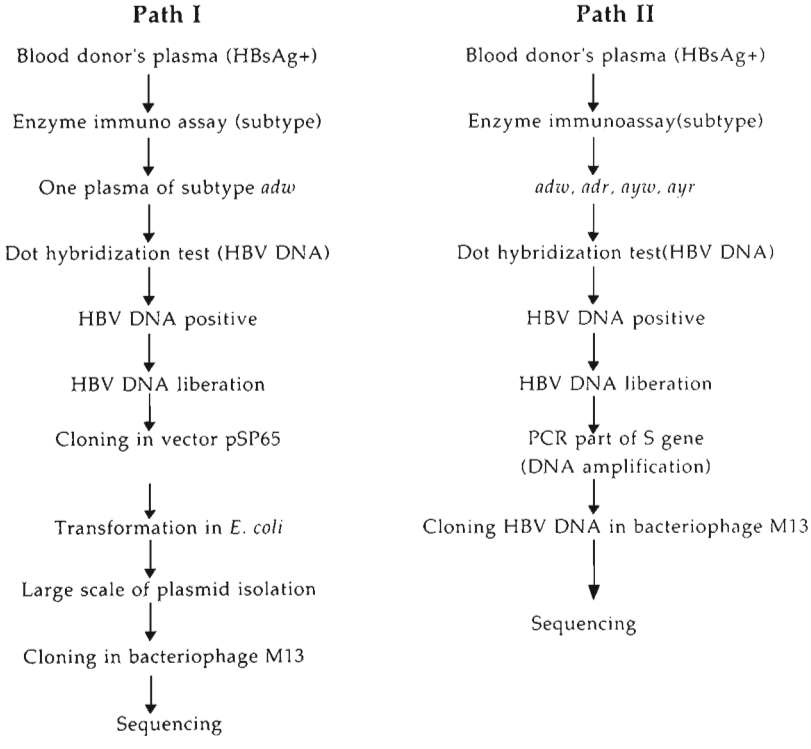

Fig. 1. Schematic diagram of sequencing the whole genome of HBV DNA (I) and part of the S gene (II).

SEQUENCES OF HBV DNA FROM INDONESIA

Path I

Three clones of HBV DNA of subtype adw were propagated and were named as pRTB 299(8), pPAD 744(9) and pMND 122(10). All of them have no EcoRI site unlike HBV DNA of same subtype published by Valenzuela (12) and Ono (6). Their length is 3215 basepairs (bp) each and possesed the eleven bp direct repeat sequence of 5'-TTCACCTCTGC at nucleotide 1590-1600 (DR2) and 1824-1843 (DR1). Clone pPAD 744 was different in the nucleotide sequence by 1.3% from pRTB 299. Both clones had the same restriction cleavage sites for Bam H1, Bgl II, Bst EII, HincII, Ava I and XdaI. Clone pMND 122 differed from pRTB 299 by 1.4% and from pPAD 744 by 1.2%, due to the accumulation of transition or tranversion type point mutation. Clone pMND 122 had no recognition sequence for Hinc II located in X gene by nucleotide substitution from adenine to cytosine at position 1686. Replacement from guanine to thymine at nucleotide 2840 in pMND 122 created another cutting site for Bgl II in P gene. These three clones, which varied each other by 1.2-1.4%, differed by 9.3% in nucleotide sequence and in genomic length from the two *adw* clones of Valenzuela (3200 bp) and Ono (3221 bp), both isolated in United States.

Path II

Two clones were propagated from each of 22 plasma samples from Indonesia carriers. One clone from each carrier without frameshift mutation was selected to represent the HBV DNA clone in them.

Sequences of XbaI-SpeI fragments of HBV DNA clones representing nt 247-683 in the S gene from those 22 carriers, from pRTB 299, pPAD 744, pMND 122, pIWD 420 and pIWK 146 were compared. There were two different categories of divergences in the sequence, one ranging from 0.5 to 4.1% and the other from 5.5 to 8.9%. There was no difference in a range between 4.2-5.4%. Based on an intra-group difference of 4.1% or less and inter-group difference of 5.5% or more, the 27 HBV DNA clones were classified into three genotypes. With the sequence homology to the four genotypes reported by Okamoto *et al.* and another described by Vaudin *et al.*, they were categorized into genotype B (12 clones, consist of 7 subtype *adw* and 5 *ayw*), C (13 clones of 1 *adw*, 10 *adr*, 1 *ayr* and 1 *ar*) and D (2 clones of *ayw*).

Different subtypes of clones in the same genotype indicated that point mutation inducing *d-y* or *w-to-r* phenotypic changes would be common among Indonesian carriers.

APPLICABLE METHODS FOR BIOTECHNOLOGY APPROACHES IN DIAGNOSIS, PREVENTION AND TREATMENT OF INFECTIOUS DISEASES

1. Dot blot hybridization test

This test is used to detect HBV DNA in serum by hibridization. It is a direct measure of quantity of virus present and probably the most accurate guidance to the presence of active viral replication. Ideally it should be used in the routine screening of all HBsAg positive serum. The HBV DNA probe can be labeled with either radioisotope or non-radioisotope materials.[1] Since the finding of pre-core defective mutants [2] in the blood of hepatitis patients with anti-HBe positive, this test could be used in the preliminary detection of such cases. Other utilization is to monitor interferon therapy to separate the responsive and non-responsive one.

2. Polymerase chain reaction (PCR)

This technique which capable of producing selective enrichment of a specific DNA sequence by 100,000 times has been used extensively in molecular biology [7]. In Hepatitis B, PCR assay for the detection of HBV DNA in serum shown to be 100,000 fold more sensitive than dot blot hybridization. Typing of Hepatitis B virus genome by a simplified PCR was carried out also [3]. Although it is an expensive procedure, PCR for the conformation diagnostic of Hepatitis C and preparing DNA/cDNA sources for sequencing are very helpful.

3. Sequencing

Sequencing is even more expensive than PCR but the benefit that could be gained from doing this work is enormous. The finding of pre-core HBV mutants [2], more reliable anti-HCV (Hepatitis C virus) test based upon the knowledge of conserved region in the sequence of the genome of HCV [5] and knowledge of genome variation in HBV [4,11] and HCV, came from sequencing.

In conclusion, it is worthwhile to set up facilities for dot blot hybridization, PCR and sequencing in every country in Asia so that parallel researches could be done which would contribute to our better understanding of infectious diseases and the agents in this region.

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MOLECULAR DIAGNOSIS OF DENGUE INFECTION (A preliminary report)

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ABSTRACT

Dengue hemorrhagic fever has become one of the major health problem in Indonesia, as well as in many other tropical countries, due to its high case fatality rate. Since the key to successful therapy in Dengue infection is early diagnosis followed by proper management, the establishment of a rapid, simple, but accurate diagnosis system is extremely required. Based on Dengue virus sequences information obtained by many researchers, we are at present developing a diagnosis method using specific DNA probes, which will be able to detect the presence of minimum amount of Dengue virus in a spot on a hybridization membrane, either directly from clinical specimen or after PCR amplified. With the approach, we are able to detect the presence of Dengue Virus in mouse brain suspension and tissue culture fluid. The study is still in progress to determine its sensitivity and specificity, as well as to establish a simplified method which will be applicable for field work.

INTRODUCTION

Dengue infection has become one of the important public health problems in many tropical and sub-tropical countries, and belongs to one of the most important mosquito born diseases [1]. Infection by dengue virus mostly lead to an acute but self limitting fever, but it could also develop to a severe febrile disease accompanied by vascular permeability and hemostasis abnormalities (Dengue hemorrhagic fever/DHF), and in some cases develop further to an acute hypovolemic shock (dengue shock syndrome/DSS) which sometimes fatal [1,2]. The key to successful management of dengue infection is early diagnosis followed by proper treatment. Since there is no specific treatment available for dengue virus infection, early diagnosis is absoultely required. Rapid laboratory diagnosis for dengue available in the market is based on immunological assay, which although it was claimed as having high sensitivity and specifity, cross-reactivity and sensitivity are still questionable, since it detects type and amount of certain antibody to dengue virus, and unfortunately, its closely related families. The presence of dengue virus in infected tissue has been demonstrated using fluorescence antibody technique, but it requires high number of virus particles. The only way to detect the presence of minimum amount of dengue virus in any kind of tissue is by using molecular approach. In this paper, an attempt to detect dengue

virus using specific DNA probe either directly in clinical specimen or after PCR amplification will be discussed.

MATERIALS AND METHODS

Virus strains and serum. Clinical virus isolates were obtained from DF, DHF, or DSS patients in Jakarta. Viruses were passaged in C6/36 cells or suckling mouse brain and were frozen in aliquots. Serum were collected from the same objects, and their activities to four dengue serotypes have been characterized.

Primers and probes. Primers and probes were generously provided by Dr. Deubel of Pasteur Institute, which sequences and characteristics have been published elsewhere.[3]

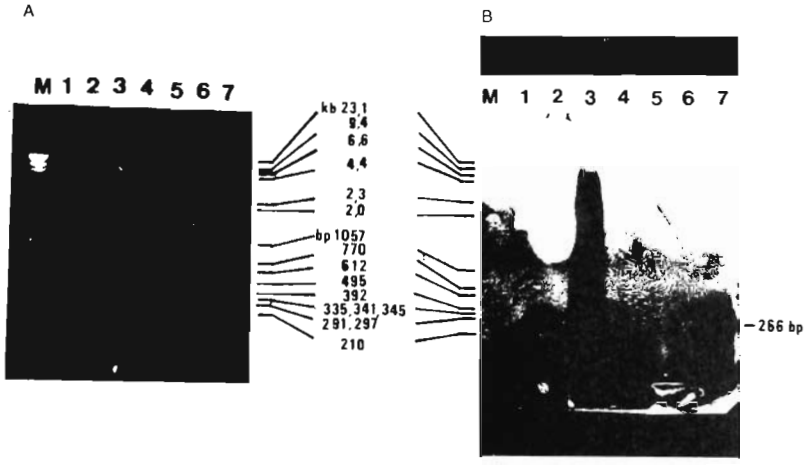
Nucleic acid targets. Several preparation of viral nucleic acid were used as hybridization targets: patient's serum, mouse brain isolates, tissue culture supernatant, and cDNA, both with and without PCR amplification. Targets were either electrophoresed and transblotted on to Nylon membrane (Hybond-N Plus, Amersham) or directly blotted on to the membrane.

Probe labeling and hybridization. DNA probes were labeled using non-radioactive reporters, i.e. Digoxygenin-dUTP (Boehringer Mannheim) and ECL system (Amersham).Labeling and hybridization reaction were performed according to the standard procedures recommended by the manufacturer.

DNA amplification

PCR was performed using primers specific to each of dengue virus serotype respectively [3]. RT-PCR was performed as described elsewhere [4,5] with minor modifications. Briefly, 5 µ l of Tissue Culture Fluid (TCF) or Patient's Serum (PS) was incubated for 60 seconds at room temperature in equal volume of PBS containing 1% of NP-40 before added with 90 µ l of RT-PCR mixture containing 8 ul of both primers, 10 µ l of Tth reaction buffer, 16 µ l of 1,25 mM dNTP mix, 2 µ l of 1U/µ l Tth polymerase, 0.5 µ l of 20 U/ µ l Reverse Transcriptase, and water. The mixture was incubated for 10 minutes at 53° C to allow the RT to react before the 30 cycles of polymerization reaction (60 seconds each of 92° C, 53° C, and 72 °C), followed by incubation for 10 minutes at 72° C and dwelling at 4° C.

Polymerase used in PCR is Tth Polymerase (Toyobo), which to our experience gives no marked difference with Taq polymerase (Boehringer Mannheim).



PCR product of DV 2. Lane M: DNA MW marker, λDNA + Hind III and ØX174+ Hinc II. Lane 1 and 2: Serum after and without RT-PCR. Lane 3 and 4: TCF after and without RT-PCR. Lane 5 and 6: DV-RNA from mouse brain after and without RT-PCR. Lane 7: DV2 cDNA after PCR. A:Ethidium bromide staining; B: Southern hybridization with DV2 E gene labeled with dUTP-DIG.

Note that in lane 3 and 7, the probe well hybridized with 266 bp sized PCR product. Unfortunately we are not yet able to demonstrate the result with the serum and other samples.

RESULTS AND DISCUSSION.

cDNA was constructed from viral RNA isolated from Dengue-2 infected mouse brain, and TCF was from Dengue-2 infected C6/36 cell culture, while acute serum was from a dengue infected patient as confirmed by Hemagglutination Inhibition. As shown in Figure 1, Digoxygenin labeled Dengue-2 probe hybridized to DNA fragments sized arround 266 bp of PCR amplified viral nucleic acid from TCF and Mouse Brain cDNA (lane 3 and 7). In fact, ethidium-bromide staining of the same gel showed that a smaller than 266 bp fragment was also appeared in lane 1, but could not hybridize with the probe. We are at present investigating, whether the fragment belongs to other serotype or completely different source. Also we have performed similar approaches using virus, probe and primers of other serotypes (datas not reported). From this preliminary results, we are convinced that this approach will be well applicable for detection of dengue virus from patient's acute serum.

ACKNOWLEDGEMENT

The authors would like to thank Dr. V. Deubel of Pasteur Institute who has generously provided the probes and primers. This work is funded by a research grant from the Indonesian Government through the Ministry of Research and Technology.

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BIOTECHNOLOGICAL APPROACHES IN THE DIAGNOSIS OF DENGUE INFECTIONS AND THE EFFECT ON PREVENTION AND MANAGEMENT OF DENGUE DISEASES

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INTRODUCTION

Infections caused by dengue viruses remain an important public health problem in many tropical and sub-tropical countries, especially in countries in Southeast Asia. The disease is endemic in countries such as Thailand, Indonesia, Myanmar, Vietnam, China, Sri Lanka, India, Singapore and Malaysia. Many other countries are at risk of severe dengue outbreaks, including Africa, the Americas, the Mediterranean region of Europe and the Caribbean islands.

There are four antigenically related serotypes of Dengue virus (Den 1-4), each having been shown to cause both classical and severe dengue infection. Studies in Thailand, Malaysia and Cuba have shown a consistently high association between Dengue type 2 infection and Dengue haemorrhagic fever (DHF) with shock syndrome. Dengue type 3 has been associated with severe dengue infection in Indonesia and Malaysia. Man is the major reservoir of the disease, though studies in Malaysia and Africa have shown that monkeys may play a role in the jungle cycle.

Aedes aegypti is the most efficient of the mosquito vectors because of its domestic habitat. Dengue outbreaks have also been attributed to *Aedes albopictus, Aedes polynesiensis* and several species of the *Aedes scutellaris* complex. Transovarian transmission has been demonstrated in limited studies, but its true importance in nature has not been established.

CLINICAL DIAGNOSIS

Clinically, dengue infections may be asymptomatic or may lead to undifferentiated fever, Dengue fever (DF), or dengue haemorrhagic fever[1]. Dengue fever in infants and young children may manifest as an undifferentiated febrile disease with a maculopapular rash. Older children and adults may have either a mild febrile syndrome or the classical incapacitating disease with abrupt onset and high fever, severe headache, pain behind the eyes, muscle and joint pains, and rash. Skin haemorrhages with a positive tourniquet test and/or petechiae are not uncommon. many

epidemics of dengue fever are accompanied by bleeding complications such as epistaxis, gingival bleeding, gastrointestinal bleeding, and haematuria.

Typical cases of DHF in Asian countries are characterised by four major clinical manifestations: high fever, haemorrhagic phenomena, hepatomegaly and often, circulatory failure. Moderate to marked thrombocytopenia with concurrent haemoconcentration is a distinctive laboratory finding. The major pathophysiological change that determines the severity of disease in DHF is the leakage of plasma, as manifested by a rising haematocrit value and haemoconcentration.

In a DHF patient who develops shock, the patient's condition suddenly deteriorates after fever of a few day's duration. The skin becomes cold, blotchy and congested, circumoral cyanosis is frequently observed and the pulse becomes rapid. Acute abdominal pain is a frequent complaint shortly before the onset of shock. Shock is characterised by a rapid, weak pulse with narrowing of the pulse pressure (20 mm Hg or less) or hypotension, with cold, clammy skin and restlessness. The duration of shock is short; the patient may die within 12-24 hours or recover rapidly following appropriate anti-shock therapy.

About a third of shock cases present as bleeding largely from the gastrointestinal tract. Disseminated intravascular coagulation is implicated as the cause. In most cases, early and effective replacement of lost plasma with plasma expander and/or fluid, fresh frozen plasma and electrolyte solution results in a favourable outcome. The prognosis of the disease depends upon the early recognition of cases and the monitoring of pre-shock conditions. Some patients with DF or DHF present central nervous system manifestations such as convulsions, spasticity, or alteration of sensorium. More recently, cases of primary dengue infection in children were reported to be associated with symptoms of fits, encephalopathy and liver failure. Another unusual clinical manifestation was seen in young adults who also had renal involvement[2]. Further studies are needed to identify what factors contributed to these unusual clinical manifestations.

PATHOGENESIS OF DHF

There has been a long standing controversy over the question of the pathogenesis of DHF[3]. One theory proposes that DHF is directly caused by a more virulent strain of the virus. The other theory suggests that it is largely a function of the host immune response. Longitudinal studies from several dengue endemic countries have lent weight to the latter theory as a large majority of DHF/DSS occurs in persons undergoing a second infection with a heterologous dengue serotype. Subneutralising or enhancing antibodies may play a role in promoting viral entry and replication within monocytes, resulting in immune elimination of these infected cells and thus to the release

of a variety of chemical mediators which then produce the symptoms of shock and haemorrhage. Neither of these theories can explain fully the clinical and laboratory findings of DHF and it is hoped that major advances in flavivirus molecular biology will help elucidate this question.

LABORATORY DIAGNOSIS

There are three approaches for the laboratory diagnosis of dengue infection. These are by:

(a) Virus isolation

(b) Serology

(c) Detection of viral RNA

(a) Virus isolation

The recovery of dengue virus from blood or tissue specimen is the most conclusive way to demonstrate dengue infection. However, this often poses a problem as the virus grows poorly in animals and conventional cell cultures. The use of mosquito cell cultures such as AP/61 and C6/36 has improved the sensitivity of isolation but the time required is about two weeks. Inoculation of adult mosquito for virus isolation has been successfully achieved and is the preferred practice in several laboratories. In our laboratory, we have developed the inoculation of *Toxorhynchites splendens* larvae for the isolation of dengue viruses[4]. The method is relatively simple, less traumatic, and the result available within 5 days as compared to the longer time required when adult mosquitoes are used.

In the past, the identification of the virus isolate requires the use of specific dengue antisera in the neutralization test. This is not only technically difficult, but laborious and time consuming. However, using the biotechnological approach of hybridoma technology, monoclonal antibodies are now available and, combined with the use of immunofluorescence, have made neutralization test obsolete. Viruses isolated in cell cultures or mosquitoes can now be typed within a few hours and this has facilitated the rapid identification of virus isolates.

(b) Serology

There are several serological techniques which have been used in the diagnosis of dengue infections. The gold standard is the haemagglutination inhibition (H.I.) test. However, there are many problems associated with this test. A reliable source of fresh goose red blood cells is essential. The test is exquisitely pH dependent and all reagents must be standardized. The

best source of antigen is acetone extracted infected suckling mouse brain and all sera have to be extracted to remove non-specific inhibitors. To demonstrate a four-fold or greater increase in H.I. antibody titres, paired serum samples are required, thus cutting down on the usefulness of the test. In addition, the test takes three days to perform. The neutralization test is too labour intensive to be considered and the complement fixation test suffers from the same constraints as the H.I.

A number of laboratories have developed the IgM antibody capture enzyme linked immunosorbent assay (IgM ELISA) to replace the other conventional serological tests. In our laboratory, we have developed such a test using monoclonal antibody as a detecting antibody[5]. To date, we have tested over 6,000 serum samples by both H.I. and IgM ELISA and there is good correlation between the tests. There are several advantages with the use of IgM ELISA. Firstly, since the test is based on the detection of early IgM, the acute specimen is often positive at the time of clinical illness. There is therefore less need to request for a second specimen. Secondly, the test is relatively simple to perform and the reading of results using the ELISA reader is objective. The whole process takes only a few hours so that the results can be fed back to the clinicians sufficiently early to influence patient management.

(c) Detection of viral RNA

The polymerase chain reaction (PCR) offers the potential for highly sensitive and specific detection of dengue viral RNA. PCR allows the *in vitro* enzymatic amplification of minute quantities of genetic material and has found increasing application for the detection of a number of pathogens, including dengue[6,7,8]. We have started to investigate the potential of this technique to detect and identify dengue viruses in patients' sera from which virus isolates have already been demonstrated. Thirty-three virus positive specimens have been tested and all correctly identified by PCR. Five other specimens from suspected dengue patients with negative isolations were similarly tested and two were positive by PCR, one strain being dengue 2 and the other dengue 3. PCR has helped to confirm a number of dengue deaths when other methods were inconclusive. The test takes only a few hours to perform, and confirmation by using cDNA probes will take slightly longer.

CONCLUSION

The rapid methods listed above are based on biotechnological approaches and offered by the WHO Collaborating Centre for Dengue Fever and Dengue Haemorrhagic Fever in Malaysia for the diagnosis of dengue infections. They have been selected because the results can be made available within a shorter

period of time than conventional methods. Rapid dengue diagnosis provides strong support for patient management and serve to provide an early warning system to predict the severity and magnitude of dengue outbreaks in the country.

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USE OF BIOTECHNOLOGY IN THE PRODUCTION OF REAGENTS FOR DIAGNOSIS AND EPIDEMIOLOGICAL STUDIES OF IMPORTANT PARASITIC DISEASES IN MALAYSIA

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ABSTRACT

An early decision was made to concentrate on the production of biological reagents using molecular biology techniques for the development of diagnostic assays for important parasitic diseases endemic in Malaysia. Some of these assays are needed as seroepidemiological tools to monitor and evaluate control programmes, as for example, an ELISA for detecting malaria antigen, while a *P. falciparum* DNA probe, in combination with nucleic acid amplification, would be useful to determine rapidly, parasite resistance to antimalarials in patients. Other biologicals like DNA probes for sporozoite identification, MABs for filaria L3 speciation, and recombinant proteins for the diagnosis of visceral larval migrans are being developed. The emphasis is to produce essiential reagents which can be used to develop specific and sensitive assays for important parasitic diseases where such assays are not commercially available or if available, are extremely expensive. Priority is given to reagents which are currently only produced with difficulty through conventional culture techniques (E.S. antigens of *T. canis*), or where production is heavily dependent on human serum and cells (*P. falciparum*), or because parasites cannot be cultured (*P. vivax*, simian malaria), or must be maintained in animal models (*B. malayi*).

INTRODUCTION

During the last few years, intense efforts and resources have been channelled to the study of infectious diseases in Malaysia using biotechnology and related techniques. As parasitic diseases still contribute significantly to morbidity and sometimes mortality in Malaysia and neighbouring countries, attention was focussed on some of the more important ones, namely, malaria, toxoplasmosis, filariasis, toxocariasis and angiostrongyliasis.

An early decision was made to concentrate on the production of reagents for the development of assays for diagnosis and use in epidemiological assessments of important parasitic diseases. Basically these include the production of monoclonal antibodies, nucleic acid probes, fusion proteins and synthetic oligopeptides. Other appropriate techniques would also be used to characterize parasite strains and species in support of the above.

The above decision was made based on a number of factors, among which were the non-availability, or if available, the extremely high cost of commercial diagnostic assays for diseases like filariasis, toxocariasis, toxoplasmosis, malaria and angiostrongyliasis. Furthermore, some of these diseases are mainly of concern to tropical countries and commercial development of diagnostic kits by multinationals based in developed countries, would be of low priority. Diseases like malaria and filariasis are well documented as important causes of ill-health in countries in the region. Others like toxoplasmosis, toxocariasis, extraintestinal amoebiasis, angiostrongyliasis, etc have not been studied systematically due to lack of suitable diagnostic assays. Our emphasis in the use of molecular biological tools in the production of reagents and development of diagnostic assays will hopefully serve to overcome this.

PRODUCTION OF REAGENTS, DEVELOPMENT AND USE OF ASSAYS

Malaria

Of the parasitic diseases common to Malaysia and the neighbouring countries, malaria is the most important. In 1991 there were 39,189 confirmed malaria infections in Malaysia, with an annual parasite incidence of 2.2 per 1000 population. Confirmed malaria deaths were 47 and most infections were due to *Plasmodium falciparum* (63.9%). Those due to *P. vivax*, *P. malariae* and mixed infections were 34.0%, 0.7% and 1.4% respectively.[1]

An important requirement in the management of malaria patients and the control program is the availability of rapid, sensitive and specific assays for patient diagnosis and case detection respectively. For the individual patient it would be necessary to identify correctly the parasite species as well as low level parasitemia especially after chemotherapy. A rapid assay would be useful not only in busy hospitals and clinics but also for control programs where the turn-over time for case detection should be rapid enough for the results to be utilized meaningfully. For this purpose, a technique which is reasonably cheap and where specimens can be batch processed is needed. The conventional giemsa stained thick and thin blood smears are extensively used in laboratories and control programs and are not only reasonably sensitive and specific but also relatively cheap. Some of the disadvantages are the necessity for well trained malaria microscopists, and the 15 minutes needed to screen each slide. However, we have found it to be more sensitive and specific than the quantitative buffy coat (QBC) technique,[2] especially for specimens where the parasite counts are less than 500 per μ l.[3] The difficulty of keeping the QBC tubes for cross-checking at a later date, the relatively greater cost per test and the unreliability in the identification of species will be factors against its use in place of the thick and thin blood smears in control programs.

The requirements of diagnostic assays for patient diagnosis and for case detection in epidemiological surveys are different in some respect. While specificity and sensitivity should be high for both, the latter needs tests which allows rapid batch processing.

Monoclonal antibodies (MABs) recognizing soluble and/or particulate antigens in those with current malaria infection will be useful if they can be utilized in the enzyme-linked immunosorbent assay (ELISA) which allows batch-processing even in the field. We have produced a number of MABs against *P. falciparum and P. cynomolgi* schizont antigens and have evaluated some for use in the detection of circulating soluble antigens in malaria patients. Two IgM MABs, one produced against *P. falciparum* (PF-IG8) and the other against *P. cynomolgi* (PC-IE12) have been evaluated in a sandwich ELISA against *P. falciparum, P. vivax and P. malariae* infections[4]. Both MABs were found to be 100% specific and 95% and 98% sensitive respectively in detecting soluble antigens in malaria patients. In the assay the capture antibody used was rabbit polyclonal antibody developed against soluble schizont antigens of *P. falciparum and P. cynomolgi.* The results showed that both MABs detected cross-reacting antigens, and could be used as panspecific reagents for detection of cases. Indeed it was found that those with parasite counts as low as 32 per μ l had sufficient circulating antigens or immune complexes to be detected as positive. It was further shown that even those with only gametocytemia had sufficient circulating antigens or immune complexes to be detected as positive. However, there was no correlation between parasite densities and optical density readings and the assay could not discriminate the malaria species involved. Further studies with these MABs to evaluate their ability to identify those with recently cured malaria infections and differentiate between endemic "normals" and those with infection are being carried out. Adaptation of the assay in a dot blot format would also be useful for individual patient diagnosis if the sensitivity can be enhanced by appropriate choice of detection systems.

Although a number of DNA probes have been developed and shown to be specific and as sensitive as direct examination for *P. falciparum* parasites,[5-8] the use of such probes at the field or laboratory level can only be justified in some special circumstances. A specific situation where DNA probes are useful especially after amplification of parasite DNA, will be in situations of extremely low (below microscopic threshold) parasitemia seen after chemotherapy and where its persistence is used to confirm resistance to the antimalarial drug given.

Another use of DNA or MAB probes will be the specific differentiation of simian from human plasmodia sporozoites, where morphological identification is unreliable. Currently we are attempting to develop DNA probes against the common simian malaria present in Malaysia as accurate epidemiological assessments based on mosquito vector sporozoite rates depend on correct identification of human sporozoites found in a locality.

Filariasis

The diagnosis of filariasis at the patient and community level is still heavily dependent on the demonstration of microfilaremia using the conventional thick blood film technique. The sensitivity of this technique depends on the volume of blood screened which, in turn, is limited by the volume of blood that can be placed on a microscope slide and the time taken to screen it. Polycarbamate membrane (3-5 μ m pore size) filtration of blood diluted with normal saline, effectively concentrates microfilariae present, and allows the rapid screening of a larger volume (usually 1 ml) of blood, thus increasing the sensitivity.

Most filariasis surveys are carried out late in the evening or at night as microfilariae of most bancroftian and brugian filaria infections are nocturnally periodic. This is rather inconvenient to the community as well as the filariasis control team. Efforts have therefore been made to detect circulating soluble antigens and/or immune complexes in sera collected during the day. MABs directed against antigens of *Brugia malayi* microfilaria, infective larva or adult parasites have been developed,[9] and studies are being carried out to determine whether any of these can be used in an ELISA for seroepidemiological purposes. We are also exploring the use of some of these MABs in an ELISA to assess the efficacy of chemotherapy by monitoring the level of circulating soluble antigens associated with living adult worms. This was suggested as being valuable for monitoring the efficacy of treatment in *Wuchereria bancrofti* infections,[10,11] and if proven, will be useful not only as a guide in the management of the individual patient but also for evaluation of the efficacy of mass drug treatment control programs.

A further problem facing filariasis control workers is the difficulty of screening large numbers of mosquitoes for infective larva (L3) and subsequently identifying those recovered. As *Brugia malayi* L3 are morphologically indistinguishable from that of *B. pahangi* which is a common parasite infecting domestic and wild animals but not man, the correct identification of L3 is essiential for accurate epidemiological assessments and identification of vectors. MABs and DNA probes can be developed to distinguish between these two species. A DNA probe based on a repeated sequence specific for *B. malayi* and named pBm 15 has been cloned[12] and shown to be useful in the identification of *B. malayi* L3 in mosquitoes.[13,14] This and another similar DNA probe[15] cannot discriminate between L3 and other immature stages of filaria larvae in mosquitoes and could therefore wrongly identify as vectors, those mosquitoes that can only support the development of the parasite to the first and second larval stages. MABs which can discriminate between these various stages in the vector are being currently developed.

Toxocariasis

Toxocariasis is believed to be common in developing countries and seroprevalence rates of 60% or more have been reported.[16] Although systematic surveys to determine its prevalence in Malaysia has not been carried out, a limited study among 480 aborigines showed a mean positive rate of 31.9%, the highest rate (45.2%) being in the age-group 0-9 years.[17]

The diagnosis of visceral larva migrans and ocular toxocariasis, due mainly to infection with the second stage larva (L2) of *Toxocara canis*, is currently based on the demonstration of specific antibodies. Occassionally, the parasite can be demonstrated in tissues obtained at biopsy or surgery.

The ELISA based on excretory-secretory (E.S.) antigens *in vitro* cultured *T. canis* L2[18,19] is now commonly used for immunodiagnosis. However, there are problems associated with the production of E.S. antigens. L2 is obtained through a complicated process. *T. canis* eggs are recovered from adult female worms obtained from autopsy of puppies, embryonated for 2-3 weeks, hatched and the L2 cultured in RPMI-1640 culture medium. Although such cultures can be maintained for months, the process is tedious and there is a case for the production of fusion proteins to substitute for E.S. antigens. A number of such recombinant proteins recognizing specific anti-*Toxocara* antibodies in sera of patients and experimentally infected animals have been produced in our laboratory and are now being characterized further for use in an ELISA in the diagnosis of visceral larva migrans.

In addition to the fusion proteins, MABs reactive to various antigens of *T. canis* have been produced and those reactive to L2 E.S. antigens are being tested for use in the ELISA to detect cirulating antigens/immune complexes in patients. These MABs recognize epitopes of 32, 38.5, 45 and 66 kDa in L2 and E.S. antigens which are also reactive with sera from toxocariasis patients and immune sera from experimentally infected animals.[20]

Angiostrongyliasis

Angiostrongylus spp. infection is common in rats in Malaysia[21-23] and although previously reported human infections[24,25] have not been confirmed, it is necessary to determine its importance to human health. As recovery of the parasite from patients is frequently not possible, it would be essiential to develop sensitive and specific immunodiagnostic assays to confirm the diagnosis. Such assays too would be useful as seroepidemiological tools to determine the prevalence of the infection in various exposed communities.

MABs have been produced against adult worm antigens of *Angiostrongylus cantonensis* (Eamsobhana *et al.*, unpublished data). Of these, an IgM MAB was found to be specific for *Angiostrongylus* antigens and immunoblot studies showed it to be reactive to antigens at 205 and 22 kDa.

We will be using this MAB for further studies and the development of an immunodiagnostic assay for the infection.

Other Parasitic Diseases

Other parasitic diseases studied include toxoplasmosis, amoebiasis and giardiasis. MABs are being raised against cyst and RH strains of *Toxoplasma gondii* for use in diagnosis. Similarly MABs are being raised against various strains of *Entamoeba histolytica* for immunotyping of pathogenic and non-pathogenic strains. Production of recombinant proteins for use in diagnosis are also being attempted. Similar studies are being carried out for *Acanthamoeba* spp.

Future studies include the development of nucleic acid probes to discriminate closely related mosquito sibling species and *Anopheles maculatus* complex is being looked at first.

ACKNOWLEDGEMENTS

I would like to thank Dato' Dr. M. Jegathesan, Director, Institute for Medical Research, Kuala Lumpur, for his permission to publish is paper. The research activities reported here received financial support from the Research and Development Fund of the Ministry of Science, Technology and Environment, Malaysia.

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MONOCLONAL ANTIBODIES FOR DIAGNOSIS OF AMOEBIC INFECTIONS

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ABSTRACT

Two free-living potentially pathogenic amoebae, *Naegleria* sp. and *Acanthamoeba* sp. were isolated from different localities in the Philippines. Monoclonal antibodies to these local isolates have been produced and characterized. Characterization involved ELISA, indirect immunofluorescent antibody tests, immunodiffusion tests, and western blot analysis.

Two types of monoclonal antibodies against *Naegleria* sp. were obtained: those that bind to the surface membrane antigens and those that bind to cytoplasmic antigens. Membrane-specific monoclonal antibodies were found to be precipitating antibodies. The locally-produced monoclonal antibodies can be used as a diagnostic tool for early detection *of Naegleria* infection.

Two monoclonal antibodies, IA1 and IB3, directed against *Acanthamoeba* are non-precipitating and recognize both membrane and cytosolic protein fractions. Western blot and immunostaining revealed that monoclone IA1 binds to a protein of MW 67 kD, while IB3 is specific to a protein of MW 76 kD.

INTRODUCTION

Among the free-living groups of amoebae, *Naegleria* and *Acanthamoeba* are known to cause human diseases. *Naegleria*, the causative agent of primary amoebic meningoencephalitis (PAME) is a free-living amoeboflagellate whose trophozoite stage may differentiate reversibly into either a non-reproductive flagellate stage or a resistant cyst (Fulton, 1977). *Acanthamoeba* on the other hand, known to cause corneal keratitis and granulomatous amoebic encephalitis (GAE) is characterized by a life cycle of active trophozoite and a dormant cyst stage. These organisms are ubiquitous being found almost everywhere, in freshwater lakes, ponds, streams, thermally polluted waters, warm springs, heated swimming pools, chlorinated water supply, soil, atmosphere and even nasal cavities.

The free-living amoebae, *Naegleria* and *Acanthamoeba* so far comprise diverse species of pathogenic and non-pathogenic strains. The strains are difficult to identify morphologically. They are distinguished mainly on the basis of biochemical characteristics such as isoenzyme patterns, lectin

sensitivity, DNA restriction patterns, and the use of probes such as monoclonal antibodies and PCR products.

The neurologic disorders caused by these organisms are difficult to diagnose much less to treat. Among the several cases of *Naegleria*-induced primary amoebic meningoencephalitis (PAME), only two are known to have survived, probably due to early detection and therapy (Flores, *et al.*, 1990). Hence, early diagnosis is very important in the successful treatment of the disease.

Use of monoclonal antibodies for diagnostic purposes against a number of parasitic protozoans has been met with much enthusiasm (Lopes and Alves, 1984). Monoclonal antibodies against *N. fowleri* have been used by Visvesvara (Flores, *et al.*, 1990) in locating and identifying trophozoites in brain sections of patients that succumbed to PAME. This antibody did not react with other species of *Naegleria*. Furthermore, Dunnebacke and Dixon (1989) produced monoclonal antibodies against amoebic lysates of *N. gruberi*, a nonpathogenic strain of *Naegleria*. Such antibodies were able to localize a cytopathogenic material at the tips of pseudopods and peripheral cytoplasm of trophozoites. Using the same antibody, the cytopathogenic material (NACM) was also found in cell cultures exhibiting cytopathic effects.

The use of monoclonal antibodies in the characterization and identification of various *Naegleria* and *Acanthamoeba* isolates as well as their potential as tools for diagnosis are the main objectives of this study. Monoclonal antibodies were produced against some local isolates *of Naegleria* and *Acanthamoeba* which have been characterized morphologically as well as biochemically. Characterization of the specificity and reactivity of these monoclonal antibodies indicates their potential as diagnostic tools for detecting *Naegleria* and *Acanthamoeba* infections.

MATERIALS AND METHODS

Amoebae culture and antigen preparation. Philippine *Naegleria* isolates (Figure 1a) were grown in serum-casein based medium while *Acanthamoeba* isolates (Figure 1b) were grown in proteose peptone-yeast extract-glucose medium in petri plates incubated at 37°C. Cultures at the logarithmic phase were harvested by washing the cells repeatedly with phosphate buffered saline. The cells were suspended in 0.1 M Tris-HCl (pH 7.2) and centrifuged in Eppendorf tubes. The supernatant was discarded and the cell pellet was stored at -70°C until use. A total of 7 ml volume of packed cells (approximately 10^7 cells) was used for antigen extraction. Pelleted cells were pooled using an extraction buffer medium (0.015 M Tris-HCl pH 7.5 containing 1 mM DTT, 1 mM EDTA, 1 mM PMSF). Trophozoites were lysed using a glass homogenizer containing acid-washed glass beads. The percentage of lysed cells was monitored under a Carl Zeiss standard 14 phase

contrast microscope. The homogenate was then centrifuged at 18000 rpm at 4°C for 1 hr using a TLA-100.3 fixed angle rotor of a Beckman TL-100 ultracentrifuge. The supernatant was further clarified by centrifugation at 32000 rpm at 4°C for 1 hr using a TLS-55 swinging bucket rotor. The clear supernatant was then aliquoted and stored at -70°C until use.

Total protein content of soluble protein was determined according to Bradford using Bovine Serum Albumin (BSA) as a standard.

Production of Monoclonal Antibodies. Balb/c mice were immunized with extracts of *Naegleria* and *Acanthamoeba* according to standard procedures (Galfre and Milstein, 1982). Approximately 15 μ g/μ l in 100 μ l of extracts were injected intravenously via the tail vein 4x at two-week intervals. 10^{-6} - 10^{-7} trophozoites were also injected intraperitoneally several times.

Myeloma SP/2 cell line, a gift from Dr. Wilfred Tiu, College of Public Health, University of the Philippines was first grown in 8-azaguanine supplemented complete medium (RPMI-1640 + 10% FBS). After three subcultures in the supplemented medium, the cells were transferred in complete medium without 8-azaguanine until use.

After fusion, cells were grown in HAT-supplemented medium for three weeks and thereafter grown in HT medium. Wells that showed signs of cell proliferation were marked positive (+) and monitored daily for growth. Culture supernatants that turned yellow from positive wells were assayed for the presence of antibodies directed against *Naegleria* or *Acanthamoeba* by ELISA (see below). Monoclonal antibodies were mass produced by injecting positive hybrids into the peritoneal cavity of primed mice. Ascitic fluid was collected and tested as above.

Indirect Immunofluorescent Antibody Test. Freshly harvested *Naegleria* or *Acanthamoeba* trophozoites were dropped onto teflon-coated glass slides. After air-drying, the slides were fixed with cold methanol (-20°C) for 10 minutes. Slides were overlayed with culture supernatant and kept in a humid chamber. One hour thereafter, the slides were once again washed as above, air-dried, and overlayed with second antibody (sheep anti-mouse IgG) labelled with fluorescein isothiocyanate (FITC). Excess or unbound FITC-labelled sheep anti-mouse IgG was removed by washing three times with PBS-Tween 20. A mounting medium (90% glycerol in 50 mM Tris-HCl, pH 8.5, with 0.15 M NaCl and 0.1% paraphenylenediamine) was used to retard fading. Fluorescence was observed under a Carl Zeiss Universal research microscope equipped with epifluorescence.

Enzyme-Linked Immunosorbent Assay. Extracts of *Naegleria* or *Acanthamoeba* were diluted in 0.1 M carbonate buffer, pH 9.6 and dispensed in a 96-well microtiter plate, each well containing 10 μ g protein in 100 μ l solution. The ELISA plate was stored at 4°C overnight. Unattached antigen

was washed off-using PBS-0.1% Tween 20. Non-specific binding sites were blocked by incubating the wells at 37°C for 60 minutes with 1% BSA in PBS-Tween 20. The wells were washed as above, dried briefly, and 100 µ l of culture supernatant from predetermined wells were added. The wells were then incubated at 37°C for 60 minutes and unbound antibodies were washed-off using PBS-Tween 20. The wells were then incubated further at 37°C for 60 minutes with a second antibody (rabbit anti-mouse IgG) bound to peroxidase diluted (1:2000) with dilution buffer. Excess and unbound antibodies were washed-off as above and the plates were incubated for 20 minutes in the dark with orthophenylenediamine (OPD) in phosphate-citrate buffer (pH 5.0) and hydrogen peroxide. A positive reaction is indicated by a change in color from yellow to orange-brown. The reaction was stopped by dispensing 50 µ l of 2.5 M H_2SO_4 to each well.

Gel Diffusion. Ascitic fluid was placed in a central well and serially-diluted antigen was placed in the peripheral wells. The set-up was placed in a humid chamber and left at room temperature for 24 hours. The gel was stained with Commassie Blue G-250 for 5 minutes and then destained in destaining solution for 10 minutes.

Western blotting and Immunostaining (for *Acanthamoeba*).

Three membrane fractions were extracted according to the method of Matthews, *et al.* (1986). These are internal vesiculated membranes (P1), internal non-vesiculated membranes (P2), and plasma membranes (P3). These membrane extracts plus whole cell extract (WC) were used in the characterization of anti-*Acanthamoeba* monoclones IA1 and IB3.

After electrophoresis, western blot transfer was performed according to Matias (1991). Immunostaining was done using both ascitic fluid-derived monoclonal antibodies as well as culture supernatants. Developed strips were air-dried and molecular weights were determined by comparison with the molecular weight markers.

RESULTS AND DISCUSSION

Among the 18 hybrids that successfully grew after fusion, 6 were found to be secreting antibodies against *Naegleria* extracts by ELISA. These 6 hybrids were then cloned in complete medium. Thirty-eight (38) out of 480 wells were found to have antibodies against *Naegleria* (Figure 2). Those 19 clones that gave the strongest reaction were again tested by indirect immunofluorescent antibody tests (IFAT). 16 out of the 19 clones were found positive for IFAT showing an 84% correlation between ELISA and IFAT. Furthermore, by means of IFAT, 2 groups of monoclonals were characterized: those that specifically bind to the surface membrane and those that bind specifically to cytoplasmic antigens (Figures 3a & b). One group of amoeba

stained with the culture supernatant from positive hybrids and visualized by FITC-labelled sheep anti-mouse IgG had localized staining reactions in the cytoplasm. In another group, staining was on the surface membrane. Furthermore, by means of immunodiffusion tests, such antibodies were also found to precipitate *Naegleria* extracts (Figure 4).

Monoclonal antibodies, specifically directed against surface membrane antigens and cytoplasmic antigens of *Naegleria* have been produced. The combination of several intravenous injections using *Naegleria* extracts followed by intraperitoneal challenge using live trophozoites proves to be a useful protocol in the production of varied types of monoclonal antibodies. These antibodies are able to differentiate the local *Naegleria* isolate from *N. fowleri* or *N. australiensis* making them a useful tool in diagnosis.

For *Acanthamoeba*, cell fusion yielded 30.16% *Acanthamoeba* monoclone secretors. Ascitic fluid induced with monoclones IA1 and IB3 were found to react with both membrane-bound and cytosolic proteins of *Acanthamoeba* isolates as demonstrated by ELISA. Culture supernatants had lower titers compared with ascitic fluid (Figure 5). Trophozoites stained with these monoclonal antibodies and visualized using FITC-labelled sheep anti-mouse IgG showed a scattered distrubution of the antigens throughout the cell, with a relatively higher concentration at the plasma membrane (Figure 6). Characterization by immunodiffusion indicates that unlike *Naegleria* antibodies, the two *Acanthamoeba* antibodies are non-precipitating (Figure 7). Finally, western blot and immunostaining revealed that monoclone IA1 binds to a protein of MW 67kD, while IB3 is specific to a protien of MW 76kD. (Figure 8).

Monoclonal antibodies have been used to diagnose a number of protozoan diseases. Visvesvara *et al.* have used such antibodies in identifying and locating *Naegleria* trophozoites in brain sections of patients that died due to amoebic encephalitis (Flores, *et al.* 1990). Whether such antibodies are specifically directed against surface membrane protein antigens or not have not been reported. In the present study, monoclonal antibodies against soluble surface membrane protein antigens of *Naegleria philippinensis* have been produced. This is shown by their ability to precipitate soluble extracts of *Naegleria* trophozoites.

Previous studies have produced polyclonal antibodies against *Naegleria* cytoplasmic antigens. Fulton, *et al.* (1989) using competitive radioimmunoassay showed that the precipitating antibodies against *Naegleria* actin did not bind to actin from *Acanthamoeba, Dictyostelium, Physarum,* sea urchin eggs and vertebrate muscles. This suggests that *Naegleria* actin although conserved in many properties is different enough to have unique antigenic determinants. Dunnebacke and Dixon (1989) have produced monoclonal antibodies against an amoebic component NACM (*Naegleria*

amoeba cytopathogenic material). Monoclonal antibodies that have been reacted with NACM neutralize their cytopathic activities. Furthermore, when used to stain trophozoites, the monoclonal antibody localizes NACM at the tips of the pseudopodia or in the peripheral cytoplasm. Results of the monoclonal antibodies produced react particularly with soluble cytoplasmic antigens.

The usefulness of these monoclonal antibodies produced against *Naegleria philippinensis* and against *Acanthamoeba sp.* remains to be seen. Pathogenic forms of these amoebae have to be characterized and used as a basis for determining whether these monoclonal antibodies are specific against the pathogenic strains or not.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the financial support given by the Philippine Council for Advanced Science and Technology Research and Development of the Department of Science and Technology, the Natural Sciences Research Institute and the Office of Research Coordination of the University of the Philippines.

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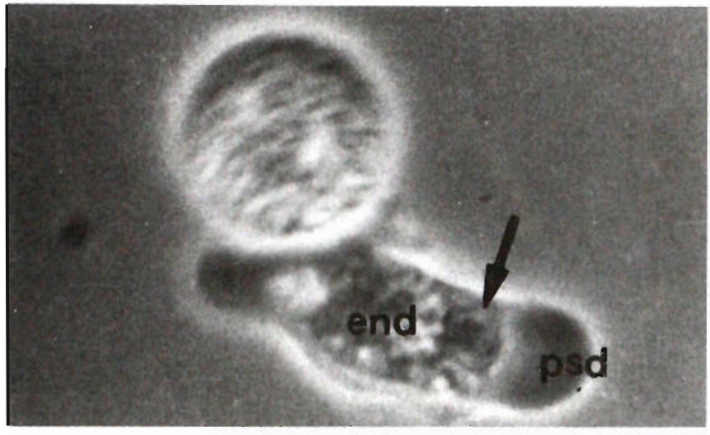


Fig. 1a. A *Naegleria* trophozoite showing the granular endoplasm with a prominent nucleus and an advancing pseudopodium (PSD) (bar=10μ m).

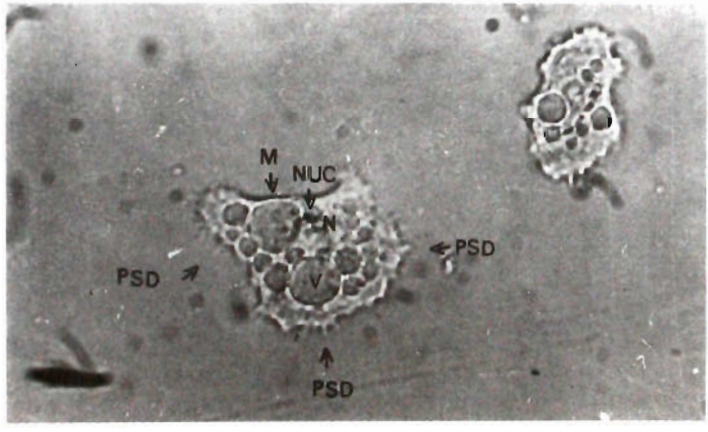


Fig. 1b. *Acanthamoeba* C13 trophozoite, wet mount. Shown are pseudopodia(PSD), membrane (M), vacuoles (V), nucleus (N) and nucleolus (NUC) (bar=10μ m).

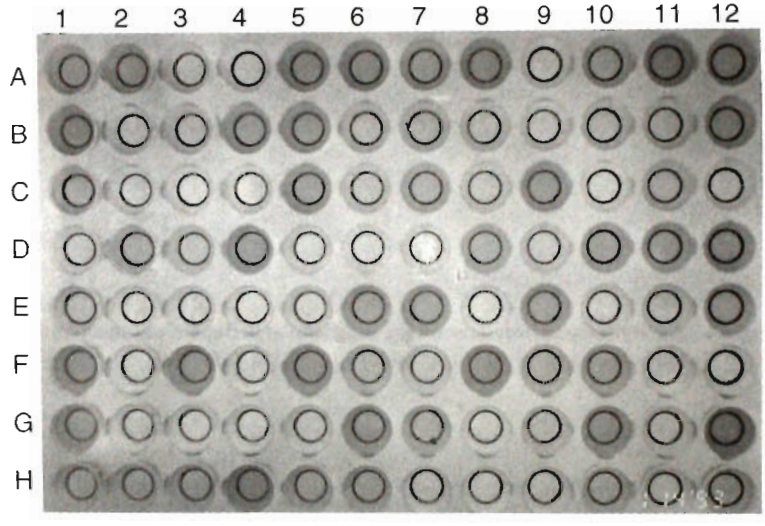
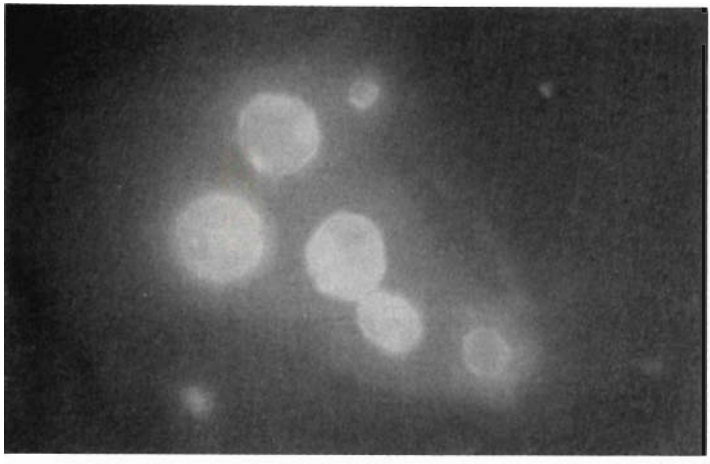
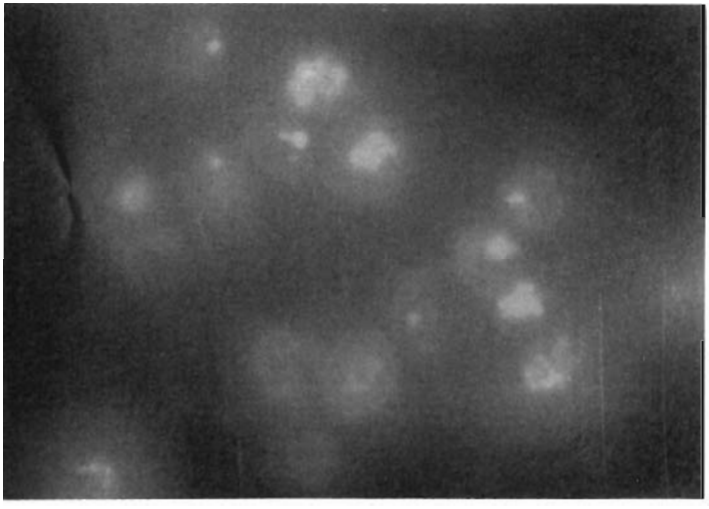


Fig. 2. Screening of cloned hybrids by ELISA. Positive wells are recognized by color reaction.



A



B

Fig. 3. *Naegleria* trophozoites stained with culture supernatant from positive clones. **(A)** The surface membrane is shown to fluoresce; **(B)** Brilliant fluorescence is observed within the cytoplasm.

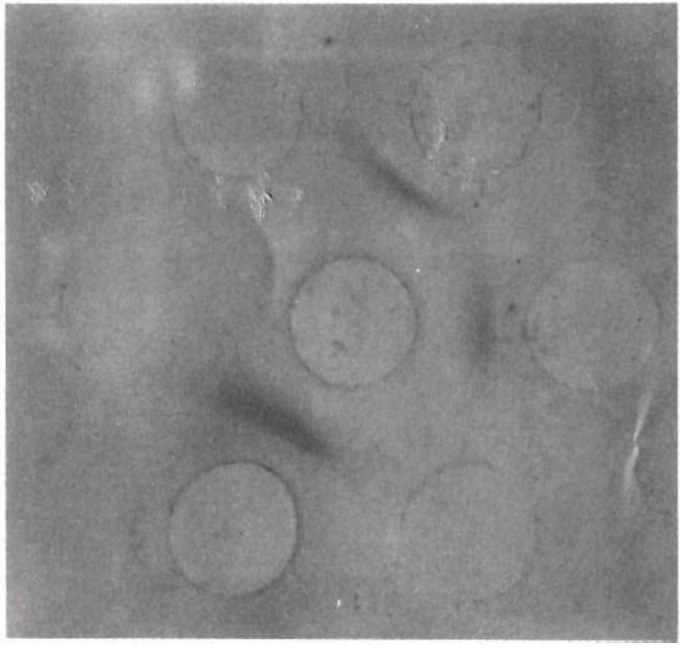


Fig. 4. Immunodiffusion showing *Naegleria* monoclonal antibody to precipitate soluble antigens. Central well contains soluble antigens and the peripheral wells contain serially-diluted monoclonal antibody.

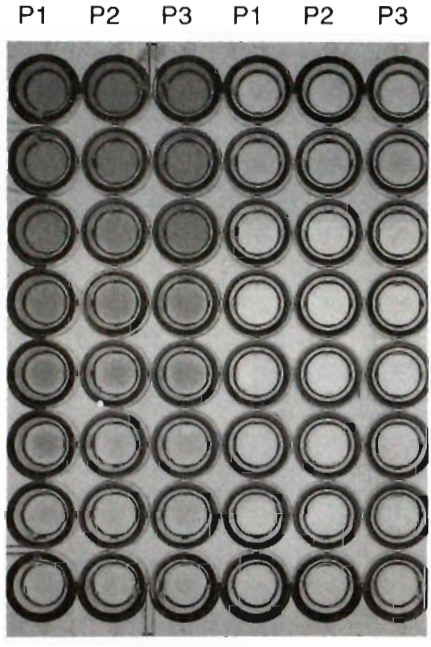
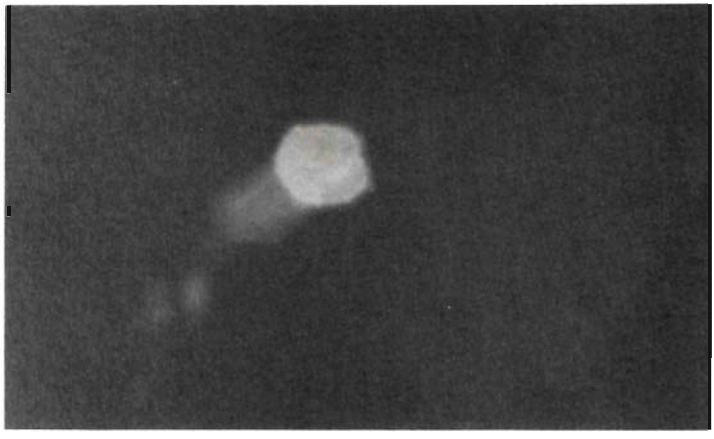


Fig. 5. Comparison of reactivity of ascitic fluid and hybridoma culture supernatants to membrane fractions of *Acanthamoeba* sp. Internal vesiculated membrane (P1), internal non-vesiculated membrane (P2) and plasma membrane (P3) were reacted against anti-*Acanthamoeba* monoclone IA1, from ascitic fluid (columns 1-3) and culture supernatant (columns 4-6).



A

B

Fig. 6. Reaction of IB3 ascitic fluid with *Acanthamoeba* trophozoite showing reactivity with membrane and cytosol. **(A)** A brilliant fluorescence observed under a fluorescence microscope. **(B)** Phase contrast micrograph of the same cell as (A).

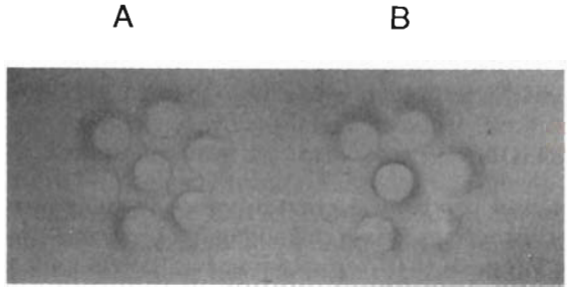


Fig. 7. Immunodiffusion of monoclonal antibodies IA1 **(A)** and IB3 **(B)** showing them to be non-precipitating antibodies. Central wells contain ascitic fluid, peripheral wells contain serially-diluted antigen.

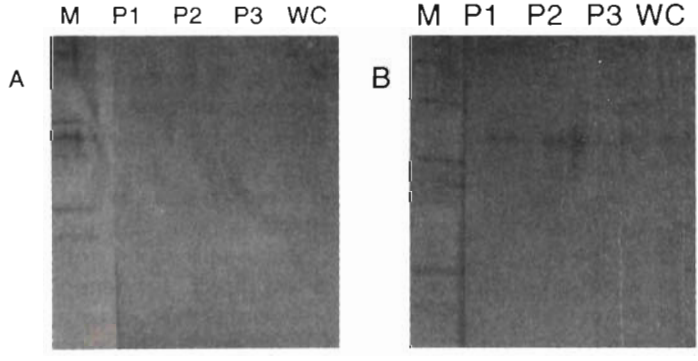


Fig. 8. Western blots of *Acanthamoeba* membrane fractions (P1, internal vesiculated membrane; P2, internal non-vesiculated membrane; and P3, plasma membrane) and soluble whole cell extract (WC) showing a single band at 67 kD recognized by monoclone IA1 (a), and a single band at 76 kD recognized by monoclone IB3 (b)

VACCINE DEVELOPMENT AGAINST THE PHILIPPINE STRAIN OF *SCHISTOSOMA JAPONICUM*

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INTRODUCTION

Praziquantel has been a great advance in the control of disease induced by schistosomes. Mass chemotherapy programs conducted in the Philippines suggest that repeated surveillance and treatment of schistosomiasis results in lower morbidity and prevalence of infection but does not appear to interrupt transmission[1]. Snail control measures or improvements in hygienic conditions have been tried in the Philippines and are not only extremely expensive but also ineffective[2]. Thus, development of other modalities, such as a vaccine, will be needed if disease eradication is contemplated in the next few decades. This is particularly important in *Schistosoma japonicum* because man is probably not the major host of the infection. Since *S. japonicum* is the only schistosome species with major domestic animal, and more importantly, wild animal reservoirs, transmission will never be blocked by chemotherapy programs alone.

Evidence for resistance to infection with schistosomes in experimental animals as well as in humans had been previously reported and provide the rationale for vaccine development in schistosomiasis. Experimental animals become partially resistant to reinfection following a primary infection with either *S. mansoni* or *S. japonicum*[3-6]; fewer cercariae from the challenge infection develop into adult worms in the presence of an active infection. This form of naturally acquired resistance has been claimed by some investigators to be specific while others conclude it to be non-specific[7]. Evidence for acquired resistance in man is less direct. Epidemiologic data demonstrate that in villages endemic for *Schistosoma mansoni*, older individuals have lower prevalence and intensities of infection[8-9]. This pattern is not observed when schistosomiasis has been recently introduced into a community[10]. Careful follow-up of drug-treated individuals with *S. mansoni* have also suggested that some humans appear to be highly "resistant" to reinfection exposed environmentally to infected water but do not become reinfected while others appear quite susceptible (reinfected with identical or even less water exposure)[11]. Such a pattern of "resistant" versus "susceptible" individuals to *S. japonicum* infections has also been suggested from an eight-year prospective village study where resistance to reinfection is suspected to develop in at least some older individuals (14-35 years of age) following chronic exposure to the parasite[12].

A variety of experimental animals, including primates, have been protected from schistosome infections by prior immunization with irradiated cercariae[13-18]. This form of artificially induced resistance is specific to the homologous species of schistosome used to vaccinate. Such preparations are, however, quite perishable, expensive, and associated with significant local side effects, making their application to humans extremely difficult[19]. Several species of rodents as well as larger mammals, including primates, have been protected by attenuated cercarial vaccines[13-18]. In rodents, resistance to reinfection can be passively transferred to naive animals by serum[20-21] or purified immunoglobulin[22] derived from vaccinated donor animals. The degree of protection induced by these antibodies is, however, always less than that observed following vaccination or natural infection.

Resistance to reinfection observed in both a natural infection and following vaccination with attenuated cercariae or passive transfer of immunoglobulin is partial. With most other infectious agents, a 50% reduction in the number of organisms would be quickly overcome by the multiplication of the surviving parasites within the host. For this reason, vaccines against viruses, bacteria and protozoa all need to be >99% effective *in vivo* to have a practical impact on disease. In contrast, helminths do not multiply in the mammalian host. Since the development of clinical disease is generally correlated with the "worm burden" of infected individuals, even a vaccine that induces partial protection would have great utility in schistosomiasis control[19]. Low levels of infection may even be necessary to maintain acquired resistance in the field setting. In addition, it is anticipated that with more purified immunologic reagents that it may be possible to improve on nature.

Very recently, exciting developments in the field of vaccine development have been reported in *S. japonicum*. The new technologies such as monoclonal antibody technology and recombinant deoxyribonucleic acid (DNA) technology have contributed to the major explosion of research in this area. This paper would like to summarize that current knowledge on vaccine development in *S. japonicum* including specific highlights on the strategies employed in the pursuit of candidate vaccine molecules in *Schistosoma japonicum*.

PROTECTIVE MONOCLONAL ANTIBODIES [23]

Monoclonal antibodies against *S. japonicum* were produced from the fusion of splenocytes of BALB/c mice hyperimmunized with soluble worm antigen preparation (SWAP) and P3-NS1 myeloma cells. Clones or hybridomas producing monoclonal antibodies that bind to the surface of living schistosomula by radioimmunoassay were subcloned. Culture supernatants or ascites fluid were collected from the hybridomas and were subsequently used for passive protection experiments in ICR mice.

Passive protection properties by the different monoclonals were tested in groups of ICR mice. Groups of mice received 0.5 ml ascites fluid intraperitoneally 24 hours prior to percutaneous cercarial challenge. Another dose of monoclonal antibodies was given 24 hours after challenge. Each mouse was challenged with 50 cercariae of *S. japonicum* using the loop-coverslip method. Five weeks after challenge infection, the mice were sacrificed and their mesenteric vasculatures perfused to recover the adult worms. The worm load of each mouse was determined by counting the number of worms harvested following saline perfusion. The significant protection levels afforded by the monoclonals is shown in TABLE 1.

One of the protective monoclonals, 200-4B9, was shown to correspond to a novel candidate vaccine molecule on the 50 kd weight range. It was also shown that protease treatment rather than periodate treatment of SWAP affected the binding capacity of 200-4B9. Current efforts are directed at screening the cDNA library for the protein that corresponds to 200-4B9.

NATIVE *S. JAPONICUM* PARAMYOSIN [24]

Based on the success achieved with *S. mansoni* paramyosin and its ability to actively protect mice from challenge infection even without adjuvant, we attempted to purify a similar fraction of antigens in *S. japonicum* adult worms.

TABLE 1. Results of passive protection experiments using murine monoclonal antibodies

Monoclonal Antibody	Isotype	Protective Level	p value
201-1E12	IgG1	16.04%	0.1609
145-4E10	IgG1	34.92%	0.0150
145-2D7	IgM	41.27%	0.0095
145-4D10	IgM	43.06%	0.0018
200-3E9	IgM	47.22%	0.0008
200-4D9	IgM	48.61%	0.0007
200-4B9	IgG1	53.97%	0.0004
201-2H3	IgG1	65.28%	0.0001
145-4F10	IgM	68.25%	0.0001

Following active immunization of ICR mice, we have demonstrated that native paramyosin, when administered even without adjuvants, afforded significant protection of 72.61% (mean of 3 experiments). This level of protection is, in fact, much higher than that reported for *S. mansoni* in mice. In addition to the induction of protection by paramyosin, we have also shown that paramyosin vaccination also results in a reduction of female worm recovery in infected immunized mice.

Recently, two clones have been isolated from a lambda ZAP cDNA expression library after screening with polyclonal anti-paramyosin rabbit sera. These clones are currently being characterized.

CLONING, CHARACTERIZATION AND EXPRESSION OF *S. JAPONICUM* ANTIGENS [25]

This strategy of screening two *S. japonicum* adult worm cDNA libraries was employed by the group at the Queensland Institute for Medical Research to be able to identify potential vaccine candidates. The first library, obtained from the Walter Eliza Hall Institute, was constructed in lambda ZAP with poly(A)+ RNA isolated from parasites of the Philippine (Sorsogon) strain of *S. japonicum* [26]. The second library was constructed at QIMR also in lambda ZAP with poly(A)+ RNA isolated from *S. japonicum* adult worms of Chinese (Anhui Province) origin. The details of the results of the immunoscreening are presented as follows.

Glyceraldehyde-3-phosphate dehydrogenase. A cDNA was sequenced and identified to encode a full-length copy of the coding region which exhibits 76% homology with a similar *S. mansoni* antigen, glyceraldehyde-3-phosphate dehydrogenase (Sm37). As previously reported in *S. mansoni*, this glycolytic enzyme is located on the schistosomular surface. With this strategic location, it has been proposed as a potential vaccine candidate for *S. mansoni*. Preliminary observations show that mice immunized with Sm37 are less susceptible than control animals to challenge infection with *S. mansoni*. Another interesting observation, this time in humans infected with *S.mansoni* in Brazil, has shown the presence of antibodies to this enzyme to correlate with enhanced resistance to reinfection. The high level of homology between *S. mansoni* and *S. japonicum* (Chinese) glyceraldehyde-3-phosphate dehydrogenase makes this reagent a useful candidate vaccine in future protection experiments.

Glutathione-S-transferase. Another cDNA that has been sequenced encodes a 26 kd glutathions-S-transferase (GST). Comparison of this Chinese *S. japonicum* 26 kd GST with that of the published sequence for the Philippine 26 kd *S. japonicum* GST[26] has shown absolute identity. This observation may have important implications when considering the relevance of vaccine candidates to be effective against both the Philippine and Chinese *S. japonicum*

strains. Considerable work has been done on the GST's by the group at the College of Public Health, University of the Philippines and other groups [27-28] and it has been shown that recombinant Sj 26 on its own does not protect but this does not preclude the possibility that Sj 26 together with other antigens may provide significant protection.

Fatty acid binding protein. A full-length copy of a cDNA encoding a fatty acid binding protein has been obtained. The cDNA that was isolated showed a high identity of greater than 80% with the cDNA sequence encoding a similar 14.8 kd in *S. mansoni*. Schistosomes intrinsically lack pathways for sterol and long chain fatty acid synthesis, and therefore, lipids are derived from the host circulation. Immunofluorescence studies have demonstrated the Sm 14.8 kd molecule to be at the dorsal surface of the adult male schistosome, as well as in the muscle layer and the body of the parasite. The presence of this antigen on the surface of the parasite makes it ideal as a candidate for further vaccine studies. Because of its importance in fatty acid intake by the parasite, a vaccine against this antigen could disrupt normal metabolism in the parasite.

22.6 kd tegumental associated antigen. Two cDNAs with good homology to a *S. mansoni* 22.6 kd tegument-associated antigen (Sm 22.6) have been isolated. Sm 22.6 corresponds to antibodies from mice immunized with purified adult *S. mansoni* tegumental membranes. One of the clones we isolated contains a full-length copy of the coding region, and experiments are currently directed to large scale expression of this antigen for vaccine studies in mice.

23 kd membrane antigen. The membrane antigen Sj 23 of *S. japonicum* containing the target epitope of monoclonal antibody I-134 has been cloned and produced as a fusion protein with Sj 26. An initial trial to determine the effect of sensitization with Sj 23 on resistance to challenge infection has resulted in 16.25% protection of the sensitized BALB/c mice. A replicate experiment with the addition of adjuvant is on-going.

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SCREENING AND PRODUCTION OF BIOLOGICALLY ACTIVE COMPOUNDS USING BIOTECHNOLOGICAL TECHNIQUES

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INTRODUCTION

At the Department of Biotechnology in Ngee Ann Polytechnic, we have a number of projects on the diagnoses of diseases, and the screening and production of therapeutics using biotechnological approaches. Our interests not only lie in human diseases but also in teaching and research in agrotechnology, with projects on diseases of poultry and fishes. Based on the theme of this Workshop, this report will emphasize the screening and production of bioactive plant compounds, in particular castanospermine, which is effective against several viruses (eg. HIV, Herpes and related viruses). A short write-up on the diagnoses and treatment for Newcastle and *Vibrio* diseases in poultry and marine fishes is also presented.

BIOACTIVE COMPOUNDS FROM PLANTS

Plants are a rich source of useful chemicals and even today, many of the world's population relies on plants as traditional medicines. In the pharmaceutical industry, 25% of the market is based on plant-derived products. The genetic diversity of plants makes them a potentially interesting source of useful chemicals. The tropical rainforests with its many, diverse plant species offers an exciting realm for exploration.

There are many interests in the production of chemicals from cultured plant cells. Extracting the compounds directly from the plants depends on the ready supply of such plants which may be rare, endangered or slow-growing. For example, it takes many years for a slow-growing woody species such as the Pacific Yew tree to be grown before the bark can be extracted to provide a dose of taxol which is used in the treatment of ovarian cancer. Secondly, there are no reliable microbial source for many plant chemicals. The *de novo* chemical synthesis of many of these secondary metabolites is either not available or is tedious with many steps involved and poor yield. These problems can be overcome by the use of plant cell cultures and bioreactors. Plant cells are likened to mini-factories and can be made to produce secondary metabolites under controlled conditions *in vitro*.

1. PRODUCTION OF CASTANOSPERMINE BY TISSUE CULTURE METHODS

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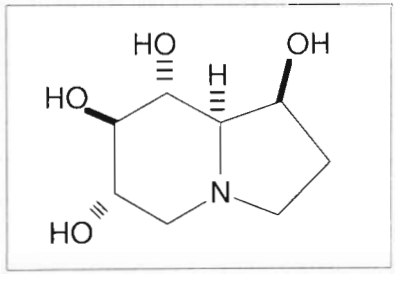
AIDS (Acquired Immune Deficiency Syndrome) is a deadly disease. In 1992, the number of reported persons with HIV infection in Singapore is over 130. There are probably many more who are infected but are unaware that they are.

AIDS is caused by the Human Immunodeficiency Virus (HIV). A glycoprotein (gp 120) on the surface of the HIV binds to a CD4+ receptor on the T4 cell surface. The virus then enters the cell. AIDS is characterised by the reduction in the number of T4 cells. There is active research into vaccines and there are now medicines such as AZT (3'-azido-3'-deoxythymidine) which can slow down the multiplication of the virus in the body and thereby prolong the lifespan of infected persons. There are also medicines to treat the infections and cancers (eg. Kaposi's sarcoma) which occur in AIDS and AIDS-related complex. We are investigating the production of some bioactive compounds, which are inhibitors of glycosylation, that can help in the treatment of the AIDS and other viral diseases.

What is castanospermine?

Castanospermine is an indolizine alkaloid present in the tropical leguminous tree, *Castanospermum australe*. It was first noticed by farmers and biologists that grazing of the fruits by cattle and sheep causes a disease known as locoism which is manifested as dizziness, excessive salivating, vomitting, gastrointestinal disorders and muscular dysfunction (Everist, 1974). The compound responsible was found to be an polyhydroxyindolizine alkaloid (fused 5/6 membered ring), which resembled glucose, and later named castanospermine. This compound is, however, not fatal to humans.



Structure of castanospermine

As viral envelope glycoproteins and host cell surface receptors play an important role in virus adsorption, penetration, syncytium formation and the spread of virus to adjacent cells, the interference with the processing of viral envelope glycoproteins can be an attractive target for chemotherapy against HIV infection (Sunkara *et al.*, 1987). The link between viral infection and cell surface glycoproteins gave castanospermine and analogues of this alkaloid additional attention due to their antiretroviral potency (Sunkara *et al.*, 1989) and their relative non-toxic nature (Burgess and Henderson, 1992).

Castanospermine prevents protein glycosylation by competitively inhibiting the endoplasmic reticulum enzyme glucosidase I. It inhibits HIV replication by modifying the glycosylation of the viral envelope protein gp 120 (Sunkara *et al.*, 1989) as well as inhibiting cell proliferation of various tumours under *in vitro* conditions (Ostrander *et al.*, 1988).

At Ngee Ann Polytechnic, our group are investigating the production of three sugar-shaped alkaloids: castanospermine, deoxynojirimycin and swainsonine. The principal compound under investigation is castanospermine and the results are discussed here.

Production of castanospermine by tissue culture methods

Castanspermine is currently extracted from *C. australe* seeds (Hohenschutz *et al.*, 1981). The trees take many years to bear fruits and fruiting is seasonal. Disease and drought can also radically alter seed supply. Therefore, an alternative source of castanospermine would be the extraction from cell cultures.

As there are no reports on tissue culture work done on *C. australe*, we have to establish an *in vitro* protocol for the growth and proliferation of *C. australe* cells. This was a two-stage process. The first stage involves the establishment of optimum culture conditions for the initiation and proliferation of cells in an unorganised form, known as callus tissues, from various tissue sources. The next stage involves the mass production of these cells in suspension cultures and subsequently in bioreactors. Results of experiments on these two stages are as follow.

a. Initiation and proliferation of callus tissues

The level of castanospermine in seedling tissues was determined first in order to select the most suitable starting material for culture. The extracts were quantified using a HPLC with refractive index detector and it was found that leaf tissues from seedlings had the highest level of castanospermine (10.1 mg/g dry weight) compared to cotyledons (5.37 mg/g), roots (3.8 mg/g) and stems (1.3 mg/g). Therefore, leaf and cotyledon tissues were used as explants (starting materials) for initiation of callus cultures.

Various parameters were tested empirically in matrix experiments to determine optimum culture conditions for proliferation of callus from leaf and cotyledon tissues.

Two nutrient formulations, Murashige and Skoog [MS] (Murashige and Skoog, 1962) and Woody Plant Medium [WPM] (Lloyd and McCown, 1981), were tested. Each matrix experiment contained either MS or WPM nutrient formulation supplemented with a combination of plant growth regulators, namely an auxin (2,4-D [2,4-dichlorophenoxyacetic acid] or NAA (naphthleneacetic acid]) and a cytokinin (BAP [benzylamino purine] or Kin [Kinetin]) at concentrations ranging from 0, 10, 20, 50, 100, 150 and 200 μ M. All cultures were incubated in the culture room at 25±2°C, under 12 h per day photoperiod and a light intensity of 30 μ $E/m^2/s$.

It was found that leaf and cotyledon explants cultured on MS medium produced less browning and formed more callus tissues compared to those on WPM. Highest proliferation of callus was obtained on media with 2,4-D and BAP or Kin combinations. Concentrations of 2,4-D ranging from 20-50 μ M and low concentration (10μ M) of cytokinin (either BAP or Kin) gave 100% of responding leaf explants, each with about 60% callus covering the explants (Fig. 1A & 1B).

Cultures were placed in two different light regimes (12h per day photoperiod, and continuous dark condition) to determine the effect of light on callus induction and proliferation stages. It was found that an initial culture of leaf segments in continuous dark condition for 5 weeks followed by 12h per day light treatment was optimal for callusing. There was less browning of the tissues in dark condition for the initiation stage but better callus proliferation occurred in light condition.

The type of callus formed under the above optimum conditions were fast-growing although they tend be slightly nodular and clumped together. The callus are formed around the cut edges of the leaf and cotyledon explants but for leaf explants more callus proliferated from the mid-rib region. Overall, leaf explants produced more callus than cotyledon segments. As with other tissues from woody trees, the tissues and callus from *C. australe* tend to turn brown easily in culture due to the oxidation of polyphenolics and tannins. There was also a heterogeneous population of callus of different textures when explants were cultured were on media with different combinations of plant growth regulators.

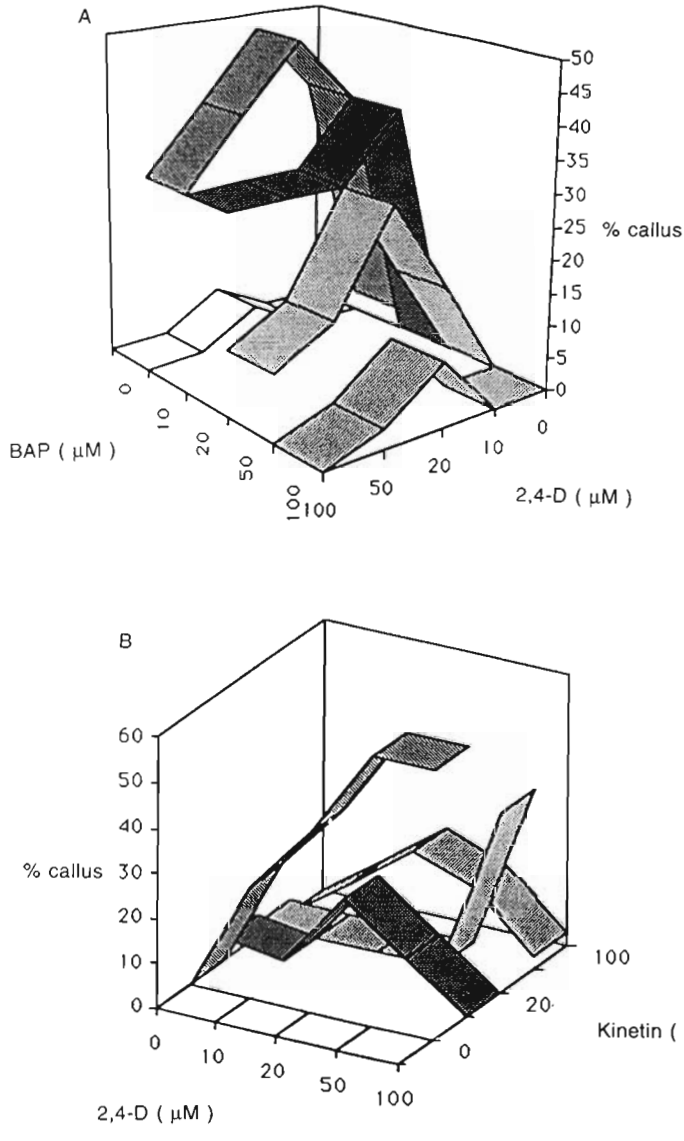


Fig.1. Degree of callus formation on leaf explants of *C. australe* seedlings cultured on MS medium with varying concentrations of 2,4-D and BAP (A) or Kin (B), after 5 weeks.

b. Cell suspension cultures

Only callus tissues which were fast-growing were selected for cell suspension cultures. It was observed that browning occurred more readily when there was fast growth of callus tissues. Therefore, the stock cultures were subcultured at 10-day intervals, to alleviate this problem.

A growth curve study was conducted to analyse the stages of growth of the callus derived from leaf tissues. Samples of media and cell extracts from each stage of growth were taken for analyses of castanospermine content. These experiments are on-going and the results are not available yet.

Further Work

Although it was initially difficult to establish *in vitro* cultures of *C. australe* due to problems of contamination, browning and slow response of the tissues in culture, we have succeeded in producing fast-growing callus tissues which are essential for cell suspension cultures. Our work now is concentrated on modification of growth conditions for the scale-up of these cells in bioreactors.

For future experiments, we intend to increase castanospermine content in cell cultures by the use of elicitors and selection of high yielding strains after mutagenic treatments as well as initiate hairy root cultures of *C. australe*. We have also initiated tests of castanospermine on virus-infected cell lines and the preliminary experiments are encouraging.

2. PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST A NEWCASTLE DISEASE VIRUS (NDV) ISOLATE

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The Newcastle Disease Virus (NDV) is the causative agent of Newcastle Disease which is a fatal disease of poultry widely prevalent in many parts of the world. NDV belongs to the Paramyxovirus group of negative-standed RNA viruses, and can be classified into several categories according to their pathogenicity. Velogenic virus strains cause severe disease with high mortality, while mesogenic strains (moderate virulence) cause reduction in egg quality and production. Lentogenic strains (low virulence) seriously affect egg production while causing little mortality.

A local disease-causing isolate of NDV was used to immunise mice in an attempt to generate virus-specific monoclonal antibodies (MAbs). Although the procedure of MAb generation is somewhat tedious, data from cross-infectivity with other NDV isolates can be useful in studying the spread and the extent of NDV infection among the poultry stocks.

3. SEROTYING FOR STRAINS CAUSING VIBRIOSIS

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The causative agent of vibriosis in marine fishes such as grouper and sea bass, is the *Vibrio* sp. bacteria. some symptoms of the disease are tail rot, haemorragic spots and ulcers. The only treatment available currently is the use of antibiotics which is costly, leaves residues in the fishes and causes development of resistance in the hardier bacterial strains.

Before an effective treatment is developed, the *Vibrio* species have to be identified and characterised. The serotying method is selected over biochemical and DNA-fingerprinting methods to distinguish between the strains and identify the most pathogenic strains.

SUMMARY

The above are some projects that staff and students of the Dept. of Biotechnology are involved in. Many of them are collaborative in nature and have potential commercial applications. We hope to be able to secure collaborative projects of common interest, not only with local institutions and industries, but also international organisations as we believe that advancement in the field of biotechnology is wide-ranging and would benefit mankind.

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INFORMATION TECHNOLOGY IN BIOTECHNOLOGY: AN ASEAN BIOCOMPUTING AND BIOINFORMATION NETWORK

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ABSTRACT

Research in the life sciences is undergoing a major paradigmatic shift driven by the advances in computing, telecommunications and information technology. Computers and computer networks are becoming so important in bioscience and biotechnology that specialised units and resource facilities have been commissioned by many institutions to keep up with the rapid developments currently taking place. National, regional and international networks specialising in biocomputing and bioinformation are already in place or being set up, notably in Europe and America. These network resources can be accessed by users worldwide through the network of networks, INTERNET, using different computer and network programs. In this paper, we describe the usefulness of these resources and explain how they may be made available to the ordinary user in order to promote current awareness in research, competitiveness in development, creativity and innovation through global discussion and to broaden perspectives. We propose that a regional resource for life scientists in ASEAN countries, and possibly beyond, be set up in order to bring the technology to currently un-networked researchers.

INTRODUCTION

Information technology on the Biotechnology agenda

According to Lander *et al.*[1], "biology is in the midst of a major paradigm shift – a shift driven by computing. Having in many respects already become an informational science, biology is rapidly becoming a computational analytical science as well. Computerized databases of genetic information, for example, are now vital in determining the potential significance of research findings". The trend in almost all future biological research is clear:

"Computing methods that allow the efficient and accurate processing of experimentally gathered data will play a crucial role". One need only try to keep up to date with the literature of any field of research to realize that the amount of information is increasing exponentially. Without computer databases and computer techniques to analyse, maintain, store, search and retrieve data, the modern scientist cannot carry out meaningful research at the frontiers of science. Moreover, research in the biosciences is increasingly carried out in collaborations and in multidisciplinary groups. Rapid exchange of information and co-ordination between members of a group, often across national boundaries is becoming a norm. Furthermore, information garnered by different research teams throughout the world is being shared by means of globally accessible databases connected by computer networks.

When the main part of this paper proposing a biocomputing resource for infectious disease research was originally presented in this Workshop[2], the implications of the above observations for the ASEAN region became self-evident. Moreover, through the discussions that ensued between ASEAN delegates, it also became obvious that the scope of the original proposal should be expanded to include other fields in which biotechnology has had impact: medical, veterinary and agricultural sciences and other life sciences.

For some of the objectives of ASEAN science and technology development to be met, the inclusion of computer networking and information technology in the biotechnology agenda is important and may have beneficial spin-offs such as:

a) continued dialogue to pursue the initiation and implementation of collaborative ventures,

b) broadened participation in collaborative projects,

c) the creation of the infrastructure and "infostructure" essential to a rich interchange of ideas and fruitful collaborations, and

d) sharing of mutually beneficial information and computing resources.

This paper seeks to survey briefly the ways in which biocomputing can and has influenced bioscience research in recent years, with particular reference to biotechnology research and development. It also provides some answers to the question - how can an ASEAN network of biocomputing and bioinformation resource foster co-operative and synergistic application of biotechnology ? Finally, we include a preliminary proposal of an internationally shared biocomputing, biotechnological and bioinformation "infostructure" based on the original paper submitted to the Bangkok Workshop and on the discussions and ideas generated at the workshop.

Table 1. List of biotechnological and bioscientific techniques and their associated computer software and hardware.

Nucleic acid DNA hybridization: DNA and RNA probes; Agarose gel electrophoresis	Biological database sequence search/retrieval; multiple sequence alignment; back translation; melting point prediction; densitometric scanning; molecular weight interpolation of electrophoretic gel bands
Oligonucleotide synthesis	Computer controlled instruments; oligonucleotide design
Polymerase chain reaction	Biological database sequence search/retrieval and multiple sequence alignment in primer design; microprocessor controlled thermocyclers
Monoclonal antibodies in Enzyme Immunoassays (EIA)	Automated data acquisition from microtiter plate readers, statistical and enzymological analysis of EIA data
Automated assays systems	Hardware and software to control the robotics
Bioreactor production of pharmaceuticals, vaccines	Real-time monitoring of bioreactor parameters; graphics display; experimental design in optimization of reactor parameters
DNA sequencing of genes	DNA/Protein sequence analysis package; biological database sequence search/retrieval; detection of regulatory sites
Protein sequencing	DNA/Protein sequence analysis package; biological database sequence search/retrieval; prediction of secondary structure; detection of protein sequence motifs; prediction of antigenic sites
Gene cloning and recombinant products	Simulation of gene cloning; restriction mapping
Synthesis of peptide antigens	Protein sequence analysis package for peptide sequence design; computer controlled instruments
Molecular modelling of target of drugs	High resolution graphics terminals; macromolecular x-ray crystallograhic analysis package; energy minimization programs
Design of drugs	Molecular modelling and manipulation software; QSAR programs
Screening for anti-microbial activity	Automated data collection; interfacing with instruments; inventory and database management using automated machines

COMPUTERS AND COMPUTER NETWORKS ARE ESSENTIAL IN BIOTECHNOLOGY

Table 1 is a selected list of techniques in modern biotechnology. For almost every technique, there are one or more stages in which computer tools may be used. These include automated data acquisition; computer-aided experiment design and planning; and computational tools for data analysis and interpretation. Ranging from diverse fields such as diagnosis, prevention and treatment of infectious diseases to biochemical engineering processes for large scale production of biotechnological products, our research and development can be improved with the inclusion of computer technology.

Advances in telecommunications and the reduction in costs of computer hardware, peripherals and networks have led to the imminent possibility of the computer-controlled, automated or robotised laboratory. Organised like a factory line for quality control and efficiency, the automated diagnostic laboratory is no more a pipe-dream for such laboratories are beginning to appear, even in the hospital context[3]. Within each laboratory, different instruments may be joined by computer cables to a computer which processes the data. Different computers may "talk" to each other in a local area network (LAN), which, in turn, can exchange data with other LANs from other research institutions and hospitals within a province, a country, a region, or across the world. One example of such an implementation is at the Bioprocessing Technology Unit of the National University of Singapore which specialises in downstream processing of biotechnological products.

THE BIOINFORMATION GLUT

Currently, there has been an unprecedented explosion in the amount of biological data produced annually-the so-called information glut has hit resesearch in the sciences associated with biotechnology. For example, DNA and protein sequence data in GenBank[4] is doubling once every two years. With the concerted effort of the many research groups associated with the global Genome Research project, this rate is expected to increase. Similarly the number of journals, research papers and reports have also increased; techniques have become more efficient and as more techniques today rely on quality-controlled kit-based technology, there is virtually a commercial kit for every procedure in molecular biology. The number of databases, too, have increased in order to cope with the organisation and storage of data. Kamel[5] recently lists the number of databases associated with molecular biology alone to well over 100, many of which are publicly available at minimal or no cost.

The challenges are, therefore, in information processing, exchange and dissemination. Advances in these new fields will increase the pace of research and lead to more data and contribute to the information glut in a positive feedback loop. For example, in the United States, a National Center for Biotechnology Information (NCBI) has been set up at the National Library of Medicine, National Institutes of Health, Bethesda, since 1988. Its mission is to develop new information technologies to aid the understanding of fundamental molecular and genetic processes that control health and disease[6]. While such lofty aims may take time to filter down to ASEAN nations, we cannot escape from the reality of this paradigmatic shift[7] in biological research, much as we cannot escape from the techniques of molecular biology that have led to the biotechnology revolution in the past two decades.

EMERGING BIOCOMPUTING AND BIOINFORMATION NETWORKS WORLDWIDE

The World Health Organization (WHO) today has at least five WHO Collaborating Centres for Health Informatics, of which the National University Hospital, Singapore, is one. The objectives of these centres include the spearheading of initiatives on a global scale for the development of informatics in health care and research, including infectious disease research.

The WHONET program comprises Microbiology and Immunology Support Services of the World Health Organization. As described by Gibbons[8] in an article exploring strategies to fight drug-resistant microbes, "WHONET's goal is to link a minimum of several hundred microbiology laboratories around the world so that information about drug resistant strains of bacteria and viruses identified in any particular lab could be shared rapidly." This network is currently four years old with connections as widely spread as two dozen laboratories in South and Central America and a dozen in Asia including China which mail, fax or hand-carry the data to Thomas O'Brien and John Stelling of the Harvard Medical School. Today, this kind of information can be rapidly disseminated via computer networks, and stored in a retrievable form for global access.

In Amsterdam, The Netherlands, the Expert Center for Taxonomic Identification (ETI) initiated an international protist network for algae and protozoa in December 1991[9]. This database, funded by the Dutch Government, the University of Amsterdam and UNESCO, may be accessed by CD-ROM or by telephone modem. These facilities are expected to expand and will no doubt be available on computer networks soon. Shalk says of developing countries that "there is a need for directly accessible taxonomic information and expertise" and that such easily accessible databases are needed to tackle agricultural problems eg. pests, diseases, and for improving public health, for wildlife, aquaculture and fisheries management.

The international Microbial Strain Data Network (MSDN) has been described by Kirsop *et al.* [10] It can now be accessed via Internet through a *telnet* remote login procedure or by dialing up to their main computer. More than a dozen different databases of microbial strains are available, including the following list.

1. MSDN directory, MSDN secretariat, Cambridge, UK.
2. ATCC American Type Culture Collection Databases, Rockvile MD, U.S.A.
   Cell Lines Catalogue; Recombinant Materials Database; Bacteria, Phages & Media Catalogue.
3. European Collection of Animal Cell Culture Database (ECACC), Porton Down, Salisbury, UK.
4. UK National Collections of Yeasts and Food Bacteria (NCYC/NCFB) Norwich, UK.
5. UK Culture Collections Databases (MiCIS).
6. UK Culture Collection of Algae and Protozoa (CCAP).
7. Deutsche Sammlung von Mikroorganismen Und Zelkulturen (DSM) database. DSM/GBF, Braunschweig, Germany.
8. Netherlands Culture Collection Databases (CBS/NCC) Centraalbureau voor Schimmel-cultures.
9. France Culture Collection Databases, MINE network, SUNIST France.
10. Czechoslovak Catalogue of filamentous fungi (CCF) Collection of Fungi, Charles University, Prague.
11. Hybridoma Data Bank directory (HDB) Canadian Scientific Numeric Database System (CAN/SND).
12. World Data Center (WDC) databases, RIKEN, Japan. A directory of culture collections worldwide and the species maintained, the HDB database, the algal collections worldwide, hybridomas and bibliographic information on plant tissue and cell cultures.
13. BDT Tropical databases Brazil, Base de Dados Tropical (BDT) Campinas Brazil.
14. Datastar databases of bibliography at Berne, Switzerland (commercial).

According to Kirsop *et al.* [10] "anyone wishing to make appropriate databases available on the network should contact the MSDN secretariat to discuss procedures. Such databases remain the property of the database provider, who may update, remove or charge for access to the database as wished. INFO, an easy-to-use software developed at the Tropical Data Base, Campinas, Brazil is now used."

At the World Data Center of Microorganisms[11] (WDC) at the RIKEN institute, Japan, 651 culture collections located in 55 countries throughout the world are registered and computerised in the World Directory of Culture Collections. Such a directory assists the researcher in locating important strains for analysis and comparison. This database of databases is now available for search and retrieval on the Internet, which means that anyone registered with WDC who has access to a computer connected to Internet can get at the data easily and nearly instantly. In the U.S.A., for instance, this means that a conservative estimate of 10 million people can potentially access that data, of which a considerable proportion may be policy makers, scientists and engineers of some kind.

Over the past decade, the United Nations Industrial Development Organization (UNIDO) initiated the foundation of the International Centre for Genetic Engineering and Biotechnology (ICGEB). Included in the ambitious agenda is the setting up of a computer resource for molecular biology called ICGEBnet[12]. This network has progressed in the past two years to include state-of-the-art biocomputing and bioinformation resources, based on a central interactive timesharing computer facility, including high performance molecular modelling graphics facilities (Silicon Graphics workstations). The principal mechanism for remote access to the ICGEB net resource is via the Public Data networks (PDNs) although recently, ICGEB can be accessed via the global computer network, Internet. Thus, many developing nations can now enjoy sophisticated biocomputing resources.

These examples highlight the trend toward international exchange and sharing of research data by electronic means. This global trend has important implications and benefits in regional contexts, as discussed in the following sections.

THE BIOCOMPUTING EXPERIENCE IN UNITED KINGDOM AND IN SINGAPORE

The overview by Rysavy *et al.*[13], outlining the computing and networking facilities available in support of the Human Genome Mapping Project in the United Kingdom, illustrates clearly how a concerted and integrated approach towards supplying computing services can be of tremendous benefit. The comprehensive infrastructure supports a wide range of research programmes in molecular biology, genetics, biochemistry, immunology and cell biology and has acquired first-class information technology software for accessing on-site, national and international molecular biology databases through global computer networking. Thus, besides the infrastructure, user service, support and training form an integral component of the network. Recently, Rouch *et al.*[14] have published proposals of guidelines for system managers and service providers to follow. An ASEAN network can distill workable ideas from

such reports and create a conducive and supportive environment for our own researchers specific to our needs based on these as models. To illustrate in concrete terms how some of these ideas have already been implemented in Singapore, the experience with using local facilities and global network connection from our university for research will be described in the next section with specific examples, some of which have been described previously[15]. These facilities can, with some effort and funding, be made available to ASEAN researchers if an ASEAN research network can be set up. Moreover, within a time-scale of one to two decades, this network may be expanded or incorporated into an Asian-Australasian network spanning the western shores of the Pacific Rim.

BENEFITS OF AN ASEAN BIOCOMPUTING AND BIOINFORMATION NETWORK

Tips on biotechnological methods from colleagues and experts.

A colleague at our Bioprocessing Technology Unit was interested in getting kits for baculovirus expression of recombinant tumour necrosis factor-B, and did not know the vendor. A simple message was composed and electronically mailed (email) to the methods-and-reagents electronic bulletin board of BIOSCI[16] (and there are more than twenty of them dealing with topics ranging from virology to molecular biology). Within a day or so, messages from all over the world flooded in, volunteering information and tips on how to get baculovirus research going, including one person who volunteered the assistance of his twin brother who was involved in the development of a kit. Although anecdotal, such assistance from the network community is widespread and occurs daily with questions ranging from molecular biology to trouble-shooting a technical procedure. This facility is free and is accessible through computer facilities already present in many institutions throughout the world, including developing nations. The key advantage in this: one has the assurance that help is available promptly whenever one needs it. For most, it is as easy as walking down the laboratory corridor for help and advice.

Information and advice on purchasing equipment and reagents.

Recently, an electronic discussion on the pros and cons of the various types of PCR thermocycler machines was most helpful for laboratories wishing to initiate PCR technology in their research but were hampered by the budget and the bewildering range of thermocyclers in the market. If one needs a rare reagent, it might just be possible that someone in the network (and there thousands of researchers who read network newsgroups and subscribe to bulletin board news) will point you in the right direction.

Daily discussion of specific topics of interest.

Discussion may not be confined to equipment or reagents, for often, intellectual discussions do take place on the network bulletin boards. These may include individual disciplines such as plant research, immunology, virology and x-ray crystallography, or techniques such as polymerase chain reaction.

Electronic conferencing and announcements of conferences.

Important conferences are periodically announced via the network, and recently, workshops have been held over the network by electronic mailing and other more sophisticated computer programs that allow realtime "conversation" between a number of participants. For instance, one of the authors (KC Lun) was involved in organising a major world congress (Seventh World Congress of Medical Informatics, MEDINFO 1992, Geneva) via this mechanism. This feature should become increasingly popular, such that before long, we may find ourselves doing away with travel to conferences and workshops except for the most crucial ones, for they will be conducted through computer networking.

Literature survey from on-line bibliographic databases and world-wide library catalogues.

To date, there are more than 300 libraries of tertiary institutions throughout the world whose on-line catalogs are available to network users. Often, associated databases such as encyclopedia and dictionary facilities are also available. In recent years, there has been a trend towards the accessibility of CD-ROMs over local area networks (LAN) or wide area networks. These will make available the wide range of bibliographic databases such as MEDLINE and Science Citation Index over computer networks. At our University, over 19 CD-ROM databases are now available on our campus-wide network called NUSNET. There are moves too, towards the installation of CD-ROM databases on servers to improve performance and we are actively studying this possibility.

Search, retrieval and browsing of biological databases; acquisition of free public domain software for biochemical, microbiological and molecular biology computational analysis for the PC and Macintosh; use of biocomputing software to facilitate molecular microbiology research locally or remotely.

In our research on the chlamydial major outer membrane protein (TW Tan), we have performed DNA sequencing on the corresponding gene. These sequences may be searched against the latest release of major sequence

databases including GenBank, EMBL[17] and after translation using DNA sequence analysis packages, searched against Swiss-Prot[18], GenPept, PIR[19] databases to pull out homologous sequences in other related serotypes, much in the same way as Boguski[20] has done for the neurofibromatosis gene. Retrieved sequences can be analysed by multiple sequence alignment packages such as CLUSTALV which are free public domain software available from the network[21]. PCR primers may then be designed using the program from the laboratory of Eric Lander, which is again public domain. Translated sequences may also be searched (ProSearch)[22] against the PROSITE database of Bairoch[23] for any protein motifs which may be important in our understanding of this immunoprotective membrane protein.

In summary, the brief examples given illustrate that information, advice and ideas can be freely exchanged over computer networks in order to overcome the intellectual and technological isolation that one experiences when working away from the major centres of bioscience research. Storage, organisation, management, search and retrieval of biological data, information and knowledge need not be confined to the privileged few but can be placed in the hands of the individual researcher. The lack of tools for processing, analysis and interpretation of data need not be a barrier to excellence in research: if an ASEAN network can be initiated, all the benefits illustrated by the above examples can become accessible to the ordinary ASEAN researchers. This may reduce the need to attend expensive courses overseas and will certainly broaden the outlook of the scientist merely by having him sit in front of a set-up as simple as a personal computer connected to a modem and a telephone line.

PROPOSAL FOR SHORT-TERM ACTION AND A SUMMARY OF LONG-TERM PLANS

As discussed in the Bangkok workshop, we have arrived at the following proposal for short-term action to initiate the long-term plan of establishing an ASEAN network (see Appendix 1).

A. Commissioning of a survey of biocomputing needs in selected representative institutions among ASEAN countries, and a study of how biocomputing may be implemented at the institution taking into account the nature of the telecommunications infrastructure of the country.

B. Initiation of exchange of experimental databases and biocomputing resources on a bilateral basis between academic or research institutions which already have the technological infrastructure.

C. Organisation of workshops and courses to raise the general awareness among ASEAN bioscience researchers of biocomputing and bioinformation software.

Furthermore, Figure 1 shows how an administrative organisation may be set up to oversee the project. This project may well involve the input from several subcommittees of the ASEAN committee on Science and Technology, including those concerned with biotechnology and information technology. At the same time, dialogue may be initiated on a bilateral basis with the appropriate bodies in Japan and Australia with a view to creating an Eastern Pacific Rim backbone of computer networks, and with the mission to exchange information and share computing resources related to the life sciences.

Concurrently, the agenda for action should include institutional, national and regional efforts towards the progressive implementation of hardware, software and training. As for the latter, workshops and regional courses may be organised to increase the awareness among ASEAN scientists of the value of such a network. Further details of this overall proposal are included as Appendix 1 of this paper.

CONCLUSION

With information technology riding on the global telecommunications infrastructure that is penetrating the remotest parts of our countries, the number of ASEAN researchers participating in regional exchange of information and collaboration need not be restricted to those who can be funded with travel. Secondly, with shared and mutually accessible databases and knowledge bases, our understanding can be greatly expanded. With computer networking to enable a rich and informal exchange of scientific ideas, the synergy of co-operation and the sum total of our joint endeavours will be far greater than the sum of our individual efforts. Finally, with a progressive implementation of computer networking in our respective countries, within ASEAN, and possibly extending to countries on the Asian-Pacific Rim, we can plug ourselves to the global information grid, create and contribute to a more global bioinfostructure and tap resources that will virtually make the barriers of time and space vanish.

Biological research today can no longer be one or two persons beavering at the laboratory bench on shoe-string budgets. In more advanced nations with a strong tradition of scientific excellence, it has even passed the stage of team work between individuals. Biological research is now capital-intensive, voluminous in data output, multi-disciplinary, multi-party and multinational, high-tech instrumentation and information-driven. Without the means of rapid exchange and extensive dissemination of information underpinning modern research, any attempt at it will very quickly be a lost cause. The time to begin thinking of an ASEAN biotechnology, biocomputing and bioinformatics computer network resource is now. And to any detractor, one need only quote the grim

reminder: anything that hinders the flow of up-to-date information and timely knowledge will condemn us to intellectual servitude to those who possess them.

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APPENDIX 1

A PROPOSAL FOR IMPLEMENTING AN ASEAN BIOCOMPUTING AND BIOINFORMATION NETWORK

In this proposal for an ASEAN biotechnology, biocomputing and bioinformatics network resource, we consider the following factors.

a) Many countries including Japan, South Korea, Taiwan, Hong Kong, and ASEAN nations such as Thailand, Malaysia and Singapore have nation-wide computer network infrastructure connected to global networks already implemented or in an advanced stage of implementation. Other nations are in the process of planning such computer infrastructure. However, not all networks are amenable to a research agenda.

b) National network infrastructure reach differing levels of penetration, usually to key personnel and not necessarily down to the scientist. Thus, although an institution may be have network facilities, these are often not accessible to the bioscientist.

c) Rural areas where field research may most appropriately be carried out may not have sophisticated telecommunications and computing facilities for rapid data analysis, gathering and dissemination.

d) The level of computer literacy is increasing rapidly amongst Asian populations, but network literacy not as widespread.

e) Although some specific databases peculiar to specialised disciplines pertaining to the ASEAN region are lacking, databases of general bioscientific interest which support biotechnology, bioscientific, medical and agricultural research are many and much sought after.

f) A regional network with initial aims limited to particular fields of research, such as infectious disease research, can immediately have effect on other areas of research and can rapidly lend itself to wider objectives. Hence, the cost-effectiveness should include an assessment of its potential spin-offs.

In view of these factors, we have devised the following proposal.

ADMINISTRATIVE INFRASTRUCTURE

We propose the setting up of the following committees and teams (Figure 1).

A) ASEAN steering committee on the development of information technology in biotechnology and biosciences, comprising high-level officials from participating countries.

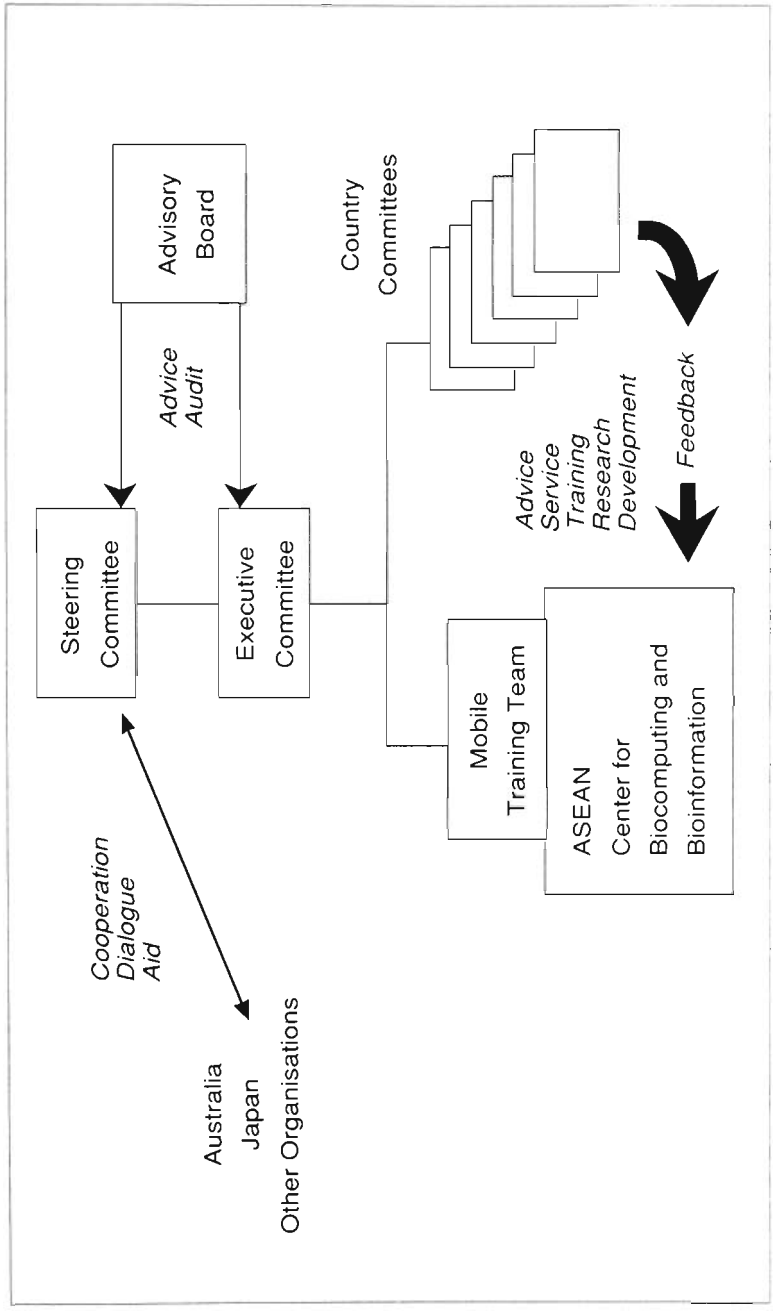

Fig. 1. Diagram of a possible administrative structure to oversee the formation of the ASEAN biocomputing and bioinformation network.

B) Expert Advisory Board comprising computer and scientific experts throughout the world to advise on the project and to audit the progress of the project, reporting to the steering committee.

C) Executive committee to carry out the proposals and to report to the steering committee.

D) Establishment of Country committees of which a representative sits with the executive committee.

E) Mobile Training Team of computer technicians and programmers in the field to assist the establishment of biocomputing facilities on site and for training users to be competent in computer and networking technology.

F) Establishment of a ASEAN Centre for Biocomputing and Bioinformation, a centre of excellence in service, training and research, to provide a focus for the project.

AGENDA FOR ACTION

Administration Agenda

a) Procurement of funds to finance the project.

b) Dialogue with other bodies such as SEAMIC which are involved in an analogous function, with the distinct possibility of co-operation and collaboration.

c) ASEAN Survey of biocomputing and bioinformation needs of each participating country.

d) Identification of national sites and key representatives/contacts of each participating nation for implementation of the network.

e) Independent evolution of national agenda for biocomputing and bioinformatics.

f) Representation at national computer infrastructure planning committees to put forward the computing interest of bioscientists, health and medical scientists and practitioners, agricultural researchers and practitioners.

g) Negotiating for usage of international telecommunications bandwidth with telecommunicaltions authorities to establish an ASEAN computer backbone for biocomputing and bioinformation.

Hardware Agenda

a) Planning for a distributed architecture of remote but INTERNET connected mainframes at national sites.

b) Purchase and installation of mainframe computers and peripheral computers for national sites which require them.

c) Pilot links (eg. using UUCP or equivalent) from un-networked computers in un-networked institutions to Internet gateways at networked institutions locally over telephone modems using low to medium speed data transmission.

d) Computer terminals to connect the laboratory of research groups to the local area network in already networked institutions.

e) Formation of computer nodes throughout ASEAN that form the ASEAN network computing backbone.

Software Agenda

a) Initiation of network by starting communications, data exchange and database sharing between Internet sites already operational in the participating nations which already have the computing infrastructure.

b) Setting up of network information retrieval systems on our machines tailored for bioinformation retrieval and the implementation of biocomputing facilities.

c) Conversion of existing electronic databases into network accessible forms.

d) Conversion of existing databases on paper medium to the electronic medium.

e) Identification of new areas for the creation of network databases.

Training Agenda

a) Discussion and mutual training of national representatives.

b) Training in biocomputing and bioinformation user support.

c) Training in conducting biocomputing training workshops

d) National and international training courses to disseminate the new technology.

Facilities Planned

In addition to bringing computing facilities to the ASEAN bioscience researcher, it may be possible to work towards the implementation of the following projects.

1. Near real-time exchange of data between international R&D collaborators in specific disciplines.

2. Dissemination of electronic publications pertaining to individual disciplines, for example, infectious disease research, control and therapy, such as those currently distributed by SEAMIC.

3. On-line surveillance data, for example for public health, a surveillance and warning system for outbreaks of infectious diseases.

4. Directory listing of

   - biological databases which are accessible;
   - services which are available;
   - resources for expert consultation, discussion etc.;
   - public domain software for computational bioscience research.

5. Creation of network bulletin boards for discussion of specific disciplines relevant to the ASEAN region.

6. Creation of specialised network accessible databases.

   for example, for infectious disease research

   - Disease register and surveillance database

     eg. Asia-Pacific Influenza surveillance;

     Antibiotic resistance surveillance.

   - International Classification of Diseases ICD-10.

   - Diagnostic techniques database.

   - Bibliographic databases.

MOLECULAR BIOLOGY TECHNIQUES APPLIED TO INFECTIOUS DISEASES : SELECTED PROJECTS IN SINGAPORE

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INTRODUCTION

The application of molecular biology techniques to the study of human pathogens has provoked a revolution in the way we look at infectious diseases. Now, more than ever the promise of better and rapid diagnosis, treatment and prevention seems closer. To control infectious diseases which are of common concern, collaboration among researchers in ASEAN countries and others should be strengthened in order to use human and economic resources efficiently and productively. This report briefly summarizes several research projects in Singapore where molecular biology techniques are being used for the diagnosis, prevention and treatment of veterinary and medically important infectious diseases.

RESEARCH PROJECTS: DIAGNOSIS

a. Molecular detection and identification of human pathogens in clinical samples

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The diagnosis of several acute human infections is complicated by the fact that current techniques do not give very rapid results and in certain cases, lack sensitivity and specificity, and require specialized containment and prepared personnel. For some of these diseases such as those caused by *Mycobacterium tuberculosis* (Tan *et al.*, 1993), *Toxoplasma gondii*, and *Chlamydia trachomatis*, traditional tests for isolation of the pathogens may require many days, and sometimes weeks. The diagnosis of *T. gondii* and *C. trachomatis* infections is further complicated by the fact that *Toxoplasma* and *Chlamydia* are obligate intracellular parasites. So, they cannot be cultivated

in non-living or cell-free media. Their isolation, thus requires cell cultures or animal inoculation, which is expensive and labour intensive.

The use of molecular, non-culture techniques such as gene amplification is therefore, highly appealing for the detection and identification of the presence of human pathogens in clinical samples. Such tools have also allowed the recognition of new microorganisms infecting AIDS patients (Relman *et al.*, 1990; Hirschel *et al.*, 1990; Bottger *et al.*, 1992). We are currently using molecular techniques for the detection of several pathogens in clinical samples.

b. Molecular techniques in the typing of pathogens and in epidemiological studies

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We are currently using several molecular biology techniques to detect and type a wide range of pathogens including *Chlamydia* spp., *Pseudomonas* spp., *Enterococcus* spp., *Klebsiella* and *Pasteurella* spp. and methicillin-resistant *Staphylococcus aureus*. The aims are to develop rapid techniques for carrying out epidemiological studies and for detection of those pathogens in a clinical setting.

Specific primer sequences targeted at the conserved 5' and 3' gene segments of the chlamydial major outer membrane protein (MOMP) gene have been used to amplify *C. trachomatis* DNA for typing from tissue culture isolates (Tan *et al.*, 1991; Chua and Tan, 1991). Restriction endonuclease analysis and direct PCR-cycle sequencing using an automated DNA sequencer (Applied Biosystems) of amplified MOMP genes have been used to distinguish between chlamydial serotypes (J.Teo *et al.*, 1992). Currently, work is in progress to increase the sensitivity of the amplification protocol to facilitate the use of these techniques in the clinical context.

The Gram-negative bacterium *Pseudomonas pseudomallei*, the causal agent for melioidosis has been suggested as a possible contributory factor to the cases of Sudden Unexplained Death Syndrome (SUDS) in Singapore among guest workers from Thailand. One pressing question is whether the protean manifestations of melioidosis might be due to different types of *P. pseudomallei*. We have used SDS-PAGE profiling of total cellular proteins as a rapid means of confirming diagnosis (Lin et al, 1990), silver-stained polyacrylamide gel electrophoretic analysis of small restriction fragments for studying the degree

of genomic DNA variation (Tan *et al.*, 1990; Chua *et al.*, 1990) and pulsed-field gel electrophoresis of genomic restriction fragments (Chua et al, 1993). Recently, we have applied the new technique, arbitrarily-primed-PCR (AP-PCR) to the "fingerprinting" of these organisms. Overall, we are beginning to identify several electrophoretypes and correlate them with host, source, type of manifestation and other factors. In view of the urban context of Singapore, we are also studying alternative reservoirs of infection other than the environment and the finding that 37% of pyaemic pigs imported to Singapore and suffering from *P.pseudomallei* is cause for concern and impetus for further epidemiological studies.

AP-PCR as mentioned above is a recently developed procedure for genetic analysis which is based on amplification of genomic DNA sequences under low-stringency conditions by using a single oligonucleotide primer. The primer is not targeted toward any specific DNA sequence in the genome. AP-PCR yields a pattern of amplification products that can be used as a genetic "fingerprint" (Welsh and McClelland, 1990, Williams *et al.*,1990). We have applied this technique to the study of a wide range of commonly occurring pathogens including *M. tuberculosis*, *Klebsiella* spp., methicillin-resistant *Staphylococcus aureus* and *Pseudomonas* spp. (Lin and Chua, 1993).

In Singapore, duck infection with *Pasteurella anatipestifer* is an economically important disease. More than a dozen serotypes are known to be prevalent, of which a few have been responsible for outbreaks in recent years. With samples obtained from the Primary Production Department, Singapore, we have carried analysis of the antigens (T.S.Teo *et al.*, 1992) and have applied PCR, restriction analysis and DNA sequencing of the 16S rDNA genes and shown that this organism should be reclassified within the Flavobacterium group (Kenneth Ban and Chua Kim Lee, in preparation). Preliminary AP-PCR analysis of the genome has also indicated the many serotypes of the organism may be subgrouped.

c. Production of monoclonal antibodies for the diagnosis of bacterial, viral and parasitic diseases and development of human monoclonal antibodies for the treatment of patients

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Mouse monoclonal antibodies to Hepatitis B virus, Human Immunodeficiency Virus, Epstein-Barr virus, human blood group antigens, liver cancer, HLA and other have been developed. Human and chimeric monoclonal antibodies are being currently developed for the purpose of treatment of liver cancer and nasopharyngeal carcinoma. Together with Singapore General Hospital we also working on radioimaging and immunotoxins on live cancer using monoclonal antibodies.

RESEARCH PROJECTS: PREVENTION

a. Live oral vaccines for typhoid fever prevention

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Typhoid fever still remains a serious health problem in some parts of the world. *Salmonella typhi* only affects human beings and this appears to be host-specific at the *in vivo* and *in vitro* levels (Vladoianu *et al.*, 1990). The targets for anti-typhoid vaccination are currently from school-age children in less-developed countries, persons from industrialized countries travelling to developing areas of the world, and clinical microbiology technicians worldwide.

Several inactivated (killed, whole-cell or purified extracts) vaccines have been used for many years for parenteral vaccination. Recently, a vaccine made of purified Vi antigen has been proposed for parenteral use (Acharya *et al.*, 1987). Parenteral whole-cell vaccines (inactivated *S. typhi*) have been shown to protect significantly against *S. typhi* infection. Vaccination with such vaccines produce local and systemic side effects ranging from painful local inflammation to headache and fever. Inactivated vaccines administered orally do not pose that problem. However, they are not very effective when used by the oral route.

Other research work has, therefore, focused in the development of attenuated, live strains of *Salmonella*, and using as a model *S. typhimurium*, the agent of murine typhoid. In animals, such strains when administered orally confer significant protection and can induce secretory, humoral and cell-mediated anti-Salmonella immune responses (Dougan *et al.*, 1986). Thus, live oral vaccines seem to be more efficacious than killed parenteral vaccines. Streptomycin-dependent live strains of *S. typhi* were the first to be proposed as candidates for oral vaccination against typhoid fever (Reitman and Iverson, 1954). Clinical trials with these strains, in spite of data from pilot studies, indicating that they were well tolerated and may confer significant protection, have not been performed. From other strains, obtained by mutagenesis, an oral human vaccine based on strain Ty21a, a *gal*E mutant of *S. typhi* Ty2 (a virulent reference strain), has been shown to be efficacious in vaccine trials (Levine *et al.*, 1987) and is currently used for the prophylaxis of typhoid fever. Further studies with this strain indicate that its efficacy is limited by the dose and the number of doses of vaccine administered. The molecular basis of its attenuation is not known.

Several mutant strains have been obtained by transposon deletion in the chromosome of *S. typhimurium*, followed by transfer of these regions into

a wild type strain of *S. typhi* (Stocker *et al.*, 1983, Levine *et al.*, 1989). These strains are defective in some metabolic pathways, are avirulent, and cannot multiply *in vivo*. Work with *S. typhimurium*, at the molecular level, have identified several genes which after deletion allow the attenuation of the bacteria (Miller *et al.*, 1989, Chatfield *et al.*, 1991). Further work with *S.typhimurium* may allow the rational attenuation of bacteria for the development of potential live vaccine candidates. However, we need to keep in mind that mutations in *S. typhimurium* may not behave the same way in *S.typhi*.

Recent studies have emphasized the use of attenuated *S. typhimurium* constructs expressing foreign antigens to stimulate immune responses to the foreign antigen (hybrid vaccines). Immunization of mice with attenuated *S.typhimurium* containing the cloned gene for the circumsporozoite protein of *Plasmodium berghei* was shown to confer significant protection to mice against *P. berghei* sporozoite challenge (Sadoff *et al.*, 1988). Interestingly, protection was afforded without enhancement of the humoral antibody response, indicating indirectly that protection was afforded by cell-mediated immune responses. *S. typhi* has also been previously used to express foreign antigens (Black *et al.*, 1987). The use of *Salmonella* as live carriers to express foreign antigens is currently a research theme of interest to many investigators.

The drawbacks of the vaccines currently used for the prevention of typhoid fever has encouraged the development of new vaccines. Some attenuated and avirulent strains have been obtained and we currently look forward to determine the molecular basis of the attenuation and avirulence of these strains.

b. Recombinant subunit vaccines against *Chlamydia* spp

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Based on the previous experience with developing recombinant vaccines against the agent of ovine enzootic abortion, *Chlamydia psittaci*, in that

a) elementary bodies protect (Anderson *et al.*, 1990);
b) the immune response is predominantly directed against major outer membrane protein, (MOMP) (Tan *et al.*, 1988);
c) MOMP purified from elementary bodies protects against disease (Tan *et al.*, 1989; Tan *et al.*, 1990a);
d) recombinant subunit MOMP preparations protect against disease (Herring *et al.*, 1990; Tan and Herring, 1991; Jones *et al.*, 1992; Herring *et al.*, 1992); and

e) that sequence analysis of the *C. psittaci* MOMP gene shows homology to the *C.trachomatis* gene at four segments which code for surface exposed antigenic variable domains (Herring *et al.*, 1989), we have embarked on cloning and expressing the corresponding gene of *C.trachomatis*.

C.trachomatis is currently one of the major sexually transmitted diseases in developed countries and the cause of blinding trachoma in developing countries. Currently no chlamydial vaccines are available to control these diseases although there have been some developments recently using MOMP peptides. Using PCR primers based on existing databank sequences, the MOMP gene of *Chlamydia trachomatis* has been cloned into pUC vectors (Chua and Tan, 1991) and expressed from the *lac* promoter (J.Teo, K.L.Chua and T.W.Tan, unpublished data).

c. High density bioreactor culture for production of duck vaccines

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Pasteurella anatipestifer vaccines are currently available but the diversity of non-cross-protecting serotypes causes problems in vaccination as a means of disease control. Conventional techniques result in dilute bacterin vaccines. We are developing high density bioreactor culture techniques to produce a denser culture to study the possibility of using multivalent vaccines within a 1ml dose to vaccinate ducklings (M.Yap, unpublished data). A high density preparation recently compared against a conventional bacterin vaccine did not produce better protection (M.Yap, T.P.Teo, H.C.Tan and H.Loh, unpublished data); we are therefore investigating the antigenic content of high density cultures to determine if protective antigens are lost during growth (T.S.Teo, T.W.Tan, unpublished data).

RESEARCH PROJECTS: THERAPEUTICS

a. Production of castanospermine by tissue culture methods

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Castanospermine is a plant alkaloid which is effective in preventing HIV infectivity as well as inhibiting cell proliferation of various tumour cells under *in vitro* conditions. The production of this compound by *de novo* chemical synthesis is tedious with many steps involved and gives low yield,

whereas extraction from the plant is not a renewable resource. Therefore, tissue culture techniques were investigated and a protocol for the production from these cultures has been established. We have also developed an extraction method for castanospermine from these cultures and are in the process of testing the efficacy of castanospermine *in vitro* on cells infected by an HIV-related virus.

b. Molecular Targets

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Parasites such as filarial parasite *Brugia malayi* and the obligate intracellular protozoan *Toxoplasma gondii* have complex and poorly understood life cycles. Application of molecular biology techniques to the cloning and characterization of key enzymes in such organisms is important for a better understanding of the pathogens and may lead to the identification of potential targets for the development of chemotherapeutic agents. The lipoamide dehydrogenase gene from *Brugia malayi* (Tan *et al.*, 1992) and protein kinase genes from *Toxoplasma gondii* are currently being cloned and characterized.

CONCLUSION

For the above selection of topics, out of the many on-going projects in Singapore, the impact of biotechnological approaches to the study and control of infectious diseases is slowly but surely increasing in importance. As new techniques are being developed, we have tried to incorporate them in our repertoire. In most cases, the projects are local multi-disciplinary collaborations in themselves. Any international collaborative effort arising from these projects will definitely provide a further boost and promote a spirit of co-operation that is the seed of mutual success.

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POLYMERASE CHAIN REACTION (PCR) AND DETECTION OF HUMAN MALARIA

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The polymerase chain reaction or PCR has caused a minor revolution in molecular biolgy. The technique was invented by Kary Mullis in 1985 while he was working for Cetus Corporation[1] and enables any given fragment of DNA to be synthesized *in vitro*, allowing a worker to obtain microgram quantity from picogram amounts of starting nucleic acid material. Mullis has just been awarded the 1993 Japan Prize in recognition for the impact that PCR has had on the field of life science.

Performing PCR involves a series of reactions that results in the geometric amplification of a sequence of DNA contained within a larger molecule. This is achieved by means of a reaction catalyzed by the enzyme DNA polymerase. The enzyme requires a single strand DNA template, a short piece of complementary DNA (called primer strand), all four deoxyribonucleoside triphosphates (substrates for new DNA strand synthesis) and an appropriate buffer solution. If another primer strand which is complementary to the opposite template strand and directs DNA synthesis towards the location of the other primer is also present, then two sets of double strand DNA whose sequence lies between the locations of the two primers can be synthesized simultaneously. If the two DNA double strands are heated, they separate into four single strands each of which in turn can act as templates in another round of enzyme catalyzed DNA synthesis.

In the original protocol, Klenow fragment of DNA polymerase I was employed in the primer extension reactions[2]. However since Klenow enzyme is unstable at the high temperature (92-97°C) needed to denature DNA, fresh lots of enzyme were required at the start of each PCR cycle. With the introduction of thermal stable *Taq* DNA polymerase (isolated from the thermophilic bacterium, *Thermus aquaticus*) a single addition of enzyme now suffices and the whole procedure can be conveniently conducted in a programmed thermal cycler[3]. The use of a thermal stable DNA polymerase in PCR also provides the following additional improvement: (1) the higher temperature used for DNA synthesis (72°C instead of 37°C) increases stringency of primer hybridization and thereby minimize chain extension from primers that are mismatched with the template; and (2) intrastrand secondary structures in single strand DNA templates which may inhibit polymerase are reduced.

For diagnostic purposes, the ability of PCR to generate large amounts of the target DNA obviates the necessity of using radiolabelled DNA probes in hybridization assays and permits the employment of nonradioactive means of detection. In many situations the presence of a DNA fragment of the expected size produced by PCR is a sufficient indicator in itself for the presence of the genome of interest provided the primers are specific and do not hybridize with host DNA. In theory, and in practice, the presence of a single copy of the genome in a sample is sufficient for amplification by PCR to a level capable of detection by eye.

Plasmodium falciparum

DNA probes constructed for detection of *Plasmodium falciparum* have nearly all been based on the existence of a 21-bp sequence present in multiple copies in the parasite genome (reviewed in ref. 4,5). Parasitemia of 0.001% can be readily detected using ^{32}P-labelled probes. Field applications in Thailand and elsewhere have produced results comparable with microscopic examination but both false negative and positive identifications were obtained, the former occuring in samples with very low parasitemia and the latter being attributed to errors in microscopic examination, espcially where there may have been mixed infection of *P. falciparum* and *P. vivax*. Synthetic oligodeoxynucleotides containing the 21-bp repeat sequence have also been used as probes, both radiolabelled and linked to alkaline phosphatase, but their sensitivities were 10-fold less than genomic DNA probes. Colorimetric visualization of hybridization has been compromized by background color of blood samples on the solid supports.

Jaureguiberry and co-workers were the first to report a PCR detection of human malaria[6]. Plasmodium-specific region of the 18S rRNA gene was amplified and hybridized with ^{32}P-labelled oligonucleotide probe for that fragment in a slot-blot format. Sensitivity is at the level of 0.01 pg of *P. falciparum* DNA (equivalent to half of the genome content of a single parasite). A detectable signal can be seen in DNA sample extracted from 50 μ l of infected blood with 0.001% parasitemia.

Amplification of parasite DNA fragments containing the 21 bp repeat sequence was employed by Barker and co-workers[7] to detect *P. falciparum* in blood samples sent from Thailand. Primers were designed from concensus sequences present in the diagnostic probe, pPF14, which was nonisotopically labelled (Genius™ system) for dot-blot hybridization with the PCR products. Blood samples were lysed, filtered onto filter papers which were allowed to dry and then placed into the PCR reaction mixture. The limit of detection is one parasite per 20 μ l of blood sample. Of 626 samples, using microscopic examination as the reference, sensitivity of the PCR method is 79%, specificity 69% and disagreement 27%. The proportion of samples not detected by PCR

increases as the parasite density in the specimen decreases. Samples of high parasite density missed by PCR method was attributed to misidentification of the cases.

In our laboratory, we have employed the insert of pBRK1-14, a *P. falciparum*-specific probe previously developed for fingerprinting of parasite clones[8], as the target for PCR amplification. Primers were designed to flank a 206 bp fragment and using standard thermal cycling conditions (30 cycles, each comprising of 94°C for 1 min, 45°C for 1 min and 72°C for 3 min), a minimum of 0.01 pg of parasite DNA could be detected in a Southern hybridization using ^{32}P-labelled pBRK1-14 insert as a probe[9]. A simple procedure has been developed to conduct PCR amplification directly on *P. falciparum*-infected red cells without prior extraction of parasite DNA enabling detection of a single infected cell by Southern hybridization, using a radiolabelled or digoxigenin-labelled probe. However, visual examination of ethidium bromide-stained gel for presence of the 206 bp fragment is possible only at the level of 1000 parasites and many nonspecific DNA ragments are also amplified. Subsequently Tirasophon modified the PCR conditions (40 cycles; 80°C for 15 sec, 50°C for 15 sec and 72°C for 15 sec) to minimize nonspecific amplified DNA fragments and was able to detect 20 parasites in 20 μ l of whole blood based on the visualization of 206 bp fragment in stained gel[10]. Over 2000 blood specimens from various malaria endemic regions of Thailand were tested by PCR and the procedure is 96% specific and 81% sensitive compared to microscopic examination, with a disagreement of 6.6%. It is worth noting that disagreement in malaria diagnosis between two microscopists can be as high as 15%.

The above PCR system (K1-14) has been evaluated in 33 *P. falciparum*-infected Thai patients over 5-7 days of curative treatment in comparison with amplification of a 800 bp fragment from the circumsporozoite protein gene (CS system)[11]. The K1-14 and CS systems detect parasite DNA in 53% and 20%, respectively, of blood films collected on the first day and 3% and 0% on the fourth day during the course of treatment when microscopic examinations are negative.

A field trial in the Solomon Islands of falciparum malaria employing a double PCR technique has recently been reported[12]. Wataya and co-workers first amplified a 410-bp fragment corresponding to the junction part of the dihydrofolate reductase-thymidylate gene of *P. falciparum* and then using another set of primers generated a 226-bp fragment from within the first PCR product which was analyzed by agarose gel-electrophoresis using ethidium bromide staining. The sensitivity is 10 parasites in 10 μ l of blood sample and there is no cross-reaction with *P. vivax*. Of 101 samples examined, 8 positive PCR results were obtained, corresponding to microscopic examination, and of these only one donor had symptoms of malaria. The clinical course of this latter patient was followed upon treatment with

chloroquine: on day 7, when no parasite was observed in blood smear, PCR diagnosis was also negative.

A rapid colorimetric assay for detection of *P. falciparum* PCR amplified DNA has been recently described.[13] Biotin and a lac operator sequence are incorporated into the targets for amplification, a region of the antigen Pf155/RESA and a region of the dihydrofolate reductase gene. The PCR product is immobilized on streptavidin-coupled magnetic beads and detected by the specific binding of *E. coli* lac repressor beta-galactosidase fusion protein using the chromogenic substrate o-nitrophenyl-beta-D-galactoside. Six blood samples from Gambia, corresponding to 0.2 μ l of packed blood cells and with parasite counts ranging from 13 to 80 parasites/100 high power fields are positive in the assay for both gene targets. However, there is no correlation between the optical density reading and parasitemia.

Plasmodium vivax

There have been a singular dearth of reports on the construction of DNA probes for *P. vivax*, presumably due to the lack of a continuous *in vitro* culture system for *P. vivax* from which to obtain a supply of parasite DNA uncontaminated with host genome. A single nucleated human cell contains over 100 times the DNA present in a malaria parasite. Using a pool of partially purified *P. vivax*-infected erythrocytes, Relf and associates[14] constructed a genomic DNA library from which they obtained a probe, VPL101, 3.2 kbp in size and containing at least two copies of a 205 bp repeat sequence. ^{32}P-labelled probe reacts positively with 73/76 microscopically diagnosed *P. vivax* samples with parasitemia as low as 0.0002% in 10-200 μ l of patients' blood obtained from various endemic regions of the world; however, there is no correlation between intensity of hybridization with extracted parasite DNA and parasitemia data. The authors have not reported using their probe in a PCR-based detection format.

Using a similarly enriched pooled samples of parasitized erythrocytes, Rajkulchai in our laboratory identified from a genomic library a *P. vivax*-specific clone, pMU-PV2, containing an anonymous insert of 1.6 kbp which is 47.5% G+C rich and has several short internal repeat sequences[15]. A primer pair was synthesized to flank a 183 bp sequence so as to generate a PCR product that is different in size from that used in detecting *P. falciparum*[9] since the ultimate goal is to employ both primer sets in the same PCR amplification condition. The sensitivity of visual detection of the 183 bp fragment in ethidium bromide-stained gel is one parasite per 20 μ l of whole blood or a parasitemia of 10^{-6}%. In testing of 233 field samples, the PCR method had a sensitivity of 76% and a specificity of 71%, with 25.3% disagreement. The rather high discrepancy between PCR and microscopy results may have been caused by problems associated with the transport

of samples from the field to the laboratory. This problem is currently being corrected.

FUTURE DIRECTIONS

Although the PCR method for detecting *P. falciparum* and *P. vivax* has proven to be as sensitive as the traditional microscopic examination of blood film, the time required (hours) before the results are known makes it unlikely to replace the microscope in case detection at malaria clinics. The advantages of the PCR approach lie in its ability to handle large number of samples at the same time, and the reduction in dependence on subjective evaluation of the data. This makes it an attractive technique for epidemiological survey of malaria endemicity and for quality control of other detection methods that may be introduced to replace the microscope. However, the current methodology which relies either on DNA hybridization or gel electrophoresis is not a familiar technique in clinical diagnostic laboratory. The report[13] of a colorimetric detection system coupled to PCR indicates that an ELISA system to detect amplified DNA products should be feasible and could be amenable to automation. Alternatively, the presence of amplified fragments from PCR could be detected by capillary electrophoresis. Of course, development of primers specific for the other human malaria parasites should not be neglected.

ACKNOWLEDGEMENTS

Work in the author's laboratory was supported by CEC STD Program, contract no. TS2-0148-TH and UNDP/World Bank/ WHO Special Program for Research and Training in Tropical Diseases.

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Appendix I

Opening Address
by
Mr. Kasem Snidvongs
Permanent Secretary
Ministry of Science, Technology and Environment
for
Workshop on Biotechnological Approaches to Diagnosis: Prevention and Treatment of Infectious Diseases
February 24-27, 1993
Bangkok, Thailand.

Good Morning, Distinguished Guests and Participants, Ladies and Gentlemen :

It gives me great pleasure to welcome all of you to "the Workshop on Biotechnological Approaches to Diagnosis, Prevention and Treatment of Infectious Diseases" which is organized by the ASEAN Subcommittee on Biotechnology and Thailand National Center for Genetic Engineering and Biotechnology (NCGEB) with generous support from the Government of Japan through Japan International Cooperation Agency (JICA). This workshop is aimed at launching an ASEAN-Japan collaboration in Biotechnology under Inter ASEAN Technical Exchange Program (IATEP).

I think you are aware that seminars or workshops, which come under the IATEP scheme, are specifically intended to promote exchanges of technologies and personnels among ASEAN utilizing the centers and technologies already existing in Japan. This particular workshop is no exception. In fact, it is geared toward the same goal by providing opportunities for ASEAN scientists who are involved with biotechnological study of infectious diseases to acquire expertises on various topics which will be discussed at the meeting.

Although biotechnology through genetic manipulation greatly contributes to better knowledge of infectious diseases, it is by no means definitive that effective treatment and diagnoses of such afflictions are imminent unless their biological complexities can be thoroughly understood. With technological breakthrough already achieved by our Japanese counterparts, we do indeed have a need to learn from them. As far as I know, this workshop will serve as a venue for both ASEAN and Japanese experts to exchange ideas and latest information on biotechnological

techniques currently employed in studying infectious diseases prevalent in the Southeast Asia. Optimistically speaking, I am confident that joint projects between ASEAN and Japan would materialize as a result of this meeting.

The commendable undertaking of ASEAN Subcommittee on Biotechnology should be acknowledged here for its effort to spearhead joint cooperation in biotechnology between Japan and the ASEAN from the very beginning. I would like to express my sincere appreciation to our Japanese and ASEAN delegates to take part in what I believe is certain to be a successful workshop. I would also like to convey my gratitude to the Government of Japan for the generous financial contribution.

Finally, I hope all of you have a pleasant stay in Bangkok and wish all the participants success. I honestly believe our joint ventures in scientific endeavour will continue to grow and mature in the years ahead. I now declare the workshop open.

Appendix II

Welcoming Address
by
Mr. Nobuji Abe
Resident Representative of JICA Thailand Office
for
Workshop on Biotechnological Approaches to Diagnosis: Prevention and Treatment of Infectious Diseases
February 24-27, 1993
Bangkok, Thailand.

Good morning, Distinguished Guests and Participants, Ladies and Gentlemen :

On behalf of JICA, I am so pleased to participate in this significant opportunity, and delighted to extend my heart-felt welcome to all participants to "the Workshop on Biotechnological Approaches to Diagnosis, Prevention and Treatment of Infectious Diseases".

I understand that in field of scientific technology, the relevant subjects related to the genetic engineering and biotechnology are highlighted in recent years. JICA Thailand Office is currently undertaking the several projects of similar fields, and the Japanese researchers and the Thai counterparts make a good collaboration to produce the advanced outcome through the research activities. For example, Japanese experts assigned to the Kasetsart University are engaged with the application of biotechnology to improve the quality of agricultural products to acquire good competiveness in the worldwide food market. Simultaneously the application to medical field is to be awaited to cope with various infectious diseases in the world.

During the latest visit around the ASEAN countries, of our Prime Minister, Mr. Kiichi Miyazawa, issued a special statement in Bangkok. He particularly emphasized the necessity to promote the exchange of science and technology and strengthen the mutual friendship, furthermore, inside the ASEAN arena. Fortunately the interest for biotechnology is completely shared among the participating countries. I am sure that the Workshop prepares a good opportunity for this purpose.

Upon the current circumstances, I suppose that time is well-matured to plan and organize this kind of Workshop under the Inter-ASEAN Technical Exchange Programme (IATEP).

JICA has already assigned two resource persons, one from Japan, Dr. Takashi Aoki, and another from one of JICA projects in Bangkok, Dr. Komi kanai, so I am sure that they assist every participant to acquire the fruitful and successful result through 4-day workshop.

To conclude, the cooperation of genetic engineering and biotechnology has quite a long sequence for the future. I wish that small breakthrough is ever achieved during this Workshop although the biological complexities upon the respective circumstances of each country cannot be throughly overcomed through short period and, most of all, this Workshop will contribute to the friendship among every participant. Thank you very much.

Appendix III

Welcoming Address
by
Dr. Yongyuth Yuthavong
Director
National Science and Technology Development Agency
for
Workshop on Biotechnological Approaches to Diagnosis: Prevention and Treatment of Infectious Diseases
February 24-27, 1993
Bangkok, Thailand.

Good morning, Distinguished Speakers and Honourable Guests, Ladies and Gentlemen :

I am extremely pleased to be given an opportunity to welcome you all here in Bangkok, Thailand to "the Workshop on Biotechnological Approaches to Diagnosis, Prevention and Treatment of Infectious Diseases". This workshop was originally conceived by ASEAN Subcommittee on Biotechnology to initiate an ASEAN-Japan cooperation in Biotechnology. In order to make you fully understand, I believe a brief history of ASEAN-Japan involvement in Science and Technology is in order. The ASEAN-Japan cooperation in Science and technology started from the 6th ASEAN-Japan Forum in May 1983. A number of potential joint scientific cooperations were identified including biotechnology. The cooperation in biotechnology, however, was limited to training of ASEAN personnels in standard techniques. Over the years, ASEAN Subcommittee on Biotechnology came to realize that joint research and development in a clearly identified area of biotechnology should be heavily emphasized instead. This particular workshop is a manifestation of our such endeavor.

I honestly believe that in the next few days, we will have an opportunity not only to learn from our ASEAN and Japanese colleagues but also to share ideas and knowledge to facilitate our research investigation. Ambitiously speaking, I would like each of us to look upon this workshop as a starting point where we can seriously consider the possibility of establishing joint scientific collaboration between ASEAN and Japan in the near future.

On behalf of the organizing committee of this workshop, I would like to extend my genuine appreciation to the Govermment of Japan for supporting this workshop under Inter-ASEAN Technical Exchange Program (IATEP). I would also like to thank the ASEAN and Japanese delegates for their valuable contributions and our invited guests for their participation. I sincerely with that you find this meeting as exciting and informative as it was intended to be.

Appendix IV

Programme
of
Workshop on Biotechnological Approaches to Diagnosis, Prevention and Treatment of Infectious Diseases
February 24 - 27, 1993
Monet Room, Novotel Hotel Bangkok, Thailand.

Wednesday February 24th, 1993

8:30 - 9:00	Registration
9:00 - 9:30	Official Opening Ceremony
9:30 - 10:30	A Parasite Cysteine Protease : Purification, Monoclonal Antibiodies, cDNA Cloning, and Possible Application **Prof. Takashi Aoki** Department of Parasitology School of Medicine, Juntendo University
10:30 - 11:00	Break
11:00 - 12:00	Review : Infectious Disease Project of JICA in Thai NIH with Special Emphasis on the Molecular and Biotechnology Approaches **Dr. Komi Kanai** National Institute of Health, Nonthaburi, Thailand
12:00 - 13:30	Lunch
13:30 - 14:30	Molecular Diagnosis of Dengue Infection **Dr. Amin Soebandrio** Department of Microbiology Medical Faculty, University of Indonesia
14:30 - 15:30	Hepatitis Virus in Indonesia : Sequence Variation of Hepatitis B Virus **Dr. Retno Iswari** Department of Microbiology Medical Faculty, University of Indonesia

15:30 - 16:00	Break
16:00 - 17:00	Biotechnological Approaches in the Diagnosis of Dengue Infections and the Effect on Prevention and Management of Dengue Diseases **Prof. Lam Sai Kit** Department of Medical Microbiology Faculty of Medicine, University of Malaya
17:00 - 18:00	Use of Biotechnology in the Production of Reagents for Diagnosis and Epidemiological Studies of Important Parasitic Diseases in Malaysia **Dr. Mak Joon Wah** Institute for Medical Research, Malaysia

Thursday February 25th , 1993

8:30 - 9:30	Vaccine Development Against the Philippine Strain of *Schistosoma japonicum* **Dr. Bernadette Ramirez** Department of Health Research Institute for Tropical Medicine
9:30 - 10:30	Monoclonal Antibodies for the Diagnoses of Amoebic Infections **Dr. Filipinas Natividad** College of Science University of the Philippines
10:30 - 11:00	Break
11:00 - 12:00	The Screening and Production of Biologically Active Compounds Using Biotechnological Technique **Dr. Goh Kiow Leng** Department of Biotechnology Ngee Ann Polytechnic, Singapore
12:00 - 13:30	Lunch

13:30 - 14:30	Computer Applications in Infectious Diseases Research : an Asian Biotechnology, Biocomputing and Bioinformatics Network Resource ? **Dr. Tan Tin Wee** Department of Biochemistry Faculty of Medicine, National University of Singapore
14:30 - 15:30	Polymerase Chain Reaction (PCR) and Detection of Human Malaria **Dr. Prapon Wilairat** Department of Biochemistry Faculty of Science, Mahidol University
15:30 - 16:00	Break
16:00 - 17:00	Diagnosis, Prevention and Treatment of Infectious Diseases : Main Issues for Possible ASEAN-Japan Cooperation **Dr. Yongyuth Yuthavong** National Science and Technology Development Agency Thailand

Friday February 26 th , 1993

9:00 - 10:30	Group Discussions Group A : Problem areas - Diagnoses - Prevention - Treatment Group B : Technology areas / Products - Recombinant DNA - Vaccine and Immunological products
10:30 - 11:00	Break
11:00 - 12:30	Joint discussion : Conclusion and Recommendation of ASEAN-Japan Project

12:30 - 13:30	Lunch
13:30 - 17:30	Field trip to NIH
19:30 - 22:30	Farewell Dinner at Yok Yor Restaurant by the Chao Phaya River

Saturday February 27th, 1993

Free

Sunday February 28th, 1993

Departure of Participants

Appendix V

List of Official Delegates and Participants

ASEAN - Japan Workshop on Biotechnological Approaches to Diagnosis: Prevention and Treatment of Infectious Diseases

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