No. 5

マレイシア
熱帯病研究プロジェクト
終了時評価報告書

平成7年6月
(1995年6月)

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国際協力事業団
医療協力部

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序　　文

マレイシア政府は、日本政府に対し熱帯病研究分野における分子生物学的手法の導入を目的として、平成２年10月に保健省医学研究所（ＩＭＲ）を拠点としたプロジェクト方式技術協力を要請してきました。日本政府はこの要請を受けて平成４年６月にＲ／Ｄを署名交換し、平成５年１月から３年間の技術協力を実施してきました。

このたび、平成７年12月31日をもって協力期間が終了するのに先立ち、当初の協力目標、計画に照らし、これまでの活動実績や目標達成度等についてマレイシア側と合同で評価を行うことを目的とした終了時評価調査団を、平成７年６月17日から６月24日まで派遣しました。

本報告書は、同調査団の調査および評価の結果について取りまとめたものです。ここに、本調査の任にあたられた団員のご努力に敬意を表しますとともに、調査に際し多大なるご協力をいただきましたマレイシア政府関係機関、在マレイシア日本国大使館、および外務省はじめ国内関係機関各位に対しまして、深甚なる謝意を表する次第です。

平成７年６月

国際協力事業団

理事　小澤　大二

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第１章　終了時評価調査団の派遣

1-1　調査団派遣の経緯と目的

1990年１月、中山外務大臣（当時）がマレイシアを訪問した際のマハティール首相との会談時に、日本側から「熱帯病に関連した両国間の医学協力の可能性について」提起した。

これを受けて同年２月、国際協力事業団（ＪＩＣＡ）はプロジェクト方式技術協力の可能性を調査するためにプロジェクト形成調査団を派遣、マレイシア側関係者と協議を行った。その後、同年10月要請書が提出されたが、協力要請分野は10項目にもわたるため、再度内容の絞り込みを行うことを目的として1991年４月に専門家チームを派遣した。その結果、要請分野のなかで保健省医学研究所(Institute of Medical Research：ＩＭＲ）を拠点とした熱帯病に関する研究協力が、ＪＩＣＡのスキーム内で実現の可能性が高いとの結論に至った。

以上の経緯を踏まえて1992年２月に事前調査団、および同年６月に実施協議調査団を派遣しＲ／Ｄを署名交換した。続いて1993年１月１日から３年間の協力が開始され、相互に同意した分野で、技術移転および研究機能強化に対する技術協力を実施してきた。

現在、長期専門家６名、短期専門家１名を派遣中である。1995年度の短期専門家派遣は計６名、研修員受入は３名を予定している。

当調査団は、当初予定した３年間の協力期間の終了を６カ月後に控え、これまで実施した協力について、① 当初計画に照らし、プロジェクトの活動実績、管理運営状況、カウンターパートへの技術移転状況等について評価を行う、② 目標の達成度を判定したうえで今後の協力方針についてマレイシア側と協議する、③ 評価結果から教訓および提言等を導き出し、今後の協力のあり方や実施方法改善に資する、ことを目的として派遣した。

1-2　調査団の構成

	（担　当）	（氏　名）	（所属）
団長	総　　括	黒川　高秀	東京大学医学部教授（前医学部長）
団員	寄生虫学	小島　荘明	東京大学医化学研究所寄生虫研究部教授
団員	疫　　学	五十嵐　章	長崎大学熱帯医学研究所分子構造解析分野教授
団員	研究協力	福原　俊一	東京大学医学部国際交流室講師
団員	医学教育	松谷　　治	文部省高等教育局医学教育課大学病院指導室病院第二係長
団員	企画調整	八重樫成寛	国際協力事業団医療協力部医療協力第一課課長代理
団員	協力計画	渡邊　聡子	国際協力事業団医療協力部医療協力第一課職員

1-3　調査団の日程

日順	月　日	曜日	移動および業務			
			黒川団長、五十嵐団員、松谷団員、八重樫団員 渡邊団員		福原団員	小島団員
1	6月17日	土	成田（JL-723）→クアラルンプール			
2	18日	日	団内打合せ			
3	19日	月	A.M　ＪＩＣＡ事務所打合せ ＥＰＵ表敬 P.M　ＩＭＲでの全体会議（評価方法、日程の合意） 田中リーダーブリーフィング			
4	20日	火	A.M　評価調査、協議（BIOTECH）（HLA） 評価調査、協議（ENT） P.M　評価調査、協議（VIRUS） ＊ＪＩＣＡ事務所長主催夕食会			
5	21日	水	A.M　日本人専門家の意見聴取 （バイオテクノロジー）渡辺専門家 （ウイルス学）　小林専門家 （医昆虫学）　森専門家 総合討論、団内協議 P.M　ＩＭＲ施設視察		福田団員 クアラルンプール ↓ 成田 （JL-724）	小島団員 クアラルンプール ↓ 成田 （MH-078）
6	22日	木	A.M　評価レポート起草会議 （ＩＭＲ各部長出席） ＊調査団長主催昼食会 P.M　レポート最終取りまとめ			
7	23日	金	A.M　合同評価報告書署名・交換 大使館報告、ＪＩＣＡ報告			
8	24日	土	クアラルンプール（MH-092） ↓ 成田	八重樫団員 クアラルンプール ↓ クチン （MH-2564）		

1-4　主要面談者

＜Economic Planning Unit, Prime Minister's Dept. : ＥＰＵ＞

Mr. K. Thillanaidarajan	Principal Assistant Director External Assistance Section

＜Institute for Medical Research : ＩＭＲ＞

Dr. Mohamad Taha bin Arif	Director, ＩＭＲ
Dr. Mak Joon Wah	Head, Department of Tropical Medicine Head, Biotechnology Centre
Dr. Sinniah Mangalam	Head, Division of Virology
Dr. Nasuruddin bin Hj. Abdullah	HLA laboratory, Head, Division of Immunology
Mr. Lee Han Lim	Head, Division of Medical Entomology
Ms. Noor Rain Abdullah	Biotechnology Center, Secretariat

＜在マレイシア日本国大使館＞

松永　大介	経済部長
米田　雅人	書記官

＜日本人専門家＞

田中　　寛	チーフアドバイザー
大田　　泉	調整員
小田　和正	長期専門家（ウイルス学）
只野　長夫	長期専門家（医昆虫学）
森　　章夫	長期専門家（分子生物学）
小林　伸好	短期専門家（ウイルス学）
渡邊　洋一	長期専門家（寄生虫学）

＜ＪＩＣＡマレイシア事務所＞

水田加代子	所長
山田　好一	次長
三角　幸子	職員
稲垣　明子	職員

1-5　終了時評価の方法

本プロジェクトにおいては、プロジェクト開始当初、ログフレームを作成し、(A) マラリア、(B) デングと日本脳炎に関して目標を設定していたので、それに従って活動別評価

調査を行った。

調査は、関連施設・活動の視察、マレイシア側関係者および日本側専門家チームからのヒアリングと、プロジェクトが取りまとめてきた実績データ、これまでに派遣した各段階の調査団の報告等をもとに、日本側は評価調査団員、専門家チーム、マレイシア側はＩＭＲ所長をはじめとする関係スタッフが出席して率直な意見交換を通じて実施された。カウンターパートは、ウイルス部、免疫部ＨＬＡラボラトリー、医昆虫部、バイオテクノロジーセンターに分かれるため、全体会議とは別に、それぞれ個別に評価会議をもった。また、各部関係スタッフおよび日本側関係者にアンケートを実施した。

1-6　協議の経緯（発言の順不同、趣旨のみを記す）

○６月19日

9:30　ＪＩＣＡマレイシア事務所

面会者：ＪＩＣＡ水田所長、三角職員、稲垣職員

（水田所長）

ＩＭＲはＪＩＣＡのいわばモデルプロジェクトとして活躍していただき、所期の目的もほぼ達成したので喜ばしく思っている。この後を受けてガン研究のプロジェクト要請もあったが、マレイシアの開発状況に鑑み、実現は難しいようである。ただし、人的交流だけでもサポートをとの要望があり、事務所として単発専門家の派遣など何らかの形で応えたいと考えている。

（小島団員）

所期の目的でいまだ達成できないのは、ＩＭＲの要望によって計画したハマダラカの感受性、抵抗性をＤＮＡレベルで区別するという研究である。

日本側の提案で計画したＨＬＡ（ヒトの白血球型）とマラリア抵抗性について、関係があるか否かという研究では、先住地域住民オラン・アスリの白血球型が、アフリカでみられるマラリア抵抗性の白血球型と近似しているという発見をしたなど、３年間の成果としては驚異的なものがある。

（水田所長）

セミナーを開催するなど、プロジェクトの成果を一般に普及する努力をしている。第三国研修は３年間の予定だが、延長もあり得る（ＩＭＲ側の中心人物であるDr. Makの定年を控え、経過をみたいためひとまず３年とした）。

（黒川団長）

第三国研修は、マレイシアが主体となって近隣諸国にトレーニングサービスをするのを日本が支援するものであり、懐の深い有意義な協力であり喜ばしい。

本プロジェクトの最終的な目的は、移転された技術と研究成果がマレイシアの保健政策のなかに生かされることではないか。ＪＩＣＡベースの協力は日本およびマレイシア研究者の共同研究が学術上の問題にとどまらず政策上に反映され、国民に寄与するという点で価値がある。レポートのなかでもこの点に関する記載をすべきであると考える。

（小島団員）

政策レベルへの貢献は当初計画でもうたっている。マラリアを例にとれば、罹患率の減少という形では成果はみえてこないが、ＤＮＡ診断が応用され、末端へ届くような工夫が可能になった。

（水田所長）

本プロジェクトは、当初から共同研究のスタンスで始まった。ＪＩＣＡが文部省から割り当ててもらっている国費留学生の枠をＩＭＲに提供する、また海外長期研修でＩＭＲに日本人研修員を派遣する、さらには大学院の課程の一部をＩＭＲで実施するなどの交流ルートづくりが考えられないものか。

（五十嵐団員）

長崎大学では特別事業費が割り当てられ、７月からＩＭＲに研究者（助手）１名を派遣することが決まっている。人的交流を絶やさないことの意義は大きい。

11:00　ＥＰＵ表敬

面会者：K. Thillainadarajan

(Principal Assistant Director External Assistance Section)

同　席：田中チーフアドバイザー、Ms. Noor Rain（ＩＭＲバイオテクノロジーセンター）、三角・稲垣（ＪＩＣＡマレイシア事務所職員）

＊ＥＰＵ：Economic Planning Unit, Prime Minister's Department

（黒川団長）

プロジェクトは所期の目的をほぼ達成し、最終段階にかかっている。協力を通じて得た貴重な人的交流はプロジェクト終了後も継続発展が望まれる。1995年から始まる第三国研修は、マレイシアによる周辺国への技術普及を日本が支援するもので、インドネシアの国際的立場に鑑みて必要かつ有意義なものである。

（ＥＰＵ）

日本からの第三国研修の提案は歓迎すべきものと認識している。人的交流の継続の重要性についてもマレイシア側としても努力したい。

（八重樫団員）

プロジェクトは所期の期間内に目的を達成し終了する運びとなったが、終了後の人

的交流の重要性についてはマレイシア事務所からも強調されている。対応について努力したい。

（黒川団長）

本プロジェクトの最終目的は、研究により得られた成果が広くインドネシア国民に寄与することであり、技術を診療面に生かしていくことが望まれる。ＩＭＲの人的充実を図り、プロジェクトが対象とした分野以外の疾病にも応用していくことが重要である。

（Ms. Noor Rain）

プロジェクトが対象とした７つの研究に関する技術移転はほぼ完了した。この成果を将来的には診療面に応用することも可能となった。

（田中チーフアドバイザー）

（チーフアドバイザーから７つの研究成果について詳細説明）

（ＥＰＵ）

マレイシアは1996年から第７次経済計画を開始するが、これは2020年を目標に、地方も含めた全マレイシアが発展し、国民生活を向上させることをめざしている。本プロジェクトが対象とした疾病研究は地方の国民にも寄与するものであり、重要な課題である。今後の進め方について意見があったらお聞かせ願いたい。

（黒川団長）

東南アジアにおけるマレイシアの立場は、医学、医療においても今後リーダーシップをとっていくべき存在であり、これら熱帯病に関する高度の研究を続けるとともに、第三国研修のような国際的貢献、人的交流は特に重要である。この点を踏まえ予算的措置にも配慮することが必要と考える。日本はこのようなマレイシアの活動をサポートしていくべき立場にあり、必要な支援については外交ルートによって要請していただきたい。

14:30　ＩＭＲ全体会議

会議出席者メンバー紹介および今後の協議の進め方について確認

① 評価の目的は批判ではなく、成果について討議し、今後の協力に生かす積極的なものとしたい。

② 目標達成度、案件の効果、発展の見通しの３点を大項目とする。

③ レポート中のリコメンデーションについては協議のなかで実現可能な案を討議していくものとする。

また、ＩＭＲ側から、コストパフォーマンスを分析し、将来に生かしたいため、可能であれば本プロジェクトにかかった全経費を、専門家派遣、研修員受入も含め公表

してほしい旨要望があった。

○6月20日

＜ＩＭＲ各部門別評価調査＞

プロジェクト対象の免疫部ＨＬＡラボラトリー、バイオテクノロジーセンター、医昆虫学部、ウイルス部の各部門別に関係者から技術的進捗状況、成果を聴取、質疑応答を行った。ＩＭＲ側から、日本の協力は日本人専門家が実際に共同作業をし、直接実務を技術移転するなど、従来経験した欧米型の技術協力にはなかった長所があり、多くを学び、また貴重な人間関係を築くことができたので、今後も関係を継続発展させていきたい旨が強調され、調査団側もこれを貴重な成果と受け止めた。また、ＩＭＲ側から、プロジェクト終了後も成果を確認する意味でモニタリングが重要ではないかとの指摘があり、（第三国研修が終了する1997年にでも）再度専門家による調査を実施してはどうかとの提案があった。

○6月21日

10:00～13:00　日本人専門家から意見聴取

（バイオテクノロジーセンター：渡辺専門家）

テクニシャンへの技術移転は順調に進んでいるが、問題はその技術を利用して何をやるかをマレイシア側が適切に選択できるかということ。これは研究指導者の能力にかかっている。こういった全体計画について部門責任者と話し合いたいと考えているが、現状では時間がとれていない。残りの期間で努力したい。

研修員として来日もしたカウンターパートが３月いっぱいで定年となり、現状ではＤＮＡ塩基配列解析の技術を移転できたのは１名のみになった。技術移転は１対１で実技指導を行うのが理想的だが、異動によって技術移転したカウンターパートがいなくなった場合、技術が伝えられない可能性もあるので、中心となるカウンターパートはあらかじめ決めるが、同時に複数人に教える講義形式も取り入れることとした。

ＤＮＡ解析技術はＩＭＲ内の他の分野にも応用できるものだが、現状では他の部との交流は不足している。

プロジェクト終了後の自立発展の可能性は、機材や試薬の購入という点では計画的に実行すれば問題ないと思われる。ＤＮＡ解析技術を応用してマラリアやガンの診断治療に応用できる研究を始めようという動きがあるが、解析技術をより多くの職員に移転する必要性がある。機会があれば再度赴任して技術移転を進めたい。

（ウイルス：小林専門家）

デングウイルスの解析については50％の進捗状況。残りの期間で所期の目的を達成できると思われる。ＰＣＲ(polymerase chain reaction)の技術移転をしたカウンタ

ーパート１名は完全に技術を習得しているが、デングの臨床診断に忙しく共同研究をする時間が十分にとれていない。

プロジェクト終了後の自立発展の可能性は、機材の購入、メンテナンスに関しても多少時間はかかるが問題はないと思われる。ただし、特殊な試薬（Ｃ-Ｔａｂなど）に関しては入手困難なものもある。デングの臨床に応用できる技術の開発が望まれているが、デングは罹患率も高く社会的インパクトも大きい。さらに技術移転を進め完成させたい分野である。所属先との調整ができ、機会があれば短期専門家として再度赴任したい。

（医昆虫学：森専門家）

部門責任者をはじめカウンターパートはルーチンワークをこなすため地方に蚊の採集などに出ることが多く、技術移転できる時間が非常に限られている。部門責任者がプロジェクト立ち上げに直接関与しておらず、プロジェクト目標にあまり協力的ではない。また、１名を除いてはモレキュラーバイオロジーに興味を持っているカウンターパートがいない。この分野は技術移転に長い時間を必要とし、受入態勢も不十分であることから、今後の協力分野として適当ではない。

14:30～16:00　ＩＭＲ内施設視察

バイオテクノロジーセンター、ウイルス部門、および医昆虫学部門の３カ所を視察した。供与機材はすべてステッカーが貼られ、よく管理・使用されており、管理状況、使用状況とも非常に良好。バイオテクノロジーセンターでは供与機材を１カ所に集中させ、共同利用する工夫もなされていた。プロジェクトによる計画的購入、管理が良好であったとともに、ほとんどが現地調達であるためメンテナンスも容易であったようである。

○６月22日

10:00～12:00　合同評価報告書起草会議

各部門の責任者、プロジェクトリーダー、および調査団による合同評価報告書の内容の最終確認。

協議の結果、当初の報告書（案）からの主な変更点は以下のとおり。

（リコメンデーション）

コンクルージョンとして、本プロジェクト実施により技術移転のみならず日本側研究者とＩＭＲ側研究者との間に強い信頼関係が築かれたことに言及、双方はプロジェクト終了後も緊密な連絡をとる努力をすることを追記した。

第三国研修の終了する1997年にセミナーを開催し、本プロジェクトの事後評価のようなモニターをすることの要望があったが、現行のＪＩＣＡスキームで対応すること

が難しいため削除した。ただし、ＪＩＣＡマレイシア事務所を通じ、ＩＭＲと緊密な連絡をとり、フォローを行うこととした。

プロジェクト終了後も単発専門家を派遣することがＩＭＲ側から強く要望されていたが、実現の可能性が確約できないことからリコメンデーションから削除した。ただし、ＩＭＲ側はプロジェクトによって移転された技術の応用、特に臨床に直接応用できる技術についてさらなる助言、技術的指導を強く希望しているため、これを追記として記載、持ち帰って検討することとした。

15:00～18:00　資料整理、報告書準備

○６月23日

9:00～10:00　合同評価報告書署名・交換

マレイシア側代表ＩＭＲ所長Dr. Mohamad Taha bin Arif、日本側代表 黒川高秀調査団長により合同評価報告書に署名・交換した。

11:00～12:00　在マレイシア日本国大使館報告

12:30～13:00　ＪＩＣＡマレイシア事務所報告

第２章　調査結果

2-1　総括

本調査団は、別項に記載されているとおり、ＥＰＵ(Economic Planning Unit)を訪問し本プロジェクトの目標および進捗状況の概要を伝えた後、カウンターパートであるマレイシアＩＭＲの所長および関係３部門（バイオテクノロジーセンター、医昆虫学、ウイルス学）の責任者を含む研究者全員と面談して、各研究課題の進捗状況と今後1995年末までに達成できる成果、ならびに本プロジェクト終了後にマレイシアに存続すると予想される効果について詳細に聴取した。それとともに、現地滞在中の派遣専門家およびチーフアドバイザーからもこれらについて聴取し、また、本プロジェクトにより購入した機材の使用・管理状況を調査した。

本プロジェクト開始時に設定した７項目の研究課題は、大部分が達成すべき目標を上回る研究が行われ、すでに論文として刊行された成果も少なくなかった。これらは延べ７名の長期派遣専門家および19名（プロジェクト終了までにさらに３名派遣予定）の短期派遣専門家による適切な指導のたまものであり、またＩＭＲ側の良好な受入体制と研究者の技術習得への熱意の表れと考えられた。本プロジェクトを通してわが国の協力機関が受け入れた８名（プロジェクト終了までにさらに３名受入予定）の研修員たちは、それぞれの分野で重要な人材となっていた。これらの人的交流によりＩＭＲの研究者および技術者とわが国の研究者との間に強い信頼関係が形成されていた。

本プロジェクトの目標は、熱帯医学の研究に役立つバイオテクノロジーを移転することであるが、目標は十分に達成され、マレイシアの医学研究ならびに国民医療に今後資するところ大と考えられる。さらに、今回の調査結果を総合すると、それ以上の効果があったと考えられる。

第一に、技術移転がマレイシア国内にとどまらず第三国に波及しようとしていることである。すなわち、ＩＭＲは、ＪＩＣＡの支援のもとに、第三国に対して本プロジェクトが取り上げた熱帯病への対策に役立つ先進技術を習得させるためのセミナーを用意しており、これを可能にした技術的基礎は本プロジェクトにより整えられたといえる。

第二は、わが国の研究者との強い信頼関係を通して生じた研究文化への影響である。マレイシアの研究者は、技術を欧米から学ぶ機会が多かったが、そこでは体験できなかった研究者としての訓練を本プロジェクトによって得ることができた。研究者が同時に技術の持ち主でもあるわが国の研究体制に接して、将来の研究の進め方について有益な示唆を得たとの発言が多かった。マレイシア側研究者の多くが、本プロジェクトにより培われた人的交流の維持と成果のフォローアップを日本側に対して強く要望したことは印象的であっ

た。

以上のような目標達成の状況と、カウンターパートを通して定着しつつある波及効果からみて、本プロジェクトの成果が失われないよう効果的な配慮を続けるべきであると考えられた。

2-2　部門別活動評価

マラリアに関するプロジェクトとしては、当初、下記の４項目にわたる活動目標が立案された。すなわち、

① マラリアのＤＮＡ診断法の開発

② マラリア原虫ミトコンドリア遺伝子の解析

③ ＨＬＡとマラリアに対する抵抗性の連関の解析

④ マラリア媒介蚊の遺伝要因の解析

の４項目である。以下には、これに従って、バイオテクノロジー部門および医昆虫学部門において、これまでに行われた研究活動を要約することとするが、その前に、プロジェクト開始時に、バイオテクノロジー部門の部長以下研究者全員を対象として、日本側専門家によって分子生物学の基礎に関する９週間に及ぶ集中講義・演習が行われたことに注意を喚起しておきたい。なぜなら、このことが、マレイシア側スタッフにとって、分子生物学の基礎的な知識と技術について「共通の」理解を深めるうえできわめて有効に作用したとみることができるからである。

（1） バイオテクノロジー部門

① マラリアのＤＮＡ診断法の開発

ＰＣＲ(polymerase chain reaction)を基礎にして、マラリアのＤＮＡ診断法として４種類の方法が考案されたが、本プロジェクトによる研究の結果、以下の３種類の方法が診断に有用と考えられるに至った。

a． nested ＰＣＲ

既知の熱帯熱マラリア原虫18S ｒＲＮＡの塩基配列をもとに作製したプライマーを用いたnested ＰＣＲでは、顕微鏡的に診断された熱帯熱マラリアの全例が検出されたが、三日熱マラリアの症例の３分の１も陽性を示した。顕微鏡的に陰性と診断された42例中５例がこの方法で陽性と診断され、そのうち１例は再検査により顕微鏡的にも診断が確認された。また、このプライマーを使用してnested ＰＣＲを行った場合、他のマラリア原虫のＤＮＡ増幅は認められず、熱帯熱マラリア原虫に特異的な増幅であることが確認された。

b． ＰＨ-ＰＣＲ (plate hybridization ＰＣＲ)

本法は、ビオチン化したプライマーを用いてサンプルＤＮＡからＰＣＲ産物を得、これをマイクロタイタープレートに固着させた熱帯熱または三日熱マラリア原虫に特異的なプローブと結合させ、さらにアビジン標識酵素と反応させたうえ基質を加えて検出するもので、鋭敏性・特異性ともきわめて高いことが確認された。

ｃ．　ＲＴ-ＰＣＲ(reverse transcriptase-ＰＣＲ)

本法では、サンプル中からＲＮＡを抽出し、これからreverse transcriptaseを用いてｃＤＮＡを合成し、さらに熱帯熱マラリア原虫の18ＳssrＲＮＡの塩基配列をもとに作製したプライマーを用いてこのｃＤＮＡからＰＣＲ産物を得るもので、アイソトープを用いることなく、鋭敏性・特異性の点で満足すべき結果が得られた。

これら３法に加えてＥＤ-ＰＣＲ（enzyme detection-ＰＣＲ）も試みられたが、満足すべき結果は得られなかった。

以上のように、このプロジェクトの活動によりどのようなプライマーやプローブを用いれば鋭敏で特異的な診断が可能になるかが明らかになり、マラリアの新しいＤＮＡ診断法の開発が行われた。その成果は、すでにいくつかの論文にもまとめられている。したがって、本項目の目標は十分に達成されており、その活動は高く評価できる。そればかりか、このようなＤＮＡ診断法の開発を通じて得られた知識と技術がすでにガン研究の領域にまで進展しつつあることは、技術移転がきわめて迅速にしかも円滑に進んだことを示している。

②　マラリア原虫ミトコンドリア遺伝子の解析

ａ．　マラリア原虫ミトコンドリアＤＮＡの検出

この目的のため、熱帯熱マラリア原虫ミトコンドリア遺伝子に特異的と考えられるＰ101/102(cytochrome oxidase Ⅲ)、Ｐ103/104(cytochrome oxidase Ⅰ)、およびＰ105/106(cytochrome b)の３種のプライマーを用いてＰＣＲを行ったところ、熱帯熱マラリア原虫の26分離株についていずれも満足すべきＰＣＲ産物が得られた。特に、ＣＯⅠミトコンドリア遺伝子を標的とするＰ103/104プライマーセットは、ＰＣＲ法による熱帯熱マラリアの診断に好適なプライマーになると考えられる。これらのプライマーセットを三日熱マラリア原虫の７分離株についても検討してみたところ、プライマー101/102および103/104については、７分離株すべてに陽性の結果が得られたが、プライマー105/106では２分離株のみが陽性であった。

ｂ．　三日熱マラリア原虫ミトコンドリアＤＮＡの研究

熱帯熱マラリア原虫の塩基配列をもとにして作製したプライマーを用いてＰＣＲを行い、ミトコンドリアＤＮＡ上にコードされている三日熱マラリア原虫ＣＯⅢ遺

伝子の塩基配列を部分的ながら決定することに成功した。その塩基配列は、熱帯熱マラリア原虫のそれと88%の相同性を示しており、それから推定されるアミノ酸レベルでは91%の相同性を示すことが判明した。この成果は、すでに学術論文として報告されたが、三日熱マラリア原虫ＣＯⅢ遺伝子に関する世界で最初の報告となった。

また、三日熱マラリア原虫ＣＯＩ遺伝子についても、同様の研究がなされた結果、部分的に決定された塩基配列から、熱帯熱マラリア原虫ＣＯＩとの間ではヌクレオチドで86%、アミノ酸レベルで91%の相同性が示されたほか、これらのマラリア原虫ＣＯＩについて酵母やヒトのそれと比較したところ、約40アミノ酸残基からなる最初の部分において相同性がより高いことが判明した。このことは、この領域が電子伝達系において重要な機能を有していることを示すものと考えられる。本研究も、三日熱マラリア原虫ＣＯＩ遺伝子に関する最初の報告となるものである。

さらに、ＣＯＩおよびＣＯⅢ遺伝子について、４種の新しいプライマー（Ｐ1101、Ｐ1102、Ｐ1103およびＰ1104）を作製し検討したところ、三日熱マラリア原虫分離株ＰＶ13については、Ｐ1102-Ｐ1104あるいはＰ1102-Ｐ1103をプライマーとした場合、それぞれ2689bpあるいは879bpのＰＣＲ産物が得られた。17種の制限酵素を用いて、後者にはＨｐａＩおよびＰｓｔ１による認識部位が存在することが判明したが、現在、その塩基配列を決定すべく解析中である。2689bpフラグメントについても、遺伝子のクローニングを行う予定となっている。

以上のことから、この分野における研究も、全長を決定するには至っていないものの、ＣＯＩおよびＣＯⅢ遺伝子についても大部分の塩基配列が決定され、世界に先駆けてその成果が学術論文として報告されるなど、目標は十分に達成されたものと評価すべきであろう。

③　ＨＬＡとマラリアに対する抵抗性の連関の解析

この研究分野に関しては、まず、免疫学研究部門のＨＬＡ研究室において、ＳＳＯＰ(sequence specific oligoprobe)法など、ＨＬＡClassⅠおよびClassⅡの新しいタイピング法についてスタッフの訓練が行われた。そのうえで、マラリア流行地に住みながら、血虫症が20～30%に達しても重篤な症状を呈することがほとんどない先住地域住民（オラン・アスリ）169名、およびこのグループの健常人62名について、ＨＬＡ-Ｂの遺伝子多型性を検討したところ、これらの住民の間では、Ｂｗ６の代わりにＢｗ４というモチーフ（エピトープ）を含むＢ75.2に相当するハプロタイプが非常に多いことが判明した。

このハプロタイプは、Ｂ77としても知られているものであり、このマレイシアの

B77様ハプロタイプをひとまずＥＣＢ-77(epitope combination ＨＬＡ-Ｂ77)と呼ぶことにしたが、その頻度はTemuan族で44%、Termiar族やSemai族では25%であり、ザイール、タイ、ジャワなどの2.5%と比較し明らかに高い頻度であった。また、これらの地域住民にはＨＬＡ-Ｂ53は認められなかった。さらに、ＳＳＣＰ(single strand conformational polymorphism)法によって検討したところ、ＰＣＲで増幅したＥＣＢ-77のａ１全体はＨＬＡ-Ｂ77と同様であることから、マレイシアのＥＣＢ-77はＨＬＡ-Ｂ77と同じタイプであることが強く示唆された。そこで、このＨＬＡ-class Ⅰ分子について抗原が結合するＢおよびＦポケットのアミノ酸配列を調べたところ、ＥＣＢ-77のＢポケットでは、ＨＬＡ-Ｂ53と比較し、第45番目のthreonineがmethionineに置換されていたが、Ｆポケット（Ｃ末端部分）ではまったくＨＬＡ-Ｂ53と同様であることが明らかとなった。ＨＬＡ-Ｂ53は、肝細胞期の原虫抗原に特異的な抗マラリア原虫細胞傷害性Ｔ細胞の誘導に関与することが報告されているので、ＥＣＢ-77についてもこのハプロタイプがマラリアに対する抵抗性の誘導に関与するものと考えられる。

このように、この分野においても、当初の期待をはるかに超える十分な成果が収められた。

④　総合評価および提言

以上に記したように、バイオテクノロジー部門では、暫定協力実施計画（ＴＳＩ）に記載されたすべての研究分野において順調に研究目標が達成されており、熱帯病研究に近代的なバイオテクノロジーの概念と手法を導入するという主要目標の主旨にも十分沿った成果が得られたことは、高く評価されるべきことである。

特に、マラリアのＤＮＡ診断に関してはnested ＰＣＲ、ＲＴ-ＰＣＲ法が感度と特異性の面で従来の顕微鏡検出法に勝ることが示され、論文もすでに１編が"Trans. Roy. Soc. Trop. Med. Hyg., 89, 271-272, 1995"誌に掲載され、他の１編も同誌に掲載されることが決定している。さらに、別の論文が投稿中であるほか、もう１編が準備中である。

ＩＭＲにおける今回の調査で、日本人専門家がみずから実験操作を行うところを見て、それまで自分自身では実技を行わなかったmedical officerたちもきわめて学ぶところが多かったようで、直接にテキストに記されていない実験の細部まで学ぶことができたこと、また、日本での研修に１人が招へいされるよりも、専門家の来所によって同時に多くの人々が学ぶことができたことに喜びを表明する所員も多く、さらに、英米の研究者と異なり日本人専門家は、高価で貴重な試薬を、所員の使用も平等に認めてくれたことに感激したと話す所員がいたことは、調査団にとっても印象的であっ

た。

一方、マラリア原虫ミトコンドリアＤＮＡの解析については、いまだ報告のなかった三日熱マラリア原虫について精力的に研究が進められ、ＰＣＲ産物のクローニングや自動ＤＮＡシーケンサーによる塩基配列決定などに関する技術移転は完全に行われた。特に、ＣＯⅢ遺伝子の塩基配列を部分的ながら決定し、その成果を"Jpn. J. Parasitol. 44, 40-43, 1995"に発表するとともに、ＩＭＲとしては、初めてその塩基配列をＤＤＢＪ（26 Jan. 1995）を通して国際的なデータベースに登録したことは、国際的な場で今後ＩＭＲがますます発展していく弾みになるものと考えられる。

なお、上記のように解析された遺伝子について、組換えたんぱくとして遺伝子の発現をみる技術に関しては、プロジェクトの後半でＩＭＲ側から希望が出され、一部技術移転中である。しかし、マラリア原虫のたんぱくを大腸菌で発現することは必ずしも容易ではないことがあり、今後も何らかの技術協力が必要であると考えられる。

いずれにしても、以上の成果は、すでにＩＭＲ自体の手によって、ガン研究や薬剤抵抗性マラリアの研究に応用展開されており、技術移転が単に研究所のレベルにとどまらず、マレイシア国民に広くその効果が広がっていくことが十分期待できる。

さらに、ＨＬＡと熱帯熱マラリアへの抵抗性に関する研究では、当初の予想に反して、マラリアに感染しても重篤な症状を呈さない先住地域住民（オラン・アスリ）に、ユニークなＨＬＡハプロタイプが高頻度に見いだされ、その抗原結合部について検討した結果、アフリカで抵抗性と関連ありとされたＢ53とアミノ酸配列が同様であることが判明したことは、今後のマラリアワクチン開発とも関連して、マラリアに対する防御免疫のエフェクター機構を考えるうえできわめて興味深くかつ重要な所見であり、本プロジェクト終了後も、両国の研究者の間で共同研究を進めることが期待される。

また、ＨＬＡハプロタイプ決定にあたって導入された新しい技術は、臓器移植等の技術にも必須のものであり、その波及効果はきわめて大きいと考えられる。

以上、総合的な評価について記したが、ＩＭＲの将来について考えると問題がないわけではない。それは、人材の問題である。現在、バイオテクノロジー関係の研究は、この部門の長であるDr. Makの力量によるところが大きい。しかし、彼は２～３年後に定年を控えているようであり、その後の研究を強力に推進する人材を早期に養成する必要がある。今後、これまでの技術協力の成果が生かされ、さらに発展することを期待するとすれば、人材の養成を考慮に入れた協力が必要であろう。

（２） 医昆虫学部門

① 蚊のマラリア感受性遺伝子の位置の検索

本研究分野では、蚊にマラリア原虫を感染させるために、熱帯熱マラリア原虫の培

養によって生殖母体を効率よく得る必要がある。この目的で、原虫の株化を試みる努力がバイオテクノロジー部門の協力も得つつ重ねられてきた結果、これにはどうやら成功し、安定して生殖母体が得られるようになった。ハマダラカとしては、Anopheles maculatusのさまざまなコロニーを用いているが、現在までのところ、感受性株が同定されるには至っていない。

一方、ネッタイシマカから作製したｃＤＮＡプローブを用いて、異なった株のハマダラカから抽出したゲノムＤＮＡをsouthern blotで検出することには成功し、これらの基本的な操作については技術移転が完了した。また、ゲノムＤＮＡとｃＤＮＡプローブのハイブリダイゼーション検出の感度を上げるため、An. maculatusのＲＮＡからｃＤＮＡを得る方法が考案された。さらに制限酵素断片サイズ多型（ＲＦＬＰ）の解析によるハマダラカの３株の選別が進められており、そのためのデータ解析用装置（ＭＡＰＭＡＲＫＥＲ／ＱＴＬcomputer package)も導入済みである。プロジェクト終了までに、何らかの成果が得られることが期待される。

なお、マレイシア産のハマダラカ３株とサバ州の１株について、遺伝学的に検討するため、アイソザイム対立遺伝子の研究がなされている。この方法については技術移転が完了し、ＪＫ(Jeram Kedah)株を標準として研究することも決定している。

② 総合評価および提言

本プロジェクトは、マレイシア側からの提案に基づくものであったが、プロジェクト開始後、部門の長が定年により交代したこともあって、研究の進展には残念ながら相当の遅れが認められる。遅れのもうひとつの原因としては、生殖母体が当初安定して得られなかったこともあるとみられる。ハマダラカの確定した株を得ることにも、技術的な困難が伴うことが想像される。このような基本的な実験系が確立できれば、本プロジェクトはかなり進展するものと考えられるが、粘り強い努力が必要であり、残りの期間では実現は困難であろう。にもかかわらず、基本的な部分の技術移転は一応完了したとみてよいであろう。今後、問題点の解決後は、バイオテクノロジー部門との密接な協力により当初の目標が達成されるよう期待したい。

（３） ウイルス学部門

① 分子生物学を含む診断法の強化

ＩＭＲウイルス部門では各種ウイルス感染症の確定診断を日常業務として、マレイシア各地からの検査依頼に対して主として血清反応による診断を行ってきた。本プロジェクトにおける対象疾患のひとつであるデングウイルス感染症は、マレイシアその他東南アジア諸国における保健衛生上の重大課題であり、依頼検体数も格段に多い。

1990年に導入されたＩｇＭ-ＥＬＩＳＡは、従来から使用されてきた血球凝集抑制

反応（ＨＩ）に代わってしだいに使用され、単一血清検体を用いた診断の可能性が増加しているが、抗体産生前に採血された検体では陰性結果を示す可能性が大きく、それらの確認試験のために回復期血清を採取することは必ずしも容易ではない。

本プロジェクトでは、デングの迅速診断を目的としたウイルス遺伝子検出のためのＲＴ-ＰＣＲの技術移転を行い、その目的は達成された。この方法を発病後５日以内に採取されたデングＩｇＭ-ＥＬＩＳＡ陰性の血清検体に適用したところ、その約30％が陽性を示し、検出されたウイルスの血清型も判定することができた。その結果、回復期血清検体を入手しなくても急性期の単一血清検体のみでデングの診断効率を上昇させることに成功している。

この成果は、臨床医に対して適切な治療法を施す指針を与え、衛生行政担当者に対しては、流行しているウイルスの血清型に関する迅速かつ正確な情報を提供することによって、当該疾患の防除に寄与するものと評価される。

今後残された期間内に、現在感染マウス脳から抽出されている診断用抗原を細胞培養法により作製し、米国に供与を依頼している単クローン抗体を自家生産することによって、ＩｇＭ-ＥＬＩＳＡに必要な試薬を自給自足できる体制を確立することが望まれる。

今ひとつの対象疾患である日本脳炎は、マレイシアではウイルスは確実に存在するにもかかわらず、衛生行政当局に報告された患者数はさほど多くない。しかしながら、髄液の日本脳炎ウイルスに対するＩｇＭ-ＥＬＩＳＡが脳炎患者の約40％で陽性を示していることは、後述する疫学的調査結果とともに、本疾患がマレイシアに広く存在していることを示している。本プロジェクトでは、技術移転されたＲＴ-ＰＣＲ法を患者髄液に適用して日本脳炎ウイルスの遺伝子の検出を試みたが、陰性であった。脳炎症状を示している患者は、すでに抗体産生によって髄液からウイルスは消失していると考えられるので、ＲＴ-ＰＣＲ よりもＩｇＭ-ＥＬＩＳＡ が適切な診断法といえる。

本課題を遂行することによって次の波及効果が得られた。すなわち、マレイシアではＷＨＯの勧告を得て日本脳炎防除国家計画を策定しており、その計画目標のひとつが当該国における日本脳炎発生数の把握であったが、その目的を達成するために、特定病院において臨床的に脳炎と診断された患者の血清と髄液はすべてＩＭＲウイルス部において確認試験を行うことになった。この確認試験では上記結果を勘案してＩｇＭ-ＥＬＩＳＡを使用することになろう。

② 疫学と伝播動態の研究

日本脳炎は蚊媒介性疾患であり、自然界ではヒト以外の複数の動物がウイルスを増

幅させ媒介蚊の感染源となる。本プロジェクトでは、マレイシアにおける日本脳炎ウイルスの媒介蚊の検索を野外採取蚊からのウイルス分離によって研究する一方、動物宿主の調査を各種動物血清の日本脳炎ウイルスに対する抗体保有状況から遂行した。

ヒトスジシマカ培養細胞C6/36を用いたウイルス分離方法の技術移転は完了し、野外蚊からの日本脳炎ウイルスの分離とともに、デング患者血清からのウイルスの分離にも応用され、分離ウイルスは後述する遺伝子解析に使用されている。ウイルス分離に使用した野外蚊材料は、衛生昆虫学部が従来から行ってきた媒介蚊調査によって採集されたものであるが、本研究の遂行によってＩＭＲの異なる部門間の共同研究が促進される契機となった。

野外蚊からのウイルス分離成績は、日本と異なりマレイシアでは複数種の蚊が日本脳炎ウイルスの伝播に関係していることを示唆しており、従来記載されていなかった種の蚊からも日本脳炎ウイルスが分離されている。

ＨＩ試験を用いた動物の血清学的調査では、従来から本ウイルスの主な増幅動物として知られているブタ以外にも、ウシ、水牛、ヒツジ、ヤギなどのほ乳動物が広く日本脳炎ウイルスの感染を受けている一方、ウズラ、スズメなどの鳥類では抗体保有率が低いことが示された。これらの動物血清はイポーのVeterinary Research Institute の協力によって提供されたものである。

マレイシアのような熱帯地域では、日本脳炎ウイルス以外にもＨＩ試験で交差反応を示す他のウイルスが存在する可能性があるので、より特異性の高い中和反応を用いて日本脳炎に対する抗体の存在を確認した結果、マレイシアにおいてもブタが日本脳炎の感染を最も頻繁に受けていることが示された。これらの研究を通じて、マイクロプレート培養細胞と酵素免疫染色法を用いた中和試験の技術移転が行われた。上述のマレイシア日本脳炎防除計画においても、ブタに関する血清疫学的監視体制が盛り込まれている。

③　ウイルスの遺伝子解析とデング病原性の検索

本プロジェクトの当初計画では、デングウイルス感染症の重症型であるデング出血熱の発病機構に関係するウイルス遺伝子を同定することを目的としていた。前記②の研究によって多数の日本脳炎ウイルスが野外蚊から分離されたので、それらの遺伝子型を解明する研究が追加された。

当初の目的であったデングウイルスの病原性遺伝子解析に使用するウイルス株は、１～２年次に臨床的に症状の異なる患者から複数株のウイルスが分離され、すべて３型であることが同定されている。予備実験として、分離ウイルス遺伝子のＥ／ＮＳ１接合領域240塩基の配列を、患者血清のＲＴ-ＰＣＲ増幅産物の直接基配列とC6/36細

胞で分離後、３代継代されたウイルスの塩基配列で比較したところ、同一結果を示したので、以後は分離ウイルスについて解析が進められた。この遺伝子領域では、重症患者から分離されたウイルス株と、軽症患者から分離されたウイルス間で顕著な差が認められなかったので、現在遺伝子の５’末端非コード領域の比較解析が進められている。この領域の塩基置換は、ポリオウイルスやシンドビスウイルスでは病原性と関係があると報告されている。

日本脳炎は、日本以外にも東アジアから東南アジアを経て南アジアに至るアジアモンスーン地域に広く存在している。温帯・亜熱帯地域では本疾患は多数の患者発生を伴う流行形式を示すのに対して、マレイシア、フィリピン、インドネシアなどの熱帯地域では、ウイルスと媒介蚊が存在するにもかかわらず患者は多発していない。

米国の研究者は多数の日本脳炎ウイルス分離株のＰｒＭ遺伝子領域240塩基の配列を比較することによって、３～４の遺伝子型に分類した結果、熱帯アジアの分離型は亜熱帯あるいは温帯の分離株と異なる遺伝子型に属することを示した。この遺伝子型の相違によって、熱帯と亜熱帯ないしは温帯における日本脳炎の疫学的相違を説明できるとする仮説が提唱されている。しかしながら、本プロジェクトによって分離された十数株の日本脳炎ウイルスの遺伝子型を解析した結果、すべての分離株は温帯から亜熱帯に存在する遺伝子型に属することが明らかとなった。この結果は上記の仮説を覆すもので、熱帯と亜熱帯ないしは温帯における日本脳炎の疫学的相違は遺伝子型以外の要因によって規定されていることを示している。

これらの研究によって、ウイルス遺伝子の塩基配列解析に必要な技術移転が完了している。

④　総合的評価および提言

本プロジェクトのウイルス学分野は、当初目的をほぼ達成し、残された未完部分についても、本プロジェクトの終了までに可能な限り目的達成に向けて努力すべき計画である。

技術移転に関してはほとんど90%近い達成度であり、研究面でもデングの迅速診断、日本脳炎の媒介蚊と血清疫学的研究、ウイルス遺伝子型の置換現象の発見などにおいてみるべき成果が得られている。デングウイルスの病原性遺伝子の解明は残されているが、世界的にも注目されている重要な課題である。

特記すべきことは、本プロジェクトを遂行することによりマレイシア、ことにＩＭＲの研究者と日本の研究者との人的交流が推進され、今後の共同研究の可能性が開拓されたことである。この成果を今後可能な方策によってさらに助成することは、本プロジェクトによって達成されたマレイシアの研究水準を今後維持するだけではなく、さらに向上させ、より高度の研究成果が得られるための必要条件である。

第3章 目 標 達 成 度

目　　標	目　標　指　標	プロジェクト開始時の状況	実　　績	目標未達成の理由
（上位目標） 熱帯病、特にマラリア、デング、日本脳炎の撲滅対策に貢献する	当該疾患の感染率、死亡率	1986～1990年における年間患者数（死亡者数）は、デング熱／デング出血熱では33,151-65,384(10-72)である。 日本脳炎は届け出感染症ではないために資料がないが、脳炎の年間患者数（死亡者数）は39-92(3-10)である。	プロジェクト終了時の患者数（死亡者数）の統計が入手されていないが、従来の数値から大きく変化しているとは想像されない。	本プロジェクトでは、対象疾患の罹患率・死亡率を直接低下させることを目的としていないために、プロジェクトの波及効果によって上位目標を達成するには今後日時を要する。
（案件目標） マラリア、デング、日本脳炎に関する分子生物学、生物技術の水準の向上	A．マラリア 1．診断の鋭敏性と特異性 2．研究成果の公表の確立 B．デングと日本脳炎 1．真の陽性率と罹患率の測定 2．ＩＭＲの診断能力の向上		A．マラリア 1．鋭敏性、特異性ともに優れたnested PCRおよびRT-PCRを開発した。 2．すでに２編が学術論文として発表され、１編が掲載受理された。他に投稿中２編、準備中１編がある。	
（アウトプット目標） A．マラリア 1．マラリアの遺伝子ＤＮＡを応用した診断 2．ミトコンドリア核酸の解析 3．ＨＬＡとマラリア抵抗性 4．蚊のマラリア感受性遺伝子の位置の検索	A．マラリア 1．診断法が確立したか、鋭敏性と特異性 2．全体の何%まで配列が決められたか 3．マラリア抵抗性のＨＬＡが発見できたか 4．蚊のマラリア感受性遺伝子の位置が決定できたか		A．マラリア 1．診断法は確立した。 3．発見できた。抵抗性を示す原住民にECB-77が高頻度に見いだされ、抗原結合部位は HLA-853とアミノ酸配列が同様であった。 4．ハマダラカのマラリア感受性遺伝子の位置については決定できなかった。	基本的な実験系の確立に問題があった。
B．デングと日本脳炎 1．分子生物学を含む診断法の強化 2．疫学と伝播動態の研究 3．ウイルスの遺伝子解析とデング病原性の検索	B．デングと日本脳炎 1．分子生物学的診断が定着したか、その他の方法の改良は進んだか 2．当該国の分布が明らかになったか、伝播する蚊の種類が決められたか 3．染色体の核酸配列が測定できるか、型分けができるか、病原性との関連がどこまで解明できるか	1．デングの確定診断法に IgM-ELISAが導入され、従来から使用されていた血球凝集抑制反応(HI)に代わり、しだいに使用されつつある。ウイルス分離はデング、日本脳炎ともにほとんど行われていなかった。RT-PCRなどの分子生物学的診断法は使用されていなかった。 2．日本脳炎の媒介蚊と増幅動物に関する情報は、1970年代に英国・米国の学者によって得られたきわめて限られたものにすぎなかった。 3．ウイルス遺伝子の塩基配列解析とそれに基づく分子疫学的研究、およびウイルスの病原性に関する研究はまったく行われていなかった。	B．デングと日本脳炎 1．デングの迅速診断にRT-PCR法が導入され、IgM-ELISA 陰性で発病後５日以内に採取された血清検体の約30%で陽性結果が得られた。 日本脳炎の確定診断にIgM-ELISA が導入され、細胞培養を用いたデングおよび日本脳炎ウイルスの分離方法が導入された。 IgM-ELISA に使用する抗原と単クローン抗体産生ハイブリドーマ細胞の培養、およびマウスにおける腹水産生は、基礎的条件の設定が終了した。 2．マレイシアでは日本と異なり、コガタアカイエカ以外に複数種の蚊が日本脳炎の媒介に関与する可能性が示された。 各種ほ乳動物、ことにブタが日本脳炎の増幅動物として重要であることが示された。 3．1992年にマレイシアで採取された野外蚊から分離された日本脳炎ウイルスは、熱帯アジアに分布している遺伝子型に属する20年前の分離株と異なり、温帯、亜熱帯地域の流行株と同じ遺伝子型に属していた。 デングウイルス分離株の遺伝子解析は現在進行中であり、1995年中にできるだけの情報を得る計画である。	1．RT-PCRの技術移転は目標を達成し、確定診断向上に寄与できた。これはマレイシア側の受入体制と検査材料の入手が円滑であったためである。C6/36 細胞培養を用いたデング、および日本脳炎ウイルス分離法の技術移転が目標を達成できたのも同じ理由による。IgM-ELISAに使用する抗原と単クローン抗体の自家生産は、本プロジェクト終了時までに完了する予定である。 2．マレイシアの研究者および衛生行政担当者が日本脳炎に対する関心を持っており、研究材料の入手と研究協力を得ることが可能であったことは、本課題の目標達成の重要要因である。 3．マレイシアに日本脳炎ウイルスが存在することは知られており、研究者がその分子生物学的研究解析に対して興味を有していたことが、本課題の目標達成に重要な要因であった。デングの病原性遺伝子の同定に使用するウイルスは１～２年次の研究で分離されたため、遺伝子解析は第３年次から開始された。
（活動目標） ＜日本側＞ 1．専門家の派遣 2．研修員受入 3．機材の供与 4．その他必要な協力 ＜マレイシア側＞ 1．カウンターパートの選出 2．保守、管理システムの確立	（インプット目標） ＜日本側＞ 1．長期専門家　３年間５～６名 短期専門家　年間８名以内 2．研修員受入　年間３名 3．供与機材　３年間１億円 ＜マレイシア側＞ 1．十分な実施人員の任命 2．消耗品経費の負担		＜日本側＞ ・長期専門家　合計７名 ・短期専門家　合計19名（追加２名予定） ・研修員受入　合計８名（追加３名予定） ・機材供与　合計１億3500万円 ＜マレイシア側＞ ・カウンターパート　マラリア　４名 デング、日本脳炎　７名 ・ローカルコスト負担　270,150 リンギット	

資　　料

1　合同評価報告書

JOINT EVALUATION REPORT ON THE PROJECT FOR RESEARCH AND DEVELOPMENT ON DIAGNOSIS OF SELECTED TROPICAL DISEASES

The Japanese Evaluation Team (hereinafter referred to as "the Team") organized by the Japan International Cooperation Agency (hereinafter referred to as "JICA") and headed by Dr. Takahide Kurokawa, visited Malaysia from 17 to 24 June 1995 in order to evaluate jointly with Malaysian authorities concerned the past achievements of Japanese Technical Cooperation for the Project for Research and Development on Diagnosis of Selected Tropical Diseases (hereinafter referred to as "the Project") in the Institute for Medical Research (IMR).

During its stay in Malaysia, the Team discussed and studied together with the Malaysian personnel concerned, a number of aspects regarding the progress and achievements of the Project, as well as fulfillment of commitments.

Through careful studies and discussions, both sides summarized their findings and observations as described in the document attached hereto.

Kuala Lumpur
Malaysia
23 June 1995

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Japan International
Cooperation Agency,
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Ministry of Health,
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I. Name List of Participants

Date : 17 - 24 June 1995

Place : Institute for Medical Research, Ministry of Health, Malaysia
Jalan Pahang 50588 Kuala Lumpur

Participants

The Evaluation Team, JICA

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Main Staff Members of IMR

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Japanese Members

Embassy of Japan
Mr. Masato Yoneda, Secretary

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Division of Medical Entomology
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HLA Laboratory

II. METHOD OF EVALUATION

1. Materials used as reference

In order to evaluate the past performance and achievements quantitatively and qualitatively the following materials were used as references;

(1) Record of Discussions with Implementation Survey Team signed on 22 June 1992
(2) Tentative Schedule of Implementation signed on 22 June 1992.
(3) Minutes of Discussions with Planning and Consultation Team signed on 19 November 1993
(4) Minutes of Discussions with Consultation Team signed on 18 August 1994
(5) The official requests made by the Government of Malaysia with respect to dispatch of Japanese experts, fellowships to IMR counterparts and provision of equipment by means of Technical cooperation Forms A-1, A-2, A-3 and A-4, respectively.
(6) Annual Report 1993
(7) Annual Report 1994
(8) Quarterly Reports from January 1993 to March 1995
(9) Other publications concerning the Project

2. Discussions and Observations

The Team discussed various aspects of the Project with main IMR staff members and Japanese experts, and observed the laboratories, machinery, equipment, facilities and utilities made available for the Project.

To recognize the impact and efficiency of strengthening research capabilities, interviews were made with researchers in Divisions concerned with the Project including those who received the training fellowships.

III. OBJECTIVE AND ACTIVITIES OF TECHNICAL COOPERATION FOR THE PROJECT

1. Objective

According to the Record of Discussions signed on 22 June 1992, the objective of the Project is to strengthen the use of biotechnology in the diagnosis and management of malaria, dengue and Japanese encephalitis.

2. Activities of Technical Cooperation

In order to accomplish the above-mentioned objectives, both sides agreed that technical cooperation should be directed to the research subjects described below with workable subtitles, that were defined in the Tentative Schedule of Implementation signed on 22 June 1992 and the Minutes of Discussions with the Planning and Consultation Team signed on 19 November 1993, through dispatch of Japanese experts, acceptance of IMR counterparts in Japanese institutions for strengthening research capability and provision of equipment.

2.1 Malaria

a. Development and use of DNA probes for malaria diagnosis

b. Analysis of mitochondrial genes

c. Identification and characterization of protective epitopes of malaria antigens
   - Human Leucocyte Antigen (HLA) and resistant to *falciparum* malaria

d. Better understanding of the vectorial status of malaria vectors at the molecular level
   - Determination of malaria susceptible locus on the chromosome of *Anopheles maculatus*

2.2 Dengue and Japanese encephalitis (JE)

a. Strengthening of diagnostic capability using molecular biology and biotechnology

b. Epidemiological and entomological studies on dengue and JE

c. Understanding of pathogenesis of dengue hemorrhagic fever (DHF) at the molecular level
   - Genome analysis for typing of JE and dengue viruses, and for pathogenicity

IV. PERFORMANCE OF THE PROJECT

1. Facilities

The space for the project office and research laboratories was provided by IMR. Other facilities provided by IMR, such as electricity, gas and water supply, sewage system, a telephone line for facsimile, fuel of an official vehicle, furnitures and so on, are highly appreciated.

2. Staffing

Most medical and science officers in 3 divisions concerned to the Project have been assigned to the Project for effective implementation and successful transfer of biotechnology. The list of Malaysian and Japanese counterparts is presented in ANNEX I

3. Management and Administration

Administrative and managerial services have been provided mostly by the Malaysian personnel.

3.1 Malaysian side

a. Director, Division of International Affairs, Ministry of Health
b. Director, Institute for Medical Research
c. Senior Administrative Officer, IMR
d. Heads of 4 Divisions of IMR connected to the Project
e. Secretariats of the Project
f. Staff members in the above 4 Divisions concerned

3.2 Japanese side

a. Chief Advisor
b. Coordinator
c. Japanese Experts dispatched by JICA
d. Staff members in JICA Malaysia Office

4. Japanese Experts

JICA has dispatched seven (7) long-term experts and nineteen (19) short-term experts as of June 1995 from the beginning of the Project, and their names and specialized fields are listed in ANNEX II. In the rest of Japanese Fiscal Year 1995, three (3) more experts will be dispatched by JICA.

5. Fellowships Provided to Malaysian Personnel

Up to now, eight (8) Malaysian counterparts have seen sent to Japan for either technical studies or observation. Their names are listed in ANNEX III. In the rest of this Japanese Fiscal Year, three (3) more fellowships will be provided by JICA.

JICA accepted the above personnel in the research fields agreed in the Record of Discussions. Their technical training in Japan was effective for strengthening research capability and acquiring the new technology and scientific information.

6. Equipment

In the period from January 1993 to the end of May 1995, the Equipment at a total amount of 135 million yens (US$ 1,200,194) for the budget of Japanese Fiscal Year (JFY) 1991 to 1994a was donated by the Government of Japan. The main items of equipment and supplies are listed in ANNEX 4. Another batch of equipment at an amount of 24.9 million yens (US$ 248,980) for the budget of JFY 1994b was purchased in March 1995 and is now being delivered to respective Divisions.

The provided equipment for the Project has been used efficiently in the activities of the Project.

7. Budget for Local Cost

Both sides made the best effort to secure the budget necessary for implementation of the Project. The budget born by the Malaysian side was from the Malaysian Governmental budget for routine operating expenditure and the grant-in-aid applied for the Research and Development Fund and New Policy Grant. Efforts by Ministry of Health and IMR to raise those funds were remarkably helpful in developing the project. The amount born by each side fractioned by the Japanese Fiscal Year (JFY) is shown below.

Fractioned by JFY	Malaysia	Japan
Jan. 1993 - Mar. 1993	RM 9,700	RM 118,468
Apr. 1993 - Mar. 1994	146,530	189,958
Apr. 1994 - Sep. 1994	113,920	37,420

V. OUTPUTS FROM THE PROJECT

1. Achievement of Technical Cooperation

After careful evaluation and discussions, both sides agreed that the technical cooperation implemented during the past 2 and a half years has accomplished the objectives mentioned in item III.1. The following seven (7) subjects written briefly were taken up as our items of research cooperation (III.2);

(1) DNA probes for malaria diagnosis
(2) Analysis of mitochondrial gene
(3) HLA and malaria resistance
(4) Malaria susceptible locus in *Anopheles* chromosomes
(5) Diagnosis of dengue and JE
(6) Epidemiology and transmission of JE and dengue
(7) Genome analysis of dengue and JE viruses

Through the cooperative activities between Japanese experts and IMR counterparts, the introduction of fundamental biotechnological techniques into each division was rapidly and successfully completed. In addition, the acquired methodology was applied to other research Projects as mentioned below. Although subjects (4) and (7) are still under way, the most other subjects are completely achieved. Subject items (1) and (6) achieved remarkable progress which was much more than initially planned. These were accomplished through careful initial planning, meticulous follow-up and implementation of these Projects. The progress of each research subject is summarized in ANNEX 5, and publications related to the research subjects are as listed in ANNEX 6 as outcomes from the project.

The fellows from IMR sent to Japan for study could acquire laboratory techniques extremely well, and the experience gained by them was invaluable for research capability strengthening of the Institute. Japanese experts

while staying in IMR, generously imparted their technical knowledge including "tricks of the trade" through intensive practical training of IMR counterparts. This has been extremely useful in increasing the pool of local scientists trained in biotechnological techniques. In addition, IMR scientists have benefited tremendously through intellectual discussions with Japanese experts.

Another important factor that contributed to the Project was the JICA's assignment of a Chief Advisor who has excellent managerial skills and strong scientific background. Close collaboration and coordination among the Director of IMR, Division Heads concerned and Chief Advisor were invaluable and instrumental in ensuring the huge success and achievement obtained during the 2 and a half years of the Project.

2. Training Programmes

2.1. Third Country Group Training, JICA

As an extended function of the Project and due to rapid development of medical biotechnology in IMR, IMR will conduct the Third Country Group Training entitled as the International Seminar on Biotechnological techniques in Tropical Medicine, to which 12 participants will be invited from Malaysia and neighbouring countries in Southeast Asia and Pacific Islands. The first seminar will be held for 4 weeks from 24 October to 18 November 1995. Similar seminars will be held yearly for 2 more consecutive years. Announcement on the Seminar to the countries concerned has already been made through diplomatic channels.

2.2. Third Country Individual Training, JICA

IMR is registered as an individual training institute for JICA. In this programme, the member in JICA projects in foreign countries will be considered by IMR for training in the 6 month Diploma Courses of Medical Microbiology (DMM) or Applied Entomology and Parasitology or in any division in IMR. A Sri Lankan medical doctor graduated from the DMM Course in March 1995.

3. Impact of the Outcomes

3.1 Effect at the division and/or IMR level

a. Improvement of diagnostic service

Molecular techniques acquired under the IMR-JICA Collaborative Project (the Project) are applicable to diagnosis of other human diseases. IMR members under the Project have now applied these techniques not only to malaria, Japanese encephalitis and dengue, but also to other infectious diseases and cancers. In malaria, other than the use of DNA probes and nested PCR techniques for malaria diagnosis, these techniques for species identification are being used, *e.g.* determining whether *P. ovale* exists in Malaysia, and these tools are used to characterize drug resistant *P.*

falciparum strains. PCR techniques for the diagnosis of toxoplasmosis and filariasis are also being used. These molecular techniques are being increasingly applied to breast and liver cancers and nasopharyngeal carcinomas.

b. Technical development of IMR staff members.

IMR officers and other technical staffs have benefited directly and indirectly from the excellent training opportunities provided under the Project. These have been achieved through the intensive courses and practical sessions conducted locally by competent Japanese experts as well as through officers receiving comprehensive training at various universities and institutions in Japan. These trained officers in turn, are now serving as resource personnel to train other officers in the IMR in the techniques they have learned.

c. Communications and collaboration among divisions inside and outside the Project.

Communications and collaboration among the divisions inside and outside the project are satisfactory. There is sharing of resources and expertise between divisions, including organizations outside IMR. Collaborative projects utilizing molecular techniques for the study of nasopharyngeal and breast cancer involving the Division of Epidemiology, Sarawak Medical Department, Hospital Kuala Lumpur and the Biotechnology Centre are being carried out. Officers in the three divisions involved in the project are also being consulted by others in the IMR regarding molecular biology techniques.

3.2 At academic level in Parasitology, Virology and Medical Entomology in Malaysia

Some post-graduate students registered for higher degrees with the University of Malaya and other institutions are conducting their research utilizing biotechnological techniques in the Biotechnology Centre and other IMR Divisions.

A PhD candidate has just completed her thesis on malaria research utilizing biotechnological techniques acquired while studying in the Project. Another PhD candidate registered with the University of Malaya, is currently conducting her research utilizing biotechnological techniques in toxoplasmosis. A WHO fellow from China was given a three month training in biotechnological techniques in the Biotechnology Centre in 1993/94.

In addition to the above, many officers who have acquired experience under the Project, are also faculty members involved in the teaching of the biotechnology module conducted under the post-graduate courses in the section of tropical medicine of the Southeast Asian Ministers of Education Organization (SEAMEO-TROPMED) leading to the Diploma in Applied Parasitology and Entomology (DAP&E) and the Diploma in Medical Microbiology (DMM). Through them, at least 14 candidates from countries inside and outside SEAMEO in Asia, Pacific Region and Africa per year in each course, have received basic training in biotechnology.

3.3 At country level of Malaysia for health administration

The greatest direct impact on health administration and policy will be through the application of rapid diagnostic tests for patient care and for control programme monitoring and evaluation. In the immediate term, the diagnostic tests now available through the application of molecular techniques in dengue and Japanese encephalitis have provided the means for rapid, sensitive and specific confirmatory tests for these diseases which will have a great impact not only on patient care and management, but also on control of these diseases.

In malaria, the use of biotechnological tools for antigenic and phenotypic characterization of parasite strains endemic in the country, will be extremely useful in decision making on the national anti-malaria drug policy as well as providing important background data needed for malaria vaccine evaluation in the future.

Another indirect but important impact on national policy is the enlargement of the science base of personnel trained in biotechnological techniques in the country. The pool of such trained scientists at the IMR and other institutions will increase as others receive training from officers trained under the Project. This enlarged science base will provide the leadership in evaluation and implementation of research utilizing biotechnological techniques, thus contributing to national health policy with regards to patient care and control programmes.

A further benefit which should be given due recognition is that obtained through the personal interactions between Japanese and Malaysian scientists. The intellectual exchange and direct observation of the work ethics of Japanese scientists, are important aspects of the Project which will benefit the scientific development of Malaysian counterparts.

4. Extended Effect of the Outcomes

4.1 Transfer of technology to other divisions in IMR and other organizations in the country levels

In-service training of officers from other Divisions of the IMR in specific biotechnological techniques is provided whenever the need arises. As mentioned in Para 3.2, the Divisions involved in the Project have been called upon to provide biotechnology training lasting 1-2 weeks in the 2 post-graduate diploma courses, *i.e.*, DAP&E and DMM in SEAMEO-TROPMED. WHO fellows are also sent to these Divisions for attachment training in biotechnology techniques. The acquired technology will be transferred to the Vector-Borne Diseases Control Programmes, Ministry of Health, Malaysia through short-duration courses.

4.2 Transfer of technology to other countries

An international seminar on biotechnological techniques in tropical medicine conducted under the "Third Country Training Programme (JICA)" is scheduled to take place at the IMR from 24 October to 18 November 1995.

As stated above in Para 3.2, candidates from SEAMEO countries, Asia, Pacific Islands and Africa, are given training in basic molecular biology through the biotechnology modules of the DAP&E as well as the DMM SEAMEO-

TROPMED Courses. It is noteworthy that one MD candidate from Sri Lanka underwent the DMM course sponsored by JICA and completed the course as the best student.

An important component of the DAP&E Course is a research project as an integral part of the Course, and on the average, 2-3 candidates per year carry out their research assignment utilizing biotechnological techniques. Such graduates will be able to disseminate their biotechnological knowledge when they return back to their own countries.

VI. SUSTAINABILITY AFTER THE PROJECT

1. Organizational perspectives

a. Existing organization of IMR and the Project:

The IMR-JICA Project is located in the Biotechnology Centre, the Virology Division, Medical Entomology Division and Immunology Division (HLA Laboratory) of the IMR. All divisions in IMR are grouped into 4 Departments, and among them, both the Virology and Entomology Divisions are under the Department of Tropical Medicine. The Immunology Division is under the Department of Clinical Pathology, while the Biotechnology Centre is a common user facility.

In the Division of Immunology, the HLA Laboratory which is involved in the Project, is also shouldering the important responsibility as the National Reference Centre for Tissue Typing.

The organization of the Project in respect to the Divisions concerned is shown in Annex 1 together with counterpart relations.

b. Human resource of expertise:

The officers and technologists involved in the project are permanent staff members of the IMR. Those who have received training are expected to and have imparted their knowledge to others through the in-house training which are being carried out to increase the pool of expertise. Intention to acquire foreign positions is low among IMR officers, and thus the problem of brain drain is not serious. Even though some officers may be transferred to other institutions, the expertise is already well established and other officers can sustain the necessary technology to continue and develop the research projects. The movement of a few technical staff members to other institutions did not affect the research activities in the divisions concerned, and should be viewed positively as enhancing the technical base of other health sectors in this country.

2 Financial perspectives

a. Funding resources:

Funds for continuation of the technology/research are more than adequate through IMR operating funds as well as through Intensification of Research in Priority Areas (IRPA), a kind of R & D Funds from the Ministry of Science, Technology and Environment, Malaysia (MOST&E), and international agencies like WHO and TDR.

b. The amount of financial support secured:

The operating budget of the IMR is expected to be in excess of RM20 million per year. Yearly R & D funds from MOST&E are expected to be in excess of RM 4 million per year. Actually, in retrospect, each division under the Project received RM 200,000 (US$ 80,000) yearly or more in recent years.

3 Technology perspectives

a. Human resources secured:

It is felt that sufficient human resources have been secured especially in those areas of biotechnology connected with the IMR-JICA Project. The quality of human resources will continue to be enhanced as further biotechnological based research projects are conducted in each division. However, we are lacking in other specialized areas of biotechnology, *e.g.* those pertaining to cancer and toxicology.

b. Continuation of training:

The IMR members in Project have been concentrating their efforts in acquiring techniques and in research implementation, and therefore could not afford to plan institutional courses while the Project is running. However, they are planning to conduct more in-service courses in each division, and short practical institutional courses in various biotechnology techniques for IMR officers. This will be in addition to *ad hoc* training requested from individual officers as when the need arises. Some IMR officers will also be selected to attend the Third Country Training Programme (JICA) as participants or observers.

VII. RECOMMENDATIONS

Resulting from the joint evaluation and discussions, both sides reached the following conclusions;

The goal and objectives of most activities in the Project as stipulated in the Record of Discussions, the Tentative Schedule of Implementation signed on 22 June 1992 and the Minutes of Discussions with the Planning and Consultation Team signed on 19 November 1993 have been realized. It was also concluded that in addition to the significant scientific accomplishments achieved through the Project, strong linkages were established between Malaysian and Japanese scientists. Furthermore, the person to person contact, sincere efforts of Japanese experts in transferring technology, as well as their work ethics and attitude towards science, impressed and strengthened the research culture of Malaysian counterparts. This was another important impact from the Project which was recognized by Malaysian leaders, although it was not planned or expected in the Project.

The period of cooperation for 2 and a half years up to now is well evaluated and found to have been successfully accomplished. Both sides agreed to the following recommendations;

1. The current excellent efforts put into the Project by both sides be continued and enhanced to complete the remaining portion of the collaborative research subjects in IMR.

2. The linkages and collaboration forged between scientists and institutions in Japan and Malaysia should be maintained and strengthened even after the end of the Project. In this respect the IMR could serve as the focal point for such exchange and collaboration.

3. It is desired that the scientific technical expertise established in IMR through the Project be further developed and extended to other appropriate projects.

4. Continued efforts be made by IMR in partnership with Japan to disseminate the medical biotechnology expertise that it has acquired, to the neighbouring countries in Southeast Asia and Pacific islands and countries in the other regions.

5. A list of publications arising from the Project in the previous year should be submitted to JICA Malaysia Office by the end of January every year.

6. It is expected that JICA continues to provide IMR the information on group training courses to be held in Japan so that IMR staff members can avail themselves of these training opportunities.

7. It is recommended that continued contacts be made between IMR and JICA for implementation of the 3rd Country Training Programme and for technical matters.

Special remark: The evaluation team takes note that IMR has strongly requested that Japanese experts be dispatched even after the Project ends, so as to strengthen the biotechnological approaches to specified fields. The team will convey this request to Tokyo.

LIST OF ANNEXES

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ANNEX Ia

LIST OF MALAYSIAN AND JAPANESE COUNTERPARTS

Field of Research	Japanese Experts	Leader	Malaysian Counterparts
Malaria			
1. DNA Probes	Dr. T. Furuta(L) (IMSUT) Dr. Y. Watanabe (L) (U.Tokyo)	Head, Biotechnology Centre	Dr. Normaznah/Ms. Noor Rain Dr. Lokman/Dr. A. Khoo
2. Mitochondrial Gene	Dr. K. Kita/ Dr. T.Furuta (IMSUT)/ Dr. Y. Watanabe (L)(U. Tokyo)		Dr. Patricia/Ms. Tan/
3. HLA and malaria resistance	Dr. K. Hirayama (Saitama MS.)	and Head, Div. of Immunology	Mr. Zaidi/Mr. Ong Kian Joo
4. Vector studies of molecular level	Dr. T. Tadano (L)/ Dr. A. Mori (L) (Nagasaki Univ.)	Head, Medical Entomology	Ms. Rohani/Mr. Chan Ms. Nazni
Dengue/JE			
5. Improvement of diagnostic methods including PCR	Prof. A. Igarashi (Nagasaki U.)	Head, Virology	Dr. Vijayamalar/Dr. Zainah/ Ms. Halimah/Mr. Apandi Yusof
6. Epidemiology and transmission dynamics of vectors	Dr. K. Oda (L) Prof. A. Igarashi (Nagasaki U.)		Dr. Vijayamalar/Ms. Choo CH/ Mr. Victor Chew Dr. Indra
7. Genome analysis for typing and Pathogenicity	Dr. K. Morita (Nagasaki Univ.) Dr. H. Tsuchie (Osaka Univ.) Dr. N. Kobayashi (Yokohama City)		Dr. Zainah/Mr. Ravindran

(L) Long term experts, (IMSUT) Institute of Medical Science, the University of Tokyo, (MS) Medical School

ANNEX Ib

LIST OF IMR MEMBERS IN IMR-JICA PROJECT

Biotechnology Centre

Head of Division	Dr. Mak Joon Wah
Officers:	Dr. Normaznah Yahaya
	Dr. Lokman Hakim Sulaiman
	Dr. Alan Khoo Soo Beng
	Dr. Patricia Lim Kim Chooi
	Ms. Noor Rain Abdullah
	Ms. Latifah Ibrahim
	Mr. Abdul Halim Abdullah
Medical Laboratory Technologists:	
	Ms. Tan Sew Kian
	Mr. Mohd Zamri Mohd Rose
	Ms. Saniah Kaspan
	Ms. Radzan Taib
	Mr. Mastuki Yunan
	Mr. Sajiri Mohd Saleh

Division of Virology

Head of Division	Dr. Sinniah Mangalam
Officers:	Dr. Vijayamalar Balasubramanian
	Dr. Zainah Saat
	Mr. Ravindran Thayan
	Ms. Halimah Mohamed
	Mr. Mohd. Apandi Yusuf
Medical Laboratory Technologists:	
	Ms. Choo Choon Hong
	Mr. Victor Chew Tong Kheong
	Ms. Teh Leok Kuen
	Ms. Aziah Ibrahim

Division of Medical Entomology

Head of Division	Mr. Lee Han Lim
Officer:	Dr. Indra Vythilingam
	Ms. Rohani binti Ahmad
	Ms. Nazni Wasi Ahmad
Medical Laboratory Technologists:	
	Mr. Chan Seng Thim

Division of Immunology (National HLA Research Centre)

Head of Division	Dr. Nasuruddin bin Hj Abdullah
Officer:	Dr. Shahnaz bt. Murad

Medical Laboratory Technologists:

Mr. Ong Kian Joo
Mr. Mohd Zaidi bin Abu Samah
Ms. Maiselamah bt. Lisut

ANNEX II.

LIST OF JAPANESE EXPERTS DISPATCHED BY JICA

(Long term)

1. Dr. Hiroshi Tanaka	13 Jan. 1993 - 22 Dec. 1995	(Chief advisor)
2. Ms. Izumi Ohta	13 Jan. 1993 - 22 Dec. 1995	(Coordinator)
Parasitology		
3. Dr. Takahisa Furuta	24 Feb. 1993 - 23 Feb. 1994	
4. Dr. Yoichi Watanabe	10 Nov. 1994 - 9 Nov. 1995	
Entomology		
5. Dr. Takeo Tadano	12 May 1993 - 11 Nov. 1995	
6. Dr. Akio Mori	16 Jun. 1993 - 15 Nov. 1995	
Virology		
7. Dr. Kazumasa Oda	27 Jan. 1993 - 26 Nov. 1995	

(Short term)

Parasitology	
1. Dr. Kiyoshi Kita	3 Mar. 1993 - 20 Mar. 1993
2. Dr. Kiyoshi Kita	18 Oct. 1993 - 2 Nov. 1993
3. Dr. Kiyoshi Kita	11 May 1994 - 26 May 1994
4. Dr. Takashi Aoki	8 Aug. 1994 - 28 Aug. 1994
5. Dr. Kiyoshi Kita	10 Nov. 1994 - 25 Nov. 1994
6. Dr. Kiyoshi Kita	14 May 1995 - 25 May 1995
Virology	
7. Dr. Akira Igarashi	27 Jan. 1993 - 21 Feb. 1993
8. Dr. Koichi Morita	14 Apr. 1993 - 12 May 1993
9. Dr. Hideaki Tsuchie	17 Jul. 1993 - 11 Sep. 1993

10. Dr. Akira Igarashi	6 Feb. 1994 -	27 Feb. 1994
11. Dr. Hideaki Tsuchie	24 Aug. 1994 -	19 Sep. 1994
12. Dr. Koichi Morita	19 Sep. 1994 -	14 Oct. 1994
13. Dr. Futoshi Hasebe	9 Nov. 1994 -	7 Dec. 1994
14. Dr. Akira Igarashi	1 Feb. 1995 -	22 Feb. 1995
15. Dr. Nobuyoshi Kobayashi	1 Mar. 1995 -	31 Oct. 1995

Biotechnology

16. Mr. Tatsuya Akaza	28 Jun. 1993 -	3 Jul. 1993
17. Dr. Katsushi Tokunaga	28 Jun. 1993 -	3 Jul. 1993
18. Dr. Kenji Hirayama	31 Jul. 1994 -	18 Aug. 1994
19. Dr. Kenji Hirayama	19 Jun. 1995 -	5 Jul. 1995

ANNEX III.

LIST OF FELLOWSHIP

Parasitology

1.	Mr. Ng. Chong Sing	17 Nov. 1992 - 22 Feb. 1993
2.	Ms. Noor Rain Abdullah	2 Jun. 1994 - 31 Aug. 1994

Virology

3.	Mr. Chew Tong Kheong, Victor	2 Dec. 1992 - 1 Mar. 1993
4.	Mr. Ravindran Thayan	10 Jan. 1994 - 29 Mar. 1994
5.	Ms. Halimah Mohamed	12 Sep. 1994 - 12 Nov. 1994

Entomology

6.	Dr. Indra Vythilingam	20 Sep. 1993 - 21 Dec. 1993
7.	Mr. Lee Han Lim (Junior Administrator)	25 Sep. 1994 - 8 Oct. 1994

Immunology

8.	Mr. Mohd Zaidi Abu Samah	12 Sep. 1994 - 3 Dec. 1994

ANNEX IV

LIST OF MAIN MACHINERY AND EQUIPMENT PROVIDED

Budget for Japanese Fiscal Year	Items of Main Equipment	Amount in Japanese Yen (JY) and US $
1991	Ultrafiltration System Ice Making Machine Refrigerated Centrifuges HPLC Waters System DNA Sequencing System with GS Automated Gel Loading System and GS Gene Reader Safety Cabinet Class 2	JY 39,998,000 (US$ 325,000)
1992	PCR Thermal Cyclers SpeedVac Centrifuge Hybridization Oven Pulse Field Electrophoresis Holten Laminair Clean Bench UV Cross Linker Submarine Gel System, Minicell Autoclave top loading Table-top centrifuges Peptide Synthesizer Ecosyn P300 ELISA Reader Micropipettors	JY 36,448,000 (US$ 313,250)
1993	DNA auto-sequencer with fluorescence, ABI Refrigerated centrifuges Deep freezers Electronic balances UV Spectrophotometer pH meter UV Transilluminators Electrophoresis systems	JY 30,277,000 (US$ 280,343)
1994a	Phast system Class 2c Safety Cabinet DNA Analysis system, DNASIS in Macintosh Speedvac Inverted microscope Genome site analysis system, with MAPMAKER and Laserprinter Hybridization oven Phase Contrast Microscope Stereomicroscope Electronic balance	JY 29,998,000 (US4 294,092

1994b	Thermal cycler, PE 9600	JY 24,898,000
	In situ PCR/ hybridization	(US$ 248,980)
	DNA Analysing System, GENETYX and Sequence Navigator in Macintosh 575 with a Laser writer	
	Biological stirrers with spinner bottle cell culture system	
	Thermomixer	
	ELISA reader	
	ELISA plate washer	
	Data plate, 5 unit hot plate stirrer	
	Modular dry bath for 1.5 and 2 ml tubes	

ANNEX V

ACHIEVEMENT OF RESEARCH SUBJECTS

1. Development and use of DNA probes for malaria diagnosis

1.1 Technology Development:

This was initiated through an intensive course in "Basic Molecular Biology" conducted by Japanese experts over a total period of 9 weeks for the Division Head and all officers in the Biotechnology Centre. This was then followed by the planning and carrying out of research activities on the use of DNA probes for malaria diagnosis.

For the development and use of DNA probes for malaria diagnosis, about 40 blood samples were obtained from patients at the Orang Asli Hospital in Gombak, Selangor and Kuala Lumpur Hospital. About 500 samples were also collected during a field survey in an Orang Asli Settlement, Betau, Pahang. In addition, *Plasmodium falciparum* isolates maintained at the Centre were used.

1.2 Restriction enzyme digestion of genomic DNA of *P. falciparum* isolates:

The restriction enzyme patterns of genomic DNA from 32 *P. falciparum* isolates with 30 restriction enzymes did not show any remarkable difference among the isolates. Further restriction analysis of the PCR products of the genomic DNA of the isolates also did not show any difference among the isolates. A similar analysis on 5 out of 30 fresh *P. falciparum* isolates from malaria positive patients also did not show any differences.

PCR amplification with *P. falciparum* specific primers, followed by restriction enzyme analysis of the PCR products, using 30 different enzymes, on a simian malaria *P. inui*, and a rodent malaria *P. yoelii*, showed that the restriction pattern of *P. inui* PCR product using restriction enzyme BST-NI was entirely different from that of *P. falciparum*. Since the asexual forms of *P. inui* and *P. falciparum* look fairly similar, restriction analysis can be used for species differentiation between the two parasites.

1.3 Polymerase chain reaction based techniques for malaria diagnosis:

a). Nested PCR: The nested PCR using primers based on the published sequence of the 18S rRNA of *P. falciparum* could detect all microscopically confirmed *P. falciparum* and was also positive in 33.3% of *P. vivax* monoinfections (diagnosed by microscopy). Six out of 43 microscopically negative samples were shown to be positive. Of these 6, one was found to be positive on microscopy on reexamination. Nested PCR using the same primers did not amplify DNA from other *Plasmodium* spp. (*P. vivax, P. malariae, P. yoelii, P. inui, P. cynomolgi*). The high sensitivity of this technique could be due to the second amplification step. The fragments of the PCR products from the microscopy negative specimens were cloned into the vector (pUC18) for sequencing and compared with *P. falciparum* sequence to determine the specificity of the technique.

b). Enzyme detection - Polymerase chain reaction (ED-PCR): This system was optimized using the specimens collected from Gombak Hospital, and was the used to analyse a total of 100 field collected specimens (12

positive and 88 negative for malaria by microscopy). However, the results obtained were not satisfactory since there was no significant difference in absorbance between the positive and the negative specimens.

c). Plate hybridization - Polymerase chain reaction (PH-PCR): This technique utilized microtitre plates with immobilized specific *P. falciparum* and *P. vivax* probes for the detection of PCR amplified malaria DNA, using biotinylated specific primers. The sensitivity and specificity using the *P. falciparum* probe were 93.3% and 100%, respectively, while using the *P. vivax* probe these were 100% and 100% respectively.

d). Reverse transcriptase-polymerase chain reaction (RT-PCR): This technique was applied on 5 *in vitro* cultured isolates as well as specimens from *P. falciparum* infected patients. Selected PCR products obtained in the experiments were sequenced and the sequences compared with those of *P. falciparum* to determine the specificity of the technique.

1.4 Molecular marker of drug resistance in *P. falciparum*:

Our earlier studies found polymorphism of the *Nsp*I restriction site (nucleotide 754) of the pfmdr1 gene. The study was carried out by polymerase chain reaction - restriction enzyme digestion of the *pfmdr*1 gene. We could not find any simple association between the particular *pfmdr*1 mutation and resistance to mefloquine, quinine or halofantrine. Our earlier study showed no simple association between the polymorphism and chloroquine sensitivity either.

1.5 Application of molecular techniques to other research fields:

1.5.1 Breast cancer study: PCR-microsatellite length polymorphism was carried out on microsatellite *D17S579* which is located on chromosome 17q2.1 (i.e. linked to the *BRCA1* gene). The PCR products were run on denaturing polyacrylamide gel and detected using the chemiluminescent detection system.

1.5.2 Sequencing of the p53 tumor suppressor gene: The p53 gene is an important tumor suppressor gene involved in many tumors. The site and type of mutation are thought to be associated with the possible aetiological factors. Inherited mutation of this gene caused inherited susceptibility to cancer in the Li-Fraumeni syndrome. Therefore, sequencing of the p53 gene would give important information to us. We are attempting to sequence the p53 gene of the DNA from certain individuals referred to us.

Achievement: The implementation of this activity was completely achieved. The techniques learned in the project have been applied not only to other research areas in malaria but also to molecular studies of cancer.

2. Analysis of mitochondrial genes - together with its characterization

2.1 Mitochondrial DNA of malaria parasites:

Detection of mitochondrial DNA of *Plasmodium* parasites was carried out using polymerase chain reaction with three primer sets specific for *P. falciparum* mitochondrial genes, namely, P101/102 (Cytochrome oxidase III), P103/104 (Cytochrome oxidase I) and P105/106 (Cytochrome b). PCR amplification was successfully carried out on 26 *P. falciparum* isolates using these primers. The primer set P103/104 of COI mitochondrial gene is considered a good candidate primer for the diagnosis of *P. falciparum* infection by PCR technique. The same sets of primers and PCR technique were also performed on 7 isolates of *P. vivax*. All the 7 isolates gave positive results with primers 101/102 and /103/104 while only 2 out of 7 isolates were positive with primers /105/106.

2.2 Studies on mitochondrial DNA of *Plasmodium vivax*:

The partial nucleotide sequence of *P. vivax* COIII gene encoded on the mitochondrial DNA (373 bp fragment) was determined after the successful amplification of DNA using primers designed from the sequence of *P. falciparum*. Alignment of the nucleotides showed 88% nucleotide homology and 91% deduced amino acid homology when compared to *P. falciparum* sequence data. This is the first report on the sequence of *P. vivax* mitochondrial COIII gene.

A part of *P. vivax* COI gene was isolated using PCR with primers designed from *P. falciparum* COI gene and sequencing was performed on the PCR product (432 bp fragment). Partial nucleotide sequence obtained (335 bp) revealed 86% nucleotide homology and 91% deduced amino acid homology when compared to *P. falciparum* COI gene. Amino acid sequence comparison between *P. vivax*, *P. falciparum*, *Saccharomyces cerevisiae* and *Homo sapiens* showed that a higher homology was observed for the first portion of the sequence (about 40 amino acids) indicating the functional importance of this region in the electron transport chain. This is also the first report on *P. vivax* COI gene.

Four new primers (P1101, P1102, P1103 and P1104) for the COI and COIII genes were designed and used in further studies. PCR on one isolate of *P. vivax* (PV13) revealed that the sizes of the PCR products with primer sets P1102-P1104 and P1102-P1103 were 2689 bp and 879 bp, respectively. Restriction enzyme analysis on the 879 bp fragment using 17 enzymes revealed the presence of two restriction sites, one for Hpa I and another for Pst 1. The 879 bp fragment was cloned for sequencing and data are being analysed. The 2689 bp fragment will also be cloned and sequenced.

Achievement: The implementation of this activity was mostly achieved except sequencing of the whole gene of COIII and COI which will be completed by the end of this project.

3. Identification and characterization of protective epitopes of malaria antigens - Association of human leucocyte antigen (HLA) and resistant to falciparum malaria.

The main objective is to study the relationship if any, between HLA and resistance to *P. falciparum* infection.

Preliminary training of staff at the HLA laboratory in the Division of Immunology, in molecular techniques of HLA typing, including RFLP for class II, SSOP for class I and SSCP for class I and II HLA DNA typing was carried out.

Blood specimens collected from 169 Orang Asli living in various malaria endemic areas were examined by PCR and hybridization methods. In these areas, although parasitaemia can be as high as 20-30%, severe clinical malaria is seldom seen. Typing of 62 unrelated healthy individuals in this group for their polymorphism in the HLA-B gene was carried out to search for another HLA-B53-like gene known to be associated with resistance to severe malaria among the west African population in the Gambia. One specific pattern that corresponded to B75.2 with Bw4 motif instead of Bw6, was found to be very common. This B75 with Bw4 epitope, is also known as B77. We have tentatively called this Malaysian B77-like DNA type as ECB-77 (epitope combination HLA-B77). The ECB-77 was found in 44% of the Temuan tribe and 25% in the Termiar and Semai tribes, compared to 2.5%, 0.4%, and 0.7% in Zairen, Thais and Javanese, respectively. In addition, no HLA-B53 was found in this population. Using SSCP method, it is strongly suggested that the Malaysian ECB-77 is the same type as HLA-B77. Amino acid sequence analysis of the antigen binding B and F (C-terminus) pockets, showed that the F pocket of ECB-77 looked completely similar with that of HLA-B53. As HLA-B53 has been shown to be effective in generating anti-malarial cytotoxic T lymphocytes due to high affinity to some major malaria antigenic peptide derived from the liver stage of the parasite, a similar situation may exist with ECB-77 as it shares a similar C-terminal peptide binding pocket (pocket F).

Achievement: This activity has been extremely successful and the item was completely achieved. It has not only strengthened the capability of HLA laboratory but also completed the planned objective of this item.

4. Better understanding of the vectorial status of malaria vectors at the molecular level - Determination of malaria susceptible locus on the chromosome of *Anopheles maculatus*

4.1 Biotechnology:

A few *Plasmodium falciparum* clones producing gametocyte frequently and at high ratio were selected from the isolates from local patients. These were *in vitro* cultured and used for selecting *Anopheles maculatus* strains susceptible to malaria. Efforts to establish stable and appropriate clones are continuing with the cooperation of the Biotechnology Centre. Selection of susceptible strains of *An. maculatus* is continuing by feeding *Anopheles* with cultured *P. falciparum* with the cooperation of the Biotechnology Centre. Until now, no susceptible strain has been found

among breeding *Anopheles* colonies, and no colonization has yet been performed. To construct a RFLP map of *An. maculatus* chromosomes, we are currently screening the cDNA clones at hand. Some of these cDNA probes made from *Aedes aegypti* (Liverpool strain) were confirmed to be useful as probes for Southern blots of genomic DNA extracted from different *An. maculatus* strains. Standardized technical procedures for Southern transfer of genomic mosquito DNA, and those for the hybridization between *An. maculatus* genomic DNA and cDNA probes from *Ae. aegypti* were established successfully in the Division. The chemiluminescence technique used in hybridization had a lower sensitivity compared to that using a radioactive marker. Therefore, to increase the hybridization sensitivity, a cDNA originating from *An. maculatus* RNA library is being constructed. We will employ two methods to process RFLP data for the identification of the malaria susceptibility loci on *An. maculatus* chromosomes. Multiplot linkage analysis and quantitative trait loci (QTL) mapping will be conducted using the MAPMAKER/QTL computer package, which has already been installed in the laboratory. The data will be also analysed using orthodox Mendelian method.

4.2 Genetics:

Out of 3 peninsular Malaysian strains and 1 Sabah strain of *An. maculatus* tested for isozyme variants, the Jeram Kedah (JK) and Kuala Milot (KM) have been selected for the fast allele (F) and slow allele (S), respectively, for 3 generations in order to purify each of the two alleles (F and S). In addition to the starch method for electrophoresis which has been employed in this Division, the method using agar gels was adapted for electrophoresis of *An. maculatus*. About 10 enzymes so far can be electrophoresed by this agar method. Three strains of *An. maculatus* are now being studied for new isozymic alleles by the modified agar method. Freshly field-collected strains have been utilized for crossing experiments to produce the heterozygotes for the above loci. So far about 85 single pairings have been performed using the JK strain as a standard strain. However, no heterozygotes at the loci concerned have been detected at the F1 hybrids between the freshly-collected and the standard strains.

Achievement: This project is the first attempt to elucidate the mechanism of malaria susceptibility in Malaysian *An. maculatus*, and experiments towards the objective are still on the way.

5. Strengthening of diagnostic capability using molecular biology and biotechnology in dengue and Japenese encephalitis

5.1 Reverse transcriptase-polymerase chain reaction (RT-PCR)

The initial evaluation of direct RT-PCR for dengue diagnosis using 20 samples showed that it was as sensitive as conventional RT-PCR. In subsequent comparison with virus isolation, sensitivity and specificity were 100%.

The rapid RT-PCR method for use in diagnosis of JE from cerebrospinal fluid (CSF) was optimised. A total of 89 CSF samples from 85 patients presenting with meningo-encephalitis and were negative for JE IgM, had been

collected and stored in 1993. Seventy five samples have been tested by direct RT-PCR using JE primers and all were negative by this technique.

5.2 Evaluation of RT-PCR compared to IgM capture ELISA

A total of 160 samples from patients with the clinical diagnosis of dengue fever were examined. Of these, 9 were positive for both PCR and ELISA-IgM, 61 were positive for ELISA-IgM only and 31 for PCR only. Both techniques gave negative results in the remaining 59 samples. By chi square test, the sensitivity of both methods is significantly different ($P<0.5\%$). It is concluded that, PCR can be performed in the central or reference laboratories as a complement to ELISA-IgM test for clinically suspected cases at early stage of infection.

As the follow-up of the above study, a total of 121 serum samples from patients suspected of having dengue with relevant clinical manifestations, but negative for dengue IgM, was put through the direct RT-PCR technique. Fourteen (14) patients were found to be positive for dengue virus infections by this technique.

5.3 Improvement of IgM capture ELISA

Comparative assay was made on dengue IgM-capture ELISA with reagents from 2 sources in which a total of 237 convalescent sera were tested by the modified IgM-capture ELISA with reagents from 2 sources. The two sets of reagents were Dengue 2 antigen raised in suckling mouse brain at IMR (SMA) and a monoclonal antibody (MCA) from CDC, Fort Collins (MCA 3H5-1-21-B71541); and a dengue antigen raised in tissue culture (TCA) and a Flavivirus group specific monoclonal antibody (MF4) courtesy of Dr. Jane Cardosa, USM (MF4/5/A5/C3-3.DEN). Both reagents were evaluated against the gold standard, haemagglutination inhibition (HI) test. The sensitivity and specificity obtained using the reagent, SMA + MCA, were 66.2% (90/136) AND 94.2% (147/156) respectively. The results obtained using the alternative reagents, TCA + MF4, are significantly more sensitive than with the reagents, SMA + MCA ($P < 0.02$), although there is no significant difference ($P > 0.10$) in specificity between the two reagents. As a result of this study we now have an option of using these alternative reagents.

Production of reagents for routine IgM-capture ELISA: The studies were conducted on antigen production in cell culture using D1, D2, D3 an D4 dengue viruses in clone C6/36 *Aedes albopictus* cell line. Four types of dengue virus were incubated at different temperatures, 28℃, 32℃ and 37℃. Antigen titres were examined by IgM-capture ELISA, haemagglutination (HA) and plaque forming unit (PFU). It was found generally that antigen production was better at higher temperature. D2 and D4 antigens reached very high titre after day 3 but D1 and D3 did so only after day 5. D2 favours temperature of 37℃ but D1 favours temperature of 32℃.

Achievement: Overall the study has been successful especially in the setting up of the rapid RT-PCR as an additional test for dengue diagnosis. The production of appropriate reagents for the IgM-capture ELISA is still on the way.

6. Epidemiological and entomological studies on dengue and Japanese encephalitis

6.1 Isolation of dengue virus from patients:

Virus isolation was carried on 150 acute sera from clinical dengue cases, and was positive in 21.3% (32/150). Eighteen of these have been typed to date by PCR as dengue 3.

6.2 Detection of JE antibodies in animal sera:

As part of the epidemiological studies on Japanese encephalitis, a total of 2,152 samples have been examined. Serum samples showing positive haemagglutination inhibition (HI), reactive at serum dilution of 1:10 or more, were defined as /positive.

The positive rates were 88.1% (156/177) in pigs, 40.0% (156/347) in buffaloes, 41.5% (237/576) in cattle, 17.9% (88/492) in sheep, 14.0% (63/449) in goats, and 0.9% (1/111) in birds. This study shows that JE is distributed in many areas in Malaysia and that the swine population is still the largest reservoir of JE virus followed by cattle.

6.3 Year round surveillance of JE activity in mosquitoes:

Virus isolation for JE was performed on 223 pools of mosquitoes collected in the Sungei Pelek area, Selangor during the period from September 1993 to October 1994 and on 36 pools collected in Sarawak in October 1994. In Sg. Pelek, one and two positive isolates were recovered from *Cx. gelidus* and *Cx. tritaeniorhynchus*, respectively. JE isolates from *Cx. gelidus* has been confirmed by PCR.

Before this Project started, the known vectors of JE in Malaysia were 4, *i.e., Cx. tritaeniorhynchus, Cx. gelidus, Cx. vishnui* and *Cx. pseudo-vishnui*. Isolation of JE virus was carried out by tissue culture method using *Aedes albopictus* clone C6/36 cells, from various species of unfed female mosquitoes at different localities, in collaboration with Division of Medical Entomology, IMR. JE virus was detected from *Cx. sitiens, Cx. bitaeniorhynchus, Cx. quinquefasciatus, Cx. fuscocephala, Aedes butleri, Ae. cancraedes and Ae. linneatopennis*, and these species are now considered as potential vectors of JE in Malaysia.

6.4 Year round surveillance of JE activity in swine in the Klang Valley:

Detection of IgM antibody against JE virus was carried out using IgM capture ELISA on swine sera collected on a monthly basis in the Klang Valley. IgM antibody positive rates were 26.1% in March, 36.7% in April, 25.0% in May, 9.5% in June, 5.0% in July, 25.0% in September and 14.3% in November, 1994. All sera from 4 month old swines also indicated the presence of neutralizing antibodies at 100% in each month from March to November.

Achievement: Overall this project has been successful and completely achieved.

7. Understanding of pathogenesis of dengue hemorrhagic fever at the molecular level - Genome analysis for typing of dengue and JE viruses, and for pathogenicity of dengue.

7.1 Genome analysis of JE isolated from mosquitoes:

Ten isolates of Japanese encephalitis virus strains isolated from mosquitoes in 1992 in Peninsular Malaysia were studied. In these isolates, 240 nucleotides from the pre-M gene region were sequenced. The results were compared with the other JE virus strains from different geographic areas in Asia. These strains studied belong to the largest genotypic group of northern Asia that includes strains isolated in temperate regions such as Japan, China and Taiwan. They differed in 32 nucleotides (13.3%) from WTP/70/22 strain isolated from Malaysia in 1970 that belonged to another distinct genotypic group.

7.2 Comparative genomic sequencing of dengue viral isolates to study pathogenesis:

Nucleotide sequencing was carried out on the envelope/ nonstructural 1 junction (E/NS1; 280 nucleotides) of 5 dengue virus isolated directly from the serum of a dengue patient and also from succeeding tissue cultures. This was intended to see if any mutation occurred after 3 passages, which are the normal number of passages to confirm the presence of dengue virus. No mutations were detected in the sequenced region. The result has shown that tissue culture isolates can be used as the source of virus for sequencing studies.

Subsequently, sequencing was conducted for the pathogenesis study where the same region (E/NS 1 junction) was chosen for 5 dengue isolates. Three of these isolates were from patients with dengue fever and 2 from patients with dengue haemorrhagic fever (DHF). The sequencing results showed almost total homology among all 5 isolates. They differ in 13 nucleotides (4.6%) from the H87, a dengue 3 isolate from the Philippines.

Currently sequencing is being continued from the 5'-end of the dengue viral genome, for a sequence of 700 nucleotides between the 5'- Non-coding region and the pre-membrane region.

Achievement: Genome analysis for typing JE and dengue have been well achieved. The analysis for pathogenicity of dengue is on the way.

ANNEX VI.

OUTCOMES FROM THE PROJECT

INTERNATIONAL REGISTRATION

Lim PKC, Kita K, Tan SK, Furuta T, Aoki T, Watanabe Y and Mak JW (26 Jan. 1995) Partial DNA sequence of 347 bp at cytochrome c oxidase subunit III gene of *Plasmodium vivax*. registered to DDBJ (DNA Data Bank, Japan) by E mail from IMR under accession no. D45369 for data bases, GSDB, DDBJ, EMBL and NCBI.

PUBLICATIONS

Published

Chew TK, Vijayamalar B, Sinniah M, Igarashi A (1993). Pre-treatment of test sera with three kinds of IgM-adsorbents did not improve sensitivity in the indirect ELISA to detect anti-dengue IgM antibodies. *Tropical Medicine* 35: 65 - 74

Chew TK, Vijayamalar B, Sinniah M, Igarashi A (1993) Comparative assay on anti-dengue IgM antibodies by the indirect ELISA and IgM-capture ELISA. *Tropical Medicine* 35: 75 - 81

Vythilingam I, Oda K, Tsuchie H, Mahadevan S, Vijayamalar B (1994) Research Note: Isolation of Japanese encephalitis virus from Culex sitiens in Selangor, Malaysia. *J. Amer. Mosq. Cont. Assoc.* 10: 228-229

Tsuchie H, Oda K, Vythilingam I, Thayan R, Vijayamalar B, Sinniah M, Hossain MM, Kurimura T, Igarashi A. (1994) Genetic study of Japanese encephalitis viruses isolated in Malaysia. *Jpn. J. Med. Sci. Biol.* 47:101-107

Vythilingam I, Morita K and Igarashi A. (1994) Nucleotide and amino acid sequence in the Pre M Region of a Japanese encephalitis virus strain isolated from a pool of *Aedes albopictus* and *Ae. butleri* mosquitoes captured in Peninsula Malaysia in 1992. *Tropical Medicine*. 36: 51-56

Zainah S, Thayan R, Vijayamalar B, Sinniah M and Igarashi A.(1994) Use of PCR for diagnosis of virus infection compared to ELISA IgM. *Tropical Medicine* 36 (3): 75-81

Lim PKC, Kita K. Tan SK, Furuta T, Kojima S, Aoki T, Watanabe Y and Mak JW (1995) Mitochodrial cytochrome oxidase III (COIII) gene of *Plasmodium vivax*. *Jpn. J. Parasitol.* 44: 40-43.

Vythilingam I, Oda K, Chew TK, Mahadevan S, Vijayamalar B, Morita K, Tsuchie H and Igarashi A. (1995) Isolation of Japanese encephalitis virus from mosquitoes collected in Sabek Bernam, Selangor, Malaysia in 1992. *J. Amer. Mosq. Cont. Assoc.* 11: 94-98

Submitted

Halimah M, Vijayamalar B, Sinniah M, Igarashi A and Tanaka H.: Comparative assay on dengue IgM capture ELISA with reagents from 2 sources. submitted to *Southeast Asian J. Trop. Med. Publ. Hlth.*

Thayan R., Vijayamalar B, Zainah S, Chew TK, Morita K, Sinniah M, and Igarashi A.: The use of polymerase chain reaction (PCR) as a diagnostic tool of dengue in the Institute for Medical Research. submitted to *Southeast Asian J. Trop. Med. Publ. Hlth.*

Lokman Hakim S, Furuta T, Noor Rain A, Normaznah Y, Mohd Zamri MR, Kojima S, and Mak JW: Diagnosis of malaria infection using plate hybridization method for detection polymerase chain reaction products. submitted to *Trans. Roy. Soc. Trop. Med. & Hyg.*

Noor Rain A, Furuta T, Radzan Taib, Kita K, Kojima S and Mak JW: Development of a new diagnostic method for *Plasmodium falciparum* infection using reverse transcriptase-polymerase chain reaction. Submitted to *Molecul. Biochem. Parasitol.*

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Hirayama K, Mohd Zaidi AS, Lokaman Hakim S, Ong KJ, Kikuchi M, Nasuruddin A and Mak JW: Anti-malaria HLA-B53 in Africa and a common DNA type of HLA-77-like gene in Malaysian aborigines share a C-terminal pocket of peptide binding groove. submitted to *Tissue Antigens*

In Preparation

Thayan R, Morita K, Vijayamalar B, Chew TK, Sinniah M and Igarashi A.: Comparison of two different methods for purification of PCR products used in direct sequencing and its application in the preliminary study of nucleotide and amino acid variation of dengue 3 virus isolated in Malaysia.

Thayan R, Vijayamalar B, Zainah S, Sinniah M and Igarashi A.: Optimizing the concentration of reverse transcriptase (RT) and DNAse inhibitor for the reverse-transcriptase polymerase chain reaction (RT-PCR), both in sensitivity and cost effectiveness.

Vijayamalar B, Zainah S, Halimah M, siiniah M and Igarashi A.: Laboratory diagnosis of Japanese encephalitis in patients with clinical encephalitis.

Khoo A, Furuta T, Noor Rain A, Ng AB, Kojima S and Mak JW : Nested polymerase chain reaction for diagnosis of *Plasmodium falciparum* infection in Malaysia

PRESENTATION AT SCIENTIFIC MEETINGS

Noor Rain, Alan Khoo and Normaznah (1994) The preliminary findings of the reverse-transcriptase-PCR (RT-PCR), Nested PCR and PCR of mitochondrial DNA of malaria parasites. presented at the Fifth Scientific Meeting of the Malaysian Society for Molecular Biology and Biotechnology on 17 - 18 May 1994 in Kuala Lumpur.

Patricia Lim Kim Chooi (1994) Partial characterization of *P. vivax* mitochondrial cytochrome oxidase III gene. presented at the First International Congress of Parasitology and Tropical Medicine held at KL, 25-28 August 1994.

Alan Khoo Soo Beng (1994) *Plasmodium falciparum* multiple drug resistance gene 1 (pfmdr I) mutations in Malaysian isolates. presented at the above meeting.

Noor Rain bt Abdullah (1994) Development of a new diagnostic method for *P. falciparum* infection using reverse transcriptase PCR. presented at the 29th Joint Conference on Parasitic Diseases, organized by the Japan-United States Cooperative Medical Programme, 27-30 July 1994 in Yamagata City, Japan.

Furuta T (1994) Nested polymerase chain reaction for diagnosis of *Plasmodium falciparum* infection. presented at the above meeting.

Indra Vythilingam (1994) Isolation of Japanese Encephalitis virus from mosquitoes collected in Sabak Bernam, Selangor, Malaysia in 1992. presented at the above meeting

Poster presentations at the National Biotechnology Seminar, Penang, Malaysia, 16-18 November 1994.

Khoo A, Furuta T, Noor Rain A, Ng AB, Kojima S, Kita K & Mak JW (1994) Diagnosis of malaria parasitaemia by nested polymerase chain reaction.

Noor Rain A, Furuta T, Mak JW, Yong HS, Kita K, Kojima S and Radzan Taib (1994) Molecular studies on simian malaria parasite, *P. inui*.

Lim PKC, Kita K, Tan SK, Furuta T and Mak JW (1994) Partial sequence of *Plasmodium vivax* cytochrome I (COI) gene.

Vijayamalar B. et al. (1994) The use of rapid RT-PCR on clinical sample.

Zainah S, et al. (1994) Evaluation of the PCR technique for diagnosis of dengue virus infections.

Thayan R, et al. (1994) Comparison of two different methods for purification of PCR products used in direct sequencing, in an Applied Biosystems 373 DNA Sequencing System.

Halimah M. (1994) Comparative assay on dengue Ig-M Capture ELISA with reagents from 2 sources.

INSTITUTE FOR MEDICAL RESEARCH AND JAPAN INTERNATIONAL COOPERATION AGENCY (IMR-JICA) RESEARCH PROJECT ON TROPICAL DISEASES

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FOURTH QUARTERLY REPORT, 1995
- Including Summary for 3 Years - 30 November 1995

Period covered: 1 October - 30 November 1995

EXECUTIVE SUMMARY

The IMR-JICA Research Project on Tropical Diseases, which started in January 1993 and is scheduled to end in December 1995, has the objectives of strengthening the IMR in the application of biotechnology in tropical diseases research. This was successfully achieved through strengthening of facilities and transfer of expertise. Transfer of expertise was through seven collaborative projects in malaria, Japanese encephalitis, dengue and the vectors involved, jointly planned and carried out by 6 long term and 22 short term Japanese consultants together with the IMR counterparts. Biotechnology equipment amounting to US$ 1,538,863 were provided under the Project. Fellowship studying in Japan was given to 10 IMR researchers and another 1 will be sent soon. In the final evaluation made by JICA in June 1995, the Project was evaluated as to be excellent.

Significant achievements include the successful introduction of molecular techniques in diagnosis of malaria by PCR and characterization of drug sensitivity in malaria infection, the important association of HLA-B*1513 or serologically defined HLA-B77 with resistance to severe malaria among Temuan, Termair and Semai tribes, and the description of the partial sequence of the *Plasmodium vivax* cytochrome oxidase III gene (Accession No. D45369 for data bases, GSDB, DDBJ, EMBL and NCBI).

In virus research, molecular techniques for the rapid diagnosis of dengue and Japanese encephalitis infections were introduced. Important findings on the epidemiology of these two diseases in the country were obtained. Diagnostic reagents for these two diseases were produced.

In entomology research, molecular techniques for the detection of dengue and Japanese encephalitis in mosquitoes, and mapping of malaria susceptibility loci in *An. maculatus* chromosomes were introduced. Genetic studies using isoenzymatic characterisation of *An. maculatus* variants were also carried out.

The biotechnological skills acquired are now being applied to other infectious diseases as well as in cancer research. In addition, the IMR is now able to disseminate the skills it has acquired to other researchers in the country and region, through the JICA Third Country Training Programme. The first training course on "International Seminar on Biotechnological Techniques in Tropical Medicine" was successfully conducted with 12 participants and 2 observers from 24 October to 18 November 1995. Subsequent courses will be held in 1996 and 1997.

Since the IMR-JICA project (the Project) will end in December 1995, this final quarterly report will also summarise important progress achieved and implemented during the last 3 years.

1. Objectives of the Project

The main objective of this project is to strengthen the use of biotechnological methods by IMR researchers on diagnosis of selected tropical diseases. The research field involves malaria, medical entomology, dengue and Japanese encephalitis, conducted in 3 divisions and a laboratory, *i.e.*, Biotechnology Centre, Division of Virology, Division of Medical Entomology and HLA laboratory of Division of Immunology. Seven research subjects as listed in Para 8 were covered, their annual plan of activities being determined at the start of Project. It was also expected from the beginning that the outcomes of the Project should have impacts on various levels of organizations inside and outside Malaysia including activities in IMR as the WHO Regional Centre.

2. Framework of technical cooperation

The Technical Cooperation of JICA follows the Colombo Plan Scheme which includes 1) dispatch of experts (A1 Form), 2) acceptance of fellows in Japan (A2-3 Form), 3) provision of necessary equipments (A4 Form) at the project site, where 4) the running cost is borne by the country assisted and 5) disbursement of the Project Budget from JICA for buying minor equipments and consumables.

3. Organization

The Project organization, headed by the Director of IMR, covers 3 divisions, a laboratory and Project Office as shown below.

Project leader; Director, Institute for Medical Research: Dato' Dr M. Jegathesan, succeeded by Dr Ismail Mohd. Noor, since 15 December 1993, and by Dr Mohamad Taha bin Arif since 16 June 1994.
Biotechnology Center: Head, Dr Mak Joon Wah
HLA Laboratory in Division of Immunology: Head, Dr Nasuruddin bin Abdullah
Division of Medical Entomology: Head, Dr Inder Singh, succeeded by Mr Lee Han Lim, since 6 March 1993.
Division of Virology: Acting Head, Dr B. Vijayamalar, replaced by Head, Dr Mangalam Sinniah, since 15 December 1993.
IMR-JICA Project Office: Chief Advisor, Dr Hiroshi Tanaka, Professor Emeritus.

Project Office

At the Project site, the project office is set up where a chief advisor and a coordinator are assigned by JICA to manage the Project implementation. The main objectives of the project

office are to provide administrative, technical and financial advice to Japanese experts and IMR members involved in the Project, and to coordinate the relevant technical and administrative matters.

Equipments to be provided by JICA based on A4 form by the request from the divisions were listed up, quotations were collected, models of the equipment were selected by IMR and Japanese members. Selected equipments were purchased and paid by the coordinator in the Project Office.

As stated above, although running costs are shouldered by IMR in principle, minor equipments, chemicals, enzymes and other consumables were purchased, and expenses of duty travels were disbursed by the Project Budget funded by JICA. In the past years, annual expenditures of both sides were nearly equal amounting between RM 150,000 to 200,000 (US$ 60,000 to US$ 80,000) annually.

TAC, Tokyo

Besides principal and financial management of JICA/HQ, Tokyo Technical Advisory Committee (TAC, Tokyo) was established to give technical advice to the Project. Advice on research planning, selection of Japanese experts, information of major equipments and other matters in the Project were given by TAC, Tokyo stationed at the Division of International Health, Faculty of Medicine, the University of Tokyo. TAC is chaired by Prof. Kiyoshi Kurokawa of Dept. of Internal Medicine, the University of Tokyo, and is composed of professors in the University of Tokyo and Nagasaki University, and an official of Department of Medical Cooperation, JICA/HQ in charge of this project. The Secretary is Ass. Prof. S. Fukuhara.

4. Results of Final Evaluation, JICA

Since this is the last year of the 3 year period, the final evaluation of the Project was performed from 17 (Sat.) to 24 (Sat.) June 1995 by 7 evaluators from JICA headed by Prof. T. Kurokawa of the University of Tokyo. They reviewed the Joint Evaluation Report prepared by the Project Office assisted by the Division Heads, IMR, and interviewed IMR Division Heads and other members, and Japanese experts. They studied the progress of Project by referring to such important points as achievement of the 7 research subjects, various aspects of the impact of Project, and sustainability of the activities. Based on the above, the Project was evaluated on the whole, as being excellent.

5. Japanese Consultants and IMR Counterparts (Experts defined in A1 Form of Colombo Plan)

5.1. Out of 6 Japanese long term consultants assigned in 1993, one has completed the assignment on 23 February 1994, and Dr. Watanabe was newly assigned from 10 November 1994 for 13 months. Therefore, the number of long term consultants in IMR was 5 or 6 in 3 years.

Long term experts during 1995 (consultant staying for 1 year

or longer)

Biotechnology Centre		
Dr. Yoichi Watanabe	10 Nov. 1994 -	9 Dec. 1995
Division of Medical Entomology		
Dr. Takeo Tadano	12 May 1993 -	11 Nov. 1995
Dr. Akio Mori	16 June 1993 -	15 Nov. 1995
Division of Virology		
Dr. Kazumasa Oda	27 Jan. 1993 -	26 Nov. 1995
Project Office		
Dr. Hiroshi Tanaka Chief Advisor	13 Jan. 1993 -	22 Dec. 1995
Ms. Izumi Ota Coordinator	13 Jan. 1993 -	30 Nov. 1995

5.2. Short term experts

The short term consultants dispatched this year were the following 7, and the total number dispatched in 3 years was 22.

Biotechnology Centre		
Dr. Kiyoshi Kita	14 May 1995 -	25 May 1995
Dr. Kiyoshi Kita	1 Sep. 1995 -	7 Sep. 1995
HLA Laboratory.		
Dr. Kenji Hirayama	19 Jun. 1995 -	5 Jul. 1995
Division of Virology		
Dr. Akira Igarashi	1 Feb. 1995 -	22 Feb. 1995
Dr. Nobuyoshi Kobayashi	1 Mar. 1995 -	15 Nov. 1995
Dr. Hideaki Tsuchie	15 Aug. 1995 -	25 Aug. 1995
Dr. Akira Igarashi	4 Sep. 1995 -	23 Aug. 1995

5.3. IMR counterparts

Most medical and science officers in 3 divisions involved in the Project have been assigned to the Project for effective implementation and transfer of biotechnology.

6. Fellowship Sent for Training in Japan
(Fellows defined in A2 and A3 Forms of Colombo Plan)

In this year, the following 2 IMR members were sent to Japan and 1 more will be sent soon. During 4 years since 1992, a total of 11 fellowships broken down as follows is provided.

Mr. Ravindran Thayan	23 Jul. 1995 - 2 Nov. 1995
Ms. Nazni Wasi Ahmad	12 Sep. 1995 - 10 Dec. 1955
Ms. Radzan Taib	14 Dec. 1995 - 12 Mar. 1996

Japanese Fiscal Year (JFY)	No. of fellowships
JFY 1992	2
JFY 1993	3
JFY 1994	3
JFY 1995	3

7. PROVISION OF MACHINERY AND EQUIPMENT

Machineries, equipments and other materials necessary for biotechnological studies have been provided based on the request

by A4 form. The spare parts of the provided equipment in an approximate amount of JY 6,804,000 (US $ 78,198) are supplied by the budget in JFY 1995. The yearly amounts for purchasing the above in Japanese Yen (JY) and US$ are as follows, totaling JY 168 million (US$ 1.5 million);

JFY 1991	JY 39,998,000	(US$	325,000)
1992	JY 36,448,000	(US$	312,250)
1993	JY 30,277,000	(US$	280,343)
1994a	JY 29,998,000	(US$	294,092)
1994b	JY 24,898,000	(US$	248,980)
1995	JY 6,804,000	(US$	78,198)
Total	JY 168,423,000	(US$	1,538,863)

8. Progress Note
(Scientific, technical and managerial progress)

The following seven subjects under the following brief titles and the divisions in charge were taken up as the research fields defined in the Project from the beginning.

(1) DNA probes for malaria diagnosis (Biotech)
(2) Analysis of mitochondrial gene (Biotech)
(3) HLA and malaria resistance (HLA, Biotech)
(4) Malaria susceptible locus in *Anopheles* chromosomes (Ent)
(5) Diagnosis of dengue and JE (Virus)
(6) Epidemiology and transmission of JE and dengue (Virus, Ent)
(7) Genome analysis of dengue and JE viruses (Virus)

The results of final Joint Evaluation indicated that the introduction of fundamental biotechnological techniques into each division was rapidly and successfully completed. In addition, the acquired methodology was applied to other research fields as described below. Although subjects (4) and (7) are still under way, the other subjects were completely achieved. Subject items (1) and (6) achieved remarkable progress which was much more than initially planned. These were accomplished through careful initial planning, meticulous follow-up and implementation of these Projects, and the result of achievement of research subjects was evaluated as to be excellent.

Biotechnological techniques which are readily performed in the divisions involved in the Project are as follows;
Restriction enzyme fragment length polymorphism (RFLP), PCR, cDNA synthesis from mRNA, reverse transcription PCR (RT-PCR), molecular cloning of PCR product into plasmid vector, cDNA cloning into lambda phage vector, DNA sequencing, primer designs for PCR and sequencing. Besides those, arbovirus isolation by tissue culture and improvement of IgM capture ELISA are worked out in the Division of Virology.

These techniques were applied to other research fields in Biotechnology Centre and readily extended to other divisions in

IMR or institutions outside IMR.

The progress of each research subject and its degree of achievement are summarized as follows;

8.1. Development and use of DNA probes for malaria diagnosis

8.1.1 Technology Development:

This was initiated through an intensive course in "Basic Molecular Biology" conducted by Japanese experts over a total period of 9 weeks for the Division Head and all officers in the Biotechnology Centre. This was then followed by the planning and carrying out of research activities on the development and use of DNA probes for malaria diagnosis.

About 40 blood samples were obtained from patients at the Orang Asli Hospital in Gombak, Selangor and Kuala Lumpur Hospital. About 500 samples were also collected during a field survey in an Orang Asli Settlement, Betau, Pahang. In addition, *Plasmodium falciparum* isolates maintained at the Centre were used.

8.1.2 Restriction enzyme digestion of genomic DNA of *P. falciparum* isolates:

The restriction enzyme patterns of genomic DNA from 32 *P. falciparum* isolates with 30 restriction enzymes did not show any remarkable difference among the isolates.

PCR amplification with *P. falciparum* specific primers, followed by restriction enzyme analysis of the PCR products, using 30 different enzymes, on a simian malaria *P. inui* and a rodent malaria *P. yoelii*, showed that the restriction pattern of *P. inui* PCR product using restriction enzyme *BST*N1 was entirely different from that of *P. falciparum*. Since the asexual forms of *P. inui* and *P. falciparum* look fairly similar, restriction analysis can be used for species differentiation between the two parasites.

8.1.3 Polymerase chain reaction based techniques for malaria diagnosis:

a). Nested PCR: The nested PCR using primers based on the published sequence of the 18S rRNA of *P. falciparum* could detect all microscopically confirmed *P. falciparum* and was also positive in 33.3% of *P. vivax* monoinfections (diagnosed by microscopy). Six out of 43 microscopically negative samples were shown to be positive. Of these 6, one was found to be positive on microscopy on reexamination. Amplification of DNA by the primer set was specific for *P. falciparum*, and this was confirmed by the sequence analysis of PCR products. Sensitivity of nested PCR was highest among the four methods tested.

b). Reverse transcriptase-polymerase chain reaction (RT-PCR): This technique was applied on 5 *in vitro* cultured isolates as well as specimens from *P. falciparum* infected patients. The sensitivity of this method was high (0.3 parasite per ul of blood), and the specificity was confirmed by sequence analysis of PCR product.

c). Plate hybridization - Polymerase chain reaction (PH-PCR): This technique utilized microtitre plates with immobilized specific *P. falciparum* and *P. vivax* probes for the detection of PCR amplified malaria DNA, using biotinylated specific primers.

The sensitivity and specificity using the *P. falciparum* probe were 93.3% and 100%, respectively. For *P. vivax* probe, it was 100% sensitive and specific.

d). Enzyme detection - Polymerase chain reaction (ED-PCR): This system was evaluated using the specimens collected from Gombak Hospital, and was used to analyse a total of 100 field collected specimens. However, the results obtained were not satisfactory since there was no significant difference in absorbance between the positive and the negative specimens.

8.1.4 Molecular marker of drug resistance in *P. falciparum*:

Our earlier studies found polymorphism of the *Nsp*I restriction site (nucleotide 754) of the *pfmdr*1 gene by PCR-restriction enzyme digestion analysis. However, no association between the particular *pfmdr*1 mutation and drug-resistance was observed.

8.1.5 Application of molecular techniques to other research fields:

8.1.5.1 Construction, characterization and immunoscreening of cDNA library from *Toxocara canis*: In an effort to develop an alternative source of specific antigens to that of *in vitro* culture of second stage (L2) larvae of *Toxocara canis* for the immunodiagnosis of visceral larva migrans, cDNA libraries from adult and L2 parasites were constructed. From the screening of 2 x 10^5 clones in the adult *T. canis* library using a clinically positive patient's serum, 8 clones were detected as positive. The partial sequencing analysis of these clones showed that these clones were classified into two groups.

8.1.5.2 Breast cancer study: PCR-microsatellite length polymorphism assay was carried out on microsatellite *D17S579* which is located on chromosome 17q2.1 (*i.e.* linked to the *BRCA*1 gene).

8.1.5.3 Sequencing of the p*53* tumor suppressor gene: The p*53* gene is an important tumor suppressor gene involved in many tumors. The site and type of mutation are thought to be associated with the possible aetiological factors. PCR-based analysis of p*53* gene of the DNA from certain individuals has been attempted.

8.2. Analysis of mitochondrial genes - together with its characterization

8.2.1 Mitochondrial DNA of malaria parasites:

Detection of mitochondrial DNA of *Plasmodium* parasites was carried out using PCR with three primer sets specific for *P. falciparum* mitochondrial genes, namely, P101/102 (Cytochrome oxidase III), P103/104 (Cytochrome oxidase I) and P105/106 (Cytochrome b). PCR amplification was successfully carried out on 26 *P. falciparum* isolates using these primers. The same sets of primers and PCR technique were also performed on 7 isolates of *P. vivax*. All the 7 isolates gave positive results with primers 101/102 and 103/104 while only 2 out of 7 isolates were positive with primers 105/106.

8.2.2 Studies on mitochondrial DNA of *Plasmodium vivax*:

The partial nucleotide sequence of *P. vivax* COIII gene was determined after the successful amplification of DNA using primers designed from the sequence of *P. falciparum* (P101/102). To obtain the full length sequence of this gene, the other 2 sets of primers were designed and used. *P. vivax* COIII gene showed 71% nucleotide homology and 80% deduced amino acid homology to those of *P. falciparum*, and deletions and insertions specific for *Plasmodium* were found. This is the first report on the sequence of *P. vivax* mitochondrial COIII gene.

A part of *P. vivax* COI gene was isolated and sequenced by the same strategy described above. Amino acid sequence comparison revealed that 40 amino acid sequence was highly conserved sequence among species, indication the functional importance of this region.

8.3. Identification and characterization of protective epitopes of malaria antigens - Association of human leucocyte antigen (HLA) and resistant to *falciparum* malaria.

The main objective is to study the relationship if any, between HLA and resistance to *P. falciparum* infection. Preliminary training of staff at the HLA laboratory in the Division of Immunology, in molecular techniques of HLA typing, including RFLP for class II, SSOP for Class I and SSCP for class I and II HLA DNA typing was carried out.

Blood specimens collected from 169 Orang Asli (Aborigine) living in the malaria endemic areas were examined by PCR and hybridization methods. In these areas, although parasitaemia was as high as 20 - 30%, severe clinical malaria is seldom observed. Typing of 62 unrelated healthy individuals in this group for their polymorphism in the HLA-B gene was carried out to search for another HLA-B53 like gene known to be associated with resistance to severe malaria among the east African population. One specific epitope combination corresponding to a serological type, HLA-B77, was revealed to be dominant. We have cloned and sequenced this DNA fragment and have confirmed that this DNA type has the same sequence as HLA-B*1513 or serologically defined HLA-B77. The HLA-B*1513 was found in 44% of the Temuan tribe and 25% in the Termiar and Semai tribes. In addition, no HLA-B53 was found in this population. Amino acid sequence analysis,showed that the F pocket of HLA-B*1513 was similar with that of HLA-B53. As HLA-53 was known to be effective in generation anti-malaria cytotoxic T lymphocytes due to high affinity to a peptide derived from the liver stage of the parasite, a similar situation may exist with as it shares a similar C-terminal peptide binding pocket.

8.4. Better understanding of the vectorial status of malaria vectors at the molecular level - Determination of malaria susceptible locus on the chromosome of *Anopheles maculatus*

8.4.1 Biotechnology:

A few *Plasmodium falciparum* clones producing gametocytes frequently and at a high ratio were selected from the isolates

from local patients. These were *in vitro* cultured and used for selecting *Anopheles maculatus* strains susceptible to malaria. Although selection of susceptible strains of *An. maculatus* was attempted through feeding of *Anopheles* with cultured *P. falciparum* clones with the cooperation of the Biotechnology Centre, no susceptible strain was found among breeding *Anopheles* colonies, and colonization was not performed. To construct a RFLP map of *An. maculatus* chromosomes, the cDNA clones at hand were screened. Some of these cDNA probes made from *Aedes aegypti* (Liverpool strain) were confirmed to be useful as probes for Southern blots of genomic DNA extracted from different *An. maculatus* strains. Standardized technical procedures for Southern transfer of genomic mosquito DNA, and those for the hybridization between *An. maculatus* genomic DNA and cDNA probes from *Ae. aegypti* were established. The chemiluminescence technique used in hybridization had a lower sensitivity compared to that using a radioactive marker. Therefore, to increase the hybridization sensitivity, cDNA originating from *An. maculatus* RNA library were constructed. For processing RFLP data for the identification of the malaria susceptibility loci on *An. maculatus* chromosomes, multiplot linkage analysis and quantitative trait loci (QTL) mapping are usually used, therefore, the MAPMAKER/QTL computer package has been installed in the laboratory. The objective initially planned, however, was not achieved in this subject.

8.4.2 Genetics:

Out of 3 peninsular Malaysian strains and 1 Sabah strain of *An. maculatus* tested for isozyme variants, the Jeram Kedah (JK) and Kuala Milot (KM) have been selected for the fast allele (F) and slow allele (S), respectively, for 3 generations in order to purify each of the two alleles (F and S). In addition to the starch method for electrophoresis which has been employed in this Division, the method using agar gels was adapted for electrophoresis of *An. maculatus*. About 10 enzymes so far have been electrophoresed by this agar method. Three strains of *An. maculatus* are now being studied for new isozymic alleles by the modified agar method. Freshly field-collected strains have been utilized for crossing experiments to produce the heterozygotes for the above loci. So far about 85 single pairings have been performed using the JK strain as a standard strain. However, no heterozygotes at the loci concerned have been detected at the F1 hybrids between the freshly-collected and the standard strains.

8.5. Strengthening of diagnostic capability using molecular biology and biotechnology in dengue and Japanese encephalitis

8.5.1. Reverse transcriptase-polymerase chain reaction (RT-PCR):

The method of direct and rapid RT-PCR was transferred to the Division of Virology. Its evaluation for dengue diagnosis on 20 sera showed equal sensitivity to the conventional RT-PCR, as well as 100% sensitivity and specificity compared with the virus isolation.

A total of 85 cerebrospinal fluid specimens from patients

with meningoencephalitis were examined by the RT-PCR and IgM-ELISA for JE virus. Twelve were positive by the IgM-ELISA, while only a single positive by the RT-PCR, indicating that IgM-ELISA is the first choice in the laboratory diagnosis on JE.

8.5.2. Evaluation of RT-PCR compared to IgM-capture ELISA:

A total of 160 serum samples from patients clinically diagnosed as dengue fever (DF) or dengue haemorrhagic fever (DHF) was examined. Positive results were obtained for 12 (8%) both by RT-PCR and IgM-ELISA, 58 (36%) by IgM-ELISA only, 36 (22%) by RT-PCR only, with negative results by both methods for the remaining 54 samples (34%). The sensitivity of the IgM-ELISA was significantly higher than the RT-PCR ($P<0.5\%$), since many sera were collected late for detecting viraemia. The results indicated that RT-PCR should be performed in the central or reference laboratories on those specimens collected at early stage of infection from clinically selected cases showing negative IgM-ELISA. The RT-PCR on a total of 121 serum samples from such cases detected 14 positives.

8.5.3. Improvement of IgM-capture ELISA:

Comparative dengue IgM-capture ELISA was carried out on a total of 237 convalescent sera using reagents from 2 sources: (1) dengue type 2 (D2) antigen prepared from suckling mouse brains at IMR and a monoclonal antibody (MCA) 3H5 from CDC, USA, and (2) mixture of 4 types of dengue antigen raised in cell culture and a flavivirus cross-reacting MCA (MF4) courtesy of Dr. Jane Cardosa, USM. Evaluation against the haemagglutination-inhibition (HI) test showed that the sensitivity of the reagents (2) was significantly higher than that of the reagents (1) ($P<0.02$), while both reagents showed similar specificities.

Production of reagents for routine IgM-capture ELISA: The production of dengue virus antigens in the infected *Aedes albopictus* clone C6/36 cell line was examined at 28°C, 32°C, and 37°C. Highest titer of D2 antigen was obtained 5 days after infection at 37°C, and dengue type 3 (D3) antigen production was better at 32°C. The maximal titer of dengue type 1 (D1) antigen was not enhanced at elevated temperature and dengue type 4 (D4) antigen remained undetectable.

Hybridoma cell lines secreting MCA were kindly supplied from the CDC, USA, and successfully cultivated in the Division of Virology.

8.6. Epidemiological and entomological studies on dengue and JE

8.6.1. Isolation of dengue viruses from patients:

Virus isolation on 150 acute sera from clinical dengue cases showed positive results in 21.3% (32/150). Eighteen isolates were identified as D3 by the RT-PCR.

8.6.2. Detection of JE antibodies in animal sera:

A total of 2,152 serum specimens were examined by the HI test. Using the cut-off titer of 1:10, positive rates were 88.1% (156/177) in pigs, 40.0% (156/347) in buffaloes, 41.5% (237/576)

in cattle, 17.9% (88/492) in sheep, 14.0% (63/449) in goats, and 0.9% (1/111) in birds. The results showed that JE virus is widely distributed in Malaysia, and swine is the most susceptible vertebrate host followed by cattle.

8.6.3. Year round surveillance of JE activity in swine in the Klang Valley:

The IgM-capture ELISA on swine sera collected in the Klang Valley showed positive rates of 26.1% in March, 36.7% in April, 25.0% in May, 9.5% in June, 5.0% in July, 25.0% in September, and 14.3% in November 1994. The result indicated year round circulation of JE virus in this area.

8.6.4. Year round surveillance of JE activity in mosquitoes:

JE virus isolation was performed in collaboration with Division of Medical Entomology, IMR. A total of 223 pools of mosquitoes was collected in the Sungai Pelek area, Selangor, from September 1993 to October 1994, while 36 pools were collected in Sarawak in October 1994. Among Sg. Pelek pools, 43 were positive (19 *Cx. tritaeniorhynchus*, 9 *Cx. gelidus*, 4 *Ae. butleri*, 3 *Cx. fuscocephala*, 1 each *Ae. cancraedes* and *Cx. quinquefasciatus*), while 5 pools of *Cx. tritaeniorhynchus* from Sarawak were positive.

So far from 1993 to 1995, besides 4 known vector mosquitoes of JE, *i.e., Cx. tritaeniorhynchus, Cx. gelidus, Cx. vishnui* and *Cx. pseudovishnui*, JE viruses were newly detected from 7 species of field-caught mosquitoes, *i.e., Cx. bitaeniorhynchus, Cx. sitiens, Cx. quinquefasciatus, Cx. fuscocephala, Ae. butleri, Ae. cancraedes* and *Ae. lineatopennis*. The results indicate that multiple mosquito species are suspected to be potential transmitters of JE virus in Malaysia.

8.7. Understanding of pathogenesis of DHF at the molecular level, and molecular epidemiology of JE and dengue viruses in Malaysia

8.7.1. Genome analysis of JE virus isolates from mosquitoes:

Ten JE virus isolates from mosquitoes in 1992 in Peninsula Malaysia were sequenced for the 240 nucleotides in their PreM gene region, and compared with the published sequence of other JE virus strains. The recent Malaysian strains belonged to the genotype 3 which contained strains from temperate to subtropical regions in Northern Asia, such as Japan, China, and Taiwan. While an old Malaysian strain WTP/70/22 isolated in 1970 belonged to genotype 2 consisting of the strains from tropical Asia.

8.7.2. Comparative genomic sequencing of dengue virus isolates in relation to the pathogenesis of DHF:

Eight strains of DEN-3 (5 from DF and 3 from DHF cases) were sequenced for their 280 nucleotides of E/NS1 and 670 nucleotides of 5'-end in the genome, although a few isolates were sequenced up to 1150 nucleotides. There are some differences in the sequence to that of H-87 virus, a reference strain on the nucleotides or amino acid sequences. The E/NS1 and 5'-end in the

present isolates showed an amino acid sequence similarity at greater than 97% to those of H-87, indicating that the sequence should be well maintained over the past 37 years. The data suggest that most virus isolates used in this study are placed together in a same subtype which belong to subtype II including THAILAND 1989. In the above studies, however, we could not determine the pathogenicity locus at the regions of DEN-3 genome.

9. Training Programmes

9.1. Third Country Group Training, JICA

As an extended function of the Project and due to rapid development of medical biotechnology in IMR, IMR conducted the Third Country Group Training entitled as the International Seminar on Biotechnological Techniques in Tropical Medicine, to which 12 participants will be invited from Malaysia and neighbouring countries in Southeast Asia and Pacific Islands. Announcement on the Seminar to the countries concerned was made through diplomatic channels.

The first seminar was held by inviting 12 participants and 2 observers for 4 weeks from 24 (Tue.) October to 18 (Sat.) November 1995 with extremely successful results. Lecturers were mainly science and medical officers in Biotechnology Centre, IMR with some in other divisions and out of IMR. Assoc. Prof. K. Kita and Dr. Rieko Shimogawara were sent by JICA to the seminar, and Dr. Y. Watanabe also assisted. Similar seminars will be held yearly for 2 more consecutive years.

9.2. Third Country Individual Training, JICA

IMR is registered as an individual training institute for JICA. In this programme, the member in JICA projects in foreign countries will be considered by IMR for training in the 6 month Diploma Courses of Medical Microbiology (DMM) or Applied Entomology and Parasitology or in any division in IMR. A Sri Lankan medical doctor graduated from the DMM Course in March 1995.

10. Other functions

Visit of H.E. Japanese ambassador: H.E. Japanese ambassador, Mr. Taizo Nakamura, accompanied by Mr. D. Mastunaga, the first secretary, Ms. K. Mizuta and Ms. A. Inagaki, JICA Malaysia Office, visited the Project at 10.00 to 11.30 on 27 July 1995. Dr. Mak Joon Wah welcomed him on behalf of the Director of IMR, and briefed the activities of IMR and the Project. Then, the ambassador went round to visit the laboratories involved in the Project. The achievement of the Project was well evaluated by him.

Project Seminar: On 5 September 1995, "The IMR-JICA Collaborative research in Tropical Disease Seminar" was held in Ming Court Hotel Kuala Lumpur. Professor A. Igarashi and Assoc. Prof. K. Kita were invited for their review papers on biotechnology in Parasitology and Virology, respectively. A total of 17

scientific papers was presented by IMR members and Japanese consultants. The morning session chaired by Dr. Mak Joon Wah dealt with topics on malaria and its vector, and the afternoon session by Dr. Mangalam Sinniah with those on dengue and Japanese encephalitis. The seminar ended with a panel discussion chaired by Dr. Mak Joon Wah and by 3 members, Dr. Ho Keong Bin, Deputy Director Disease Control Division (Vector), Ministry of Health, Prof. Lam Sai Kit, University of Malaya, Head, WHO Collaborating Centre for Arbovirus Reference and Research (Dengue & Dengue Haemorrhagic Fever) and Prof. H. Tanaka, Chief-Advisor, IMR-JICA Project. Scientific and technical details achieved in each research subject were presented individually, and frank opinions were exchanged at the panel discussion.

Handing Over Ceremony of the Project: It was held at Asia Hotel, KL on 5 October 1995. The Ceremony was officiated by Dato' Dr. M. Jegathesan, Deputy Director General (Research and Technology Support), Ministry of Health, Malaysia by the arrangement of IMR with 60 invited members. It started at 11.30 with Welcome Address by Dr. Mohamad Taha Arif, Director of IMR, followed by address by Ms. K. Mizuta, Resident Representative, JICA Malaysia office. Then, Dato' Dr. M. Jegathesan officially opened the ceremony by expressing the overview of the successful achievement of the Project which was initiated by him when he was the Director of IMR, and expressed appreciation to all who were involved. At the ceremony, a handing over note, which documented that the machineries and equipment provided are officially handed over from JICA to IMR, attached with a list of the above inventory, was signed between JICA representative and Director of IMR, and the Deputy Director General as the witness. Then, a commemorative key was transferred from JICA representative to the Director, IMR as the symbol of functional hand over. After the ceremony, lunch was served by IMR.

11. Publications in 1995

INTERNATIONAL REGISTRATION

Lim PKC, Kita K, Tan SK, Furuta T, Aoki T, Watanabe Y and Mak JW (26 Jan. 1995) Partial DNA sequence of 347 bp at cytochrome c oxidase subunit III gene of *Plasmodium vivax*. registered internationally by accessing to DDBJ (DNA Data Bank, Japan) by E mail from IMR under accession no. D45369 for data bases, GSDB, DDBJ, EMBL and NCBI.

PUBLICATIONS

Published

Lim PKC, Kita K. Tan SK, Furuta T, Kojima S, Aoki T, Watanabe Y and Mak JW (1995) Mitochondrial cytochrome oxidase III (COIII) gene of *Plasmodium vivax*. *Jpn. J. Parasitol.* **44**: 40-43.

Vythilingam I, Oda K, Chew TK, Mahadevan S, Vijayamalar B, Morita K, Tsuchie H and Igarashi A. (1995) Isolation of Japanese encephalitis virus from mosquitoes collected in Sabek Bernam,

Selangor, Malaysia in 1992. *J. Amer. Mosq. Cont. Assoc.* 11: 94-98

Thayan R, Morita K, Vijayamalar B, Chew TK, Sinniah M and Igarashi A (1995) Comparison of two different methods for purification of polymerase chain reaction (PCR) products used in direct sequencing, in an Applied Biosystems 373A DNA sequencing system. *Tropical Medicine* 37: 29-33

Lokman Hakim S, Furuta T, Noor Rain A, Normaznah Y, Mohd Zamri MR, Kojima S and Mak JW (1995) Diagnosis of malaria infection using plate hybridization method for detection polymerase chain reaction products. submitted to *Trans. Roy. Soc. Trop. Med. & Hyg.* 89: 271-272

Halimah M, del Carmen Castillo L, Sinniah M and Igarashi A (1995) Elevated incubation temperature enhance antigen production of dengue type 2 and 3 viruses in the infected *Aedes albopictus* clone C6/36 cultures. *Tropical Medicine* 37: 21-27

Submitted

Khoo A, Furuta T, Noor Rain A, Ng AB, Kojima S and Mak JW : Nested polymerase chain reaction for diagnosis of *Plasmodium falciparum* infection in Malaysia. *Trans. Roy. Soc. Trop. Med. Hyg.* (in press)

Noor Rain A, Furuta T, Radzan Taib, Kita K, Kojima S and Mak JW: Development of a new diagnostic method for *Plasmodium falciparum* infection using reverse transcriptase-polymerase chain reaction. *Am. J. Trop. Med. Hyg.* (in press)

Hirayama K, Mohd Zaidi AS, Lokaman Hakim S, Ong KJ, Kikuchi M, Nasuruddin A and Mak JW: Molecular analysis of HLA-B in the Malaysian aborigines. *Tissue Antigens* (accepted)

Halimah M, Vijayamalar B, Sinniah M, Igarashi A and Tanaka H.: Comparative assay on dengue IgM capture ELISA with reagents from 2 sources. submitted to *Tropical Medicine.*

Thayan R., Vijayamalar B, Zainah S, Chew TK, Morita K, Sinniah M, and Igarashi A.: The use of polymerase chain reaction (PCR) as a diagnostic tool of dengue in the Institute for Medical Research. submitted to *Southeast Asian J. Trop. Med. Publ. Hlth.*

Oda K, Igarashi A, Chew TK, Choo CH, Vijayamalar B, Sinniah M, Sharifah SH and Tanaka H: Cross sectional serosurvey for Japanese encephalitis specific antibody from animal sera in Malaysia 1993. submitted to *Southeast Asian J. Trop. Med. Publ. Hlth.*

Compiled by Dr. H. Tanaka
and Dr. Mak Joon Wah

Selangor, Malaysia in 1992. *J. Amer. Mosq. Cont. Assoc.* 11: 94-98

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