Army Malaria Institute – its Evolution and Achievements Third Decade (1st Half): 1985-1990

Karl H. Rieckmann, Anthony W. Sweeney, Michael D. Edstein, Robert D. Cooper, Stephen P. Frances

Abstract

The first half of the third decade (1985-1990) after the establishment of the Malaria Research Unit was characterised by a substantial increase in laboratory and field activities. Various new procedures were developed to discover and counter the increasing problem of parasite resistance to established antimalarial drug regimens. After early findings that the currently-used pyrimethamine/dapsone (Maloprim®) prophylaxis was no longer effective against *Plasmodium falciparum* malaria, *in vitro*, *ex vivo* and pharmacological investigations were undertaken with proguanil and a low dose of dapsone. This led to a field study in Papua New Guinea (PNG) which indicated that such a drug combination might be more effective than Maloprim®. Before the effectiveness of this combination could be determined further, it became obvious that Maloprim®/ chloroquine had become inadequate for the protection of ADF soldiers on exercises in PNG. During one of these exercises, the landmark discovery that *P. vivax* had developed resistance to chloroquine was the first documented evidence that this previously successful standard medication had lost its efficacy against vivax malaria. These findings in 1988/1989 led to the evaluation of mefloquine and doxycycline as alternative prophylactic regimens for ADF personnel serving in malarious areas. In efforts to identify and assess other potential antimalarial drugs, *in vitro* studies were continued with various strains of *P. falciparum*. These studies were supplemented by investigations with *P. falciparum* and *P. vivax* in non-human primates and by malaria transmission studies with *Anopheles farauti* mosquitoes. The survey of anopheline mosquitoes in northern Australia indicated the widespread presence of three isomorphic species of *An. farauti* and the ever-present possibility of re-introducing malaria into northern Australia. Investigations relating to the biological control of mosquitoes were gradually phased out and emphasis was given to the assessment of novel mosquito repellent measures for improved personal protection against malaria.

Background

The global malaria situation had generally not improved during the previous decade. Following the principles of primary health care outlined at the Alma Ata Conference in 1978, increasing emphasis was given to applying strategies of malaria control tailored more specifically for different epidemiological conditions.[1,2] Although basically an eminently sensible approach, lack of funding and decreased prioritisation of malaria activities in many malarious countries meant that there was generally little or no improvement in the malaria situation. The extension of chloroquine-resistant falciparum malaria to other areas, including Africa and the Southwest Pacific region was a contributing factor hindering malaria control activities. In some countries, a combination of pyrimethamine and sulfadoxine (Fansidar®) had largely replaced chloroquine for malaria treatment.[3] However, since most infections still responded clinically to chloroquine and related drugs, they continued to be used as first-line treatment because they were affordable and largely effective in suppressing malaria in populations with some degree of background immunity to malaria. In a few areas, though, travellers or residents with little or no immunity to malaria were not responding adequately to prophylaxis or treatment with standard drugs. This raised the possibility of using alternative drugs, such as the tetracyclines and mefloquine, for malaria prophylaxis and treatment.[4]

Research activities at the Army Malaria Research Unit (AMRU), commenced in the mid-1960s,[5] were able to be increased following its relocation from the University of Sydney to the Ingleburn military facility in 1974.[6] This was possible because improved housing facilities enabled the gradual acquisition of more suitable equipment for the laboratories, the establishment of an appropriate mosquito insectary and animal quarters, and a gradual increase in staff from 9 to 23 positions by the end of the decade. Significant progress was made in assessing *in vitro* drug resistance and screening potential antimalarial

drugs by short-term and long-term culture of *Plasmodium falciparum*, in determining the synergistic activity of antifolate drug combinations in rodent malaria, and in developing sensitive methods for estimating drug concentrations in body fluids with a view to optimising drug doses used for malaria prophylaxis and treatment.[6] Following the Unit's documentation of chloroquine-resistant falciparum malaria in Papua New Guinea (PNG) at the start of the second decade, increasing attention was given to the investigation of various antifolate drug combinations. Although studies with dapsone/proguanil combinations continued for a while, greater attention was placed on dapsone combined with another dihydrofolate reductase (DHFR) inhibitor – pyrimethamine. This pyrimethamine/dapsone combination, widely known as Maloprim®, was registered and approved for malaria prophylaxis in Australia in 1979.

By 1980, standard malaria prophylaxis for Australian military personnel consisted of weekly Maloprim® and chloroquine while on deployment overseas and for 4 weeks after return to Australia to suppress falciparum and vivax malaria. In addition, a 14-day course of primaquine (7.5 mg base 3 times a day) was taken to eradicate any residual liver stages of vivax malaria. With good drug compliance, these regimens appeared to be largely effective in preventing malaria in the rather limited number of military personnel deployed overseas during this period.

The accidental discovery of a fungus killing mosquito larvae in the Unit insectary generated considerable interest in the possible use of *Culicinomyces clavisporus* as a potential fungal larvicide to control mosquito breeding, although its practical application in the field remained in doubt. In addition to preliminary studies with another mosquito pathogen, other entomological activities included speciation and malaria transmission studies with *Anopheles farauti*, the major malaria vector in the Southwest Pacific region, and the initiation of annual surveys to map the distribution of this vector in northern Australia.[6]

Staff and facilities

In January 1985, Colonel Eric Donaldson became Director of AMRU, following the promotion of Colonel Graham Maynard to Brigadier and his transfer to Army Headquarters in Canberra. Colonel Donaldson inherited a unit which had grown considerably since its inception about 20 years earlier and which was destined to contribute significantly to international efforts to control drug-resistant malaria. During 1985 and 1986, there was a substantial extension of primate, mosquito and parasitology facilities. In addition, office space was acquired from 2nd Military Hospital to accommodate a refurbished library, staff offices and a small laboratory. In late 1985, Professor Karl Rieckmann was recruited as Director of Medical Research, following a 25-year absence from Australia during which he conducted laboratory and field investigations to control the increasing problem of parasite resistance to antimalarial drugs. After the transfer of Lieutenant Colonel John Twartz to 2nd Military Hospital in 1986, Major Andrew Taylor was posted to the Unit as medical officer for 12 months. Following Colonel Donaldson's re-assignment to the Army Aviation Centre at Oakey, Queensland, in August 1987, Lieutenant Colonel Sweeney became Commanding Officer and Acting Director of the Unit. In 1988, Ms Barbara Kotecka was appointed Parasitologist following the resignation of Dr Haydn Scott, and Lieutenant Colonel Donald Davis was posted to the Unit as Medical Officer for a short period of time.

Figure 1: Army Malaria Research Unit Staff (1986).

Standing (L to R): Mr H. Scott, PTE A.Topping, SGT C. Rogers, SGT J. Staley, CAPT R. Cooper, CPL M. Baker, Mr T. Haddon, CPL M. Sellars, SGT K. Newman, CPL G. Owen, Mrs J Turl, LT S. Frances.

Sitting (L to R): MAJ M. Edstein, Prof K. Rieckmann, BRIG G. Maynard (DMS-A), COL E. Donaldson, MAJGEN W. Rodgers (DGAHS), LTCOL A. Sweeney, MAJ R. Veenendaal.

Absent: MAJ J. O'Brien, CAPT W. Backhouse, CAPT A. Taylor, WO2 P. Flannery,

In 1988, Emeritus Professor (Colonel) Black died after a distinguished career in tropical medicine during which he was also the motivating force in re-establishing the Army's active participation in malaria research activities. In the same year, Karl Rieckmann was appointed Professor of Medicine at the University of Sydney. After his appointment as Director of AMRU in 1989, the long-standing association with the University of Sydney was strengthened when AMRU was accorded departmental status within the Faculty of Medicine during the following year. In

1989, Major Michael Edstein commenced a 3-year posting to the Armed Forces Research Institute of Medical Research (AFRIMS), Thailand, and contributed to joint efforts by AMRU and US and Thai Army scientists to counter the increasing problem of drug- resistant malaria.

Malaria situation

Upsurge in malaria during military exercises in PNG. Current malaria prophylaxis appeared to control malaria in the ADF quite well until the deployment of 163 members of the Special Air Services (SAS) regiment to PNG for 3 to 4 weeks during 1988 and 1989. Five soldiers developed malaria (3 *P. falciparum*, 2 *P. vivax*) while taking prophylaxis and 36 of them had attacks of malaria (6 *P. falciparum*, 30 *P. vivax*) after completing prophylaxis.[7] The malaria attack rates were similar to those observed during the Pacific campaign of World War II and higher than those observed during the Malayan emergency or the Vietnam conflict. This suggested very strongly that weekly Maloprim®/chloroquine prophylaxis was no longer able to protect soldiers adequately against falciparum and vivax malaria and that the current primaquine eradication regime was becoming less effective in preventing relapses of vivax malaria. The gravity of the situation was heightened even further by the documentation that chloroquine was unable to suppress vivax malaria in 2 soldiers who were treated with chloroquine after their return from PNG.[8] There was obviously an urgent need for improved protection of soldiers against malaria.

Drug resistance

***In vitro* test for drug resistance.** The increased likelihood of ADF personnel acquiring drug-resistant infections highlighted the importance of forwarding parasitised blood samples to AMRU to assess the *in vitro* sensitivity of parasites to chloroquine and other drugs.[9] By determining the presence and degree of drug resistance in patients with falciparum malaria, medical personnel could modify their treatment regimens, if necessary, for patients whose infections had been acquired in the same geographical area. These tests could also be helpful in formulating more effective prophylactic and therapeutic drug regimens for particular areas of deployment.

***P. falciparum* resistance to pyrimethamine/dapsone (Maloprim®).** The first documented evidence that *P. falciparum* was becoming resistant to Maloprim® was in 1987 when high performance liquid chromatography (HPLC) analysis revealed that high plasma concentrations of pyrimethamine and dapsone did not prevent an Australian soldier from developing falciparum malaria while on prophylaxis in PNG.[10] Furthermore, adequate plasma concentrations of pyrimethamine, dapsone and chloroquine were observed in 3 of the men who developed falciparum malaria while they were still on weekly prophylaxis following the 1988/1989 SAS exercise in PNG.[7] Six other men became ill with falciparum malaria within 4 weeks after completing prophylaxis, suggesting that parasites were only partially suppressed by Maloprim®/chloroquine prophylaxis.

***P. vivax* resistance to chloroquine.** Following the 26-day deployment of SAS soldiers to PNG during 1989, 2 soldiers developed acute attacks of vivax malaria 3 to 15 days after return to Australia.[8] Both men were still on weekly chloroquine and Maloprim® prophylaxis, and their plasma chloroquine levels were considerably higher than those generally regarded as therapeutically effective against *P. vivax*. About 30 years after the emergence of chloroquine-resistant *P. falciparum*, this was the first documented evidence that *P. vivax* was also capable of developing resistance to chloroquine. Chloroquine-resistant *P. vivax* was also observed in a traveller returning to Australia from PNG and the Solomon Islands.[11]

***P. vivax* tolerance to primaquine.** Relapses of vivax malaria acquired in the southwest Pacific area have traditionally been treated with a total daily primaquine dose of 22.5 mg base, rather than 15 mg base, because parasites from this area were considered to be more tolerant to the drug than in other parts of the world. The unacceptably high number of vivax infections observed in the SAS soldiers after their return to Australia suggested that the parasites were becoming even more tolerant to primaquine than previously reported.[7]

Malaria diagnosis. The increasing problem of drug resistance highlighted the importance of early microscopic diagnosis of malaria and correct identification of malaria species. The diagnostic verification service provided by the Unit revealed that the wrong plasmodial species was being identified in up to one in 5 military personnel, raising the likelihood that some military personnel had received inappropriate treatment. This emphasised the need to forward duplicate blood films to AMRU as quickly as possible to enable treatment to be modified if necessary. As species identification by microscopic examination of blood films can sometimes be notoriously difficult, it was hoped that this problem could be partly resolved by reviving 2-4 week training courses for pathology technicians working at defence and other laboratories.

Proguanil/Dapsone as a possible alternative to pyrimethamine/dapsone (Maloprim®)

Earlier favourable field and laboratory findings with proguanil/dapsonel[5,6] led to a reconsideration of using proguanil, in combination with a low-dose of dapsone, for malaria prophylaxis. Further studies were carried out with proguanil, chlorproguanil, and their active triazine metabolites to gain a better understanding of the pharmacokinetics of these drugs.[12,13] Addition of proguanil, dapsone or monoacetyl-dapsone (principal metabolite of dapsone) to the pyrimethamine-resistant K1 isolate of *P. falciparum* showed little or no antimalarial activity *in vitro*. On the other hand, cycloguanil (active metabolite of proguanil) was several orders of magnitude more active than its parent compound, with 90% parasite growth of the drug-resistant K1 isolate being inhibited at a concentration of 80 ng/mL. However, only 20 ng/mL cycloguanil was required to inhibit parasite growth when 40 ng/mL dapsone was added to the culture. Parasite growth was also inhibited when 20 ng/mL cycloguanil was added to sera collected from volunteers 24 hours after administration of a low dose of 10 mg dapsone.[14]

These encouraging results led to the use of a bioassay (see below) to assess the activity of proguanil and dapsone by incubating sera, collected at various times after drug administration, with *in vitro* cultured parasites. By determining serum antimalarial activity *in vitro*, preliminary information about the duration and synergy of drug activity could be obtained *ex vivo*, before proceeding to *in vivo* assessment of such activity in individuals exposed to or infected with malaria. Thus, 6 healthy Army volunteers, not serving in a malarious area, received 200 mg proguanil daily for 7 days and, after an interval of at least a month, 10 mg dapsone daily for 7 days.[14,15] Serum specimens were collected at various intervals up to 24 hours after the end of proguanil or dapsone administration. None of the dapsone sera showed any antimalarial activity against the K1 isolate and, although sera from a few of the proguanil-treated volunteers (containing cycloguanil) showed partial inhibition of parasite growth for up to 12 hours, none of them did so 24 hours after drug administration. On the other hand, when dapsone- and cycloguanil-containing sera were combined with each other, parasite inhibition varied between 77% and 99% in the samples collected from volunteers 24 hours after medication.[16]

The favourable *ex vivo* antimalarial activity of proguanil combined with dapsone prompted a further study to determine the steady-state pharmacokinetics of 200 mg proguanil co-administered daily with 10 mg dapsone in 6 healthy male soldiers.[17] After the seventh and last daily dose, mean plasma maximum (minimum) concentrations of proguanil, cycloguanil and dapsone were 151 (21), 56 (15) and 285 (125) ng/mL, respectively. Respective elimination half-lives were 23, 15 and 18 hours. When serum samples collected between 4 and 24 hours were incubated with parasites of the K1 isolate, no parasite growth was observed in any of the cultures.

The complete inhibition of parasite growth following daily co-administration of proguanil and dapsone was in marked contrast to observations in 6 soldiers following weekly administration of pyrimethamine/dapsone (Maloprim®).[18] Although sera collected from the 6 volunteers inhibited parasite growth of the drug-sensitive FC27 isolate completely at trough concentrations of both drugs, even maximum serum concentrations failed to inhibit the growth of K1 parasites. Infections with less resistant parasites might still be able to be suppressed at peak concntrations, but this would be unlikely for serum samples collected towards the end of the weekly dose of Maloprim® This is due to the ill-matched elimination half-lives and clearance of pyrimethamine and dapsone, with mean half-lives in the volunteers being 105 hours and 23 hours, respectively.[18] The considerably more rapid elimination of dapsone implied that residual levels of pyrimethamine would not be able to suppress parasites without the synergistic activity of dapsone. This very significant difference in the pharmacokinetics of the two drugs probably explained the decreasing effectiveness of Maloprim® in areas where parasites were becoming increasingly resistant to pyrimethamine.

Field study with proguanil and low-dose dapsone.

The results of these findings were shared with the PNG Medical Research Advisory Committee and it approved a joint proposal by Ramu Sugar Limited (RSL) and AMRU to evaluate the effectiveness of proguanil/dapsone among seasonal workers at RSL, PNG. Some consideration had been given by RSL to using proguanil alone for malaria prophylaxis because chloroquine prophylaxis was not protecting their relatively non-immune workers from highland provinces against malaria. However, because *in vitro* tests in 1987 had shown parasites in this malarious area to have a degree of resistance to cycloguanil similar to that observed during studies at AMRU, the sugar company was keen to evaluate the effectiveness of a 12-week supervised course of proguanil in combination with a low dose of dapsone. As a result, 280 out of 1800 workers living in highly malarious villages indicated their willingness to participate in a supervised 12-week course of proguanil/dapsone.

Three weeks after receiving a standard 3-day course of amodiaquine to eliminate any circulating asexual parasites, they received 200 mg proguanil once a day and 25 mg dapsone twice a week (10 mg tablets for daily administration were unavailable). Finger-tip blood specimens were collected every two weeks for examination of malaria parasites and white cell counts. In addition, blood specimens were examined for malaria parasites from any villagers (participants and non-participants) who developed fever or reported to the RSL medical clinic with any illness. None of the 225 participants who completed the 12-week proguanil/dapsone course showed any parasites in their blood films during the period of drug administration, but 16 of them developed symptomatic infections 2 to 12 weeks after the end of drug administration. During the 12-week proguanil/dapsone course, 72 symptomatic infections were detected in the 1,500 or so non-participants, indicating continuous malaria transmission during the period of the trial.[16]

These findings suggested that this prophylactic regimen was effective in preventing malaria in an area with proven resistance to chloroquine and proguanil. The cumulative weekly dose of dapsone (50 mg) was one-half the weekly dose of dapsone (100 mg) in Maloprim® and less than one-third the cumulative weekly dose given to Australian and American soldiers during the Vietnam conflict (175 mg).[4] No decrease in white blood cells was observed throughout the course of the study and, in reality, agranulocytosis would be unlikely to manifest itself after such low doses of dapsone. Due to the marked drop in malaria cases and no discernible drug toxicity, the company expressed its interest in promoting the wider use of this drug combination among its workers to enhance their health and to reduce the number of man-hours lost by malaria illness.

Although favourable results were obtained at Ramu, further studies were obviously required before this drug combination could be considered for use as an alternative to Maloprim®. Therefore, field studies were started in 1989 with two possible alternative drugs – doxycycline (a tetracycline drug) and mefloquine.

Doxycycline prophylaxis

In 1970, the tetracyclines had been found to be effective against both the pre-erythrocytic and asexual erythrocytic stages of chloroquine-resistant *P. falciparum* malaria.[19] Following that early discovery, clinical and field studies with various tetracyclines, including doxycycline, had confirmed that this group of drugs, when given in combination with a rapidly-acting blood schizontocide, was highly effective in curing drug-resistant falciparum malaria.[20] However, despite indications that the selective use of the tetracyclines might be beneficial for "personal prophylaxis by non-immune individuals visiting or working temporarily in areas with a high transmission of multidrug resistant strains",[20] the first field study to determine the prophylactic effectiveness of doxycycline had not been conducted until 1986.[21, 22]

During 1989, 184 Australian soldiers volunteered to take 100 mg doxycycline daily during their deployments to PNG for 3 to 6 weeks. In addition to doxycycline prophylaxis, 69 men also took a daily dose of 7.5 mg primaquine base. Medication was generally well-tolerated and, although about 1 in 20 soldiers experienced some photosensitivity, the incidence of gastrointestinal disturbances was similar to that observed with mefloquine or Maloprim®/chloroquine prophylaxis.[7] All of the men were protected against *P. falciparum* and *P. vivax* while they were on medication. None of them developed falciparum malaria after returning to Australia but, despite a 14-day primaquine eradication course, 13% (15 out of 115) of the soldiers taking doxycyline alone experienced delayed primary attacks of vivax malaria, usually within 2 to 3 weeks after returning to Australia. On the other hand, none of the 69 soldiers taking doxycycline, in combination with a low-dose of primaquine, developed vivax malaria.[7] Leaving aside these preliminary findings with primaquine, it seemed likely that short-term administration of doxycycline might play a useful role in protecting healthy non-pregnant adults against falciparum malaria and in suppressing vivax malaria during medication with this drug.

Mefloquine (Lariam®) prophylaxis

Mefloquine is a 4-quinolinemethanol drug which had been developed under the auspices of the antimalarial drug development program of the US Army. Initial clinical studies with this drug in 1974 had shown it to be very effective in the prophylaxis and treatment of multidrug-resistant falciparum malaria.[23,24] After extensive field studies over the next decade, this long-acting drug had progressively been approved for use as an antimalarial by various countries, and was registered in Australia in 1988. The following year, 40 soldiers agreed to take a weekly dose of 250 mg mefloquine for 4 weeks during their deployment to PNG; all of them remained well and the medication was tolerated as well as Maloprim®/chloroquine prophylaxis.[7] After return to Australia, despite taking the primaquine eradication course, 10% (4 out of 40) of the soldiers suffered acute attacks of vivax malaria. As expected, mefloquine had acted against

the asexual erythrocytic stages of *P. falciparum* and *P. vivax* but not against the liver stages of *P. vivax*. These results provided further evidence that 22.5 mg primaquine a day for 2 weeks was not always able to eliminate the latent tissue stages (hypnozoites) of *P. vivax* acquired in PNG.

Assessment of other potential antimalarial drugs

***In vitro* assessment of drug activity against drug-resistant isolates of *P. falciparum*.** Additional studies were performed with various pyrimidine and purine antimetabolites in collaboration with the Department of Biochemistry, University of New South Wales. Some synergistic activity against *P. falciparum* was observed between pyrazofurin and tubercidin, but none was observed in most combinations of these compounds or when they were combined with dihydrofolate reductase (DHFR) inhibitors such as pyrimethamine or cycloguanil (active metabolite of proguanil).[25] These investigations were followed by collaborative studies with the Department of Biochemistry at the University of Sydney to determine the activity of TDHO-Me, BW566C80 (a new antimalarial undergoing clinical trials), and pyrazofurin, which inhibit the third, fourth and sixth step of the de novo pyrimidine pathway, respectively. Unfortunately, no synergism was observed between any of these compounds nor between them and dihydrofolate reductase (DHFR) inhibitors, such as cycloguanil. This was in marked contrast to the intense synergistic activity observed between DHFR inhibitors and dapsone.[25] Because of these findings, further studies with pyrimidine and purine antimetabolites were discontinued. By contrast, collaborative studies with the Australian National University, started in 1984, continued to show that Mannich bases exerted significant antimalarial activity against drug-resistant isolates of *P. falciparum*.[26,27]

Bioassay for assessing drug activity. After establishing the continuous culture of several different strains of *P. falciparum*, it became possible to use a bioassay method[28] to complement HPLC drug analysis of serum specimens. In addition to estimating the concentration of some drugs, such as the active metabolite of proguanil (cycloguanil),[29] the bioassay could provide comparative data of biological activity against parasite strains with defined drug susceptibilities. The bioassay could also be used to detect the presence of unrecognised active drug metabolites not yet detectable by HPLC analysis. But perhaps the most important contribution of the bioassay was that now the synergistic antimalarial activity of various serum concentrations could be assessed against different strains of *P. falciparum* after drug administration to either uninfected non-human primates or human volunteers. Thus, vital information about the potential value of various drug combinations could be obtained before formulating drug regimens for malaria prophylaxis and treatment.[15]

Effectiveness of amodiaquine against chloroquine-resistant *P. vivax*. Historically, chloroquine and amodiaquine had been used interchangeably for malaria prophylaxis and treatment because it was considered that malaria parasites were equally susceptible to both these 4-aminoquinoline drugs. However, in 1969, amodiaquine was shown to be more effective than chloroquine in treating multidrug-resistant infections of *P. falciparum*.[30] Would chloroquine-resistant *P. vivax* respond in the same way? The findings in 2 *Aotus* monkeys inoculated with the AMRU 1 isolate indicated that this isolate was also more susceptible to amodiaquine and that this 4-aminoquinoline drug could play a useful role in the treatment of chloroquine-resistant vivax infections.[31]

Malaria transmission studies

Malaria transmission via mosquitoes using cultured parasites

As part of AMRU's collaboration with the Walter and Eliza Hall Institute (WEHI) and the Australian Malaria Vaccine Joint Venture (Saramane Pty Ltd), colony specimens of *An. farauti* 1 needed to be infected with *in vitro* cultured *P. falciparum* gametocytes. This would enable immunisation studies to assess potential vaccine candidates as well as future trials to evaluate the efficacy of antimalarial drugs. The *An. farauti* 1 colony was chosen for this work as, unlike the *An. farauti* 2 and *An. farauti* 3 colonies, it was self-mating and sufficient numbers to support the work could be readily maintained. The procedure of producing cultured gametocytes and infecting anophelines was being routinely carried out by Dr Imogene Schneider's team at Walter Reed Army Institute of Research (WRAIR) using the NF54 (WR) strain of *P. falciparum* and *An. stephensi*. During February and March 1987 Captain Bob Cooper learnt the technique at WRAIR and then transferred it to AMRU. Despite being able to induce *P. falciparum* cultures to produce healthy gametocytes, there was a persistent failure to infect *An. farauti* 1 past the ookinete stage. To determine whether *An. farauti* 1 was indeed refractory to cultured *P. falciparum* gametocytes, colony *An. farauti* 1 material was sent to Dr Schneider who ran parallel infection studies with the same batch of gametocytes fed to both *An. farauti* and *An. stephensi*. The results showed that while 70% of *An. stephensi* became infected to the

sporozoite stage, only 2.1% of *An. farauti* 1 were infected and this only to the oocyst stage.[32] The importation of *An. stephensi* into Australia was not possible due to quarantine regulations and, in the absence of a competent vector, these studies were abandoned pending changes in the quarantine regulations.

Malaria transmission in *Saimiri* and *Aotus* monkeys

Aotus monkeys, first received at the Unit in 1982, continued to be regarded as the best experimental host for human malaria parasites. Although breeding pairs of *Aotus* monkeys were producing live births, older monkeys were dying from old age, so that the colony was not increasing in size. As further *Aotus* monkeys could not be obtained from overseas, *Saimiri sciureus* monkeys were acquired from the Commonwealth Serum Laboratories (CSL), Melbourne, to determine whether they could be used as an alternative host for human malaria parasites, especially *P. vivax*.

Commencing in 1989 attempts were made to infect *Saimiri* and *Aotus* monkeys with various strains of *P. vivax* (Chesson and Salvador 1). Following isolation of the chloroquine resistant strain - designated as AMRU 1 - from an ADF soldier exercising in Papua New Guinea, studies using this strain became the main focus of the work. The aim was to adapt and characterise the natural course of infection of this strain in *Saimiri* and *Aotus* monkeys so that a monkey malaria model could be used to evaluate the efficacy of antimalarial drugs.

Studies with *Saimiri* monkeys showed that these animals were refractory to the chloroquine resistant AMRU 1 strain of *P. vivax*, though they were infected with the AMRU 2 strain which was a chloroquine sensitive strain isolated from another ADF soldier who had become infected in PNG.[33]

Aotus monkeys, on the other hand, could be infected quite readily with the AMRU 1 strain. Over a period of a year 12 monkeys were inoculated with AMRU 1 parasites, either by blood passage or using ccryopreserved parasites. Parasites appeared in the peripheral blood of all monkeys within 3-18 days (mean 7.5 days) following inoculation, with maximum parasitaemias (range 1200 - 68800/μL; mean 20587) being achieved within 19 days of inoculation. However, within 13 days, parasitaemias in all monkeys fell to less than 500/μL. Gametocytes were produced during the course of the infection and appeared to be most infective 3-4 days prior to peak parasitaemia. Infection rates in *An. farauti* 1, *An. farauti* 2, and *An. farauti* 3 were 16.8%, 8.9%, and 16.9%, respectively, though rates as high as 96% were observed in some batches. Attempts to transmit infections via the bites of these infective mosquitoes were achieved in 9 out of 15 monkeys, with an average prepatent period of 32 days (range 15-79 days).[34] The ability to use sporozoites to initiate infections in monkeys offered the potential to develop a cyclical model for the evaluation of antimalarial drugs against the liver stages of *P. vivax*.

Distribution of Anopheline mosquitoes in northern Australia

Operation Anopheles, initiated in 1984 to determine the distribution of the *An. farauti* sibling species in northern Queensland, was extended during 1985-1990 to cover all of northern Australia where malaria outbreaks had previously occurred and to include all species of anophelines. Cape York Peninsula was surveyed in 1985 and 1986, the Gulf of Carpentaria in 1987 and 1988, and the Northern Territory in 1989 and 1990. The work was supported by members of the 1st Preventive Medicine Company. Kiowa light observation helicopters from 162 Reconnaissance Squadron, Army Aviation, provided access to the remote and isolated areas of the region. Adult anophelines, collected by using CO_2 baited light traps, were identified morphologically. This was also done with adult mosquitoes which were reared from larvae that had been collected from breeding sites throughout the survey area.

Specimens identified as *An. farauti* were cryopreserved and transported back to AMRU where the isomorphic species were separated using isoenzyme electrophoresis or P^{32} DNA labelled probes. The isoenzyme electrophoresis method, developed by Dr Rod Mahon (Division of Entomology, CSIRO, Canberra), worked well for specimens collected in northern Queensland but failed to identify *An. farauti* specimens collected in the Northern Territory. The latter specimens were examined using species specific DNA probes designed to separate the isomorphic species - *An. farauti* 1, *An. farauti* 2, and *An. farauti* 3.[35] This method, developed in collaboration with Dr Tom Burkot and Leanne Cooper of the Queensland Institute of Medical Research, does not require field specimens to be preserved as stringently as those collected for analysis by isoenzyme electrophoresis. This is due to the DNA molecule being quite robust and less susceptible to degradation than enzymes.

An. farauti 1 could also be identified by a simple field technique not requiring the sophisticated equipment used for isoenzyme electrophoresis and DNA hybridisation. It relied on the fact that larvae of this species breed in brackish water and can tolerate higher levels of salinity than either *An. farauti* 2 or 3. Thus, exposure of first instar larvae to sea water for 1 hour killed 0.5% of *An. farauti* 1 larvae but >99.1% of *An. farauti* 2 and 3 larvae.[36]

Of the 12 species of anophelines collected from 620 sites, the 9 most common were *An. farauti* 1 (128 sites), *An. farauti* 2 (67 sites), *An. farauti* 3 (93 sites), *An. annulipes* (335 sites), *An. bancroftii* (181 sites), An. meraukensis (162 sites), *An. hilli* (88 sites), *An. amictus* (93 sites), and *An. novaguinensis* (70 sites). All species were found in areas with rainfall above the 1200 mm p.a. isohyet, but only *An. annulipes* and *An. amictus* were found below the 1000 mm p.a. isohyet. While the breeding of most species was dependent on adequate rainfall, *An. farauti* 1 and *An. hilli* also required brackish water. Geographically, the distribution of the three isomorphic species of *An. farauti* varied from one area to another. *An. farauti* 1 was common along all coastal areas of northern Australia. *An. farauti* 2 was the most common and widespread of the three isomorphic species in Cape York Peninsula, while *An. farauti* 3 was the most abundant species in inland areas of the Northern Territory.[37-39]

Operation Anopheles indicated the wide distribution of various species of the *An. farauti* taxon, the main malaria vectors in northern Australia. As vast tracts of the Northern Territory and northern Queensland within the range of these species are frequently used by the ADF for training exercises, they remain a potential threat to ADF personnel if malaria is re-introduced into Australia. In 1989-1990, about 1000 malaria cases were imported annually into Australia, of which about 1 in 20 were in Army personnel returning from overseas exercises. A small malaria outbreak did, in fact, occur in northern Queensland. Although it was not related to any movement of military personnel, it illustrated the importance of prompt malaria diagnosis and treatment to decrease the likelihood of gametocytes infecting local vector mosquitoes.

Potential of Microsporidia for mosquito control.

Earlier field observations at Mildura conducted by AMRU had discovered a microsporidian – *Amblyospora* - parasitising *Culex annulirostris* mosquitoes in Mildura, Victoria.[5] With the support of research grants from the National Health and Medical Research Council and the World Health Organization, the complete life cycle of *Amblyospora* in mosquitoes and the copepod intermediate host was established. This provided the opportunity to evaluate the potential value of these parasites as biological control agents of mosquitoes.

The particular *Amblyospora* species infecting *Cx. annulirostris* mosquitoes and *Mesocyclops albicans* copepods was described as *Amblyospora dyxenoides*.[40] Its infection rates in copepods were directly proportional to the concentration of spores (derived from dead infected mosquito larvae) to which they were exposed.[41]

Subsequent studies with another microsporidium - *Amblyospora indicola* – infecting Culex sitiens (with *Apocyclops* as the copepod intermediate host) showed that its life cycle was similar to that of *Amblyospora dyxenoides*. Furthermore, the developmental stages of these two microsporidia looked to be the same in both mosquito hosts and also in the different copepod species.

The possibility that these two microsporidia were the same species was investigated by host specificity experiments in which spores produced in both mosquitoes were exposed separately to the two copepods. The results indicated that spores from *Cx. annulirostris* were not able to infect *Apocyclops* sp. copepods and that spores from *Cx. sitiens* were not infectious to *M. albicans*. Further experiments showed that copepod spores produced in *M. albicans* were infectious to *Cx. annulirostris* larvae but not to *Cx. sitiens* larvae. Similarly, spores from *Apocyclops* copepods were infectious to *Cx. sitiens* but not to *Cx. annulirostris* larvae. These findings suggested that there was a high level of specificity of these microsporidia in their invertebrate hosts.[42]

A computer simulation model of the dynamics of *A. dyxenoides* in its mosquito and copepod hosts showed that the biological complexity of this host-parasite system made it difficult to adequately assess its potential for control of mosquito populations.[43] Moreover, there were technical problems in the production of inocula for field release that would have to be solved before this approach could be implemented, including the artificial culture of microsporidia and the mass laboratory rearing of spores in copepods or mosquito larvae.[44] Even though the specificity experiments were based on only two mosquito/copepod combinations, each *Amblyospora* species probably only infects a single mosquito host species, thereby preventing its biocontrol potential against a range of disease vectors. For these reasons it was concluded that, on the basis of current knowledge, such parasites could not be considered as promising biological control candidates of mosquitoes.

No further investigations with any other potential biological agents were carried out, including some that were discovered accidentally during Operation Anopheles in northern Queensland. They included *Culicinomyces bisporalis*, *Crypticola clavulifera* and *Lagenidium giganteum*, observed in mosquito and biting midge larvae living in water contained in plant leaf axils near Millaa Millaa.[45-48]

Figure 2: Entomology staff at Cowley Beach Training Area, Queensland.

(L to R) LTCOL A. W. Sweeney, CAPT R. D. Cooper, SGT C. Rogers, LT S. P. Frances.

Insect repellents

Deet – applied to skin. The topical application of mosquito repellents was (and continues to be) an important means of protecting oneself against malaria. The ADF mosquito repellent, a liquid formulation containing 95% diethylmethylbenzamide (also known as diethyltoluamide, commonly called deet) in ethanol, felt oily when applied to skin and was not particularly well accepted by soldiers in the field. As there was also some concern about the safety of using such high concentrations of deet, studies were undertaken by AMRU to evaluate alternate approaches to reducing vector-human contact.

A novel approach, developed by a chemist in Melbourne, was to incorporate 20% deet and 0.5% permethrin in a bar of soap, and using it as a repellent by rubbing moistened soap onto the surface of the skin. When field studies were undertaken at Homebush Bay, Sydney and the Cowley Beach Training Area in Queensland. the soap formulation provided protection against mosquitoes for only one hour, much shorter than the ADF mosquito repellent.[49]

Permethrin – impregnated in uniforms and bednets. Another field study at Cowley Beach was conducted using an alternative approach for repelling mosquitoes – the impregnation of clothing with permethrin, a synthetic pyrethroid compound. The results showed that impregnated jungle green uniforms prevented more mosquitoes from biting than untreated ones.[49]

In 1989, permethrin-impregnated uniforms and bednets were first used by ADF personnel during their service with the UNTAG Mission to Namibia. The effectiveness of permethrin impregnation was monitored by placing swatches of fabric in the pockets of Disruptive Pattern Combat Uniforms (DPCU) and by attaching them to ADF bednets before treatment. Samples returned to AMRU by the health officer for chemical analysis showed that the treatment was carried out successfully. Furthermore, bioassay of the samples showed a marked knockdown and mortality of *An. farauti* mosquitoes. However, due to operational limitations, it was not possible to determine how long the permethrin treatment persisted in DPCU.

Also in 1989, the persistence of permethrin in bednets was investigated in collaboration with the World Health Organization entomologist in Honiara, Solomon Islands. Net samples used in the field evaluation of impregnated nets on Guadalcanal were sent to AMRU before treatment and 2, 5, and 8 months after treatment. Chemical analysis and bioassays showed that permethrin persisted in treated nets for 8 months, but was lost more readily from the bottom of nets, probably due to abrasion after handling.

Deet combined with permethrin. In a collaborative project with the Letterman Army Institute of Research, US Army, the efficacy of 2 new 'controlled release' formulations of deet and the US Army's standard repellent (75% deet in ethanol) were compared with each other at the Cowley Beach Training Area.[50] In addition, mosquito biting activity was determined when each of these topical skin applications were used in combination with permethrin-impregnated uniforms. This was the first US Army/ Australian Army entomological research undertaken since World War II. The 3 deet formulations and the impregnated uniforms had similar repellent activity. However, when any of the deet repellents were used in combination with the impregnated uniforms, there was a marked decrease in mosquito biting activity.[50]

Repellents against "Chiggers". Studies were also carried out to evaluate the activity of various repellents against the chigger mite, Eutrombicula hirsti. Trombiculid mites can transmit scrub typhus, a potential public health problem for soldiers in northern Australia. They are also responsible for 'Scrub Itch', a painful condition which is often encountered by soldiers in the wet tropics. After establishing a colony of these mites and studying their biology,[51] repellent studies showed that low concentrations of deet, dimethylphthlate, benyl benzoate and permethrin were highly toxic to chiggers.[52] A subsequent field study conducted in 1990 at Cowley Beach Training Area showed that Disruptive Pattern Combat Uniforms treated with permethrin and dibutylphthalate provided excellent protection against *E. hirtsi* chiggers.[53]

These investigations represented the first Australian research in this field since World War II.

Conclusion

The first half of the third decade (1985-1990) was characterised by greater emphasis on practical problems facing ADF personnel in malarious areas. *In vitro*, *in vivo* and *ex vivo* tests and procedures were used successfully to investigate the increasing prevalence of *P. falciparum* and *P. vivax* to standard antimalarial drugs. These investigations revealed, for the first time, that *P. vivax* could develop resistance to chloroquine, the standard drug used world-wide for the prevention and treatment of vivax malaria. Laboratory and field studies were also able to identify potentially useful drugs and drug combinations for preventing drug-resistant malaria infections. Although wider biological control of mosquitoes proved difficult, significant advances were made in identifying repellent formulations and procedures for providing better personal protection against mosquitoes and other arthropods. As northern Australia remains receptive to malaria, the Unit conducted the most extensive survey of anopheline mosquitoes and characterisation of malaria vectors ever performed in that region.

Acknowledgement

The opinions expressed are those of the authors and do not necessarily reflect those of the Joint Health Command or any extant Australian Defence Force Policy.

Highlights

1985

- Colonel Eric Donaldson appointed fourth Director (up to 1987).
- Professor Karl Rieckmann appointed Director of Medical Research.
- *Plasmodium falciparum in vitro* studies with Mannich base compounds (up to 1990).
- Annual surveys and characterisation of anopheline mosquitoes in northern Australia (up to 1990).
- Experiments with microsporidia (*Amblyospora*) for biocontrol of mosquitoes (up to 1990).

1986

- Ex vivo and pharmacological studies with proguanil and low-dose dapsone.
- Trials with topical mosquito repellents and insecticide-impregnated clothing and bed nets (up to 1990).

1987

- First documented evidence of *P. falciparum* resistance to Maloprim in PNG.
- Attempts at transmission of cultured *P. falciparum* via mosquitoes (up to1988)

1988

- Field study with proguanil and low-dose dapsone.
- Upsurge in malaria during ADF exercises in PNG.

1989

- Professor Karl Rieckmann appointed fifth Director (up to 2006)
- Discovery that *P. vivax* is able to develop resistance to chloroquine.
- Course of chloroquine-resistant *P. vivax* infections in non-human primates and their cure by amodiaquine.
- Evaluation of doxycycline and mefloquine for malaria prophylaxis

Authors' affiliation: Australian Army Malaria Institute
Corresponding author: Karl Rieckmann
Email: krieckmann@iprimus.com.au

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