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REGIONAL WORKSHOP FOR MONITORING P. FALCIPARUM RESPONSE TO ANTI-MALARIAL DRUGS

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I. INTRODUCTION

A Regional Workshop on monitoring *P. falciparum* response to anti-malarial drugs was held at the National Training Centre, Lahore, Pakistan, from 3 to 20 August 1986. Fifteen participants from eight countries of the Eastern Mediterranean Region attended the workshop as shown in Annex I. Resource personnel are also listed in the same annex. The workshop was based on a series of introductory lectures, discussions, seminars and practical sessions in the laboratory and exhaustive field experience in villages around Lahore. The Agenda and Programme of Work appear as Annexes II and III respectively of this report.

The workshop was officially opened by Lt. Gen. Mohammed Abu Zafar Mohydin, representing the Ministry of Health, Pakistan, and Dr Nabil S. Al Tawil, WHO Representative in Pakistan. Dr Hussein A. Gezairy, Director, WHO Regional Office for the Eastern Mediterranean, in his opening message emphasized that *P. falciparum* resistance to anti-malarial drugs is considered to be one of the most important problems facing malaria control programmes in various countries. He mentioned that there is an urgent need to fill in the gaps in our knowledge about the distribution of resistant strains in the Region and to develop models for anti-malarial regimens and drug combinations that could be used effectively at affected foci or, when necessary, country-wide. Such information would be most valuable to public health specialists, clinicians and malariologists engaged in the prevention and control of malaria.

The Regional Director expressed his special thanks to AGFUND for its recognition of the seriousness of the drug-resistant malaria problem and the threat posed to many countries of the Eastern Mediterranean Region. AGFUND has shown its interest by making a generous contribution to this workshop and it will, in the future, be assisting with national workshops aimed at monitoring and following up the pattern of drug resistance in the Region.

II. OBJECTIVES

The main objectives of the workshop were to familiarize participants with the resistance phenomenon and how to monitor *P. falciparum* response to anti-malarial drugs by *in-vivo* and *in-vitro* methods.

III. THE IMPORTANCE OF STUDYING DRUG RESISTANCE IN MALARIA

Drug resistance in malaria has been defined as the "ability of a parasite strain to survive or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject" (WHO Technical Report Series No. 246).

Of the four *Plasmodium* species that affect man only *P. falciparum* has demonstrated the ability to develop isolates or strains, possibly through mutation, resistant to chloroquine, the most commonly used anti-malarial drug. Once such a strain has developed it propagates gradually through drug pressure, replacing the drug-sensitive strains. Other human *Plasmodium* species have also shown resistance to pyrimethamine and closely related drugs.

The emergence and spread of drug-resistant strains of human malaria parasites may constitute a serious problem for national health services. This is reflected by the necessity of changing malaria chemotherapy and anti-malarial operations. Drug-resistant cases may either be imported from an existing focus or emerge locally as a result of mutation and selection, particularly under excessive drug pressure. Rapid identification and notification of such cases are essential for the timely administration of appropriate treatment and the institution of remedial measures.

The data collected are first evaluated at country level. Communication of information within the country is a national responsibility. The exchange of information must be continuous and based on medical practitioners' awareness of the danger presented by the appearance of drug-resistant strains of malaria. Alertness should be maintained by means of medical literature, circulars and the distribution of data from national and international sources.

At the international level, each government has been requested by the World Health Assembly Resolutions WHA10.32, WHA13.55 and WHA22.48 to provide WHO with detailed information on the status of sensitivity or resistance of *P. falciparum* to anti-malarial drugs. This information from countries is first collected and evaluated in the WHO Regional Offices and then at global level at WHO Headquarters. The global data are published once a year in the WHO Weekly Epidemiological Record. This publication is sent on a regular basis to the national authorities concerned. The information is also circulated in the form of other WHO literature published by Regional Offices and Headquarters in regular WHO serial publications.

IV. SITUATION ANALYSIS

Chloroquine-resistant *falciparum* malaria was first identified in a border area between Vietnam and Thailand more than 30 years ago. At first, it was thought to be of relatively little operational significance. However, the resistant parasites spread with astonishing rapidity, gaining wide geographical distribution and developing an ever greater degree of resistance with increasing frequency. Malaria parasite resistance to chloroquine has now been documented in almost all countries of the Western Pacific and South-East Asia Regions of WHO, as well as in some countries of its African Region.

The situation in some parts of the world is alarming. In Thailand, 95% of all *P. falciparum* cases studied were found to be resistant to chloroquine. Moreover, resistance has been demonstrated to other anti-malarial drugs, for example the combination of long-acting sulphanomides with pyrimethamine.

The main factors that appear to influence the spread of *P. falciparum* resistant strains are:

(a) proximity to areas affected by resistant strains;
(b) intensity of transmission in the area;
(c) the ability of the local vector to transmit a particular *Plasmodium* strain;
(d) the number of workers, immigrants or international travellers moving between an affected area and a given country;

(e) the amount of drug pressure exercised by a national control programme;
(f) the efficiency of the control programme in a country and the awareness of the problem.

Resistance, however, is not an "all or none" phenomenon. Chloroquine can still be effective and provide clinical cure, especially if there is some immunity in the patient. The presence of resistant parasites in a given country does not necessarily mean that they are widespread, although they may rapidly become so.

On the basis of the above mentioned factors, most of the countries in the Eastern Mediterranean Region are vulnerable to the spread of chloroquine-resistant *falciparum* strains.

In Pakistan, the most easterly country of the Region, the first resistant cases were reported in 1980, from one district in Punjab Province. Since then, resistance has spread to other districts and it is thought to be more widespread and more intense and severe than originally estimated.

A recent study in Sudan has revealed the presence of chloroquine-resistant malaria in Khartoum Province.

More recently, chloroquine-resistant *P. falciparum* strains were detected in Afghanistan, Islamic Republic of Iran and Somalia.

V. THE REGIONAL PLAN FOR CONTAINMENT OF DRUG-RESISTANT MALARIA

The Regional Plan envisages the following approaches to detecting and containing resistant *P. falciparum* strains:

1. The training of medical officers and technicians in the detection of *P. falciparum* resistant strains by both *in-vivo* and *in-vitro* testing techniques. Following the Regional Workshop in Lahore, eight national workshops have been planned to be held to train more teams. The Regional Office is providing necessary test kits and is willing to collaborate with countries in conducting national workshops.

2. Monitoring of the response of *P. falciparum* to anti-malarial drugs will be increased in areas where malaria transmission is intensive as well as in areas bordering countries where resistance has been confirmed. National authorities are being asked to report routinely to WHO full details of any resistant case detected in the country.

3. Efforts to be made to prevent the introduction of resistant strains. Vulnerable countries should devise mechanisms by which travellers coming from endemic areas could be screened within one week of their arrival. As far as practically possible, parasite carriers should not be allowed to settle in or visit areas where transmission exists until they have undergone radical cure.

4. Spread of resistant strains must be prevented by intensifying control measures in areas where such cases are detected.

Of particular importance are the measures taken against the vector to interrupt transmission. Mass surveys and radical cure of patients should be undertaken if the area is limited. Monitoring to detect resistant cases

should be intensified, not only in the area concerned but also in neighbouring areas.

5. As a general rule, intensive control measures should be undertaken in:

(i) areas where resistance has been confirmed;
(ii) areas bordering countries in which resistance has been confirmed;
(iii) areas in the country where *P. falciparum* transmission is high.

VI. THE WHO STANDARD *IN-VITRO* MICRO-TEST SYSTEM

Although requiring rather more sophisticated equipment and manipulatory skill, the micro-test system is much more rapidly adaptable to the screening of the large numbers of isolates needed to establish the response of *P. falciparum* to standard anti-malarial drugs since the test requires only small quantities of blood (100 µL) obtainable from the finger-tip. Furthermore, the system is refined and simplified to make testing feasible at the basic field level.

VI-1. Application of the test system

WHO standard test systems are not primarily intended as individual diagnostic tests but rather as an epidemiological tool for determining the status of drug susceptibility of *P. falciparum* in a given population and for monitoring (or detection of) the development and spread of resistance within the community.

A Global Monitoring Programme has been established under the aegis of the Malaria Action Programme and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

The decision to develop a *P. falciparum* monitoring programme within a specific country is usually based on one or more of the following criteria (not necessarily in order of importance):

- evidence of failure of treatment with the recognized curative dose, as revealed by reports from hospitals, medical practitioners and outpatient clinics;
- the failure of previously effective treatment regimens in non-immunes;
- reports of resistance from adjacent countries/areas;
- the movement of non-immunes and semi-immunes in and out of malarious areas (usually associated with refugees or mobile labour forces);
- the screening of immigrants and visitors coming from malarious areas with resistance and about to enter areas of potential malaria transmission;
- the need to establish a routine monitoring programme of currently used and alternative anti-malarials with a view to developing a rational programme of drug regimens.

VI-2. Size of field team

The following considerations should be borne in mind in the staffing requirements for a field team:

Team leader - should be, ideally, a medical officer with a good background in clinical pharmacology of anti-malarials, parasitology, demography and

statistics, and with experience of the *in-vitro* and *in-vivo* test systems for drug response.

Field operations supervisor - should be a field operations worker with a good knowledge of the study area, able to read maps, and qualified to organize all aspects of the screening of populations for malaria and the selection of suitable malaria patients. A good understanding of the *in-vivo* test system is also essential.

Laboratory operations supervisor - should be a laboratory worker conversant with all aspects of running a parasitological laboratory for the study of malaria and with the use, application and interpretation of *in-vitro* and *in-vivo* test systems.

Assistants - should be persons trained in all aspects of the *in-vivo* and *in-vitro* test systems and able to perform tasks in either the operational or laboratory spheres.

A field team with the potential for 50 *in-vitro* tests per working week would consist of:

1 medical officer (team leader)
1 field operations supervisor
1 laboratory operations supervisor
2 polyvalent workers
1 driver

VI-3. Selection of study areas

The micro-test is the test of choice for larger-scale studies.

The selected area should meet the following minimal requirements:

- it should be reasonably accessible to the team and the supportive elements that may be required;

- it should have been approved by a prior agreement with and enjoy the support of the interested institutions and bodies in the area of operations and, where necessary, the appropriate written authorizations should have been obtained;

- it should have an adequate population for sampling purposes and a prevalence rate of *P. falciparum* sufficient to provide an adequate supply of positive cases suitable for test purposes;

- it should have facilities for the establishment of an adequate laboratory or be within reasonable proximity to suitable facilities.

To attempt studies in areas which have not been adequately researched can lead to expensive loss of time, effort and material.

VI-4. Selection of cases

The principal sources of positive malaria cases are:

(a) Hospital in-patients and clinic out-patients

In areas of endemic malaria, positive cases visiting hospitals and clinics have often taken anti-malarials on their own initiative; therefore only a relatively small proportion may prove suitable for testing. However, such sources may be highly productive in malaria epidemic situations.

(b) Schoolchildren in areas with relatively high malaria endemicity

Whenever possible, this is the subject group of choice, as schoolchildren can be readily identified, easily relocated when required, grouped by age, and have relatively high prevalence rates, particularly in the more junior classes.

(c) Pre-school children, pre-natal and post-natal clinics

Both young children and pregnant women have high malaria prevalence rates in endemic and epidemic situations. However, the general reluctance of mothers to participate in a trial, the difficulty of handling very small children, the problem of follow-up and, not least, the ethical considerations, make these two groups difficult to work with.

VI-5. Sample and screening of study populations

VI-5.1. Sample size

Sample size will depend on the prevalence of resistance in the population; this is usually an unknown quantity. Therefore, a "rule of thumb" is to test a sample of 50 selected *P. falciparum* patients with the aim of obtaining a successful test in 30 or more individuals from the group. If prevalence of resistance is known, or can be estimated, a more precise sample size can be determined in advance, using standard statistical sampling criteria.

VI-5.2. Screening of study populations

VI-5.2.1. Hospitals and clinics

Some idea of the incidence of the new malaria cases can be obtained from current medical records or those for a comparable period in previous years. Unless these records show a daily average of at least 10 to 12 cases of clinical malaria, it is likely that the few cases found suitable for testing will not justify the effort involved since the following minimum criteria must be met:

- no history of taking anti-malarials (some antibiotics are parasiticidal or parasitistatic) during the previous 14 days (28 days for sulfanomides and pyrimethamine; 56 days for mefloquine);
- a urine negative for chloroquine and sulfanomides;
- a pure *P. falciparum* parasitaemia of at least 1000 asexual parasites per mm^3 of blood, but less than 80 000 mixed infections should not be used.

VI-5.2.2. Schools

With adequate advance preparation large-scale screening surveys can be made of schoolchildren; highly satisfactory collections can be made when the rate of *P. falciparum* parasitaemia in the target group equals or exceeds 40%. In such groups the screening of 300 children will produce 120 or more positive *P. falciparum* cases of which about one-half/one third will have parasitaemias of 1000 or more asexual parasites per mm^3 of blood.

VI-5.2.3. General population

Unless there is a particularly strong group and/or discipline or an epidemic malaria situation warranting anxiety, it is generally very difficult to screen sufficient numbers from other suitable age groups by this method.

VI-5.3. The screening process

Apart from ethical considerations, it is not reliable to base collection of *P. falciparum* isolates on the results of blood slide examinations done many hours before the patients are selected and blood withdrawn; tests carried out on the basis of blood slides collected the previous day are even less reliable as many isolates will subsequently be found to have sub-critical parasitaemias. Accordingly, the screening process should be as rapid as possible and only parasitaemias with 1000 - 80 000 asexual parasites per mm^3 of blood considered as acceptable.

VI-6. Preparation for the test

VI-6.1. Identification of the selected cases

Once suitable cases have been selected and the corresponding donors identified, the necessary data are transferred to the form "Response of *P. falciparum* to chloroquine and mefloquine (*in-vitro* test)" or the similar form for amodiaquine and quinine. These forms were specifically developed for the WHO Global Programme for the Monitoring of Drug Response in *P. falciparum*.

The donor is then:

- questioned about his/her movements over the past 12 months;
- asked to give any history of treatment or prophylaxis for malaria over the previous 28 days (56 days if mefloquine is being used in the area);
- tested for the presence of chloroquine and/or sulfanomides in the urine;
- weighed and the appropriate malaria treatment calculated (the treatment is not given until after the blood sample for the *in-vitro* test has been taken).

VI-6.2. Preparation of RPMI 1640 medium

Once the number of samples to be selected is known, an appropriate quantity of RPMI 1640 medium is prepared and aliquoted into holding tubes of 6 mL capacity which are labelled with the case numbers. When feasible, the most efficacious system is obviously to do tests in multiples of twelve (each pre-dosed plate takes twelve isolates).

It is not advisable to aliquot the medium until just before it is required; fresh medium should be prepared for each test day.

Predosed plates. These have a minimum shelf life of 24 months at ambient temperatures at 20°C or below. If necessary store at +4°C.

HEPES/$NaHCO_3$. The sealed solution is stable at normal room temperatures, but in areas where very high ambient temperatures are usual, store at +4°C. Once opened, the solution should be used or discarded within 14 days.

RPM 1640. Lyophilized medium must be stored at all times at 4°C; it quickly deteriorates at room temperatures. Normal life under these conditions is 12 months.

All these components of the test kit should be allowed to return to room temperature before use.

VI-6.3. Blood sample withdrawal

The principal dangers at the early stage of the micro-test are clotting and contamination by bacteria and fungi.

The former can be avoided by ensuring a rapid flow of blood from the puncture site so that it is not necessary to manipulate the finger of the donor too much. The "Autolet" automatic pricker provided with the test kit greatly facilitates the process as it gives a clean prick of adequate size and is relatively painless.

The problem of contamination can be overcome by ensuring that the puncture site is carefully cleaned with 70% alcohol and that the heparinized tube is kept sterile during manipulation.

Experiments show that the heparinized microcapillary tube will prevent clotting for at least five minutes but transfer to the medium-holding tube should be as rapid as possible.

VI-6.4. Transportation of the test sample

All evidence points to higher success rates if the transportation period is kept to a minimum. Moreover, there are indications that *P. falciparum* isolates resistant to chloroquine travel much better than sensitive ones. Accordingly, in areas with a high proportion of sensitive isolates a reduction in transportation time may significantly improve the test results.

Temperatures higher than 40°C must be avoided or averted by the use of refrigerated containers and incubators.

Almost all isolates travel well for three hours when carried in RPMI 1640 medium at ambient temperatures between 25° and 40°C. Transportation on wet ice is feasible and successful tests have been made on isolates so carried after 24, 48 and 72 hours.

Ideal transportation time seems to fall within the 0-3 hours range and maintenance of the isolate/medium mixture at near body temperature (such as obtained by carrying the vials in a pocket) also seems to encourage successful test results.

VI-7. Conducting the test

VI-7.1. Aliquoting

The main difficulties encountered in the aliquoting of blood are: the maintenance of a homogeneous suspension of the blood cells throughout the range of wells, the risk of contamination of the test by bacteria or fungi and the risk of self-inoculation with pathogens in the blood.

The 50 µL Eppendorf pipettes supplied with the micro-test kit are extremely reliable and accurate. From time to time, they require the simple servicing described in the leaflet provided with each pipette.

When contamination occurs in the micro-test it is usually due to a contaminated blood sample. However, talking, coughing or sneezing during the performance of the test or careless pipetting technique will also cause gross contamination which is incompatible with an adequate growth of the parasite during the 24-hour incubation period.

VI-7.2. Incubation

The environmental requirements for the micro-test are more exacting, since it not only requires a controlled temperature (ideally 38°C $\pm$ 0.5°C) but also a CO_2-enriched and O_2-reduced atmosphere. This can be obtained even at field level by means of the standard "candle jar" technique.

Suitable conditions for the micro-test can be obtained with any of the following: an incubator in which CO_2 and O_2 can be regulated; a standard incubator with a candle jar, a waterbath with the lid sealed to provide an air-tight chamber equivalent to the candle jar; or a waterbath with a candle jar.

Most trophozoites will have matured into schizonts after 24 hours. When trophozoites are very young, 26 or even 28 hours may give suitable growth but there is an increasing danger that some of the schizonts will rupture, releasing merozoites producing re-invasion. This will make a reliable count difficult. Incubation times exceeding 30 hours change the test conditions and such results are not valid for the Global Monitoring Programme.

The addition of 5/10 mL of distilled water to the base of the candle jar largely prevents evaporation of the contents of the wells of the test plate during incubation.

The ideal incubator should be robust, portable and reliable; it should provide a stable temperature and have low power requirements; it should be long-lasting and moderately priced.

Proper calibration of the incubators is essential for ensuring consistent success. Work programmes must always allow ample time for achieving this before testing begins.

Finally, temperatures inside the candle jars which are used with the very small portable incubators may be lower by 1° to 2°C than the temperature indicated by the incubator thermometer; these internal temperatures should be established and the appropriate adjustment made.

Candle jars

Maturation of the trophozoite to the schizont in the micro-test requires an enhanced CO_2 and a reduced O_2 tension. If incubators in which CO_2 and O2 can be regulated are not available, an adequate degree of CO2 enrichment can be obtained by burning a pure stearate candle in a vented gas-tight jar and then, when the candle is at the point of extinction, closing the vent.

VI-7.3. Harvesting, staining, examination and counting

The procedures for the post-incubation preparation of the micro-test system are as follows:

VI-7.3.1. Harvesting

The cellular elements of the blood settle at the bottom of the test-wells. If they are re-suspended with the supernatant the mixture will be too dilute to produce a good thick film; the supernatant should therefore be carefully withdrawn with a micro-pipette and absorbed on absorbent paper. The remaining cells, plus a small volume of the supernatant, should then be carefully re-suspended and transferred to the test slide to make up the series of the control and seven concentrations.

VI-7.3.2. Staining

Adhesion of cultured blood to the standard glass slide has been found to be inferior to that of normal blood. Consequently, the drying and staining of the slides require considerably more care. If air-dried these films are not usually ready for staining before 48 hours and are liable to be attacked by insects. To overcome this problem, the use of a low-power airblower (not more than 1000 watts) will rapidly dry the slides and, if care is taken, will not "heat-fix" the thick films. Alternatively, the slides can be placed overnight in an incubator at about 37°C. However, the airblower technique is preferred as it will permit rapid processing of the material.

The best staining results are obtained with a Giemsa stain (Merck Art 9204) at a pH of 7.2 for the micro-test slides. The best dilution is 1% for 30 minutes.

After staining comes the most crucial step: the removal of the slides from the staining jar without contaminating them with the scum produced by the oxidation of the stain nor washing off the very fragile thick films. The most effective procedure is to add, very gently, distilled water to the staining vessel so that it floods and the golden scum floats away. When this is completely flushed off, the slides are carefully removed one by one and placed on a flat surface for drying (face up).

Drying is best effected with an airblower held at a sufficient distance from the slides which must not be flipped over.

Once the slides are dry the films are much more robust but should not be wiped or touched with the fingers.

VI-7.3.3. Examination

The stained and dried slides are ready for examination starting with the control(s) and proceeding through the ascending concentrations. Experience indicates that a total magnification of 700X is optimal and that the use of an ocular graticule with 5 x 5 grid considerably facilitates the counting process.

VI-7.3.4. Counting

A baseline count of the schizonts (with three or more nuclei) is made in the control and the effect of the test drug is measured at the predetermined test concentrations by comparative counts. Hand tally counters are used to tally the counts.

The basecount in the micro-test is a set number of asexual parasites which, of course, include the schizonts. The number of schizonts (with three nuclei or more) counted among 200 asexual parasites forms the basis of comparison between the control and the various concentrations. Obviously, the maximum possible count is 200 schizonts. Occasionally, the counts in the lower concentrations are higher than in the controls.

The minimum acceptable number of schizonts for a satisfactory control in both test systems is 20. If the control growth does not attain this level, the test is not suitable for inclusion in the Global Monitoring Programme.

Schizont counts may also be expressed as a percentage of the control growth, using the following formula:

$$\frac{\text{schizont count at a given concentration}}{\text{schizont count in the control}} \times 100$$

The degree of schizont maturation inhibition can be expressed by subtracting the above from 100, ie.:

schizont maturation = 60% of the control growth
schizont maturation inhibition = 40%

VI-8. Records and recording

Once the principle of a standard WHO *in-vitro* test was established, it became imperative that the data collected using the test system be collated in a standard and comparable way. Accordingly, the Global Monitoring Programme for the determination of the drug response of *P. falciparum* was launched under the auspices of the Malaria Action Programme and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

The programme uses a computer-coded form covering tests with chloroquine and mefloquine. The forms are of a "one time-four copy format"with the original to be retained by the investigator and the other three copies to be sent to (i) WHO Malaria Action Programme, Geneva; (ii) the appropriate WHO Regional Office; and (iii) the inter-country national institute responsible for the coordination of the studies.

WHO Headquarters in Geneva transfers the results of all correctly performed tests on to computer tapes and from this data prepares printouts, analyses and comparative studies for global circulation. Eventually, it is hoped to produce malaria maps indicating the status of resistance in all participating countries.

VI-9. Analysis and interpretation

Instructions for the micro-tests, included in all test kits, provide a graph on which can be plotted the individual test results or, more usually, the group data of a series of tests the sample size of which has been determined by known (or estimated) prevalence of resistance. This simple graphic representation can be used to make a rough estimate of the EC_{50}, EC95, EC99 or any intermediate value.

For a preliminary evaluation it will be appropriate to use the cut-off point of the inhibition of schizont maturation. For instance, schizont maturation in well E of the micro-test plate which is dosed with 5.7×10^{-12} mol chloroquine/blood is indicative of *in-vivo* drug resistance in non-immunes.

VII. PROCEDURES FOR ASSESSING THE RESPONSE OF MALARIA PARASITES TO DRUGS *IN-VIVO*

The first step in assessing drug response is to collect baseline data on the sensitivity of *P. falciparum* to chloroquine, not only in localities from which reports of suspected resistance have been received but also from areas of distribution of this parasite where the drug response appears to be normal. Several tests are available. Selection of the appropriate one should take into account the level of immunity of the subjects to be tested, their clinical condition, and the period of time within which they can reasonably be followed up. A further factor to be considered is the local epidemiological situation that will determine the likelihood of the subjects becoming re-infected during the course of the observation period. The options available are the following:

(a) The WHO Standard Field Test consisting of the administrtion of 25 mg of chloroquine base per kilogram of body weight over three days, with a 7-day observation period (sometimes referred to as the "7-day test").

(b) The same test with the observation period extended to a total of 28 days, referred to as the "extended test".

(c) The single-dose test, or "alternative test", consisting of the administration of 10 mg of chloroquine base per kilogram of body weight. This test is applicable:

  (i) where for any reason treatment cannot be pursued for 3 days;
  (ii) in areas of high endemicity where, owing to the elevated level of immunity in the population, a single dose of chloroquine has been accepted as the standard form of treatment, or
  (iii) as a preliminary screening procedure prior to applying the standard 3-day treatment.

All these procedures are designed for use under field conditions, although the extended test often presents difficulties in the field. They all give an indication of the response of the local *P. falciparum* strains to the dosage of chloroquine used. In principle, they should exclude various cases of drug failure that might otherwise lead to the erroneous belief that chloroquine resistance is present in the area.

Although there is some possibility of vomiting after the first dose when chloroquine is administered by mouth, oral administration is preferred to injection because of its safety, ease and uniformity.

The test must be evaluated by the examination of thick blood films.

Since transmission cannot always be excluded under field conditions, recrudescences cannot always be distinguished from re-infections. A determination of resistance at the RII or RIII level is therefore based on the response of asexual parasitaemia during the first week of treatment. Only if new infections can be excluded will further observations over an additional three weeks yield more conclusive evidence as to recrudescence of parasitaemia, thus permitting the observer to distinguish between sensitivity (S) and the RI level of resistance.

Experience has confirmed that all steps of the test must be carried out, or at least supervised, by responsible and qualified technical staff.

(1) Field test for response to a standard regimen of chloroquine

The field test may determine the response of a strain of malaria parasite to a standard test dosage of chloroquine (25 mg/kg over three days, starting on day 0). The test may be performed on subjects irrespective of age, parasite count, and previous suppressive therapy. However, it should not be carried out on a person who is seriously ill.

(2) Procedure for standard and extended field tests

One dose of chloroquine is given on each of three successive days (a total of 1.5 g of base for a 60 kg adult) according to the following schedule:

day 0: first dose - 10 mg/kg (600 mg of base for a 60 kg adult)

day 1: second dose- 10 mg/kg (600 mg of base for a 60 kg adult)

day 2: third dose - 5 mg/kg (300 mg of base for a 60 kg adult)

The chloroquine tablets must not be coated and must comply with the standards laid down by the International Pharmacopoeia or the national pharmacopoeia of the country. At the time of each drug administration precautions must be taken to ensure that the drug is swallowed and retained. To avoid nausea or vomiting, the drug should not be taken on an empty stomach. Subjects who vomit should not be used for the test.

For obvious reasons, severely ill patients should be excluded from the test. Those with mixed infections should also be excluded so as to avoid confusion over species identification. It is desirable, where possible, to include persons with high parasite counts; in practice, this will mean young children in highly endemic regions.

At all times the clinical condition of the patient must take precedence over the conduct of the test. If the parasite count is excessively high or the patient is ill at any time, it is advisable, in areas of suspected chloroquine resistance, to administer drugs of other types, such as quinine.

The subjects of the test should be observed daily for seven days after the first day (day 0) of drug administration. Even a 7-day observation period may be impracticable under field conditions but should be insisted upon if possible. The standard 7-day field test does not permit distinction between sensitivity (S) and resistance at the RI level. Extended observation for an additional 21 days will usually distinguish between sensitivity and RI resistance; this is the extended field test.

The results of the test may be recorded on WHO form 4518.1 EME/MAP.

Duplicate thick and thin blood films should be made immediately before the first test dose and repeated daily for at least seven days, one of each set being kept for reference. Parasite counts should be made and the species of parasite identified because *P. malariae* trophozoites may persist seven days after the start of the test procedure. A thick film is considered negative when the examination of 100 fields fails to reveal any asexual parasites. Whenever possible the urinary excretion of chloroquine should be determined by a suitable method.

Urine should be collected prior to drug administration on day 0 or the previous day, and at least once during days 1-3 after the beginning of treatment (preferably on day 1 or 2).

The number of persons with symptomatic and asymptomatic asexual parasitaemia subjected to the test will depend upon the circumstances. The test can, of course, be used individually, but if information on the baseline sensitivity of the local parasite is being sought, proper sampling methods are required to provide confidence in the interpretation of the results. As a working guide, tests should be made on at least 30 persons in a given locality whenever possible. If a detailed search is being made for the presence or absence of resistant strains, larger numbers should be tested. It is advisable that the results of blood examinations be available within 12 hours at the latest, or sooner if patients with clinical malaria are included in the test. Tests carried out on partially immune asymptomatic carriers with fewer than 1000 trophozoites per mm^3 of blood probably do not provide a sound basis for the thorough assessment of the action of the drug on non-immune subjects.

(3) Interpretation of the WHO Standard Field Test (7-day test)

(a) If no asexual parasites are found by day 7, the infection may be either sensitive (S) or resistant at the RI level.

(b) If asexual parasites disappear for at least 2 consecutive days but return and are present on day 7, they are resistant at the RI level.

(c) If asexual parasitaemia does not clear but is reduced to 25% or less of the original pre-test level during the first 48 hours of treatment, the parasites are resistant at the RII level.

(d) If asexual parasitaemia is reduced by less than 75% during the first 48 hours or if it continues to rise, the parasites are resistant to the standard dose of the drug at the RIII level.
Note: Resistance at the RIII level may exist when the count on day 2 markedly exceeds the count on day 0. In this case the test should be suspended and the patient given effective treatment if his clinical condition so demands.

(4) Interpretation of an extended test

The test will distinguish between sensitivity (S) and the kind of resistance that is demonstrable only by recrudescence following a normal initial response. It is interpreted as follows:

(a) If no asexual parasites are found by day 6 and parasites do not reappear by day 28, the parasites are sensitive (S).

(b) If asexual parasites disappear as in (a) but return within 28 days, re-infection having been excluded, the parasites are resistant at the RI level.

(c) If the asexual parasitaemia does not clear but is reduced to 25% or less of the original pre-test level during the first 48 hours of treatment, the parasites are resistant at the RII level.

(d) If asexual parasitaemia is reduced by less than 75% during the first 48 hours or if it continues to rise, the parasites are resistant at the RIII level (see cautionary note under 3(d) above).

VIII. MALARIA CHEMOTHERAPY

VIII-1. Malaria chemoprophylaxis

Several recent reports on adverse reactions in travellers who were taking amodiaquine for chemoprophylaxis of malaria infections stress again the fact that there is no drug which is entirely satisfactory for the prevention of malaria. According to these reports, the use of amodiaquine has led to neutropaenia and agranulocytosis, and several deaths have occurred. The frequency of adverse reactions under amodiaquine prophylaxis appears to be relatively high, i.e. approximately 1 in 2000. In view of the considerable risk which seems to be associated with the prophylactic use of the drug, amodiaquine should be used with great caution, if at all, for chemoprophylaxis against malaria. Considering that there are no drugs that guarantee full malaria suppression, prevention of malaria should mainly be based on personal protection from mosquito contact. Chemoprophylaxis is advisable only when there is a substantial risk of infection and with drugs that are known to be safe. If chemoprophylaxis is needed, travellers to any endemic area should preferably use chloroquine in a weekly adult dosage of 300 mg base. Travellers have to be warned of possible breakthroughs and side-effects under any prophylactic medication. Travellers in areas where chloroquine-resistant *falciparum* infections are prevalent, should carry a therapeutic dose (for longer exposure, several treatment doses) of sulfadoxine pyrimethamine or mefloquine with them in case severe febrile illness occurs and access to prompt diagnosis and medical attention is not available.

VIII-2. Malaria regimens at national levels

It is the responsibility of each malaria programme to develop its treatment regimens using the types of drugs required. These differ from one programme to another, depending on many factors such as:

- country population
- malaria prevalence
- *P. falciparum* ratio
- status of immunity of the population to malaria
- organization of the malaria services
- organization of basic health services
- response of *P. falciparum* to anti-malarial drugs
- population movements
- socio-economic conditions of the country.

WHO is always ready to assist governments in evaluating their drug policies whenever required.

A question which is often asked is: when to consider replacing chloroquine by another drug should *in-vivo* and *in-vitro* tests confirm the existence of strains of *P. falciparum* resistant to chloroquine in a given population or country. There is no simple answer to this question. All the above mentioned factors have to be considered. However, as a general rule and in areas where *P. falciparum* is dominating the picture, one should consider the use of an alternative drug when the frequency of resistant cases lies between 20 and 30% of cases investigated.

VIII-3. Severe and complicated malaria

The WHO publication "Severe and Complicated Malaria" was introduced to the participants of the workshop during one lecture. It is beyond the scope of this report to go into details of the management of severe and complicated malaria.

IX. CONCLUSIONS

The objectives of the workshop were met and a cadre of trained workers is now available in the eight countries covered by it to implement the plan of work for both *in-vivo* and *in-vitro* studies on *P. falciparum* malaria.

The plans of work produced by country representatives will provide a solid basis for future development of *in-vivo* and *in-vitro* studies in each of the eight countries. These plans of work also contain detailed requirements of resources both in manpower and material for the next two years. These plans of work form the basis of future planning activities of the respective countries of the Region.

With the availability of these trained workers it is now imperative that immediate action be taken to hold national workshops in each of the eight countries, according to the time-table stipulated in the relevant plans of work.

For the monitoring and assessment of *P. falciparum* response to 4 amino-quinolines and other anti-malarial drugs, the procedure described for the WHO Standard Field Test should be strictly followed.

In-vivo and *in-vitro* tests should be carried out simultaneously. While *in-vivo* tests show the degree of resistance, *in-vitro* tests confirm the existence of resistant strains. Immunity developed in patients in endemic areas may play a role in *in-vivo* tests, rendering them less sensitive, but does not interfere in the *in-vitro* tests.

The quality of drug, its proper ingestion and absorption as well as careful parasitological examination must be ensured to avoid premature conclusions. If drug resistance is strongly suspected by any medical officer, the health authorities should be notified immediately. In order to confirm findings, both *in-vivo* and *in-vitro* investigations should be carried out by specially trained teams, without delay. These teams should carry out susceptibility studies on a regular basis in order to assess the sensitivity of malaria parasites to anti-malarial drugs and to monitor the spread, frequency and degree of drug resistance. Such teams can also carry out investigation of alleged cases of drug resistance reported by medical practitioners.

X. RECOMMENDATIONS

At the concluding session of the workshop, the resource persons and the participants made the following recommendations.

(1) To conduct as early as possible national workshops on the monitoring of *P. falciparum* response to anti-malarial drugs as a follow-up of the Regional Workshop.

(2) National workshops should be conducted during the transmission season, preferably towards its end.

(3) In national workshops priority should be given to on-the-job field training and more laboratory technicians should be trained for conducting both *in vivo* and *in vitro* tests.

(4) It is recommended that WHO standardize the supplies and equipment provided to countries of the Region to ensure comparable results.

(5) Cooperation between bordering countries should be strengthened for the early detection of possible foci of *P. falciparum* resistance along border areas.

(6) Exchange of information on drug-resistant malaria and the appearance of new foci of *P. falciparum* resistant to chloroquine should be encouraged between countries either through bilateral mechanisms or through WHO.

(7) It is recommended that countries of the Region restrict the use of sulfadoxine pyrimethamine combination to the treatment of *P. falciparum* cases proven to be resistant to chloroquine.

(8) Mass drug administration using chloroquine should be limited to the control of epidemics, particularly in their early stages.

(9) Governments are encouraged to conduct seminars on the management of severe malaria cases, particularly in areas where chloroquine resistance is confirmed. Treatment regimens should also be revised according to the epidemiological situation.

(10) WHO is requested to send relevant publications on drug-resistant malaria to the Directors of all malaria projects in the Region and to the participants in the Regional Workshop.

(11) The countries and WHO should adhere to the Plans of Action developed by representatives of each country and more attention should be given to this important and emerging problem in the Region.

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Sincere appreciation is expressed for the hospitality so warmly shown by the host country and the dedication of Dr I.H. Shah and the staff of the National Malaria Training Centre in Lahore in ensuring the smooth running and success of the workshop.

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ANNEX I

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ANNEX II

AGENDA

1. Opening of the Workshop.

2. Election of Officers.

3. Adoption of Agenda.

4. Status of *P. falciparum* resistance to chloroquine in the Eastern Mediterranean Region.

5. Drugs used as anti-malarials in the Eastern Mediteranean Region.

6. Mefloquine and other new anti-malarials.

7. Supplies and equipment necessary to carry out susceptibility tests of *P. falciparum* resistance.

8. Recording of results of *in-vivo* tests.

9. Recommended regimens for use of anti-malarials in areas where there is no resistance, where resistance has just emerged and where resistance is well-established.

10. Operational methods to prevent the introduction of *P. falciparum* resistant strains in an area or a country.

11. Methods of control and containment of *P. falciparum* resistant strains.

12. Recommendations of the Workshop.

13. Closing session.

ANNEX III - REGIONAL WORKSHOP ON P.FALCIPARUM SENSITIVITY, 3.- 20 AUGUST 1986, LAHORE, PAKISTAN

Working Day	ONE	TWO	THREE	FOUR	FIVE	SIX	SEVEN
HOUR DATE	SUNDAY 3.8.86	MONDAY 4.8.86	TUESDAY 5.8.86	WEDNESDAY 6.8.86	THURSDAY 7.8.86	FRIDAY 8.8.86	SATURDAY 9.8.86
8.00 - 9.00	Registration/ Administrative arrangements	Lecture/Revision Counting malaria parasites	Lecture Introduction to in-vivo studies	Field Screening for test subjects for in-vitro/ in-vivo test	Field In-vivo Day 1 follow-up	Field In-vivo Day 2 follow-up	Field In-vivo Day 3 follow-up
9.00 - 10.00	Inaugural Address	Lecture Antimalarial drugs and therapy	Lecture Introduction to in-vitro studies	Field Screening for test subjects for in-vitro/ vivo test			
10.00 - 11.00	(10.30-11.30) Review of Global and Regional Resistance	Lecture/Revision Staining	Lecture/Demonstration Microtest Kit components	Field Screening for test subjects for in-vitro/vivo test			
11.30 - 12.30	Seminar Discussions: problems of Drug Resistance	Practical Staining (Slides prepared on 3.8.86)	Practical In-vitro test procedure	Practical Setting-up in-vitro tests	Lecture Harvesting in-vitro tests		
12.30 - 13.30	Lecture Use of Microscope in Drug sensitivity	Practical Counting Malaria parasites	Lecture Organization of screening surveys	Practical Setting up in-vitro tests	Practical Harvesting in-vitro tests	Practical Reading in-vitro tests	Practical Reading in-vivo tests
13.30 - 14.30	Lecture Bloodslides as used in Drug Sensitivity Studies	Practical Counting Malaria parasites	Field Advance Visit to study area	Practical Setting-up in-vitro tests	Practical Staining in-vitro	Continued Reading in-vivo tests	Continued

REGIONAL WORKSHOP ON P.FALCIPARUM SENSITIVITY 3 - 20 AUGUST 1986, LAHORE, PAKISTAN

Working Day HOUR DATE	EIGHT SUNDAY 10.8.86	NINE MONDAY 11.8.86	TEN TUESDAY 12.8.86	ELEVEN WEDNESDAY 13.8.86	TWELVE THURSDAY 14.8.86	THIRTEEN FRIDAY 15.8.86	FORTEEN SATURDAY 16.8.86
08.00 - 09.00	Field In-vivo Day 4 follow-up In-vitro case selection	Field In-vivo Day 5 follow-up	Field In-vivo Day 6 follow-up	Field In-vivo Day 7 follow-up	HOLIDAY	Lecture/Revision Human Plasmodium in thin film	HOLIDAY
09.00 - 10.00	Field	Field	Field	Field		Lecture Human Plasmodium in thick film	
10.00 - 11.00	Field	Field	Field	Field		Lecture Management of severe malaria	
11.30 - 12.30	Field	Field	Field	Field		Practical Human Plasmodium in thick and thin films	
12.30 - 13.30	Practical -Setting up in-vitro cases - Reading in-vivo cases	Practical -Harvesting in-vitro cases -Reading in-vivo cases	Practical -Staining in-vitro cases -Reading in vitro cases	Practical -Reading in-vitro cases -Reading in-vivo cases		Practical Human Plasmodium in thick and thin films	
13.30 - 14.30			Reading in-vivo cases			Practical Human Plasmodium in thick and thin films	

REGIONAL WORKSHOP ON P.FALCIPARUM SENSITIVITY 3-20 AUTUST 1986, LAHORE, PAKISTAN

Working Day HOUR DATE	FIFTEEN SUNDAY 17.8.86	SIXTEEN MONDAY 18.8.86	SEVENTEEN TUESDAY 19.8.86	EIGHTEEN WEDNESDAY 20.8.86
08.00 - 09.00	Continued Examination in-vivo material	Continued Examination in-vitro material	SEMINAR Presentation of results of all in-vivo/vitro results	EXAMINATION Practical
09.00 - 10.00	Continued Examination in-vivo material	Continued Examination in-vitro material	SEMINAR Ditto	EXAMINATION Ditto
10.00 - 11.00	- ditto -	- ditto -	SEMINAR Organization of in-vivo/in-vitro studies in home countries of partici-pants	SEMINAR Results of written examination
11.30 - 12.30	Lecture Preparation of in-vivo data for analysis	Lecture Preparation of in-vitro data for analysis	SEMINAR Discussion and analysis of all in-vivo/in-vitro results	SEMINAR Review of course: objectives and attainments
12.30 - 13.30	Practical Preparation of in-vivo data for analysis	Ditto	SEMINAR Ditto	SEMINAR Ditto
13.30 - 14.30	Ditto	Ditto	EXAMINATION Written multiple choice	CLOSURE of the Course