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Report Submitted on monoclonal Antibody based elisa for detection of P. Falciparum and P. vivax Antigens in malaria endemic populations in Southern Nepal

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ON

MONOCLONAL ANTIBODY BASED ELISA FOR DETECTION OF *P.FALCIPARUM* AND *P.VIVAX* ANTIGENS IN MALARIA ENDEMIC POPULATIONS IN SOUTHERN NEPAL

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ABSTRACT

Two indirect enzyme-linked immunosorbent assay (ELISA) systems based on the monoclonal antibodies (MAbs) originated from the native Thai strains of *P.vivax* (McPV1) and from *P.falciparum* (F2W22C1) and the polyclonal antibodies (PAbs) raised against local Nepali strains of *P.vivax* and *P.falciparum* in rabbits were developed for the detection of local Nepali strains of *P.vivax* and *P.falciparum* antigens in lysed red cell lysates. The MAb-PAb indirect ELISA for the detection of *P.vivax* antigens was specific since it was positive only with *P.vivax* infected erythrocytes and was negative when erythrocytes from forty healthy individuals and forty *P.falciparum* infected erythrocytes were tested. Similarly, the MAb-PAb indirect ELISA developed for the detection of *P.falciparum* antigens was specific since it was positive only with *P.falciparum* infected erythrocytes and was negative when blood from forty healthy individuals and forty *P.vivax* infected erythrocytes were tested. When these two MAb-PAb indirect ELISA systems were applied to 203 vivax blood samples and 154 falciparum blood samples already proven by microscopic examination before anti-malarial treatment collected from Dhalkebar Health Post, Godar Health Post and Lalghad Health Post of Dhanusha district of southern Nepal, the assays could detect *P.vivax* antigens and *P.falciparum* antigens with high degree of sensitivity (100%), specificity (100%) and accuracy (100%). Furthermore, the lowest number of *P.vivax* and *P.falciparum* parasite detections in blood samples were 6 parasites/10^6 erythrocytes and 9 parasites/10^6 erythrocytes, respectively. The ability to detect vivax antigens in the present study were closed to the level of microscopic examination, but were superior to those of previous two site monoclonal antibody sandwich ELISA (MAb-MAb ELISA) and two site polyclonal-monoclonal antibody sandwich ELISA (PAb-MAb ELISA) where the same monoclonal antibody (McPV1) was used and could detect *P.vivax* antigens with the ability of 6.68 parasites/10^6 erythrocytes and 2.69 parasites/10^6 erythrocytes, respectively. Furthermore, the assay systems developed in this study either for *P.vivax* antigen or for *P. falciparum* antigen detection showed significant correlation between optical density values and the parasitemia ($r = 0.649$; $p = 0.018$ for *P.vivax* system and $r = 0.872$; $p = 0.013$ for

P.falciparum system). Although the results in this study indicated that the MAb-PAb based indirect ELISA systems developed for detection of both *P.vivax* and *P.falciparum* antigens using the monoclonal antibodies raised against Thai strains and polyclonal antibodies raised against local Nepali strains offer high degree of advantages of sensitivity, specificity and accuracy. However, the tests developed should be used to evaluate more blood samples from the people who have been living in such malaria endemic areas before any conclusions could be made.

CHAPTER I
INTRODUCTION

The malaria causative agents in humans are four species of *Plasmodium* protozoa namely *Plasmodium falciparum*, *P.vivax*, *P.ovale and P.malariae*. Of these, *P. falciparum* accounts for the majority of infections and is the most lethal. The majority of cases approximately 85% are caused by *P.falciparum* worldwide (WHO, 1983) and the global vivax malaria incidence is approximately 14% and the rest of one percentage is covered by ovale and malariae malaria. *P.vivax* is the commonest species in Africa and in Asia. These two species account for the most of the human sufferings and economic loss. These parasites are transmitted by biting of the female mosquito of genus *Anopheles*. Malaria is a curable disease if promptly diagnosed and adequately treated.

More than 70% of the total population and 64 districts out of 75 districts are at malaria risk in Nepal with varying percentage of Malaria Parasite Incidence Rate/1,000. The highest incidence rate is in Bhojpur district 13.02% (Annual Report, Department of Health Services, 2055/2056 (1998/1999). Out of five development regions of the country, the central development region has been found to be the highest number of malaria cases. His Majesty's Government of Nepal, Ministry of Health had initiated a programme to combat this disease in 1954 as a part of Insect Borne Disease Control Programme. Since then, the disease has been found outbreaks and even endemics in some southern parts of Nepal. This disease was felt long existed as *aulojoro* (meaning high fever in hot areas) existing particularly in hot climatic regions.

The overall malaria situation of Nepal has been deteriorated since last decade because of the lack of resources, intersectoral collaboration and trained professional staffs. Moreover, the country has been seriously facing insufficient health infrastructures, uncontrolled population migration and civil unrest. Besides these problems, identification of chloroquine resistant *P.falciparum* and insecticide resistant parasite vectors aggregate

the gravity of the problems.

Normally, the microscopic examination for malaria parasite diagnosis is very popular and convenient for the microscopists. The sensitivity of this method is high: an experienced microscopist can detect in a 3-minute examination as little as one parasite per 10^6 RBCs (WHO, 1986). But time limitation and eye fatigue after many hours of microscopic examination may cause inaccuracy. With this concern, as a part of malaria eradication program launched by His Majesty's Government of Nepal, rechecking and cross checking of blood films were done in all the malaria offices of the districts, zones and regions of the country. Apart from the district offices, checking was carried out at the zonal and regional malaria offices on all positive blood films and on 10% of randomly selected negative blood films. The misdiagnosis include, negative to positive, positive to negative, mis-species especially in low-grade parasitemia and mixed infection. It was estimated that more than three thousand patients, for each year, would not receive the radical treatment or improperly received radical treatment (Annual Report of Malaria, Nepal, 1987). In view of the possible utilization of malaria vaccine in the future, it is therefore necessary that accurate and reliable test be developed to detect antigens as a supplement or even replace the microscopic examinations.

In order to address the above-mentioned problems, techniques-with higher sensitivity, rapidity and cheaper alternative methods have to be sought which can replace the microscopic examination or at least competitive enough. There are no other malaria diagnostic methods employed in such rural areas of Nepal apart from the conventional microscopic diagnostic methods.

Many serological methods which have been chosen in some epidemiological studies provided adequate information with regard to exposure to malaria, but by virtue of persistence of malaria antibodies following disappearance of malaria parasites from the blood of the infected subjects. Therefore, such tests do not accurately discriminate between the current and the past infections. Demonstration of malaria parasite antigens

has become increasingly popular in the diagnosis of malaria infections because they are more closely related to the current illness than antibodies detection which show the previous or recent past infection.

An immunological method, enzyme linked immunosorbent assay (ELISA) so as to detect malaria antigens were concerned because this method provides such efficiencies as described above. Enzyme linked immunosorbent assay (ELISA) was selected in this research study because it offers the aforementioned attractive advantages of the use of affordable apparatus. Moreover, a large number of samples could be tested at the same time. Enzyme labeled reagents are cheaper, easy to prepare and are stable at the room temperatures and yet retain a sensitivity approaching to that of radioimmunoassay (Mackey *et al.*, 1980, 1982). The assay can be partially automated so that many samples can be carried out simultaneously and the results can be read either visually or with an ELISA Reader. Moreover, the assay can be modified to detect malaria antigens in blood samples under field conditions.

A two-site monoclonal antibody sandwich IRMA (MAb-IRMA), two-site polyclonal antibody-monoclonal antibody sandwich IRMA (PAb-MAb-IRMA), and two-site polyclonal antibody sandwich IRMA (PAb-IRMA), were developed by Khusmith *et al.*, (1987) to detect low grade infections with *P.falciparum*. The assays showed good correlation with parasitemia when tested against parasites from *in vitro* cultures (r = 0.996, 0.994, and 0.998 for MAb-, PAb-MAb-, and PAb-IRMA, respectively) with the ability to detect as few as 0.24, 0.67, and 1.82 parasites/10^7 erythrocytes, respectively; a number of ELISA procedures have been developed to detect *P.falciparum* antigen include inhibition ELISA (Bidwell *et al.*, 1981, Mackey *et al.*, 1992), non inhibition ELISA (Bidwell *et al.*, 1981), DOT-ELISA (Londner *et al.*, 1987), two site sandwich ELISA (Khusmith *et al.*, 1992), polyclonal-monoclonal two-site sandwich ELISA (Khusmith *et al.*, 1987).

However, the detection of blood stage malaria parasite antigens of *P.vivax* and

P.falciparum in the malaria patients by immunological test have not been established anywhere in Nepal so far. Therefore, in view of this prospective, the present study aimed to develop the indirect ELISA using monoclonal antibodies produced against Thai isolates of *P.vivax* or *P.falciparum* and polyclonal antibodies produced against Nepali strains of *P.vivax* or *P.falciparum*. These MAb-PAb based indirect ELISA were applied for the detection of *P.vivax* and *P.falciparum* antigens in the blood samples collected from the people who have been infected in three health posts of malaria endemic Dhanusha district of Nepal.

However, the monoclonal-polyclonal antibody based indirect ELISA for the detection of *P.vivax* and *P.falciparum* antigens needs to be further developed and applied to detect parasite antigens in more blood samples from the people who have been living in malaria endemic areas in Nepal with or without symptoms before the conclusion could be made.

CHAPTER II
REVIEW OF THE LITERATURES

Malaria is still the most prevalent and serious infectious disease in tropical and subtropical areas of the world. More than two billion persons are potentially exposed to malaria each year (WHO, 1985). The majority of cases approximately 85% are caused by *P. falciparum* worldwide (WHO, 1983). The mortality and morbidity directly attributable to malaria constitute major constraints on economic progress in the tropic and subtropical areas of the world. Failure of global eradication is attributable primarily to the practical difficulties in countries that have practical problems have been compounded by the spread of insecticide resistance in mosquitoes, by alterations in vector behavior and especially by the widespread appearance of chloroquine resistant strains of malaria parasites (Bruce-Chwat, 1980). *P.vivax* is the commonest species in the Americas and Asia. These two species account for most of the human suffering and economic loss due to malaria in the world today.

Resistance of *P. falciparum* to drugs has probably become the most important threat to effective control of disease. It has arisen largely through a combination of massive antimalarial drug development and a failure to combat transmission of the disease (WHO, 1985). *P. falciparum* asymotomatic cases appear to play a major role in the persistence of malaria in endemic foci and are difficult to eradicate. The asymptomatic parasitemia and low-grade parasitemia are very difficult to detect and deal with, and thus special attention, should be given to the epidemiological studies of these carriers. Thick blood film examination is still the best technique now available, but the method has its limitation when the parasitemia is low (Ittiravivongs, 1983).

1. History of malaria

Hippocrates was the first to describe the manifestations of the disease, and relate them to the time of year and to where the patients lived-before this, the supernatural was

blamed. The association with stagnant water (breeding grounds for *A nopheles*) led the Romans to begin drainage programs, the first intervention against malaria. The first recorded treatment dates back to 1600, where the bitter bark of the cinchona tree in Peru was used by the native Peruvian Indians. By 1649, the bark was available in England, as 'jesuits powder' (Bruce-Chwatt, 1980), so that those suffering from 'agues' might benefit from the quinine it contained. Malaria in the U.K. (known as agues) would have been clustered around stagnant marshes, and the invading Roman soldiers would certainly have brought the disease with them. There have been no recent cases due to an infective mosquito bite received within the U.K. so this is certainly not an endemic region.

Not until 1889 was the protozoal cause of malaria elicited by Laveran working in Algeria, and only in 1897 was the *Anopheles* mosquito demonstrated to be the vector for the disease. At this point the major features of the epidemiology of malaria seemed clear, and control measures started to be implemented.

Thoughtless man-made irrigation schemes and damps provided new habitats for *Anopheles*, and resulted in 'man-made' malaria. The extension of urban areas lead to epidemics in the peripheries of the growing cities. Mass migrations of non-immune populations into endemic areas for political reasons further complicate matters. Africa, where the majority of malarial disease is manifested was not even included in the global control mechanism, as it had insufficient infrastructure to support the policy (WHO, 1980).

Despite the setbacks, up until 1969, when the global eradication policy was finally abandoned, the following European countries had managed to completely eradicate their endemic malaria by interrupting transmission: Hungary, Bulgaria, Romania, Yugoslavia, Spain, Poland, Italy, Netherlands, Portugal. From the early 1970's the malaria situation has slowly and progressively deteriorated, and reduced control measures between 1972 and 1976 due to financial constraints lead to a massive 2-3 fold increases in cases globally. Spraying never truly eradicated the mosquitoes anywhere, and the reduction in the more persistent *P.vivax* infections were much less than for *P.falciparum*, though the

latter returned in much greater strength as control measures waned. The growing interchange of populations between malarious countries and malaria free countries is responsible for the continuous increase in the number of imported malaria cases in European countries, and causes serious concern because of possible epidemic focal resurgence in receptive areas such as the Mediterranean. Since 1976, several new pockets of malaria transmission have evolved, and a WHO 1980 report recommended that countries which had become non-malarious should maintain at least one malaria vigilance unit. The morphology of *Plasmodium* species was first of all studied by a French Colonial Surgeon Alphonse Laveran more than a century ago (Bruce-Chwatt, 1988) while working in Algeria. He first described the malaria parasite. He observed the exflagellation of male gametocytes of *Plasmodium* species from a malaria patient.

2. The global picture of malaria

Approximately 27% of the world's population live in areas where malaria never existed or disappeared without antimalarial intervention, 32% live in areas where malaria was reduced or eliminated but has now re-emerged and 9% live in areas where there is intense transmission in many parts and where no antimalarial programs are fully implemented. This situation is represented mainly by tropical Africa (WHO, 1991).

The fact sheet further states that malaria kills one child in every thirty seconds. Malaria remains one of the leading causes of morbidity and mortality in the tropics. An estimated 300-500 million cases of malaria each year result in about one million deaths, mainly children under five in Africa. Malaria has been estimated to present 2.3% of the overall global disease burden and 9% in Africa, ranking third among major infectious disease threats, after pneumococcal acute respiratory infections (3.5%) and tuberculosis (2.8%). Pregnant women and children under five continue to constitute one of the most important risk groups. The disease is often linked to the movement of refugees, mining and water development projects. In Africa alone, the estimated annual direct and indirect costs of malaria were US$ 800 millions in 1987, and were expected to exceed US$ 1800 millions by 1995.

3. WHO global malaria strategy

The Current WHO Global Malaria Control Strategy emphasizes early diagnosis, treatment with effective antimalarials and the selective use of preventive measures, including vector control. Another strategy is to reduce malaria mortality by the year 2000 at least 20% compared to 1995 in at least 75% of affected countries (WHO, 1995).

4. Malaria situations of Nepal

4.1 Past situation (before 1997)

In 1954, a largescale malaria control project named Insect Borne Disease Control supported by USAID (then USOM) was started in Nepal. However, all malarious areas were not covered by this control project. Another planned malaria control project was taken up in Rapti Valley by HMG/WHO/USAID during 1956-1958 to obtain baseline data and to reccommend an appropriate strategy for a largescale malaria eradication programme in the country. Up to that period, it was estimated that approximately, two million cases of malaria occurred annually and 10 to 15 percentage among them resulted in deaths (The Annual Internal Assessment of the Malaria and Kala-azar control activities of the year 1997, Department of Health Services, Epidemiology and Disease Control Division, His Majesty's Government of Nepal, Ministry of Health). By 1963, malarious areas of east, central and west regions and by 1965 all of the malarious areas of the country were under malaria surveillance and thus started recording malaria c ases i n a s ystematic m anner. F rom t he d ata a vailable s ince 1 963, the malaria dynamics in Nepal reveals periodic upward lifts followed by sharp falls in next two years and then the period of stagnation with slight flactuations between 2,500 to 4,000 cases lasting for 5-6 years (1971). From 1972, the cases started rising and in 1973 and 1974 due to the epidemics in the west region the number of cases reached to 14,000. With great effort of mobilisation of the limited resources, and replacing DDT with malathion for few more years upto 1977, the NMEO achieved some progress in detecting the malaria cases from 10,000 to 13,000 cases annually.

From 1978, the real deterioration of the situation started when the cases reached to 14,212 from 11,615 in 1977. With steady increase every year, the number of cases reached to 16,719 in 1983. There was steep rise to 29,388 cases in 1984 which again escalated to 42,321 in 1985 with major outbreaks in far-western region and minor ones in many parts of the country. After 1985, the cases in the country started decreasing due to the massive spraying of insecticides in such epidemic outbreak areas. By 1989, the cases again increased to 22,366 which reached to 22,856 in 1990. In 1991, there was an exacerbation of malaria in eastern, western and mainly on central region thus the number of cases again escalated to 29,135. After 1991, the number of cases gradually decreased and reached to 8,957 in 1997.

The cyclic pattern for the whole country does not reflect similar specific dynamics of malaria due to the climatological distinctions, prevailing levels of malaria endemicity, patterns of population migration and extent of malaria control activities. The impact of insecticide spraying on malaria dynamics is fairly evident. Since 1977, the central region which has been contributing more than one third of the total malaria cases annually (with the exception in 1985-1986) got into the status of exacerbation of malaria in 1990-1991. Repeated exacerbation of malaria every third or fourth year is typical for the central region.

Review of the programme objectives in 1978 was consequential to conversion of eradication programme to control malaria according to the WHO global strategy of malaria control. During the years 1987 and 1988 malaria control programme in all the districts was integrated into the basic health services as a conmponent of primary health care at district/health post levels. Simultaneously, regional malaria offices were amalgamated into Regional Health Services Directorates. The national headquarters of malaria control programme at the central level in Kathmandu became a division in the Ministry of Health as Malaria Control Division in July 1990.

Since the inception of the Malaria Eradication Programme in Nepal in 1958, remarkable progress was achieved in controlling malaria in the late 1960s. In 1970, only

2,500 cases of malaria were recorded in the country. In 1978, the eradication programme was converted into malaria control programme. Despite the concerted efforts which have been kept the disease under control in the country, the programme has been plagued by focal outbreaks or epidemics of malaria from time to time. The sudden changes in physical and ecological factors, population movemnets, development projects like roads and damp constructions and resettlement projects were noticed responsible for those focal epidemics/outbreaks. Keeping in view that one of the main objectives of the Global Malaria Control Strategy is "Prevention and control epidemics" Nepal has also enmphasized this objective in the National Plan of Operations for Malaria Control, 1994.

In some areas the number of blood films and the number of microscopists are unbalanced, with a consequent inevitable extension of the time-lag (the time between blood slide taken and radical treatment), especially in rural area the time-lag should not be more than 7 days. In all malaria endemic regions of Nepal, there have routinely, been a decrease in a number of officials from the partial integration areas and an increase in the control areas. The imbalance between the number of blood films and the person each of whom could examine on the average 70 films/day contributes to the time-lag being longer than 7 days (Annual Report of Malaria, Department of Health Services, 2044/45, HMG, Nepal).

4.2 District-wise malaria cases in Nepal

Nepal is a land locked country situated in between Tibet of China to the North and the South, the East and the West of India. The Southern part is a low-lying alluvial plain (known as "Terai") and a long lying area boardering to India. The large density and population of Terai region is due to the huge population migration within the country from the mountainous and hilly regions and migration from Indian states as well (Shrestha SH, 1989). Because of the open border between Nepal and India, malaria cases have been increased in each year imported from these badly affected areas of India (Shrestha SH, 1989). The Southern Terai region of Nepal consists of 16 districts and the Incidence Rate of malaria in such areas is different from each district. Some of these districts have even up to Annual Parasite Incidence (API) of 4.46/1,000 populations like

in Bardia district (Annual Report, Department of Health Services 2055/56, Nepal) (Figure 1).

4.3 Initiation of malaria eradication programme in Nepal

The first attempt to control malaria in Nepal was initiated in 1954 through the Insect Borne Disease Control Programme supported by USAID. In 1958, the malaria eradication programme had launched as a vertical programme (the first national public health programme in the country) with an ultimate objective of an absolute eradication of malaria from the country within a limited period. It remained satisfactory until 1970 when only 2,518 malaria cases were detected from the areas under this programme (Shrestha SL *et al.*, 1980).

During early seventies due to various problems such as technical, financial, administrative and logistic support, the programme suffered setbacks and the malaria situation was deteriorated. One of the major changes brought in this programme during 1970th decade was to gradually integrate malarious areas with API 1% or below into the Primary Health Care Programme.

The post eradication epidemics of malaria took place in Nepal in 1974 when about 15,000 cases were reported (Kondrashin AV *et al.*, 1981). In 1975, the government augmented its own resources with the financial support of some international organizations and bilateral agencies and reverted back the programme to the attack phase. Due to technical, operational, administrative and logistical constraints, the above target could not be achieved and consequently the program reverted back to malaria control in 1978 prevailing ecological, epidemiological and socio-economic factors required bringing about changes in the malaria control strategy and as a result the current strategy has been revised in accordance with the Global Malaria Control Strategy (GMCS) of WHO, 1993.
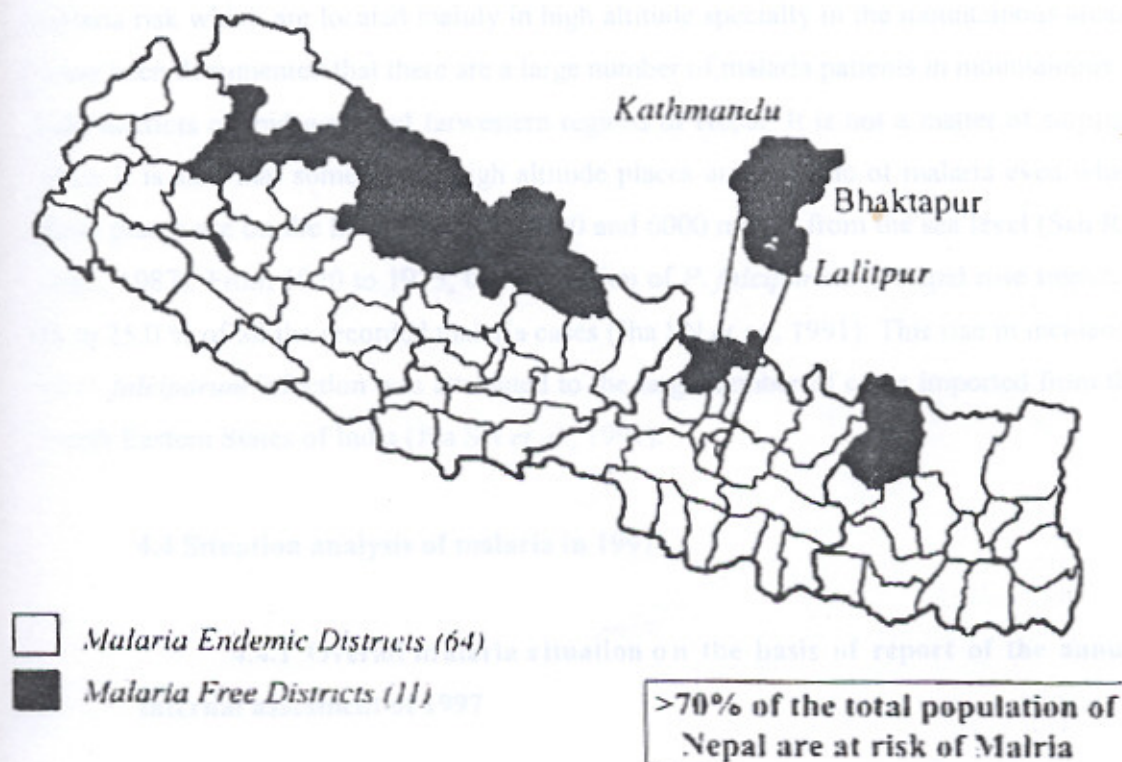
Figure 1. District-wise distribution of malaria in Nepal showing malaria endemic districts (64) and malaria free districts (11). (Source: Annual Report, Department of Health Services 2055/56 (1998/99), His Majesty's Government of Nepal, Ministry of Health, Department of Health Services, p. 125).

Malaria control services are being provided to approximately 15.6 million people in malaria risk areas of 64 districts of the country. Only 11 districts out of 75 are free of malaria risk which are located mainly in high altitude specially in the mountainous areas. It has been documented that there are a large number of malaria patients in mountainous high districts of mid-west and farwestern regions of Nepal. It is not a matter of surprise when it is said that some of the high altitude places are endemic of malaria even when these places are on the height between 4000 and 6000 meters from the sea level (Sah RL et al., 1987). From 1970 to 1975, the proportion of *P. falciparum* in Nepal rose from 6.2 % to 25.0 % of all the recorded malaria cases (Jha SN et al., 1991). This rise in incidence of *P. falciparum* infection was attributed to the large number of cases imported from the North Eastern States of India (Jha SN et al., 1991).

4.4 Situation analysis of malaria in 1997

4.4.1 Overall malaria situation on the basis of report of the annual internal assesment of 1997

A total of 160,293 slides were collected which 8957 malaria cases were detected in 1997. There was a decrease in number of slides collected by 21.27% as well as malaria cases by 0.70% in 1997 as compared to 1996. Annual Parasite Incidence was 0.57% as against 0.6% in 1996. Slide Positive Rate (SPR) was increased to 5.59% as against 4.41% while Blood Examination Rate (BER) was only1.03% compared to 1.23% in 1996. Likewise, SPR Slide Falciparum Rate (SFR) was also increased to 0.72% in 1997 as compared to 0.47% in 1996 (The Annual Internal Assessment of the Malaria and Kala-azar control activities of the year 1997, HMG, Nepal) (Figure 2). Analysis of malaria in the regionwise situation revealed that as usual out of 8957 malaria cases, the highest number of malaria cases were recorded in central region (43.0%) followed by western region (21.0%), far western region (15.0%), eastern region (10.0%) and midwestern region (10.0%). Regarding *P.falciparum* cases, out of total 1150 cases, the highest was recorded in western region (63.0%) followed by farwestern region (14.0%), eastern region (12.0%), central region (8.0%) and mid western region (2.0%) (Figure 3).
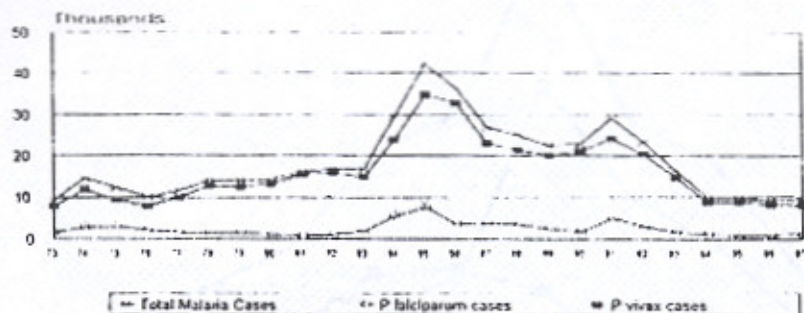
Figure 2. Species-wise malaria cases from 1973 to 1997 (Source: His Majesty's
Government of Nepal, Ministry of Health, Department of Health Services,
Epidemiology and Disease Control Division, The Aannual Internal Assessment
of Malaria and Kala-azar Control activities of the year 1997. Vector Borne
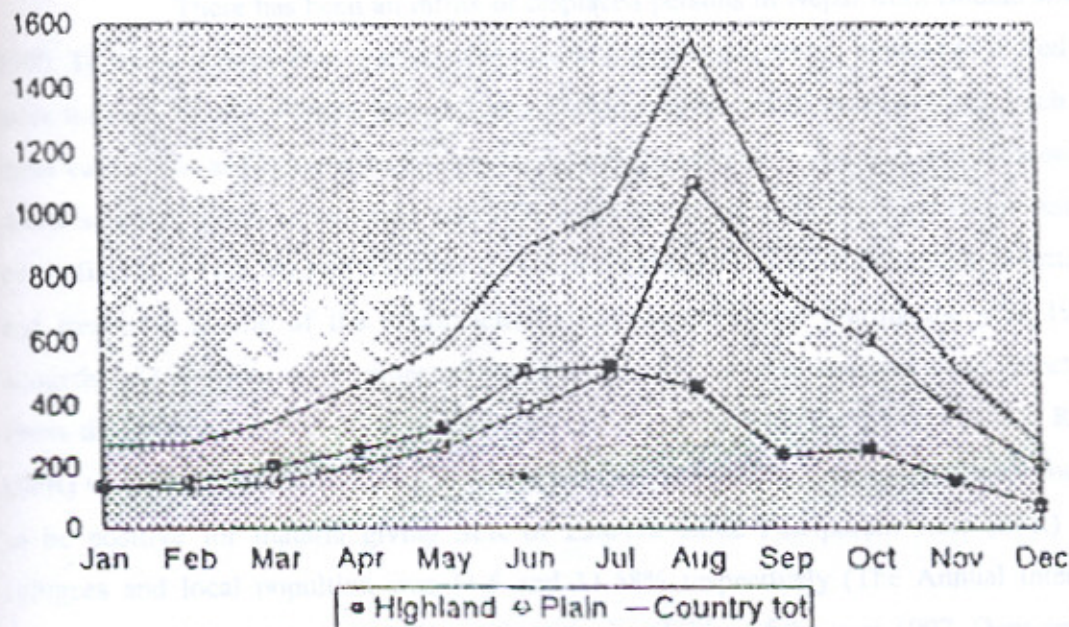Disease Research and Training Center, Bhutandevi, Hetauda, p. 13).

Figure 3. Comparative trend of malaria cases in highland, plain area and country in 1997
(Source: His Majesty's Government of Nepal, Ministry of Health,
Department of Health Services, Epidemiology and Disease Control Division,
The Aannual Internal Assessment of the Malaria and Kala-azar Control
activities of the year 1997, Vector Borne Disease Research and Training
Center, Bhutandevi, Hetauda, p.23).

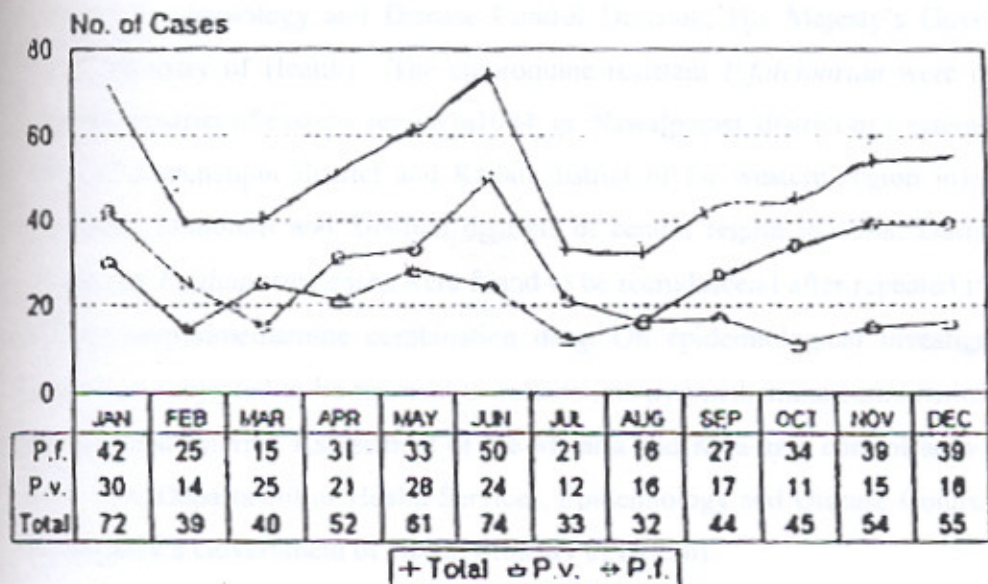## 4.5 Malaria situation in refugee camps

There has been an influx of displaced persons in Nepal from Bhutan since 1990. To the date more than one hundred thousand displaced persons have been settled in more than six camps of Jhapa and one camp in Morang district of eastern Nepal. Each of these camps has at least one health centre rendering preventive, curative and promotive health services to this community under the aegis of United Nation's High Commission for Refugees (UNHCR) and Save the Childern Fund (SCF) U.K. Malaria case detection and treatmnet is one of the major activities of these centres. During the year 1997 altogether 3350 slides were collected out of which 601 malaria positives were detected. From the refugees alone 2924 slides were malaria positives. The Slide Positive Rate (SPR) was 17.13%. Out of 423 slides collected from the local population 100 were found to be positive for malaria giving SPR of 23.64%. Slide Falciparum Rate (SFR) for refugees and local popultion was 11.5 and 11.58% respectively (The Annual Internal Assessment of the Malaria and Kala-azar control activities of the year 1997, Department of Health Services, Epidemiology and Disease Control Division, His Majesty's Government of Nepal, Ministry of Health).

4.6 Seasonality of malaria cases recorded

Monthwise distribution of malaria cases in 1997 for the country showed the peak shift to August as compared to the peak in July in previous years. Similar to the country trend in plain areas the peak was in August. In highland area the peak was observed in June forming almost a plateau up to August (Figure 4).

4.7 Drug resistance malaria

Until 1983, the sensitivity status of locally transmitted *P.falciparum* cases could not be determined by *in vitro* and *in vivo* methods because of the sporadic nature of *P.falciparum* incidence. Only the imported *P.falciparum* cases from the north eastern parts of India were subjected to *in vivo* and *in vitro* tests and found to be resistant to

No. of Cases

	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
P.f.	42	25	15	31	33	50	21	16	27	34	39	39
P.v.	30	14	25	21	28	24	12	16	17	11	15	16
Total	72	39	40	52	61	74	33	32	44	45	54	55

+ Total ↔ P.v. ↔ P.f.

Total: P.v. - 229, P.f. - 372

Figure 4. Month and species-wise malaria cases detected in refugee camps in 1997
(Source: His Majesty's Government of Nepal, Ministry of Health, Department
of Health Services, Epidemiology and Disease Control Division, The Aannual
Internal Assessment of the Malaria and Kala-azar Control activities of the year
1997, Vector Borne Disease Research and Training Center, Bhutandevi,
Hetauda, p. 35)

chloroquine. In 1984, the first indigenous case of chloroquine resistant *P.falciparum* was recorded in Makawanpur district of central region (The Annual Internal Assessment of the Malaria and Kala-azar control activities of the year 1997, Department of Health Services, Epidemiology and Disease Control Division, His Majesty's Government of Nepal, Ministry of Health). The chloroquine resistant *P.falciparum* were recorded in Udayapur district of eastern region in1984, in Nawalparasi district of western region in 1986, in Kanchanpur district and Kailali district of far western region in1987 and in Dhanusha, Mahottari and Sindhuli districts of central region in 1988. During 1993 to 1995 some *P.falciparum* cases were found to be recrudescend after repeated treatment of sulfadoxine/pyrimethamine combination drug. On epidemiological investigtions these cases were subjected to be resistant to sulfadoxine/pyrimethamine combination treatment (The Annual Internal Assessment of the Malaria and Kala-azar control activities of the year 1997, Department of Health Services, Epidemiology and Disease Control Division, His Majesty's Government of Nepal, Ministry of Health).

4.8 Border malaria

Out of the 64 malarious districts of the country 26 districts are bordered with India. A total of 10.43 million population (67% of the country population at malaria risk) reside in such areas. With the free and opened border between the two countries local cross border mobility is very much common. There are certain entry/exit points (Kakarvitta, Bhadrapur and Jogbani in the eastern, Birgunj.in the central, Sunauli in the western, Rupaidiha in the midwestern and Gauriphanta and Banbasa in the far western region) which have high movement of population not only from bordering district but also from the northern parts of the country. The exact magnitude of population movement between the borders is difficult to estimate due to the common free border between Nepal and India and also the the people from countries do not have to submit any travel documents for crossing the border to and fro.

5. Malaria vectors

Malaria transmission was proven entomologically in Nepal in 1955 for the first time (Peters *et al.*, 1955). The species incriminated then was *An. fluviatilis* from the valley of Chitwan. Later in the same valley, it was proved that *An. minimums* is the primary vector (Brydon *et al.*, 1961). In Nepal, Darsie and Pradhan (1989) published an extensive account of the mosquitoes of Nepal recording 130 species in 14 genera. Previously, Joshi and Pradhan (1965) reported the mosquitoes of *An. fluviatilis*, *An. ammoniums*, *An. maculates*, *An. willmori* and Darsie (1989) had reported on the mosquito fauna of Nepal. However, among five vector species *An. annularis* have been found to be mainly responsible for the malaria transmission in Nepal. The distribution of *An. annularis* has been found from the southern plain cultivated Terai up to mountains valley at 6,500 ft above the sea level. Likewise, *An. fluviatilis* is the primary vector of malaria in the hilly region which is mostly in the northern sector where as *An. minimums* is the primary vector and *An. fluviatilis* is the secondary vector in the forest fringe areas which comprise the mid-terai regions which pass predominantly through the middle sector (Harold WB *et al.*, 1961).

6. Life cycle of *Plasmodium* parasite

Infection is initiated by the injection of sporozoites into the blood stream during blood feeding by female *Anopheles* mosquitoes. These parasites undergo essentially the same of life cycle, which comprises of an exogenous sexual phase (sporogony) with multiplication in certain female *Anopheles* mosquito, the invertibrate host and an endogenous asexual phase (schizogony) with multiplication in the human the vertebrate host (Figure 5).

The incubation period which denotes the first appearance of clinical symptoms of disease from the time of infection varies between the strains, the longest period being found in *P.malariae* infection. Likewise, the prepatent period, which is the interval between the date of the infection to the time when malaria parasites are detectable in the peripheral blood, is also different from strains to strains.
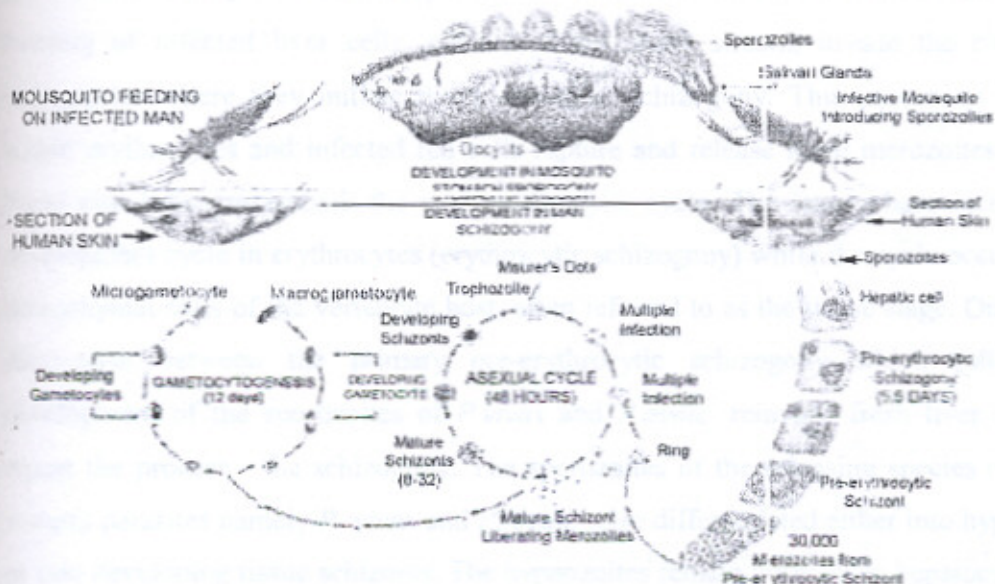
Figure 5. Life cycle of *P. falciparum* parasite in mosquito and in human host (From Rollback Malaria, home page photo gallery).

Following inoculation of sporozoites to the human body during blood sucking by the mosquito, the sporozoites migrate to the liver, invade the hepatocytes and undergo a phase of maturation and asexual reproduction. The merozoites are liberated soon after the bursting of infected liver cells, enter into the blood stream, invade the circulating erythrocytes where they initiate the erythrocytic schizogony. This process is repeated within erythrocytes and infected red cells rupture and release more merozoites into the blood circulation to embark the next erythrocytic cycle. The latter phase includes the development cycle in erythrocytes (erythrocytic schizogony) while the cycle occurs in the parenchymal cells of the vertebrate host, often referred to as the tissue stage. One should distinguish between the primary pre-erythrocytic schizogony which follows the development of the sporozoites of *P.vivax* and *P.ovale*, reinvade fresh liver cells and repeat the process - the schizogony. The sporozoites of the relapsing species of human malaria parasites namely *P. vivax* and *P. ovale* were differentiated either into hypnozoites or into developing tissue schizonts. The hypnozoites remain dormant in hepatocytes as an uni-nucleated form for a considerable periods. At a predetermined time the hypnozoites begin to grow and undergo exo-erythrocytic schizogony, forming a wave of merozoites that invade the blood, and produce a clinical relapse. Additional populations of hypnozoites may cause further relapses in the same way, according to the relapse pattern of the species and strain of *Plasmodium*. There is no evidence of true relapse in *P. falciparum* and in *P.malariae* since their sporozoites do not differentiate into hypnozoites but develop directly into pre-erythrocytic schizogony in the liver. The growth of the latter unpigmented tissue forms takes between 6-16 days, and this period varies according to the parasite species.

At the end of pre-erythrocytic stage, the mature schizont bursts and releases thousands of merozoites into the blood circulation. The merozoite consists of a single nucleus and adjacent cytoplasm with a diameter of approximately 1 μm. It invades the erythrocytes almost immediately after its release from the schizont. Each mature schizont of *P.vivax* contains 14-24 merozoites. In *P.malariae*, the number of merozoites varies from 6-12 which is equal to those of *P.ovale* while the *P.falciparum* schizont possesses 8-32 merozoites in each erythrocytic schizont. A proportion of invading merozoites

differentiate into male or female gametocytes which when ingested by the mosquito during human blood sucking, emerge to form gametes by a process known as gametogenesis. Fertilization occurs soon after the fusion of female gametes (macrogametes) by the male gametes (microgametes) following their exflagelletion. The two nuclei fuse each other and the zygote is formed (fertilization). The zygote being very active, it migrates across the mid gutwall of the mosquito. The zygote matures (Sporogony) within the body cavity of the invertibrate host - the mosquito. Within 18 hours or less, the zygote become elongated and transform into a slowly motile ookinate which then transverses the blood clot and peritropic membrane to reach the epithellium of the mid gut of the mosquito host. Later on, the ookinate comes to the rest on the external surface of the midgut where it develops into the oocyst. The oocyst expands by repeated nuclear division and gives rise to as many as 10,000 sporozoites. Each sporozoite measures 10-15 µm in length and 1 µm in diameter, curved, narrow ends and is freely motile. Each sporozoite escapes through the oocysts and travel across the haemocoelomic fluid to accumulate in the acinars of the salivery glands waiting for the next invasion when the opportunity comes.

The asexual erythrocytic cycle causes the symptoms and pathology of malaria. The malaria fever and associated symptoms of headache, nausea and muscular pain occur at the time of schizont-infected erythrocytes rupture and new ring forms appear.

7. Diagnostic tests of malaria

Malaria can be detected by several methods for the research as well as diagnostic purposes. These methods include from conventional techniques o recent molecular methods. Malaria diagnosis includes the detection of malaria parasites either in man or in mosquito by microscopic, detection of either antigens or antibodies by immunological tests or by genetic probes.

7.1 Microscopic examination

Romanowsky's stain, first described in 1891, is extensively employed for both thick and thin smears in order to demonstrate the parasite inside the infected red cells. This stain is made of a combination of methylene blue and eosin. This stain achieves a much improved definition of the parasites particularly the nuclear structure. It obtains a deep-red nuclear staining while the cytoplasm is stained blue.

The conventional method for malaria diagnosis is the demonstration of the parasites by microscopic examination of stained blood films. This technique is considered as the Gold Standard method in every aspect. When performed by a skilled microscopist, the limits of detection are about 1-20 parasites/µl blood (Bruce-Chwat, 1984). This technique allows for differentiation of the *Plasmodium* species responsible for the actual malaria infection. In theory, thick film should be thirty times more efficient than thin film in the detection of the infection (Payne, 1988), but the loss of the parasites which occur during thick film staining reduces this advantage ten times. A microscopist must routinely spend 30 min reading a thin smear. This level of sensitivity can be improved at a 0.0002% parasitemia (10 parasites/µl) by examining the entire smear of the slide.

Malaria parasites induce a variety of structural and antigenic changes in the membrane and cytoplasm of their host erythrocytes including the development of caveolae, caveolae-like complexes, clefts and knobs (Trager *et al.*, 1966; Luse *et al.*, 1971; Aikawa *et al.*, 1983; Sterling *et al.*, 1975; and Leech *et al.*, 1984). By light microscopy such structures usually recognized as a stipping are composed of fine, brick red dots, in the cytoplasm of *P.vivax* infected erythrocytes. However, little is known about the functional significance of these complexes. In Romanowsky stained blood smears, it is termed Schuffner's dots (Schuffner, 1984) which can be demonstrated in asexual stages of the parasite as well as gametocytes and are used as a diagnostic criteria of *P.vivax* or *P.ovale* infection when they are present.

7.2 DNA probe and polymerase chain reaction (PCR)

In 1985, Mullis *et al.*, introduced the technique for specific synthesis of DNA fragments *in vitro* system via enzymatic amplification using large fragments of DNA. PCR is an *in vitro* enzymatic replication of DNA by a repetitive cyclical process that results in approximately a billions fold amplification of a specific DNA sequence. The sensitivity and specificity of this technique is much higher than the conventional techniques. Jaurequiberry *et al.*, (1990) were the first who reported on PCR detection of human malaria. *Plasmodium* specific region of the 18s rRNA gene was amplified and hybridized with ^{32}P-labeled oligonucleotide for that fragment in slot-blot format. Sensitivity was at the level of 0.01 pg of *P. falciparum* DNA. The detection limit of microscopy is 5-20 parasites/μl (Bruce-Chwat, 1884) and the sensitivity of rapid diagnostic tests such as a simple immunochromatic test have been reported to be above 90% if more than 100 parasites/μl are present (Thomas and Martin, 2002), where as PCR assays could detect parasitemias of less than 5 prarasites/μl and probably as low as 0.004 parasites/μl (WHO, 2000; Moody, 2002).

Based on the premise that within any organism there are unique DNA sequences which differentiate that organism from closely related organisms permit the development and use of such DNA probes for diagnosis. DNA probes containing cloned repetitive sequences from *P. falciparum* used to identify malaria-infected blood samples by spot hybridization assay have been developed (Frazen *et al.*, 1984). This technique could detect at the parasitemia levels of 0.001% with no cross reaction with three other human *Plasmodium* species. Followed by several published reports, the DNA probes produced from *P. falciparum* isolates were used for the detection of *P. falciparum* antigens in the blood (Coppel *et al.*, 1985, Pollack *et al.*, 1985, Oquendo *et al.*, 1986, Barker *et al.*, 1986). In 1985, Mclaughlin and his colleagues have been established the use of synthetic 21-nucleotide repeated unit of *P. falciparum* DNA(5'-AGGTCTTAACTTGACTAACAT-3') as a probe. This synthetic DNA probe showed no cross-hybridization with host DNA or with DNA isolated from *P. vivax* or *Babesia* species and it could be detected as few as 0.1 ng of *P. falciparum* DNA. Since synthetic DNA is easily prepared, the observed sensitivity and specificity suggests that synthetic DNA probes would be generally useful in the malaria diagnosis.

7.3 Immunological tests

The success in epidemiological studies of malaria and in malaria control programmes requires the application of adequately reliable and precise diagnostic methods. This approach is particularly useful in the areas where the basic malaria diagnostic facilities are very primitive and the available resources are also very limited. There are two general approaches used for the diagnosis of malaria that comprise of either the demonstration of the parasite or parasitic antigen or the detection of antibodies in the human vertibrate host serum elicited against such parasites as an immunological aspect.

Immunological tests have also a diagnostic role in malaria infections. In the past, these tests had the disadvantage of using the crude antigen(s) or antibodies. The recent isolation of purified antigens, however, from all stages of the parasite and the development of the specific monoclonal antibodies have shown highly sensitive and specific results for the diagnosis of malaria. Attempts have also been made to develop an antigen detection test based on monoclonal antibodies or polyclonal antibodies raised against a specific malaria antigen. By the use of labeled monoclonal antibodies (MAbs) inhibition assay, using *P.berghi* as the antigen, had a sensitivity of 68.3% in detecting *P. falciparum* with 99.8% specificity (Avidor *et al.*, 1987). The higher sensitivity has been reported by Khusmith *et al.*, (1988) using immunoradiometric sandwich assays to a population in an endemic area in Thailand. The sensitivity and specificity achieved were 100% and 65.9%, respectively.

An improved pan-species monoclonal and polyclonal antibody to detect blood stage *P. vivax* by sandwich ELISA gave sensitivity range of 87-98% and the limitation of the detection was greater than 1 parasite/100 red blood cells (Gao *et al.*, 1991). An IgM monoclonal antibody was employed in the detection of *P. falciparum* in the whole blood. The test was based on IgM captured MAb on solid phase and IgG monoclonal antibody conjugated to peroxidase (Dianne and Voller, 1993). They claimed that as few as 0.001-0.0002% of parasitemias could be detected.

Apart from the red blood cell associated antigens, the free soluble parasite antigens can be detected by using ELISA. In a study reported by Fortier et al., (1987), a four step ELISA based on the antigen capture by MAb against *P. falciparum* antigen with a relative molecular mass of 50,000 was used to detect parasitemias between 0.001 and 25% without false positivity. One sandwich MAb-based antigen detection assay has been developed for the specific purpose of screening blood donors (Duberry et al., 1990). Using two different monoclonal antibodies combination, its sensitivity in detecting the antigen in patients suffering from their first attack of malaria was 72.2% with 99.2% specificity. The sensitivities obtained of the sandwich ELISA using MAb against *P faciparum* and *P. cynomogi* were 95% and 98% respectively (Lim et al., 1992). The specificity of this assay with both monoclonal antibodies was 100%.

Until the successful production of monoclonal antibody against all blood stages of *P. falciparum* including gametocytes by Khusmith and colleagues, in 1986, the solid phase competitive binding RIA has been established. Using dilution of red blood cells from *in vitro* cultures of *P.falciparum*, the tests were found to detect parasites at levels equivalent to 13 and 2.2 parasites/10^6 erythrocytes when the IgG fraction from human immune sera and monoclonal antibody (MAb) are used respectively. A higher proportion of tests were positive with RIA than with microscopic examination particulary when MAb was used with the correlation of 0.06, $P < 0.0001$ as assessed by regression analysis (Khusmith et al., 1986).

It is likely that the monoclonal antibody (MAb) could bind to the parasite antigen more specifically than could the polyvalent antibody. In an atempt to improve the sensitivity of the antigen detection assay, three systems of two-site sandwich immunoradiometric assay (IRMA): (i) a two-site MAb sandwich IRMA (MAb-IRMA), in which the plates were coated with unlabeled MIgG and detected with labeled MIgG; (ii) a two-site PAb-MAb sandwich IRMA (PAb-MAb-IRMA), in which the plates were coated with unlabeled PIgG and detected with labeled PIgG (Khusmith et al., 1987), has been developed to detect low-grade infection with the ability to detect as few as 0.24, 0.67, and 1.82 parasites/10^6 erythrocytes, respectively. The assays showed good

correlation with parasitemia when tested against *P. falciparum*-infected erythrocytes from *in vitro* culture as well as from clinical specimens, especially when the MAb-IRMA was used. MAb-IRMA was therefore recommended for use in the detection of low-grade parasitemia (Khusmith *et al.*, 1987). By virtue of its higher sensitivity and the ease in processing of a large number of specimens a two-site MAb-IRMA for the detection of *P. falciparum* antigen have been applied to the field successfully (Khusmith *et al.*, 1988). Unfortunately, immunoradiometric assays require the use of short-lives radioactive reagents that have inherent drawbacks associated with transportation and disposal after use. These problems permit the development of enzymatic techniques which are being extensively used in almost all biological fields for both routine clinical and advanced rsearch purposes.

Thus, to overcome these problems, the alternative diagnostic methods to detect malaria cases, especially those with lowgrade parasitemia to supplement or perhaps to replace microscopy are required. Recent studies on the parasite genome together with the production by the genetic manipulation of pure malarial antigens and the production of specific monoclonal antibodies have the potentials for providing both more extensive and detailed information on the parasite in the infected human and the insect vector.

7.3.1 Enzyme linked immunosorbent assay (ELISA)

Enzyme immunoassay (EIA) methods are based on reactions of enzyme-labeled immunoreactants, especially antibodies, are playing an increasingly important role in the diagnostic laboratory. The technique was derived from the work of Nakane and Pierce (1967) which showed that antibodies could be labeled with enzymes for use in histochemical staining of tissues. The enzyme immunoassay is based on the promise that antibodies or antigens are conjugated to enzymes in such a manner that both the enzymatic reactvity and the immunoreactivity of this conjugate are retained. The degradation of a substrate by the enzyme is measured spectrophotometrically which is proportional to the concentration of the unknown "antibody" or "antigen" in the test solution. Enzyme immunoassays can be sub-divided into two broad catagories, either

homogeneous enzyme immunoassay or enzyme multiplied immunoassay technique (EMIT), or, heterogeneous enzyme immunoassay or enzyme linked immunosorbent assay (ELISA).

ELISA technique has been used extensively in the diagnosis of clinical specimens as well as in the research purposes because of its simplicity and inexpensiveness in nature. This technique was first of all described by Engvall and Perlmann (1971, 1972), and by Von Weemen and Schuurs (1971, 1972). Application of ELISA using *P.knowlesi* antigens t o m easure m alarial a ntibody i n h uman p opulation h as b een r eported i n 1975 (Voller *et al.*, 1975). However, the microplate ELISA method using *P.falciparum* antigen was more convenient and sensitive (Voller *et al.*, 1974). Demedts and colleagues simultaneously use *P. falciparum* crude antigen and red blood cell control antigen in the ELISA to evaluate the reactivity of each serum to RBC contaminants of crude malarial antigen, which seemed to be interfered in the interpretation of the test (Demedts *et al.*, 1987). Their results also indicated that ELISA using *P.falciparum* antigen displayed a considerable degree of species-specificity, which was in agreement with the results described by Wahlgren *et a l.*, (1983). T he s imultaneous u se o f r ed blood c ells c ontrol antigen with crude *P.falciparum* antigen become even more important in the light of their observation that sera from patients with *P.malariae*, *P.ovale*, or *P.vivax* malaria reacted only weakly with the *P.falciparum* antigen, while displayed strong reaction with normal red blood cells.

ELISA method has been found to have a very high sensitivity and precision, but for this to be achieved, a reliable reproducible soluble antigen preparation is essential. In the context of malaria diagnosis, the results have been somewhat disappointing and this probably reflects t he use o f crude antigen p reparations t o d ate. A lthough a ntibody h as been readily detected within a few days of *Aotus* monkeys being infected with falciparum malaria it has been difficult to detect the early antibody response in people with natural infection (Voller & Houba, 1980).

Double-antibody-sandwich ELISA and inhibition ELISA for the diagnosis of *P. falciparum* in blood from infected *Saimiri* monkeys have been developed by Bidwell & Voller, in 1981 with the sensitively of $1/10^3$ erythrocytes and $1/10^4$ erythrocytes, respectively. However, these methods are not yet as sensitive as conventional blood-film examinations, in which a well-trained microscopist might be expected to detect one parasite/10^6 erythrocytes. In addition, the antibody-binding inhibition in a solid-phase system developed in a murine malaria model has been described (Mackey *et al.*, 1980). It was shown to have a high degree of sensitivity with a level of parasite detection of $1/10^6$ erythrocyte.

In human, an ELISA inhibition test has also been carried out by Macky and his colleagues, in 1982, using human immune serum and infected red blood cells. The test was applied to the detection of parasites in red blood cells from *in vitro* cultures of *P. falciparum* and in red blood cells from infected Gambians. In titration experiments, the degree of antibody inhibition correlated with the number of parasites in the test red blood cells with the sensitivity of 8 parasites/10^6 erythrocytes. In infected blood, 86% of cases gave positive results and the levels of parasitemia ranged from 10 to 125,000/µl of blood. A few years ago, DOT-ELISA for rapid detection of *P. falciparum* antigens and antibodies had been developed (Londner *et al.*, 1987). It was shown that *P. falciparum* antigens could be detected by their ability to inhibit the binding of antibody to the filter paper. The erythrocyte infected with *P. falciparum in vitro* could be detected at a level of 0.001% parasitemia.

Fortier *et al.*, (1987) developed enzyme immunoassay (EIA) for detection of antigen in acute *Plasmodium falciparum* malaria by using a monoclonal antibody specific for an epitope of a 50 kDa *falciparum* exo-antigen in culture supernatant and in the sera of 31 patients suffering from acute malaria. The assay was specific for *P. falciparum* and did not appear to be strain restricted. A parasitemia level below 1 parasite/10^3 erythrocytes could be detected, and the sensitivity of the test render this EIA useful for diagnosis of *P.falciparum* malaria with a very low level of parasitemia. This work was in

progress to design a simpler test suitable for the field applications which would make microscopic examination unnecessary.

Given the advantage of ELISA to detect malarial antigen as was described above, many references of this assay will be reviewed as follow: Bidwell *et al.*, (1981) developed ELISA for detecting malarial parasites in blood of uninfected monkeys and in monkeys infected with *Plasmodium falciparum*. The assay was carried out in two types: (a) a double antibody sandwich ELISA which could detect one malaria parasite per 10^3 uninfected erythrocytes and (b) an inhibition ELISA which could detect one parasite per 10^4 uninfected erythrocytes. Mackey *et al.*, (1982) adapted the methodology from a competitive binding radio-immunoassay (RIA) using polyclonal antibodies (PAb) with a sensitivity of detecting 8 parasites/10^6 erythrocytes to an ELISA inhibition test system, using human immune serum and infected RBC. The test was applied to the detection of parasites in RBC from *in vitro* of *P. falciparum* with parasites were detected at a level of 8 parasites/10^6 RBC. The test was applied to detect parasites in RBC from infected Gambians with parasites were detected in the range of 10 to 125,000/μl of blood. Londner *et al.*, (1987) developed DOT-ELISA for rapid detection of *P.falciparum* antigens and antibodies. The reasons for the development of DOT-ELISA were because the conventional ELISA procedures require large amount of soluble antigen (Inhibition ELISA to detect antigen), overnight adsorption of antigen to microplate wells, longer incubation periods and photometers to determine titer endpoints. They showed, *P.falciparum* antigens were detected by their ability to inhibit the binding of antibody to the filter paper. RBC infected with *P.falciparum in vitro* can be detected at a level of 1 parasite/10^3 erythrocytes. This report explored the feasibility of an assay for detecting malaria antigens in blood sample which is easily applicable even in the field conditions.

A longitudinal serological study of *P.falciparum* malaria in the West African Savanna using an ELISA technique showed a lower seropositive rate in the protected individuals than that in the unprotected group of the same age (Voller *et al.*, 1980). Comparison between ELISA and IFA performed with cultured *P. falciparum* as antigen for measuring antibody have been established (Spencer *et al.*, 1979). The major

differences observed occurred in younger persons, seropositive rates by ELISA were singificantly higher than those found by IFA. However, even in older persons whom the seropositivity rates observed with the two tests were similar, many of the specimens were positive by one test but not by both. Fifteen persons have slightly demonstrated infected with *P.falciparum*; all 15 persons were positive by IFA and only 12 were positive by the ELISA.

Londner *et al.*, (1987) have described an ELISA method for the rapid detection of *P.falciparum* antibodies and antigens based on the application of antigen as a spot to nitrocellulose filters, DOT-ELISA, with the ability to detect pools of sera from patients with a recent disease or from individuals with a history of malaria containing antibodies up to a dilution of 1:64,000. Negative results were obtained when the normal red blood cells were used for clotting the filters.

7.4 Monoclonal antibody

7.4.1 Strategies of monoclonal antibody production

The strategy of the production of antibody-secreting cell lines "hybridomas" is accomplished initially by the fusion of myeloma cells with spleen cells from immunized or infected donors permits the production of potentially unlimited amount of homogeneous antibodies of unique specificity. These homogeneous antibodies secreted by cell lines derived from clonal expansion of single hybrid cells have been termed "Monoclonal Antibodies" (MAbs) and characteristically demonstrate high specificity for a certain epitope of an immunogen. The "hybridmyeloma' or "hybridoma" cells inherit the immortal character of neoplastically-transformed continuous cell line from their myeloma parents and the genetic code determining a given antibody specificity from an immune splenocyte parents.

One of the most important variables determining the outcome of a hybridization encountered in selecting a hybrid cell line producing antibodies of the desired specificity

and properties is the immunization protocol. Since in the lymphocyte population used for fusion only a small percentage of the cells are specific for the immunizing antigens. This is about 1 out of 10 antibody bearing cells in the repertoire of inbred mice that show the ability to recognize a single antigenic determinant (Kreth & williamson, 1973; Klinman & Press, 1975; Kohler, 1976 and; Kohler, 1986). Therefore, only a fraction of the hybrids produced secreted antibodies of the desired specificity. However, the frequency of positive hybrids depends not only on that of the antigen specific B-cells in the spleen cell populations but also on the maturation stage of these cells. It is clear that the activated B-cells hybridize more efficiently than the resting cells. With the aim of maximizing the frequency of antigen specific activated lymphocytes in the spleen, a massive dose of antigen is applied intravenously two to four days prior to fusion.

Only such myelomas that secrete antibody were used in the previous development of hybridoma techniques and then these myelomas may secrete molecules which are made up of heavy and/or light chains from both parent cells. Such molecules may have a much lower avidity for the specific antigen than the ones consisting only of spleen cell derived chains, and may thus reduce the antibody titer. Thus, the immunoglobulin non-secreting and non-producing myeloma cell lines have therefore been introduced (Shulman et al., 1978; Kearney et al., 1979) and used to obtain hybrids that secrete only antibody molecules from parent spleen cells but not from the parent myelomas even when they are crossed with spleen cells.

An initial step in the production of somatic cell hybrids is cell fusion where as spontaneous fusion of cells is usually rare. Fusion can be accomplished when the cells are treated with Sendai virus (Kao et al., 1974) or high concentration of polyethylene glycol (PEG), a chemical fusogen (Gefter et al., 1977). PEG has been preferred recently because it has greatly simplified and increases the efficiency of cell fusion protocol. However, the mechanism of fusion is poorly understood (Westerwoudt, 1986). The fusion of the two cell membranes results in the formation of multinucleated cells called heterokaryons. At the next cell division, the nuclei of heterokaryons fuse, and the daughter cells possess a more or less equal share of the genetic material (Goding, 1980).

The process of fusion is a relatively rare event and consequently the culture can easily and rapidly overgrown by unfused myeloma cells. Thus, if it is anticipated to produce a long-term hybrid cell line from two cell types, a selection procedure is always required.

The most common selection procedure is that devised by Littlefield in 1964. This selection is based on the inhibition of the main biosynthetic pathways for thymidylate and the purine nucleotides by the folate antagonist, aminopterine (A). When the main synthetic pathways are blocked with the folic acid analogue aminopterine, the cell must depend on the "salvage" enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK) if hypoxanthine (H) and thymidine (T), the respective substrates for these enzymes are provided in the medium. Thus, the cells lacking either enzyme die in a HAT medium because both the main and the salvage pathways are blocked. However, mutant myeloma cells (HGPRT⁻ or TK⁻) can be made to grow in HAT medium if they are provided the missing enzyme by fusion with spleen cell which possess HGPRT and TK, but has short life in culture.

Selection of mutant myeloma cell (HGPRT⁻, TK⁻) can be accomplished by growing in medium containing the toxic base analogues 6-thioguanine or 8-azaguanine, which are incorporated into DNA in cells expressing the corresponding exzyme and only HGPRT⁻, TK⁻ cells survive. Another selective mechanism exploits deficiency of the enzyme adenosine phosphoribosyl transferase (APRT⁻). The medium contains azaserine which blocks *de novo* purine synthesis. Thus, the myeloma cell line possess APRT⁻ could not complete their nucleic acid synthesis and die out while those hybrid cells contain this enzyme can survive and grow.

It was found in the early study of Kohler and Milstein's discovery (Kohler & Milstein, 1975) that unlimited quantities of absolutely specific and uniform antibodies recognizing only one epitope could be produced, even if the immunizing antigen was weakly immunogenic or grossly impure. All that is needed is an appropriate way of screening for antibody with the desired properties, but does not require exact quantitation.

The most commonly used methods are solid phase radioimmunoassay (Tsu & Herzenberg, 1980), ELISA (Kearney *et al.*, 1979) and immunofluorescence (Ledbetter & Herzenberg, 1979). There are at least two considerations in selecting the screening assays. One consideration is the nature of the antigen and the other is the convenience of the test that should be quick and capable enough to be set up daily since hybrid clones usually grow at different rates.

By virtue of the high probability of chromosome loss in the hybrids, the relative high probability of growth of nonproducer variants and to make sure that the antibodies are indeeded monoclonal, hybridoma lines should be cloned at least twice to make absolutely certain that each is a true monoclone. However, after two cycles of cloning, the rate of chromosome loss is little, although the risk of overgrowth by non-producer cells never ceases completely.

Myeloma cell lines and their hybrids can be cloned either by growing in soft agar (Kohler & Milstein, 1975) or by limiting dilution. The latter procedure is generally preferred because of its ease and convenience, but it does not guarantee clonality. If cells are grown in small numbers, the fraction of wells with growth should follow the Poisson distribution (Fazekas *et al.*, 1980; De Blas *et al.*, 1983). In order to obtain optimal growth of hybrids after fusion and to clone myelomas or hybrids by limiting dilution, the feeder layers that include thymocytes, normal spleen cells and peritoneal macrophages are commonly used (Goding, 1980). The exact function of feeder cells is poorly understood. It is suggested that they reduce the toxicity of the plastic tissue culture plates. However, several hybridoma laboratories have well success without using feeder layer (Zola & Brook, 1982; Khusmith *et al.*, 1984).

Once the active hybrids have been identified and cloned, they are left to grow *in vitro* in a larger volume for antibody production. Typical antibody levels in the culture supernatant ranges from 5-50 μg/ml, and can be secreted up to 100 μg/ml. However, this varies from line to line and can be achieved only under optimal culture conditions. An alternative way to produce large quantities of hybridoma products takes advantage of the

fact that these cell lines, are tumorogenic as their myeloma parents and it is generally easier to grow the cells as tumors in animals. The serum or ascites will usually contain around 1000 times the antibody concentration in culture.

7.4.2 Special properties of monoclonal antibodies

The properties and advantages of monoclonal antibodies (MAbs) have been discussed extensively (Pearson *et al.*, 1977; Willams *et al.*, 1977). Essentially, they are chemically homogeneous reagents which bind to single antigenic sites on a molecule and can be produced in unlimited quantities without using pure antigens for immunization. The result is that each monoclonal antibody is a unique protein with its own biochemical and immunochemical characteristics unlike conventional antisera which are composed of antibodies of different classes, affinities and specificities. This biochemical uniformity of the monoclonal reagents results in their unique specificity and high titers obtained after growth of cloned hybridomas.

SPECIFIC OBJECTIVES

1. To develop monoclonal and polyclonal antibody (MAb-PAb) based indirect ELISA using monoclonal antibodies originated from native Thai strains of *P.vivax* and *P.falciparum* and the polyclonal antibodies raised against native Nepali strains of *P.vivax* and *P.falciparum*.

2. To apply the established techniques to detect *P.vivax* and *P.falciparum* antigens in individuals who have been living in malaria endemic areas in southern Nepal.

3. To evaluate the sensitivity, specificity and accuracy of monoclonal and polyclonal antibody (MAb-PAb) based indirect ELISA.

CHAPTER III

MATERIALS AND METHODS

1. Study areas

The topography of Nepal (Figure 6) is dominated by a series of northwest to southeast mountain ranges that arises from the plains of outer Terai (altitude, <100 m) to Mt. Everest (altitude, 8448 m). The weather is characterized by cool, dry winter and hot, wet summer dominated by a southeast monsoon which begins in May or early June in the east and June or July in the west.

Nepal is a landlocked country situated on the southern slopes of the Himalayan Mountains between an autonomous region of China (Tibet) in north and in India in the south, east and west. It is located between 26° 22' and 30° 27' north latitudes and 80° 04' and 88° 12' east latitudes with a total surface area of approximately 147,181 sq km. On an average, the east-to-west length is 885 km; the north-south width is not uniform, the maximum being 241 km and the minimum is 145 km.

The field study was conducted in Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district (Figure 7), Janakpur Zone in the southern tropical areas of Nepal from July 2001 to July 2002 (Shrawan 2058 to Shrawan 2059). Dhanusha district extends from 85° 52' to 86° 20' east longitude and from 26° 36' to 30° 27' north latitude. The total area of this district is 1,180 square kilometers, and the elevation of the land ranges from 500 to 800 ft in plain areas and up to 2,000 ft for Chure hill (Sherchand et al., 1996). The area covered by forests in this district is 3,039.7 hectares i.e. 24.9% of the total land. The annual rainfall is in between 650 to 2,000 mm. The annual average maximum temperature is 26°C to 41°C and the monthly minimum temperature range is 10°C to 20°C. The Dhanusha district is bordered to the north by Sindhuli district, to the west by Mahottari district, to the east by Sagarmatha Zone and to the south by India (Sherchand et al., 1996). Most people were local permanent residents. The Annual Parasite Incidence (API) of malaria in Dhanusha district in the year 1997 has been documented 1.8/1,000 population and the total slide positive cases of malaria was

Nepal

Figure 6. Map of Nepal showing Dhanusha district where field research was conducted. (Source: Annual Report, Department of Health Services 2053/54 (1996/97), His Majesty's Government of Nepal, Ministry of Health, Department of Health Services).
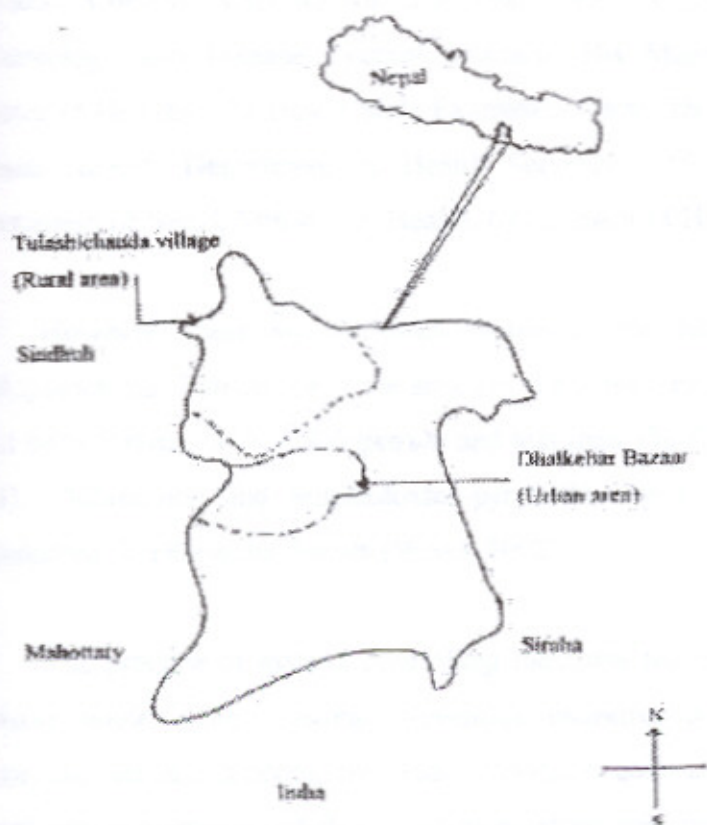
Figure 7.   Map of Dhanusha district of Nepal where the enrolled health posts are situated.

1144 representing 12.8% of vivax malaria positive and 4.09% of the falciparum malaria positive cases of the whole country (The Annual Internal Assessment of the Malaria and Kala-azar Control Activities of the Year 1997, Department of Health Services, Epidemiology and Disease Control Division, His Majesty's Government of Nepal, Ministry of Health). The Blood Slide Examination Rate (BSER)/100 population is 1.20% (Annual Report, Department of Health Services 2055/56 (1998/99), His Majesty's Government of Nepal, Ministry of Health, Department of Health Services).

However, there are no exact figures of the relative importance of *P.vivax*, *P.falciparum* and *P.malariae* in the area and it has been estimated that the proportions are about 65% *P.vivax*, 34% *P.falciparum* and less than 1% *P.malariae* (Shrestha JPB *et al.*, 1991). Chloroquine and Sulphadoxine-pyrimethamine (SP) resistant cases have been documented in Dhanusha district (WHO, 2002).

The practice of malaria controlling measures has not been exercised by the rural residents while some personal preventive measures such as the use of bed-nets, commercial aerosol spraying repellents, mosquito coils and other substances that when burned produce smoke and thus repel mosquitoes are popular mostly in the urban areas rather than in the rural areas. Local village people do not use the insecticide spraying not only because of the poverty but also due to the displeasure of the fumes produced by the insecticides. Therefore, the number of malaria cases in this district have been found to be escalated annually.

2. Informed consent and ethical clearance

A written informed consent was obtained from each patient either from the patient himself or herself or from his or her closed relative or preferably from his or her own family members who know the patient very well and take care well as a part of ethical clearance. Those patients who did not give permission to give blood were not included in this study. The statements given by each patient were maintained strictly confidential as a part of ethical clearance. Likewise, a formal ethical approval/ethical clearance letter was obtained from the Nepal Health Research Council, the highest government body of Nepal

regarding research and ethical clearances before embarking to conduct research in the targeted field sites of this research.

3. Blood collection

Blood were collected from patients with vivax malaria and falciparum malaria separately. Two to three ml of blood were collected in a screw caped tubes containing 10 µl of 10 % EDTA per ml of blood from each patient. Blood were gently and thoroughly mixed with the anticoagulant to prevent the coagulation. Blood from controls groups were also collected in the same way. Thick and thin blood films were prepared by the usual ways on a clean and greasy free microscopic slides, stained with Giemsa's staining method and were examined by bino-occular light microscopy. The number of infected erythrocytes/10^3 erythrocytes was determined by counting the number of parasitized cells in 10^3 erythrocytes in thin blood films. For light infections, the number of parasites in thick blood films were counted and expressed per 200 white blood cells (WBC). The collected blood were centrifuged at 1000 rpm for 10 min at room temperature to remove the buffy coat and plasma and the remaining packed red cells were aliquoted immediately and stored at -70 °C until use.

4. Subjects

4.1 Patients with vivax malaria

The blood collection of *P.vivax* parasites from the infected patients was carried out from July 2001 to April 2002 (Shrawan 2058 to Baisakh 2059) from Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district.

Two hundred and six blood were collected individually from patients aged ranged from 1.0 to 82 years. All were microscopically confirmed *P.vivax* positive patients with informed consent from each and individual patient as a part of ethical clearance. Those who had already taken anti-malarial medications were not included and there was no

discrepancy of sexes on blood sample collection. Three blood samples were found to be clotted so these three blood samples were not included and thus remained two hundred and three samples were only included in this research.

4.2 Patients with falciparum malaria

The sample collection of *P.falciparum* parasites from the infected patients was carried out from July 2001 to July 2002 (Shrawan 2058 to Shrawan 2059) from Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district.

One hundred and fifty four blood were collected from patients aged ranged from 1.0 to 73 years. All were microscopically confirmed cases of *P.falciparum* positive patients with informed consent from each and individual patient as a part of ethical clearance. Those who had already taken anti-malarial medications were not included and there was no discrepancy of sexes on blood sample collection.

4.3 Healthy controls

Forty blood samples from healthy O blood group persons were taken residing in Kathmandu where malaria is not endemic. Those persons without any history of malaria and denied traveling to any malaria endemic areas at least for two years, hence they must unlikely to have been exposed to malaria during the time of study.

5. Preparation of red cell lysates

The packed red cells from the test and control groups were treated with 9 volumes of 0.01 M Phosphate Buffered Saline (PBS), pH 7.4 containing 0.05% Tween 20 and 0.5% Nonidet P-40 (PBST-NP40) for 10 min at room temperature on the testing day.

6. Preparation of polyclonal antibodies (PAbs)

6.1 Preparation of anti-*P.vivax* polyclonal antibodies (PAbs)

The polyclonal antibodies were produced against local Nepali *P.vivax* strains in the rabbit system. A blood sample with high parasitemia (32,800 parasites/μl) was collected in a sterilized tube containing 10 μl of 10% EDTA/ml of blood. This whole blood was centrifuged at 1000 rpm at room temperature for 10 min and both plasma and buffy coat were removed. The portion of the red cells was sedimented and was reconstituted to be 50% suspension and proceeded to discontinuous percoll gradient centrifugation (Pharmacia, Fine Chemicals, Uppsala, Sweden). The percoll concentrations used in the gradient were 75%, 60%, 50% and 40%, respectively. After centrifugation at 4°C for 30 min in 2500 rpm, the interfaces between each percoll concentration was collected and the blood smears were prepared and stained with Giemsa's stain and the parasite concentrations were observed by microscopic technique. The parasite concentrations of 75-60% percoll interface, 60-50% percoll interface, 50-40% percoll interface and 40% percoll interface were 35%, 52%, 32% and 18%, respectively. The interface between 50% and 60% percoll was shown to contain the highest parasite concentration, thus the parasites were collected from this interface, washed with PBS pH 7.4 and centrifuged at 4°C for 30 min in 2500 rpm and then stored at -70°C until use. The content of these parasitized red cells was used as an antigen for rabbit immunization in the steps of production of polyclonal antibodies.

A healthy male rabbit was immunized intramuscularly with the 0.5 ml of the previously prepared Nepali strains of *P.vivax* parasitized red cells with equal volume of Freunds' Complete Adjuvant (Difco Laboratories, Michigan, USA) and subsequently with two boosts of the same amount of *P.vivax* parasitized red cells in Freunds' Incomplete Adjuvants (Difco Laboratories, Michigan, USA) at an intervals of three weeks. The blood samples were taken from the ear veins of the immunized rabbit before the first immunizing dose and after each booster dose. The serum was separated aseptically by centrifugation at 1000 rpm at room temperature for 10 min and was stored at -20°C.

The immune rabbit sera were inactivated at 56°C for 30 min. The globulin fractions of the rabbit sera were precipitated by saturated ammonium sulphate as described by Voller and Bidwell, (1986). Equal volumes of cold neutral saturated ammonium sulphate were slowly added to the rabbit sera with gentle stirring at 4°C for at least 45 min, followed by centrifugation at 12,000 rpm at 4°C for 30 min. The pellet was resuspended with distilled water to make the final volume approximately twenty times lesser than the original volume of the supernatant and the concentrated supernatant was dialysed extensively against distilled water and against 0.1 M phosphate buffer, pH 8.0 subsequently at 4°C overnight and were stored in a sterile container at -20°C for future use. The total IgG concentrations were determined directly at 280 nm (Spectronic Unicam Genesystem machine) using PBS pH 7.4 as a blank at the Everest Biotech laboratory of the British Biotechnology unit located at the Tribhuvan University, Central Campus, Maharajgunj, Kathmandu, Nepal. The IgG concentrations of the rabbit sera before the first immunizing dose, second dose and the third dose were 11.29 µg/ml, 29.01 µg/ml and 96.37 µg/ml, respectively. The preparation with the highest concentration of IgG was used in the subsequent experiments.

6.2 Preparation of anti-*P.falciparum* polyclonal antibodies (PAbs)

The polyclonal antibodies were produced against local Nepali *P.falciparum* strains in the rabbit system. A blood sample with high parasitemia (307,200 parasites/µl) was collected in a sterilized tube containing 10 µl of 10% EDTA/ml of blood. This whole blood was centrifuged at 1000 rpm at room temperature for 10 min and both plasma and buffy coat were removed. The portion of the red cells was sedimented and was reconstituted to be 50% suspension and proceeded to discontinuous percoll gradient centrifugation (Pharmacia, Fine Chemicals, Uppsala, Sweden). The percoll concentrations used in the gradient were 75%, 60%, 50% and 40%, respectively. After centrifugation at 4°C for 30 min in 2500 rpm, the interfaces between each percoll concentration were collected and the blood smears were prepared and stained with Giemsa's stain and the parasite concentrations were observed by microscopic technique. The parasite concentrations of 75-60% percoll interface, 60-50% percoll interface, 50-

40% percoll interface and 40% percoll-PBS interface were 15%, 43%, 28% and 19%, respectively. The interface between 50% and 60% percoll was shown to contain the highest parasite concentration, thus the parasites were collected from this interfaces, washed with PBS pH 7.4 and centrifuged at 4°C for 30 min in 2500 rpm and then stored at -70°C until use. The content of these parasitized red cells was used as an antigen for rabbit immunization in the steps of production of polyclonal antibodies.

A healthy male rabbit was immunized intramuscularly with the 0.5 ml of the previously prepared Nepali strains of *P.falciparum* parasitized red cells with equal volume of Freunds' Complete Adjuvant (Difco Laboratories, Michigan, USA) and subsequently with two boosts of the same amount of *P.falciparum* parasitized red cells in Freunds' Incomplete Adjuvants (Difco Laboratories, Michigan, USA) at an intervals of three weeks. The blood samples were taken from the ear veins of the immunized rabbit before the first immunizing dose and after each booster dose. The serum was separated aseptically by centrifugation at 1000 rpm at room temperature for 10 min and was stored at -20°C.

The immune rabbit sera were inactivated at 56°C for 30 min. The globulin fractions of the rabbit sera were precipitated by saturated ammonium sulphate as described by Voller and Bidwell, (1986). Equal volumes of cold neutral saturated ammonium sulphate were slowly added to the rabbit sera with gentle stirring at 4°C for at least 45 min, followed by centrifugation at 12,000 rpm at 4°C for 30 min. The pellet was resuspended with distilled water to make the final volume approximately twenty times lesser than the original volume of the supernatant and the concentrated supernatant was dialysed extensively against distilled water and against 0.1 M phosphate buffer, pH 8.0 subsequently at 4°C overnight and were stored in a sterile container at -20°C for future use. The total IgG concentrations were determined directly at 280 nm (Spectronic Unicam Genesystem machine) using PBS pH 7.4 as a blank at the Everest Biotech laboratory of the British Biotechnology unit located at the Tribhuvan University, Central Campus, Maharajgunj, Kathmandu, Nepal. The total IgG concentrations of rabbit sera before the first immunizing dose, second dose and the third dose were 7.29 µg/ml, 36.11

μg/ml and 64.57 μg/ml, respectively. The preparation with the highest concentration of IgG was used in the subsequent experiments.

7. Preparation of anti-*Plasmodium* monoclonal antibodies (MAbs)

7.1 Preparation of anti-*P. vivax* monoclonal antibodies (MAbs)

Murine hybridoma namely McPV1 secreting antibodies against *P. vivax* was kindly provided by Prof. Dr. Srisin Khusmith, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. The hybridoma was produced by fusion of Sp 2/0 myeloma cells with spleen cells from BALB/c mice immunized with the concentrated *P. vivax* antigens according to the method described previously (Khusmith *et al.*, 1984). Supernatants from the culture of growing hybrids were collected and tested for anti-malarial antibody by indirect fluorescent antibody test (IFA). All positive clones were further subcloned twice by the limiting dilution method, followed by further propagation and the clone products analysed by the IFA. Positive monoclones were cryopreserved in liquid nitrogen for further use. The monoclonal antibodies were characterized based on the immunofluorescent reactivities (Khusmith *et al.*, 1984) and by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blot analysis. The monoclonal antibodies reacted specifically to *P. vivax* and did not react with *P. falciparum*. This monoclonal antibody was belonged to IgG$_1$ and produced uniformly bright generalized staining of all blood stages including gametocytes.

7.2 Preparation of anti-*P. falciparum* monoclonal antibodies (MAbs)

Murine hybridoma namely F2W22C1 secreting antibodies against *P. falciparum* was kindly provided by Prof. Dr. Srisin Khusmith, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. The hybridoma was produced by fusion of Sp 2/0 myeloma cells with spleen cells from BALB/c mice immunized with the concentrated *P. falciparum*

antigens according to the method described previously (Khusmith *et al.*, 1984). Supernatants from the culture of growing hybrids were collected and tested for anti-malarial antibody by indirect fluorescent antibody test (IFA). All positive clones were further subcloned twice by the limiting dilution method, followed by further propagation and the clone products analysed by the IFA. Positive monoclones were cryopreserved in liquid nitrogen for further use. The monoclonal antibodies were characterized based on the immunofluorescent reactivities (Khusmith *et al.*, 1984) and by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blot analysis. This monoclonal antibody was belonged to IgG_1 and produced uniformly bright generalized staining of all blood stages including gametocytes.

8. Large-scale production of monoclonal antibodies

Both monoclones namely McPV1 and F2W22C1 were thawed out by putting the cell ampoules at $37^{\circ}C$ water bath immediately after being taken from liquid nitrogen tank. The cells were washed in 10 ml of RPMI IV medium, pelleted by centrifugation and suspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 15 mM HEPES, 5×10^{-2} mM hypoxanthine, 100 units/ml of penicillin (Dumex), 100 µg/ml of streptomycin (Dumex), 100 µg/ml of kanamycin, 30% glucose and 100 mM pyruvate (H-medium). Thereafter, the cells were pipetted into 50 ml tissue culture flask (Costar) and placed in humidified 5% CO_2 incubator at $37^{\circ}C$. The cultures were allowed to grow to exponential phase before passage. About 80-90% of the cell populations were removed before going to the next cycle.

The supernatant of the cultures were harvested when the colour of the medium began yellow. The culture of each monoclone was centrifuged at 1000 rpm for 10 min at room temperature to remove the cells and then the supernatant was collected and retested for antibody activity by means of an indirect fluorescent antibody test. All hybridomas that showed positive IFA were expanded subsequently in 600 ml tissue culture flask (Costar) containing 150 ml of complete RPMI medium. The culture was allowed to grow

A monoclonal-polyclonal antibody based indirect ELISA in which the plates were coated with monoclonal antibodies and with the detecting polyclonal antibody reacting with anti-rabbit IgG labeled with HRP enzyme, were developed in order to test the field samples of both the *P.vivax* and *P.falciparum* infected erythrocytes collected previously from the patients in the fields.

The optimum conditions of MAb-PAb based indirect ELISA were standardized in the following ways.

12. Optimization of monoclonal antibody for plate coating, anti-rabbit IgG labeled with horse radish peroxidase conjugate, incubation time for MAb-PAb based indirect ELISA

To optimize the concentration of coating monoclonal antibody and the dilution of anti-rabbit IgG labeled with Horse Radish Peroxidase conjugate, different concentrations of monoclonal antibody varied from 2.5, 5, 10 and 20 μg/ml suspended in carbonate-bicarbonate buffer, pH 9.6 (coating buffer) were made. The substrate-enzyme reaction was allowed to develop in different time intervals in 30 min and in 1 hr at room temperature.

The polystyrene micro ELISA plates (Costar) were coated with 100 μl of monoclonal antibody of varied concentrations from 2.5, 5, 10 and 20 μg/ml in the wells of microtitre plate. The plates were incubated at 37°C for 2 hr and further at 4 °C overnight. The unbound monoclonal antibody in the well was washed away three times with PBST and the wells were filled with 200 μl of PBST-2.5% milk to block the unbound sites for one hr at 37°C. After washing, 100 μl of *P.vivax* antigens and *P.falciparum* antigens of 2,200 parasites/10^6 erythrocytes and 2,260 parasites/10^6 erythrocytes, respectively were added to each coating concentration followed by incubation for 2 hr at 37°C. Likewise, after washing, 100 μl of anti-*P.vivax* and anti-*P.falciparum* polyclonal antibodies concentrations of 2.5, 5, 10 and 20 μg/ml were used

as the detecting polyclonal antibodies (PAbs) in each system followed by incubation for 1 hr at 37°C. The plates were washed again with PBST and then 100 µl of goat anti-rabbit IgG labeled with HRP conjugate (Dako Company, Denmark) diluted to 1:2000 or 1:1000 prepared with the conjugate buffer were added to each wells and incubated at 37°C for another 1 hr. After washing away the excess conjugate, the substrate solution (Orthophenylenediamine-OPD prepared in Citrate buffer at pH 5.0) was added to each well and the reaction in each plate was allowed to complete at room temperature and then stopped with the addition of 50 µl of 0.5 M H_2SO_4. The O.D. of each well was measured by ELISA reader (Titertek Multiskan MCC/340) at 492 nm after 30 min and then 1 hr of reactions at room temperature.

13. Detection of *P. vivax* antigens in blood specimens using MAb-PAb based indirect ELISA

Two hundred and three vivax malaria blood specimens, forty healthy controls and forty falciparum malaria cases collected from different groups were tested by using MAb-PAb based indirect ELISA.

The flat bottom polystyrene micro ELISA plates (Costar) were coated with 100 µl of 10 µg/ml of McPV1 monoclonal antibody and were incubated at 37°C for 2 hr and then at 4°C overnight. The unbound components were washed away with PBST, three times, 3 min in each time. Then the non-reactive sites were blocked with 150 µl of 2.5% milk prepared in PBS 0.05% Tween 20 at 37°C for 1 hr. It was then washed three times with 200 µl of PBST. After washing, 100 µl of lysed samples from each different groups namely *P.vivax* test group, *P.falciparum* control group and healthy control groups were added to the appropriate wells. The binding between antigens and antibodies was allowed to occur at 37°C for 2 hrs. Then the plates were washed extensively with 150 µl of PBST three times, three min in each time. Then, 100 µl of 10 µg/ml anti-*P.vivax* polyclonal antibodies (PAbs) was added in each well and were incubated at 37°C for one hr and the plates were also washed three times with 150 µl of PBST in each time. Later on, 100 µl of goat anti-rabbit IgG with Horse Radish Peroxidase conjugate (Dako Company,

Denmark) diluted to 1:2000 in conjugate buffer was added to each well. After incubation at 37°C for 1 hr, the unbound conjugates were washed away with 150 μl of PBST three times three min in each time, then, 100 μl of substrate solution (Orthophenylenediamine-OPD) was added to each well. The enzyme-substrate reaction was allowed to develop at room temperature for 30 min keeping the plates in the perfectly dark place. The enzyme substrate reaction was then stopped following the addition of 50 μl of 0.5 M H_2SO_4 in each well. The O.D. of each well was measured by ELISA reader (Titertek Multiskan MCC/340) at 492 nm. The colour development in the test system and in the control systems were easily distinguished by the observation of the naked eyes. PBS was used instead of lysed blood as a reagent control. All blood samples were tested in duplicates.

14. Detection of the *P.falciparum* antigens in blood specimens using MAb-PAb based indirect ELISA

One hundred and fifty four falciparum malaria infected cases, forty healthy controls and forty vivax malaria infected cases collected from different groups and sites were tested by using MAb-PAb based indirect ELISA.

The flat bottom polystyrene micro ELISA plates (Costar) were coated with 100 μl of 10 μg/ml of monoclonal antibody namely F2W22C1 and were incubated at 37°C for 2 hr and then at 4°C overnight. The unbound components were washed away with PBST, three times, 3 min in each time. Then the non-reactive sites were blocked with 150 μl of 2.5% milk prepared in PBS 0.05% Tween 20 at 37°C for 1 hr. It was then washed three times with 200 μl of PBST. After washing, 100 μl of lysed samples from each different groups namely *P.falciparum* test group, *P.vivax* control group and the healthy control group were added to the appropriate wells. The binding between antigens and antibodies was allowed to occur at 37°C for two hrs. Then the plates were washed extensively with 150 μl of PBST three times, three min in each time. Then, 100 μl of anti-*P.falciparum* polyclonal antibodies at the concentration of 10 μg/ml was added in each well and were incubated at 37°C for 1 hr and the plates were also washed three times with 150 μl of

PBST in each time. Later on, 100 μl of goat anti-rabbit IgG with Horse Radish Peroxidase conjugate (Dako Company, Denmark) diluted to 1:2000 in PBST-0.05% was added to each well. After incubation at 37°C for 1 hr, the unbound conjugates were washed away with 150 μl of PBST three times three min in each time, then, 100 μl of substrate solution (Orthophenylenediamine-OPD) was added to each well. The enzyme-substrate reaction was allowed to develop at room temperature for 30 min keeping the plates in the perfectly dark place. The enzyme substrate reaction was then stopped following the addition of 50 μl of 0.5 M H_2SO_4 in each well. The O.D. of each well was measured by ELISA reader (Titertek Multiskan MCC/340) at 492 nm. The colour development in the test system and in the control systems were easily distinguished by the observation of the naked eyes. PBS was used instead of blood specimen as a reagent control. All blood samples were tested in duplicates.

15. Data analysis

SPSS 10.0 for Windows was used for the statistical analysis of data. Pearson's Correlation was applied for the estimation of correlation in between the number of parasites/μl and the O.D. values (at 492 nm) of the developed MAb-PAb based indirect ELISA method for the detection of both *P.vivax* and *P.falciparum* antigens.

In order to calculate the sensitivity, specificity and accuracy of the test, the following approaches were carried out.

Indirect ELISA results	Microscopic Examination		Total		
	+	-			
Positive	TP	FP	TP	+	FP
Negative	FN	TN	FN	+	TN
Total	TP + FN	FP+TN	TP+FP+FN+TN		

$$\text{Sensitivity (\%)} = \frac{TP}{\text{--------------}} \times 100$$

$$TP + FN$$

$$\text{Specificity (\%)} = \frac{TN}{TN + FP} \times 100$$

$$\text{Accuracy (\%)} = \frac{TP+TN}{TP+FP+TN+FN} \times 100$$

FP: False Positive
FN: False Negative
TP: True Positive
TN: True Negative

CHAPTER IV
RESULTS

1. Age and sex distribution and month-wise collection of vivax infected patients

The total number of *P. vivax* infected patients were 206, collected in Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district of Nepal. Three blood samples were found to be clotted before carrying out the test, so, these three samples were excluded and thus only 203 microscopically confirmed *P. vivax* infected blood were included in this research. Vivax infected patients were mostly 33.9% (69/203) in the age group of 16-30 years, 33.0% (67/203) in age group of 1-15 years, 19.7% (40/203) in age group of 31-45 years, 10.3% (21/203) in age group of 46-60 years. However, there was no different prevalences [(2.9%) (6/203)] in more than 60 years age group in both male and female (Figure 8).

The number of males who were infected with *P. vivax* was 58.1% (118/203) while those of female was 41.9% (85/203). The males infected with vivax malaria were higher than the females infected with vivax malaria in this study (Figure 9). The number of vivax patients from Dhalkebar Village Developement Committee was 27.0% (55/203), from Begadabar was 23.0% (47/203), from Hariharpur was 12.0% (25/203), from Lalghad was 10.0% (20/203), from Nactajhij was 8.0% (16/203), from Pushpabalpur was 7.0% (14/203), from Godar was 7.0 % (14/203) and from Umabalpur was 6.0% (12/203) (Figure 10).

The samples were collected during July 2001 to April 2002 (Shrawan 2058 to Baisakh 2059) from Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district of Nepal. The highest prevalence (31.0% [63/203]) was found in July 2001 and the lowest (3.4% [7/203]) was found in December 2001 and in January 2002 (Figure 11).
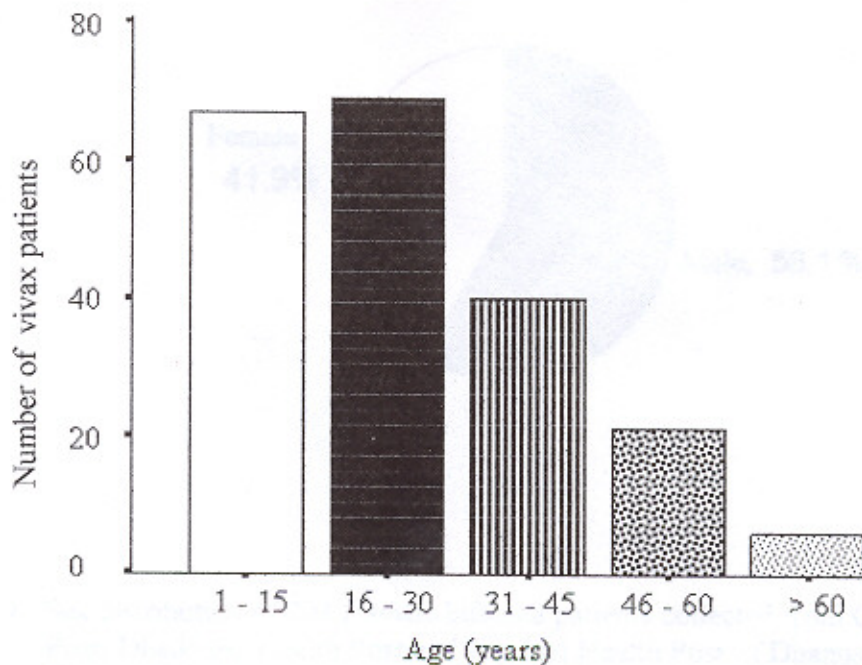
Figure 8. Age distribution of 203 *P.vivax* infected patients collected from Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district. The highest number of vivax infected patients was 33.9% (69/203) in 16-30 years age group, while the others were 33.0% (67/203) in age group of 1-15 years, 19.7% (40/203) in age group of 31-45 years and the lowest number of vivax infected persons was 2.9% (6/203) in the age group of more than 60 years.

Figure 9. Sex distribution of 203 *P. vivax* infected patients collected from Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district. The overall male group was 58.1% (118/203) and the overall female group was 41.9% (85/203). The males infected with vivax malaria were higher than the females infected with vivax malaria.
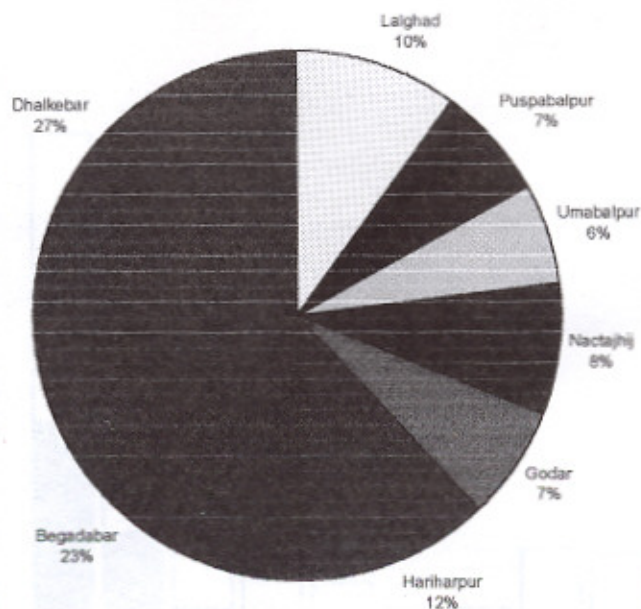
Figure 10. The number of *P. vivax* infected patients in Godar Health Post, Dhalkebar
Health Post and Lalghad Health Post of Dhanusha district. The highest
number of vivax patients was from Dhalkebar Health Post (27.0%) (55/203)
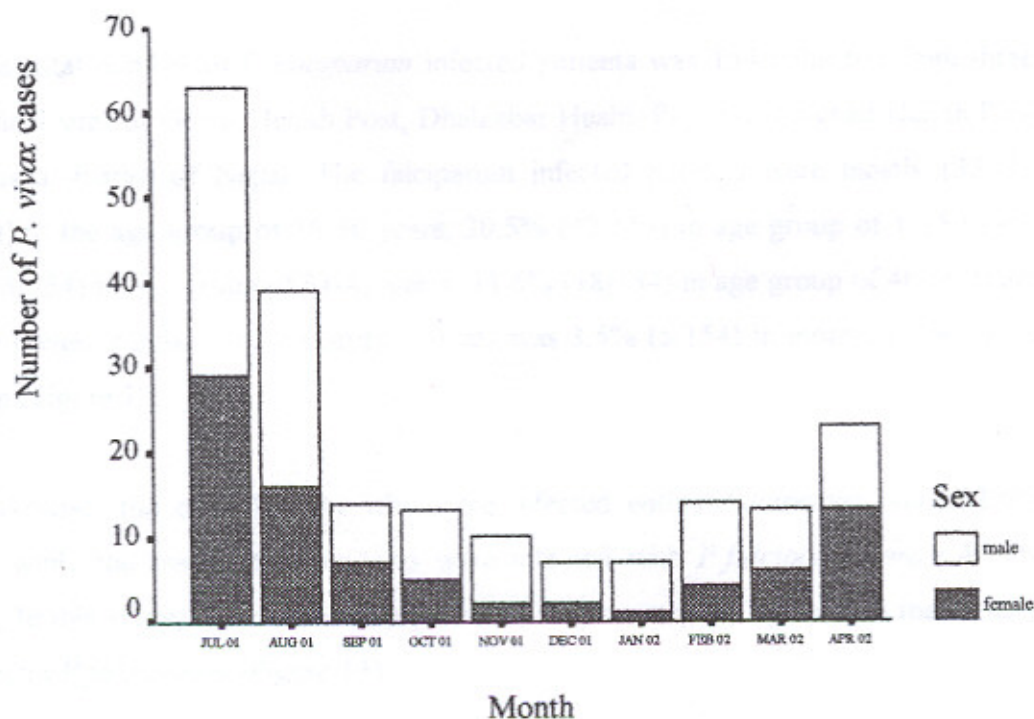while the lowest number was from Umabalpur VDC (6.0 %) (12/203).

Figure 11. Month-wise detection of 203 *P. vivax* cases in Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district. The highest number of vivax malaria case was 31.0% (63/203) detected in July 2001 while the lowest number was 3.4% (7/203) detected in December 2001 and in January 2002.

2. Age and sex distribution and month-wise collection of falciparum infected patients

The total number of *P.falciparum* infected patients was 154 collected from three enrolled field sites of Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district of Nepal. The falciparum infected patients were mostly (33.1% [(51/154)] in the age group of 16-30 years, 30.5% (47/154) in age group of 1-15 years, 21.4% (33/154) in age group of 31-45 years, 11.6% (18/154) in age group of 46-60 years while the lowest number of falciparum patients was 3.5% (5/154) in more than 60 years age group (Figure 12).

Likewise, the overall males who were infected with *P.falciparum* were 52.6% (81/154) while the overall females who were infected with *P.falciparum* were 47.4% (73/154). In this study, the males infected with *P.falciparum* were higher than the females infected with *P.falciparum* (Figure 13).

The number of falciparum patients were from Begadabar Health Post 22.7% (35/154), Dhalkebar Health Post 22.1% (34/154), Hariharpur Health Post 14.9% (23/154), Godar Health Post 11.7% (18/154), Nactajhij Health Post 7.8% (12/154), Pushpabalpur Health Post 7.8% (12/154), Umabalpur Health Post 7.1% (11/154) and the lowest number of falciparum infected patients was from Lalghad Health Post 5.8% (9/154) in both sexes (Figure 14).

The blood were collected with informed consent during July 2001 to July 2002 (Shrawan 2058 to Shrawan 2059) from Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district of Nepal. The highest prevalence (12.9% (20/154) was found in July while the lowest 3.8% (6/154) was found in February 2002 (Figure 15) from the different enrolled field sites in Dhanusha district of southern malaria endemic area of Nepal.
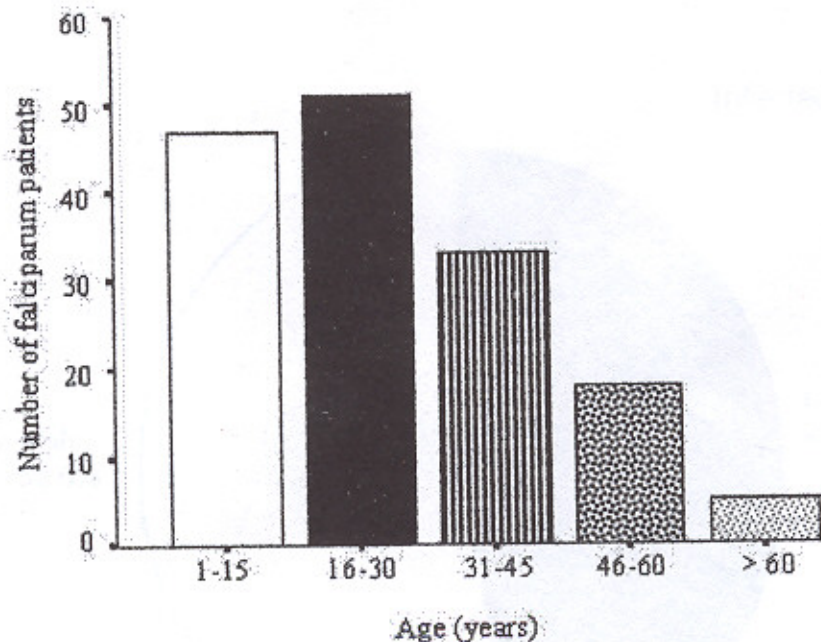
Figure 12. Age distribution of 154 *P.falciparum* infected patients in Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district. The highest number of falciparum infected patients was from age group of 16-30 years (33.1%) (51/154) while the lowest number was from age group of more than 60 years (3.5%) (5/154).
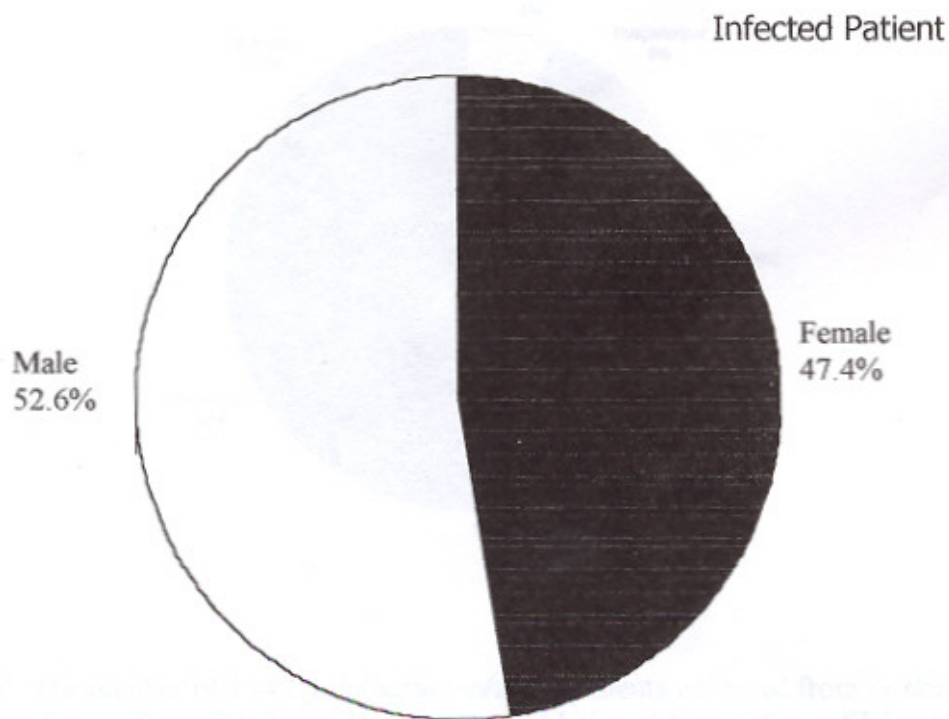
Figure 13. Sex distribution of 154 *P. falciparum* infected patients in Godar Health
Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district.
The number of falciparum infected males was 52.6% (81/154) and the
number of falciparum infected females was 47.4% (73/154). Male sex group
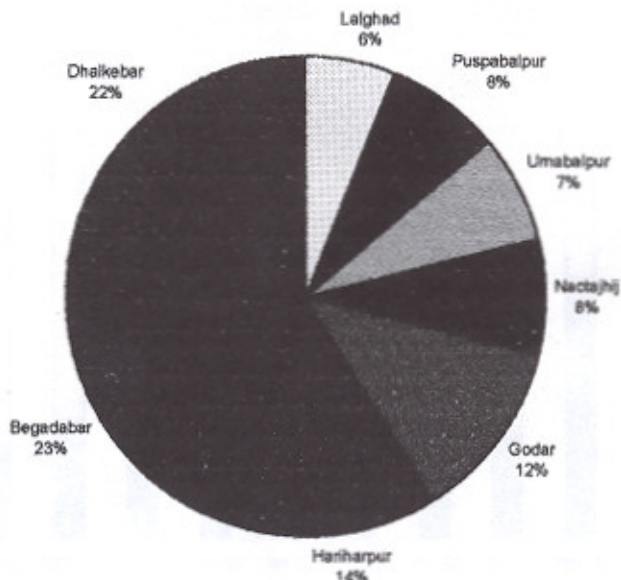was higher than the female sex group of falciparum infected patients.

Figure 14. The number of 154 *P.falciparum* infected patients collected from Godar
Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha
district. The highest number of falciparum patients was from Begadabar
Village Development Committee (22.7%) (35/154) and the lowest number
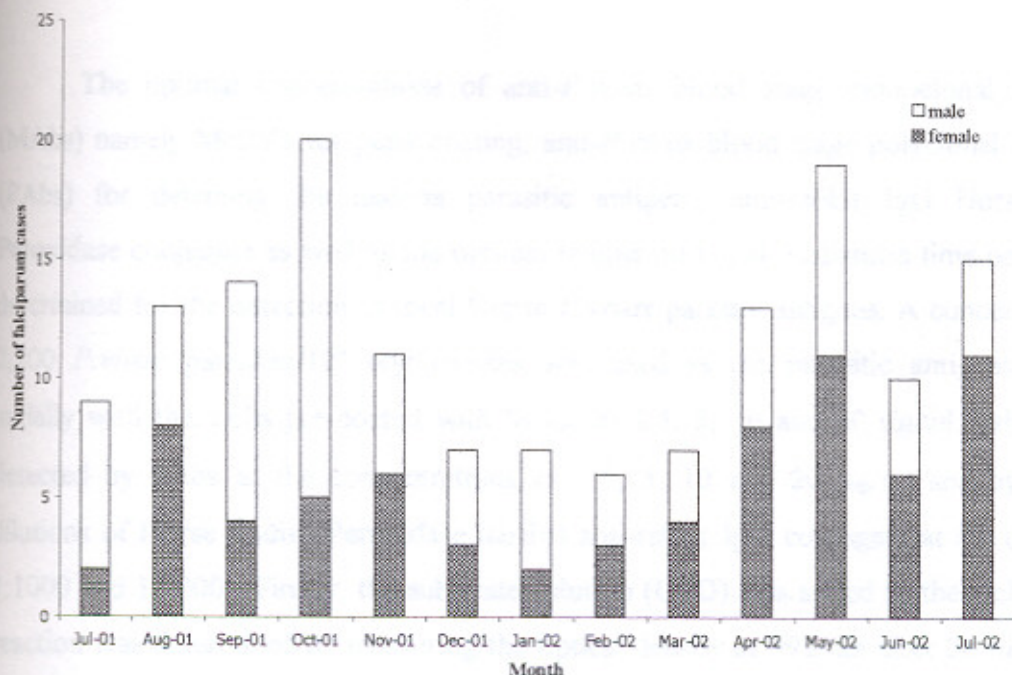was collected from Lalghad Village Development Committee (5.8%) (9/154).

Figure 15. Month-wise detection of 154 *P.falciparum* cases collected from Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district. The highest number of falciparum cases was 12.9% (20/154) collected in October 2001 while the lowest number was 3.8% (6/154) collected in February 2002.

3. Optimization for *P. vivax* detection

The optimal concentrations of anti-*P. vivax* blood stage monoclonal antibodies (MAbs) namely McPV1 for plate coating, anti-*P. vivax* blood stage polyclonal antibodies (PAbs) for detecting the malaria parasitic antigens, anti-rabbit IgG Horse Radish Peroxidase conjugate as well as the optimal temperature and incubation time period were determined for the detection of local Nepali *P. vivax* parasite antigens. A concentration of 2,200 *P. vivax* parasites/10^6 erythrocytes was used as the parasitic antigens to react initially with the wells pre-coated with MAbs at 2.5, 5, 10 and 20 µg/ml, subsequently detected by PAbs at the concentrations of 2.5, 5, 10 and 20 µg/ml and by the two dilutions of Horse Radish Peroxidase labeled anti-rabbit IgG conjugate at the dilution of 1:1000 and 1:2000. Finally, the substrate solution (OPD) was added to the wells and the reaction was determined by measuring the optical density at 492 nm after 30 min (Figure 16) and 1 hr (Figure 17). The level of colour intensity was detected visually as well. It was observed that the absorbance value (O.D.) was higher when the 1:2000 conjugate dilution was applied than when the conjugate dilution of 1:1000 was used.

In the test system developed, the optimal concentrations of coating anti-*P. vivax* MAb was 10 µg/ml and of detecting anti-*P. vivax* PAb was 10 µg/ml since the optical density values were measured higher. The appropriate dilution of anti-rabbit IgG Horse Radish Peroxidase conjugate was 1:2,000. The enzyme substrate reaction period of 30 min at room temperature (in dark place) was used for the detection of *P. vivax* blood stage antigens in clinical samples.
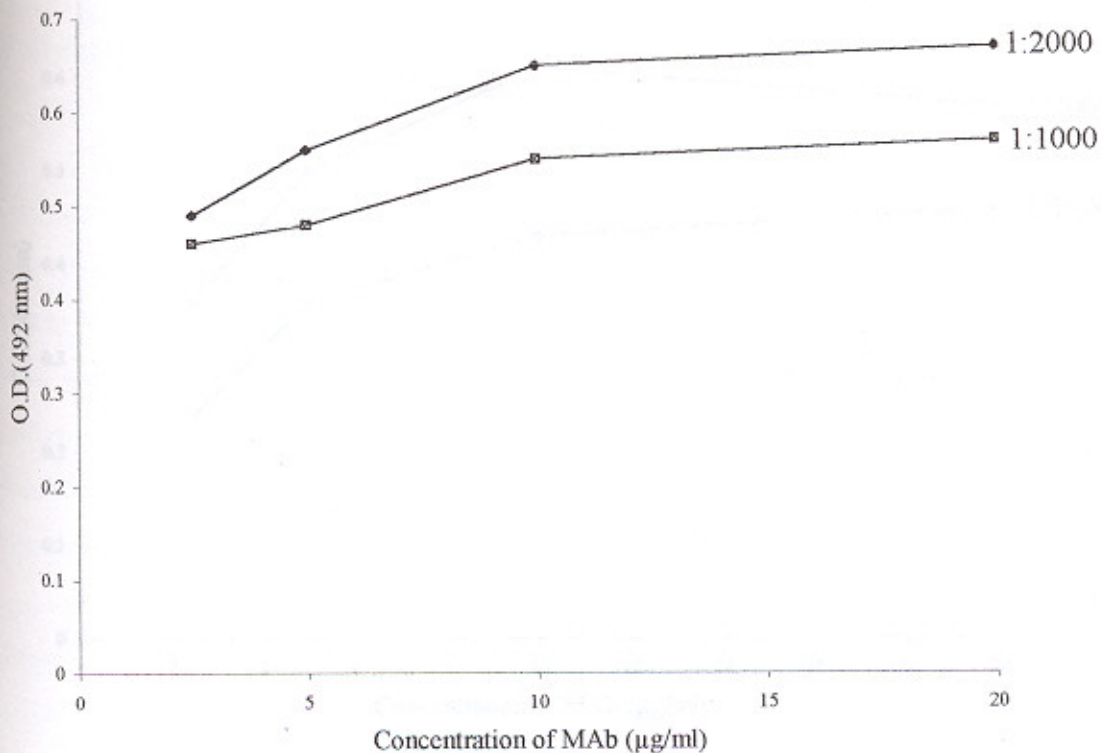
Figure 16. Optimization of the concentration of coating MAb and anti-rabbit IgG
conjugate with HRP for the establishment of MAb-PAb based indirect
ELISA. The plates were coated with 2.5, 5, 10 and 20 µg/ml of
anti-*P. vivax* MAbs and reacted with antigen concentrations of 2,200 *P. vivax*
parasites/10^6 RBC, detected with the concentrations of 2.5, 5, 10 and 20
µg/ml of anti-*P. vivax* PAbs then reacted with 1;1,000 (----) and 1:2,000
(----) dilution of conjugate. The O.D. was measured at 492 nm after 30 min
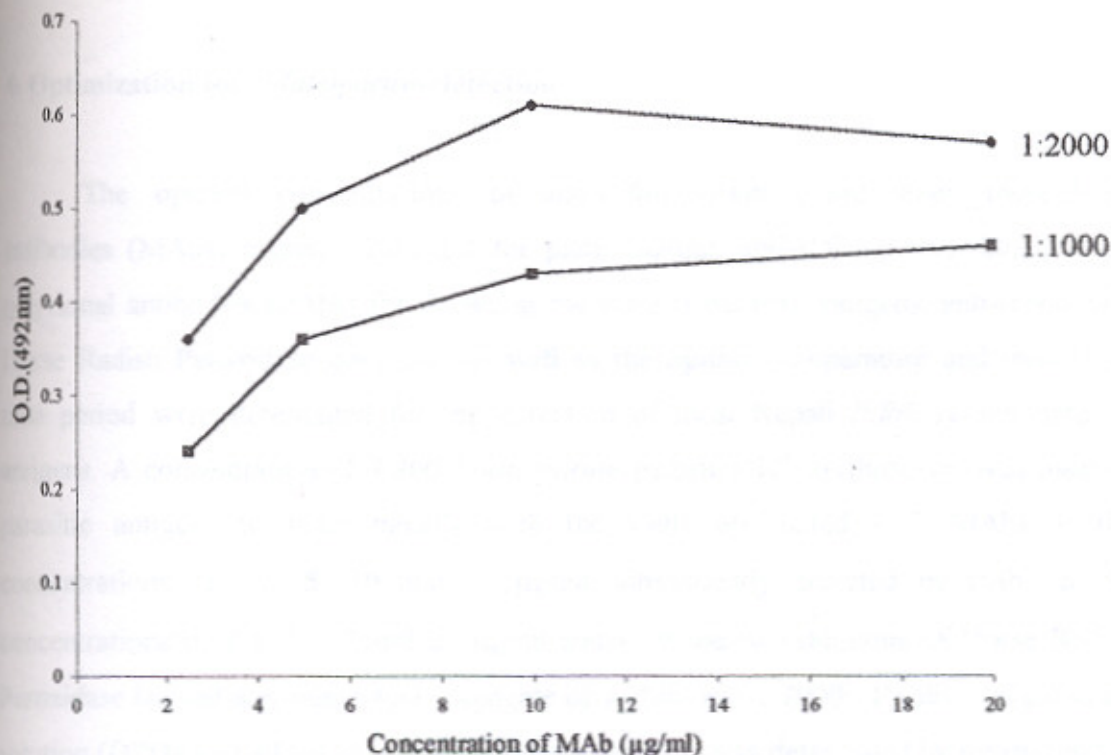incubation at room temperature.

Figure 17. Optimization of the concentration of coating MAb and anti-rabbit IgG conjugate for the establishment of MAb-PAb based indirect ELISA. The plates were coated initially with 2.5, 5, 10 and 20 µg/ml of anti-*P.vivax* MAb and reacted with antigens of 2,200 *P.vivax* parasites/10^6 RBC, detected with 2.5, 5, 10 and 20 µg/ml of anti-*P.vivax* PAbs then reacted with 1;1,000 (----) and 1:2,000 (----) dilution of conjugate. The O.D. of the reaction was measured at 492 nm after 1 hr incubation at room temperature.

4. Optimization for *P.falciparum* detection

The optimal concentrations of anti-*P.falciparum* blood stage monoclonal antibodies (MAbs) namely F2W22C1 for plate coating, anti-*P.falciparum* blood stage polyclonal antibodies (PAbs) for detecting the malaria parasite antigens, anti-rabbit IgG Horse Radish Peroxidase conjugate as well as the optimal temperature and incubation time period were determined for the detection of local Nepali *P.falciparum* parasite antigens. A concentration of 2,260 *P.falciparum* parasites/10^6 erythrocytes was used as parasitic antigens to react initially with the wells pre-coated with MAbs at the concentrations of 2.5, 5, 10 and 20 µg/ml subsequently detected by PAbs at the concentrations of 2.5, 5, 10 and 20 µg/ml and with the two dilutions of Horse Radish Peroxidase labeled anti-rabbit IgG conjugate of 1:1000 and 1:2000. Finally, the substrate solution (OPD) was added to the wells and the reaction was determined by measuring the optical density at 492 nm after 30 min (Figure 18) and 1 hr (Figure 19). The level of colour intensity was detected visually as well. It was observed that the absorbance value (O.D.) was higher when the 1:2000 conjugate dilution was applied than when the conjugate dilution of 1:1000 was used.

In the test system developed, the optimal concentrations of coating anti-*P.falciparum* MAb was 10 µg/ml and of detecting anti-*P.falciparum* PAb was 10 µg/ml since the optical density values were measured higher. The appropriate dilution of anti-rabbit IgG Horse Radish Peroxidase conjugate was 1:2,000. The enzyme substrate reaction period of 30 min at room temperature (in dark place) was used for detection of *P.falciparum* blood stage antigens in clinical samples.

Figure 18. Optimization of coating MAb and anti-rabbit IgG conjugate with HRP for the establishment of MAb-PAb based indirect ELISA. The plates were coated initially with 2.5, 5, 10 and 20 μg/ml of anti-*P.falciparum* MAb and reacted with antigen concentrations of 2,260 *P.falciparum* parasites/10^6 RBC, detected with 2.5, 5, 10 and 20 μg/ml of anti-*P.falciparum* PAb then reacted with 1:1,000 (----) and 1:2,000 (----) dilution of conjugate. The O.D. of the reaction was measured at 492 nm after 30 min incubation at room temperature.
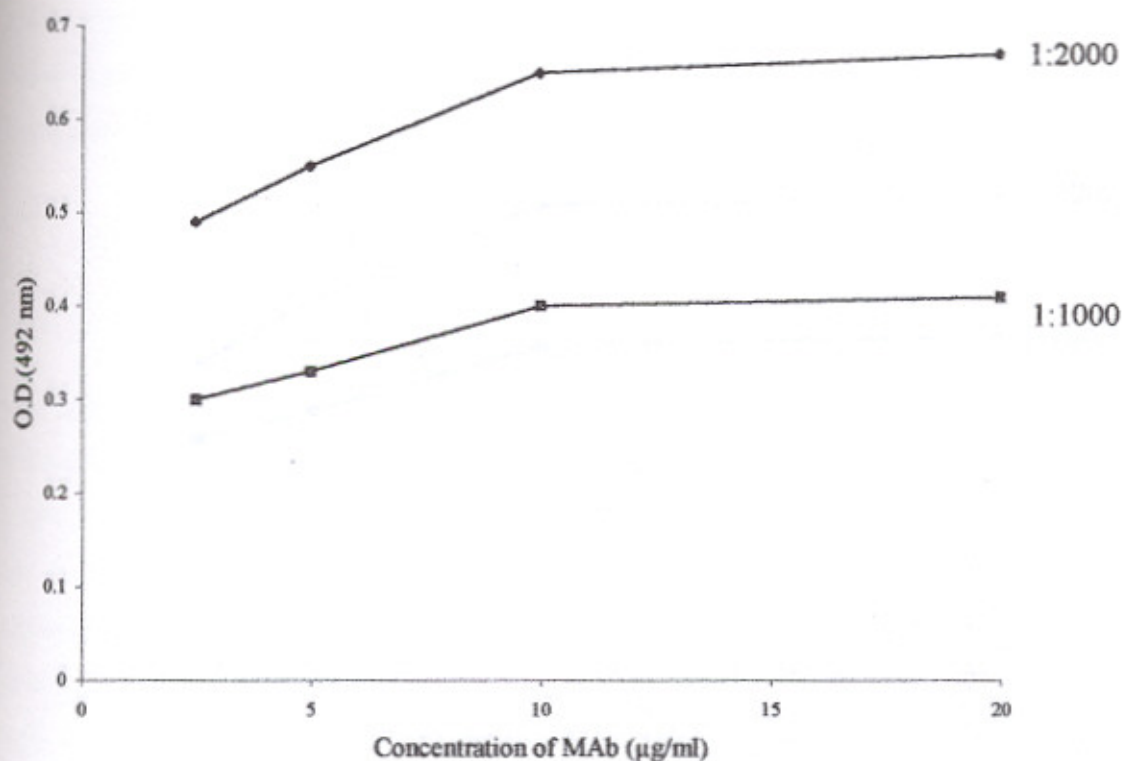
Figure 19. Optimization of coating MAb and anti-rabbit IgG labeled with HRP for the establishment of MAb-PAb based indirect ELISA. The plates were coated initially with 2.5, 5, 10 and 20 µg/ml of anti-*P.falciparum* MAb and reacted with antigen concentrations of 2,260 *P.falciparum* parasites/10^6 RBC, detected with 2.5, 5, 10 and 20 µg/ml of anti-*P.falciparum* PAb then reacted with 1:1,000 (----) and 1,2000 (----) dilution of conjugate. The O.D. of the reaction was measured at 492 nm after 1 hr incubation at room temperature.
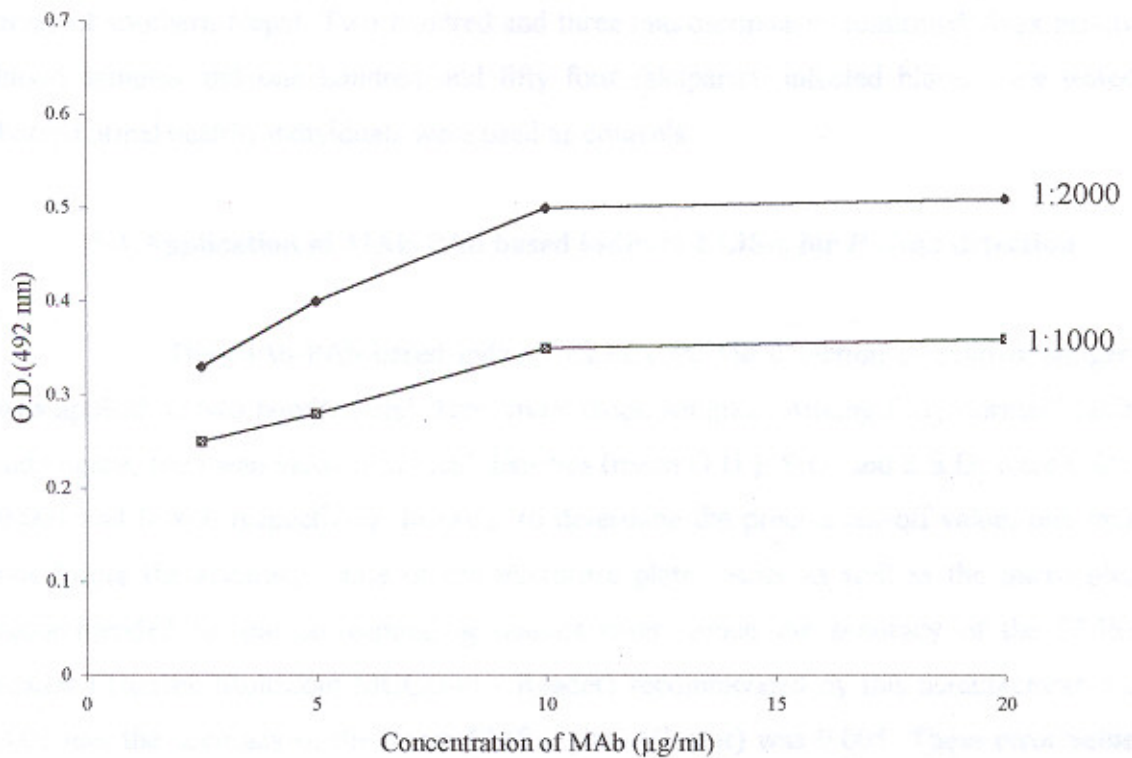
5. Application of MAb-PAb based indirect ELISA in blood specimens

The established MAb-PAb based indirect ELISA test systems were used for detection of both *P.vivax* and *P.falciparum* antigens in blood samples obtained from vivax and falciparum malaria patients, recpectively, collected from the malaria endemic areas of southern Nepal. Two hundred and three microscopically confirmed vivax malaria blood samples and one hundred and fifty four falciparum infected blood were tested. Forty normal healthy individuals were used as controls.

5.1 Application of MAb-PAb based indirect ELISA for *P.vivax* detection

The MAb-PAb based indirect ELISA for the detection of *P.vivax* antigens was applied to two hundred and three vivax blood samples. Among forty normal healthy individuals, the mean value of optical densities (mean O.D.), S.D. and 2 S.D. were 0.055, 0.004 and 0.009, respectively. In order to determine the precise cut-off value, one must not forget the accuracy value of the microtitre plate reader as well as the micro plate recommended by the corresponding manufacturer. Since the accuracy of the ELISA reader (Titertek Multiskan MCC/340 - Reader) recommended by this manufacturer was 0.05 and the accuracy of the micro ELISA plate (Costar) was 0.005. These error values should be included to define the precise cut-off values in the test system. Therefore, the samples were considered positive when the O.D. values were higher than the mean O.D. values of normal healthy controls + 2SD + error values of ELISA Reader + error values of the micro ELISA plate which was 0.120.

5.1.1 Sensitivity, specificity and accuracy of MAb-PAb based indirect ELISA for detection of *P.vivax*

The established cut-off levels were used to determine the sensitivity, specificity and accuracy of MAb-PAb based indirect ELISA when all 203 samples of

P.vivax infected blood were allowed to react with the anti-*P.vivax* MAb and anti-*P.vivax* PAb. All 203 *P.vivax* infected blood were positive while forty healthy controls and forty falciparum malaria cases were negative as shown in (Figure 20). Therefore, the sensitivity of the test was 100% for the detection of *P.vivax* antigens. Since the range of *P.vivax* parasites count from vivax blood samples collected for this study was 6-32,920 parasites/10^6 erythrocytes. This established MAb-PAb based indirect ELISA was able to detect as low as 6 parasites/10^6 erythrocytes.

When the *P.falciparum* infected erythrocytes from forty cases collected from the rural endemic areas of Nepal were tested, the O.D. value was below the established cut-off value. Therefore, the MAb-PAb based indirect ELISA for the detection of *P.vivax* antigens was 100% specific. In addition, no false positives or false negatives were observed. The test was considered 100% accuracy for the detection of *P.vivax* parasites.

5.1.2 Correlation between parasite examination and O.D. of MAb-PAb based indirect ELISA for detection of *P.vivax*

The correlation between the number of *P.vivax* parasitized cells/10^6 erythrocytes and the O.D. values of MAb-PAb based indirect ELISA is shown (Figure 21). When the number of parasitized cells counted per microliter in all the 203 vivax malaria patients were plotted against the O.D. values, the significant correlation was observed ($r = 0.649$; $p = 0.018$).
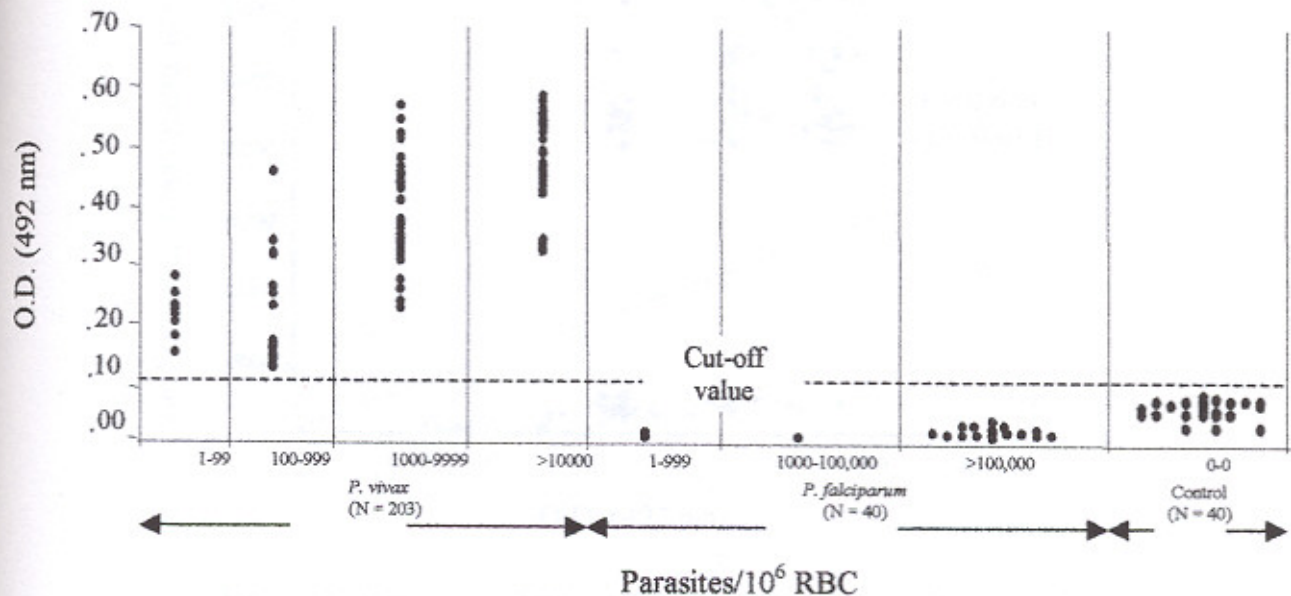
Figure 20. Scatter diagram of O.D. values of the MAb-PAb based indirect ELISA for detection of *P. vivax* antigen in 203 vivax malaria cases. Controls are 40 healthy individuals and 40 falciparum malaria cases.
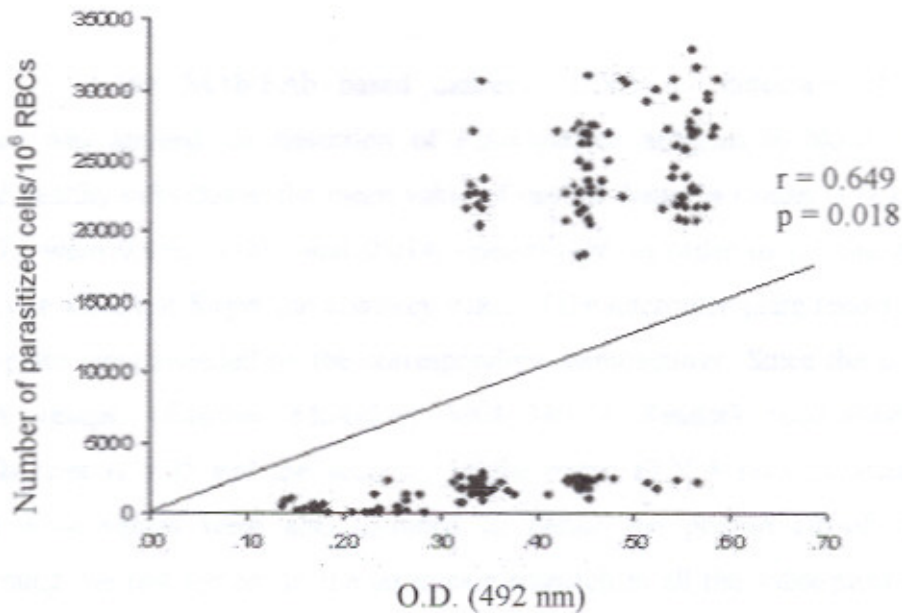
Figure 21. Correlation between degree of *P.vivax* parasitemia and
O.D. of MAb-PAb based indirect ELISA (r = 0.649, p=0.018).

5.2 Application of MAb-PAb based indirect ELISA to *P.falciparum* detection

The MAb-PAb based indirect ELISA for detection of *P.falciparum* antigens was applied for detection of *P.falciparum* antigens in blood. Among forty normal healthy individuals, the mean value of optical densities (mean O.D.), (1 S.D.) and (2 S.D.) were 0.056, 0.007, and 0.014, respectively. In order to get the precise cut-off value, one must not forget the accuracy value of the microtitre plate reader as well as the micro plate recommended by the corresponding manufacturer. Since the accuracy of the ELISA reader (Titertek Multiskan MCC/340 - Reader) recommended by this manufacturer is 0.05 and the accuracy of the micro ELISA plate (Costar) was 0.005. These error values were also included to define the precise cut-off values before performing the test system in the largescale research in all the subsequent tests batches. Therefore, the samples were considered positive when the O.D. values were higher than the mean O.D. + 2 S.D. + error values of ELISA Reader + error values of the micro ELISA plate which was 0.125.

5.2.1 Sensitivity, specificity and accuracy of MAb-PAb based indirect ELISA for detection of *P.falciparum* antigens

The established cut-off levels were used to determine the sensitivity, specificity and accuracy of MAb-PAb based indirect ELISA when all 154 samples of *P.falciparum* infected blood were allowed to react with the anti-*P.falciparum* MAb and anti-*P.falciparum* PAb. All 154 *P.falciparum* infected blood were positive while forty healthy controls and forty vivax malaria cases were negative as shown in (Figure 22). Therefore, the sensitivity of the test was 100% for the detection of *P.falciparum* antigens. Since the range of *P.falciparum* parasites count from falciparum blood samples collected for this study was 9-325,680 parasites/10^6 erythrocytes. This established MAb-PAb indirect ELISA was able to detect as low as 9 parasites/10^6 erythrocytes.

When the *P.vivax* infected erythrocytes from forty cases collected from the rural endemic areas of Nepal were tested, the O.D. value was below the established cut-off value. Therefore, the MAb-PAb based indirect ELISA for the detection of *P.falciparum* antigens was 100% specific. In addition, no false positives or false negatives were observed. The test was considered 100% accuracy for the detection of *P.falciparum* parasites.

5.2.2 Correlation between parasite examination and O.D. of MAb-PAb based indirect ELISA for detection of *P.falciparum*

The correlation between the number of *P.falciparum* parasitized cells/10^6 erythrocytes and the O.D. values of MAb-PAb based indirect ELISA is shown (Figure 23). When the number of parasitized cells counted per microliter in all 154 falciparum malaria patients was plotted against the O.D. values, the significant correlation was observed (r = 0.872; p = 0.013).
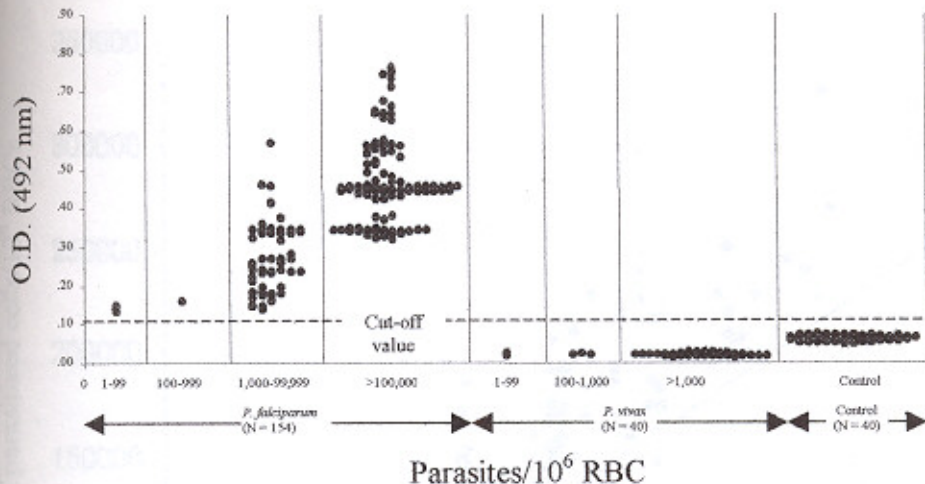
Figure 22. Scatter diagram of O.D. values from the MAb-PAb based indirect ELISA for the detection of *P.falciparum* antigen in 154 falciparum malaria cases. Controls are 40 healthy individuals and 40 vivax malaria cases.
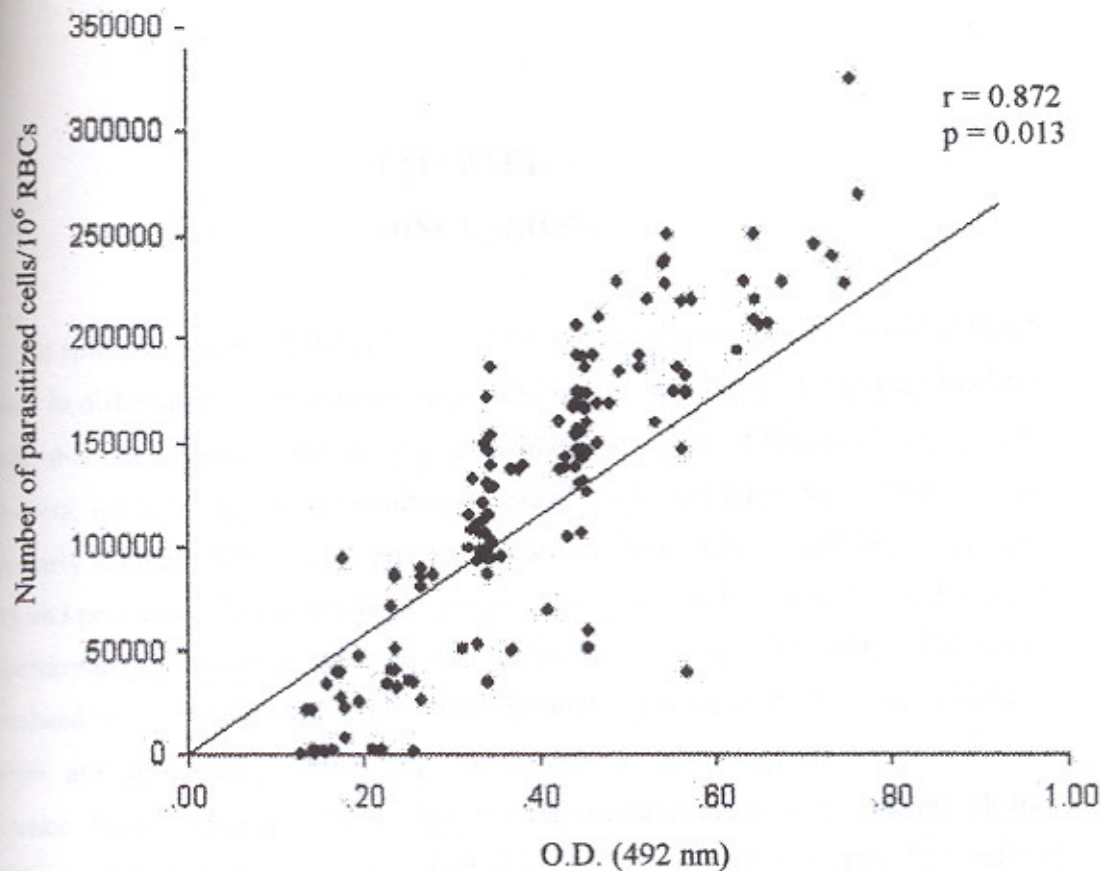
Figure 23. Correlation between degree of *P.falciparum* parasitemia and O.D. (492 nm) of MAb-PAb based indirect ELISA (r = 0.872, p= 0.013).

CHAPTER V
DISCUSSION

In spite of several years of control attempts, malaria remains a major public health problem in different areas of southern Nepal. During the last four decades, there has been considerable flactuations in the status of the malaria situations of Nepal. During the pre-control era, malaria was mesoendemic and hyperendemic, but large parts of the country, particularly southern Nepal, were prone to epidemics. This transmission used to be very heavy and perennial in areas where more than one vector was found. In Nepal, *P.vivax* is the predominant species in most of the malarious areas by 10:1 with *P.falciparum* (Sherchand *et al.*, 1999). More than 70% of the total population and 64 districts out of 75 districts are at malaria risk in Nepal with varying percentage of Malaria Parasite Incidence Rate/1,000 populations. The highest incidence rate is in Bhojpur district (13.02%). Malaria control services are being provided to approximately 15.6 million people in malaria risk areas of 64 districts of the country. Only 11 districts out of 75 are free of malaria risk which are located mainly in high altitude specially in the mountainous areas. The overall malaria situation of Nepal has been deteriorated since last decade because of the lack of resources, intersectoral collaboration and trained professional technicians. Moreover, the country has been seriously facing insufficient health infrastructures, uncontrolled population migration and civil unrest. Besides these problems, identification of chloroquine resistant *P.falciparum* and insecticide resistant parasite vectors aggregate the gravity of the pressing problems.

In southern Nepal, most patients having fever are treated with anti-pyretics or with and anti-malarial drugs usually chloroquine. Despite the importance of the problem, whenever a patient comes with fever, a diagnosis of malaria is empirically made without microscopic confirmation of the presence of malaria parasites in the blood. The reasons of failure to do blood test are several varying from inaccessibility of patient to the medical care due to the limit of medical staff and equipment. Hospital admission cases

are grossly under-diagnosed for malaria. Similarly, cerebral malaria tend to be misdiagnosed in the urban area of Dhanusha district, southern Nepal (Sherchand JB *et al.*, 1998).

Microscopic examination which is considered as the gold standard technique for diagnosis of malaria parasites in the stained blood films is still extensively and routinely employed not only in the malarious areas but also in the non malarious areas if the patient has been travelling to malarious areas within a year. This method is satisfactory in terms of sensitivity and specificity as well as for the species differentiation of the parasites. Moreover, the method provides information about the viability of parasites present in peripheral blood which may be helpful when the response to anti-malarial treatment is being assessed (WHO, 1986). The technique permits the quantitation of blood parasitemia levels. However, the method is time consuming and requires skilled laboratory personnel for the precise identification and interpretation of the parasites particularly when one has to perform a large number of samples in the field epidemiological survey or when the number of malarial parasites are scarce or with low grade parasitemia or even absent during blood examination thereby reducing the accuracy of results particularly in such low grade parasitemia and in mixed malaria infections. As a consequence, alternative methods have to be sought which can replace the microscopic examination or at least competitive enough to introduce confidentially even in the rural settings.

In general, many serological methods which have been chosen in some epidemiological studies provided adequate information with regard to exposure to malaria, but by virtue of persistence of malaria antibodies following disappearance of malaria parasites from the blood of the infected subjects, therefore, such tests do not accurately differentiate between the current and the past infections. Demonstration of malaria parasite antigens in different test systems has been increasingly applied for the

diagnosis of malaria infections because they are more closely related to the current illness than antibodies detection which show the previous or recent past infection.

The pressing need of an introduction of the immunological diagnostic methods for the rapid detection of malaria parasite antigens in the rural settings of Nepal have been considered since long ago. The frequent problems encountered in the National Malaria Eradication Programme of Nepal are due to the imbalance in between the number of blood films to be examined and the number of trained microscopists in many areas and the lengthy time taken from the moment of blood drawn from the patients until the result of the examination dispatched to the patients or the results are delivered to the concerned clinicians. The patients themselves and the patient's visitors expect to receive the blood report within the same day of blood drawn or as soon as possible because of the prompt treatment with anti-malarial drugs and extreme geographical difficulties of the country. It is somewhere so difficult to reach the desired site that can take days or even weeks to access to the nearest health center. The situation is further deteriorated because of the lack of proper transportation system. By virtue of the large number of the blood samples and the shortage of the technical manpower, the delivery of the blood report may take days or even weeks in some remote parts of Nepal. Therefore, a need of new techniques with higher sensitivity, specificity, accuracy and rapidity apart from the thick and thin blood film examination could be developed and applied in such subtle rural settings; which would be highly beneficial to those malaria sufferers and ultimately would be helpful to the National Malaria Eradication Programme of Nepal.

The highest number of vivax infected patients in the present study was 33.9% (69/203) in age group of 16-30 years, 33.0% (67/203) in age group of 1-15 years, 19.7% (40/203) in age group of 31-45 years, 10.3% (21/203) in age group of 46-60 years and the lowest number was 2.9% (6/203) in age group of more than 60 years in both sexes. Likewise, the highest number of falciparum infected patients was 33.1% (51/154) in age group of 16-30 years, 30.5% (47/154) in age group of 1-15 years, 21.4% (33/154) in age

group of 31-45 years, 11.6% (18/154) in age group of 46-60 years and the lowest number of falciparum infected patients was 3.5% (5/154) in more than 60 years of age. These results showed that the highest number of both vivax and falciparum malaria cases were found in patients of 16-30 years age group. This might be due to the levels of natural IgG and IgM levels in individuals with different age groups. However, from the quantitative study of immunoglobulins in Nepalese population by nepherometric technique, it was indicated that the reference range of IgG, IgM and IgA concentrations showed a gradual rise with the increase of age. The IgG concentration attained the adult level at about the age of 10-14 years, while the IgA concentration was still increasing with the age of 20-29. However, the IgM increases continuously up to the age of 15-19 years (Nakanishi M and Shrestha HG, 1990). Therefore, the higher malaria cases in the age group of 16-30 years in both sexes were due to the significant reduction of non specific IgM concentration, the protective immunity to malaria. In this study, the number of malaria cases were gradually reduced with the increase of the age which could be due to the development of protective immunity from the previous exposures with malaria as the recruited subjects were all local people residing in malaria endemic areas of Nepal.

The *P.vivax* infected patients of 58.1% male (118/203) and of 41.9% (85/203) female were found. Likewise, *P.falciparum* infected patients were 52.6% male (81/154) and 47.4% female (73/154). Therefore, both *P.vivax* and *P.falciparum* recruited in this study were more male than female. Thus, the numbers were consistent with the predominence of male sex patients than female sex patients in terms of sex-wise distribution of total malaria cases of 1997 (The Annual Internal Assessment of the Malaria and Kala-azar Control Activities of the Year 1997, HMG, Nepal) which reported more malaria cases among males (66%) and less among females (34%). According to the Central Bureau of Statistics, HMG, Nepal, the final tally of the national census held in June 1991, the male and female ratio of Nepal is 49.87 and 50.13%, respectively. Interestingly, the malaria infected patients were higher in male than in female. Recently, Nakanishi and Shrestha (1990) have reported that the total non specific IgM

concentration w as s hown t o b e s ignificantly h igher i n g irls t han i n b oys and total IgG concentrations were also found slightly higher in girls than in boys in different geographical areas of Nepal. Therefore, the higher incidence of malaria cases in male may be possibly due to the significant reduction of total IgM concentrations and slight reduction of total IgG concentrations in male sex group than in female sex group in overall populations of Nepal thereby reducing the natural protective immunity against infections in male sex groups than in the female sex groups in different geographical locations of Nepal. Moreover, the outdoor household activities including forest works are carried out by male sex groups than the female sex groups in Nepal thereby leading to high chances of *Anopheles* vector bites male than female.

The highest number of vivax patients was from Dhalkebar Village Developement Committee 27.0% (55/203), while 23.0% (47/203) from Begadabar, 12.0% (25/203) from Hariharpur, 10.0% (20/203) from Lalghad, 8.0% (16/203) from Nactajhij, 7.0% (14/203) from Pushpabalpur, 7.0% (14/203) f rom Godar w ere reported. The lowest number of vivax infected patients was 6.0% (12/203) from Umabalpur. In addition, the highest number of falciparum infected patients was 22.7% (35/154) from Begadabar Village Developement Committee, while 22.1% (34/154) from Dhalkebar Village Developement Committee, 14.9% (23/154) from Hariharpur Village Developement Committee, 11.7% (18/154) from Godar V illage D evelopement C ommittee, 7 .8% (12/154) f rom N actajhij Village Developement Committee, 7.8% (12/154) in Pushpabalpur Village Developement Committee, and 7.1% (11/154) from Umabalpur Village Developement Committee were reported. The lowest number of falciparum infected patients was detected from Lalghad Health Post (5.8%) (9/154). From these results, two possibilities could be drawn, (i) the higher number of *Plasmodium* parasites isolated mainly from Dhalkebar Village Development Committee and Begadaber Village Development Committee was due to the proximity of forest of both village development committees leading to the higher vector habitat and (ii) it could be possible that the relatively number of local residents is higher than the villagers of other village developement committees.

The optimum concentrations of monoclonal antibodies for capturing the antigen used in this study were 10 µg/ml for McPV1 and 10 µg/ml for F2W22C1 raised against Thai strains of both *P.vivax* and *P.falciparum* parasites, respectively and 10 µg/ml of polyclonal antibodies as the detecting antibodies raised in the rabbit model against local Nepali strains of both *P.vivax* and *P.falciparum* antigens. For enzyme conjugate, several different kinds of enzymes can be used in general as a label for antibodies or antigens in immunoassays. However, the two most enzymes used are horse radish peroxidase and alkaline phosphatase (Cheng, 1987). In order to choose an appropriate enzymes for the labeling predominantly, four general criteria were considered (Avrameas, 1978) (1) high specific activity and turnover rate; (2) readily available in highly purified forms from the local commercial sources or ease of preparation; (3) no substantial loss of activity following coupling; and (4) relatively good stability at room temperature. In the present study, the goat anti-rabbit IgG conjugated with horse radish peroxidase enzyme was chosen because of its readily availability in the local markets. Although it might interfere the test results because of its endogeneous content in the red cells from which the parasitic antigens are extracted. However, the orange coloured product was quite stable and could be observed by naked eyes, hence it could be applied where the ELISA Reader or the other optical density reading devices are not available. Moreover, the other enzyme such as alkaline phosphatase gives an unstable and unsoluble substrate product at room temperature which is not applicable in the field conditions.

The optimum concentration of coating MAb and the working dilution of the conjugates are best determined by checkerboard titration using reference reagents (Voller *et al.*, 1979). It was found that the optical densities increased proportionally when the coating antibodies are increased from 1 to 10 µg/ml which corresponded to the previous study (Cheng, 1987). So, the MAb for coating and PAb for detecting used in these test systems were consistent with the previous report (Cheng, 1987). Higher concentrations of protein used for coating tended to increase desorption during incubation and washing. In addition, the enzyme horse radish peroxidase (HRP) labeled anti-rabbit IgG at the

dilution of 1:2000 in the test system were selected instead of 1:1000 because (1) it gave relatively low background in control preparations; (2) its orange colour produced by the enzyme on its substrate-orthophenylenediamine (OPD) was visually distinguished from the colourless negative reaction after 30 min incubation at room temperature.

By virtue of the application of the monoclonal antibodies (MAbs) that specifically bind to the particular epitopes, the established MAb-PAb indirect ELISA showed sensitivity, specificity and accuracy values which represented the validity of the assays were 100% for the detection of both P.vivax and P.falciparum antigens since the MAbs used in the assays were the antibodies that gave broad reactivity against blood stage antigens including gametocytes in almost all isolates of both P.vivax and P.falciparum in Thailand (Khusmith et al., 1987, 1988). T herefore, w hen the established MAb-PAb based indirect ELISA for the detection of P.vivax antigens was applied to the vivax blood samples collected from the remote malaria endemic areas of Nepal, the assay was sensitive (100%) because it could detect all 203 cases of vivax malaria and specific since it was negative when reacted with lysed red cells from 40 healthy individuals or from 40 P.falciparum infected blood. The assay showed neither false positive results nor false negative results so the assay was considered accurate (100%). Simillarly, MAb-PAb based indirect ELISA for the detection of P.falciparum antigens was also sensitive (100%) because it was positive only with 154 cases of falciparum malaria and specific since it was negative when erythrocytes from 40 healthy individuals and 40 P.vivax infected blood were tested. Thus, neither false positive nor false negative reactions were observed. So, the test was considered accurate (100%). In the present study, although the MAb-PAb based indirect ELISA showed a very low non-specific background reactions using healthy individuals residing in non-malaria endemic areas of Nepal. However, the positive cut-off levels should be considered carefully when the assay is applied to detect malaria parasites in the endemic areas. It has been proposed that soluble malaria antigens are present in the serum during infection as well as during drug suppression (Perrin, et

al., 1979). Therefore, higher backgrounds could be obtained when the whole blood samples collected especially from the malaria endemic areas were tested.

Regarding the correlation between the number of parasites/µl and the optical density values in the *P.vivax* positive cases, it showed significantly correlation ($r = 0.649$; $p = 0.018$). Similarly, the correlation between the number of parasites/µl and the optical density values in the *P.falciparum* positive cases also showed significantly correlation ($r = 0.872$; $p = 0.013$). It is assumed that the lower degree of correlation in vivax cases may be due to (i) the stages variations of parasites found among vivax parasites which leads to the different amount of protein contents, while *P.falciparum* in blood specimen usually contained only ring stage, (ii) the binding capacity of the monoclonal antibodies to parasites antigen is also considered one of the factors responsible to the degree of sensitivity. If the binding site is altered, the antibody may or may not continue to bind (Burnette, 1981). (iii) the possible effect during blood collection, preparation and storage of the specimens particularly in the field conditions where interruption of electric power supply and extreme temperature flactuations were usually encountered.

Taken together, the detection of blood stage antigens of both *P.vivax* and *P.falciparum* in malaria patients using specific monoclonal antibodies (MAbs) and polyclonal antibodies (PAbs) by an enzyme immunoassays (MAb-PAb based indirect ELISA) has been established and applied to clinical specimens, for the first time in Nepal. The anti-*P.vivax* and anti-*P.falciparum* monoclonal antibodies (MAbs) raised against local Thai strains of both *P.vivax* and *P.falciparum* and the polyclonal antibodies (PAbs) raised against local Nepali strains of *P.vivax* and *P.falciparum* were used as capture and detecting antibodies, respectively in these two system. These MAb-PAb based indirect ELISA showed highly specific and sensitive enough to detect the lowest number of both *P.vivax* and *P.falciparum* parasites as low as 6 parasites/10^6 erythrocytes and 9 parasites/10^6 erythrocytes, respectively. These levels of detection were closed to the parasite detection limit by microscopic examination by skilled microscopist with the

limits of detection of about 10-20 parasites/µl of blood (Bruce-Chwatt, 1984). In addition, the level of detection by MAb-PAb indirect ELISA were also corresponded to the previous detection of *P.fapciparum* antigens by several techniques (Avraham *et al.*, 1981,1983; Mackey *et al.*, 1982; Frazen *et al.*, 1984; Barker *et al.*, 1986) which could detect the falciparum parasite varying from 1-10 parasites/10^6 erythrocytes. Moreover, previous studies using the same monoclonal antibodies (McPV1) against *P.vivax* Thai isolates, two systems of sandwich enzyme-linked immunosorbent assay (ELISA). Those were a two-site monoclonal antibody sandwich ELISA (MAb-MAb sandwich ELISA) and a two site polyclonal-monoclonal antibody sandwich ELISA (PAb-MAb sandwich ELISA) could detect *P. vivax* antigens as few as 6.68 parasites/10^6 erythrocytes and 2.69 parasites/10^3 erythrocytes, respectively (Khusmith *et al.*, 1992). Additionally, such assays showed good correlation with the level of parasitemia when tested against serially diluted *P.vivax* parasites (r = 0.937, and 0.997 for MAb-MAb- and PAb-MAb- sandwich ELISA, respectively). Such MAb-MAb sandwich ELISA was specific, since it was positive only with *P.vivax*-infected erythrocytes from vivax malaria patients and negative when erythrocytes from 34 healthy individuals and 30 falciparum malaria cases were tested. The results of the present study were consistent with such previous results regarding the parasite detection levels and the specificity of the two-site MAb-MAb sandwich ELISA. However, when the two site PAb-MAb sandwich ELISA for the detection of *P.vivax* antigens were compared, the MAb-PAb based indirect ELISA seem to be superior in detecting the lower number of vivax parasites.

Furthermore, other two-site pan-species monoclonal antibody sandwich ELISA (MAb-MAb ELISA) developed recently to detect *P.vivax* and *P.falciparum* antigens in whole blood (Gao *et al.*, 1992) by coating the plate with pan-species MAb 3F9 and the another pan-species MAb M26-32 conjugated with alkaline phsophatase, the sensitivity of the assay was 5, 10 and 10 parasites per 10^6 erythrocytes for cultured *P.falciparum*, patient-derived *P.vivax* and *P.falciparum*, respectively and the specificity of such assay was 93% for healthy individuals, 92.4% for *P.vivax* and *P.falciparum* malaria cases. The

specificity of the present assays for detection of both *P.vivax* and *P.falciparum* was 100% which was superior to their tests. The lower specificity in their assays could be due to the use of rabbit anti-*P.cynomolgi* in detecting the human strains of *P.vivax* and *P.falciparum* antigens in such assay.

However, the development of monoclonal-polyclonal antibody based indirect-ELISA for the detection of both *P.vivax* and *P.falciparum* antigens needs to be further developed and applied to detect parasite antigens in a large number of blood samples from the people who have been living in malaria endemic area of rural Nepal with or without symptoms before the definite conclusion could be made. In addition, the patients having fever with unknown origin from the recruited malaria endemic areas should also included in this study as controls, it might be useful in analyzing the sensitivity and the specificity of the results if such patients are infected with malaria and could not be diagnosed by conventional diagnostic methods. Furthermore, if the assay could be proven to be sensitive and specific enough, it could be used in the field in addition to the parasitological examination since the method is considered as practicable enough for a large scale survey to monitor malaria endemicity in the rural settings which might be use in the National Malaria Eradication Program (NMEP), Ministry of Health, His Majesty's Government of Nepal in a broader scale.

CHAPTER VI
CONCLUSION

Malaria is still one of the major global tropical diseases that causes of 1-2 million deaths every year. The disease has been found affecting even in the south Asian sub-continent including Nepal. Both vivax and falciparum malaria are predominantly found in Nepal especially in the southern tropical geographical areas bordering with India. More than two third of the population of Nepal has been affected with malaria. The malaria situation in Nepal has been further deteriorated because of the current civil unrest and financial constraints thereby leading the overall malaria situation further worsening. In spite of the several control measures undertaken by Ministry of Health, His Majesty's Government of Nepal with the aide of international agencies since 1958, the disease has not been under control because of the lack of development and subsequent application of the reliable malaria diagnostic methods with higher sensitivity, spcificity and accuracy which could be confidentially used in the malaria control program throughout the country.

In realizing this national problem of Nepal, a simple and affordable immunodiagnostic method of MAb-PAb indirect ELISA was developed using monoclonal antibodies (MAbs) as capture antibodies, produced against Thai strains of P.vivax and P.falciparum and the polyclonal antibodies (PAbs) as detecting antibodies, produced against the native Nepali strains of P.vivax and P.falciparum in the rabbit model were. The conditions of MAb-PAb based indirect ELISA were optimized before application to the blood samples collected from the fields. Blood from normal healthy individuals were used as controls. The established MAb-PAb based indirect ELISA when applied to 203 vivax and 154 falciparum blood samples proven microscopically showed high degree of sensitivity, specificity and accuracy. Moreover, the assays could detect the lowest number of both P.vivax and P.falciparum parasites as low as 6 parasites/10^6 erythrocytes and 9 parasites/10^6 erythrocytes, respectively. These levels of detection were

closed to the parasite detection limit by microscopic examination by a skilled microscopist. The established assay is rapid and more practicable particularly when processed in a large number of samples simultenously. By applying other highly advanced recent techniques such as molecular biological techniques using the sophisticated equipments and the expensive reagents in the rural field of Nepal is almost impossible at the current situation so the MAb-PAb based indirect ELISA could be the method of choice for diagnosis of malaria in such underprevileged people of the malaria endemic sites of Nepal. Furthermore, the MAb-PAb based indirect ELISA developed for the first time in Nepal seems to be affordable by the rural common people of Nepal.

The development of monoclonal-polyclonal antibody based indirect ELISA for the detection of both *P.vivax* and *P.falciparum* antigens, however, needs to be further developed and applied to detect parasite in more blood samples from the people who have been living in malaria endemic areas in Nepal with or without symptoms, before the conclusion could be made. If the test was sensitive and specific enough, it could be applied in the field in addition since the method is quite practicable for a large scale survey to monitor malaria endemicity in the rural settings. Such test may have adequate potentiality for use in the National Malaria Eradication Program (NMEP), Ministry of Health, His Majesty's Government of Nepal in a broader scale.

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