A Study on the Plasmodium vivax Relapse Pattern and Identification of Dominant Genotype in Far-Western Nepal

NEPAL HEALTH RESEARCH COUNCIL (NHRC)

2011

A Study on the Plasmodium vivax Relapse Pattern and Identification of Dominant Genotype in Far-Western Nepal

Study Team

Prof. Dr. Chol Lal Bhusal (NHRC)

Dr. Gajananda Prakash Bhandari (NHRC)

Mr. Umesh Ghimire (NHRC)

Dr. Sameer M Dixit (CMDN)

Ms. Sulochana Manandhar (CMDN)

Government of Nepal

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Executive Summary

Altogether, six health centers were chosen from the Kailali and Kanchanpur district; three from each district. The respective health centers were selected on the basis of maximum number of malaria positive cases reported preceding year. The study tried to find out the relapse pattern of *P. vivax* for the period of six months from December 2010 to May 2011.

In total, 137 malarial blood samples were accessed from the four health centers. Two health centers from Kanchanpur district were excluded due to unavailability of malaria positive cases during the study period. Age-group 21- 30 years had the highest distribution with 34 percent participation. Twenty nine percent and 17 percent of the patient belonged to the age-group 10 to 20 and 31 to 40 years respectively. Only 4 percent cases were observed in the age group 60 above years. In our study, 81 percent were male and 19 percent were female. Out of total malaria blood samples, 23 cases (17%) were relapsed during six months period.

Random Fragment Length Polymorphism (RFLP) is a method used in distinguishing between selected genotypes within a species. RFLP was carried out on 100 *P. vivax* species identified in Nepali malarial cases. The study looked into distinguishing two major genotypes of *P. vivax*-namely VK210 and VK247 within the *P. vivax* species which have been identified in South Asian cases, especially in neighboring India. Results of 100 samples showed a net success rate in positive identification of 84% samples whereby all the isolates were found to be VK210 genotype. This is a preliminary baseline genotyping study targeting only known gene sequences pertaining to the genotypes. A thorough genome sequencing of all strains in this study would provide detailed genetic variation data amongst these and other isolates.

Acknowledgement

I would like to express my sincere gratitude to the Tropical Disease Research (TDR) Sub-Committee of Nepal Health Research Council (NHRC) for initiating this research. A warm gratitude to Dr. Gajananda Prakash Bhandari, who had led the Tropical Disease Research Project and supported us in each and every step during the research. I would like to thank Dr. Shanker Pratap Singh, Member-Secretary NHRC for his coordination during field work.

I am thankful to Mr. Umesh Ghimire, Assistant Research Officer, who handled all the activities of this research program. I would like to appreciate the support of Center for Molecular Dynamic Nepal (CMDN) for its great role in molecular level laboratory analysis. My sincere thanks goes to all the Chief Officers of health institutions who permitted us to conduct the research in their institution. The health workers of respective health institutions were the part of the team who coordinated us during the entire field works.

Last but not the least; I am grateful to the team of Nepal Health Research Council and to all the persons who had directly or indirectly supported in this research to make it successful.

Prof. Dr. Chop Lal Bhusal
Executive Chairman
Nepal Health Research Council

Table of Content

List of Figure

List of Table

Acronyms

ACT	:	Artemisinin-based Combination Therapy
CMDN	:	Center for Molecular Dynamic Nepal
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
DPHO	:	District Public Health Office
G6PD	:	Glucose-6-phosphate dehydrogenase
GCLP	:	Good Clinical Laboratory Practice
NHRC	:	Nepal Health Research Council
PCR	:	Polymerase chain reaction
PHCC	:	Primary Health Care Center
RFLP	:	Restriction Fragment Length Polymorphism
SEA	:	South-east Asia
SHP	:	Sub-health Post
SP	:	Sulfadoxine-pyrimethamine
SPSS	:	Statistical Package for Social Sciences
TDR	:	Tropical Disease Research
ULV	:	Ultra-low Volume
WHO	:	World Health Organization

CHAPTER ONE

1.1 Introduction

Malaria is a disease of public health importance and caused by parasites that are transmitted through the bites of infected mosquitoes called "malaria vectors", which bite mainly at night time. In the world, about 20 different anopheles species have been found. *Plasmodium falciparum* and *Plasmodium vivax* are the most common species for its transmission. *Plasmodium falciparum* is the most deadly one.(1) Globally an estimated 75 to 90 million cases of *vivax* malaria occur each year. *P. vivax* tends to be co-endemic with *P. falciparum* in the tropics, and occurs in an increasing proportion towards higher latitudes.(2)

1.2 Global Scenarios

Malaria continues to be one of the main public health problems in the world, especially in the majority of African countries. Sub-Saharan Africa is reported to have ninety percent of all malaria cases where it is the main cause of death and a major threat to child health. Malaria is caused by *Plasmodium* parasites and transmitted by anopheles mosquitoes. Human is classified as the malaria donor and recipient. The bloodsucking parasite infects one out of 10 world's population and present in 90 countries. Worldwide, a child dies of malaria every 30 seconds interval. It particularly affects young children, young adults engaged in economic development activities, pregnant women and international itinerant groups of population, moving into endemic areas. Greater resistance of parasites to the drugs has even hindered the desires for, global malaria elimination and is still posing a great threat to the global health. Further, proliferation of drug resistance is closely related to massive population movements, inadequate health services, improper use of anti-malarial drugs, limited resources and operational difficulties in implementing malaria control activities. The economic impact of malaria is felt by various social groups of the society particularly by the poorest countries of the world and especially among the people living under the most difficult conditions. (3, 4)

Fifty percent of the world's population is still living in malaria endemic areas and every year 200 million new cases with 2 million deaths are reported. Children less than 5 years old are at greater risk of malaria and the malaria deaths are recorded high in this age group Although malaria endemicity exists mainly in tropical region, global initiation towards malaria eradication scaled up during 1950's rather increased its movement towards higher region than diminishing its prevalence.(5) Being found in tropical areas, its geographical distribution is worldwide, throughout Sub-Saharan Africa and to a lesser extent in South Africa, South-east Asia, the Pacific Islands, India and Central and South America.(2) Nowadays, malaria vectors are found surviving in the higher altitudinal range due to the rapid climate change.

In WHO sub-regions 1 and 2, which are bearing around 87.9% of the current global malaria burden, around 9.4 million people are estimated to live near large dams and irrigation schemes. In contrast, the remaining sub-regions concentrate an estimated 15.3 million people are found living near large dams and up to 845 million near irrigation sites, while here only 12.1% of the global malaria burden is concentrated. The establishment and operation of water projects has had a history of facilitating a change in the frequency and transmission dynamics of malaria, but analyses of these environmental risk factors are sparse.(6)

Best estimates currently describe the annual global burden of malaria with 300-500 million cases and 1-2 million deaths. Over 90% of the disease burden is in Sub-saharan Africa.(7) The World Health Organization defines malaria as a disease of poverty. Pregnant women infected with malaria usually have more severe symptoms and outcomes, with premature delivery, low-birth-weight neonates and neonatal death, higher rates of miscarriage and intrauterine demise; they are also at a higher risk for severe anemia and maternal death.(8)

Globally, malaria is the most wide spreading infectious disease affecting humans. Malaria parasites kill up to 2 million populations; infecting more than 500 million annually, and causing at least 100 million cases of acute illness. Malaria is a highly dynamic infection and disease and therefore an endemic public health plague for almost one-third of the world's population.(9)

The current burden of *Plasmodium vivax* malaria is increasing worldwide and causing great concerns in public health systems. The global burden of malaria due to *Plasmodium vivax* is approximately 70-80 million cases annually and approximately 10-20% of the world's cases of *P.*

vivax infection occur in Africa, south of the Sahara. In eastern and southern Africa, *P. vivax* represents around 10% of malaria cases but < 1% of cases in western and central Africa. Outside of African, *P. vivax* accounts for > 50% of all malaria cases.(10)

P. vivax infections affect people of all ages, however the effects of repeated attacks of *P. vivax* through childhood and adult life are only rarely directly lethal, they can have major deleterious effects on personal well-being, growth, and development, and on the economic performance at the individual, family, community, and national levels.(10)

The report published by WHO linked malaria with changes in land use related to road building, mining, logging, and agricultural and irrigation projects. Global climatic change, disintegration of health services, armed conflicts, and mass movements of refugees also contribute to the increasing risk for malaria.(11)

The Roll Back Malaria Partnership has developed the Global Malaria Action Plan for a substantial reduction in the burden of malaria and its eradication in the long term. In south Asia, there are many efficient vectors causing epidemics of both *Plasmodium vivax* and *Plasmodium falciparum*. The interruption of *P. vivax* transmission is difficult to achieve due to liver-stage infection. New drugs, insecticides and other intervention tools with sufficient coverage are the uttermost requirements to achieve the goal of malaria control or elimination.(12)

Relapse pattern in *P. vivax* malaria is an emerging issue in malaria control program. Numbers of studies have given insightful evidence that the relapse of malaria is a serious threat to global public health. In an endemic area, relapse of *Plasmodium vivax* cases as well as its re-infection is creating hurdles in achievements as desired from malaria control program. New strains of *Plasmodium vivax* vary in their sensitivity to primaquine, Different drugs are required to prevent relapse and provide reservoir reduction as per the sensitivity of *Plasmodium vivax* to drugs. A 14-day course of primaquine (PQ) is effective however it cannot used safely in routine practice because of its interaction with glucose-6-phosphate dehydrogenase (G6PD) deficiency. A study "A randomized trial in Northwest Frontier Province, Pakistan" suggests that a practical radical treatment for *vivax* malaria is essential for control and elimination of the disease. The 8-week PQ course was found more effective at preventing relapse than current treatment with chloroquine

alone.(13) Another study suggest that, prevention of relapse after effective therapy for the acute attack requires a standard daily dose of primaquine administered over 14 days.(14)

The rise in the average temperature of earth is believed to incur several harmful effects on human health, both directly and indirectly. Since malaria is greatly influenced by climatic conditions because of its direct relationship with the mosquito population, it is widely assumed that its incidence is likely to increase in coming days. As many studies have portrayed direct relationship between the recent increase in malaria incidence and global warming.(15)

Study on effect of global warming and local social and economic conditions on the malaria transmission, suggests that the level of malarial risk depends on rate of malaria transmission which can be predicted by the ambient temperature and socio-economic conditions.(16)

The global fight against malaria has been continually challenged by poor access to affordable & effective medicine. Artemisinin, the successor therapy to chloroquine, is at least ten times more costly than the older drug. Finally those drugs manufactured to fight against poverty disease are found killing ailing people due to their high prices.(17)

The global campaign towards malaria eradication has received a tremendous boost with the addition of artemisinin compounds to the therapeutic armament. Artemisinin drugs are now being recommended in combination with existing anti-malarial, a pairing often referred to as artemisinin -based combination therapy (ACT). The World Health Organization considers ACT first-line treatment for uncomplicated malaria in endemic regions.(18)

1.3 South East Asia

Being highly seasonal, with long relapse intervals along with occurrence of its epidemic at higher latitudes, the epidemiology of *P. vivax* malaria is found varying across the region, while *falciparum* malaria transmission is found more recurrent in the south.

By its nature as a vector-borne disease, and with its long persistence in the human host, *P. vivax* malaria readily spreads within countries and across national borders. The reasons for the increase in its prevalence are still unclear; however climatic factors, economic and social factors, and

changing agricultural practices are mainly coined. Approximately 56% of all *P. vivax* malaria cases occur in SEA.(2)

Despite years of continual efforts, malaria is still one of the major causes of morbidity and mortality in third-world countries. Malaria is highly prevalent in South-east Asia where large numbers of tourists visit each year. A remarkable rise in the number of imported cases of malaria has been reported mainly due to the increasing worldwide travel to regions where there is high risk of malaria transmission. Nowadays, cases of malaria acquired from international travelers of developed countries exceed 25,000 cases per year, with 10,000 of them reported annually and approximately 150 deaths per year.(7).

Mixed *P. falciparum/P. vivax* infections are common in South-East Asia. After several weeks of follow-up of treated *P. falciparum* malarial cases, a significant proportion of cases were found to have developed *P. vivax* malaria. In a report of Cambodia, 47% of those with *P. falciparum* gametocytes on admission were later found to have developed *P. vivax* malaria upon follow-up after day 28. The presence of both sexual and asexual forms of *P. falciparum* on blood smear with acute *falciparum* malaria serves as a marker for possible *P. vivax* co-infection and subsequent relapse.(19)

The morbidity and mortality both shares a huge economic, health and social burden. *Plasmodium vivax* malaria constitutes about 60 - 65% of total malaria cases in India with, pronounced morbidity particularly in the economically weaker sections of the society.(20) The distinctive epidemiology of malaria in India, where *P. vivax* predominates over *Plasmodium falciparum*, renders India ideal for studying the dynamics of co-infections.(21)

Relapse infections are barrier to successful treatment and control of *Plasmodium vivax* malaria but the nature of the relapse pattern is still in vague. Therefore, it is important to specify the pattern of *vivax* malaria relapses and to try to discriminate efficiently re-infections from relapses. Finding of the study "Molecular analysis of strains of *Plasmodium vivax* from paired primary and relapse infections", came to the conclusion that most relapses are caused by the same parasite populations that circulate during the primary infection and do not arise from a genetically distinct sub-population.(22) Due to the greater drug resistivity in malarial parasites, it

has emerged as havoc in most of the malaria endemic countries. The disease is now on the rise again since it is appearing in areas where it had disappeared. (2)

1.4 Country Situation

Malaria is a major public health problem in Nepal. Out of 27.3 million populations of the country, 22.5 million people still live in malarias endemic areas. In Nepal, around 70 percent of the total populations are believed to be at risk to malaria. It's difficult to garner the precise data on morbidity and mortality due to low reporting of the cases, poor health care seeking practices, non-availability of diagnostic facilities. (23)

The first attempt to control malaria in Nepal was initiated in 1954 through the Insect Borne Disease Control Program supported by the U.S. Agency for International Development (USAID). Later in 1958, the malaria eradication program was launched as a vertical program with an ultimate objective of eradicating malaria from the country, but it could not be achieved due to technical, operational and administrative constrains, and consequently the program reverted back to malaria control in 1978.(24) The low lands of Southern belt, which is also known as the Terai region, Indo-Nepal boarder area, and middle hills below 1,000 m elevation possess the most favorable environment for the malarial vector to survive. Most of the malaria in the middle hills are believed to have imported by local Nepalese arriving from the Terai, where almost of the cases were engaged in big engineering projects. The majority of malaria transmission in Nepal occurs in 12 districts and these have been labeled as priority districts by Ministry of Health and Population. These districts are Dadeldhura, Kanchanpur, Kailali, Kavrepalanchowk, Bardia, Nawalparasi, Sindhuli, Mahottari, Dhanusha, Morang, Jhapa and Ilam. (7)

Since 1950, chloroquine was used as first line drug, but sulfadoxine-pyrimethamine (SP) was introduced after the emergence of *P. falciparum* resistant to chloroquine, and later established it as the first line drug for the treatment of microscopically confirmed uncomplicated *P. falciparum* malaria. But choloroquine remained a standard drug for the treatment of *P. vivax* malaria in Nepal due to maximum side effects of SP. Thus, the 80% of the malaria cases are treated with chloroquine. However, the Government of Nepal has recommended 5 days treatment

with primaquin for *vivax* malaria. But the standard treatment with primaquin requires 14 days to prevent relapse except in those cases with G6PD deficiency, and in infants and pregnant women (8). Finding of different studies show that the relapse rate in *P. vivax* malaria is highly variable, ranging between 2% to 44% even after treating them with chloroquine. And the relapse mainly occurred during the first three months after the first attack.

The present study is an attempt to understand the rate of relapses in Nepalese context to elucidate their transmission dynamics for planning vector control strategies and chemotherapeutic measures in *P. vivax* foci.

1.5 Rational of the study

Controlling vector and preventing vector borne diseases is itself a difficult task for the economically weak countries like Nepal. Such action requires multi-dimensional approaches, inter-sectoral co-ordination to anchor its propagation. Similarly in Nepal, preventing vector borne diseases are possessing great challenge to the efforts of entire health system. Vector borne diseases have remained a leading cause of morbidity and mortality in Nepal. So these scenarios reveal a dire need of action oriented research in this field. Research is a vital tool to find out measures to handle and control the vector borne diseases like malaria.

This study tends to find out the relapse pattern of *P. vivax* that is also found especially in Far-Western Nepal. Findings the relapse pattern of malaria can be helpful in early management of malarial cases. Due to open border with India, relapse and re-infection of the *P. vivax* is more frequent. The people residing near border, more often visit India for short term employment and when they return, they bring back with malaria. That's why there is a need of continuous study of malaria to identify as well as to treat the imported cases of malaria.

Molecular level analysis is helpful in finding out the genotype of malaria species found in Nepal and knowledge of genetic diversity of the parasite in such malaria endemic country like Nepal can assist in understanding the dynamics of the disease transmission which eventually can help design effective malaria control measures. Further since the two genotypes VK210 and VK247

of the malarial parasite *P. vivax* have shown to have differences in their drug susceptibility, the knowledge on genetic makeup of the parasite can also help in the anti-malarial drug choice.

CHAPTER TWO

2.1 Objectives

- To assess the relapse rate of *Plasmodium vivax* malaria during six months follow-up period in two districts of Far-Western Nepal.

- To assess PCR genotyping of *Plasmodium vivax* malaria from the same district.

CHAPTER THREE

3.1 Methodology

3.1.1 Study Design

Prospective longitudinal study by observing the *Plasmodium vivax* malaria cases to assess relapse rate.

3.1.2 Study Area

The study was carried out in two districts of Far-Western Region, Kailali and Kanchanpur. Altogether six health centers (Annex), three from each district were selected on the basis of maximum number of cases reported last year. However, the two centers did not report any malaria cases during the study period and hence only four centers were included in the analysis.

3.1.3 Study Period

In most of the Terai districts, including Kailali and Kanchanpur, *Plasmodium vivax* infections are prevalent and are recorded throughout the year. Their prevalence & infectivity are found to rise & fall in cyclic trends. It shows a gradual rising trend from March onwards, reaching a peak in July and August soon after the rainy season, and then decreasing sharply to very low levels in September. Considering their cyclic trend, the study was conducted from August 2010 to May 2011 for the period of ten months. Malarial blood samples were collected from respective health centers From August 2010 to November 2010 and from November 2010 onwards to May 2011, the cases were followed-up to see the relapse pattern.

3.1.4 Study Population

All patients of age group more than 6 months to 65 years of age during the study period of the study area were considered eligible for the study.

3.1.4.1 Inclusions:

Patient with auxiliary temperature $\geq 37.5°C$ at visit and those who are able to come for follow-up visit were included in the study. A slide confirmed infection with *P. vivax* (i.e. no mixed infections) was involved in the study.

3.1.4.2 Exclusions:

The patient with mixed infection with *P. falciparum* malaria and any feature of severe malaria were not included in the study. Pregnant mother or lactating mother and the patients with existence of underlying chronic severe illness were also excluded from the study.

3.1.5 Sample size consideration

Determination of sample size was based on desired confidence level (95%) and precision (10%). Assuming the possible relapse rate of 30%, the sample size required will be a minimum of 81 patients in order to be representative. Sample size was adjusted by 10% for the follow-up losses and withdrawals. The final sample size will be 90 eligible patients.

3.1.6 Screening Evaluation

Initially, demographic profile of the patients was recorded. Later clinical examination, axillary temperature measurement, blood slide examination was done to screen the patient. A record book was kept in which all the relevant information regarding age, sex, address, temperature, blood film etc. of the screened cases was entered.

3.1.6.1 Initial Clinical evaluation/enrolment evaluation

General physical examination was done by research officer. Special care was taken to detect the presence of febrile diseases other than malaria.

3.1.6.2 Parasitological examination

The day that the patient was enrolled and received the first dose of medicine was marked Day 0. A thick films was prepared before treatment on Day 0 and then on 6 months, and any other day that the patient was brought to the health institution for rise in temperature. The thick film was used for rapid staining (10-15 min in 10% Giemsa stain). Parasitaemia (per microlitre) = number of parasites x 8,000/number of leucocytes counted. Two trained laboratory technicians were engaged and both used to examine all the slides independently to assure quality. All the slides were sent to an independent laboratory for quality control.

5 ml of whole blood was taken in a container for molecular analysis.

3.1.7 Informed Consent

Formal informed consent was obtained from all patients or guardians in case of minors meeting the enrollment criteria.

3.1.8 Indicator

Relapse Rate = Total number of Relapse cases of Malaria due to *Plasmodium vivax*/ Total number of malaria cases due to *Plasmodium vivax* treated by choloroquine standard regime

3.1.9 Treatment

Anti-malarial treatment (chloroquine 25 mg/kg body weight in three days) was given under the direct observation by study team members using established treatment regimens. After diagnosis patients were asked to complete the dose of chloroquine and visit the same health institution. The patients were also asked not to take any other medications during the period without informing the treating physician. Ancillary treatments, such as antipyretics, was administered and given to patient as "take home" as per requirement and was provided to patients by the study team.

Once the complete enrollment and treatment procedure was finished, the patients were advised for follow-up visit after 3 days and then after 6 months or anytime if they experience rise in temperature. Patients were also advised to visit same health institution at any time during the follow-up period if they experience any other health problems. For ethical reasons, all patients, irrespective of symptoms were treated with the alternative anti-malarial drug (oral primaquine) at the end of the follow-up period or after they are diagnosed as relapse. But it was done only after the careful screening of G6PD deficiency (to prevent the risk of hemolysis) at Center for Molecular Diagnosis Neal (CMDN), Kathmandu.

3.1.10 Follow-up schedule

A monthly visit/call was given with the intention to remind the patient for the follow-up. Patients were advised to visit any day during the follow-up period if any unwanted symptoms were experienced.

Blood films for parasite count were collected and examined in between day 0 and 6 months period or on any other day if the patient returns with any symptoms.

Considering the patient's safety reason, blood films were collected whenever the research officer requested for parasitological reassessment. The exact address of patients was recorded to locate their houses if they did not attend as per the request. The schedule for treatment and follow-up examination, given in this protocol, was followed rigorously to ensure data integrity. Patients who failed to appear within 6 months were traced by their home address.

3.1.11 Case Definition

Primary *P. vivax* infection: Cases with no past history of malaria but those presenting with fever and microscopic evidence of *P. vivax* infection at the time of study. Some patients in this group who were present with no clinical symptoms of malaria or parasitological evidence of *P. vivax* infection following their primary infection during the entire study period were considered non-relapse cases. Those patients who reported back to the clinic within 1.5 to 6 months with renewed clinical symptoms (mild) along with a periodic alternate day fever (not observed in the primary cases) and those who were found to be microscopically positive for *P. vivax* infection were considered relapse cases.

3.1.11 Rescue Treatment

The rescue treatment was as per the national guidelines. Patients presenting with relapse and all other patients who complete 6 months of follow up were given 14 days anti-relapse regime of primaquine after screening for G6PD deficiency. Patients experiencing severe deterioration in clinical status during the study period were referred immediately for appropriate inpatient care to tertiary level health institution.

3.1.12 Definition of study end-points

Valid study end-points includes relapse and completion of the follow-up period without treatment relapse, loss to follow-up, withdrawal from study (voluntary and involuntary), and protocol violation.

3.1.12.1 Relapse

Those patients who returned back to the clinic within 1.5 to 6 months with renewed clinical symptoms (mild) along with a periodic alternate day fever (not observed in the primary cases) and with microscopically positive for *P. vivax* infection were considered relapse cases.

3.1.12.2 Loss to follow-up

Loss to follow-up defines when, despite all reasonable efforts, an enrolled patient cannot be found for study.

3.1.12.3 Withdrawal from study

As per the consent, all subjects participating in the study were always free to end their participation in the study at any time. An involuntary withdrawal means withdrawal of sample, who have developed a concomitant illness interfering the clear interpretation of study outcomes.

3.1.12.4 Protocol violation.

A protocol violation in this study defines all the following conditions from missing treatment dose, detection of a mixed infection during follow-up, or a credible report of additional anti-malarial drug used outside the study protocol (such as self-medication).

3.1.13 Determination of study outcomes

Two categories were used to demonstrate the study outcome: Relapse and Treatment success without relapse till 6 months. During the entire study period, the patient's welfare was taken priority and all the procedures were done considering the standard guidelines of Good Clinical Laboratory Practice (GCLP).

3.1.14 Data Analysis

The demographic and other data were entered in Ms-Excel 2007 and analyzed in SPSS software 13.0 version. Relapse rate was calculated to see the relapse pattern.

3.1.15 Indicator

- Relapse Rate

3.2 Methodology for Molecular Analysis

3.2.1 Sample storage

Whole blood samples obtained from NHRC were stored at -20^{o}C till were used for DNA extraction.

3.2.2 Optimization

Before the real analysis of samples, 6 random samples were picked to optimize all the steps from DNA extraction to PCR for identification of *Plasmodium* genus, *vivax* species, and differentiation of genotype of *P. vivax*.

Further to confirm the result of microscopic detection of *P. vivax*, a rapid immunochromatography test detecting *P. vivax* specific antigen was subjected to random 6 samples. All samples tested positive for the immunology based test.

3.2.3 DNA Extraction

DNA from the samples was extracted using a commercial kit specific for whole blood sample. Briefly, whole blood was firstly dehemolysed with a lysis buffer, which was followed by cell digestion. The crude DNA in the lysate was purified by spin column method and finally eluted in Tris-EDTA buffer. The DNA was stored at -20^{o}C till used for PCR.

3.2.4 *Plasmodium* genus and *vivax* species specific PCR

Malarial parasite of *Plasmodium* genus was detected in microscopically confirmed malarial samples by *Plasmodium* Genus specific PCR using rPLU6 (TTAAAATTGTTGCAGTTAAAACG) and rPLU5(CCTGTTGTTGCCTTAAACTTC) primer pair (25). The PCR condition used was 400nM of each of the primers, 2mM of $MgCl_2$, 800nM of dNTPs, 0.1µg/µl BSA and 0.4 units of Taq Polymerase. The PCR thermo cycling condition was initial denaturation at 95°C for 5 minutes followed by 24 cycles of annealing at 58°C for 2 minutes, extention at 72°C for 2 minutes and denaturation at 94°C for 1 minute, which was followed by one cycle of each annealing at 58°C for 2 minutes and final extension at 72°C for 5 minutes. The result was read in 1% agarose gel electrophoresis.

Subsequently, the *Plasmodium* genus positive samples were then tested for species *vivax* by Species specific PCR by using primer pair rVIV1(CGCTTCTAGCTTAATCCACATAACTGATAC) and rVIV2(ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA).(25) The PCR condition was same to that for genus PCR except that the concentration of primers was 250nM. The PCR thermo cycling condition was same to that of 1st round genus PCR except that the cycle was repeated for 30 times. The result was read in 2% agarose gel electrophoresis.

Both of the above steps of nested PCR for detecting *Plasmodium vivax* were carried out in only 6 random samples in order to ensure our technical capability of genetically identifying *Plasmodium vivax* species in the biological samples.

3.2.5 *Plasmodium vivax* genotyping PCR and Restriction analysis

All the samples that were presumed to be *Plasmodium vivax* species on the basis of microscopic examination were tested for identifying two major genotypes of *Plasmodium vivax* circulating in Asian world, namely: VK210 and VK247.

The genotyping was carried out by two rounds of nested PCR using two sets of primer pairs VCS-OF(-ATGTAGATCTGTCCAAGGCCATAAA) / VCS-OR(TAATTGAATAATGCTAGGACTAACAATATG) and VCS-NF(GCAGAACCAAAAAATCCACGTGAAAATAAG)/ VCS-NR(-CCAACGGTAGCTCTAACTTTATCTAGGTAT) (26) targeting the polymorphic central repeat region of the circumsporozoite protein (pvcs).

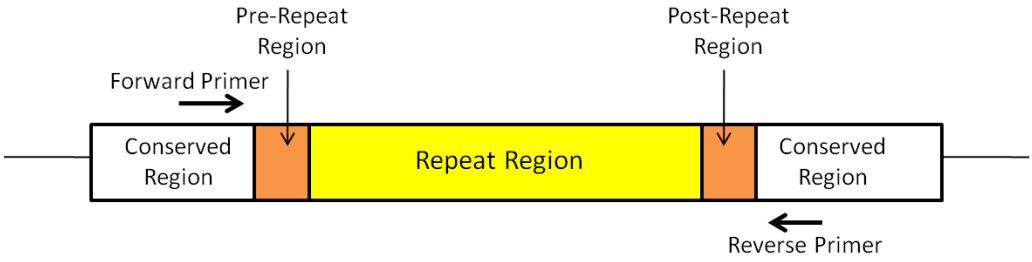


Figure 1 Pvcs Gene

The central repeat region is composed of a nucleotide repetitive element 27 bp (or 9 amino acid) long. And the repeat element found in the central region is composed of two exclusive patterns; either repeats of GDRADGQPA which is categorized as VK210 type or 19 repeats of ANGAGNQPG that is found in VK247 type.

Variation in this repeat element in the central region of Pvcs gene is exploited in distinguishing between two genotypes by digesting the 2nd round nested PCR products with two restriction enzymes AluI and BstNI in parallel. The digested products were analyzed in 2% agarose gel electrophoresis and two major genotypes VK210 and VK247 were identified based on the differences in the digestion pattern observed.

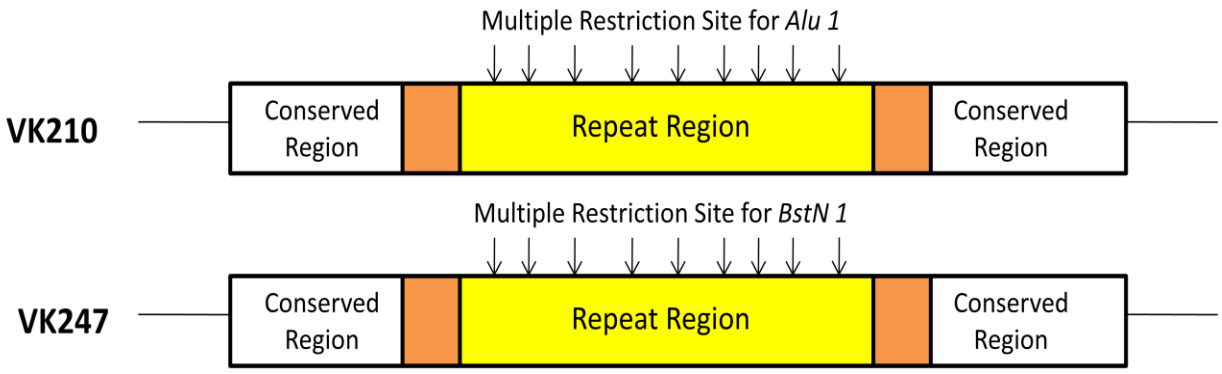


Figure 2 Restriction digestion sites in the repeat region of Pvcs gene by two different restriction enzymes

The 1st round genotyping PCR using VCS-OF and VCS-OR primer set was done with final concentrations of 400nM of each primers, 1mM of MgCl2, 800nM of dNTPs and 0.4 unit Pfu Taq polymerase with 1µl of DNA template, The thermo cycling condition for 1st round genotyping was exactly same to that of 1st round genus specific PCR for Plasmodium.

The subsequent 2nd round genotyping nested PCR using VCS-NF and VCS-NR primer set was done with 250nM of each primer, 1mM of MgCl2, 800nM of dNTPs and 0.4 unit Pfu Taq polymerase with 1µl of 1st round PCR product as template. The PCR thermocycling condition used was initial denaturation at 95°C for 5 minutes followed by 30 cycles of annealing at 62°C for 2 minutes, extention at 72°C for 2 minutes and denaturation at 94°C for 1 minute, which was followed by one more cycle of each annealing at 62°C for 2 minutes and final extension at 72°C for 5 minutes.

The overall schematic workflow is given below:

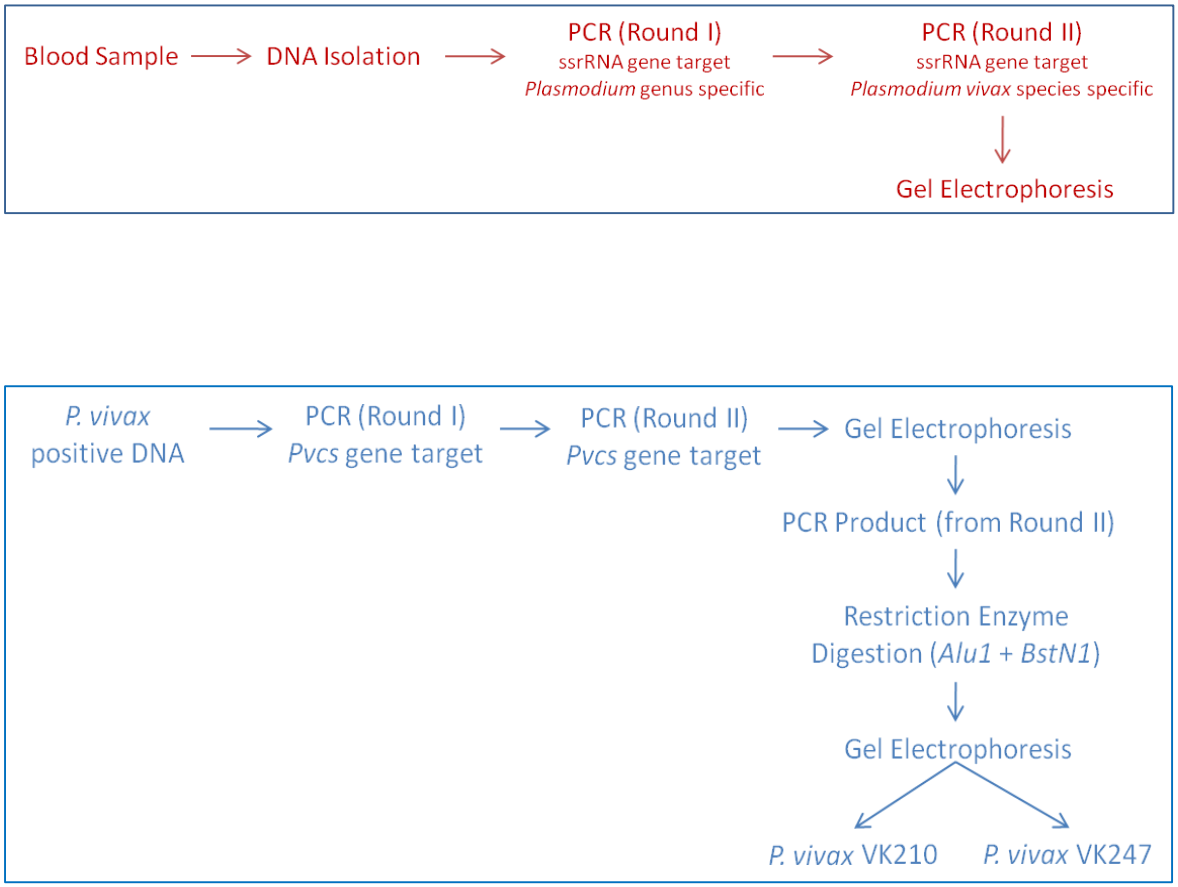


Figure 3 Systematic Workflow of Gel Electrophoresis

CHAPTER FOUR

4.1 Result

4.1.1 Malarial Patient in Health Institution

Altogether six health centers from Kailali and Kanchanpur district were selected for the study; three from each district (see annex) on the basis of occurrence of malaria cases in previous fiscal year. However, the two centers (Beldani PHCC and Jhalari SHP) did not report any cases during the study period and therefore only four centers were included in the analysis.

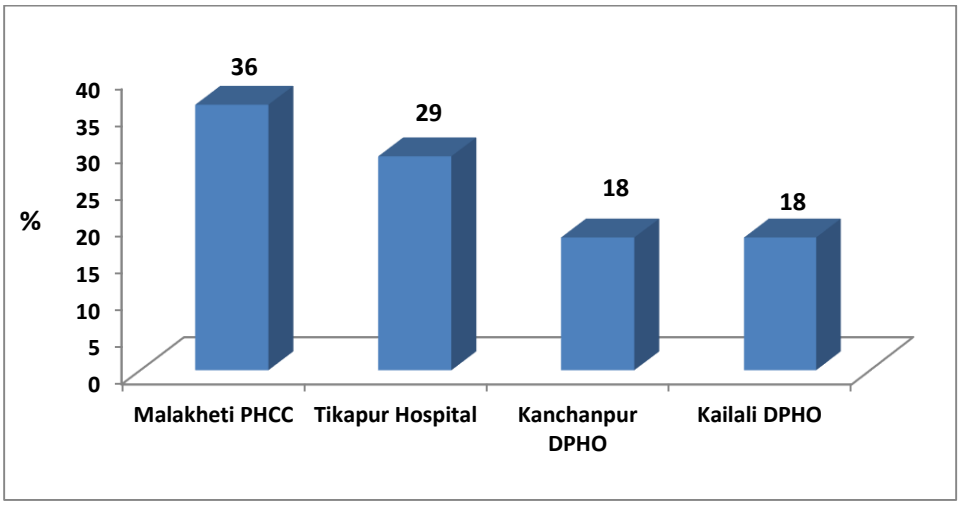


Figure 4 Malaria Cases in Different Health Centers

In total, 137 malarial blood samples were accessed from the four health centers. Maximum number of cases were seen in Malakheti Primary Health Care Center (PHCC) center comprising 36 percent of total cases. Out of total cases, 18 percent of cases were from District Public Health Office of Kanchanpur and Kailali district each. Almost 29 percent of the cases were received from Tikapur hospital.

4.1.2 Age distribution

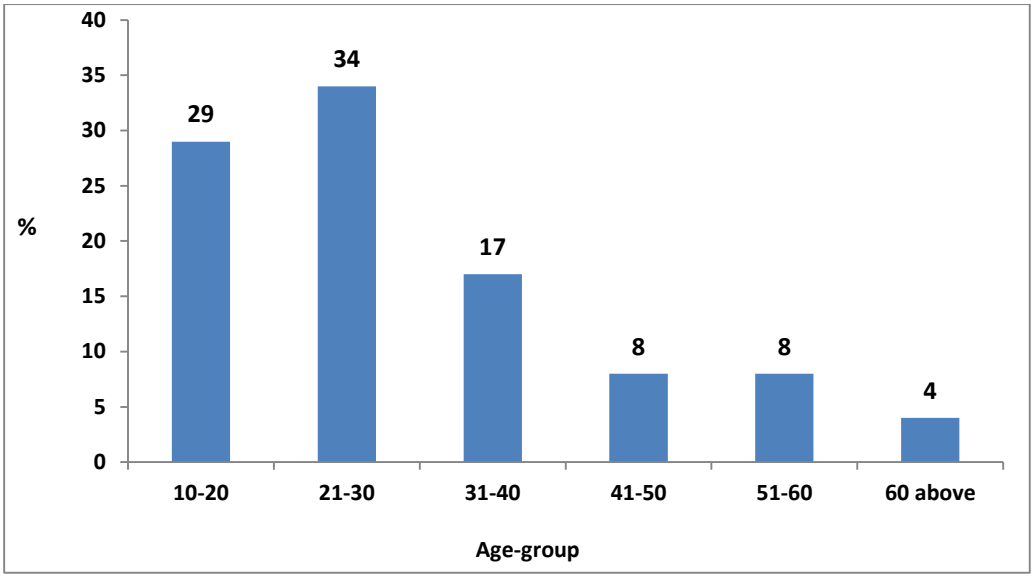


Figure 5 Age distribution of Cases

Total of 137 malaria cases were involved in the study, of which the age distribution ranged from 10 to 60 years and above. Age-group 21- 30 years had the highest distribution with 34 percent participation. Twenty nine percent and 17 percent of the patient belonged to the age-group 10 to 20 and 31 to 40 years respectively. Only 4 percent cases were observed in the age group 60 above years.

4.1.3 Male Female Ratio

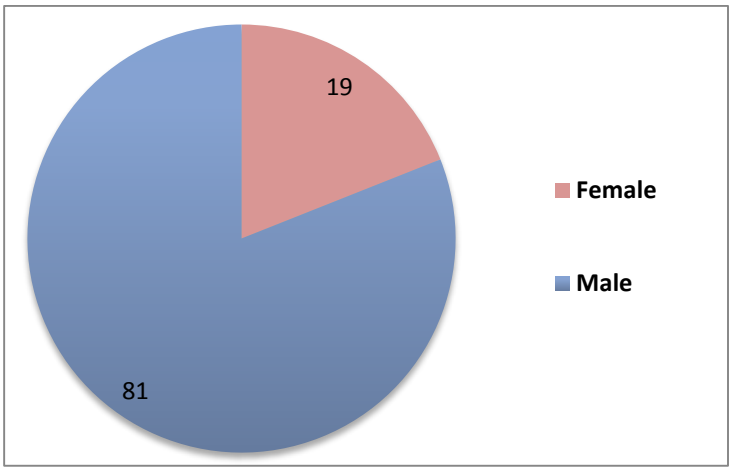


Figure 6 Male Female Ratio

Amongst the total 137 cases, 81 percent of malaria patients were male and 19 percent were female.

4.1.4 Relapse by Male and Female

During the follow up period of six months, 23 cases out of total 137 were relapsed. Hence, in the study the relapse rate was observed to be 17 percent. In case of gender-wise distribution of relapse rate, the proportion of relapse rate is higher in male (19%); than in females (8%). However the difference in gender-wise distribution of relapse rate is not more than 10% with p value of more than 0.05.

Table 1 Relapse Pattern by Gender

	Male	Female	Total
Relapsed	21(19%)	2 (8%)	23 (17%)
Not Relapsed	90 (81%)	24 (92%)	114 (83%)
Total	111 (81 %)	26 (19 %)	137

p>0.05 (Fisher exact test applied)

4.2 Molecular Analysis

4.2.1 Plasmodium genus and *vivax* species specific PCR

Of six random samples tested by Genus- and *vivax* Species-specific PCR, all samples tested positive for *Plasmodium vivax* (Fig. 7 - 8).

Based on this information, all the samples were presumed to be of *vivax* species as diagnosed by microscopic analysis. Confirming the parasite to be of *vivax* species was important in this study because the subsequent genotyping PCR was specific only for the *vivax* species.

4.2.2 Plasmodium *vivax* genotyping PCR and Restriction analysis

Among 100 samples tested for genotyping PCR, 84 samples showed successful PCR amplification and upon restriction analysis, were found to be of genotype VK210. None of the samples tested till turned up to be of genotype VK247 (Table 2).

Of rest 16 samples, some showed PCR failure while others showed some ambiguous restriction digestion pattern. The results of these samples have been indicated as *'No Result'* in the Table 2. The analysis of these samples will be repeated soon which can answer many possible avenues which have been explained in the discussion section. Rest 16 samples showed PCR failure.

The gel pictures for Genotyping-RFLP PCR of presumed *Plasmodium vivax* in selected samples are given in the figures below (Fig. 9 - 10).

Table 2 Results of genus *Plasmodium*, species *vivax* and genotyping PCR

S.N	Sample Coding	Results for *Plasmodium vivax* Genotyping-RFLP PCR	
		Genotypes	
		VK210	VK247
1	MR001	-	-
2	MR002	+	-
3	MR003	+	-
4	MR004	+	-
5	MR005	+	-
6	MR006	+	-

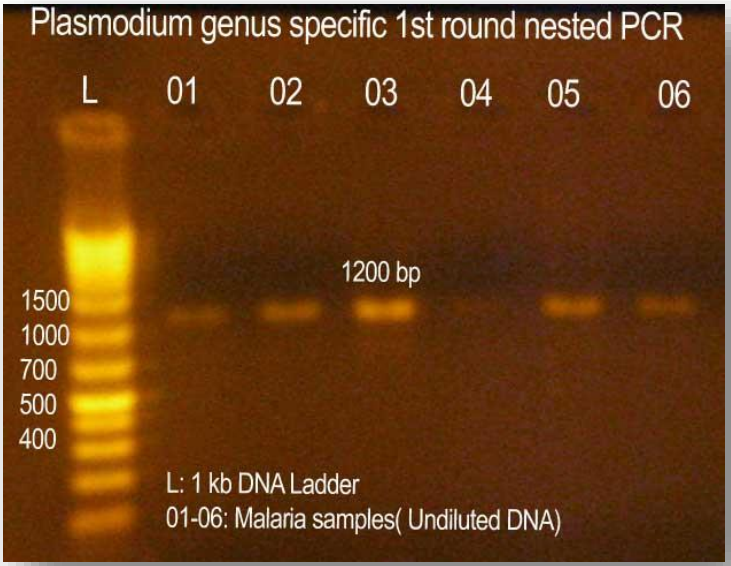
7	MR007	+	-
8	MR008	+	-
9	MR009	+	-
10	MR010	+	-
11	MR011	+	-
12	MR012	+	-
13	MR013	+	-
14	MR014	+	-
15	MR015	-	-
16	MR016	+	-
17	MR017	+	-
18	MR018	+	-
19	MR019	+	-
20	MR020	+	-
21	MR021	+	-
22	MR022	+	-
23	MR023	+	-
24	MR024	+	-
25	MR025	+	-
26	MR026	+	-
27	MR027	+	-
28	MR028	+	-
29	MR029	-	-
30	MR030	+	-
31	MR031	+	-
32	MR032	+	-
33	MR033	+	-
34	MR034	+	-
35	MR035	+	-
36	MR036	+	-
37	MR037	+	-
38	MR038	-	-
39	MR039	+	-
40	MR040	+	-
41	MR041	+	-
42	MR042	+	-
43	MR043	-	-
44	MR044	+	-
45	MR045	-	-

46	MR046	+	-
47	MR047	+	-
48	MR048	+	-
49	MR049	+	-
50	MR050	+	-
51	MR051	+	-
52	MR052	+	-
53	MR053	+	-
54	MR054	+	-
55	MR055	+	-
56	MR056	-	-
57	MR057	+	-
58	MR058	+	-
59	MR059	-	-
60	MR060	+	-
61	MR061	+	-
62	MR062	+	-
63	MR063	+	-
64	MR064	-	-
65	MR065	-	-
66	MR066	+	-
67	MR067	+	-
68	MR068	-	-
69	MR069	+	-
70	MR070	+	-
71	MR071	-	-
72	MR072	-	-
73	MR073	+	-
74	MR074	+	-
75	MR075	+	-
76	MR076	+	-
77	MR077	+	-
78	MR078	+	-
79	MR079	+	-
80	MR080	-	-
81	MR081	+	-
82	MR082	-	-
83	MR083	+	-
84	MR084	-	-

85	MR085	+	-
86	MR086	+	-
87	MR087	+	-
88	MR088	+	-
89	MR089	+	-
90	MR090	+	-
91	MR091	+	-
92	MR092	+	-
93	MR093	+	-
94	MR094	+	-
95	MR095	+	-
96	MR096	+	-
97	MR097	+	-
98	MR098	+	-
99	MR099	+	-
100	MR100	+	-

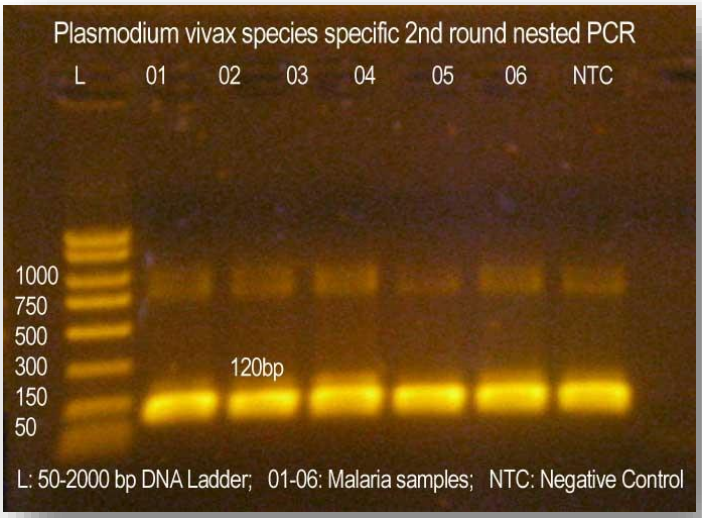
** 16 samples failed to yield PCR products*

Figure 7 Gel picture of *Plasmodium* genus specific 1 round nested PCR



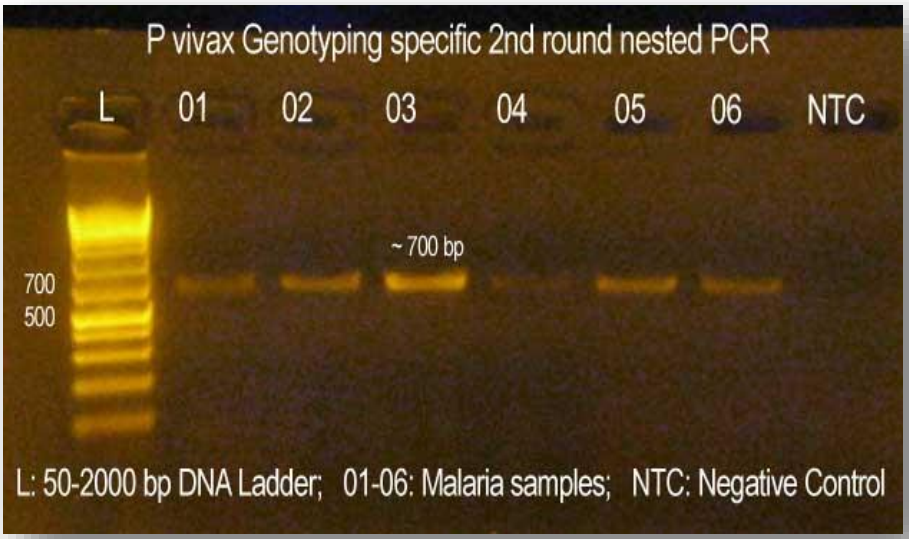
The gel picture shows the result of malaria samples MR001 to MR006 for *Plasmodium* genus specific PCR. The presence of 1200 bp PCR product in all samples shows the presence of malarial parasite *Plasmodium* sps.

Figure 8 Gel picture of *P. vivax* species specific 2nd round nested PCR



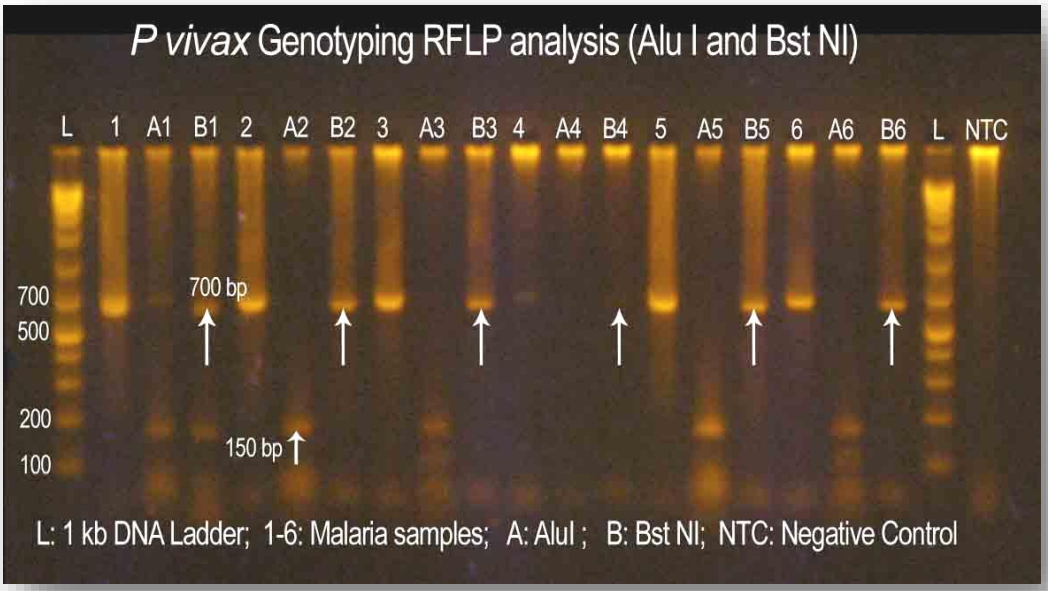
The gel picture shows the result of malaria samples MR001 to MR006 for *P. vivax* species specific PCR. The presence of 120 bp PCR product in all samples shows the presence of malarial parasite *Plasmodium* of species *vivax*.

Figure 9 Gel picture of *P. vivax* genotyping 2nd round nested PCR



The gel picture shows the result of malaria samples MR001 to MR006 for 2nd round Genotyping PCR of *P. vivax*. The presence of 700 bp PCR product in all samples shows the presence of malarial parasite *Plasmodium* of species *vivax*.

Figure 10 Gel picture of Restriction analysis (RFLP) based genotyping of *P. vivax*



The gel picture shows the result of Restriction analysis for *Plasmodium vivax* genotyping of malaria samples MR001 to MR006. 1-6 are the 2nd round genotyping PCR products (700 bp) for samples MR001 to MR006 respectively. A1-A6 are the samples digested by AluI enzyme and B1-B6 are that digested by BstNI enzyme for the same samples. All samples showed digestion by AluI but not by BstNI, indicating all of them belonging to the *Plasmodium vivax* of genotype VK210.

CHAPTER FIVE

5.1 Discussion

The study had incorporated only 137 blood samples, collected from four health institutions of Kailali and Kanchanpur district of Far-Western Development Region.

Malarial blood samples were collected from August 2010 to November 2010 and then the cases were followed-up from December 2010 to May 2011 for the period of six months. Most of the relapses were observed in between the February 2011 to May 2011. It shows that the relapse rate increased when the days get warmer and warmer. Such direct relationship supports the statement that the hot temperature is favorable for malaria agent to breed faster. A study, "Is Global warming likely to cause an increased incidence of Malaria?", reflected a fact that the malaria is greatly influence by climatic condition which furthers favor rapid breeding of mosquito population, and the incidence is likely to increase in a future warmer world.(15)

The time duration taken to relapse shows that the incubation period of the *P. vivax* may prolong for longer time. A study in India reported that the relapse can occur up to 4 years after primary attack but would be less frequent in 3rd and 4th year.(27) A similar finding was presented in the study on the *Plasmodium vivax* relapse pattern in Delhi, India represents that there were distinct incubation periods and the possible existence of *P. vivax*, characterized by primary long incubation periods.(20) The study showed that there was a differentiation of primary attack versus relapse or re-infection, particularly during the peak transmission season. It is seen that the relapse cases were recorded at the end of winter season or the start of summer season.

In our study, out of 137 cases those who were prescribed chloroquine drug during the first visit, 23 cases patients returned to the health center for the follow-up. After the blood test, it was confirmed to be relapsed. That conferred a relapse rate of 17 percent. The same study conducted in Delhi, India shows that in 1988, among 316, 176 patients did not have any relapses, whereas

140 patients had relapsed in five year follow-up study, giving a relapse rate of 44.3%. Similarly, for the years 1989–1992, the relapse rates calculated were 30.2%, 26.6%, 28.4% and 23.3%, respectively, and 29.04% for the five-year period.(20) The relapse rate seemed to be quite higher than our study suggested.

In our study, chloroquine was the drug that was prescribed to the malarial patient who visited the health institution for the first time. If the patient relapsed with the malaria, then the patient were prescribed primaquine drug. Most of the studies found that depending on the duration of follow-up of patients, five days treatment of primaquine was inadequate to prevent relapses. The relapse rates were highly variable, ranging between 2% and 30% in such condition. The study "Relapse Pattern in *P. vivax*" revealed that 70% of the patients never had a relapse after the primary infection without any primaquine treatment.(28) The WHO stresses the curability of malaria in spite of the occurrence of drug resistance. If the anti-malarial drugs are used properly and targeted to those at risk, malarial disease and death can be reduced.(11)

Genotyping

The genotyping in this study targeted on a polymorphic gene Pvcs coding for circumsporozoite protein.(26) This region is indicative of at least two major *P. vivax* types circulating in South Asia. This is also the first time that a Nepali organization has carried out the entire genotyping study in the country.

In this study on 100 samples, 84 sample showed PCR success, all of which i.e., 100% of them being of genotype VK210. Failure in PCR amplification in rest 16 samples might be because DNA of the parasite in the blood sample was either degraded or the malarial parasite in the sample was of the species other than *P. vivax*.

Our result of predominance of VK210 type closely corresponds to the identical PCR-RFLP based study on genetic diversity of *vivax* species in Kolkata, India (29) where 99.33% (150/151) of samples were of VK210 genotype while only 0.66% (1/151) sample was found to be of VK247 type. In another study done in Pakistan and Iran by similar molecular tool of PCR RFLP on *Pvcsp* gene, Pakistani samples showed predominant type of VK210 (95.7%, 179/187), while the Iranian samples showed relatively lower but yet a predominant proportion of VK210 69.3% (104/150).(30) In contrast to our study, this study reported also the presence of mixed infection (VK210 and VK247) in minority of samples i.e. 1.6% in Pakistani and 8% in Iranian samples. The similar VK210 predominance has been reported in other malaria endemic countries like in Western Thailand 77% (31), Azerbaijan 100% (32) and Brazil 86%.(33) In a study in Myanmar (34), 50% of isolates were of VK210 type and just 1% of VK247 type while 24.5% showed positive result for both types.

CHAPTER SIX

6.1 Conclusion

In conclusion, among total 137 malarial cases, 17 percent of the cases were relapsed. Majority of the relapsed cases were male (19 percent) while only 8 percent were female during the six months duration. Out of 137, male patient represented with maximum number of malaria cases with 81 percent while female were 19 percent. Age-wise category shows that 21-30 age-group people were mostly affected by the malaria.

In genotyping, cent percent of the isolates of *Plasmodium vivax* has been found to be of monotype ie. VK210 corresponding to the situation in neighboring country India and other South Asian endemic countries. However, because this study has taken only two districts into consideration, extending the study in other endemic districts of Terai belt of Nepal taking representative samples can reflect the true picture of the genetic heterogeneity of *P. vivax* in entire Nepal which can help understand the detailed malarial epidemiology in Nepal.

6.2 Challenges

After world learned that eradication and elimination of malaria is not possible, thereafter malaria control program was introduced. All these things indicate that dealing with the malaria is not an easy task. It's a very challenging task for the entire world later its infectivity and endemicity have pounced back with the global warming.

Proliferation of drug resistance malaria and roll back malaria are the major challenges. Improper use of anti-malarial drugs as well as the resistance developed by the parasites has geared up the situation even worse. The limited resources in implementing malaria control activities, massive population movements, and inadequate health services are the additional factors that favor its endemicity.

6.3 Recommendation

In Nepal, there is no such advance techniques available at present which could be used to analyze the genetic diversity of the *P. vivax* population and correlate this with epidemiological finding. Therefore, there is a strong need for laboratory and field studies as well as the use of statistical tools to interpret the complex transmission dynamics of *P. vivax* so that appropriate control strategies, including chemotherapeutic measures can be devised.

There should be increased efforts to evaluate alternative treatments for *P. vivax* strains that are resistant to chloroquine. Instigating *in vitro* culture of *P. vivax* could be one of the actions to be taken to permit the assessment of drug susceptibility. Therefore, molecular research is very important to improve understanding of the molecular mechanisms of drug resistance, and the development of better tools for genotyping *P. vivax*. Systematic reviews of malaria research should be used to guide policy.

Intensified measures to control the mosquito population including larviciding and adulticiding with pyrethroids (conventional and ULV spraying) should be implemented by the local health authorities to prevent local malaria transmission. Similarly greater attention should be given for controlling replase and re-infection side by side. A task force team should be mobilized to the malaria endemic areas of the country where there is a possibility of malaria and other tropical disease outbreak. Health promotion campaign should be massively spread throughout the malaria endemic areas and in the malaria low risk areas as well. Insecticide treated bed net should be made available to the poor class of population as well and subsidies should be given for them.

Genotyping

It is necessary to increase the genotyping resolution of Pvcs marker to discriminate further allelic variants among VK210 and VK247 genotypes by targeting the sequence variation observed in the pre- and post-repeat regions. Supplement the Pvcs gene with the commonest polymorphic markers like pvmsp1 and pvmsp3-alpha to access the genetic diversity.

The number of target loci and number of samples should be increased for greater accuracy. Gene sequencing should be done to identify exact genotype of all strains including those returning unknown results. There should be a coordinative approach with the concerned laboratory for the immediate transfer of samples for better results.

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Annex

List of Selected Health Centers

A. Kailali District

1. District Public Health Office, Dhangadi, Kailali
2. Malakheti Primary Health Care, Malakheti, Kailali
3. Tikapur Hospital, Tikapur, Kailali

B. Kanchanpur District

1. District Public Health Office, Mahendranagar
2. Beldandi Primary Health Care, Beldandi, Kanchanpur
3. Jhalari Sub-Health Post, Jhalari, Kanchanpur