MOLECULAR EPIDEMIOLOGY OF MALARIA IN NEPAL

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CONTENT

Molecular Diagnosis of Malaria in Nepal

ABSTRACT

During study period from July 2003 to December 2003, a total 676 blood samples were collected from individual malaria suspected patient attending Malaria Clinic, District Public Health Office (DPHO) and different set of camps (Mahendranagar Municipality and six different VDCs). Among the total, 302 patients attended in the camps were from five different areas (Rampur-Bilashpur, Krishanpur, Jhalari and, Daijee VDCs and Mahendranagar municipality) where the positive cases for malaria were 68, in which 27 cases were of *Plasmodium falciparum* and 41 cases were of *Plasmodium vivax.*

Among the total 374 patients attended in malaria clinic (Mahendranagar Municipality, Rampur-Bilaspur, Krishnapur, Parasan and Daije) the positive cases for malaria were 80, in which 10 cases were of *Plasmodium falciparum* and 70 cases were of *Plasmodium vivax*. Microscopic test revealed the parasitemia count ranges from 3076-42,333/µl of blood.

The total of 302 patients attended in the camp, 90 patients were selected for Rapid Diagnostic Tests (RDT, optiMAL assay). 34 (37.77%) RDT positive malaria cases in which 22 (64.70%) cases were *vivax* malaria and 12 (35.30%) were of *falciparum.* Among 90 patients 40 (44.44%) were microscopically positive for malaria in which 26 (65%) were positive for *P. vivax* and 14 (35%) were positive for *P. falciparum* However, there was no significant difference between malaria positivity by RDT and microscopic examination. The RDT was not able to detect 4 vivax cases, which was positive in the microscopy. The parasitemia level is all these cases was <10,000 /µl but in case of falciparum malaria all positive cases were identified by the RDT as positive cases which had also the parasitemia <10,000/µl of blood. The optiMAL was unable to detect 2 falciparum cases which had the parasitemia level >10,000 parasites/µl.

The sensitivity of the optiMAL when compared to the microscopy was found to be 85% and the specificity was found to be 100%. Similarly, the positive predictive value (PPV) was 100% and the negative predictive value (NPV) was 89.28%. Likewise the sensitivity,

specificity, positive predictive value (PPV) and negative predictive value (NPV) of the optiMAL test for the diagnosis of *P. vivax* was found to be 84.61%, 100%, 100% ans 77.78% respectively. Similarly, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the optiMAL test for the diagnosis of *P. falciparum* was found to be 85.71%, 100%, 100% and 92. 85% respectively.

Ten of the positive slides followed by the dipstick positive was chosen for PCR work. Five of each was positive for *P. falciparum* and *P. vivax,* on which the parasitemia was between 10,000 to 20,000/ul of blood. Template DNA was prepared from EDTA containing whole blood and filter paper strip. DNA isolated from the filter paper strip and by saponin lysis were run in 0.8 % agarose gel. During visulization DNA was conformed. PCR of saponin lysis method was inhibited probably due to high concentration of the template. Filter paper method somehow is reasonable and feeble amplified bands were observed. A Sharp band(s) was obtained in positive controls no band was obtained for any of the negative controls. The band for standard plasmodium species amplified by PLF, the forward primer, was 783-821 (Depending upon the plasmodium species) for parasitaemia above 3%/ul of blood. Genomic DNA prepared from healthy individual with no history of malaria, living in non malarious areas of the country have been included as negative controls in all PCR diagnosis assay.

During this, saponin lysis method was inhibited. So far the filter paper strip method was followed feeble bands. Saponin lysis method has to optimize by quantitating the amount of the DNA and successive dilution has to follow so that the PCR is not inhibited.

While performing the standard test, the plasmodium species identification reaction, specific reverse primer of VIR amplified *Plasmodium vivax* with the product size of 499bp and of FAR for *Plasmodium faciparum* of 395 bp. In mixed infection both of these bands will be obtained.

CAPTER: ONE

INTRODUCTION

Malaria is global health problem for half of the world's population. Every year more than 500 million people are infected with malaria and at least one million peoples are dying annually as the result of malaria (Tham J. et al, 1998). One fifth of the world's populations (40 %) are at malaria risk. More than three billion people are living in malaria endemic region. A number of factors have contributed for the global resurgence of malaria, including insecticide resistance, rapid spread of anti-malarial drug resistance and increased movement of populations secondary to increase in international travel and migration.

Malaria situation in South East Asia has not shown appreciable change in the last few years (WHO bull., 2000). Out of total population of Nepal, approximately 15.62 millions are at malaria risk (HMG, 1997) and is the fifth major infectious disease in Nepal. Malaria epidemic is the major cause for death, reduces in the agriculture productivity, hinders travel and tourism and affects external investment. Malaria is spreading in new area each year, which initially was free from this disease. Mean time malaria parasites are become increasingly resistant to commonly used anti-malarial drugs.

Study site, Kanchanpur district lies on the Far Western part of Nepal. The malarial trend in this region is forest- related malaria and the geography of district related with Stratum I of forest related malaria. (Annual Report, EDCD, HMG. 2002)

World Health Organization demands early diagnosis, high sensitivity and specificity for the detection of malaria to facilitate treatment on the time. Even though there are various laboratory methods for the diagnosis of malaria, at present Giemsa stained thick and thin blood films through microscopy is routine one. Though microscopy is gold standard, it is conventional and has subjective variation. Mean time, it is time consuming, labour intensive and accurate species identification is problematic in patients with low levels of parasitemia or those with having mixed infections. Several alternative tests like Rapid Diagnosis Test (RDT) and Polymerase Chain Reaction (PCR) are in practice. In recent year, several diagnostic kits based on antigen detection of *P. falciparium* and *P. vivax* has

been developed with various specificity and sensitivity. PCR test for diagnosis of *P. falciparum* and *P. vivax* malaria may be as specific and sensitive as other diagnostic tools in addition to microscopy. As few as one *P. falciparum* and/or *P. vivax* parasite per microliter of blood is detectable by PCR method. This method is sensitive and superior than that of thick blood film microscopy. The high sensitivity, rapidity, and simplicity of the method should make it attractive for a large-scale epidemiological study to follow-up of drug treatment, and immunization trials. It is also useful for measurement of drug sensitivity and efficacy.

This study is related with the molecular epidemiology of malaria in Nepal where for diagnostic purpose PCR was used focusing only on *P. vivax*. Microscopy, Rapid Diagnosis Test (RDT) and PCR methods were applied for the diagnosis purpose of *P. vivax*. Samples were collected from the malaria suspected patients from different VDCs of Kanchanpur including malaria clinic at zonal hospital. Verbal Consent was taken prior to sample collection and samples were collected after filling up the questionnaire and prejudging the patient's history and recent anti-malarial drugs if taken.

Giemsa stained thin and thick blood smears were examined microscopically and was compared with the Rapid Diagnosis Test which was finally followed by PCR. The observations thus obtained were documented / analyzed and comparative study was performed.

In this study, species-specific primer was used to amplify the relevant *Plasmodium spp* through Polymerase Chain Reaction (PCR). This was done with the concept that the collected samples could have possibility of mixed infection and/or low parasitemia, which may be escaped out during microscopy. Rapid Diagnostic Test Kit- was also performed to confirm it. On the other hand, the distribution and the molecular finger print of the existing strain of the malaria and the indigenous inherent species was not yet disclosed. In this regard, molecular epidemiology of malaria in the context of Nepal is an essential area to be studied.

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CHAPTER: TWO

OBJECTIVES

General Objective

Molecular epidemiological study of malaria in Nepal and Use of PCR and Standardization of PCR primers for the diagnosis of malaria in Nepal

Specific Objectives

1. Standardization of PCR primers for the diagnosis of *Plasmodium vivax* Malaria in Nepal.
2. Use of PCR method for the diagnosis and molecular epidemiology of malaria in Nepal.
3. Comparative evaluation of PCR and Microscopy in the diagnosis of vivax malaria in Nepal.

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CHAPTER: THREE

MATERIALS AND MEHODS

Study site:

Kanchanpur District, a far Western Region of Nepal

Study Duration

From July, 2003 to December, 2003

Study population:

With the permission obtained from the District Health Office, Kanchanpur a total of 676 patents enveloped. Samples were collected from individual malaria suspected patient attending Malaria Clinic, District Public Health Office (DPHO), and different sets of camps. Camps were established where Mahendranagar Municipality and six different VDCs population were participated.

Sample collection site

1. Mahendra Nagar Municipality
2. Rampur Bilashpur VDC
3. Jhalari VDC
4. Krishnapur VDC
5. Daijee VDC
6. Parasan
7. Dekhatuvali

Collections of blood samples and smear preparation:

Two milliliters of venous blood was collected in EDTA vial. Three drops of blood were placed on Whattman No. 1 filter paper for PCR. At the same time, thin and thick blood

smears were prepared for microscopy. During blood sample collection, rapid diagnostic test was performed by using OptiMAL assay (DiaMed).

Microscopic examination:

Malaria parasites were identified through 10 % Giemsa stained thick and thin blood smears under the microscope at thousand times magnification (Cheesbrough M., 2000). Smears were considered negative if no parasite was seen in 200 consecutive fields in a thick blood smear. It was also confirmed by positive control of the preserved *Plasmodium* spp positive slide.

Parasites Count:

Rapid Diagnostic Test (RDT):

Ninety samples were tested with the OptiMAL® assay. OptiMAL® dipstick was performed following the manufactures instruction. Diagnosis Kit which can detect parasitaemia levels of 100 - 200 parasites / µl of blood (corresponding to a parasitemia of 0.002 %). All whole blood samples were tested with dipstick (OptiMAL®) assay. Briefly, 1 drop of blood was mixed with 4 drops of reagent buffer in a conjugated well, and dipstick was placed vertically, the sample was allowed to migrate to the top of the OptiMAL® dipstick. After 10 min, placing it in wash well containing a droop of reagent cleared the strip. The appearance of a dark band on the strip indicates a positive reaction for any one of the four major malarial species infecting humans. The monoclonal antibody attached at this area of the strip is against an enzyme common to the four targets *Plasmodium* species. If *P. falciparum* was present in the test sample, a second band appeared on the strip. The monoclonal antibody at this site is specific for *P. falciparum* only. A mixed infection with *P. falciparum* and another Plasmodium species is indicated when both genus and species specific bands appear and the genus specific band is present with mixed infections, since all stages of the parasite are present in the blood during *P. falciparum* infection. Since the pLDH enzyme is present in all stages and since there are more stages present during *P. vivax* infections, there is strong response with the

OptiMAL® stick. A positive control band appears on each strip as an indicator that the test is working correctly.

Sample treatment and Polymerase Chain Reaction (PCR), DNA amplification:

DNA extraction:

Extraction of DNA was done described by Evengard B. *et al*, 1988 and extraction of DNA from whole blood was done as described on Methods in Malaria Research, Marth. S. *et al* 2000. Briefly, DNA was extracted from the 6 mm blood spot of filter paper incubating 2 hours at room temperature in 500 µl of PBS containing 0.05 % Tween and 0.5 % BSA and the supernatant was use for PCR.

From the whole blood the DNA was extracted by saponin lysis method. Red blood cell was pelleted at 3000 g for 2 min and was washed with PBS which was mixed with 5 % saponin so that to have final concentration of 0.05 %. Immediately, it was centrifuged at 6000 g / 5 min. The pellet was lysed on lysis buffer (40 mM Tris–HCl, 80 mM EDTA, 2 % SDS, pH 8.0 and add 0.1 mg / ml Protenase K) and was used as template DNA for PCR work..

DNA Amplification:

A semi-nested, multiplex Polymerase Chain Reaction (PCR) based on the amplification of the sequences of the 18s ribosomal RNA (ss rRNA) was adapted. In first reaction, universal reverse primer (UNR) and two forward primers (PLF specific for *Plasmodium* and HUF specific for mammals) were adopted. In second step, amplification was carried out by using forward primers PLF and two specific reverse primers FAR for *P. falciparum* and VIR for *P. vivax*.

The first reaction included primers UNR, and PLF each were expected to yield two products: a 231- base-pair (bp) band from UNR and a 753 - 821–bp band (depending on the *Plasmodium* species) from UNR and PLF that should detect the presence of any

malaria. The second amplification is a multiplex PCR that is the *Plasmodium* species identification reaction. It incorporates the products of the first reaction along with those of primers PLF, FAR and VIR. Infections with different human *Plasmodium* species yield products of different sizes. A band of 269 bp from PLF and VIR indicates a *P. vivax* infection. Mixed infections would be expected to show all the appropriate bands. (Rubio et *al*, 1999) The PCR mixture for first reaction was contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ 0.001% (W/V) gelatin, 1 % glycerol 200 µM of each dNTP, PCR primers, 2.5 U AmpliTaq DNA polymerase (Genei, India) and 41.1 µl of template DNA in a final volume of 50 µl. The concentrations of primers used were 25 p moles for UNR and PLF. In the multiplex PCR (second reaction) the reaction mixture contained 10 mM Tris-HCL (pH8.3), 50 mM KCl, 2.5 mM $MgCl_2$ 0.001 % (W/V) gelatin, 1 % glycerol 200 µM of each dNTP, the PCR primers, one units AmpliTaq DNA polymerase (Genei, India) and template DNA in a final volume of 25 µl. The concentration of primers used was empirically determined: PLF 25 p moles, FAR = 15 p moles and VIR = 2.5 p moles. The amount of template used was 2 µl of the PCR product of the first reaction diluted to a final volume of 1 ml in distilled water and 2 µl was added to the PCR. Then thermal cycler (GeneAmp 9700) was set at 94^0 C for 5 min. followed by (first reaction) 40 cycles at 94^0 C for 45 sec, 62^0 C for 45 sec, and 72^0 for 60 sec and (multiplex PCR reaction) 35 cycles at 94^0 C for 20 sec, 62^0 C for 20 sec, and 72^0 for 30 sec. The final cycle was followed by an extension time at 72^0 for 10 min. (Rubio J. *et al*, 1999)

Gel electrophoresis:

The PCR amplified products from first and second step was analysed after electrophoresis on 2% agarose gel and staining with ethidium bromide. (Ruibo J. et al, 1999)

PRIMER USED IN PCR TEST

Company	BIOBASIC INC (Canada), OLIGOSYNTHESIS		
3900 DNA SYNTHESIZE REPORT			
Primers	*Universal*	*P. falciparum*	*P. vivax*
Code	UNVERSIAL /E6349	PLF / E 6350	VIR / E 6352
Synthesis scale	0.2 Micro mol.	0.2 Micro mol.	0.2 Micro mol.
Tm	57.8	56.3	
Sequence length	20 mer	22 mer	19 mer
Molecular weight	6076	6715	5785
Sequence base	GAC ggT ATC TgA TCg TCT TC	AgT gTg TAT ATC Gag TTT C	Agg ACT TCC Aag CCg Aag C
10 OD_{260} / Tube	54.3 n mols /Tube	59.1 n mols/ ube	47.0 n mols / Tube

Note: 1 O 260 Unit = 33 μg, MW = (A X 312) + G* X 288) + (T* X 303) – 61.*

FLOW CHART

Data Collection

Blood Sample Collection

- Whole blood sample in EDTA vial
- 3 drops in different spot filter paper sample

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Dipstick Test (OptiMAL) Assay

- Pre-preparation of processing
- pLDH performed
- Species identification

↓

Microscopic Observation and Species Identification

- Thick and thin smear preparation
- Staining (Giemsa Stain)
- Observation

↓

Polymerase Chain Reaction (PCR) tests:

1. Preparation of Reagents chemicals, glassware and instrumnents
2. DNA extraction (Whole Blood and Filter paper)
3. Preparation of DNA template
4. Settling up the Thermal Cycler and running the PCR:
5. Detecting of PCR product: Preparation of Agarose Gel electrophoresis:
6. Documentation and result analysis

CHAPTER: FOUR

RESULTS

During this study period, from July 2003 to December 2004, there were total 676 participants, among which 302 patients were attended in camp from five different areas (Bilashpur, Krishanpur, Jhalari and, Daijee VDCs and Mahendranagar municipality) and 374 patients were also attended in Malaria Clinic, DPHO, Kanchanpur from five different areas (Mahendranagar Municipality, Rampurbilaspur, Krishnapur, Parasan and Daije). Microscopic exam was performed for identification of species and parasite levels. Rapid Diagnosis test was done form patient attend in camps. PCR test was done from malaria positive and negative cases from microscopic and RDT positive cases.

The results of different diagnostic test by different methods and the area-wise distribution and the species thus detected are as tabulated below.

PATIENTS ATTENDED IN CAMP FROM DIFFERENT AREAS

CASES FROM MAHENDRANAGAR MUNICIPALITY

During our study from Mahendranagar municipality total numbers of cases were 78 in which positive cases for malaria were 11 comprising 8 *Plasmodium falciparum* and 3 were *Plasmodium vivax*. Maximum numbers of positive cases were from 16-45 years and 1 case was under 5 years.

Table: 1. Distribution and species identification of malaria parasites in Mahhendranagar Municipality

Age Groups	Patients		Total Positive Cases	
	Male	Female	*P. falciparum*	*P. vivax*
0-5	4	5	1	0
6-15	7	5	0	0
16-45	37	15	6	3
46-60	1	2	1	0
61+	1	1	0	0
Total	50	28	8	3

CASES FROM RAMPUR BILASHPUR VDC

From Rampurbilashpur VDC total numbers of cases were 82 in which positive cases for malaria were 12 comprising 3 *Plasmodium falciparum* and 9 were *Plasmodium vivax*. Maximum numbers of positive cases were from 16-45 years age group and 3 cases were of under 5 years.

Table: 2. Distribution and species identification of malaria parasites in Bilshapur VDC

Age Groups	Patients		Total Positive Cases	
	Male	Female	*P. falciparum*	*P. vivax*
0-5	18	12	0	3
6-15	8	6	1	2
16-45	7	23	2	4
46-60	3	3	0	0
61+	1	1	0	0
Total	37	45	3	9

CASES FROM KRISHNAPUR VDC

From Krishnapur VDC, numbers of cases were 58 in which positive cases for malaria were 14 comprising 7 *Plasmodium falciparum* and 7 were *Plasmodium vivax*. Maximum numbers of positive cases were from 16-45 years and 2 cases were under 5 years.

Table: 3. Distribution and species identification of malaria parasites in Krishnapur VDC

Age Groups	Patients		Total Positive Cases	
	Male	Female	*P. falciparum*	*P. vivax*
0-5	2	5	2	0
6-15	6	11	0	3
16-45	15	15	4	4
46-60	1	1	0	0
61+	1	1	1	0
Total	25	33	7	7

CASES FROM JHALARI VDC

Among the total 52 patients from Jhalari VDC, the positive cases for malaria were 25, in which 7 were *Plasmodium falciparum* and 18 were *Plasmodium vivax*. Maximum numbers of positive cases were from 16-45 years and 1 case was below 5.

Table: 4. Distribution and species identification of malaria parasites in Jhalari VDC

Age Groups	Patients		Total Positive Cases	
	Male	Female	*P. falciparum*	*P. vivax*
0-5	1	1	1	0
6-15	6	4	2	3
16-45	19	17	3	14
46-60	2	1	1	0
61+	1	0	0	1
Total	29	23	7	18

CASES FROM DAIJEE VDC

Among the total 32 patients, from Daijee VDC, the positive cases for malaria were 6, in which 2 cases were of *Plasmodium falciparum* and 4 cases were of *Plasmodium vivax*. Maximum numbers of positive cases were from 16-45 years and 1 case was below 5.

Table: 5. Distribution and species identification of malaria parasites in Daijee VDC

Age Groups	Patients		Total Positive Cases	
	Male	Female	*P. falciparum*	*P. vivax*
0-5	2	1	0	1
6-15	2	3	0	0
16-45	10	7	1	3
46-60	3	2	1	0
61+	0	2	0	0
Total	17	15	2	4

PATIENTS ATTENDED IN MALARIA CLINIC, DPHO, KANCHANPUR FROM DIFFERENT AREAS

POSITIVE CASES DETECTED IN MALARIA CLINIC, DPHO KANCHANPUR

Among the total (676) the 374 patients were attended in malaria clinic, DPHO Kanchanpur in which 80 cases were malaria positive. Among the positive cases 10 cases were of *Plasmodium falciparum* and 70 cases were of *Plasmodium vivax*. The patients attended in clinic included Mahendranagar Municipality, Rampurbilaspur, Krishnapur, Parasan and Dekhatuvuli which are as tabulated below:

MAHAENDRANAGAR MUNICIPALITY

Total positive cases for malaria from this area attend in malaria clinic, DPHO Kanchanpur were 32 in which 3 cases were of *Plasmodium falciparum* and 29 cases were of *Plasmodium vivax*. Maximum numbers of positive cases were from 16-45 years and 1 case was below 5.

Table: 6. Distribution and species identification of malaria parasites in Mahendranagar Municipality

Age Groups	*Plasmodium vivax*		*Plasmodium falciparum*	
	Male	Female	Male	Female
0-5	0	1	0	0
6-15	8	3	0	1
16-45	11	6	2	0
46-60	0	0	0	0
61+	0	0	0	0
Total	19	10	2	1

RAMPURBILASHPUR VDC

Total positive cases for malaria from this area were 28 in which 3 cases were of *Plasmodium falciparum* and 25 cases were of *Plasmodium vivax*. Maximum numbers of positive cases were from 6-15 years and 8 cases were below 5.

Table: 7. Distribution and species identification of malaria parasites in Rampurbilashapur VDC

Age Group	*Plasmodium vivax*		*Plasmodium falciparum*	
	Male	Female	Male	Female
0-5	2	6	0	0
6-15	7	4	0	0
16-45	2	3	1	1
46-60	0	1	1	0
61+	0	0	0	0
Total	11	14	2	1

KRISHANPUR VDC

Total positive cases for malaria from this area were 7 in which 2 cases were of *Plasmodium falciparum* and 5 cases were of *Plasmodium vivax*. Maximum numbers of positive cases were from 16-45 years.

Table: 8. Distribution and species identification of malaria parasites in Krishnapur VDC

Age GroupS	*Plasmodium vivax*		*Plasmodium falciparum*	
	Male	Female	Male	Female
0-5	0	0	0	0
6-15	0	0	1	0
16-45	2	1	1	0
46-60	1	1	0	0
61+	0	0	0	0
Total	3	2	2	0

PARASHAN VDC

Total positive cases for malaria from this area were 8 in which 2 cases were of *Plasmodium falciparum* and 6 cases were of *Plasmodium vivax*. Maximum numbers of positive cases were from 16-45 years. Maximum numbers of positive cases were from 16-45 years.

Table: 9. Distribution and species identification of malaria parasites in Parashan VDC

Age Groups	*Plasmodium vivax*		*Plasmodium falciparum*	
	Male	Female	Male	Female
0-5	1	0	0	0
6-15	1	0	1	0
16-45	2	2	1	0
46-60	0	0	0	0
61+	0	0	0	0
Total	4	2	2	0

DEKHATVULI VDC

Total positive cases for malaria from this area were 5. All cases were of *Plasmodium vivax*. Maximum numbers of positive cases were from 16-45 years.

Table: 10. Distribution and species identification of malaria parasites in Dekhatvuli VDC

Age Groups	*Plasmodium vivax*		*Plasmodium falciparum*	
	Male	Female	Male	Female
0-5	0	1	0	0
6-15	1	0	0	0
16-45	0	2	0	0
46-60	0	0	0	0
61+	1	0	0	0
Total	2	3	0	0

TOTAL NUMBERS OF POSITIVE CASES IN DIFFERENT VDC, MAHENDRA NAGAR MUNICIPALITY AND PATIENTS ATTENDED MALARIA CLINIC OF DPHO KANCHANPUR DITRICT

Table: 11. Distribution positive and negative malaria cases and species identification those patients attended in camp and malaria clinic from different VDC and Mahendranagar Municipality.

Studied area	Negative cases		Positive cases		*Plasmodium vivax*		*Plasmodium falciparum*		Total Positive cases
	Camp	Clinic	Camp	Clinic	Camp	Clinic	Camp	Clinic	
Mahendranagar	67	294	11	32	3	29	8	3	43
Rampurbilashpur	70		12	28	9	25	3	3	40
Jhalari VDC	27		25	-	18	-	7	-	25
Krishnapur VDC	44		14	7	7	5	7	2	21
Daijee VDC	26		6	-	4	-	2	-	6
Parashan	-		-	8	-	6	-	2	8
Dekhatvuli	-		-	5	-	5	-	0	5
All total	234	294	68	80	41	70	27	10	
	528		148		111		37		148
Grand total	676								

RAPID DIAGNOSTIC TEST (RDT)

Among the total 302 patients (Bilashpur, Krishanpur, Jhalari and, Daijee VDCs and Mahendranagar municipality), 90 patients presenting fever were selected and Rapid diagnostic tests were performed then compared with microscopic. In this study, among the total 90 patients 40 (44.44%) were microscopically positive for malaria in which 26 (65%) were positive for *P. vivax* and 14 (35%) were positive for *P. falciparum* The dipsticks positive were 34 (37.77%) in which 22 (64.70%) cases were of *P. vivax* and *P. falciparum* were of 12 (35.30%). However, there was no significant difference between malaria positivity was found from microscopic examination. These results are shown in Table 12 and 13.

Table No. 12. Distribution of malaria suspected patients

S.N.	Identity	Microscopic examination	optiMAL
1	Total patients	90	90
2	Total malaria positive	40 (44.44%)	34 (37.77%)
3	Total *P. vivax*	26 (65%)	22 (64.70%)
4	Total *P. falciparum*	14 (35%)	12 (35.30%)

PARASITE COUNT

During smear analysis, the thick – thin films showed the parasitemia ranges from 3076-42,333/µl of blood. In this study, the optiMAL assay was not able to detect 4 vivax cases which was positive in the microscopy. The parasitemia level is all these cases was <10,000 /µl but in case of Falciparum malaria all positive cases were identified by the optiMAL as positive cases which had also the parasitemia <10,000/µl of blood. The optiMAL was unable to detect 2 falciparum cases which had the parasitemia level >10,000 parasites/µl. This was shown in Table 2.

Table No. 13: Parasites levels detected by microscopic examination and by OptiMAL assay

Nos. of parasites/µl of blood	Microscopic positive		OptiMAL test positive	
	P. falciparum	*P. vivax*	*P. falciparum*	*P .vivax*
1-10000	3	17	3	13
10001-20000	6	8	5	8
20001-30000	4	1	3	1
30001-40000	0	0	0	0
> 40001	1	0	1	0
Total	14	26	12	22

The sensitivity of the optiMAL when compared to the microscopy it was found to be 85% and the specificity was found to be 100%. Similarly, the positive predictive value (PPV) was 100% and the negative predictive value (NPV) was 89.28%. Likewise the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the optiMAL test for the diagnosis of *P. vivax* was found to be 84.61%, 100%, 100% ans 77.78% respectively. Similarly, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the optiMAL test for the diagnosis of *P. falciparum* was found to be 85.71%, 100%, 100% and 92. 85% respectively. The specificity and sensitivity of the optiMAL test for *P. vivax* and *P. falciparum* are shown in Table 3 (a) (b) and 3(c).

Table 14 (a): Validity of optiMAL tests (Sensitivity and specificity of the optiMAL and microscopic examination) for the diagnosis of malaria.

	Microscopy		
optimal	Malaria Positive	Malaria Negative	Total
optiMAL Positive	34	0	34
optiMAL Negative	6	50	56
Total	40	50	90

OptiMAL	
Sensitivity	85%
Specificity	100%
Positive predictive value (PPV)	100%
Negative predictive value (NPV)	89.28%

Table 3 (b): Validity of optiMAL tests (Sensitivity and specificity of the optiMAL and microscopic examination) for the diagnosis of *P. vivax*

	Microscopy		
	Positive	Negative	Total
Positive	22	0	22
Negative	4	14	18
Total	26	14	40

optiMAL	
Sensitivity	84.61%
Specificity	100%
Positive predictive value (PPV)	100%
Negative predictive value (NPV)	77.78%

Table 3 (b): Validity of optiMAL tests (Sensitivity and specificity of the optiMAL and microscopic examination) for the diagnosis of *P.faciparum*

	Microscopy		
	Positive	Negative	Total
Positive	12	0	22
Negative	2	26	28
Total	14	26	40

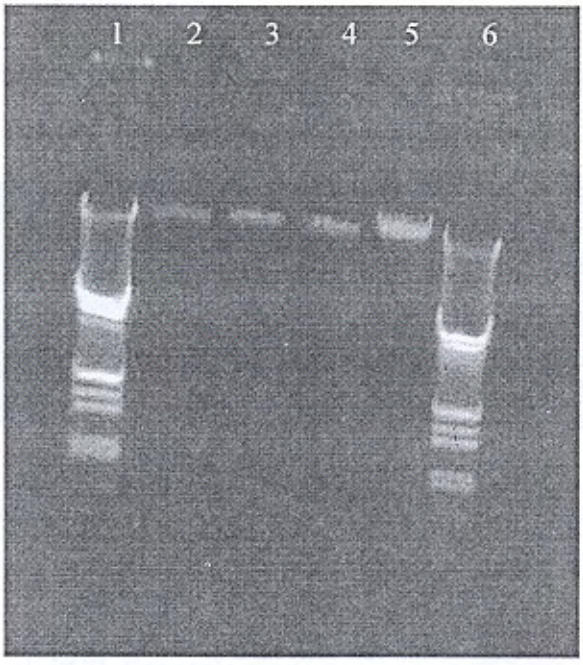
optiMAL	
Sensitivity	85.71%
Specificity	100%
Positive predictive value (PPV)	100%
Negative predictive value (NPV)	92.85%

DNA WORK

For molecular work, ten of the slide - positive samples followed by the dipstick positive was chosen. Five of each was positive for *P. falciparum* and *P. vivax,* on which the parasitemia was between 10,000 to 20,000/ul of blood. EDTA containing whole blood and filter paper strip of them was taken and followed the extraction processes to prepare template DNA.

DNA ELECTROPHORESIS

Initially isolated DNA from the filter paper strip and DNA isolated by saponin lysis were run in 0.8 % agarose gel. During visulization DNA was flourished which conform that the DNA was present in the template sample (shown in following figure).



Lane 1 & 6: Marker with base pairs 21.2kb, 5.1kb, 4.2kb, 3.5kb, 2.0kb, 1.9kb, 1.5 kb, 1.3 kb, 0.947 kb from well

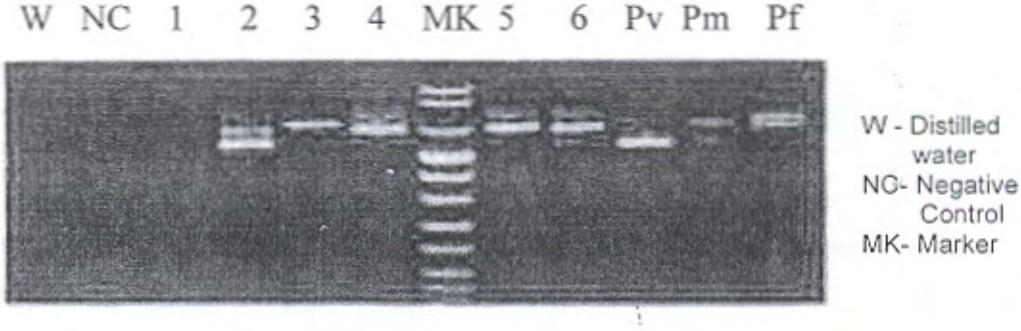
Lane 2 &3: From paper elution

Lane 4 & 5: From saponin lysis

POLYMERASE CHAIN REACTION (PCR)

THE FIRST CYCLE OF PCR

During this, the DNA extracted from the saponin lysis method was inhibited probably due to high concentration of the template. Filter paper method, somehow is reasonable and feeble amplified bands were observed. A Sharp band(s) can be seen in positive controls and samples (lanes 2 to 6), while a faint band is seen in lane 1. No band is seen for any of the negative controls. The band for standard plasmodium species amplified by PLF, the forward primer, is 783-821 (Depending upon the plasmodium species). Distinct band could be obtained when the parasitemia was above 3%.

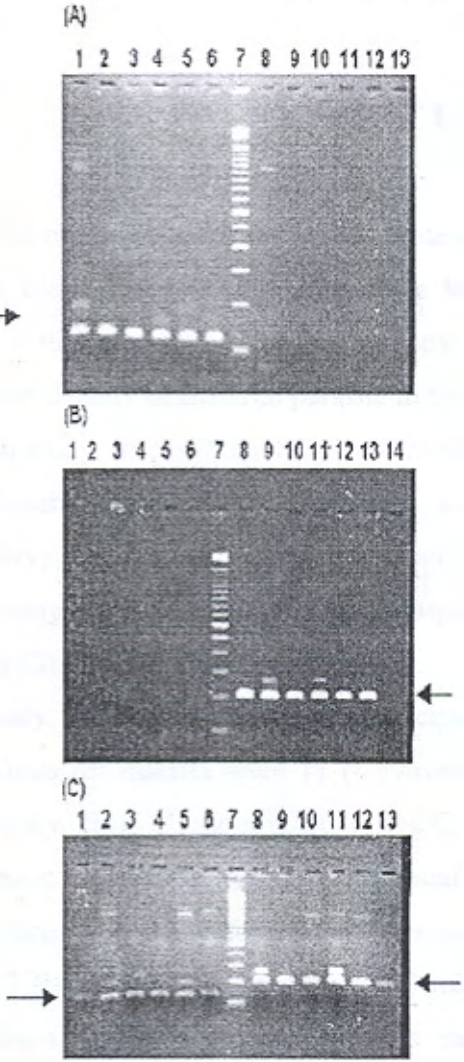


During PCR, even though the DNA was present in the extracted sample, the PCR product was not much satisfactory. Genomic DNA prepared from healthy individual with no history of malaria, living in non malarious areas of the country have been included as negative controls in all PCR diagnosis assay.

THE SECOND ROUND OF PCR:

During this, the product of the first PCR taking as template was amplified. Saponin lysis method cannot give any result. So far the filterpaper strip method was followed feeble bands were obtained. Saponin lysis method has to optimize by quantitating the amount of the DNA and successive dilution has to follow so that the PCR is not inhibited.

While performing the standard test, the plasmodium species identification reaction, specific reverse primer of VIR amplified *Plasmodium vivax* with the product size of 499bp and of FAR for *Plasmodium faciparum* of 395 bp. In mixed infection both of these bands will be obtained.



Agarose gel electrophoresis

Fig. A (*P. vivax*),
Fig. B (*P. falciparum*),
Fig. C (mix *P. vivax* & *P. falciparum*),
Marker is 100 bp ladder

A. The representative microscopically *P. vivax* diagnosed samples, which were positive by using *P. vivax*-specific primers (left panel), and negative by *P. falciparum*-specific primers (right panel).

B. The representative microscopically *P. falciparum* diagnosed samples, which were positive by using *P. falciparum*-specific primers (right panel), and negative by *P. vivax*-specific primers (left panel).

C. Demonstrates the representative samples diagnosed by species-specific primers as mix *P. falciparum* and *P. vivax*. Lane 5 and 2 has mixed infection.

CHAPTER: FIVE

DISCUSSION

The method of detection of malaria parasite is the examination of Giemsa stained thick and thick blood smears. This method is labor-intensive and time consuming. It is, however, well documented that microscopy has limitations, its sensitivity decrease in parallel with density of malarial parasite in the blood.

During our study, there were total 676 respondents, in whom 302 were from five different areas (Bilashpur, Krishanpur, Jhalari and, Daijee VDCs and Mahendranagar municipality) were attended in camp and 374 were also from five different areas (Mahendranagar Municipality, Rampurbilaspur, Krishnapur, Parasan and Daije) attended in Malaria Clinic, DPHO, Kanchanpur.

In this study, from Mahendranagar municipality (total numbers of cases 78) in which positive cases for malaria were 11 (8 *Plasmodium falciparum* and 3 were *Plasmodium vivax)*. Likely, from Rampurbilashpur VDC total numbers of cases were 82 in which positive cases for malaria were 12 (3 *Plasmodium falciparum* and 9 *Plasmodium vivax)*. From Krishnapur VDC, numbers of cases were 58 in which positive cases for malaria were 14 (7 *Plasmodium falciparum* and 7 were *Plasmodium vivax)*. Among the total 52 patients from Jhalari VDC, the positive cases for malaria were 25 (7 *Plasmodium falciparum* and 18 *Plasmodium vivax)*. Likely, from Daijee VDC 32 patients were involved, on which the positive cases for malaria were 6 (2 *Plasmodium falciparum* and 4 *Plasmodium vivax)*.

Among the total (676 patients) the 374 were attended in malaria clinic, DPHO Kanchanpur in which 80 cases were malaria positive. The patients attended in clinic included Mahendranagar Municipality, Rampurbilaspur, Krishnapur, Parasan and Dekhatuvuli.

Here, during our study both in the field camp and in the Malaria Clinic, DPHO, Kanchanpur, the most infected populations were 16-45 years and child below 5 years

were also positive for both *P. falciparum* and *P. vivax*, so it is very remarkable result and give proper insight to study malaria infection in the age group below 5 years.

The method of detection of malaria parasite is the examination of Giemsa stained thick and thick blood smears. This method is labor-intensive and time consuming. It is, however, well documented that microscopy has limitations, its sensitivity decrease in parallel with density of malarial parasite in the blood.

Out of total population of Nepal, approximately 15.62 millions are at malaria risk (3). At present the parasite is routinely detected by microscopy of Giemsa stained thick and thin blood films. This technique is cheap and easy to performed allow quantification but it has detection limit about 20 parasite per micro liter a substantial number of patients with low level of parasitemia may be missed by this technique (4).

For the confirmatory and rapidity of the test procedure for that selected area 90 samples were reandomly choosen and OptimaL test was performed. The results obtained from the optiMAL tests were compared with those of the traditional Giemsa stained thin and thick smears. Among the studied 90 samples, the microscopy could detect 40 (44.44%) malaria cases in which 26 (65%) were *P. vivax* and 14 (35%) were *P. falciparum*. The optiMAL assay successfully detected 34 (37.77%) malaria cases among which 22 (64.70%) were vivax and 12 (35.30%) were falciparum. optiMAL assay show the sensitivity of 85% and the specificity of 100% with positive predictive value (PPV) of 100% and the negative predictive value (NPV) of 89.28%. The sensitivity, specificity, PPV and NPV of optiMAL test for the detection and identification of *P. vivax* was found to be 84.61%,100%,100% and 77.78% respectively whereas for *P. falciparum* it was found to be 85.71%, 100%, 100% and 92.85% respectively. OptimaL test can be used in the diagnosis of malaria, in the differentiation of the plasmodium species and to monitor the responses of the antimalarial treatment even in peripheral setup without experienced microscopists. But there are different companies for the distribution of the dipstick and different researcher use different of them. It is really very necessary to do a trail for all the possible kit that are in practice and should give a demarcation for the best one. Parallel study of microscopy and rapid test give proper insight for the study of malaria. In our study, rapid test in field and microscopy in the center (Central Department of Microbiology, Tribhuvan University) provide a concise picture of the comparative study

and proper insight for detection of level of parasitemia. It is very suggestive, that if parallel study in field can operate a prompt suggestion can be set for national regimen. Rapid test method is simple, does not require highly equipped facilities, and in most cases enables differentiation of 4 parasites causing malaria in humans (*P. falciparum, P.vivax .P. ovale P malariae*) when performed by a skilled microscopist. However microscopy is often time consuming and labour intensive and it is estimated that even a skilled person can evaluate only 30-40 specimens per day under field conditions (8). This method can sometimes be misleading in identifying parasite species, specially in the case of a low level of parasitemia, a mixed parasite infection, or modification by drug treatment, but microscopy still remains as the "Gold standard "for the diagnosis of Malaria *(5,12)*. An easily perfomed, rapid and accurate test for the detection of plasmodial infections is needed in laborious lacking trained miscroscopists not only where malaria is endemic but also where it is not endemic. Such a test could facilitate the early diagnosis and an appropriate therapy in patients with malarias, thereby reducing mortality (2,13). Due to the above listed limitations of the microscopy, alternate methods for the diagnosis of malaria are required. The aims of the current study was to compare the performance of optiMAL, an immunochromatographic antigen detection assay for the diagnons of malaria using parasite specific lactate dehydrogenase (pLDH) with standard microscopy in patients with suspected Malaria . The optiMAL assay is used to differentiate both the *P. falciparum* and the *P. vivax*. The OptimaL test has thus special significance in a country like Nepal where most of the rural health centers are unable to diagnose malaria due to the lack of microscopes, microscopic slides, stain and trained technicians. The malaria is endemic in Nepal in the rural villages, mostly far distant from the Highway. Due to the poor economic condition, the distance from health centers, Lack of Transportation facility, the patients can not go to the health center thus, increasing mortality. The follow up is also very difficult in the malaria patients. Therefore the prompt diagnoses and treatment in a must for the rural malarias patients. The cost of the new non-microscopical optiMAL test is expensive than the microscopy to diagnose malarias. In the many places, where microscopy is not possible and malaria is diagnosed presumptively from clinical symptoms (shown repeatedly to be unreliable), the savings in drugs costs more than offset the cost of optiMAL test,particularly in areas of drug

resistance where expensive second line anti-malarial drugs may need to be used. Besides the financial savings from unnecessary treatments, the use of optiMAL tests is of value in the early investigations and management of malarial epidemics.

Polymeraze chain reaction (PCR) was done for the 10 selected positive samples comprising 5 of each positive cases for *P. falciparum* and *P. vivax,* on which the parasitemia was between 10,000 to 20,000/ul of blood.

In diagnostic specimens especially those from patients diagnosed by microscopy as having possible mixed infections, and those demonstrating low parasitemia or that those are parasite negative.

In these years low parasitemia, multiple infection of malarial parasite and multi drug resistance are problems of malaria treatments. In this regards, PCR is the best possible detection phenomena. But in our national context, not much work has done and the selection of the primer and optimization has no clear picture. This study, probably, is the first try. For this, primers of single set, the product of BIOBASIC, Canada for *Plasmodium vivax* and *Plasmodium falciparum* was used for optimization. Multiplex PCR with two steps were performed by taking negative control, positive control and the template DNA from fiter-paper strip and saponin lysis.

Various studies in different country shows PCR are more sensitive for the diagnosis of malaria in molecular biology. It is suggested to standardize PCR primer in own country Nepal and whether it is useful or not for the diagnosis of malaria in Nepal, In this issue molecular epidemiology of malaria in Nepal is an essential subject to be studies.

Plasmodium falciparum and *P. vivax* are the major cause of malaria morbidity and mortality in the world Biologic and antigenic diversity is a characteristic of this parasite and infections can consist of several genetically diverse parasites (6). A highly sensitive, rapid and simple method PCR to detect human malaria in blood samples was developed (5). The polymerize chain reaction (PCR) was employed for detection and strain identification of faliciparum in a comparative field study of Indian isolate. The primer were selected from highly conserved regions flanking the variable, tandemly repeated regions of highly polymorphic surface of antigens, major merozoite surface antigen-1 (MSP-1), major surface antigen-2 (MSP-2) circumsporozoite surface antigen (CSP) and

ring infected erythrocyte surface antigen (RESA). There is a specific pattern for each strain and this could be utilized to identify a particular field isolate (11). PCR based method was used as the reference standard due to its established sensitivity an specificity and its advantages over microscopy, particularly in cases with low level parasitemia (1). Detection and identification of the parasites, solely by electrophoretic analysis of the PCR products, has proven to be more sensitive and accurate than by routine diagnostic microscopy (9).

A high proportion of mixed species infections were brought to light by the PCR assay. Malaria parasite DNA in blood from a finger prick was directly amplified by the polymerize chain reaction (PCR) using two sets of primers to yield a 206-basepair (bp) product for *Plasmodium falciparum* and a 183-bp product for *P. vivax*. Both were easily visualized in an ethidium bromide-stained agarose gel, allowing identification of the two human malaria species in a single amplification reaction. As little as a one *P. falciparum* and / or *P. vivax* parasite per micro liter of blood was detectable by this method, a sensitivity superior to that of thick blood film microscopy. The high sensitivity, rapidity, and simplicity of the method should make it attractive for a large-scale epidemiology study, follow-up of drug treatment, and immunization trials (6).

Seventeen pairs of published primer sets were compared for their relative sensitivity to detect malaria DNA extracted from blood samples, which were obtained from Pakistani patient suffering from malaria. The primer sets investigated consisted of : (i) 9 pairs of direct primers an 3 sets of nested primers for detecting *Plasmodium falciparum*, (ii) 2 pairs of direct primers and sets of nested primers for detecting *P. vivax*, and (iii) 1 set of multiplex primers for detection both *P. falciparum* and *P. vivax*, simultaneously. After a miniscreen of 9 DNA-extracted blood samples using the 17 primer sets stated above, 5 primer sets were short-listed (based on their superior sensitivity) and used for a maxi-screen of DNA extracted from 126 microscopy-positive blood samples from Pakistan (8). The PCR assay using the genus-specific oligonucleotide primer set (pg PCR) was able to detect 52.4% infection and showed a sensitivity of 100% and specificity 100% when compared Microscopy (7).

The ability to detect the presence parasite during the course of treatment demonstrated by PCR amplification it has been observed that PCR-positive result obtained only when the

DNA was extracted from sample containing live parasites. Dead parasites cleared by drug treatment of immune system pressure did not register as positive by PCR amplification.(4) The sensitivity of the PCR varied significantly ($P<00.001$) in three survey villages (between 63.2% and 83.9% for the primer pair K1-14-1 and between 37.9% and 69.9% for the primer pair MSP-1) and was highly linked to geographic difference and social exchanges of the inhabitants with other area of the district. It is advisable not to use a single primer pair epidemiological field studies for the detection of falciparum malaria. The use of combine primer pairs and the frequent confirmation of the results by microscopic are recommended (10).

Regarding this, in our research, though result somehow is appreciable elaborate work should more be conducted. Various primer for same species should more be conducted on the samples of various geographical location ranges from Hilly region, forest fringe to the low land of the Terai. This study in fact set the procedure and give ideas for the elaborated study in this aspect.

CHAPTER: SIX

CONCLUSSION

During this study period, there were 676 respondents. While setting five camps in different areas (Bilashpur, Krishanpur, Jhalari and, Daijee VDCs and Mahendranagar municipality) 302 samples were collected and 374 were from five different areas (Mahendranagar Municipality, Rampurbilaspur, Krishnapur, Parasan and Daije) attended in Malaria Clinic, DPHO, Kanchanpur.

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RECOMMENDATION

1. Microscopy in combination of RDT should be done in other malaria endemic region of Nepal
2. Comparative evaluation of the different diagnostic kit should be standardized in the context of Nepal
3. Extensive study of the malaria as molecular epidemiology should be conducted for the determination of low parasitaemia and multiple infections
4. HRD should be done in governmental level for the early diagnosis and prompt treatment of malaria in molecular level

CHAPTER: SEVEN

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ANNEXES

Data Collection Instruments including Questionnaires

Questionnaires

Serial No: **Lab Code No:**

Patients Name: Age / Sex:

Address:

Clinical Notes:

Fever:	Continuous	[ ]	Intermittent	[ ]
Chills and Rigors		[ ]	Nausea / Malaise	[ ]
Sweating		[ ]	Headache	[ ]
Anemia		[ ]	Muscular Pain	[ ]
Splenomegali		[ ]	Haepatomegali	[ ]

Other Clinical Data:

1. .
2. .
3. .

PHOTOGRAPHS

1: SAMPLE COLLECTION BY VEIN PUNCTURE

2: VIEW OF FORESTED AREA NEAR RESIDENCS

3. PADDY FIELD NEAR RESIDENCS AREA

4. ENVIRONMENTAL CONDITION INSIDE THE HOUSE

5. CAMP SET ON VDC AND DATA COLLECTION

6. ENVIRONMENTAL CONDITION ARROUND RESIDENCS

7. TWO SISTERS LIVING (BUHARI) TOGETHER: ONE WAS SUFFERED FORM *P. vivax* AND NEXT FROM *P. falciparum*

DISTRICT : KANCHANPUR

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