Sulfadoxine-pyrimethamine resistant molecular markers in *Plasmodium falciparum* from Eastern and Central Nepal

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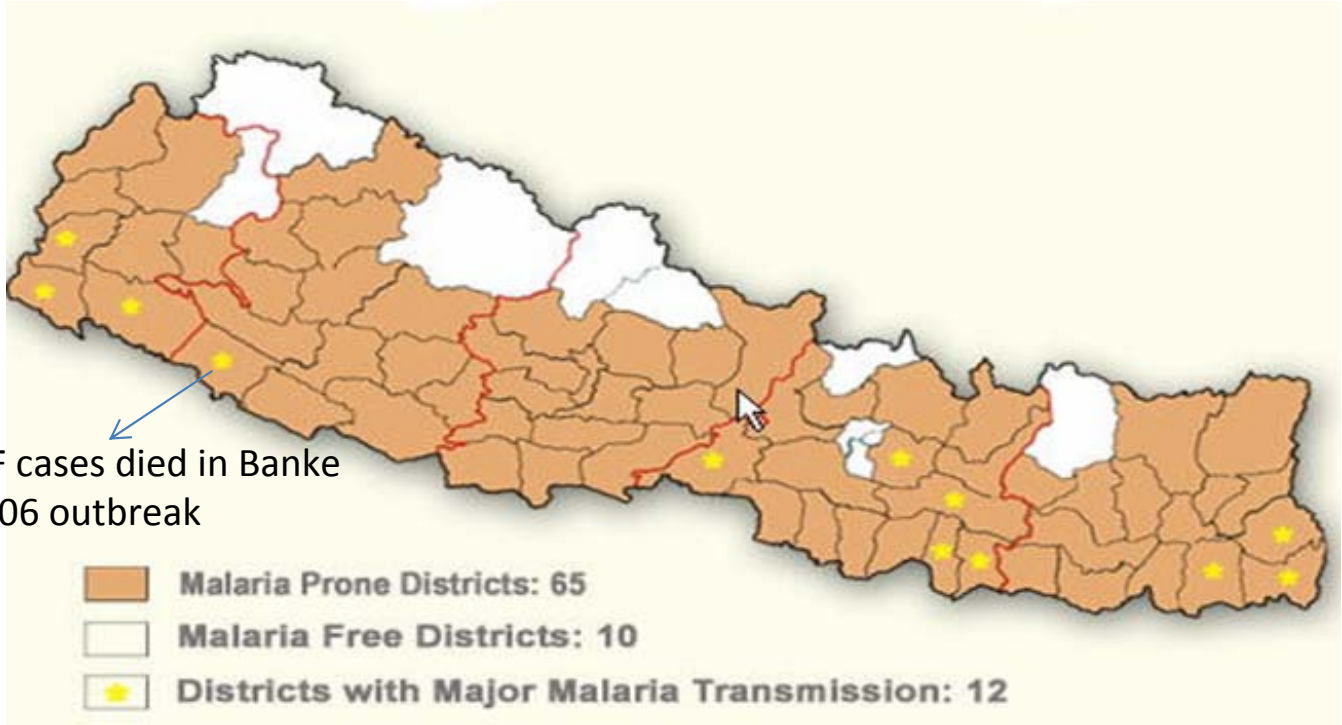
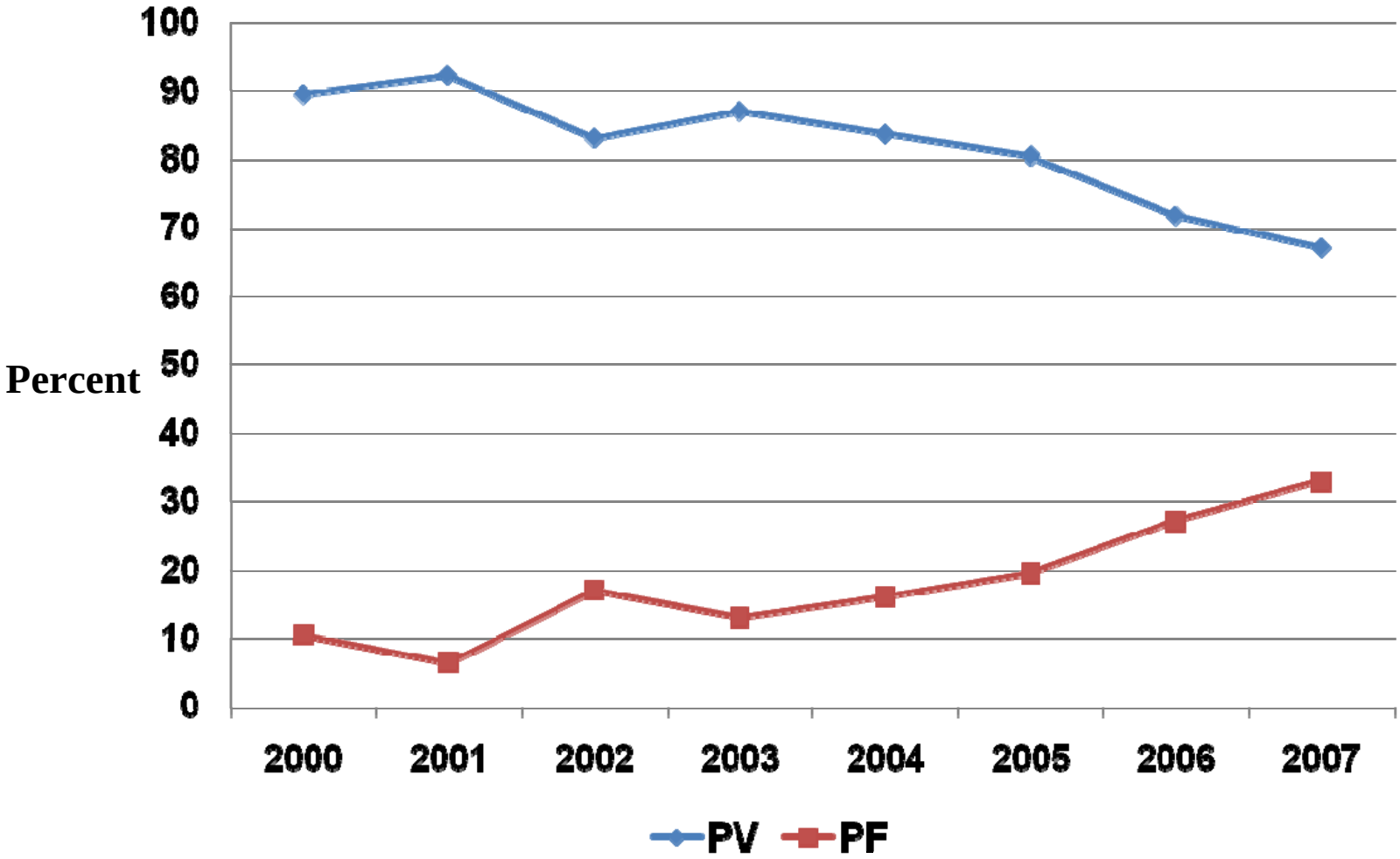
Malaria in Nepal

- Annual malaria cases : 8,000-10,000

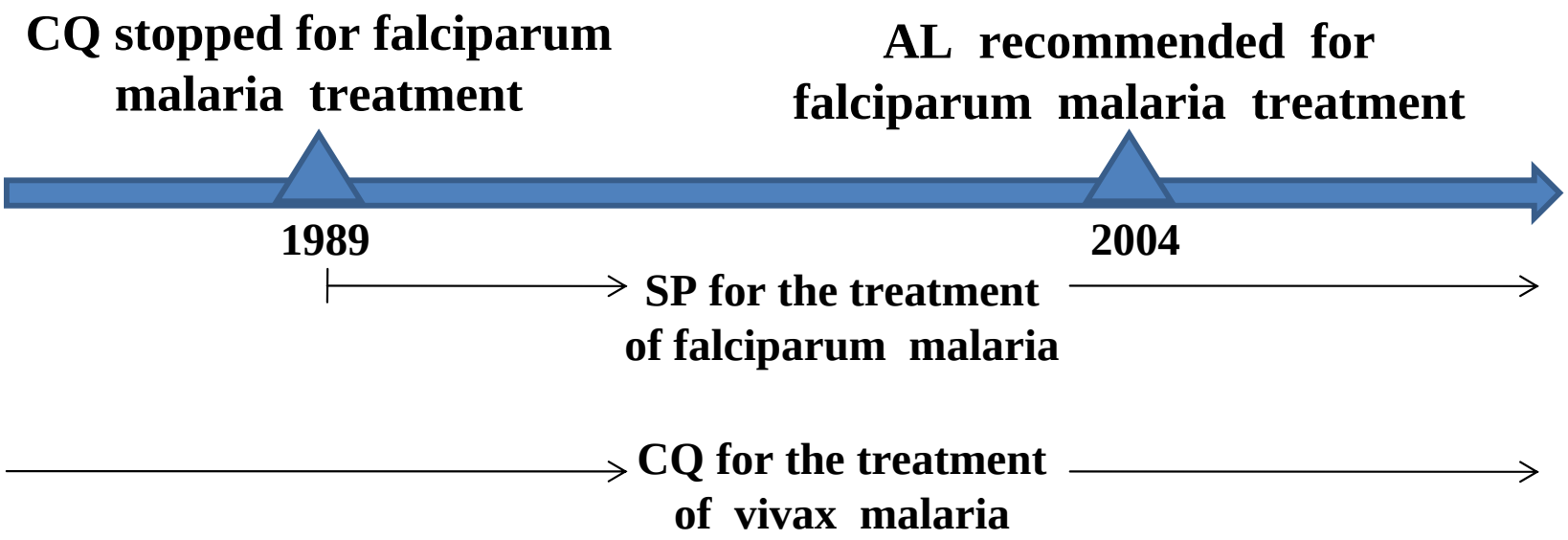
- *Plasmodium vivax* : 80%
  P. falciparum :15-20%



- *P. falciparum* cases are increasing over the recent years



Antimalarials used in Nepal



Rationale

Two *in vivo* drug efficacy studies in the Eastern region:

Thapa et al., 2007
Wijeyaratne et al., 2005

} Contrasting results

Malaria cases:

In Eastern region- importation and local transmission

In Central region- considered indigenous

No study in the Central region

Rationale

SP for treatment - > 20 years

Limited information on:

- molecular markers to assess drug resistance
- population structure of *P. falciparum* in Nepal

Hypothesis

Difference in prevalence of molecular markers of *P. falciparum* resistant to SP in the Eastern and Central Nepal

Objective

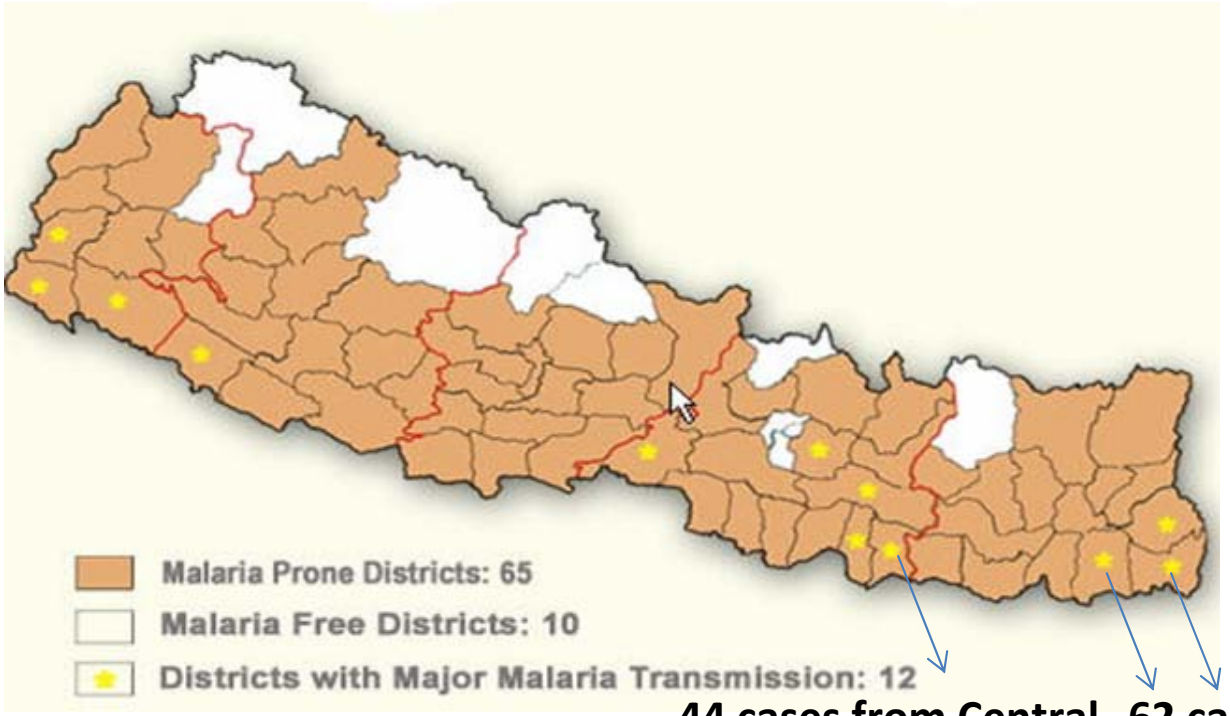
To determine the prevalence of SP resistant molecular markers of *P. falciparum* from the Eastern and Central region of Nepal

Methods

Cross-sectional comparative study

Health facility based study:

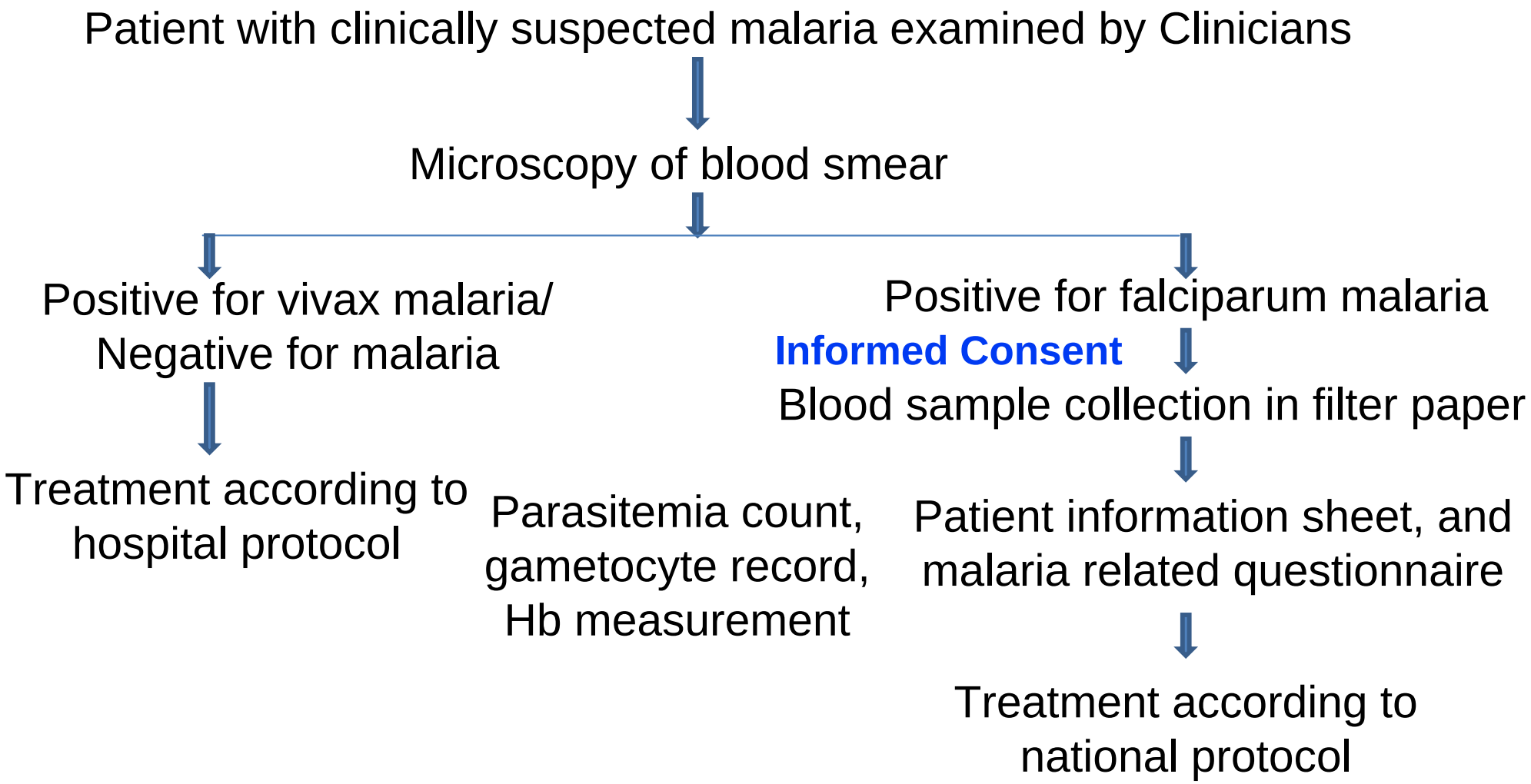
- Jhapa and Morang - Eastern region
- Dhanusha - Central region



44 cases from Central Nepal: Dhanusha

62 cases from Eastern Nepal: Jhapa, Morang

Patient Selection and Sample Collection



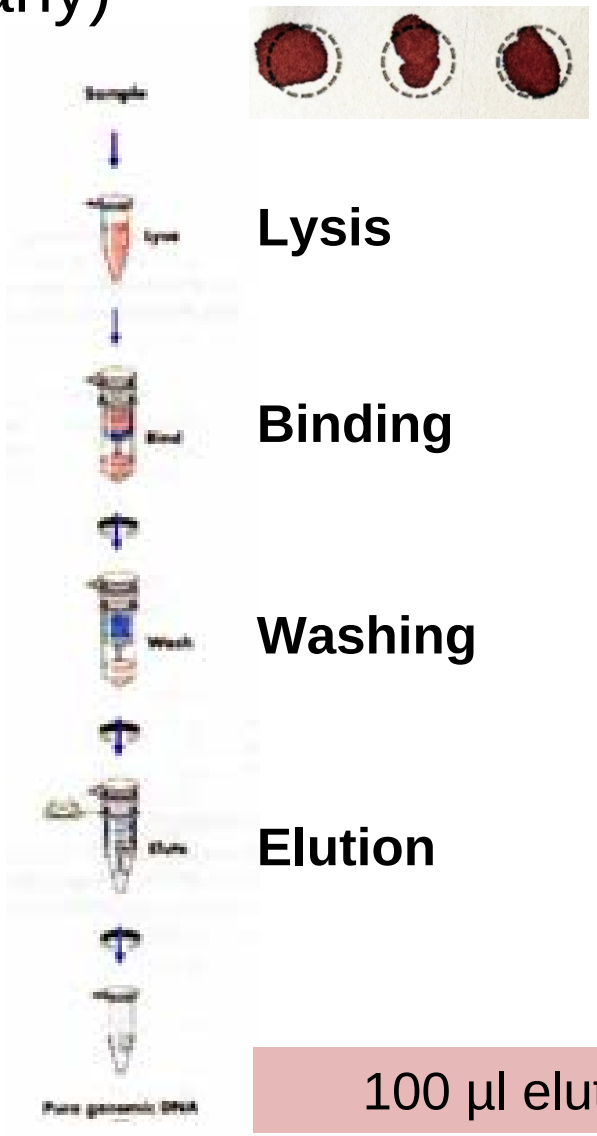
Protocol review and ethical approval:

EC Faculty of Tropical Medicine, Mahidol University

IRB Institute of Medicine, Tribhuvan University

DNA Extraction

DNA extracted using QIAamp DNA Mini Kit (QIAGEN, Hilden Germany)



Blood spot on filter paper

Lysis

Binding

Washing

Elution

100 µl elute → Storage at -70^{0}C

PCR of *dhfr* and *dhps*

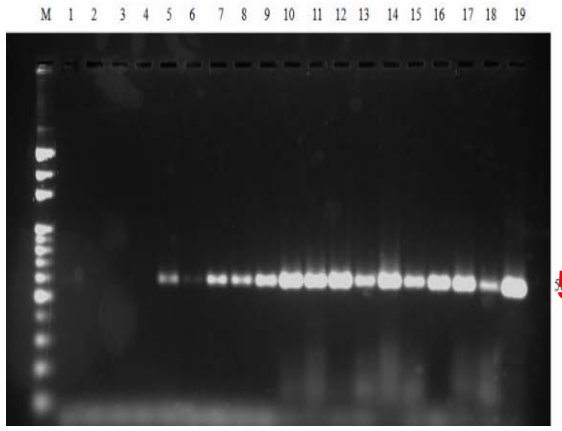
Primers (Nair *et al*., 2002)

dhfr

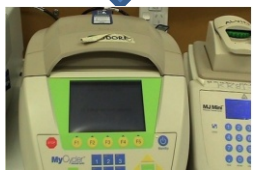
dhps

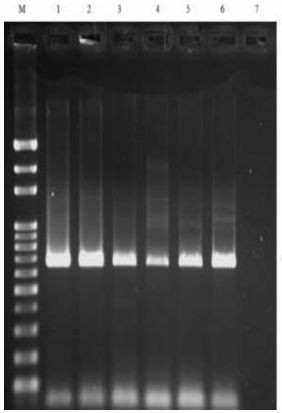
Primers - 0.3 µM each
KAPPA HiFi GC rich buffer with 2mM Mg^{2+}-1X
dNTPs- 0.3 mM
HiFi Taq polymerase – 0.5U
DNA template- 5 µl
Total volume- 50 µl

Initial denaturation- 95°C (5 min.), 98°C (3 min.)
Annealing- 57°C (15 sec.)
Extension- 72°C (30 sec.)
Final extension- 72°C (1 min.)
Cycles- 40



562 bp





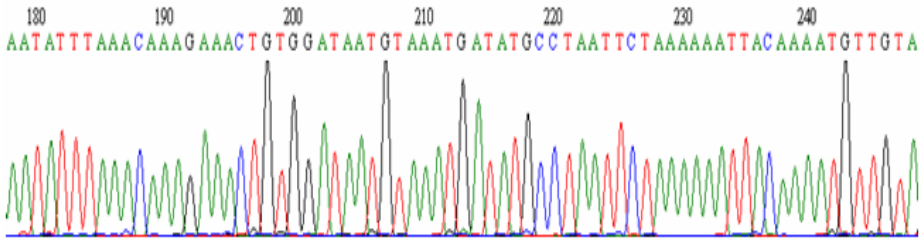
672 bp

Gel electrophoresis
1.5% gel

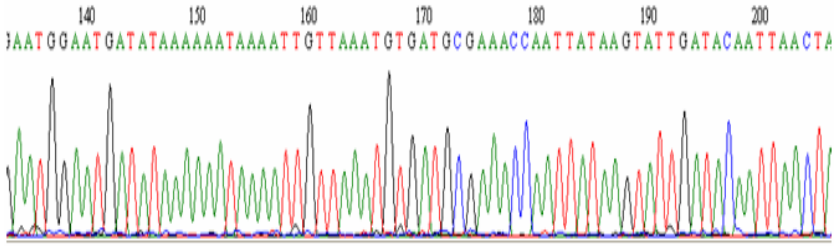
Purification of PCR product
using Nucleospin Extract II Protocol (Macherey-Nagel)

Sequencing and Identification of Mutations

Direct sequencing
ABI 3100 Genetic Analyzer



dhfr

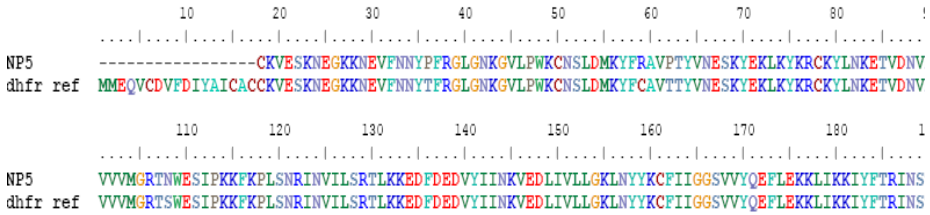


dhps

ExPASY DNA Translate Tool

Amino acid sequences

Alignment with reference by Bio-edit software



Dhfr: 51, 59, 108, 164

Dhps: 436, 437, 540, 581

Data analysis

SPSS version 11.5 software
Chi-square and Fisher Exact Test

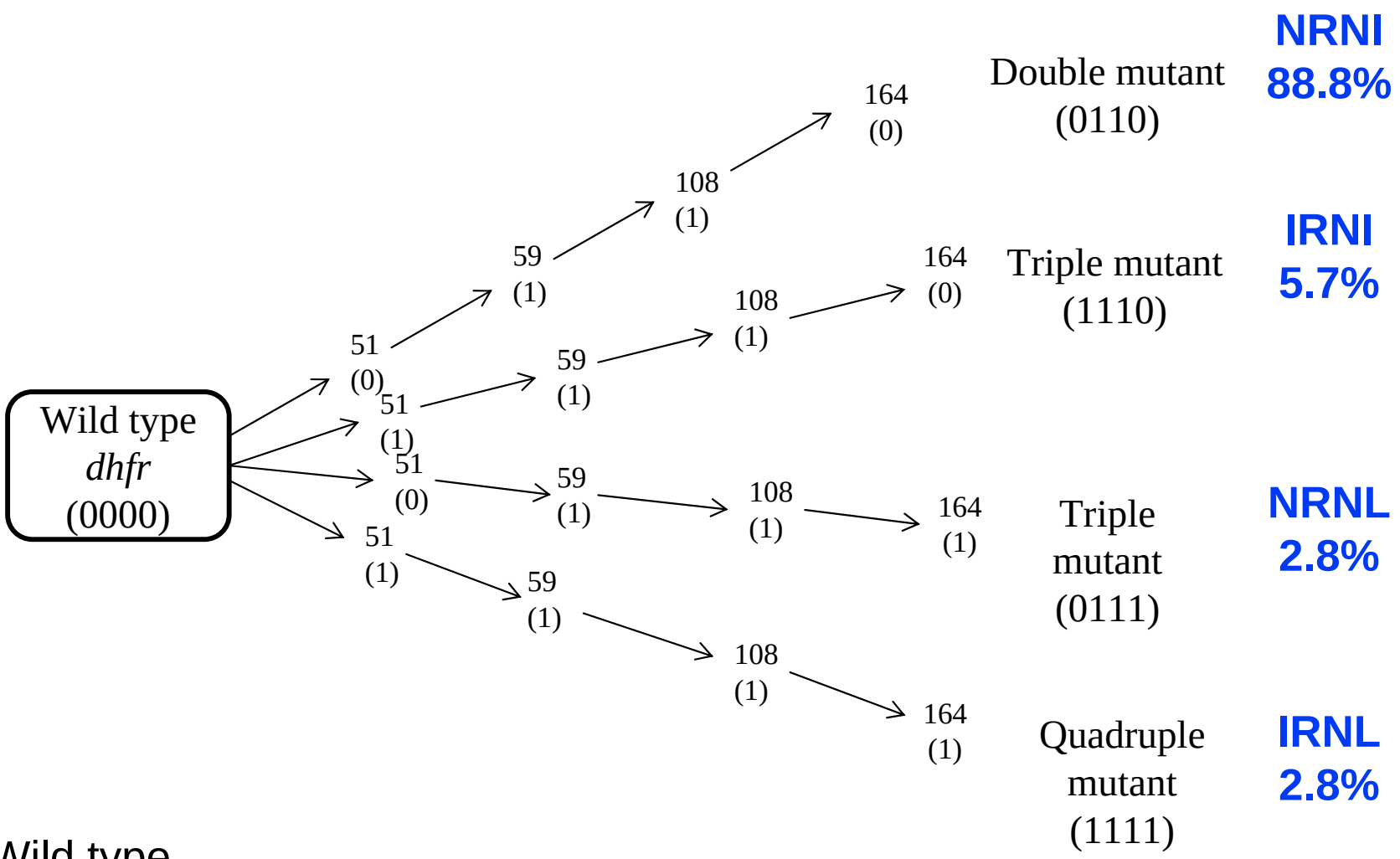
Results and Discussion

Mutations at *dhfr*

Positions	Types	Eastern region	Central region	p-value
51 (n=70)	Wild type-N	30 (90.9)	34(91.9)	1.000*
	Mutant type-I	3 (9.1)	3 (8.1)	
59 (n=72)	Wild type-C	0 (0.0)	0 (0.0)	-
	Mutant type-R	33 (100.0)	39 (100.0)	
108 (n=72)	Wild type-S	0 (0.0)	0 (0.0)	-
	Mutant type-N	33 (100.0)	39 (100.0)	
164 (n=72)	Wild type-I	29 (87.9)	39 (100.0)	0.040*
	Mutant type-L	4 (12.1)	0 (0.0)	

Mutations at 108 and 59 positions- fixed and saturated

Dhfr Mutant Alleles Found in Nepal



0- Wild type
1- Mutant type

Triple and quadruple mutants still low although SP used >20 years

Regional Distributions of *dhps* Genotypes

Genotypes	No. of mutations	Eastern region (n=31)	Central region (n=35)	Total (n=66)
SAKAA(wild type)	0	7 (22.6)	1 (2.9)	8 (12.1)
S**G**KAA	1	9 (29.0)	33 (94.2)	42 (63.6)
S**G**K**G**A	2	5 (16.1)	0 (0.0)	5 (7.6)
S**GE**AA	2	0 (0.0)	1 (2.9)	1 (1.5)
AGEAA	3	10 (32.3)	0 (0.0)	10 (15.2)

Mutation at 437 position is high in Central region, but other mutations are very low or none.

Distribution of *dhfr-dhps* Two Locus Genotypes

Genotypes *Dhfr-dhps*	No. of mutations	Eastern region (n=30)	Central region (n=34)	Total (n=64)
NRNI-SAKAA	2 (2, 0)	7 (23.3)	0 (0.0)	7 (10.9)
IRNI-SAKAA	3 (3, 0)	0 (0.0)	1 (2.9)	1 (1.6)
NRNI-SGKAA	3 (2, 1)	8 (26.7)	30 (88.2)	38 (59.4)
NRNI-SGEAA	4 (2, 2)	0 (0.0)	1 (2.9)	1 (1.6)
NRNI-SGKGA	4 (2, 2)	5 (16.7)	0 (0.0)	5 (7.8)
IRNI-SGKAA	4 (3, 1)	0 (0.0)	2 (5.8)	2 (3.1)
NRNI-AGEAA	5 (2, 3)	6 (20.0)	0 (0.0)	6 (9.4)
NRNL-AGEAA	6 (3, 3)	2 (6.7)	0 (0.0)	2 (3.1)
IRNL-AGEAA	7 (4, 3)	2 (6.7)	0 (0.0)	2 (3.1)

Mutations Associated with SP Resistance

Mutations	Eastern region n (%)	Central region n (%)	Total n (%)	p-value
Triple mutations in *dhps* (436A+437G+540E)	10/31 (32.3)	0/35 (0.0)	10 (15.2)	<0.001
Triple mutations in *dhfr* (51I+59R+108N)	1/33 (3.0)	3/37 (8.1)	4 (5.7)	-
Quadruple mutations in *dhfr* (51I+59R+108N+164L)	2/33 (6.1)	0/37 (0.0)	2 (2.8)	0.219
Quadruple mutation (triple *dhfr*+*dhps* 437G)	2/30 (6.7)	1/34 (2.9)	3 (4.7)	0.596
Quintuple mutation (triple *dhfr*+double *dhps*)	2/30 (6.7)	0/34 (0.0)	2 (3.1)	0.216

Conclusion

Regional bias in mutations:

- *Dhfr* double (S108N+C59R) mutation- fixed and saturated in both the regions
- *Dhfr* triple mutation – relatively low in both the regions
- *Dhfr* quadruple mutation – only in Eastern region

- A437G mutation at *dhps*- high in both regions
- Mutations at other positions of *dhps*- low or none in Central region

Regionwise different antimalarial policy can be used

- Quintuple (*dhfr+dhps)* mutation – only in Eastern region but not in Central region
- AL drug in Eastern region for falciparum malaria treatment

Acknowledgements

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THANK YOU