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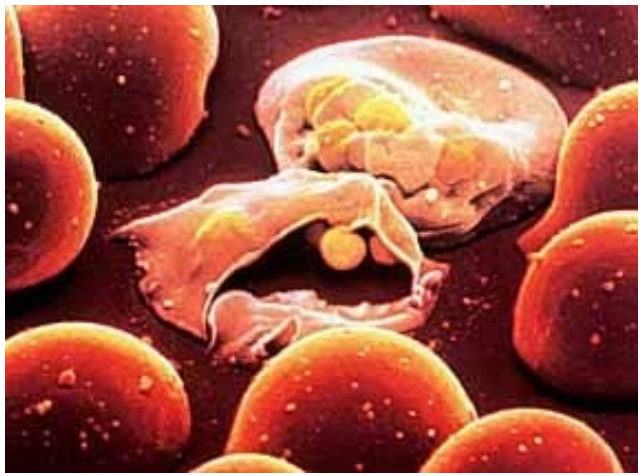
Nucleotide polymorphisms of *pfcrt* gene in Thai isolates of *Plasmodium falciparum*



Setthaudom Ch.[1], Tan-Ariya P.[1], Mungthin M.[2]

[1]Department of Microbiology, Faculty of Science, Mahidol University
[2]Department of Parasitology, Phramongkutklao College of Medicine

Malaria situation

- 300-500 million people of world's population suffer from malaria.
- 1-2 million children under than 5 years old die from malaria annually.

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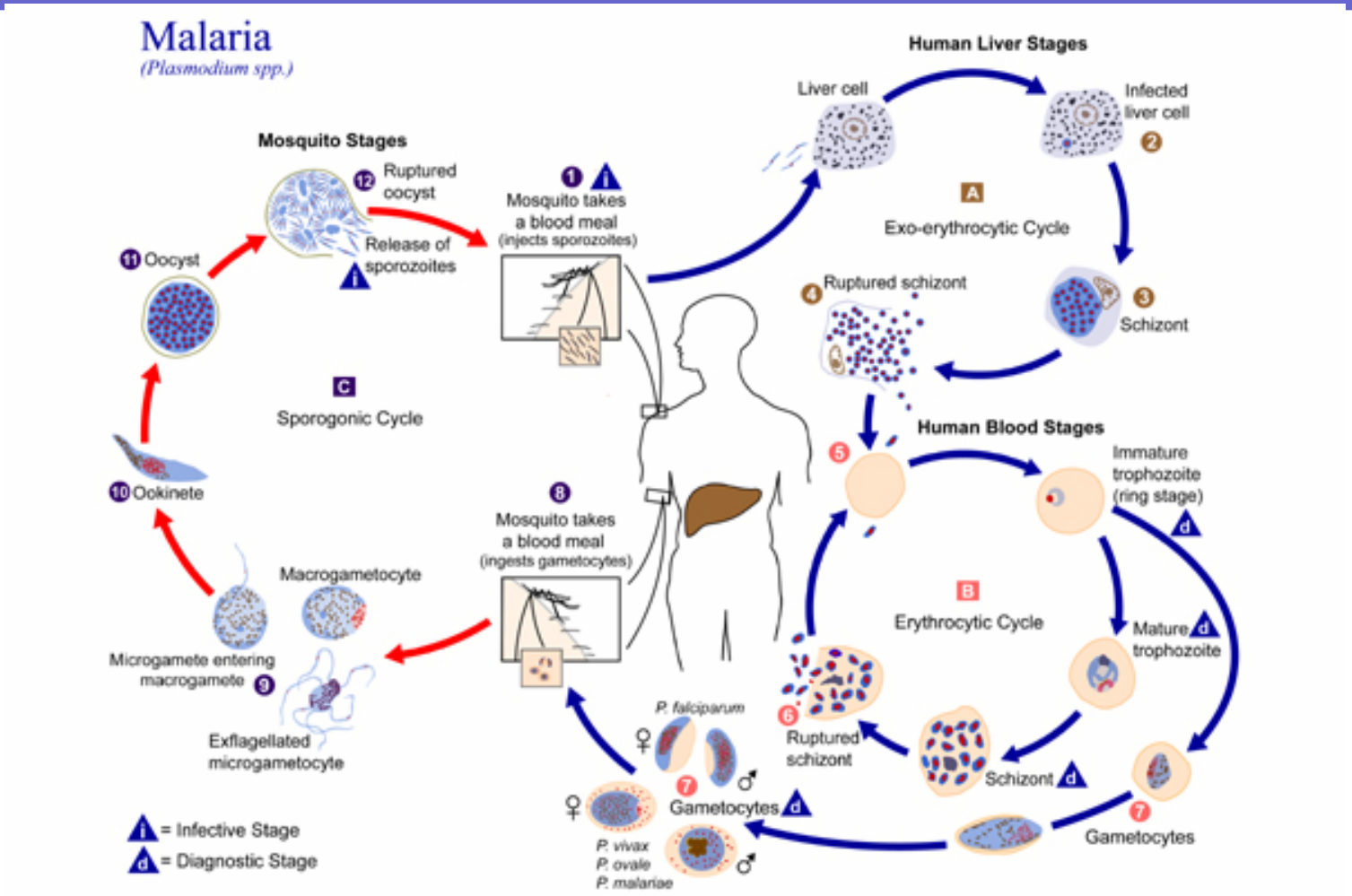
Plasmodium falciparum



http://www.wmin.ac.uk/biosciences/images/Malaria.jpg

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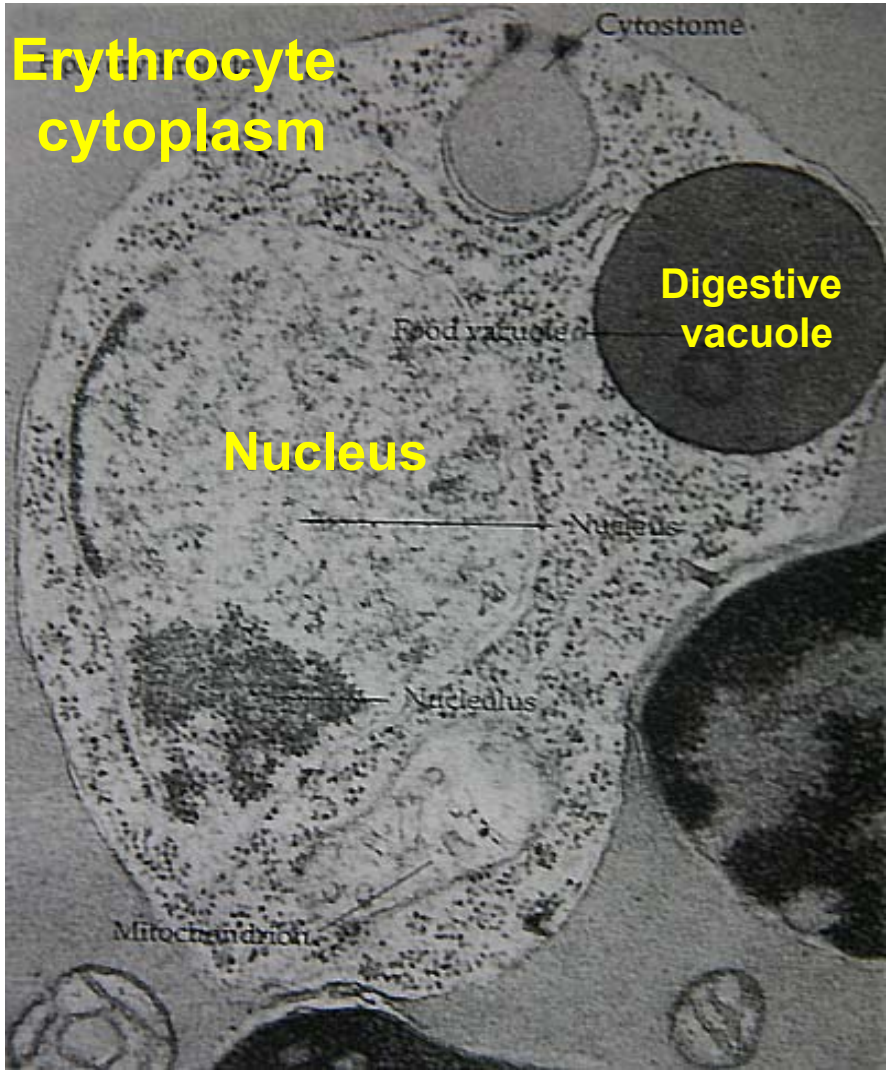
Asexual erythrocytic stage of *Plasmodium falciparum*



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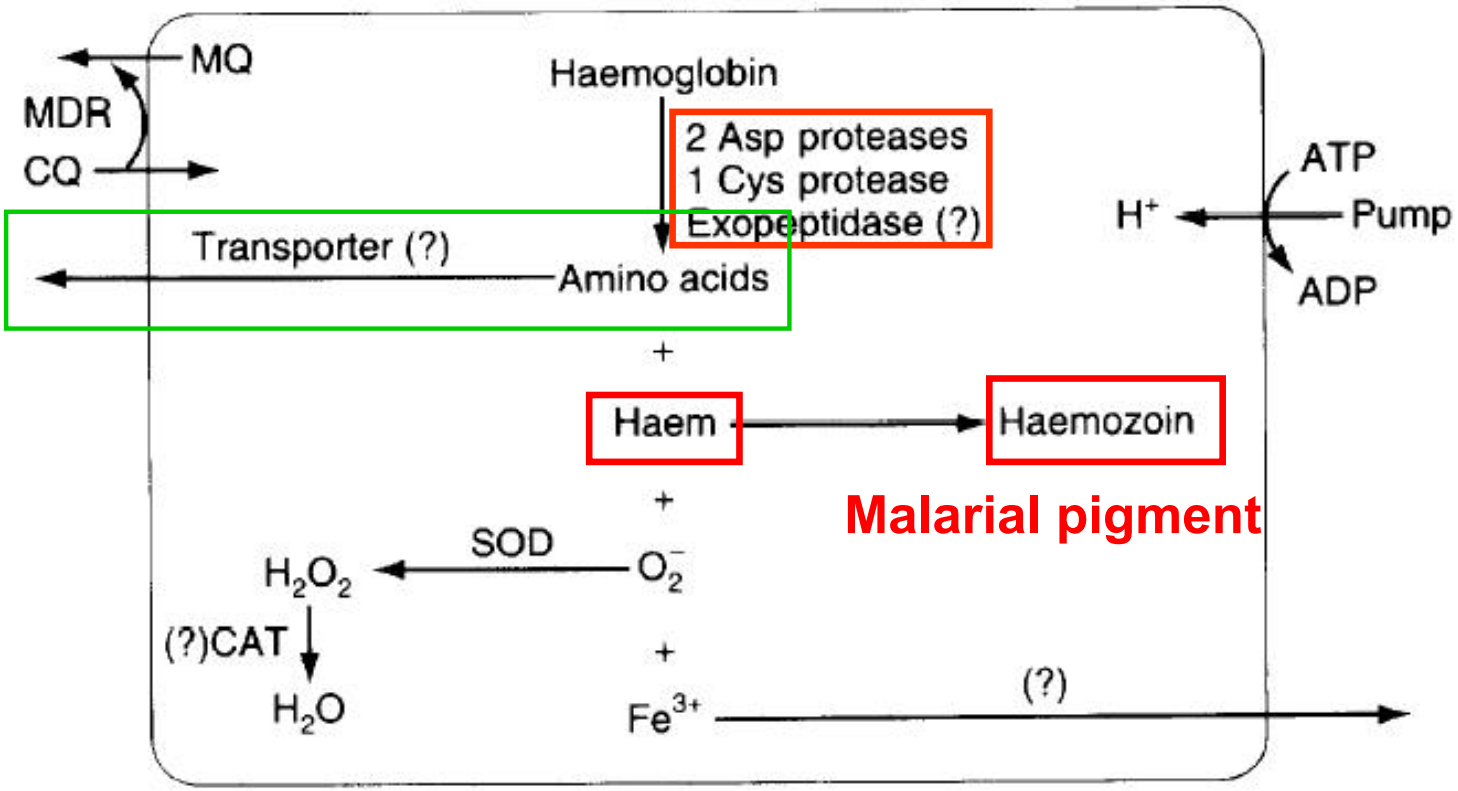
Plasmodium Digestive Vacuole



P.L. Olliaro and D.E. Goldberg, 1995

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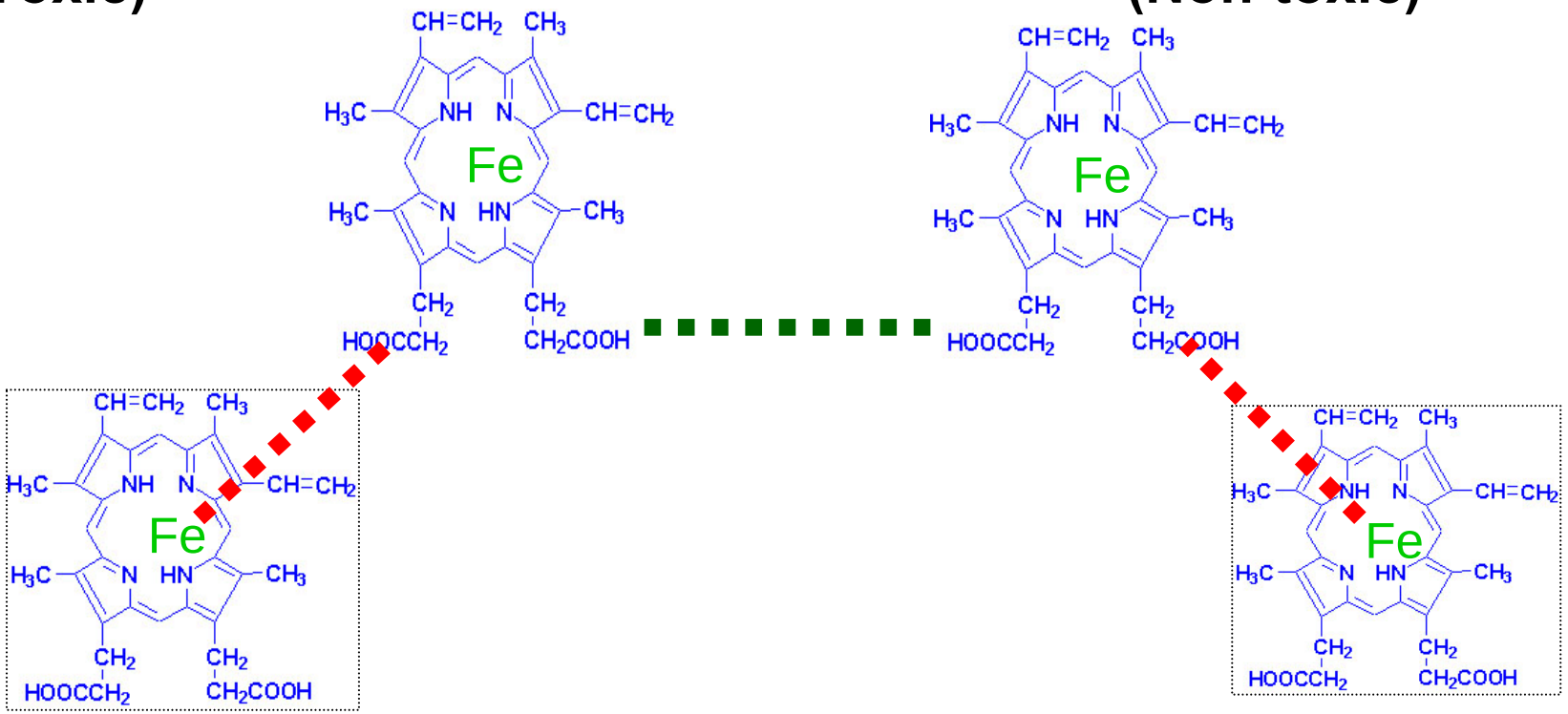
Detoxification of Ferriprotoporphyrin (FP)

Ferriprotoporphyrin (Toxic) → **β-hematin** → **Hemozoin** (Non-toxic)



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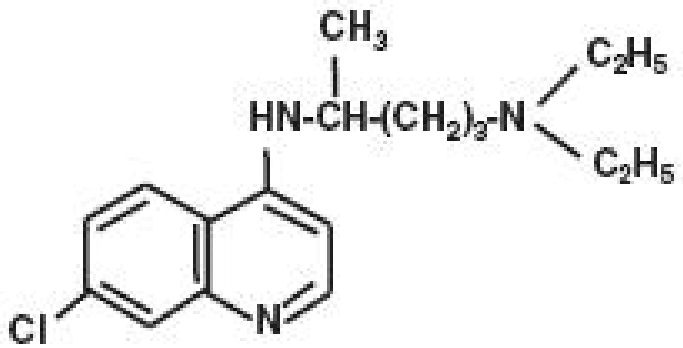
Chloroquine

- A schizontocidal 4-aminoquinoline
- Rapid action, Low cost, Low incidence of side effects
- It is still effective against *P. vivax, P. malariae, P. ovale* and CQ-sensitive *P. falciparum.*



Chloroquine

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Action of Chloroquine in digestive vacuole (DV)

- CQ is a weak base, accumulated as CQ^{2+} in DV of the parasite up to 1000-fold higher than in the cytoplasm.
- CQ inhibits heam detoxification by binding to ferriprotoporphyrin. The FP-CQ complex will increase membrane permeability of digestive vacuole and cause death of parasite.

CQR *Plasmodium falciparum*

- CQR parasites showed a markedly reduced concentration of CQ in their digestive vacuoles.

Fidock et al. Mol Cell, 2000

- The putative transporter PfCRT was identified through the analysis of genetic cross between a chloroquine sensitive (CQS) and chloroquine resistant (CQR) clones.

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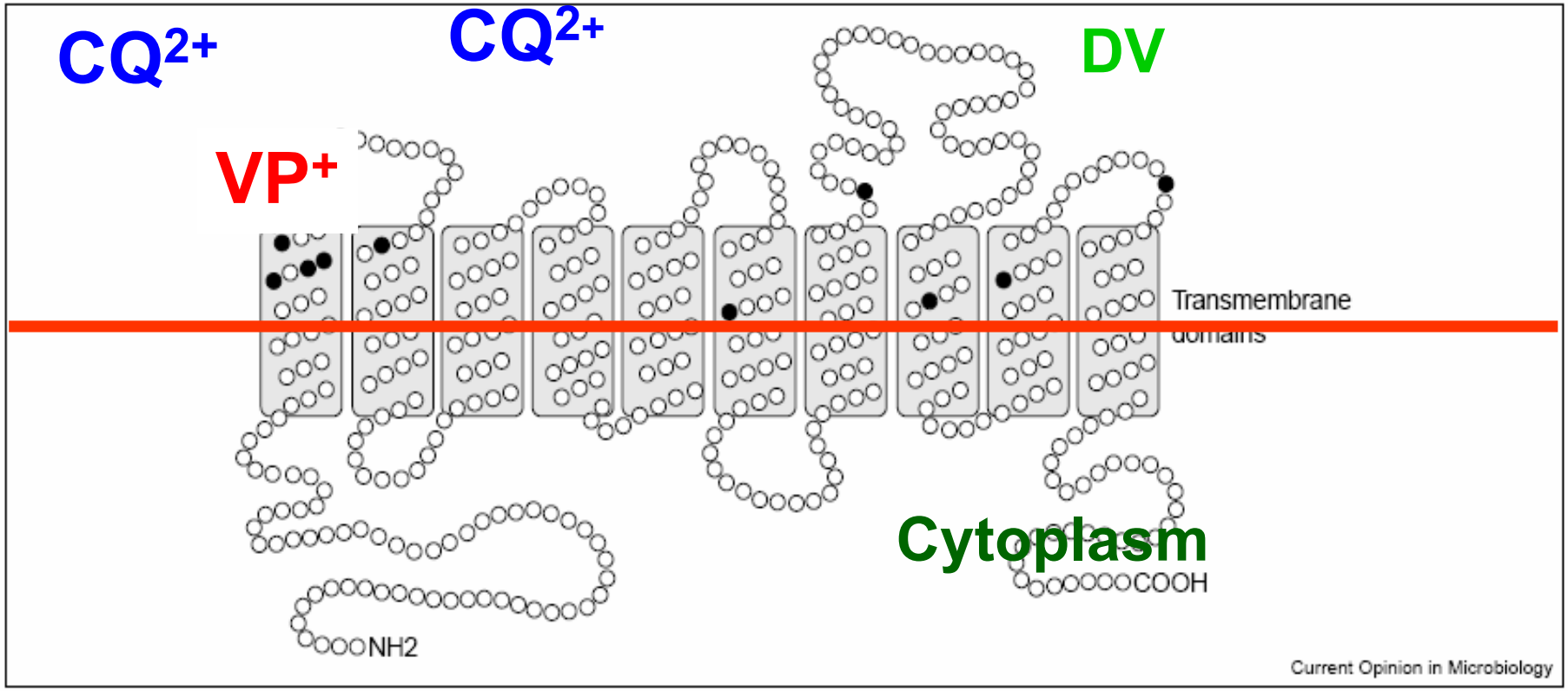
pfcrt is a major gene of CQR

- This gene encodes 45 kDa peptide which contains 10 predicted transmembrane domains, localized on DV membrane.

K76T mutation in PfCRT

- Point mutation at K76T in PfCRT protein is a key of CQ resistance. This mutation is called
  “Charge loss mutation”

Charge loss mutation of *PfCRT*



The schematic structure of the protein product of the *pfcrt* gene, PfCRT, showing the ten predicted transmembrane domains. The positions of all of the different mutations identified from the analysis of forty geographically diverse isolates from the Eastern and Western hemispheres are indicated by filled circles. The K (lysine) → T (threonine) change at position 76 (indicated by the arrow) is critical to CQ resistance in *P. falciparum*.

Calton et al., 2001

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K76T is major mutation in PfCRT (Cooper *et al*., 2005)

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Geographic distribution	Parasite clone/isolate	PfCRT amino acid positions										
		72	74	75	76	77	97	144	148	152	160	163
CQS parasites												
Honduras	HB3[a]	C	M	N	K	I	H	A	L	T	L	S
CQR parasites Africa												
Mali	S35CQ[d]	C	I	E	T		H	A	L	T	L	S
South Africa	RB8[a]	C	I	E	T	I	H	A	L	T	L	S
Southeast Asia												
Thailand	Dd2[a]	C	I	E	T	I	H	A	L	T	L	S
Thailand	TM93-C1088[f,g]	C	I	E	T		L	A			L	
Cambodia	783[h]	C	I	E	T		H	A	L			
Cambodia	738[h]	C	I	D	T		H	A	I			
Indonesia												
Lombok	Field isolate[j]	C	M	N	N							
South America												
Ecuador	Ecu1110[a]	C	M	N	T	I	H	A	L	T	L	S
Colombia	Jav[a]	C	M	E	T	I	Q	A	L	T	L	S
Brazil	7G8[a]	S	M	N	T	I	H	A	L	T	L	S

“Charged drug leak” hypothesis

- From the “Charge loss mutation” to the “Charged drug leak” hypothesis

K1
CQ IC50= 101nM

CQR

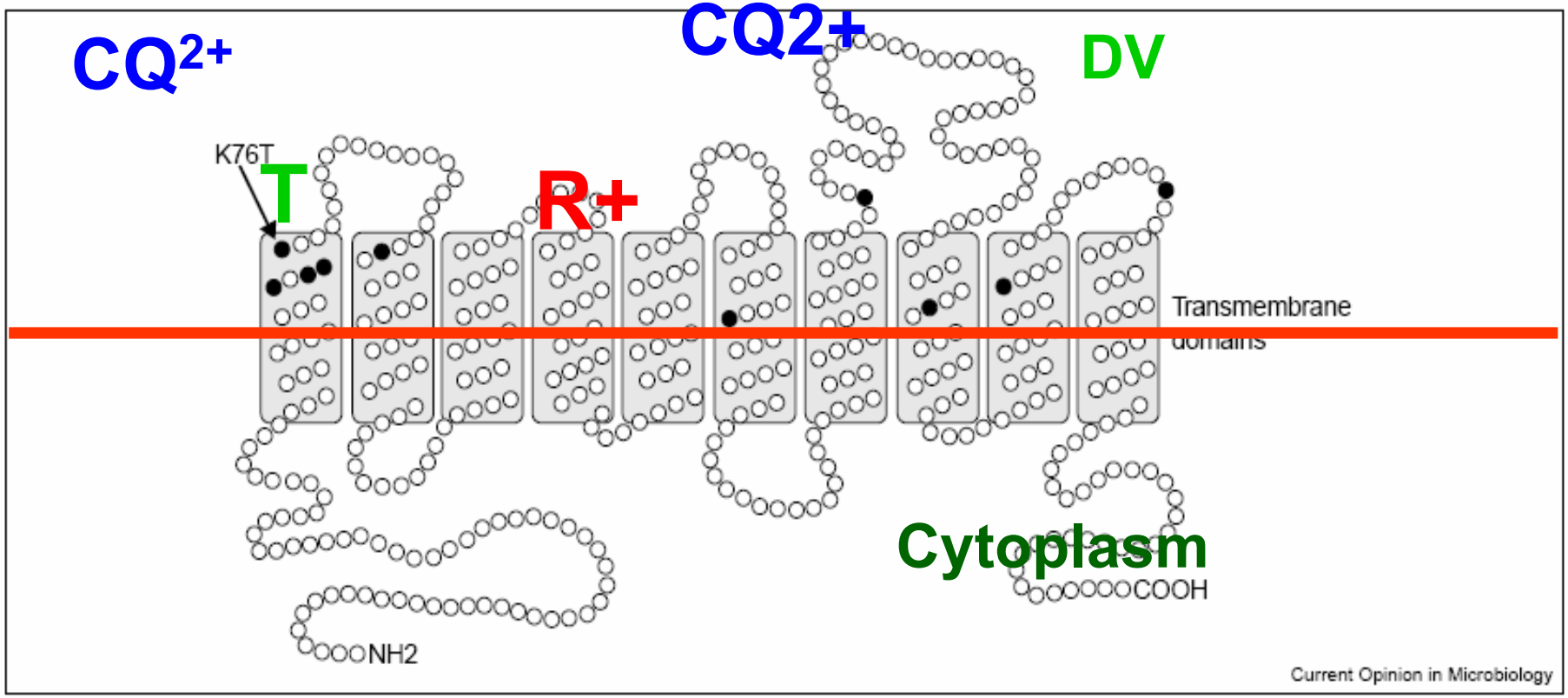
→

K1 AM
CQ IC50= 26nM

K1HF
CQ IC50=38nM

CQS

“Charged drug leak” hypothesis



The schematic structure of the protein product of the *pfcrt* gene, PfCRT, showing the ten predicted transmembrane domains. The positions of all of the different mutations identified from the analysis of forty geographically diverse isolates from the Eastern and Western hemispheres are indicated by filled circles. The K (lysine) → T (threonine) change at position 76 (indicated by the arrow) is critical to CQ resistance in *P. falciparum*.

Calton et al., 2001

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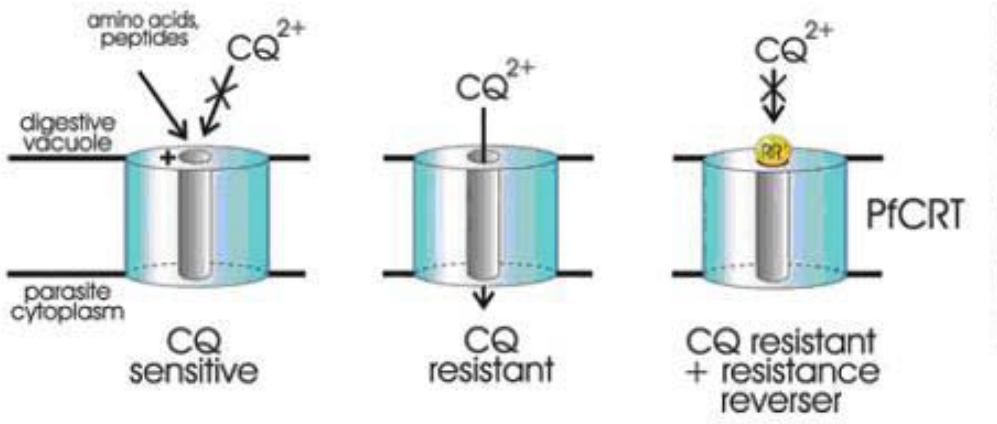
“Charged drug leak” hypothesis

- “Charged drug leak” hypothesis was proposed by Johnson et al. by using selected lines.



Bray PG. et al., 2005

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Objectives

- To identify nucleotide polymorphisms of pfCRT in Thai isolates.
- To determine the polymorphisms of pfCRT and changing of CQ resistant levels in Thai isolates.

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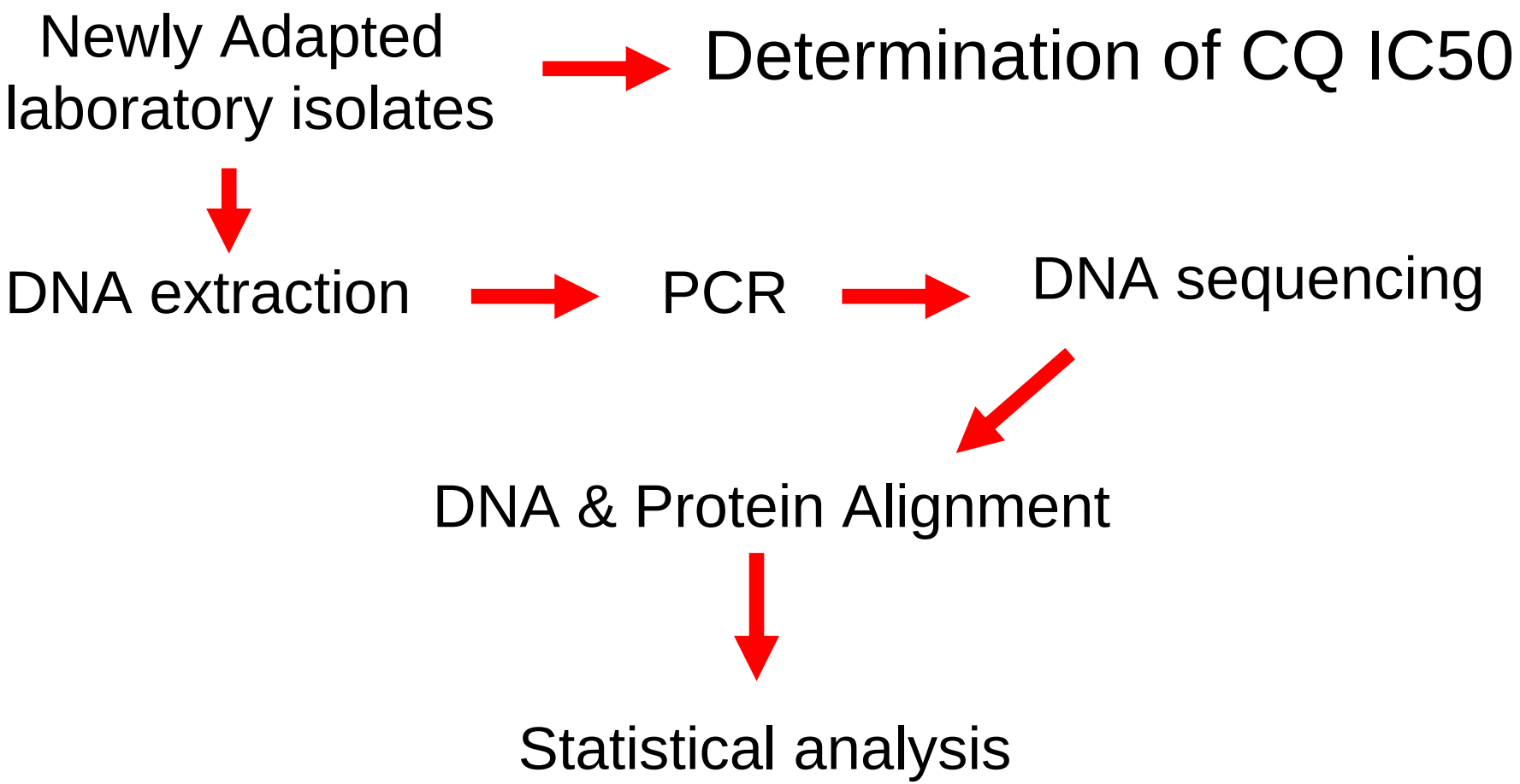
Materials and methods



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Samples

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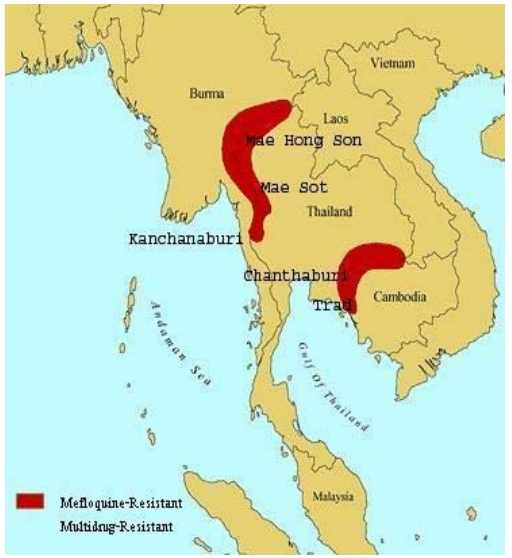
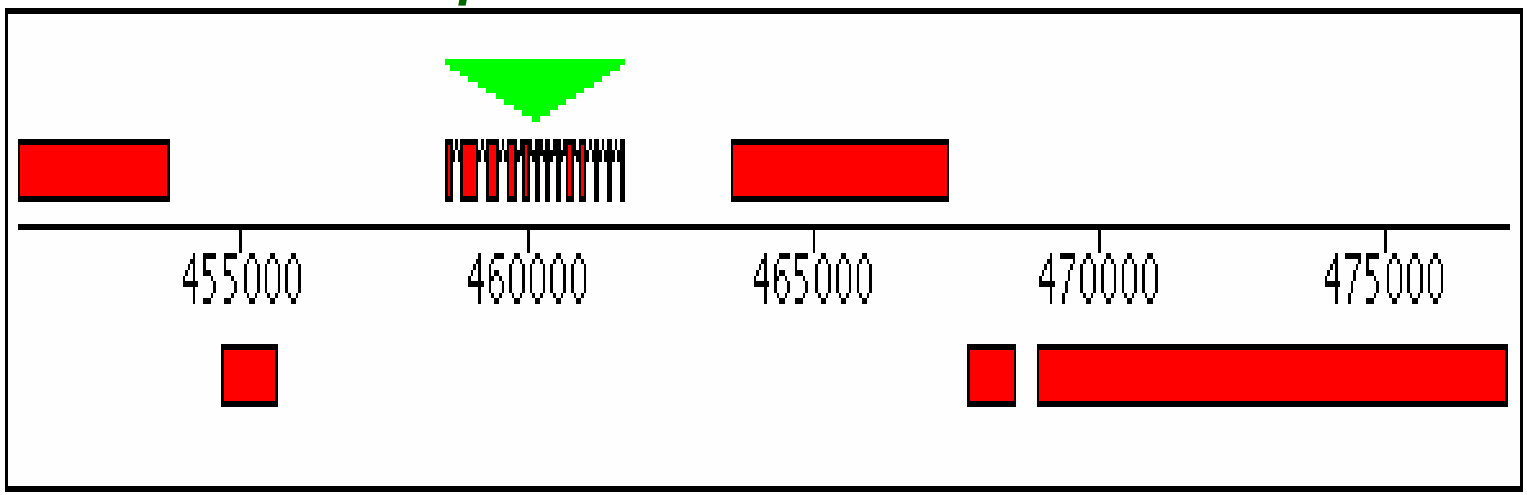
- Ninety isolates of *P. falciparum* from different malaria endemic areas in Thailand were used.
- CQ IC50 for all samples were determined.



PCR

pfcrt



Length 3096 bp
Spliced length 1275 bp

13 exons

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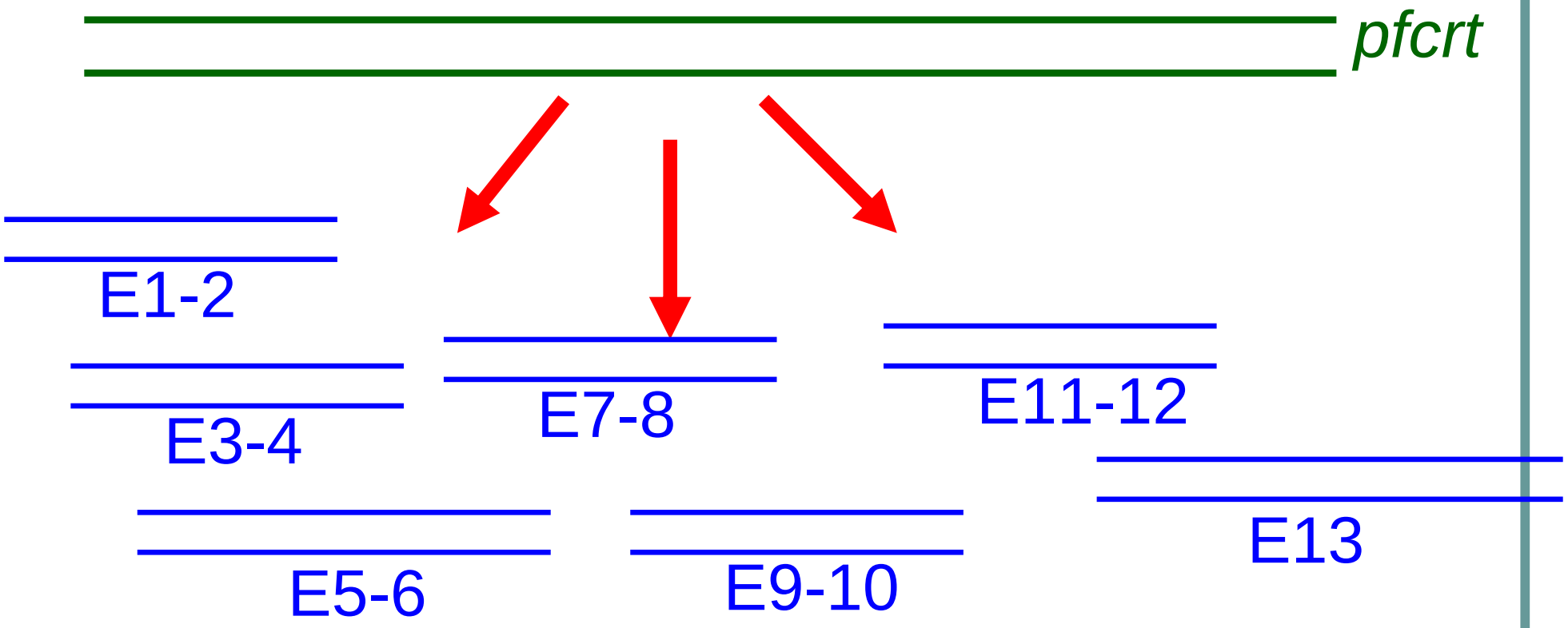
PCR



This gene is separated into 7 fragments

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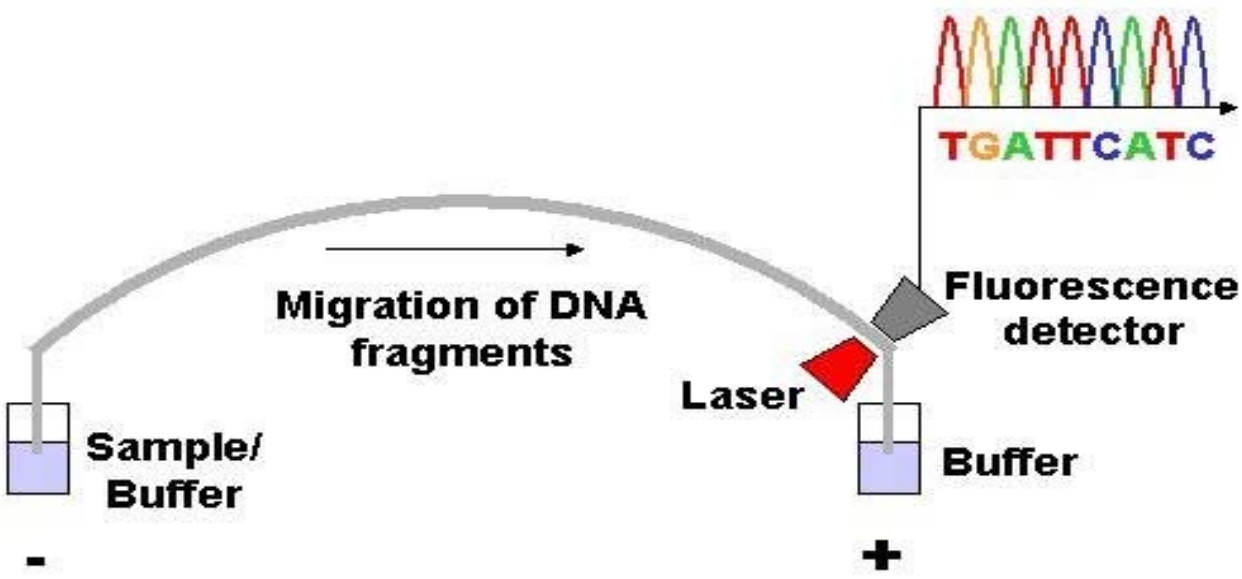
DNA sequencing

- Dye-terminator seqencing

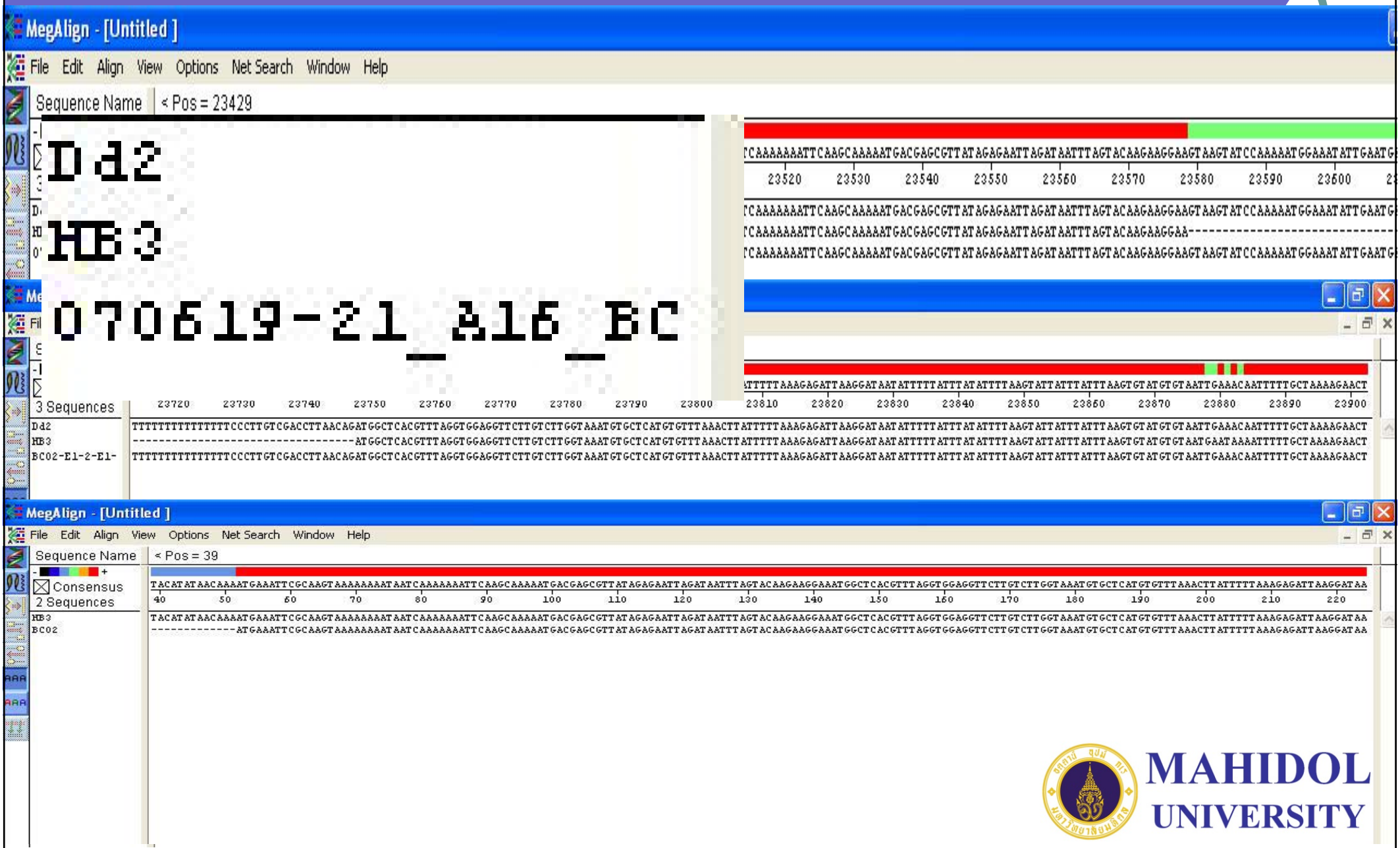


Exon positions of Dd2 line

- In 2000, Su et al. submitted the 47,573 bp of Dd2 line which contain *pfcrt* gene at positions 23488 to 26557. The position of 13 exons were indicated.

Exon	start pos.	end pos.
1	23488	23578
2	23748	24016
3	24180	24352
4	24522	24654
5	24816	24887
6	25011	25086
7	25192	25274
8	25401	25451
9	25589	25645
10	25788	25880
11	26075	26119
12	26257	26311
13	26481	26557

DNA alignment (Megalign)



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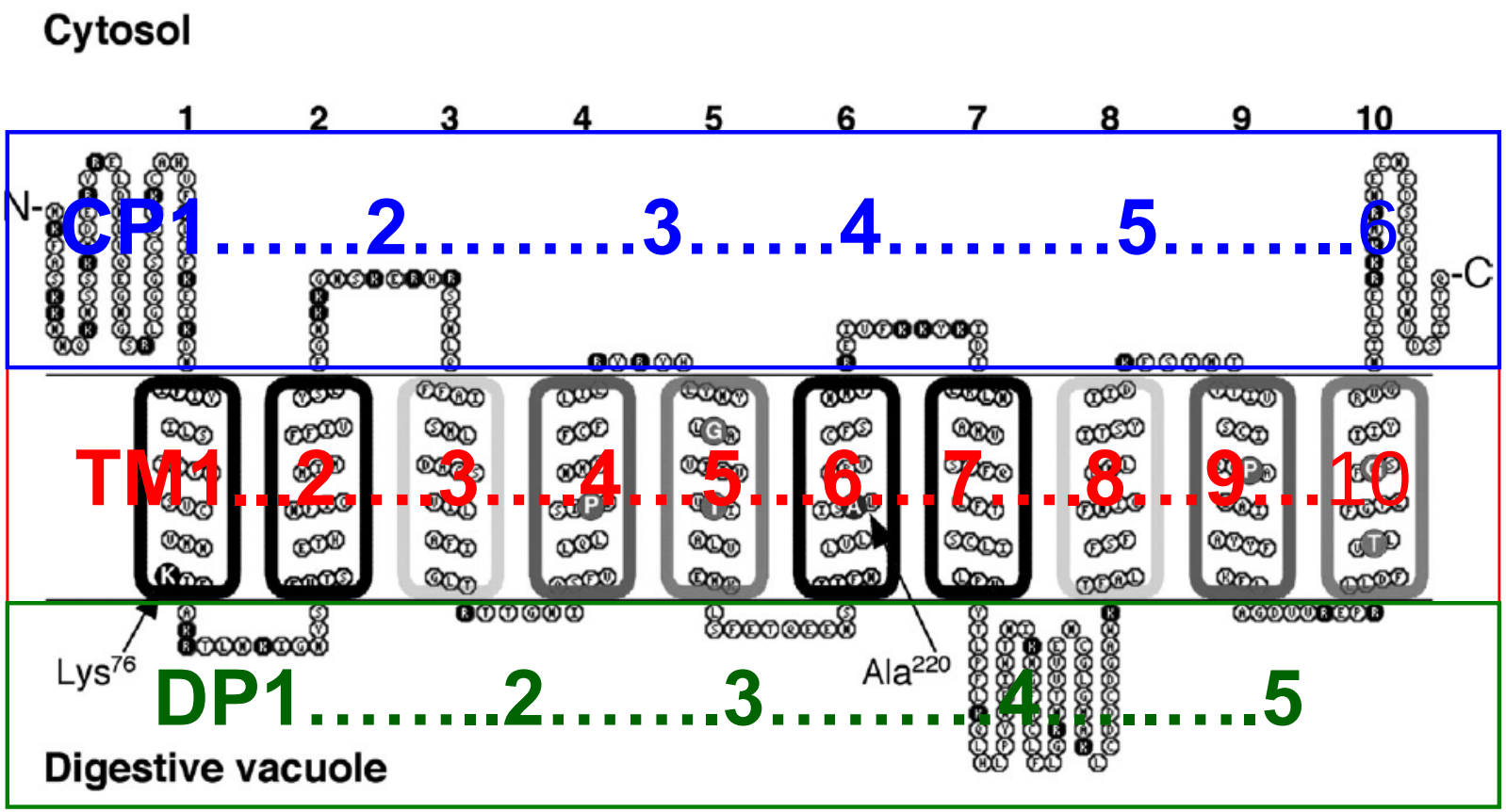
Predicted 10 transmembrane domains

Peptide	Amino acid position	Nucleotide position
TM1	59-78	175-234
TM2	91-113	271-339
TM3	126-148	376-444
TM4	158-175	472-525
TM5	180-197	538-591
TM6	212-229	634-687
TM7	242-264	724-792
TM8	315-337	943-1011
TM9	344-366	1030-1098
TM10	376-398	1126-1194

http://v4-4.plasmodb.org/plasmodb/servlet/sv?page=gene&source_id=MAL7P1.27&view=proteinset

Predicted 10 transmembrane domains



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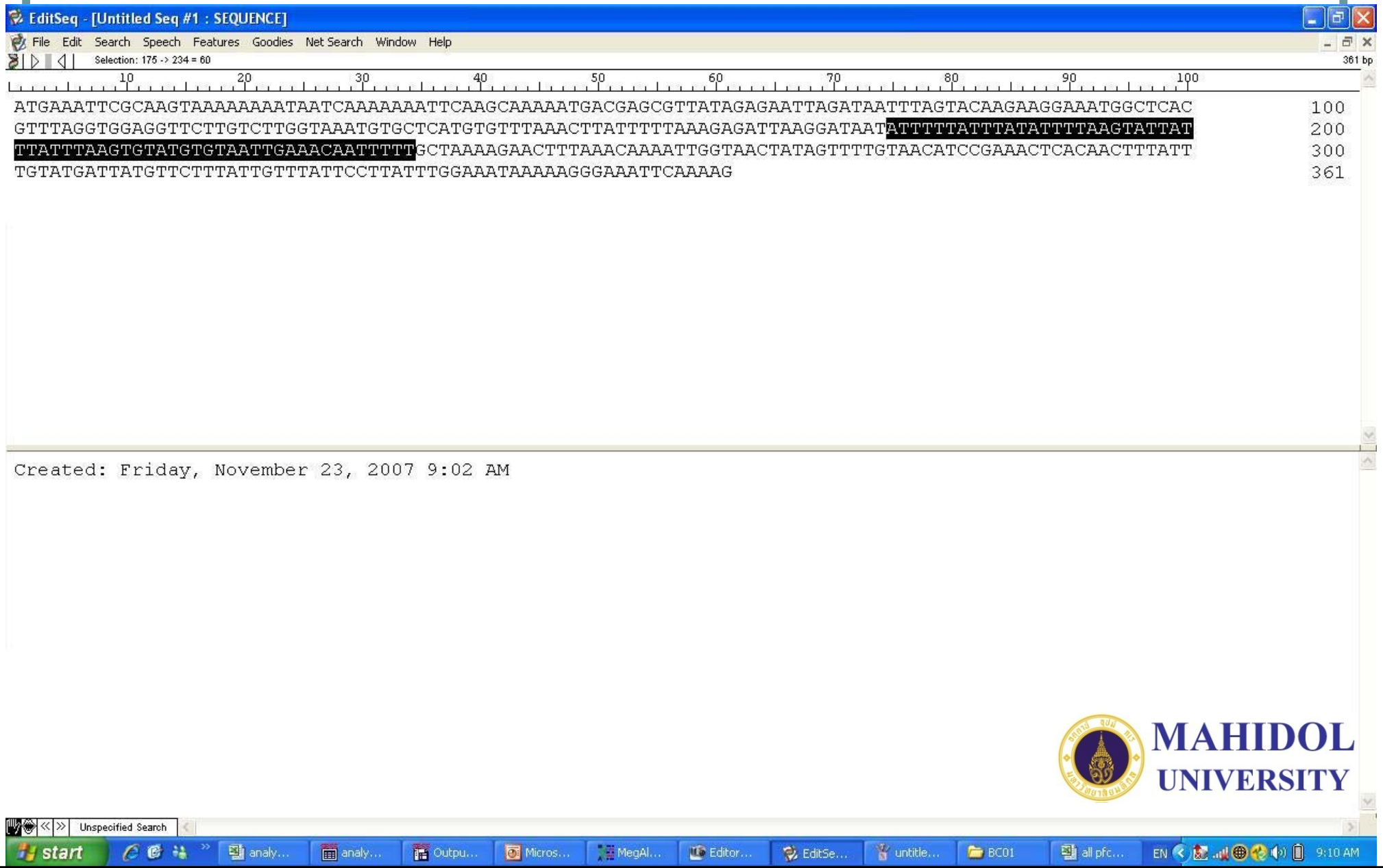
EditSeq



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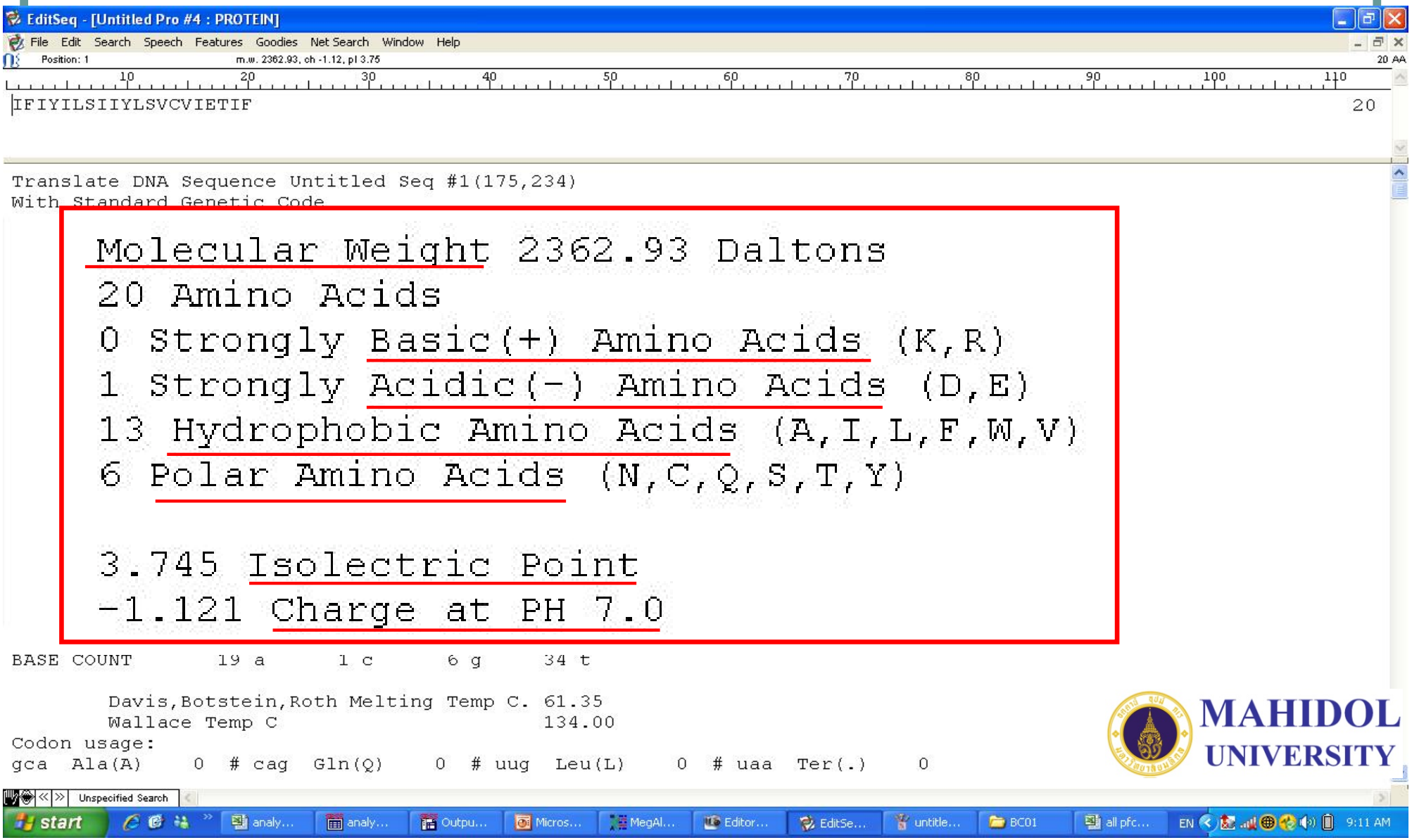
DNA translation from selected sequence



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Data Table

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Samples	CQ IC_{50} (nM)	TM1 (20 amino acid)						
		pI	Charge	M.W.	Basic(+) AA.	Acidic(-) AA.	Hydrophobic AA.	Polar AA.
BC36 (76T)	99. 9	3.745	-1.121	2362. 93	0	1	13	6
J10 (76K)	14	6.151	-0.122	2390	1	1	13	5
BC38 (76T)	160. 1	3.745	-1.121	2362. 93	0	1	13	6
BC39 (76T)	124. 8	3.745	-1.121	2362. 93	0	1	13	6
BC1 (76T)	75. 8	3.745	-1.121	2362. 93	0	1	13	6
BC11 (76T)	123.7	3.745	-1.121	2362. 93	0	1	13	6
BC12 (76T)	49. 2	3.745	-1.121	2362. 93	0	1	13	6

Data analysis

- Each peptide of PfCRT was analyzed with the level of CQ IC50 by independent *t*-test and one-way ANOVA.

Analysis of the pfcrt sequence

Group Statistics						
	TM1 Charge group	N	Mean CQ_IC50 (nM)	Std. Deviation	Std. Error Mean	*p* value
CQ_IC50	76T (-1.12)	83	85.3	40.9	4.5	0.02
	76K (-0.12)	2	16.0	3.1	2.2	

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Analysis of the pfcrt sequence

Group Statistics

	TM2 charge group	N	Mean CQ IC50	Std. Deviation	Std. Error Mean	*p* value
CQ_IC50	97H (-0.95)	82	81.0	39.6	4.4	0.01
	97L (-1.12)	3	157.8	38.0	21.9	

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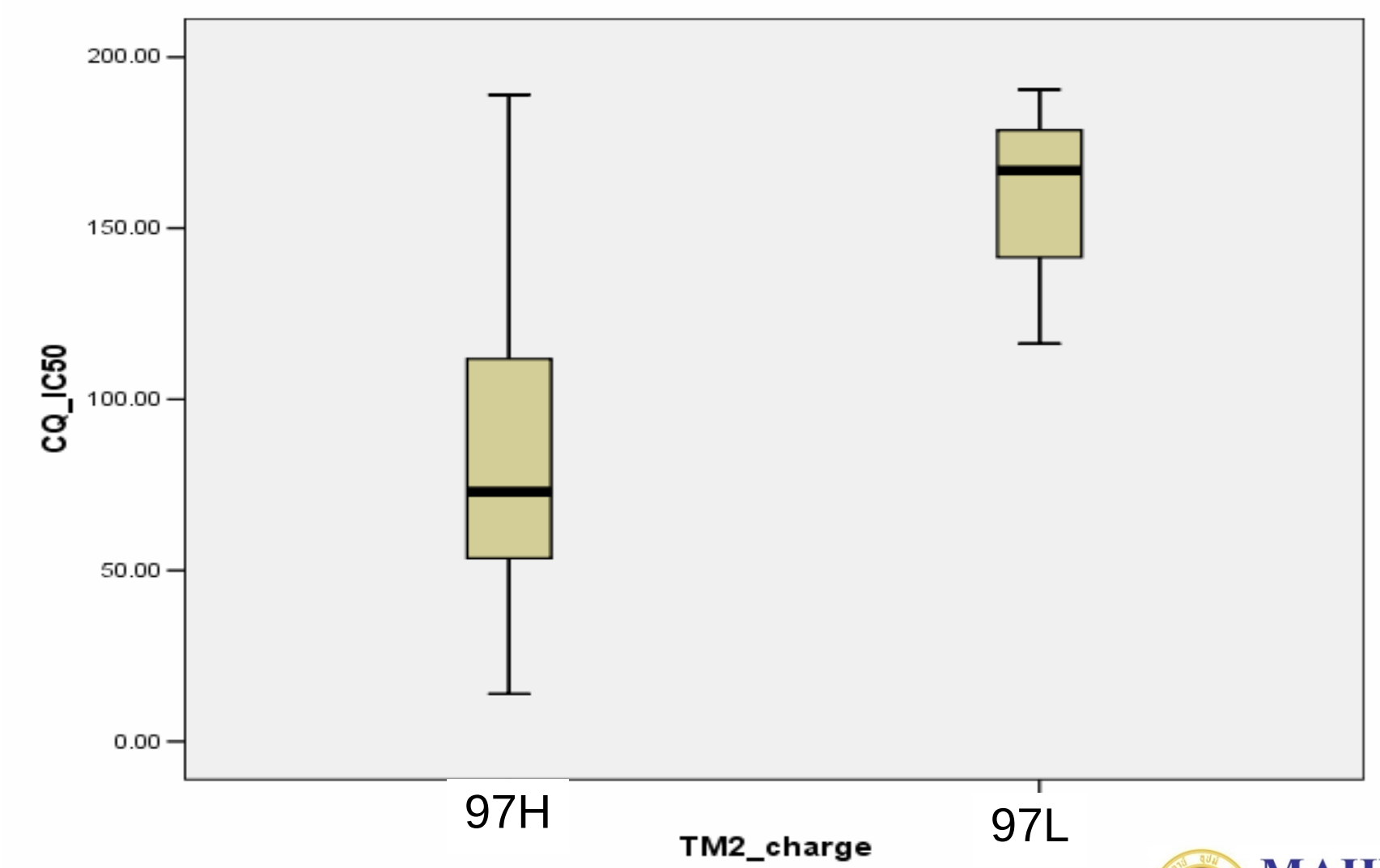
Boxplot of charge TM2 and CQ IC50



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Results of TM1 and TM2

	TM1 polymorphism		
	76K	76T	
Charge	- 0.12	- 1.12	decreased
CQ IC50	16.03	85.31	increased

	TM2 polymorphism		
	97H	97L	
Charge	- 0.95	- 1.12	decreased
CQ IC50	80.97	157.83	increased

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Conclusions

- Polymorphisms of exon 1 and 2 of the pfcrt sequence were identified.
- These polymorphic sequences caused the changing in the charge of the peptides in TM1 and TM2. And it would influence the CQ IC50 level.

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Acknowledgements

- Department of Microbiology, Faculty of Science, Mahidol University
  - Prof. Peerapan Tan-ariya
- Department of Parasitology, Phramongkutklao College of Medicine
  - Col. Assoc. Prof. Mathirut Mungthin
  - Staffs of Department of Parasitology, Phramongkutklao College of Medicine

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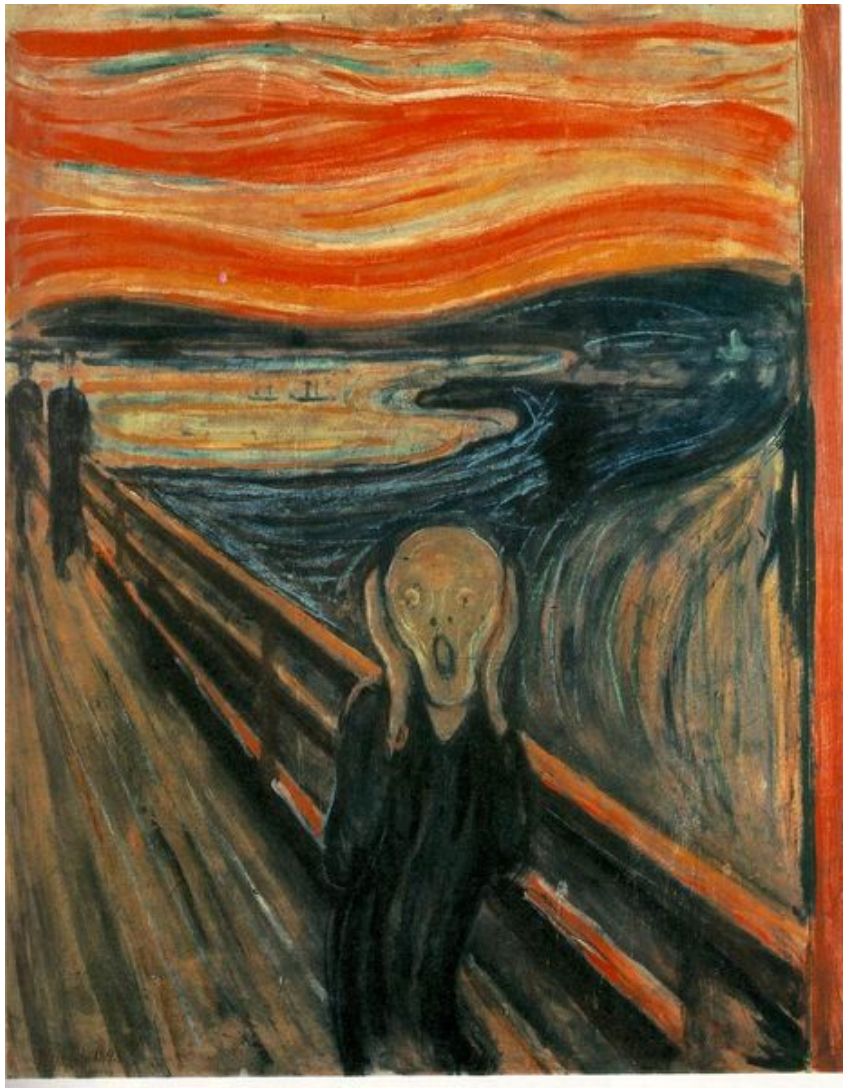
Thank you



Mechanisms of CQR

Two hypotheses

- The first hypothesis: CQR parasite regulates physiological condition in DV by changing pH which then reduces CQ accumulation.

- The second hypothesis: CQR parasite reduces an accumulation of CQ in DV by increased CQ efflux.

CQ makes a comeback

MALARIA

Chloroquine Makes a Comeback

Chloroquine, a malaria drug rendered useless in most of the world by drug-resistant parasites, is once again effective in Malawi. In a study in the 9 November *New England Journal of Medicine*, researchers report that chloroquine cured 99% of 80 malaria cases in Blantyre, the country's commercial capital.

Cheap, easy to administer, and with few side effects, chloroquine was once considered a miracle drug. But by the 1980s, resistance had spread, and in 1993, Malawi became the first African country to officially discourage its use. Few suspected that natural susceptibility would return. But in 2001, molecular studies in Malawi suggested that the resistance mutation had nearly disappeared, and studies of adults hinted that the drug could again clear the parasite.

The new study shows that chloroquine can also work in children with acute infections. Miriam Laufer and Christopher Plowe of the University of Maryland, Baltimore, and their colleagues treated children suffering from uncomplicated malaria with either chloroquine or sulfadoxine-pyrimethamine (SP), the standard first-line drug in Malawi. Chloroquine was effective in 79 of 80 children who received it. In contrast, SP failed in 71 of 87 children. (Those children received backup treatment, and all made full recoveries.)

The result does not mean that Malawi should go back to using chloroquine, Plowe stresses. "Malawi is a little island of sensitivity surrounded by a sea of resistance," he says. "Resistance would come washing back in" if the drug were widely used.

But knowing that the drug can regain its usefulness after a prolonged absence gives researchers hope that the same might be true for other resistance-plagued drug regimes. The result "is another argument for getting chloroquine out of Africa," says malaria expert Thomas Wellems of the U.S. National Institute of Allergy and Infectious Diseases in Bethesda, Maryland. **–GRETCHEN VOGEL**

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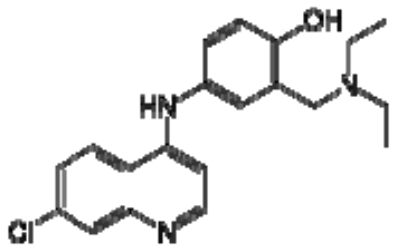
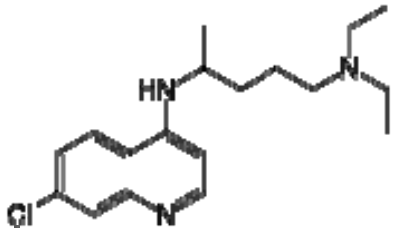
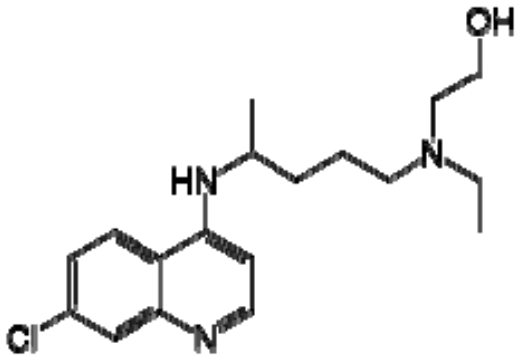
Published by AAAS

4-Aminoquinoline

- Amodiaquine
- Chloroquine
- Hydroxychloroquine



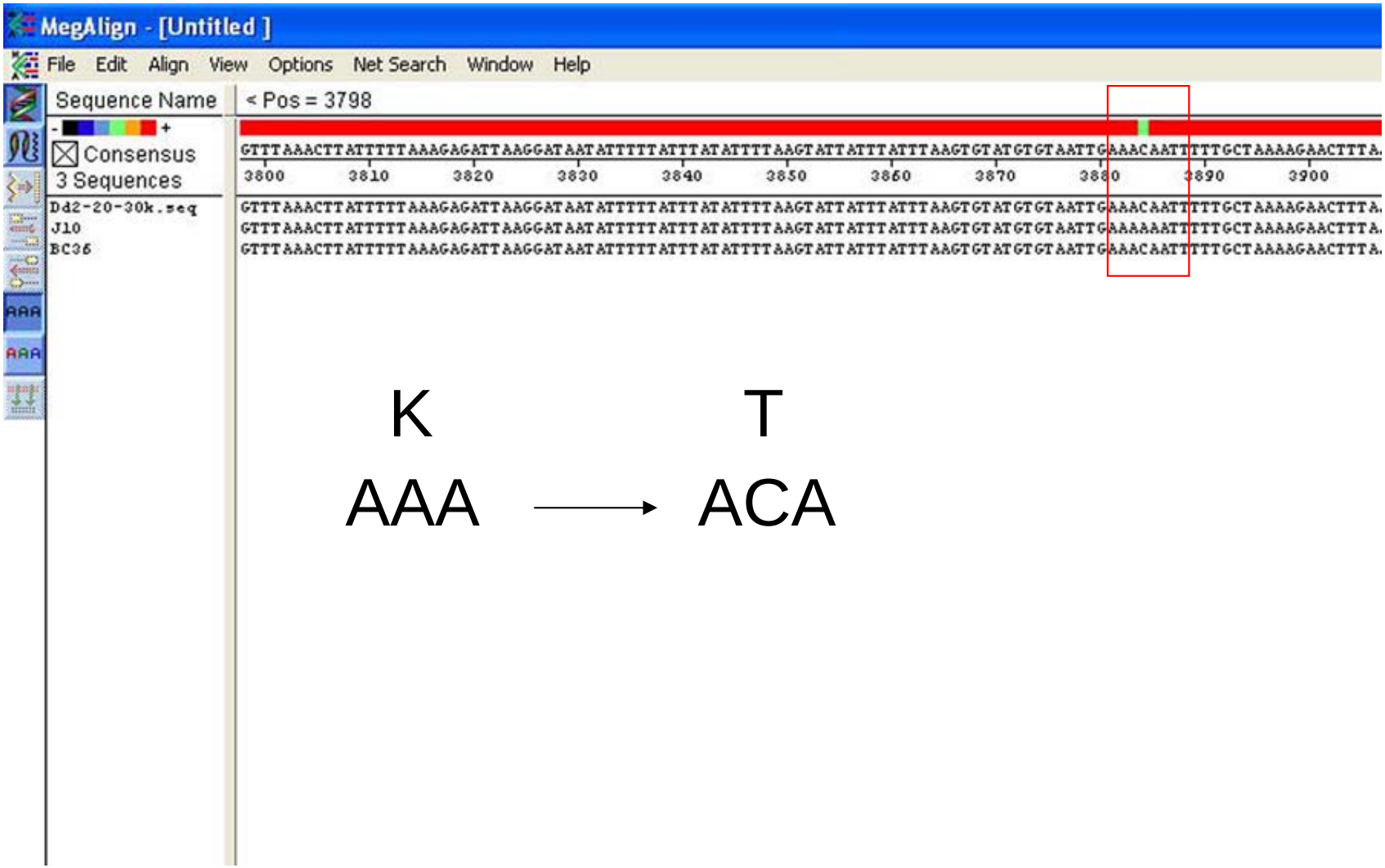




Reistance haplotype region	*72*	*73*	*74*	*75*	*76*	*77*	*97*	*220*	*271*	*326*	*356*	*371*
SE Asia ,Africa RB8	C	V	I	E	T	I	H	S	E	S	I	I
Sudan stain ,Dd2 Thailand	C	V	I	E	T	I	H	S	E	S	T	I
106/1^{K76}	C	V	I	E	K	I	H	S	E	S	I	I
7G8 Brazil, FCQ22 PNG , INDO 19 Thailand	S	V	M	N	T	I	H	S	Q	D	L	R

Sequence of *P. falciparum* lines 106/1 from Fidock, D.A.

J10 vs BC36


MegAlign - [Untitled]
File Edit Align View Options Net Search Window Help
Sequence Name
< Pos = 3798
Consensus
3 Sequences
Dd2-20-30k.seq
J10
BC36
3800 3810 3820 3830 3840 3850 3860 3870 3880 3890 3900
K
T
AAA
ACA

Results from DNA alignment

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Samples	Nucleotide position	nucleotide change	amino acid change	peptide
BC28	16	A>C	K6Q	CP1
BC35	12	A>C	A4A	CP1
	16	A>C	K6Q	CP1
	29	A>G	Q10R	CP1
	77	T>G	L26S	CP1
PCM6	12	A>C	A4A	CP1
	16	A>C	K6Q	CP1
PCM12	12	A>C	A4A	CP1
J10	227	C>A	T76K	TM1
RN28	227	C>A	T76K	TM1
BC33	198	T>C	I66I	TM1
J6	290	A>T	H97L	TM2
PCM11	290	A>T	H97L	TM2
KS25	290	A>T	H97L	TM2

Overview

- Introduction
  - Basic knowledge of malaria
  - Antimalarial drug i.e., Chloroquine
  - Chloroquine resistant *P.falciparum* and *pfcrt* gene
- Research
  - Materials and Methods
  - Results
  - Conclusions
  - Future plan

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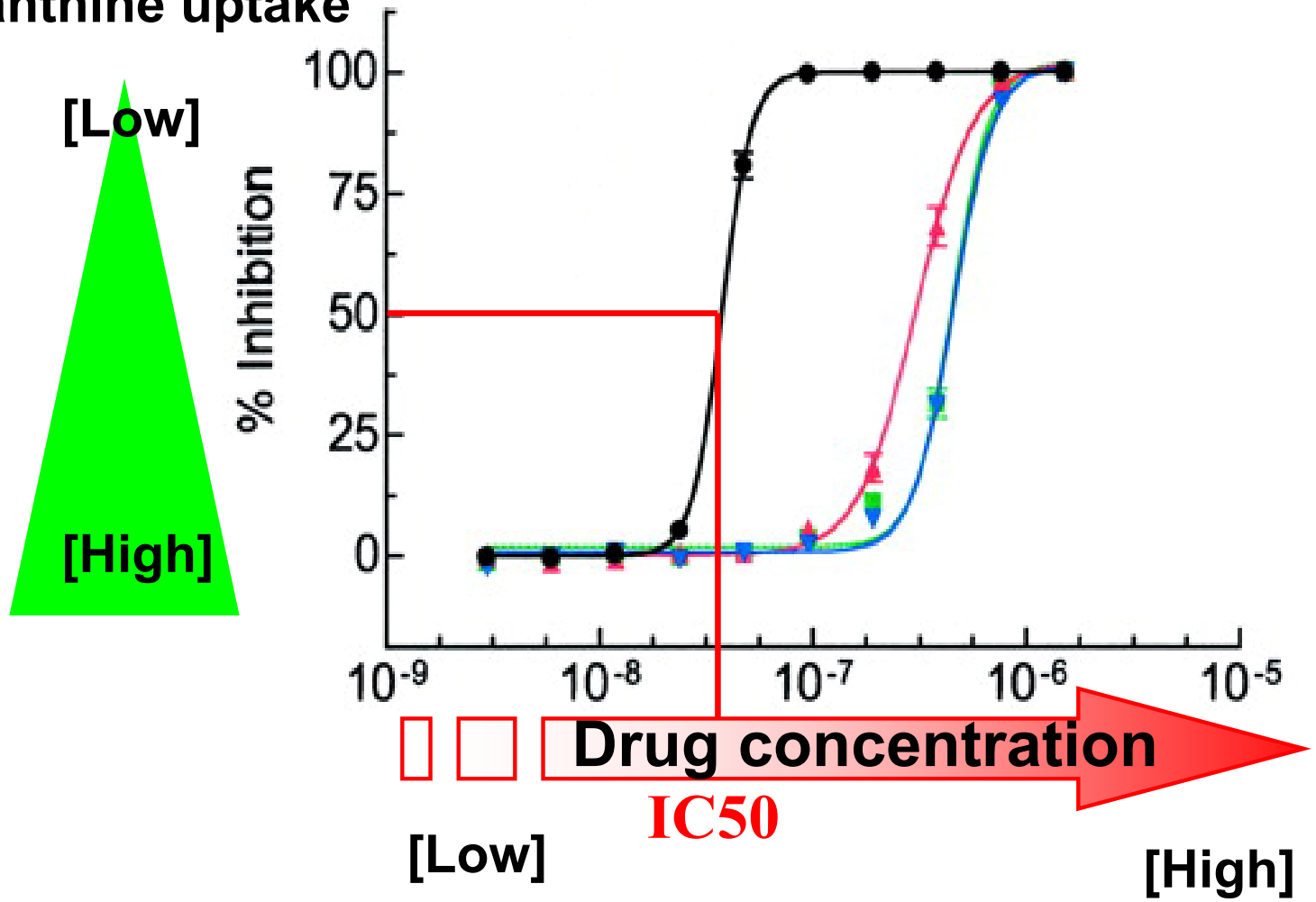
Antimalarial drug response assays

[3H]-hypoxanthine uptake



Steps to CQR

- Under CQ pressure, malaria parasites acquired *pfcrt mutation* sequentially.
- As many as 8 to 9 *pfcrt* mutations are associated with CQR in some geographical regions whereas only 4 mutation was required for the other regions. The number of acquired mutations for CQR was varied with genetic background of *P. falciparum* strains)
- However, the mutation at position 76 from K to T was definitely required for CQR.

Characters of *pfcrt* gene in *Plasmodium falciparum*

	pfcrt						
Codon	76	163	220	271	326	356	371
Wild type (%)	3 (2.7)	110 (100)	2 (1.8)	2 (1.8)	2 (1.8)	6 (5.3)	2 (1.8)
Mutant (%)	110 (97.3)	0	111 (98.2)	111 (98.2)	111 (98.2)	105 (92.9)	111 (98.2)
Mixed (%)	-	-	-	-	-	2 (1.8)	-
Total	113 (100)	113 (100)	113 (100)	113 (100)	113 (100)	113 (100)	113 (100)

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Results from DNA alignment

Samples	position	nucleotide change	amino acid change	peptide
BC4	16	A>T	K6stop	CP1
	34	A>T	N12Y	CP1
BC6	13	A>T	S5C	CP1
	16	A>T	K6stop	CP1
BC9	16	A>T	K6stop	CP1
BC10	16	A>T	K6stop	CP1

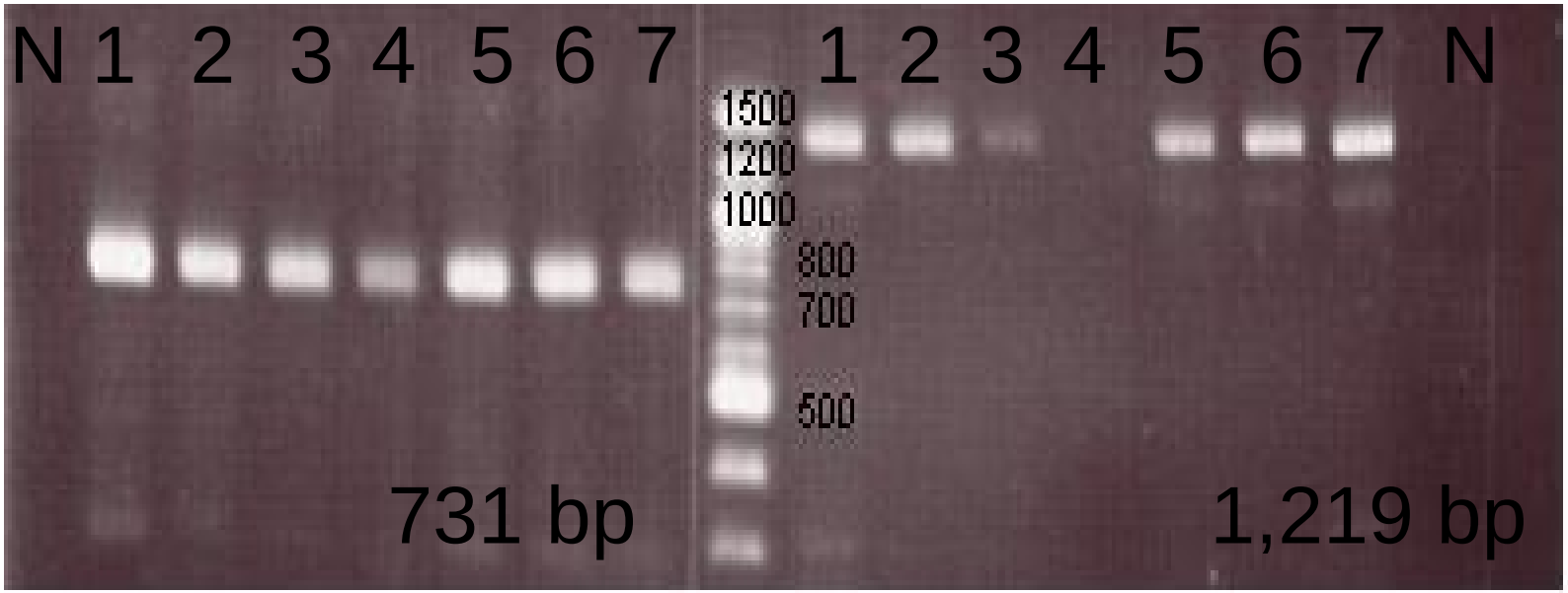
- A **point mutation**, or **single base substitution**, is a type of mutation that causes the replacement of a single base nucleotide with another nucleotide.

- **Genetic polymorphism** is the occurrence together in the same locality of two or more discontinuous forms of a species in such proportions that the rarest of them cannot be maintained just by recurrent mutation [5]. It is sometimes called balancing selection, and is intimately connected with the idea of heterozygote advantage.

TM analysis program

- Protscal
- TMMHM 2.0

PCR results from E1-2 and xxx



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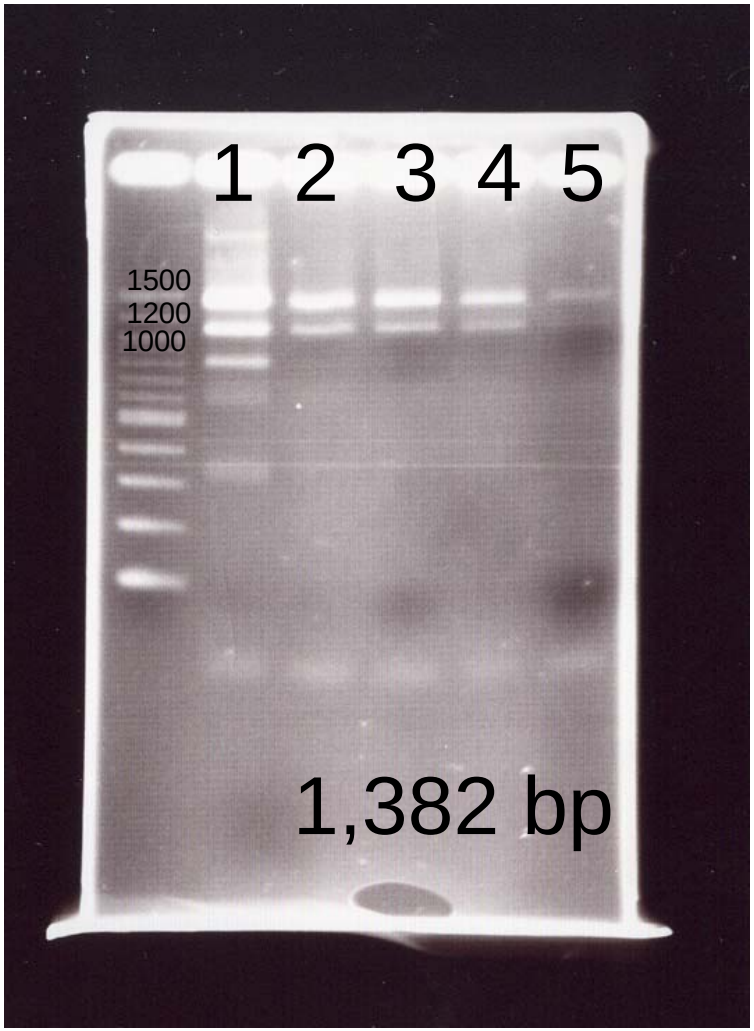
PCR results from xxxx



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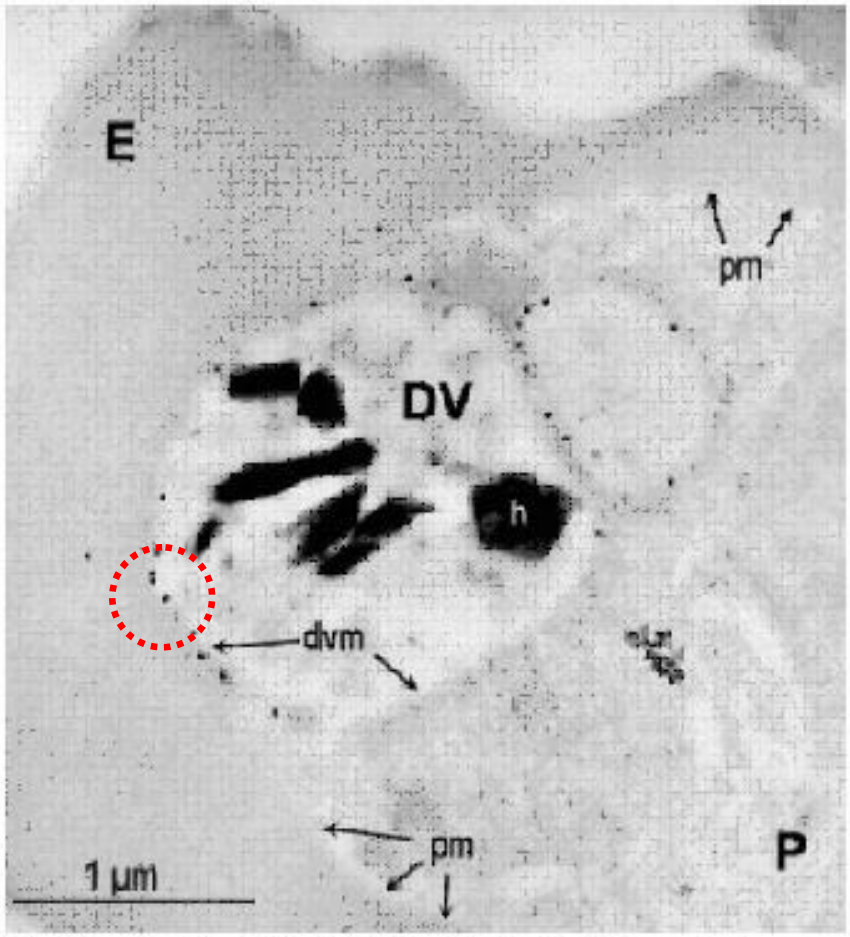
Mechanisms of drug resistance

- Mutations in drug target
- Increasing of drug target
- Decreasing of drug accumulation (includes increasing efflux)
- Drug inactivation (physiological change at drug action site)
- Using alternative pathway

Position of PfCRT on DV



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