

Nucleotide polymorphisms of pfcrt gene in Thai isolates of Plasmodium falciparum



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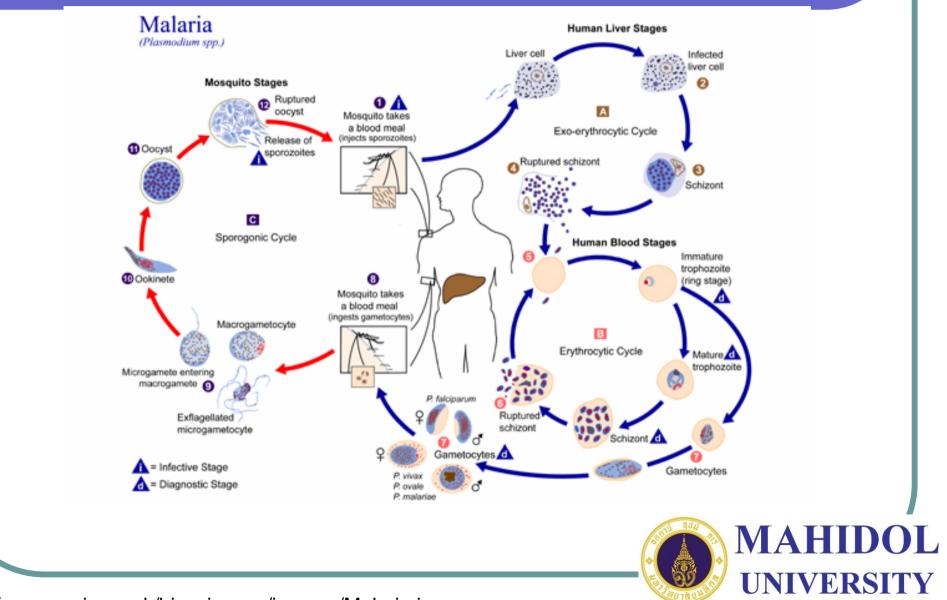
Malaria situation

 300-500 million people of world's population suffer from malaria.

 1-2 million children under than 5 years old die from malaria annually.

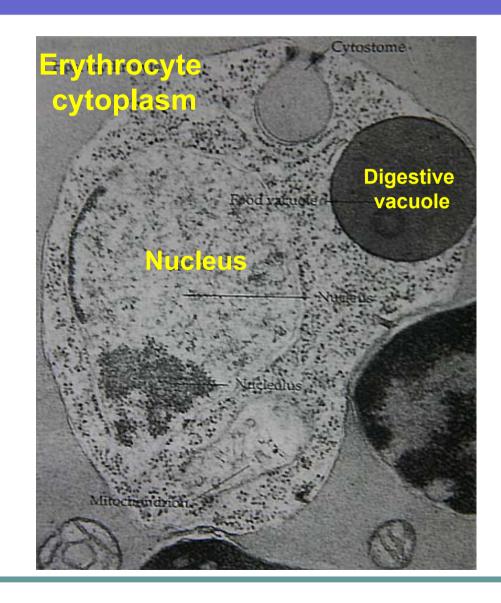


Plasmodium falciparum



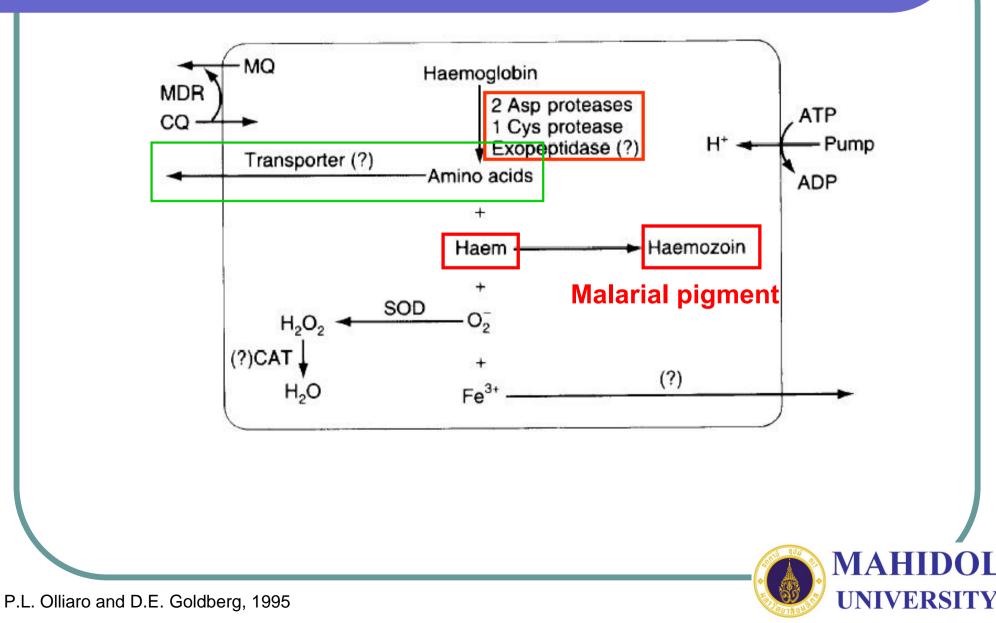
http://www.wmin.ac.uk/biosciences/images/Malaria.jpg

Asexual erythrocytic stage of Plasmodium falciparum

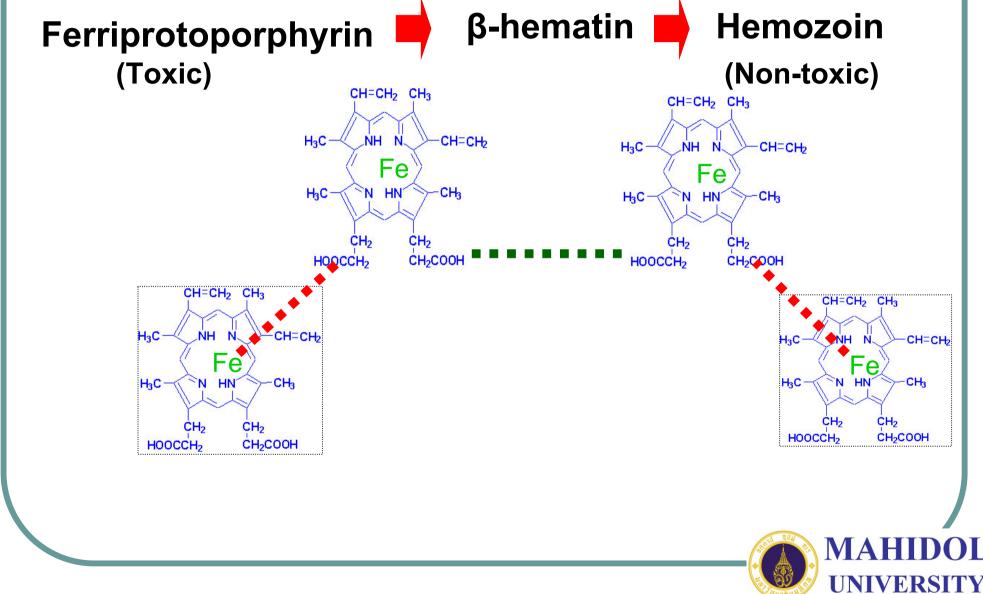




Plasmodium Digestive Vacuole

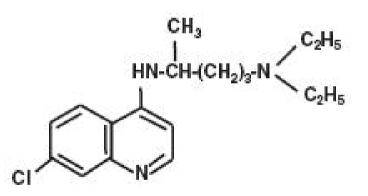


Detoxification of Ferriprotoporphyrin (FP)



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



Action of Chloroquine in digestive vacuole (DV)

- CQ is a weak base, accumulated as CQ²⁺ 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.



pfcrt is a major gene of CQR

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

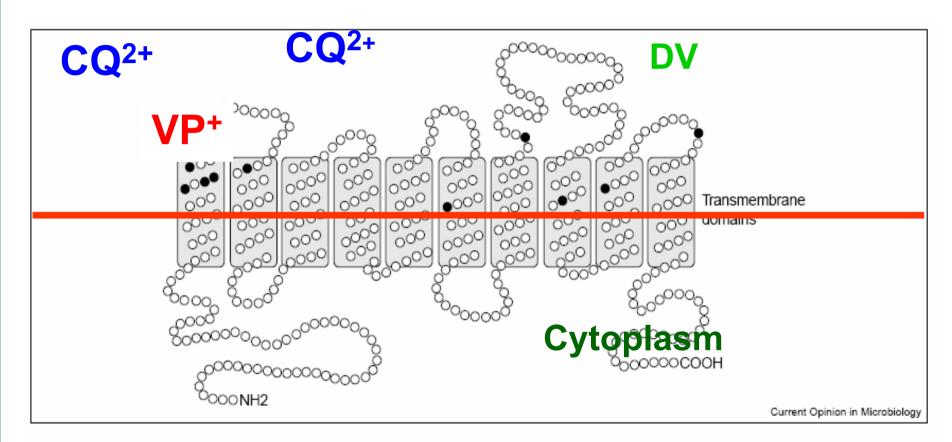


K76T mutation in PfCRT

 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) \rightarrow T (threonine) change at position 76 (indicated by the arrow) is critical to CQ resistance in *P. falciparum*.

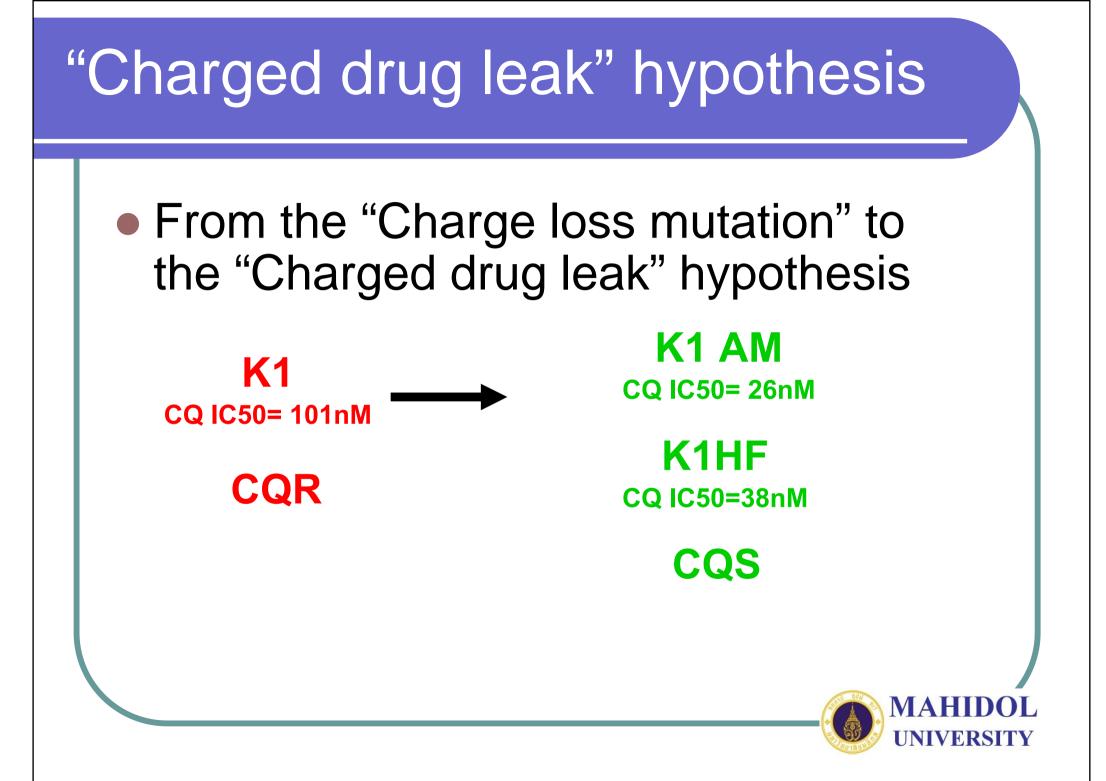


Calton et al., 2001

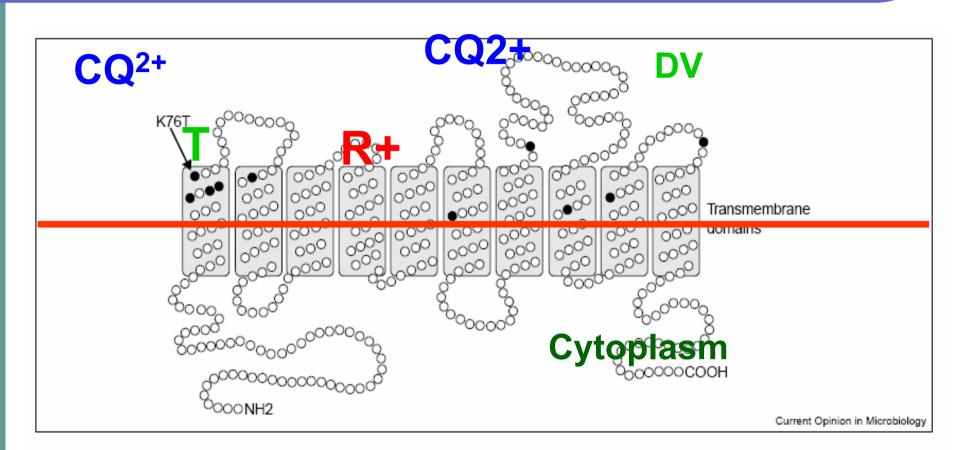
K76T is major mutation in PfCRT (Cooper et al., 2005)

-				-		-			-	- <u>60</u>	J UNI	VERSITY
Geographic	Parasite	PfC	RT a	mine	o acio	i po	sitior	15				
distribution	clone/isolate	72	74	75	76	77	97	144	148	152	160	163
CQS parasites						1						
Honduras	HB3 ^a	С	Μ	Ν	Κ	Ι	Η	Α	L	Т	L	S
CQR parasites A	Africa											
Mali	S35CQ ^d	\mathbf{C}	1	E	Т		н	А	L	Т	L	s
South Africa	RB8 ^a	С	1	E	Т	Ι	н	Α	L	Т	L	s
Southeast Asia												
Thailand	Dd2ª	\mathbf{C}	I	E	Т	Ι	H	Α	L	Т	L	S
Thailand	TM93-C1088 ^{f,g}	С	I	E	Т		L	Α			L	
Cambodia	783 ^h	С	1	E	Т		н	Α	L			
Cambodia	738 ^h	С	1	D	Т		Η	Α	1			
Indonesia					<u> </u>							
Lombok	Field isolate ^j	С	Μ	N	Ν	1						
South America					· _ ·							1
Ecuador	Ecu1110 ^a	\mathbf{C}	м	N	Т	Ι	н	А	L	Т	L	s
Colombia	Jav^{a}	\mathbf{C}	М	E	Т	Ι	Q	А	L	Т	L	s
Brazil	7G8 ^a	S	\mathbf{M}	Ν	Т	I	н	А	L	Т	L	S

MAHIDOL



"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

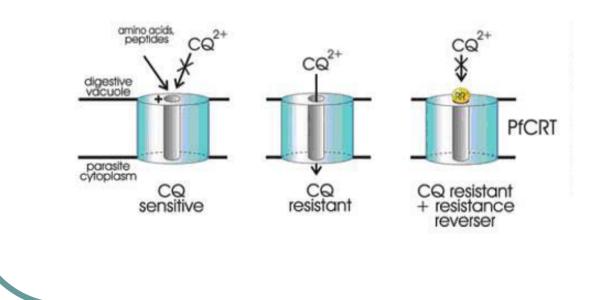
diverse isolates from the Eastern and Western hemispheres are indicated by filled circles. The K (lysine) \rightarrow T (threonine) change at position 76 (indicated by the arrow) is critical to CQ resistance in *P. falciparum*.



Calton et al., 2001

"Charged drug leak" hypothesis

 "Charged drug leak" hypothesis was proposed by Johnson et al. by using selected lines.





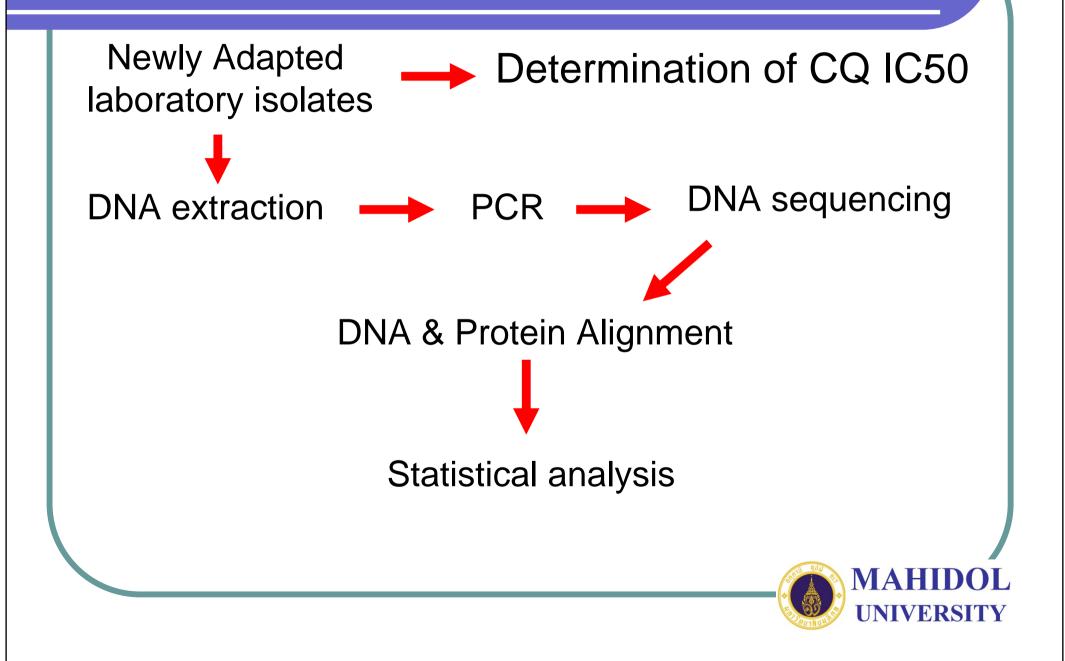
Bray PG. et al., 2005



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



Materials and methods



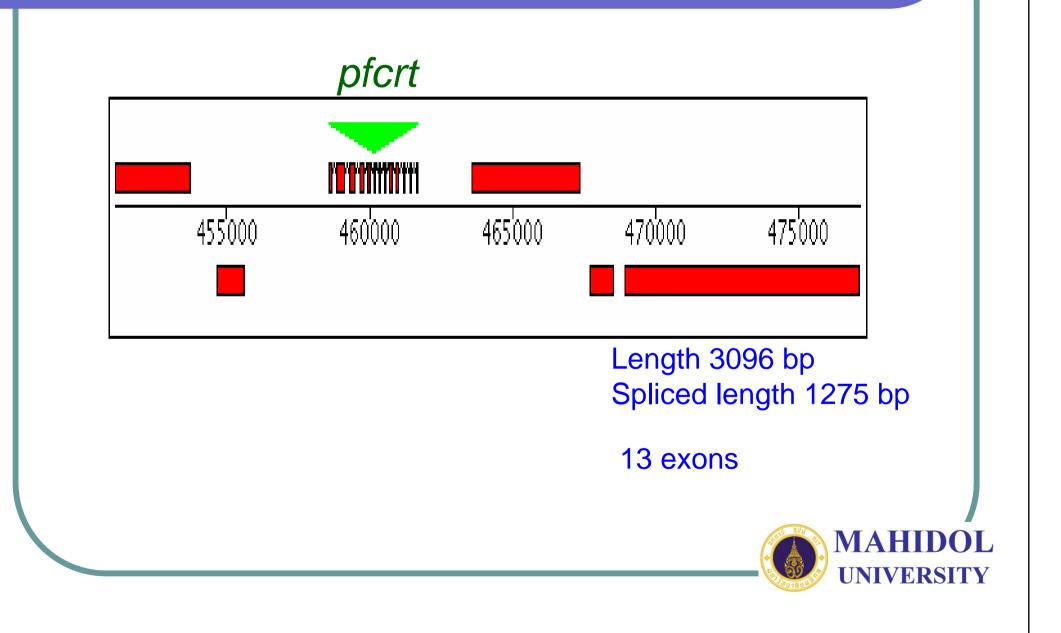
Samples

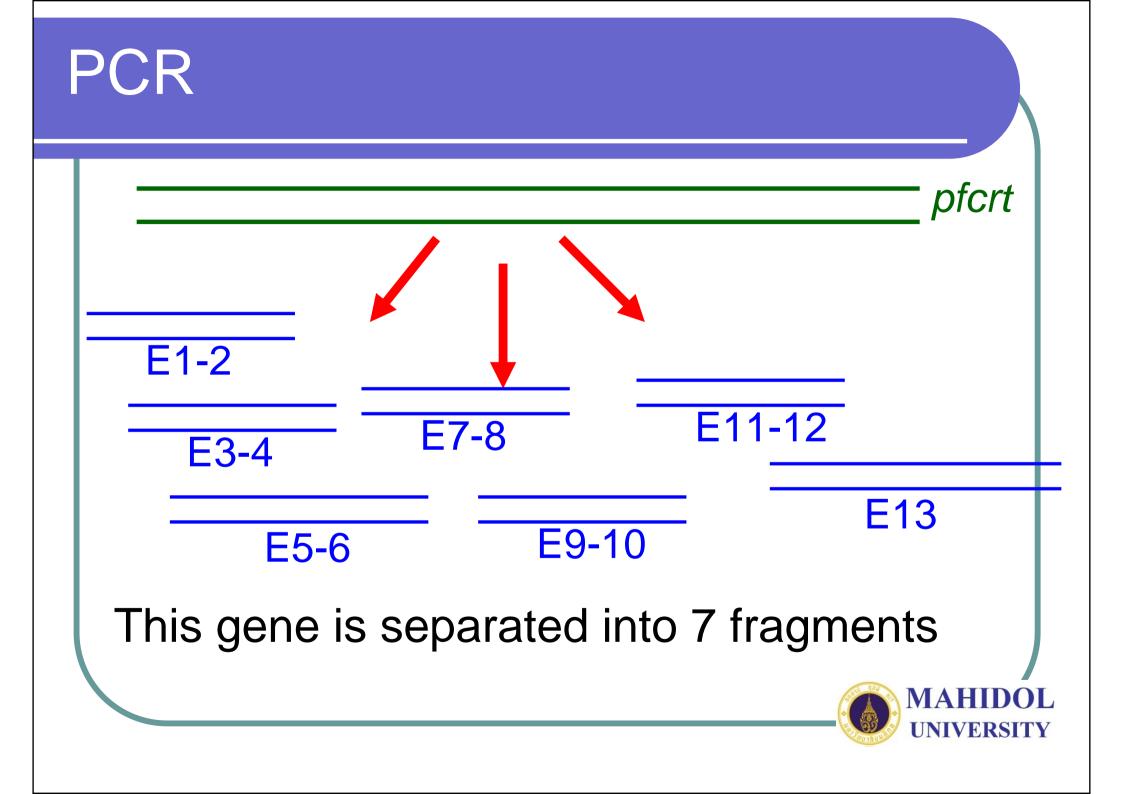


- Ninety isolates of *P*.
 falciparum from different malaria endemic areas in Thailand were used.
- CQ IC50 for all samples were determined.

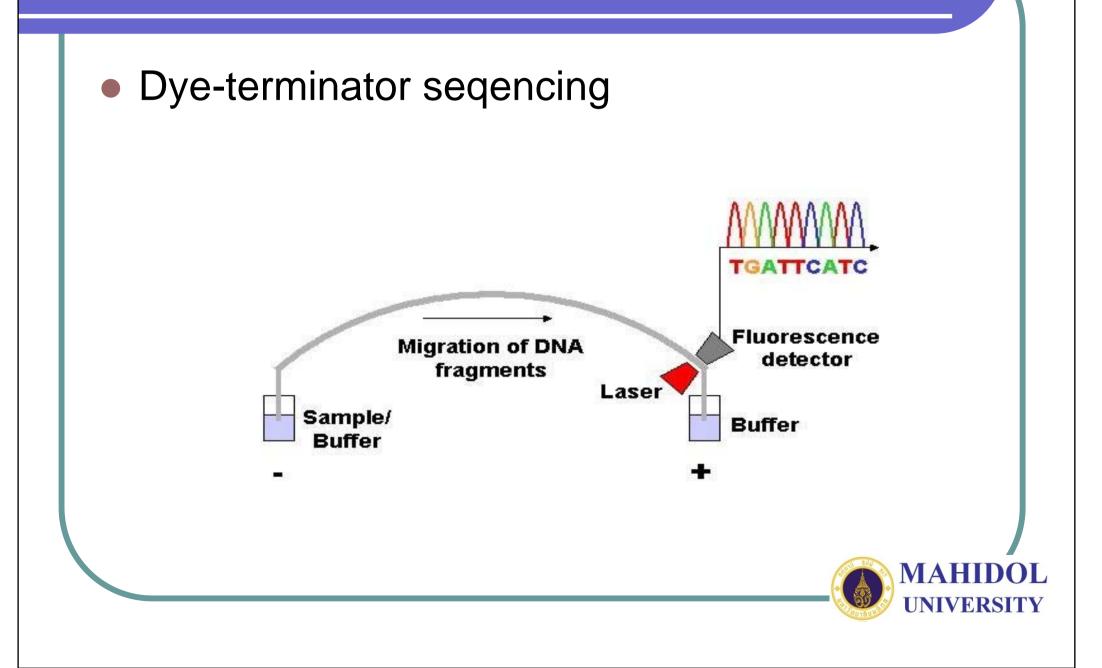


PCR





DNA sequencing



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



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07)	0619-21_	A16	вс	ATTTT &	AAGAGATTAAGGAT	0			0			
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Sequences 42 53 C02-E1-2-E1-	23720 23730 23740 23750 23 TTTTTTTTTTTTCCCTTGTCGACCTTAACAGATGGCTCACGTT	2760 23770 23780 TTAGGTGGAGGTTCTTGTCTTGG TTAGGTGGAGGTTCTTGTCTTG	0 23790 TAAATGTGCTCATGT TAAATGTGCTCATGT	23800 23810 TGTTT AAACTT ATTTT A	23820 : AAGAGATTAAGGAT AAGAGATTAAGGAT	23830 238 AATATTTTTATT AATATTTTTATT	840 2383 TATATTTTAAG TATATTTTAAG	50 2385 TATTATTTATT TATTATTTATT	0 23870 TAAGTGTATGI TAAGTGTATGI) 23880 FGTAATTGAAA FGTAATGAATA	23890 CAATTTTTGC	CTAAAAGA.) 239 CTAAAAGA. CTAAAAGA.
Sequences	23720 23730 23740 23750 23 TTTTTTTTTTTTTCCCTTGTCGACCTTAACAGATGGCTCACGTT ATGGCTCACGTT	2760 23770 23780 TTAGGTGGAGGTTCTTGTCTTGG TTAGGTGGAGGTTCTTGTCTTG	0 23790 TAAATGTGCTCATGT TAAATGTGCTCATGT	23800 23810 TGTTT AAACTT ATTTT A	23820 : AAGAGATTAAGGAT AAGAGATTAAGGAT	23830 238 AATATTTTTATT AATATTTTTATT	840 2383 TATATTTTAAG TATATTTTAAG	50 2385 TATTATTTATT TATTATTTATT	0 23870 TAAGTGTATGI TAAGTGTATGI) 23880 FGTAATTGAAA FGTAATGAATA	23890 CAATTTTTGC	CTAAAAGA.) 239 CTAAAAGA. CTAAAAGA.
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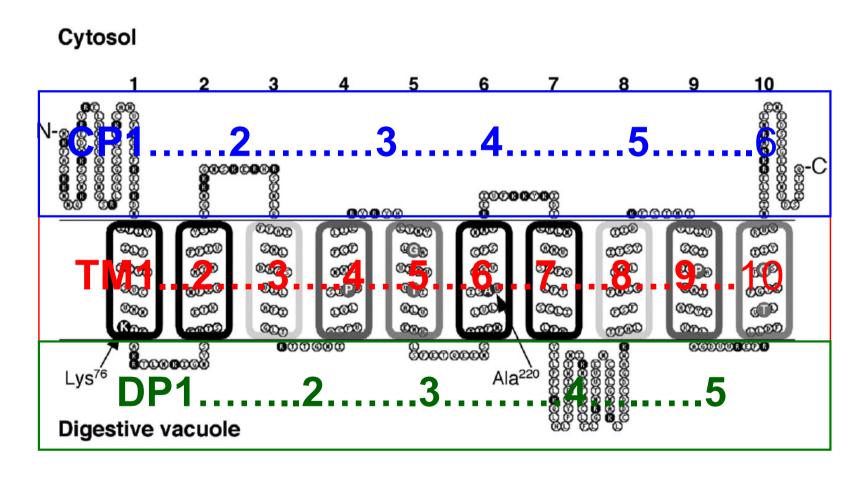
Predicted 10 transmembrane domains

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



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Predicted 10 transmembrane domains





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I <mark>ATTTAAGTGTATGTGTAATTGAAACAATTTTT</mark> GCTAAAAGAACTTTAAACAAAATTGGTAACTATAGTTTTGTAACATCCGAAACTCACA	
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DNA translation from selected sequence 😻 EditSeg - [Untitled Pro #4 : PROTEIN] 🕏 File Edit Search Speech Features Goodies Net-Search Window Help m.w. 2362.93, ch -1.12, pl 3.75 30 40 50 60 70 80 10 90 100 110 IFIYILSIIYLSVCVIETIF 20 Translate DNA Sequence Untitled Seq #1(175,234) With Standard Genetic Code Molecular Weight 2362.93 Daltons 20 Amino Acids 0 Strongly Basic(+) Amino Acids (K,R) 1 Strongly Acidic (-) Amino Acids (D,E) 13 Hydrophobic Amino Acids (A, I, L, F, W, V) 6 Polar Amino Acids (N,C,Q,S,T,Y) 3.745 Isolectric Point -1.121 Charge at PH 7.0 BASE COUNT 19 a 1 c6 a 34 t Davis, Botstein, Roth Melting Temp C. 61.35 МАНП Wallace Temp C 134.00 Codon usage: UNIVERSI gca Ala(A) 0 # cag Gln(Q) 0 # uug Leu(L) 0 # uaa Ter(.) 📲 🛞 🔍 🔊 Unspecified Search EN < 🎊 📲 🔀 🍪 🗊 analy. Micros.. MegAl. Me Editor. 🕏 EditSe.. 🖌 untitle. CO1 8C01 all pfc... (C) +4 Outpu... start

Data Table



					TM1 (20 am	ino acid)		
Samples	(nM)	pl	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

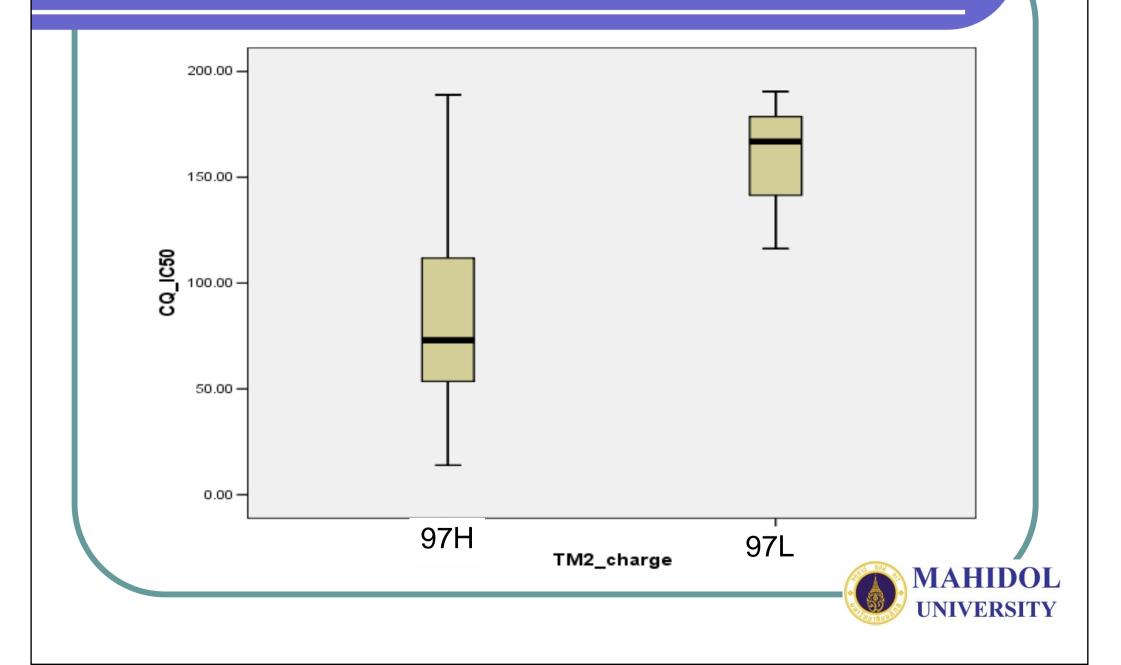
			Group Stati	stics		
	TM1 Charge group	N	Mean CQ_IC50 (nM)	Std. Deviation	Std. Error Mean	<i>p</i> value
CQ_IC50 _	76T (-1.12)	83	85.3	40.9	4.5	0.02
	76K (-0.12)	2	16.0	3.1	2.2	



Analysis of the pfcrt sequence

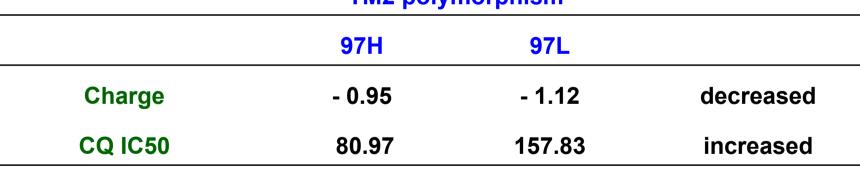
	TM2 charge group	N	Mean CQ IC50	Std. Deviation	Std. Error Mean	<i>p</i> value
CQ_IC50 97	97H (-0.95)	82	81.0	39.6	4.4	0.01
	97L (-1.12)	3	157.8	38.0	21.9	

Boxplot of charge TM2 and CQ IC50



Results of TM1 and TM2

	TM1 polyı	norphism	
	76K	76T	
Charge	- 0.12	- 1.12	decreased
CQ IC50	16.03	85.31	increased
	TM2 polyn	norphism	
	97H	97L	





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.

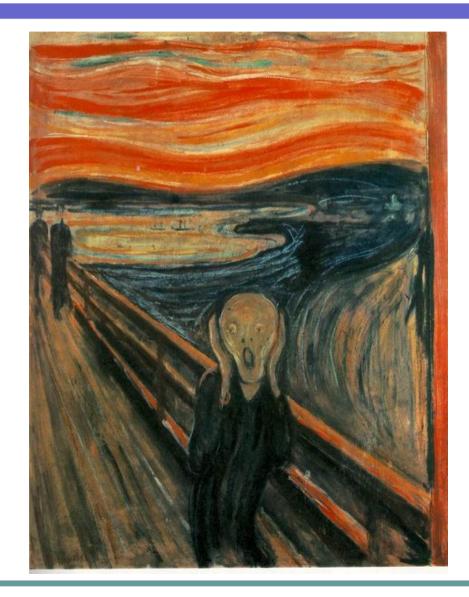


Acknowledgements

- 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



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.

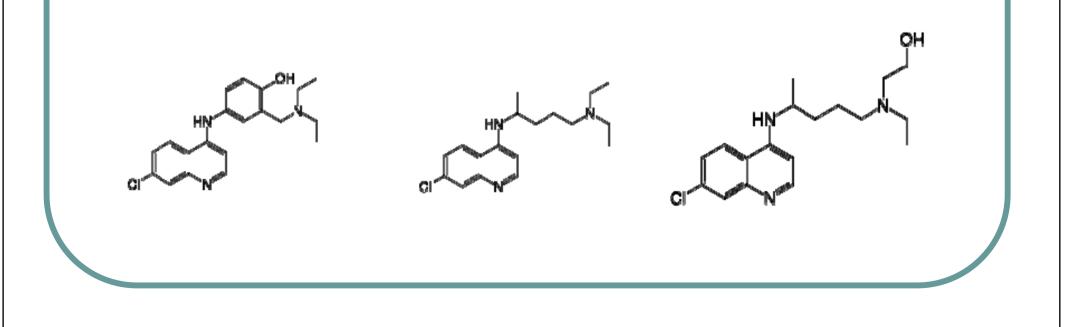
But knowing that the drug can regain its usefulness after a prolonged absence gives researchers hope that the same might be true for other resistance-plagued drug regimes. The result "is another argument for getting chloroquine out of Africa," says malaria expert Thomas Wellems of the U.S. National Institute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

904

10 NOVEMBER 2006 VOL 314 SCIENCE www.sciencemag.org 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	С	V	I	E	Т	I	Η	S	E	S	I	Ι
Sudan stain ,Dd2 Thailand	С	V	I	E	Τ	I	Η	S	E	S	Т	Ι
106/1 ^{K76}	С	v	Ι	Е	к	I	н	S	E	S	I	I
7G8 Brazil, FCQ22 PNG , INDO 19 Thailand	S	V	м	N	T	I	H	S	Q	D	L	R

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

J10 vs BC36

Sequence Name	< Pos = 3798									
- Consensus	GTTTAAACTTATTT		GGATAATATT 3830	TTTATTTATA 2840	TTTTAAGTAT	TATTTATTTA 3860	AGTGTATGTG 3870	TAATTGAAAC 3880	AATTTTTGCT 3890	AAAAGAACTTTA. 3900
3 Sequences D42-20-30k.seq J10 BC36	GTTT AAACTT ATTT GTTT AAACTT ATTT GTTT AAACTT ATTT	ITAAAGAGATTAA ITAAAGAGATTAA	GGAT AAT ATT GGAT AAT ATT	TTT ATTT AT A TTT ATTT AT A	TTTT AAGT ATT TTTT AAGT ATT	FATTTATTTA FATTTATTTA	AGT GT AT GT G AGT GT AT GT G	TAATTGAAAC TAATTGAAAA	AATTTTTGCT AATTTTTGCT	AAAAGAACTTTA. AAAAGAACTTTA.
		K AA		. Λ	T CA					
		AA		→ <i> </i> -						

Results from DNA alignment



Samples	Nucleotide position	nucleotide change	amino acid change	peptide
BC28	16	A>C	K6Q	CP1
	12	A>C	A4A	CP1
BC35	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	1661	TM1
J6	290	A>T	H97L	TM2
PCM11	290	A>T	H97L	TM2
KS25	290	A>T	H97L	TM2

Overview

Introduction

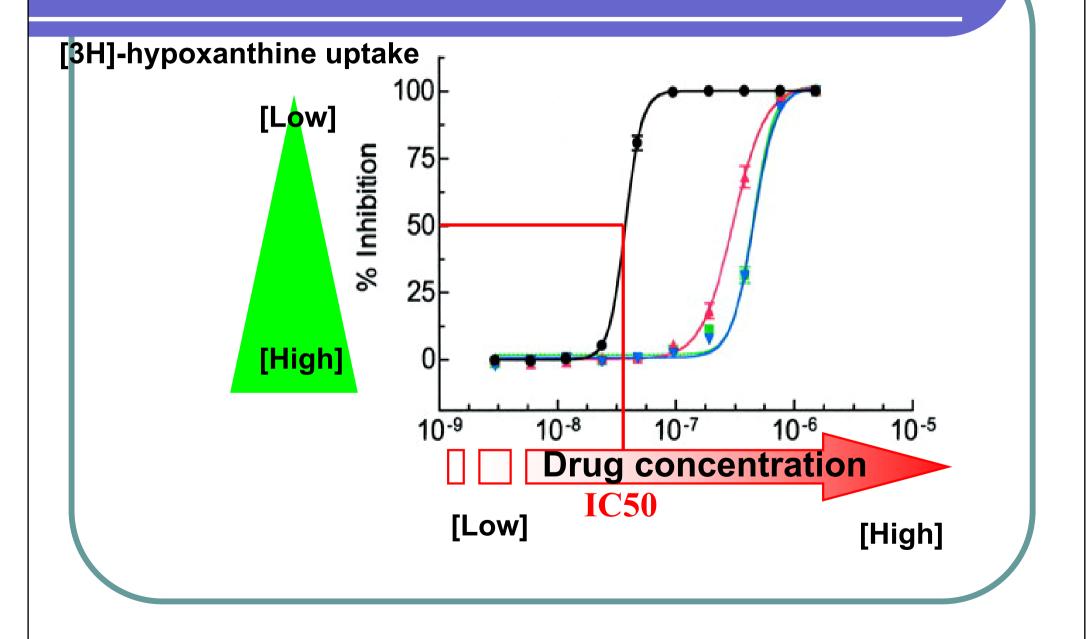
- Basic knowledge of malaria
- Antimalarial drug i.e., Chloroquine
- Chloroquine resistant *P.falciparum* and *pfcrt* gene

Research

- Materials and Methods
- Results
- Conclusions
- Future plan



Antimalarial drug response assays



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)	



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

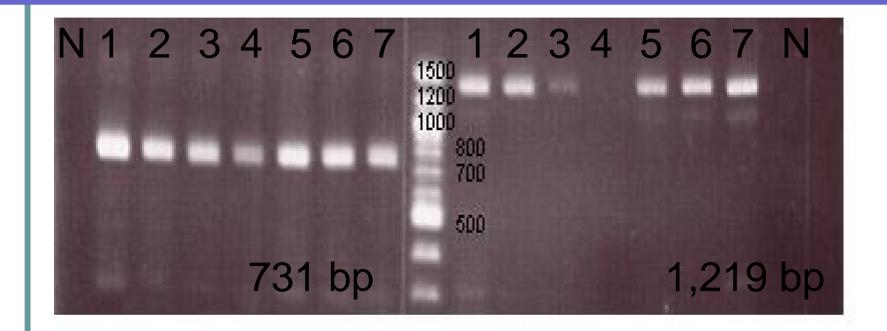
- A point mutation, or single base substitution, is a type of mutation that causes the replacement of a single base nucleotide with another nucleotide.
- Genetic polymorphism is the occurrence together in the same locality of two or more discontinuous forms of a species in such proportions that the rarest of them cannot be maintained just by recurrent mutation [5]. It is sometimes called <u>balancing selection</u>, and is intimately connected with the idea of <u>heterozygote</u> <u>advantage</u>.

TM analysis program

Protscal

• TMMHM 2.0

PCR results from E1-2 and xxx





PCR results from xxxx



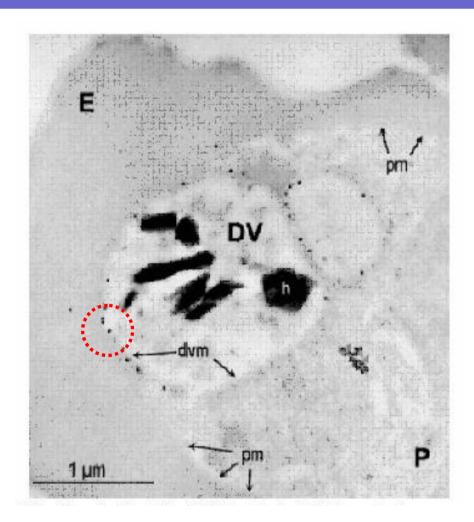


Mechanisms of drug resistance

- 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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