CHAPTER FOUR

4.0 RESULTS

4.1 Socio- demographic characteristics of respondents

The socio-demographic profile of the respondents revealed (31.0%) were in age group of 6-10 years and (71.8%) of the 135 respondents were students. A large number of respondents (73.8%) were single (Table 1).

4.2 Respondents' awareness of signs and symptoms of malaria

Fever (71.4%), vomiting (64.3%), headache (69%) and loss of appetite (83.3%) were the common symptoms of malaria mentioned among the respondents while paleness of the eyes (2.4%) and body weakness (2.4%) were the least mentioned (Figure 5).

4.3 Drug Acquisition by Respondents

As regards the drug the respondents took when they had fever in the two weeks preceeding the survey, a larger percentage (over 50%) reported that it was prescribed by a qualified health practitioner while the least percentage (7.1%) owed it to their friend's advice (Fig. 6).

4.4A Drug use pattern of respondents

Half of the respondents reported having fever two weeks preceding the study. Of the 135 respondents, half reported to have taken ACT while (23.8%) reported to have taken nothing. Most of the respondents, 81% reportedly bought their drug from patent medicine vendors (PMV). Dosage completion was high as (64.3%) reported completing the drug dosage (Table 2).

Table 1: Socio-demographic characteristics of respondents

Characteristic	Number (N)	Percentage (%)
Age Group		
≤5	32	23.8
6-10	42	31.0
11-20	16	11.9
21-30	19	14.3
≥31	26	19.0
Total	**135**	**100**
Sex		
Male	74	54.8
Female	61	45.2
Total	**135**	**100**
Occupation		
Student	97	71.8
Trading	32	23.7
Farming	6	4.4
Total	**135**	**100**
Marital Status		
Single	100	73.8
Married	35	26.2
Total	**135**	**100**

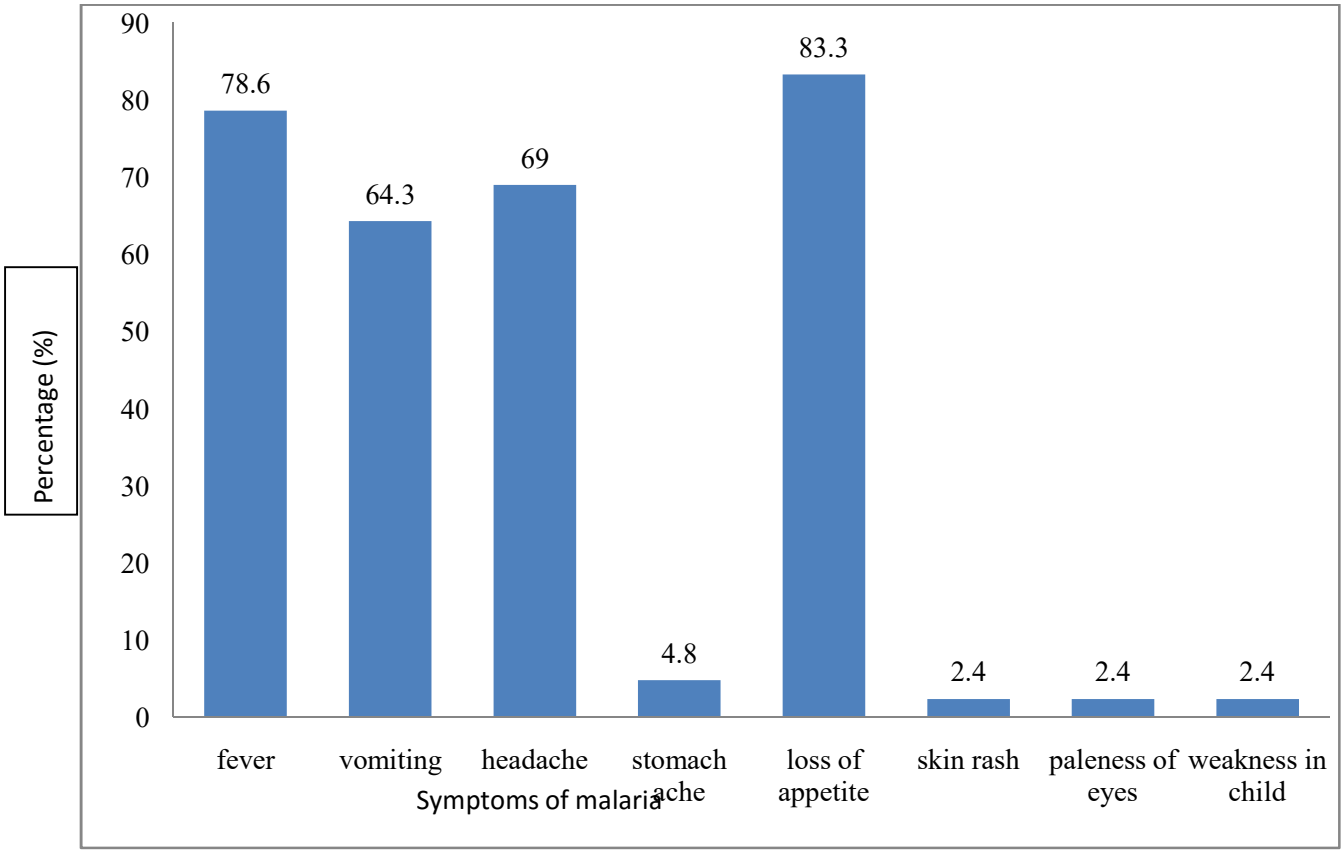


Figure 5: Symptoms of malaria mentioned by the respondents

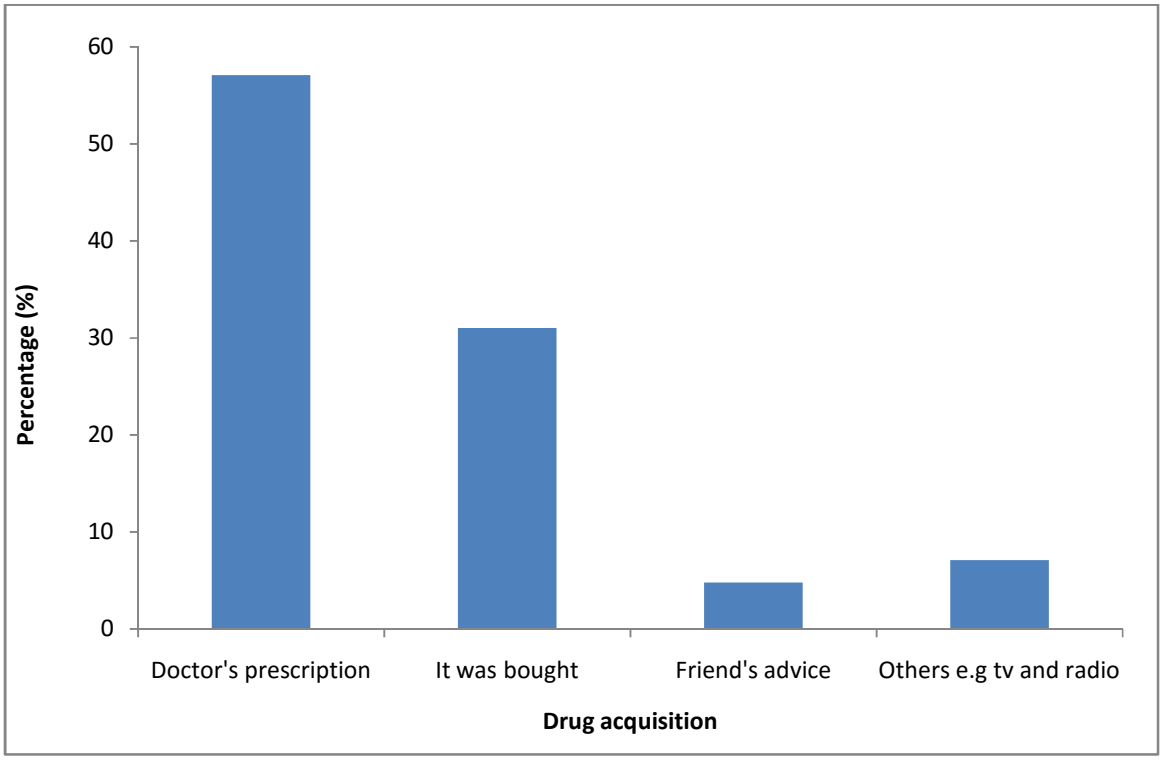


Figure6:Route of drug acquisition by respondents

Table 2: Type of drugs used for malaria treatment by respondents

Variable	Number (N)	Percentage (%)
Medication taken		
ACT	68	50.0
SP	16	11.9
Paracetamol	13	9.5
Herbal medicine	6	4.8
No medication taken	32	23.8
Total	**135**	**100**
Place of drug acquisition		
PMV	109	81.0
Roadside	26	19.0
Total	**135**	**100**
Dosage completion		
Yes	87	64.3
No	48	35.7
Total	**135**	**100**
Reasons for non-completion of drug		
Fever subsided	13	27.1
Drug not convenient	9	18.7
No improvement	10	20.8
Reaction to drug	3	6.3
No reason	13	27.1
Total	**48**	**100**

PMV = Patent Medicine Vendors, SP = Sulphadoxine-Pyrimethamine, ACT = Arthemesinin-based Combination Therapy

B. Adverse reaction to drug and drug preference

Eighty-three percent of the respondents claimed they did not react to the drug while (59.5%) expressed their willingness to take the drug again due to its effectiveness. Medication taken, source of drug prescription, drug convenience, days fever subsided and reaction to drug all had statistical significance ($p<0.05$) with the choice of drug taken as shown in Table 3.

C.Factors affecting ACT Uptake

Age, occupation, source of drug prescription, subsiding of fever, days fever subsided and convenience of drug all had statistical significance with the choice of drug taken as shown in Table 4.

D. Dosage Completion

Drug convenience, source of drug prescription, occupation, if fever subsided, all had statistical significance with willingness to complete drug dosage as shown in Table 5.

E. Drug Preference among respondents

Medication taken, source of drug prescription, drug convenience, days fever subsided and reaction to drug all had statistical significance with willingness to take that drug again as shown in Table 6.

Table 3: Distribution of respondents who have had episode of adverse reactions

Variable	Number (N)	Percentage (%)
Episode of adverse reaction		
Yes	23	16.7
No	112	83.3
Total	135	100
Types of reactions		
Drowsiness	9	39.1
Nausea	5	21.7
Headaches	6	26.1
Vomiting	3	13.1
Total	23	100
Would you like to use drug again for malaria treatment?		
Yes	80	59.5
No	55	40.5
Total	135	100
Why would you treat malaria with the same drug?		
Doctor's recommendation	3	2.4
It is effective	77	57.1
Total	80	59.5
Why would you not treat malaria with the same drug?		
Has side effects	3	2.4
No improvement	13	9.5
No response	39	28.6
Total	55	40.5

Table 4: Factors influencing Artemisinin-based Combination Therapy (ACT) Usage

Variable	Drug				
	ACT N(%)	**Others N(%)**	**χ^2**	**df**	***p*-value**
Age			**12.092**	**4**	**0.017**
Less than 5	22(70.0)	10(30.0)			
5-10	26(61.5)	16(38.5)			
11-20	13(80.0)	3(20.0)			
21-30	0(0.0)	19(100.0)			
31-42	6(25.0)	20(75.0)			
Occupation			**8.633**	**3**	**0.035**
Student	61(63.3)	36(36.7)			
Trading	3(4.4)	29(90.6)			
Farming	3(50.0)	3(50.0)			
Source of drug prescription			**19.808**	**3**	**0.000**
Doctor's prescription	61(79.2)	16(20.8)			
Self prescription	3(7.7)	39(92.3)			
Friend's prescription	0(0.0)	6(100.0)			
Others	3(33.3)	7(66.7)			
Did fever subside?			**9.882**	**2**	**0.007**
Yes	52(61.8)	32(38.2)			
No	0(0.0)	51(100.0)			
Days fever subsided			**12.548**	**1**	**0.000**
≤2	11(13.3)	69(86.7)			
3 days	39(70.4)	16(29.6)			
Was the drug convenient for you?			**9.722**	**1**	**0.002**
Yes	62(80.1)	15(19.2)			
No	13(22.20)	45(77.8)			

ACT = Artemisinin-based Combination Therapy. *Significant difference (p<0.05)

Table 5: Dosage Completion amongst the respondents

Variable	Was the dosage completed?				
	Yes	**No**	χ^2	**df**	***p*-value**
Occupation			**8.281**	**3**	**0.041**
Student	71(73.3)	26(26.7)			
Trading	10(31.3)	22(68.7)			
Farming	6(100)	0(0.0)			
Source of drug prescription			**24.871**	**3**	**0.000**
Doctor's prescription	74(95.8)	3(4.2)			
Self-prescription	10(23.1)	32(76.9)			
Friend	0(0.0)	6(100)			
Others	3(33.3)	7(66.7)			
Was the drug convenient?			**13.144**	**1**	**0.000**
Yes	67(87.5)	10(12.5)			
No	19(33.3)	39(66.7)			
Did fever subside?			**11.543**	**1**	**0.001**
Yes	83(76.5)	26(23.5)			
No	3(12.5)	24(87.5)			

*Significant difference ($p<0.05$)

Table 6: Drug preference among the respondents

Variable	**Would you like to take the drug again?**				
	Yes	**No**	χ^2	**df**	***p*-value**
Medication taken			22.235	**1**	**0.000**
ACT	64(80.0)	16(20.0)			
Others	3(5.9)	52(94.1)			
Source of Drug Prescription			21.314	**3**	**0.000**
Doctor	65(84.0)	12(16.0)			
Self	15(35.3)	27(64.7)			
Friend	0(0.0)	6(11.8)			
Others	6(8.0)	4(5.9)			
Drug Convenience			18.192	**1**	**0.000**
Yes	65(84.0)	12(17.6)			
No	10(16.0)	48(82.4)			
Reaction to drug			7.135	**1**	**0.008**
Yes	27(4.0)	8(35.3)			
No	108(96.0)	15(64.7)			

ACT = Artemisinin-based Combination Therapy

*Significant difference (p<0.05)

4.5Haematological parameters in malaria infected individuals

The WBC and lymphocyte had mean values that were not significantly (p<0.05) different from the control before and 14 days after treatment with ACT. While the Neutrophil had mean values significantly (p<0.05)lower than the control. The mean Neutrophil values remained lowered 14 days post ACT treatment.The mean platelet was only significantly (p<0.05) lowered on day 0 and subsequently normalized by days 7 and 14. The Eosinophil had mean values that were significantly (p<0.05) higher than the control before and 14 days post treatment with ACT (Table 7)

The mean PCV values were significantly (p<0.05) lower in patients with malaria parasite compared to the normal control range for both male and female groups before treatment. These values remained significantly (p<0.05) lower up to 14 days after treatment with ACT (Table 8)

4.6 Changes in liver function biomarkers in malaria infected individuals

There was a significant (p<0.05) change and elevation of mean liver function enzymes activities (AST, ALT and ALP) compared with control individuals. Treatment of patients with ACT had no significant corrective effect within the study period (Table 9).

4.7 Amplifications curve of 18S rRNA, *pfATPase*6 and K13 transcript in malaria parasite samples

Real-time PCR method was used for expression studies. Different expression patterns of target genes were observed in malaria parasite transcript. Each sample was represented by two repeats in the same qPCR run.Figure 7 show the standard curve of the analysed transcript. Allelic discrimination (Fig. 8) show both wild and mutant genes while amplification plot (Fig 9)depicted the various genes present in malaria parasite samples from Lagos and Osun States.

Table 7: Haematological parameters in malaria infected individuals

Duration After Treatment	**Haematological Parameters**				
	WBC (µ)	LYMPH(%)	EOSINOPHIL(%)	NEUTROPHIL(%)	PLT (µ)
Control	8.94 ± 0.11	41.98 ± 1.92	4.49 ± 0.55	56.1 ± 0.97	301.5 ± 2.33
Day 0	7.41 ± 0.62	37.26 ± 4.40	*18.25 ± 3.70	*36.39 ± 3.53	*179.93 ± 12.70
Day 7	8.73 ± 0.89	34.99 ± 5.89	*18.65 ± 0.23	*31.90 ± 5.00	239.38 ± 26.16
Day 14	9.46 ± 1.94	46.80 ± 2.87	*19.31 ± 3.90	*33.74 ± 3.04	211.64 ± 18.62

WBC=White Blood Cell, LYMPH=Lymphocyte, PLT=Platelet

*Significant difference (p<0.05)

Table 8: Packed Cell Volume (PCV) in malaria infected individuals

Treatment Days	**PCV Values (%)**	
	Male	Female
Control Range Values	40-50	37-47
Day 0	*36.8875±5.64432	*29.6143±4.54695
Day 7	*35.9800±1.24579	*31.0333±2.58908
Day 14	*37.6286±2.12502	*31.0143±5.17476

PCV=Packed Cell Volume

*Significant difference (p<0.05)

Table 9: Changes in liver function enzymes in malaria infected patients

Duration After Treatment	**Liver Function Biomarkers**		
	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Control	10.01 ± 0.16	8.94 ± 0.17	42.72 ± 0.94
Day 0	*57.14 ± 9.06	*30.08 ± 7.88	*202.52 ± 22.82
Day 7	*29.81 ± 2.94	*17.6 ± 2.26	*159.15 ± 20.04
Day 14	*35.09 ± 7.10	*25.92 ± 8.82	*179.73 ± 34.82

AST=Aspartate aminotransferase, ALT=Alanine aminotransferase, ALP=Alkaline phosphatase

*Significant difference (p<0.05)

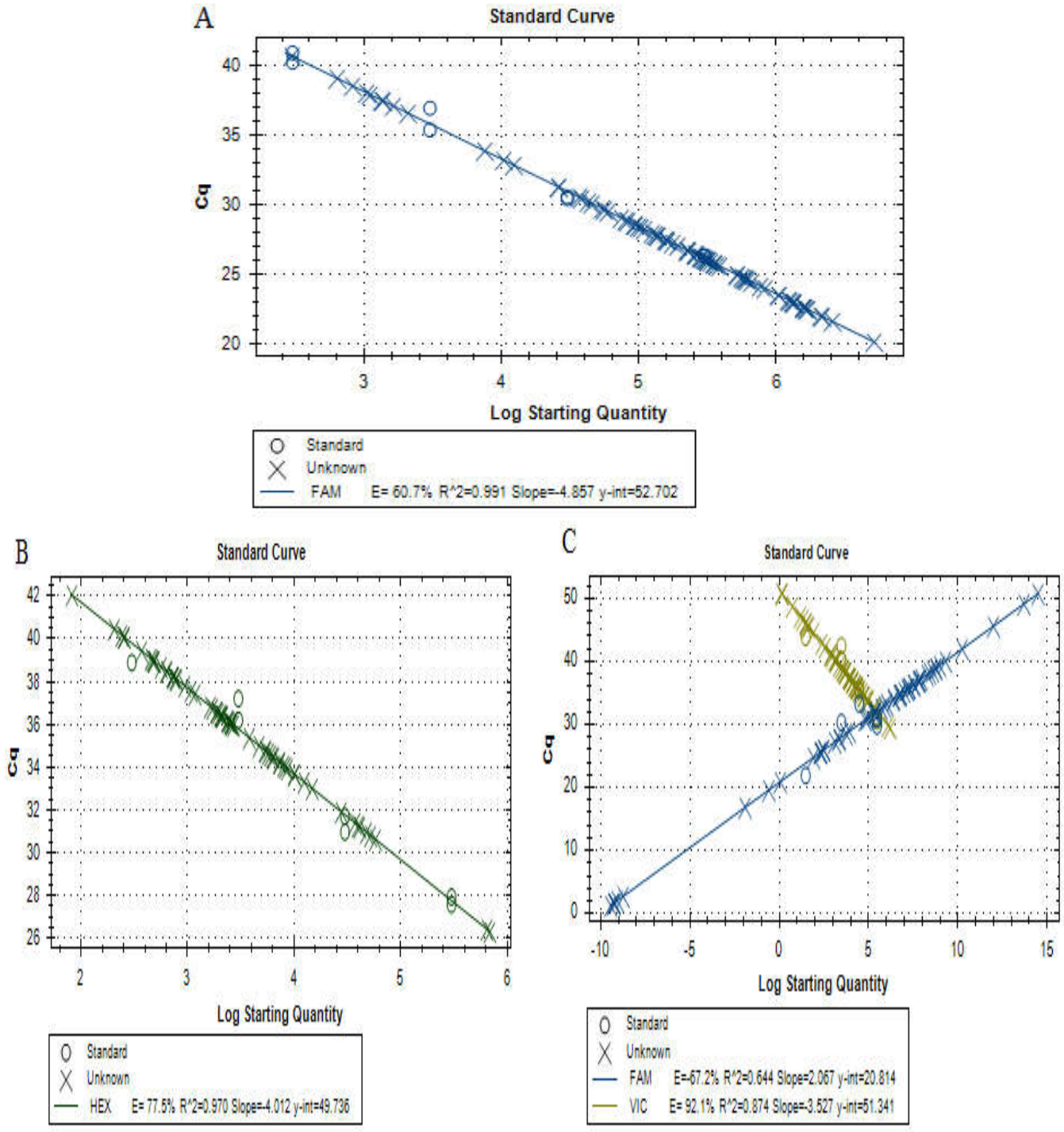


Figure 7: Standard curve of transcript in malaria parasite samples: (A) 18S rRNA (B)K-13 (C) *pfATPase*

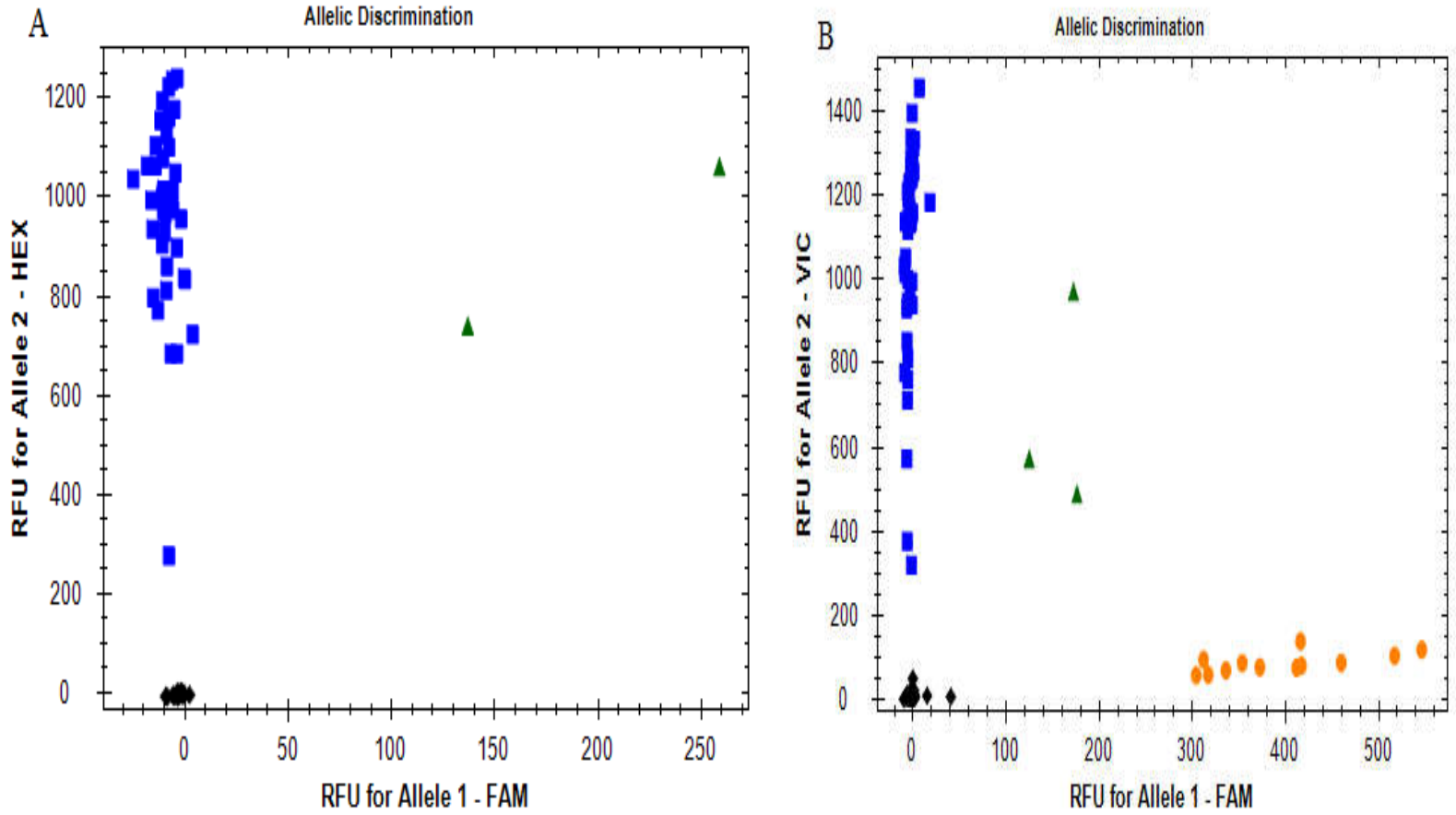


Figure 8: Allelic discrimination of wild and mutant genes in parasite samples: (A) K-13 (B) *pfATPase*

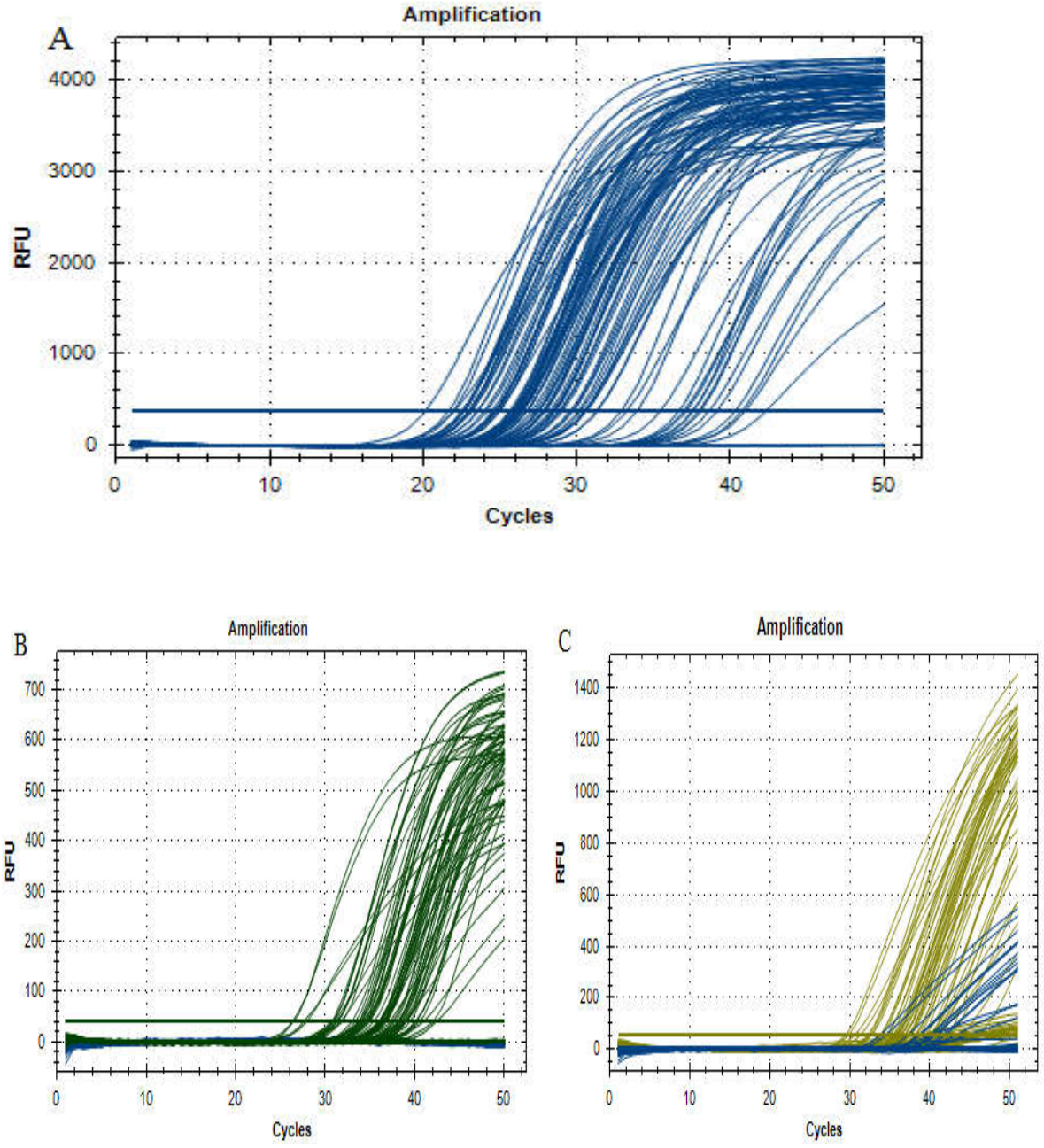


Figure 9: Amplification plots of transcript in malaria parasite samples: (A) 18S rRNA (B)K-13 (C) *pfATPase*. *each colour is as shown in Figure 7.

4.8 Gene expression studies showing the mean fold change in expression of target genes

The mean fold change in expression of targeted gene was analysed using $2^{-\Delta\Delta Ct}$. Only the wild strain of K13 gene was found in the parasite population while *pfATPase* 6 had very low expression generally; both the wild and mutant strains were however expressed (Figure 10).

4.9 Species identification of 383 samples by multiplex PCR method at different time points

Extracted DNA was quantified using NanoDrop 1000 Spectrophotometer showing a very good yield with high purity (Figure 11).Species identification using multiplex PCR method showed that all 83 samples obtained before treatmentwere positive to*Plasmodium falciparum* (Plate 1). *P. falciparum*positive samples were also found on days 2 and 3 after treatment with ACT has started (Table 10). K13, *pfATPase* 6, *pfmdr* 1 and *pfcrt* genes were amplified before sequencing was carried out (Plate2-5).

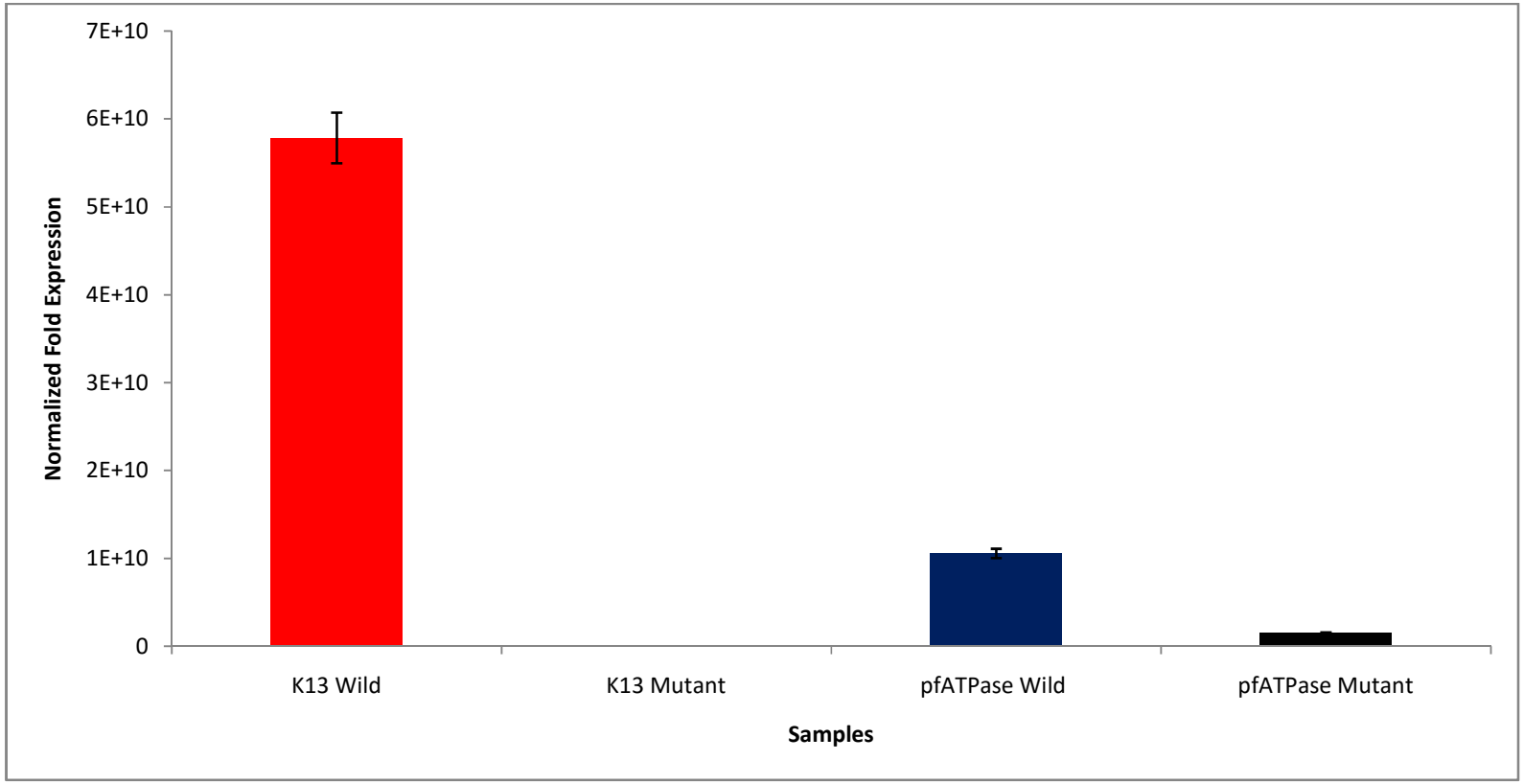


Figure 10: Gene expression profile of K13 and *pfATPase* genes using $2^{-\Delta\Delta Ct}$

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
1	Default	1/1/2006	12:09 AM	13.01	0.260	0.147	1.77	1.46	50.00	230	0.179	0.061
2	Default	1/1/2006	12:10 AM	5.28	0.106	0.084	1.26	1.26	50.00	230	0.084	0.026
3	Default	1/1/2006	12:11 AM	1.67	0.033	0.014	2.45	0.62	50.00	230	0.054	0.039
4	Default	1/1/2006	12:12 AM	11.27	0.225	0.121	1.86	1.77	50.00	230	0.127	0.032
5	Default	1/1/2006	12:13 AM	15.16	0.303	0.173	1.76	2.22	50.00	230	0.137	0.022
6	Default	1/1/2006	12:14 AM	1.76	0.035	0.013	2.78	0.38	50.00	230	0.092	0.025
7	Default	1/1/2006	12:15 AM	4.08	0.082	0.045	1.83	0.52	50.00	230	0.156	0.048
8	Default	1/1/2006	12:21 AM	6.05	0.121	0.083	1.47	0.43	50.00	230	0.283	0.283
9	Default	1/1/2006	12:22 AM	0.46	0.009	-0.004	-2.23	-17.54	50.00	230	-0.001	0.018
10	Default	1/1/2006	12:22 AM	1.90	0.038	0.018	2.12	2.14	50.00	230	0.018	0.034
11	Default	1/1/2006	12:23 AM	1.63	0.033	0.012	2.83	0.47	50.00	230	0.070	0.025
12	Default	1/1/2006	12:24 AM	4.46	0.089	0.057	1.58	0.31	50.00	230	0.287	0.082
13	Default	1/1/2006	12:25 AM	1.85	0.037	0.017	2.13	1.80	50.00	230	0.021	0.001
14	Default	1/1/2006	12:26 AM	6.40	0.128	0.089	1.43	0.42	50.00	230	0.305	0.073
15	Default	1/1/2006	12:27 AM	10.45	0.209	0.153	1.36	0.50	50.00	230	0.414	0.481
16	Default	1/1/2006	12:28 AM	3.09	0.062	0.076	0.82	1.15	50.00	230	0.054	0.504
17	Default	1/1/2006	12:29 AM	1.16	0.023	0.009	2.66	0.30	50.00	230	0.077	0.011
18	Default	1/1/2006	12:30 AM	5.47	0.109	0.089	1.23	0.23	50.00	230	0.476	0.095
19	Default	1/1/2006	12:31 AM	4.29	0.086	0.057	1.51	0.59	50.00	230	0.147	0.065
20	Default	1/1/2006	12:32 AM	2.99	0.060	0.028	2.16	0.46	50.00	230	0.130	0.045
21	Default	1/1/2006	12:33 AM	17.78	0.356	0.216	1.65	0.54	50.00	230	0.656	0.994
22	Default	1/1/2006	12:34 AM	2.13	0.043	0.023	1.89	1.68	50.00	230	0.025	0.032
23	Default	1/1/2006	12:34 AM	6.52	0.130	0.096	1.36	0.34	50.00	230	0.386	0.373
24	Default	1/1/2006	12:35 AM	38.63	0.773	0.778	0.99	0.47	50.00	230	1.651	2.815
25	Default	1/1/2006	12:36 AM	1.80	0.036	0.023	1.54	0.54	50.00	230	0.067	0.038

Figure 11: Spectrophotometric quantification of Extracted DNA samples

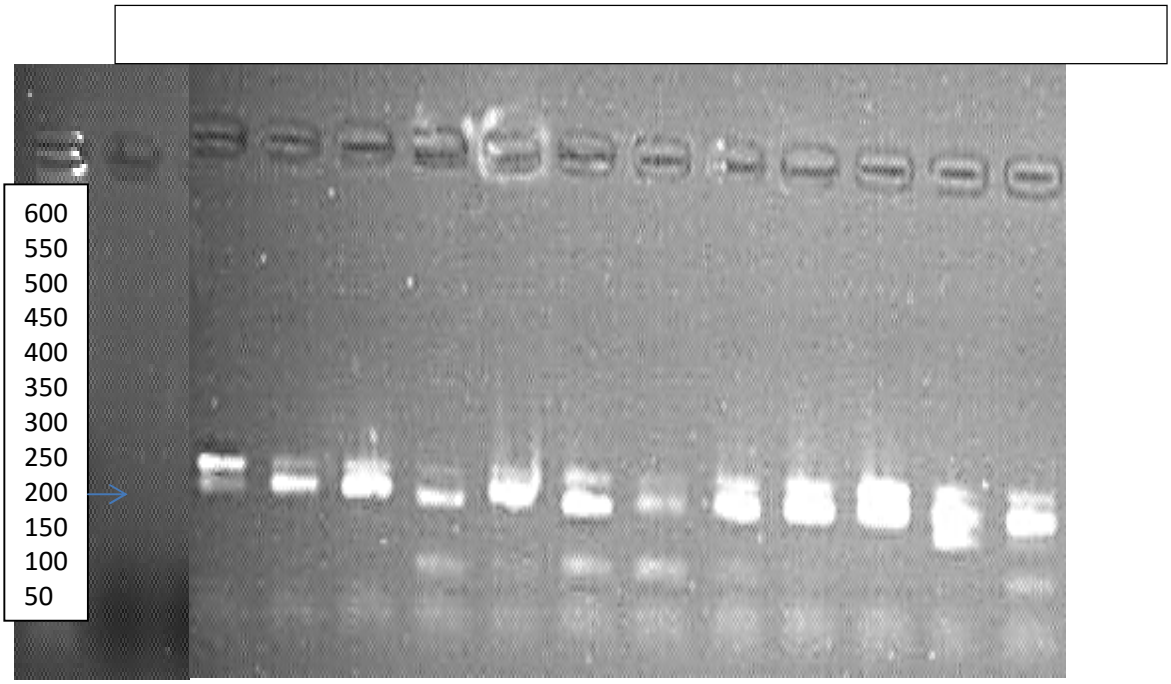


Plate 1: Multiplex Nested PCR gel picture for *Plasmodium* species identification showing *P. falciparum* with 205 base pair

L = Molecular size ladder (50bp ladder), N = Negative Control, P = Positive Control (3D7), Test samples: Lane 1,2,3,4,5,6,7,8,9,10 and 11

Table 10: Species identification by multiplex Polymerase Chain Reaction method at different time points

Treatment Days	**Day 0**	**Day 2**	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Total (%)**
Positive samples	83	39	18	0	0	0	0	140 (36.6%)
Negative samples	0	11	32	50	50	50	50	243 (63.4%)
Total	83	50	50	50	50	50	50	383 (100%)

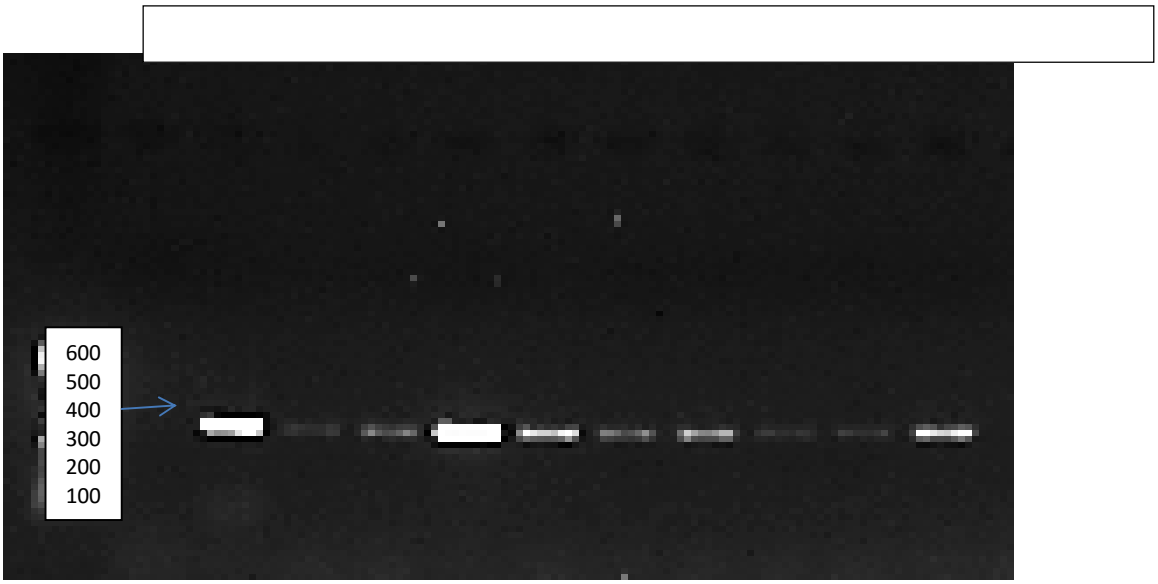


Plate 2: The PCR amplification of K-13 genes of extracted gDNA.K13 gene amplified by conventional PCR method to give a product of 380 base pair.

L = Molecular size ladder (100bp ladder), N = Negative Control, P = Positive Control (3D7), Test samples: Lane 1,2,3,4,5,6

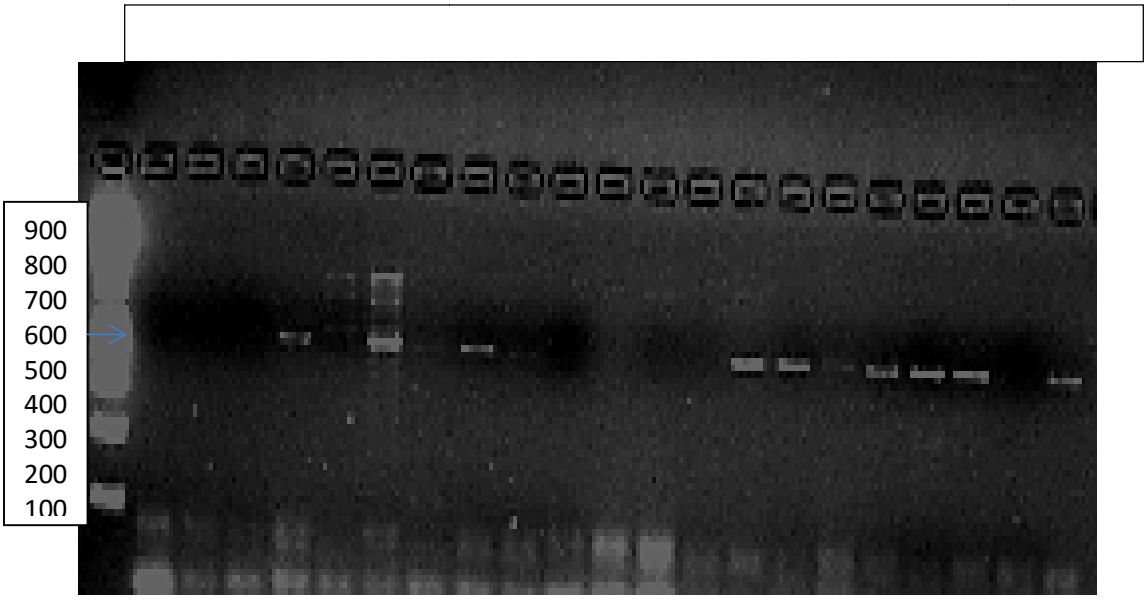


Plate 3: The PCR amplification of *pfATPase* 6 genes of extracted gDNA.*pfATPase* 6 gene amplified by conventional PCR method to give a product of 650 base pair.

L = Molecular size ladder (100bp ladder), N = Negative Control, P = Positive Control (3D7), Test samples: Lane 1,2,3,4,5,6

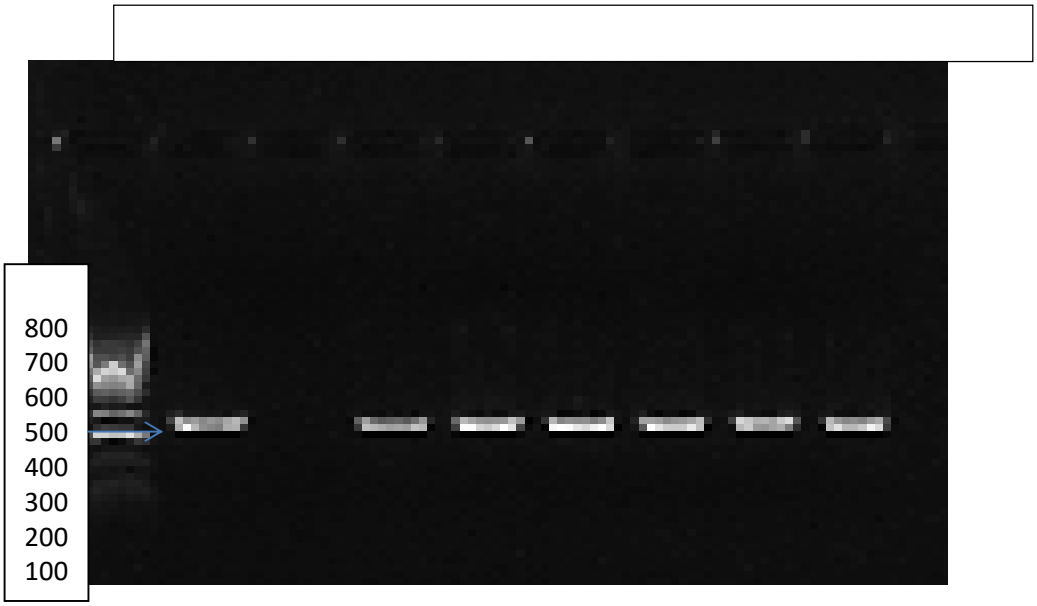


Plate 4: The PCR amplification of *pfmdr* 1 genes of extracted gDNA.*pfmdr* 1 gene amplified by nested PCR method to give a product of 530 base pair.

L = Molecular size ladder (100bp ladder), N = Negative Control, P = Positive Control (3D7), Test samples: Lane 1,2,3,4,5,6

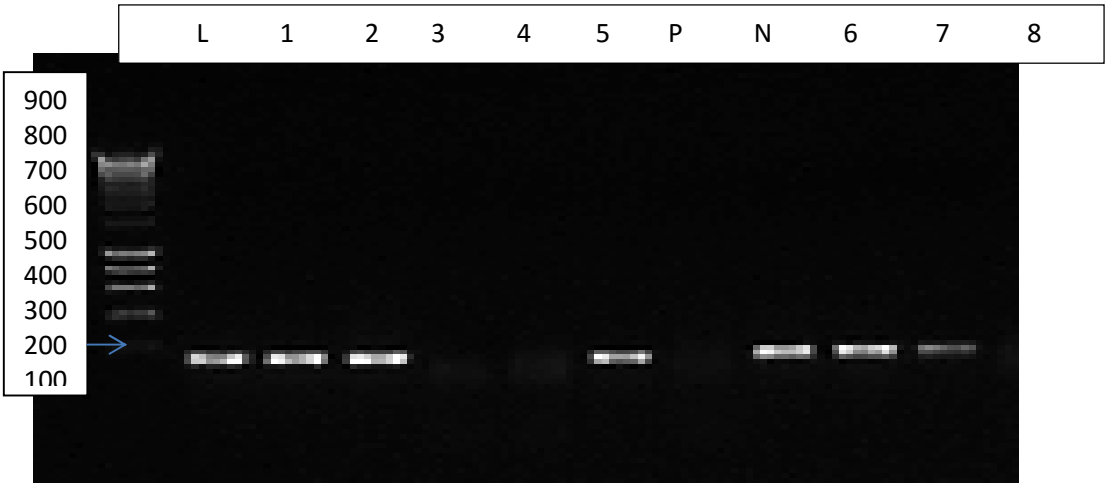


Plate 5: The PCR amplification of *pfcrt* genes of extracted gDNA.*pfcrt* gene amplified nested PCR method to give a product of 160 base pair.

L = Molecular size ladder (100bp ladder), N = Negative Control, P = Positive Control (3D7), Test samples: Lane 1,2,3,4,5,6

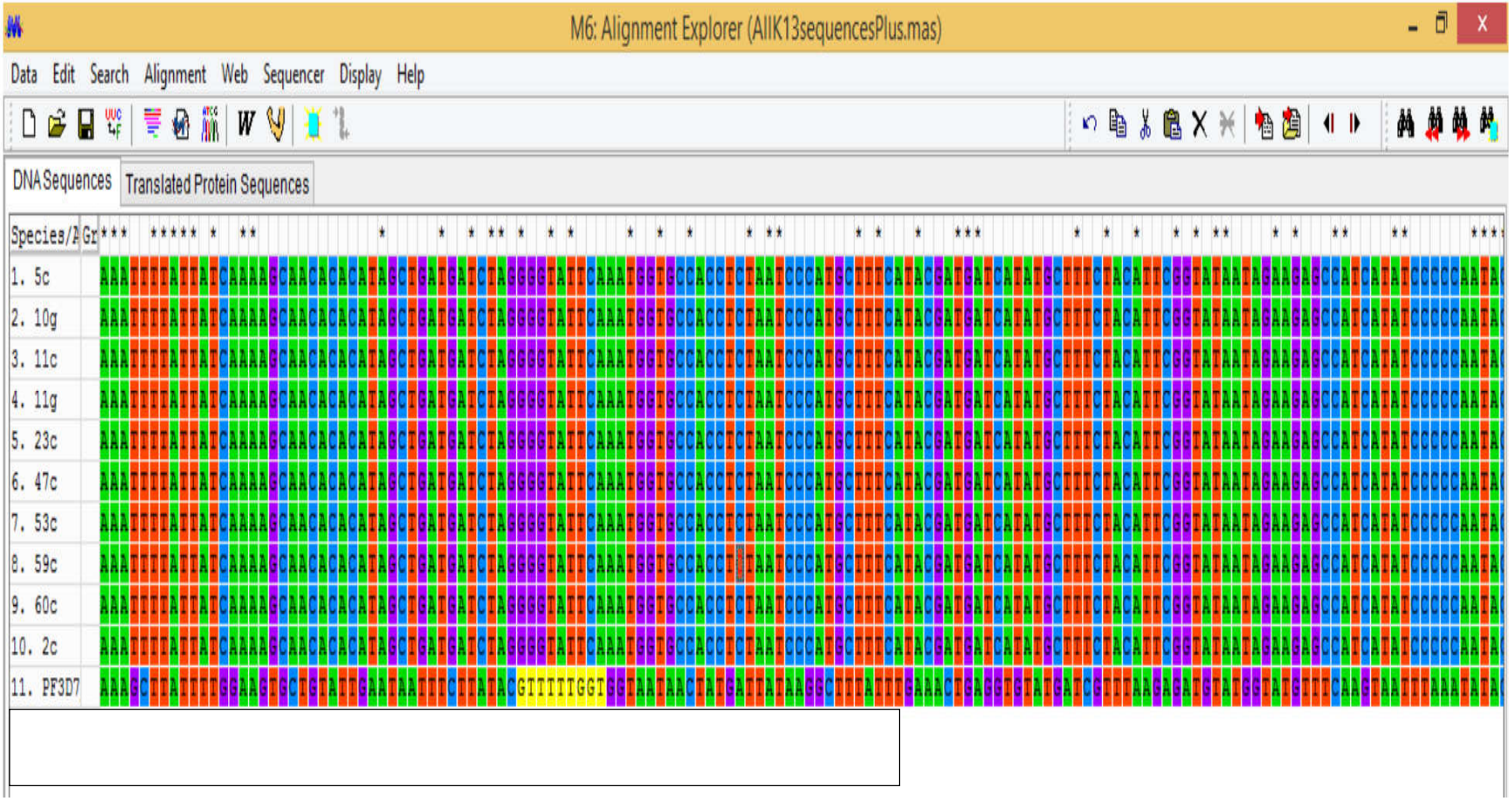
4:10 Distribution and prevalence of K13, *pfATPase* 6, *pfmdr* 1 and *pfcrt* polymorphism

The aligned sequence of K13 gene with *P. falciparum*reference gene is shown in fig. 12. From analysis, 8 SNPs were detected (Table 11). All the 8 mutation detected in K13 gene are non-synonymous with A557 having the highest prevalence (3.60%) in the parasite study population.

Figure 13 show the aligned sequence of *pfATPase* 6 gene with *P. falciparum*reference gene. The analysis of the sequence detected 3 SNPs,a synonymous and two non-synonymous mutations were detected in the *pfATPase* 6 gene (Table 12). The most reported ACT resistance related SNP (S769) had the lowest prevalence of the 3 reported (S679, M699 and S769) in this study.

Figure 14 show the aligned sequence of *pfmdr* 1 gene with *P. falciparum*reference gene. The study population had 2 SNPs, N86 SNP (56%), which has been reported to contribute to resistance of malaria parasite to artemisinin while Y184 had very low prevalence as shown in Table 13.

Figure 15 show the aligned protein sequence of *pfcrt* gene with *P. falciparum* reference gene. *pfcrt* haplotype for antimalarial drug resistance common in Africa is the CVIET. Analysis of the studied sequences of *pfcrt* showed that CVIET had prevalence as high as 45% in the parasite study population (Figure 16).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Data Edit Search Alignment Web Sequencer Display Help
DNA Sequences
Translated Protein Sequences
Species/P Gr
1. 5c
2. 10g
3. 11c
4. 11g
5. 23c
6. 47c
7. 53c
8. 59c
9. 60c
10. 2c
11. PF3D7

Table 11: Prevalence of K13-propeller polymorphisms

Codon Position (SNPs)	**Reference AA**	**Reference Nucleotide**	**Mutant AA**	**Mutant Nucleotide**	**N**	**Prevalence (%)**
496	G	ggt	S	tca	1	1.20
539	R	aga	F	ttt	1	1.20
543	I	att	V	gta	1	1.20
557	A	gca	?	-aa	3	3.61
566	V	gta	K	aaa	1	1.20
578	A	gct	K	aaa	2	2.41
580	C	tgt	Y	tat	1	1.20
584	D	gat	I	att	1	1.20

SNP = Single Nucleotide Polymorphism
N = Number of SNPs
= Nucleotide deletion
? = Not an amino acid

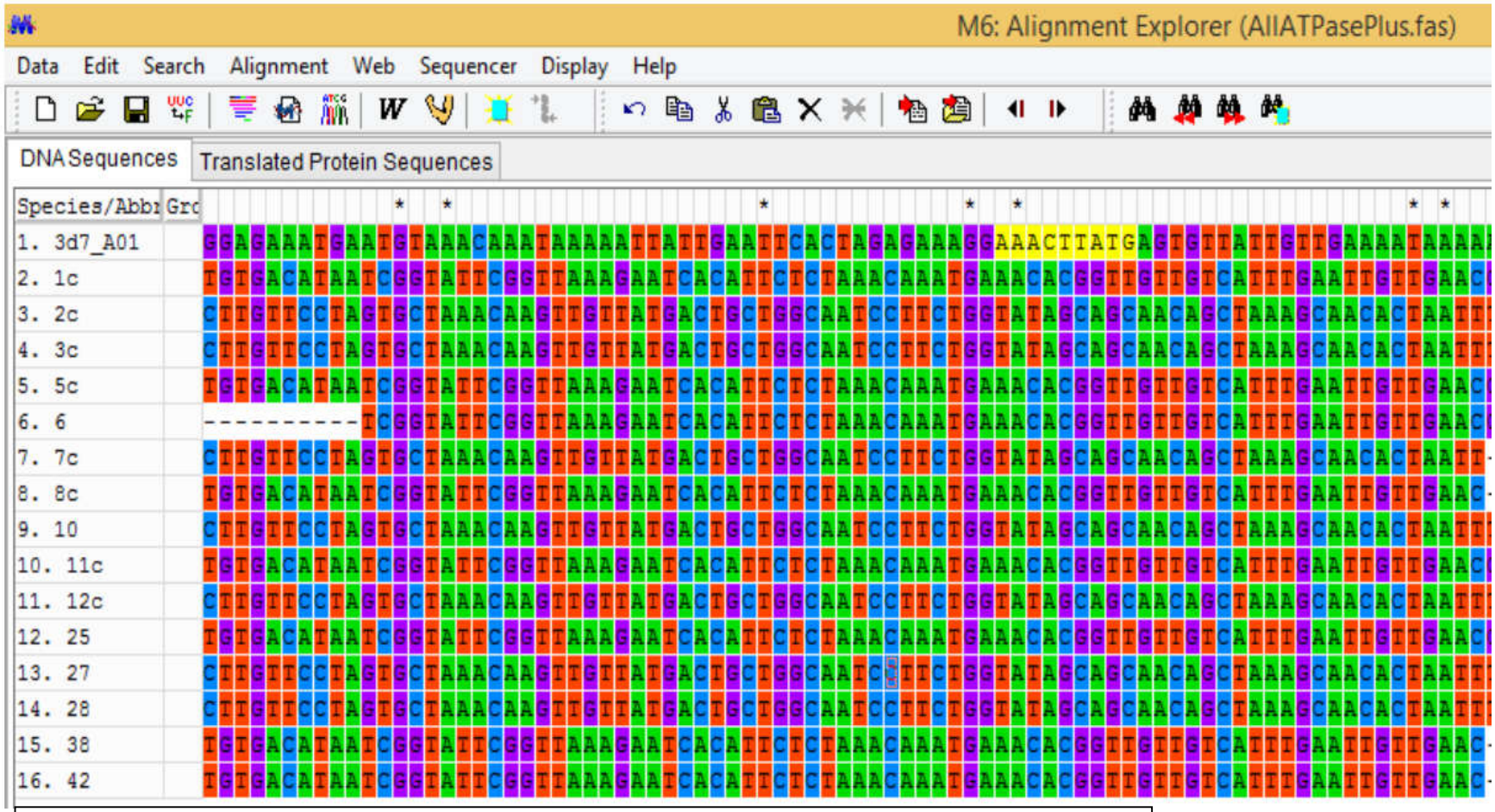


Figure 13: Aligned nucleotide sequences of *pfATPase* 6 gene with reference sequence showing SNP

Table 12: Prevalence of *pfATPase* 6 polymorphisms

Codon Position (SNPs)	**Reference AA**	**Reference Nucleotide**	**Mutant AA**	**Mutant Nucleotide**	**N**	**Prevalence (%)**
679	S	tct	S	tcc	7	8.43
699	M	atg	V	gtt	8	9.63
769	S	agt	M	atg	3	3.61

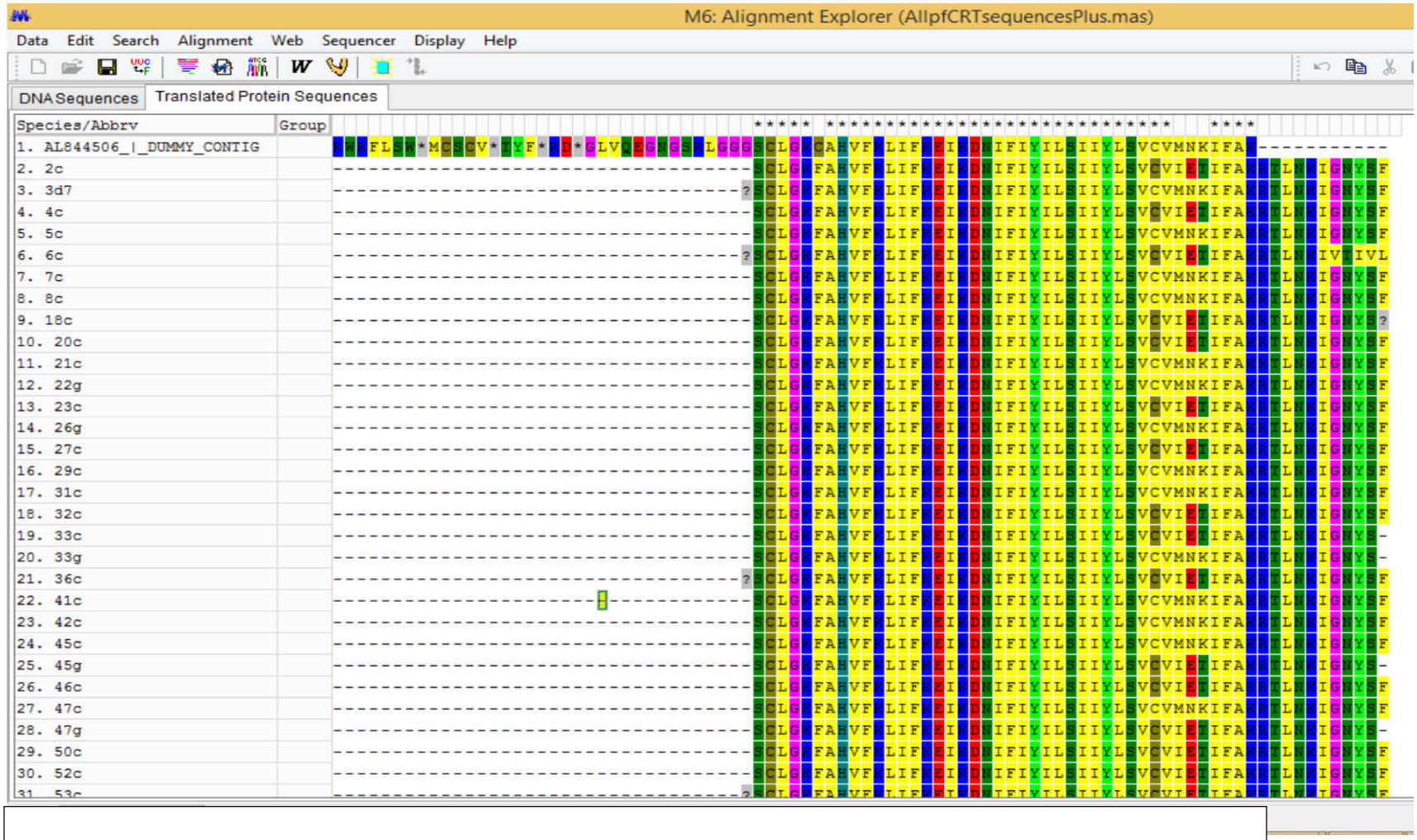
SNP = Single Nucleotide Polymorphism
N = Number of SNPs

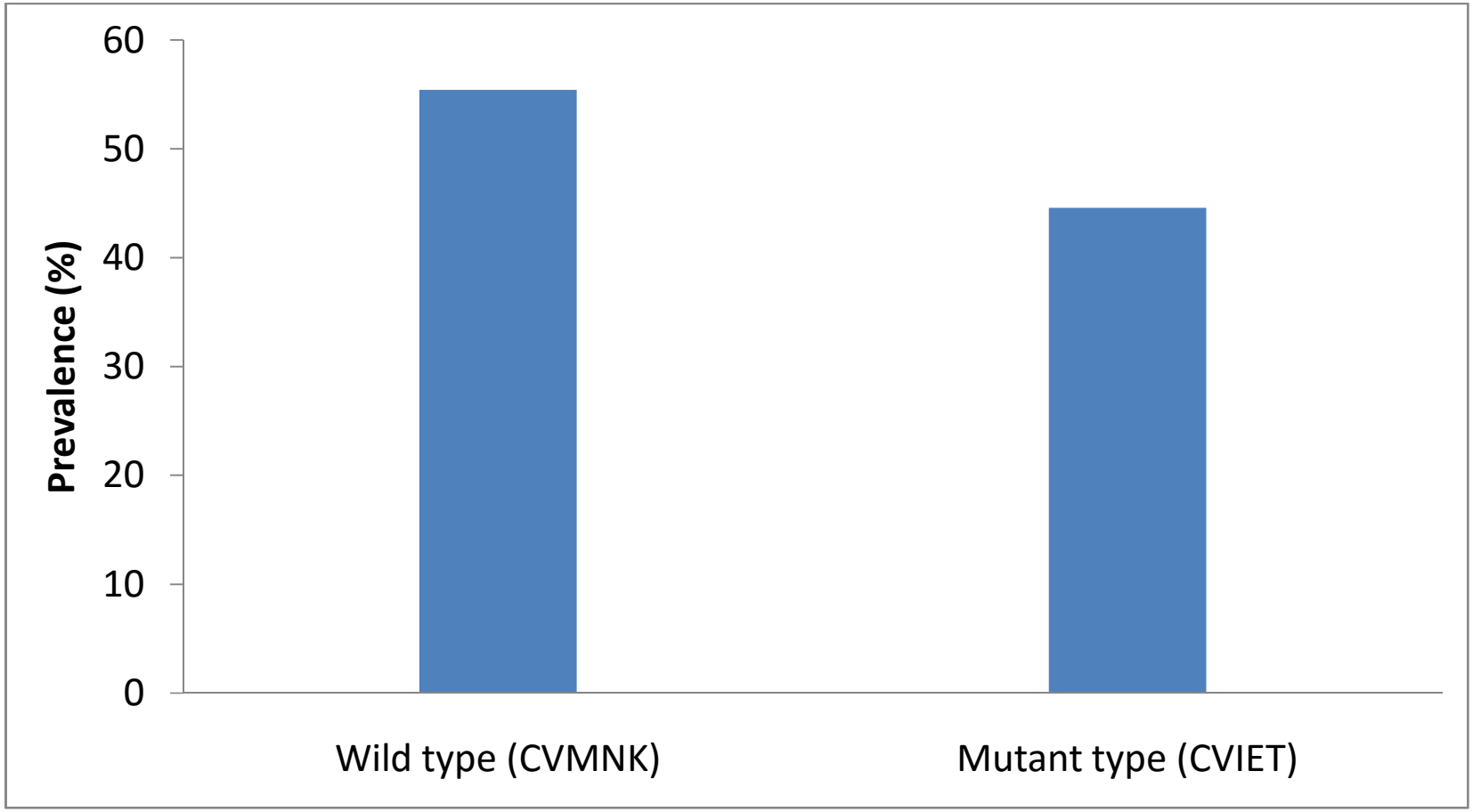

M6: Alignment Explorer (AllpfMDRsequences.mas)
Data Edit Search Alignment Web Sequencer Display Help
DNA Sequences
Translated Protein Sequences
Species/Gr
1. 3D7
2. 2c
3. 4c
4. 5c
5. 6c
6. 7c
7. 8c
8. 8g
9. 10c
10. 11c
11. 20c
12. 23c
13. 36c
14. 41c
15. 47c
16. 50c
17. 51c
18. 53c
19. 54c
20. 55c
21. 57c
22. 59c
23. 63c

Table 13: Prevalence of *pfmdr*1 polymorphisms

Codon Position (SNPs)	**Reference AA**	**Reference Nucleotide**	**Mutant AA**	**Mutant Nucleotide**	**N**	**Prevalence (%)**
86	N	aat	K	aaa	47	56.63
184	Y	tat	F	taa	11	13.25

SNP = Single Nucleotide Polymorphism
N = Number of SNPs


M6: Alignment Explorer (AllpfCRTsequencesPlus.mas)
Data Edit Search Alignment Web Sequencer Display Help
DNA Sequences
Translated Protein Sequences
Species/Abbrv
Group
1. AL844506_|_DUMMY_CONTIG
2. 2c
3. 3d7
4. 4c
5. 5c
6. 6c
7. 7c
8. 8c
9. 18c
10. 20c
11. 21c
12. 22g
13. 23c
14. 26g
15. 27c
16. 29c
17. 31c
18. 32c
19. 33c
20. 33g
21. 36c
22. 41c
23. 42c
24. 45c
25. 45g
26. 46c
27. 47c
28. 47g
29. 50c
30. 52c



CVMNK = Cysteine-Valine-Methionine-Asparagine-Lysine, CVIET = Cysteine-Valine-Isoleucine-Glutamic acid-Threonine

Figure 16: Distribution and prevalence of *pfcrt* polymorphisms