A seven-year surveillance of epidemiology of malaria reveals travel and gender are the key drivers of dispersion of drug resistant genotypes in Kenya

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Malaria drug resistance is a global public health concern. Though parasite mutations have been associated with resistance, other factors could influence the resistance. A robust surveillance system is required to monitor and help contain the resistance. This study established the role of travel and gender in dispersion of chloroquine resistant genotypes in malaria epidemic zones in Kenya. 1,776 individuals presenting with uncomplicated malaria at hospitals selected from four malaria transmission zones in Kenya between 2008 and 2014 were enrolled in a prospective surveillance study assessing the epidemiology of malaria drug resistance patterns. Demographic and clinical information per individual was obtained using a structured questionnaire. Further, 2mL of blood was collected for malaria diagnosis, parasitemia quantification and molecular analysis. DNA extracted from dried blood spots collected from each of the individuals was genotyped for polymorphisms in *Plasmodium falciparum* chloroquine transporter gene (*Pfcrt* 76), *Plasmodium falciparum* multidrug resistant gene 1 (*Pfmdr*1 86 and *Pfmdr*1 184) regions that are putative drug resistance genes using both conventional polymerase chain reaction (PCR) and real-time PCR. The molecular and demographic data was analyzed using Stata version 13 (College Station, TX: StataCorp LP) while mapping of cases at the selected geographic zones was done in ArcGIS version 10. Chloroquine resistant (CQR) genotypes across gender revealed an association with chloroquine resistance by both univariate model (*p = 0.027*) and by multivariate model (*p = 0.025*), female as reference group in both models. Prior treatment with antimalarial drugs within the last 6 weeks before enrollment was associated with carriage of CQR genotype by multivariate model (*p = 0.034*). Further, a significant relationship was observed between travel and CQR carriage both by univariate model (*p = 0.001*) and multivariate model (*p = 0.002*). These findings suggest that gender and travel

are significantly associated with chloroquine resistance. From a gender perspective, males are more likely to harbor resistant strains than females hence involved in strain dispersion. On the other hand, travel underscores the role of transport network in introducing spread of resistant genotypes, bringing in to focus the need to monitor gene flow and establish strategies to minimize the introduction of resistance strains by controlling malaria among frequent transporters.

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Abstract

40   Malaria drug resistance is a global public health concern. Though parasite mutations have been

41   associated with resistance, other factors could influence the resistance. A robust surveillance

42   system is required to monitor and help contain the resistance. This study established the role of

43   travel and gender in dispersion of chloroquine resistant genotypes in malaria epidemic zones in

44   Kenya. 1,776 individuals presenting with uncomplicated malaria at hospitals selected from four

45   malaria transmission zones in Kenya between 2008 and 2014 were enrolled in a prospective

46   surveillance study assessing the epidemiology of malaria drug resistance patterns. Demographic

47   and clinical information per individual was obtained using a structured questionnaire. Further,

48   2mL of blood was collected for malaria diagnosis, parasitemia quantification and molecular

49   analysis. DNA extracted from dried blood spots collected from each of the individuals was

50   genotyped for polymorphisms in *Plasmodium falciparum* chloroquine transporter gene (*Pfcrt*

51   76), *Plasmodium falciparum* multidrug resistant gene 1 (*Pfmdr*1 86 and *Pfmdr*1 184) regions that

52   are putative drug resistance genes using both conventional polymerase chain reaction (PCR) and

53   real-time PCR. The molecular and demographic data was analyzed using Stata version 13

54   (College Station, TX: StataCorp LP) while mapping of cases at the selected geographic zones was

55   done in ArcGIS version 10. Chloroquine resistant (CQR) genotypes across gender revealed an

56   association with chloroquine resistance by both univariate model ($p = 0.027$) and by multivariate

57   model ($p = 0.025$), female as reference group in both models. Prior treatment with antimalarial

58   drugs within the last 6 weeks before enrollment was associated with carriage of CQR genotype

59   by multivariate model ($p = 0.034$). Further, a significant relationship was observed between

60   travel and CQR carriage both by univariate model ($p = 0.001$) and multivariate model ($p =

61   0.002$). These findings suggest that gender and travel are significantly associated with

62   chloroquine resistance. From a gender perspective, males are more likely to harbor resistant

63   strains than females hence involved in strain dispersion. On the other hand, travel underscores

64   the role of transport network in introducing spread of resistant genotypes, bringing in to focus the

65   need to monitor gene flow and establish strategies to minimize the introduction of resistance

66   strains by controlling malaria among frequent transporters.

67 Introduction

68   In 2006, the World Health organization (WHO) recommended artemisinin-based combination

69   therapy (ACTs) use as first-line drug for treatment of uncomplicated malaria following

70   widespread failure of sulfadoxine-pyrimethamine combination treatment. The strategy relied on

71   the fast acting artemisinin derivatives rapidly to bring down the parasite biomass, relieves

72   symptoms while the partner drug with longer half-life clears the residual parasites therefore

73   cushioning the artemisinin derivative from emerging resistance (Nosten & White, 2007). The

74   most widely used combinations globally include artemether-lumefantrine (AL, Coartem),

75   dihydroartemisinin-piperaquine (DHAPPQ), artesunate-amodiaquine (ASAQ) and artesunate-

76   mefloquine. These treatments alongside other intervention have contributed to about 37%

77   decline in malaria cases and 60% reduction in mortality during the last fifteen years (Dhiman,

78   2019). However, the recent reports of resistance to artemisinin derivative of the ACTs in

79   Southeast Asia (Woodrow & White, 2017) and its pattern of migration (Shetty *et al.*, 2019)

80   threaten this trajectory due to fears that the global dissemination of these strains would follow

81   historical pattern of drug resistant strain dispersal. Africa parasite diversity studies show that

82   these strains have not yet been detected in Africa (Kamau *et al.*, 2015), but shows extant

83   subpopulations of *P. falciparum* in sub-Saharan Africa (Amambua-Ngwa *et al.*, 2019) suggestive

84   of parallel emergence of ACT resistance in the region given the varying first-line drugs in across

85   Africa. Notwithstanding the origin of the initial resistance, it is essential to understand patterns of

86   the dispersal present-day characterized strains in natural infections at local level as a stop-gap for

87   containment should ACT resistant strains emerge.

88   Non-synonymous mutations in the kelch propeller domain (K13-propeller) in *Plasmodium*

89   *falciparum* have been associated with artemisinin resistance in samples from Southeast Asia

90   (Witkowski *et al.*, 2013). Further, the waning efficacy of ACTs has been partly associated with

91   failure of the quinoline partners in the combination (Borrmann, Matsiegui, Missinou, &

92   Kremsner, 2008). Though most of these drugs have not been policy recommended, cross-

93   resistance has been shown to occur owing to similarity in mechanisms of actions (Veiga *et al.*,

94   2016). A case in point is the diminished mefloquine sensitivity in Kenya (Eyase *et al.*, 2013) yet

95   mefloquine had neither been first-line nor second-line treatment in the country. Single

96   Nucleotide Polymorphisms (SNPs) in the *P. falciparum* multidrug resistance protein (*Pfmdr1*)

97   and *P. falciparum* chloroquine resistance transporter (*Pfcrt*) genes confer resistance to a number

98   of anti-malaria drugs. Chloroquine resistance has been associated to *Pfcrt* K76T SNP

99   (Mohammed et al., 2013; Zhao et al., 2019). *Pfmdr1* 86Y and Y184 haplotypes have been shown

100   to modulate chloroquine (CQ) resistance in the presence of *Pfcrt* 76T mutation *(Venkatesan et*

101   *al.*, 2014). Mefloquine (MQ) and lumefantrine (LU) sensitivities are linked to *Pfmdr*1 86Y point

102   mutation. Additionally, emerging *Pfcrt* K76 allele carrying parasites carrying *Pfmdr*1 N86 and

103   184F in the last decade have been linked to declining susceptibility to LU, part of the AL

104   treatment that is recommended in Kenya (Achieng *et al.*, 2015; Eyase *et al.*, 2013; Venkatesan *et*

105   *al.*, 2014), and a reciprocal rise in parasite susceptibility to CQ and amodiaquine (ADQ)

106   (Achieng *et al.*, 2015; Veiga *et al.*, 2016). Conversely, our previous studies showed that the

107   prevalence of *Pfcrt* K76 and N86 increased from 6.4% in 1995-1996 to 93.2% in 2014 and 0.0%

108   in 2002-2003 to 92.4% in 2014 respectively (Achieng *et al.*, 2015), a period when the first and

109   second-line malaria drugs for Kenya are AL and DHAPPQ respectively. A study done in

110   Zanzibar reported that treatment with AL selects for chloroquine-susceptible *Pfcrt* K76 allele

111   while re-infecting parasites harbor *Pfmdr*1 N86 and 184F alleles (Sisowath *et al.*, 2009).

112   Development of resistance is often attributed to acquisition of survival advantage of parasites

113   against drugs (Hastings & Donnelly, 2005). The rate and pattern of spread of initial resistant

114   strain is often dependent on multiple confounding factors that should be monitored alongside

115   drug resistant surveillance programs concurrently (Mbugi *et al.*, 2006). The confounding factors

116   includes initial prevalence of mutations, population movement between transmission regions among

117   others (Bloland, 2001). Distribution, control and prevention of malaria has been reported to be

118   influenced by human population movement (HPM) from areas with high transmission rates to

119   malaria free areas (Cohen *et al.*, 2012). Similarly, HPM has been shown to contribute to spread

120   of drug resistant parasite strains elsewhere (Lynch & Roper, 2011). Other factors like age,

121   gender, location, history of past malaria infection and previous malaria treatment have been

122   shown to affect malaria prevention and control. These factors could consequently be associated

123   with the dispersal and distribution of chloroquine resistance.

124   To combat drug resistance, the WHO recommends a robust surveillance system to monitor and

125   contain the drug resistance (WHO, 2009). In areas with documented success and declining

126   disease burden, there are calls for initiation of pre-elimination/elimination phase in malaria

127   control and management (Whittaker, Dean, & Chancellor, 2014; WHO, 2015). However,

128   elimination process is facing numerous challenges. A study done in Nigeria reported research,

129   political will/funding, attitude/behavior change, conflicts, terrorism/migration, climate change,

130   insecticide resistance, drug resistance and treatment as challenges facing malaria elimination

131   (Aribodor D.N, Ugwuanyi I.K., & Aribodor O.B, 2016), highlighting the importance of

132   understanding the malaria drug resistance patterns alongside demographics/clinical factors and

133   parasite characteristics among symptomatic malaria cases as a prerequisite for entry in pre-

134   elimination phase.

135   As countries move towards the elimination phase, enhanced surveillance systems are required

136   to ensure that every infection is detected, treated and reported (WHO, 2016a). However, there is

137   lack of sufficient information to feed such a trend of reduced malaria burden in Kenya. Most

138   studies done in Kenya often dwell on one site mostly endemic regions, with a smaller sample

139   size and no detailed information on demographics. Therefore, this study aimed at determining

140   the role of gender and travel in dispersion of chloroquine resistant genotypes among individuals

141   with symptomatic malaria in epidemic zones, Kenya.

142   # Materials & Methods

143   **Ethics Statement, Study Protocol, Sites and Subjects**: This study was approved by the Kenya

144   Medical Research Institute (KEMRI), Walter Reed Army Institute of Research (WRAIR) and

145   Human Research Protection office institutional review boards (protocol numbers: KEMRI

146   #1330, WRAIR #1384 and HRPO Log #A-19306.3) respectively. Seven participating clinical

147   centers were all Ministry of Health facilities located in various malaria epidemic regions in

148   Kenya (Fig. 1); Lake endemic region (Kisumu East and Kisumu West/Kombewa District

149   Hospitals) both situated in Kisumu County (0°14'60.00" N 34°54'59.99" E), Highland epidemic

150   region had (Kericho District Hospitals and Kisii teaching/referral hospital) situated in Kericho

151   County (0°22'2.3"S 35°16'52.72"E) and Kisii County (0° 40' 49.7352"S 34° 46' 37.4196" E)

152   respectively, Coast endemic region (Malindi District Hospital) situated in Kilifi County (

153   3°13′25″S 40°7′48″E) and Semiarid seasonal region (Isiolo District, Isiolo county (0°52'60.00" N

154   38°40'0.12" E) and Marigat Sub district Hospital, Baringo County (0°28′N 35°59′E). At each

155   participating facility, training of medical staff, capacity building, and facility upgrades were

156   provided by the Global Emerging Infections Surveillance (GEIS) Program, U.S. Department of

157   Defense. Subjects attending outpatient clinics from 2008 to 2014, at least 6 months old and

158   suspected of having non-complicated *P. falciparum* malaria were invited to participate. Written

159   informed consent was obtained from adult subjects ($\geq$ 18 years of age) or legal guardians for

160   subjects < 18 years of age. Infants weighing less than 5 kilograms were excluded.

161   **Sample Collection and Preparation:** 2ml of blood was collected from eligible subjects who

162   had tested positive by rapid diagnostic test (RDT; Parascreen H (Pan/*Pf*), Zephyr Biomedicals,

163   Verna Goa, India) for *P. falciparum* malaria. Additionally, FTA filter paper (Whatman Inc.,

164   Bound Brook, New Jersey, USA) was used to collect three blood spots of about 100 µl each for

165   *P. falciparum* DNA extraction and molecular analysis. Two blood films on glass slides were

166   made for Giemsa staining at the laboratory for microscopic examination, to confirm RDT results

167   and determine parasitemia. For discrepancies between RDT and microscopy, microscopy

168   determined the final result. Collected specimens for molecular analysis were stored in -20 °C

169   freezers at the respective hospitals temporarily, before being transported to the

170   KEMRI/USAMRD-A Kenya research laboratory in Kisumu for long-term storage at -80 °C. The

171   remainder ~1.5mL of blood was cryopreserved in liquid nitrogen for future culture and in vitro

172   susceptibility testing.

173   Subjects' demographic information, malaria history, symptoms for current infections and

174   physical examination findings were recorded in case report forms (CRFs). Subjects were treated

175   with oral artemether-lumefantrine (AL; Coartem) administered over three consecutive days, a

176   standard of care for *P. falciparum* malaria in Kenya. The first dose was observed by the study

177   team and remaining doses were self-administered at home.

178   **Microscopy:** Thick and thin smears were prepared for low and high parasitemia respectively.

179   The smears were stained in fresh working Giemsa stain (diluted 1:10 in buffered water from

180   stock) for 15-20 minutes and air dried. The parasite count was recorded as parasites per 200

181   white blood cells (WBCs) for thick smear and per 2000 red blood cells for the thin smear. The

182   two were standardized to parasites per microliter. An assumed WBCs count of 8000/µl that has

183   been accepted as reasonably accurate in estimating malaria parasite densities was used (Afrane,

184   Zhou, Githeko, & Yan, 2014; Gyasi *et al.*, 2015). To convert parasite count per 2000 RBCs to

185   parasites/µl an assumed RBCs count of 4.5 X 10^6/ µL was used (Desai *et al.*, 2015).

186   **DNA extraction:** DNA was extracted from blood spotted onto FTA filter papers using the

187   QIAamp DNA Mini Kit (QIAGEN sciences, Maryland, USA) in accordance with the

188   manufacturer's protocol for dried blood spots. The purified DNA was stored at -20 °C until

189   required.

190   *Pfcrt* **K76T SNP analysis:** A fragment of *Pfcrt* gene spanning K76T SNP was amplified by

191   conventional polymerase chain reaction (PCR) on an Applied Biosystems' GenAmp PCR system

192   9700 (Foster City, CA, USA). The primer sequences used for the analysis are as described in

193   (Wang *et al.*, 2005). The primers were purchased from Applied Biosystems (Foster City, CA,

194   USA). The primary reaction was performed in a 15 µL reaction mixture which consisted of

195   0.5µL AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 2.5µL of 10 µM forward

196   primer and reverse primers, 2.5 µL of 10X PCR Buffer, 2.5µL of 10mM dNTPs, 2.0 µL of

197   25Mm $MgCl_2$, 1µL of DNA sample, and 4µL water. The primary amplification was performed at

198   the following conditions: 94 °C, 3minutes for initial denaturation, 94 °C, 30 seconds for

199   secondary denaturation and 45 cycles consisting of 56 °C, 30 seconds annealing, 60 °C for 30

200   seconds elongation followed by 64 °C for 3 minutes of final elongation and held at 4 °C. The

201   primary PCR amplicon was then used as a template in the secondary PCR. The Secondary

202   reaction was performed in a 25µL reaction mixture which consisted of 0.5µL AmpliTaq Gold

203   (Applied Biosystems, Foster City, CA, USA), 2.5µL of 1 µM forward primer and reverse

204   primers, 2.5µL of 10X PCR Buffer, 2.5µL of 10mM dNTPs, 1.5µL of 25mM $MgCl_2$, 3.7µL of

205   primary PCR product, and water 11.8µL. The secondary amplification was performed at the

206   following conditions: 94 °C, 3minutes for initial denaturation, 94 °C, 30 seconds for secondary

207   denaturation and 20 cycles consisting of 47 °C, 30 seconds annealing, 64 °C for 30 seconds

208   elongation followed by 64 °C for 3 minutes of final elongation and held at 4 °C. The samples

209   were run in duplicate with one set containing primers targeting the wild type allele (K76) while

210   the second had primers specific for the mutant allele (76T). 5µL of the amplicon was mixed with

211   5µL of gel loading dye on a parafilm and run concurrently with reference DNA ladder on a 2%

212   gel. The two products per sample from secondary PCR were run concurrently on agarose gel to

213   discriminate between the wild type, mutant and mixed alleles. Ethidium bromide dye was used to

214   stain the agarose gel for visualization under UV light. The electrophoresis was performed at 97

215   volts for 30 minutes for both primary and secondary products. The bands on the gels were

216   visualized immediately under UV light system (Ultra Violet Products Inc. Transilluminator).

217 DNA from W2 and D6 *Plasmodium falciparum* clones were used as positive controls for mutant

218 and wild type respectively while distilled water was used as a negative control. The negative

219 control was not expected to show any band on gel after electrophoresis while positive control

220 and samples were expected to have sizes of 560 base pairs (bps) for primary product, 342 bps for

221 both mutant and wild type of the secondary product.

222 **Pfmdr1 N86Y and Pfmdr1 Y184F SNP analysis.** A fragment of *Pfmdr*1 gene spanning N86Y

223 and Y184F SNPs was amplified to determine mutations by real-time PCR machine, Applied

224 Biosystems' prism 7500 Fast real-time PCR system (Foster City, CA, USA). The primer and

225 probe sequences used are described in (Purfield *et al.*, 2004). The probes were labeled with

226 6FAM (for wild type) or VIC (for Mutant), at their 5' ends and non-fluorescent quencher

227 (TAMRA) covalently attached at the 3' end for each. Each well on PCR plate contained both the

228 wild type and mutant type primers and probes. The presence of either or both alleles was showed

229 from the increase of the FAM or VIC fluorophores in distinguishing wild, mutant or mixed

230 alleles. These primers and probes were purchased from Applied Biosystems (Foster City, CA,

231 USA). The reactions were performed in a 25 µL reaction mixture made of 18.75 µL of the master

232 mix and 6.25 µL of the *Plasmodium* DNA. The master mix consisted; 6.55 µL of water, 5 µL of

233 25mM $MgCl_2$, 20 µL of Amplitaq Gold, 2.5 µL of 10X PCR buffer, 2.5 µL of 100Mm, 0.5 µL

234 of 100 µM of *Pfmdr1* (86 or 184,) forward primer, 0.5 µL of 100 µM of *Pfmdr1* (86 or 184)

235 reverse primer, 0.25 µL of 100 µM of *Pfmdr1* (86 or 184) wild type probe and 0.25 µL of 100

236 µM of *Pfmdr1* (86 or 184) mutant probe. Amplification was performed at the following

237 conditions: 1 cycle at 50 °C for 1 minute, 1 cycle at 95 °C for 10 minutes and 45 cycles

238 consisting of 95 °C for 15 seconds followed by 58 °C for 1 minute.

239 Quality control was done by running samples and controls in triplicates. Additionally, quality

240 control amplification results were valid only when the positive control (clones W2-mutant and

241 D6-wild type) generated the acceptable average cycle threshold (C_T) value ≤ 30 while the

242 negative control (distilled water) remained unamplified therefore undetected.

243 **Data management and analysis:** Numerical data were expressed as proportions and compared

244 using Pearson's Chi-square. Parasitemia expressed as parasites/microliter were log transformed

245 to the natural log and presented as medians with inter quartile range (IQR) and range.

246 Comparison of parasitemia across age and regions was determined by Kruskal–Wallis test by

247 ranks or one-way analysis variance (ANOVA) on ranks. Dunn's pairwise multiple comparison

248 test was used for post hoc analysis. Univariate and multivariate Logistic regression was used to

249 assess the association of demographic and clinical factors to chloroquine resistance molecular

250 markers. Significance levels were set at 0.05 at 95% confidence interval. Statistical analyses

251 were performed using Stata version 13 (Stata Corp LP, College Station, Texas) while mapping

252 was done in ArcGIS version 10.

Results

254 A total of 1,776 subjects presenting with uncomplicated malaria from four malaria epidemic

255 zones across Kenya between 2008 and 2014 were evaluated. The lake endemic zone enrolled the

256 highest number of subjects owing to high disease endemicity followed by highland epidemic

257 zone while Semi-arid seasonal had the least. Their demographic and clinical presentation

258 characteristics are summarized in table 1. Both genders were equally represented with 48.8% (n

259 = 867) male and 51.2% (n=909) female. Majority 56.7% (n=999) were children below five years

260 followed by 6-15 years 21.9% (n=386) and above 16 years 21.3% (n=376).

261 All subjects enrolled by this study had a mean temperature of 38.1 ± 1.21, mean ± standard

262 deviation (SD). The chief complaint was fever followed by headache that is in line with classical

263 malaria presentation. At least 62.4% (n=1,109) subjects had not travelled from their residence

264 within two months prior to enrolment to the study. 80.2% (n=1,422) reported to have had at least

265 one episode of malaria before date of enrollment. Of these, 10.6% (n=188) had been treated for

266 malaria in the last 6 weeks prior to enrollment.

267 **Malaria parasite density**

268 The log transformed parasitemia for the 1,776 samples assessed as number of parasites

269 diagnosed by microscopy per microliter of whole blood was median 10.4, IQR 9.1-11.5 and

270 range 3.7-14.2. Using Kruskal–Wallis test by ranks, there was a significant difference [F (3) =

271 282.4, *p < 0.0001*] in parasitemia across the seven study sites located in the four malaria

272 endemicity zones of Kenya. The lake endemic region had the highest parasitemia median 10.8

273 IQR 9.9-11.6 range 3.7-14.2 followed by coast endemic region median 9.9 IQR 8.8-10.7 range

274 4.8-11.7 while highland epidemic had the least median 8.9, IQR 7.7-10.4, range 4.4-14.2 (Fig.

275 2). Post hoc analysis by Dunn's test showed that the lake endemic region has a significantly

276 higher parasitemia than the rest of the transmission regions (*p <0.05*) as shown in figure 2.

277 When comparing parasite burden across age groups, individuals in age group < 5 years had

278 marginally higher parasitemia median 10.6, IQR 9.3-11.6 range 3.7-13.9 followed by that of age

279 group 6-15 years, median 10.4, IQR 9.2-11.4 range 4.4-14.2. There was a significant difference

280 [F (2) = 54.94, *p < 0.0001*] in parasite density among the age groups. Age group ≥ 16 years

281 parasitemia differed significantly from those of both age groups of <5 years and 6-15 years (*p <*

282 *0.05*) (Fig. 3).

283 **Trends of *Pfcrt* 76, *Pfmdr*1 86 and *Pfmdr*1 184 polymorphisms**

284 We established trends and distribution of chloroquine resistant genotypes; *Pfcrt* 76, *Pfmdr*1 86

285 and *Pfmdr*1 184 across Kenya between 2008 and 2014. The average frequency of the mutant

286 genotype, *Pfcrt* 76T was 28.3% (n=446), Wild-type *Pfcrt* K76 was 49.4% (n=780) while mixed

287 infection comprising both wild-type and mutant accounted for 22.3% (n = 353) infections

288 countrywide (Table 2). Notably, there was a decline in frequency of *Pfcrt* 76T single nucleotide

289 polymorphism (SNP) between 2008 and 2014 by 66% (n = 125) to 5.5% (n=27) respectively

290 (Fig. 4). There was a significant difference among the *Pfcrt* 76 polymorphisms across the years

291 by Chi-square test [χ^2 (6) = 39.55, *p < 0.0001*].

292 The distribution by the current years in the study (2012 to 2014) mutant genotype *Pfcrt* 76T by

293 malaria endemicity zones showed that Coast endemic region had the highest 34.8% (n = 8)

294 frequency as compared to highland epidemic 11.2% (n = 36), Lake endemic 26.2% (n = 39) and

295 semi-arid seasonal with 5.0% (n = 3) as shown in figure 5. There was a significant difference in

296 frequency of *Pfcrt* 76T polymorphism across the zones (*p < 0.0001*) across the study period.

297 Distribution by age was 27.6% (n = 243), 26.9% (n = 94), 29.9% (n = 100) for ages ≤ 5, 6-15, ≥

298 16 years respectively during the study period. There was no difference in *Pfcrt* 76T frequency

299 across the age groups.

300 The average frequency of the mutant genotype, *Pfmdr*1 184F was 32.3% (n = 507), wild type

301 *Pfmdr*1 Y184 was 47.1% (n = 739) while mixed infection accounted for 20.6% (n = 324)

302 infections countrywide (Table 2). A three-fold increase in *Pfmdr*1 184F, SNP was noted between

303 2008 and 2014 with frequency of 13.7% (n = 27) to 37.2 % (n = 191) (Fig. 4). There was a

304 significant difference among the *Pfmdr*1 Y184F polymorphisms across the years [χ^2 (6) = 17.07,

305 *p < 0.009*].

306 The distribution by the current years in the study (2012 to 2014) of mutant genotype *Pfmdr*1

307 184F by malaria transmission zones showed a marginally higher frequency in the Semi-arid

308   seasonal 45.0% (n = 9) followed by Lake endemic 38.4% (n=161), Highland epidemic 36.8% (n

309   = 118) and Coast endemic 13.0% (n = 3) as shown in figure 5. There was a significant difference

310   in frequency of *Pfmdr*1 184F SNP among the zones (χ^2 = 17.1, *p < 0.009*), (Table 2) across the

311   study period. The *Pfmdr*1 184F, SNP was comparable across all age groups 31.4% (n=280)

312   during the study period, 34.3% (n=118), 32.9% (n = 105) for ages ≤ 5, 6-15, ≥ 16 years

313   respectively. There was no difference in *Pfmdr*1 184F genotype mutation across the age groups.

314   The average frequency of the mutant genotype, *Pfmdr*1 86Y was 13.7% (n = 229), wild type

315   *Pfmdr*1 N86 was 73.9% (n = 1238) while mixed infection comprising both wild-type and mutant

316   accounted for 12.5% (n=209) infections countrywide (Table 2). Notably, there was a decline of

317   the *Pfmdr*1 86Y SNP from 41.8% (n=81) to 1.4% (n = 7) between 2008 and 2014 countrywide

318   (Fig. 4). There was a significant difference among the *Pfmdr*1 N86Y polymorphism across the

319   years [χ^2 (6) = 21.87, *p < 0.001*].

320   The distribution by current years in the study (2012 to 2014) of mutant genotype, *Pfmdr*1 86Y by

321   malaria transmission zones showed that the coastal endemic had the highest 30.4% (n=7)

322   frequency followed by Semi-arid seasonal 10% (n = 2), Lake endemic 3.3% (n = 14) and

323   Highland epidemic 2.2% (n = 7) (Fig.5). There was a significant difference in frequency of

324   *Pfmdr*1 86Y SNP (Table 2) across the study period and was comparable among all age groups

325   12.8% (n = 122), 13.4% (n = 49**),** 16.3% (n = 56) for ages ≤ 5, 6-15, ≥ 16 years respectively

326   during the study period. There was no difference in *Pfmdr*1 86 genotype mutation across the age

327   groups.

328   **Demographic and clinical factors associated with chloroquine resistance**

329   To explore the association between demographic factors and chloroquine resistance (CQR) based

330   on *Pfcrt* 76 allele, a CQR primary marker, univariate and multivariate logistic regression was

331   used (Table 3). Demographic factors included sex, age group, and travel in the last 2 months,

332   ever had malaria, treated for malaria within the last 6 weeks and the malaria transmission zones.

333   Sex was significantly associated with CQR, *p < 0.027* by univariate model and *p < 0.025* by

334   multivariate model. Males were more likely to have CQR, 1.28 times by univariate model and

335   1.29 times by multivariate model. On the other hand, age was not significantly associated with

336   CQR, though it's important to note that subjects above 6 years of age were about one more time

337   likely to harbor resistant genotypes. There was a significant relationship between having

338   travelled in the last two months and CQR both by univariate model (*p < 0.001*) and multivariate

339   model (*p < 0.002*), with subjects who had travelled being one or more time likely to be

340   chloroquine resistant than those who had not. Having had malaria infection prior to the present

341   visit was not associated with CQR, but individuals who had suffered from malaria before were

342   one more time likely to be CQR than those who had no malaria infection prior to study

343   enrollment. On the contrary, treatment in the last 6 weeks before visit was significantly

344   associated with CQR by multivariate model (*p < 0.034*). Individuals who had treatment six

345   weeks prior to the visit were one more time likely to acquire CQR. No association between

346   living in malaria epidemic zones and CQR was observed. However, individuals in Lake endemic

347   region are more likely to be CQR by 1.52 times (univariate model) and 1.87 times (multivariate

348   model). Lake endemic region was followed by highland epidemic while people living in semiarid

349   seasonal were less likely to be CQR.

350

351   # Discussion

352   Antimalarial resistance has significantly hindered malaria control efforts. Since effective

353   vaccines for malaria are still way off, and drugs are most relied upon, success of malaria control

354   strategies are dependent on understanding factors that influence dispersion of malaria drug

355   resistance genotypes. Most studies on CQR appear inconclusive on methods of containing it's

356   spread due to insufficient demographic and clinical information (Akala et al., 2014; Angira,

357   Otieno, Muga, & Abong'o, 2010; Zhong et al., 2008). This study showed that travel, gender and

358   clinical factors were associated with chloroquine resistance among symptomatic malaria cases in

359   four of the five malaria epidemic zones in Kenya. Additionally, this study established that while

360   *Pfcrt* 76 and *Pfmdr*1 86 SNPs had reduced, the *Pfmdr*1 184 SNP was on the rise.

361   Travel and gender were significantly associated with CQR among symptomatic malaria

362   individuals by both univariate and multivariate model (Table 3). The association between gender

363   and CQR implies that males are more likely to be chloroquine resistant than females, though

364   literature indicates that gender norms exposes females and children to the risk of malaria

365   (Ministry of Health, 2015). On the contrary, recent studies in Yemen and Malaysia did not show

366   association between CQR and gender (Atroosh, Al-Mekhlafi, Mahdy, & Surin, 2012; Bamaga,

367   Mahdy, & Lim, 2015) despite compelling evidence of potential role of gender in epidemiology

368   of malaria elsewhere (Diiro *et al.*, 2016; WHO, 2007). The contrary findings could be attributed

369   to the social cultural values around gender. A study done on prevalence of asymptomatic and

370   submicroscopic malaria infections on Islands in Lake Victoria, Kenya, reported to have a

371   significant prevalence of malaria in males than in females ($p < 0.005$) (Idris *et al.*, 2016).

372   Globally, gender dimensions also influence access to treatment, care for malaria and use of

373   preventive measures (WHO, 2007). Females play the primary role of care giving to other

374   members in the household, including leading the majority of health care seeking role for the rest

375   of the family members (Diiro et al., 2016; Roll back malaria partnership, 2016) though males

376   still dominate decision making on health and economic issues in the households (Diiro *et al.*,

377   2016). Malaria is "gender blind" as mosquitoes bite indiscriminately (Ministry of Health, 2015).

378   However, in endemic regions, males may delay seeking treatment and risk accumulating super

379   infection. Research has shown that mutant strains that develop drug resistance enhance virulence

380   (Shahinas, Folefoc, & Pillai, 2013) wanton utilization of resources and therefore eliciting

381   symptoms that distorts the host/parasite balance that would allow wild type parasites to remain

382   asymptomatic (Akala *et al.*, 2014; Deroost, Pham, Opdenakker, & Van den Steen, 2016).

383   The current finding on association of travel and CQR is concurrent with similar studies reported.

384   A study done in Ethiopia reported effects of migration on parasite gene flow promoting

385   transmission of chloroquine resistance (Lo *et al.*, 2017). At a smaller scale, another study in

386   Congo, reported evidence of imported resistant *P. falciparum* strains into Guatemala from Congo

387   given globalization and international travels. Travelers from endemic regions with malaria

388   symptoms should be suspected of harboring chloroquine resistant strains (Patel *et al.*, 2014)

389   Human population movement has been shown to contribute to spread of drug resistant parasite

390   strains elsewhere (Lynch & Roper, 2011).

391   It was also noteworthy that treatment in the last 6 weeks before enrollment was not associated

392   with CQR by univariate model but was associated by multivariate model. This is an implication

393   that other factors contribute to CQR. Literature shows that irrational use of drugs (Nsubuga *et*

394   *al.*, 2011) gender dimensions (WHO, 2007), travel (Juliao et al., 2013), number of parasites

395   exposed to a drug, the drug concentration to which the parasites are exposed, and the

396   simultaneous presence of other antimalarials in the blood to which the parasite is not resistant

397   influence the emergence and spread of drug resistant malaria parasites (WHO, 2018). These

398   multiple factors argue for deployment of integrated research for effective malaria control. Such a

399   strategy would include systems thinking, out-come-oriented monitoring and evaluation

400 approaches, collaboration - across disciplines, sectors and regions -, multi-stakeholder

401 engagement, and sensitivity to social equity (Wiese, 2012).

402 Other factors such as age, ever had malaria and location by epidemic zone were not associated

403 with CQR. However, age has been shown to be a risk factor of malaria with a vast majority of

404 malaria cases occurring under age of 5 years (WHO, 2016b). Though children who live in

405 endemic areas may have parasites with drug resistance to *Plasmodium falciparum*, they often

406 recover after chemotherapy. This is an indication that acquired immunity works in synergy with

407 antimalarials (Enevold *et al.*, 2007). On the other hand, the history of malaria is important in

408 determining recurrent malaria, recrudescence of malaria, relapse and infection (Velho, Alves-jr,

409 Ribatski-silva, & Gomes, 2014). Recurrence has been reported to be due to one or many of the

410 following: therapeutic failure resulting from non-adherence to treatment, resistance of the

411 parasite to the drug-use, poor quality of the medication, or sub-therapeutic doses of the drugs; (b)

412 reactivation of hypnozoites; and (c) exposure to new infection by the mosquito vector (Hedt,

413 Laufer, & Cohen, 2011; White, 2011). Additionally, though location by epidemic zones was also

414 not associated with CQR, determining location of origin of patients who present with malaria is

415 key for successful intervention strategies (Pindolia *et al.*, 2013).

416 High parasitemia was noted for samples from across all sites (Fig.2) and age groups (Fig.3) in

417 Kenya regardless of the difference in disease transmission burden. Studies suggest that residents

418 of holoendemic regions are often immune to malaria (Rolfes *et al.*, 2012). They therefore can

419 harbor parasitemia for long prior to showing symptoms than those in non-immune individuals

420 residing in highland epidemic and semi-arid seasonal transmission zones pf Kenya. It is however

421 worth noting that microscopy that is the Kenya ministry of health's guidelines recommended

422 diagnosis method is less sensitive for detection of low parasitemia (Lo *et al.*, 2015), a factor that

423 could have excluded individuals with low parasitemia. Additionally, a study done in Tanzania

424 reported a two-fold difference in parasite density when samples were collected at two time points

425 (-2 and 0 hours) prior to treatment (Carlsson *et al.*, 2011), It is therefore possible that parasitemia

426 for low transmission region would be higher than that reported here if all sub-microscopic

427 parasitemia's were depicted using sensitive methods and if two samples were collected at

428 different time points. Further, the study design that included only symptomatic individuals with

429 detectable malaria could have been biased towards individuals with higher load across all sites

430   though similar studies in Southeast Asia have reported significantly lower parasitemia

431   (Chaorattanakawee *et al.*, 2013).

432   Analysis of trends and patterns of distribution of chloroquine resistant genotypes revealed a

433   notable decline by 56.7% in the *Pfcrt* 76T and 38.8% for the *Pfmdr*1 86Y mutant genotypes

434   between 2008 and 2014 (Fig.4),. This presents a possibility for future introduction of chloroquine

435   use for malaria treatment as a combination therapy. On the other hand, this observation indicates

436   an increased risk of reinfection after treatment with AL. A study done to determine effects of

437   *Pfcrt* and *Pfmdr*1 gene polymorphisms on therapeutic responses to artesunate-amodiaquine

438   (ASAQ) and AL reported that parasites with the *Pfmdr*1 N86, and *Pfcrt* K76 alleles re-infected

439   patients earlier after AL treatment (Venkatesan *et a*l., 2014), due to selection of less susceptible

440   parasites (Sisowath *et al.*, 2009). Another study done on genetically engineered *P. falciparum*

441   lines reported that *Pfmdr1* N86Y mutation increased parasite susceptibility to a wide range of

442   first line antimalarials including Lumefantrine and Dihydroartemisinin (DHA) while decreasing

443   parasite susceptibility to CQ. *Pfmdr1* N86Y mutation was reported to alter parasite response

444   DHA when compared to N86 parasites (Veiga *et al.*, 2016).The mixed genotype *Pfcrt* K76T

445   mutant and wild type allele is directly linked to both in vitro and clinical resistance. Therefore, it

446   can still be used as a biomarker for CQ resistance (Wellems & Plowe, 2001).

447   On the other hand, there was a reciprocal increase for the *Pfmdr*1 184F mutant genotype from

448   13.7% to 37.2% representing a 23.5% rise between 2008 and 2014 (Fig.4). The implication of

449   *Pfmdr*1 Y184F mutation has been reported to be minimal on antimalarial drug susceptibilities

450   (Veiga *et al.*, 2016). This observation was contrary to a study reported in Grande Comore Island,

451   Comoros between 2006-2014 where *Pfmdr*1 184F mutations dropped from 52.2 to 30.0%

452   (p<0.01) (Huang *et al.*, 2016). Another study done in Guinea-Bissau between 2003 and 2012

453   reported that there was no significant change in the *Pfmdr*1 184F mutant allele (Jovel, Kofoed,

454   Rombo, Rodrigues, & Ursing, 2015). A study done in Yemen, Tehama region reported a high

455   prevalence of *Pfmdr*1 184F mutant allele (99%) (Atroosh *et al.*, 2016)and an increase from 19.6

456   to 22.7% in 2010 -2012 in Maputo after introduction of ACT (Lobo *et al.*, 2014). In Kenya, a

457   significant association between polymorphisms at the *Pfmdr*1 184 and lumefantrine have been

458   reported (Achieng *et al.*, 2015). However, a previous study from Odisha has reported the high

459   prevalence of *Pfmdr*1 86Y mutation and low prevalence of *Pfmdr*1 184F mutation (Antony, Das,

460   Parija, & Padhi, 2016). Additionally, increased *Pfmdr1* copy number has been associated with

461   treatment failure after short 4-dose AL therapy (Sisowath *et al.*, 2009). The increased *Pfmdr1*

462   copy numbers has been reported to be a rare observation in African population due to little

463   exposure to MQ (Venkatesan *et al.*, 2014).

464   Significant variation in genotype distribution was noted; the Coast and Lake endemic regions

465   had the highest frequency of 31.6% and 29.6% for the *Pfcrt* 76T mutant allele respectively

466   (Table 2). Genotype distribution for year 2014 (Fig.5) by spatial analysis also showed varied

467   distribution (Fig.5). This observation could be partly due to inadequate enforcement on the ban

468   of CQ and that CQ is still in use after its official withdrawal in Kenya (Rebelo, Tempera,

469   Fernandes, Grobusch, & Hänscheid, 2015). The coastal region had the highest *Pfmdr*1 86Y

470   mutant allele with 23.4%. CQ selects for parasites with *Pfmdr*1 86Y mutation (Li *et al.*, 2014).

471   Semi-Arid seasonal had the highest *Pfmdr*1 184F mutant allele with 32.5% while the coastal

472   endemic region had the highest *Pfmdr*1 Y184 allele at 57.8% suggesting that the population in

473   the Semi-Arid seasonal zone have a higher risk to parasite tolerance to some antimalarials. It has

474   been reported that polymorphism in the *Pfmdr*1 gene is associated with an increase in parasite

475   tolerance/resistance to some anti-malarials (Atroosh *et al.*, 2016). This would also imply

476   increased use of lumefantrine in the region. It has been reported that existence of *Pfmdr*1 184F

477   mutant allele increases with the use of lumefantrine (Malmberg *et al.*, 2013; Sisowath *et al.*,

478   2007). An increase of 18.4% to 23.5% access to AL from within 48 hours of fever on set had

479   been reported in 2015 (Wasunna *et al.*, 2015), after implementation of a new drug policy in

480   Kenya 2004 (Gitonga *et al.*, 2008; Watsierah, Onyango, Ombaka, Abong 'o, & Ouma, 2012).

481   The high parasitemia means that individuals living in the Lake endemic region will consequently

482   have high rates of transmission of CQR genotypes as compared to other regions.

483   Previous studies show increasing chloroquine sensitivity (Eyase *et al.*, 2013; Kiarie, Wangai,

484   Agola, Kimani, & Hungu, 2015; Mohammed *et al.*, 2013) after it was replaced by sulfadoxine-

485   pyrimethamine in 1998 (Eyase *et al.*, 2013). A study done in Tanzania reported > 90% recovery

486   of CQ susceptibility ten years after its withdrawal (Mohammed *et al.*, 2013). Two studies done

487   in Western Kenya reported a significant difference to CQ sensitivity in the *Pfcrt* 76 codon

488   between 2008 and 2011 p<0.001 (Eyase et al., 2013), and a decline from 76% to 6% of *Pfcrt*

489   76T prevalence from 2003 to 2015 (Vardo-Zalik *et al.*, 2018). Another study done in Msabweni

490   Kenya reported a 41% (n-99) in the *Pfcrt* 76T mutant allele in the year 2013 representing a

491   significant decline in frequency compared to 2006 (p≤ 0.05) (Kiarie *et al.*, 2015). Similarly, this

492   study shows increasing frequency of *Pfcrt* K76 and *Pfmdr1* N86 genotypes that are suggestive of

493   increasing CQ sensitivity in the four out of five malaria endemicity zones of Kenya heralding

494   possible reintroduction of CQ based combination treatment. On the other hand, the prevalence

495   *Pfmdr*1 184F genotype increased during the 2008-14 period. The *Pfmdr*1 184F mutation has

496   been associated with AL resistance (Antony *et al.*, 2016), and poses a threat to the sensitivity of

497   lumefantrine and MQ (Ministry of Health, 2010). Lumefantrine in combination with artemether

498   has been the recommended first-line treatment for uncomplicated malaria in Kenya since 2006

499   (Gitonga *et al.*, 2008; Lucchi *et al.*, 2015; Ministry of Health, 2010), while MQ is used as a

500   chemoprophylaxis in Kenya (Ministry of Health, 2010).

501   Studies have shown that understanding the patterns of parasite dispersal from local hotspots of

502   transmission can aid the design of additional targeted control by identifying both the regions

503   where imported infections originate and where they may contribute substantially to transmission

504   (Wesolowski *et al.*, 2012). It will therefore be of great importance to establish gene flow

505   patterns of *Plasmodium falciparum* resistant genotypes within the Kenyan population. This will

506   be crucial in designing strategies towards control and prevention of malaria per epidemiological

507   zone.

508   The limitation to this study was using a convenience sample. This implies that the sample size

509   used in some regions could not be a representative of the whole population. Univariate and

510   multivariate regression was used to analyze data that was stratified by age, gender and location to

511   ensure that the analysis outcome is not due to confounding factors, bias or effect modifiers.

Conclusions

513   According to this study gender and travel have contributed to the dispersion of CQR genotypes

514   in Kenya. The coast and lake endemic region bears the brunt of the burden among other regions.

515   Additionally, children under the age of 5 years remain to be at a higher risk of malaria burden.

516   Decline in CQR based on *Pfcrt* 76T SNP, is an indication that CQ can be re-considered for

517   treatment of *Pf* malaria in future. An increase in *Pfmdr*1 N86 wild type implies tolerance to

518   lumefantrine. On the other hand, increased *Pfmdr*1 184F mutant genotype frequency indicates a

519   reduced sensitivity to lumefantrine. This study recommends continuous monitoring of CQR with

520   wholesome information. Further studies should be done to determine migration patterns of *Pf*

521 CQ resistant genotypes and gender factors associated with CQR in the malaria epidemic zones in

522 Kenya.

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794

Figure 1

Map of Kenya showing the different malaria endemicity zones and locations of various surveillance hospitals.

Lake endemic region: Kisumu East and Kisumu West District Hospitals in Kisumu county, Semi-Arid, seasonal: Marigat District hospital in Baringo county and Isiolo District Hospital in Isiolo county. Coast endemic: Malindi District Hospital, Kilifi County. High land Epidemic: Kisii teaching and referral Hospital and Kericho District Hospital (QGIS version 2.18).

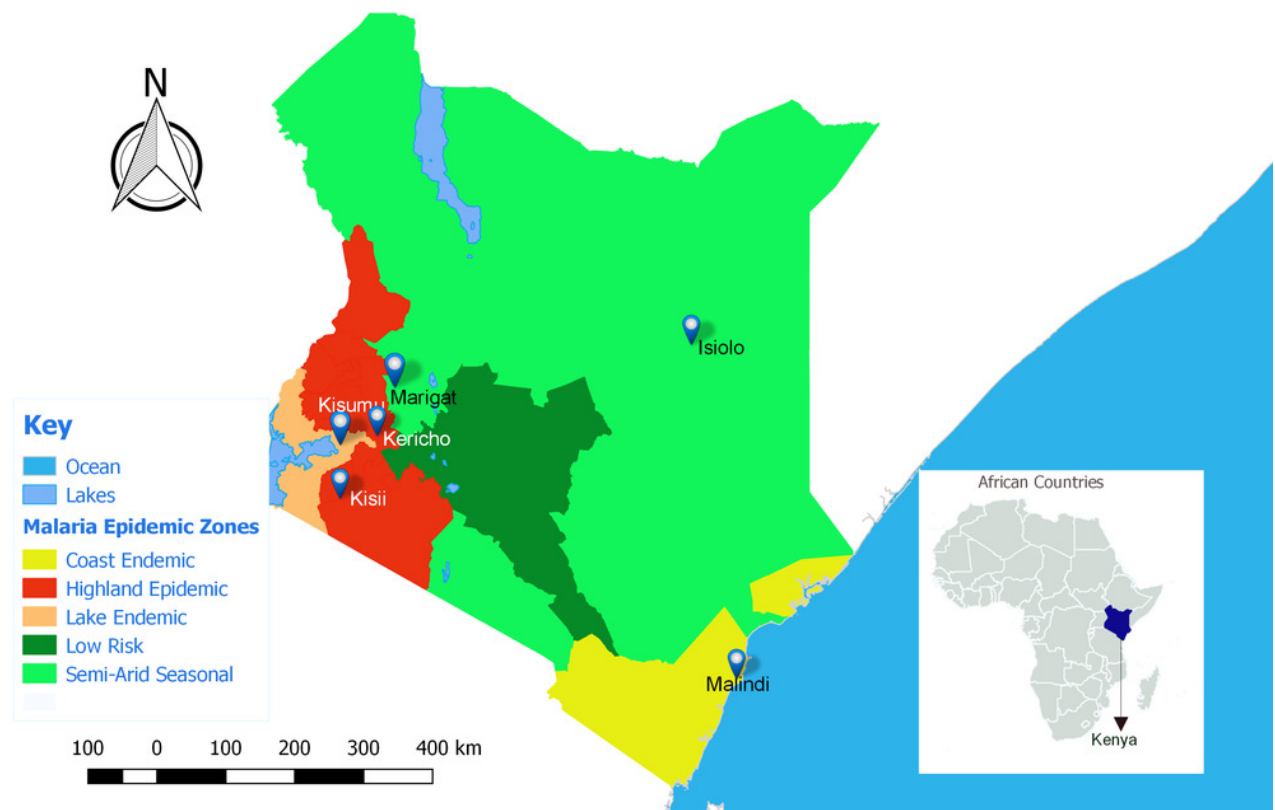



Figure 2

Comparison of parasite density by malaria epidemic zones.

Parasite density (natural log transformation). Dots represent outliers. P value < 0.05 was considered significant.

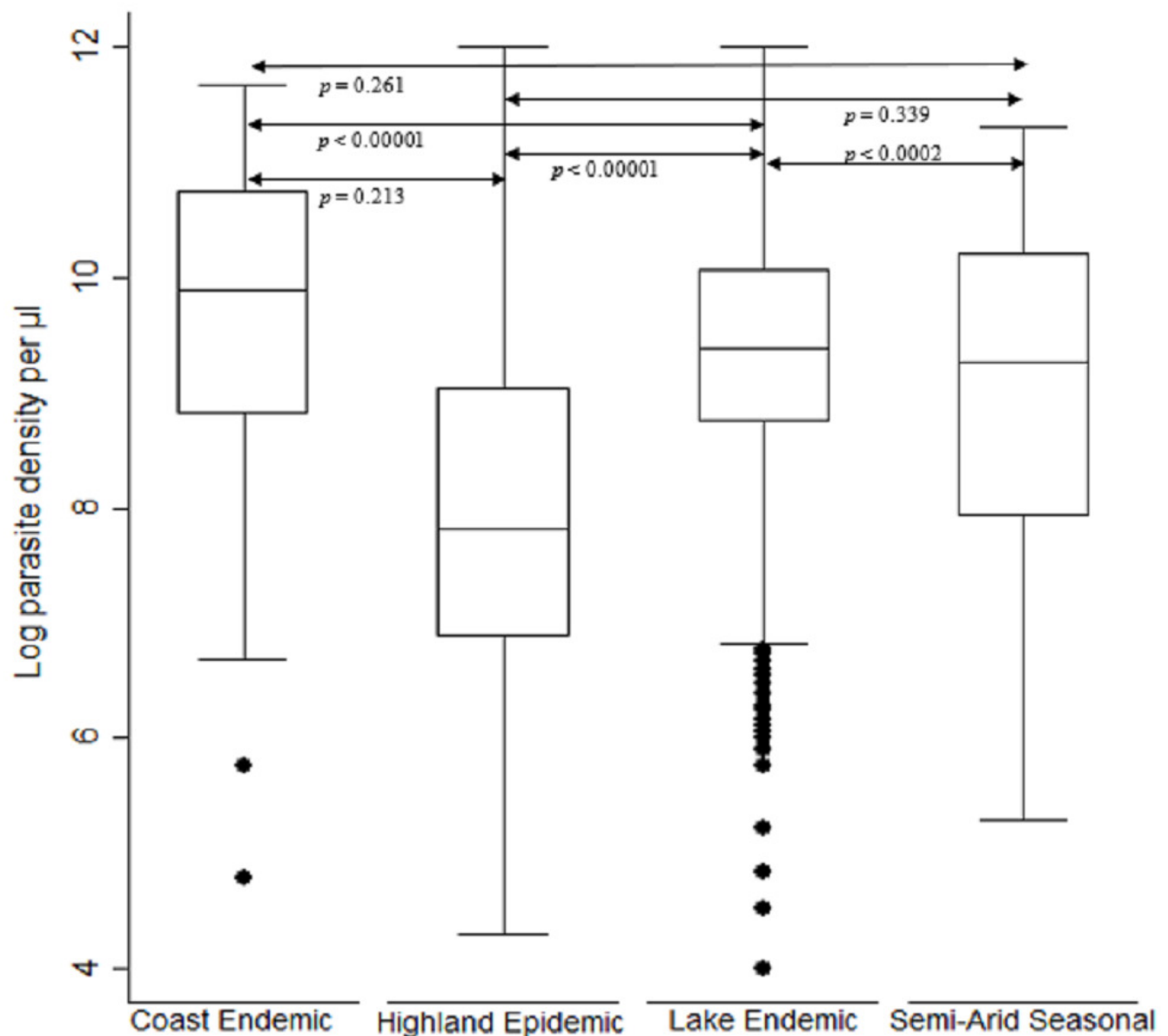



Figure 3

Comparison of parasite densities by age groups.

Parasite density (natural log transformation). Dots represent outliers. P value < 0.05 was considered significant.

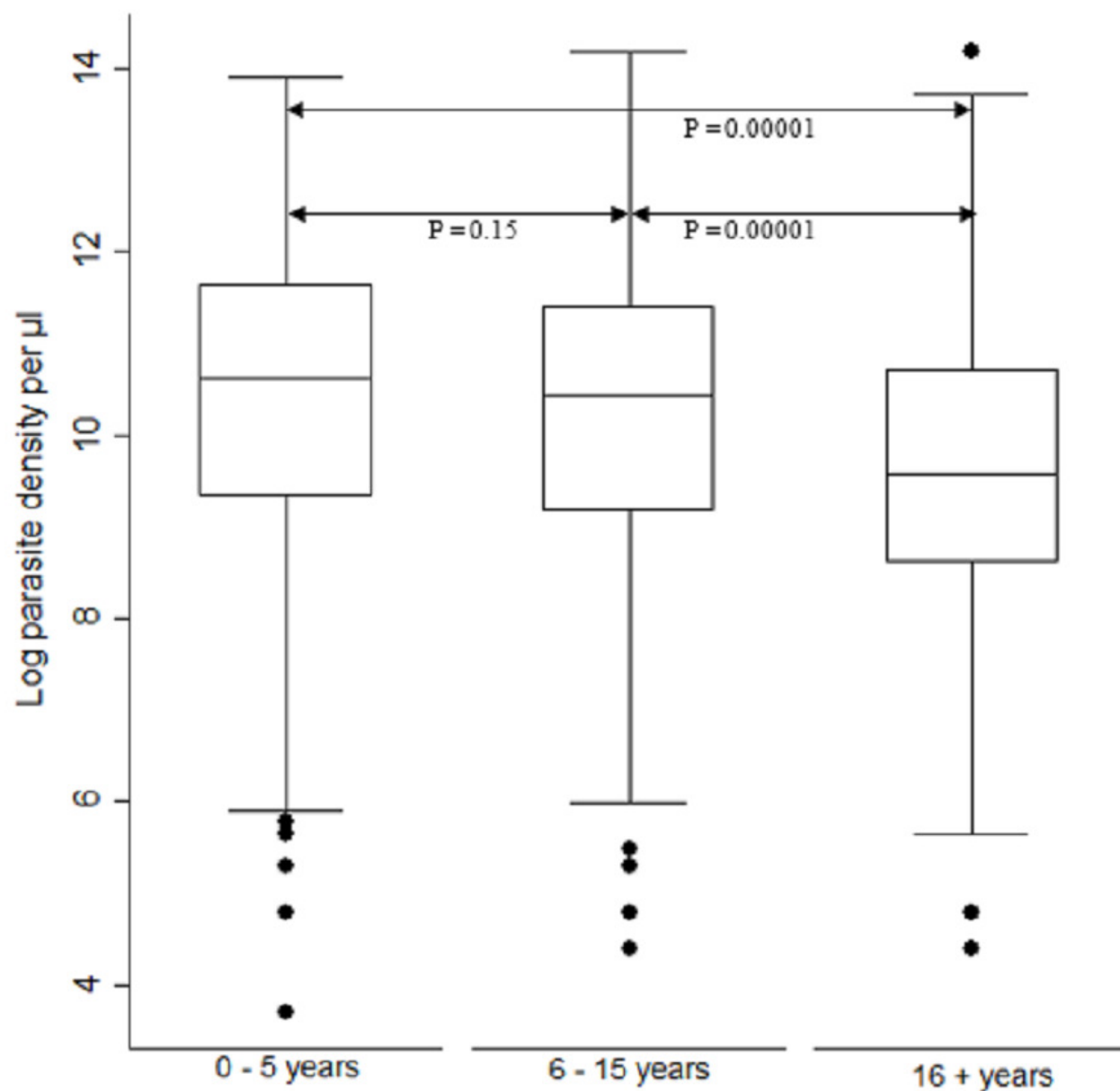



Figure 4

Trends of genotype frequencies of *Pfcrt* 76T, *Pfmdr*1 184F and *Pfmdr*1 86Y polymorphisms between 2008 and 2014.

There was a significant difference (*p* < 0.0001) for each of the *Pfcrt* 76T, *Pfmdr*1 184F and

*Pfmdr*1 86Y SNPs with χ^2 of 354, 780, 103 respectively from 2008 to 2014. *Pfcrt* 76 (n=1579),

*Pfmdr*1 86 (n= 1676) and *Pfmdr*1 184 (1570).

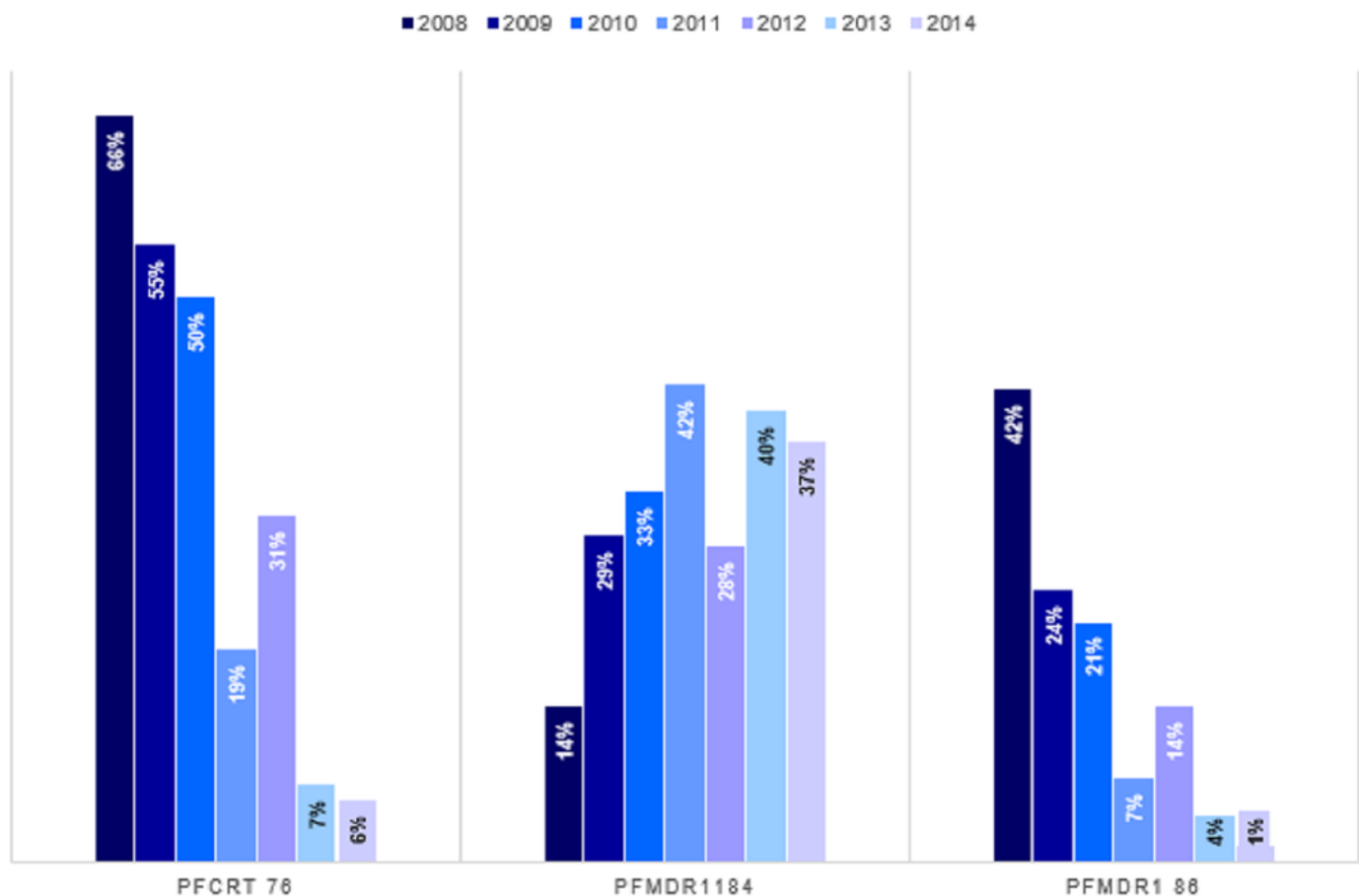



Figure 5

Spatial distribution patterns of A) *Pfcrt 76*T, B) *Pfmdr*1 184F and C) *Pfmdr*1 86Y SNPs by malaria epidemic zones using ArcGIS version 10.

There was a significant difference among the *Pfcrt* 76T, *Pfmdr1* 184F and *Pfmdr1* 86Y SNPs across the epidemic zones (χ2 (6) = 39.5, *p < 0.0001*), (χ2 (6) = 21.9, *p < 0.001*) and (χ2 (6) = 17.1, *p < 0.009*) respectively.

A

Key

Lakes

Pfcrt 76T SNP

No data : Low Risk
5.0 % : Semi-Arid Seasonal
9.3% : Lake Endemic
11.2% : Highland Epidemic
34.8% : Coast Endemic

B

Key

Lakes

Pfmdr1 184F SNP

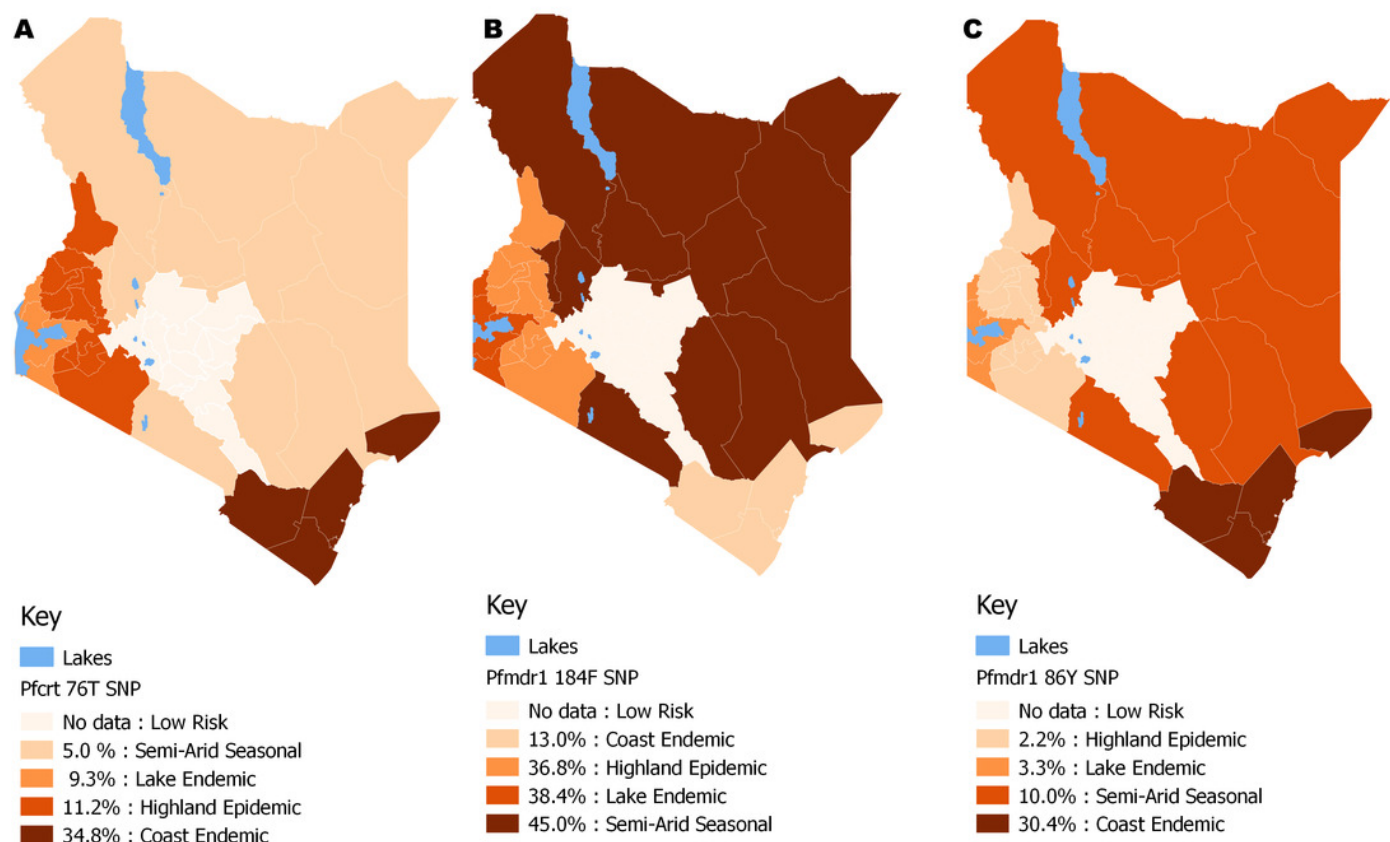
No data : Low Risk
13.0% : Coast Endemic
36.8% : Highland Epidemic
38.4% : Lake Endemic
45.0% : Semi-Arid Seasonal

C

Key

Lakes

Pfmdr1 86Y SNP

No data : Low Risk
2.2% : Highland Epidemic
3.3% : Lake Endemic
10.0% : Semi-Arid Seasonal
30.4% : Coast Endemic

Demographic and clinical characteristics of subjects enrolled by resistance.

** Not applicable for chi-square test. *P* value < 0.05 was considered significant. SD- standard deviation.

Characteristics	Malaria Resistant Status		Total n (%) n=1,776	Chi Square	p - value
	Resistant n (%) n=941	Non-Resistant n (%) n=835			
Sex				1.44	0.23
Male	472(54.4%)	395(45.6%)	867(48.8%)		
Female	469(51.6%)	440(48.4%)	909(51.2%)		
Age group, years				0.4	0.82
≤ 5	520(52.1%)	479(47.9%)	999(56.7%)		
6 - 15	206(53.4%)	180(46.6%)	386(21.9%)		
≥ 16	202(53.7%)	174(46.3%)	376(21.3%)		
Mean body temperature ±SD	38.1±1.24	38.1±1.18	38.1±1.21	**	**
Ever travelled in the last 2 months					
Yes	368(55.3%)	297(44.7%)	665(37.5%)	2.36	0.125
No	572(51.6%)	537(48.4%)	1,109(62.5%)		
Ever had malaria					
Yes	746(52.5%)	676(47.5%)	1,422(80.2%)	0.79	0.372
No	194(55.1%)	158(44.9%)	352(19.8%)		
Treated for malaria in the last 6 weeks					
Yes	118(62.8%)	70(37.8%)	188(10.6%)	8.07	0.004
No	822(51.8%)	764(48.2%)	1,586(89.4%)		
Chief Complain					
Fever	611(52.5%)	552(47.5%)	1,163(66.2%)	0.46	0.978
Headache	200(54.1%)	170(45.9%)	370(21.1%)		
Coughing	10(55.6%)	8(44.4%)	18(1.0%)		
Joint pains	20(50.0%)	20(50.0%)	40(2.3%)		
Others	87(52.4%)	79(47.6%)	166(9.4%)		

Distribution of *Pfcrt* 76, *Pfmdr1* 86 and 184 single nucleotide polymorphisms by zone.

Notes: Samples with mutant, wild-type and a mixture of mutant/wild-type were identified for each epidemic zone. Undetermined samples contained neither mutant, Wild-type nor a mixture of mutant/Wild-type. $P < 0.05$ was considered significant.

Polymorphisms (n=1,676)	Lake Endemic	Highland Epidemic	Semi-Arid Seasonal	Coast Endemic	Total	Chi Square	p - value
Pfcrt-76							
Mutant	302(29.6%)	129(26.2%)	3(11.1%)	12(31.6%)	446(28.3%)	39.5	0.0001
Wild-type	467(45.7%)	282(57.2%)	21(77.8%)	10(26.3%)	780(49.4%)		
Wild-type/mutant	252(24.7%)	82(16.6%)	3(1.1%)	16(42.1%)	353(22.3%)		
*Pfmdr*1-86							
Mutant	155(14.4%)	56(10.8%)	3(12.5%)	15(27.3%)	229(13.7%)	21.9	0.001
Wild-type	769(71.5%)	415(79.7%)	19(79.2%)	35(63.6%)	1,238(73.9%)		
Wild-type/mutant	152(14.1%)	50(9.6%)	2(8.3%)	5(9.1%)	209(12.5%)		
*Pfmdr*1 184							
Mutant	319(33.1%)	168(32.3%)	13(32.5%)	7(13.5%)	507(32.3%)	17.1	0.009
Wild-type	434(45.0%)	255(49.0%)	13(32.5%)	37(71.2%)	739(47.1%)		
Wild-type/mutant	212(21.9%)	97(18.7%)	7(17.5%)	8(15.4%)	324(20.6%)		

Association of demographic and clinical factors with CQR.

Ref- Reference group, OR- Odds ratio, CI-confidence interval. *P* < 0.05 was considered significant.

Parameter	Chloroquine			Univariate		Multivariate	
	Resistant (n=446)	Non-Resistant (n=1,330)	Total (n=1776)	OR(95%CI)	*p*-value	OR(95%CI)	*p*-value
Sex							
Female	208(22.8%)	701(77.1%)	909(51.2%)	ref		ref	
Male	238(27.4%)	629(72.6%)	867(48.8%)	1.28(1.03 - 1.58)	0.027	1.29(1.03 - 1.61)	0.025
Age group, years							
≤ 5	243(24.3%)	756(75.7%)	999(56.7%)	ref		ref	
6 - 15	94(24.4%)	292(75.7%)	386(21.9%)	1.00(0.76 - 1.32)	0.991	1.08(0.82- 1.43)	0.577
≤ 16	100(26.6%)	276(73.4%)	376(21.4%)	1.13(0.86 - 1.48)	0.386	1.20(0.90 - 1.59)	0.207
Ever travelled in the last 2 months							
Yes	198(29.7%)	467(70.2%)	665(37.5%)	1.47(1.18 - 1.83)	0.001	1.44(1.14 - 1.79)	0.002
No	248(22.4%)	861(77.6%)	1109(62.5%)	ref		ref	
Ever had malaria							
Yes	354(24.9%)	1068(75.1%)	1422(80.2%)	0.94(0.72 - 1.22)	0.631	0.81(0.60 - 1.08)	0.155
No	92(26.1%)	260(74.6%)	352(19.8%)	ref		ref	
Treated for malaria in the last 6 weeks							
Yes	58(30.9%)	130(69.1%)	188(10.6%)	1.38(0.99 - 1.92)	0.056	1.46(1.03 - 2.06	0.034
No	388(24.5%)	1198(75.5%)	1586(89.4%)	ref		ref	
Epidemic Zones							
Coast Endemic	12(19.4%)	52(80.7%)	62(3.5%)	ref		ref	
Highland Epidem	129(23.3%)	424(76.7%)	553(31.1%)	1.27(0.65 - 2.45)	0.027	1.44(0.72 - 2.9)	0.302
Lake Endemic	302(26.8%)	823(73.2%)	1125(63.3%)	1.52(0.8 - 2.91)	0.184	1.87(0.93 - 3.74)	0.077
Semi-Arid Seasonal	3(8.3%)	33(91.7%)	36(2.0%)	0.38(0.09 - 1.45)	0.124	0.38(.09 - 1.52)	0.174