Impact of Resistance to Pyrehroids on the Incidence of Malaria into *Anopheles gambiae* (Giles, 1902) in the Southern Forest Area in Côte d'Ivoire

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Abstract: ***The Levels and mechanisms of resistance to pyrethroids and DDT in field Anopheles gambiae populations and their impact on malaria incidence were investigated from December 2016 to May 2017 in four sites in the southern part of Côte d'Ivoire. Among these sites; two have high incidences of malaria; Jacqueville and Toumodi and two other ones with low incidences of malaria; Tiassale and Adzope. The results showed resistance to pyrethroids and DDT with mortality rates ranging from 32% to 67% with alphacypermethrin 0.05%, from 27% to 64% with deltamethrin 0.05%, from 22% to 47% with permethrin 0.75% and 18% to 28% with DDT 4%. This resistance was not only due to the metabolic mechanisms of detoxification including single oxygen cytochrome P_{450} activities and esterases, but also to the kdr mutation. The two identified species; An. coluzzii and An. gambiae were sympatric in Tiassalé and Toumodi, while only An. coluzzii was present in Jacqueville. The frequencies of the resistant alleles at the kdr mutation L1014F were high ranging from 0.53 in Tiassale to 0.95 in Toumodi and thoses at the Ace-1^R mutation G119S were very low ranging from 0.25 in Toumodi to 0.03 in Tiassalé. This research revealed a link between the kdr mutation and the incidence of malaria and also a link between the Ace-1^R mutation and the malaria incidence. Indeed, the kdr and the Ace-1^R mutations frequencies were higher into field Anopheles gambiae populations from high malaria incidence areas than those from low malaria incidence areas. These mutations may be favorable for a high malaria transmission.***

Keywords: *Anopheles gambiae*, malaria, incidence, resistance, pyrethroids, DDT, Côte d'Ivoire

1. Introduction

Malaria remains a public health concern with 216 million cases and some 445, 000 deaths in 2016 [1] due to the parasite, *Plasmodium* resistance to drugs and also due to mosquitoes resistance to insecticides. Indeed, mosquito resistance is widespread in sub-Saharan Africa and especially in Côte d'Ivoire. The main mechanism is the kdrmutation conferring cross-resistance to pyrethroids insecticides and DDT. However, the operational efficiency of pyrethroid-treated mosquito nets is maintained when resistance is due to the kdr mutation [2-4]. But, with metabolic resistance, the effectiveness of the LLINs is sometimes reduced [5].

In fact, metabolic resistance had less been investigated and the link between levels of resistance and the incidence of malaria in Côte d'Ivoire has never been reported. The current study was carried out in four different sites in the southern forested area in Côte d'Ivoire.

This study aimed to evaluate resistance levels with pyrethroids and DDT and also to research for the resistance mechanisms in *Anopheles gambiae* populations and their impact on the incidence of malaria in four sites in the southern forested area in Côte d'Ivoire.

2. Materials and Methods

2.1 Study Sites

This study was carried out in four sites in southern forested in Côte d'Ivoire: Jacqueville (05°09' N and 04°24' W), Toumodi (06°55' N and 05°03' W), Tiassale (05°88' N and 04°38' W) and Adzopé (06°10' N and 03°87' W) (Figure 1), during six (6) months from December 2016 to May 2017 (Fig1). In the context of this work, the following was considered:

- the low incidence zone comprising the localities of Adzopé with respective incidences of 78.4 ‰ in 2015 and 150.78 ‰ in 2016 and Tiassalé with incidences of 128.46 ‰ in 2015 and 113.73 ‰ in 2016;
- the high incidence zone comprising the localities of Toumodi with respective incidences of 264.35 ‰ in 2015 and 273, 19 ‰ in 2016 and Jacqueville with impacts of 429.97 ‰ in 2015 and 331, 26 ‰ in 2016 [6, 7].

2.2 *A Gambiae* Collection

The larvae of *An. gambiae* were collected in paddles and rice lockers. They were then raised in the insectarium until adult stages for testing according to WHO insecticides susceptibility tubes test.

In Adzopé, we did not collected enough larva of *An. gambiae*, necessary to perform the WHO tubes test. Thus, the susceptibility to insecticides of those field mosquitoes from Adzopé could not be evaluated.

2.3 Mosquito Breeding in the Laboratory

In the laboratory the larvae were grouped by site and collection day. Then they were sorted at first, second, third and fourth stages and placed in separate breeding containers.

Larvae were fed with finely ground dog biscuit.

Emerging adults of *An. gambiae* were transferred to cages and fed with a 10% diluted honey solution.

2.4 Who tubes tests to detect mosquitoes susceptibility to insecticides

Mosquito susceptibility levels were evaluated basing on the WHO tubes revised protocol of 2016 [8], using three pyrethroids i.e. alphacypermethrin 0, 05%; deltamethrin 0, 05% and permethrin 0, 75% and one organochlorine i. e. DDT 4%. An inhibitor of oxidizes and esterase i. e. the piperonylbutoxide (PBO) was used to detect metabolic resistance mechanism into mosquitoes.

Bioassays were performed with four replicates batches of 25 unfed females of *An. gambiae* aged between two and five days. The tests were performed at a temperature of 25 ± 2 °C, within a relative humidity of 70-80%.

After one-hour exposure to insecticide papers, the mosquitoes were transferred into observation tubes and fed with 10% diluted honey solution. Mosquitoes' mortality was read 24 hours after observation.

In order to assess the involvement of detoxifying enzymes in a potential resistance into the field *An. gambiae* tested, additional bioassays were performed with one hour pre-exposition to PBO before exposition to pyrethroids insecticides only.

The specimen mosquitoes used as controls were individually conserved in the Eppendorf tubes containing cotton-coated and silica gel. Those mosquitoes were stored at-20 °C and were used to perform molecular biology tests.

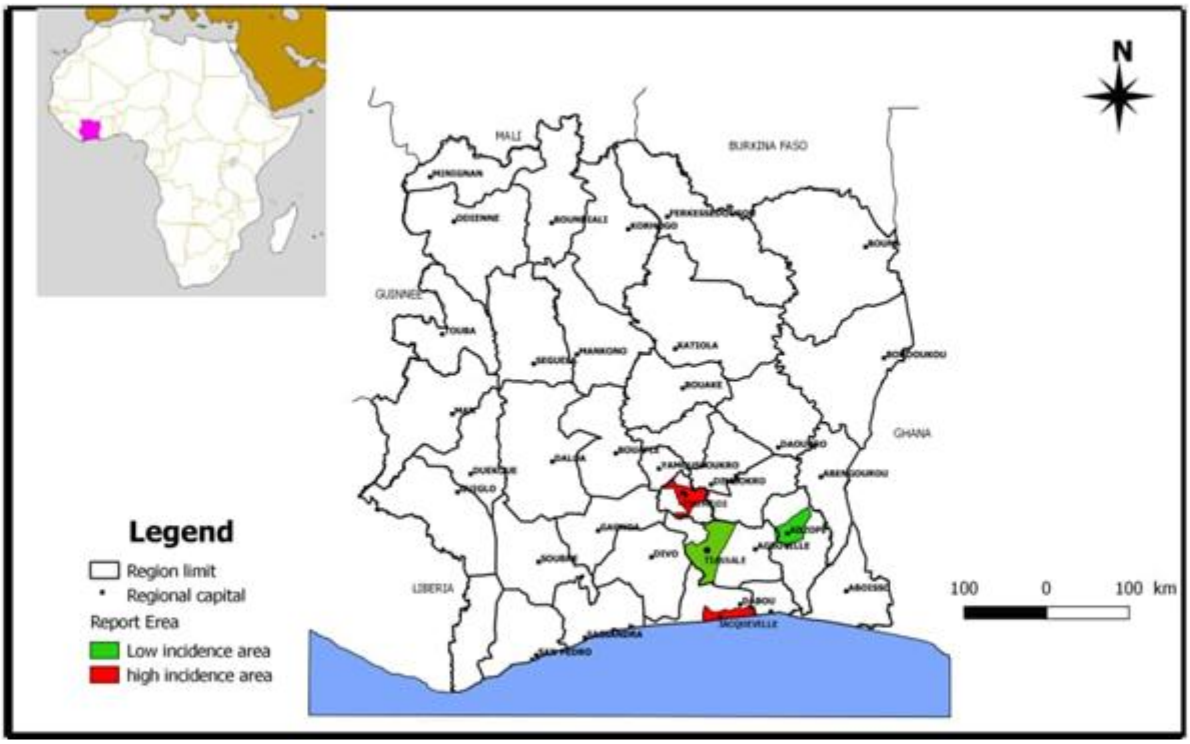

Figure 1: Study sites in Côte d'Ivoire

2.4 Molecular Biology Tests to Detect Resistance Due to Single Nucleotid Mutation and Speciation

Extraction of the total DNA of each individual mosquito was done according to the protocol of Collins [9]. The knockdown mutation conferring cross resistance to pyrethroids and DDT was investigated by test-detecting the L1014F kdr using the protocol of Martinez-Torres [10]. The G119S mutation conferring resistance to carbamates and organophosphorus insecticides was also investigated [11]. Then, *An. gambiae* species found in studies sites were designed according to the method of Favia [12].

2.5 Data Analysis

Mortality rates were calculated and analyzed according to the WHO criteria to find out whether samples populations were susceptible or resistant [6].

The proportional relationship's test was used to compare the mortality rates for each insecticide with and without pre-exposure to the PBO.

The Student's T-test was used to compare the mortality of mosquitoes with or without prior exposure to PBO between low and high incidence areas of malaria for each insecticide [13].

Allelic and genotypic frequencies at both the kdr and ace-1^R locus were calculated using GENEPOP, version 4.6.

Genotypic differentiation was investigated with Goudet G test [14]. Hardy-Weinberg equilibrium was tested [15] and Fis estimating deviation to panmixia was calculated basing on Weir and Cockerham model [16].

The Fischer Test was used to compare the proportions of the different species in each locality. The significance level was set at 5% (p=0.05)

3. Results

3.1 Mortality Rate of Field Populations of *An. Gambiae*

The mortality rates of field mosquitoes used as control ranged from 1% to 4%. Therefore, no correction by the Abott formula was necessary to be done.

Bioassays performed with the three pyrethroids showed low mortality rates (< 90%), revealing resistant mosquitoes (Table 1). The mortalities ranged from 32% to 67% with alphacypermethrin 0.05%; from 27% to 64% with deltamethrin 0.05% and from 22% to 47% with permethrin 0.75%.

With DDT 4%, the mortalities of those field *An. gambiae* populations were also very low. They ranged between 18% and 28 %. All mortalities rates observed were below 90%, indicating those field mosquitoes were resistant to DDT.

Table I: Mortality rate of field populations of *An. gambiae* exposed to pyrethroid and DDT,

Insecticides	**Jacqueville**			**Toumodi**			**Tiassale**			**Adzopé**		
	N	% Mort	Status	N	% Mort	Status	N	% Mort	Status	N	% Mort	Status
Alphacypermethrin 0, 05 %	128	67	**R**	103	32	**R**	102	56	**R**	0	-	-
Deltametrhin 0, 05 %	129	44	**R**	98	27	**R**	101	34	**R**	47	64	**R**
Permethrin 0, 075 %	122	23	**R**	45	22	**R**	101	47	**R**	30	30	**R**
DDT 4 %	109	28	**R**	100	18	**R**	107	20	**R**	-	-	-

N: number of mosquitoes tested, % Mort: mortality rate / percentage of mortality, **R**: Resistant

3.2 Mortality Rate after A Pre-Exposition tO PBO

After pre-exposition to PBO, the mortalities with Alphacypermethrin increased in Jacqueville and Tiassalé, from 67% and 56% to 97 % and 98%, respectively (P=0, 000). With deltamethrin the mortalities increased in Jacqueville from 44% to 94%, in Toumodi from 27 % to 98% and in Tiassalé from 34% to 98% (P=0, 000).

With permethrin the mortalities increased in Jacqueville and Tiassalé, from 23% and 47% to 69% and 97%, respectively (P=0, 000).

A sharp increase in mortality rates was observed with pre-exposition to PBO, with all those pyrethroids (P=0, 000 for all) revealing the presence of metabolic resistance into field *An. gambiae* in Jacqueville, Tiassalé and Toumodi (Fig2).

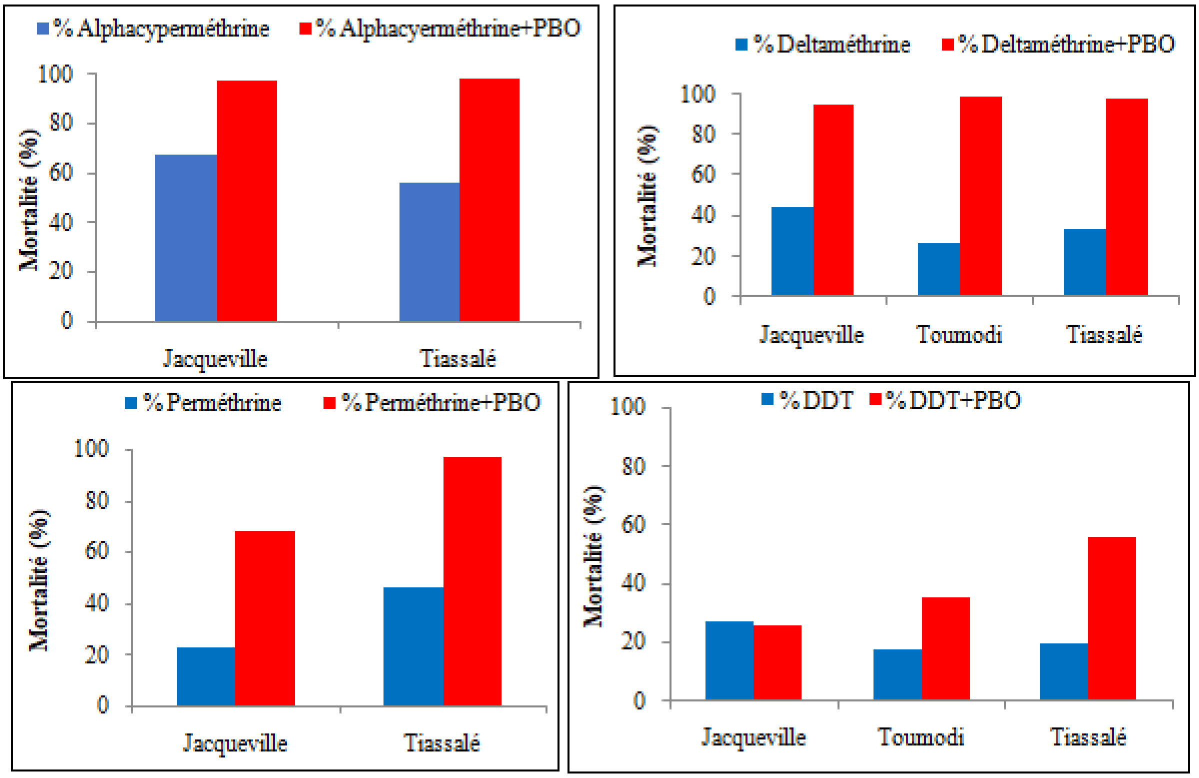

Figure 2: Difference of mortality rates after pre-exposition to PBO of field mosquitoes populations collected in Jacqueville, Toumodi and Tiassalé in Côte d'Ivoire

3.3. Comparison of Metabolic Resistance Levels between Sites With High and Low Incidence of Malaria

Only with permethrin, the mortality observed in the low incidence zone (43%) is significantly higher than that observed in the high incidence zone (23%) (p=0.0001). With other insecticides; alphacypermethrin (p=0, 2316), deltamethrin (p= 0, 982) and DDT (p=0, 751). No significant difference was recorded between mortality between the two areas (Table II).

After pre-exposure to PBO the mortalities observed in the high incidencearea were higher than those in the high incidence area with permethrin and DDT. These mortalities went respectively from 97% to 69% for deltamethrin (p=0.000) and from 50% to 30% for DDT (p=0.000) with other insecticides; alphacypermethrin (p=0, 3442) and deltamethrin (p= 0, 2426). No significant difference was recorded between mortality of the two areas (Table III).

Table II: Comparison of insecticides induced mosquito mortality between areas with high and low malaria incidence

	PYRETHROIDS						ORGANOCHLORINES	
	Alphacypermethrin		Deltamethrin		Permethrin		DDT	
	N	%M	N	%M	N	%M	N	%M
Low Incidence	102	56	148	43	131	43	107	20
High Incidence	231	52	227	37	167	23	209	23
p-value	**0, 2316**		**0, 982**		**0, 0001**		**0, 751**	

N: Number of individual tested, % M: Percentage of mortality

Table III: Comparison of insecticides induced mosquito mortality after pre-exposure to PBO between areas with high and low malaria incidence

	PYRETHROIDS						ORGANOCHLORINES	
	Alphacypermethrin + PBO		Deltamethrin + PBO		Permethrin + PBO		DDT+ PBO	
	N	% M	N	% M	N	% M	N	% M
Low Incidence	116	98	127	98	111	97	109	56
High Incidence	78	97	185	96	105	69	195	30
p-value	**0, 3442**		**0, 2426**		**0, 0000**		**0, 0000**	

N: Number of individual tested, % M: Percentage of mortality

3.4 Genetic Analysis at both KDR and ACE-1^R Mutations Locus according to the Malaria Incidence Levels

- **KDR LOCUS**

Over all allele frequencies were high. The weakest was detected in Tiassalé (0, 53). There was a significant genotypic differentiation between field mosquitoes collected in Jacqueville with high malaria incidence and those collected in Tiassalé with low malaria incidence (P (dG) = 0, 00018).

There was also a significant genotypic differentiation between field *An. gambiae* from Tiassale and those from Toumodi with High malaria incidence (P (dG) =0, 0000).

Moreover, there was a significant genotypic differentiation between field *An. gambiae* from Jacqueville and from Toumodi, but this difference was weak (P (dG = 0, 0105).

The kdr frequencies were higher in high malaria incidence sites i. e. Jacqeville and Toumodi than in the low malaria incidence one, i. e. Tiassalé, revealing a link between the kdr mutation and the incidence of malaria.

Only the population of Tiassalé is not at Hardy-Weinberg equilibrium (P (HW) =0, 0025) and Wright index measuring deviation from panmixis (Fis) shows a lack of heterozygous (Fis (W& C) > 0). In the populations from Jacqueville and Toumodi the Fis shows an excess of heterozygous at kdr locus (Fis (W& C) < 0).

- **ACE-1^R LOCUS**

As at the kdr locus, there was a significantgenotypic differentiation between field *An. gambiae* collected in Jacqueville and Tiassalé (P (dG) = 0, 00589) and also between those from Tiassalé and Toumodi at the Ace-1^R locus (P dG) = 0, 0000).

However, there was no significant genotypic differentiation between mosquitoes from Jacqueville and those from Toumodi (P (dG) > 0, 06708).

The Ace-1^R mutation was higher in high malaria incidence localities i. e. Jacqeville and Toumodi than in low incidence i. e. Tiassalé. These results suggested a link between the Ace-1^R mutation and malaria incidence.

The *An. gambiae* populations from Jacqueville (P (HW) = 0, 568) and Tiassalé (P (HW) = 1, 000) were in Hardy-Weinberg equilibrium (P (HW) > 0.05), while, those of Toumodi (P (HW) = 0, 021) were not in Hardy-Weinberg equilibrium (P (HW) < 0.05).

At the Ace-1^R locus, all study sites revealed an excess of heterozygotes (Fis (W & C) < 0).

Table IV: Genotypic differentiation and Hardy Weinberg equilibrium at the kdr and ace-1^R locus

	Locus kdr				Locus Ace-1^R				
Populations	F (kdr)	P (dG)	P (HW)	Fis (W&C)	F (Ace-1^R)	P (dG)	P (HW)	Fis (W&C)	N
Jacqueville	0, 83		0, 327	-0, 189	0, 15		0, 568	-0, 173	45
		0, 0018				0, 00589			
Tiassalé	0, 53		0, 0025	0, 47	0, 03		1, 000	-0, 023	45
		0, 000				0, 000			
Toumodi	0, 95		1, 000	-0, 04	0, 25		0, 021	-0, 33	49

F (kdr): Allelic Frequencies of the kdr mutation; P (dG): Genotypic differentiation test a cording to Goudet; P (HW): Hardy Weinberg exact probability Value; Fis (W&C): Wright index measuring the deviation from panmixis according to weir and cockerham; F (Ace-1^R): Allelic Frequencies of the G119S mutation; N: Number of individual analyzed

3.5 Different species into *An. gambiae* complex in study sites

During this study two species were identified; *An. gambiaes. s*. and *An. Coluzzii* (Table IV). *An. Coluzzii* was found in all three study sites; ranging from 100% in Jacqueville to 45% in Toumodi and 7% in Tiassalé, while *An. gambiae. s. s.* was found in Tiassale and Toumodi, with 93% and 53%, respectively. Hybrid individuals were observed only in Toumodi (2%).

In Tiassalé and Toumodi the species An. gambiaes. s. (93% and 53%) was more abundant than An. Coluzzii (7% and 45%) (p=0.000)

Table V: Distribution of *An. gambiae*species in Jacqueville, Tiassalé and Toumodi

Localities	Species & Molecular form	Number (%)	p-value
Jacqueville	*An. gambiaes. s*	0 (0)	
	An. coluzzii	45 (100)	
	Total	45 (100)	
Tiassale	*An. gambiaes. s*	42 (93)	0.000
	An. coluzzii	3 (7)	
	Total	45 (100)	
Toumodi	*An. gambiaes. s*	26 (53)	0.000
	An. coluzzii	22 (45)	
	Hybrid *An. gambiaes. s – An. coluzzii*	1 (2)	
	Total	49 (100)	
Total		139	

4. Discussion

The current study revealed resistance to pyrethroids and DDT into field *An. gambiae*populations from Tiassalé, Toumodi, Jacqueville and Adzopé in Côte d'Ivoire. The mortalities ranged between 22% and 67% with pyrethroids and between 18% and 28% with DDT. In fact, resistance to pyrethroids and DDT was already reported in several sites in Côte d'Ivoire [17-22].

Bioassays with pre-exposure to PBO shown significant increase in mortality rates revealing the presence of metabolic resistance in study sites. Indeed, PBO improves the insecticidal effects of pyrethroids by inhibiting two major metabolic enzyme systems, cytochromes P_{450} and esterases in insect [23, 24, 25]. Those enzymes are reported to play a key role in metabolic resistant of insects to insecticides such as to pyrethroids, carbamates and organophosphorus [23, 26]. These results supported those of Chouaïbou [27] who observed a significant increase in post-exposure mortality of PBO with deltamethrin suggesting the presence of metabolic resistance in field *Anopheles* populations of Tiassalé. In Jacqueville and Toumodi, this metabolic resistance could be the result of the use of insecticides in agricultural activities (rice growing, market gardening, etc.),

The kdr mutation was detected in the three sites. The high kdr frequencies in Toumodi (0.95) and Jacqueville (0.83), were linked to the presence of many resistant homozygous individuals. This suggested an ecological advantage associated with homozygosity at the kdr locus.

The Ace-1^R mutation, was present in all three field mosquitoes populations with low frequencies between 0, 03 and 0, 25. These low frequencies seem to be the consequence of the absence of homozygous resistant individuals at this locus, indicating a potential ecological disadvantage associated with homozygosity at the Ace-1^R locus. However, the Ace-1^R mutation may enhance resistance to other insecticides such as organophosphates and carbamates that were not considered in this research.

The weak selection of Ace-1^R in *An. gambiae* was already reported in Côte d'Ivoire [28] with only 2 homozygous individuals resistant over 298 individuals tested.

This study showed a link between the kdr mutation and malaria incidences and also between the Ace-1^R mutation and the malaria incidences. In fact, kdr and Ace-1^R mutations are higher in field mosquitoes populations in high Malaria incidence locations than in low ones.

These mutations conferring insecticide resistance to mosquitoes, seem to reinforce malaria transmission in study sites. It clearly appears that the *Anopheles* populations studied have several resistance mechanisms to pyrethroids and DDT, not only metabolic but also linked to the kdr and Ace-1^R mutations. Insects multiple resistances are suspected to be the basic cause of entomological inefficacy of Malaria vector control with lambda-cyhalothrin-treated mosquito nets [28].

During this research, kdr mutation was observed in both *An. gambiaes. s.* and *An. coluzzii* species found in study sites and.

5. Conclusion

The study showed multiple resistance mechanisms into *Anopheles* populations to pyrethroids and DDT. Resistance was not only linked to enzymatic metabolic mechanisms but also to the kdr mutation. This resistance is a real gap to the main malaria control strategy, which is based on vector control using LLINs. There was no difference in levels of metabolic resistance between low and high incidence areas. On the other hand, kdr and ace 1^R mutations were higher in localities with high incidence.

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