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The L1014F *Kdr* mutation is associated with a higher prevalence and load of the *Plasmodium*-blocking symbiont *Microsporidia* MB In *Anopheles Gambiae* s.l. In Benin

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Abstract

Introduction The *Plasmodium* transmission-blocking endosymbiont *Microsporidia* MB was previously identified in *Anopheles gambiae* s.l., but its association with the carriage of the genotypes of the L1014F *kdr* mutation, as well as the ecological factors driving its geographical distribution remain understudied.

Methods Adult mosquitoes were field-collected using human landing catches (HLCs) across 60 villages in the Covè, Ouinhi, and Zangnanado communes of southern Benin. After morphological identification, a sub-sample of *An. gambiae* s.l. were molecularly speciated, and genotyped for the L1014F *kdr* mutation by Polymerase Chain Reaction (PCR). Enzyme-Linked Immunosorbent Assay (ELISA) and qPCR were also used to assess infection with *Plasmodium falciparum* sporozoites and *Microsporidia* MB, respectively. The environmental variables that drive the habitat suitability for *Microsporidia* MB were also assessed using Maximum Entropy (MaxEnt) modelling.

Results The *An. gambiae* complex ($N = 1040$) was composed of 93.7% *An. coluzzii*, 4.4% *An. gambiae* s.s., 0.2% *An. gambiae* s.s./*coluzzii* hybrids, while the rest failed to amplify. Infection prevalence with *Microsporidia* MB was 1.6% (95% CI: 0.7–3.3) in *An. coluzzii* and 2.2% (95% CI: 0.1–13.2) in *An. gambiae* s.s. The *P. falciparum* sporozoite rate was 2% (95% CI: 1.2–3.1, $N = 974$) in *An. coluzzii*, and null in *An. gambiae* s.s. ($N = 46$). None of the mosquitoes infected with *Microsporidia* MB were infected with *P. falciparum*. The frequency of the L1014F *kdr* mutation was 75.1% (95% CI: 73.1–76.9) in *An. coluzzii* and 91.3% (95% CI: 83.1–95.9) in *An. gambiae* s.s. *Microsporidia* MB was absent in *kdr*-SS mosquitoes but was present in low proportions in both *kdr*-RS and *kdr*-RR mosquitoes (1.9%, 95% CI: 0.6–5.1). The

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mean load of *Microsporidia* MB DNA was higher in *kdr*-RR (23.23 ng/μl, 95% CI: 18.77–28.48) compared to *kdr*-RS (10.13 ng/μl, 95% CI: 7.36–13.91) mosquitoes. The elevation and soil contributed to explain, at 78% and 20% respectively, the habitat suitability for *Microsporidia* MB.

Conclusion In this study, we demonstrated that *An. gambiae* s.l. mosquitoes bearing the L1014F *kdr* resistant allele was associated with a higher prevalence and load of *Microsporidia* MB than their susceptible counterparts. Moreover, the geographical distribution of *Microsporidia* MB was found to be influenced by certain environmental conditions, which warrant further large-scale investigations.

Keywords *Microsporidia* MB, Plasmodium, L1014F *Kdr* mutation

Background

Malaria, transmitted by *Anopheles* (*An.*) mosquitoes, remains a global public health issue with approximately 263 million annual cases, mostly occurring in Africa [1]. Globally, the malaria case incidence declined between 2000 and 2015, stalled between 2016 and 2022, and increased slightly in 2023 compared to 2022 [1]. The stagnation of control progress between 2000 and 2015 is partially attributable to vector insecticide resistance jeopardizing the efficacy of insecticide-based vector control tools [2–4]. This stresses the vital need for complementary control tools, including novel biotechnological ones.

Although some microbes were found to substantially impair pathogen transmission in mosquitoes [5]; the exact pathogen blocking molecular mechanisms have not been elucidated so far. Recently, it has been discovered that *Microsporidia* MB—a naturally-occurring bacterial endosymbiont—can block the infection of *An. arabienensis* with *Plasmodium* (*P.*) *falciparum* [6]. Moreover, it has been shown that *Microsporidia* MB does not induce any major negative impacts on host longevity, development, fecundity, and fertility [6, 7]. These characteristics make *Microsporidia* MB a promising biological tool for blocking the *Plasmodium* transmission cycle in *Anopheles* mosquitoes. Thus, the endosymbiont-based control tools, which prevent pathogen transmission, could be deployed alone or together with traditional insecticide-based control tools to reduce malaria transmission. The potential of endosymbiont-based control strategies was recently shown by the positive effect of the deployment of *Wolbachia*-based strategies in controlling dengue fever in Brazil and Indonesia [8, 9]. This success was due to the ability of the *Wolbachia* endosymbiont to impair viral replication and dissemination, and to reduce pathogen transmission by *Aedes* mosquitoes [10].

A recent study conducted in Nigeria and Niger Republic reported that insecticide phenotypic-resistant mosquitoes displayed a significantly higher prevalence of infection with *Microsporidia* MB than susceptible ones [11]. In the south of Benin, a recent cross-sectional study reported the natural presence of *Microsporidia* MB in both *An. coluzzii* and *An. gambiae* s.s. with respective infection prevalences of 57.0% (95% CI: 45.4–67.9) and

41.0% (95% CI 25.9–57.8) at the mosquito pools level [12]. Moreover, the L1014F knockdown resistance (*kdr*) mutation – a well-validated pyrethroid resistance marker involving a structural change in the voltage-gated sodium channel – was found close to fixation with the presence of three genotypes (RR, RS, and SS) nationwide, particularly in the Covè, Ouinhi, and Zangnanando districts [13, 14]. The present study aimed to assess the association between L1014F *kdr* genotypes and the *Microsporidia* MB infection in *An. gambiae* s.l. In addition, ecological niche models designed based on certain environmental variables (minimum, maximum, and average temperature; elevation; soil; aspect; slope; normalized differential vegetation index) were used to map the occurrence of *Microsporidia* MB, and predict the most suitable areas for its development.

Methods

Study area

Mosquitoes used for the present study were collected between March and May 2023 (onset of the rainy season), during the last round of entomological collection for a three year long randomized controlled trial (RCT). The RCT aimed to evaluate how pyrethroid-pyriproxyfen and pyrethroid-chlorfenapyr long-lasting insecticidal nets (LLINs) perform on malaria transmission, compared to pyrethroid-only LLINs [15]. The current study occurred during the final trial round to ensure that mosquito infection with *P. falciparum* sporozoites was not influenced by insecticide incorporated into nets, which could be a major confounding factor that would obfuscate *Microsporidia* MB detection. The majority of insecticide in the trial LLINs was lost only two years post-deployment [16, 17].

Of note, the RCT was conducted in 60 villages, in the Covè (07°13'08.0400''N, 02°20'21.8400''E), Ouinhi (07°05'00''N, 02°29'00''E), and Zangnanando (07°16'00''N, 02°21'00''E) communes of the Zou department, situated 154 km away from Cotonou, the economic capital of Benin (Fig. 1). Agriculture, livestock, hunting, fishing, hospitality industry and trade are the main economic activities in the area. In the study districts, the climate is subequatorial, with two rainy seasons (April to July and

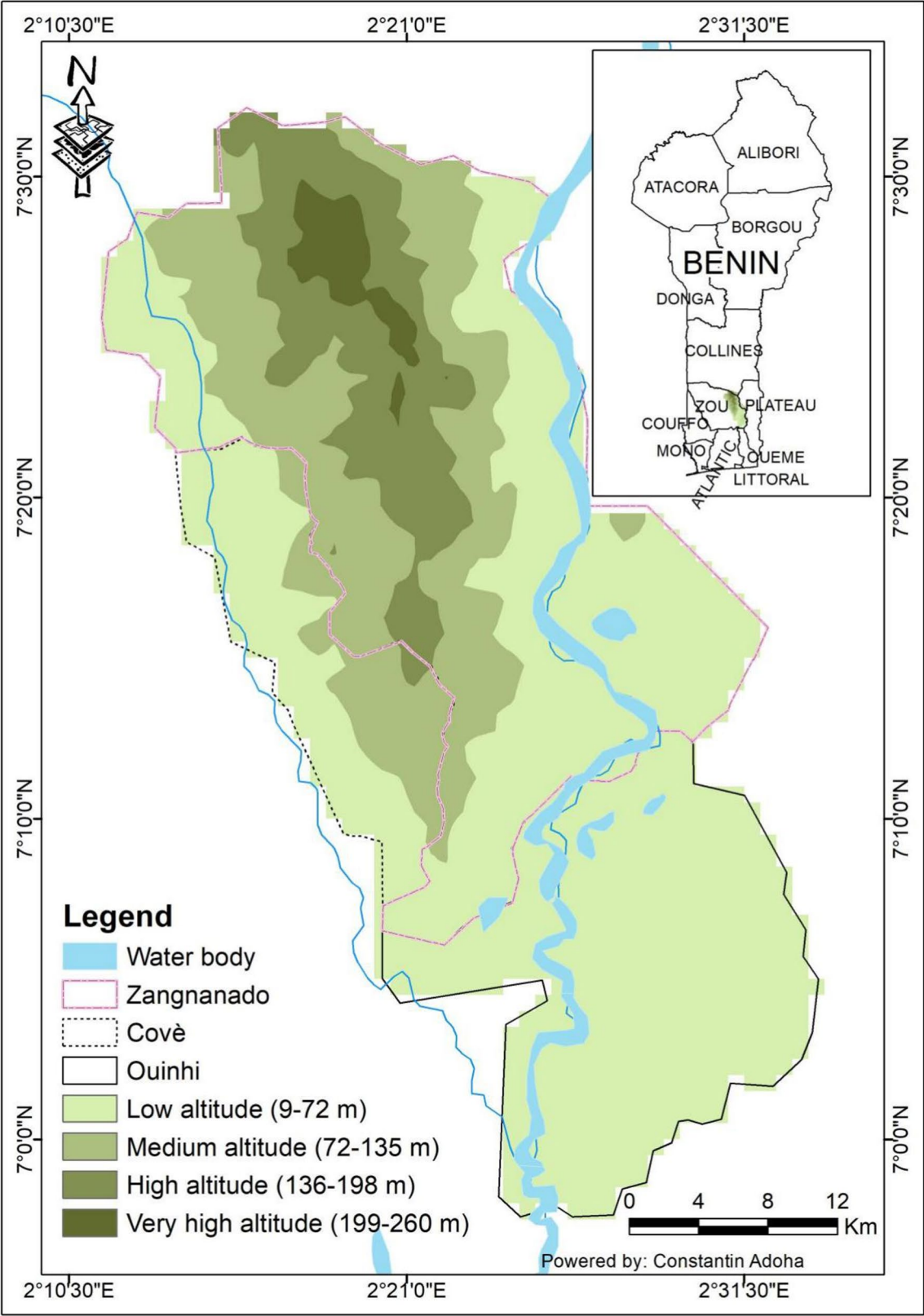


Fig. 1 Map of the study area by Adoha et al. [18]

September to October), and two dry seasons (November to March and July to August). The malaria transmission, principally by *An. coluzzii* and *An. gambiae* s.s. in the area was intense [13].

Mosquito sampling

In each of the 60 surveyed villages, 4 houses (1 chosen at random, and 3 others selected 15–20 m around the first one) were sampled from 07:00 p.m. to 06:00 a.m. using human landing catches (HLCs). All collections were performed over 12 nights, with 5 villages surveyed simultaneously during a single night. Each village was surveyed once. In each house, there were 2 volunteers (one positioned indoors, and the second outdoors) who used haemolysis tubes and flashlights to collect all mosquitoes which landed on their legs. In each village, the first group of 8 volunteers who collected from 07:00 p.m. to 01:00 a.m. was replaced by a second group 8 other volunteers from 01:00 a.m. to 06:00 a.m.

The same sampling effort (4 collections × 60 villages = 240 collections) was performed both indoors and outdoors.

Collected mosquitoes were morphologically identified to species-level using a stereomicroscope and the taxonomic keys of Gillies and De Meillon [19] and Coetzee [20]. Collections were then stored on silica gel at room temperature for further molecular analyses.

Molecular analyses

Molecular identification of species, L1014F *kdr* mutation and *P. falciparum* sporozoite infection The head-thoraces of 16–18 individuals of *An. gambiae* s.l. were randomly selected and screened for *P. falciparum* sporozoite infection using circumsporozoite protein (CSP) ELISA [21]. Their legs, wings, and abdomens were also used for DNA extraction using the DNeasy Blood & Tissue Kit (QIAGEN, GERMANY). The extracted DNA served to identify molecular species [22] and to assess the frequency of the L1014F *kdr* mutation [23] by PCR.

Molecular detection and quantification of *Microsporidia* MB

Due to limited funding, DNA from only 494 *An. gambiae* s.l. sampled from all the 60 villages (about 8–9

mosquitoes per village) were used for the molecular identification of *Microsporidia* MB. These samples include DNA of all the mosquitoes carrying the *kdr*-SS genotype ($N=72$), all those infected with *P. falciparum* sporozoites ($N=18$), and all those detected as *An. gambiae* s.s. ($N=46$), while the rest ($N=358$) were randomly selected across all surveyed villages (Table 1).

Molecular detection and quantification of *Microsporidia* MB were performed using specific primers (MB18SF, 5'-CGCCGGCCGTGAAAAATTTA-3'; MB18SR, 5'-CCTTGGACGTGGGAGCTATC-3') designed to detect this bacterium [6, 24] using a MIC qPCR cycycler (Step One plus). A 10 µL mixture consisting of 5 µL of SYBR green, 1 µL of nuclease-free PCR water, 1 µL of each primer (forward and reverse), and 2 µL of DNA were prepared. Reactions were run in technical duplicate. The reaction conditions were: 95 °C for 15 min, 40 cycles (95 °C for 60s, 65 °C for 90s, 72 °C for 60s), 72 °C for 5 min, followed by a melting curve. The efficiency of the primers was determined following linear regression, and the standard curve was generated. The data collected following the quantification of *Microsporidia* MB data were normalized using primers from the housekeeping gene *RpS7* (S7F, TCCTGGAGCTGGAGATGAAC; S7R, GACGGGTCTGTACCTTCTGG) [25].

Ecological niche modelling for *Microsporidia* MB

Occurrence data for *Microsporidia* MB was recorded at the level of the georeferenced study villages. Fourteen environmental predictors, including climatic and topographic variables (Supplemental file, Table S1), were collected for the three months study period, from the Shuttle Radar Topography Mission (SRTM), the Normalized Difference Vegetation Index (NDVI), and the WorldClim database at 1 km spatial resolution. All raster datasets were clipped to the study area extent, resampled using bilinear interpolation, and projected to the WGS 1984 World Mercator system. Of the 14 environmental predictors initially used to run the first model, 11 showed no contribution and were removed (Supplemental file, Table S1).

The MaxEnt algorithm (version 3.4.4) was used for modelling the ecological niche for *Microsporidia* MB. This model, based on the principle of maximum entropy,

Table 1 Number of mosquitoes tested according to species, L1014F *Kdr* genotype, and status of infection with *P. falciparum*

Molecular species and L1014F kdr genotypes									
	An. coluzzii				An. gambiae s.s.				
Infection status with Pf	RR	RS	SS	Total 1	RR	RS	SS	Total 2	Total 1 + Total 2
Negative	159	199	72	430	38	8	0	46	476
Positive	13	5	0	18	0	0	0	0	18
Grand Total	172	204	72	448	38	8	0	46	494

Pf *Plasmodium falciparum*, RR Homozygous resistant, RS Heterozygous resistant, SS Homozygous susceptible

estimates the probability of a species' presence based on occurrence data and environmental variables.

The “cloglog” format was used to set the model output and produce a prediction map with values varying between 0 and 1, showing habitat suitability. A k-fold cross-validation (allowing cross-evaluation of model performance) with 10 replicates was used to enable the contribution of all presence records to the training and the testing of the model. The model was set up to a maximum number of 5,000 iterations, with quadratic and linear features, as well as a regularization multiplier set to 2 in order to limit overfitting due to the small number of points.

Permutation importance analysis was used to evaluate the contribution of each predictor variable. The model performance was assessed through the Area Under the receiver operating characteristic Curves (AUC). Of note, the closer the AUC value is to 1, the better performing the model is. Presence probability maps were produced in logistic format (values from 0 to 1), indicating the environmental suitability of different areas for the presence of *Microsporidia*.

ArcGIS software (version 10.8) was used to generate the final habitat suitability map for *Microsporidia* MB.

Statistical data analyses

In *An. gambiae* s.l., the *P. falciparum* sporozoite rate, and infection prevalence with *Microsporidia* MB was calculated by dividing the number of infected mosquitoes by the total tested. The frequency of the L1014F *kdr* mutation was calculated using the following formula: $F(L1014F\ Kdr) = (2 \cdot RR + RS) / (2 \cdot (RR + RS + SS))$.

The confidence intervals of the sporozoite rate, infection prevalence with *Microsporidia* MB, and frequency of the L1014F *kdr* mutation was calculated using the exact

binomial test. The Chi-square test of comparison of proportions was used to compare all three outcomes.

The load of DNA of *Microsporidia* MB was estimated in positive samples from their mean Ct values by drawing a standard curve from known dilutions of the positive control. A linear regression was performed between the Ct values and the decimal logarithm of the loads according to the formula: $Ct = a \times \log_{10}(\text{load}) + b$, where *a* is the slope and *b* the y-intercept of the line. The load of DNA of *Microsporidia* MB was thus calculated using the following formula: $\text{DNA load} = 10^{((Ct - b)/a)}$. The Poisson method was used to compare and estimate the confidence intervals of mean DNA load per L1014F *kdr* genotype. All analyses were performed using R statistical software, version 4.4.3.

Results

Anopheles species composition

Of the 8,855 *Anopheles* mosquitoes collected during the last round of the entomological data collection of the RCT, 44.8% (*N*=3971) were sampled indoors, and the rest outdoors. *An. gambiae* s.l. accounted for 96.6% (3837/3971) of the indoor collection and 97.9% (4785/4884) of the outdoor collection, with a mean of 97.4% (8622/8855) for the total collection (indoor + outdoor). Other *Anopheles* mosquitoes, including *An. funestus*, *An. pharoensis*, and *An. ziemanni*, were also found but at lower frequencies both indoors and outdoors (<3%) (Fig. 2).

Of the 1040 *An. gambiae* s.l. molecularly speciated, 93.7% (*n*=974) were *An. coluzzii*, 4.4% (*n*=46) were *An. gambiae* s.s., 0.2% (2/1040) were hybrids (*An. gambiae* s.s./*coluzzii*), while 1.7% (18/1040) did not amplify. The same trend was observed both indoors (Fig. 3A) and outdoors (Fig. 3B).

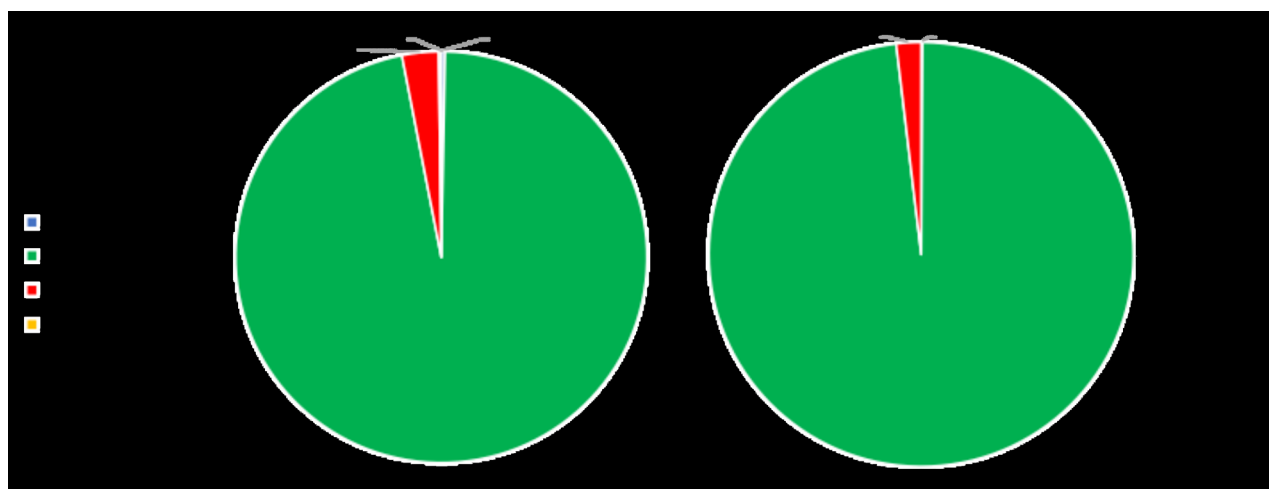


Fig. 2 *Anopheles* species composition in the study area

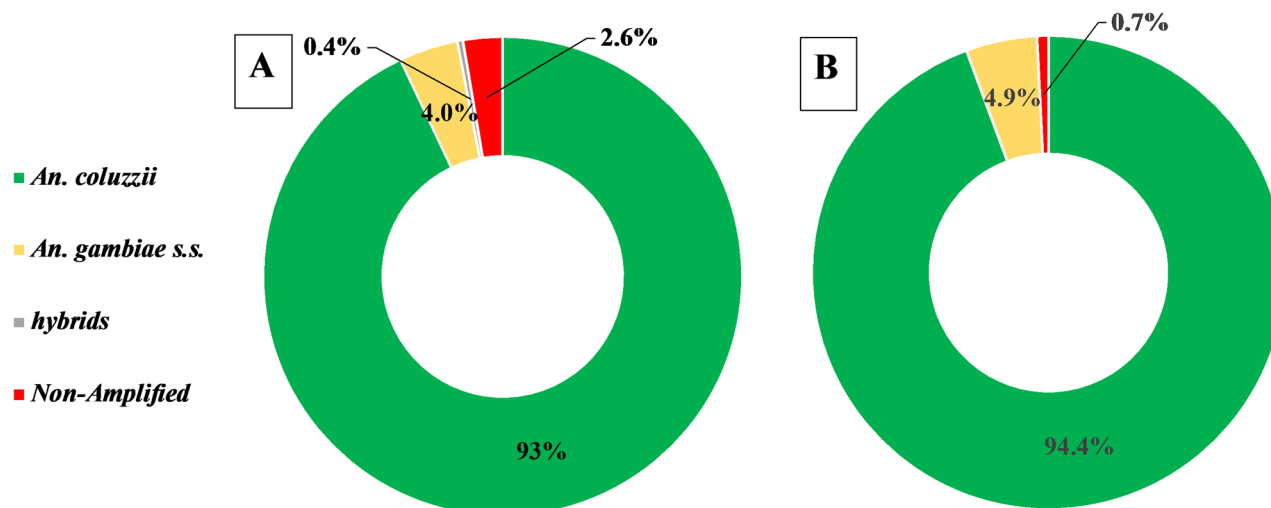


Fig. 3 Molecular species composition in the *Anopheles gambiae* complex indoors (**A**; $N=526$), and outdoors (**B**; $N=514$)

Table 2 Infection prevalence (IP) with *Microsporidia* MB in *Anopheles Gambiae sensu stricto* and *Anopheles coluzzii*

Mosquito Species	N identified	N tested	N Positive	IP (%)	95% CI
<i>An. coluzzii</i>	974	448	7	1.6	0.7–3.3
<i>An. gambiae</i> s.s.	46	46	1	2.2	0.1–13.2

An *Anopheles*, N Number of, IP Infection prevalence, CI Confidence interval

Table 3 Sporozoite rate in *Anopheles Gambiae sensu stricto* and *Anopheles coluzzii*

Mosquito Species	N tested	S+	SR (%)	95% CI
<i>An. coluzzii</i>	974	18	1.8	1.0–2.9.0.9
<i>An. gambiae</i> s.s.	46	0	0	0.0–9.6.0.6
<i>An. gambiae</i> s.s./ <i>coluzzii</i>	2	0	0	0.0–80.2.0.2
<i>An. gambiae</i> s.l.	1022	18	1.7	1.0–2.7.0.7

An *Anopheles*, N Number of, S+ Number of sporozoite positive mosquitoes, SR Sporozoite rate, CI Confidence interval

Infection prevalence with *Microsporidia* MB in *An. gambiae* s.s. and *An. coluzzii*

Of 493 specimens of *An. gambiae* s.l. tested for the presence of *Microsporidia* MB, 8 were positive, which equates to a mean infection prevalence of 1.6% (95% CI: 0.8–3.3).

At the molecular species level, the infection prevalence was 1.6% (95% CI: 0.7–3.3, 7/448) in *An. coluzzii*

versus 2.2% (95% CI: 0.1–13.2, 1/46) in *An. gambiae* s.s. (Table 2).

P. falciparum sporozoite rate (SR) in *An. gambiae* s.l. and its molecular species

Of 1,022 specimens of *An. gambiae* s.l. tested, 18 were positive for *P. falciparum* sporozoites, equating to an average SR of 1.7% (95% CI: 1.0–2.7.0.7) (Table 3).

At the molecular species level, the SR was 1.8% (95% CI: 1.0–2.9.0.9) in *An. coluzzii*, and null in *An. gambiae* s.s. and hybrids (*An. gambiae* s.s./*coluzzii*) (Table 3).

Of the eight individual *An. gambiae* s.l. found positive with *Microsporidia* MB, none harboured *P. falciparum* sporozoites.

Frequency of the L1014F *kdr* mutation in *An. gambiae* s.l. and its molecular species

Of the 1,022 specimens of *An. gambiae* s.l., 58.8% ($N=600$) were *kdr*-RR genotype, 34.2% ($N=350$) *kdr*-RS genotype, and 7% ($N=72$) *kdr*-SS genotype. The mean frequency of L1014F *kdr* mutation for *An. gambiae* s.l. was 75.8% (95% CI: 73.9–77.7) (Table 4).

At the molecular species level, the frequency of L1014F *kdr* mutation was 75.1% (95% CI: 73.1–76.9) in *An. coluzzii* and 91.3% (95% CI: 83.1–95.9) in *An. gambiae* s.s. (Table 4).

Table 4 Frequency of the L1014F *Kdr* mutation in *Anopheles Gambiae sensu lato* collected from villages in Covè, Ouinhi and Zangnanado communes in Zou Department, Southern Benin

Mosquito Species	Total	RR	RS	SS	F(L1014F <i>kdr</i>)	95% CI
<i>An. coluzzii</i>	974	561	341	72	75.1	73.1–76.9
<i>An. gambiae</i> s.s.	46	38	8	0	91.3	83.1–95.9
<i>An. gambiae/coluzzii</i>	2	1	1	0	75	21.9–98.7
<i>An. gambiae</i> s.l.	1022	600	350	72	75.8	73.9–77.7

An *Anopheles*, CI Confidence interval, RR Homozygous resistant, RS Heterozygous resistant, SS Homozygous susceptible

Table 5 Infection prevalence with *Microsporidia* MB per L1014F *Kdr* genotype (SS, RS, and RR) in *Anopheles Gambiae* sensu Lato populations collected from Covè, Ouinhi and Zangnanado communes in Zou Department, Southern Benin

L1014F <i>kdr</i> Genotypes	N tested	N Positive	IP (%)	95% CI
SS	72	0	0	0.0–63.03
RS	212	4	1.9	0.6–5.1
RR	210	4	1.9	0.6–5.1

N Number of, IP Infection prevalence, CI Confidence interval, RR Homozygous resistant, RS Heterozygous resistant, SS Homozygous susceptible

Infection prevalence with *Microsporidia* MB per L1014F *kdr* genotype (SS, RS, and RS) in *An. gambiae* s.l.

Overall, the infection prevalence with *Microsporidia* MB was null in *kdr*-SS, and 1.9% in both *kdr*-RS (95% CI: 0.6–5.1, $N=212$), and *kdr*-RR (95% CI: 0.6–5.1, $N=210$) genotypes of *An. gambiae* s.l. (Table 5).

The mean load of DNA of *Microsporidia* MB was significantly higher in the *kdr*-RR genotype (23.23 ng/μl, 95% CI: 18.77–28.48) than in the *kdr*-RS genotype (10.13 ng/μl, 95% CI: 7.36–13.91) (Table 6).

Overall, *Microsporidia* MB was absent in *kdr*-SS genotype, and had low prevalence with lesser load in *kdr*-RS genotype, and low prevalence with higher load in *kdr*-RR genotype.

Ecological niche model for *Microsporidia* MB

The ecological niche model that was developed showed good predictive power of the potential distribution of *Microsporidia* MB, with an average AUC value of 0.825, indicating satisfactory performance.

The modelling results showed that the most suitable areas for the development of *Microsporidia* MB were the southwest of Covè, the south of Zangnanado, and the entire western part of Ouinhi (Fig. 4). Of the sixty investigated villages, the seven where *Microsporidia* MB was detected are identified on Fig. 4.

Contribution of environmental variables to the habitat suitability for *Microsporidia* MB

Of all the evaluated environmental variables (Supplemental file, Table S1), the highest contributors were elevation (78%), followed by the soil (20%), and the slope (2%) (Fig. 5). The contribution of all other environmental variables used in the model was null.

Areas with low elevation were found to be particularly conducive for the development of *Microsporidia* MB (Fig. 6a). Furthermore, the soil also showed a negative

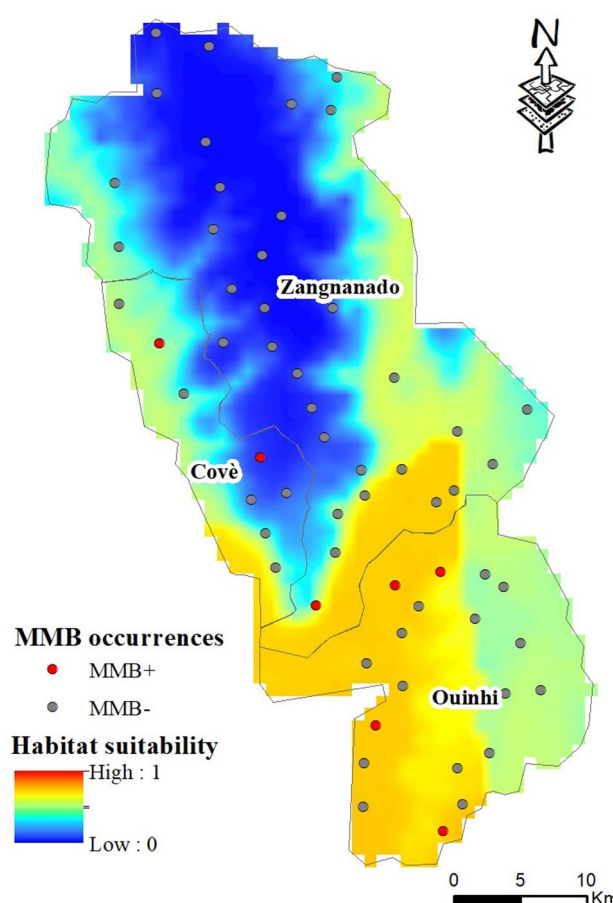


Fig. 4 Ecological niche model for *Microsporidia* MB (MMB occurrences: Status of *Microsporidia* MB occurrences in villages, MMB- : Villages with *Microsporidia* MB negative samples, MMB+ : Villages with *Microsporidia* MB positive samples)

correlation with the habitat suitability for *Microsporidia* MB (Fig. 6b).

Discussion

Microsporidia MB is a bacterial endosymbiont that disrupts *Plasmodium* development cycle, and whose transmission occurs vertically in *Anopheles* mosquitoes [6]. The present study provides information on its infection prevalence, relationship with L1014F *kdr* genotypes (RR, RS, and SS), and ecological factors that drive its geographical distribution in the Covè, Ouinhi and Zangnanado communes in southern Benin. It was carried out over the last round of data collection of a three year long randomized controlled trial (RCT) assessing the efficacy of dual active-ingredient LLINs. During the third year of

Table 6 Load of *Microsporidia* MB in each specimen bearing the L1014F *Kdr* mutation

Genotypes	Load of DNA of <i>Microsporidia</i> MB (ng/μl)					95% CI
	Mosquito 1	Mosquito 2	Mosquito 3	Mosquito 4	Mean	
RS	0.06	5.27	0.06	35.13	10.13	7.36–13.91
RR	12.20	18.40	28.54	33.77	23.23	18.77–28.48

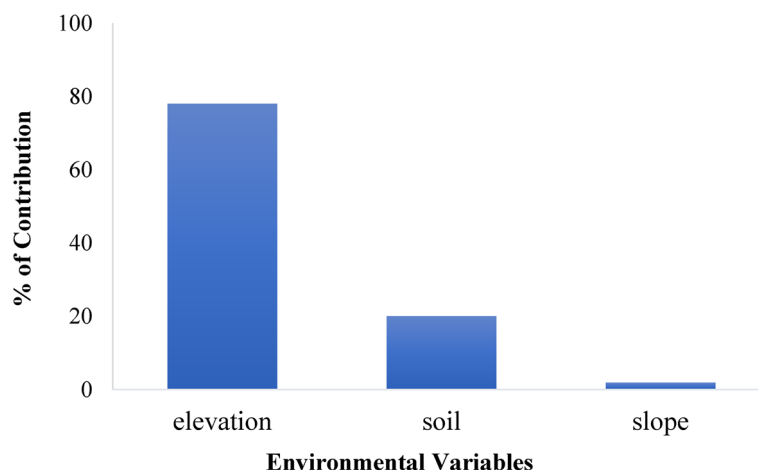


Fig. 5 Contribution of environmental variables in the habitat suitability for *Microsporidia* MB

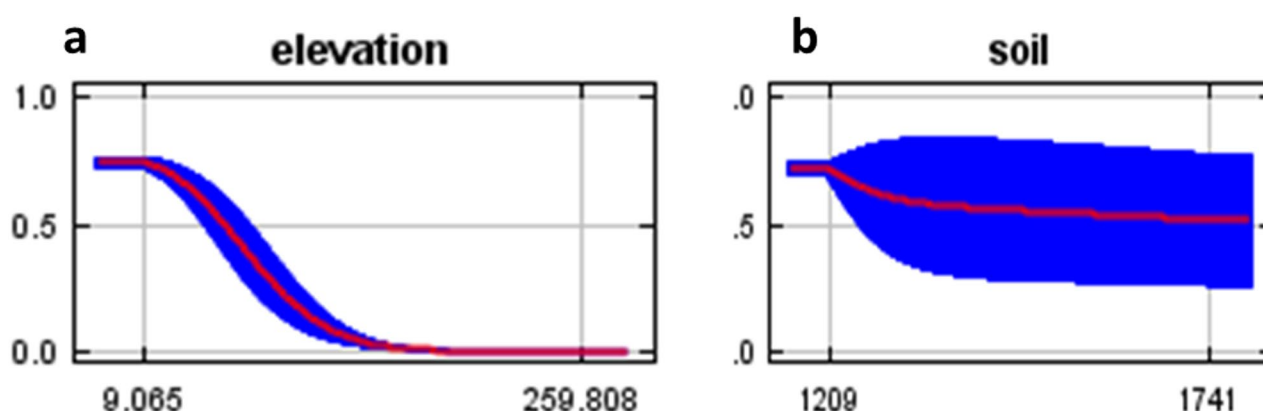


Fig. 6 Effect of environmental variables according to the Maxent prediction (Elevation (a), and Soil (b))

the trial, pyrethroid-pyriproxyfen and pyrethroid-chlorfenapyr LLINs performed similarly on both entomological [26] and epidemiological [16] outcomes, compared to the standard-pyrethroid only LLIN.

The most frequent *Anopheles* species in the study area was *Anopheles gambiae* s.l., while all other *Anopheles* species were found at very low frequencies. This confirms *Anopheles gambiae* s.l. as the main malaria vector in the study area. Molecular species identification performed in *An. gambiae* s.l. revealed that the mosquito complex was mostly composed of *An. coluzzii* followed by *An. gambiae* s.s. Previously published results by Koukpo et al. [27] suggested that climatic conditions of southern Benin may be more conducive to the development of *An. coluzzii*. Moreover, the numerous permanent and semi-permanent larval habitats derived from the Oueme and Zou rivers in the study area could have been favorable to the emergence of *An. coluzzii*, as previously demonstrated by Diabate et al. [28] in a study conducted in Burkina Faso. Overall, the *Anopheles* species composition was similar to what was previously observed in some other southern locations of the country [12].

The mean infection prevalence of *An. gambiae* s.l. with *Microsporidia* MB was 1.6%, which is relatively low and could be due to the fact that the mosquito collection occurred at the onset of the wet season. A previous study conducted in the Koussin-Lélé village, commune of Covè, showed a higher infection prevalence of *Microsporidia* MB in the dry season (14%) compared to the wet season (0.7%) [29]. Similarly, Herren et al. [6] showed that the highest prevalence of infection of mosquitoes with *Microsporidia* MB was observed after the peak rainfalls.

The two molecular species had similar infection prevalence with *Microsporidia* MB (*An. coluzzii*: 1.6%, and *An. gambiae* s.s.: 2.2%), though there was a higher number of *An. coluzzii* tested (*An. coluzzii* (N = 448), and *An. gambiae* s.s. (N = 46)). The association of *Microsporidia* MB with both species in the present study corroborates previous findings from Akorli et al. [7] in Ghana, and Moustapha et al. [11] in Nigeria and Niger.

The *P. falciparum* sporozoite rate was 1.8% in *An. coluzzii* and null in *An. gambiae* s.s., and none of the mosquitoes found positive with *Microsporidia* MB contained sporozoites of *P. falciparum*. Although this finding

does not have enough power because of the low number of mosquitoes found positive with *Microsporidia* MB, the absence of malaria parasites could be due to *Microsporidia* MB impairing transmission of *P. falciparum*, as previously demonstrated by Herren et al. [6].

Given, the infection prevalence with *Microsporidia* MB was found to be season-dependent [6, 29], it was important to investigate environmental drivers of its presence. The ecological niche model showed that the most suitable areas for the development of *Microsporidia* MB were the southwest of Covè, the south of Zangnanado, and the whole western part of Ouinhi. This could be due to the fact that these areas have a low elevation, which favors the accumulation of water that serves as habitat for the development of mosquito aquatic stages that could harbour *Microsporidia* MB. The amount of nutrients and heavy metals in the soil can also drive the contamination level of mosquito breeding sites and affect the survival and transmission of *Microsporidia* MB, as previously mentioned by Tchigossou et al. [29]. While a recent laboratory study revealed that temperature modulates the dissemination potential of *Microsporidia* MB [30], the present field study showed no contribution of temperature to the bacteria habitat suitability. This may be because the temperature data of the current study was only collected during three months, so we suggest a larger *Microsporidia* MB ecological niche modelling study before drawing any conclusions.

Overall, the L1014F *kdr* mutation was close to fixation because the combined genotypic frequency for both *kdr*-RR and *kdr*-RS carrying mosquitoes equated to 93%, and the mean allelic frequency of L1014F *kdr* mutation was 75.8% for *An. gambiae* s.l. This situation could be due to the triennial mass distribution campaigns of pyrethroid-based LLINs in the region, combined with the uncontrolled use of pyrethroid insecticides for agricultural purposes.

A recent study conducted in Nigeria and Niger showed a possible association between infection of *An. gambiae* s.l. with *Microsporidia* MB, and phenotypic resistance to pyrethroids [11]. Indeed, this study revealed a significantly higher infection prevalence with *Microsporidia* MB in resistant mosquitoes than in susceptible ones. Similarly, the assessment of the infection prevalence with *Microsporidia* MB per L1014 *kdr* genotype in the present study revealed an infection prevalence of 1.9% in both *kdr*-RR and *kdr*-RS genotypes, while no mosquito with *kdr*-SS genotype harboured *Microsporidia* MB. Moreover, the mean load of DNA of *Microsporidia* MB was significantly higher in mosquitoes with the *kdr*-RR genotype than in those with the *kdr*-RS one. These results that show a graded effect for the prevalence/load of *Microsporidia* MB (absence in *kdr*-SS genotype, low prevalence with lesser load in *kdr*-RS genotype, and low prevalence

with higher load in *kdr*-RR genotype), which suggests a possible genotype-dependent association. Thus, the carriage of the R resistant allele of the L1014F *kdr* mutation might have conferred an advantage to the mosquitoes of becoming more infected with *Microsporidia* MB. One potential explanation could be that *Microsporidia* MB stimulates the mosquito's immune system or alters its metabolism, making it more susceptible to infection, or because the resistance process weakens the mosquito's defence against parasitic infections. These hypotheses deserve confirmation through a larger *Microsporidia* MB-focused study. Another study demonstrated an association between overabundance of *Asaia* and *Serratia* Bacteria and deltamethrin resistance in Côte d'Ivoire [31]. Indeed, there was increased evidence that resistant mosquitoes bear those bacteria (*Asaia* and *Serratia*) as they can degrade insecticides [31]. All these display the pivotal role played by mosquito microbiota.

Although, the method used for selecting *An. gambiae* s.l. mosquitoes screened (all *kdr*-SS, all *P. falciparum* positive, all *An. gambiae* s.s., and a random selection of the rest) to identify *Microsporidia* MB is a pragmatic approach given funding limits, it introduces a potential bias and may affect statistical power of the analyzes, which is the main limitation for the study. The overall low prevalence and the used sampling method mean that the observed association, while suggestive, require validation with a larger, randomly selected sample set to confirm causality and generalizability. Moreover, it would also be interesting to investigate how the physico-chemical characteristics of mosquito breeding sites (pH, turbidity, dissolved oxygen, conductivity, total dissolved solids, salinity) could influence the dissemination of *Microsporidia* MB.

Conclusions

Findings of the present study show that the carriage of the L1014F *kdr* resistance allele by *An. gambiae* s.l. mosquitoes was associated with greater prevalence and load of *Microsporidia* MB, compared to homozygous susceptible ones. Moreover, elevation and soil were the ecological drivers that influence infection with *Microsporidia* MB, which requires further investigation.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12866-026-04810-5>.

Supplementary Material 1

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Authors' contributions

AS, MJA, RA, GST, NP, JC, MCA, LAM, and CJA wrote the main study protocol and designed it. AS, MJA, AD, and CJA performed the statistical and spatial analysis. MJA, GST and LAM and conducted the molecular analyzes. BY, GST, SZH, DMZ, AKK, and CJA performed the field work. AS, RA, NP, JC, GGP, MCA, and LAM supervised the study data collection. NP, JC MCA, and GGP provided administrative support. AS, TS, MJA, AD, and CJA wrote the original draft of the manuscript that was edited by BY, UT, RCM, TS, RA, CZK, SZH, DMZ, AKK, HS, VG, RO, AAO, NP, JC, GGP, MCA, and LAM. All authors read and approved the final manuscript.

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Data availability

The datasets generated and/or analysed during the present study are available on reasonable request from the corresponding author.

Declarations

Ethics approval and consent to participate

The protocol of the present study was reviewed and approved by the Benin's Comité National d'Éthique pour la Recherche en Santé (N°30/MS/DC/SGM/DRFMT/CNERS/SA, Approval n°6 of 04/03/2019), and the Ethics Committee of the London School of Hygiene and Tropical Medicine (16237-1). Prior to their involvement in the study, all community members and leaders provided their informed consent. At the onset of the study, collectors were trained on how to catch mosquitoes on their lower limbs while avoiding being bitten. They were also immunized against yellow fever and taken care of at the nearest health facility when they suffered from malaria or other illnesses that had similar symptoms. The study was conducted in compliance with the Helsinki Declaration, and all national health guidelines and regulations were enforced.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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