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Adan Oviedo Date

Spatial Cluster Analysis of *P. vivax* and *P.malariae* Exposure Among Haitian School Children Sampled Between 2014 And 2016

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B.S., Georgia Gwinnett College, 2015

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Abstract

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By Adan Oviedo

Malaria is a parasitic disease associated with flu-like symptoms. Haiti's failure to eradicate malaria during the 1955-1969 global intervention effort testifies to the need for accurate surveillance methods in sub-microscopic low-transmission settings. Protocol within the Transmission Assessment Survey (TAS) employs multiplex bead technology to determine exposure to *Plasmodium* Merozite Surface Protein 1 (MSP1). The goal of the project is to describe the distribution of *P. malariae* and *P. vivax* exposure as well as identify significant hot-spot foci in Haiti. Access to the de-identified TAS was granted by the CDC. Environmental variables were attained from open source sites and analyzed using univariate logistic regressions. K-functions were calculated using a maximum radius of 50km with 2.5km intervals and confidence intervals using 999 permutations. Moran's I was calculated using inverse distance weights, 999 permutations, and a threshold distance of 20km. Kulldorff's Scan Statistic was calculated using discrete purely spatial Poisson modeling and 999 Monte Carlo simulations. 21,670 children were tested for *P. malariae* in 679 schools. 24,510 children were tested for *P. vivax* in 787 schools. 275 (1.27%) and 113 (0.46%) children were determined seropositive for *P. malariae* and *P. vivax*, respectively. K-functions suggested school aggregation at all intervals up to 50 km. Weighted K-functions in north Haiti suggested aggregation at limited intervals. Weighted k-functions in south Haiti suggested spatial randomness at all intervals. Moran's I in the north suggested autocorrelation for P. malariae ($I=0.153$, $p<0.01$) but not for P. vivax ($I=-0.016$, $p>0.05$). Moran's I in the south suggests no autocorrelation for P. malariae ($I=-0.001$, $p>0.05$) or P. vivax ($I=0.062$, $p<0.10$). Kulldorff's Spatial Scan suggested five hot-spots for non-falciparum malaria exposure. No environmental variables were associated with seropositivity. Cluster A ($LR=20.8$, $p<0.001$) was determined a significant hot-spot of interest. Due to limitations, clusters B (12.3, $p<0.01$) and E ($LR=10.1$, $p<0.05$) are potentially of interest but need further review. Clusters C ($LR=22.1$, $p<0.001$) and D (10.4, $p<0.05$) likely result directly from limitations in methods. This study serves as a baseline distribution of non-falciparum malaria within Haiti's borders intended to provide suggestions for focused and thorough active surveillance projects conducted in the near future.

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BACKGROUND

Malaria is a parasitic disease typically associated with flu-like symptoms such as chills, headaches, and fever. Cases of malaria typically occur in tropical and sub-tropical regions where exposure to anopheline vectors is common. In 2016 the World Health Organization (WHO) reported that 216 million clinical cases of malaria occurred worldwide with a majority of cases occurring in developing countries. Although there are various forms of affordable medication available for this disease, malaria is a burden on society, inducing bouts of fever and even death in extreme cases (1-2). The cycle of human transmission begins when a female *Anopheles* mosquito consumes a blood meal from an infected human, ingesting parasitic *Plasmodium* gametocytes in the process (39). The parasite gametes undergo fertilization and development in the mosquito midgut before migrating to the salivary glands of its vector host (39, 44). During the next human blood meal, the *Plasmodium* sporozoites are ejected from the mosquito salivary gland into the bloodstream of the new human host. Once inside the human host, the latent period of development is dependent on species, but generally, the parasite migrates to the liver and invades hepatocytes where they replicate and develop into merozoites (40). The parasite is eventually released into the bloodstream where they invade erythrocytes and undergo asexual reproduction (40, 44). Once they reach a certain threshold, the human immune response is triggered, leading to clinical symptoms. Some of the merozoites will develop into gametocytes in the bloodstream, where the cycle of transmission will continue if an uninfected vector ingests them (39, 44).

Various species of *Plasmodium* parasites, such as *Plasmodium falciparum, Plasmodium malariae, Plasmodium vivax,* and *Plasmodium ovale*, have been previously documented to infect humans. However, the focus of this study will center on the species *Plasmodium malariae* and

Plasmodium vivax. The biological mechanisms of infection between these two species suggest clinically important differences (44, 45). For example, *P. malariae* displays no evidence of a latent period during liver cell infection (44). In addition, asexual propagation in the erythrocyte lasts longer compared, with an infection cycle of 72 hours, compared to 48 hours in other species. The combination of biological tendencies causes *P. malariae* to have a relatively low parasite count and an earlier immune response (44). *P. vivax* on the other hand, has dormant forms of liver hpynozoites, which can induce relapse infections without further bites from infected mosquitoes (45). In addition, this species is distinct in its reliance on the Duffy blood group antigen found on the erythrocyte surface. Research suggests that *P. vivax* only tends to invade blood cells expressing this surface protein. However, recent studies have suggested that the parasite can still infect Duffy-negative individuals, but at a lower capacity (46).

Both of these species rely on a vector for reproduction and transmission. Thus, the parasite distribution is highly reliant on the movement and behavioral factors of its host. The first requirement for the life cycle of the mosquito host is dependent on a standing water source for reproduction and development its early larval stages. Upon emergence, females have a species dependent latency period of maturation before mating and the need for a blood meal (42). However, once the females become adults they enter a cycle of mating, blood-feeding, and egg-laying for the duration of their lives (42). Different species of Anopheline mosquitoes have varying preferences in environment, flight behavior, and feeding habits. For example, *A. gambie* and *A. funestus* tend to be endophagic and have peak biting times around midnight, while *A. farauti* mostly feeds outdoors in the early evening (41). One of the more clinically significant vectors of interest in certain regions of the tropics such as the Island of Hispaniola is *Anopheles albumanus.* This is a primarily coastal species of mosquito normally found below an elevation of

500m (16, 24). This species typically prefers to feed outdoors and has a zoophilic feeding preference, making rural environments ideal. Its gonadotrophic cycle is roughly between 2-5 days and can lay its eggs in permanent and semi-permanent groundwater sources. In addition, *A. albumanus* larvae can co-inhabit pools of water with other species of mosquito (16). The clinical significance of this species is that it can harbor and transmit *Plasmodium* parasites such as *P. falciparum, P. malariae,* and *P. vivax.*

Surveillance of sub-microscopic *Plasmodium* reservoirs in low transmission settings require novel tools. One tool in particular takes advantage of nucleic acid, protein, tissue, or even chemical biological responses. As an example, the *Plasmodium* Merozite Surface Protein 1, subunit 19 ($MSP1_{19}$) plays a role in the initial binding of merozoites to an erythrocyte (48). Blood serum immunoglobulin G (IgG) antibodies are useful for detecting past exposure to a *Plasmodium spp* by detecting antibodies specific to the *Plasmodium* suface proteins such as the MSP1. Antibodies in the blood-stream are produced by B-cells as a protective measure against blood-stage *Plasmodium* merozoites. Their main role is to inhibit erythrocyte infection by blocking adherence to surface proteins and stimulating phagocytosis by monocytes and macrophages (47-48). A B-cells memory retains the ability to produce antigen specific antibodies for a prolonged period of time. Research testing B-cell memory to malaria infections have recorded short and long-lived persistence (47). One study by Wipasa et al suggests a half-life of 7.6 years for certain *P. vivax* specific IgG antibody production (47). Multiplex bead technology can evaluate these immunological responses with a focus on specific and multiple analytic testing. The prime benefit offered by multiplex bead technology is an inexpensive and robust method of data generation for parasite transmission in low endemic regions (3, 7, 19, 27, 29). Multiplex technology allows for a lower titration capacity thanks to the three dimensional

bead structure and flow cytometry emission detectors which can read a wide range of fluorescent intensities (29). Results allow for continuous data collection or a reliable dichotomization into seropositive and seronegative categories. This dichotomization simplifies interpretation to allow for prevalence measure comparisons between sampling regions. In addition, because serological samples contain long lived IgG responses, data do not denote incident cases but can determine exposure for as long as antibodies are present in the human body (47, 48). Although, there is generally an overlap between incidence and exposure data (31), the acquisition of exposure data explores a different aspect of malaria transmission. Exposure is meant to represent historic or consistent transmission sites; where-as incident surveillance is concerned with active infection within a specific period of time. Unfortunately, because exposure is a historical description of transmission, newer transmission regions can be missed if antibody responses have not developed in the local population (31). In addition, readings and results are highly dependent on protocol, reagents, and on sample size for which there is no internationally accepted standard (29). A study by Stresman et al also suggests that when a sample size is ≤ ~20% of the local population an average of 38% of transmission hot-spots were misclassified. This would suggest that one third of the regions needing intervention would be missed due to low sample size collection. Eradication efforts need to be preceded by accurate surveillance systems in order to effectively incorporate interventions. The largest scale example of global intervention efforts occurred from 1955-1969, when the WHO embarked on the Global Malaria Eradication Program (GMEP) (24-25, 49). The Hattian malaria elimination efforts in the early 1960's are a good example of why accurate surveillance is a need (22, 24-25). During these efforts, overlooked sites and sites deemed parasite free still harbored enough parasites to re-introduce infection within the intervention regions (25, 49).

The county of Haiti on the Island of Hispaniola rests between the Caribbean Sea and the Atlantic Ocean, sharing a boarder with the Dominican Republic and located southeast of Cuba. Haiti has a landmass of about 27,000 km^2 with a width of 177km along the eastern border with the Dominican Republic and a maximum length of 290 km along the southern peninsula (24). Haiti contains two major islands, the Island of Tortue to the north, and the Island of Gonâve between the two peninsulas. Four fifths of the country are mountainous with ranges running east to west. The remainder of the country consists of coastal plains and valleys. Roughly 40% of the landmass is above 500m in elevation, 30% between 300-500m and the remainder below 300m (24). Due to the mountain ranges, rainfall varies across the country, but consistent patterns are noted. April through May and September through October experience heavy rainfall along the southern peninsula, central plateau, and northern coast. December through March mark the dry season in these same regions. It is important to note that the Island of Hispaniola is nested in the hurricane belt which is usually active June through November (24). According to the World Bank, the geopolitical divisions of the country consist of 10 departments, 42 arrondissements, 145 communes, and 571 communal sections with a total population of roughly 10.85 million as of 2016.

Haiti's history with malaria is an unsuccessful story of elimination attempts followed by a loss of commitment (24-25, 49). A joint collaboration between the Haitian Government, UNICEF, the US Agency for International Development, and WHO/PAHO agreed to undertake a full scale eradication program in 1961 (24). With a start date of January 1962, mass DDT spraying became a common practice to wipe out the vector. By 1964 the drug program was added to distribute chloroquine-pryimethamine, an effective anti-malarial. The efforts were initially well accepted with an estimated 90% acceptance of the anti-malarial drug in the first 15

cycles of treatment (24). With these combined efforts prevalence dropped from 15% to 1% by April of 1965. Pilot studies even suggested signs of eradication in regions such as the Petit-Goave Commune located in the eastern portion of Ouest. Efforts were briefly terminated in September of 1965 but reinstated when cases began to reappear in December of the same year (24). Once the program officially ended in 1969 funding took a sharp decline and was shut down almost completely due to a major financial crisis by 1988 (22). It wasn't until Haiti's first Global Fund Grant was awarded that control efforts could resume between 2004 and 2009. Unfortunately, not only were these merely control efforts, as opposed to prevention, but setbacks were common due largely to the political coup in 2004, which resulted in a two year wait for elections to take place. By 2009 however, progress and collaboration between Haiti and the Dominican Republic lead to the development of the binational plan for malaria elimination by the year 2020 (26, 50). Unfortunately, a devastating earthquake killed an estimated 220,000 people and disrupted the healthcare infrastructure of the entire nation the following year (22). Several humanitarian organizations arrived on the scene and caused large scale logistical issues with coordination due to a lack of familiarity with the country. These organizations did commonly test for malaria, but employed rapid detection tests (RDT) which were not of approved by the Ministry of Health at the time (22). Light microscopy (LM) was a preferred method but not feasible in emergency settings. Because RDT was the most readily available tool, the Ministe` re de la Sante´ Publique et de la Population (MSPP) and the CDC quickly revised goals to include certain RDTs for surveillance (22). Since the 2010 earthquake, recuperation efforts, infrastructure rebuilding, and major displacement have made active surveillance or even a census difficult.

It is known that six species of Anopheline mosquitos are documented *Plasmodium* carriers in Haiti; *A. albimanus, A. argyritarsis, A. crucians, A. grabhamii, A. pseudopunctipennis, and A. vestitipennis* (16). The most common malarial vector of these in Haiti is A. albimanus (22). This species has been documented in Ouest, Nippes, Sud, and Grande Anse which align with previous literature reports of transmission (1, 5, 22-25, 32). There have been previous reports of vector resistance to dieldrin, DDT, and fenitrothion but recent studies have not validated these results (16). Peak biting season in Haiti tends to center around October through December directly after the rainy season while minimal biting rates occur during January through May, in line with the dry season (5). In a systematic review by Frederick et al. the researchers note that between 2010 and 2015, only four published papers and three-scientific meeting abstracts on malaria vector entomology in Haiti could be found. Post-earthquake published literature on the topic is scarce due to the major economic, environmental, and population shifts the country has experiences since then.

The most recent studies suggest that malaria transmission levels are still relatively low. The Clinton Health Access Initiative for example suggests that the national prevalence was below 1% as of 2012 while Lemoine et al suggested a prevalence of 0.04%. Literature suggests a varying distribution of each species with *P. falciparum* being the best documented and isolated in the Ouest, Nippes, Sud, Arbonite, Grand' Anse, and Sud-Est departments (1, 5, 22-25, 32). Studies exploring either *P. vivax* or *P. malariae* suggest low transmission, but unfortunately do not suggest geographic distributions with the same detail as *P. falciparum*. Only a handful of studies document *P. vivax* or *P. malariae* infections and are usually conducted in the mid-section of the southern peninsula where *P. falciparum* is commonly found (16, 23, 25). In one study by Frederick et al, the team suggests that *P. vivax* is mostly imported rather than locally transmitted.

Relatively few studies have been conducted recently to suggest current distributions in Haiti. In addition, the relatively low parasitemia and pyrogenic threshold of *P. malariae* and *P. vivax* make detection difficult and further complicate the accuracy of routine diagnostic methods (44-45). The commonly used methods (RDT and LM) used from previous studies may not be the most robust tools to illustrate parasite prevalence and distribution, especially at such low transmission levels. LM, for example, underestimates prevalence when compared to diagnostic methods such as PCR and microarrays because it is insensitive to low levels of parasitaemia (2, 10, 51). Similarly, RDTs yield an estimated 80% sensitivity and 92% sensitivity compared to PCR, but the only RDT's in use in Haiti are currently specific for *P. falciparum* (10).

A recent effort for malaria surveillance in Haiti includes protocol within the Transmission Assessment Survey (TAS) (47). The TAS was developed by the WHO with the aim of providing measurements to determine a critical threshold of lymphatic filariasis (LF) infection prevalence, where the threshold suggests the point at which transmission is no longer feasible (35-36). The results of the survey were designed to provide evidence for the discontinuation of mass drug administration for LF in conjunction with Global Program to Eliminate LF launched by the WHO in 2000 (35-36). The most recent cross-sectional survey was executed between 2014 and 2016 in efforts to contribute to the World Health Assembly 2020 goal of parasitic disease elimination specific to LF. School children were selected as a proxy to determine filarial parasite carriage and exposure because this age range serves as an indicator of the effectiveness of the mass drug administration conducted about 6 years prior to the survey. But in addition, school children provide a readily accessible at risk population with a biological peak determinacy of intensity and prevalence (36). Although the TAS was employed to determine community LF levels, the methods are readily transferable to malaria exposure surveillance. As previously

mentioned, malaria surveillance in Haiti has a history of low quality passive surveillance which does not include asymptomatic infections. The uncertainty associated with the data has only been magnified by the major displacement and toll on the healthcare infrastructure from the 2010 earthquake. Without a reliable recent baseline of parasite distribution, the need for community level data to identify geographic populations with increased risk is vital to malaria elimination. For these reasons malarial immunoassay protocol was introduced during the recent TAS in Haiti. Immunoassays for the presence of IgG antibodies as a marker for malaria exposure were used to determine seropositive participants in this endemic low transmission setting. Luminex multiplex bead assays were chosen for the TAS to evaluate IgG antibody responses specific to *P. vivax* or *P. malariae*. The goal of the current study is it to detect significant hot-spot foci for malaria exposure with a focus on *P. vivax* and *P. malariae* using the geospatial location of schools and immunoassay results from the TAS.

Spatial analysis can provide helpful insight into the exposure distribution of malarial parasites such as *P. vivax* and *P. malariae* in Haiti through the use of the malaria antibody relevance through the TAS samples. These two species have been largely overlooked during Haitian elimination efforts in the 60's and remain overlooked in the current body of literature. In addition, the devastating earthquake, political unrest, and ineffective diagnostic methods have made conditions difficult for surveillance. The overall goal of this project is to identify significant hot-spot foci for *P. vivax* and *P. malariae* exposure sites in Haiti. The application of multiplex bead immunoassay technology surveillance in the country of Haiti has not been previously described in literature for these two species. The high sensitivity in low transmission sub-microscopic settings make this tool helpful to Haiti's malaria eradication goals. This paper is not intended to suggest the best intervention locations. Instead, using the MSP1 antigen as an

exposure marker and school children as a community proxy, this study is intended to serve as a baseline distribution of non-falciparum malaria within Haiti's borders. Results are intended to provide suggestions for focused and thorough active surveillance projects conducted in the near future.

MATERIALS AND METHODS

Data source. Access to the de-identified data was granted by the Centers for Disease Control and Prevention's Division of Parasitic Diseases and Malaria. Study protocol was determined to be IRB exempt by Emory University's eIRB board. The data was a subset of the cross-sectional school-based Transmission Assessment Survey for the surveillance of Lymphatic filariasis (43). Funding for the execution of the TAS was provided from IMA World Health, UND, CDC, and The Carter Center (47). The particular subset of data obtained from the CDC contained dried blood spot assay results of IgG antibody responses assayed through Luminex® multiplex bead technology. Assay results tested for a participant's previous malaria exposure through the presence of $MSP1_{19}$ specific to *P. vivax* and *P. malariae* IgG antibodies. This survey was administered to primary school children focusing on 6 and 7 year olds across the country of Haiti between November 2014 and August 2016. Other variables included in the dataset were Latitude and Longitude coordinates of schools, school name, sex, age, corresponding mean fluorescent intensity (MFI) values, and dichotomized seropositivity/negativity results. Inclusion in the analysis required that children be between the ages of 6-10 years of age, have immunoassay results for *P. vivax* or *P. malariae*, and have a GPS location for their corresponding school. Individual participant data were reformatted to consolidate participant count, seroprevalence, average MFI-bg intensity, average age, and proportion of males per school location. Additional geographic variables were attained from open source shape files. Major bodies of water, department, arrondissement, commune, and sub-communal section geopolitical boundaries were attained from Haitidata. Elevation raster files were attained from Trimble Data Marketplace. Normalized difference vegetation index (NDVI) was acquired from

Google Earth Engine. Population per square hectare was attained from WorldPop. Elevation, distance to nearest water source, NDVI value, and population per hectare was calculated for each school using ArcMap's (Version 10.5.1) Euclidian distance tool.

Immunoassays. Multiplex bead technology immunoassay was conducted by the CDC prior to the start of this project. This assay works by coating microfluorescent beads with malaria-specific antigens. These antigens capture the IgG of interest. A biotinylated secondary antibody is added to form an antibody-antigen "sandwich." A streptavidin-linked phycoerythrin dye is then bound to the secondary antibody in order to attain a quantitative MFI value (29). The recombinant antigen chosen for this study was the merozoite surface protein 1 subunit 19kDa ($MSP1_{19}$) isoforms produced by *P. vivax* and *P. malariae*. A previous study by Rogier et al in 2015 tested the utility of US citizen samples as a seronegative population in order to determine $MSP1_{19}$ seropositivity in malaria endemic regions. The results suggested that US citizens serve a good true negative when using multiplex bead assays. The same study suggests that all MSP1 subtypes have a positive skew where high IgG titers are indicative of active or repeated exposure. In addition, the multiplex $MSP1_{19}$ MFI values previously studied suggest higher titers among children due to repeated exposure and a higher seroprevalence with increasing age (3, 18, 28).

Spatial analysis. Latitude and Longitude coordinates were geocoded and converted from World Geodetic System (WGS) 1984 into Universal Transverse Mercator (UTM) Zone 18 projections using ArcMap (Version 10.5.1). Spatial randomness measures were calculated using Ripley's K-function through ArcMap (Version 10.5.1). Ripley's K-function quantifies how often events are found within a certain distance of one another and the level of aggregation, randomness, or dispersion within the boundaries of a set of observations (37-38, 52). If

observations are not spatially random, it would suggest evidence of biological, environmental, or sampling association with the clustering of cases. K-function permutations allow for confidence interval calculation around an expected random distribution (52). K-function in this study was calculated using a maximum radius of 50 km with 2.5 km intervals. Weighted K-function was calculated using seropositive counts per school with the same parameters. Confidence intervals were calculated using 999 permutations for weighted and unweighted K-functions. Autocorrelation was calculated using Moran's I in ArcMap (Version 10.5.1). Autocorrelation measures such as Moran's I allow for the quantification of the degree to which values spatially correlate to similar values (52). If there is no spatial autocorrelation, Moran's I is assumed to equal zero. Values above zero indicates autocorrelation of data values among the locations. Moran's I was calculated using inverse distance weights, 999 permutations, and a threshold distance of 20km. If values are significantly autocorrelated, an additional analysis, the Local Indicator of Spatial Association, will then identify at which locations autocorrelation is occurring. Cluster analysis was performed by Kulldorff's spatial scan statistic using Satscan (Version 9.5) to identify clusters with an increased relative risk of malaria exposure. Kulldorff's scan statistic considers moving "windows" with variable radii ranging from the smallest observed distance to a pre-determined upper bound (52). The goal of this multiple testing to determine collections of cases inconsistent with the average distribution and calculate Likelihood Ratio (LR) significance. Kulldorff's scan was calculated using elliptical windows with a maximum of 50% of the population, using discrete purely spatial Poisson modeling, 999 Monte Carlo simulations, non-overlapping windows, and maximum likelihood estimations with an alpha level of 0.05. All analyses were performed on the entire country of Haiti and then again divided into north and south regions. The north region included the departments Nord, Nord-Est,

Nord-Ouest, Arbonite, and Centre. The southern region included the departments, Grand' Anse, Sud, Nippes, and Sud-Est. *P. malariae* excludes Sud-Est due to a lack of *P. malariae* MSP1 antigen when data was collected for that department.

Statistical analysis. All statistical analysis was performed using SAS (Version 9.4 (32)). Environmental and demographic variables were tested using univariate selection through logistic regression to test for their association to seropositive school children. Reference category for departments was chosen for the closest mean seroprevalence to national mean. Residuals for the regressions were also spatially analyzed using Moran's I using inverse distance weights, 999 permutations, and a threshold distance of 25km to determine autocorrelation of high residual values.

RESULTS

A total of 21,670 children were tested for *P. malariae* $MSP1_{19}$ exposure in 679 schools (Table 1.), meanwhile a total of 24,510 children were tested for *P. vivax* $MSP1_{19}$ exposure in 787 schools (Table 2). Of those children tested 275 (1.27%) and 113 (0.46%) children were determined seropositive for *P. malariae* and *P. vivax*, respectively, meaning they have IgG against the specified antigens. 21,415 students were tested for both species, of which only 12 (0.06%) were seropositive for both *P. malariae* and *P. vivax*. Of the schools surveyed, 187 (27.54%) and 93 (11.82%) schools contained one or more seropositive children for *P. malariae* or *P. vivax*, respectively. When divided by department, *P. malariae* prevalence remained relatively similar to national seroprevalence in the Nord (1.37%), Nord-Ouest (1.47%), and Sud (1.05%) departments, but varied in other regions (Table 1). When divided by department, *P. vivax* prevalence remained relatively similar to national seroprevalence in the Centre (0.4%), Nord (0.4%), and Nord-Ouest (0.3%) but varied in other regions (Table 2). The 12 participants seropositive for both species were widely dispersed across the departments of Sud, Centre, Nord, Nord-Ouest, and Nord-Est. Average age among seropositive children was almost identical across species and regions, with an average participant age of 6.6 years of age. A similar homogenous trend was observed for proportion of male to female students across species and regions, with an average proportion of 0.5. Spatial distribution of schools surveyed for *P. malariae* and *P. vivax* is detailed in figure 2A-B. The number of children sampled per school ranged from 1 up to 309; meanwhile, the number of seropositive children per school ranged from 0 up to 7. Few to no schools were surveyed in the central eastern portion of the country bordering the Dominican Republic. Additionally, no children from the department of Sud-Est were able to provide data for *P. malariae*.

Cluster analysis. Ripley's K function for spatial randomness of schools determined a significant spatial clustering at all intervals tested up to 50 km for both species in the northern and southern Haiti (Figure 1 - A, C, E, and G). Weighted K-function of schools tested for *P. malariae* in northern Haiti using seropositive counts as weights suggests a significant spatial aggregation of cases per school at all intervals between 2.5 km and 15 km with a peak at 12.5 km (Figure 1 - B). The weighted K-function of schools tested for *P. vivax* in northern Haiti also suggest a significant spatial aggregation of cases at all intervals between 2.5 km and 17.5 km with a peak at 12.5 km and again between 30 km and 50 km with a peak at 45 km (Figure 1 - F). Weighted k-functions for both species suggest spatial randomness at all distances in southern Haiti (Figure 1 - D and H). Although seropositive counts for *P. vivax* have no statistically significant aggregation at any interval, a very definitive arch can be noted between 5 and 50 km with a peak difference at 17.5 km (Figure 1 – H). This could suggest a borderline significant aggregation that has been underestimated by the spatial configuration of schools in the south (Figure 1 – G). Spatial autocorrelation analysis using Moran's I resulted in values above zero for both *P. malariae* ($I = 0.16$, $p < 0.001$) and *P. vivax* ($I = 0.04$, $p > 0.05$), suggesting spatial autocorrelation on a national level for *P. malariae* but potentially not for *P. vivax* samples (Figure 3). Moran's I using spatial points is especially susceptible to variability in distance and empty space. For this reason, the test was again conducted for northern and southern regions. Moran's I for *P. malariae* suggests a significant autocorrelation of high seropositive counts in the north with an I-value of 0.153 ($p < 0.001$) and no autocorrelation in the south with an I-value of -0.001 ($p > 0.5$) (Figure 4). *P. vivax* Moran's I in the north does not suggest autocorrelation with an I-value of -0.016 ($p > 0.05$) (Figure 5). *P. vivax* Moran's I in the south however does suggest autocorrelation but not to a significant level with an I-value of 0.062 ($p < 0.10$) (Figure 5).

The results of Moran's I suggest that cases are clustered, but it does not define where they are clustered. Cluster location analysis of the entire country using Kulldorff's Bernoulii scan suggested five hot-spots with a significant likelihood for non-*falciparum* malaria exposure. Cluster A (LR=20.8, $p<0.001$), an ellipse with a minor diameter of 9 km and major diameter of 14 km, and cluster B (12.3, $p<0.01$), an ellipse with a minor diameter of 2 km and major diameter of 10 km, were hot-spot foci for *P. malariae.* Cluster A and B were located in the southeastern portion of the Centre department and in the northeastern portion of the Nord-Est department respectively (Figure 3 – A). Three significant clusters were suggested for *P. vivax* (Figure 3 - B). Cluster C (LR=22.1, $p<0.001$), a circular hot-spot with a diameter of 3 km was located in the Nord-Est department. Cluster D (LR=10.4, $p<0.05$), an ellipse with a minor diameter of 56 km and major diameter of 112 km was found in the southern department of Sud-Est. And finally, cluster E (LR=10.1, $p<0.05$), an ellipse with a diameter of 15 km was found in the southern department of Nippes. Clusters D and E in the southern portion were significant, but had the highest p-values of all hot-spots determined. When Kulldorff's Poisson Spatial Scans were re-run in the northern and southern regions independently, clusters were verified with the exception of cluster D (Figure 4 and 5). Cluster D was not indicated in the same location by the regional scan, but instead another cluster of roughly the same size and angle was suggested due east closer to the border with the Dominican Republic (Figure 5).

Statistical Analysis. Logistic regression analysis was performed to test the association between environmental factors and seropositive children. Using Nord as the reference category for departments surveyed for *P. malariae,* a statistical difference was suggested when compared to the Arbonite, Centre, and Nord-Est departments (Chi-Square =77.5, p-value<0.001) (Table 1). Using Nord again as the reference category for departments surveyed for *P. vivax* a statistical

difference was suggested when compared to the Nippes, Nord-Est, and Sud-Est departments (Chi-Square = 52.7, p-value<0.001) (Table 2). In addition, elevation was determined to be associated with children seropositive for *P. vivax* (OR=0.88, 95% CI: 0.7,1.00). All other associations were determined to have a p-value above 0.05 (Table 1 and 2). Autocorrelation analysis of both models' residuals suggest no significant autocorrelation with an I-value of 0.038 ($p<0.10$) for *P. malariae* and I=0.012 ($p>0.05$) for *P. vivax*.

DISCUSSION

Despite massive elimination efforts in the 1960's malaria remains endemic in the country of Haiti. Even throughout these elimination efforts (24-25, 49), studies focused on non-*P. falciparum* species have been largely ignored. Although the TAS was employed to determine LF intensity in certain areas (35, 36), the methods of school based sampling are readily transferable to malaria exposure surveillance. The use of school children as a readily accessible at risk population with a biological peak determinacy of intensity and prevalence, which is equally beneficial for malaria survey purposes (36). With an extensive sample size of 21,670 children tested for antibodies against *P. malariae* and 24,510 children tested for *P. vivax,* this study is the most complete recent study of these two species in Haiti. The results using multiplex bead technology immunoassay protocol to detect IgG $MSP1_{19}$ specific antibodies suggest a relatively low nationwide seropositivity prevalence of both *P. malariae* (1.27%) and *P. vivax* (0.48%) among Haitian school children. In addition, cluster analysis identified 5 geospatial exposure hot-spots, only three of which are of high statistical interest.

The culmination of a visual inspections, autocorrelation, and ellipse likelihood in north Haiti suggest that cluster A determined through Kulldorff's spatial scan is a significant *P. malariae* exposure hot-spot of interest. Exposure hot-spot results for clusters B and E on the other hand are potentially of interest but could use further review through future surveillance. Beginning with cluster B, logistic regression analysis suggests that the Nord-Est department, where cluster B was suggested, has a 1.6 (95% CI: 1.1, 2.4) times higher odds of seropositive individuals compared to the reference category. Analysis describing the distribution of the schools through un-weighted K-functions determined whether any clustering was not simply a result of the spatial location of the sample sites. Ripley's K-function for spatial randomness

determined that schools are spatially clustered at all intervals tested for north and south Haiti for both species. Therefore, any clustering of cases could potentially be dependent on school location and therefore be over-estimated due to the aggregation of school sample locations (37-38). However, it should be noted that when the same K-functions were also calculated through Point Pattern Analysis (PPA) (Version 1.0b) developed by Dongmei Chen, Jared Aldstadt, and Arthur Getis in San Diego University. The suggested unweighted spatial distribution of schools calculated through this software was significantly dispersed at all intervals for both species (Appendix A). The reason calculations were contrasting one another was undetermined. However, as noted in Appendix A, the L(d) scale on the graphs was unusually high. For this reason, ArcMap generated calculations were used. With this in mind, *P. malariae*'s weighted K-function only marginally suggests the presence of seropositive aggregation, while Moran's I suggests a highly significant autocorrelation. The main issue of interest with cluster B is its proximity to the coast. Proximity to empty space on the edges of the sampling area is a factor that increases hot-spot likelihood due to the neighboring empty space and a subsequent increase in the weighting of values (52). *P. vivax*'s cluster E experiences the same concern with coastal proximity, but on the other hand, a visual inspection of seropositivity in the Nippes department suggest that the cluster is located at the center of a secluded island of seropositive schools. In addition, the odds of seropositivity in the Nippes department was 2.2 (95% CI: 1.2, 4.0) times higher than the reference category. However, this cluster resulted in mixed aggregation and autocorrelation results. In south Haiti, the weighted k-function suggests spatial randomness at all intervals (38). Because of the previously mentioned over-estimation of aggregation, it's important to take note of the weighted k-function curve. Specifically, as it nears the upper bound of significance. This could again be over-estimated. However, the near significance could

additionally correlate with the Moran's I value above zero, which indicated autocorrelation in the south but did not meet the alpha value cutoff of 0.05.

Lastly, the combination of results for *P. vivax*'s clusters C and D are relatively unconvincing and potentially explained by the limitations of methods and sampling (38, 52). Cluster C was suggested in the Nord-Est department, where the odds of seropositivity was actually 0.4 (95% CI: 0.3, 0.6) times that of the reference category. Although the weighted K-function for north Haiti suggests borderline aggregation of cases across nearly all intervals, autocorrelation measures clearly suggest that high seropositive counts do not neighbor other high counts. In addition, a visual inspection of cluster C suggests that the likelihood is heavily weighted by one school with 5 seropositive students. At a glance, cluster D can be slightly convincing. Logistic regression suggests that the Sud-Est department, where Cluster D was suggested, has 7.2 (95% CI: 2.8, 18.3) times higher odds of seropositive individuals compared to the reference department. In addition, Cluster D shares the same K-function and autocorrelation interpretation as cluster E. However, the first concern is the total of 5 seropositive individuals and the total of 153 participants surveyed in the entre department. This sample size is extremely low in comparison to the other departments causing a higher weight per seropositive individual and thus statistically significant results. There was also a lack of validation during Kulldorff's Poisson spatial scan in the south. Although Cluster D was suggested in the Sud-Est department, it was not verified in the same location but instead was suggested due west, closer to the border with the Dominican Republic when only the south departments were tested. This cluster is unlikely due to the presence an exposure hot-spot but instead can be explained by the surrounding empty coastal space and small sample size in the department.

The clusters suggested are based off of serological data testing for the presence of IgG antibodies specific to the blood stage $MSP1_{19}$ antigen (48). The biological mechanisms of each species must also be considered when interpreting clusters. For example, *P. malariae* does not have latent period between liver and erythrocyte infection and has a longer erythrocyte propagation cycle (44). These tendencies cause an earlier onset of immune responses. In terms of immunological surveillance, this means that seropositive individuals are more likely to represent recent infections compared to other species. *P. vivax*, on the other hand, has dormant forms that cause relapse infections which subsequently increase the immune response even without further exposure to infected vectors (45). The difference between the two species could partially explain the higher *P. malariae* seroprevalence found in Haiti. In addition, it could partially change the interpretation between species where *P. malariae* clusters are potentially more recent exposure sites compared to *P. vivax*. Either way, clusters A through E do not classify an aggregation of current transmission sites. Instead, results detail regions where the human immune system has developed a short and long term B-cell antibody persistence, suggesting a historical exposure of *Plasmodium* parasites. The presence of the parasite is assumed to lasting long enough for the human immune system to develop antibodies to block adherence of merozoites to surface proteins and stimulate phagocytosis by monocytes and macrophages (47-48). A major benefit to this method is that the school children did not have to be infected at the time of testing to produce antibodies (47) allowing greater exposure detection compared to incidence case detection. Using children as a community proxy we can assume that infections occurred within the last 6 to 10 years from the collection date, narrowing the time interval of parasite presence. But at the same time, limitations to school-based immunology methods include the inability to distinguish the exact time or location of incidence infections. There is no direct evidence to

suggest that the community in which the school is located harbors parasites at the time of collection.

It is important to note that the spatial tests mentioned thus far only serve as a descriptive measure of case distribution and do not associate the disease to any environmental or behavioral factor. The environmental factors included in this study have been associated with malaria incidence and exposure in previous studies (5, 7-8, 18). However, univariate analysis did not suggest association between any environmental factor and seropositivity with the exception of elevation. And even though *P. vivax* seropositivity was inversely associated with increasing elevation ($p<0.05$) the odds ratios 95% confidence interval included the null value of 1.00. These results were expected due mostly to the low parasite exposure prevalence in Haiti.

Limitations to this project mainly include the sampling distribution of schools (31-38) and the low parasite exposure of non-falciparum species in Haiti (44-45). Although sampling was extensive and covered nearly every department, the schools sampled were significantly aggregated across the nation. Measures such as dividing the country into northern and southern regions were taken to reduce the distribution bias by reducing the scale (52). Unfortunately, little could be corrected regarding a lack of sampling between directly adjacent sub-communes in the same general regions. As suggested by Stresman et al., sampling $\leq 20\%$ of a local population can lead to hot-spot misclassification (31). This is especially the case in the Sud-Est department where only 153 participants were tested yet a cluster was identified. Concerning the low exposure levels, although multiplex bead technology is able to detect cases in low transmission and sub-microscopic environments (3, 7, 19, 27, 29), the results of this study suggest that the exposure prevalence is still relatively low for non-falciparum species in the country of Haiti. This is an ideal result in terms of eradication goals but it further highlights the need for highly

sensitive tools. This low exposure prevalence makes cluster determination analytically difficult, [31] and could partially explain the lack of significance during univariate model analysis. In addition, decreasing exposure rates mean exposed individuals increasingly carry more weight during cluster determination (52). This issue was mainly noted with *P. vivax* seropositive cases in clusters C and D. These clusters were suggested using Kulldorff's Discrete Poisson Spatial Scan. But upon further inspection of Ripley's K, Moran's I, and a visual inspection, the clusters were determined to be a biased by either relatively high seropositive counts or having sparse sampling. Applying other distributional models, such as the Bernoulli, for Kulldorff's Scan may be more appropriate in a setting with sparse seropositivity.

P. malariae and *P. vivax* have been largely overlooked during Haitian elimination efforts in the 60's and remain overlooked in the current body of literature (24-25, 49). A devastating earthquake and political unrest have made conditions difficult for surveillance. In addition, the relatively low parasitemia and pyrogenic threshold of non-*falciparum* malaria make detection difficult and further complicate routine diagnostic methods (44-45). This project described seropositive exposure distribution and identified 5 hot-spot foci for *P. vivax* and *P.malariae* across in Haiti. Of the 5 identified geospatial hot-spots, one (cluster A) was verified through visual inspection, weighted k-functions, autocorrelation, and likelihood measures for *P. malariae.* Two of the remaining four foci (cluster B and E) are potentially of interest but could use further review through future surveillance due to potential biased results stemming from limitations of the study. The remaining two foci (cluster C and D) likely result directly from limitations in methods and sampling and are therefore not likely clusters of interest. High sensitivity in low transmission, sub-microscopic settings make multiplex bead technology helpful to Haiti's ultimate five-year goal of malaria eradication within its borders. The

application of multiplex technology in the country of Haiti has not been previously described in literature for *P. malariae* and *P. vivax*. This paper is not intended to suggest the best intervention locations. Instead, the results presented here are to serve as a baseline for non-falciparum malaria distribution for a more focused and thorough active surveillance conducted in the near future. Using the descriptions from this study, the next step is to integrate malaria into the TAS with a focus on regions suggested in by these results to maximize surveillance quality and cost.

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TABLES AND FIGURES

Table 1. Summary of Descriptive Variables and Associations for *Plasmodium malariae* Serology Samples Collected in Haiti Between 2014-2016.

	Total	Seropositive			
Varaiable	N	n (%)	OR	95% CI	p-value
Schools [a]	679	187 (27.54)	-	-	-
Participants	21,670	275 (1.27)	-	-	-
Department/Island					
L'Arbonite	3,186	14 (0.44)	0.36	0.21 - 0.63	
Centre	1,213	46 (3.79)	3.2	2.26 - 4.53	
Grand'Anse	1,666	12 (0.72)	0.59	0.32 - 1.07	
Nippes	44	0	-	-	
Nord	9,363	114 (1.22)	Ref	-	<0.001 [b]
Nord-Est	1,625	31 (1.91)	1.58	1.06 - 2.36	
Nord-Ouest	2,368	35 (1.48)	1.22	0.83 - 1.78	
Ouest	15	0	-	-	
Sud-Est	-	-	-	-	
Sud	2,190	23 (1.05)	0.86	0.55 - 1.35	
Age			0.91	0.72 - 1.16	0.455
6	8,999	123 (1.37)			
7	12,151	152 (1.25)			
>7	4	0			
Elevation			0.99	0.91 - 1.07	0.830
<300 m	15,194	192 (1.26)			
300-500 m	4,425	49 (1.11)			
>500 m	2,025	31 (1.53)			
Gender			0.92	0.73 - 1.17	0.514
Male	10,646	133 (1.25)			
Female	10,510	142 (1.35)			
Distance to water			0.96	0.88 - 1.05	0.385
<2km	8,382	115 (1.37)			
2 -10km	9,676	115 (1.19)			
>11km	3,480	45 (1.29)			
NDVI			0.66	0.35 - 1.24	0.198
<0.2	1,790	25 (1.42)			
0.2 -0.3	5,496	74 (1.36)			
>0.4	14,384	176 (1.22)			
People per Hectare			1.00	0.93 - 1.07	0.955
<50	17,273	219 (1.27)			
50-200	1,776	22 (1.24)			
>200	2,284	33 (1.44)			

[a] One or more students determined seropositive categorizes a school as seropositive

b Chi-square p-value comparing all departments using the Nord department as the refference category

Table 2. Summary of Descriptive Variables and Associations for *Plasmodium vivax* Serology Samples Collected in Haiti Between 2014-2016.

	Total	Seropositive			
Varaiable	N	n (%)	OR	OR (95% CI)	p-value
Schools [a]	787	93 (11.82)	-	-	-
Participants	24,510	113 (0.46)	-	-	-
Department/Island					
Arbonite	2,663	0		-	
Centre	1,213	5 (0.41)	0.88	0.35 - 2.21	
Grand'Anse	1,666	2 (0.12)	0.25	0.06 - 1.05	
Nippes	1,366	14 (1.02)	2.19	1.20 - 4.01	
Nord	9,138	43 (0.47)	Ref	-	<0.001 [b]
Nord-Est	3,115	37 (1.18)	0.39	0.25 - 0.61	
Nord-Ouest	2,368	6 (0.25)	0.54	0.23 - 1.26	
Ouest	71	0	-	-	
Sud-Est	153	5 (3.27)	7.15	2.79 - 18.30	
Sud	2,234	0	-	-	
Averge Age			1.22	0.93 - 1.59	0.151
6	10,136	46 (0.45)			
7	13,103	54 (0.41)			
>7	741	10 (1.35)			
Elevation			0.88	0.77 - 1.00	0.042
<300 m	17,329	83 (0.48)			
300-500 m	4,857	20 (0.41)			
>500 m	2,298	10 (0.44)			
Gender			1.04	0.72 - 1.51	0.826
Male	11,958	56 (0.47)			
Female	12,024	54 (0.45)			
Distance to water			0.96	0.88 - 1.105	0.385
<2km	9,704	42 (0.43)			
2 -10km	11,019	53 (0.48)			
>10km	3,655	18 (0.49)			
NDVI			1.94	0.71 - 5.31	0.198
<0.2	2,230	8 (0.36)			
0.2 -0.3	6,230	21 (0.34)			
>0.4	16,050	84 (0.52)			
People per Hectare			0.98	0.88 - 1.10	0.772
0-50	19,909	95 (0.48)			
50-200	2,082	10 (0.48)			
>200	2,182	6 (0.27)			

a One or more students determined seropositive categorizes a school as seropositive

b Chi-square p-value comparing all departments using the Nord department as the refference category

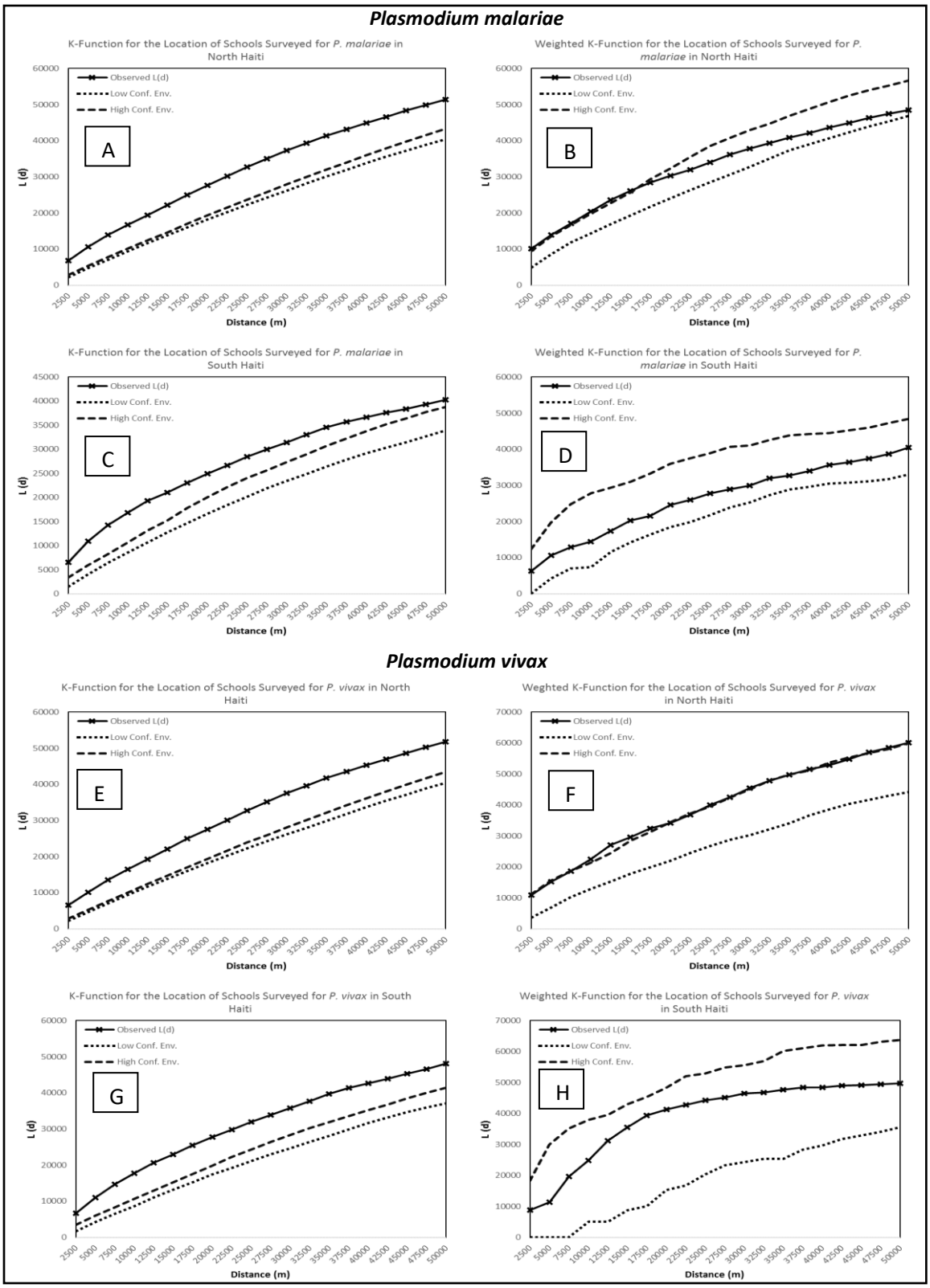

Figure 1. K-functions and weighted K-functions detailing spatial randomness of schools surveyed for *P. malariae* (A-D) and *P. vivax* (E-H) in Haiti. Spatial scan area was divided into northern and southern regions in order to avoid empty space bias caused by lack of sampling in the Ouest department.

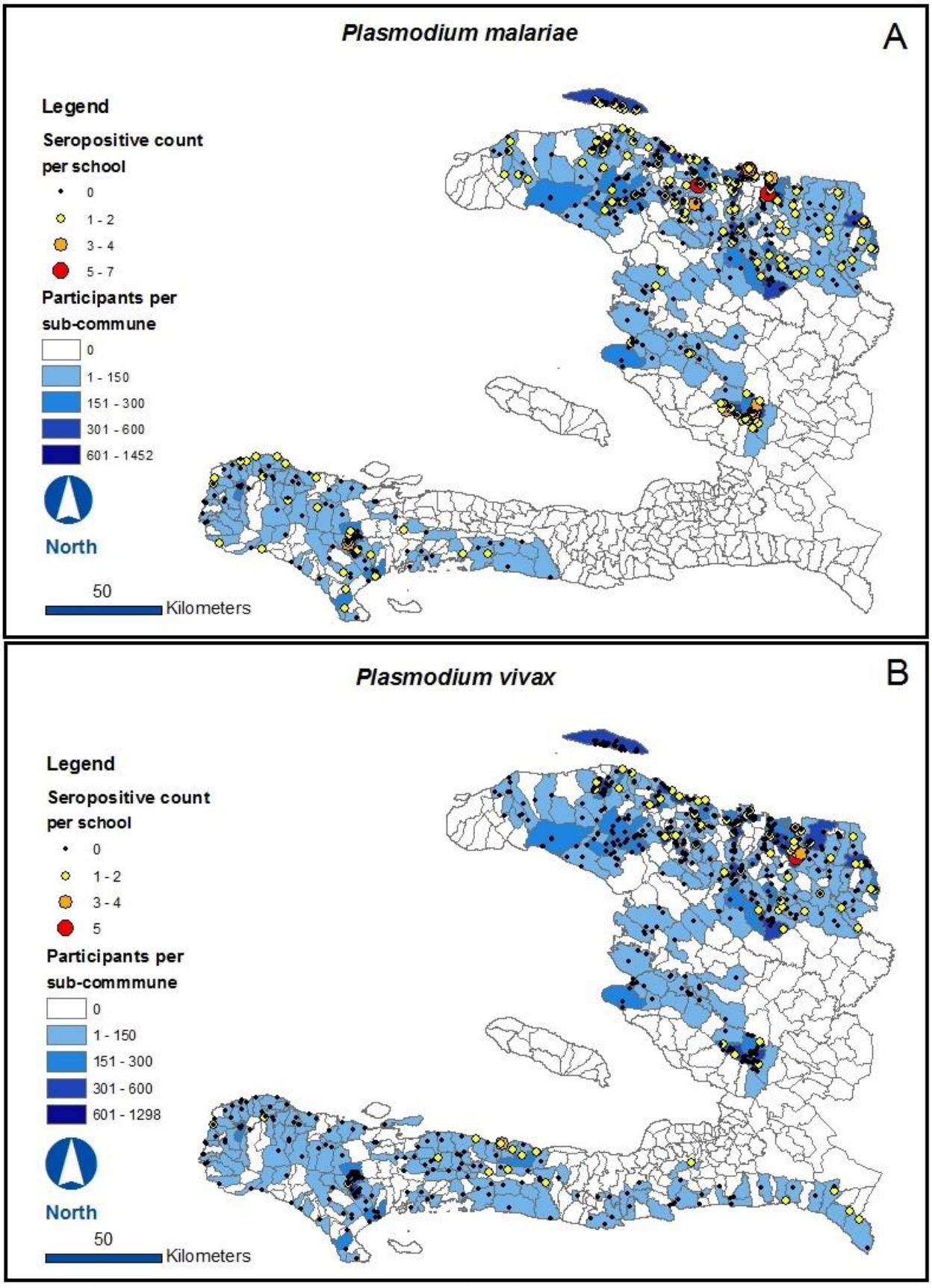

Figure 2. Spatial distribution of schools surveyed for P. malariae (A) and P. vivax (B) between 2014 and 2016. Each point on the map represents an individual school and its corresponding sero-positive count. Shading of sub-commune denotes the cumulative number of students surveyed within the sub-commune boundry.

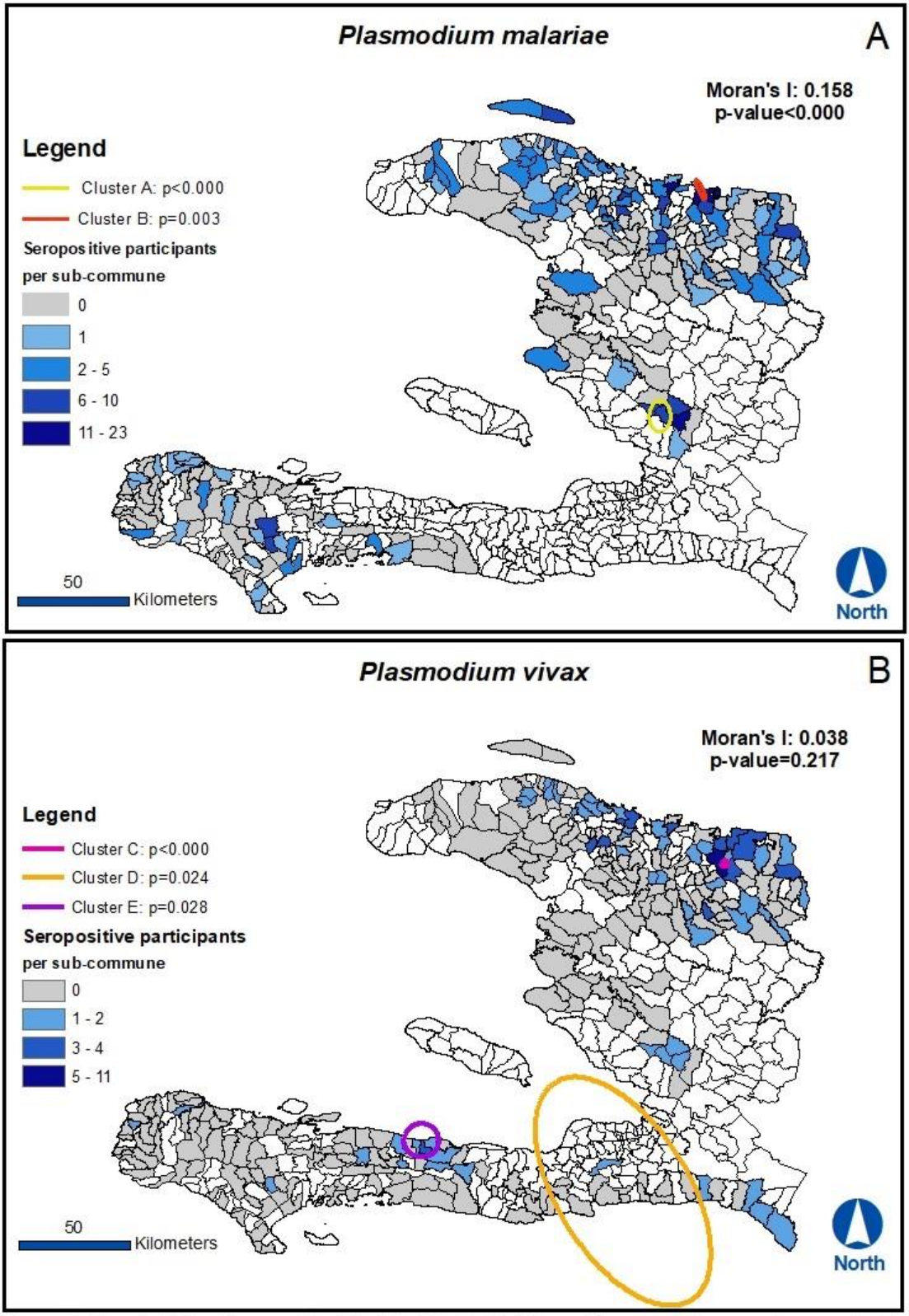

Figure 3. Map detailing the results of Kulldorff's Spatial Scan of seropositive counts per school for P. malariae (A) and P. vivax (B) between 2014 and 2016. Ellipses denote cluster boarders. Shading of sub-commune denotes the cumulative number of seropositive students surveyed within the sub-commune boundry.

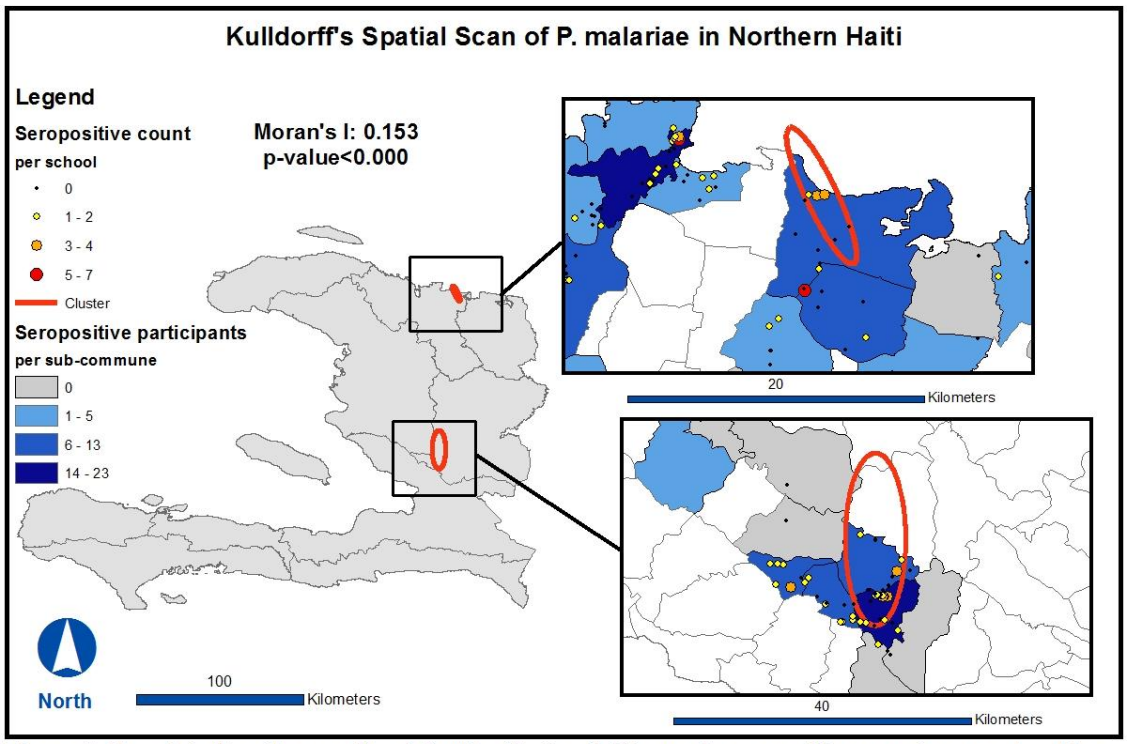

Figure 4. Map detailing the results of Kulldorff's Spatial Scan of seropositive counts per school for P. malariae in Northern Haiti between 2014 and 2016. Ellipses denote cluster boarders. Shading of sub-commune denotes the cumulative number of seropositive students surveyed within the sub-commune boundry.

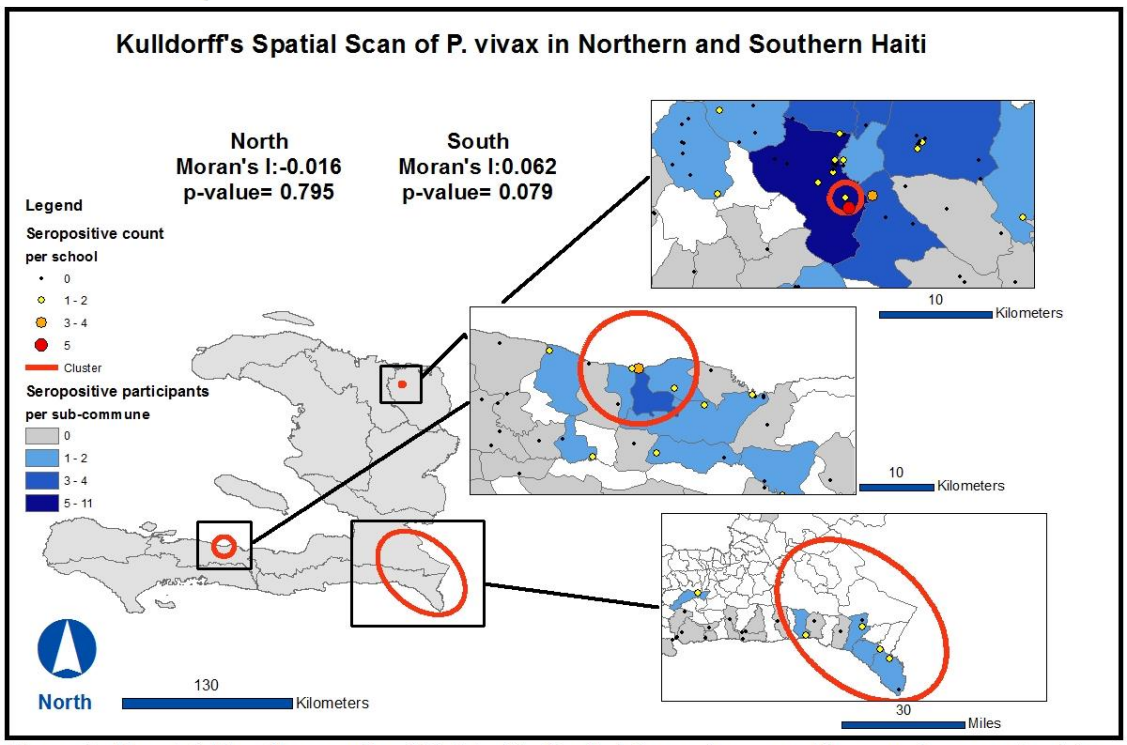

Figure 5. Map detailing the results of Kulldorff's Spatial Scan of seropositive counts per school for P. vivax in Northern Haiti between 2014 and 2016. Ellipses denote cluster boarders. Shading of sub-commune denotes the cumulative number of seropositive students surveyed within the sub-commune boundry.

APPENDIX A

K-functions and weighted K-functions calculated using Point Pattern Analysis (PPA) (Version 1.0b)