Anopheles stephensi is implicated in an outbreak of *Plasmodium falciparum* parasites that carry markers of diagnostic resistance and candidate artemisinin resistance in Dire Dawa City, Ethiopia, January–July 2022

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Abstract

Anopheles stephensi, an Asian urban malaria vector, continues to expand across Africa. We investigated the role of *An. stephensi* in malaria transmission following a dry season outbreak in Dire Dawa, Ethiopia, from April to July 2022, using a prospective case control design. *Plasmodium falciparum* microscopy-positive febrile patients (n=101) and microscopy-negative controls (n=189) and their contacts (n=662) were identified and screened. Spatial clustering of *P. falciparum* infections was observed among case contacts but not among controls and was strongly associated with detection of *An. stephensi.* In combination with the detection of *Plasmodium* sporozoites in *An. stephensi,* this study provides the strongest evidence to date for a role of *An. stephensi* in driving an urban malaria outbreak in Africa. Importantly, this outbreak involved clonal propagation of drug and diagnostic resistant parasites. This study provides the first direct epidemiological evidence linking *An. stephensi* with increase in malaria transmission in Africa, highlighting the major public health threat of this fast-spreading invasive mosquito.

Background

The promising decline in malaria burden has slowed since 2015. This is particularly evident in Africa, the continent that carries the largest malaria prevalence[1]. Malaria control programs in Africa traditionally focus on rural settings, where most infections occur, but malaria is of increasing concern in urban settings[2]. Given the rapid urbanization in Africa[3], urban malaria transmission can result in a considerable health burden[4]. Urban malaria is classically associated with importation from areas of intense transmission[5] but can be exacerbated by the adaptation of existing malaria vectors to urban environments[6] and the emergence of urban malaria vectors such as *Anopheles stephensi*[7].

An. stephensi is distinct from other *Anopheles* species that are traditional vectors in (rural) Africa with its preference for artificial water storage containers that are common in urban settings[8,9]. Native to the Indian sub-continent and the Persian Gulf[10], *An. stephensi* is now rapidly expanding its geographic range westward (Fig. 1a)[7]. First detected in Africa in Djibouti in 2012[11], *An. stephensi* is rapidly expanding its range in the Horn of Africa including Ethiopia (2016)[12], Sudan (2016)[13], Somalia (2019)[14],

Eritrea (2022)[15] and beyond: Yemen (2021)[16], Kenya (2022)[17], Ghana (2022)[15], and Nigeria (2020)[15]. In recognition of the potentially devastating consequences of *An. stephensi* spreading across Africa, the World Health Organization (WHO) urgently requested more data on its distribution and released a strategy to mitigate its spread[18].

In addition to being an efficient vector for both *Plasmodium falciparum* and *Plasmodium vivax* in its native geographical range[10], *An. stephensi* was recently confirmed to be susceptible to local parasites in Ethiopia (Fig. 1a)[9,19] and a resurgence of malaria was reported in Djibouti following its detection[20], although direct evidence for a role of *An. stephensi* in this resurgence was unavailable. Following a report of a dry-season upsurge in malaria cases in Dire Dawa City, Ethiopia, where *An. stephensi* was recently documented[8], we prospectively investigated its role in malaria transmission through responsive epidemiological and entomological surveillance (Fig. 1b).

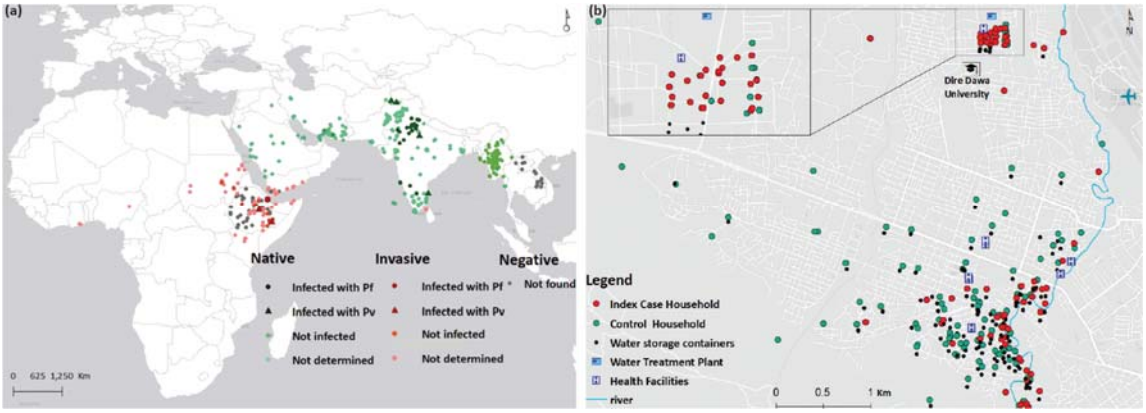

Figure 1. Global distribution of *An. stephensi* and the study location. (A) The global distribution of *An. stephensi* where it is native (green) and invasive (red) is shown together with the sporozoite infection detection outcomes where it was found infected and not infected with *P. falciparum* (*Pf*) and *P. vivax* (*Pv*). Sites where *An. stephensi* was observed but mosquitoes were not tested for the presence of sporozoites are also shown (Not determined). Settings where dedicated entomological surveillance did not detect *An. stephensi* mosquitoes are indicated in grey circles (Negative). (B) The locations of case (red) and control (green) households/dormitories surveyed in this study are shown together with water storage containers (black), water treatment plant (in the university campus), health facilities (H) and Butiji river in Dire Dawa city. Source: The global map (A) was modified based on the malaria threats map[7] (https://apps.who.int/malaria/maps/threats/#/maps?theme=invasive&map) of the World Health Organization.

Results

Malaria outbreaks in Dire Dawa city and its university

Clinical malaria incidence data (diagnosed by microscopy) collected from public and private health facilities (n=34) showed a 12-fold increase (Supplementary Table 1 and Fig. S1) in malaria incidence in Dire Dawa during the dry months (January – May) of 2022 (2,425 cases) compared to 2019 (205 cases). An overall statistically significant trend of increasing number of malaria positive cases was observed between 2019 and 2022 (Mann-Kendall statistical test $\tau = 0.42$, $p<0.001$). Patients reported at both public and private health facilities with the latter contributing to 15.8% of patients diagnosed for malaria in the last four years with an increasing trend from 17.7% in 2019 to 25.9% in 2021 which later declined to 5.7% during the outbreak (2022). In 2022, 76% of all reported malaria cases originated from only three public health facilities: Dire Dawa University (DDU) students' clinic (42%), Sabiyan Hospital (19%) and Goro Health Center (15%). At DDU campus, 94% (1,075/1,141) of clinical malaria episodes occurred in the male student population living in the university single-sex dormitories.

We conducted a prospective case control study to identify risk factors associated with this sudden rise in malaria in the city (Goro Health Center) and DDU (Fig. 1b). In the city, we recruited 48 microscopy malaria confirmed febrile cases plus 125 case-household members and 109 febrile controls without microscopy confirmed malaria who attended the same clinic within 72 hours plus 241 control-household members. At DDU, we recruited 53 students with clinical malaria and 110 dormmates and 80 uninfected febrile students with 186 dormmates. Details of individual and household characteristics are presented in the extended Data Table 1. Fever was detected in additional family/dormitory members of the controls (1.4%, 6/424) and index cases (6.0%, 14/233) (Supplementary Table 2). The responsive case control study unit was household/dormitory; no plausible risk factors were defined *a priori* and neither a sex/gender nor *Plasmodium* species stratification was considered in the study design. The outbreak at the university campus happened at a fine spatial scale (20 dormitory buildings in a 45,450 m^2 area); the dormitories affected by malaria were occupied by male students only (Extended Data Table 1). Despite Dire Dawa being historically co-endemic for *P. falciparum* and *P. vivax*, the proportion of cases that were due to *P. falciparum* increased from 61% in 2015 to 93% in 2022 (Fig. 2a). All the index cases we recruited (n=101) and the additional infections detected (n=102) in this study were found to be *P. falciparum* except only two *P. vivax* infections detected by 18S based qPCR. *Plasmodium* infection was detected in 14 controls by 18S based qPCR. The parasite density in these infections was very low (median parasitemia was 21 parasites/µL) and thus lie below the detection limits of the conventional diagnostics. Only two of these infections had parasitemia above 100 parasites/µL (278 and 1,822 parasites/µL).

Household and dormitory members of malaria cases experience higher mosquito exposure and higher infection prevalence

The results obtained from case control analysis showed that members of the index cases and controls had different levels of mosquito exposure (Extended Data Table 2). Members of a case household/dormitory were more likely to be living close to *An. stephensi* positive sites, defined as the presence of larvae within a 100-meter radius from the household/dormitory (odds ratio [OR] 5.0, 95% confidence interval [CI] 2.8-9.4, $p<0.001$), or adult mosquitoes resting sites (OR 1.9, 95% CI 0.9-4.0, $p=0.068$), or to natural/manmade waterbodies in general (OR 1.6, 95% CI 1.2-2.2, $p=0.002$). The odds of using an aerosol insecticide spray were 58% lower among members of the index cases compared to controls (OR 0.42, 95% CI 0.23-0.72, $p<0.001$).

In the city, *P. falciparum* qPCR detected infections were significantly more common (OR 12.0, 95% CI 5.8-25.1, $p<0.001$; Fig. 2b) among case household members (35.3%, 43/122) than control household members (4.3%, 10/233), with a similar trend for microscopy (OR 42.4, 95% CI 5.6-320.8, $p<0.001$) and RDT detected infections (OR 8.0, 95% CI 3.1-20.4, $p<0.001$). At DDU, despite all students living in close proximity (20 buildings in a 45,450 m^2 area), dormmates of malaria cases were thrice as likely (OR 3.0, 95% CI 1.2-7.4, $p=0.020$; Fig. 2b) to be *P. falciparum* positive by RDT (11.8%, 13/110) compared to dormmates of controls (4.3%, 8/185). A quarter of microscopy-positive infections (34/136) were negative by HRP2-based RDT (sensitivity 75.0, 95% CI 72.2-77.8, specificity 97.0, 95% CI 95.9-98.1; Supplementary Table 3) with different proportions of HRP2-based RDT negative infections in the city (10.3%, 7/68) and the university (39.7%, 27/68). qPCR detected considerably more infections with the likelihood of infections being missed by RDT (Fig. 2c) or microscopy (Fig. 2d) being dependent on parasite density and, for RDT, *pfhrp2* gene deletion status (Supplementary Table 4, Fig. S2). As expected, parasitemia was higher in the index cases (geometric mean 669 *P. falciparum* parasites/µL, 95% CI 442-1012; Fig. 2e) compared to malaria-infected controls (21.1, 6.9-68.6, $p<0.001$), malaria-infected control family members (29.2, 8.8-96.8, $p=0.005$), and malaria-infected index family members (53.4, 26.7-107.0, $p<0.001$).

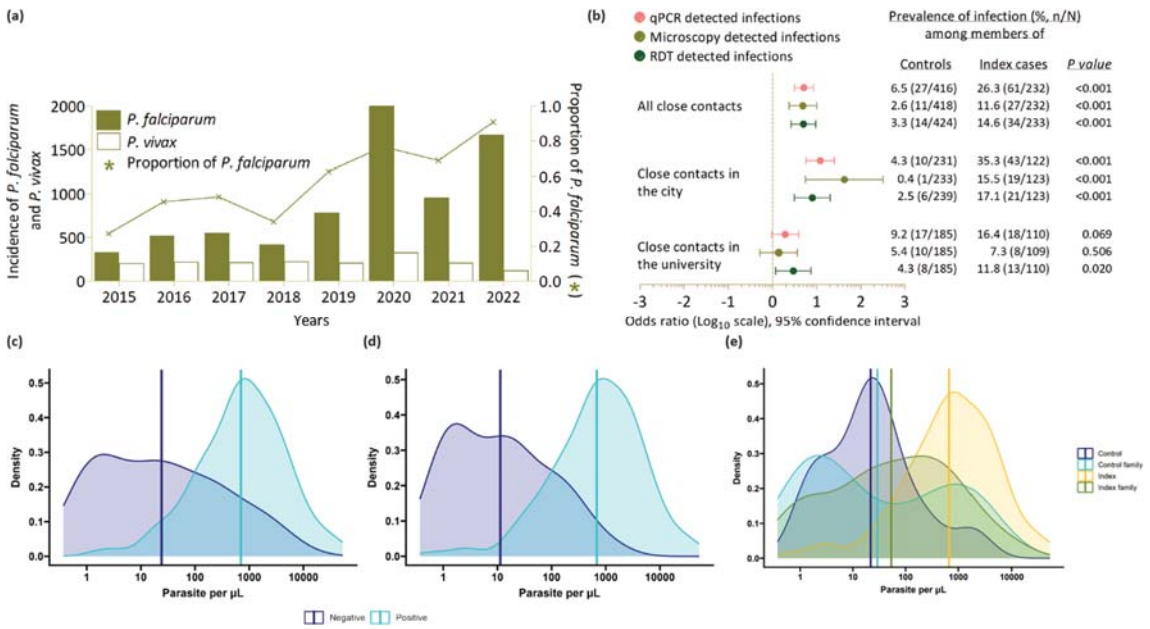

Figure 2. Temporal trend in malaria burden and parasite density distributions in Dire Dawa. Malaria trends using district health information system 2 (DHIS2) data (A) are shown with the prevalence and odds of detecting additional infections in close contacts of cases compared to controls in Dire Dawa, separately for all close contacts, contacts in the city and the university (B). The odds ratios are shown on a $\log_{10}$ scale (X-axis) together with their 95% confidence interval. The numbers to the right of the forest plot indicate the proportion of positive cases by the respective diagnostic test (color coded and embedded in the figure) among control and index household/dormitory members with the respective *p* value. Parasite density distributions determined by 18S based qPCR among HRP2-based RDT (C) positive (n=113) and negative (n=88) infections and microscopy (D) positive (n=129) and negative (n=71) infections is shown together with the distribution among index cases (n=99), contacts of index cases (n=61), controls (n=14), and contacts of controls (n=27) (E).

An. stephensi* is the predominant mosquito detected and the only one infected with *P. falciparum

In entomological surveillance, all households and dormitories were surveyed for adult mosquitoes (indoor, outdoor, and animal shelter) and immature stages of *Anopheles* in waterbodies that were present within a 100-meter radius. *Anopheles* larvae were detected in 3% (26/886) of aquatic habitats, which were either artificial (n=17) or natural (n=9). *An. stephensi* was the only species detected in the artificial containers (n=414 larvae) and was the predominant species detected at the stream edges (57% larvae, 160/280; Supplementary Table 5). Adult *Anopheles* spp. mosquitoes were detected in the majority of examined animal shelters (18/24), water storage tankers (4/4), manholes (7/7), inside (22/508) and outside (7/305) the index and control households/dormitories using Prokopack® aspirators, with nearly all identified as *An. stephensi* (97%, 599/618; Supplementary Table 6). All mosquitoes that were morphologically identified as *An. stephensi* and tested molecularly (n=90) were confirmed to be this species except 4 for which the ITS2 based PCR experiment failed (Fig. S3). Fully engorged adult caught *An. stephensi* (195/599) and *An. gambiae* (5/16) mosquitoes (Supplementary Table 7) were tested for bloodmeal sources: for cow, dog, goat, and human. Goats or cows were the main recent blood meal sources of *An. stephensi* (98%, 96/98) and *An. gambiae s.l.* (80%, 4/5), but only *An. stephensi* (2/98) had recently fed on humans. Blood meal source was undetermined for half (n=96) of the *An. stephensi* mosquitoes that were tested in this study. *P. falciparum* sporozoites, indicative of transmission upon natural blood-feeding, were detected only in *An. stephensi* (0.5%, 3/599).

Areas with higher *P. falciparum* prevalence and higher *An. stephensi* abundance overlap

Spatial analysis of *P. falciparum* infection localities within the city demonstrated significant evidence for clustering (Global Moran's *I* 0.020; $p<0.001$; Fig. 3a) in the study area, and 11 significant clusters of *P. falciparum* infections were detected. *An. stephensi* larvae and/or adult mosquitoes were more often detected near the index cases (14.9%) than controls (4.3%, $p=0.020$; Fig. 3b) and this overlapped with clusters of *P. falciparum* infections (Fig. 3c). The sporozoite infected mosquitoes were also found in close proximity and clustered (Moran's *I* 0.198, $p<0.001$; Fig. 3b). In the city, the clusters of households with higher infection prevalence were all situated within 200-meter of the river.

An. stephensi presence is strongly linked with being _P. falciparum_ positive

We next evaluated risk factors for being infected with *P. falciparum* (Table 1). Male sex (OR 3.0, 95% CI 1.7-5.4, $p=0.001$) and being above 15 years of age (OR 4.3, 95% CI 1.2-15.7, $p=0.029$) were risk factors associated with *P. falciparum* infection positivity whilst using aerosol insecticide sprays was found protective from malaria (OR 0.3, 95% CI 0.1-0.8, $p=0.016$). The results further show that those individuals residing in households/dormitories with *An. stephensi* positivity (larvae/adult/indoor/ outdoor) had a higher risk of malaria infection (OR 3.7, 95% CI 1.7-6.5, $p<0.001$) compared to individuals in households/dormitories where *An. stephensi* was not detected.

Clonal expansion of parasites with genetic signatures of partial artemisinin resistance and _pfhrp2/3_ gene deletions

We attempted to sequence 18S qPCR positive samples and of these the sequencing was successful for 71% (n=131) of the samples. All blood samples were collected from patients before treatment was provided, and thus represent the composition of parasites in the blood. Genotyping of 131 infections at 162 microhaplotype loci by amplicon sequencing uncovered that 90% of infections were monoclonal and nearly all were closely related to other detected infections, with 98% falling into one of two distinct, nearly clonal lineages. Lineage 1 was the most common, almost completely homogeneous, observed throughout the study period, and distributed widely throughout both study sites (Fig. 3d and 3e, Table 2, and Supplementary Table 10). Lineage 2 comprised 15% of infections and contained some genetic diversity, with only 13 of 20 infections highly related to each other. Highly related infections within lineage 2 were not detected until May, with most (11/13) detected at DDU (Fig. 3c). Infections within dormitories were not restricted to a single lineage; half (7/14) of all dormitories with more than one infection had infections from both lineages detected. Of concern was that 14 out of 20 lineage 2 infections carried the R622I mutation in the *kelch13* gene – which has been associated with reduced *ex vivo* susceptibility to artemisinins in Eritrea[21] – along with evidence of *P. falciparum* histidine rich protein 2 (*pfhrp2*) and *pfhrp3* gene deletions. Consistent with evidence of deletions of these genes, the majority of lineage 2 parasites (70.0%, 14/20) tested negative on HRP2-based RDT but were positive by microscopy. Lineage 1 infections did not contain *pfhrp2* deletions, most were detectable by RDT (71.6%, 78/109), and only 2.8% (n=3) contained the *kelch 13* R622I mutation, but all had evidence of *pfhrp3* deletions and the quintuple mutation in *pfdhfr* and *pfdhps* associated with antifolate resistance. Of the successfully sequenced microscopically detectable but RDT negative infections (n=24), some were found *pfhrp2* and *pfhrp3* double gene deleted (37.5%, 9/24) whilst the rest were only *pfhrp3* gene deleted (62.5%, 15/24). Interestingly, most infections from lineage 2 containing the R622I mutation (11/14) exhibited incomplete antifolate resistance, lacking the *pfdhfr* 59 mutation. A single monoclonal infection with low relatedness within lineage 2 showed unique features: elevated *pfmdr* copy number, heterozygous for the *pfmdr1* 184 mutation, whilst being the only infection with a wildtype *pfcrt* genotype. There was no significant association between lineage 1 and 2 with self-reported uptake of vector control measures (bed net utilization, insecticide residual spray, using repellents), travel history, age, sex, educational level, occupation or infection detection by microscopy (Supplementary Table 11). In contrast, a larger proportion of lineage 2 infections were undetected by RDT, as described above. These data, showing primarily clonal transmission of two distinct parasite lineages that did not intermix, are consistent with increased transmission occurring on the background of an exceedingly small parasite population, with more recent spread of a parasite lineage containing mutations that are concerning for drug and diagnostic resistance.

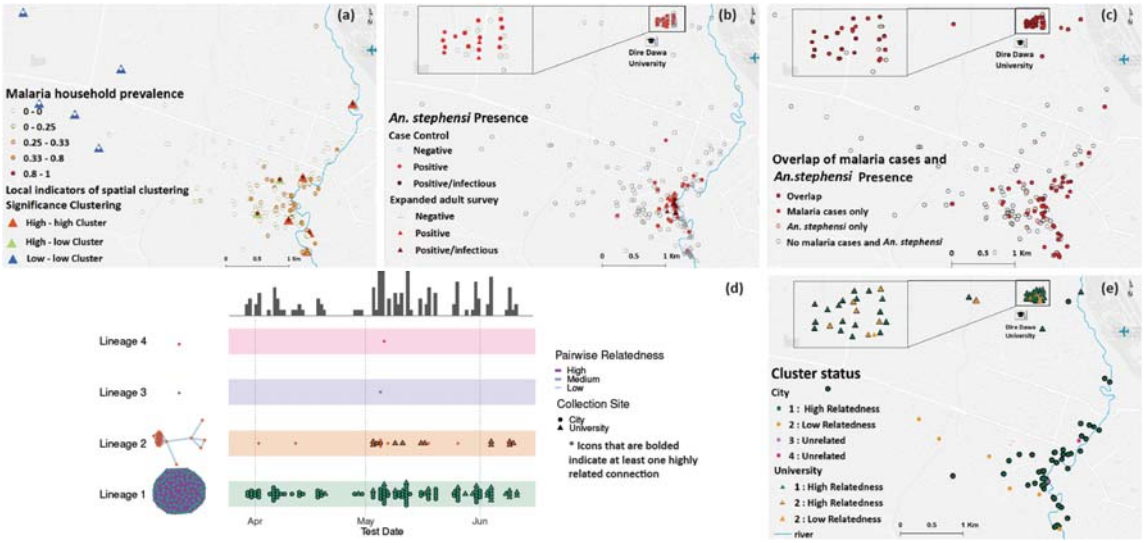

Figure 3. Spatial distribution and clustering of *P. falciparum* parasites and *An. stephensi*. Statistically significant evidence for global spatial clustering of household *P. falciparum* infections prevalence (A) and *An. stephensi* mosquitoes (B), and an overlap between the two (C) were observed. Eleven clusters of households were found (A) in the city (p<0.05) by local Moran's *I* test: high-high (n=6) whereby households had high *P. falciparum* prevalence, low-low clusters (n=5) whereby households had low *P. falciparum* prevalence, and high-low outlier clusters (n=2) whereby high *P. falciparum* prevalence households were surrounded by low *P. falciparum* prevalence households, or vice-versa. Locations of *An. stephensi* mosquitoes found infected (n=3) are shown in dark red circles and triangles (B). A map displaying case incidence colored by genetic cluster (lineage 1 in green and lineage 2 in orange) are shown along with timelines that cases were identified (D) and their spatial distribution I.

Discussion

Our findings are a reason for concern about urban malaria associated with the presence of *An. stephensi.* First detected in 2018 in Dire Dawa[8], *An. stephensi* is now perennially present in the city and was found infected with *P. falciparum*[19]. In 2014*,* no *Anopheles* developmental stages were detected in containers in Dire Dawa[22], supporting the notion of its recent introduction in the area. In the years following its first detection (between 2019 and 2022), a 12-fold increase in malaria incidence that was predominantly *P. falciparum* was observed in the city. The spatial overlap and association between malaria infection and the presence of *An. stephensi,* the detection of sporozoites in adult mosquitoes and the clonal propagation of parasites that we report here, provide the strongest evidence to date for a role of *An. stephensi* in driving an urban malaria outbreak in Africa. This, to our knowledge, is the first direct evidence of the role of *An. stephensi* in transmitting malaria in Africa and corroborates recent reports from Djibouti of exponential increases in malaria cases in the years following detection of the species[20].

The outbreak in the university campus Was localised and the dormitories affected by malaria were occupied by male students only. However, in the population of Dire Dawa city, male sex and older age were predictors of malaria positivity. Higher parasite prevalence in males compared to females has been reported in Ethiopia[23], other African countries[24], Brazil[25] and is commonly described in South East Asia[26]. Common explanations are increased risk due to employment and socio-behavioral factors (e.g. use of preventive measures, sleeping times, and forest work). There may be other behavioral differences between males and females involving crepuscular activities consistent with biting times for *An. stephensi,* which is exophilic and exophagic[27]. In our setting, chewing khat outdoors is done predominately by men[28] again increasing exposure to vectors. There is limited evidence for sex associated biological differences in infection acquisition or infection consequences; with the exception of the well-established role of pregnancy in malaria risk[29]. The recently described longer infection

duration in males compared to females[30] suggests that there may be differences in infection kinetics/responses to infections between sexes that may in turn impact the epidemiology of malaria infection.

Interestingly, this outbreak only involved *P. falciparum* infections despite the co-occurrence of *P. vivax* in the region. We previously demonstrated that *An. stephensi* is highly susceptible to Ethiopian *P. vivax* isolates[9] and an increase in *P. vivax* cases coincided with a rise in *An. stephensi* mosquitoes in Djibouti[20]. Epidemiological circumstances at the start of the outbreak, notably the extent of the human infectious reservoir for *Plasmodium* infections, may have been more favorable for *P. falciparum* in our setting. In sympatric settings, it is well known that *P. falciparum* is more prone to epidemic expansion than *P. vivax*[31,32]. There is a large and increasing body of evidence (including our own work from Ethiopia)[33,34] showing that asymptomatic *P. falciparum* infections can be highly infectious to mosquitoes and that the level of infectivity depends on the circulating parasite biomass (i.e., parasite density in asymptomatic carriers). Related studies on the human infectious reservoir for *P. falciparum* have also demonstrated that a limited number of individuals, sometimes with asymptomatic infections, may be highly infectious to mosquitoes[34]. This hypothesis is supported by the limited genetic diversity of parasites detected in this study. We speculate, that at the start of the outbreak, the asymptomatic infectious reservoir for *P. falciparum* was larger than for *P. vivax* and that a small number of infected individuals may have been responsible for initiating the current outbreak. The continued increase in the proportion of *P. falciparum* infections between 2015 and 2022 in Dire Dawa and the timing of the outbreak supports this notion. Although sporozoite rates are difficult to compare between sites, times and species, since they depend on many factors including mosquito age and survival, the 0.5% *P. falciparum* sporozoite positivity that we observed is similar to that observed in *An. arabiensis,* a native malaria vector in Ethiopia[35]. We consider a comparison with other areas with markedly different parasite populations and transmission intensity less relevant although sporozoite rates of *An. stephensi* in Afghanistan (0.8%) and India (0.6%) are in the same range as we observed[36]. Higher sporozoite rates are more likely to be associated with sustained endemicity (with entomological inoculation rate >1) and are typically associated with microscopy parasite prevalence between 10 and 40%[37]. Continuous entomological and clinical surveillance would provide further evidence if this was the case in Dire Dawa. In contrast, asymptomatic *P. vivax* infections are typically too low parasite densities to infect mosquitoes[33,38]. Since *P. vivax* sporozoites have been detected in *An. stephensi* mosquitoes previously from the same setting[19], it is possible that future malaria outbreaks caused by *An. stephensi* would also involve *P. vivax*.

The trends in increased parasite carriage among individuals living in proximity of malaria cases was most apparent for conventional diagnostics (RDT and microscopy) but not for qPCR. This is likely to reflect the age of infections with recent infections (i.e., acquired during the outbreak under examination) being more likely to be of higher parasite density while low-density infections that are detectable by qPCR to mainly reflect old infections that may have been acquired many months prior to the study[39]. Historical transmission levels influence the size of the submicroscopic reservoir through acquired immunity[40]. As Dire Dawa was previously endemic[41] some low density infections may persist and affect the interpretation of the extent of the outbreak. The relatively high-density (microscopy-detected) asymptomatic infections provided a better description of the current outbreak[33].

In addition to the role for the invasive *An. stephensi,* two other biological threats for the control of *P. falciparum* were identified in our study: drug resistance and diagnostic resistance. The high prevalence of parasites with the R622I mutation in the *kelch13* gene is of particular concern. Although it should be noted that parasite strains were not directly tested for resistance *ex vivo* in the current study, a recent WHO strategic meeting on tackling emerging antimalarial drug resistance in Africa identified this as variant linked with partial drug resistance in Eritrea[21]. Following the first report in 2016 from northwest Ethiopia[42], parasites carrying the R622I variant are being reported expanding in the same setting[43], more widely in the country[44] and elsewhere in the Horn of Africa[45]. In addition to evidence for

artemisinin-resistant parasites, mutations conferring chloroquine and anti-folate resistance were common in the outbreak parasite population. Similarly, *pfhrp2* and *pfhrp3* gene deletions with phenotypic evidence of RDT negativity were detected in our study. Despite its first report from Peru[46], the Horn of Africa (Ethiopia[47], Eritrea[48], Sudan[49], South Sudan[50], and Djibouti[51]) is disproportionately affected by the emergence of parasites with *pfhrp2/3* deletions. Co-occurrence of parasites with *pfhrp2/3* gene deletions and the R622I mutation was recently reported from other sites in Ethiopia[44]. To date, no evidence exists if the drug resistance conferring *kelch13* mutation (R622I) and *pfhrp2/3* gene deletions co-evolved in the region or if this is a matter of coincidence. Even without the evidence of co-evolution, the convergence of the three biological threats (*kelch13* mutation, *pfhrp2/3* gene deletion, and *An. stephensi* playing a role in sustaining transmission of these parasites) is concerning for the region and the entire continent at large.

In this study we concurrently examined parasite carriage and spatial clustering in humans and mosquitoes as well as genetic linkage analysis to demonstrate a highly plausible role for *An. stephensi* in an outbreak of *P. falciparum* infections that carry diagnostic and drug resistance markers in Ethiopia. Our data, demonstrating *An. stephensi* being abundant both in artificial and natural aquatic habitats in the driest months of the year, highlights how well-adapted the mosquito is to perennial persistence and urban ecology. Whilst our outbreak investigation was performed shortly after the mosquito species was first detected in the area[8], routine vector surveillance was sparse and we cannot draw firm conclusions on the timing of *An. stephensi* introduction in the area. Additionally, limited methodologies for sampling exophagic adult mosquitoes may have resulted in an underestimate of mosquito exposure and precision of sporozoite prevalence estimates. Common adult mosquito collection methods have limited sensitivity for this invasive exophilic/exophagic species. Enhanced surveillance in this study revealed outdoor resting sites (manholes, water storage tankers and animal shelters) that offer opportunities for targeted vector control and highlight the behavioral plasticity of this invasive mosquito which makes it less amenable to conventional control approaches. Our data on the use of protective measures (e.g. repellents) was insufficiently detailed to explore how effective these measures are against *An. stephensi*. Future studies should address this. Considering the very high level of resistance of *An. stephensi* to the major insecticides in Ethiopia[19,52], the repellent effect of the aerosol sprays is one explanation for the protective association observed in this study[53].

In terms of public health consequences, the spread of *An. stephensi* in rapidly expanding urban settings could pose a challenge to malaria control programs in Africa for four main reasons: i) its year round persistence due to its ability to exploit manmade containers that are abundantly present in rapidly expanding urban settings; ii) its ability to evade standard vector control tools given its unique ecology and resistance to many of the currently available insecticides; iii) its ability to efficiently transmit both *P. falciparum* and *P. vivax* in the region; and iv) its confirmed role in sustaining the transmission of drug and diagnostic resistant parasites demonstrated in this study that highlights a concerning convergence of biological threats for malaria control in the Horn of Africa and beyond. There is an urgent need for intensified surveillance to identify the extent of the distribution of this vector and to develop and implement tailored control measures. Whilst there is an increasing body of high-quality evidence of the spread of *An. stephensi* across the African continent, pragmatic studies on how to address this novel malaria threat are largely absent. Given increasing reports of *An. stephensi* in West and East Africa, the time window during which elimination of this mosquito from (parts of) Africa is possible is rapidly closing.

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Table 1. Results from a multi-level logistic regression model with nested random effects for being infected with *P. falciparum* in Dire Dawa City

Factors	Category	Proportion parasite positive, % (n/N)	Unadjusted		Adjusted	
			OR (95%CI)	P value	OR (95%CI)	P value
Sex	Female (Ref.)	10.3 (29/281)				
	Male	20.2 (134/665)	2.3 (1.4-3.9)	0.001	3.0 (1.7-5.4)	<0.001
Age in years	< 5 years (Ref.)	5.3 (3/57)				
	5 -15 Years	16.4 (18/110)	4.1 (1.1-15.3)	0.036	3.7 (0.9-14.9)	0.071
	Above 15 Years	15.2 (142/779)	3.8 (1.1-13.0)	0.035	4.3 (1.2-15.7)	0.029
An. stephensi larvae and/or adult presence	Absent (Ref.)	15.3 (132/269)				
	Present	36.5 (31/85)	3.2 (1.8-5.8)	<0.001	3.3 (1.7-6.5)	<0.001
Natural waterbody presence	Absent (Ref.)	11.2 (32/269)				
	Present	19.7 (133/677)	2.0 (1.2-3.3)	0.007	1.8 (0.9-3.4)	0.089
Usage of aerosol insecticide spray	Not Using (Ref.)	18.6 (147/790)				
	Using	7.4 (7/95)	0.3 (0.1, 0.8)	0.013	0.3 (0.1-0. 8)	0.016

Results from univariate and multivariate generalized linear mixed model. Study site, household and case/control were included as nested random effects after adjusting sex and age for study sites. Only those risk factors with p-values lower than 0.1 in univariate analyses were considered for multivariate analysis. The estimated variance between nested household and case control for the final model was 1.06, which corresponds to intra cluster correlation (ICC) of 0.24. Ref. reference category; OR odds ratio; 95% CI 95% confidence interval.

Table 2. Summary of diagnostic results and drug resistance genotype prevalence stratified by lineage, clonality, and within lineage relatedness.

Lineage	Overall	1			2				
Subset		**All**	**Monoclonal**	**Polyclonal**	**All**	**Monoclonal**	**Polyclonal**	**High Relatedness**	**Low Relatedness**
N	131	109	105	4	20	13	7	13	7
RDT+ (%)	**84 (64.1)**	**78 (71.6)**	78 (74.3)	0 (0)	**6 (30)**	3 (23.1)	3 (42.9)	2 (15.4)	4 (57.1)
Microscopy+ (%)	**97 (74)**	**82 (75.2)**	82 (78.1)	0 (0)	**15 (75)**	11 (84.6)	4 (57.1)	11 (84.6)	4 (57.1)
***pfhrp2* deleted (%)**	**12 (9.2)**	**0 (0)**	0 (0)	0 (0)	**12 (60)**	11 (84.6)	1 (14.3)	11 (84.6)	1 (14.3)
***pfhrp3* deleted (%)**	**127 (96.9)**	**109 (100)**	105 (100)	4 (100)	**16 (80)**	12 (92.3)	4 (57.1)	13 (100)	3 (42.9)
qPCR Geometric Mean, parasite/µL (IQR)	**220 (48 - 1800)**	**210 (51-1700)**	240 (76-1700)	6.1 (3.4-17)	460 (87-3400)	950 (280-2900)	120 (2.1-6300)	470 (280-2200)	440 (19-20000)
***pfk13* 622I (%)**	**17 (13.4)**	**3 (2.8)**	3 (2.9)	0 (0)	**14 (73.7)**	9 (75)	5 (71.4)	12 (100)	2 (28.6)
***pfdhps* 437/540 (%)**	**128 (99.2)**	**107 (100)**	103 (100)	4 (100)	**19 (95)**	12 (92.3)	7 (100)	13 (100)	6 (85.7)
***pfdhfr* 51/108 (%)**	**128 (99.2)**	**107 (100)**	103 (100)	4 (100)	**19 (95)**	12 (92.3)	7 (100)	13 (100)	6 (85.7)
***pfdhfr* 59/108 (%)**	**116 (89.9)**	**107 (100)**	103 (100)	4 (100)	**7 (35)**	2 (15.4)	5 (71.4)	1 (7.7)	6 (85.7)
***pfdhfr* 51/59/108 (%)**	**116 (89.9)**	**107 (100)**	103 (100)	4 (100)	**7 (35)**	2 (15.4)	5 (71.4)	1 (7.7)	6 (85.7)
***pfdhps* 437/540 + *pfdhfr* 51/59/108 (%)**	**115 (89.1)**	**107 (100)**	103 (100)	4 (100)	**6 (30)**	1 (7.7)	5 (71.4)	1 (7.7)	5 (71.4)
***pfcrt* CVIET* (%)**	**130 (99.2)**	**109 (100)**	105 (100)	4 (100)	**19 (95)**	12 (92.3)	7 (100)	13 (100)	6 (85.7)
***pfmdr1* 184Y (%)**	**1 (0.8)**	**0 (0)**	0 (0)	0 (0)	**1 (5)**	1 (7.7)	0 (0)	0 (0)	1 (14.3)

Lineage 3 (monoclonal) and lineage 4 (polyclonal) infections were *pfhrp3* deleted, negative both by microscopy and RDT, and mutated for all drug resistance variants (except *pfk13* 622I and *pfmdr* 184Y). **pfcrt* CVIET = *pfcrt* 72Cys-73Val-74Ile-75Glu-76Thr; IQR = interquartile range.

Methods

Description of the study area

Dire Dawa, located 515km southeast of Addis Ababa (capital of Ethiopia) and 311km west of Djibouti, is a logistics hub for transportation of goods and cargo (Fig. 1b). Of its total population (445,050), 74% live in an urban area which is only 2.3% of the 1,288 km^2 Dire Dawa city administrative land (UN-HABITAT, 2008). The area has a warm and dry climate with low level of precipitation (annual average rainfall of 624mm), and an annual temperature ranging from 19^oC to 32^oC. Malaria incidence has historically been low (an annual parasite clinical incidence of <5 per 1,000 people between 2014 and 2019), with strong seasonality (August to November being the peak season), and sympatric *P. falciparum* and *P. vivax* infections.

Public health data collected through the district health information system 2 (DHIS2) was obtained to analyze the trend in malaria cases between 2015 to 2022. In the Ethiopian malaria case management guideline, microscopy is recommended for diagnosis at the health center level and above. Rapid diagnostic tests (RDTs) are recommended to be used only at the health post level by community health extension workers, in rural settings. In all of the facilities located in Dire Dawa, microscopy was used for diagnosis. The DHIS2 data does not capture cases detected at private health facilities. The recent "Global framework for the response to malaria in urban areas" by the World Health Organization (WHO)[54] states that "In some urban settings, the private sector is a major source of malaria diagnosis and treatment. However, it is poorly integrated into the surveillance system." To give context on how much is being managed by the private sector in Dire Dawa, we have collected four years data (January 2019 to May 2022) from 34 out 39 health facilities (both private and public) that are located within the city administration. This included two public and five private hospitals, 15 health centers (funded publicly), and 17 clinics (private). Some private clinics (n=5) refused to provide data or provided incomplete data. Goro health center and Dire Dawa University (DDU) students' clinic were selected for the current study based on the highest number of cases they reported prior to the start of the study (January – February 2022). In fact, together, the two health facilities reported 56% of the total cases in the city in 2022 (January – May). As in all public universities in Ethiopia, students live within campus with full and shared accommodation provided by the government. At DDU, an average of six students of the same sex and year of study share a dormitory on a three-story building that has an average of 67 dormitories. Routine healthcare service is provided in a university dedicated students' clinic.

Study design and procedure

To ascertain the effect of exposure to *An. stephensi* on malaria, we employed a case control study where identification of patients was done prospectively to capture co-occurrent characteristics in terms of exposure and risk factors. We recruited consecutive patients with criteria described below in a 1:2 ratio (one case: two controls) unmatched study design.

Recruitment of participants: Patients with (history within 48 hours) fever that presented at the two health facilities and tested positive for malaria by microscopy were recruited as index cases (index) from April to July 2022. Febrile patients who attended the same clinic and tested negative for malaria were recruited as controls within 72 hours of when the index was identified. The index and controls were followed to their homes and their household/dormitory members were tested for malaria and their households/dormitories were screened for *Anopheles* mosquitoes (larvae and adult). It is noticeable that although the study was unmatched due to the difficulty in recruiting matched controls in geographical proximity of the cases, their general characteristics were very similar. Detailed characteristics of study participants are presented in Extended Data Table 1.

Sample size: We planned an unmatched case:control ratio of approximately 1:2[55] with prospective case identification until the stopping rule was achieved. The choice of the ratio was based on a logistic regression model aimed to detect an odds ratio (OR) of at least 2, assuming an exposure of 20% in controls at household level, where the exposure was defined as presence of *An. stephensi*. The power

analysis was conducted in epiR package (R-cran software), and the stopping rule was set to a power of 70% for the study to be sufficiently powered to detect differences between the presence of malaria on *An. stephensi* exposure at household level. The controls were selected from the same population as the cases and post-stratification applied. Data from cases and controls were reviewed regularly, and final sample size was set to 290 with 101 cases and 189 controls. The recruitment of case-household and control-household members was done to include reactive case detection and improve the power of the study (as well as the OR minimum detection).

Blood samples collection: Finger prick blood samples (~0.5mL), collected in BD K_2EDTA Microtainer® tubes, were used to diagnose malaria using rapid diagnostic test (RDT) (ABBOTT BIOLINE Malaria Ag Pf/Pv HRP2/LDH, India) and microscopy, and to prepare dried blood spots (DBS) on 3MM Whatman filter paper (Whatman, Maidstone, UK). The remaining blood was separated into cell pellet and plasma. Slide films were confirmed by expert microscopists. Socio-demographic, epidemiological, intervention utilization, and history of travel and malaria were collected from all study participants.

Entomological surveys: Immature stages of *Anopheles* mosquitoes were surveyed within a 100-meter radius of the index and control houses/dormitories targeting both manmade water storage containers and natural habitats including riverbeds and stream edges. Each aquatic habitat was checked for 10 minutes from 9:00-11:00AM and 3:00-5:00PM for the presence of *Anopheles* mosquitoes' larvae or pupae aiming for ten dips per habitat (using a standard dipper with 350mL capacity). Characteristics of water holding containers (permanency of habitat, lid status, purpose, volume, presence of shade, type, turbidity, temperature, and water source) were recorded for each habitat (Supplementary Table 5). Adult mosquitoes were searched using Prokopack® aspirators for 10 minutes between 06:00-08:00AM indoor, outdoor, and in animal shelters located within the compound of the household or inside and outside the dormitories at the university (Supplementary Table 6). Mosquito surveys (immature and adult) were done within 48-72 hours of when the index/control was recruited.
Conventional adult mosquito collection methods such as CDC light traps and pyrethrum spray sheet have limited sensitivity for this invasive species mainly related with its unique resting behaviour[21]. To supplement the evidence generated from the case control study and examine the resting sites of the adult *Anopheles* mosquitoes in detail in the study area, additional adult mosquito surveys were done targeting potential resting sites including animal shelters and manholes within the study time and area. Informed by these preliminary findings, surveys were systematized in three fortnightly rounds during the study period. In the city, households with (n=15) and without (n=15) animal shelters were included (Supplementary Table 6). At DDU, two dormitory buildings which reported the highest number of malaria cases, and their surroundings were selected. Adult mosquitoes were surveyed indoor, outdoor, in animal shelters, overhead tanks, and manholes using Prokopack® aspirators for 10 minutes between 06:00-08:00AM. Animal shelters were not available at DDU. Adult caught mosquitoes (sorted based on their abdominal status) and those raised from aquatic stages, were morphologically identified to the species level[22] (Supplementary Table 7). *Anopheles* mosquitoes were individually preserved in tubes that contained silica gel desiccant in zipped bags and transported to the lab at the Armauer Hansen Research Institute (AHRI) for further analysis. The global positioning system (GPS) coordinates of the households and immature and adult mosquito collection sites were recorded using GARMIN handheld GPS navigator (GARMIN GPSMAP 64S, Taiwan).

Laboratory procedures

Nucleic acid extraction from whole blood and parasite quantification, and genotyping: Blood samples in EDTA tubes were used to extract genomic DNA using MagMAX™ magnetic bead-based technology DNA multi-sample kit on KingFisher Flex robotic extractor machine (Thermo Fisher Scientific™). 50µL of whole blood input was eluted in a 150µL low-salt elution buffer. Multiplex quantitative PCR (qPCR) targeting the 18S rRNA small subunit gene for *P. falciparum* and *P. vivax* was run using primer and probe sequences described by Hermsen[56] and Wampfler[57] using TaqMan Fast Advanced Master Mix (Applied Biosystems). *P. falciparum* parasites were quantified using standard

curves generated from a serial dilution of NF54 ring stage parasites (10^6 – 10^3 parasites/mL). For *P. vivax*, parasite quantification was done using plasmid constructs to infer copy numbers by running serial dilutions (10^7 – 10^3 copies/µL) of plasmids having the amplicon. Serial dilutions of the standard curves were generated in duplicate on each plate. Multiplexed amplicon sequencing was performed on qPCR positive samples with reagents and protocol as in Tessema et al.[58]. DNA was amplified for 15 or 20 cycles in multiplexed PCR, depending on parasitemia and ability to amplify, and for 15 cycles for indexing PCR. The primer pools used in this study comprised high-diversity microhaplotype targets (n=162), polymorphisms associated with drug resistance, and targets in and adjacent to *pfhrp2* and *pfhrp3* to assess for gene deletion (Primer pools 1A and 5 as described in protocols.io repository)[59]. Amplified libraries were sequenced in a NextSeq 2000 or a MiniSeq instrument using 150PE reads with 10% PhiX.

Nucleic acid extraction from mosquitoes, assessment of infectivity and blood-meal source and confirmation of morphological species identification: Wild-caught adult *Anopheles* mosquitoes were bisected on the second and third leg under a stereo microscope using sterile scalpels[60]. The head and thoraces were stored separately from the abdomen of the mosquitoes. The heads and thoraces of the mosquitoes were homogenized in 150µL molecular grade water that contains 0.2g Zirconium bead (1mm diameter) using a Mini-Bead Beater 96 (Bartlesville, OK, USA). Part of the homogenate (50µL) was used for nucleic acid extraction using Cetyl trimethyl ammonium bromide (CTAB)[61]; 100µL grinding buffer (0.5% w/v Cas–in - Sigma, 0.1N NaOH in 10mM PBS, pH 7.4, and 0.5% IGPAL CA-630) was added to the remaining that was used to screen samples for circumsporozoite in bead-based assay. Colony maintained *An. arabiensis* and *An. stephensi* mosquitoes fed on sugar solution and infectious blood of patients' blood in direct membrane feeding assays that were used as negative and positive controls, respectively, for the downstream assays were processed the same way. *Plasmodium* infected mosquitoes were used as positive controls along with sugar-fed mosquitoes as negative controls in every extraction round (Fig. S4, Supplementary Table 8 and 9). Antibody-coupled magnetic beads and Biotinylated secondary antibodies obtained from the Center for Disease Control and Prevention (CDC), Division of Parasitic Diseases and Malaria, Entomology Branch, Atlanta, GA, USA were used to screen the presence of sporozoites in heads and thoraces as described before[62] using MagPix immunoanalyzer (Luminex Corp, CN-0269-01) after boiling the homogenate at 100°C on a thermocycler to avoid false positive signals. Samples with higher mean fluorescence intensity (MFI) signal than the negative controls plus 3 standard deviations and a representative set of mosquitoes that gave low signal were re-run to confirm the observations. Genomic DNA extracted from the head and thoraces of all mosquitoes was tested on a PCR that targeted 18S small ribosomal subunit gene as a confirmatory test. Only mosquito samples positive by the CSP based assays and 18S based PCR were considered infected. Nucleic acid was extracted from the abdomen of fully engorged mosquitoes for blood meal source identification following the same procedure[61]. A multiplex PCR assay that amplifies the cytochrome b gene based on Kent and Norris 2005[63] was used for blood meal source analysis. We have introduced slight modifications to improve product size separation on gel electrophoresis. The multiplex of cow and dog was separately done from the multiplex of goat and human. The optimized PCR thermal cycler conditions were: 5 minutes at 95°C as an initial denaturation followed by 40 cycles of denaturation at 95°C for 60 seconds, annealing at 56°C for 60 seconds for cow and dog multiplex, and 62°C for goat and human multiplex, followed by an extension at 72°C for 60 seconds, and 1 cycle of the final extension at 72°C for 7 minutes.

Confirmation of the *Anopheles* morphological identification was done following a recently published protocol that targets the ITS2 gene[64]. *An. stephensi* diagnostic amplicon of 438 bp size was expected whilst a universal amplicon of varying sizes (>600 bp), depending upon the length of ITS2 in a particular species, was expected in this multiplex protocol. The universal amplicon was used to serve as an internal control to rule out PCR failure.

Data management and analysis

Data management: Study data collection tools (mobile application version 5.20.11) were prepared and managed using REDCap electronic data capture tools hosted at AHRI. CSV files exported from REDCap were analyzed using STATA 17 (StataCorp., TX, USA), RStudio v.2022.12.0.353 (Posit, 2023), QGIS v.3.22.16 (QGIS Development Team, 2023. QGIS Geographic Information System. Open Source Geospatial Foundation Project), and GraphPad Prism 5.03 (Graph Pad Software Inc., CA, USA). RStudio with packages lme4 (generalized linear mixed models) and dcifer (Pairwise relatedness analysis on P. falciparum genotypes in diverse loci).

Bioinformatic analysis: FASTQ files from multiplexed amplicon sequencing of *P. falciparum* were subjected to filtering, demultiplexing and allele inference using a Nextflow-based pipeline (https://github.com/EPPIcenter/mad4hatter). We used cut adapt to demultiplex reads for each locus based on the locus primer sequences (no mismatches or indels allowed), filter reads by length (100 base pairs) and quality (default NextSeq quality trimming). We used dada2 to infer variants and remove chimeras. Reads with a PHRED quality score of less than 5 were truncated. The leftmost base was trimmed and trimmed reads of less than 75 base pairs were filtered out. Default values were used for all other parameters. We then aligned alleles to their reference sequence and filtered out reads with low alignment. We masked homopolymers and tandem repeats to avoid false positives.

Genetic analysis: Pairwise relatedness analysis was performed on *P. falciparum* genotypes in diverse loci using Dcifer with default settings. Pairwise relatedness was only considered between samples where the lower 95% confidence interval of estimated relatedness was greater than 0.1. Point estimates of pairwise relatedness that satisfied this threshold were then binned into low, medium, and high relatedness at greater than 0.2, 0.5, and 0.9 respectively. Samples were then clustered based on pairwise relatedness. Drug resistance marker genotypes were extracted from loci of interest. Evidence of *pfhrp2* and *pfhrp3* deletions were identified from a drop in normalized coverage in amplicons within and surrounding *pfhrp2* and *pfhrp3*. Complexity of infection was estimated by taking the 0.97 quantile (5^{th} highest number) of observed alleles across loci to minimize the impact of false positives on estimates.

Epidemiological analysis: We used standard Case-Control analyses to examine the association between risk factors and malaria infection. It calculates point estimates and confidence intervals for the OR along with the significance level based on the chi squared test. Continuous variables were presented as median and interquartile range (IQR). Tests of association between two categorical variables were performed using contingency tables. Mann-Kendall statistical test was used to test for monotonic (increasing or decreasing) trends of malaria cases using the secondary data obtained from the private and public health facilities at the city and DDU.

Spatial data analysis: As the dormitories within the university study site were located within a small area (20 buildings in 45,450m^2 area), clustering of prevalence data was assessed in the city only. The prevalence of malaria by RDT and/or microscopy was calculated for each household. Global and local Moran's *I* calculations were used to estimate the level of spatial autocorrelation within household prevalence data. The statistical strength of association for global Moran's *I* was calculated using Monte-Carlo methods based on 9,999 times permutations of the prevalence data. The Euclidean distance from the river to every site where adult or larval *An. stephensi* were located were calculated in meters.

Statistical analysis: To identify the association of *An. stephensi* and other risk factors for malaria positivity in Dire Dawa, we employed a multilevel logistic regression model with nested random effects to account for intra-class correlation (ICC) and quantify the variation in a parasite positive outcome that accounted for the household and case control group variances (nested random effects)[65]. After model selection with several model outcomes and distribution (Supplementary Table 12), the binomial model with outcome represented by malaria positivity (Positive/Negative) using RDT and/or microscopy best represented the relationship between malaria and risk factors (Supplementary Table 13)[66]. The employment of geographic unit's effects such as household and area setting (city vs university) enabled us to control for unknown variations by including them as random effects in the model. In fact, individuals living in the same household may share exposures that can determine similarities in malaria transmission as well as in the larger setting (city versus university).

Let y_{ij} denote the malaria outcome of the i^{th} individual in the j^{th} household or cluster, identified by the (RDT and/or microscopy) with probability π_{ij}, where y_{ij} = 1 denotes the individual tested positive, while y_{ij} = 0 denotes the individual tested negative for malaria. A multilevel logistic regression model with random effects for the outcome y_{ij} is given by.

$$logit(\pi_{ij}) = \beta_{0j} + \beta X_{ij} + u_j$$

Where X_{ij} = (1, x_{1ij}, ..., x_{pij}) is vector of *p* explanatory variables or covariates measured on the *i* individual and on the *j* household (cluster), β vector of fixed regression coefficients or parameters and u_j is a random effect varying over household and case control.

Ethical statement. Study protocol was approved by the Institutional ethical review board of AHRI/ALERT ethics review committee (AF-10-015.1, PO/07/19).

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Competing interests: All authors declare that they do not have competing interests

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Data and code availability: All the data used in the manuscript are available on dryad (linked with the ORCID: https://orcid.org/0000-0003-1931-1442). Sequence data are deposited on NCBI with the BioProject accession number PRJNA962166. Raw data of the study will be available in the future upon reasonable request. The R codes used to run the analyses reported in this study can be found at https://github.com/legessealamerie/DD-Stephensi and https://github.com/EPPIcenter/mad4hatter.

Disclaimer: The findings and conclusions in this paper are those of the authors and do not necessarily represent the official position of the U.S. Centers for Disease Control and Prevention.

Anopheles stephensi is implicated in an outbreak of *Plasmodium falciparum* parasites that carry markers of diagnostic resistance and candidate artemisinin resistance in Dire Dawa City, Ethiopia, January–July 2022

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Abstract

Anopheles stephensi, an Asian urban malaria vector, continues to expand across Africa. We investigated the role of *An. stephensi* in malaria transmission following a dry season outbreak in Dire Dawa, Ethiopia, from April to July 2022, using a prospective case control design. *Plasmodium falciparum* microscopy-positive febrile patients (n=101) and microscopy-negative controls (n=189) and their contacts (n=662) were identified and screened. Spatial clustering of *P. falciparum* infections was observed among case contacts but not among controls and was strongly associated with detection of *An. stephensi.* In combination with the detection of *Plasmodium* sporozoites in *An. stephensi,* this study provides the strongest evidence to date for a role of *An. stephensi* in driving an urban malaria outbreak in Africa. Importantly, this outbreak involved clonal propagation of drug and diagnostic resistant parasites. This study provides the first direct epidemiological evidence linking *An. stephensi* with increase in malaria transmission in Africa, highlighting the major public health threat of this fast-spreading invasive mosquito.

Background

The promising decline in malaria burden has slowed since 2015. This is particularly evident in Africa, the continent that carries the largest malaria prevalence[1]. Malaria control programs in Africa traditionally focus on rural settings, where most infections occur, but malaria is of increasing concern in urban settings[2]. Given the rapid urbanization in Africa[3], urban malaria transmission can result in a considerable health burden[4]. Urban malaria is classically associated with importation from areas of intense transmission[5] but can be exacerbated by the adaptation of existing malaria vectors to urban environments[6] and the emergence of urban malaria vectors such as *Anopheles stephensi*[7].

An. stephensi is distinct from other *Anopheles* species that are traditional vectors in (rural) Africa with its preference for artificial water storage containers that are common in urban settings[8,9]. Native to the Indian sub-continent and the Persian Gulf[10], *An. stephensi* is now rapidly expanding its geographic range westward (Fig. 1a)[7]. First detected in Africa in Djibouti in 2012[11], *An. stephensi* is rapidly

expanding its range in the Horn of Africa including Ethiopia (2016)[12], Sudan (2016)[13], Somalia (2019)[14], Eritrea (2022)[15] and beyond: Yemen (2021)[16], Kenya (2022)[17], Ghana (2022)[15], and Nigeria (2020)[15]. In recognition of the potentially devastating consequences of *An. stephensi* spreading across Africa, the World Health Organization (WHO) urgently requested more data on its distribution and released a strategy to mitigate its spread[18].

In addition to being an efficient vector for both *Plasmodium falciparum* and *Plasmodium vivax* in its native geographical range[10], *An. stephensi* was recently confirmed to be susceptible to local parasites in Ethiopia (Fig. 1a)[9,19] and a resurgence of malaria was reported in Djibouti following its detection[20], although direct evidence for a role of *An. stephensi* in this resurgence was unavailable. Following a report of a dry-season upsurge in malaria cases in Dire Dawa City, Ethiopia, where *An. stephensi* was recently documented[8], we prospectively investigated its role in malaria transmission through responsive epidemiological and entomological surveillance (Fig. 1b).

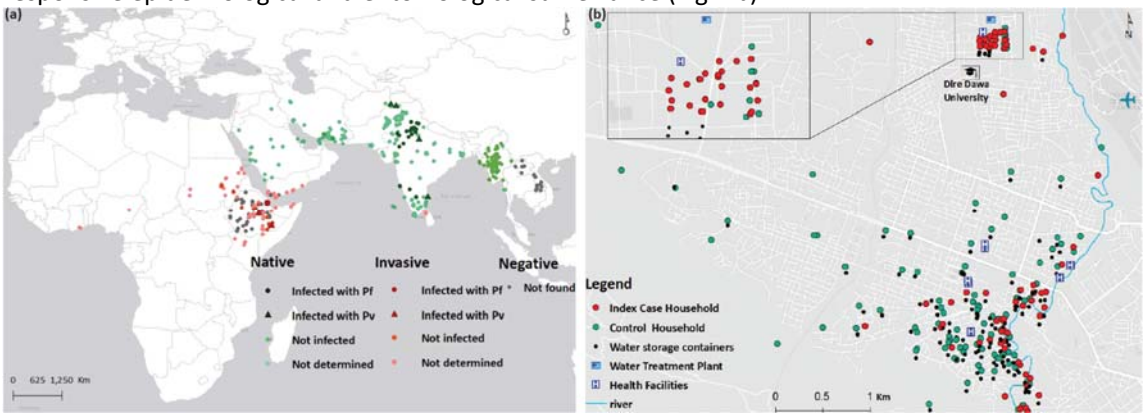

Figure 1. Global distribution of *An. stephensi* and the study location. (A) The global distribution of *An. stephensi* where it is native (green) and invasive (red) is shown together with the sporozoite infection detection outcomes where it was found infected and not infected with *P. falciparum* (*Pf*) and *P. vivax* (*Pv*). Sites where *An. stephensi* was observed but mosquitoes were not tested for the presence of sporozoites are also shown (Not determined). Settings where dedicated entomological surveillance did not detect *An. stephensi* mosquitoes are indicated in grey circles (Negative). (B) The locations of case (red) and control (green) households/dormitories surveyed in this study are shown together with water storage containers (black), water treatment plant (in the university campus), health facilities (H) and Butiji river in Dire Dawa city. Source: The global map (A) was modified based on the malaria threats map[7] (https://apps.who.int/malaria/maps/threats/#/maps?theme=invasive&map) of the World Health Organization.

Results

Malaria outbreaks in Dire Dawa city and its university

Clinical malaria incidence data (diagnosed by microscopy) collected from public and private health facilities (n=34) showed a 12-fold increase (Supplementary Table 1 and Fig. S1) in malaria incidence in Dire Dawa during the dry months (January – May) of 2022 (2,425 cases) compared to 2019 (205 cases). An overall statistically significant trend of increasing number of malaria positive cases was observed between 2019 and 2022 (Mann-Kendall statistical test $\tau = 0.42$, $p<0.001$). Patients reported at both public and private health facilities with the latter contributing to 15.8% of patients diagnosed for malaria in the last four years with an increasing trend from 17.7% in 2019 to 25.9% in 2021 which later declined to 5.7% during the outbreak (2022). In 2022, 76% of all reported malaria cases originated from only three public health facilities: Dire Dawa University (DDU) students' clinic (42%), Sabiyan Hospital (19%) and Goro Health Center (15%). At DDU campus, 94% (1,075/1,141) of clinical malaria episodes occurred in the male student population living in the university single-sex dormitories.

We conducted a prospective case control study to identify risk factors associated with this sudden rise in malaria in the city (Goro Health Center) and DDU (Fig. 1b). In the city, we recruited 48 microscopy malaria confirmed febrile cases plus 125 case-household members and 109 febrile controls without microscopy confirmed malaria who attended the same clinic within 72 hours plus 241 control-household members. At DDU, we recruited 53 students with clinical malaria and 110 dormmates and 80 uninfected febrile students with 186 dormmates. Details of individual and household characteristics are presented in the extended Data Table 1. Fever was detected in additional family/dormitory members of the controls (1.4%, 6/424) and index cases (6.0%, 14/233) (Supplementary Table 2). The responsive case control study unit was household/dormitory; no plausible risk factors were defined *a priori* and neither a sex/gender nor *Plasmodium* species stratification was considered in the study design. The outbreak at the university campus happened at a fine spatial scale (20 dormitory buildings in a 45,450 m^2 area); the dormitories affected by malaria were occupied by male students only (Extended Data Table 1). Despite Dire Dawa being historically co-endemic for *P. falciparum* and *P. vivax*, the proportion of cases that were due to *P. falciparum* increased from 61% in 2015 to 93% in 2022 (Fig. 2a). All the index cases we recruited (n=101) and the additional infections detected (n=102) in this study were found to be *P. falciparum* except only two *P. vivax* infections detected by 18S based qPCR. *Plasmodium* infection was detected in 14 controls by 18S based qPCR. The parasite density in these infections was very low (median parasitemia was 21 parasites/µL) and thus lie below the detection limits of the conventional diagnostics. Only two of these infections had parasitemia above 100 parasites/µL (278 and 1,822 parasites/µL).

Household and dormitory members of malaria cases experience higher mosquito exposure and higher infection prevalence

The results obtained from case control analysis showed that members of the index cases and controls had different levels of mosquito exposure (Extended Data Table 2). Members of a case household/dormitory were more likely to be living close to *An. stephensi* positive sites, defined as the presence of larvae within a 100-meter radius from the household/dormitory (odds ratio [OR] 5.0, 95% confidence interval [CI] 2.8-9.4, $p<0.001$), or adult mosquitoes resting sites (OR 1.9, 95% CI 0.9-4.0, $p=0.068$), or to natural/manmade waterbodies in general (OR 1.6, 95% CI 1.2-2.2, $p=0.002$). The odds of using an aerosol insecticide spray were 58% lower among members of the index cases compared to controls (OR 0.42, 95% CI 0.23-0.72, $p<0.001$).

In the city, *P. falciparum* qPCR detected infections were significantly more common (OR 12.0, 95% CI 5.8-25.1, $p<0.001$; Fig. 2b) among case household members (35.3%, 43/122) than control household members (4.3%, 10/233), with a similar trend for microscopy (OR 42.4, 95% CI 5.6-320.8, $p<0.001$) and RDT detected infections (OR 8.0, 95% CI 3.1-20.4, $p<0.001$). At DDU, despite all students living in close proximity (20 buildings in a 45,450 m^2 area), dormmates of malaria cases were thrice as likely (OR 3.0, 95% CI 1.2-7.4, $p=0.020$; Fig. 2b) to be *P. falciparum* positive by RDT (11.8%, 13/110) compared to dormmates of controls (4.3%, 8/185). A quarter of microscopy-positive infections (34/136) were negative by HRP2-based RDT (sensitivity 75.0, 95% CI 72.2-77.8, specificity 97.0, 95% CI 95.9-98.1; Supplementary Table 3) with different proportions of HRP2-based RDT negative infections in the city (10.3%, 7/68) and the university (39.7%, 27/68). qPCR detected considerably more infections with the likelihood of infections being missed by RDT (Fig. 2c) or microscopy (Fig. 2d) being dependent on parasite density and, for RDT, *pfhrp2* gene deletion status (Supplementary Table 4, Fig. S2). As expected, parasitemia was higher in the index cases (geometric mean 669 *P. falciparum* parasites/µL, 95% CI 442-1012; Fig. 2e) compared to malaria-infected controls (21.1, 6.9-68.6, $p<0.001$), malaria-infected control family members (29.2, 8.8-96.8, $p=0.005$), and malaria-infected index family members (53.4, 26.7-107.0, $p<0.001$).

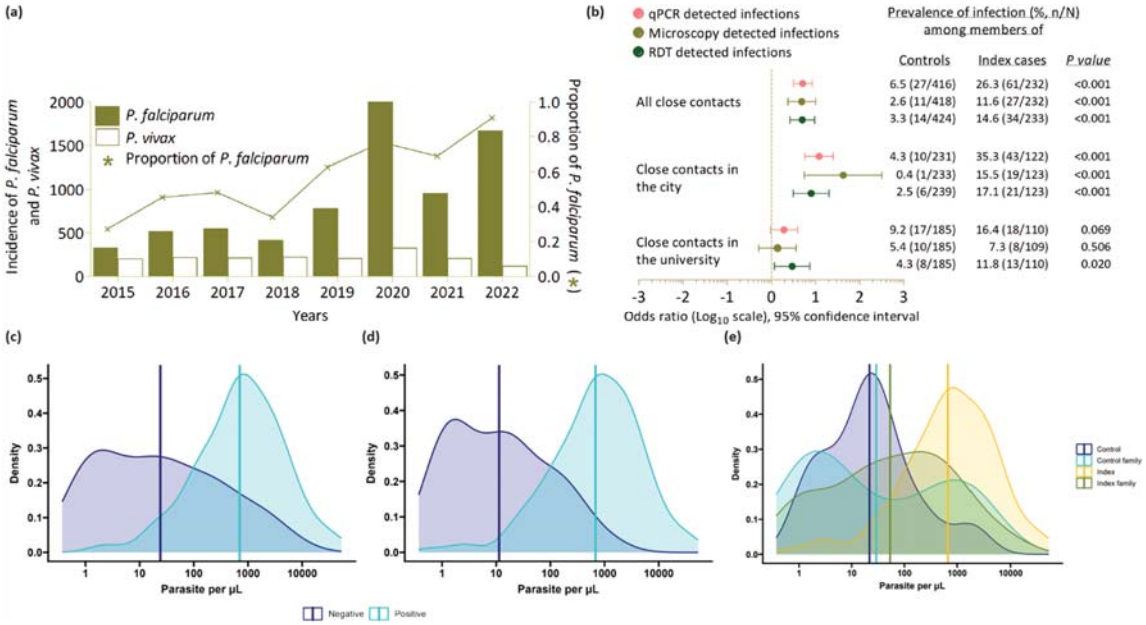

Figure 2. Temporal trend in malaria burden and parasite density distributions in Dire Dawa. Malaria trends using district health information system 2 (DHIS2) data (A) are shown with the prevalence and odds of detecting additional infections in close contacts of cases compared to controls in Dire Dawa, separately for all close contacts, contacts in the city and the university (B). The odds ratios are shown on a $\log_{10}$ scale (X-axis) together with their 95% confidence interval. The numbers to the right of the forest plot indicate the proportion of positive cases by the respective diagnostic test (color coded and embedded in the figure) among control and index household/dormitory members with the respective *p* value. Parasite density distributions determined by 18S based qPCR among HRP2-based RDT (C) positive (n=113) and negative (n=88) infections and microscopy (D) positive (n=129) and negative (n=71) infections is shown together with the distribution among index cases (n=99), contacts of index cases (n=61), controls (n=14), and contacts of controls (n=27) (E).

An. stephensi* is the predominant mosquito detected and the only one infected with *P. falciparum

In entomological surveillance, all households and dormitories were surveyed for adult mosquitoes (indoor, outdoor, and animal shelter) and immature stages of *Anopheles* in waterbodies that were present within a 100-meter radius. *Anopheles* larvae were detected in 3% (26/886) of aquatic habitats, which were either artificial (n=17) or natural (n=9). *An. stephensi* was the only species detected in the artificial containers (n=414 larvae) and was the predominant species detected at the stream edges (57% larvae, 160/280; Supplementary Table 5). Adult *Anopheles* spp. mosquitoes were detected in the majority of examined animal shelters (18/24), water storage tankers (4/4), manholes (7/7), inside (22/508) and outside (7/305) the index and control households/dormitories using Prokopack® aspirators, with nearly all identified as *An. stephensi* (97%, 599/618; Supplementary Table 6). All mosquitoes that were morphologically identified as *An. stephensi* and tested molecularly (n=90) were confirmed to be this species except 4 for which the ITS2 based PCR experiment failed (Fig. S3). Fully engorged adult caught *An. stephensi* (195/599) and *An. gambiae* (5/16) mosquitoes (Supplementary Table 7) were tested for bloodmeal sources: for cow, dog, goat, and human. Goats or cows were the main recent blood meal sources of *An. stephensi* (98%, 96/98) and *An. gambiae s.l.* (80%, 4/5), but only *An. stephensi* (2/98) had recently fed on humans. Blood meal source was undetermined for half (n=97) of the *An. stephensi* mosquitoes that were tested in this study. *P. falciparum* sporozoites, indicative of transmission upon natural blood-feeding, were detected only in *An. stephensi* (0.5%, 3/599).

Areas with higher *P. falciparum* prevalence and higher *An. stephensi* abundance overlap

Spatial analysis of *P. falciparum* infection localities within the city demonstrated significant evidence for clustering (Global Moran's *I* 0.020; $p<0.001$; Fig. 3a) in the study area, and 11 significant clusters of *P. falciparum* infections were detected. *An. stephensi* larvae and/or adult mosquitoes were more often detected near the index cases (14.9%) than controls (4.3%, $p=0.020$; Fig. 3b) and this overlapped with clusters of *P. falciparum* infections (Fig. 3c). The sporozoite infected mosquitoes were also found in close proximity and significantly clustered (Moran's *I* 0.198, $p<0.001$; Fig. 3b). In the city, the clusters of households with higher infection prevalence were all situated within 200-meter of the river.

***An. stephensi* presence is strongly linked with being *P. falciparum* positive**

We next evaluated risk factors for being infected with *P. falciparum* (Table 1). Male sex (OR 3.0, 95% CI 1.7-5.4, $p=0.001$) and being above 15 years of age (OR 4.3, 95% CI 1.2-15.7, $p=0.029$) were risk factors associated with *P. falciparum* infection positivity whilst using aerosol insecticide sprays was found protective from malaria (OR 0.3, 95% CI 0.1-0.8, $p=0.016$). The results further show that those individuals residing in households/dormitories with *An. stephensi* positivity (larvae/adult/indoor/outdoor) had a higher risk of malaria infection (OR 3.7, 95% CI 1.7-6.5, $p<0.001$) compared to individuals in households/dormitories where *An. stephensi* was not detected.

Table 1. Results from a multi-level logistic regression model with nested random effects for being infected with *P. falciparum* in Dire Dawa City

Factors	Category	Proportion parasite positive, % (n/N)	Unadjusted		Adjusted	
			OR (95%CI)	P value	OR (95%CI)	P value
Sex	Female (Ref.)	10.3 (29/281)				
	Male	20.2 (134/665)	2.3 (1.4-3.9)	0.001	3.0 (1.7-5.4)	<0.001
Age in years	< 5 years (Ref.)	5.3 (3/57)				
	5 -15 Years	16.4 (18/110)	4.1 (1.1-15.3)	0.036	3.7 (0.9-14.9)	0.071
	Above 15 Years	15.2 (142/779)	3.8 (1.1-13.0)	0.035	4.3 (1.2-15.7)	0.029
An. stephensi larvae and/or adult presence	Absent (Ref.)	15.3 (132/269)				
	Present	36.5 (31/85)	3.2 (1.8-5.8)	<0.001	3.3 (1.7-6.5)	<0.001
Natural waterbody presence	Absent (Ref.)	11.2 (32/269)				
	Present	19.7 (133/677)	2.0 (1.2-3.3)	0.007	1.8 (0.9-3.4)	0.089
Usage of aerosol insecticide spray	Not Using (Ref.)	18.6 (147/790)				
	Using	7.4 (7/95)	0.3 (0.1, 0.8)	0.013	0.3 (0.1-0. 8)	0.016

Results from univariate and multivariate generalized linear mixed model. Study site, household and case/control were included as nested random effects after adjusting sex and age for study sites. Only those risk factors with p-values lower than 0.1 in univariate analyses were considered for multivariate analysis. The estimated variance between nested household and case control for the final model was 1.06, which corresponds to intra cluster correlation (ICC) of 0.24. Ref. reference category; OR odds ratio; 95% CI 95% confidence interval.

Clonal expansion of parasites with genetic signatures of partial artemisinin resistance and *pfhrp2/3* gene deletions

We attempted to sequence 18S qPCR positive samples and of these the sequencing was successful for 71% (n=131) of the samples. All blood samples were collected from patients before treatment was provided, and thus represent the composition of parasites in the blood. Genotyping of 131 infections at 162 microhaplotype loci by amplicon sequencing uncovered that 90% of infections were monoclonal and nearly all were closely related to other detected infections, with 98% falling into one of two distinct, nearly clonal lineages. Lineage 1 was the most common, almost completely homogeneous, observed throughout the study period, and distributed widely throughout both study sites (Fig. 3d and

3e, Table 2, and Supplementary Table 10). Lineage 2 comprised 15% of infections and contained some genetic diversity, with only 13 of 20 infections highly related to each other. Highly related infections within lineage 2 was not detected until May, with most (11/13) detected at DDU (Fig. 3c). Infections within dormitories were not restricted to a single lineage; half (7/14) of all dormitories with more than one infection had infections from both lineages detected. Of concern was that 14 out of 20 lineage 2 infections carried the R622I mutation in the *kelch13* gene – which has been associated with reduced *ex vivo* susceptibility to artemisinins in Eritrea[21] – along with evidence of *P. falciparum* histidine rich protein 2 (*pfhrp2*) and *pfhrp3* gene deletions. Consistent with evidence of deletions of these genes, the majority of lineage 2 parasites (70.0%, 14/20) tested negative on HRP2-based RDT but were positive by microscopy. Lineage 1 infections did not contain *pfhrp2* deletions, most were detectable by RDT (71.6%, 78/109), and only 2.8% (n=3) contained the *kelch 13* R622I mutation, but all had evidence of *pfhrp3* deletions and the quintuple mutation in *pfdhfr* and *pfdhps* associated–with antifolate resistance. Interestingly, most infections from lineage 2 containing the R622I mutation (11/14) exhibited incomplete antifolate resistance, lacking the *pfdhfr* 59 mutation. A single monoclonal infection with low relatedness within lineage 2 showed unique features: elevated *pfmdr* copy number, heterozygous for the *pfmdr1* 184 mutation, whilst being the only infection with a wildtype *pfcrt* genotype. There was no significant association between lineage 1 and 2 with self-reported uptake of vector control measures (bed net utilization, insecticide residual spray, using repellents), travel history, age, sex, educational level, occupation, or infection detection by microscopy (Supplementary Table 11). In contrast, a larger proportion of lineage 2 infections were undetected by RDT, as described above. These data, showing primarily clonal transmission of two distinct parasite lineages that did not intermix, are consistent with increased transmission occurring on the background of an exceedingly small parasite population, with more recent spread of a parasite lineage containing mutations that are concerning for drug and diagnostic resistance.

Table 2. Summary of diagnostic results and drug resistance genotype prevalence stratified by lineage, clonality, and within lineage relatedness.

Lineage	Overall	1			2				
Subset		**All**	**Monoclonal**	**Polyclonal**	**All**	**Monoclonal**	**Polyclonal**	**High Relatedness**	**Low Relatedness**
N	131	109	105	4	20	13	7	13	7
RDT+ (%)	**84 (64.1)**	**78 (71.6)**	78 (74.3)	0 (0)	**6 (30)**	3 (23.1)	3 (42.9)	2 (15.4)	4 (57.1)
Microscopy+ (%)	**97 (74)**	**82 (75.2)**	82 (78.1)	0 (0)	**15 (75)**	11 (84.6)	4 (57.1)	11 (84.6)	4 (57.1)
***pfhrp2* deleted (%)**	**12 (9.2)**	**0 (0)**	0 (0)	0 (0)	**12 (60)**	11 (84.6)	1 (14.3)	11 (84.6)	1 (14.3)
***pfhrp3* deleted (%)**	**127 (96.9)**	**109 (100)**	105 (100)	4 (100)	**16 (80)**	12 (92.3)	4 (57.1)	13 (100)	3 (42.9)
qPCR Geometric Mean, parasite/µL (IQR)	**220 (48 -1800)**	**210 (51-1700)**	240 (76-1700)	6.1 (3.4-17)	460 (87-3400)	950 (280-2900)	120 (2.1-6300)	470 (280-2200)	440 (19-20000)
***pfk13* 622I (%)**	**17 (13.4)**	**3 (2.8)**	3 (2.9)	0 (0)	**14 (73.7)**	9 (75)	5 (71.4)	12 (100)	2 (28.6)
***pfdhps* 437/540 (%)**	**128 (99.2)**	**107 (100)**	103 (100)	4 (100)	**19 (95)**	12 (92.3)	7 (100)	13 (100)	6 (85.7)
***pfdhfr* 51/108 (%)**	**128 (99.2)**	**107 (100)**	103 (100)	4 (100)	**19 (95)**	12 (92.3)	7 (100)	13 (100)	6 (85.7)
***pfdhfr* 59/108 (%)**	**116 (89.9)**	**107 (100)**	103 (100)	4 (100)	**7 (35)**	2 (15.4)	5 (71.4)	1 (7.7)	6 (85.7)
***pfdhfr* 51/59/108 (%)**	**116 (89.9)**	**107 (100)**	103 (100)	4 (100)	**7 (35)**	2 (15.4)	5 (71.4)	1 (7.7)	6 (85.7)
***pfdhps* 437/540 + *pfdhfr* 51/59/108 (%)**	**115 (89.1)**	**107 (100)**	103 (100)	4 (100)	**6 (30)**	1 (7.7)	5 (71.4)	1 (7.7)	5 (71.4)
***pfcrt* CVIET* (%)**	**130 (99.2)**	**109 (100)**	105 (100)	4 (100)	**19 (95)**	12 (92.3)	7 (100)	13 (100)	6 (85.7)
***pfmdr1* 184Y (%)**	**1 (0.8)**	**0 (0)**	0 (0)	0 (0)	**1 (5)**	1 (7.7)	0 (0)	0 (0)	1 (14.3)

Lineage 3 (monoclonal) and lineage 4 (polyclonal) infections were *pfhrp3* deleted, negative both by microscopy and RDT, and mutated for all drug resistance variants (except *pfk13* 622I and *pfmdr* 184Y). **pfcrt* CVIET = *pfcrt* 72Cys-73Val-74Ile-75Glu-76Thr; IQR = interquartile range.

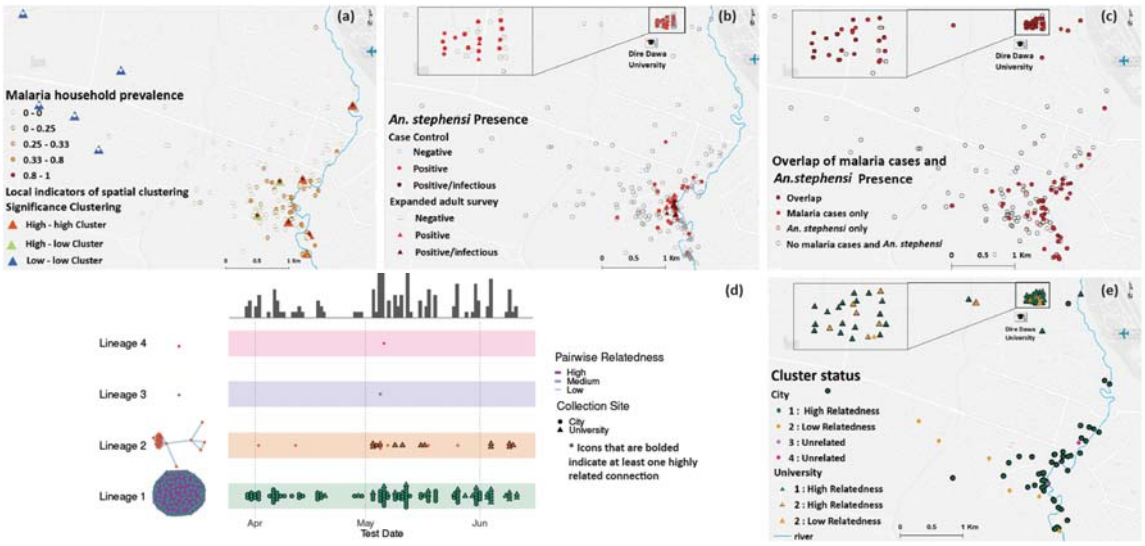

Figure 3. Spatial distribution and clustering of *P. falciparum* parasites and *An. stephensi*. Statistically significant evidence for global spatial clustering of household *P. falciparum* infections prevalence (A) and *An. stephensi* mosquitoes (B), and an overlap between the two (C) were observed. Eleven clusters of households were found (A) in the city (p<0.05) by local Moran's *I* test: high-high (n=6) whereby households had high *P. falciparum* prevalence, low-low clusters (n=5) whereby households had low *P. falciparum* prevalence, and high-low outlier clusters (n=2) whereby high *P. falciparum* prevalence households were surrounded by low *P. falciparum* prevalence households, or vice-versa. Locations of *An. stephensi* mosquitoes found infected (n=3) are shown in dark red circles and triangles (B). A map displaying case incidence colored by genetic cluster (lineage 1 in green and lineage 2 in orange) are shown along with timelines that cases were identified (D) and their spatial distribution (E).

Discussion

Our findings are a reason for concern about urban malaria associated with the presence of *An. stephensi.* First detected in 2018 in Dire Dawa[8], *An. stephensi* is now perennially present in the city and was found infected with *P. falciparum*[19]. In 2014*,* no *Anopheles* developmental stages were detected in containers in Dire Dawa[22], supporting the notion of its recent introduction in the area. In the years following its first detection (between 2019 and 2022), a 12-fold increase in malaria incidence that was predominantly *P. falciparum* was observed in the city. The spatial overlap and association between malaria infection and the presence of *An. stephensi,* the detection of sporozoites in adult mosquitoes and the clonal propagation of parasites that we report here, provide the strongest evidence to date for a role of *An. stephensi* in driving an urban malaria outbreak in Africa. This, to our knowledge, is the first direct evidence of the role of *An. stephensi* in transmitting malaria in Africa and corroborates recent reports from Djibouti of exponential increases in malaria cases in the years following detection of the species[20].

The outbreak in the university campus Was localised and the dormitories affected by malaria were occupied by male students only. However, in the population of Dire Dawa city, male sex and older age were predictors of malaria positivity. Higher parasite prevalence in males compared to females has been reported in Ethiopia[23], other African countries[24], Brazil[25] and is commonly described in South East Asia[26]. Common explanations are increased risk due to employment and socio-behavioral factors (e.g. use of preventive measures, sleeping times, and forest work). There may be other behavioral differences between males and females involving crepuscular activities consistent with biting times for *An. stephensi,* which is exophilic and exophagic[27]. In our setting, chewing khat outdoors is done predominately by men[28] again increasing exposure to vectors. There is limited evidence for sex

associated biological differences in infection acquisition or infection consequences; with the exception of the well-established role of pregnancy in malaria risk[29]. The recently described longer infection duration in males compared to females[30] suggests that there may be differences in infection kinetics/responses to infections between sexes that may in turn impact the epidemiology of malaria infection.

Interestingly, this outbreak only involved *P. falciparum* infections despite the co-occurrence of *P. vivax* in the region. We previously demonstrated that *An. stephensi* is highly susceptible to Ethiopian *P. vivax* isolates[9] and an increase in *P. vivax* cases coincided with a rise in *An. stephensi* mosquitoes in Djibouti[20]. Epidemiological circumstances at the start of the outbreak, notably the extent of the human infectious reservoir for *Plasmodium* infections, may have been more favorable for *P. falciparum* in our setting. In sympatric settings, it is well known that *P. falciparum* is more prone to epidemic expansion than *P. vivax*[31,32]. There is a large and increasing body of evidence (including our own work from Ethiopia)[33,34] showing that asymptomatic *P. falciparum* infections can be highly infectious to mosquitoes and that the level of infectivity depends on the circulating parasite biomass (i.e., parasite density in asymptomatic carriers). Related studies on the human infectious reservoir for *P. falciparum* have also demonstrated that a limited number of individuals, sometimes with asymptomatic infections, may be highly infectious to mosquitoes[34]. This hypothesis is supported by the limited genetic diversity of parasites detected in this study. We speculate, that at the start of the outbreak, the asymptomatic infectious reservoir for *P. falciparum* was larger than for *P. vivax* and that a small number of infected individuals may have been responsible for initiating the current outbreak. The continued increase in the proportion of *P. falciparum* infections between 2015 and 2022 in Dire Dawa and the timing of the outbreak supports this notion. Although sporozoite rates are difficult to compare between sites, times and species, since they depend on many factors including mosquito age and survival, the 0.5% *P. falciparum* sporozoite positivity that we observed is similar to that observed in *An. arabiensis,* a native malaria vector in Ethiopia[35]. We consider a comparison with other areas with markedly different parasite populations and transmission intensity less relevant although sporozoite rates of *An. stephensi* in Afghanistan (0.8%) and India (0.6%) are in the same range as we observed[36]. Higher sporozoite rates are more likely to be associated with sustained endemicity (with entomological inoculation rate >1) and are typically associated with microscopy parasite prevalence between 10 and 40%[37]. Continuous entomological and clinical surveillance would provide further evidence if this was the case in Dire Dawa. In contrast, asymptomatic *P. vivax* infections are typically too low parasite densities to infect mosquitoes[33,38]. Since *P. vivax* sporozoites have been detected in *An. stephensi* mosquitoes previously from the same setting[19], it is possible that future malaria outbreaks caused by *An. stephensi* would also involve *P. vivax*.

The trends in increased parasite carriage among individuals living in proximity of malaria cases was most apparent for conventional diagnostics (RDT and microscopy) but not for qPCR. This is likely to reflect the age of infections with recent infections (i.e., acquired during the outbreak under examination) being more likely to be of higher parasite density while low-density infections that are detectable by qPCR to mainly reflect old infections that may have been acquired many months prior to the study[39]. Historical transmission levels influence the size of the submicroscopic reservoir through acquired immunity[40]. As Dire Dawa was previously endemic[41] some low density infections may persist and affect the interpretation of the extent of the outbreak. The relatively high-density (microscopy-detected) asymptomatic infections provided a better description of the current outbreak[33].

In addition to the role for the invasive *An. stephensi,* two other biological threats for the control of *P. falciparum* were identified in our study: drug resistance and diagnostic resistance. The high prevalence of parasites with the R622I mutation in the *kelch13* gene is of particular concern. Although it should be noted that parasite strains were not directly tested for resistance *ex vivo* in the current study, a recent WHO strategic meeting on tackling emerging antimalarial drug resistance in Africa identified this as variant linked with partial drug resistance in Eritrea[42]. Following the first report in 2016 from northwest Ethiopia[43], parasites carrying the R622I variant are being reported expanding in the same

setting[44], more widely in the country[45] and elsewhere in the Horn of Africa[46]. In addition to evidence for artemisinin-resistant parasites, mutations conferring chloroquine and anti-folate resistance were common in the outbreak parasite population. Similarly, *pfhrp2* and *pfhrp3* gene deletions with phenotypic evidence of RDT negativity were detected in our study. Despite its first report from Peru[47], the Horn of Africa (Ethiopia[48], Eritrea[49], Sudan[50], South Sudan[51], and Djibouti[52]) is disproportionately affected by the emergence of parasites with *pfhrp2/3* deletions. Co-occurrence of parasites with *pfhrp2/3* gene deletions and the R622I mutation was recently reported from other sites in Ethiopia[45]. To date, no evidence exists if the drug resistance conferring *kelch13* mutation (R622I) and *pfhrp2/3* gene deletions co-evolved in the region or if this is a matter of coincidence. Even without the evidence of co-evolution, the convergence of the three biological threats (*kelch13* mutation, *pfhrp2/3* gene deletion, and *An. stephensi* playing a role in sustaining transmission of these parasites) is concerning for the region and the entire continent at large.

In this study we concurrently examined parasite carriage and spatial clustering in humans and mosquitoes as well as genetic linkage analysis to demonstrate a highly plausible role for *An. stephensi* in an outbreak of *P. falciparum* infections that carry diagnostic and drug resistance markers in Ethiopia. Our data, demonstrating *An. stephensi* being abundant both in artificial and natural aquatic habitats in the driest months of the year, highlights how well-adapted the mosquito is to perennial persistence and urban ecology. Whilst our outbreak investigation was performed shortly after the mosquito species was first detected in the area[8], routine vector surveillance was sparse and we cannot draw firm conclusions on the timing of *An. stephensi* introduction in the area. Additionally, limited methodologies for sampling exophagic adult mosquitoes may have resulted in an underestimate of mosquito exposure and precision of sporozoite prevalence estimates. Common adult mosquito collection methods have limited sensitivity for this invasive exophilic/exophagic species. Enhanced surveillance in this study revealed outdoor resting sites (manholes, water storage tankers and animal shelters) that offer opportunities for targeted vector control and highlight the behavioral plasticity of this invasive mosquito which makes it less amenable to conventional control approaches. Our data on the use of protective measures (e.g. repellents) was insufficiently detailed to explore how effective these measures are against *An. stephensi*. Future studies should address this. Considering the very high level of resistance of *An. stephensi* to the major insecticides in Ethiopia[19,53], the repellent effect of the aerosol sprays is one explanation for the protective association observed in this study[54].

In terms of public health consequences, the spread of *An. stephensi* in rapidly expanding urban settings could pose a challenge to malaria control programs in Africa for four main reasons: i) its year round persistence due to its ability to exploit manmade containers that are abundantly present in rapidly expanding urban settings; ii) its ability to evade standard vector control tools given its unique ecology and resistance to many of the currently available insecticides; iii) its ability to efficiently transmit both *P. falciparum* and *P. vivax* in the region; and iv) its confirmed role in sustaining the transmission of drug and diagnostic resistant parasites demonstrated in this study that highlights a concerning convergence of biological threats for malaria control in the Horn of Africa and beyond. There is an urgent need for intensified surveillance to identify the extent of the distribution of this vector and to develop and implement tailored control measures. Whilst there is an increasing body of high-quality evidence of the spread of *An. stephensi* across the African continent, pragmatic studies on how to address this novel malaria threat are largely absent. Given increasing reports of *An. stephensi* in West and East Africa, the time window during which elimination of this mosquito from (parts of) Africa is possible is rapidly closing.

Methods

Description of the study area

Dire Dawa, located 515km southeast of Addis Ababa (capital of Ethiopia) and 311km west of Djibouti, is a logistics hub for transportation of goods and cargo (Fig. 1b). Of its total population (445,050), 74% live in an urban area which is only 2.3% of the 1,288 km^2 Dire Dawa city administrative land (UN-HABITAT, 2008). The area has a warm and dry climate with low level of precipitation (annual average rainfall of 624mm), and an annual temperature ranging from 19°C to 32°C. Malaria incidence has historically been low (an annual parasite clinical incidence of <5 per 1,000 people between 2014 and 2019), with strong seasonality (August to November being the peak season), and sympatric *P. falciparum* and *P. vivax* infections.

Public health data collected through the district health information system 2 (DHIS2) was obtained to analyze the trend in malaria cases between 2015 to 2022. In the Ethiopian malaria case management guideline, microscopy is recommended for diagnosis at the health center level and above. Rapid diagnostic tests (RDTs) are recommended to be used only at the health post level by community health extension workers, in rural settings. In all of the facilities located in Dire Dawa, microscopy was used for diagnosis. The DHIS2 data does not capture cases detected at private health facilities. The recent "Global framework for the response to malaria in urban areas" by the World Health Organization (WHO)[55] states that "In some urban settings, the private sector is a major source of malaria diagnosis and treatment. However, it is poorly integrated into the surveillance system." To give context on how much is being managed by the private sector in Dire Dawa, we have collected four years data (January 2019 to May 2022) from 34 out 39 health facilities (both private and public) that are located within the city administration. This included two public and five private hospitals, 15 health centers (funded publicly), and 17 clinics (private). Some private clinics (n=5) refused to provide data or provided incomplete data. Goro health center and Dire Dawa University (DDU) students' clinic was selected for the current study based on the highest number of cases they reported prior to the start of the study (January – February 2022). In fact, together, the two health facilities reported 56% of the total cases in the city in 2022 (January – May). As in all public universities in Ethiopia, students live within campus with full and shared accommodation provided by the government. At DDU, an average of six students of the same sex and year of study share a dormitory on a three-story building that has an average of 67 dormitories. Routine healthcare service is provided in a university dedicated students' clinic.

Study design and procedure

To ascertain the effect of exposure to *An. stephensi* on malaria, we employed a case control study where identification of patients was done prospectively to capture co-occurrent characteristics in terms of exposure and risk factors. We recruited consecutive patients with criteria described below in a 1:2 ratio (one case: two controls) unmatched study design.

Recruitment of participants: Patients with (history within 48 hours) fever that presented at the two health facilities and tested positive for malaria by microscopy were recruited as index cases (index) from April to July 2022. Febrile patients who attended the same clinic and tested negative for malaria were recruited as controls within 72 hours of when the index was identified. The index and controls were followed to their homes and their household/dormitory members were tested for malaria and their households/dormitories were screened for *Anopheles* mosquitoes (larvae and adult). It is noticeable that although the study was unmatched due to the difficulty in recruiting matched controls in geographical proximity of the cases, their general characteristics were very similar. Detailed characteristics of study participants are presented in Extended Data Table 1.

Sample size: We planned an unmatched case: control ratio of approximately 1:2[56] with prospective case identification until the stopping rule was achieved. The choice of the ratio was based on a logistic regression model aimed to detect an odds ratio (OR) of at least 2, assuming an exposure of 20% in

controls at household level, where the exposure was defined as presence of *An. stephensi*. The power analysis was conducted in epiR package (R-cran software), and the stopping rule was set to a power of 70% for the study to be sufficiently powered to detect differences between the presence of malaria on *An. stephensi* exposure at household level. The controls were selected from the same population as the cases and post-stratification applied. Data from cases and controls were reviewed regularly, and final sample size was set to 290 with 101 cases and 189 controls. The recruitment of case-household and control-household members was done to include reactive case detection and improve the power of the study (as well as the OR minimum detection).

Blood samples collection: Finger prick blood samples (~0.5mL), collected in BD K_2EDTA Microtainer® tubes, were used to diagnose malaria using rapid diagnostic test (RDT) (ABBOTT BIOLINE Malaria Ag Pf/Pv HRP2/LDH, India) and microscopy, and to prepare dried blood spots (DBS) on 3MM Whatman filter paper (Whatman, Maidstone, UK). The remaining blood was separated into cell pellet and plasma. Slide films were confirmed by expert microscopists. Socio-demographic, epidemiological, intervention utilization, and history of travel and malaria were collected from all study participants.

Entomological surveys: Immature stages of *Anopheles* mosquitoes were surveyed within a 100-meter radius of the index and control houses/dormitories targeting both manmade water storage containers and natural habitats including riverbeds and stream edges. Each aquatic habitat was checked for 10 minutes from 9:00-11:00AM and 3:00-5:00PM for the presence of *Anopheles* mosquitoes' larvae or pupae aiming for ten dips per habitat (using a standard dipper with 350mL capacity). Characteristics of water holding containers (permanency of habitat, lid status, purpose, volume, presence of shade, type, turbidity, temperature, and water source) were recorded for each habitat (Supplementary Table 5). Adult mosquitoes were searched using Prokopack® aspirators for 10 minutes between 06:00-08:00AM indoor, outdoor, and in animal shelters located within the compound of the household or inside and outside the dormitories at the university (Supplementary Table 6). Mosquito surveys (immature and adult) were done within 48-72 hours of when the index/control was recruited.
Conventional adult mosquito collection methods such as CDC light traps and pyrethrum spray sheet have limited sensitivity for this invasive species mainly related with its unique resting behaviour[21]. To supplement the evidence generated from the case control study and examine the resting sites of the adult *Anopheles* mosquitoes in detail in the study area, additional adult mosquito surveys were done targeting potential resting sites including animal shelters and manholes within the study time and area. Informed by these preliminary findings, surveys were systematized in three fortnightly rounds during the study period. In the city, households with (n=15) and without (n=15) animal shelters were included (Supplementary Table 6). At DDU, two dormitory buildings which reported the highest number of malaria cases, and their surroundings were selected. Adult mosquitoes were surveyed indoor, outdoor, in animal shelters, overhead tanks, and manholes using Prokopack® aspirators for 10 minutes between 06:00-08:00AM. Animal shelters were not available at DDU. Adults caught mosquitoes (sorted based on their abdominal status) and those raised from aquatic stages, were morphologically identified to the species level[22] (Supplementary Table 7). *Anopheles* mosquitoes were individually preserved in tubes that contained silica gel desiccant in zipped bags and transported to the lab at the Armauer Hansen Research Institute (AHRI) for further analysis. The global positioning system (GPS) coordinates of the households and immature and adult mosquito collection sites were recorded using GARMIN handheld GPS navigator (GARMIN GPSMAP 64S, Taiwan).

Laboratory procedures

Nucleic acid extraction from whole blood and parasite quantification, and genotyping: Blood samples in EDTA tubes were used to extract genomic DNA using MagMAX™ magnetic bead-based technology DNA multi-sample kit on KingFisher Flex robotic extractor machine (Thermo Fisher Scientific™). 50µL of whole blood input was eluted in a 150µL low-salt elution buffer. Multiplex quantitative PCR (qPCR) targeting the 18S rRNA small subunit gene for *P. falciparum* and *P. vivax* was run using primer and probe sequences described by Hermsen[57] and Wampfler[58] using TaqMan Fast

Advanced Master Mix (Applied Biosystems). *P. falciparum* parasites were quantified using standard curves generated from a serial dilution of NF54 ring stage parasites (10^6 – 10^3 parasites/mL). For *P. vivax*, parasite quantification was done using plasmid constructs to infer copy numbers by running serial dilutions (10^7 – 10^3 copies/µL) of plasmids having the amplicon. Serial dilutions of the standard curves were generated in duplicate on each plate. Multiplexed amplicon sequencing was performed on qPCR positive samples with reagents and protocol as in Tessema et al.[59]. DNA was amplified for 15 or 20 cycles in multiplexed PCR, depending on parasitemia and ability to amplify, and for 15 cycles for indexing PCR. The primer pools used in this study comprised high-diversity microhaplotype targets (n=162), polymorphisms associated with drug resistance, and targets in and adjacent to *pfhrp2* and *pfhrp3* to assess for gene deletion (Primer pools 1A and 5 as described in protocols.io repository)[60]. Amplified libraries were sequenced in a NextSeq 2000 or a MiniSeq instrument using 150PE reads with 10% PhiX.

Nucleic acid extraction from mosquitoes, assessment of infectivity and blood-meal source and confirmation of morphological species identification: Wild-caught adult *Anopheles* mosquitoes were bisected on the second and third leg under a stereo microscope using sterile scalpels[61]. The head and thoraces were stored separately from the abdomen of the mosquitoes. The heads and thoraces of the mosquitoes were homogenized in 150µL molecular grade water that contains 0.2g Zirconium bead (1mm diameter) using a Mini-Bead Beater 96 (Bartlesville, OK, USA). Part of the homogenate (50µL) was used for nucleic acid extraction using Cetyl trimethyl ammonium bromide (CTAB)[62]; 100µL grinding buffer (0.5% w/v Casein - Sigma, 0.1N NaOH in 10mM PBS, pH 7.4, and 0.5% IGPAL CA-630) was added to the remaining that was used to screen samples for circumsporozoite multiplex bead assay (CS-MBA). Colony maintained *An. arabiensis* and *An. stephensi* mosquitoes fed on sugar solution and infectious blood of patients' blood in direct membrane feeding assays that were used as negative and positive controls, respectively, for the downstream assays were processed the same way. *Plasmodium* infected mosquitoes were used as positive controls along with four sugar-fed mosquitoes as negative controls in every extraction round (Fig. S4, Supplementary Table 8 and 9). Antibody-coupled magnetic beads and Biotinylated secondary antibodies obtained from the Center for Disease Control and Prevention (CDC), Division of Parasitic Diseases and Malaria, Entomology Branch, Atlanta, GA, USA were used to screen the presence of sporozoites in heads and thoraces as described before[63] using MagPix immunoanalyzer (Luminex Corp, CN-0269-01) after boiling the homogenate at 100°C on a thermocycler to avoid false positive signals. Samples with higher mean fluorescence intensity (MFI) signal than the negative controls plus 3 standard deviations and a representative set of mosquitoes that gave low signal were re-run to confirm the observations. Genomic DNA extracted from the head and thoraces of all mosquitoes was tested on a PCR that targeted 18S small ribosomal subunit gene as a confirmatory test. Only mosquito samples positive by the CSP based assays and 18S based PCR were considered infected.

Nucleic acid was extracted from the abdomen of fully engorged mosquitoes for blood meal source identification following the same procedure[62]. A multiplex PCR assay that amplifies the cytochrome b gene based on Kent and Norris 2005[64] was used for blood meal source analysis. We have introduced slight modifications to improve product size separation on gel electrophoresis. The multiplex of cow and dog was separately done from the multiplex of goat and human. The optimized PCR thermal cycler conditions were: 5 minutes at 95°C as an initial denaturation followed by 40 cycles of denaturation at 95°C for 60 seconds, annealing at 56°C for 60 seconds for cow and dog multiplex, and 62°C for goat and human multiplex, followed by an extension at 72°C for 60 seconds, and 1 cycle of the final extension at 72°C for 7 minutes.

Confirmation of the *Anopheles* morphological identification was done following a recently published protocol that targets the ITS2 gene[65]. *An. stephensi* diagnostic amplicon of 438 bp size was expected whilst a universal amplicon of varying sizes (>600 bp), depending upon the length of ITS2 in a particular species, was expected in this multiplex protocol. The universal amplicon was used to serve as an internal control to rule out PCR failure.

Data management

Data management: Study data collection tools (mobile application version 5.20.11) were prepared and managed using REDCap electronic data capture tools hosted at AHRI. CSV files exported from REDCap were analyzed using STATA 17 (StataCorp., TX, USA), RStudio v.2022.12.0.353 (Posit, 2023), QGIS v.3.22.16 (QGIS Development Team, 2023. QGIS Geographic Information System. Open-Source Geospatial Foundation Project), and GraphPad Prism 5.03 (Graph Pad Software Inc., CA, USA). All statistical analyses were performed in RStudio with packages lme4 (generalized linear mixed models) and dcifer.

Bioinformatic analysis: FASTQ files from multiplexed amplicon sequencing of *P. falciparum* were subjected to filtering, demultiplexing and allele inference using a Nextflow-based pipeline (https://github.com/EPPIcenter/mad4hatter). We used cut adapt to demultiplex reads for each locus based on the locus primer sequences (no mismatches or indels allowed), filter reads by length (100 base pairs) and quality (default NextSeq quality trimming). We used dada2 to infer variants and remove chimeras. Reads with a PHRED quality score of less than 5 were truncated. The leftmost base was trimmed and trimmed reads of less than 75 base pairs were filtered out. Default values were used for all other parameters. We then aligned alleles to their reference sequence and filtered out reads with low alignment. We masked homopolymers and tandem repeats to avoid false positives.

Genetic analysis: Pairwise relatedness analysis was performed on *P. falciparum* genotypes in diverse loci using Dcifer with default settings. Pairwise relatedness was only considered between samples where the lower 95% confidence interval of estimated relatedness was greater than 0.1. Point estimates of pairwise relatedness that satisfied this threshold were then binned into low, medium, and high relatedness at greater than 0.2, 0.5, and 0.9 respectively. Samples were then clustered based on pairwise relatedness. Drug resistance marker genotypes were extracted from loci of interest. Evidence of *pfhrp2* and *pfhrp3* deletions were identified from a drop in normalized coverage in amplicons within and surrounding *pfhrp2* and *pfhrp3*. Complexity of infection was estimated by taking the 0.97 quantile (5^{th} highest number) of observed alleles across loci to minimize the impact of false positives on estimates.

Epidemiological analysis: We used standard Case-Control analyses to examine the association between risk factors and malaria infection. It calculates point estimates and confidence intervals for the OR along with the significance level based on the chi squared test. Continuous variables were presented as median and interquartile range (IQR). Tests of association between two categorical variables were performed using contingency tables. The Mann-Kendall statistical test was used to test for monotonic (increasing or decreasing) trends of malaria cases using the secondary data obtained from the private and public health facilities at the city and DDU.

Spatial data analysis: As the dormitories within the university study site were located within a small area (20 buildings in 45,450m^2 area), clustering of prevalence data was assessed in the city only. The prevalence of malaria by RDT and/or microscopy was calculated for each household. Global and local Moran's *I* calculations were used to estimate the level of spatial autocorrelation within household prevalence data. The statistical strength of association for global Moran's *I* was calculated using Monte-Carlo methods based on 9,999 times permutations of the prevalence data. The Euclidean distance from the river to every site where adult or larval *An. stephensi* were located were calculated in meters.

Statistical analysis: To identify the association of *An. stephensi* and other risk factors for malaria positivity in Dire Dawa, we employed a multilevel logistic regression model with nested random effects to account for intra-class correlation (ICC) and quantify the variation in a parasite positive outcome that accounted for the household and case control group variances (nested random effects)[66]. After model selection with several model outcomes and distribution (Supplementary Table 12), the binomial model with outcome represented by malaria positivity (Positive/Negative) using RDT and/or microscopy best represented the relationship between malaria and risk factors (Supplementary Table 13)[67]. The employment of geographic unit's effects such as household and area setting (city vs university) enabled us to control for unknown variations by including them as random effects in the

model. In fact, individuals living in the same household may share exposures that can determine similarities in malaria transmission as well as in the larger setting (city versus university).

Let y_{ij} denote the malaria outcome of the i^{th} individual in the j^{th} household or cluster, identified by the (RDT and/or microscopy) with probability π_{ij}, where y_{ij} = 1 denotes the individual tested positive, while y_{ij} = 0 denotes the individual tested negative for malaria. A multilevel logistic regression model with random effects for the outcome y_{ij} is given by.

$$logit(\pi_{ij}) = \beta_{0j} + \beta X_{ij} + u_j$$

Where X_{ij} = (1, x_{1ij}, ..., x_{pij}) is vector of *p* explanatory variables or covariates measured on the *i* individual and on the *j* household (cluster), β vector of fixed regression coefficients or parameters and u_j is a random effect varying over household and case control.

Ethical statement. Study protocol was approved by the Institutional ethical review board of AHRI/ALERT ethics review committee (AF-10-015.1, PO/07/19).

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Author contributions: FGT, TB, CD, JET, SZ, EG, GA, HSA, JH, MY, SG, PM, SC, HT, HD, and TE conceived the study; TE, DG, MGB, GJ, TT, and FGT executed the study data and sample collection; MM, MA, WC, AE, ENV, AAD, LA, SWB, AS, and FGT run the laboratory experiments; TE, MM, LS, LAE, MGB, IB, MD, CD, BG, TB, and FGT analyzed the data; TE, DG, MM, LS, LAE, MGB, IB, MD, JH, MY, AS, SZ, JET, CD, BG, TB, and FGT drafted the manuscript. All authors read and approved the final version.

Competing interests: All authors declare that they do not have competing interests.

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Data and code availability: All the data used in the manuscript are available on dryad (linked with the ORCID: https://orcid.org/0000-0003-1931-1442). Sequence data are deposited on NCBI with the BioProject accession number PRJNA962166. Raw data of the study will be available in the future upon reasonable request. The R code used to run the analyses reported in this study can be found at https://github.com/legessealamerie/Stephensi_Outbreak_DireDawa_ETH_For_Publication and https://github.com/EPPIcenter/mad4hatter.

Disclaimer: The findings and conclusions in this paper are those of the authors and do not necessarily represent the official position of the U.S. Centers for Disease Control and Prevention.

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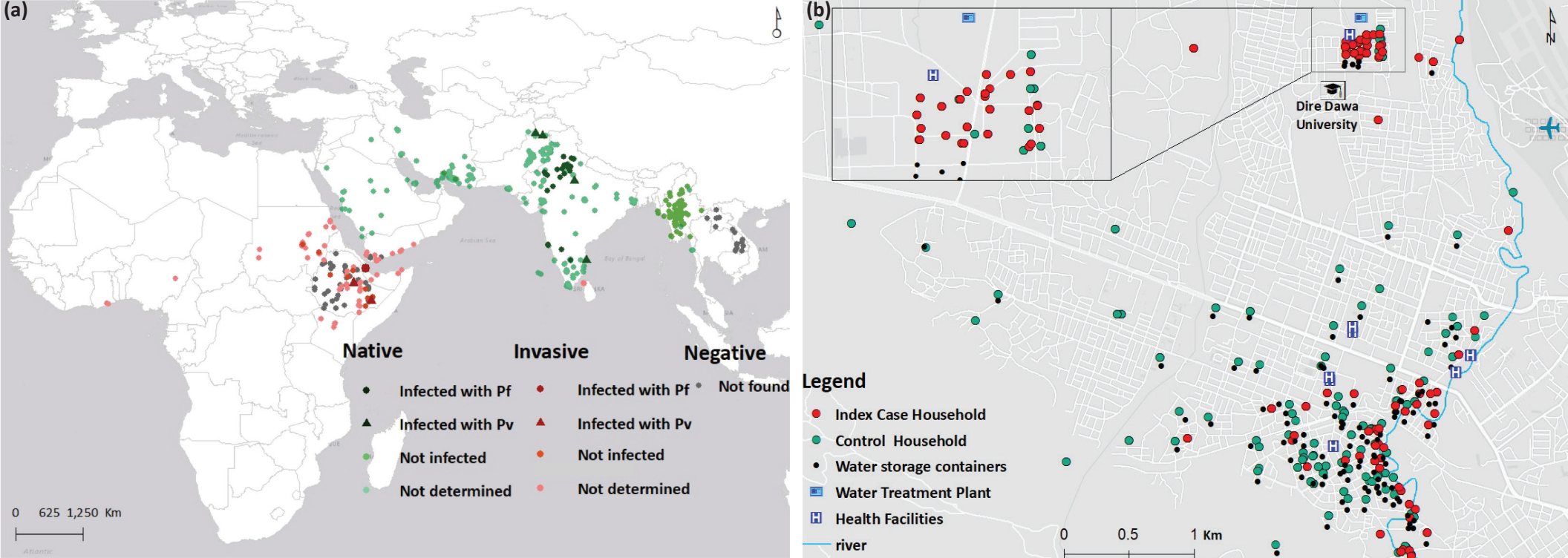
Extended Data Table 1: Summary statistics of individual and household level characteristics for members of the cases and controls in the two settings in Dire Dawa.

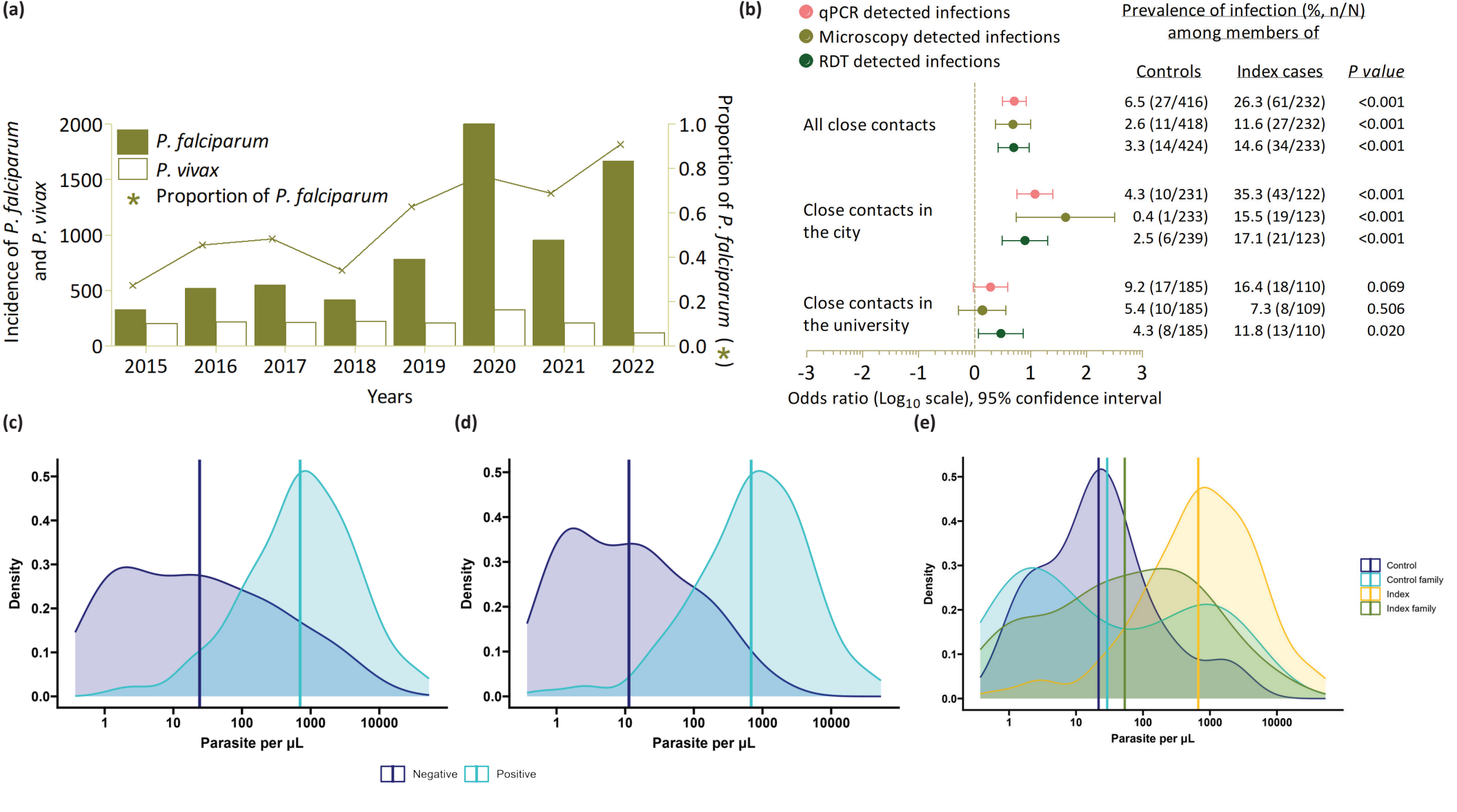
Characteristics	City		University	
	Cases	Controls	Cases	Controls
Individual characteristics, %(n/N)				
Number of participants (n)	173	350	163	266
Malaria incidence	42.1(72/173)	2.3(8/350)	42.9(70/163)	5.3(14/266)
Fever (axillary temperature ≥ 37.5°C)	0.9(1/110)	1.(2/186)	10.6(13/123)	1.7% 94/238)
Male sex	47.9(83/173)	45(157/349)	100(163/163)	100(266/266)
Age (years), median (interquartile range)	23(14,35)	22(11,35)	22(21,23)	21(20,22)
Travel history last month	9.3(16/173)	9.5(33/349)	9.8(16/163)	6.4(17/266)
Long lasting insecticide treated nets use	41.9(67/160)	50.9(169/332)	41.4(67/162)	41.7(105/252)
Use of aerosol insecticide sprays	12.3(19/155)	23.7(75/316)	0.0(0/160)	0.4(1/259)
Repellent use	25.3(39/152)	21.3(68/320)	0.0(0/163)	0.0(0/266)
Household characteristics, %(n/N)				
Number of households (n)	48	109	53	80
Larvae positivity around household	14.6(7/48)	4.6(5/109)	17.0(9/53)	5.0(4/80)
Adult *An. stephensi* presence (indoor/outdoor)	2.1(1/48)	0.0(0/109)	13.2(7/53)	10.0(8/80)
An. stephensi positivity (larvae and/or adult)	16.7(8/48)	4.6(5/109)	30.0(16/53)	15.0(12/80)
Livestock presence	31.9(15/47)	38.3(36/94)	0.0(0/53)	0.0(0/80)
Average distance to river (meter)	666.9	488.9	385.3	394.8
Average distance to artificial containers (meter)	688.7	661.5	68.5	65.2
Eave opened	4.7(2/43)	6.2(6/97)	54.9(28/51)	52.1(37/71)
Modal water body type	Stream	Stream	Pond	Pond
Water body presence in the neighborhood	47.9(23/48)	44.0(48/109)	96.2(51/53)	98.8(79/80)
Insecticidal residual spray in the last twelve month	2.3(1/44)	0.0(0/104)	26.9(14/52)	13.2(10/76)

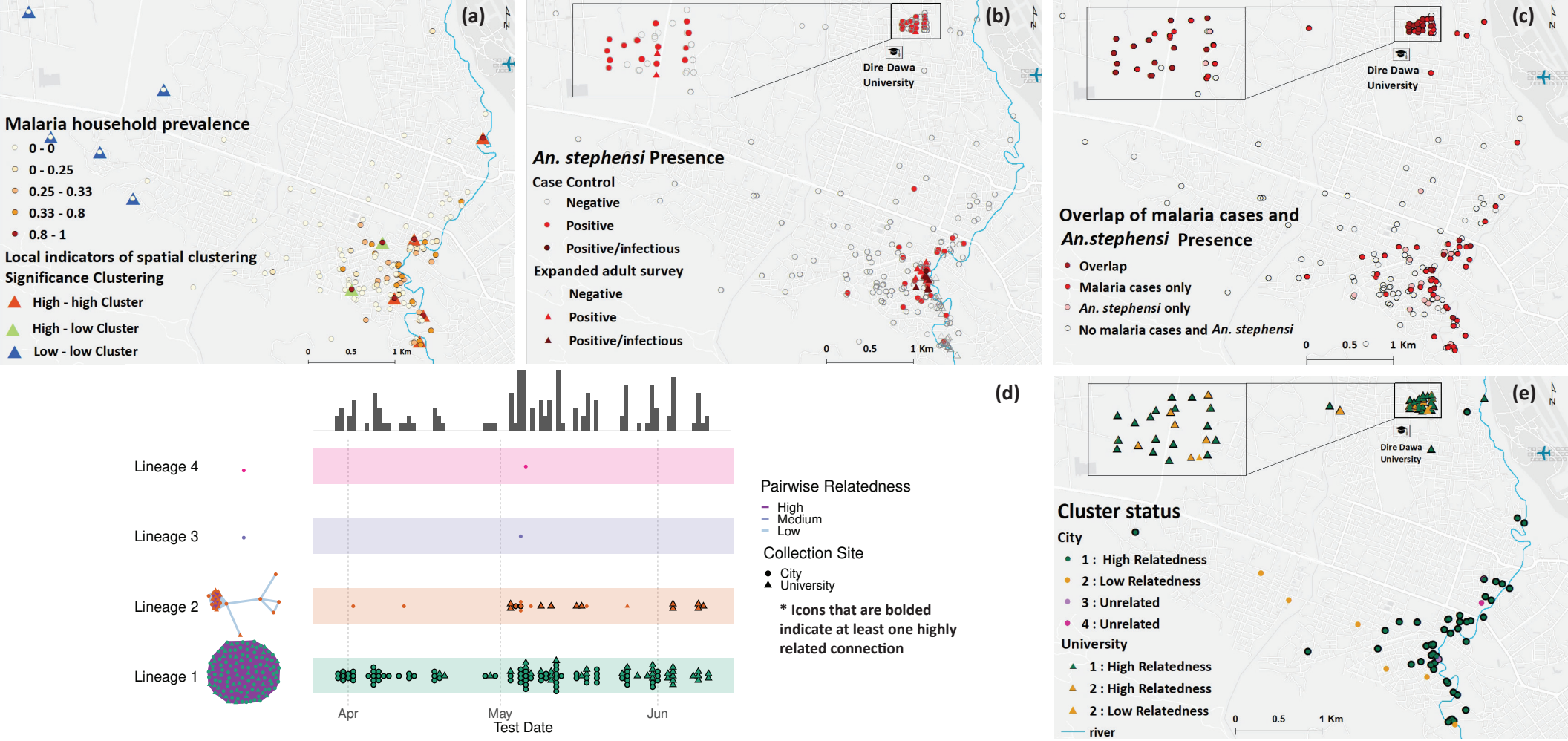
Extended Data Table 2: Case control analysis, risk factors associated with the index cases and their family members.

Exposure	OR (95% CI)	P value
Male sex	2.2(1.4,3.4)	<0.001
Natural water body presence	1.6(1.2,2.2)	0.002
Usage of aerosol insecticide spray	0.4(0.2,0.7)	0.001
An. stephensi larvae presence	5.0(2.8,9.4)	<0.001
An. stephensi adult presence	1.9(0.9,4.0)	0.068
An. stephensi larvae/adult presence	3.8(2.3,6.3)	<0.001
Long lasting insecticide net use	0.8(0.6,1.1)	0.125
Travel history in the last month	1.2(0.7,1.9)	0.464
Open eaves	0.8(0.6,1.1)	0.118
Livestock presence	1.2(0.9,1.7)	0.254
Distance from manmade container	0.7(0.5,0.9)	0.018

OR odds ratio; 95% CI 95% confidence interval


(a)
Native
Infected with Pf
Infected with Pv
Not infected
Not determined
Invasive
Infected with Pf
Infected with Pv
Not infected
Not determined
Negative
Not found
0 625 1,250 Km
(b)
Dire Dawa University
Legend
Index Case Household
Control Household
Water storage containers
Water Treatment Plant
Health Facilities
river
0 0.5 1 Km


(a)
Incidence of P. falciparum and P. vivax
2000
1500
1000
500
0
P. falciparum
P. vivax
Proportion of P. falciparum
1.0
0.8
0.6
0.4
0.2
0.0
Proportion of P. falciparum (*)
2015
2016
2017
2018
2019
2020
2021
2022
Years
(b)
qPCR detected infections
Microscopy detected infections
RDT detected infections
Prevalence of infection (%, n/N) among members of
Controls
Index cases
P value
All close contacts
6.5 (27/416)
26.3 (61/232)
<0.001
2.6 (11/418)
11.6 (27/232)
<0.001
3.3 (14/424)
14.6 (34/233)
<0.001
Close contacts in the city
4.3 (10/231)
35.3 (43/122)
<0.001
0.4 (1/233)
15.5 (19/123)
<0.001
2.5 (6/239)
17.1 (21/123)
<0.001
Close contacts in the university
9.2 (17/185)
16.4 (18/110)
0.069
5.4 (10/185)
7.3 (8/109)
0.506
4.3 (8/185)
11.8 (13/110)
0.020
-3
-2
-1
0
1
2
3
Odds ratio (Log10 scale), 95% confidence interval
(c)
(d)
(e)
Density
0.5
0.4
0.3
0.2
0.1
0.0
1
10
100
1000
10000
Parasite per µL
Negative
Positive
Control
Control family
Index
Index family


(a)
Malaria household prevalence
0 - 0
0 - 0.25
0.25 - 0.33
0.33 - 0.8
0.8 - 1
Local indicators of spatial clustering
Significance Clustering
High - high Cluster
High - low Cluster
Low - low Cluster
0 0.5 1 Km
(b)
Dire Dawa University
An. stephensi Presence
Case Control
Negative
Positive
Positive/infectious
Expanded adult survey
Negative
Positive
Positive/infectious
0 0.5 1 Km
(c)
Dire Dawa University
Overlap of malaria cases and An.stephensi Presence
Overlap
Malaria cases only
An. stephensi only
No malaria cases and An. stephensi
0 0.5 1 Km
(d)
Lineage 4
Lineage 3
Lineage 2
Lineage 1
Apr
May
Jun
Test Date
Pairwise Relatedness
High
Medium
Low
Collection Site
City
University
* Icons that are bolded indicate at least one highly related connection
(e)
Dire Dawa University
Cluster status
City
1 : High Relatedness
2 : Low Relatedness
3 : Unrelated
4 : Unrelated
University
1 : High Relatedness
2 : High Relatedness
2 : Low Relatedness
river
0 0.5 1 Km