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Evidence for a role of *Anopheles stephensi* in the spread of drug and diagnosis-resistant malaria in Africa

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Abstract

Anopheles stephensi, an Asian malaria vector, continues to expand across Africa. The vector is now firmly established in urban settings in the Horn of Africa. Its presence in areas where malaria resurged suggested a possible role in causing malaria outbreaks. Using a prospective case control design, we investigated the role of *An. stephensi* in transmission following a malaria outbreak in Dire Dawa, Ethiopia in April-July 2022. Screening contacts of malaria patients and febrile controls revealed spatial clustering of *P. falciparum* infections around malaria patients in strong association with *An. stephensi* presence in the household vicinity. *Plasmodium* sporozoites were detected in these mosquitoes. This outbreak involved clonal propagation of parasites with molecular signatures of artemisinin and diagnostic resistance. This study provides the strongest evidence to date for a role of *An. stephensi* in

driving an urban malaria outbreak in Africa, highlighting the major public health threat this fast-spreading mosquito poses.

Introduction

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* has spread across the Horn of Africa; its range now includes 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 the Horn of Africa, the vector was found firmly established[18] and abundantly present in manmade aquatic habitats in the driest months of the year, when endemic vectors like *An. arabiensis* are largely absent, demonstrating how well-adapted the mosquito is to perennial persistence and urban ecology. This poses a risk of year-round malaria transmission. In recognition of the potentially devastating consequences of *An. stephensi* advancing across Africa, the World Health Organization (WHO) urgently requested more data on its distribution and released a strategy to mitigate its spread[19].

In addition to the invasive *An. stephensi* and widespread high prevalence of insecticide resistance, the Horn of Africa region is disproportionately affected by other emerging biological threats for malaria control including the emergence of *P. falciparum* parasites with drug resistance (Uganda[20], Rwanda[21], Eritrea[22]) and histidine rich protein 2 (*pfhrp2*) and *pfhrp3* gene deletions (Ethiopia[23], Eritrea[24], Djibouti[25]) that could compromise the utility of widely used rapid diagnostic tests (RDT). Because of its abundant and species-specific expression by *P. falciparum* parasites, HRP2-based RDTs are commonly used for the diagnosis of *P. falciparum*. Recent reports of expansion of parasites with *pfhrp2/3* gene deletions and drug resistance together with a highly efficient invasive mosquito in the region threaten the major gains documented in recent decades.

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)[9,18] and a resurgence of malaria was reported in Djibouti following its detection in 2012[26], 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).

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 an overall statistically significant trend of increasing number of malaria positive cases between 2019 and 2022 (Mann-Kendall statistical test $\tau = 0.42$, $p<0.001$). A 12-fold increase was observed (Extended Data 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). Similar increasing trend was observed using the district health information system 2 (DHIS2) data (Fig. 2a and Fig. S1). 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 confirmed febrile malaria 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 Table 1. The index cases and controls were febrile either at the time of recruitment or within 48 hours (self-reported) prior to attending the clinics. The family members/dormmates were recruited irrespective of symptoms. Fever was detected in a minority of recruited family/dormitory members of the controls (1.4%, 6/424) and index cases (6.0%, 14/233) (Extended Data Table 2). The responsive case control study unit was the household/dormitory; no plausible risk factors were defined *a priori* and neither a sex/gender nor *Plasmodium* species stratification were 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 malaria 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* with the exception of 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 which were all *P. falciparum* 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).

Mosquito exposure and infection prevalence in malaria contacts

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 3). In entomological surveillance, all households and dormitories were surveyed for adult mosquitoes (indoors, outdoors, and in animal shelters if present) and immature stages of *Anopheles* in waterbodies that were present within a 100-meter radius. 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 three times 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; Extended Data Table 4) with different proportions of HRP2-based RDT negative infections in the city (10.3%, 7/68) and the university (39.7%, 27/68). HRP2-based RDTs are the most commonly used RDTs

for the diagnosis of *P. falciparum* in the area. Recent reports of expansion of parasites with *pfhrp2/3* gene deletion threaten the important role these RDTs have in diagnosing malaria. 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 (Extended Data Table 5). Parasite densities were higher in the RDT positive infections (geometric mean 702 parasites/µL, 95% CI 495-997) than RDT negative infections (24, 14-42, p<0.001). Similarly, parasite densities were higher in microscopy positive infections (683 parasites/µL, 488-956) than in microscopy negative infections (11, 7-19, p<0.001) (Extended Data Table 5). The median parasite density by qPCR for infections that were RDT negative, but microscopy positive was 357,236 parasites/µL (interquartile range [IQR] 51,440-1,790,966, n=31), strongly suggestive of *pfhrp2/3* gene deletion in these infections. Parasite density distributions were not different between university students (geometric mean 158 parasites/µL, 95% CI 94-265) and city residents (163 parasites/µL, 91-291, p=0.132, 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, 7-67, p<0.001), malaria-infected control family members (29, 9-97, p=0.005), and malaria-infected index family members (53, 27-107, p<0.001).

An. stephensi dominates and carries _P. falciparum_ sporozoites

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) of which the majority were metal and plastic barrels and Jerrycans and was the predominant species detected at the stream edges (57% larvae, 160/280; Extended Data Table 6). 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; Extended Data Table 7). All mosquitoes that were morphologically identified as *An. stephensi* and tested molecularly (n=90) were confirmed to be this species except four for which the ITS2 based PCR experiment failed (Fig. S3) – which might be the result of loss of genetic material during extraction. Fully engorged adult caught *An. stephensi* (195/599) and *An. gambiae* (5/16) mosquitoes (Extended Data Table 8) were tested for bloodmeal sources in a multiplex PCR assay that amplifies the cytochrome b gene: 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 fully engorged (n=199) *An. stephensi* mosquitoes that were tested in this study. *P. falciparum* sporozoites, indicative of transmission upon natural blood-feeding, based on sporozoite and PCR-based detection were confirmed only in *An. stephensi* (0.5%, 3/599).

Overlapping clusters of _P. falciparum_ and _An. stephensi_ abundance

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 (detectable by microscopy and/or RDT) 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 (Fig. 3b). In the city, the clusters of households with higher infection prevalence were all situated within 200-meter of Butiji river.

An. stephensi presence is strongly linked with _P. falciparum_ positivity

We next evaluated risk factors for being infected with *P. falciparum* (Table 2). 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.

Parasites with signatures of artemisinin and diagnostic resistance

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 3). 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, 2022, 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 susceptibility to artemisinins in Eritrea[22] – along with evidence of *P. falciparum pfhrp2* and *pfhrp3* gene deletions – which are associated with false negativity of HRP2-based RDTs. 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 to be *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 (Extended Data Table 9). 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.

Discussion

Our findings raise 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*[18]. In 2014, no *Anopheles* developmental stages were detected in containers in Dire Dawa[27], 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[26].

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[28], other African countries[29] and Brazil[30], and is commonly described in South East Asia[31]. 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[32]. In our setting, chewing khat outdoors is done predominately by men[33] 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[34]. The recently described longer infection duration in males compared to females[35] 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[26]. 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*[36,37]. There is a large and increasing body of evidence (including our own work from Ethiopia)[38,39] 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[39]. 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 previously in *An. stephensi* in Dire Dawa and other areas in Ethiopia[18] as well as sporozoite rates in *An. arabiensis,* a native malaria vector in Ethiopia[40]. 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[41]. 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%[42]. Continuous entomological and clinical surveillance would provide further evidence if this was the case in Dire Dawa. In contrast, asymptomatic *P. vivax* infections have typically too low parasite densities to infect mosquitoes[38,43]. Since *P. vivax* sporozoites have been detected in *An. stephensi* mosquitoes previously from the same setting[18], 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[44]. Historical transmission levels influence the size of the submicroscopic reservoir through

acquired immunity[45]. As Dire Dawa was previously endemic[46] 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[38].

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 study identified this as a variant linked with partial drug resistance in Eritrea[22]. Following the first report in 2016 from northwest Ethiopia[47], parasites carrying the R622I variant are reported to be expanding in the same setting[48], more widely in the country[49] and elsewhere in the Horn of Africa[50]. In addition to evidence for artemisinin-resistant parasites, mutations conferring chloroquine and anti-folate resistance were common in the parasite population responsible for this outbreak. Similarly, *pfhrp2* and *pfhrp3* gene deletions with phenotypic evidence of RDT negativity were detected in our study. Despite its first report from Peru[51], the Horn of Africa (Ethiopia[23], Eritrea[24], Sudan[52], South Sudan[53], and Djibouti[25]) is disproportionately affected by diagnostic challenges of infections 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[49]. To date, no evidence exists if the drug resistance conferring *kelch13* mutation (R622I) and *pfhrp2/3* gene deletions have 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 sensitivity of methodologies for sampling exophagic adult mosquitoes may have resulted in an underestimation of mosquito exposure and reduced 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[18,54], the repellent effect of the aerosol sprays is one explanation for the protective association observed in this study[55]. Most sprays contain repellents such as DEET (N,N-diethyl-meta-toluamide) or permethrin. Permethrin and DEET have strong repellent effects on both *Plasmodium*-infected and uninfected *An. stephensi* mosquitoes[55].

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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401 **Table 1: Summary statistics of individual and household level characteristics for members of the**
402 **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^{o}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)
Wood smoke the house the previous night	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 within 100meter radius 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 within 100meter radius around household	47.9(23/48)	44.0(48/109)	96.2(51/53)	98.8(79/80)
Insecticidal residual spray in the last twelve months	2.3(1/44)	0.0(0/104)	26.9(14/52)	13.2(10/76)

403 **Counts over totals for each characteristic are shown in brackets.**

Table 2: 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 3: 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. Proportions are shown within brackets.

Figure Legends

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.

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). Odds ratios were obtained from univariate logistic regression with diagnostic test results as outcome and site as predictor. Univariate logistic models were fitted for each diagnostic test. Odds ratios are shown on a $\log_{10}$ scale (X-axis) together with their 95% confidence interval bars and respective *p* values (estimated from a Wald test). 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. Parasite density per µL distributions and their respective averages 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).

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 one-sided local Anselin Moran's *I* test (pseudo p-values calculated from 9,999 random permutations): 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).

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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.

We obtained public health data, collected through the district health information system 2 (DHIS2), 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)[4] 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. Study protocol was approved by the Institutional Ethical Review Board of AHRI/ALERT ethics review committee (AF-10-015.1, PO/07/19). We obtained informed written consent from all participants and guardians or parents for minors.

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. We recruited febrile patients who attended the same clinic and tested negative for malaria 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). Household/dormitory members of cases and controls who were willing to participate in the reactive case detection were included irrespective of their symptoms. Households were surveyed for mosquitoes when the head of the household and members of the dormitory gave consent to allow the study team to use mosquito collection methods in their houses/dormitories. Families of cases or controls who were not available within 72 hours of recruitment of the cases or controls irrespective of their symptoms were excluded as well as individuals or family members who were unwilling/refused

to give informed written consent. 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 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 case:control 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).

Data collection: Data on the socio-demographic, epidemiological, intervention, and travel history were collected verbally using pre-tested questionnaires which were uploaded to mobile tablets using REDCap tools. The entomological survey data and intervention availability were scored by the study data collectors. Malaria case incidence data (from January 2019 to May 2022) were collected from the records of both private and public health facilities (n=34).

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: We surveyed immature stages of *Anopheles* mosquitoes 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. We checked each aquatic habitat 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 (Extended Data Table 6). We searched adult mosquitoes 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 (Extended Data Table 7). 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 (Extended Data Table 7). 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] (Extended Data Table 8). *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: Infection detection in wild caught mosquitoes is commonly based on an ELISA based protocol that targets circumsporozoite protein (CSP) which is expressed on the surface of *Plasmodium* sporozoites. Low level expression of CSP at stages of sporogony before the parasites migrate to the salivary gland might interfere with signal detected[61]. Several studies have reported false positive results when targeting CSP especially in zoophilic mosquitoes[62,63]. The false positive results could lead to an overestimation of mosquito infection rates. To achieve a conservative estimate of mosquito infection rates, we implemented stringent steps as indicated below:

i). **Bisected mosquitoes**: We observed previously[61] that a signal detected from an earlier stage of sporogony might interfere with interpretation of sporozoite detection, likely causing false positive results. We bisected the mosquitoes anterior to the thorax-abdomen junction under a stereo microscope before processing them for infection detection[64]. The head and thoraces were processed and stored separately from the abdomen of the mosquitoes. We only used the head and thorax part for infection detection following homogenization in a robust semi-high-throughput mini-bead beater protocol we developed previously[65]. 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 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.

ii). **Circumsporozoite bead-based assay**: We adopted the most advanced (highly sensitive) bead-based assay for infection detection in mosquitoes[66] by targeting CSP. Antibody-coupled magnetic beads and biotinylated secondary antibodies were obtained from the Center for Disease Control and Prevention (CDC), Division of Parasitic Diseases and Malaria, Entomology Branch, Atlanta, GA, USA and

implemented as described before[7] and were run using MagPix immunoanalyzer (Luminex Corp, CN-0269-01).

iii). **Quality control to reduce cross-reactivity**: The bead-based assay we adopted may eliminate false negatives due to lower limit of detection than previous ELISA based assays[66] but also brings a challenge of enhanced detection of cross-reacting proteins. To reduce this chance, mosquito homogenate was boiled at 100°C before processing to eliminate false positives that may be caused by heat-unstable cross-reactive proteins to strengthen the validity of the results. To ascertain this specificity issue, we have included colony-maintained *An. arabiensis* and *An. stephensi* mosquitoes fed on sugar solution and patients' blood in direct membrane feeding assays (had infection status determined morphologically in the same mosquito batches) that were used as negative and positive controls, respectively. *Plasmodium* infected mosquitoes were used as positive controls along with sugar-fed mosquitoes as negative controls in every extraction round (Fig. S3, Table S1 and S2).

iv). **Retesting and confirmatory 18S based species specific PCR**: 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[67]. A multiplex PCR assay that amplifies the cytochrome b gene based on Kent and Norris 2005[68] 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[69]. *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 (Fig. S4). 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).

Description of study variables: We collected the following variables in this study:

- **Socio demographic:** sex, age, educational level, occupation
- **Household characteristics:** main materials used for building the household, fuel source, water source and presence of water bodies near the household/dormitory, presence of livestock.
- **Intervention:** presence, number, and condition of bed nets, use of bed nets, use of smoke repellents or aerosol mosquito spray, history of insecticide residual spray (IRS)
- **Diagnosis and treatment:** malaria test result by rapid diagnostic test (RDT) and microscopy, temperature, presence of symptoms and treatment history, pregnancy status

- **Human behavior**: travel history, health seeking behavior, sleeping and waking time, sleeping place
- **Entomological survey:** mosquito collection method and time of collection, mosquito species detected and density, *Anopheles* species detected and density, abdominal status of mosquitoes detected, type of aquatic habitat near the household/dormitory, type and characteristics of water sources detected within 100-meter radius around the household/dormitory.

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 Chi-squared test on 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 with heterogeneous household and case control group variances (nested random effects)[70]. The covariates included for the multi-level logistic regression analysis with random effect are listed in detail in Table S3. Having more than 30+ potential covariates associated to malaria, more than one billion models for exhaustive best model searching (excluding interactions between covariates), we reduced the number of covariates to a manageable size by considering univariate generalized mixed models (with case index as random effect instead of setting which were not contributing to the differences in malaria positivity for cases and controls) and considering only the covariates with *p*-value lower than 0.3 within these models (Table S3). The decision to use case/control as random effect instead of fixed

effect came from preliminary analysis that considered the best candidate(s) for random effects. Variable selection was performed by testing 2000+ binomial logistic mixed models (number of tested models depending on initial screening). During the initial screening, a candidate variable was selected if its *p-value*, obtained from a Wald test applied to the variable's estimated coefficient in logistic regression, was lower than 0.3. The models were ranked based on their Akaike Information Criteria (AIC) and the Bayes information criteria (BIC) values, with the top model being the one with the lowest AIC value[71]. Variable selection was repeated for three different response variables: Model 1 with response RDT/Microscopy, Model 2 with response RDT/Microscopy/qPCR, and finally, Model 3 with response qPCR. As a result, only five of the 12 factors assessed for individual and household characteristics (sex, age, *An. stephensi* larvae and/or adult presence, atural waterbody existence, and use of aerosol insecticide spray) were included for the final model (Table S4). We also explored interactions between gender, age and site.

After model selection with several model outcomes and distribution (Table S4), 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 (Table S4)[72]. In this model, 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.

Materials and Correspondence: correspondence and material requests can be addressed to Fitsum G Tadesse, Malaria and NTD directorate, Armauer Hansen Research Institute (AHRI), Ministry of Health, Jimma Road, PO Box 1005, Addis Ababa, Ethiopia. Email: fitsum.tadesse@ahri.gov.et; Telephone: +251912627540

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 request following signing of data sharing agreement, abiding to institutional and international data sharing guidelines. 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.

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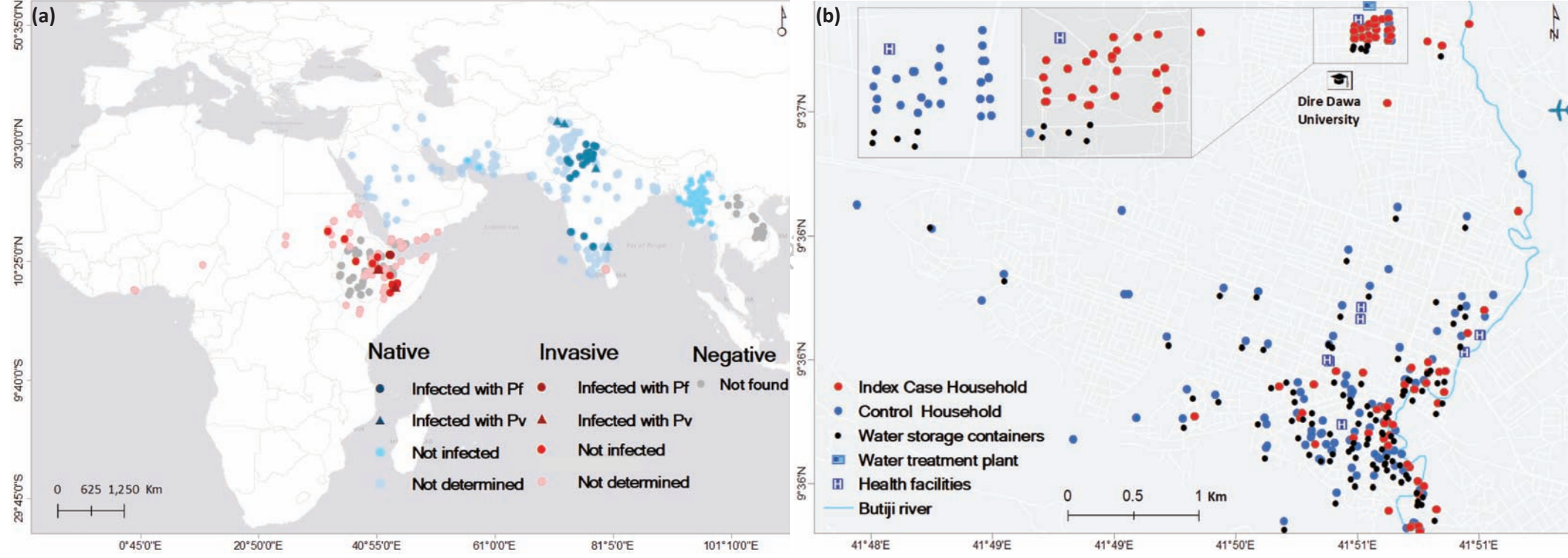
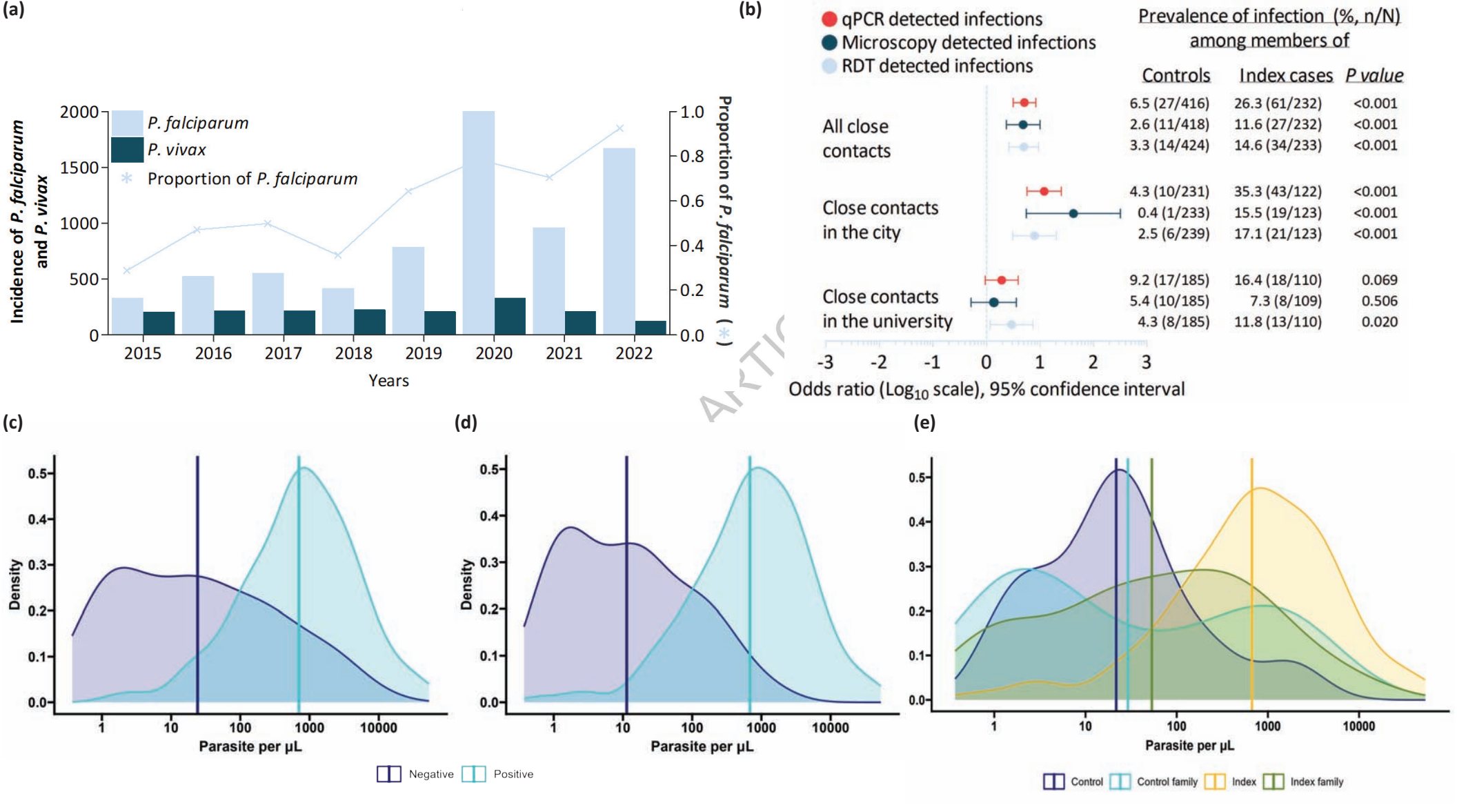
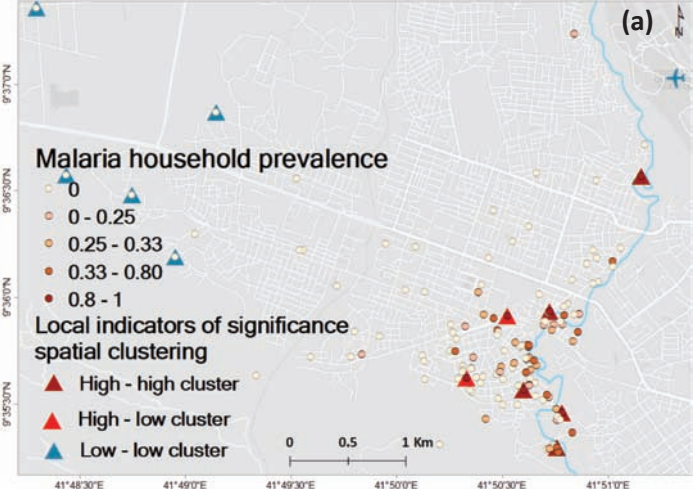
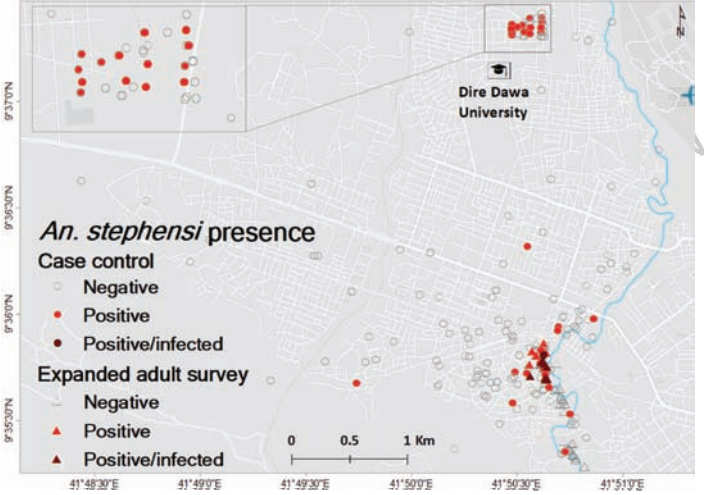
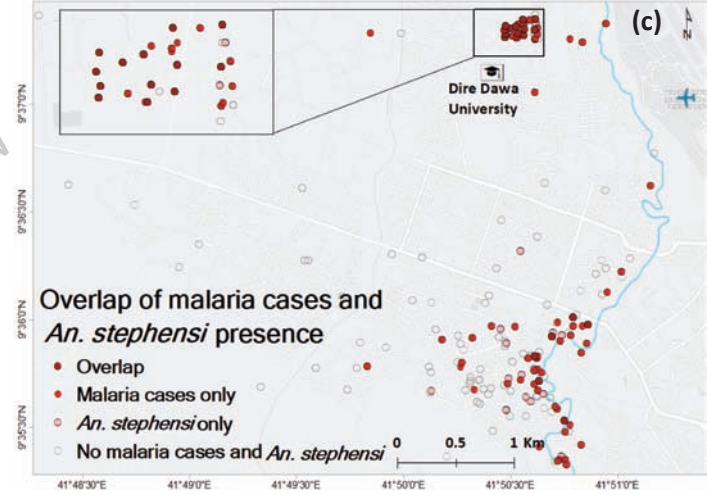

(a)
Native
Infected with Pf
Infected with Pv
Not infected
Not determined
Invasive
Infected with Pf
Infected with Pv
Not infected
Not determined
Negative
Not found
0 625 1,250 Km
50°35'0"N
30°30'0"N
10°25'0"N
9°40'0"S
29°45'0"S
0°45'0"E
20°50'0"E
40°55'0"E
61°0'0"E
81°5'0"E
101°10'0"E
(b)
Dire Dawa University
Index Case Household
Control Household
Water storage containers
Water treatment plant
Health facilities
Butiji river
0 0.5 1 Km
9°37'N
9°36'N
9°36'N
9°36'N
41°48'E
41°49'E
41°49'E
41°50'E
41°51'E
41°51'E

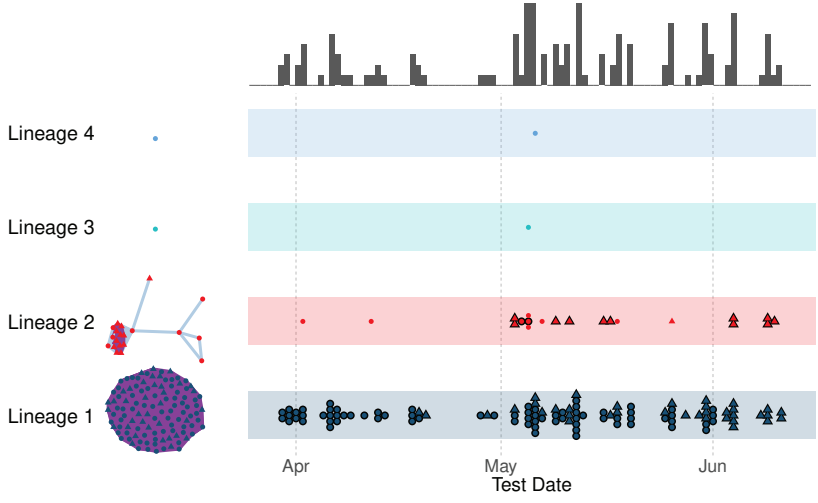

(a)
Incidence of P. falciparum and P. vivax
2000
1500
1000
500
0
P. falciparum
P. vivax
Proportion of P. falciparum
1.0
0.8
0.6
0.4
0.2
0.0
Proportion of P. falciparum ( )
2015
2016
2017
2018
2019
2020
2021
2022
Years
(b)
qPCR detected infections
Microscopy detected infections
RDT detected infections
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)
Density
0.5
0.4
0.3
0.2
0.1
0.0
1
10
100
1000
10000
Parasite per µL
Negative
Positive
(d)
Density
0.5
0.4
0.3
0.2
0.1
0.0
1
10
100
1000
10000
Parasite per µL
(e)
0.5
0.4
0.3
0.2
0.1
0.0
1
10
100
1000
10000
Parasite per µL
Control
Control family
Index
Index family







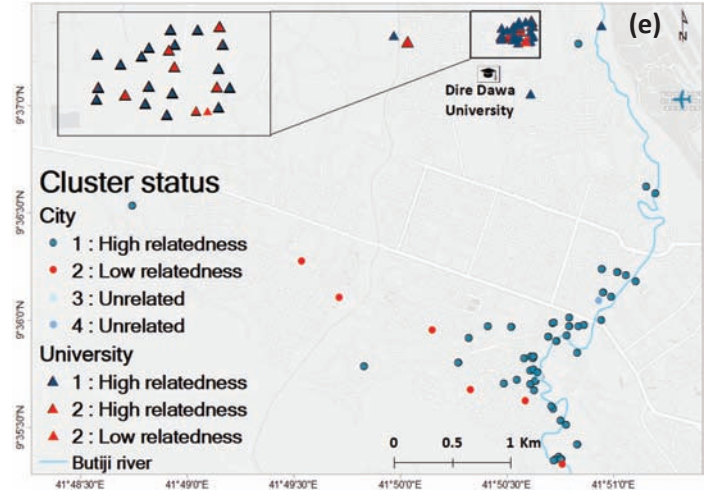
(d)



Pairwise Relatedness
- High
- Medium
- Low

Collection Site
- City
- University

*** Icons that are bolded indicate at least one highly related connection**



Months	Year, % (total positive/total tested)			
	2019	2020	2021	2022
January	2.3(43/1884)	5.1(153/3022)	6.5(73/1131)	9.9(371/3735)
February	3.0(45/1507)	5.3(152/2843)	6.3(66/1044)	12.5(432/3454)
March	3.5(52/1476)	7.0(187/2676)	7.2(75/1048)	9.6(368/3850)
April	2.3(31/1374)	11.6(190/1642)	6.4(52/809)	16.6(646/3902)
May	2.4(34/1442)	11.8(262/2212)	6 .0(54/895)	12.2(608/5003)
June	3.6(74/2035)	6.1(122/1995)	9.0(131/1449)	-
July	3.9(108/2747)	6.5(164/2520)	8.9(131/1474)	-
August	3.1(123/3988)	5.9(140/2382)	9.8(176/1794)	-
September	2.3(82/3574)	6.4(190/2948)	7.8(137/1756)	-
October	6.3(165/2638)	5.4(160/2948)	10.6(183/1732)	-
November	6.1(171/2790)	3.4(92/2671)	10.9(186/1712)	-
December	5.5(211/3834)	3.7(110/2992)	12.3(253/2052)	-
Total	3.9(1139/29289)	6.2(1922/30851)	9.0(1517/16896)	12.2(2425/19944)

Site	Control family	Index family	Control	Index
University	1.7%, 4/238	10.6%, 13/123	22.0%, 24/109	45.8%, 22/48
City	1.1%, 2/186	0.9%, 1/110	8.8%, 7/80	39.6%, 21/53

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

Study Site	Test Characteristic	Microscopy as a reference	qPCR as a reference	
		RDT	RDT	Microscopy
City	True Positive (n)	61	66	66
	False Positive (n)	9	4	2
	True Negative (n)	433	396	398
	False Negative (n)	7	39	39
	Sensitivity (95% CI)	89.7 (87.1-92.3)	62.7 (58.6-67.1)	62.9 (58.6-67.1)
	Specificity (95% CI)	97.9 (96.7-99.2)	99.0 (98.1-99.9)	99.5 (98.9-100.0)
	Accuracy	96.7 (494/510)	91.5 (432/505)	91.9 (464/505)
University	True Positive (n)	41	47	63
	False Positive (n)	15	10	5
	True Negative (n)	342	322	325
	False Negative (n)	27	49	32
	Sensitivity (95% CI)	60.3 (55.6-64.9)	48.9 (44.2-53.7)	66.3 (61.8-70.8)
	Specificity (95% CI)	95.8 (93.9-97.7)	96.9 (95.4-98.6)	98.5 (97.3-99.7)
	Accuracy	90.1 (383/425)	86.2 (369/428)	91.2 (388/425)
All	True Positive (n)	102	113	129
	False Positive (n)	24	14	7
	True Negative (n)	775	718	723
	False Negative (n)	34	88	71
	Sensitivity (95% CI)	75.0 (72.2-77.8)	56.2 (53.0-59.4)	64.5 (61.4-67.6)
	Specificity (95% CI)	97.0 (95.9-98.1)	98.1 (97.2-99.0)	99.0 (98.4-99.7)
	Accuracy, % (n/N)	93.8 (877/935)	89.1 (831/933)	91.6 (852/930)

Variables	Category	Geometric mean	95%CI	P value
Case category	Control (n=14)	21.7	(6.9-68.6)	-
	Control family (n=27)	29.2	(8.8-96.8)	
	Index (n=99)	669.0	(442.0-1012.0)	
	Index family (n=61)	53.4	(26.7-107.0)	
Study site	City (n=105)	163.0	(90.9-291.0)	0.132
	University (n=96)	158.0	(94.3-265.0)	
RDT	Negative (n=88)	24.1	(13.8-42.2)	0.001
	Positive (n=113)	702.0	(495.0-997.0)	
Microscopy	Negative (n=71)	11.3	(6.9-18.7)	0.001
	Positive (n=129)	683.0	(488.0-956.0)	

Habitat type	Habitats positive for *Anopheles*, n/N	Number of larvae collected			
		An. stephensi	*An. gambiae* s.l.	*An. pretoriensis*	*An. turkhudi*
Cemented cistern	1/4	23	0	0	0
Metal barrel	1/86	6	0	0	0
Plastic drum	1/27	16	0	0	0
Plastic barrel	12/238	230	0	0	0
Stream (Butiji river)	9/17	160	67	42	11
Ditch (Water leak)	1/1	38	0	0	0
Jerrycan	0/504	0	0	0	0
Water treatment plant	1/1	101	0	0	0
Total		574	67	42	11

Place of collection	Resting site positivity, %(n/N)	Survey days	Number of adult mosquitoes collected							
			An. stephensi	*An. gambiae*	*An. turkhudi*	*An. pretoriensis*	*An. funestus*	Un-identified	*Aedes aegypti*	Culex
City indoor	0.6(1/157)	54	1	0	0	0	0	0	14	0
City outdoor	0.6(1/157)	54	0	0	1	0	0	0	15	0
DDU indoor	7.5(10/133)	30	17	0	0	0	0	0	0	0
DDU outdoor	3.8(5/133)	30	12	0	0	0	0	0	0	0
Animal shelter	100(9/9)	5	249	3	0	0	0	0	0	0
Manholes/Ditch	100(6/6)	4	28	13	0	1	0	0	0	0
Empty water storage tankers	100(4/4)	3	30	0	0	0	0	0	0	0
Indoor (without animal shelter)	0.0(0/15)	4	0	0	0	0	0	0	0	13
Outdoor (without animal shelter)	6.7(1/15)	4	0	0	0	0	1	0	0	3
Indoor + Animal Shelter	6.7(1/15)	6	4	0	0	0	0	0	1	5
Outdoor + Animal Shelter	60.0(9/15)	6	223	0	0	0	0	0	2	1
Indoor***	4.2(13/313)	9	12	0	0	0	0	1	0	0
Outdoor	100(6/6)	3	23	0	0	0	0	0	0	0
Total			599	16	1	1	1	1	32	22

Place of collection	Resting site positivity, %(n/N)	Survey days	Number of adult mosquitoes collected							
			An. stephensi	*An. gambiae*	*An. turkhudi*	*An. pretoriensis*	*An. funestus*	Un-identified	*Aedes aegypti*	Culex
City indoor	0.6(1/157)	54	1	0	0	0	0	0	14	0
City outdoor	0.6(1/157)	54	0	0	1	0	0	0	15	0
DDU indoor	7.5(10/133)	30	17	0	0	0	0	0	0	0
DDU outdoor	3.8(5/133)	30	12	0	0	0	0	0	0	0
Animal shelter	100(9/9)	5	249	3	0	0	0	0	0	0
Manholes/Ditch	100(6/6)	4	28	13	0	1	0	0	0	0
Empty water storage tankers	100(4/4)	3	30	0	0	0	0	0	0	0
Indoor (without animal shelter)	0.0(0/15)	4	0	0	0	0	0	0	0	13
Outdoor (without animal shelter)	6.7(1/15)	4	0	0	0	0	1	0	0	3
Indoor + Animal Shelter	6.7(1/15)	6	4	0	0	0	0	0	1	5
Outdoor + Animal Shelter	60.0(9/15)	6	223	0	0	0	0	0	2	1
Indoor***	4.2(13/313)	9	12	0	0	0	0	1	0	0
Outdoor	100(6/6)	3	23	0	0	0	0	0	0	0
Total			599	16	1	1	1	1	32	22

Variables	Chi square (X^2)	P value
LLINs utilization	0.45	0.52
IRS	1.26	0.26
Repellent	0.20	0.65
Travel history	0.24	0.62
Age	1.85	0.39
Sex	1.22	0.27
Educational level	6.26	0.18
RDT	12.84	0.001
Microscopy	0.001	0.99

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
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☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐	☒	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Study data collection tools (mobile application version 5.20.11) were prepared and managed using REDCap electronic data capture tools
Data analysis	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 Geo spatial Foundation Project), and Graph pad prism 5.03 (Graph Pad Software inc., CA, USA). Rstudio v.2022.12.0.353(Posit,2023) was used , with packages lme4 (generalized linear mixed models) and dcifer (pairwise relatedness analysis on P. falciparum genotypes in diverse loci). The R codes can be found at https://github.com/legessealamerie stephensi_outbreak_DireDawa_ETH_For_Publication.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All the data used in the manuscript are available on dryad (linked with the ORCID: https://orcid/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/stephensi_outbreak_DireDawa_ETH_For_Publication and https://github.com/EPPIcenter/mad4hatter.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	We followed the guidance on sex and gender reporting of the journal. Sex was self-reported in this study. We presented data disaggregated by sex when analyzed malaria risk in population. Individual level data included in the link provided contains sex as a variable at an individual level. Sex or gender was not considered in the study design. The research findings in this study apply in box sexes. Since the outbreak in the university campus happened at a fine spatial scale the dormitories affected by malaria were occupied by male students only. In the city, we had a predictable mix of sex. In the city 240 male and 282 females participated in the study whilst all participants in the university (n=249) were male students. Sex based malaria risk was analyzed in this study for the population in the city. Consent was obtained to share data not linked with personal identifiers.
Reporting on race, ethnicity, or other socially relevant groupings	N/A
Population characteristics	The median age in the city was 23 years (interquartile range 12-35) and in the university it was 22 years(20-22).
Recruitment	Patients with (history in 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 households/dormitory members were tested for malaria and their households or dormitories were screened for anopheles mosquitoes (larvae and adult). Patients were requested to join the study as far as the capacity of the field team allowed on a first come first served basis.
Ethics oversight	Study protocol was approved by the institutional ethical review board of AHRI/ALERT ethics review committee (AF-10-015.1,PO/07/19). We provided a statement of informed consent in the manuscript.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	We planned an unmatched case control ratio 1:2 with prospective case identification until 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 the presence of An. stephensi. The power analysis was conducted in epiR package (R-cran software) and 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. An. stephensi exposure at household level. The controls were selected from the same population as the cases 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).
Data exclusions	No data was excluded from the final analysis

Replication	Infection detection in wild caught mosquitoes that relies on an ELISA based protocol that targets circum sporozoite protein (CSP)is one of the highly informative tools to estimate malaria transmission. However several studies reported false positive results when targeting CSP specially in zoophilic mosquitoes. The false positive results could lead to an overestimation of the entomological inoculation rate. To circumvent this, we implemented stringent steps to determine infection status of mosquitoes. One of the approaches we followed is to rerun the bead based assays and test mosquitoes on a confirmatory PCR. Of the mosquitoes that were scored positive (n=4) and tested again all were confirmed to be positive in the rerun. when tested with 18s based PCR, only 3 of these mosquitoes were confirmed to be positive. We thus implemented a stringent criteria for determining infection status of a mosquito was considered infected only when it was found both CSP and 18S PCR positive which ended up to be only 3 infected An. stephensi mosquitoes.
Randomization	N/A: We recruited consecutive 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) in a 1:2 ratio (one case: two controls) unmatched study design. So randomization is not relevant for our study since we employed a case control study design.
Blinding	Grouping was determined by the infection status as determined by microscopy slide film investigation for the cases and controls. Once the case or control were identified, the investigators test all their members of the family/dormitory irrespective of their symptoms. Due to this blinding of investigators was not relevant to our study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging