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ABSTRACT

Circumsporozoite protein enzyme-linked immunosorbent assays (CSP-ELISA) are widely used for malaria vector identification throughout the world. However, several studies have reported false-positive results when using this method. The present study was conducted to estimate the frequency of false positives among anopheline species in malaria endemic areas of Bangladesh. In total, 4,724 *Anopheles* females belonging to 25 species were collected and tested for *Plasmodium falciparum*, *Plasmodium vivax*-210, and *P. vivax*-247 CSP. Initially, 144 samples tested positive using routine CSP-ELISA, but the number of positive results declined to 85 (59%) when the samples were tested after heating at 100^0 C for 10 min to remove false-positive specimens. Ten species, *Anopheles annularis*, *An. baimaii*, *An. barbirostris*, *An. jeyporiensis*, *An. karwari*, *An. kochi*, *An. minimus* s.l., *An. peditaeniatus*, *An. philippinensis*, and *An. vagus* were CSP-positive. The highest and lowest infection rates were found in *An. baimaii* (4/25, 16.0%) and *An. jeyporiensis* (1/139, 0.67%), respectively. A significant correlation was found (regression analysis, $R^2 = 0.49$, $F = 8.25$, $P < 0.05$) between human blood index results and the true CSP-positive ratios in 15 *Anopheles* species. We confirmed that false-positive reactions occurred more frequently in zoophilic species. The relatively high proportion of false positives (40%) that was found in this study should warn malaria epidemiologists working in the field to be cautious when interpreting ELISA results.

Keywords: CSP; ELISA; False positive; Malaria; Vector incrimination; Bangladesh

1. Introduction

Malaria vector identification involves establishing the relationship between *Anopheles* populations and the transmission of *Plasmodium* (Eldridge et al., 2000). Vector identification is a critical activity because effective control is only possible if potential vectors are accurately determined. The methods used to identify malaria vectors range from classic microscopic examinations to modern molecular techniques. The detection of *Plasmodium* sporozoites in anophelines has relied on dissection and microscopic examination of mosquito salivary glands. Although this method is referred to as the "gold standard," it is not convenient for examining large numbers of samples because it is very laborious and samples need to be tested when fresh. Therefore, an enzyme-linked immunosorbent assay (ELISA) was developed and has proven useful for detecting *Plasmodium* in mosquito populations (Burkot et al., 1984; Wirtz et al., 1985). Its use has progressively increased in malaria studies in several countries since the mid-1980s (Burkot et al., 1984; Wirtz et al., 1985; Wirtz et al., 1992). These assays can be easily carried out on preserved samples and can differentiate between *Plasmodium falciparum* and *Plasmodium vivax*. Monoclonal antibodies (mAbs) are highly specific with no or little cross-reaction between different *Plasmodium* species (Collins et al., 1988). Many studies have reported good correlations between infection rates detected by ELISA and salivary gland dissections, with the sporozoite rate being overestimated by ELISA by up to 1.1–1.5 times (Sokhna et al., 1998). However, several studies in many countries have reported false-positive CSP-ELISA results when using bovine and swine blood (Durnez et al., 2011; Koekemoer et al., 2001; Lochouarn and Fontenille, 1999; Mouatcho et al., 2007; Somboon et al., 1993).

In a study conducted to identify malaria vectors in malaria endemic areas in Bangladesh using CSP-ELISA, we observed positive results in zoophilic *Anopheles* species. This was unexpected because these mosquitoes have never been confirmed as malaria vectors in Bangladesh (Elias et al., 1982; Khan and Talibi, 1972; Maheswary et al., 1992; Maheswary et al., 1993; Maheswary et al., 1994) and their vector capacity has not been confirmed.

Recently, Durnez et al. (2011) described a new method for verifying true CSP-positive results. In fact, a similar heating technique was initially described by Somboon (unpublished) and quoted by Lochouarn and Fontenille (1999). They demonstrated that the ELISA reacting antigen of *Plasmodium* was heat-stable but false-positive causal agents were not. Therefore, they recommended that positive results be confirmed using a second CSP-ELISA test during which the samples are heated to 100°C for 10 min. In this study, we aimed to estimate the frequency of false positives among anopheline species collected from four malaria endemic areas of Bangladesh using the method described by Durnez et al. (2011).

2. Materials and methods

2.1. Study areas and collection period

The study areas were sites at which the Directorate General of Health Services (DGHS) of Bangladesh had conducted entomological studies in the recent past (10 years) and all of the sites were reported as malaria endemic villages. The areas were Kumari (Lama) of Bandarban district (21°44´ N, 92°8´ E), Srimangal of Moulvibazar district (24°19´ N, 91°46´ E), Durgapur of Netrokona district (25°11´ N, 90°39´ E), and Dhobaura of Mymensing district (25°9´ N, 90°0´ E) (Fig. 1). Mosquitoes were captured from Kumari throughout 1 week from August to September 2010 (as described by Bashar et al., 2012) and twice each month from January to July 2011. A large portion of Kumari is vegetated by secondary forests with interspersed rubber plantations. Mosquito breeding sites within the village include a narrow slow-running stream and many wells, pools, ponds, and rice fields. In Srimangal, monthly collections were made in the Lawachara rain forest from February to July 2011. This site consisted of tea gardens, semi evergreen coniferous forests, and mixed deciduous forests of the tropical and subtropical moist broadleaf forest biome (Banglapedia, 2010). A few slow-running streams acted as mosquito breeding sources. Collections were carried out in Durgapur (Netrokona) and in Dhobaura (Mymensing) in June 2011. Both areas have nearly the same climatic conditions, with plains and dense secondary forests. Many ponds, narrow slow-running streams, rivers, wells, pools, and rice fields served as potential mosquito breeding places. The geographical locations

of the sampling houses were recorded using a handheld GPS (Garmin Oregon 550). ArcView GIS 3.3 and Arc GIS 9.2 software were used to map the sampling area.

2.2. Mosquito collections and identification

Anophelines were collected indoors and outdoors at 86 randomly selected houses using light traps (LTs), pyrethrum spray (PS), and human landing (HL) following World Health Organization procedures (WHO, 1975). LTs were set 1.5 m above the ground at indoor and outdoor locations. HL collections were made at both indoor and outdoor locations, but PS collections were only conducted inside houses. Ethical clearance was obtained from the head of each village and the office of the local government (No. PU/203/11). Collectors and individuals serving as human landings were given antimalarial drugs (atovaquone/proguanil) for disease prevention.

Collected mosquitoes were brought to a field laboratory for processing and identification. Mosquitoes were killed with chloroform and identified morphologically under stereomicroscopes within 12 h of sampling using taxonomic keys (Christophers, 1933; Harrison and Scanlon, 1975; Puri, 1960). Specimens were stored in Eppendorf tubes with soft tissue paper and silica gel desiccant to store stably at room temperature for further processing. Collected mosquitoes were transported to a laboratory at Jahangirnagar University and preserved in freezers. These mosquitoes were later brought to Kanazawa University to confirm the species identification and for CSP-ELISA.

2.3. Sample preparation and CSP-ELISA

Each mosquito was bisected into head–thorax and abdomen. Anterior parts, heads, and thoraxes of mosquitoes were tested in pools of one to five (maximum) specimens, retaining information about the species, sampling method, sampling time, and sampling location (house number and whether indoor or outdoor). Samples were tested for circumsporozoite proteins (CSPs) of *P. falciparum* (Pf), *P. vivax*-210 (Pv-210), and *P. vivax*-247 (Pv-247) using ELISA as described by Burkot et al. (1984) and Wirtz et al. (1991), with slight modifications. All monoclonal antibodies were supplied by the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA. These were used to detect and distinguish the CSP of *P. falciparum* and the VK-210 and VK-247 polymorphs of *P. vivax*. Four

negative controls (each a triturated whole body of a laboratory reared *Aedes aegypti*) and one positive control (supplied by the CDC) were included in each microtiter plate. ELISA results (i.e., changes in color) were visually interpreted and were scored as 0 (no color change, negative result), 1, 2, or 3, with 1 being the lowest color intensity and 3 being the highest color intensity as compared to the positive control. A score of 0 was considered a negative result. All positive samples were retested to confirm the result. We considered a sample to be CSP-positive if both tests were positive. We retested positive samples to determine if they consisted of heat-sensitive antigens that caused a false-positive result, as described by Durnez et al. (2011).

2.4. Data analysis

Traditional analyses were used to calculate the minimum infection rate (MIR) and to estimate the population infection rate from pooled samples. When a pool was positive, only one individual in that pool was considered to be positive. The relationship between the degree of zoophily, using the human blood index (HBI), and the true positive ratio was determined through regression analysis. HBI values were used from a recently published article (Bashar et al., 2012).

3. Results

In total, 4,724 female anopheline specimens belonging to 25 species or species groups were collected from the four malaria endemic areas of Bangladesh. *Anopheles vagus* Doenitz was the predominant species (*n* = 1978, 41.87%), followed by *An. philippinensis* Ludlow (*n* = 1169, 24.75%), *An. karwari* **(**James) (*n* = 244, 5.17%), and *An. peditaeniatus* (Leicester) (*n* =238, 5.04%) (Table 1). *Anopheles vagus* was collected from all of the study areas. *Anopheles philippinensis* was also captured from all of the areas except Dhobaura (Mymensing), where only 31 *Anopheles* mosquitoes were collected. All 25 species were collected in Kumari (Lama, Bandarban), but only four (*An. vagus*, *An. annularis* Van der Wulp, *An. kochi* Doenitz, *An. umbrosus* (Theobald)) were found in Dhobaura, seven (*An. vagus*, *An. philippinensis*, *An. minimus* s.l. Theobald, *An. umbrosus*, *An. kochi*, *An. karwari*, *An. jamesii* Theobald) were collected in Durgapur and seven species (*An. peditaeniatus*, *An. annularis*, *An. jeyporiensis* James, *An. philippinensis*, *An. vagus*, *An. karwari*, *An. umbrosus*) were encountered in Srimangal. *Anopheles peditaeniatus* was dominant only in Srimangal.

All female anophelines were tested for *P. falciparum*, *P. vivax* 210, and *P. vivax* 247 CSP; 10 species were found to be positive. The CSP-positive species were *An. annularis*, *An. baimaii* Sallum and Peyton, *An. barbirostris* Van der Wulp, *An. jeyporiensis*, *An. karwari*, *An. kochi*, *An. minimus* s.l., *An. peditaeniatus*, *An. philippinensis*, and *An. vagus*. Thirty-two (0.68%) mosquitoes belonging to nine species were positive for Pf, 34 (0.72%) mosquitoes belonging to five species were positive for Pv-210, and eight (0.17%) mosquitoes belonging to four species were positive for Pv-247. Mixed infections were found in 11 females (0.23%). Pf-positive specimens included one *An. annularis*, two *An. baimaii*, one *An. barbirostris*, two *An. karwari*, one *An. kochi*, two *An. minimus* s.l., one *An. peditaeniatus*, nine *An. philippinensis*, and 13 *An. vagus*. Pv-210 positive specimens included two *An. baimaii*, four *An. karwari*, 10 *An. minimus* s.l., 10 *An. philippinensis*, and eight *An. vagus*. Pv-247-positive specimens included one *An. jeyporiensis*, four *An. karwari*, one *An. peditaeniatus*, and two *An. philippinensis*. Mixed CSP-ELISA-positive specimens belonged to *An. karwari*, *An. minimus* s.l., *An. peditaeniatus*, *An. philippinensis*, and *An. vagus* (Table 1). Among all of the *P. falciparum* and *P. vivax* infections, the highest infection rate was observed in *An. baimaii* (4/25, 16.0%) followed by *An. minimus* s.l. (13/211, 6.16%), *An. karwari* (11/244, 4.51%), *An. annularis* (1/37, 2.7%), *An. kochi* (1/44, 2.27%), *An. philippinensis* (25/1169, 2.14%), *An. vagus* (25/1978,1.26%), *An. peditaeniatus* (3/238, 1.26%), *An. barbirostris* (1/140, 0.71), and *An. jeyporiensis* (1/139, 0.67%). No *Anopheles* collected from other study sites (Dhobaura, Durgapur, and Srimangal), were found to be CSP-positive.

Initially, 144 samples tested positive by standard CSP-ELISA, but the number of positives declined to 85 when the samples were tested after heating at 100^{o}C for 10 min. False positives were found in 50% and 37.78 % of the Pv-210 and Pf results, respectively. The rate of *Plasmodium falciparum* false positives was highest in *An. barbirostris*, whereas the Pv-210 false-positive rate was highest in *An. jeyporiensis*, *An. kochi*, and *An. peditaeniatus*. Nearly 100% of the Pv-247 false positives were observed in *An. vagus* (Table 2). The circumsporozoite rates in Kumari (Bandarban) were 3.05% in ELISA when applying non-heated samples and 1.80% in ELISA when the samples were heated. A significant correlation was found between the HBI and the true-positive ratio using data for 15 *Anopheles* species ($R^2 = 0.49$, $F = 8.25$, $P < 0.05$), meaning that high rates of false positivity were found more in zoophilic species (Fig. 2).

4. Discussion

Recent studies in Bangladesh (Alam et al., 2010), India (Prakash et al., 2004), Thailand (Somboon et al., 1993), Vietnam (Manh et al., 2010), South Africa (Koekemoer et al., 2001; Mouatcho et. al. 2007), Cameroon (Bigoga et. al. 2007), Gabon (Sylla et. al. 2000), Senegal (Lochouarn and Fontenille, 1999), and Papua New Guinea (Cooper et al., 2009) have reported that several vector species are involved in the transmission of malaria based on CSP-ELISA results alone. In these articles, most of the authors argue that false positives could have occurred in their results. We confirmed that in Bangladesh, zoophilic species have a higher probability of producing false-positive CSP-ELISA results, as Durnez et al. (2011) described for Vietnam and Cambodia. They did not identify the heat-unstable cross-reacting agent in CSP-ELISA, but they confirmed that the false-positive reaction was not caused by other *Plasmodium* species or by parasites belonging to Haemogregarina, Piroplasmida, or Trypanosomatidae. Since the 1980s, CSP-ELISA has been widely used to identify malaria vectors (Burkot et al., 1984; Wirtz et al., 1985; Wirtz et al., 1992). Our results confirm that the application of CSP-ELISA alone is risky in vector identification because false positives occur more frequently in zoophilic species, which are less likely to be malaria vectors. In previous studies that used dissection as the gold standard, mosquitoes were often collected by human landing collections, in which case the probability of collecting anthropophilic species is higher. We reported previously that mosquito species composition can vary drastically according to sampling methods (Bashar et al., 2012). We found more zoophilic species in light trap collections and indoor resting collections than in human landing collections (Bashar et al., 2012). Recently, human landing collections have been replaced more and more frequently by alternative methods because of ethical and logistical reasons (Alam et al., 2010; Bashar et al., 2012). Therefore, the risk of incorrectly identifying malaria vectors is increasing through the combined effects of changes in sampling methods and the presence of cross-reacting agents in zoophilic species. In the literature, different hypotheses have been proposed regarding the source(s) of cross-reacting antigens: cross-reactions with a protein within the mosquito itself (Koekemoer et al., 2001), cross-reactions with an antigen in the blood of the host, and that the antigen is present in the head–thorax portion of zoophilic mosquitoes (Durnez et al., 2011).

We found true-positive CSP-ELISA specimens in 10 species; the highest infection rate occurred in *An. baimaii*, followed by *An. minimus* s.l., *An. karwari*, *An. annularis*, *An. kochi*, *An. philippinensis*, *An. vagus*, *An. peditaeniatus*, *An. barbirostris*, and *An. jeyporiensis.* So far, seven anopheline species have been identified as competent malaria vectors in Bangladesh (Elias et al., 1982; Maheswary et al., 1992; Maheswary et al., 1993; Maheswary et al., 1994). Four of the seven species (*Anopheles baimaii*, formerly known as *An. dirus* species D, *An. philippinensis*, *An. sundaicus* (Rodenwaldt), *An. minimus* s.l.) have been considered primary vectors (Elias et al., 1982). The other three species, *An. aconitus* Doenitz, *An. annularis*, and *An. vagus*, transmit malaria during outbreak situations (Maheswary et al., 1992; Maheswary et al., 1993; Maheswary et al., 1994; Sharma, 1996). Recently, Alam et al. (2010) reported high malaria infection rates in *An. karwari*, *An. barbirostris*, *An. maculatus*, *An. nigerrimus*, and *An. subpictus* using CSP-ELISA in Bangladesh. However, in that study, the number of CSP-ELISA-positive results was probably overestimated since the ELISA results were not confirmed by PCR and ELISA-lysates were not heated before analysis.

We found false CSP-ELISA-positive results for both *P. vivax* and *P. falciparum* in many anopheline species in Bangladesh. Somboon et al. (1993) used ELISA to test pools of mosquitoes captured in animal enclosures in Thailand and reported false-positive results when detecting sporozoites of *P. vivax* and *P. falciparum*. Our findings support these results, but Durnez et al. (2011) reported high rates of false positives when testing for *P. falciparum* in vectors having a zoophilic biting trend. Cow blood also reacted to produce false-positive results in CSP-ELISA that was used to detect *P. malariae* and *P. ovale* sporozoites in Senegal (Lochouarn and Fontenille, 1999).

We conducted entomological surveys at four locations in four districts in malaria endemic regions of Bangladesh, but CSP-positive mosquitoes were only found in Kumari (Lama, Bandarban district). No CSP-positive mosquitoes were found in the other three sampling areas. *Anopheles baimaii* was not found during our survey in Srimangal, where the species was reported as the major vector in the 1980s (Rosenberg, 1982; Rosenberg and Maheswary, 1982). Our study shows that false-positivity rates were higher in zoophilic species, i.e., *An. karwari*, *An. barbirostris*, *An. kochi*, *An. jeyporiensis*, and *An. peditaeniatus*. True-positive CSP-ELISA specimens were observed in *An. kochi*, *An. jeyporiensis*, and *An. peditaeniatus;* this is the first time that these species were reported as being

positive for *Plasmodium* in Bangladesh. These species are zoophilic and prefer to feed on bovine blood (Bashar et al., 2012; Ifteara et al., 1998; Sinka et al., 2011); they are considered to be non-vectors on the Indian subcontinent (Rao, 1984). In laboratory, *Anopheles hyrcanus* group and *An. barbirostris group* were refractory to *P. falciparum* but susceptible to *P. vivax* (Somboon et al., 1994; Thongsahuan et al., 2011). We endorse Durnez et al. (2011) that all ELISA-positive specimens should be confirmed by a *Plasmodium* specific PCR before definite conclusion of vector status can be made.

Our study showed that malaria vector species and high malaria endemic areas have changed. Several factors are associated with the changing vector composition and behaviors: irrigation for agriculture, deforestation, insecticide pressure, and climate change. Irrigation is very suitable for breeding mosquitoes. The high population growth rate in Bangladesh has led to an increased demand for food and an increase in irrigation. High numbers of malaria vector *Anopheles* mosquitoes resulting from irrigation have inevitably led to increased malaria in local communities in Africa (Ijumba and Lindsay, 2001) and Bangladesh. Deforestation is one of the most potent factors at work in the emergence and reemergence of infectious diseases (Patz et al., 2004; Taylor, 1997). Several researchers (Kondrashin et al., 1991; Patz et al., 2000; Walsh et al., 1993) have described the influence of deforestation and subsequent land use on the local density of mosquito vectors. Heavy use of insecticides and insecticide treated nets (ITNs) has created insecticide resistance in vectors (Antonio-Nkondjio et al., 2011). Moreover, mosquitoes have shifted their time of biting, feeding sites, and blood hosts because of insecticidal pressure (Bashar et al., 2012; Taken, 2002). Climate change has taken place worldwide and some of the impacts are now obvious.

We emphasize the need to conduct entomological surveys that apply multiple sampling methods and to identify malaria vector species through dissection, or CSP-ELISA with heated samples and confirm by PCR methods, in Asian countries where more zoophilic species can act as vectors.

5. Conclusions

The high number of CSP false-positive mosquitoes found in this study should warm malaria epidemiologists working in the field to be cautious when interpreting sporozoite rates that are obtained by CSP-ELISA with non-heated mosquito lysates. We need to realize that standard CSP-ELISA methods should be modified according to Durnez et al. (2011). Different vector species are

likely present compared to vector communities that were reported in the 1980s to 1990s. We need to conduct longitudinal entomological surveys that cover several consecutive years in different areas of the country, and we need to evaluate environmental changes that could be the cause of changes in malaria conditions in Bangladesh.

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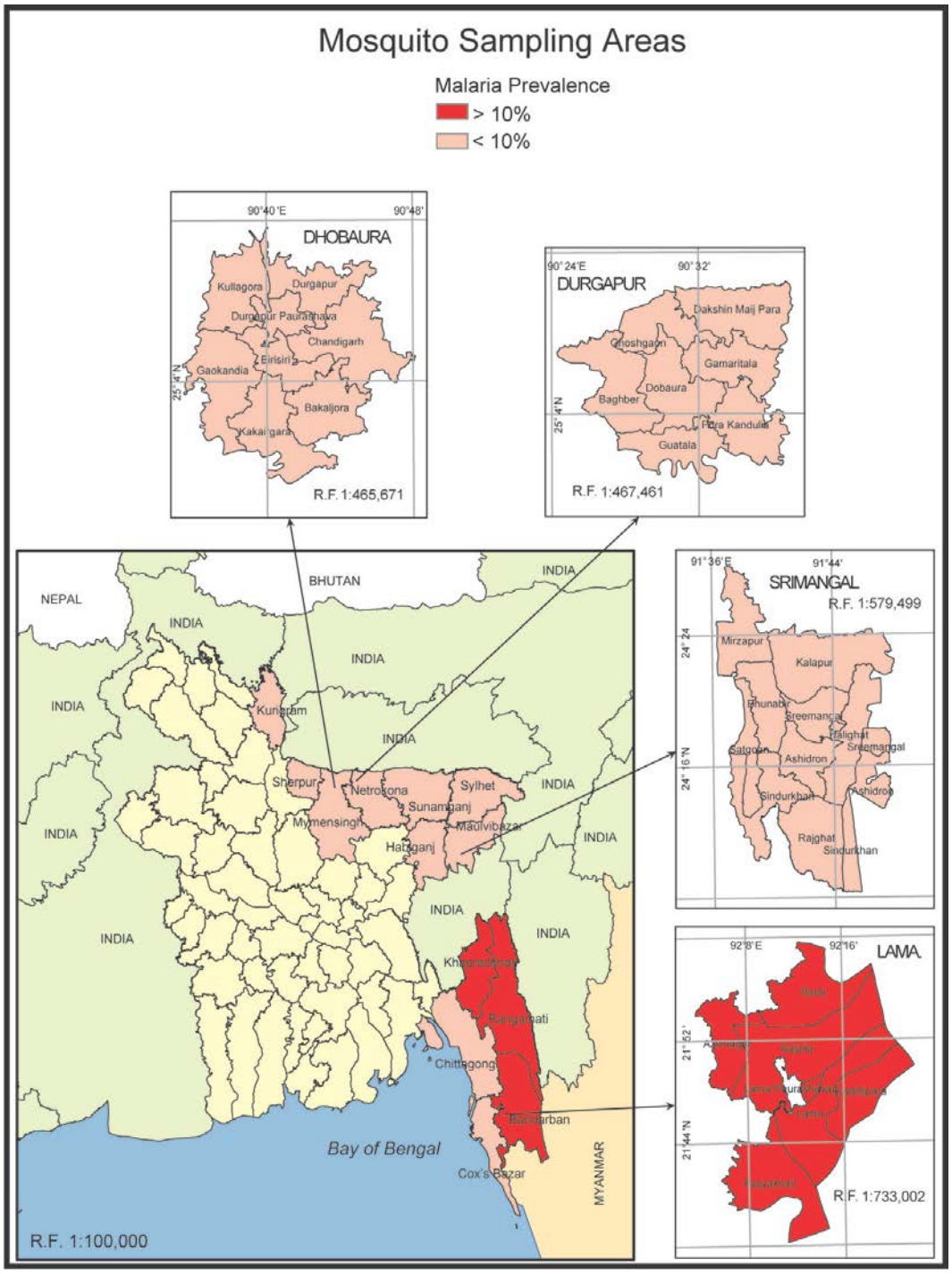

Figure 1

Mosquito sampling areas in Bangladesh.

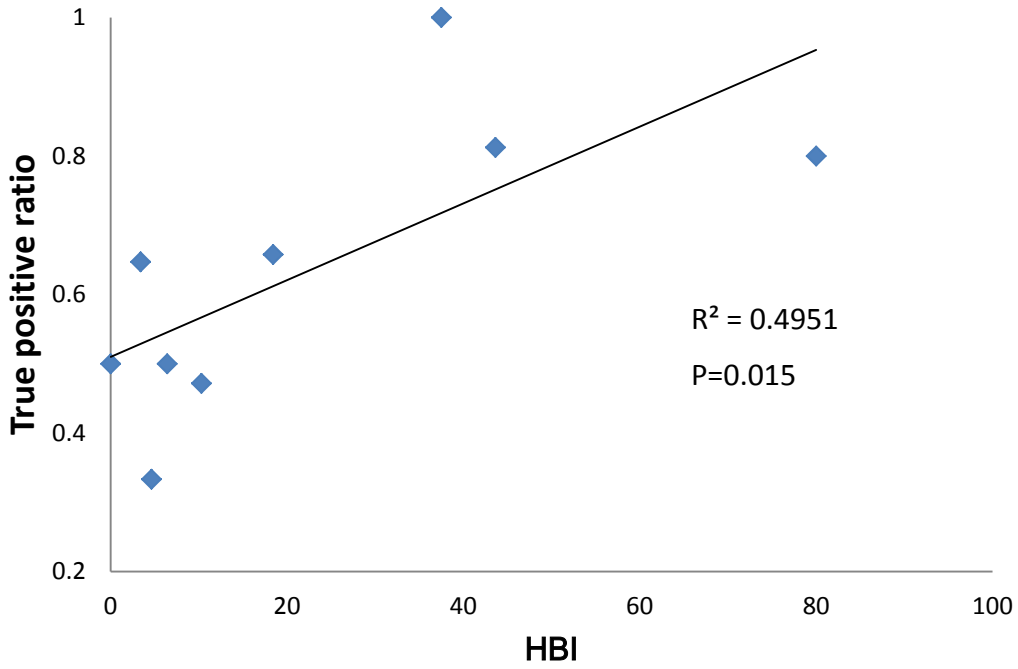

Figure 2

Correlation between the human blood index (HBI) and true-positivity ratios for 15 *Anopheles* species or species complexes.

Table 1. Number of Anopheline with true positive reaction by CSP-ELISA assay

Anophelines species	N[1]	Number of mosquito with true positive reaction				
		Pf	Pv210	Pv247	Mixed	Total
Anopheles aitkenii group	6	0	0	0	0	0
An. annularis	37	1	0	0	0	1
An. baimaii	25	2	2	0	0	4
An. barbirostris	140	1	0	0	0	1
An. fluviatilis	1	0	0	0	0	0
An. hyrcanus group	58	0	0	0	0	0
An. jamesii	61	0	0	0	0	0
An. jeyporiensis	149	0	0	1	0	1
An. karwari	244	2	4	4	1	11
An. kochi	44	1	0	0	0	1
An. maculatus group	28	0	0	0	0	0
An. minimus s.l.	211	2	10	0	1	13
An. nigerrimus	55	0	0	0	0	0
An. nivipes	4	0	0	0	0	0
An. pallidus	32	0	0	0	0	0
An. peditaeniatus	238	1	0	1	1	3
An. pseudojamesi	4	0	0	0	0	0
An. philippinensis	1169	9	10	2	4	25
An. splendidus	1	0	0	0	0	0
An. subpictus	79	0	0	0	0	0
An. tessellatus	8	0	0	0	0	0
An. umbrosus	148	0	0	0	0	0
An. vagus	1978	13	8	0	4	25
An. varuna	2	0	0	0	0	0
An. willmori	2	0	0	0	0	0
Total	4724	32	34	8	11	85

[1]Number of mosquito tested

Table 2. Number of false and true positive cases in anophelines tested for *Plasmodium* by CSP-ELISA

		P. falciparum			*P. vivax* 210			*P. vivax* 247			Total	
Species	N[1]	P[2]	TP[3]	% of FP[4]	P[2]	TP[3]	% of FP[4]	P[2]	TP[3]	% of FP[4]	P[2]	TP[3]
An. annularis	19	1	1	0.00	-	-	-	-	-	-	1	1
An. baimaii	24	3	2	33.33	2	2	0.00	-	-	-	5	4
An. barbirostris	140	3	1	66.67	-	-	-	-	-	-	3	1
An. jeyporiensis	142	-	-	-	1	0	100.00	2	1	50.00	3	1
An. karwari	244	4	2	50.00	10	6	40.00	2	2	0.00	17	11
An. kochi	44	1	1	0.00	1	0	100.00	-	-	-	2	1
An. minimus s.l.	211	4	2	50.00	10	9	10.00	-	-	-	16	13
An. peditaeniatus	139	2	1	50.00	2	0	100.00	1	1	0.00	6	3
An. philippinensis	1169	16	9	43.75	14	9	35.71	3	2	33.33	38	25
An. vagus	1978	18	13	27.78	26	8	69.23	4	0	100.00	53	25
Total	4110	45	28	37.78	64	32	50	12	6	50	144	85

[1]Number of mosquito tested
[2]Number of mosquito with positive reaction
[3]Number of mosquito with true positive reactions of CSP-ELISA, the samples were heated at 100℃ for 10 minutes before the assay
[4] Percentage of false positive

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