Stratifying Malaria Receptivity In Bangladesh Using Archived Rapid Diagnostic Tests

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Abstract

Background Surveillance on low-density infections and exposure to vectors is crucial to understand where malaria elimination might be feasible, and where the risk of outbreaks is high. Archived rapid diagnostic tests (RDTs), used by national malaria control and elimination programs for clinical diagnosis, present a valuable, yet rarely used resource for in-depth studies on malaria epidemiology. Methods We screened 1,022 RDTs from two sub-Districts in Bangladesh (Alikadam and Kamalganj) by qPCR for low-density Plasmodium falciparum and Plasmodium vivax infections, and by ELISA for Anopheles salivary gland antibodies as a marker for exposure to vectors. Results Concordance between RDT and qPCR was moderate. qPCR detected 31/1022 infections compared to 36/1022 diagnosed by RDT. Exposure to Anopheles was significantly higher in Kamalganj despite low transmission, which could be explained by low bed net use. Conclusions Archived RDTs present a valuable source of antibodies for serological studies on exposure to vectors. In contrast, the benefit of screening archived RDTs to obtain a better estimate of test positivity is moderate. Kamalganj could thus be prone to outbreaks.

Background

Where malaria transmission is low, robust surveillance is needed to focus efforts on reducing transmission in remaining foci, while preventing reemergence in areas where malaria has been eliminated. Many malaria infections among febrile patients remain below the limit of detection of light microscopy and rapid diagnostic test (RDT) [1, 2]. Molecular screening is required to diagnose these infections and reveal the true infection rates. To capture exposure to vectors, and thus to stratify transmission potential, entomological surveys are conducted.

Molecular surveillance in malaria elimination settings across large geographical scales is challenging. If incidence of infection becomes very low, large research programs are required to obtain sufficient specimens to identify risk factors and determine spatial heterogeneity [2]. As an alternative, specimens routinely collected by control and elimination programs might be used. In the past two decades, RDTs have become a crucial tool for malaria diagnosis, with over 300 million tests used each year [3]. RDTs are lateral-flow devices that detect parasite proteins in a droplet of blood of approximately 5 µL by immunohistochemistry. DNA can be extracted from archived RDTs for PCR [4, 5, 6, 7], as can antibodies for serological surveys to estimate past exposure to malaria parasites [8].

As an alternative to entomological surveys, exposure to Anopheles vector bites can be assessed by human biomarkers [9]. During blood uptake, the Anopheles mosquito injects saliva containing immunogens into the host. Antibody titers to mosquito saliva are a proxy for the intensity of mosquito bites received by the individual. This biomarker has been used to assess the risk of malaria transmission in low-level exposure/transmission areas to accurately evaluate the effectiveness of interventions [10, 11, 12, 13].
In Bangladesh *Plasmodium falciparum* and *Plasmodium vivax* are endemic along the north-eastern and south-eastern borders. In recent years, a significant decrease of malaria cases and deaths was achieved, with only 10,523 confirmed cases in 2018 compared to 39,719 cases in 2015 (NMEP MIS report). Transmission within Bangladesh is heterogenous. Most cases are reported from the three districts collectively known as Chittagong Hill Tract (CHT) in the south-eastern area [14].

Each year, over 300,000 RDTs are used by the Bangladesh Malaria Elimination Program (NMEP) to screen febrile patients. We screened 1,022 archived RDTs from two Upazilas (sub-districts) by PCR to estimate the proportion of infections missed by routine diagnosis. For the first time, we have used RDTs to study heterogeneity in exposure to *Anopheles* mosquitoes using an antibody-based salivary biomarker.

**Methods**

**Field specimens**

 Archived RDTs and routine demographic data collected during diagnosis were obtained from the NMEP from 2 Upazilas in Bangladesh: Alikadam (Bandarban District, n = 522, highest transmission in Bangladesh), and Kamalganj (Moulvibazar District, n = 500, low transmission). RDTs had been used in August-December 2018 and since been stored at ambient temperature.

**DNA extraction and qPCR**

For initial testing, RDTs were prepared with whole blood spiked with cultured *P. falciparum* at densities of 20,000 and 10,000 parasites/µL. After 10 days of storage at 4 °C, the RDT cassettes were opened, and fragments of the following components were sampled: sample pad and conjugate pad, nitrocellulose strip 1 (between the conjugate pad and the 1st test band), nitrocellulose strip 2 (between the two test bands), nitrocellulose strip 3 (between the 2nd test band and the filter paper), and filter paper. DNA was extracted with the NucleoMag whole blood kit (Macherey-Nagel) and screened by qPCR [15]. *P. falciparum* DNA was detected in almost all regions of the RDT, with the highest concentration DNA found in the first half of the RDT strip between the sample pad and the first test band. Thus, this part was used for screening of archived RDTs.

DNA was eluted in 25 µl volume, and 4 µl was used for screening for *P. falciparum* using the varATS assay [15] and for *P. vivax* using the *cox1* assay [16]. Both assays target multiple gene copies per parasite.

**Indirect Enzyme-Linked Immunosorbent Assay from RDTs**

For antibody elution, the second half of the RDT strip was cut into small pieces, placed in 1.5 ml microcentrifuge tubes, and immersed in 250 µl PBS supplemented with 0.05% (v/v) Tween-20 (VWR, USA) (PBS/T). RDT eluates were equivalent to a 1/100 dilution of whole blood or 1/200 of serum (assuming a hematocrit of approximately 50%). Tubes were incubated overnight at 4 °C, after which RDT eluates were separated from solid RDT components and stored at -20 °C until assayed.
ELISA conditions were standardized as described elsewhere [17]. Testing of serial dilutions of the eluate (1:10, 1:20 and 1:40 in PBS/T) showed optimal performance of the ELISA using a 1:10 dilution.

Nunc-Maxisorp 96-well plates were coated with 100 µL/well of gSG6-P1 peptide (2 µg/mL) diluted in 1 × PBS. Plates were incubated at 37 °C for 2:30 h and blocked with 3 × 300 µL of SuperBlock (TBS) Blocking Buffer. 100 µL of 1:10 diluted sample was added to each well. Plates were incubated overnight at 4 °C, then washed three times and incubated 1:30 h at 37 °C with 100 µL/well of a 1/500 dilution of mouse monoclonal anti-human IgG conjugated with biotin (BD Pharmingen). Peroxidase conjugated extravidine (GE Healthcare Life Sciences) was added at a 1/1000 concentration and incubated 1 h at 37 °C. After four final washes, colorimetric development was carried out using ABTS (2.2-azino-bis (3 ethylbenzthiazoline 6-sulfonic acid) diaminonum (Sigma) in 50 mM citrate buffer (pH = 4, containing 0.003% H₂O₂), and absorbance (OD) was measured at 405 nm. All samples were tested in duplicate. Each microplate contained in duplicate: positive control, negative control, and blank wells. The positive control was a pool of DBS of people with recent travel to a malaria endemic country. The negative control was a sample of people from US (n = 2) with no exposure to malaria vectors.

OD normalization and plate-to-plate variation were performed as described elsewhere [18]. Briefly, antibody levels were expressed as the ΔOD value: ΔOD = ODx – ODb, where ODx represents the mean of the technical replicates of each RDT, and ODb the mean of the blank wells. Positive controls of each plate were averaged and divided by the average of the ODx of the positive control for each plate to obtain a normalization factor. Each plate normalization factor was multiplied by the plate sample ΔOD to obtain normalized ΔODs that were used in statistical analyses. The mean ΔOD of negative US controls plus 3 standard deviations (SD) was used to determine cut-off value for responsiveness to the gSG6-P1 peptide. Results are reported as seroprevalence and as median antibody titers.

Results

Infection status

Table 1 describes the characteristics of the study population. The prevalence of *P. falciparum* and *P. vivax* combined was 3.52% (36/1,022) by RDT and 3.3% (31/1,022) by qPCR screening of archived RDTs. The majority of infections by RDT (91.7%, 33/36) and qPCR (90.3%, 28/31) were *P. falciparum*, the remaining three were *P. vivax*. All positive RDTs were from Alikadam. RDT positivity was comparable between individuals aged 18 years and older (3.6%, 25/683) and those younger (3.2%, 11/339; p = 0.7).

Concordance between RDT and qPCR was low for *P. falciparum*. Among the 33 positive RDTs, only 20 were positive by qPCR. On the other hand, qPCR detected *P. falciparum* DNA from 11 negative archived RDTs. While all RDTs from Kamalganj were negative, qPCR diagnosed 1 low-density *P. falciparum* infection (3.9 copies/µL eluted DNA).
8/11 RDT-negative/qPCR-positive tests were from adults aged 18 years and older. As the majority of RDTs were from adults, the probability of sub-patent infections did not differ between age groups (8/658 in adults, 3/325 in minors, p = 0.671). The mean \textit{P. falciparum} density of RDT-negative/qPCR-positive tests (12.9 copies/µL eluted DNA, CI95: 9.4–35.3) was significantly lower than of RDT-positive tests (154.6 copies/µL eluted DNA, CI95: 2.1-311.2; p < 0.0001).

Results were in full concordance for \textit{P. vivax}. All 3 positive RDTs were confirmed by qPCR and no additional infections were detected.

### Table 1

| Characteristics                  | Alikadam (n = 522) | Kamalganj (n = 500) |
|----------------------------------|--------------------|---------------------|
| Age, years, median (range)       | 22 (0.5–70)        | 27 (2.5–75)         |
| Female sex                       | 46% (240/522)      | 49.8% (249/500)     |
| LLIN use                         | 98.1% (469/478)    | 26.4% (132/500)     |
| RDT positivity\(^1\)             | 6.9% (36/522)      | 0.0% (0/500)        |
| qPCR positivity\(^1\)            | 5.7% (30/522)      | 0.2% (1/500)        |
| Immune responders                | 9.9% (52/522)      | 7% (35/500)         |
| Antibody titers, median (range)  | 0.035 (0.0–2.1)    | 0.062 (0.0–0.70)    |

\(^1\) \textit{P. falciparum} and \textit{P. vivax} combined

### Exposure to Anopheles

The sero-prevalence of specific IgG to \textit{Anopheles} gSG6-P1 salivary peptide did not vary significantly between Alikadam (9.9%, 52/522) and Kamalganj (7.0%, 35/500, p = 0.08, Table 1, Supplementary File S1). In contrast, the median titers significantly varied between the two study sites and was higher in Kamalganj, the low transmission area (p < 0.0001, Mann-Whitney test; Fig. 1A). This result could be explained by lower bed net use in Kamalganj. The rate of mosquito net use 15 days prior the RDT collection was significantly higher in Alikadam (98.1%, 469/478) compared to Kamalganj (26.4%, 132/500, p < 0.0001, Table 1). People who declared having slept under a LLIN 15 days prior the RDT collection had significantly lower anti-gSG6-P1 IgG levels compared to those who did not (all p < 0.05, Mann-Whitney test, Fig. 2C). Median antibody titers did not differ across age groups (p = 0.053, Kruskal Wallis test; Fig. 1D).

In Kamalganj, gSG6-P1 antibody titers remained constant from August to October, and then gradually increased until December (p < 0.0001, Kruskal Wallis test; Fig. 1B). Titers did not significantly vary from October to December in Alikadam (p = 0.421, Kruskal Wallis test; Fig. 1B).
Discussion

Molecular screening for low density infections and entomological surveys on vector exposure can greatly enhance our understanding of malaria transmission. Sample collection for such surveys spanning large geographical areas is often logistically difficult. This study showed that archived RDTs can present a valuable alternative for surveillance.

As observed in other studies [5], screening of archived RDTs by qPCR yielded few additional infections. In contrast, comparing RDT for diagnosis of febrile patients to qPCR using properly stored blood samples, RDT sensitivity is often below 50% [1]. The low volume of blood applied to the RDT and further dilution during DNA extraction, combined with long storage at ambient temperature resulted in markedly reduced sensitivity by qPCR. DNA degradation can also explain the high number of positive RDTs that were negative by qPCR, along with other factors such as persistence of the antigen after parasite clearance or cross-reactivity of the antibody bound to the RDT with non-Plasmodium antigens [19]. In summary, the benefit of qPCR-screening of archived RDTs to obtain a better picture of case numbers was moderate.

Measuring vector density as surrogate marker for the risk of malaria re-emergence is costly [2]. Measuring exposure to Anopheles vectors through salivary gland antibody biomarkers presents a cost-efficient alternative, in particular when archived RDTs are analyzed.

Our results show that exposure to Anopheles mosquitoes was higher in Kamalganj where transmission is low, compared to Alikadam, a high transmission area. This result can be explained by much lower bed net use. Only a quarter of patients in Kamalganj reported sleeping under a bed net, compared to nearly all in Alikadam. Antibody titers in individuals from Kamalganj using a bed net did not differ from those in Alikadam. The exposure to Anopheles increased significantly from October to December in Kamalganj, likely representing seasonal changes in transmission potential [20]. Given the high human-vector contact in Kamalganj in November and December, this site could be prone to outbreaks. The risk is particularly high as hrp2 deletions have been reported from this area, hampering diagnosis by RDT [21].

The seropositivity values determined in this study cannot be compared directly to values measured in population surveys elsewhere [10, 11, 12, 13]. First, samples included only febrile patients. Their exposure might not be representative for the entire population. Second, the volume of blood on the RDT is minimal, and storage at ambient temperature resulted in degradation of antibodies. Analyzing a larger blood volume stored in a freezer would likely yield higher antibody titers. Nevertheless, results obtained from different sites or across age groups can be compared among each other.

Conclusions

Our study demonstrated the usefulness of archived RDTs for quantifying human-vector contact, and in general for serological surveys in elimination settings. While the low number of infections in Kamalganj precluded analysis of the benefits of bed net usage, the serological data clearly showed that exposure to vectors is high among those not using bed nets. The presence of a sub-patent P. falciparum infection
detected by qPCR further highlighted the potential for outbreaks. Thus, malaria prevention interventions need to be maintained in low transmission settings in Bangladesh.

Declarations

Ethical approval

IRB approval was obtained from the University of Notre Dame Office of Research Compliance (approval number 19-04-5329).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data is available within the manuscript or in Supplementary file S1

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Authors' contributions

CK and MSA conceived the study and acquired the funding, MGK, SN, SI, and MMA obtained the RDTs and recorded the demographic data, ABS and MGK conducted the lab work, ABS, MSA and CK analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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**Figures**

**A**

**Sites**

- P value $< 0.001$

| Site       | ΔOD |
|------------|-----|
| Alikadam   | 2.5 |
| Kamalganj  | 1.5 |

(n=52/522) (n=35/500)

**B**

**Season**

- P value $0.421$
- P value $< 0.0001$
- P value $0.118$

| Month     | ΔOD |
|-----------|-----|
| Oct       | 0.3 |
| Nov       | 0.2 |
| Dec       | 0.1 |
| Aug       | 0.5 |
| Sep       | 0.4 |

(n=52/522) (n=35/500)

**C**

**Bed net use**

- P value $0.002$
- P value $< 0.0001$

| Use        | ΔOD |
|------------|-----|
| Yes        | 2.5 |
| No         | 1.5 |
| Yes        | 0.3 |
| No         | 0.2 |

(n=469) (n=9) (n=132) (n=368)

**D**

**Age**

- P value $0.053$

| Age (Years) | ΔOD |
|-------------|-----|
| 0-5         | 0.3 |
| 6-10        | 0.2 |
| 11-15       | 0.1 |
| 16-20       | 0.5 |
| 21-25       | 0.4 |
| 25+         | 0.0 |

**Figure 1**

Specific IgG levels to the Anopheles gSG6-P1 salivary peptide in Alikadam and Kamalganj, Bangladesh. Black dots indicate individual IgG responses, and bars represent median values in each group. Statistically significant differences between the two (nonparametric Mann-Whitney test) and three groups (nonparametric Kruskal Wallis test) are indicated.
Supplementary Files

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- SagnaSupplementaryFileS1.xlsx
- SagnaRDTBangladeshGraphicalAbstract.pdf