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Research Note

Genotyping of chloroquine resistant *Plasmodium falciparum* in wild caught *Anopheles minimus* mosquitoes in a malaria endemic area of Assam, India

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Abstract. We validated the feasibility of using *Plasmodium falciparum*, the human malaria parasite, DNA present in wild caught vector mosquitoes for the characterization of chloroquine resistance status. House frequenting mosquitoes belonging to *Anopheles minimus* complex were collected from human dwellings in a malaria endemic area of Assam, Northeast India and DNA was extracted from the head-thorax region of individual mosquitoes. *Anopheles minimus* complex mosquitoes were identified to species level and screened for the presence of *Plasmodium* sp. using molecular tools. Nested PCR-RFLP method was used for genotyping of *P. falciparum* based on K76T mutation in the chloroquine resistance transporter (*pfcrt*) gene. Three of the 27 wild caught *An. minimus* mosquitoes were harbouring *P. falciparum* sporozoites (positivity 11.1%) and all 3 were had 76T mutation in the *pfcrt* gene, indicating chloroquine resistance. The approach of characterizing antimalarial resistance of malaria parasite in vector mosquitoes can potentially be used as a surveillance tool for monitoring transmission of antimalarial drug resistant parasite strains in the community.

Vectored mainly by *Anopheles baimaii* and *An. minimus*, control of malaria in the north-east of India is challenging due to the complex vector behaviour and abundance of drug resistant malaria parasite strains, especially that of *Plasmodium falciparum* (Prakash *et al.*, 2010). The first report of chloroquine (CQ) resistance to *P. falciparum* in the north-east India came from Karbi Anglong district of Assam state (Sehgal *et al.*, 1973). Presently, while CQ resistance is wide spread in all the seven north-east Indian states, resistance to other antimalarials, eg. sulfadoxine/pyrimethamine and quinine, is also quite common (Mohapatra *et al.*, 2005). Operational challenges posed to the Indian National Vector Borne Diseases Control Programme by the ever increasing CQ resistant malaria parasites in the north-east India calls for regular spatial and temporal monitoring of resistant malaria parasite strains for better management and control. Studies on antimalarial resistance are mostly carried out either on human host (*in vivo*) or on the parasite isolates drawn from them (*in vitro*). Of these, *in vivo* therapeutic evaluation of antimalarials in malaria infected patients is considered as the standard monitoring tool. The limitations of *in vivo* (eg. time consuming, expensive) and *in vitro* (eg. parasite cultivation facility, unreproducible results) antimalarial resistance monitoring methods have led to the development of rapid and efficient molecular assays for genotyping of malaria parasite strains based on drug resistance

molecular markers (Plowe *et al.*, 2007). A majority of these assays used nested polymerase chain reaction (PCR) of parasite DNA followed by restriction fragment length polymorphism (RFLP) that have been successfully deployed for surveillance of drug resistant malaria parasites (Wernsdorfer & Noedl, 2003; Schonfeld *et al.*, 2007).

Parasite DNA for molecular genotyping assays can be obtained either from its human host (malaria patient) or from mosquito host (vector *Anopheles*). In spite of potential advantages of obtaining parasite DNA from the vector mosquitoes such as no need of skilled medical/paramedical staff due to non-involvement of human subjects, non-invasive procedures and quick processing time, this approach has largely remained unexplored except for sporadic attempts made by Mohanty *et al.*, 2009 in Orissa, India and Temu *et al.*, 2006 in Tanzania. This may be partly due to the low chances of getting adequate infection in mosquito vector and lack of proper parasite DNA isolation strategy.

Herein we describe our experience of using parasite DNA present in wild caught *An. minimus* mosquitoes to characterize the chloroquine resistance status of circulating *P. faciparum* strains in a malaria endemic area of Assam state in the north-east India. The idea was to explore the possibility of monitoring the transmission of CQ resistant *P. falciparum* strains by screening vector mosquitoes.

House frequenting mosquitoes were collected from 2 human-dwellings each in 3 malaria endemic villages in North Lakhimpur district of Assam state using standard Centers for Disease Control (CDC) light traps from dusk to dawn. Morphologically identified *An. minimus* complex mosquitoes were stored individually in beam capsules along with silica beads until processed. In the laboratory, genomic DNA was isolated from the head-thorax part of the individual mosquitoes using Chelax-100 based protocol (Lardeux *et al.*, 2008). The DNA pellet was dissolved in 20 µl 0.1X TE buffer and stored at 4ºC for further analysis.

Individuals of *An. minimus* complex mosquitoes were identified to species level using allele specific polymerase chain reaction (ASPCR) method of Phuc *et al.* (2003). Furthermore, in nested-PCR assay for the detection of *Plasmodium* species following Snounou *et al.* (1993) was carried out. For these assays, genomic DNA of 5 to 6 individual mosquitoes was pooled together (approximately 5 µl DNA perindividual), mixed thoroughly and 5 µl of this pooled DNA was used as template in a 15µl reaction volume. All mosquitoes of *Plasmodium*-positive pools were subsequently processed individually to pinpoint the infected mosquito and for identification of malaria parasite species. Nested PCR-RFLP assay was used for genotyping of the malaria parasite based on K76T mutation in the *pfcrt* gene of *P. falciparum* infected mosquitoes. The first round PCR was performed using CRTP1 (5'-CCGTTAATAATAAATACACGCAG-3') and CRTP2 (5'-CGGATGTTACAAAACTATAGTTA CC-3') primers and 3 µl of first round PCR product was subsequently used in the second PCR with CRTD1 (5'-TGTGCTCATGTGTTT AAACTT-3') and CRTD2 (5'-CAAAACTATA GTTACCAATTTTG-3') primers in a final volume of 25 µl as described by Djimde *et al.* (2001) and Olukosi *et al.* (2005). The first round PCR produced an amplicon size of 260 bp while the second round produced 145 bp product of the *pfcrt* gene. An aliquot of 8 µl from the 2nd round PCR product was incubated with *Apo I* (New England Biolabs, Beverly, MA) at 50ºC for 5 hours. Bands were resolved in 2.5% agarose gel. Genomic DNA of RKL2 (CQ resistant) and 3D7 (CQ sensitive) strains of *P. falciparum* were used concurrently as positive controls.

A total of 123 anophelines belonging to 5 species, including 27 individuals of *An. minimus* complex were captured in 6 trap night collections. In ASPCR, all 27 individuals were found to be *An. minimus* (earlier species A of Minimus complex). Grouped in 5 pools, these 27 individuals of *An. minimus* were processed for the detection of malaria parasite by nested PCR, which yielded 2 positive pools for *Plasmodium*. Assay of individual mosquitoes belonging to the *Plasmodium*-positive pools revealed 3 individuals (coded as MA 44, MA 62 and MA 68) positive for sporozoites of *P. falciparum* (sporozoite rate 11.1%) (Figure 1a).

Plasmodium falciparum of these 3 infected mosquitoes was genotyped to determine K76T mutation in the *pfcrt* gene using nested PCR-RFLP. In RFLP, *Apo I* restriction enzyme did not cut the 145 bp amplicon of nest 2 PCR into 111 and 34 fragments (Figure 1b), indicating that all the 3 *P. falciparum* isolates were resistant to chloroquine.

Monitoring of the spread of drug resistant malaria often gets neglected because of the cumbersome, time consuming and resource demanding *in vivo* and *in vitro* drug resistance monitoring techniques. As an alternative approach, molecular genotyping of malaria parasites using drug resistance markers is increasingly being used due to their accuracy, low cost and ability to process large numbers of samples in a short time with relative ease. Infected vector mosquitoes collected from the field could be a good source for obtaining parasitic DNA to achieve this goal. Since PCR-based assays are widely used in vector incrimination studies i.e. detecting the presence of malaria parasite in mosquitoes (Temu *et al.*, 2006; Bass *et al.*, 2008; Hasan *et al.*, 2009; Bhattacharyya *et al.*, 2010), it should not be difficult to genotype the malaria parasite for molecular drug resistance markers using the same DNA as successfully demonstrated in this study. Our results demonstrated the circulation of CQ resistant *P. falciparum* strains and transmission by *An. minimus* in the study villages. This finding was supported by the detection of nearly 100% CQ resistant strains when genotyped from malaria positive patients' blood during the same time and from the same area (RMRC unpublished data). Though not attempted in this study, resistance to other antimalarials can also be monitored using the same DNA sample and by targeting specific markers such as *dhps* gene (for sulphadoxine), *dhfr* gene (for pyrimethamine) and *mdr* gene (for quinine) as demonstrated by Mendes *et al.* (2013). Another advantage of this approach is that drug resistance can be detected in vector mosquitoes well in advance before its transmission/clinical manifestation in human hosts. Therefore, screening of vector mosquitoes as a tool to monitor the transmission of antimalarial drug resistant malaria parasite strains should be encouraged to supplement the efforts of antimalarial drug resistant monitoring teams.

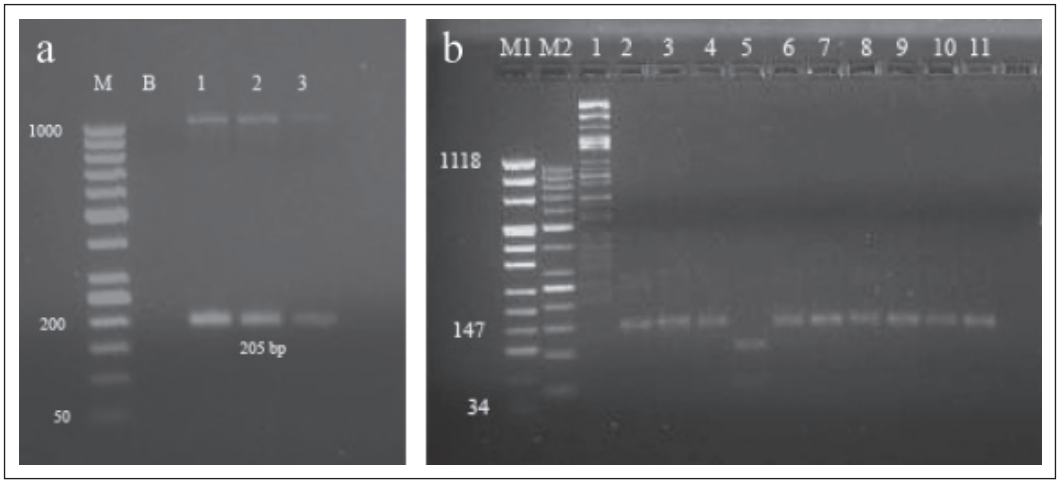

Figure 1. PCR based detection and genotyping of *pfcrt*K76T mutation in wild caught *An. minimus* mosquitoes. (a) *Plasmodium* species specific PCR, M: 100 bp marker, B: Blank, Lane 1-3: MA44, MA62 & MA68 showing *P. falciparum* specific band (205 bp). (b) PCR-RFLP of *pfcrt* nest 2 PCR amplicon, M1: pUC Mix Marker, M2: 50 bp marker, Lane 1: *Apo* I digested λ-DNA, Lane 2, 4, 6, 8 & 10: *pfcrt* nest 2 amplicons of RKL2, 3D7, MA44, MA62 and MA68 without *Apo* I digestion, Lane 3, 5, 7, 9 &11: *pfcrt* nest 2 amplicon of RKL2, 3D7, MA44, MA62 and MA68 with *Apo* I digestion

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