MULTIPLEX PCR ASSAY FOR THE DETECTION OF ANOPHELES FLUVIATILIS SPECIES COMPLEX, HUMAN HOST PREFERENCE, AND PLASMODIUM FALCIPARUM SPOROZOITE PRESENCE, USING A UNIQUE MOSQUITO PROCESSING METHOD

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Abstract. A multiplex PCR assay has been developed for detection of Anopheles fluviatilis cryptic species, their human host preference, and Plasmodium falciparum presence in the mosquito. PCR conditions were optimized using primer sets specific for A. fluviatilis cryptic species, Homo sapiens, and P. falciparum and evaluated with field-collected mosquitoes. A unique mosquito processing method was used for screening P. falciparum carrying capacity and human host preference of A. fluviatilis mosquitoes in first-round multiplex PCR. The vectorial status of the mosquito for P. falciparum parasite was confirmed in second-round PCR. Of the 121 collected mosquitoes, 92 were of S type, 26 of T type, and 3 were of other types. Human host preference was dominant in S type, of which 4% were P. falciparum sporozoite positive. This assay and processing method can also be used to evaluate vector competence of other anophelines.

INTRODUCTION

Malaria remains one of the most important pathogen, endemic in more than 107 countries, inhabited by some 3.2 billion people—almost half of the world’s population—and causes 1.2 million deaths each year.1,2 Malaria prevention through vector control has immense potential. There are ~40 species of Anopheles mosquitoes transmitting human malaria, differing in their transmission potential.2 Both the potential vectors and the non-vectors of the genus Anopheles exhibit similar features and overlapping characteristics. Therefore, vector identification certainly has a role to play in vector control measures. It is evident from several studies that most malaria vectors comprise morphologically indistinguishable groups of species, cryptic species, or sibling species,3-4 having vectorial and behavioral variations. It becomes important to distinguish closely related groups of anophelines using methods other than morphologic taxonomy. Among the available alternative methods, the cytologic method of polytene chromosome-based identification has a few problems, namely, it identifies only female mosquitoes; further, it cannot be used in females that are unfed or fully gravid; and finally, the method requires a high level of technical expertise. The polymerase chain reaction (PCR)-based diagnostic assay reflects its own advantage in taxonomy, as it is reliable and rapid. PCR methods have been effectively used to distinguish Anopheles cryptic species.5-9 To gain a quick grasp of the malaria vector composition in a particular geographical area, it may be very useful to scan the anopheline population using a PCR-based method. As the Anopheles prevalence and its attributes, such as feeding habit, host preference, sporozoite rate, and insecticide resistance, vary greatly from one cryptic species to another; we must identify these parameters along with the species types.10,11 To have a better perspective of malaria epidemiology of an area, these parameters must be included in PCR detection of Anopheles species differentiation.

To provide useful inputs to vector control strategy, it is essential to study different attributes of the entire anopheline species of a particular area. For this work, we have selected Anopheles fluviatilis James, as it is a primary vector in the hilly and foothill regions of India and ranks second in contributing to the total malaria cases of the country.2 It is widely distributed in eastern Asia and in some parts of western Asia.13 A. fluviatilis exists as three distinct, reproductively isolated cryptic species, viz. S, T, and U, which are distinguished by the cytologic method on the basis of fixed inversion genotypes in polytene chromosome arm 2.14 Marked differences were observed in the distribution pattern,4-14 feeding preferences,15 and role in disease transmission15,16,17 among these three cryptic species. Species S is primarily anthropophagic (~ 90%)15,16 and is active in transmitting malaria, whereas species T and U are mainly zoophagic (~ 99%)15 and are normally considered as non-vectors. For identification of the A. fluviatilis species complex, apart from the cytologic method, DNA-based PCR assays are also available.16,15 The feeding preference of anophelines on humans constitutes a significant aspect of their vectorial capacity. The knowledge of feeding preference of A. fluviatilis becomes more crucial as the species complex exhibit some peculiar patterns of feeding habits.15,16 Proper recognition of blood meals, particularly from the human host, of the A. fluviatilis species complex provides information on the respective anthropophilic index and demonstrates changing patterns in host preferences, if any. There are different methods available to analyze the mosquito blood meal, such as ELISA,20-22 and gel diffusion techniques23; however, PCR-based detection24,25 is more convenient. Evaluation of sporozoite-carrying capacity of anophelines is significant in determining their vectorial potential. Although the mosquito feeds on human, it cannot be regarded as a potential malaria vector unless it transmits Plasmodium sporozoite. Therefore, in addition to determining the feeding preference, the PCR-based method for detection of sporozoite within the mosquito has been implemented here due to its sensitivity and specificity in com-
parison to other available methods, such as microscopy and ELISA.26–30

To determine the host preference and sporozoite rate simultaneously with species identification, we developed a multiplex PCR to detect *A. fluviatilis* species complex, their human host preference, and *P. falciparum* sporozoite presence. In addition, we have adopted a unique mosquito processing method to facilitate the detection.

**MATERIALS AND METHODS**

**Mosquito maintenance.** *Anopheles stephensi* were reared in an insectary of Regional Medical Research Center, Bhubaneswar, India, at 27°C and 80% relative humidity under a 12-hour light/dark cycle. Adult mosquitoes were maintained on a 6% glucose solution. Test mosquitoes were fed on human blood from the forearm of the human adult volunteers who had provided informed consent. The ethical committee of the Regional Medical Research Center, Indian Council of Medical Research, Bhubaneswar, India, approved both the procedures for the maintenance and care of the experimental animals and mosquito blood feeding on human volunteers. Blood-fed mosquitoes were kept under the same rearing conditions for 12 hours and freeze-killed prior to DNA extraction using the FTA Classic card protocol (Whatman, NJ) as per the manufacturer’s instructions.

**Mosquito collection and sampling.** Indoor resting *Anopheles* mosquitoes were collected from different malaria-endemic areas from the state of Orissa, India (Table 3) with the help of mechanical aspirators and CDC light traps. Attempts were made to collect the *A. fluviatilis* specimen based on its behavioral biting habits. To separate the *A. fluviatilis* mosquitoes from the other species, preliminary morphologic identification was carried out soon after the catch, as per the standard key of Christophers32 and Nagpal.33

**Mosquito processing and DNA isolation.** For the rapid screening of vectors from the pool of non-vectors, we have adopted a unique mosquito processing method (Figure 1). Immediately after the identification, each individual *A. fluviatilis* mosquito was homogenized in two separate parts in two different 0.5 μL microcentrifuge tubes containing 30 μL of phosphate-buffered saline, with the help of a micropestle. In one of the microcentrifuge tubes, only the head-thoracic region and in the other, the rest of the body was homogenized. Homogenates were then dispensed onto properly coded spots on FTA Classic cards (Whatman) separately. Half of the head-thoracic region homogenate was dispensed to an area on the card coded as spot A, and the rest part to another area coded spot B for the same mosquito; then half of the homogenate of the rest body parts was dispensed to spot B and the rest part to another spot C. The punched discs obtained from the coded spots on FTA cards were subjected to DNA isolation using the FTA Classic card protocol, followed by PCR. DNA isolation from the blood of *Plasmodium falciparum* infected persons was carried out using QIAaamp DNA Mini kit (Qiagen, Hilden, Germany) protocol.

**Primer design.** Primers were designed using the DNA sequences from the rDNA region of *P. falciparum* and *Homo sapiens*, which were downloaded from GenBank. Primers specific for those sequences were designed using PerlPrimer v1.1.7 software (Owen Marshall, http://perlprimer.sourceforge.net). During the software operation, different preferences were set to suit the need for the multiplex PCR. Primers, sequences, and melting temperatures ($T_m$) are shown in Table 1.

**Polymerase chain reaction assays.** *A. fluviatilis* species-specific polymerase chain reaction assay. For identification of *A. fluviatilis* species S and T from the other types, species-specific primers from the D3 domain of 28S rDNA region were used as per Singh31 with minor modifications. The PCR reaction mixture contained 1× PCR buffer containing 1.5 mM MgCl$_2$ (Sigma, St. Louis, MO), 200 μM each dNTP, 1.5 μM D3a primer, 1.6 μM D3b primer, 1.5 μM AFS primer, 1.5 μM AFT primer, and 0.6 unit of Taq DNA polymerase (Jump-Start Taq, Sigma) in a 25 μL reaction volume. Punched discs from spot C of a representative number of samples were used as templates for the reaction. Amplification was performed in

![Figure 1. Mosquito processing method. The head-thoracic portion and remaining abdominal parts of each mosquito were homogenized separately. Half of the homogenate from the head-thoracic region was dispensed to spot A and the other half to spot B. Similarly, half of the homogenate from the abdominal part was dispensed to spot C and the other half to spot B. Punched discs from these spots were then subjected to DNA isolation and PCR.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>No. of bases</th>
<th>$T_m$ (°C)</th>
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<tr>
<td>PF1</td>
<td>AGC GTG ATG AGA TGG AAG TCA G</td>
<td>22</td>
<td>63.3</td>
</tr>
<tr>
<td>PF2</td>
<td>CCC TAA ACC CTC TAA TCA TGG TC</td>
<td>23</td>
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<tr>
<td>HUM1</td>
<td>GGA GAG TTC TCT GGA AGA AAT GA</td>
<td>23</td>
<td>63.5</td>
</tr>
<tr>
<td>HUM2</td>
<td>TGA TAC CCT GGA AGT GAC AAA AT</td>
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</tr>
</tbody>
</table>
a thermocycler (MJ Research, Watertown, MA) under the following conditions: initial denaturation at 95°C for 4 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 8 minutes. A negative control was also included, containing all the ingredients in the PCR mixture except the DNA template. PCR-amplified products (10 μL) were subjected to gel electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized in an UltraLum gel documentation system (Claremont, CA).

**Multiplex PCR.** Using the designed, specific primers for the identification of *P. falciparum* and *H. sapiens*, respectively, PCR was performed independently under the identical conditions as used above for the *A. fluviatilis* cryptic species detection. There is a great deal of difficulty is involved in rearing *A. fluviatilis* in laboratory conditions. Therefore, during standardization of the *H. sapiens-* and *P. falciparum*-specific primers, the DNA isolated from the captive-bred *A. stephensi* fed with human blood and the DNA isolated from the blood of *P. falciparum*-infected persons were used, respectively. The PCR reaction mixture contains 1× PCR buffer containing 1.5 mM MgCl₂, 200 μM each dNTP, either 1.5 μM HUM1 primer and 1.5 μM HUM2 primer or 1.8 μM PF1 primer and 1.8 μM PF2 primer, and 1 unit of Taq DNA polymerase (JumpStart Taq, Sigma) in a 30 μL reaction volume. The amplification was performed in a thermocycler (MJ Research) under the following conditions: initial denaturation at 95°C for 4 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 8 minutes. The multiplex PCR was carried out with *A. fluviatilis* DNA (punched disc) mixed with the DNA isolated from the *P. falciparum*-infected person’s blood. The PCR reaction mixture contains 1× PCR buffer containing 1.5 mM MgCl₂, 200 μM each dNTP, 1.5 μM D3a primer, 1.6 μM D3b primer, 1.5 μM AFS primer, and 1.5 μM AFT primer for the *A. fluviatilis* species differentiation, and simultaneous addition of 1.5 μM HUM1 primer and 1.5 μM HUM2 primer for human host preference determination. 1.8 μM PF1 primer and 1.8 μM PF2 primer for the *P. falciparum* detection, and 1 unit of Taq DNA polymerase (JumpStart Taq, Sigma) in a 30 μL reaction volume. The PCR condition was as follows: initial denaturation at 95°C for 4 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 8 minutes. To test HUM primers’ specificity, DNA isolated from *A. stephensi* fed with human blood was used as positive control and DNA from *A. stephensi* fed with rabbit blood as negative control. Similarly, for PF primers’ specificity, DNA isolated from the blood of *P. falciparum*-infected persons was used as positive control and DNA from non-infected persons as negative control. Another negative control was also included containing all the ingredients in the PCR mixture without any DNA template. To prevent sample-to-sample cross-contamination, during all the steps of sample preparation for PCR, filter tips with an aerosol barrier (Axygen, Union City, CA) were used. PCR-amplified products (10 μL) were subjected to gel electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized in an UltraLum gel documentation system.

**Evaluation of multiplex PCR.** The efficiency of the multiplex PCR was evaluated on the *A. fluviatilis* specimen collected from different malaria-endemic parts of Orissa, India. For the initial screening round of multiplex PCR, DNA from the spot B was taken. Then only those samples that showed a *P. falciparum*-positive result were subjected to a second round of PCR using the spot A DNA and PF primers.

**RESULTS**

**Identification of *A. fluviatilis* cryptic species.** To identify the different cryptic species of *A. fluviatilis* viz. S and T from the other species, PCR was carried out following the method of Singh.19 DNA isolated from a representative number of mosquitoes from the selected areas was subjected to PCR. Twenty-nine of the 40 *A. fluviatilis* specimens tested were positive by PCR for S-type species with an amplified 295-bp product, whereas 11 were positive for T type with a 128-bp product. Besides, all the samples were positive for the D3 region of the rDNA and amplified a 375-bp product (Figure 2).

**Multiplex PCR.** To detect the *P. falciparum* and *H. sapiens* DNA within the *A. fluviatilis* mosquitoes, the collected samples were scanned by multiplex PCR. Multiplex PCR amplified the *A. fluviatilis* species S- and T-specific bands of 295 and 128 bp, respectively, with the common band at 375 bp. *P. falciparum*-specific PF1 and PF2 primers amplified a 205-bp PCR product, and *H. sapiens* specific HUM1 and HUM2 primers amplified a 519-bp product in the presence of respective templates (Figure 2). The cryptic species-specific bands were well separated from the *P. falciparum*-specific 205-bp PCR product and the *H. sapiens*-specific 519-bp product. Thus, non-interference of the products contributes to better readability of the multiplex PCR gel. As the *A. fluviatilis* DNA was mixed with the DNA isolated from the *P. falciparum*-infected person’s blood in the PCR reaction mixture, the gel of all the species types of *A. fluviatilis* exhibited *P. falciparum-* and *H. sapiens*-specific bands irrespective of their normal habit.
Testing multiplex PCR. Results of the multiplex PCR of the *A. fluviatilis* mosquitoes collected from different malaria endemic areas of Orissa, India, has been shown in Figure 3. Using the DNA from the spot B, we did the initial screening PCR of all the mosquitoes. This provided a preliminary idea of *P. falciparum* sporozoite presence and human host feeding habit of the respective types of *A. fluviatilis* mosquitoes. Any of the mosquito found to be sporozoite-positive in multiplex PCR was then subjected to second-round PCR with a DNA template from spot A using only PF primers. Table 2 gives details of the multiplex PCR results from the entire field of collected mosquitoes. Of the total 121 *A. fluviatilis* mosquitoes, 92 were S type and 26 were of T type. Three mosquitoes were of other types, for which no molecular markers are presently available. In first-round multiplex PCR (spot B analysis), of the total 92 species S mosquitoes, 22 showed a positive result for *P. falciparum* rDNA-specific PCR product; among them, 17 gave a positive result for human blood preference. Additionally, of the 70 *P. falciparum*-negative mosquitoes, 32 were positive for human blood preference. Of the total 26 T type mosquitoes, none was positive for *P. falciparum* whereas only two were positive for human blood preference. Three specimens that were of other types of *A. fluviatilis* (presumably U type) were negative for *P. falciparum* as well as for human blood preference. Second-round PCR was carried out using the DNA from spot A from the 22 species S mosquitoes found to be positive for *P. falciparum* in first-round multiplex PCR. Only four were positive for *P. falciparum*. These results indicate that these mosquitoes positively carried *P. falciparum* sporozoite in their salivary glands and that the remaining 18 mosquitoes probably had *P. falciparum* in their gut as gametocytes or as developing oocysts. The result of different types of *A. fluviatilis* cryptic species collected from endemic parts of Orissa, India is provided in Table 3.

**DISCUSSION**

PCR-based assays to differentiate cryptic species have received wide acceptance in epidemiologic studies worldwide. The speediness, sensitivity, and specificity of these assays proved to be extremely useful in scanning a bulk amount of sample in a short time. PCR assays are very effective in elucidating the role of individual species of anophelines in malaria transmission, and thus are helpful in devising control strategies at the earliest possible time. In addition, increased insecticide resistance among anophelines calls for urgent study and control of vector populations using rapid and effective methods.\(^4\)–\(7\) These assays can determine different attributes of the anopheline vector population together with the species identification very effectively. Here, we have incorporated two essential attributes, namely, human host preference and sporozoite presence, in the species-diagnostic PCR assay of *A. fluviatilis*. For this PCR assay, we have designed primers specific for *H. sapiens* and *P. falciparum*. The primer-design approach was targeted at the ribosomal DNA. The obvious reason is its variability across the species and the presence of thousands of copies throughout the genome, which lead to specificity and a high amplification signal, respectively.\(^8\)–\(38\)

<table>
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<tr>
<th>Area</th>
<th>Species S</th>
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<td>9</td>
<td>3</td>
<td>39</td>
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<tr>
<td>Total</td>
<td>92</td>
<td>26</td>
<td>3</td>
<td>121</td>
</tr>
</tbody>
</table>

**Figure 2.** Ethidium bromide-stained gel electrophoresis of multiplex PCR products of the *A. fluviatilis* species complex, *P. falciparum*, and human host preference during standardization. Lane M, 100-bp DNA ladder (NEB); Lanes 1 and 2, species S; Lanes 3 and 4, species T; Lanes 5 and 6, other type of *A. fluviatilis*. Lane 7 is a negative control with no DNA. A 375-bp band appears in all of the samples (1–6). Species S-specific bands of 295 bp appear in Lanes 1 and 2. Species T-specific bands of 128 bp appear in Lanes 3 and 4. The 375-bp band in only Lanes 5 and 6 indicates the species were of other types of *A. fluviatilis*. In samples 2, 4, and 6, the mosquito DNA was mixed with DNA isolated from *P. falciparum*-infected person’s blood, thus giving human and *P. falciparum*-specific bands of 519 and 205 bp, respectively.

**Figure 3.** Ethidium bromide-stained gel electrophoresis of multiplex PCR products of the *A. fluviatilis* species complex, *P. falciparum* sporozoite presence, and human host preference in the field-collected mosquitoes. Lane 1, negative control without any DNA template; Lane 2, negative control with DNA from unfed *A. stephensi*; Lane 3, another negative control with DNA from *A. stephensi* fed with rabbit blood; Lane 4, DNA isolated from the blood of *P. falciparum*-infected persons; Lane 5, blood DNA from non-infected persons; Lanes 6, 7, 9, and 11, *A. fluviatilis* species S (295 bp S-specific product); Lane 6, presence of *P. falciparum* sporozoite (205 bp) as well as human DNA (519 bp) is indicated; Lane 10, *A. fluviatilis* species T (128 bp T-specific product); Lanes 8 and 12, other types of *A. fluviatilis*; Lane M, 100-bp DNA ladder (NEB); Lanes 4, 5, and 6, human-specific 519-bp PCR product; Lanes 4 and 6, *P. falciparum*-specific 205-bp product; Lanes 6–12, common 375-bp product from the D3 domain of rDNA.
The blood meal analysis of mosquitoes in the field is the first step to establish their relationship with disease transmission. Anophelines that feed upon human beings carry the probability of transmitting malaria parasite. Therefore, it is imperative to check for the host preferences of Anopheles mosquitoes, particularly human host preference. This can be done by PCR using human DNA-specific primers. Any trace of human DNA present in the blood meal of the Anopheles mosquito can give a positive PCR result and would establish the malaria-carrying potential of that particular mosquito. PCR-detectable human DNA is present in the abdomen of mosquitoes for up to 24–33 hours post-feeding. This is particularly useful in the field when the collected specimen is normally processed after a time gap of 8–12 hours post-collection. Comparatively less degradation of human DNA takes place within the mosquito mid-gut during this time interval. Additionally, if field-collected mosquitoes are processed as described herein, the storage life of the DNA can be further enhanced.

To become a potential malaria vector, apart from the human host preference, an Anopheles mosquito must carry the Plasmodium parasite. Plasmodium sporozoite presence in anophelines was previously carried out by the dissection of salivary glands of individual mosquitoes and visualization under microscope, which is relatively laborious, time consuming, and requires technical expertise. This method is seldom used in vector control programs in areas where vector density is too high and highly zoophilic vectors are prevalent. Due to limitations of microscopy in epidemiologic studies, other more practical techniques, like serological (ELISA) and molecular techniques, such as PCR and RAPD, have been used for detection of sporozoites in recent years. The PCR method can detect as few as 10 sporozoites in the salivary glands of the anophelines as compared with 200–400 sporozoites required for CS antigen detection. The reported sporozoite load in the salivary gland of Anopheles gambiae s.l. ranged from 125 to 79,875, with a geometric mean of 1,743 sporozoites per infected mosquito; the geometric mean sporozoite loads in A. stephensi ranged from 6,608 to 17,702. This implies that anophelines with low sporozoite rates can be efficiently scanned for sporozoite presence by PCR. This assay is also very useful in entomological inoculation rate (EIR) determination, which is another essential parameter for the study of malaria epidemiology of an area.

Of the four species of Plasmodium that infect humans, P. falciparum causes most of the severe disease and deaths attributable to malaria and is most prevalent in Africa (south of the Sahara) and in certain areas of South-East Asia and the Western Pacific. Falciparum malaria causes more than 1.2 million deaths each year worldwide, thus it is deadlier than other forms of malaria. Taking into account the severity of falciparum malaria, it is vital to identify those vectors that carry P. falciparum with subsequent adoption of control strategy in a specifically targeted approach. Therefore, we have opted here for detection of anophelines that carry P. falciparum.

This unique method of processing mosquito samples, separately on filter papers with the three-spot approach, helps in rapid screening of malaria vectors from a heterogeneous population of anophelines in a particular area. This also aids in long-term storage of DNA by minimizing the degradation of DNA during transportation and storage under normal conditions. One or more persons with routine laboratory skills can easily manage immediate processing of the collected mosquitoes in the field. Once initial processing of the mosquitoes is done, the rest of the process can be easily carried out in a regular molecular biology laboratory. Rapid scanning of vectors is possible by using the DNA from spot B in multiplex PCR, in the first round of screening. This examines mainly the blood meal of the mosquito, specifically, for its human preference or lack of it. The test results showed that, of the total 121 A. fluviatilis collected from different malaria-endemic areas of Orissa, 49 mosquitoes from the species S and 2 from species T exhibited human blood preference. The anthropophagic nature of species S is evident from that result. The human preference of species T deviating from its normal zoophilic habit may be due to either accidental feeding of human being or a shifting pattern of host preference from animals to human. More mosquitoes are required to be analyzed to establish their link to human feeding habit. Once the human preference is detected for a mosquito, the next step would be the need to examine the same mosquito for the probable occurrence of P. falciparum. Simultaneous detection of host and parasite determines the vectorial capacity of an Anopheles mosquito. Those mosquitoes whose spot B analysis gives a P. falciparum-positive PCR have either ingested P. falciparum (gametocyte)-infected blood or carried P. falciparum (sporozoites) in the salivary gland. In our test, the number was 22, all from A. fluviatilis species S. Those positive samples are again subjected to another confirmatory PCR with a DNA template from spot A using only PF primers. If the second-round PCR gives a P. falciparum-negative result (18 mosquitoes in our test), then it can be inferred that P. falciparum malaria-infected persons dwell in the respective locality; a positive P. falciparum in first-round PCR may be due to developing oocysts or ingestion of P. falciparum (gametocyte) during blood feeding of the mosquito from an infected person. More mosquitoes from the area need to be tested for the presence of P. falciparum sporozoites in their salivary glands to establish their vectorial efficiency. A positive second-round PCR result for P. falciparum (four A. fluviatilis species S mosquitoes in our test) establishes sporozoite presence in the salivary gland of the respective mosquito, which may be responsible for active P. falciparum malaria transmission in that area. A flow chart depicting the inferences, which can be drawn based on the test results, is provided in Figure 4.

Along with such inherent advantages as speediness, sensitivity, and specificity, this multiplex PCR assay carries a limitation in the overall number of mosquitoes that can be processed. However, this can be overcome by devising different strategies depending upon the study objectives. For example, this assay can be used as an efficient method to screen a representative number of mosquitoes, which may reflect the vector competence of a larger population of a given area. The proper strategy can help in selecting the mosquito collection sites, the time of collection, etc. that aid in obtaining a representative sample to subject to this assay. The mosquito processing method adopted here provides a scope for better storage and processing of DNA samples on cards and also helps in rapid screening of malaria vectors of a particular area. This representative assay, which has been done with A. fluviatilis mosquitoes, can be applied to almost any type of mosquitoes by excluding the A. fluviatilis cryptic species-specific primers.
in multiplex PCR to detect the potential malaria vectors of an area. This assay, which offers the scope for simultaneous detection of \textit{A. fluviatilis} cryptic species types, their human host preference, and \textit{P. falciparum} susceptibility status, can be used as a tool in determining the malaria endemicity of a particular area, thus inviting intervention of a control strategy with better pace and precision.

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\textbf{FIGURE 4}. Flow diagram showing the inferences that can be drawn based on the result of the multiplex PCR assay.


