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The development and evaluation of a single step multiplex PCR for simultaneous detection of *Anopheles annularis* group mosquitoes, human host preference and *Plasmodium falciparum* sporozoite presence

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Summary The *Anopheles annularis* group mosquitoes, subgenus Cellia Theobald (Diptera: Culicidae), includes five recognized species: *An. annularis* Van der Wulp, *An. nivipes* Theobald, *An. pallidus* Theobald, *An. philippinensis* Ludlow and *An. schueffneri* Stanton. From these five, the three most common species found in Orissa were considered for this study because of their remarkable vectorial and behavioral variation and the important role they play in malaria transmission. To identify and understand their role in malaria transmission we developed a single multiplex PCR-based assay. This assay included the detection of human blood feeding habit and *Plasmodium falciparum* sporozoite presence. Of the 186 *An. annularis* mosquitoes collected, morphological character-based identification showed that 94 were *An. annularis*, 54 were *An. philippinensis* and 38 were *An. pallidus*. However, the multiplex PCR assay confirmed that 91 were *An. annularis*, 56 were *An. philippinensis* and 39 were *An. pallidus* individuals after adjustments were made for misidentified specimens in the morphological method. *Anopheles annularis* and *An. philippinensis* were found positive for human blood, and two samples of *An. annularis* species were positive for *P. falciparum* sporozoites. This one-step PCR-based method constitutes a very powerful tool in large surveys of anopheline populations.

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1. Introduction

The proper identification of *Anopheles* species, which are actively involved in malaria transmission, is essential in devising malaria control strategies in a particular area. There are 444 formally named and 40 unnamed members of species complexes recognized as distinct species of *Anopheles*.[1] There are about 40 species of anophelines that transmit human malaria, each differing in their transmission potential.[2] The *An. annularis* group consists of potential malaria vector species classified in the Neocellia series of the subgenus Cellia.[1] The group currently comprises five recognized species: *An. annularis* Van der Wulp, *An. nivipes* Theobald, *An. pallidus* Theobald, *An. philippinensis* Ludlow and *An. schueffneri* Stanton. The last species is restricted to Java and Sumatra,[3] and *An. nivipes* is mainly found in the northeastern region of India.[4] The remaining three species, *An. annularis*, *An. philippinensis* and *An. pallidus*, are fairly widespread. *Anopheles annularis* has been incriminated as a malaria vector in Sri Lanka, Bangladesh, Myanmar, Malaysia, India, Indonesia and China.[5–8] In India, its role in malaria transmission has been established in Orissa, Assam, West Bengal and Andhra Pradesh.[9–11] *Anopheles philippinensis* and *An. pallidus* are typically more limited in density and distribution and are regarded as secondary vectors of malaria in India.[12,13] The adults of the species are morphologically very similar and often difficult to distinguish, especially those of *An. philippinensis*, *An. nivipes* and *An. pallidus*, which cannot always be identified reliably unless accompanied by larval and pupal exuviae.[3] This results in misidentification in the field, where they often occur in sympatry. The vectorial and behavioral variations found among the species[13] constitute the major reason for needing accurate and precise identification.

Species identification in the past has mainly been performed using either morphological or cytogenetic methods. Morphological identification is dependent on various characters found in different life stages. Examination of these characters entails holding wild-caught blood-fed females for egg laying and rearing of larvae to fourth instars. The latter is by no means simple, as it is difficult to sustain the larvae under laboratory conditions. Mortality is high during the rearing process, which is also time-consuming, taking between 10 and 12 d before identification can be made. Polytene chromosome identification is much faster than using morphology, but the disadvantages of using cytogenetic tools routinely for identification purposes are that they are limited to half gravid females only. However, there is a variety of circumstances in which the molecular approach has greatly improved the accuracy of species identification.[14]

Molecular identification methods for species have mainly used rDNA because it is one of the multigene families frequently distributed in the genome. In this study, we report the use of a PCR-based assay that rapidly identifies the three most commonly found members of the *An. annularis* group of mosquitoes: *An. annularis*, *An. pallidus* and *An. philippinensis*.

Along with the species type there is a need to analyze the vectorial attributes of the *An. annularis* group to define their role in malaria transmission. The feeding preference of *Anopheles* mosquitoes on humans constitutes a significant aspect of their vectorial capacity. There are different methods available to analyze the mosquito blood meal, such as ELISA[15] and gel-diffusion techniques;[16] however, PCR-based detection[17] is more useful when multiple parameters are simultaneously evaluated. A mosquito feeding on a human 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 due to its sensitivity and specificity in comparison to other available methods, such as microscopy and ELISA.[18,19] Therefore the determination of species type, human blood feeding habit and *P. falciparum* presence within the members of the *An. annularis* group is essential to ascertain their role as malaria vectors in a particular area.

Our objective was to develop a rapid and effective assay that can clearly differentiate between species in the *An. annularis* group and determine their vectorial attributes. Therefore, in this article we report the development and use of a multiplex PCR assay to identify three of the most commonly found members of the *An. annularis* group along with human host preference and sporozoite presence.

2. Materials and methods

2.1. Study area

The study was carried out in specific areas of five districts of Orissa, India, i.e. Boudh, Gajapati, Phulbani, Cuttack and Keonjhar. As the state of Orissa is divided into four distinct physiographical regions, the districts were chosen from each region based on high endemicity of malaria reported by the state government. For instance, Keonjhar belongs to the northern plateau, Boudh to the central tableland, Cuttack to the coastal tract, and Phulbani and Gajapati to the Eastern Ghats of Orissa.

2.2. Mosquito collection

Adult resting mosquitoes were collected at dusk and dawn from both human dwellings and cattle sheds from August 2006 to February 2007, i.e. after the decline of the monsoon season and during winter. Mosquito collection was done in the morning hours, i.e. from 06:00 to 09:00 h, and in the evening, i.e. from 18:00 to 21:00 h.

2.3. Field processing and morphological identification of mosquitoes

The mosquito samples used in the study were from various localities in Orissa (Table 1). After collection, all mosquitoes were identified, based on their morphology as per the standard key.[20,21] After identification, each individual specimen from the *An. annularis* group was dissected into two parts; the head-thoracic part was kept in one microcentrifuge tube and the rest of the body in another. Then the mosquito body parts were preserved in iso-propanol for DNA isolations.