GENETIC CHANGE IN RELATION TO VECTORIAL CAPACITY OF ANOPHELINES IN ODISHA-REVIEW

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Abstract— In India, malaria is a major public health problem in states having predominantly tribal population. Anopheles fluviatilis is widespread in mainland India and is considered to be an important vector in hills and foothills contributing ~15% of reported cases annually. It has been extensively studied and recognized a species complex comprising three sibling species, i.e., S, T, U and a form 'V' based on cytotaxonomic study for fixed chromosomal inversions readable in the polytene chromosomes.

Keywords: PCR Amplification, Genetic, Malaria in Odisha, Plasmodium, Cytogenetic

I. INTRODUCTION

Malaria cases in India are reported throughout the year, since a perfect combination of average temperature (15–30 ◦C), rainfall and precipitation-inducing conditions persist across different parts of the country over all the seasons. With increasing ecological and man-made environmental change (e.g. urbanization, construction of dams, agricultural intensification, deforestation) malaria in India is exhibiting general trends from rural to urban malaria, from forest to plain malaria, and from industrial to travel malaria (Sharma et al., 2006). The highest incidence of malaria in India occurred in the 1950s, with an estimated 5 million cases and 0.8 million deaths per year (World Health Organization, Country Office for India). The launch of the National Malaria Control Program (NMCP) in 1953 resulted in a significant decline in the number of reported cases toand no reported mortality, by 1961. Despite its near elimination in the mid-1960s, malaria resurged to ~ 6.45 million cases in 1976. Since then, confirmed cases have gradually decreased to 1.6 million cases and ~1100 deaths in 2009. Recently, it has been suggested that the malaria incidence is between 9 and 50 times greater than reported (reviewed in Hay et al., 2010), with a ~13-fold under-estimation of malaria-related mortality (Dhingra et al., 2010). Such claims reinforce the need for robust and comprehensive epidemiological surveillance studies across the country (Singh et al., 2009) to determine the actual burden.

Vectors of Malaria in Odisha

Anopheles fluviatilis is widespread in mainland India and is considered to be an important vector in hills and foothills contributing ~15% of reported cases annually (Sharma, 1998). It has been extensively studied and recognized a species complex comprising three sibling species, i.e., S, T, U and a form 'V' based on cytotaxonomic study for fixed chromosomal inversions readable in the polytene chromosomes arm 2 (Nagpal and Sharma 1995; Subbarao, 1998; Raghvendra et al., 2011;Manguin et al., 2008; Rao, 1984; Subbarao et al., 1994); differentiation of S and T, however, not possible due to diagnostic inversion polymorphism but can be characterized by distinct biological characteristics and regional distribution.

Molecular Methods:

DNA or RNA probes: Advancements in DNA recombinant technology have facilitated the development of simple and rapid molecular tools for the identification of sibling species. The first of the DNA methods used to identify species was the use of DNA probes. Clones containing specific DNA segments of the undefined highly repeated component of the genome are identified by differential screening of genomic libraries with homologous and heterologous genomic DNAs. DNA segments from these clones are labelled and used as probes. The paper of Post and Crampton (1988) on DNA probes for the Simuliumdamnosum Complex and Black and Munstamann (2004) give procedures (with illustrations) used in the isolation of species-specific DNA probes for the identification of sibling species. Initially, radioactive probes were used. Simple nonradioactive probe assays for squash-blot hybridizations have been developed for the identification of members of the Gambiae (Hill et al., 1991), Punctulatus (Cooper and Burkot, 1991) and Dirus (Audtho et al., 1995) Complexes. Johnson, Cockburn and Seawright (1992) have improved the procedure to clean up the background in squash-blot hybridizations. Non-radioactive probe methods remove the hazards of radioisotopes and make the assays simple and usable under field conditions. The advantage with DNA probes, as with isozymes, is that species can be identified at all stages of the mosquito life-cycle. And if kits are developed, as they have been for the Gambiae Complex (Hill et al., 1992), probes can be used with much more ease in field laboratories.

Allele specific polymerase chain reaction (ASPCR): Allele-specific PCR (ASPCR) assays mostly exploit variation in the rDNAcistron. In anophelines this is X-linked (Rai and Black, 1999). It consists of tandem repeated arrays of conserved genes (18S, 5.8S and 28S) punctuated by rapidly evolving non-coding internal transcribed spacers, ITS1 between 18S and 5.8S and ITS2 between 5.8S and 28S. Each gene cluster is separated by intergenic spacers (IGS). Within interbreeding populations the arrays undergo rapid homogenization through concerted evolution, which drives new sequence variations to fixation, leading to species specific differences. For members of many species complexes, differences in ITS2 and variable regions within 28S rDNA gene have been used to develop ASPCR assays for members of the Culicifacies Complex (Curtis and Townson, 1998; Singh et al., 2004a), for the Fluviatilis Complex (Manonmani et al., 2001; Singh et al., 2004b), or the Dirus Complex (Walton et al., 1999) and for several nother species complexes prevalent in the Afrotropical, and Neotropical regions. Portions of the genes from mitochondrial genome, COI and COII, are also used to develop diagnostic PCR assays as has been done for members of the Culicifacies

Complex (Goswamy et al., 2006). For An. minimus species A and C, Kengue et al. (2001) used the RAPD marker assays of Sucharit and Komalamisra (1997) to develop a robust multiplex ASPCR. This assay distinguished other anophelines, *An. aconitus*, *An. varuna* and *An. pampanai*, which are morphologically very close to An. minimus and are found sympatric with *An. minimus*.

Morphological variation: Morphological characters that are often used to identify adults of anopheline species are largely confined to scale pattern and colour and their distribution. Characters that are used in the description of immature stages are sculpture of eggs, setation and pigmentation of larvae, and the forms of paddles and trumpets as well as chaetotaxy of pupae. Spermatheca and spiracular morphology are also used in the identification of species. In addition to light microscope examination for the specific characters, scanning and transmission electron microscopes are also used to study morphological variations. Morphometrics has proved useful in studying some species complexes when used in conjunction with statistical analyses.

Mitotic and meiotic Karyotypes: All anophelines studied so far have three pairs of chromosomes two pairs of autosomes which are either metacentric or submetacentric and a pair of sex chromosomes which are homomorphic (XX) in females and heteromorphic (XY) in males. X- and Y-chromosomes have been found as telocentric, acrocentric or subtelocentric, or submetacentric (depending on the position of the centromere in the chromosome) in different species of anophelines. The best mitotic chromosomes are found in the neurogonial cells of the brain in early IV instar larvae and meiotic chromosomes in the reproductive organs of newly-emerged adults (Breeland, 1961; French et al., 1962; Baimai, 1977).

Structural variants: Structural variations due to the position of centromere and quantitative variations in heterochromatin blocks are commonly observed. The variation in autosomes and X-chromosomes, which are found in the homozygous state, demonstrate reproductive isolation between the populations if heterozygotes for the variation are not found or are found in deficient numbers. This is similar to the situations described for paracentric inversions under polytene chromosomes. Unlike the banding pattern due to paracentric inversions, variations at a given position in the chromosome can exist as more than two alternatives.

Cytogenetic techniques:

Polytene chromosomes: Anopheline females in the semi-gravid stage have the best polytene chromosomes in ovarian nurse cells (Coluzzi, 1968). Larvae at the IV instar stage have polytene chromosomes in salivary glands. For those anopheline species which do not have good ovarian polytenes, larval salivary chromosomes can be used (but salivary gland polytene chromosomes are not very good in most anophelines). The advantage with adult females is that ovaries can be removed and fixed in modified Carnoy's fluid (1:3 glacial acetic acid: methanol) and can be used at any time. Another advantage is that the same female can be studied for other parameters such as host preference, presence of sporozoites/sporozoites antigen, susceptibility to insecticides. Hunt and Coetzee (1986) describe storing of field collected mosquitoes in liquid nitrogen for correlated cytogenetic, electrophoretic and morphological studies. The preparation of polytene chromosomes from adult females is not difficult. Polytene chromosomes are the result of repeated replication of chromosomes at interphase without nuclear division, the process being known as endo mitosis. Chromatids after division remain attached, causing thickening of chromosomes which results in the appearance of long ribbon-like structures with dark and light horizontal portions representing band and inter band regions respectively. The dark and light regions represent differential condensation of chromosomes. The banding pattern of each chromosome is specific in a given species; thus, each species differs from others in characteristic banding pattern. Any changes in the pattern can be easily detected. In the polytene chromosome complement, only euchromatic regions are seen and the heterochromatic portions of the chromosomes which are under-replicated are not seen.

Electrophoretic variations: Enzyme electrophoresis is extensively used in the study of species complexes. The technique involves the detection of the protein bands of an enzyme system with different mobilities as a function of electric charge and molecular structure. On a gel zymogram of an enzyme system, electrophoretic variations in the form of bands with different mobilities represent proteins coded by different alleles (allozymes). These alleles, being co-dominant, behave like paracentric inversions and the two homozygotes and heterozygotes can be differentiated. Variations at a locus thus enable the detection of the reproductive isolation between populations resulting from positive assortative matings within a population. Because of the simplicity of the procedures for the processing and interpretation of data, this technique permits large scale sampling of natural populations and is very useful as a diagnostic tool in the routine identification of species.

Molecular characteristics of sibling species

Since sibling species A, B, C, D and E of *An. culicifacies* are morphologically indistinguishable at any stage of life and due to practical difficulties associated with classical cytotaxonomic method for the identification of members of the complex; The ASPCR is essentially cheaper and quicker than the other methods and does not involve special treatment following PCR. However, the position of neucleotide variations is critical in designing primers for ASPCR so that all the allele-specific amplicons can be distinguished separately on a gel. Black and Munstermann (2004) show in detail the steps involved in PCR assay with illustrations.

Allele-specific PCR (ASPCR) assays mostly exploit variation in the rDNA cistron. In anophelines this is X-linked (Rai and Black, 1999). It consists of tandem repeated arrays of conserved genes (18S, 5.8S and 28S) punctuated by rapidly evolving non-coding internal transcribed spacers, ITS1 between 18S and 5.8S and ITS2 between 5.8S and 28S. Each gene cluster is separated by intergenic spacers (IGS). Within interbreeding populations the arrays undergo rapid homogenization through concerted evolution, which drives new sequence variations to fixation, leading to species specific differences. For members of many species complexes, differences in ITS2 and variable regions within 28S rDNA gene have been used to develop ASPCR assays for members of the Culicifacies Complex (Curtis and Townson, 1998) for the Fluviatilis Complex (Manonmani et al., 2001), for the Dirus Complex (Walton et al., 1999) and for several other species complexes prevalent in the Afrotropical, and Neotropical regions. In recent studies species E was identified by ASPCR technique from Odisha (Das et al., 2013) and Madhya Pradesh, (Sharma et al., 2014). The targeted region, D3 domain of 28 S rDNA, was amplified by PCR using universal primers, D3A and D3B designed for platyhelminth (Litvaitis et al. 1994) and later used for An. minimus (Sharpe et al. 1999) and for both *An. fluviatilis* and *An. culicifacies* (Singh et al. 2004a, b). An

allele-specific polymerase chain reaction (ASPCR) assay targeted to the D3 domain of 28S ribosomal DNA was developed. The assay discriminates *An. culicifacies* species A and D from species B, C and E. The assay was validated using chromosomally identified specimens of *An. culicifacies* from different geographical regions of India representing different sympatric associations. The assay correctly differentiates species A and D from species B, C and E. (Singh et al., 2004a). A polymerase chain reaction (PCR) assay based on the D3 domain (D3-PCR) of 28S rDNA and a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay involving ITS2 of rDNA are available for the discrimination of the members of the An. culicifacies complex. However, both these can only differentiate species A and D from species B, C, and E. Two allele specific PCR assays (AD-PCR and BCE-PCR) using sequence differences in the mitochondrial cytochrome oxidase II (CO II) subunit. The AD-PCR assay distinguishes species A and D, whereas the BCE-PCR assay distinguishes species B, C, and E. Thus, with a combination of two PCR assays, namely the D3-PCR/ITS2- RsaI assay, followed by either the AD-PCR or the BCE-PCR assay, it is possible toidentify individual specimens of any of the species of this complex. This assay system is the first, and the best available at present to distinguish all sibling species and especially to discriminate non-vector, species B from all the vector species, A, C, D, and E, of the An. culicifacies complex. Until another DNA-based method involving fewer steps is developed, this assay system can be used in all malaria epidemiologic studies where An. culicifacies is prevalent (Gowswami et al., 2006).

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