GENETIC CHANGE IN RELATION TO VECTORIAL CAPACITY OF ANOPHELINES IN ODISHA

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***Abstract*—**Malaria is a disease of significance to human health. Five species of Plasmodium, the protozoan parasite that causes malaria, are infectious to humans and are transmitted by Anopheles mosquitoes. Mosquitoes vector a variety of essential human pathogens with filarial, viruses and apicomplexan parasites. Traditional vector-control methods have been used to control these diseases for many years, they have yet to make lasting inroads in the battle to eliminate diseases such as malaria and dengue fever.In human malaria, transmission strength is highly reliant on the vectorial capacity as well as competence associated with local mosquitos. In this study to understand the factors of vectorial capacity and competence has greatly progressed in recent years; however, some aspects have been overlooked and the evolutionary pressures that affect them often neglected.

Keywords: PCR Amplification, Genetic, Malaria, Plasmodium

I. INTRODUCTION

A variety of factors contribute to the ability of a mosquito to transmit a pathogen successfully and to the efficiency of disease transmission, referred to as the vectorial capacity. The inherent capability of a mosquito to transmit the pathogen, or vector competence, is determined by genetic components of the mosquito and pathogen as well as by environmental components, including temperature. Other variables involved in vectorial capacity include the vector population density, the extrinsic incubation period required for vectors to become infectious and the daily survival rate of competent vectors. The advent of molecular biology, genomics and functional genomics has provided unprecedented opportunities to elucidate the complex interactions that take place between the mosquito vector and the pathogens it transmits. This new technology has led to significant advances in our understanding of how the mosquito's innate immune system is actively involved in eliminating large fractions of these human pathogens, sometimes rendering the mosquito vector completely refractory to infection (Garver et al. 2009). The progress made in basic research, together with the development of mosquito transgenic methodologies, has opened the way for the development of novel disease control strategies that are based on blocking pathogen transmission in genetically modified super-immune mosquitoes (Dong et al. 2011).

Odisha, an eastern Indian coastal state, occupying only 4% of land mass and 3% of national population, contributed about 27 % of total malaria incidence and approximately 18 % of reported deaths in the country in year 2013, making it the highest contributor to national malaria burden. Over the last decade, Odisha has drawn national and international attention

and has intensified anti-malaria activities in line with national initiatives. This initiative is intended to study the epidemiology of malaria in the state with exclusive focus on its temporal and spatial distribution over the last 11 years, thereby helping in the characterization of the disease trends in Odisha; which will be indicative of the effectiveness of the anti-malarial measures implemented over this period, and help predict trends.

Material and Methods

Study area, mosquito sampling and species identification

The study was conducted in the 5 districts of the Odisha state. Dry summer's rainy season and dry winters are the three prevailing seasons. *P. falciparum* is the predominant species, accounting for more than 90% of the total number of malaria cases. The occurrence of malaria is high level in the month of July-September and from November-December. *An.fluviatilis* was considered to be the main vector of malaria. Streams and rice terraces are the main breeding grounds of *An.fluviatilis*. The required number of female mosquitoes from *An. fluviatilis* was collected in the early morning time with the help of oral aspirator and a flashlight trap in the selected villages.

PCR Amplification and Sequencing

For determining the relative positions of *An. fluviatilis* cryptic species S, T, U and V in reference to *An. fluviatilis* individuals from other parts of the globe and as well as with *An. minimus* specimens, we have sequenced their (Internal Transcribed Spacer 2) ITS2 intronic region of the ribosomal RNA gene and compared with previously submitted sequences of *An. fluviatilis* and *An. minimus*. Genomic DNA was extracted from individual mosquitoes using FTA Classic cards (Whattman, NJ, USA) as per manufacturer's instruction. Punched discs from the samples were used as template for the reaction. Effort was to amplify the ITS2 intronic region of the ribosomal RNA gene.A ~515bp PCR product was amplifiedwith the forward primers ITS2a (5' - TGT GAA CTG GAG GAC ACA T - 3') and reverse ITS2b (5' - TAT GCT TAA ATT CAG GGG GT - 3'). The PCR reaction mixture contained 1X PCR buffer containing 1.5 mMMgCI£ (Sigma, St. Louis; MO, USA), 200 |iM each dNTP (Sigma), 1.7 pM ITS2a primer, 1.7 pM ITS2b primer and 1 unit of Taq DNA polymerase (JumpStart™ Taq, Sigma) in 25 pi reaction volume. The amplification was performed in a thermo cycler (MJ Research, Watertown, MA, USA) under the following condition; initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 1 minute; and a final extension at 72°C for 10 minutes. Negative controls were also included, containing all the ingredients in the PCR mixture except the DNA template. All the PCR amplified products (25 pi) were subjected to gel electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized in UltraLum gel documentation system (Claremont, CA, USA). The PCR products were purified from the gel with lllustra™ DNA and Gel Band Purification kit (GE Healthcare, UK). These purified PCR products were then processed for automated sequencing by dideoxy chain termination method using Genome lab DTCL-Quick Start Kit (Beckman Coulter, USA) utilizing the CEQ 8000 Genetic Analysis System (Beckman Coulter, USA), genetic analyzer. Sequencing was done in repeats for each sample and sequence analysis was performed with the software, provided in the instrument.

Sequence Alignment and Phylogenetic Analysis

Multiple sequence alignment of ITS2a, D3, COI and COII was carried out with Clustal W 1.7 multiple sequence alignment program (Thompson et al., 1994). We used the ITS2, D3, COI and COII sequences available from GenBank database for the comparison. Multiple sequence alignment of different species are given in figure 1(rDNA-D3), (ITS2) arranged as per species name followed by accession numbers of the sequences considered for the comparison. From the aligned sequences regions of maximum similarity were selected from D3, ITS2, COI and COII genes respectively, for further analysis. The DNA sequence-based phylogenetic analyses were performed using distance methods [Neighbour-joining (NJ)] and discrete character methods [Maximum Parsimony (MP)] included in the Molecular Evolutionary Genetic Analysis 4 (MEGA 4) programs version 4.0 (Tamura et al., 2007). Genetic distances were estimated using Tamura-Nei distance (Tamura and Nei, 1993) and Jukes-Cantor (Jukes and Cantor, 1969) method. The reliability of the branching order was determined by 1000 bootstrap replications (Felsenstein1985). The number of polymorphic, transition, and transversion sites was calculated from the program. Phylogenetic trees for all genes were reconstructed by the NJ and MP method. Gaps and missing data are considered as complete deletion and numbers of nucleotide substitutions per site were estimated. Phylogenetic trees were drawn using Tree Explorer (Tamura and Nei, 1993). *Aedes aegypti* and *Culex pipienspipiens* were chosen as out group while building tree.

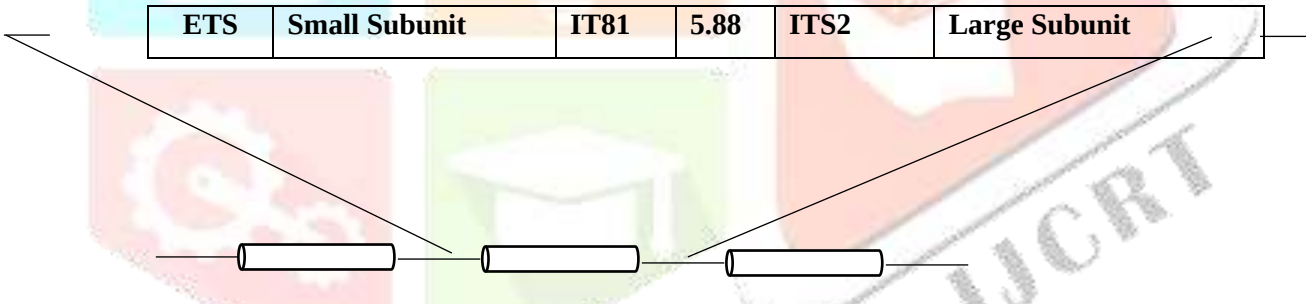

Figure 1. Ribosomal gene clusters in tandem repeats, separated by intergenic spacer

Discussion

Determination of anopheline vectors those particularly transmit malaria is an urgent need for malaria control strategy. Finding out the vectorial capacity of potential malaria vectors in a locality is always a challenge in formulating the control strategy.The molecular phylogeny obtained in this work matches the classicalmorphological taxonomy reasonably well. This varies considerably, however, at different gene levels. The proper arrangement of various anophelines taxa as per the traditional morphological character based classification of anophelines has been shown when we considered the ribosomal RNA gene in particular D3 region of 28S rDNA and Intergenic Transcribed Spacer 2 (ITS2) intronic region of rDNA. However the arrangement of the taxa did not match to that of morphological classification in some aspect, when we consider the COI and COII region of mitochondrial DNA. It may be due to variable degree of rate evolution of different genes and appropriateness of certain genes from the evolutionary point of view. Thus the selection of those particular genes which evolve at the rate which is reflected at the species differentiation level will help in constructing

proper phylogenetic relationship among anophelines and could be used to correlate with the grouping pattern done at morphological classification point of view. The analysis will be valuable in studies involving molecular taxonomy, particularly for those species that are difficult to identify using morphologic characteristics and in epidemiological research. Sequence comparisons of different geographic populations will give estimates of their genetic relatedness and provide information about vector movement. Comparison of sequences' of pathogen carrying and refractory strains of the same species should provide clues about the vectorial capacity of various strains of anophelines.

COI and COII are among the fastest-evolving mitochondrial genes and for this reason its phylogenetic signal is likely to be degraded by multiple changes when more diverged taxa are compared. Parsimony may be an inconsistent estimator of relationships under such conditions due to inability of the method to detect multiple substitutions on long branches. Performance of parsimony, however, can be improved by implementation of more realistic evolutionary models via differential weighting based on the information about potential saturation drawn from the data. Substantial phylogenetic information was retrieved from D3 and ITS2 despite the large sequence divergence observed among more distantly related taxa and the short fragment studied. These sequences provided very high support for relationships within Anopheles. A striking feature of D3 is the conservation of its sequences in closely related species of Anopheles. For example, the sequence of *An. minimus* C and *An. fluviatilis* S shows complete similarity. This may result in clustering of the species in the same clade. However this did not create any problem in drawing the affinity among the groups as per the cladistics parameters.

Therefore, to approach a better picture of anopheline evolution, additional information from independent loci is needed. Protein-coding single-copy nuclear genes are very promising candidates for the purpose and may add valuable information for the present trends in malaria vector divergence. While studying the drug resistance patterns of *P. falciparum* in an area, normally the clinical data is referred. This is only possible after a clear manifestation of the drug resistance parasites inside the human host, thereafter detected by healthcare persons. In this process the detection of drug resistant parasites in transmission cannot be detected unless symptoms from the patients are carefully observed. It will be of great use, if we can detect the spreading of drug resistant *P. falciparum* into a population, before any pathological symptoms are detected in humans. This can be possible by analyzing the anophelines vectors, transmitting malaria. Here, the result of the study indicates that by adopting the mentioned methodologies, we can detect the spreading of the drug resistant strains of P. falciparum in its transmission. This approach of studying the anophelines to search for drug resistant parasites may also help in tackling the sporadic cases of drug resistance due to spreading of malaria. The very nature of the present study is unique in its aspect and may reveal some interesting facts of malaria transmission by the survey of vector population of a particular area when wide scale evaluations will be undertaken. This will give a preliminary knowledge of status of drug resistance pattern of the studied area and help in devising control strategy with a proactive role, and enable a better drug regimen after having knowledge of resistant parasites.

Recently our understanding of the molecular interactions between Plasmodium and Anopheles has increased tremendously. Factors affecting vectorial capacity both positively and negatively have been identified in *An. gambiae* and research into the mechanisms of their mode of action is ongoing. The underlying genetics of the phenotypes of mosquito

refractoriness to Plasmodium infection, either through melanization or lysis of parasite midgut stages, is complex. From the identification of individual molecules that influence the outcome of an infection we are now beginning to understand the complex network and interplay of these molecules and their regulation. The enormous technological advances over recent years with the completion of the genome sequence and the establishment of haemocyte-like cell lines, RNAi, and genome-wide arrays, means that we are now in the position to not only identify a more and more complete picture of the interactions between the malaria parasite and its vector but also to identify and develop strategies to manipulate these interactions for disease control purposes.

The malarial parasite Plasmodium has to traverse the gut wall of the Anopheles mosquito in order to complete its lifecycle and to be transmitted between hosts. At the midgut stage of infection, the mosquito activates immune responses to eliminate most invading parasites. The features of these immune responses are not very well understood and have mainly been examined using the rodent parasite model *P. berghei*. The mosquito responses against these pathogens were quite diverse, and the defense against the two malaria parasite species involved both common and species-specific components. Malaria infected blood was sufficient to activate anti-Plasmodium immune responses, even in the absence of midgut invasion. Through this mechanism, the mosquito can initiate its defense against Plasmodium prior to invasion of the gut. Mosquito genes that could negatively influence Plasmodium development were also capable of regulating the resistance to bacterial infection, but several of the antibacterial genes had no effect on Plasmodium; thus, the mosquito apparently utilizes its antibacterial defense systems against the malaria parasite. During the result analysis of the immune genes we have considered for the present work, viz. DefA, CecA, CecB, CecC and Ppo, it was found that there are very rare non-synonymous mutations occurred in the coding sequence and most of the mutations are of synonymous nature resulting less deleterious effects on the polypeptide. The non-synonymous mutations are marked in *An. vagus* which is a non-vector for malaria as compared to vector species. This also suggests a high degree of selection pressure on this gene due to Plasmodium presence. We can further investigate towards the direction of in vitro and in vivo studies on the effects of the identified mutations on the overall effect of Plasmodium development inside the vector. Thus this will lead to the deciding factors of vector competency in anophelines.

Identification of both parasite antagonists and agonists in the vector is an important conceptual advance that sets the stage for dissecting the molecular mosquito-parasite interactions in detail. It also suggests a novel avenue for potential control of the malaria parasite in the mosquito. Establishment of the infection in Anopheles is essential for disease transmission and undoubtedly depends on multiple interactions of the parasite with other blood-meal components and with multiple mosquito tissues that it encounters sequentially. Of these interactions, the ones that occur during the transition from ookinete to oocyst, when parasite numbers are at a minimum, are especially important and most probably reflect a fine balance between positive and negative mosquito factors. Such a balance may prove a favorable point for chemical intervention to reduce malaria transmission.

REFERENCES

1. National Vector Borne Disease Control Programme, Directorate General of Health Services, Ministry of Health & Family Welfare, Govt. of India; 2013. [accessed on September 16, 2013]. National Vector Borne Disease Control Programme. *Malaria situation in India*.

2. Bhuwaneshwar: National Vector Borne Disease Control Programme, Ministry of Health & Family Welfare, Govt. of Odisha; 2013. Annual Report 2011-2012; p. 36.

3. Sharma SK, Upadhyay AK, Haque MA, Singh OP, Adak T, Subbarao SK. Insecticide susceptibility status of malaria vectors in some hyper-endemic tribal districts of Odisha. Curr Sci. 2004;87:1718–26.

4. Gunasekaran K, Sahu SS, Jambulingam P, Das PK. DDT Indoor residual spray, still an effective tool to control *Anopheles fluviatilis - transmitted Plasmodium falciparum* malaria in India. Trop Med Int Health. 2005;10:1–9.

5. Das M. A note on susceptibility status of some *Anopheles* to chlorinated hydrocarbon insecticides in Odisha. Bull Indian Soc Malaria Commun Dis. 1966;3:323–9.

6. Sahu SS, Gunasekaran K, Jambulingam P, Das PK. Susceptibility status of *Anopheles fluviatilis*, *An. annularis* and *An. culicifacies* to insecticides in Koraput district, Odisha. Indian J Malariol. 1990; 27:51–3.

7. Sahu SS, Patra KP. A study on insecticides resistance in *Anopheles fluviatilis* and *An. culicifacies* to HCH and DDT in Malkangiri district of Odisha. Indian J Malariol. 1995;32:112–8.

8. Chand SK, Yadav RS. Insecticide susceptibility of mosquito vectors in Sundargarh district, Odisha. Indian J Malariol. 1991; 28:65–8.

9. Sahu SS, Parida SK, Sadanandane C, Gunasekaran K, Jambulingam P, Das PK. Breeding habitats of malaria vectors: *An. fluviatilis*, *An. annularis* and *An. culicifacies* in Koraput district, Orissa. Indian J Malariol. 1990; 27:209–16.

10. Geneva, Switzerland: WHO; 1998. World Health Organization. Report of the WHO informal consultation. Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of monitoring insecticides on treated surfaces. WHO/CDS/CPC/MAL/98/12; p. 43.

11. Abbott WS. A method of computing the effectiveness of an insecticide. J Econ Entomol. 1925;18:265–7.

12. Kumari R, Thapar BR, Dasgupta RK, Kaul SM, Lal S. Susceptibility status of malaria vectors to insecticides in India. J Commun Dis. 1998; 30:179–85.

13. Singh RK, Dhiman RC, Mittal PK, Das MK. Susceptibility of malaria vectors to insecticides in Gumla district, Jharkhand state, India. J Vector Borne Dis. 2010; 47:116–8.

14. Singh RK, Dhiman RC, Kumar G, Sinha ATS, Dua VK. Susceptibility status of malaria vectors to insecticides in Koderma, Jharkhand. J Commun Dis. 2011;43:273–6.

15. Singh N, Khare KK. Forest malaria in Madhya Pradesh: changing scenario of disease and its vectors. Indian J Parasit Dis. 1999;23:105–12.

16. Vittal M, Deshpande LB. Development of malathion resistance in a DDT, HCH resistant *Anopheles culicifacies* population in Thane district (Maharashtra) J Commun Dis. 1983;15:144–5.