Vector Biology and Control

An Update for Malaria Elimination Initiative in India

Edited by

Vas Dev

M.Sc. (Hons.), Ph.D (Notre Dame), FNASc

The National Academy of Sciences, India

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Contributors

Sylvie Manguin, Vas Dev, Surya Kant Sharma, Rajpal Singh Yadav, Kamaraju Raghavendra, Poonam Sharma Velamuri, Vaishali Verma, Sreehari Uragayala, Susanta Kumar Ghosh, Khageswar Pradhan, Vijay Veer, Varun Tyagi, Manoj Kumar Das, Pradyumna Kishore Mohapatra, Ashwani Kumar, K. Hari Krishan Raju, Anupkumar Anvikar, Chazhoor John Babu, Virendra Kumar Dua, Tapan Kumar Barik, Usha Rani Acharya, Debojit Kumar Sarma, Dibya Ranjan Bhattacharyya, Anil Prakash, Nilanju Pran Sarmah

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Dedicated to

Vinod Prakash Sharma
(1938 – 2015)

In loving memory of Padma Bhushan Dr. Vinod Prakash Sharma for his laudable contributions in 'Malariology' and promoting 'Bio-environmental Control of Disease Vectors'. India stands better equipped with added knowledge in 'Vector Biology and Control' and above all skilled workforce to accelerate towards malaria elimination. His services and contributions will continue to inspire younger generations to strive hard innovating newer technologies for sustainable control of disease vectors in making malaria 'history'.

Reminiscing the Great Scientist and Mentor

"My mentor said, 'Let's go do it,' not 'You go do it.' How powerful when someone says, 'Let's!'" — Jim Rohn

Ever since the passing away of Dr. V. P. Sharma, Founder Director of the National Institute of Malaria Research, the fraternity of Parasitologists is still grieving. Dr. Sharma was a very special person who made an impact on the lives of many people who came in contact or worked in association with him. I am sure everyone who met him would have a story to tell; I wish to write a few lines about my reminiscences of him.

In the summer of 1995, a 3-day conference on Hill Science Research, organized at the summer resort-cum-lab of Sir JC Bose in Darjeeling (West Bengal) by a small group of scientists, gave me the first opportunity of meeting Dr. V. P. Sharma. He was a towering figure, globally known and recognized for his scientific achievements in the field of malaria research, while I was a little-known entity with my research interest limited to helminth parasites, though the common link between us was, of course, Parasitology. Meeting him was an overwhelming experience for me. Thereafter, I had a continued association with him for many years (till his final departure) on many fronts- National Academy of Sciences India (NASI) and Indian Society for Parasitology (ISP), in particular; I looked up to him as a mentor, whose guidance was always there whenever needed.

After the establishment in the year 2002 of the NASI North-Eastern Region Local Chapter in NEHU, Shillong (for which I was the convener), beginning 2006 Dr. Sharma initiated a NASI-sponsored Special Science Promotion Program for school students of North-East India. And together with a dedicated team of NASI Fellows and Members from the region, every year we conducted many hugely successful events for popularization of science among young minds throughout the north-eastern states. For every such event, Dr. Sharma always made it a point to join the team as a 'let's go and do it'approach, notwithstanding the remote, difficult terrains and travel ordeals of the northeast. I cannot forget the scary experience of one such program that we had organized in the far-flung area of Along (Arunachal Pradesh); we had to cross the mighty River Brahmputra via a ferry from Dibrugarh to Pasighat and travel further for hours by road in the Monsoon season. All this ordeal- for the sake of interaction with school children in facility-deprived locations!!

Dr. Sharma's contributions to ISP can never be forgotten. He was President of the Society for two consecutive terms. It was under his leadership that ISP organized the centenary celebration (in 2000) of Roland Ross in Hyderabad. Reposing much confidence in me, Dr. Sharma handed over the responsibility of ISP to me in 2007. He was Editor-in-Chief of Journal of Parasitic Diseases- the official journal of ISP published by Springer-India, since 2009 till his last days. What a moment of great joy and elation it was when Dr. Sharma's name was announced as the (first) recipient of the World Federation of Parasitologists-Distinguished Parasitologist Award during the 12th International Congress of Parasitology 2010 held in Melbourne, Australia!!

For me, Dr. Sharma was not only a great scientist, but also a caring, engaging, and thoughtful colleague, a mentor and above all a great human being. I count many blessings from him; perhaps, my nomination for Padma award (2016) was his parting gift to me.

Veena Tandon
(Formerly Professor of Zoology, NEHU, Shillong)
NASI Senior Scientist Platinum Jubilee Fellow
Editor-in-Chief, Journal of Parasitic Diseases (Springer)
Biotech Park, Janki Puram Sector G, Kursi Road,Lucknow-226021, India

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Foreword

As an medical entomologist, specialized on malaria vectors across three continents including Asia, it is a great honor for me to write the foreword of this book on 'Vector Biology and Control, An Update for Malaria Elimination Initiative in India' by Dr Vas Dev. I have known Dr Dev for few years now, especially as we co-edited the recent book "Towards malaria elimination – A leap forward" published in July 2018 [1]. This joint experience allowed me to interact with him -an accomplished scientist with vast field-based research experience in malaria control in India which made our collaboration both productive and fulfilling. Therefore, this is with immense pleasure that I wrote this 'foreword' and having an opportunity to share current knowledge and distribution maps of the dominant malaria vectors occurring in India.

The world is moving fast, and huge strides have recently been made inventing newer intervention technologies to lower the burden of some infectious diseases such as malaria. Since 2000, eight countries have been declared malaria-free by WHO including Maldives in 2015 and Sri Lanka in 2016, while other Asian countries have reported zero indigenous cases since 2017, such as China and Timor-Leste [2]. The initiative towards malaria elimination is underway globally and fast accelerating in 21 countries identified as E-2020 by WHO, which are on the move defeating malaria by 2020.

These optimistic results rely on great efforts at the national level on prompt and reliable malaria diagnosis concomitantly to new insights for targeting efficient malaria vectors helping control programs in active foci [3]. The fight against malaria will be successful only if these two modes of intervention are sustained and implemented universally. However, two major challenges may prevent malaria elimination and even escalate the risk of re-establishment of the disease transmission. One is the gradual attrition of expertise to correctly diagnose malaria while the disease becomes rare or absent and the second is the crucial lack of skilled medical entomologists, an expertise that is getting scarce. Therefore, this book on malaria vectors in India is step forward that I fully approve, as it will provide solid background information to students, as well as essential knowledge to medical entomologists, malaria control program managers and scientists alike interested in mosquito-borne diseases.

This book provides an updated information on malaria control in India along with illustrated account of bionomical characteristics of vectors in all ecotypes including rural, forested and urban areas. Status of resistance to insecticides, innovative vector control methods and integrated disease vector control approaches are also discussed. This book offers a clear picture of current knowledge on malaria transmission in India and clearly brings out the information gaps to be addressed for achieving malaria elimination by target date of

2027. The challenges for India to eliminate malaria are numerous, but integrated vector control tools and great expertise of Indian scientists that exists, when combined, should attain this cherished goal of freedom from malaria in foreseeable future. Let alone India, ending malaria transmission in this country of billion plus population would be a hallmark achievement in public health for the entire South-East region preventing re-establishment in malaria-free territories [4].

Prof. Sylvie Manguin
Institute of Research for Development
Montpellier, France

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Preface

Malaria elimination is a buzz word. Between 1955 – 2019, as many 38 countries and territories have been certified to be malaria-free by the World Health Organization and several others are targeting elimination by 2020 [1]. Aims and aspirations are high; the world is now aspiring malaria eradication by 2050, a mission that is considered ambitious but achievable [2]. In Southeast Asia, while Bhutan is reaching malaria elimination in the foreseeable future, countries like Sri Lanka and Maldives have already been declared malaria-free. Joining alliance with the Asian Pacific Malaria Elimination Network (APMEN) group of countries, India has registered appreciable decline in malaria transmission over the past few years [3], and accelerating towards elimination by 2027 in keeping pace with the World Health Organization Global Elimination Strategy [4].

Given the multiple challenges including climate change, deforestation, ecological succession, population migration, continued urbanization, paradigm shift in vector behaviour and growing menace of insecticide resistance; vector biology is regaining eminence as an integral component of the National Malaria Control Strategy [5]. To inform programme and policy managers, this book 'Vector Biology and Control: An Update for Malaria Elimination Initiative in India' includes updated information on distribution and bionomical characteristics of prevalent mosquito vector species specific to India, thus helping formulate appropriate intervention strategies in place and time to end transmission and prevent re-establishment of the disease in malaria-free territories mitigating the disease onslaught. It is projected that this compendium would be of immense value in putting together information on this very important topic, encouraging research on emerging issues and strengthening control interventions for achieving malaria-free status of the country by the target date of 2027 [6].

The book comprises 20 chapters split into six sections. Section-I relates to introduction giving distribution of major vector taxa in the country and significance of prioritizing species-specific interventions in context of malaria elimination initiative; Section-II includes an in-depth review of bionomical characteristics of the dominant mosquito vector species and disease transmission relationships; Section-III includes information on vectors of secondary importance and implications in the context of malaria elimination; Section-IV provides a present-day account of malaria transmission and control strategies inclusive of newer intervention tools for sustainable control; Section-V illustrates molecular taxonomic approaches and phylogenetic relationships of some important mosquito vector species; and Section-VI includes the executive summary giving salient findings and specific recommendations with reference to vectors of malaria in India for their effective control. Each chapter is based on credible field experiences giving updated first-hand information

on eco-biological characteristics of the respective vector species and provides good reference material for further reading.

The compilation is dedicated in loving memory of Padma Bhushan (late) Dr. V. P. Sharma, the founder Director of the National Institute of Malaria Research, New Delhi in recognition of his services promoting field-based research on malaria vectors and bio-environmental interventions for sustainable control. Dr. Sharma rejuvenated research on 'Malariology' post-resurgence and contributed significantly in this field of research evidenced by scholarly record of publications and level of expertise and services benefiting the world community. His stewardship and research efforts have culminated in number of intervention technologies, which have been field-evaluated and duly incorporated in healthcare services resulting in substantial decline in malaria transmission in areas hitherto considered intractable. He had extraordinary zeal for documenting research findings and promoting research at the grassroots for increased community awareness and participation in disease prevention and control programs. His relentless efforts have borne fruit for the National Vector Borne Diseases Control Programme helping formulate informed policies based on evidence-based interventions for effective vector control. India today stands better poised and equipped with skilled workforce and appropriate interventional technologies accelerating towards ambitious goal of malaria elimination, which is seen as the single largest achievement of this millennium in the domain of public health.

Dr. Sharma had been a constant source of inspiration for many research scholars. I drew my strength (to put together this compendium) from interactions with him for a long period of time spanning over 25 years. This volume is an outcome of my cherished association with him and a befitting tribute reminiscing his vision and contributions in the discipline of 'Vector Biology & Control', which would continue to inspire younger generation of scientists for field-based research and invent newer interventions to conquer malaria.

I am particularly thankful to Professor Sylvie Manguin, Institute of Research for Development, Montpellier, France for having agreed to write the 'Foreword' for me and her specific inputs sharing latest information on disease vectors and their distribution maps, and above all to my colleagues V. K. Dua, Anupkumar Anvikar, R. S. Yadav, Ashwani Kumar, S. K. Sharma, Anil Prakash, D. R. Bhattacharyya, S. K. Ghosh, Tapan Barik, K. Pradhan, P. K. Mohapatra, C. J. Babu and Vijay Veer (all have had significant interaction with Dr. Sharma) for contributing chapters and sharing valued information on disease vectors of importance in their respective field of expertise.

I also wish to accord due credits to Prof. Veena Tandon, Prof. U. C. Srivastava, Dr. Niraj Kumar and Dr. Chitranjan Sharma for extending cooperation and unstinted support, and to the National Academy of Sciences, India for accepting my proposal to publish this book as part of NASI's Publications. Thanks are also due to Dr. Purnima Sharma, Ms. Shreya Malik and Mr. Rajesh Kumar Sagar from Biotech Consortium India Limited (BCIL), New Delhi for their team effort and coordination all throughout the publication process.

Vas Dev, Ph.D.
Senior Scientist (Retired)
[Formerly at the ICMR - National Institute of
Malaria Research, New Delhi]

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Introduction

Chapter 1

Mosquito vectors of human malaria and geographical distribution

Vas Dev

Ex - Scientist, ICMR -National Institute of Malaria Research, New Delhi – 110 077, India

*Correspondence: mrcassam@hotmail.com

Abstract

In India, malaria transmission is supported by multiple vector species spread across varied eco-epidemiological regions. This chapter gives an brief overview of distribution of mosquito vectors and relative contribution of cases helping formulate species-specific intervention strategies to mitigate the disease onslaught. Priority areas of research and emerging challenges are identified which should be addressed to defeat malaria.

Keywords: Malaria, mosquito vectors, distribution range, malaria transmission, vector control, India

India is malaria endemic and disease epidemiology is complex on account of diverse ecological determinants and multiplicity of disease vectors [1]. Mosquito fauna is rich and as many as 61 different anopheline mosquito species have been reported to occur in India [2]. Of these, six species namely *Anopheles culicifacies s.l.*, *An. fluviatilis s.l.*, *An. minimus*, *An. baimaii*, *An. stephensi* and *An. sundaicus s.l.* are the predominant mosquito vectors with regional distribution (Figure 1) [3]. Among these, *An. culicifacies s.l.* is the most widespread breeding in diverse habitats in rural India generating about 65% of cases; *An. fluviatilis s.l.*, instead is the predominant vector in foothills of east-central and north-western states breeding in seepage water streams contributing 15% of cases annually. *An. minimus* and *An. baimaii* (sibling species of the *An. dirus* complex), prevalent in northeast India, are proven efficient vector species for high predilection for human host contributing 5% of cases each. *An. stephensi* is a vector of urban malaria generating 10% of cases, while *An. sundaicus s.l.* is a brackish species restricted to Andaman and Nicobar Islands contributing few hundred cases annually.

Most of these vector taxa are species complexes designated as *s.l.* (*sensu lato*), opposed to *s.s.* (*sensu stricto*), represented by one species only. These complexes comprise morphological

similar sibling species, except *An. stephensi*, which is known to have only species variants [4]. Ever since initial faunistic surveys of Christophers [5] and Puri [6], anopheline fauna has been updated periodically giving illustrated keys for easy morphological identification of mosquito adults [7-10]. But in the past decade, there has been significant added information on sibling-species composition of individual vector taxa based on molecular taxonomy and their bionomical characteristics related to disease transmission and control [11, 12]. Bionomical information on vector species relates to the correct species identification, which is crucial for implementing an efficient and appropriate vector control program. Vector control is an integral part of the National Vector Borne Diseases Control Programme and gaining eminence (formerly neglected) given the clarion call for malaria elimination by 2027 ahead of target date of 2030 [13, 14].

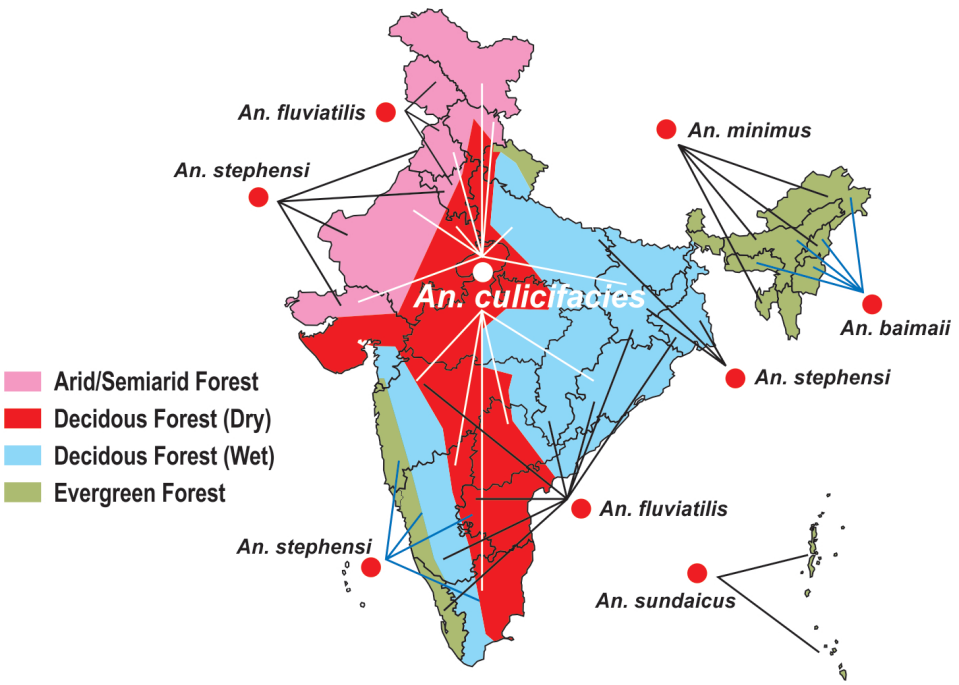

Figure 1: Regional distribution of the dominant mosquito vectors of human malaria in relation to physio-geographic regions encompassing evergreen tropical forest (wet zone receiving rainfall >200 cm), deciduous wet forest (monsoon forests receiving rainfall 100-200 cm), deciduous dry forest (scrub forest receiving rainfall 50-100 cm), and desert forest (arid and semi-arid area receiving rainfall <50 cm) annually. Source Reference [3]

Malaria elimination has become a reality with many countries certified to be malaria-free by the World Health Organization (WHO) [15]. In the Southeast Asia region, while Bhutan, China, Malaysia, Timor-Leste are heading for malaria elimination, Sri Lanka (2016) and Maldives (2015) have already been certified malaria-free. India alone contributes >70% malaria cases reported in Southeast Asia and has been identified among 11 high-burden nations under flagship initiative of WHO to catalyse the pace of progress towards elimination. Given the present-day intervention tools, India has made laudable progress in reducing malaria transmission to less than a million cases in 2017 (24% decline compared to 2016) and down to nearly half a million cases in 2018, yet there are multiple challenges ahead to achieve zero transmission [16, 17].

Inter alia, it is the diversity and diversification of the Asian mosquito fauna and changing transmission dynamics in the altered ecology which is considered detrimental and of paramount importance. With the rapid economic boom, infrastructure development, population migration, deforestation and urbanization, there has been ecological succession of some mosquito species accessing newer territories as well as paradigm shift in mosquito behaviour establishing outdoor transmission [18-20]. Vector species once considered of lesser significance, e.g., *An. subpictus* is getting recognition as vector of importance in western and southern Indian states resulting in perennial transmission in urban cities [21]. Indoor-residual spraying has become operationally difficult for poor community acceptance and diminishing returns [22]. Over and above, emergence of insecticide resistance in disease vector species has made control a formidable task and cost prohibitive [23]. Funding is far from adequate and intervention coverage of population at any risk remains off target [24]. Health infrastructure has not kept pace with the population expansion/migration from rural to urban/town areas permitting vector proliferation and increased receptivity for not only malaria but also other vector-borne diseases, dengue in particular [25].

In keeping with malaria elimination initiative in India [26], vector control should go together with anti-parasite measures on equal footing. Entomological surveillance for monitoring both population dynamics and insecticide resistance in targeting high-risk areas for focussed interventions should be the corners tone at the zonal level [27]. Cross-border initiative should be strengthened for data sharing and coordinated vector control with neighbouring countries preventing impending disease outbreaks and re-establishment of transmission in malaria-free territories; this activity would be even more important post-elimination [28, 29]. Insecticide resistance has emerged as the largest debacle not only locally but globally [30]. For management of insecticide resistance and sustained vector control, community-based interventions should be promoted and newer innovative technologies, viz., mosaic insecticide-treated nets, eave-tube, attractive toxic sugar-based baits should all be considered for operational feasibility [31]. Under country-led response in 'high burden to high impact' group of countries, WHO has advocated: (i) political will for greater allocation of resources in reducing malaria-attributable deaths, (ii) strategic application of tools for maximum impact, (iii) evidence-based policies and strategies, (iv) a coordinated national malaria response complemented by other partners making judicious use of resources for common goal to defeat malaria [32].

In the following pages, an attempt has been made giving up-to-date account of each mosquito vector species inclusive those of lesser significance including taxonomic status, distribution, sibling-species composition and disease transmission relationships, larval ecology, insecticide resistance and control options for species-sanitation. Follow up to bionomical characteristics of vector species, a full section is dedicated to malaria transmission control giving current status of insecticide resistance in disease vectors and modern-day intervention tools for disrupting transmission. A special section is added giving detailed account of molecular approaches for sibling-species identification and phylogenetic relationships using representative vector species for benefit of young researchers. It is projected that this volume would help programme officials developing informed policy for sustainable vector control to realize the ambitious goal of malaria elimination in due time.

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Bionomics of dominant malaria vectors and disease transmission relationships

Chapter 2

Emergence and spread of insecticide-resistant *Anopheles (Cellia) culicifacies*: its bionomics and control in the context of malaria elimination initiative in India

Sylvie Manguin[1] and Vas Dev[2*]

[1]Hydro Sciences Montpellier (HSM), Institut de Recherche pour le Développement (IRD), CNRS, Université Montpellier, F-34093 Montpellier, France

[2]Ex - Scientist, ICMR -National Institute of Malaria Research, New Delhi – 110 077, India

[*]Correspondence: mrcassam@hotmail.com

Abstract

Anopheles culicifacies s.l. is a predominant mosquito vector in rural India generating bulk of malaria cases (>60%) annually and maintains endemic malaria in areas of its influence. It is the most studied species complex for its sibling-species composition, distribution and their bionomical characteristics having implications in vector control. It is a highly adaptive and robust mosquito virtually resistant to all available insecticides including pyrethroids in large tracts of mainland India and fast invading new territories thwarting malaria elimination efforts. Control of *An. culicifacies* is a formidable challenge and containing its spread and transmission of drug-resistant malaria deserves utmost priority. Innovative technologies are warranted for sustained control of this vector species for achieving malaria elimination.Vector surveillance and monitoring insecticide resistance should be the continuing activity for formulating evidence-based and doable intervention strategies for effective vector management.

Keywords: *Anopheles culicifacies*, sibling-species complex, vector bionomics, insecticide resistance, malaria transmission, vector control, India

Introduction

India is malaria endemic and recently has been enlisted amongst 11 high burden countries by the World Health Organization for contributing 4% of the global estimated cases in 2017 [1]. The transmission is heterogenous across its landscape due to multiplicity of disease vectors, diverse ecology and contextual determinants [2]. Among six predominant mosquito vectors in India, *Anopheles culicifacies s.l.* is the most dominant one contributing >60% of the reported cases in the country [3, 4]. Historically, it was held responsible for devastating malaria epidemics in domain of its distribution in rural India and much of the malaria control efforts relate to containment of *An. culicifacies* alone even in the context of present-day malaria [5]. Malaria resurgence in 1970s may be attributed to the failure to control *An. culicifacies s.l.* inter-alia due to inadequate interventions and emergence of insecticide resistance resulting in rising densities and consequent predilection for human host [6]. Over the past three decades, a great deal of information has been generated in understanding its population genetics and bionomics for the benefit of the control programme helping devise appropriate control strategies [7-9]. It stands out to be the most studied mosquito species complex for its sibling-species composition, distribution, bionomical characteristics and role in disease transmission. India is currently experiencing rapid economic boom, population explosion/ migration, deforestation and infrastructure development resulting in expansion and ecological succession of other vector species in the altered ecology. *An. culicifacies* is one such species which is invading new territories and has grown multi-resistant inviting attention of programme planners and policy managers alike for its effective control [10-13]. Given the clarion call for malaria elimination in India by 2027 [14, 15], control of the malaria vector *An. culicifacies* deserves priority averting disease outbreaks and spread of drug-resistant malaria. We, hereby, present the overview of bionomics of this species complex in the context of malaria elimination for achieving sustainable control of this dominant mosquito vector in India.

Taxonomic considerations, sibling-species identification & distribution

An. culicifacies s.l is widely distributed in South and South-East Asia extending from Afghanistan to the far east to Thailand and Vietnam, with an eastern extension into the southern Arabic Peninsula and eastern Africa (Figure 1) [16]. It is a medium sized mosquito, adults of which can easily be distinguished from other species of the subgenus *Cellia* by diagnostic morphological characters (Figure 2) [17, 18]. Genetical investigations of this taxon revealed it to be a complex of five sibling species informally designated as species A, B, C, D and E based on species-specific diagnostic fixed paracentric inversions suggestive of pre-mating barriers in natural populations [7-9]. The five-sibling species are morphologically indistinguishable but can be substantiated by wide array of techniques including mitotic karyotype, Y- chromosome polymorphism, gene-enzyme variations, cuticular hydrocarbon profiles and diagnostic molecular assays. These sibling species are spread across India (Figure 3) and characterized to have distinct bionomical characteristics and distribution record with obvious implications for malaria transmission control [19]. Among these, species B is spread throughout rural India and occurs in sympatry with A, C and D. Whereas, species A and B are sympatric in north and south India; species A is

more abundant in the north than species B and vice versa in the south. In states of Uttar Pradesh, Bihar and north-east, species B is the only species that is prevalent or the most predominant. Species B and C are predominant in the west and east which overlap with species D in central and western Indian states. Species E has been exclusively recorded in south India and believed to be spreading to other endemic states.

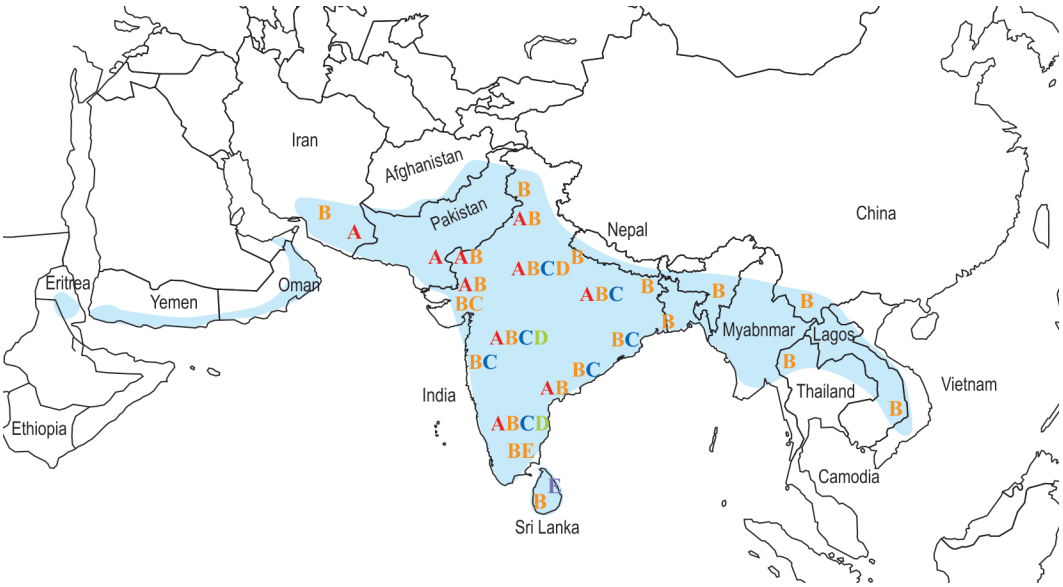

Figure 1: Distribution of sibling-species of *Anopheles culicifacies s.l.* in the world [sketch map not necessarily in conformity with political boundaries].

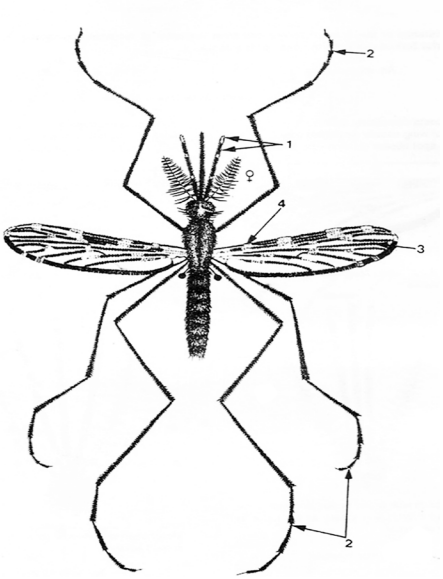

Figure 2: *Anopheles culicifacies:* morphological distinguishing characters of mosquito adult: (1) apical band nearly equal to pre-apical dark band on palpi, (2) tarsomeres without bands, (3) vein - 3 mainly dark, (4) inner costa interrupted. Source Reference [18].

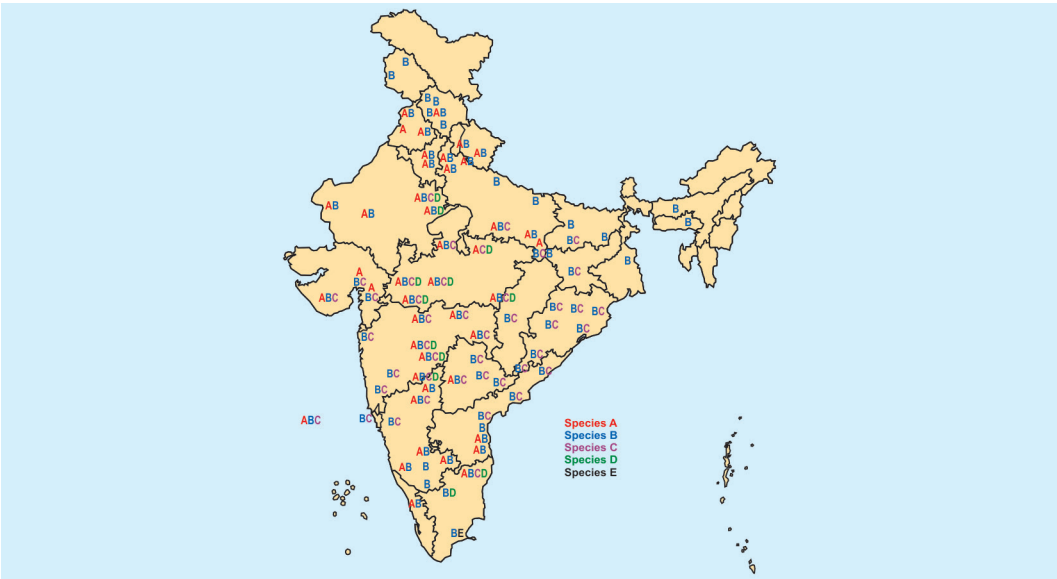

Figure 3: Geographical spread of sibling-species of *Anopheles culicifacies* complex (species A,B,C,D,E) in India. Source Reference [19].

Seasonal abundance, breeding and resting habitats

An. culicifacies s.l is widely abundant during monsoon and post-monsoon months in rural and peri-urban India. It occurs in low densities at high altitudes (1000 - 2000 msl), but most commonly found in plains receiving heavy to moderate rainfall. It is responsible for unstable malaria in large tracts of forested tribal belts [20], and given the ideal climatological conditions, it attains high densities resulting in focal/regional epidemics. It is a prolific breeder and habitats are diverse and numerous. These include irrigation channels, seepage-water streams, unused wells, river-bed pools, rice fields, miningand borrow pits, rocky pools and other fresh water collections (Figure 4). All member species rest indoors in human dwellings but known to rest outdoors as well in cattle sheds.

Host preferences, infectivity and disease transmission

All sibling species are predominantly zoophilic, except species E which is observed to be highly anthropophilic in Rameswaram Islands of Tamil Nadu and incriminated as malaria vector having high sporozoite infectivity [21]. Species A is reported to have relatively high anthropophilic index compared to B and D, and C having intermediate level of human blood index [7-9]. All member species of the Culicifacies complex are night biting, peak activity hours, however, varied anywhere between 18:00 – 23:00 h. *An. culicifacies s.l* has been repeatedly incriminated as vector by detection of gut and salivary gland infections across range of its distribution [18]. Sporozoite infection rates, however, varied amongst sibling species in range of their distribution. Among these, species A, C and D were proven vectors by immunoradiometric assays by cumulative infection rates of 0.51%, 0.3% and

0.4% respectively [22, 23]. However, species B is a non-vector or poor vector evidenced by low prevalence of malaria in areas of its occurrence. These observations were further substantiated by fitness studies for reproductive potential, sporogony and insecticide susceptibility [24]. Disease transmission is largely seasonal during monsoons and post-monsoon months corresponding to build up of high vector density.

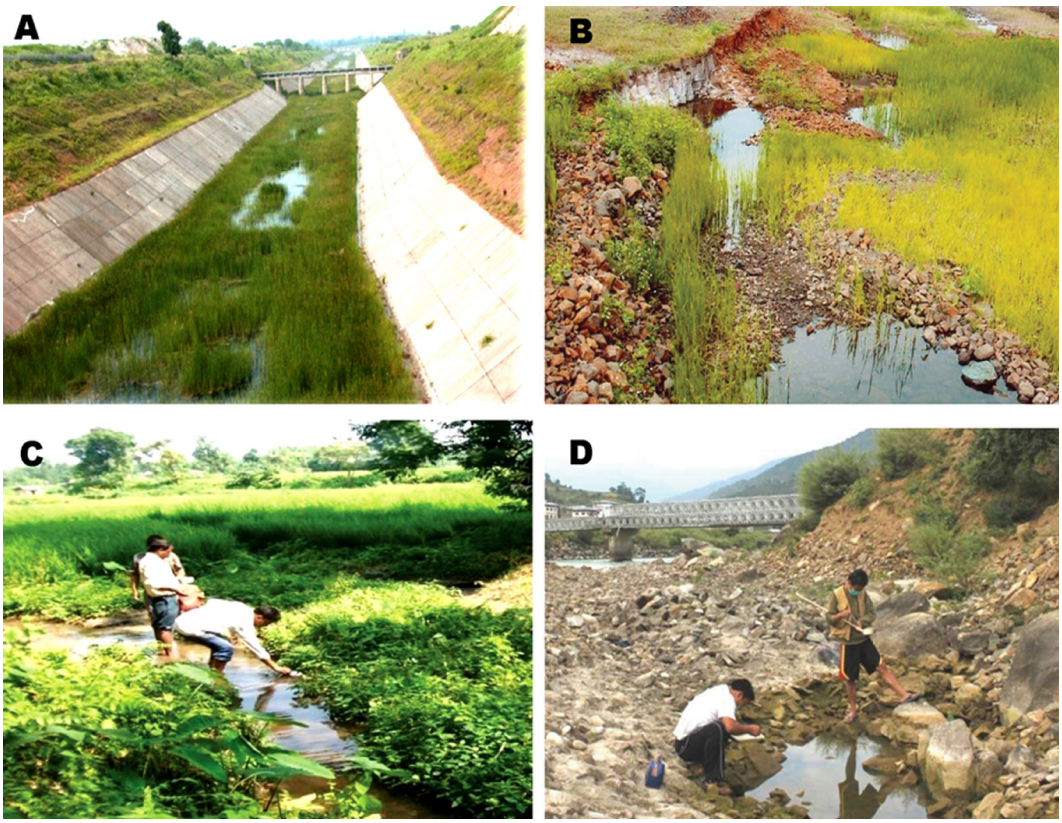

Figure 4: Larval habitats of *Anopheles culicifacies*: (A) Irrigation channels with residual water pools, (B) Rainwater collections in ditches/pools (courtesy: Neeru Singh), (C) seepage-water streams, (D) river-bed pools (courtesy: Rinzin Namgay).

Insecticide resistance and vector control

Insecticide resistance in malaria vectors is a global phenomenon [25, 26]. The Indian National Vector Borne Disease Control Programme relies heavily on application of residual insecticides for vector control [27]. DDT proved to be an "angel" during 1953 – 1960 resulting in dramatic decline of cases from 75 million in pre-DDT era to less than one hundred thousand cases in 1960s in India [28]. However, in 1970s, malaria re-emerged with vengeance to six million cases on record, which was largely attributed to development of resistance to DDT in *An. culicifacies* maintaining endemic malaria [6]. The phenomenon of emerging resistance was unstoppable across arsenal of insecticides including cross-resistance with organo-chloride compounds (Dieldrin), and other classes of insecticides, i.e., organophosphate (malathion) and more recently pyrethroids (deltamethrin) making

control of rural malaria a difficult enterprise (Figure 5) [29-31]. These data are corroborated by low frequency of the *kdr* allele (mostly in heterozygous condition) in field populations that confer resistance to DDT and pyrethroids [32]. *An. culicifacies* is presently resistant to virtually all available insecticides and worse that multi-resistant populations are now proliferating and spreading to newer territories. It is reported in high densities in degraded forests of north-east India replacing susceptible populations of *An. minimus* resulting in ecological succession sharing similar resting and larval habitats [10-13, 33].

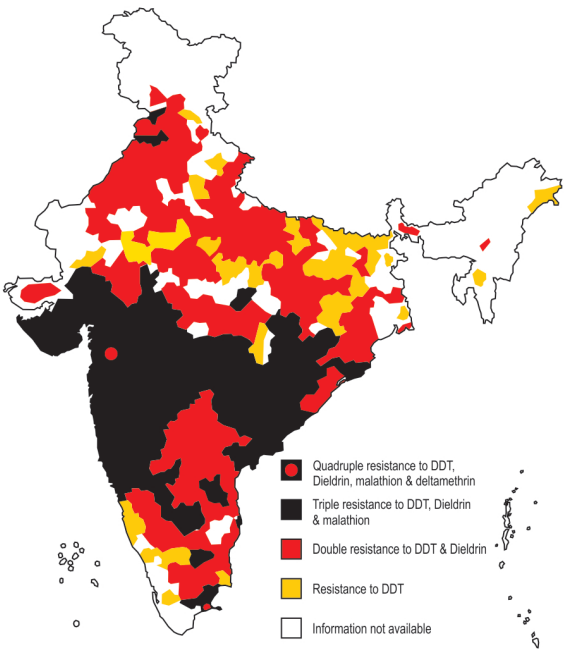

Figure 5: Status of insecticide resistance in *Anopheles culicifacies* in India for data based on 2006. Since then, *Anopheles culicifacies* has invaded north-east India and is resistant to DDT and Dieldrin in almost all parts of the country, and to DDT, Dieldrin and Malathion in large parts of central and western states, and to all insecticides including pyrethroids in certain parts of Gujarat and Tamil Nadu.Source Reference [29].

Among high-burden countries, India holds the distinction for reporting steady decline in cases over the past few years registering 24% decline in 2017 compared to 2016 [1, 34]. It can be largely attributed to the combination of interventions including induction of insecticide-treated netting materials/long-lasting insecticidal nets for vector control, artemisinin-based combination therapies (ACT) circumventing the development and spread of drug-resistant malaria, dipsticks for on-the-spot rapid diagnostic tests (RDT) ensuring early case detection, human-resource development in strengthening healthcare services in the periphery, and above all external financial assistance by international agencies for enhanced coverage that made the difference in rolling back malaria to less than a half million cases in 2018, and counting towards malaria elimination in the foreseeable future [15]. However, threat looms large for expansion of insecticide-resistant populations of *An. culicifacies*, which would spell doom to the programme evidenced by rising proportions of *Plasmodium falciparum* each passing year to presently >60% of total reported cases in the country [35]. There is an imperative need for monitoring insecticide resistance and developing risk-maps for focussed coverage for keeping its populations at bay.

Sibling species paradox and implications in malaria vector control

It is ironic that given the wealth of added information in understanding the sibling-species composition and bionomics of *An. culicifacies*, nowhere this information has been utilized in the national control programme. The techniques applied for confirmed identification for sibling-species A, B, C and D are not diagnostic except that of fixed inversions readable in the polytene chromosome karyotype (Table 1). Besides, there being a great deal of overlap in the geographical range of distribution of sibling species (Figure 3); identification is far more a skilled exercise well beyond the capacity of field-health workers making it an operationally difficult choice for control interventions in resource-poor settings.

Table 1. Techniques applied for characterization of sibling-species of *Anopheles culicifacies* complex

Sibling species	Polytene chromosome inversion genotype	Mitotic karyotype Y- chromosome	LDH enzyme alleles*	Species specific DNA probes	PCR-RFLP**	ASPCR***
A	$X+^{a}+^{b}$; $2+g^{1}+h^{1}$; $+i^{1}/i^{1}$	Submetacentric	Fast	Yes	Yes	Yes
B	Xab; 2g1+h1	Acrocentric, Submetacentric	Slow	Yes	Yes	Yes
C	Xab; 2+g1h1	Acrocentric, Submetacentric	Slow	Same as B	Same as B	Same as B
D	X+a+b; 2i1+h1	Submetacentric	Fast	Not tested	Same as A	Same as A
E	Xab; 2g1+h1	Submetacentric	Slow	Not tested	Same as B	Same as B

LDH: lactate dehydrogenase; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; ASPCR: allele specific polymerase chain reaction. Adapted from source Reference [9].

Seemingly, there are lapses in reaching correct identification except the existence of A and B (initially proposed by Chris Green) validated by post-zygotic fertility data marked by hybrid male sterility, reduced fertility and atrophied gonads [36]. However, similar dataon reciprocal crosses between remaining sibling species are far from adequate, *inter-alia*, which would have been a clinching evidence to prove their existence beyond doubt [37]. Moreover, application of PCR based r-DNA diagnostic assays clearly clubbed sibling-species into two distinct groups, i.e., group I (species A/D) and group II (species B/C/E) [38]. Besides, based on the cumulative data on mitotic chromosomes karyotype, gene-enzyme system, species-specific DNA probes and distribution records, it is imperative that true prevalence of species C, D and E requires introspection (Table 1). It seems as if species A and D are similar; species C is akin to B (except polytene chromosome karyotype); instead species B and E are the same corroborated by homosequential polytene chromosome karyotype and sequencing of ITS2 and D3 regions of 28S rDNA [39]

In essence, species A and B are only the two true breeding species requiring species-specific control interventions for effective vector management. The existence of possible morphological differences between sibling-species have not been explored and need to

be prioritized enabling binomial nomenclature. Apparently, there are glaring gaps of information, which have been clearly overlooked [16]. In summary, *An. culicifacies* species complex is far from being resolved and for control of its populations, irrespective of prevalence of any of its sibling-species in given area, intensification of interventions has become of paramount importance in order to eliminate residual populations preventing re-establishment of active transmission in malaria-free territories.

Priority areas of research

The expanding range of distribution of *An. culicifacies* in north-east India is an emerging threat to the control programme for continued transmission and spread of multi-drug resistant malaria. The proportions of drug-resistant *P. falciparum* malaria are steadily risingfor which north-eastern region is considered high-risk zone for proliferation and spread to peninsular India [35, 40]. Low-grade artemisinin resistance has already surfaced in north-east along international borders evidenced by detection of *kelch-13* mutations [41-43]. *An. culicifacies* is robust, invasive and highly adaptive species in varied ecological contexts for which interventions should be strengthened for universal coverage ensuring maximal compliance. The emergence and spread of this species must be contained by innovative technologies aiming reducing its density below threshold and defeating insecticide resistance. There exists scope for alternate mechanisms for insecticide resistant management like insecticide rotation [44, 45], and newer technologies, viz., eave-tubes, attractive toxic sugar baits (ATSB), mosaic long-lasting insecticidal nets, which must be put to field-evaluation in different transmission settings [46]. These interventions combined with other bio-environmental approaches, e.g., larvivorous fish (guppy and *Gambusia*) and above all behavioural change communication educating communities would help achieve sustainable control of vector populations [40]. Early biting behaviour of mosquito vectors associated with out door transmission are seen as crucial challenges, which must be addressed by appropriate technologies, viz., insecticide-treated plastic sheeting/ hammocks, use of repellents, to cite a few, in context of malaria elimination efforts in the South-East Asian region of the World Health Organization [47, 48]. North-east India shares wide international border with Myanmar to the east, Bangladesh to south and Bhutan to the west having similar ecology and disease vectors. These borders are porous and ill equipped to meet the complex emergencies permitting mix of parasite strains and resultant focal disease outbreaks and propagation of drug-resistant strains. Cross-border initiative with these member countries deserves priority for coordinated action for vector control operations to arrest the spread of drug-resistant varieties of malaria emanating across borders [49].

Conclusions

An. culicifacies has grown multi-resistant to the insecticides. The emergence of spread of this invasive speciesis a formidable challenge control of which has regained importance in the wake of malaria elimination efforts in the Asia Pacific Malaria Elimination Network

of countries (APMEN). To contain its spread, country-led response is mandated for vector surveillance, monitoring insecticide resistance, data sharing and cross-border initiative for coordinated action; all these should be the core-activities for mitigating impending disease outbreaks and spread of drug-resistant malaria [50]. In keeping with 'high burden to high impact' flagship initiative of WHO, much more can be achieved in transmission reduction by strategic application of technologies that are evidence-based, community-oriented and doable, and above all political commitment for strengthening healthcare services in the periphery/high-risk foci ensuring universal access to realize the envious goal of malaria elimination in India in due time [51-53].

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Chapter 3

Anopheles (Cellia) fluviatilis James 1902: an efficient vector of malaria in hills and foothills of India

Khageswar Pradhan*

ICMR-National Institute of Malaria Research (Field Station), Rourkela – 769 002, Odisha, India

*Correspondence: kpradhan4@gmail.com

Abstract

Anopheles fluviatilis has been characterized to be species complex comprising sibling-species S, T and U, of which species 'S' is proven an efficient vectorof malaria in the hills and foothills of India. It acts as relay transmitter in winter months for its seasonal abundance and infectivity supplementing transmission by *An. culicifacies* in large tracts of mainland India, and *An. minimus* in east-central and north-eastern states maintaining hyperendemic malaria. Its control is of paramount importance for generating 15% of total reported cases in the country annually for which strengthening entomological surveillance and vector management strategies are mandated averting impending disease outbreaks. For sustainable vector control, universal coverage of insecticide-treated nets is advocated for their proven efficacy in reducing vector density and transmission intensities in high-risk states.

Keywords: *Anopheles fluviatilis*, sibling-species, foothill malaria, epidemiology, relay transmission, bionomics, vector control, India

Introduction

Anopheles fluviatilis is an efficient vector for maintaining hyperendemic malaria in the hills and foothills of rural India generating about 15% of reported cases in the country annually [1]. It continues to inflict high degree of morbidity in the outreach forest-fringe/hill tract population groups and the problem is compounded due to logistics, poor access, inadequate healthcare services and little awareness on disease prevention and control. Among the dominant mosquito vector species [2], *An. fluviatilis* is ranked second to *An. culicifacies* for contributing bulk of cases and has been the subject for in-depth investigations for its bionomical characteristics and disease transmission relationships. What initially thought to

be comprising races for differing population characteristics in seasonal abundance, ecotype and varied infectivity [3], it has now been characterized to be species complex throughout its range of distribution having implication in control strategies [4]. Control of *An. fluviatilis* transmitted malaria is vital to the programme to prevent disease outbreaks due to its high anthropophily (strong predilection for human host) and consequent propagation of drug-resistant malaria for which India is considered corridor for spread to rest of the World. This species occurs in sympatricity with *An. minimus* in the north-east and east-central state of Odisha (formerly Orissa), as well as *An. culicifacies s.l.* in rest of India, and acts as relay transmitter evidenced by seasonal abundance and infectivity in cooler months maintaining perennial transmission [2, 4]. The trio including *An. culicifacies*, *An. fluviatilis* and *An. minimus* together contribute nearly 85% of cases in the country requiring prioritization for vector control. Included in this chapter is review of the available knowledge on this important vector species to help formulate species-specific interventions for sustainable control.

Taxonomic considerations & distribution

Anopheles fluviatilis s.l. is small to medium sized mosquito and can be easily distinguished from other members of the subgenus *Cellia* by given morphological characters (Figure 1).

Aided by cytotaxonomic and molecular tools, it has now been recognized to be comprised of morphologically indistinguishable sibling-species, S,T and U [2, 4]. These sibling-species can be characterized by diagnostic fixed paracentric inversions readable on polytene chromosome arm 2 as well as distinct biological characteristics including relative abundance, resting and feeding preferences, prevalence record in ecotype and infectivity

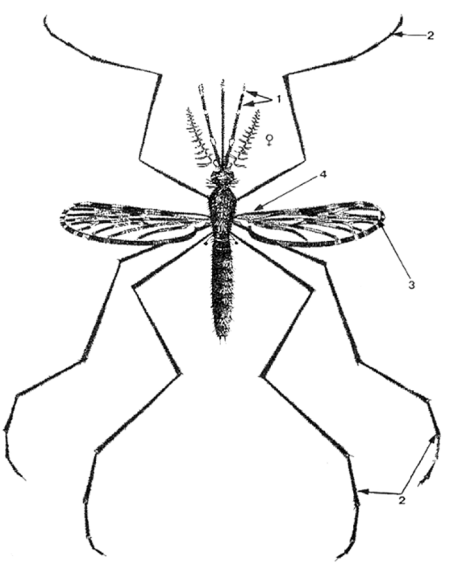

Figure 1: *Anopheles fluviatilis*, morphological distinguishing characters of mosquito adult: (1) apical pale band nearly equal to pre-apical dark band, (2) tarsomeres without bands, (3) vein 3 mainly pale, (4) inner costa completely dark.Source Reference [5].

(Table 1) [6]. In addition, these sibling species can be identified unequivocally by molecular techniques based on rDNA-ITS2-PCR as well as differences in nucleotide sequences within the D3 domain of 28S rDNA [7,8]. Based on these tools, earlier identified populations of *An. fluviatilis* in north-eastern state of Assam are now characterised to be hyper-melanic seasonal variant of *An. minimus s.s.* [9]. However, conspecificity of Indian populations of *An. fluviatilis* species 'S' with that of *An. harrisoni* (species C of *An. minimus*) proposed by Garros *et al* [10] and Chen *et al* [11] could not be validated [12].

Table 1. Inversion genotype and biological characteristics of *Anopheles fluviatilis* sibling species complex in India. Source Reference[13]

Sibling Species	Inversion genotypes on chromosome arm 2	Mosquito densities (per person hour)	Feeding preference	Preferred adult habitat	Prevalence	
					Ecotype	Endemicity
S	+q′+r′	Low-to-moderate (1-40)	Anthropophilic	Human dwellings	Hilly forests & foothills	Hyperendemic
T	q′+r′	High (up to 200)	Almost totally zoophilic	Cattle sheds	Foothills & plains	Hypo-mesoendemic
U	+q′r′					

An. fluviatilis is widely distributed in the Oriental region and parts of the West Asian subregion; the latter includes Pakistan, Afghanistan, Iraq, Iran, Saudi Arabia, Oman, Bahrain (Figure 2). In the Oriental region, it is prevalent in India, Nepal, South China, Bangladesh, Myanmar, Indochina and Thailand [3, 4]. Within India, it is wide spread in mainland and has sympatric distribution with populations of *An. culicifacies*, yet sibling-species of both have variable distribution pattern (Figure 3). It is not reported in Union Territories of Andamans and Lakshadweep.

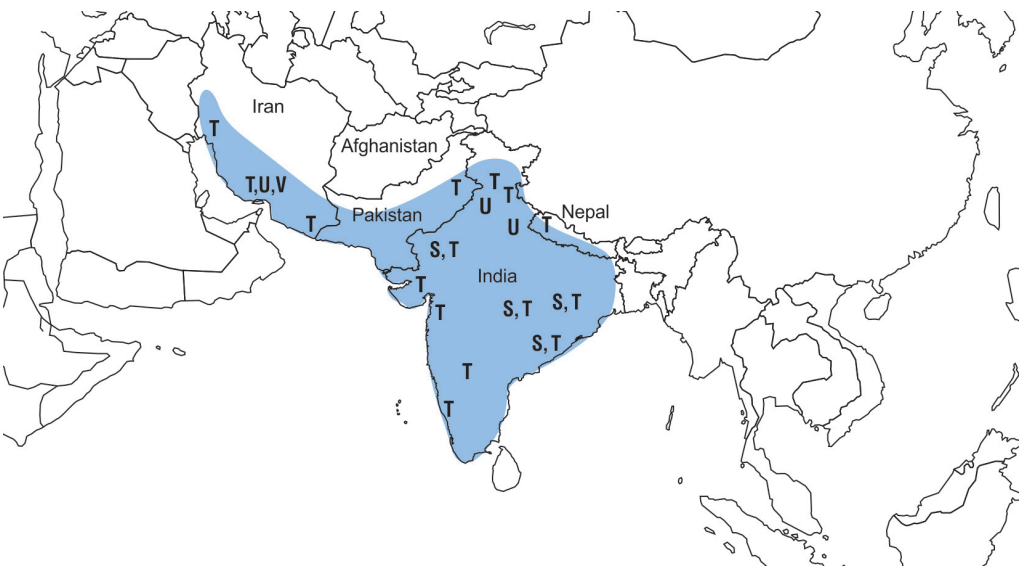

Figure 2: Distribution of *Anopheles fluviatilis* complex (S, T, U, form V) in in India and Western Asia (Courtesy: Sylvie Manguin, Montpellier, France) [Sketch map not necessarily in conformity with political boundaries].

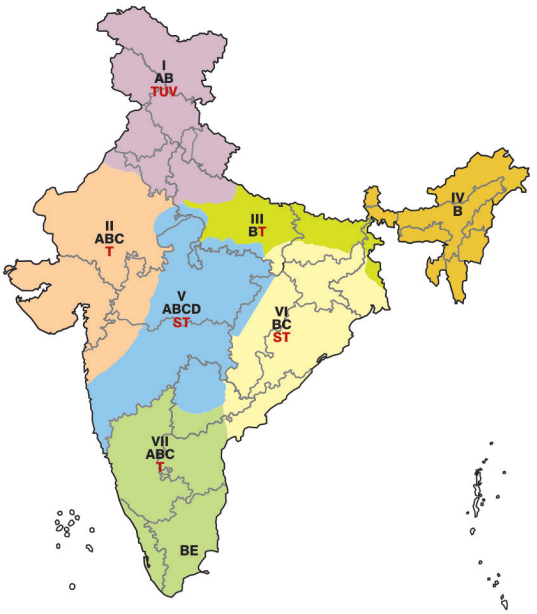

Figure 3: Sympatric distribution of sibling species of *Anopheles fluviatilis* complex (S,T,U, form V) and *Anopheles culicifacies* complex (A,B,C,D,E) in different geographical regions of India (divisions I – VII). While sibling species T, U and form 'V' occur in northern states (division I), species 'T' is predominant in western and southern India (division II, III, VII), and species 'S' and 'T' coexist in central and eastern India (division V, VI). In north-east India (division IV), formerly identified populations of *Anopheles fluviatilis* are now characterized to be hyper-melanic variant of *Anopheles minimus*; its occurrence, however, cannot be denied and needs introspection. Source Reference [13].

Seasonal abundance, infectivity and disease transmission

An. fluviatilis is essentially a species of the hills and foothills occurring up to 2500 metres above mean sea level (msl) and has been incriminated by detection of gland and/or gut infections across India [3, 5]. It is prevalent throughout the year, yet seasonal abundance largely rests on rainfall pattern and terrain ecotype. Sibling species of this taxon (S,T/U), however, have distinct bionomical characteristics and disease transmission relationships (Table 1). Among these, sibling-species 'S' is a winter species prevalent in high density during post-monsoon season (November - March/April) up in hills and foothills maintaining hyperendemic malaria [14,15]. It is highly anthropophilic all throughout in range of its distribution; anthropophilic index, however, reportedly varied anywhere from 60%-90% in high-risk districts of Odisha and Chhattisgarh [16-18]. The high predilection for human host is further corroborated by sporozoite infectivity in malaria-endemic hill districts throughout India including states of Andhra Pradesh, Arunachal Pradesh, Assam, Bihar, Gujrat, Haryana, Jammu & Kashmir, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Nagaland, Rajasthan, Tamil Nadu, Uttar Pradesh and West Bengal; infection rate, however, varied between locations [3, 5]. Generally, sporozoite infection rates ranging from 03–10 per cent were of common occurrence between states but occasionally high infectivity >10% has also been cited [5].

Bionomics of species 'S' is akin to *An. minimus s.s.* for being highly anthropophilic, endophilic and endophagic in areas where both species co-exist [19-21]. Indoor-resting characteristic

of both these species is further affirmed by record of high proportions of semi-gravid and gravid females in human dwellings (Table 2). In foothill districts of Uttarakhand and that of north-east India, *An. fluviatilis* acts as relay vector to *An. minimus* for continued transmission during winter months maintaining hyper-endemicity [19, 22]. In Arunachal Pradesh, *An. minimus* and *An. fluviatilis*; both together constituted fair proportion of mosquito collection in December (min temp 3.7^{0}C; elevation 1570 msl) and were incriminated with high sporozoite infectivity rate (Table 2). Both were held responsible for focal disease outbreak evidenced by high parasite rate (424/1190, 35.6%), majority of which were *P. falciparum* cases (344/424, 81%). Similar observations were reported in hill district of east-central state of Odisha on relative abundance of *An. minimus* and *An. fluviatilis* in different seasons (the latter being more abundant in later part of the year), both supplementing contribution of cases constituting active transmission throughout year [14.15, 23]. Yet another study reported greater role of *An. fluviatilis* in relation to *An. culicifacies* in high-risk districts of Odisha reporting seasonal abundance, high infectivity and receptivity for malaria in hill-top/foot-hill villages during cooler months [24-26]. In all these study reports, species 'S' was identified as the predominant fraction and incriminated. Conversely, in central Indian state of Madhya Pradesh, *An. fluviatilis* species 'T' was predominant compared to 'S' in plain ecotype villages but relative contribution of cases was higher in comparison to *An. culicifacies* evidenced by high infectivity [27], whereas in Baster district of Chhattisgarh (highly forested), *An. fluviatilis* species 'S' was the predominant collection in human indoor dwellings and incriminated [18].

In contrast to sibling 'S'; instead 'T' and 'U' are species of the plains, and cattle sheds are the preferred resting habitat [27, 28]. Both these siblings are largely zoophilic [29] and considered poor vector or of lesser significance in malaria transmission but have shown inherent ability to support normal sporogony in laboratory feeding experiments [30]. Of these, species 'T' is further characterized to have different haplotypes (T1, T2, Y) implicating the existence of additional taxa of which 'form V' has been recorded to occur in Uttarakhand, north India [31].

Table 2. Seasonal abundance and infectivity of anopheline mosquito species in day-resting collections indoors human dwellings in Yazali, Lower Subansiri district of Arunachal Pradesh*

S. N.	Mosquito species (*Anopheles* = *An.*)	No. mosquitoes collected	Abdominal condition**				Vector density per person hour (person hours: 27)	Vector incrimination	
			UF	FF	SG	G		No. mosquitoes dissected	No. (%) gland positive
1	*An. aconitus*	1	0	1	0	0	0.04	1	0
2	*An. culicifacies*	5	0	0	0	5	0.18	5	0
3	*An. fluviatilis*	56	3	3	19	31	2.07	54	5 (9.3)
4	*An. jeyporensis*	1	0	0	1	0	0.04	1	0
5	*An. maculatus*	1	0	0	1	0	0.04	0	0
6	*An. minimus*	17	0	2	5	10	0.63	17	3 (17.7)
7	*An. varuna*	19	1	1	10	7	0.70	19	0

* Study period: 6th – 20th December 1995; ** UF= Unfed, FF = Fully fed, SG = Semi-gravid, G = Gravid

Feeding behaviour and larval breeding ecology

An. fluviatilis is a nocturnal indoor biter; peak biting activity, however, occurred between 20:00–24:00 hours (before midnight) particularly during winter months, but varied between locations due to climatic and contextual determinants [15, 23, 32]. The species apparently lives long enough for more than 10 days to epidemiological significant level permitting sporogonic development in the mosquito host evidenced by parity >2 in majority adult population [33]. The flight range, however, is estimated to be about half a km from human habitations; larval breeding, however, was recorded up to 1500 metres above mean sea level but there was negative correlation between resting places and breeding habitat [34, 35]. This species was recorded breeding preferentially in slow-flowing seepage water streams; others included irrigation channels, terraced rice-fields and swamps with perceptible flow of water [36-38]. Breeding was observed to be intense during winter months and early part of summer in relation to remainder of the year. Heavy rains and floods were detrimental to breeding resulting in depletion of mosquito density restricting larval positivity to shallow wells and other miscellaneous habitats. Invariably shaded places with grassy margins, vegetation, bushes etc were the choice for oviposition opposed to sunlit areas.

Vector Control

An. fluviatilis has been assessed to susceptible to all three insecticides used in the control programme, i.e., DDT, malathion and pyrethroids in hyper-endemic districts of Odisha [39-41]. However, while it is held susceptible to malathion and pyrethroids across India, it is reported to be resistant to DDT in states of Chhattisgarh, Himachal Pradesh, Jharkhand, Karnataka, and Maharashtra, Tamil Nadu and Uttarakhand [42]. Nevertheless, choice insecticide for control of *An. fluviatilis* transmitted malaria should be based on susceptibility status to *An. culicifacies* for their sympatric distribution and sharing similar resting habitats in large tracts of India (Figure 2). In north-eastern states, however, wherein both *An. fluviatilis* and *An. minimus* are prevalent, DDT should continue to be used for vector control [19,20]. Despite the fact that *An. culicifacies* is virtually resistant to all available insecticides, there is evidence of decreased susceptibility in *An. fluviatilis* to DDT necessitating the need for regular monitoring and management for effective control in areas of its dominance to check transmission [43]. Nevertheless, the insecticide-treated nets seem to offer sustainable solutions for transmission control in areas of their co-dominance for proven efficacy in reducing vector density and longevity reinforcing personal protection against infective mosquito bites in high-risk areas [44-46].

Priority areas of research

Vector populations of *An. fluviatilis s.l.* are apparently in the process of diversification in relation to control interventions and changing agroclimatic contextual determinants. There is evidence of emergence of additional haplotypes of species 'U' for which entomological surveillance should be the continuing activity for their epidemiological significance and

control [11,31]. With proven existence of sibling species within this taxon, time is now ripe to accord binomial nomenclature to individual species supported by identification of morphological differences and cross-fertility data between sibling species.

Conclusions

An. fluviatilis is proven efficient vector of malaria in the hills and foothills of India maintaining perennial transmission. Its bionomical characteristics are very similar to *An. minimus* for being highly anthropophilic and sharing similar resting and breeding habitats. Given the declining transmission trends in domain of its seasonal abundance (Odisha in particular reporting >80% decline in 2018 compared to 2017), control of *An. fluviatilis* should be accorded priority by strengthening healthcare services in the periphery and large-scale implementation for 'universal coverage' of sustainable interventions to end transmission for good [47,48].

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Chapter 4

Bionomics and control of *Anopheles (Cellia) minimus* in the context of disappearing malaria in India

Vas Dev[1*] and Sylvie Manguin[2]

[1]Ex - Scientist, ICMR -National Institute of Malaria Research, New Delhi – 110 077, India

[2]HydroSciences Montpellier (HSM), Institut de Recherche pour le Développement (IRD), CNRS, Université Montpellier, F-34093 Montpellier, France

[*]Correspondence: mrcassam@hotmail.com

Abstract

Malarial threat is receding in India and for achieving malaria-free status, vector biology is regaining its due importance for targeting species-specific interventions in place and time. *Anopheles minimus*, the major vector of malaria in north-east India, is disappearing fast evidenced by reduced levels of malaria transmission and morbidity. *An. minimus* is a species complex comprising three sibling species of which *An. minimus s.s.*, formally named as *An. minimus*, is recorded to occur in east and north-east India. It is highly anthropophilic and responsible for fulminating outbreaks of malaria evidenced by incrimination records in range of its distribution. For its control, DDT continues to be the insecticide of choice for indoor residual spraying; however, populations are highly resilient in response to residual insecticides for paradigm shift in vector behaviour from indoor resting to outdoors resulting in continued transmission. Extra-domiciliary transmission of malaria is a challenge for which newer interventions, viz., attractive toxic sugar bait, eave-tube technology, nano-synthesized pesticides, new adult repellents, oviposition deterrents need to be field-evaluated under local geo-epidemiological conditions. To keep vector populations at bay, it is advocated to upscale interventions for 'universal coverage' of human populations at risk to check malaria transmission and spread of drug-resistant malaria.

Keywords: Anopheles, malaria elimination, sibling-species, bionomics, vector control, outdoor transmission, India

Introduction

India is reporting declining transmission of malaria and targeting elimination by 2027 – three years ahead of global target date of 2030 [1-4]. Given the mandate, vector biology is regaining its lost ground for being an integral component of the national control strategy. A lot of new information has been generated on malaria vectors, aided by molecular taxonomy tools, including sibling-species composition, distribution and disease transmission relationships helping formulate species-specific control interventions [5, 6]. Amidst myriad of challenges including climate change, deforestation, population migration, cross-border population movement, galloping urbanization, paradigm shift in vector behaviour, and growing menace of insecticide resistance; monitoring vector abundance and bionomical characteristics in the altered ecology has become important in different eco-epidemiological zones of the country. Amongst dominant vectors of malaria in India, *Anopheles minimus* is reckoned as the major vector in north-east region contributing ~5% of the recorded positive cases in the country annually [7]. History is replete with records of devastating malaria outbreaks characterised by high rise in cases and deaths across all age groups attributed to this dreaded vector [8-10]. Due to its medical importance, it has been the subject of extensive investigations and review for its bionomical characteristic both in pre-independent and post-independent India for formulating intervention strategies. It has become increasingly clear that containment of populations of this vector species is of paramount importance to forbid entry and spread of drug-resistant malaria and check ongoing transmission specific to northeast India (the gateway to Southeast Asia). Included in this chapter are the bionomical characteristics of this species (in brevity), highlighting significance for sustained interventions for keeping its populations below threshold for malaria elimination at national/sub-national level.

Taxonomic considerations & distribution

Anopheles (*Cellia*) *minimus s.l.* is an Oriental species and has been genetically characterised to be a complex of three designated formally named species namely *An. minimus* Theobald, *An. harrisoni* Harbach & Manguin, and *An. yaeyamaensis* Somboon & Harbach with distinct bionomical characteristics and distribution records (Figure 1) [11, 12]. Of these, exclusively *An. minimus* is encountered in north-east India with records of its disappearance and re-appearance after decades in erstwhile domains of its distribution including eastern state of Odisha (formerly Orissa) [13, 14]. It is a small sized mosquito that is morphologically similar to sympatric populations of *An. varuna* and requires an experienced eye for its correct identification due to subtle morphological differences (Figure 2) [8, 15], but can be identified by molecular tools unequivocally [11, 12]. Aided by molecular tools, earlier identified populations of *An. fluviatilis*, prevalent during winter months (November – March) in Assam, are now characterised as hyper-melanic seasonal variant of *An. minimus* with history of malarial outbreaks in hill ranges (~3000 feet above mean sea level) [16].

Seasonal abundance, infectivity and disease transmission

An. minimus is recorded throughout the year, however, its density is seen rising beginning March/April with the onset of pre-monsoon showers and recorded to occur in good numbers till cessation of rainy season in September/October; it occurs in low numbers during winter months (November–February) [10, 13, 17]. The disease transmission is perennial and persistent that follows suit with rising density of *An. minimus* beginning April/May marked with maximum malaria cases occurring during May till September/October (months of heavy rainfall), while the rest of the year is the period of low transmission season. *An. minimus* mosquitoes were repeatedly incriminated for most part of the year evidenced by detection of live sporozoites in salivary glands, with an overall infectivity rate of about 3% (Table 1) [13, 17]. The relative risk of infection, however, varied across landscape, the highest being in forest-fringe foothill villages in closer proximity to mosquito breeding sources and the minimal to healthcare facility within <5 km [18]. In these villages, all age groups of both sexes were recorded parasite positive mostly for *Plasmodium falciparum* (70%), the remaining were *P. vivax* cases (data not shown).

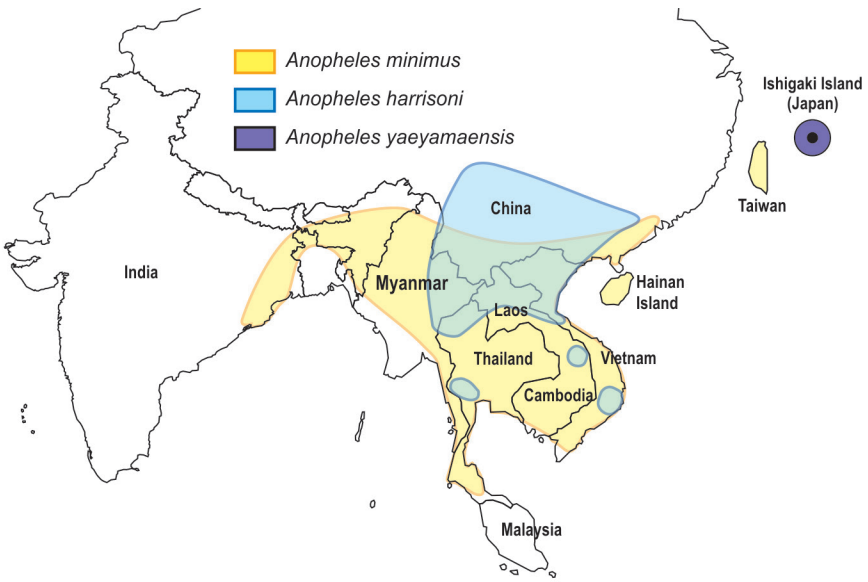

Figure 1: Distribution map of sibling species of the *Anopheles minimus* complex in Southeast Asia based on molecular identification. *Anopheles minimus* has a wide distribution extending from eastern to north-east India and eastwards to Myanmar, Thailand, Laos, Vietnam, Cambodia, China including Taiwan and occurs in sympatry with *Anopheles harrisoni* over large areas in southern China, Vietnam, Laos and Thailand. *Anopheles yaeyamaensis* is restricted to the Ishigaki Island of the Ryukyu Archipelago of Japan. Populations of *Anopheles minimus* are fast depleting in north-eastern states of India and seemingly have disappeared from Bangladesh [sketch map not necessarily in conformity with political boundaries].

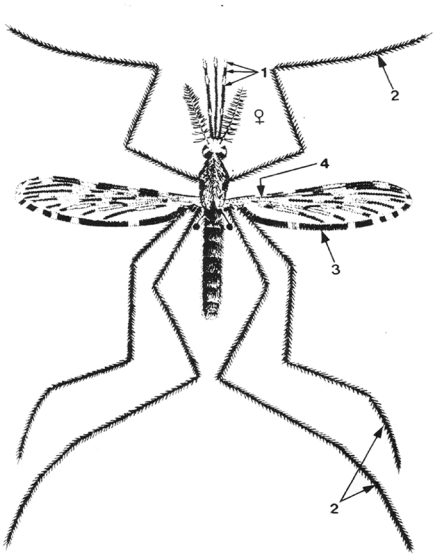

Figure 2: *Anopheles minimus* Theobald 1901, adult morphological distinguishing characters: (1) Apical and sub-apical pale bands equal separated by a dark band, (2) Tarsomeres without bands, (3) Fringe spot absent on wing vein 6, (4) Presence of pre-sector pale spot and humeral pale spot on the costa. These characters do not allow identification of the sibling species of the *An. minimus* complex. Source Reference [8]

Table 1. Seasonal sporozoite infection rate in *Anopheles minimus* in the Sonapur Primary Health Centre of Dimoria Block in Kamrup district of Assam, north-east India for data based on 1989-1991. Source Reference [13]

Month, year	No. mosquitoes dissected for salivary glands	No. gland positive for sporozoites	Infection rate (%)
1989			
July	802	39	4.86
August	284	12	4.22
September	386	11	2.85
October	114	8	7.02
November	38	0	0
December	28	0	0
1990			
January	106	1	0.94
February	223	3	1.35
March	282	2	0.71
April	217	2	0.92
May	328	11	3.35
June	172	7	4.07
July	52	1	1.92
August	25	0	0
September	5	0	0
October	94	8	8.51
November	227	7	3.08

December	182	3	1.65
1991			
January	112	0	0
February	38	1	2.63
March	96	0	0
April	245	7	2.86
May	334	13	3.89
June	245	17	6.94
August	15	1	6.67
September	30	1	3.33
Total	4680	155	3.31

Host choice, resting and breeding characteristics

An. minimus is a formidable foe in maintaining hyper-endemic malaria throughout north-east India where it shows a strong predilection for human host (anthropophilic index >90%) making it an efficient vector substantiated by sporozoite infectivity for all months of the year ranging from anywhere from <1% to 7% (Table 1) [13, 17]. All night mosquito landing catches revealed that it actively searched for a host throughout the night beginning 19:00 hours with pronounced feeding activity during 01:00–4:00 hours that ceased at break of the dawn. The mosquito biting rate per person night (02–23) and entomologic inoculation rates (0.12–0.71) varied between locations representative of low-to-moderate transmission intensities [18]. It is largely an endophilic and endophagic (resting and feeding indoor human dwellings) species recorded resting indoors often in lower half of the walls in darker corners of the house (much away from sunlit areas) and seen exit at break of the dawn. Houses made of split-bamboo with thatched roofing is the preferred resting habitat opposed to RCC (Reinforced Cement Concrete) structures [19]. The flight range of this mosquito is estimated to be just about one km resulting in focal disease outbreaks often with case clusters in given locality yielding more than one case per household (Figure 3). It is a perennial species breeding in slow-flowing foothill seepage water streams in all seasons/months [20]. Larvae of *An. minimus* are recorded breeding along the shaded grassy banks sans sunlit areas in good numbers for most part of the year including winter season both in hills (~3000 feet above mean sea level) and valleys (Figure 4).

Figure 3: Top: Resting habitat - typical housing made of split bamboo and thatched roofing; mosquitoes are collected in good numbers inside house premises in dark/ shady corners resting on clothes, umbrella and other articles. Bottom: Infants and children are the vulnerable population groups with record of multiple cases within single household.

Figure 4: Top: A typical village hamlet in the forest-fringe at risk of malaria transmitted by *Anopheles minimus* in north-east India. Bottom: Typical larval habitat of *Anopheles minimus* in foothill perennial seepage water stream marked with grassy banks. Households located nearer to breeding habitat (<1 km) are at greater risk of malaria

Vector Control

An. minimus is reported to be highly susceptible to DDT despite decades of its application in areas of its occurrence over space and time [10]. It has innate abilities evading exposure (behavioural resistance) to the sprayed surfaces and tend to maintain extra-domiciliary transmission by changing its resting habitat from indoors to outdoors. Due to repeated applications of DDT during 1960s, it was believed to have disappeared, but resurfaced decades apart (due to inadequate spray coverages years together) resulting in fulminating outbreaks across its range of distribution [21]. The advent of insecticide-treated netting materials, i.e., long-lasting insecticidal nets (LLINs) proved to be big boon for its effectivecontrol disrupting transmission. Based on field evaluation of this technology, LLIN-based intervention was held appropriate against *An. minimus* transmitted malaria and well received by the communities and programme official alike [22]. With the increasing coverage of LLINs, the populations of *An. minimus* are once again depleting in erstwhile domains of its distribution corroborated by evidences of reducing levels of transmission [10,23]. The populations of this species are presently at lowest ebb and scarce restricted to remote areas rendered inaccessible due to some logistic reasons, viz., recurrent flash floods, poor communication, insurgency in the preceding years. There is strong body of evidence that the niche, thus, vacated by *An. minimus* is being accessed by *An. culicifacies s.l.*, which is multi-resistant to available insecticides including pyrethroids [24-26]. It is the high time to ensure blanket coverage of the populations at any risk by appropriate interventions to ward off this vector species below threshold.

Priority areas of research

Vector control programme in India largely rests on indoor residual spraying (IRS) and distribution of LLINs in communities most at risk. The largest challenge in vector control, however, is the emerging paradigm shift in mosquito behaviour towards outdoor transmission [27]. Mosquitoes are shifting outdoors in response to application of indoor residual insecticides rendering less amenable to control interventions [28]. *An. minimus* is one such classic example stymieing the control authorities by phenomenon of disappearing and re-appearance decades apart. Little is known of its outdoor population resting characteristics, which needs to be investigated for spearheading interventions. North-east is experiencing rapid ecological changes on account of population migration, deforestation and developmental project activities permitting sub-structuring of vector populations. *An. minimus* is proven genetically diverse evidenced by nucleotide diversity suggesting population expansion and possible existence of other sibling species with obvious implications for control interventions [29, 30]. There is an urgent need to devise appropriate technologies that are community-based and doable to capture residual as well as outdoor resting populations. Development of newer interventions like attractive toxic sugar baits, eave-tubes technology, nano-synthesized pesticides, new adult repellents, oviposition deterrents; all need to be evaluated in local ecological transmission settings possibly as component of integrated vector management for containment of vector populations [31]. Equally important would be to prioritize: (i) vector surveillance (that remained neglected), which should be the cornerstone activity to check unusual build-up of vector density [32],

(ii) monitor current insecticide susceptibility status, (iii) ecological succession by other vector species and their bionomical characteristics, (iv) promote information, education and communication activities for enhanced community participation and compliance, and above all (v) seek political commitment for sustained allocation of resources for 'universal coverage' of interventions to end malaria for good. For malaria elimination in India, it is of utmost importance to invest heavily strengthening interventions in north-eastern corridor for reducing populations of this vector species at sub-optimal levels averting impending disease outbreaks and spread of drug-resistant malaria.

Conclusions

An. minimus is an invincible mosquito vector with characteristics of its innate ability to avoid sprayed surfaces and establish extra-domiciliary transmission. However, with the available current tools for vector control, *An. minimus* is seen fast disappearing in north-east India. Nevertheless, for outdoor transmission control (an emerging paradigm in Southeast Asia), field-evaluation of newer interventions targeting residual populations and spotting the residual malaria foci should be the priority [33]. What concerns most is the ecological succession by yet another vector, *An. culicifacies s.l.*, which is multi-resistant to available arsenal of insecticides. We strongly advocate 'vector surveillance' and 'universal coverage' of evidence-based interventions for control of vector populations to defeat malaria.

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Chapter 5

Anopheles (Cellia) baimaii (sibling-species of the Dirus complex): the invincible vector of forest malaria in north-east India

Vijay Veer[1*] and Varun Tyagi[1]

[1] Defence Research Laboratory, Defence Research & Development Organization (DRDO), Tezpur – 784 501, Assam, India

[*] Correspondence: vijayveer50@yahoo.com

Abstract

Anopheles baimaii is an efficient vector of human malaria in north-eastern states of India with its predominance along borders with neighbouring countries. It is a member species of the *Anopheles dirus* complex comprising eight sibling-species seven of which have been assigned Latin name. These include *An. dirus s.s., An. cracens, An. scanloni, An. baimaii, An. elegans, An. nemophilous* and *An. takasagoensis* spread across Southeast Asia. *An. baimaii* is a monsoon species with its peak abundance coinciding with months of rainfall (March/April – September/October). It is a sylvatic species, outdoor resting, predominantly endophagic and highly anthropophilic species for having strong predilection for human host. It is night biting mosquito, peak feeding activity, however, varied between locations extending from second to third quartile. Species-specific breeding sources included transient jungle water pools shaded with dense foliage and elephant footprints. Even though it is highly susceptible to DDT, its control remains challenge for being exophilic species requiring newer innovative technologies which are community-based and sustainable. Continued attack for containment of vector populations is mandated for which entomological monitoring and capacity building should be the cornerstone. The control of *An. baimaii* is of paramount importance for averting transmission and spread of drug-resistant malaria to peninsular India and beyond westwards.

Keywords: *Anopheles baimaii*, forest malaria, vector bionomics, sibling-species, vector control, outdoor transmission, India

Introduction

Among six dominant mosquito vectors of human malaria in India, *Anopheles baimaii* has significance for its regional distribution and distinct bionomical characteristics [1, 2]. Its role was overlooked until the second World War for persistent transmission in areas with good control of *An. minimus* inflicting heavy morbidity in waring forces [3]. It is a sibling-species of the *An. dirus* complex, species of which are implicated in disease transmission related to forest malaria throughout Southeast Asia [4, 5]. With the available modern morphological, molecular, cytological and ecological tools, *An. dirus* is presently known to comprise eight sibling species, seven of which have assigned Latin name [6]. Among these, *An. baimaii* (formerly recorded as *An. balabacensis balabacensis/An. dirus*) is implicated in malaria transmission in north-east India [7-9], whereas *An. elegans* with restricted distribution in southwest is not known to transmit human malaria, but instead incriminated as vector of simian malaria [10]. *An. baimaii* is an efficient vector of human malaria for contributing nearly 5% of total reported cases in the country annually exclusively from north-east India [11]. It is a forest dweller and relates to malaria transmission in forest-fringe population groups having strong predilection for human host [12]. It is a species of undisturbed forest reserve, but now its populations are reported to be dwindling owing to depletion of forest cover at expense of population migration, increased acreage for agriculture, expanding infrastructure and industrialization [13, 14]. This chapter includes updated information on its taxonomical position and bionomical characteristics of *An. baimaii* related to malaria transmission and control options specific to India in the context of malaria elimination efforts.

Taxonomic considerations, distribution and evolutionary relationships

An. dirus species-complex belongs to the Leucosphyrus subgroup under Leucosphyrus group of Neomyzomyia series of subgenus *Cellia* and has been the subject of extensive investigations for sibling-species composition and disease transmission relationships [15]. It is widely spread across Southeast Asia and its sibling-species are occurring in India, Nepal, Bangladesh, Myanmar, Thailand, Indonesia, Malaysia, Vietnam, Cambodia, south China, and Taiwan [2-5]. Now with the help of wide array of taxonomic tools, *An. dirus* has been characterized and found comprising eight sibling-species with regional distribution across Southeast Asia (Figure 1). Among these, seven sibling-species presently have been individually named as *An. dirus s.s.* Peyton & Harrison (species A), *An. cracens* Sallum & Peyton (species B), *An. scanloni* Sallum & Peyton (species C), *An. baimaii* Sallum & Peyton (species D), *An. elegans* James (species E), *An. nemophilous* Peyton & Ramalingam (species F), and *An. takasagoensis* Morishita [6], while *An. aff. takasagoensis* is cryptic species morphologically similar to *An. dirus* and *An. takasagoensis* but phylogenetically distinct from either of the two having restricted distribution in northern Vietnam (S. Manguin, pers. commun.).

All sibling-species have been well characterized by several techniques including cross-mating experiments and karyotypic studies [16-20], gene-enzyme variation [21], DNA probes [22, 23] and egg morphology [24]. In addition, number of molecular assays have

been developed for rapid identification of individual sibling-species based on polymerase chain reaction (PCR) techniques using specific primers [25, 26], restriction fragment length polymorphism (RFLP) [27], polymerase chain reaction assay based on second internal transcribed spacer (ITS2) sequences of the ribosomal DNA (rDNA) [28], allele-specific PCR (ASPCR) assay [29], microsatellite markers [30-32], species-specific SCAR (sequence characterized amplified region) markers which distinguishes sibling-species unambiguously [33].

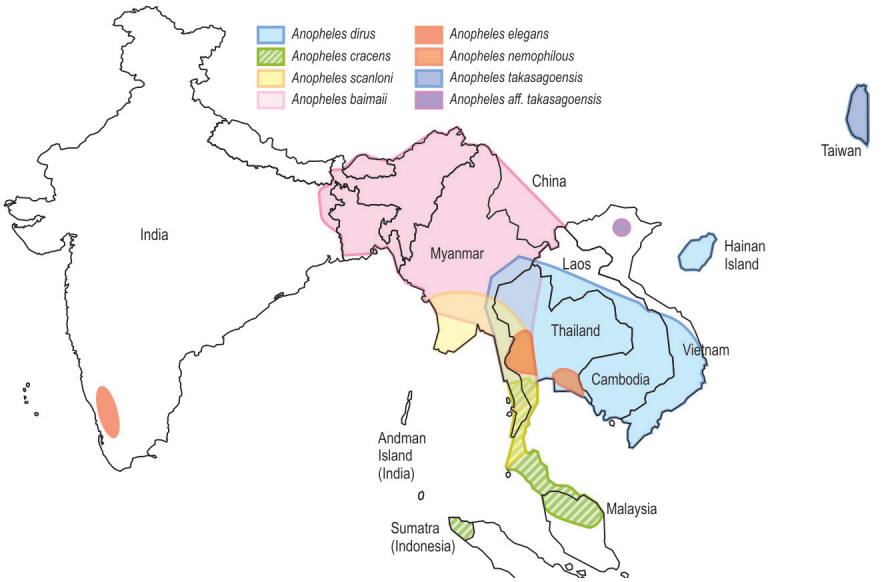

Figure 1: Distribution of sibling-species of the *Anopheles dirus* complex in Southeast Asia. *An. dirus* has a wide distribution in eastern Asia including Myanmar, Thailand, Cambodia, Laos, Vietnam and Hainan Island. *An. cracens* occurs in southern Thailand, peninsular Malaysia and Sumatra (Indonesia). *An. scanloni* distribution is restricted along border of southern Myanmar and western Thailand. *An. baimaii* distribution extends from southwest China to north-east India through western Thailand, Myanmar, Bangladesh and Andaman Islands (India). *An. elegans* distribution is restricted to hilly forests of south-western India. *An. nemophilous* has a patchy distribution along Thai-Malaya peninsula and Thai border with Myanmar and Cambodia. *An. takasagoensis* is restricted to Taiwan and *An.* aff. *takasagoensis* has recently been reported from northern Vietnam (Courtesy: Sylvie Manguin, Montpellier, France) [Sketch map not necessarily in conformity with political boundaries].

Among these sibling-species, *An. dirus* and *An. baimaii* are the primary vectors of human malaria in Southeast Asia [2, 4, 34]. Of these two species, it is exclusively *An. baimaii* (except for a focal presence of another unnamed species *An. dirus*`X' in Jatinga Hills, Dima Hasao, district of Assam) which is widely abundant in north-eastern states with records of its prevalence in forest-fringe villages particularly in interstate and inter-country border areas of north-east India [35, 36]. The only other member species recorded to occur in India is *An. elegans* but restricted to hilly forests of south-western India [37]. *An. baimaii* is a medium sized mosquito spotted by distinct broad white band on the tibio-tarsal joint on hind legs and can easily be characterized from other species of the subgenus *Cellia* by distinct morphological characters [38] (Figure 2). In earlier records of its prevalence in India prior to 1980s, it was invariably identified as *An. balabacensis balabacensis* [3,8], but later up until 1990s same populations were referred to as *An. dirus* [9].

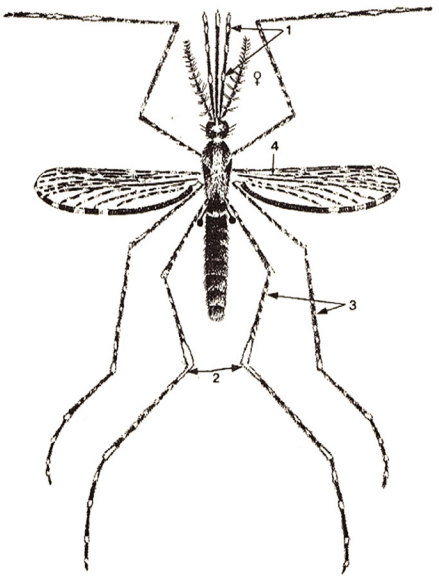

Figure 2: *Anopheles baimaii*, adult morphological distinguishing characters: (1) four banded palpi, (2) broad white band at tibio-tarsal joint, (3) legs with speckling, (4) pre-sector dark mark on vein 1 (R1) basally extended up to humeral dark mark of costa. Source Reference [38].

An. baimaii is believed to be the ancestral surviving species from which all other sibling species have diverged over evolutionary time scale [36, 39, 40]. However, *An. baimaii* was observed to have closer genetic affinities with *An. dirus* compared to all other sibling-species [41-43]. Populations of *An. baimaii* from north-east India even though observed to have greater genetic diversity but were in panmixia; nevertheless, were highly differentiated from those of Bangladesh, Myanmar and Thailand possibly due to hill rages acting as barrier to gene flow [44]. Interestingly, the unnamed species '*An. dirus* X' from Assam state was found genetically closer to that of *An. dirus* found in China, which was subsequently named as *An. dirus* species X [45]. *An. baimaii* is also known to inhabit forests of Andaman and Nicobar Islands but there is dearth of data on its population genetic structure and role in malaria transmission. *An. elegans*, instead, is genetically proven distinct from all species of the *An. dirus* species-complex and seem to have evolved from it's geographically nearest relative, *An. baimaii* by allopatric speciation [19].

Seasonal abundance, infectivity and disease transmission

An. baimaii is a monsoon species with abundance coinciding with months of rainfall during March/April–September/October [46-48]. The transmission season followed suit corresponding to vector density and receded with the onset of winter in November/December. It is widely prevalent in forested hills and foothills of north-eastern states frequently encountered in rubber plantations and recorded up to 1000 metres above mean sea level [3, 49]. *An. baimaii* is a largely a forest dweller with population expansion during rainy season associated with increased breeding resources later retracting to mother foci in winter months [12] and reckoned as the most efficient vector that even at very low densities

it can maintain heavy transmission. It is the often the most common species along inter-country border areas of north-east India and believed to be the carrier of drug-resistant malaria evidenced by high parasite rate predominated by *P. falciparum* (Table 1, 2) [50, 51]. It equals *An. minimus* for its high anthropophilic index and often both co-exist and supplement transmission in foothill areas resulting in devastating disease outbreaks [52]. *An. baimaii* have been repeatedly incriminated by records of salivary and gut infections in places of its occurrence [53-56]. Sporozoite infection rates on an average of 1% to 3% were of common occurrence but higher infectivity of even up to 7.8% was recorded during post-monsoon season [56]. Parity rate as high as 60% have been recorded in peak transmission season suggestive of high longevity in natural population. It is a hygrophilic species and flight range is estimated to be 1.5 km resulting in clustering of cases invariably of *P. falciparum* cases in given locality [50].

Table 1. Relative abundance of anopheline mosquito species in Lawngtlai district of Mizoram, north-east India bordering Bangladesh (Study period: July – August 2015)

Mosquito species	Day-resting (Human dwellings Indoors)		Cattle biting evening collection mosquito density person hours	CDC Trap (No. Trap nights)		Mean mosquito landing rate per person night	
	Hand catch (Person hours 32)	Total catch (5 rooms)		Outdoor (5)	Indoor (4)	Outdoor	Indoor
An. aconitus	0	0	0	2	0	0	0
An. baimaii	0	0	0	1	5	2	1
An. barbirostris	0	0	6	0	0	0	0
An. jamesii	0	0	8	4	0	0	0
An. kochi	0	0	17	3	0	0	0
An. maculatus	0	0	4	2	6	2	1
An. minimus	0	0	0	0	1	0	0
An. nigerrimus	0	0	9	0	0	0	0
An. nivipes	0	0	9	4	0	0	0

Table 2. Prevalence of malaria in forest-fringe communities along Indo-Bangladesh border districts of north-east India

S. N.	Study Location, District, State	Study period	No. of fever cases examined for malaria parasite	No. of blood-smears +ve (%) for any malaria parasite	No. of blood-smears +ve for *Plasmodium falciparum* (% of total +ve cases)
1.	Silachari, Gomti, Tripura	July – October 2013	1224	126 (10.3)	101 (80)
2.	Tlabung, Lunglei, Mizoram	July – August 2014	885	220 (25)	209 (95)
3.	Chawngte, Lawngtlai, Mizoram	June – August 2015	1058	408 (38.6)	338 (83)
4.	Gandachara, Dhalai, Tripura	June – August 2015	736	212 (28.8)	200 (94)
5.	Darangirri, West Garo hills, Meghalaya	August – September 2015	864	65 (7.5)	64 (98)

Host choice, resting and breeding characteristics

An. baimaii is predominantly anthropophilic species having strong predilection for human host (>90%) [56], and often seen as the single largest catch in all night human-landing collections [46]. It is even though predominantly an endophagic species [13,57], outdoor feeding has also been recorded to occur in the Indian ecological context [58]. It has inherent tendencies to leave house premises just after bloodmeal and observed resting often clinging to thatched roofing and nearby structures for sometimes before leaving for interior of the forest. *An. baimaii* is principally an exophilic species often collected resting from dark moist corners of large tree trunks from <2 metre distance from ground level and rarely encountered indoors/cattle sheds during day-time [59]. It is nocturnal in habit searching human host for blood meal all through the night but feeding activity varied between locations and seasons, the highest being during 23:00 – 03:00 hours (Table 3) [60,61]. In typically *An. baimaii* receptive endemic area, annual average mosquito landing rate per person night was 6.31, but varied seasonally being lowest <1 during winter months (November – February) and the highest (48/person/night) during hot summer/monsoon (May – September); the record number compared to any other vector species in Assam [56]. Accordingly, the vectoral capacity of *An. baimaii* was well above the threshold value of 0.01 to maintain transmission in given locality.

Table 3. Human-bait mosquito landing rate of *Anopheles baimaii* in malaria endemic locations of north-eastern states of India

Location, District, State (Study period)	Average number of mosquitoes collected per person during hours of										Mean mosquito landing rate per person night
	18-19	19-20	20-21	21-22	22-23	23-24	00-01	01-02	02-03	03-04	
Sonapur, Kamrup, Assam (June-October 1988)*	0.25	0.25	1	0	0	0	0	1	0	1	0.39
Jairampur, Changlang, Arunachal Pradesh (June-September 1990)**	0	1	0	0	0	3	3	3	1	1	1.09
Parva, Lawngtlai, Mizoram (March 2005)	0	0	0	1	1	0	1	2	0	0	2.50

*Source Reference [48], ** Source Reference [13]

The risk of infection, however, was the highest in typical setting of forest/forest fringe villages in juxta position to reserve forest areas (Figure 3). The species-specific breeding habitats of *An. baimaii* included rain fed transient jungle water pools/puddles with densely shaded foliage and elephant's footprints (Figure 4), however, during winter months (November- December) larval breeding was recorded to occur in pools associated with streams [59, 62, 63].

Figure 3: A typical high-risk forest-fringe human population settlement receptive for *Anopheles baimaii* transmitted malaria, Lawngtlai district of Mizoram located along Indo-Bangladesh border in north-east India. Seen in the picture is canopy of forest receiving high rainfall during monsoons serving an ideal habitat for resting and breeding of vector mosquito.

Figure 4: Breeding habitats of *Anopheles baimaii* (top - a stagnant rain fed water pool surrounded by densely shaded foliage; bottom - rainwater collection in elephant footprint in forest (Courtesy: Anil Prakash, ICMR - National Institute for Research in Environmental Health, Bhopal, Madhya Pradesh)

Vector Control

An. baimaii has been assessed to be highly susceptible to DDT, the commonly used residual insecticide in vector control operations in domains of its distribution, but transmission continues unabated [64-66]. Control of vector populations, however, is difficult proposition for species innate abilities to avoid contact with sprayed surfaces and having outdoor resting characteristics [67, 68]. Anti-larval operations are just not practicable even though clearing forests have resulted in depletion of breeding resources, but the niche thus vacated is being accessed by *An. culicifacies*, populations of which are multi-resistant rendering less amenable to control [14, 69]. Nevertheless, personal protection from mosquito bites can be

ensured by applying repellents such as DEPA (N, N-diethyl phenyl acetamide) or DEET (N, N-diethyl-m-toluamide) based cream or spray formulations on the exposed body parts or using insecticide-treated fabrics. DEPA based spray formulation is commonly used by the defence service personnel on routine patrolling. Long-lasting insecticidal nets (LLINs) are proven successful in reducing vector host contact and thus disrupting malaria transmission to a greater extent; however, net coverage remains dismal given the huge requirement [70]. There is an imperative need for alternate technologies which are community-based and self-sustainable to contain outdoor resting populations [71]. The advent of newer interventions like 'eave-tubes' and 'attractive toxic sugar baits' (ATSB) seem to offer sustainable solution but need to be validated in the local epidemiological situations (Figure 5, 6).

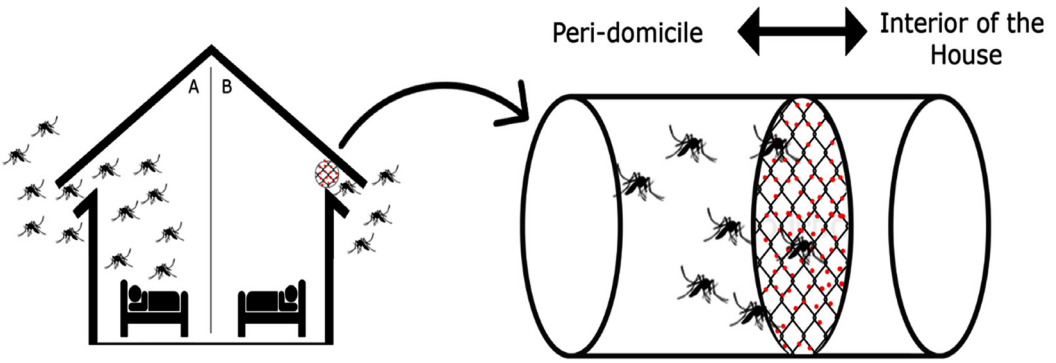

Figure 5: The "eave tubes" technology comprises the use of plastic tubes with adulticide-coated mesh under the roofline and the installation of a screen to close the remaining gap. (A) Graphic representation of a house without "eaves tubes" and (B) with "eaves tubes" (Courtesy: John Beier, Miami, USA).

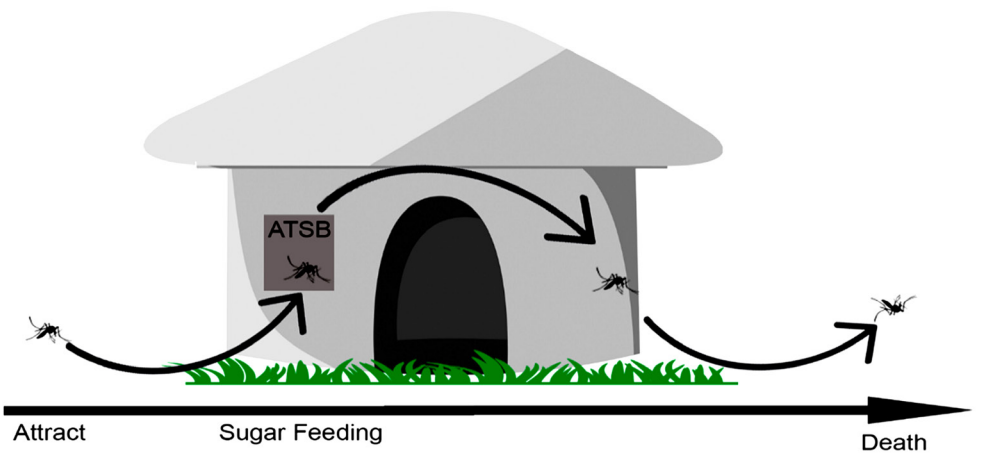

Figure 6: Attractive toxic sugar baits (ATSB) employing an "attract and kill" strategy. This technique consists of using natural attractants such as fruit or flower scent to lure mosquitoes to sugar feeding in a solution containing toxic substances that will lead to its death (Courtesy: John Beier, Miami, USA).

Both these technologies are target-specific based on "attract and kill" posing minimal threat to non-target insects and environment contamination. With the installation of eave-tubes, the mosquitoes attracted to the human host indoors get killed while coming in contact with adulticide-coated mesh providing protection against infective mosquito bites. The ATSB strategy is based on similar principle and can be easily applied killing both males and females that feed on sugar source (Figure 6). Most other intervention technologies, viz., genetically modified mosquitoes are still in pipeline and it would be long before these are put into the programme [72, 73].

Priority areas of research

Distribution of *An. baimaii* is presently considered restricted exclusively to north-eastern region of India, however, given the favourable ecological and climatological conditions, occurrence of this species has also been predicted in parts of central and north-western India which call for extensive faunistic surveys to formulate appropriate control interventions [74]. Existence of additional sibling-species is highly probable given the diverse ecology, terrain and changing landscape epidemiology specific to India [2, 4]. Control of *An. baimaii* is of paramount importance for two reasons: (i) firstly to avert impending disease outbreaks for being most efficient transmitter of malaria parasite, (ii) secondly to arrest the development and spread of drug-resistant malaria. This species is invariably linked to transmission of drug-resistant malaria evidenced by its distribution range and prevalence of multidrug-resistant varieties across Southeast Asia [75, 76]. The proportions of *P. falciparum* are seen steadily rising presently constituting >50% of reported cases in the country largely attributed to emerging drug-resistance [77]. The spread of multi-drug resistant malaria would spell doom to the programme for rising costs and logistics reaching the outreach population groups. Outdoor transmission and shifts in mosquito behaviour are another emerging paradigm which need to be addressed adequately by appropriate interventions preventing infective mosquito bites particularly in mobile/displaced population groups including military surveillance personnel [78]. High morbidity and case fatality have been recorded in defence posts along international border with Myanmar, Bhutan and Bangladesh [79]. Innovative technologies are mandated involving communities and intersectoral convergence for maximal compliance in forest-goers at high-risk to prevent spread of drug-resistant malaria [80]. More importantly, strengthening cross-border initiative for shared information and coordinated vector control operations are tantamount for malaria elimination initiative in the Southeast Asia region [81]. Monitoring vector density and building entomological capacity are critical to vector control operations not only in the present-day context but also post-elimination preventing re-establishment of malaria transmission in malaria-free territories.

Conclusions

It is clearly established that of seven sibling-species of the *An. dirus* species-complex, interventions should be targeted against *An. baimaii*, the only sibling-species that relates to transmission of drug-resistant malaria in India. It is a sylvatic species widely prevalent

in north-eastern states along inter-state and inter-country border areas inflicting heavy morbidity in forest-fringe human populations, forest-goers and defence service personnel. To realize the goal of malaria elimination in due time [82], control of *An. baimaii* transmitted malaria is of immediate importance for which interventions should be prioritized in high-risk population groups ensuring blanket coverage and increased awareness for case detection and treatment, investments for which yield rich dividends in defeating drug-resistant malaria. There is an imperative need for continued resource mobilization and increased domestic funding to address funding gap to sustain the end game for malaria-free status [83,84]. Innovative technologies should be applied to identify risk-areas in response to climate change and changing forest cover for intensified control interventions to check rising vector densities and species-specific interventions to mitigate disease onslaught [85]. Strengthening cross-border initiative with neighbouring countries holds the key for sharing data and coordinated action to thwart spread of drug-resistant malaria and end malaria transmission for good.

Acronyms	
ASPCR	Allele Specific Polymerase Chain Reaction
ATSB	Attractive toxic sugar baits
DDT	Dichloro-diphenyl-trichloroethane
GIS	Geographical Information System
ITS 2	Internal Transcribed Spacer 2
LLIN	Long-lasting insecticidal net
PCR	Polymerase Chain Reaction
rDNA	Ribosomal DNA
RFLP	Restricted Fragment Length Polymorphism
SCAR	Sequence Characterized Amplified Region
s.l.	Sensu lato
s.s.	Sensu stricto

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Chapter 6

Anopheles (Cellia) sundaicus: a vector of malaria in the Andaman & Nicobar Islands, India

Manoj Kumar Das[1] and Vas Dev[2]*

[1]ICMR - National Institute of Malaria Research (Field Station), Ranchi – 835 301, India

[2]Ex-Scientist, ICMR - National Institute of Malaria Research, New Delhi – 110 077, India

*Correspondence: mrcassam@hotmail.com

Abstract

Anopheles sundaicus has retracted from eastern coastal states of India and presently confined to Andaman and Nicobar group of islands. It is a complex of four sibling species of which 'species D' occurs in India and has been incriminated as vector maintaining endemicity in the Andaman & Nicobar archipelago. It is largely zoophilic, exophilic and breeds preferentially in brackish water. Among insecticides used in the national control programme, it is susceptible to DDT, malathion and pyrethroids, however, monitoring is mandated for effective control of vector populations for its varied response to DDT in place and time. For its sustainable control, bio-environmental interventions including application of larvivorous fish, bio-larvicides and installation of sluice gates have been held appropriate and should be enforced to prevent mosquito proliferation. Given these tools, transmission of the causative parasites is declining presenting window of opportunity for strengthening interventions for 'universal coverage' inclusive of itinerant labour force to end malaria transmission in the Andaman & Nicobar Islands. At present, Car Nicobar Island is accelerating towards malaria elimination in the archipelago.

Keywords: *Anopheles sundaicus*, Island malaria, brackish-water species, bionomics, vector control, Andaman & Nicobar Islands, India

Introduction

The Andaman and Nicobar group of Islands are historically endemic for malaria [1]. Mosquito fauna is rich and breeding habitats are numerous associated with heavy rainfall and evergreen tropical rainforest [2-4]. Among mosquito vectors of human malaria in the South-East Asia, *Anopheles sundaicus* is characteristically a brackish water species and proven vector in the coastal belts of the Oriental region of countries [5, 6]. In India, it has retracted from erstwhile distribution in the eastern states of West Bengal, Odisha, Andhra Pradesh and Tamil Nadu, and presently confined to group of Andaman and Nicobar Islands (A & N Islands) and has been considered a vector of regional importance [7-10]. In these islands, even though transmission intensities are presently assessed to be low [11], yet there is possibility of flare up cases and spread with trans-migration from mainland India and abroad associated with tourism and developmental activities. For instance, during 2005-2008 post-tsunami, a significant increase in case incidence was observed for nearly two-fold rise in *Plasmodium falciparum* cases associated with labour influx from mainland and increased receptivity for malaria [12, 13]. With malaria elimination high on the agenda, control of *An. sundaicus* transmitted malaria deserves priority averting impending outbreaks and check local transmission. This is an updated review on biology of this vector species for benefit of the control programme formulating informed policy for species-specific interventions to end transmission in the A & N Islands.

Taxonomic considerations & distribution

An. sundaicus belongs to subgenus *Cellia* and Ludlowae group in the Pyretophorus Series. This species, what initially thought to comprise different races for varied breeding and ethological population characteristics [7], has now been recognised to be a complex comprising four morphologically indistinguishable sibling-species, i.e., *An. epiroticus* Linton & Harbach (formerly species A), *An. sundaicus s.s.*, *An. sundaicus* D and *An. sundaicus* E based on cytogenetic, enzymatic profiles and molecular markers [14-19]. Member species of this species complex are widely distributed in the coastal belts of the Oriental region including countries of India, Bangladesh, Myanmar, China, Thailand, Cambodia, Laos, Vietnam, Malaysia, Singapore, Indonesia (Figure 1).

Among these, it is exclusively 'species D' which is known to be prevalent in the A & N Islands [20]. Molecular characterization of island populations from varied habitats did not reveal any genetic isolation but were distinct from *An. epiroticus* of Vietnam and *An. sundaicus s.s* from Borneo [21]. It is a medium sized mosquito and can easily be distinguished from other species of the subgenus '*Cellia*' by diagnostic morphological characters (Figure 2).

Seasonal abundance, resting habitats, infectivity and disease transmission

An. sundaicus is a predominant mosquito species in the A & N Islands constituting >50% of the total collection and is the sole vector proven by record of gut and gland infections

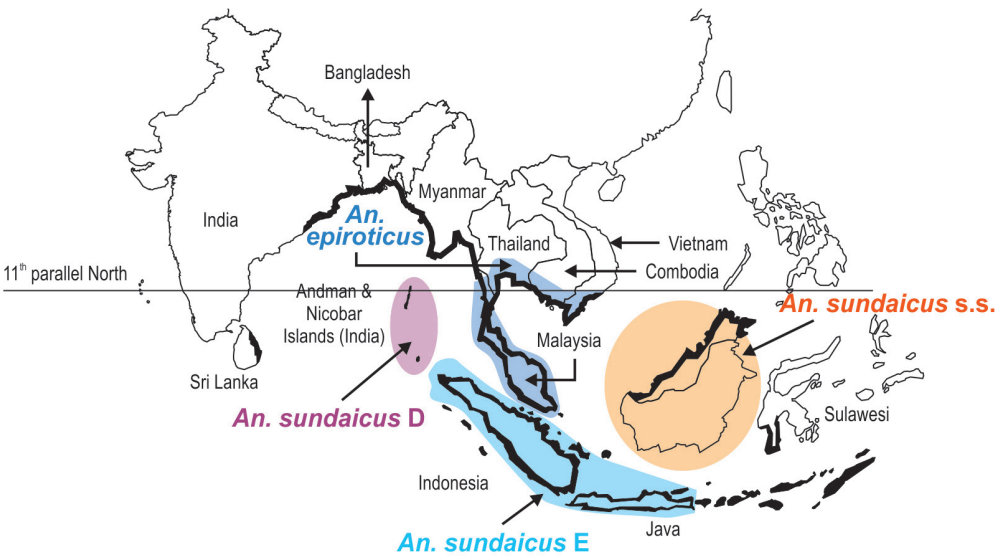

Figure 1: Distribution map of sibling-species of the *Anopheles sundaicus* complex in South-East Asia (courtesy: S. Manguin, Montpellier, France). *An. sundaicus s.s.* is distributed along the coast of Borneo; *An. epiroticus* occurs in coastal brackish water sites extending from southern Vietnam to peninsular Malaysia; *An. sundaicus* E occurs in Sumatra and Java (Indonesia); *An. sundaicus s. l.* instead has retracted from mainland India populations of which presently restricted to Andaman and Nicobar Islands have been characterized to be *An. sundaicus* D. Source Reference[6] [Sketch map not necessarily in conformity with political boundaries]

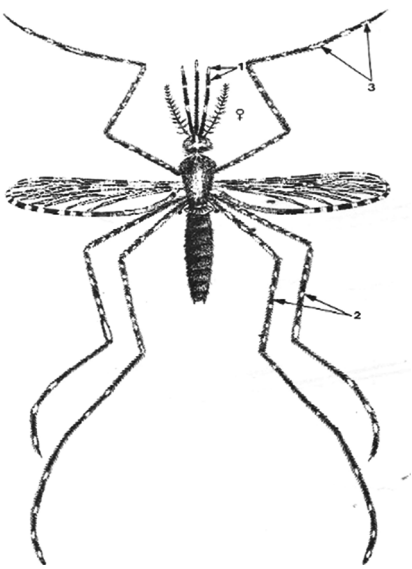

Figure 2: *Anopheles sundaicus*: morphological distinguishing characters of mosquito adult, (1) apical pale band nearly equal to pre-apical dark band, (2) legs with speckling, (3) fore-leg tarsomeres with broad bands. Source Reference [8]

maintaining endemic malaria [3]. It was widely incriminated in its earlier domains of distribution in West Bengal, Odisha and Andhra Pradesh [7, 8], however, infection rates in its present distribution range of A & N Islands remained <1% [22]. Majority populations

are exophilic resting in coconut stumps (*Cocos nucifera*), bushes, dry leaves of banana (*Musa paradisiaea*) and Keori bush (*Pandanus larum*); indoor resting sites included mixed dwellings inclusive of cattlesheds and houses (Copra Machan) [23]. These islands are co-endemic for both *Plasmodium vivax* and *P. falciparum*, but former is the predominant infection constituting >70% of total cases (Table 1) [24, 25]. Transmission is perennial and persistent evidenced by record of malaria cases for all months with seasonal peak in June of each year investigated [12]. Active transmission was corroborated by malaria positivity in infants and younger age population groups. The endemicity was further established by asymptomatic malaria in 10% population of the local tribes compared to migrant population groups. The increase in malaria receptivity in Nicobar group of islands post-tsunami during 2005-2008 was largely attributed to increased vector mosquito productivity and exposure to infective mosquito bites in non-immune labour migrant force and indigenous tribes alike for lack of interventions [12, 13].

Table 1. Malaria transmission in the Andaman and Nicobar Islands, India*

Year	No. of blood-smears examined for malaria parasite	Malaria +ve cases for any parasite (parasite rate)	No. +ve cases with *Plasmodium falciparum* (% of +ve cases)
2013	77943	1005 (1.29)	334 (33)
2014	74905	557 (0.74)	109 (20)
2015	56012	409 (0.73)	77 (19)
2016	57452	485 (0.84)	140 (29)
2017	55430	505 (0.91)	141 (28)
2018	42034	259 (0.61)	29 (11)

*Data source: National Vector Borne Disease Control Programme, India(https://www.nvbdcp.gov.in)

Breeding ecology, biting and feeding behaviour

An. sundaicus recorded breeding preferentially in brackish water but also observed to thrive in freshwater bodies as well. The common breeding habitats included sea-shore tidal brackish water pools, creeks, coral cavities, ponds infested with putrefying algal mosses, cement tanks, rock pools, disused wells, marshy area and mangrove swamps etc (Figure 3). *An. sundaicus* is largely zoophilic feeding on cattle and pigs except for indoor-resting populations in human dwellings having predilection for human host; anthropophilic index, however, remained <1% ranging from 0.5 – 0.87 per cent [22, 26]. Biting activity was observed to be bimodal with first peek between 20:30 – 24:00 hrs and second in between 02:00 – 03:00 hrs, however, peak biting occurred during 21:00 – 22:00 hrs. Indoor biting activity was observed to be slightly higher than outdoors (unpublished observations). Due to varied biting activity, there remains possibility of existence of another sibling or yet another species closely related to *An. sundaicus* in Car Nicobar Island yet to be explored. Contrary to some mosquito species, *An. sundaicus* was recorded breeding exclusively in sunlit still water bodies [7]. Filamentous floating algae and aquatic plants appeared to be crucial for development of the mosquito larvae. Mosquito adults are estimated to be living 2-3 weeks evidenced by high parity (73%) and believed to be strong fliers for record of breeding up to 9 km off breeding resources [7, 8].

Figure 3: Typical resting and breeding habitats of *Anopheles sundaicus* in Andaman and Nicobar Islands. Top left: mosquito breeding in sea-shore tidal brackish water pool; Top right: mosquito breeding in mangrove brackish water pools; Bottom left: mosquito resting on dry leaves of Keori bush (*Pandanus larum*); Bottom right: indoor day-resting in houses made of split bamboos (courtesy: I.P. Sunish, ICMR - Regional Medical Research Centre, Port Blair).

Vector Control

An. sundaicus is susceptible to DDT, malathion and pyrethroid class of insecticides [27]. However, response to DDT has been reported to be variable in certain places mandating periodic monitoring for effective control of vector populations [28]. Application of bio-environmental control interventions including large-scale introduction of larvivorous fish, installation of sluice gates to stop inward flow of saline water and bio-larvicides all have been held appropriate to check mosquito proliferation [29]. What is tantamount to control programme is the blanket coverage of interventions of populations at any risk to check build-up of vector density disrupting transmission which clearly remained off target largely attributed to high refusal rates (>60%) and lack of awareness in the communities [30]. For sustainable control of vector population, strengthening healthcare services for targeting interventions in species-specific mosquito breeding habitats, monitoring vector density and insecticide susceptibility status, ensuring universal coverage including itinerant labour force, and enhanced community participation and compliance should all be considered [31].

Priority areas of research

A & N Islands are house to primitive tribes yet unexplored presenting an opportunity to study host parasite interactions with possible outcome in understanding malaria vaccine responses. For instance, for the very first time in India, the Jarawa tribe of Andamans are discovered to harbour only *P. falciparum* associated with absence of Duffy antigen Fy(a) and Fy(b) restricting invasion of *P. vivax* [32, 33]. Low-grade resistance to chloroquine for treatment of *P. falciparum* was reported way back in 1994 [34]. However, follow up investigations for study based in 2012 revealed high treatment failures to standard chloroquine therapy (60%) evidenced by higher prevalence of mutated marker gene (*PfCRT*) [35]. Furthermore, in non-responders to chloroquine therapy with high early treatment failure (ETF), anti-folate resistance marked by *PfDHFR* and *PfDHPS* mutations was also recorded consequent to which artemisinin-based combination therapy (ACT) was recommended for treatment of falciparum malaria.

Even though *P. vivax* is the predominant parasite in the islands, there was unprecedented rise of *P. falciparum* cases post-tsunami years coinciding with labour influx from mainland endemic areas for which drug-resistance monitoring should be of paramount importance for instituting appropriate policy for treatment. Equally important would be to screen labour force both at entry and exit points for confirmed diagnosis and radical cure to avert spread of drug-resistant malaria. Emergence of zoonotic malaria is seen as possible threat for islands being in close proximity to other South-East Asian countries reporting virulent *P. knowlesi* cases [36-38]. Sporadic cases of simian malaria have been reported to occur on A & N Islands in the past [39]. Recently, possibility of transmission of *P. knowlesi* infection by *An. sundaicus* has been projected to occur in A & N Islands [40].

An. sundaicus is apparently a highly adaptive species for breeding in brackish with varying degree of salinity as well as freshwater bodies. The species was colonized at Car Nicobar Island presenting host of opportunities for laboratory-based investigations [41]. Given the vast chain of islands yet untraversed, there is possibility of existence of other sibling species of the complex for which understanding disease transmission and control options is critical to render islands malaria-free [5, 18]. Besides subsidence of South A & N Islands post-tsunami, these islands are prone to natural disasters, viz., hurricanes, cyclones resulting in increased brackish water bodies and mosquito productivity lending high risk of disease transmission for which early warning system should be in place for concerted action in place and time [42].

Conclusions

An. sundaicus, 'species D' is proven unequivocally the sole vector of malaria in the A & N Islands. This vector species is highly adaptive for breeding both in brackish and freshwater bodies alike, and for resting characteristics outdoors as well as human dwellings indoors. Given the multiple interventions in force for vector control, disease transmission trends are steadily declining each passing year presenting an unprecedented opportunity for investments ensuring 'universal coverage' and educating communities for enhanced complianceto move forward with the malaria elimination agenda [42, 43].

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Chapter 7

Anopheles (Cellia) stephensi Liston 1901: the vector of urban malaria - an imminent threat to malaria elimination in India

Susanta Kumar Ghosh*

Ex-Scientist, ICMR - National Institute of Malaria Research (Field Unit), Bengaluru, Karnataka, India

* Correspondence: ghoshnimr@gmail.com

Abstract

Anopheles stephensi is a proven efficient vector of human malaria in urban India and its sub-continent. It is an invasive species for establishing in newer areas and malaria-free territories thus threatening control and elimination efforts. With the current ongoing rapid urbanization, transmission of malaria is expanding unabated in cities and suburban settlements. The disease burden due to malaria in urban metropolitan cities is grossly underestimated and requires prioritization by policy and programme managers for added health infrastructure and continued interventions. For sustainable control of vector populations, bio-environmental approaches are advocated which are community-based and doable for saving operational costs.

Keywords: Urban malaria, *Anopheles stephensi*, vector bionomics, malaria transmission, vector control, India

Introduction

Malaria in India was considered a rural problem up until its resurgence in 1970s with substantial rise in cases in urban metropolitan cities accounting for 10% of reported cases in the country [1]. To contain spread of cases to adjoining malaria-free territories, the Urban Malaria Scheme (UMS) was launched in 1971 in selected cities having population >40,000 and reporting annual parasite incidence of two or above per thousand population [2,3]. Consequent to the record number of over six million cases in 1976, the modified plan of operation (MPO) was implemented in 1977 for strengthening primary healthcare services both in urban and rural areas aiming at reducing transmission and preventing deaths due to malaria. At present, 130 million population of 131 towns in 19 states and Union Territories are protected from malaria and other vector-borne diseases under UMS. Under this scheme, besides parasite control (based on case detection and

treatment by passive agencies established in government, public and private sectors), vector control remains the mainstay in preventing mosquito breeding by multiple interventions including promulgation of civic bylaws by the local municipal corporations. Currently, with the ongoing economic reforms, increased infrastructure and work opportunities, urban population is growing by phenomenon of 'rural push' for earning livelihood and 'urban pull' for availing healthcare services and educational opportunities. The intervention measures undertaken by municipal and health authorities have, however, not kept pace with rapid urban development. Satellite townships are mushrooming, and moquito vector breeding sources have multiplied due to water storage practices, unplanned growth and swelling urban slums; all these have contributed to an upsurge in cases of not only malaria but also dengue as similar breeding habitats are shared by mosquito vector species [4-5].

Among the six dominant mosquito vectors of malaria in India [6], *Anopheles stephensi* is held as the culprit and reckoned as the major vector in urban/semi-urban areas as established by records of its prevalence and incrimination data in major cities and towns [7, 8]. Present-day India stands better poised by added healthcare services, monitoring and evaluation, and has embarked on malaria elimination by 2027 much before the WHO mandate of 2030 [9-11]. Included in this chapter is the updated information on vector bionomics, distribution and disease relationships that would help devise interventions for sustainable control of this vector species in urban India.

Taxonomic considerations and distribution

Anopheles stephensi is a commonly encountered mosquito species in metropolitan cities and adjoining suburban town areas [7]. It can easily be identified from other member species of the subgenus *Cellia* by a set of distinguishing morphological characters (Figure 1) [8]. Unlike other dominant vector species, it is not considered as a species complex, and rather comprises of three ecological variants, i.e., 'type form', 'intermediate form' and variety '*mysorensis*' characterized by egg morphometrics (egg length, width and number of ridges on the egg float), cytological features [12], Y–chromosome variation for being metacentric (Y1) and sub-metacentric (Y2) [13], and spiracular index [14]. In India, the 'type form' is an efficient vector of malaria in urban areas, while variety '*mysorensis*' and 'intermediate form' recorded in rural/peri-urban areas are largely zoophilic and have no role in malaria transmission [7,15]. All three ecological variants are reported to occur in India with 'type form' exclusively restricted to urban areas.

An. stephensi is widespread in South and Southeast Asia extending from West of India to Pakistan, Afghanistan, Iraq, Iran, Bahrain, Oman and Saudi Arabia, and to the East in South China and Myanmar (Figure 2) [7, 8, 15]. It is widespread throughout India but has not been reported in large tracts of north-eastern states of India (except for a presumed narrow band connecting to Bangladesh/ Myanmar) [16-18]. There is no record of its prevalence in neighbouring countries Bhutan [19] and Nepal [20], except that of Maldives [21]. There are reports of its invasion to Djibouti (the horn of Africa), Ethiopia and Sudan thus presenting the possibility of its spread to neighbouring countries and threatening malaria control and elimination efforts (S. Manguin, Pers. Commun.). Recent reports of *An. stephensi* invading Sri Lanka post-elimination is a matter of grave concern for preventing re-establishment of malaria transmission [22].

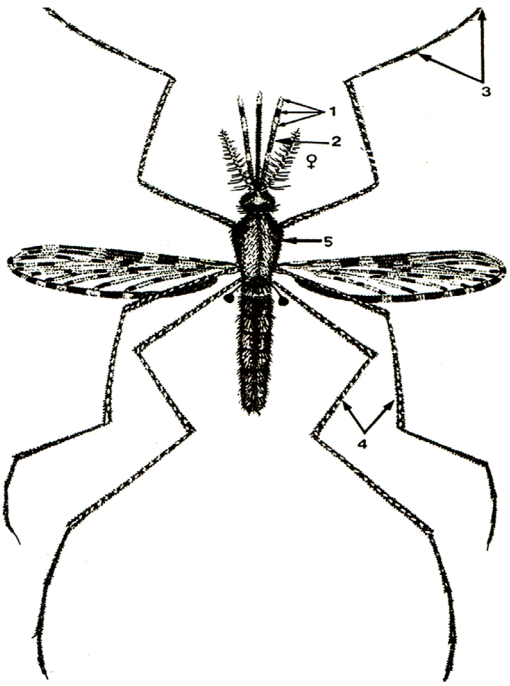

Figure 1: *Anopheles stephensi*: morphological distinguishing characters of mosquito adult: (1) apical and subapical pale band equal separated by dark band, (2) palpi with speckling, (3) fore-legs tarsomeres without broad bands, (4) legs with speckling, (5) thorax with broad scales. Source Reference [8].

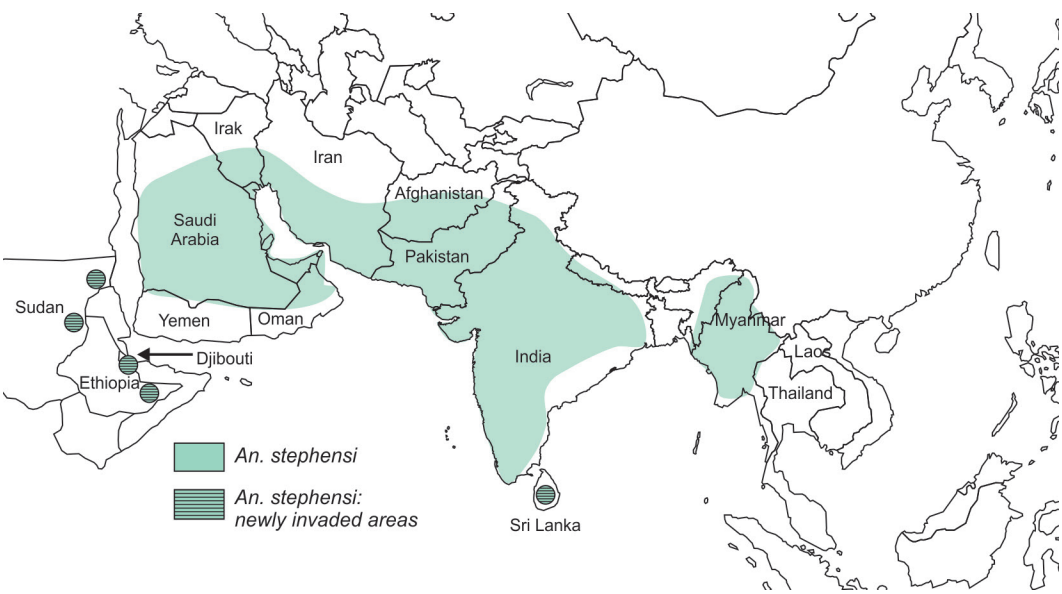

Figure 2: Distribution of *Anopheles stephensi* in South and South-East Asia. There is probable small corridor of distribution between India to South-East Asian country of Myanmar. It has been spotted in Djibouti (the horn of Africa) and known to have invaded island country of Sri Lanka, Ethiopia and Sudan (Courtesy: Sylvie Manguin, Montpellier, France) [sketch map not necessarily in conformity with political boundaries].

Breeding characteristics and seasonal prevalence

Breeding of *An. stephensi* 'type form' has been recorded in diverse habitats including domestic overhead tanks, wells, underground cement tanks, cisterns, desert coolers, ornamental fountains, water storage reservoirs/curing water at building construction sites and parapets on terraces and windows in urban areas (Figure 3) [23-26]. Of these, overhead tanks and water storage reservoirs at the building construction sites were the most common breeding habitat in big metropolitan cities, viz., Chennai, Mumbai (formerly Bombay) and Mangaluru (formerly Mangalore). *An. stephensi* is fast invading desert-ecosystem and its proportions are seen far exceeding those of *An. culicifacies* breeding and resting exclusively in 'tankas' (underground water reservoirs) resulting in focal disease outbreaks (Figure 4).

An. stephensi variety mysorensis, instead is recorded in rural areas breeding in a wide variety of habitats including seepage water streams and irrigation channels, ponds/lakes and abandoned wells [7]. It normally prefers fresh water, but breeding has also been recorded in polluted as well as brackish water [27]. *An. stephensi* mosquitoes are recorded to be prevalent throughout the year but most abundant during months of rainfall coinciding with the transmission period [28-30].

Figure 3: Breeding habitats of *Anopheles stephensi* in urban metropolitan cities. Upper left: masonry water reservoir in building construction projects; Upper right: ornamental garden fountain (Courtesy: Ashwani Kumar, ICMR - National Institute of Malaria Research, Field Unit, Goa); Lower left: high-rise buildings with innumerable water reservoirs (often left without intervention); Lower right: curing water over freshly laid cement slabs

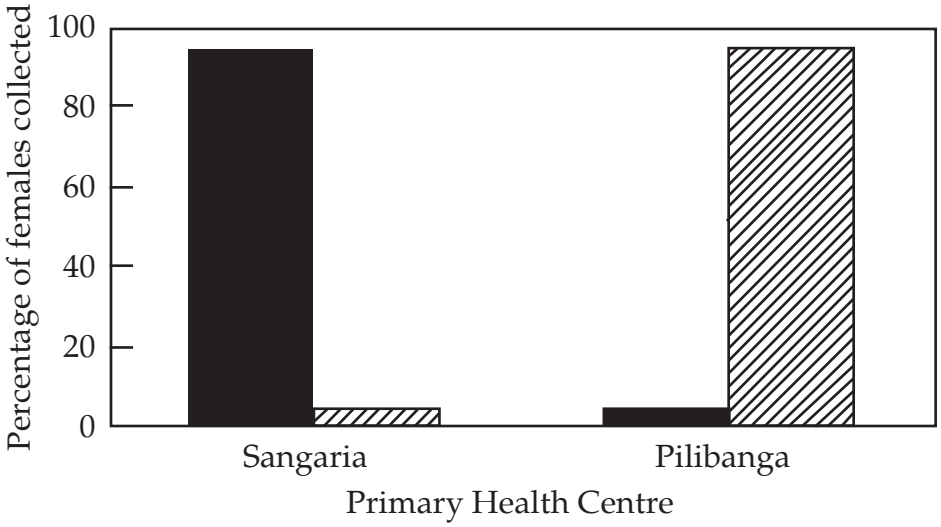

Figure 4: Relative abundance of *Anopheles stephensi* and *Anopheles culicifacies* in Sri Ganganagar district of Rajasthan. *Anopheles stephensi* proportions far exceeded those of *Anopheles culicifacies* in Pilibanga Primary Health Centre collected breeding and resting exclusively in Tankas (xeric ecosystem). The reverse was true in Sangaria Primary Health Centre (highly irrigated terrain) wherein densities of *Anopheles culicifacies* exceeded those of *Anopheles stephensi* found breeding in variety of resources (Courtesy: B.K. Tyagi, Jodhpur).

Genetics, evolution and speciation

An. stephensi 'type form' is easier to colonize and ideal for laboratory-based investigations including teaching demonstrations and evolutionary relationships. It has been thoroughly studied for cytology including chromosome karyotype, polytene chromosomes, chromosomal polymorphism and allied investigations [31-39]. The chromosome karyotype comprises three pairs of chromosomes (2n=6) including a pair of heteromorphic sex-chromosomes (XX/XY), and two homomorphic pairs of autosomes, chromosome 2 and 3. Of these, chromosome 2 is sub-metacentric (the longest) and chromosome 3 is metacentric. The sex-chromosome pair (the shortest of all three) is acrocentric, i.e., XX in females and XY in males (Y chromosome is heterochromatic). The polytene chromosomes both in salivary glands and ovarian nurse cells are well spread and have been extensively examined for inversion polymorphism by independent investigators across range of its distribution [35-38]. The urban populations, i.e., 'type form' were observed to be highly polymorphic for inversions, while variety '*mysorensis*' was comparatively less polymorphic in peri-urban/rural areas [35,37].

In populations from urban India, majority of these inversions were concentrated in chromosome 2R (sub-zones 11A–16A), the longest arm of the autosomal pair; 2L, however, was completely devoid of any such aberration [36]. The inversions, however, were equally prevalent in both arms of chromosome 3, i.e., 3R (sub-zones 32C-36B) and 3L (39A-43B), but to lesser frequency compared to chromosome 2. The sex-chromosome pair (XX), however, was monomorphic. Consistent with observations in Indian population, Mahmood and Sakai reported several additional inversions in urban populations of Pakistan [37]. All recorded inversions were only paracentric involving

variable segments of the chromosomes. Nevertheless, in addition to simple paracentric inversions, Sharma et al [36] reported complex chromosome re-arrangements and overlapping inversion heterozygotes (inversion within inversion) in laboratory inbred population apparently conserving blocks of genes together (supergenes) to possibly provide adaptive advantage.

An. stephensi was initially believed to be a species taxon based on population differences in habitat and biological characteristics [40]. Based on these observations, Rao et al [41] hypothesised the existence of two distinct biological races, i.e., 'type form' and 'variety *mysorensis*' evidenced by egg morphometrics and urban/rural divide. These observations were further substantiated by crossing experiments reporting reduced fecundity, fertility and hybrid sterility [42]. Later, Puri [43] and Stone et al [44] accepted them as subspecies. However, Rutledge et al [45] considered them as variants based on which Knight and Stone [46] labelled them as synonyms in World Mosquito Catalogue. Subsequently, Subbarao et al [47] also reported successful reciprocal crosses and backcrosses between 'type form' and '*mysorensis*' colonies, there being no evidence of post-copulatory isolation. These authors, in addition to 'type form' and variety '*mysorensis*', established the existence of 'intermediate form' based on having median egg float ridge numbers, i.e., 14-22, 12-17 and 9-15 in 'type form', 'intermediate form' and variety '*mysorensis*' respectively; and considered all three as ecological variants.

Host preferences, infectivity and disease transmission

An. stephensi 'type from' is primarily an endophilic and endophagic (resting and feeding indoors in human dwelling) in urban settings but has also been recorded resting in cattle sheds, construction sites/labour huts and barracks closer to human dwellings/breeding sources [48-50]. The species is largely zoophilic and feeds on cattle; anthropophilic indices, however, varied from 0.09 to 3.4 per cent between locations [7]. It is a thermophilic species and has a flight range between 0.8 - 2.5 km maintaining high degree of contact with human host. It is reckoned as a sturdy mosquito with high longevity and parity compared to other sympatric mosquito vector species [7, 8]. Peak biting activity is recorded between 22:00 to 24:00 hours (the first two quarters of the night) but varied seasonally in different localities [7, 8, 48-50]. It is proven highly susceptible to induced human plasmodial infection and has been widely incriminated in all major metropolitan cities and towns of India, Pakistan, Iran and Iraq by detection of gut and gland infections, but overall infection rate remained <2% [7, 8]. It is largely the 'type form' that maintains endemic malaria in urban metros and often held responsible for malaria outbreaks in building construction sites associated with congregation of migratory labour force hailing from malaria endemic areas as evidenced by periodic high rise of cases in Mumbai and Mangaluru [7, 15, 23, 26]. Transmission by *An. stephensi* is often recorded to be supplemented by *An. subpictus* and *An. culicifacies* in urban/peri-urban [51-53], and *An. culicifacies* and *An. fluviatilis* in arid/semi-arid/foothills respectively [54-57]. *An. stephensi* variety '*mysorensis*' in rural areas is predominantly zoophilic and rests outdoors in cattle sheds, barracks and poorly constructed houses [7, 8]. In Iran, however, it is variety '*mysorensis*' that is widely abundant and considered as a vector of importance [58].

Insecticide resistance and vector control

An. stephensi is resistant to multiple insecticides including DDT, dieldrin, malathion in most parts of the country except pyrethroids (variable response) [59]. However, conversely reduced susceptibility to pyrethroids and good response to DDT was reported from the southwest coastal city of Mangaluru, Karnataka [60]. Indoor residual application is not in practice in urban settlements except for containing disease outbreaks restricted to pyrethrum space-spraying in high-risk blocks to cull vector populations. Instead, control interventions are applied against larval breeding sources which include: (i) source reduction by minor engineering works by earth filling of ditches, pits, low-lying areas, streamlining, canalizing, desilting, de-weeding, emptying water containers (desert coolers) and observing weekly dry-day and the like, (ii) recurrent anti-larval application of larvicides including Temephos, *Bacillus sphaericus* and *Bacillus thuringiensis* israelensis H-14 (Bti) at weekly intervals; these interventions have been field evaluated to be effective for control of mosquito breeding in urban settings [61, 62], (iii) biological control by application of larvivorous fish (Guppy and Gambusia) in ponds, drains, irrigation wells etc.; fish-based intervention has been successfully applied in targeting mosquito breeding in variety of breeding habitats in Karnataka and Gujarat [63-65], (iv) enforcement of legislative civic bylaws for preventing mosquito breeding in household premises, construction projects and industrial belts earlier applied in Bombay [23]. However, what is seen in practice that these interventions are rarely enacted in true essence permitting proliferation of vector species and propagation/spread of malaria [66].

Urban malaria and its magnitude

In urban India, the maximum numbers of malaria cases are reported from major metropolitan cities including Chennai, Vishakhapatnam, Vadodara, Ahmedabad, Kolkata, New Mumbai, Vijayawada and New Delhi annually with large concentration of cases in industrial/mining belts, slums and water scarcity areas [2,3]. In Tamil Nadu, 70% of cases were reported from Chennai alone majority (96%) of which were *P. vivax* cases [50]. In Karnataka, similar situation has been observed where almost 70% of malaria cases are reported from Mangaluru city, of which 90% are vivax cases [26]. Contrary to rural India, *P. vivax* (the relapsing malaria) is the most common infection in urban cities, the remaining are *P. falciparum* cases [2, 3, 66-68]. The morbidity due to *P. vivax* malaria, however, is a serious concern due to its relapsing characteristics and consequent anaemia compromising health and work productivity [69 ,70]. Instead, *P. falciparum* is solely responsible for malaria-attributable death cases [67]. *P. vivax* malaria by and large remains susceptible to chloroquine therapy [71,72], yet drug-resistant vivax cases have been documented in India and other endemic countries [73-75]. Given the interventions in force under UMS, malaria transmission is unabated and disease outbreaks are happening despite healthcare services [68]. In 2010, disease outbreak was witnessed in Mumbai related to building construction project sites claiming 145 lives [3]. However, rise in malaria-attributable deaths cases was invariably associated with the increased migration from *P. falciparum* endemic rural areas and associated drug-resistance [66, 67, 76]. Overall, malaria cases in urban areas are grossly underestimated due to shortages of skilled staffs and inadequate healthcare services to address the rising population pressure (77, 78).

Priority areas of research

For effective containment of vector populations, entomological surveillance for monitoring susceptibility status of both adult and larval populations to adulticides and bio-larvicides respectively should be the cornerstone [79, 80]. Intersectoral linkages should be prioritized and training and re-orientation of health staff should be a continuing activity keeping abreast with the latest technologies [78]. Application of Remote Sensing (RS) and Geographical Information System (GIS) should be explored for early warning and targeting interventions in real time and place to avert disease outbreaks [81, 82]. Equally important would be monitoring the therapeutic efficacy of anti-malarial drugs for radical treatment of both *P. vivax* and *P. falciparum* malaria and monitoring compliance with the national drug policy for malaria [83]. Most critical is the screening of migratory labour force/active surveillance at construction project sites, slum areas and adjoining townships/peri-urban areas for malaria parasite in order to mitigate impending outbreaks and spread of drug-resistant malaria.

Conclusions

India is moving towards malaria elimination yet there are multiple issues requiring attention of programme and policy managers to conquer malaria [84,85]. Amidst myriad challenges, *An. stephensi* transmitted malaria is seen as an emerging threat to the control programme. Urban malaria is a growing menace with a larger share of *P. vivax* cases that have extended morbidity on account of multiple relapses. With the continuing phenomenon of urbanization, there is a growing need for strengthening healthcare services for disease surveillance thus ensuring early case detection and treatment, implementation of appropriate interventions for species-sanitation and enforcement of civic bylaws for keeping vector populations in check [86, 87]. *An. stephensi* is an invasive species and its entry to new towns and settlements evidenced by first record of its occurrence in malaria-free territories of Sri Lanka [88,89], Lakshadweep [90,91], Andamans & Nicobar group of Islands [92], and offshores in Djibouti and Ethiopia [93] threatens re-establishment of transmission in malaria-free territories. Vector control should be the major stay to interrupt transmission for which integrated vector management methods, environment-based user-friendly interventions, and human resource development should all be considered to keep pace with the population explosion/urbanization. Health education and behavioural change communication campaigns should be continuing activities for enhanced community compliance to prevent mosquito breeding and to seek treatment well in time to minimize transmission.

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Section III

Bionomics of secondary malaria vectors and implications in malaria elimination

Chapter 8

Anopheles (Cellia) annularis group: species composition, bionomics and risk of malaria transmission in India

Surya Kant Sharma*

ICMR – National Institute of Malaria Research, New Delhi – 110 077, India

*Correspondence: suryaksharma@gmail.com

Abstract

Mosquito species of the *Anopheles annularis* species group comprising of *An. annularis, An. philippinensis, An. nivipes* and *An. pallidus* are widely abundant in north-east, east and south-eastern coastal states of India and considered vectors of secondary importance for records of occasional malarial parasite infectivity and some degree of predilection for human host. These species are closely related for their taxonomical and shared bionomical characteristics but can be identified unequivocally aided by molecular taxonomic tools. Whereas, *An. annualris* is resistant to DDT, *An. philippinensis/nivipes* mosquitoes are susceptible to all available insecticides including DDT, malathion and synthetic pyrethroids. There are indications of incipient resistance in these mosquito species for which monitoring population abundance, insecticide resistance and ecological succession is considered important to keep pace with malaria elimination efforts and beyond to maintain malaria-free status.

Keywords: malaria, transmission risk, Anopheles, sibling-species, vector control, ecological succession

Introduction

India is malaria endemic and transmission is maintained by multiple vector species spread across its varied landscape and agroclimatic zones [1]. Besides six dominant mosquito vectors of human malaria [2], several other species are also implicated in malaria transmission evidenced by some degree of anthropophagy and occasional records of sporozoite infectivity [3, 4]. Among these, some member species of the *Anopheles annularis*

group, i.e., *An. philippinensis/nivipes* and *An. annularis* are of significance for having incriminated and contributing to transmission intensities in north-eastern and eastern coastal states of West Bengal and Odisha in particular [5, 6]. These member species even though are of lesser significance for being predominantly zoophagic but may assume greater role in maintaining ongoing transmission or may re-establish transmission in malaria-free territories in context of population migration, deforestation and changing agricultural practices. There is every possibility of ecological succession by some member species of this species group given the niche vacated by some zoophilic species having no role in malaria transmission specific to north-east India [7, 8]. Included in this chapter is an updated account of species composition and bionomical characteristics of *An. annularis* group for formulating appropriate intervention strategies for containment of vector populations in time and space.

Taxonomic considerations, sibling-species composition and distribution

Anopheles annularis group belongs to the Neocellia seriesof the subgenus *Cellia*, member species of which are widespread in the oriental region from Afghanistan, Pakistan through India to 'Indochina' and the Philippines, south to Sri Lanka and north to China [9]. It includes *An. annularis* Van der Wulp, *An. philippinensis* Ludlow, *An. nivipes* (Theobald), *An. pallidus* Theobald and *An. schueffneri* Stanton. Except *An. schueffneri* (with restricted distribution in Java and Sumatra), all other species have been reported from India and implicated in malaria transmissionin their distribution range [5,10].

These are all medium sized mosquito species each having morphological description and are closely related except for subtle morphological differences in larval and adult stages [11,12]. Aided by molecular tools, all member specieshave been characterized unequivocally based on sequence variation of internal transcribed spacer 2 (ITS2) and domain–3 (D3) regions of rDNA [13, 14]. Polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) of D3 region produced distinctive pattern for four member species of this group including *An. annularis*, *An. philippinensis, An. nivipes* and *An. pallidus*. Among these, *An. annularis* has been identified to be species complex comprising of two cryptic species provisionally designated as'A' and 'B'based on fixed paracentric inversion readable on ovarian polytene chromosomes [15]. These two sibling-species were further characterized by sequencing variation of ITS2 and D3 domainof rDNAas well as PCR-RFLP method corresponding with cytogenetic form 'A' and 'B' [16]. Of these, species A is more abundant across Indian states and implicated in malaria transmission [15, 16].

Similarly, *An. philippinensis* and *An. nivipes* instead are virtually difficult to identify correctly for sympatric distribution and overlapping morphological differences [17]. *An. nivipes*, formerly considered only a variant form of *An. philippinensis* [18], was accorded species status based on crossing experiments and hybrid sterility [19]. Earlier taxonomic records of these two species referred exclusively to *An. philippinensis*; and *An. nivipes* was thought not to exist in India [5]. However, at the turn of century, polytene chromosome analysis revealed that earlier described *An. philippinensis* were actually *An. nivipes* based on chromosome homologies and diagnostic inversions described in populations from Thailand [20,21]. Subsequently, aided by molecular tools, both *An. philippinensis* and *An.*

nivipes are now confirmed to coexist, however, relative abundance varied in range of their distribution [22, 23]. Of these two species, *An. nivipes* has been confirmed to be more abundant in north-eastern states except for hilly-forested regionsof Arunachal Pradesh and Mizoram where *An. philippinensis* exceeded the proportions of the former [23].

Seasonal abundance, infectivity and disease transmission relationship

Among member species of the Annularis group, *An. philippinensis* and *An. nivipes* are seasonal with peak density corresponding with months of rainfall, viz., April-September/ October. These two species are commonly encountered constituting major proportion of mosquito collection in north-eastern states [24-27], Andaman and Nicobar Islands [28] and adjoining country of Bangladesh [29] and have been implicated in playing some role in malaria transmission [4, 30, 31]. However, presently *An. philippinensis/nivipes* reportedly have disappeared from the adjoining state of West Bengal (erstwhile area of dominance) [5, 32, 33] and getting scarce westwards [34]. Instead, *An. annularis* is species of the post-monsoon season (September-April) and widely recorded in winter months (melanic form) and constitutes fair proportion of the mosquito collection across its distribution range (Table 1) [24, 35, 36]. Its seasonal density, however, varied between 0.05 to 7.72 per person hour with predominance in eastern states of West Bengal, Odisha, Jharkhand and Chhattisgarh [6,32-36]. Relative abundance of *An. philippinensis* and *An. annularis*, however, varied across states, e.g., in north-eastern states of India, *An. philippinensis* predominated compared to *An. annularis* [3, 24-26]. On the contrary, in eastern state of Odisha, proportion of *An. annularis* exceeded those of *An. philippinensis* [35]. Nevertheless, both these mosquito species have been incriminated in Assam, West Bengal, Odisha by detection of salivary gland and midgut infections [4, 5, 12, 30, 37-40]. Comparatively, seasonal infectivity of both these species remained insignificant given the major role of *An. minimus*, *An. baimaii* and *An. fluviatilis* in areas of their dominance [2, 41]. The seasonal abundance of *An. pallidus* remained invariably much lower than that of *An. philippinensis* more so in north-east [25]; conversely, it constituted fair proportion of the fauna exceeding *An. philippinensis* in rest of India (35, 36). However, role of *An. pallidus* in malaria transmission is considered only marginal, if any.

Table 1. Seasonal abundance of *Anopheles annularis* group of species by CDC light trap sampling method in Kamrup district of Assam, north-east India (1990-1991)*

Anopheles Species	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	Total	% of total collection**
An. annularis	0	0	0	0	13	2	5	0	1	0	2	11	34	4.89
An. philippinensis	8	0	6	0	45	7	28	0	2	2	72	30	200	28.74

*Source Reference [24], **Data based on 44 light trap collections in human dwellings indoors during 18:00-05:00 hours

Host choice, resting and larval breeding characteristics

Member species of the *An. annularis* group are largely cattle biting/zoophilic but reported to have some predilection for human host as well [25,42-44]. The anthropophilic

index for *An. annularis* reportedly varied from 0.23 in Gujarat to high of 13.1% in Chhattisgarh [6]. The seasonal mosquito human landing rates varied between months for *An. annularis*, *An. nivipes* and *An. pallidus*, and mean biting rate per person night was much higher for *An. nivipes* and the least for *An. pallidus* (Table 2). All member species are nocturnal and actively search human host all through the night, but peak biting activity occurred in first half till midnight [41, 42]. *An. annularis* is often recorded resting in mixed dwellings both indoors as well as cattle sheds [43]. Instead, *An. philippinensis*, *An. nivipes* and *An. pallidus* are largely recorded resting in cattle sheds during evening collections [25, 27].

Table 2. Mosquito landing rate of *Anopheles annularis* group of mosquito species in Kamrup district of Assam, north-east India*

Anopheles species	April	May	June	July	Aug	Sept	Oct	Nov	Mean bites/person/ night
An. annularis	0.00	0.25	2.67	1.00	1.50	1.33	0.67	0.00	1.15
An. nivipes	0.50	0.00	2.67	1.00	3.25	4.50	4.33	0.50	2.59
An. pallidus	0.00	0.25	0.33	0.00	0.00	0.17	0.00	0.00	0.12

*Source reference [3]

Mosquito species of the *An. annularis* group are known to breed in variety of habitats including ponds, ditches, streams and paddy fields (Table 3) [45]. *An. annularis* mosquitoes in particular are recorded breeding in ponds/water reservoirs, ditches, streams and barren paddy fields/with plant growth ≤30 cm often in association with *An. barbirostris*, *An. nigerrimus* and *An. vagus*. Instead, *An. philippinensis/nivipes* are truly species of the rice-agroecosystem found breeding in all stages of plant growth including post-harvest and barren fields; density of these mosquito species invariability corresponds with paddy cultivation/wet season.Typical epidemiological settings supporting breeding of these two species and associated risk of malaria transmission is depicted in Figure 1. *An. pallidus*, instead has been recorded breeding exclusively in ponds only.

Table 3. Larval breeding characteristics of *Anopheles annularis* group of mosquito species in Assam, north-east India*

Anopheles species	Ponds	Ditches	Streams	Paddy fields at various stages of plant growth					
				Barren	Saplings	≤30 cm	>30 cm	Ready to harvest	After harvest
An. annularis	+	+	+	+	-	+	-	-	-
*An. nivipes***	+	-	+	+	+	+	+	+	+
An. pallidus	+	-	-	-	-	-	-	-	-

*Source Reference [45]; **Data distinguishing breeding habitats of *Anopheles philippinensis* and *An. nivipes* are not available; (+) denotes positive for larval breeding and (-) for absence

Figure 1: Landscape epidemiology supporting breeding of *Anopheles nivipes* and *Anopheles philippinensis* and malaria transmission associated with paddy cultivation. Upper: Typical mud-plastered housing structure made up of split bamboos with Tin/thatched roof seen with closely annexed cattle-shed. *Anopheles philippinensis/nivipes* mosquito species are largely zoophilic/cattle biting and can be collected in good numbers in evening collections resting in cattle-sheds. Lower: Paddy fields often in close vicinity of human habitation are the preferred breeding habitats of *Anopheles philippinensis/nivipes*. These mosquito species are recorded breeding at all stages of plant growth including after harvest and barren fields

Insecticide susceptibility status and control options

Among species of the *An. annularis* group, *An. annularis s.l* is resistant to DDT, but susceptible to malathion and pyrethroids [46-48]. There are indications of developing resistance against malathion for which verification is mandated. Instead, *An. philippinensis* and *An. nivipes* are susceptible to all three categories of insecticides, *i.e.*, DDT, malathion and pyrethroids [43]. There is no available information on insecticide susceptibility status of *An. pallidus*. In summary, the comparative bionomical characteristics of member species are presented in Table 4.

Taking cognizance of their potential in malaria transmission and available intervention tools, mass scale distribution of long-lasting insecticidal nets is advocated to minimize the risk of malaria infection in areas of their dominance.

Priority areas of research

Even though member species of the *An. annularis* group are considered vectors of secondary importance for malaria transmission, these species can assume greater role in absence of major vectors, viz., *An. minimus* and *An. baimaii*, populations of which are reportedly

Table 4. Comparative bionomical characteristics of *Anopheles annularis* group of mosquito species specific to India

Anopheles species/taxa	Number of sibling-species identified	Molecular diagnostic tools*	Breeding habitats	Feeding behaviour (peak biting activity)	Seasonal abundance & resting habitats	Incrimination status (average sporozoite infectivity rate)	Insecticide susceptibility status	Distribution range
An. annualaris s.l.	A & B	ITS-2, PCR-RFLP of D3 region	Ponds, ditches, streams, barren paddy fields and plant growth up to<30 cm	Mostly zoophilic, AI = 0.23 – 13.1%, MBR=1.15 (till midnight)	Post-monsoon months/ winter season (Sept-April), both indoors and outdoors	Incriminated (<0.01 %), MPI=5.8%	Resistant to DDT but susceptible to malathion and pyrethroids	Predominant in north-east, east and south-eastern coastal states
An. philippinensis	None	ITS-2, PCR-RFLP of D3 region	Predominantly paddy fields	Predominantly cattle-biting, MBR=2.59 (22.00-01.00 hours)	Monsoon season/ wet season (March – Oct/ Nov), mostly outdoors	Incriminated (0.51%), MPI=0.94%	Susceptible to DDT, malathion and pyrethroids	Predominant in north-eastern states
An. nivipes	None	ITS-2PCR-RFLP of D3 region	Predominantly paddy fields	Predominantly cattle-biting, MBR=3.16 (21.00-24.00)	Monsoon season/ wet season (March – Oct/ Nov), mostly outdoors	Incriminated (<0.01 %)	Susceptible to DDT, malathion and pyrethroids	Predominant in north-eastern states
An. pallidus	None	ITS-2PCR-RFLP of D3 region	Ponds	Predominantly cattle-biting, MBR=0.12 (no data)	Monsoon season, mostly outdoors	No data	No data	North-eastern states, coastal sates of Odisha and South India

*All species can be characterized by polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) of ribosomal DNA-D3 region; ITS-2: Internal transcribed spacer; AI= Anthropophilic index; MPI=Minimum prevalence of infection; MBR = Mosquito biting rate per person/night

depleting [7, 8]. Deforestation, population migration and growing acreage under paddy cultivation are resulting in ecological succession of disease vectors creating increased breeding resources. There are indications of incipient insecticide resistance in *An. annularis s.l* for which monitoring population abundance and insecticide resistance is mandated for appropriate policy in time and space for effective vector control. This information would be of vital importance in preventing re-establishment of transmission in malaria-free territories.

Conclusions

An. annularis group of species are widely abundant in north-east, eastern and south-eastern coastal states and hold potential for malaria transmission. Member species including *An. annularis*, *An. philippinensis*, *An. nivipes* and *An. pallidus* are closely related but can easily be identified aided by molecular diagnostic tools based on sequencing of ITS2 and D3 domain of rDNA. Monitoring population abundance, infectivity and current status of insecticide resistance is mandated for containment of vector populations helping accelerate towards the set goal of malaria elimination by 2030 and beyond for maintaining malaria-free status [49, 50].

Acronyms	
AI	anthropophilic index
D3	D3 domain of 28S rDNA
DDT	dichloro-diphenyl-trichloroethane
ITS 2	Internal Transcribed Spacer 2
MPI	minimum prevalence of infection
MBR	mosquito biting rate
PCR	Polymerase Chain reaction
rDNA	ribosomal DNA
RFLP	restricted fragment length polymorphism

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Chapter 9

Anopheles (Cellia) maculatus group: species composition, distribution and role in the transmission of malaria in India

Pradyumna Kishore Mohapatra*

Ex-Scientist, ICMR – Regional Medical Research Centre, Dibrugarh – 786 001, Assam, India

*Correspondence: pkmdibr@gmail.com

Abstract

Anopheles maculatus group is known to comprise nine species of which six namely *An. sawadwongporni, An. maculatus (s.s.), An. dravidicus, An. pseudowillmori, An. willmori,* and *An. rampae* have been characterized to occur in India. Of these member species, *An. pseudowillmori* is most abundant in hills/foothill areas and had some degree of predilection for human host. These member species rest both indoors and outdoors but largely are exophilic and exophagic, and recorded breeding in variety of habitats including ponds, seepage streams, pits and paddy-fields. There is virtual lack of data on insecticide susceptibility status and bionomical characteristics of individual member species specific to India for possible role in malaria transmission. These data would be of significance for assuming possible role in residual transmission in the wake of disappearing malaria and strengthening cross-border initiative in achieving malaria-free status in Southeast Asia.

Keywords: Maculatus group, species composition, cross-border malaria, malaria elimination, bionomics, residual transmission, India, Southeast Asia

Introduction

Malaria transmission in India is characterized by multiplicity of disease vectors and varied ecological characteristics [1]. Many mosquito species are implicated in disease transmission of which six species including *Anopheles culicifacies, An. fluviatilis*, *An. dirus, An. minimus, An. sundaicus* and *An. stephensi* are held dominant vectors for records of seasonal sporozoite infectivity in field populations in areas of their receptivity [2]. Besides these, member species of *An. maculatus* along with those of *An. annularis* group as well as sibling-species of *An. subpictus* complex are also implicated in malaria transmission but considered of lesser significance for sporadic observations of incrimination [3]. Presently, India is witnessing economic boom with expanding infrastructure, industrialization and urbanization resulting in ecological changes affecting fauna and flora. There is body of evidence that deforestation and increase in agricultural acreage is resulting in ecological succession of some of these mosquito species hitherto considered of little importance [4]. Building densities of *An. maculatus* have been documented in erstwhile domain of *An. dirus* and *An. minimus* in districts sharing international border in north-east India having implication in formulating intervention strategies related to cross-border malaria [5-8]. Included in this chapter is the information on species composition of *An. maculatus* group, distribution and disease transmission relationships for strengthening cross-border initiative to end transmission in Southeast Asia region.

Taxonomy, species composition and distribution

An. maculatus (s.l). is a medium sized mosquito and can be distinguished from other member species of the subgenus *Cellia* by given morphological characters (Figure 1) [9]. What formerly considered to be single species with two varietal forms [10], *An. maculatus (s.l.)* is now characterized to be group of nine formally recognized species [11]. These include: *Anopheles maculatus (s.s.), An. dravidicus, An. notanandai, An. rampae, An. sawadwongporni, An. dispar, An. greeni, An. pseudowillmori* and *An. willmori* [12]. Most of these species can be distinguished by adult and egg morphological characters with some degree of certainty, yet all these species can now be identified unequivocally by number of techniques. These include mitotic chromosomes karyotype (X and Y polymorphism) and polytene chromosome karyotypic studies for fixed diagnostic paracentric inversions [13], electrophoretic variation/cuticular hydrocarbon profile [14], PCR/RFLP method [15], PCR-based assays for interspecific variation in internal transcribed spacer (ITS2) and D3 domain of ribosomal DNA (rDNA) [16,17], and microsatellite markers (Table 1) [18, 19]. Member species of this group are widespread in West Asia and the Oriental region ranging from Pakistan to Indonesia and recorded to occur in Bhutan, Nepal, Bangladesh, Myanmar, South China, Thailand, Cambodia, Malaysia, Philippines, Taiwan (Figure 2) [20]. *An. maculatus* is characteristically a species of hills and foothills and recorded to be prevalent throughout mainland India including Andaman & Nicobar Islands except Lakshadweep [3]. While no intraspecific genetic variation was observed in inbreeding populations of member species within India, but some degree of isolation seems to have occurred between species compared to those of neighbouring countries for data based on sequencing of ITS2 of rDNA [21]. Phylogenetically, *An. pseudowillmori* appeared to be ancestral species for being in basal position showing distant relationships with all other member species.

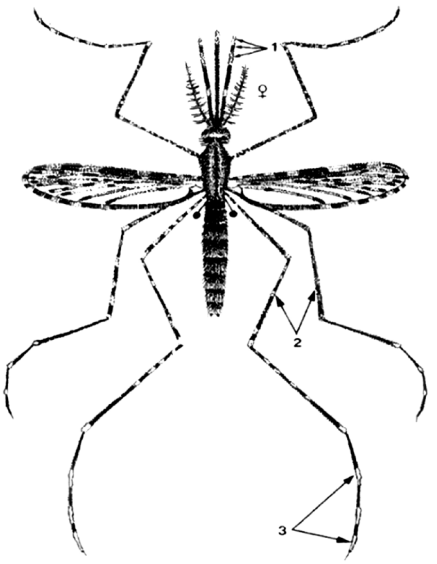

Figure 1: Morphological distinguishing characters of *Anopheles maculatus (s.l.)*: (1) apical and subapical pale bands equal separated by dark band, (2) legs with speckling, (3) hand leg tarsomeres banded. Source Reference [9]. The taxonomic keys have been upgraded providing distinguishing characters between *An. maculatus (s.s.)*, *An. pseudowillmori* and *An. willmori* [22]

Seasonal abundance, infectivity and disease transmission relationships

Very little information is available on species composition and disease transmission relationships of prevalent member species in India except for that of north-eastern states [21]. Literature search by and large invariably refer to *An. maculatus (s.l.)* (nominotypical form with reduced abdominal scaling) without any sibling-species break up except for prevalence of *var. willmori* (with heavy abdominal scaling) in the Himalayas and eastern India [3]. Amongst nine known member species of the Maculatus group, as many six species namely *An. pseudowillmori, An. maculatus (s.s.), An. sawadwongporni, An. willmori, An. dravidicus* and *An. rampae* were recorded to occur in north-east India in varying proportions [21]. Of these, *An. pseudowillmori* (59.5%) and *An. maculatus (s.s.)* (32%) were most abundant and constituted bulk of mosquito collection; distribution of other member species was patchy and occurred in insignificant numbers. Among these six species, morphological taxonomic keys are now upgraded to distinguish *An. pseudowillmori, An. willmori* from that of *An. maculatus (s.s.)* based on scaling pattern of abdominal terga and palpi [22]. The relative abundance of member species, however, was much higher in hill/foothill areas of eastern Himalayas (Indo-Myanmar border) than plain valleys and recorded to occur at varying elevations ranging anywhere from 100 – 2000 metres (m) above mean sea level (amsl) [6, 21, 23, 24]. In South India, instead, *An. maculatus (s.s.), An. dravidicus* and *An. willmori* were reported to occur in Western Ghats at elevations ranging from 400– 1000 m amsl [25]. No such information is available in other malaria-endemic states of India. *An. maculatus (s.l.)* is a pre-monsoon species with seasonal abundance during (March - May) and constituted significant proportion of the mosquito collection compared to other prevalent anopheline species in foothills of Arunachal Pradesh bordering Myanmar [6].

However, in plain valleys, these mosquitoes occurred in much lesser densities compared to foothill areas evidenced by various sampling devices including CDC miniature light traps, whole-night human landing catches, indoor day-resting and cattle-biting collections (Table 2).

An. maculatus (s.l.) has been suspected to play some role in malaria transmission in India but there is no substantial body of evidence for lack of data on incrimination records. None of its member species were found sporozoite positive in the recent past [21] except for historic data of pre-DDT era dating back to 1940s for study based in erstwhile Assam [26, 27]. However, *An. pseudowillmori* and *An. willmori* have been implicated for malaria transmission in adjoining countries of Bhutan and Nepal respectively [28-30]. *An. pseudowillmori* has also been incriminated in Thailand with sporozoite infections of both *P. falciparum* and *P. vivax* [31]. *An. maculatus*, instead has been held playing an important role in peninsular Malaysia [32] and have been implicated in Afghanistan [33] and Indonesia [34].

Host choice, resting and larval breeding characteristics

An. maculatus group of mosquito species are largely zoophilic and exophagic and found

Table 1. Diagnostic inversion genotypes and other methods available for the identification of Maculatus group of species. Source Reference [20]

Mosquito species [Reference No]	Distribution	Cytotaxonomic designation	Diagnostic inversion genotypes on arm 2*	6-Pgd electromorphs	PCR-RFLP	ITS2-based PCR assay
Anopheles. sawadwongporni Rattanarithikul & Green 1986 [12]	Myanmar, China, India, Cambodia, Thailand and Viet Nam	A	$pt^1u^1v^1w^1$	130	-	yes
An. maculatus (s.s) Theobald 1901[10]	Bangladesh, Myanmar, China, India, Indonesia, Cambodia, Malaysia, Nepal, Pakistan, Sri Lanka, Taiwan, Thailand and Viet Nam	B,E,F	j	100	-	yes
An. dravidicus Christophers 1924[10]	Myanmar (Kaley valley), India, China and Thailand	C	$x^1y^1z^1$	-	-	yes
An. greeni Rattanarithikul & Harbach1990 [42]	Philippines	D	q	-	yes	-
An. notanandai Rattanarithikul & Green 1986[12]	Thailand	G	xy	-	-	-
An. willmori (James) 1903[10]	India, Nepal,Pakistan, China and Thailand (Chiang Mai)	H	-	-	-	yes
An. pseudowillmori (Theobald) 1910[10]	China, India, Nepal, Thailand and Viet Nam	I	$o^1p^1q^1$	70	-	yes
An. dispar Rattanarithikul & Harbach 1990[42]	Philippines	J	r^1	-	yes	-
An. rampae Harbach & Somboon2011 [43]	Thailand, India	K	-	-		yes

*These inversions are used to distinguish *An. maculatus* sibling-species from *An. stephensi*. In addition to these, in all species of the Maculatus group, inversions **a** on arm 3, x on arm 4, and c and d on arm 5 are fixed, an exception is *An. pseudowillmori* in which the +x arrangement on arm 4 is seen as in *An. stephensi. An. willmori* is distinguished from *An. stephensi* only by 3a, 4x and 5cd.

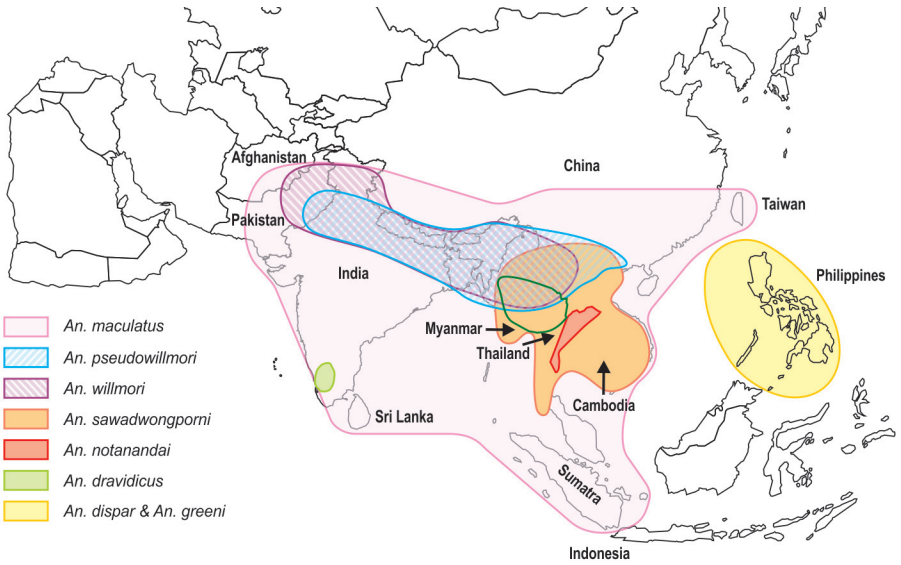

Figure 2: Distribution of member species of the *Anopheles maculatus* group in West Asia and the Oriental region (Courtesy: Sylvie Manguin, Montpellier, France) [sketch map not necessarily in conformity with political boundaries].

Table 2. Relative abundance of *Anopheles maculatus (s.l.)* in different ecotypes of malaria-endemic blocks of north-eastern states of India

Study location (district, state) [Reference No]	Ecotype	Study period	Mosquito density per person hour		Mosquito-biting rate per person night		No. mosquitoes per CDC light trap* night	
			Day-resting	Cattle-biting	Out-doors	In-doors	Out-doors	In-doors
Dimoria block (Kamrup, Assam) [23]	Plain valleys	Oct.1988 - Sept.1990	0.06	0.24	-	0.18	-	0.09
Silachari (South Tripura, Tripura)[24]	Indo- Bangladesh border	June-Sept., 2012	0	0.73	1.33	0.50	5	3
Jairampur (Changlang, Arunachal Pradesh) [6]	Foothills of Indo - Myanmar border	Feb-Sept., 1990	-	2.44	0.67	0.55	2.52	-

*CDC light trap = Centre for Disease Control light trap

resting in cattle sheds more abundantly than house dwellings [35]. The relative abundance was nearly fourfold high in cattle sheds than indoors, viz., it ranged from 0.06 (indoors) to 0.24 (outdoors) per person hour in plain valleys of Assam [23], and nearly zero (indoors) to 0.73 (outdoors) in plains of Tripura [24]. Mosquitoes sought human host both indoors and outdoors and biting rate varied from 0.18–1.33 per person night between locations but had marked activity outdoors than indoors (Table 2). Much of the biting activity occurred before midnight beginning soon after dusk (08:00–24:00) hours [6]. However, host-blood meal analysis of the *An. maculatus* group of mosquitoes those collected indoors human dwellings revealed that 50% (17/34) and 65% (13/20) of *An. pseudowillmori* and *An. willmori* were positive for human blood respectively suggestive of possible role in malaria transmission [21]. *An. maculatus* mosquitoes were recorded breeding in variety of

habitats including ponds, seepage streams, roadside pits/pools and paddy-fields [36], but reportedly had marked preferences for sunlit areas [3, 37]. The flight range is estimated to be between 0.5 to 1.6 km for record of mosquito breeding from human habitation and expected to live two weeks substantiated from parity rate that ranged between 38-53 per cent [3, 38].

Insecticide susceptibility status and control options

There is dearth of information on insecticide susceptibility status of *An. maculatus* species group specific to India. In other Southeast Asian countries, however, it exhibited low degree of resistance to all test insecticides including DDT, malathion, deltamethrin, permethrin [39, 40]. Given the available data, it is believed that for control of *An. maculatus* transmitted malariain India, present interventions in force along with mass-scale distribution of long-lasting insecticidal nets (LLINs) would suffice to reduce transmission risk [41].

Priority areas of research

There is virtual lack of data on distribution and composition of member species of the *An. maculatus* group in different climatic zones of India. Information on bionomics of member species and disease transmission relationships would gain eminence in wake of disappearing malaria for possible role in residual transmission. There is virtually no information on insecticide susceptibility status throughout rage of its distribution specific to India. All these aspects of biology of this fast-evolving species group deserves due priority in context of malaria elimination to prevent re-establishment of disease transmission in malaria-free territories in India.

Conclusions

An. maculatus is a fast-evolving species group given the range of agroclimatic zones in the South-East Asia region of the world with periodic reports of new species hitherto undescribed [42,43]. While nine species have been formally recognized, yet another tentatively designated as *An. maculatus var. menorah* has been reported to occur in Central Java, Indonesia[44]. Among these, six species have been reported to occur in varying proportions having implications in malaria transmission control in India. Except for that of Western Ghats in South India [25], no such information is available in other malaria endemic states with reference to species composition, relative prevalence and malarial infectivity. Even though member species are primarily zoophagic but there exists possibility of *An. pseudowillmori* playing some role in malaria transmission given its abundance and some degree of predilection for human host in foothill areas of north-east India sharing international border with Myanmar and Bhutan. However, there is paucity of information on insecticide susceptibility status of member species for targeting appropriate control

interventions. Given the vector status of some member species of *An. maculatus* group and possibility of emerging zoonotic malaria in other Southeast Asian countries [45], additional information on distribution, species composition and bionomical characteristics

Acronyms	
amsl	above mean sea level
DDT	dichloro-diphenyl-trichloroethane
D3	domain 3 region of 28S rDNA
ITS2	Internal Transcribed Spacer 2
LLIN	long-lasting insecticidal net
m	metres
PCR	Polymerase Chain Reaction
rDNA	Ribosomal DNA
RFLP	Restricted Fragment Length Polymorphism

is warranted specific to India for formulating species-specific intervention to avert return of malaria and helping accelerate towards malaria elimination in the foreseeable future.

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Chapter 10

Anopheles (Cellia) subpictus Grassi 1899: an emerging vector of malaria in urban India

Ashwani Kumar[1*], K. Hari Krishan Raju[1] and Vas Dev[2]

[1] ICMR -Vector Control Research Centre, Indira Nagar, Puducherry-605 006, India

[2] Ex - Scientist, ICMR -National Institute of Malaria Research, New Delhi – 110 077, India

[*] Correspondence: ashwani07@gmail.com

Abstract

Anopheles subpictus has been characterized to be a complex comprising sibling-species provisionally designated A, B, C and D. It is the most abundant mosquito species throughout India breeding in a variety of habitats, and fast establishing as a vector of human malaria in coastal urban cities evidenced by rising densities and parasite infectivity. It is sibling-species B which has been incriminated in the coastal region breeding in brackish water bodies. Elsewhere in South-East Asia, *Anopheles subpictus* is a well-recognized vector of *Plasmodium* species in Indonesia, Malaysia and Sri Lanka. However, very little information is available on the distribution of its sibling-species and bionomics of vector species related to disease transmission across Indian states. Further more, there is a paucity of information on insecticide susceptibility status and response to the mass-scale distribution of insecticide-treated nets/long-lasting insecticidal nets to contain this vector species, hitherto, considered to be of low priority. The vectoral role of this species may be linked to the rapid ecological changes due to urbanization and climate change. It is high time to generate more evidence and recognize the impending threat by this ubiquitous species as a vector of malaria and integrate appropriate control strategies to contain this mosquito. Also, strengthening healthcare services in slum agglomerations helping monitor and formulate species-specific interventions targeting this emerging vector to avert its spread would be necessary accelerating towards malaria elimination in urban and peri-urban India.

Keywords: Urban malaria, sibling-species, vector bionomics, urbanization, ecological succession, transmission dynamics

Introduction

India is reporting steady decline in malaria cases over the past few years targeting elimination by 2027 and certification by 2030 [1, 2]. Vector control is the cornerstone to disrupt malaria transmission, efforts for which are largely focused on six dominant mosquito vector species including *Anopheles culicifacies*, *An. fluviatilis, An. minimus, An. dirus, An. stephensi* and *An. sundaicus* [3]. Besides these, *An. annularis, An. philippinensis/ nivipes, An. maculatus* and *An. subpictus* are known to play secondary role in malaria transmission but not targeted specifically in the control operations [4]. In the present-day context of expanding urbanization, population migration on account of 'rural push' and 'urban pull', deforestation and changing agricultural practices are inadvertently resulting in the increased number of breeding habitats and ecological succession of disease vectors once considered inefficient to sustain transmission [5-7]. Given the dwindling populations of some of the dominant vector species in high-risk areas on one hand and invasion and establishment and expansion of mosquito species such as *An. subpictus* on other, implies that the malarial parasite is either invading new mosquito vector host once considered of low priority or this phenomenon has been little explored; thus opening new vistas for research, policy and planning for overall effective vector management [8,9]. Apparently, the malaria parasite and the mosquito host have co-evolved considerably exhibiting paradigm shift in mosquito behaviour towards human host for blood-meal and enormous breeding potential amounting to rising population density and continued transmission. In keeping with the malaria elimination efforts, it is time to underscore the bionomics of vector species of lesser significance (largely zoophilic and zoophagic) to formulate a comprehensive intervention strategy to conquer malaria. Among these, *An. subpictus* which has long been considered of low priority is assessed to be assuming greater role in malaria transmission in urban settings [10,11]. Given in this chapter is the current knowledge on biological attributes of *An. subpictus*, the emerging vector in urban India helping formulate appropriate interventions for benefit of control programme and policy managers in reducing transmission risk.

Taxonomic considerations, sibling-species composition and distribution

An. subpictus belongs to subgenus *Cellia*, series Pyretophorus [12]. It is widely distributed in the Oriental region extending from west of India in Pakistan, Afghanistan, Iran and to the east as far as Papua New Guinea, Australia and Mariana islands including Bangladesh, Myanmar, Thailand, Malaysia, Vietnam. Philippines and Indonesia(Figure 1) [13]. It is abundant throughout India including Lakshadweep and neighbouring countries of Sri Lanka, Maldives, Nepal, Myanmar and south China. *An. subpictus s.l.* is a medium-sized mosquito and can be distinguished from other species of the subgenus *Cellia* by given morphological characters (Figure 2) [14]. This taxon has evolved to be species complex comprising four sibling-species provisionally designated as species A, B, C and D identified by series of techniques including morphological differences, distinct polytene chromosome karyotype with fixed paracentric inversions in X-chromosome, and larval breeding characteristics [15]. Among these, inversions readable on polytene chromosome-X (*two distinct inversions - one small towards the tip of the chromosome designated 'a' and another big in middle designated 'b'*) served as diagnostic markers for sibling-species identification for mere absence of heterozygotes in natural populations [15]. Sibling-species 'A' with

standard polytene chromosome karyotype (X+a, +b) was predominant in inland villages while species 'B' with fixed inversions (Xa, b) was observed to be sympatric with species 'A' in coastal villages of South India. These findings were corroborated by egg, larval, pupal and adult morphological differences (Table 1). In northern India, while in villages near to Delhi, species A was reported to be prevalent [16]; instead sibling-species A, C and D, all reported to co-exist in nearly equal proportions in adjoining township to Delhi breeding in river-bed pools [17]. No such data are available from other regions of India giving sibling-species composition and relative abundance. In Sri Lanka, however, both species A and B are reported to occur, the former being more abundant and endophilic than species B [18].

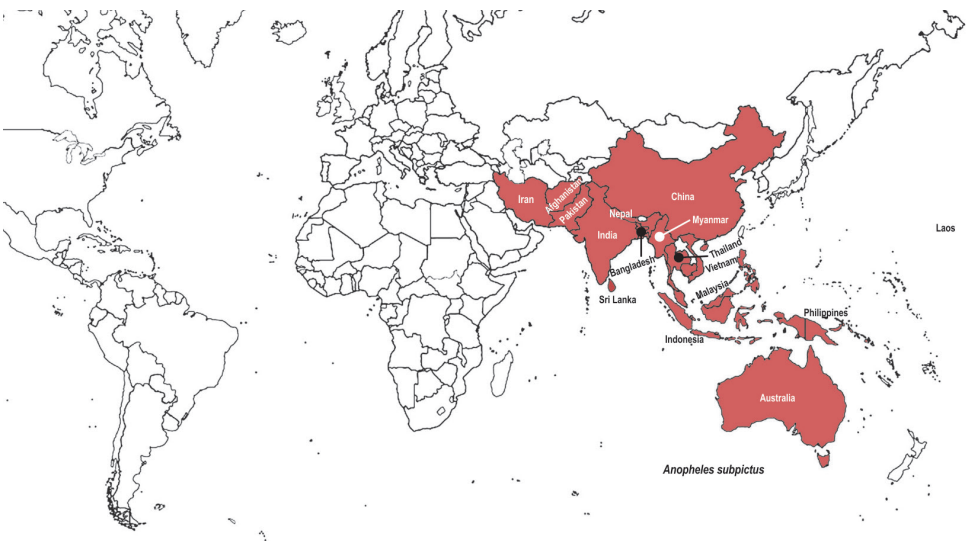

Figure 1: Geographical range of distribution of *Anopheles subpictus* in the world [sketch map not necessarily in conformity with political boundaries].

Table 1. Morphological, cytological and larval breeding characteristics of sibling-species of *Anopheles subpictus* complex. Source References [13,15]

Sibling-species	Polytene chromosome karyotype	Egg morphology		Larval chaetotaxy	Pupal chaetotaxy	Adult morphology	Breeding habitat (salinity range)
		Mean ridge No. (range)	Frill	Mesothoracic Seta 4 M	Setae 7-I	Length of apical pale band on female palpi	
A	X+a, +b	35 (31-36)	Opaque	2-branched (rarely 3)	Simple; as long as setae 6 & 9	Longer than sub-apical dark band	Paddy fields (0.0054 - 0.2636), Riverine pools (0.0247 - 0.7827) Back waters (0.5574 - 5.3554)
B	Xa, b	18 (16-20)	Transparent	2-branched (rarely 1)	Branched 4-5; shorter than setae 6 & 9	Shorter than Sub-apical dark band	Back waters (0.5574 - 5.3554)
C	Xa, +b	27 (25-29)	Semi-transparent	3-branched (rarely 2)	Branched - 2; medium length	Equal to sub-apical dark band	Same as A
D	X+a, b	22 (21-24)	Semi-transparent	3-branched (rarely 2)	Branched - 3; medium length	Equal to sub-apical dark band	Same as A

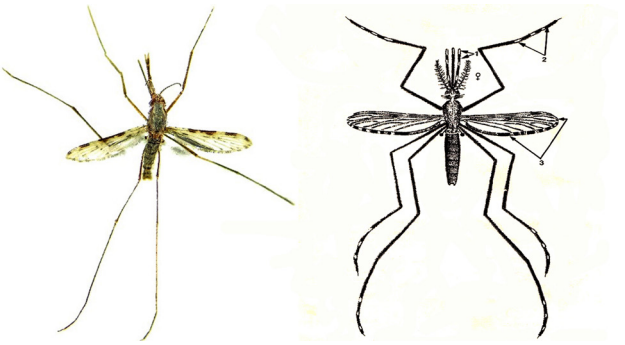

Figure 2: Left: *Anopheles subpictus s.l.* mosquito adult (Courtesy Mr. Elango, ICMR-Vector Control Research Centre, Puducherry); Right: diagrammatic sketch showing morphological distinguishing characters, (1) apical pale band nearly equal to the pre-apical dark band, (2) fore-leg tarsomeres with broad pale bands, (3) fringe-spots on all the veins. Source Reference [14]. Taxonomic keys have been updated to identify sibling-species A, B, C and D [15]

Seasonal abundance, infectivity and disease transmission relationships

An. subpictus is the most abundant mosquito species widely prevalent in mainland India all through north, west, central and south Indian states, however, its relative abundance decreases eastwards (Figure 3). It is recorded to occur up to 1900 metres above mean sea level in hill districts of Uttarakhand and Tamil Nadu but not in Sikkim and West Bengal [19]. Invariably, it occurs in close association with *An. stephensi, An. culicifacies* and *An. vagus* having implications in disease transmission control. *An. subpictus* is typically a monsoon species and occurs in varying density between regions/seasons, viz., it ranged from 98 - 132 per person hour in villages near Delhi [20], 66 - 84 in Madhya Pradesh [21], 4.04 - 20.02 between post-monsoon and monsoon months respectively in Chhattisgarh [22], while in north-eastern states its prevalence was recorded to be <1.00 [23,24]. In Goa, not only the relative prevalence of *An. subpictus* exceeded that of *An. stephensi* (the known vector) but also recorded to be prevalent for the most part of the year including post-monsoon season facilitating perennial transmission [10]. Similarly, in the metropolitan city of Chennai, besides *An. stephensi; An. subpictus* constituted a significant proportion of mosquito collection (43.6%) and incriminated for the very first time in the city [11]. *An. subpictus* mosquitoes have also been incriminated in Madhya Pradesh [21], Pondicherry [25], Odisha [26], Goa [10], and the neighbouring country of Sri Lanka [27,28] by detection of Plasmodial infections of both *P. falciparum* and *P. vivax* individually as well as mixed infection. Among its sibling-species, it is species B (brackish water species) which has been implicated for its role in malaria transmission in coastal villages of South India [10,25]. Even though seasonal infection rates remained well below 1.00% (0.01– 0.52) in most study locations [29], yet in one study based in Goa, the infection rate in *An. subpictus* (2.8%, 14/501) exceeded that of *An. stephensi* (2.1%, 7/334) [10]. Disease transmission potential of *An. subpictus* is further substantiated by laboratory-based infectivity studies showing susceptibility range to plasmodial species comparatively higher in sibling-species B than A [30, 31].

Host choice, resting and larval breeding characteristics

An. subpictus is largely a zoophilic and outdoor species found resting predominantly in

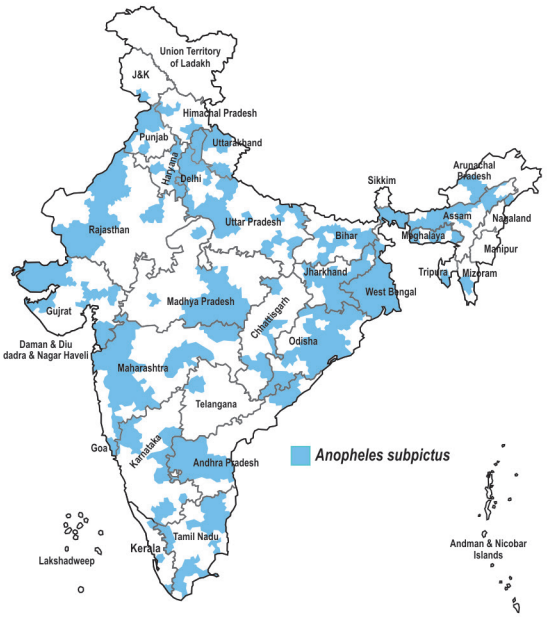

Figure 3: Geographical range of distribution of *Anopheles subpictus* based on records of prevalence in India.

cattle sheds/barns across its distribution range [11,23,24]. Nevertheless, there are records of its resting indoors human dwellings and seeking blood-meal from human host to variable extent ranging anywhere from 3% - 44%; the higher ranges being of brackish water populations [29]. In the fast-emerging metropolitan city of Goa, the anthropophilic index of *An. subpictus* (26.9%) was reported to be comparable to that of *An. stephensi* (29.9%) [10]. Like many other anopheline vector species, *An. subpictus* mosquitoes are also nocturnal biting with pronounced activity during first quarter of the night beginning just after dusk with occasional pre-dawn activity [32,33]. *An. subpictus* mosquitoes are considered strong fliers ranging from 1.5– 6 km [4] and believed to live long enough (>10 days) to support sporogony [34-35]. *An. subpictus* is ubiquitous of all species for breeding in a variety of resources including both fresh and brackish water bodies (Figure 4). These mosquitoes are recorded breeding profusely in temporary rainwater collections in burrow pits, roadside ditches, wheel ruts, puddles; other breeding habitats included shallow ponds, fallow and freshly flooded rice fields, cement tanks etc [25,36,37]. Among its sibling-species, species A, C and D besides being freshwater species are known to withstand salinity ranging from 0.004 to 0.734% NaCl, while species B invariably recorded breeding in salinity ranges >0.40% [36]. These mosquitoes also observed to withstand pollution/turbid water bodies marked by low dissolved oxygen content and reported breeding in association with variety of aquatic plants [38].

Insecticide susceptibility status and control options

An. subpictus mosquitoes are resistant to DDT and other organochlorine compounds across India [39]. Furthermore, there are reports of resistance to malathion and increased tolerance to synthetic pyrethroids [40-44]. However, there are no data on insecticide susceptibility

status specific to sibling-species for targeting them selectively for intervention in place and time. Additional data are warranted from many other endemic states of India and response to the mass-scale introduction of insecticide-treated nets/long-lasting insecticidal nets for effective vector control.

Figure 4: Typical resting and breeding habitats of *Anopheles subpictus* in north-west India: Top: Resting habitat – a barn in rural villages used for housing cattle and fodder storage; Bottom: Breeding habitats; Right–fresh water-logged agricultural fields in close vicinity to human habitations (Courtesy: S.K. Sharma, ICMR - National Institute of Malaria Research, New Delhi); Left – a brackish water body in coastal area (Courtesy: A.K. Mohanty, ICMR - National Institute of Malaria Research, Field Unit, Goa).

Priority areas of research

The emergence of *An. subpictus* as a vector of malaria in urban India has opened new vistas of research to understand its bionomics in major metropolitan cities for which there exists little data on prevalence of sibling-species, infectivity and disease transmission relationships. Additional data are mandated on specific-specific larval breeding habitats in urban areas in relation to those of *An. stephensi* for application of anti-larval interventions. Urbanization is a growing phenomenon resulting in faunistic changes and enhanced receptivity in construction projects and associated migratory labour force agglomerations. Interestingly, molecular characterization of *An. subpictus s.l.* populations of Sri Lanka have revealed genetic homologies of sibling-species B with that of cytotype D of *An. sundaicus* complex (brackish water species in both countries) [45,46]. However, there is a virtual lack of data on the application of molecular assays for characterization of the sibling-species composition and bionomics in many other malaria-endemic Indian states for instituting appropriate vector control interventions. Besides malaria, *An. subpictus* has

also been implicated in transmission of Japanese Encephalitis (JE) in South India for which additional data are mandated in other JE endemic states of India for comprehensive vector management [47].

Conclusions

From the available body of evidence, it is evident that *An. subpictus*, what formerly considered to be a vector of low priority, is establishing rapidly in urban metropolitan cities. It is fast invading urban territories with its vast potential for breeding in a variety of habitats including both freshwater as well as brackish water bodies. Mosquito densities are seen rising in urban cities (formerly regarded as rural species) and hold inherent capacities to host-parasite development evidenced by infection rate comparable to that of *An. stephensi* (the known vector in urban India). *An. subpictus* in conjunction with *An. stephensi* is resulting in perennial transmission what formerly used to be only seasonal corresponding to months of rainfall. Given the clarion call for malaria elimination, it is time to target all vector species inclusive of minor significance to disrupt transmission as well as preventing re-establishment of malaria post-elimination [48]. For a decisive attack on disease vectors, the programme should address strengthening healthcare services in keeping pace with growing urban agglomerations to contain the spread of not only malaria but also JE (an emerging zoonotic disease), which would require local and regional malaria elimination strategies [49, 50].

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Section IV

Malaria transmission and vector control options

Chapter 11

Malaria transmission in India: disease distribution and prevalence of mosquito vectors in different physiographic zones

Anupkumar Anvikar[1] and Vas Dev[2]*

[1] ICMR - National Institute of Malaria Research, New Delhi – 110 077, India

[2] Ex- Scientist, ICMR - National Institute of Malaria Research, New Delhi – 110 077, India

* Corresponding author: mrcassam@hotmail.com

Abstract

India is co-endemic for both *Plasmodium falciparum* and *P. vivax* malaria, distribution of cases, however, varied across different physiographic zones. Among these, East, Central and North-East zones contributed bulk of cases (70%) with large concentration of *P. falciparum* cases. These are also the region with vast forest cover and high tribal/ marginalized population groups living in improvised conditions. Transmission is characterized by multiplicity of mosquito vector species of which *Anopheles culicifacies* is the most commonly spread throughout India generating nearly 65% of reported cases. *An. fluviatilis* is species largely of hills and foothill areas contributing 15% of cases, while *An. minimus*, *An. baimaii* are of regional significance spread in East and North-East zones contributing 5% of cases each. Others included *An. stephensi* (an urban vector) and *An. sundaicus* (a vector in Andaman & Nicobar Islands) contributing about 10% of cases. Most of these except *An. stephensi* are species complexes having distinct bionomical characteristics and disease transmission relationships. Among these, while *An. culicifacies* (multi-insecticide resistant) is spreading; populations of *An. minimus*, *An. baimaii* (highly susceptible to DDT), however, depleting in response to up scaling of current interventions resulting in ecological succession by vectors considered of lesser significance, viz., *An. subpictus*, *An. maculatus*, *An. annularis* group of species. Of available intervention options, larger provision of long-lasting insecticidal nets (LLINs) and roll out of artemisin in-based combination therapy (ACT) have resulted in tangible transmission reduction. Malarial threat is receding yet there is array of challenges given the current parasite load in the endemic communities and resources which are far from adequate for universal coverage. Among these, development and spread of multiple insecticide resistance in vector populations and emergence of drug-resistant malaria are some of the major concerns to 'end malaria'.

Keywords: malaria transmissions, vector control, parasite, malaria elimination, drug-policy, insecticide resistance, Southeast Asia

Introduction

India is a vast country with varied ecology and climatic determinants. Malaria is historically endemic transmitted by multiple mosquito vector species specific to different physiographic regions with varied receptivity [1]. Nearly a billion population is estimated to be living at risk of malaria with disproportionally high burden in low socio-economic/marginalized population groups living below poverty line. Both *Plasmodium falciparum* and *P. vivax* are the predominant infections; parasite formula, however, varied between regions skewed much in favour of *P. falciparum* transmission [2]. With continued urbanization, industrialization, population movement and changing landcover utilization, malaria has diversified into various ecotypes between urban and rural divide, viz., tribal/forest malaria, industrial malaria, desert malaria, mining malaria [3]. India has come long way combating malaria dating back to pre-independence era (1940s) with record number of 75 million cases to near elimination in 1960s (<0.1 million cases) and no deaths, resurgence in 1976 with six million cases to present day malaria reporting about less than a million cases annually. Continued research on parasite biology and vector bionomics post-resurgence have culminated in number of evidence-based technologies that made significant impact on disease transmission control making malaria elimination an achievable goal [4]. Given the implementation of newer intervention tools, viz., long-lasting insecticidal nets (LLINs) for vector control and artemisin in-based combination therapy (ACT) for radical treatment, malaria is fast disappearing in areas once reckoned intractable. Of late, India has made a laudable progress in malaria control reporting steady decline in cases over past few years (>50% decline in 2018 compared to 2016) and embarked upon elimination by 2027 (three years ahead of international agenda) [5, 6]. Yet, it still contributes most cases in the South-East Asia region (80% of total reported cases) and has been grouped among high burden country including 10 others in sub-Saharan Africa [7]. Malarial parasite and mosquito host are continually evolving making malaria control a complex paradox. Disease transmission is heterogenous in intensity and distribution for many regions contributing proportionally far more cases than others [8]. Included in this chapter is distribution of cases in different physiographic regions of India in relation to prevalent mosquito vectors helping formulate informed policy and prioritizing interventions in high-risk zones to end transmission.

Morbidity due to malaria: distribution of cases

Malaria is by and large seasonal in most parts of India corresponding with months of rainfall (monsoon season) except that of perennial transmission in East and North-Eastern states [9-11]. Transmission intensities, however, are assessed to be low-to-moderate in large tracts of land [12]. All Indian states and Union Territories (UTs) are reporting malaria cases annually, distribution of cases and parasite formula, however, varied in different physiographic zones characterized by geographical, ecological and climatic determinants (Table 1) [13]. Of total six different zones, group of states in the East, Central and North-East zones collectively carried the highest disease burden contributing nearly 70% of the total reported cases for data based on 2018. Of the two prevalent parasite species, cumulatively *P. falciparum* was the predominant infection (83%) in these three zones, whereas in North, South and West zones, *P. vivax* predominated. Most states and UTs reported declining transmission trends 2001 onwards from about 2 million cases in the late 1990s to less than

a million cases in the present-day malaria (Figure 1) [14]. These data were corroborated by steady decline in parasite rate (% positive cases) and annual parasite incidence (number of cases per thousand population) in the corresponding reporting period (Figure 2) [15]. Conversely, proportions of *P. falciparum* cases had risen significantly from what was 10% in 1977 to high as 65% in 2016 of total malaria cases (data not shown); majority of which were contributed by East (52%), Central (68%) and North-East zones (90%) with large concentration of *P. falciparum* cases (Table 1).

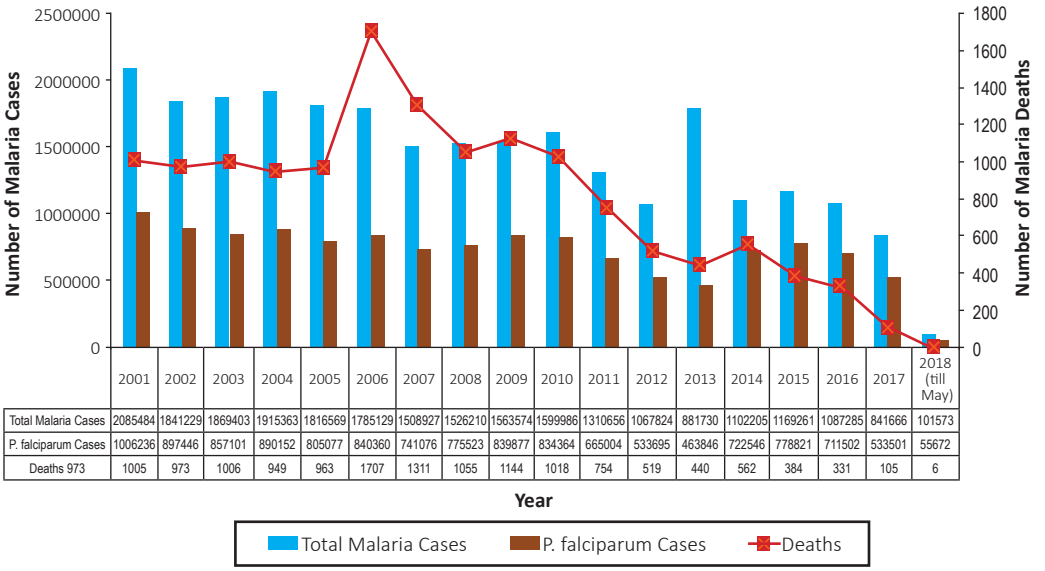
Malaria-attributable mortality: contribution by zone

Malaria-attributable deaths were reported annually and invariably all cases were due to *P. falciparum* infection. From nearly 1000 deaths a year in the 1990s, the numbers declined considerably (except that of epidemic year in 2006 in North-East zone) each passing year presently to less than record 200 in 2017 and decreasing (Figure 1). Of total confirmed death cases in 2018, 82% (79/96) were contributed cumulatively by East, Central and North-East zones in proportion to relatively high abundance of *P. falciparum* cases (83%) in these regions. Comparatively, no deaths due to malaria was recorded in the North-zone whereas a lone case was reported in the South-zone, however, as many 16 deaths were in the West-zone (Table 1). Unlike Africa, in India deaths were recorded in all age groups of both sexes in high transmission zones [9].

Table 1. Relative contribution of malaria cases and prevalence of mosquito vector species in different physiographic zones of India for data based on 2018*

Physiographic Zone	Group of states	No. of reported malaria cases (% of total +ve cases)	No. of *Plasmodium falciparum* cases (% of +ve cases)	No. of malaria attributable deaths	Anopheles vector species	
					Primary vectors	Secondary vectors
North Zone	Jammu and Kashmir, Haryana, Himachal Pradesh, Punjab, Uttarakhand, Uttar Pradesh, National Capital Territory of Delhi	91451 (21)	22679 (25)	0	*An. culicifacies, An. fluviatilis, An. stephensi*	*An. subpictus*
South Zone	Andhra Pradesh, Telangana, Karnataka, Kerala, Tamil Nadu, Andamans & Nicobar Island	18537 (4)	6892 (37)	1	*An. culicifacies, An. fluviatilis, An. stephensi, An. sundaicus*	*An. subpictus*
East Zone	Bihar, Jharkhand, Odisha, and West Bengal	151382 (35)	78268 (52)	15	*An. culicifacies, An. fluviatilis, An. minimus, An. annularis, An. stephensi*	*An. nivipes (philippinensis)*
West Zone	Goa, Gujarat, Rajasthan and Maharashtra	39221 (9)	4897 (12)	16	*An. culicifacies, An. fluviatilis, An. stephensi, An. subpictus*	
Central Zone	Chhattisgarh and Madhya Pradesh	100996 (24)	68818 (68)	40	*An. culicifacies, An. fluviatilis*	
North-East Zone	Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura	28341 (7)	25644 (90)	24	*An. baimaii, An. minimus, An. culicifacies*	*An. nivipes (philippinensis), An. annularis*
Total	**All states**	429928	207198	96		

*Source Reference [13]



	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018 (till May)
Total Malaria Cases	2085484	1841229	1869403	1915363	1816569	1785129	1508927	1526210	1563574	1599986	1310656	1067824	881730	1102205	1169261	1087285	841666	101573
P. falciparum Cases	1006236	897446	857101	890152	805077	840360	741076	775523	839877	834364	665004	533695	463846	722546	778821	711502	533501	55672
Deaths 973	1005	973	1006	949	963	1707	1311	1055	1144	1018	754	519	440	562	384	331	105	6

Figure 1: Declining trends of malaria transmission in India (2001-1017). The cases have declined consistently from 2.08 in 2001 to 0.84 million in 2017. Similarly, *Plasmodium falciparum* cases have declined from 1.0 to 0.53 million during the corresponding period. Malaria-attributable death cases were also seen declining from 1707 in 2006 to 105 in 2017. Source: National Vector Borne Disease Control Programme, Government of India. Source Reference [14]

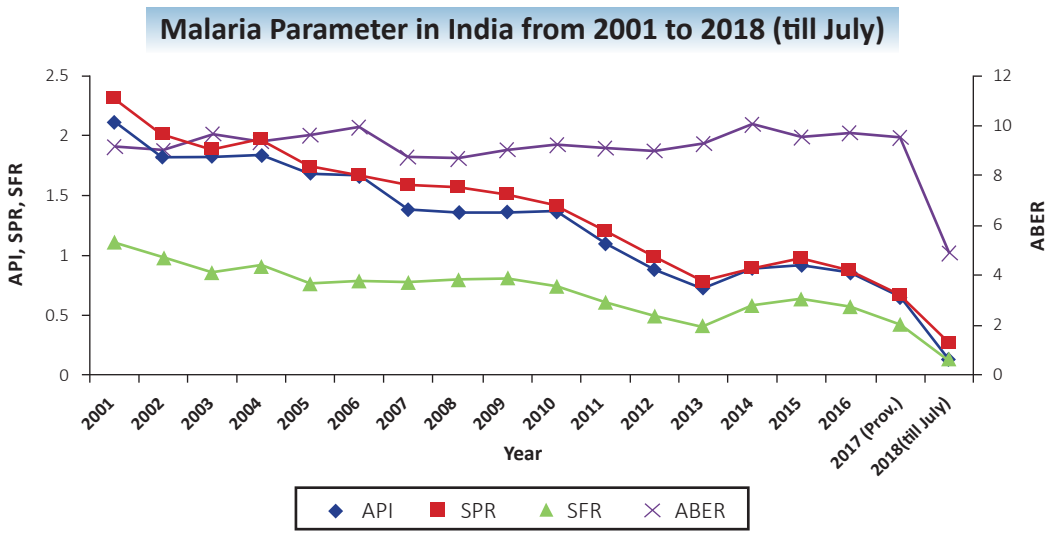


Figure 2: Declining malaria endemicity in the country evidenced by reducing parasite rate and case incidences. SPR and SFR denotes percent smear positives for any malaria parasite and *Plasmodium falciparum* respectively. API (Annual Parasite Incidence) and ABER (Annual Blood Examination Rate) corresponds to number of cases per thousand population and percent population screened for malaria respectively. SPR has declined from 2.31 in 2001 to 0.87 in 2016 and SFR from 1.11 to 0.57 in the corresponding period. Source: National Vector Borne Disease Control Programme, Government of India. Source Reference [15]

Emergence of drug-resistant malaria and treatment policy

Malaria control in India largely rests on two pillars, i.e., vector control and disease surveillance for radical treatment of malaria cases. In this context development of national drug-policy in force has played significant role in reducing parasite load and case management [16]. Ever since inception of the control programme in 1953, Chloroquine (CQ) remained the drug of choice for treatment of both *P. falciparum* and *P. vivax* cases for its efficacy to eliminate blood stage infection. While CQ continued to be effective for treatment of *P. vivax* malaria till date [17], resistance had surfaced in *P. falciparum* first ever detected in Assam in 1973 to standard regimen of chloroquine therapy [18]. Since then, drug-resistant foci had multiplied throughout North-East sector and spread to rest of India over space and time [19]. To contain spread of drug-resistant varieties of *P. falciparum* to peninsular India, national drug policy (first formulated in 1982) has been reviewed periodically and evolved a great deal switching from CQ to sulfadoxine-pyrimethamine (SP) in chloroquine resistant foci in 1995; artemisinin monotherapy in 1998 for cases not responding to SP/complicated cases, and artemisinin-based combination therapy (ACT) beginning 2005 in select districts to delay the emergence of artemisinin resistance (Figure 3). Of various available combinations of ACTs, artesunate+ sulfadoxine-pyrimethamine (AS+SP) was implemented in North-Eastern sector and select districts of other endemic states reporting >10% chloroquine resistant malaria. Artemisinin monotherapy was withdrawn in 2009, instead AS+SP was instituted for treatment of every single case of *P. falciparum* malaria in the country in 2010 replacing SP alone. Soon enough, declining therapeutic efficacy of AS+SP was sighted in North-East sector in 2013 resulting in switch over to artemisinin + lumefantrine (AL) presently in practice in North-East (*seen as corridor for spread of drug-resistant malaria emanating from Cambodia*) as an exception to rest of the country[20]. The rising proportions and growing resistance in *P. falciparum*, however, are still perceived as persistent threat to the control programme.

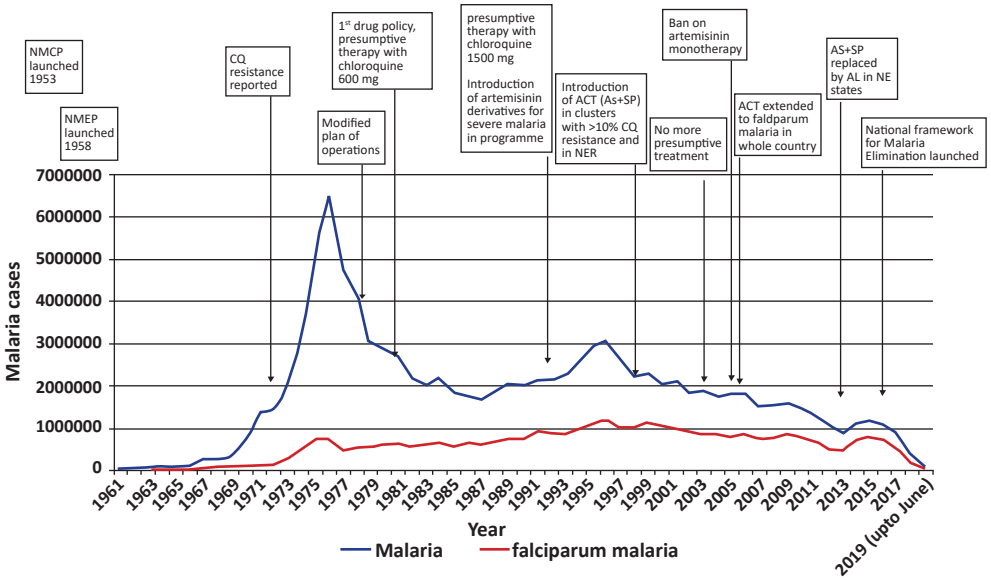


Figure 3: Malaria cases and evolution of antimalarial drug policy in India. Acronyms NMCP, NMEP, CQ, ACT (AS+SP) and NER denote National Malaria Control Programme, National Malaria Eradication Programme, chloroquine, artemisinin-based combination therapy (artesunate + sulfadoxine-pyrimethamine) and North-East Region respectively(Data source: NVBDCP)

The distribution of mosquito vectors and relative contribution of cases

Transmission of malaria in India is characterized by multi-species complexes of mosquito vector taxa having distinct bionomical characteristics and regional distribution (Figure 4) [21]. Of six dominant mosquito vector species, *An. culicifacies* is the most widespread and alone contributes >65% of reported cases in the country [22]. It is a species complex comprising informally designated sibling species A, B, C, D and E having varied distribution and disease transmission relationships. It is rural vector and have grown to resistant virtually to all available insecticides including DDT, malathion and pyrethroids [23]. It is highly adaptive species and has established its foothold evidenced by rising densities and records of incrimination in North-East sector accessing habitats consequent to ecological changes on account of depleting forest cover, population migration across borders and changing land use pattern [24-26]. *An. fluviatilis*, instead is a foothill species spread throughout India having over lapping distribution with that of *An. culicifacies* [27]. It is also a species complex comprising S, T and U, of which sibling species 'S' is highly anthropophagic and proven efficient vector in range of its distribution contributing nearly 15% of reported cases annually. Besides these, *An. minimus* (*An. minimus s.s* of the Minimus complex in India) and *An. baimaii* (member species of the *An. dirus* complex) are the most efficient vectors having regional distribution in east and North-East zones of the country each one contributing 5% of the total reported cases. These two species are exclusively anthropophilic and result in perennial transmission evidenced by incrimination records for all months of the year in their range of distribution. Both these species along with *An. fluviatilis* (species S) are highly susceptible to DDT, malathion and pyrethroids but are invincible for their behavioural characteristics avoiding sprayed surfaces. *An. minimus* is commonly abundant in foothill valleys breeding in seepage water streams, whereas *An. baimaii* is a forest dweller (a jungle pool breeder) linked to transmission of drug-resistant malaria across borders. Unlike most other vector species, *An. stephensi* is an urban vector, a species of the metropolitan cities across India and generates~10% of cases annually. It is considered invasive species and spreading with increased urbanization. Other than that, *An. sundaicus* is a brackish water species prevalent exclusively in the Andamans & Nicobar Islands. It is also a species complex of which 'species D' has been incriminated and known to contribute few hundred cases annually. It has reportedly retracted from erstwhile distribution in coastal Indian states.

Besides aforementioned dominant vector species, *An. annularis, An. subpictus, An. nivipes/philippinensis*, *An. maculatus*, and to some extent *An. jeyporiensis* are also implicated in malaria transmission evidenced by seasonal abundance, host-bloodmeal analysis and sporadic records of sporozoite infectivity [28]. Among these, *An. subpictus* is deemed to be emerging vector quite at par with *An. stephensi* in urban areas evidenced by record of its prevalence and seasonal infectivity resulting in perennial transmission, the former serving as relay transmitter in the post-monsoon season [29].

The evolution of vector control intervention tools

Vector control in large part relies on insecticide residual spraying (IRS) in rural India using DDT, malathion or pyrethroids. Among these, DDTone time considered angel for

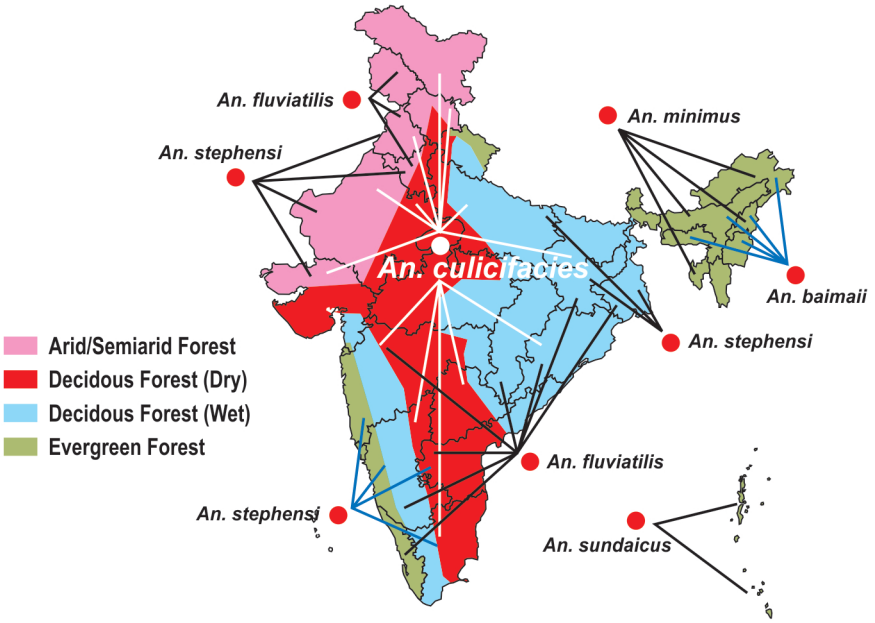


Figure 4: Regional distribution of the dominant mosquito vectors of human malaria in relation to physio-geographic regions encompassing evergreen tropical forest (wet zone receiving rainfall >200 cm), deciduous wet forest (monsoon forests receiving rainfall 100-200 cm), deciduous dry forest (scrub forest receiving rainfall 50-100 cm), and desert forest (arid and semi-arid area receiving rainfall <50 cm) annually. Source Reference [21]

vector control during 1950-1960 had fallen resulting resurgence in 1970s [30]. *An.culicifacies*, the most abundant vector species throughout India has grown resistant not only to DDT but also to malathion and pyrethroids [31]. In India, much of the budgetary allocation for vector control relates to control of *An. culicifacies* transmitted malaria [32]. Nevertheless, the regional vector species including *An. minimus s.s.*, *An. baimaii*, *An. fluviatilis* (species S) and *An. sundaicus* are still susceptible to all three available options including DDT, malathion and pyrethroids [23]. Instead, *An. stephensi*, the urban vector is resistant to DDT and malathion but still susceptible to pyrethroids. Yet given the robust health infrastructure, the programme had still fallen short of expectations giving diminishing returns resulting in continued transmission in many high transmission areas [33]. What ails the programme is the inadequate coverage of population at risk, poor quality spray due to lack of supervision, high refusal rates for variety of reasons, and for not keeping with spray schedule timings and logistics [34]. Many far-flung areas are invariably left unsprayed for years together due to poor accessibility resulting in unusual build-up of vector populations and consequent focal disease outbreaks. In any case, IRS is getting impractical in future India for population explosion, rising costs and continued urbanization making it obsolete strategy.

The advent of insecticide-treated netting materials/long-lasting insecticidal nets (LLINs), however, has proven boon to the vector control programme globally in addressing most of the inadequacies. LLINs have been evaluated to be the appropriate technology in high-risk transmission settings and assessed to be operationally feasible for community-wide acceptance that is environment-friendly, sustainable and cost-savvy [35]. LLINs have become order of the day for large scale procurement and supplies. The

logistic requirement, however, is huge, but coverage remains miniscule of what is mandated. What needed is the continued political commitment for adequate allocation of resources and innovative delivery mechanisms to reach the outreach population groups most at risk. Many more technologies are in the offing, viz., combination nets incorporating more than one class of insecticide to beat multi-resistant vector populations,'eave-tubes' and 'attractive toxic sugar baits (ATSB)'to contain outdoor resting vector populations [36]; but it would long before these are operationalized. Till then, LLINs hold the answer to plethora of issues and should be promoted towards universal coverage making it a common household good.

Malaria elimination: checks and balances

India is a huge country with population a billion plus living at varied risk of malaria. With the currently available tools, many states/UTs are reporting less than 1000 cases while several others have registered substantial decline, Odisha in particular, reporting 80% transmission reduction in 2018 compared to the preceding year [37]. Still, the parasite load is high with close to half a million cases, 50% of which are due to *P. falciparum* (the killer parasite). More so, the estimates for malarial morbidity are well above the board for many sustainable reasons, viz., firstly inadequate disease surveillance in hard-to-reach areas and secondly there being no mechanism to capture cases those recorded in private sector which normally not included [7,8]. Countdown has begun for achieving malaria elimination by 2027 [6], yet the road ahead is bumpy beset with many challenges [38-40]. Some of these include: (i) a sea of asymptomatic malaria/sub-patent parasitaemia which is not included in the disease surveillance least the treatment, (ii) development and spread of multi-insecticide resistant vector populations, *An. culicifacies* in particular, (iii) emergence of multi-drug resistant malaria; the parasite is continually evolving and the armamentarium is running out and it would be a while before new drug regimens come into being, (iv) cross-border malaria for sharing long international border with Myanmar, and Bangladesh, Nepal and Bhutan which are porous with little access to healthcare services in inter border areas. In Southeast Asia, while Sri Lanka is already granted malaria-free status, Bhutan and Nepal are fast approaching elimination for which strengthening cross-border initiative would be of paramount importance to prevent re-establishment of transmission in malaria-free territories.

Despite these challenges, India is all set to meet the cherished goal of elimination given the following inputs: (i) first and foremost is the research support by host of institutions providing evidence-based technologies, monitoring and evaluation services [41,42], (ii) structured strategic action plan strengthened by intensive disease surveillance down to the village level by Accredited Social Health Activists (ASHA) for on-the-spot diagnosis and instituting treatment at door-step, Integrated Disease Surveillance Project (IDSP) for monitoring to prevent impending disease outbreaks and contain by rapid response, (iii) provisions for additional work force and logistics under National Health Mission (NHM); all set to defeat malaria [43]. India has joined hands with Asia Pacific Malaria Elimination Network (APMEN) and signatories to the Asia Pacific Leaders Malaria alliance (APLMA) for shared experience and political commitment for increased funding towards malaria 'end-game' in the Asia Pacific region.

Conclusions

India stands better equipped with host of evidence-based technologies and human resource to meet the malaria challenge. Stakeholders and communities alike are better informed in disease prevention and control lending community support and much needed compliance. There is renewed hope and optimism for living in malaria-free world in the foreseeable future. The advent of LLINs and ACTs have done wonders in reducing malaria transmission substantially. It is high time to universalize these interventions for communities at any risk both against disease vectors and parasite by stronger health systems beginning at the primary health care by increased spending [44]. The requirement is huge but the funding gap, however, is enormous to meet the logistics. What concerns most is the evolution of multi-drug resistant malaria for which disease surveillance and monitoring therapeutic efficacy are critical in averting spread of drug-resistant malaria. With the dwindling populations of efficient mosquito vector species, parasites are invading alternative hosts for continued transmission inclusive of zoonotic malaria making inroads in Southeast Asia. With malaria elimination high on agenda, it is opportune time to target all parasite species and disease vectors including those of lesser significance to end malaria for good [45].

Acronyms	
ABER	annual blood examination rate
ACT	artemisinin based combination therapy
IDSP	Integrated Disease Surveillance Project
AL	artemisinin + lumefantrine
API	annual parasite incidence
APMEN	Asia Pacific Malaria Elimination Network
APLMA	Asia Political Leaders Malaria Alliance
AS+SP	artesunate + supfadoxine-pyrimethamine
ASHA	Accredited Social Health Activist
ATSB	attractive toxic sugar baits
CQ	Chloroquine
DDT	dichloro-diphenyl-trichloroethane
ICMR	Indian Council of Medical Research
LLIN	long-lasting insecticidal net
NHM	National Health Mission
NMCP	National Malaria Control Programme
NMEP	National Malaria Eradication Programme
SP	supfadoxine-pyrimethamine
SPR	smear positivity rate
SFR	smear falciparum rate
UT	Union Territory

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Chapter 12

Insecticide Resistance in Malaria Vectors and Management Strategies in India

Rajpal Singh Yadav[1]*, Kamaraju Raghavendra[2], Poonam Sharma Velamuri[2], Vaishali Verma[2] and Sreehari Uragayala[2]

[1] Department of Control of Neglected Tropical Diseases, World Health Organization, Geneva, Switzerland

[2] ICMR- National Institute of Malaria Research, New Delhi – 110 077, India

* Correspondence: yadavraj@who.int

Abstract

Insecticides are the mainstay for vector control and elimination of malaria. Insecticide resistance of varying levels has appeared globally against most of the malaria vectors in different geographical areas with active transmission. In certain areas with high burden of malaria, its intensity has increased manifold. In India that is poised to eliminate malaria by 2030, multiple insecticide resistance has surfaced in principal malaria vectors in different endemic states. The intensity of resistance, however, varies by vector species to different insecticides, although three malaria vector species namely *Anopheles fluviatilis, An. minimus* and *An. baimaii* are largely susceptible to DDT, malathion and pyrethroids. There is inadequate monitoring of insecticide resistance in terms of coverage and quality; datasets are far too weak and sparse. Major reasons of the under performing vector surveillance system include low public health entomology capacity, inadequate resources and logistic supplies. An integrated national database on insecticide resistance in malaria vectors should be established to provide evidence-base for selection of insecticide-based products and resistance management. As such an insecticide resistance monitoring and management network for the Indian subcontinent would be a useful platform to support the national control programme in capacity building and data sharing for coordinated vector control operations across borders.

Keywords: Malaria, mosquito vector, insecticide resistance, vector control, resistance management

Introduction

Ever since the resurgence of malaria in India in 1976 [1], there has been a steady decline in the incidence of malaria and attributable deaths. India is currently reporting less than half a million cases and targeting elimination by 2027 [2-4]. Presently, control of malaria vectors rests heavily on the effectiveness of insecticide-based vector control tools, i.e., long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) in rural India, and larvicides for larval source management in select situations largely in urban metropolitan cities. Different insecticide products and larvicide formulations that are recommended by the World Health Organization (WHO) for vector control are given in Table 1. Among these, DDT (dichloro-diphenyl-trichloroethane) was widely used in terms of the quantity applied (71% of total), while the use of the pyrethroids dominated in terms of the surface area covered (81% of total) globally [5]. Currently, most of the LLIN products use pyrethroids, except for combination nets, viz., Interceptor G2 LN that incorporates a mixture of a pyrethroid and chlorfenapyr, a pyrrole class of compounds [6]. Among the most effective insecticides that are currently available for IRS include the pyrethroid formulations as well as two recently introduced products containing clothianidin – a neonicotinoid– as a new active ingredient (i.e., SumiShield®50WG and Fludora® Fusion WP-SB) [7]. Overall, in the past 15 years, the use of pyrethroid-based products in vector control operations has increased significantly. Reliance on insecticides for vector control over several decades has resulted in development of resistance in mosquitoes of varying frequency and intensity. Considering pyrethroids that are the widely used adulticides, development of pyrethroid resistance in malaria vectors specifically poses a serious biological threat to global malaria elimination efforts.

Table 1: Insecticide products and larvicide formulations recommended by the World Health Organization for vector control

S. N.	Prodcuts	Indoor residual spraying	Long-lasting insecticidal nets	Insecticides for net treatment	Larvicides
1	Pyrethroids	Alpha-cypermethrin SC, WP, WG-SB; Bifenthrin WP; Cyfulthrin WP; Deltamethrin SC-PE, WP, WG, WG-SB; Etofenprox WP; Lambdacyhalothrin SC, WP	Alpha-cypermethrin; Deltamethrin; Permethrin	Alpha-cypermethrin SC; cyfluthrin EW, Deltamethrin SC, WT; Lambda - cyhalothrin CS; Permethrin EC; Icon Maxx	
2	Organochlorines	DDT WP			
3	Organophosphates	Malathion WP; Fenitrothion WP; Pirimiphos - methyl EC, WP, CS			Chlorpyrifos EC; Fenthion EC; Pirimiphos-methyl EC; Temephos EC, GR
4	Carbamates	Bendiocarb WP, WP-SB; Propoxur WP			
5	Neonicotinoids	Clothianidin WG			
6	Bacterial products				Bti strain AM65-52 WG, GR; Bti AM65-52 + Bs 50 Bsph

7	Benzoylureas		Diflubenzuron DT, GR, WP; Novaluron EC
8	Juvenile hormone mimics		Pyriproxyfen GR; Pyriproxyfen 2 MR
9	Spinosyns		Spinosad DT, EC, GR, SC; Spinosad 83.3 monolayer DT; Spinosad 25 extended release GR
10	Pyrroles	Chlorfenapyr[a]	
11	Synergist	PBO[b]	

CS= capsule suspension; EC= emulsifiable concentrate; EW= emulsion, oil in water; SC= suspension concentrate; SC-PE= polymer enhanced suspension concentrate; WG= water dispersible granules; WG-SB= water dispersible granules in sealed water soluble bags; WP= wettable power; WP-SB= wettable power in sealed water soluble bags; WT= water dispersible tablet.

[a] Mixed with alpha-cypermethrin for net treatment.

[b] Incorporated in nets in combination with alpha-cypermethrin or deltamethrin or permethrin.

Global Overview of Insecticide Resistance

The available information on insecticide resistance frequency, intensity and mechanisms in different malaria endemic geographical areas on various malaria vector species is scanty and far from complete. This problem is more acute in areas outside Africa. Analysis of data made available to WHO between 2010 - 2016 indicated that 77% (56/72) countries and 64% of sites (1375/2145) confirmed resistance in at least one malaria vector to one pyrethroid molecule; that among three main insecticide classes (i.e., organochlorine, organophosphates or carbamates) used in indoor residual spraying, resistance was reported to one class in 12 countries, two classes in 13 countries, three classes in 19 countries, and against all four classes including pyrethroids in 18 countries [8]. Studies indicated that the intensity of insecticide resistance, as measured by the exposure time required to kill 50% of the exposed population, has increased. In Africa that contributes majority of global malaria burden, high intensity resistance, stronger mechanisms and an increasing trend have been observed [9, 10]. A study based in 2016 indicated that pyrethroid resistant *Anopheles gambiae* are present across central and western Africa (Kenya, Tanzania, Zambia and Zimbabwe) [11]. While little is known regarding the spread of resistance in populations of *An. funestus*, the trends reported are thought to be like those of *An. gambiae*. In Asia including the Mekong sub-region, the Latin America and the Pacific, multiple insecticide resistance has become widespread showing an increasing frequency [12, 13].

Insecticide Resistance in India

In India, malaria is transmitted mainly by six principal vectors namely *An. culicifacies, An. fluviatilis, An. stephensi, An. dirus, An. minimus* and *An. sundaicus* [14]; other vectors including *An. annularis, An. philippinensis/nivipes, An. jeyporiensis* and *An. subpictus* are

Table 2: Distribution of malaria vectors in different zones of India

	Zones	States	Principal malaria vectors	Secondary malaria vectors
1.	North zone	Jammu and Kashmir, Haryana, Himachal Pradesh, Punjab, Uttarakhand, Uttar Pradesh, National Capital Territory of Delhi	*An. culicifacies, An. fluviatilis, An. stephensi*	*An. subpictus*
2.	South zone	Andhra Pradesh, Telangana, Karnataka, Kerala, Tamil Nadu, Andamans & Nicobar Island	*An. culicifacies, An. fluviatilis, An. stephensi, An. sundaicus*	*An. subpictus*
3.	East zone	Bihar, Jharkhand, Odisha, and West Bengal	*An. culicifacies, An. fluviatilis, An. minimus, An. stephensi*	*An. annularis, An. nivipes (philippinensis)*
4.	West zone	Goa, Gujarat, Rajasthan and Maharashtra	*An. culicifacies, An. fluviatilis, An. stephensi,*	*An. annularis, An. subpictus*
5.	Central zone	Chhattisgarh and Madhya Pradesh	*An. culicifacies, An. fluviatilis, An. stephensi*	*An. subpictus*
6.	North-East zone	Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura	*An. culicifacies, An. baimaii, An. minimus*	*An. nivipes (philippinensis), An. annularis*

Table 3: Insecticide susceptibility status of malaria vectors in India for data based on 1995 - 2018

Zone	States/ Union territory	*An. culicifacies*	*An. stephensi*	*An. fluviatilis*	*An. annularis*	*An. baimaii*	*An. minimus*	*An. nivipes (philippinensis)*	*An. subpictus*	*An. sundaicus*
North	Delhi	RSS#	RRS	–	–	–	–	–	–	–
	Haryana	RRS	–	–	–	–	–	–	–	–
	Himachal Pradesh	–	–	RSN	–	–	–	–	–	–
	Punjab	–	–	–	–	–	–	–	NRV	–
	Uttar Pradesh	RSS	RSS	–	–	–	–	–	–	–
	Uttarakhand	RRS	–	RNS	–	–	–	–	–	–
East	Jharkhand	RVS	–	RSS	RVS	–	SSN	SSN	–	–
	Odisha	RRR	–	SSS	RNS	–	RNS	–	–	–
	West Bengal	RRS	RRS	–	–	–	–	–	–	–
Central	Chhattisgarh	RRR	–	RNS	–	–	–	–	–	–
	Madhya Pradesh	RRR	–	-	–	–	–	–	–	–
South	Andhra Pradesh	RRR	–	SSS	–	–	–	–	–	–
	Karnataka	NVR	RRR	RSN	–	–	–	–	–	–
	Tamil Nadu	RSR	–	RSN	–	–	–	–	–	–
	Telangana	RRR	–	–	–	–	–	–	–	–
	A & N Islands	–	–	–	–	–	–	–	–	RSN
West	Goa	–	RRN	–	–	–	–	–	–	–
	Gujarat	RRR	RRV	–	–	–	–	–	RRR	–
	Maharashtra	RRR	RSN	RVV	RVV	–	–	–	–	–
	Rajasthan	RSN	RRV	–	RVN	–	–	–	RRV	–
North-East	Assam	RNR	–	–	RNV	SSN	SSS	RSN	–	–
	Meghalaya	–	–	–	–	–	SNN	–	–	–
	Tripura	–	–	–	–	–	VNS	VSS	–	–

The first alphabet is for DDT, the second is for malathion and the third is for deltamethrin. R, resistance; V, possible resistance; S, susceptible; N, not done; – denotes data not available. Source reference [19]

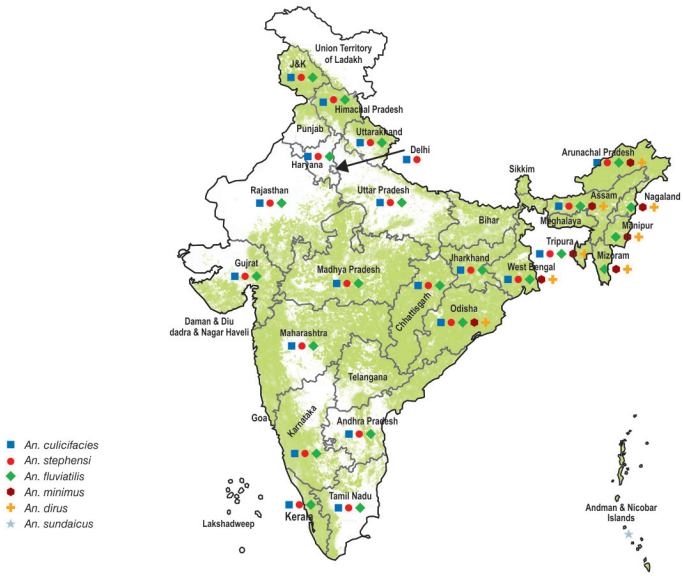

Figure 1: Distribution of principal vectors of malaria in India

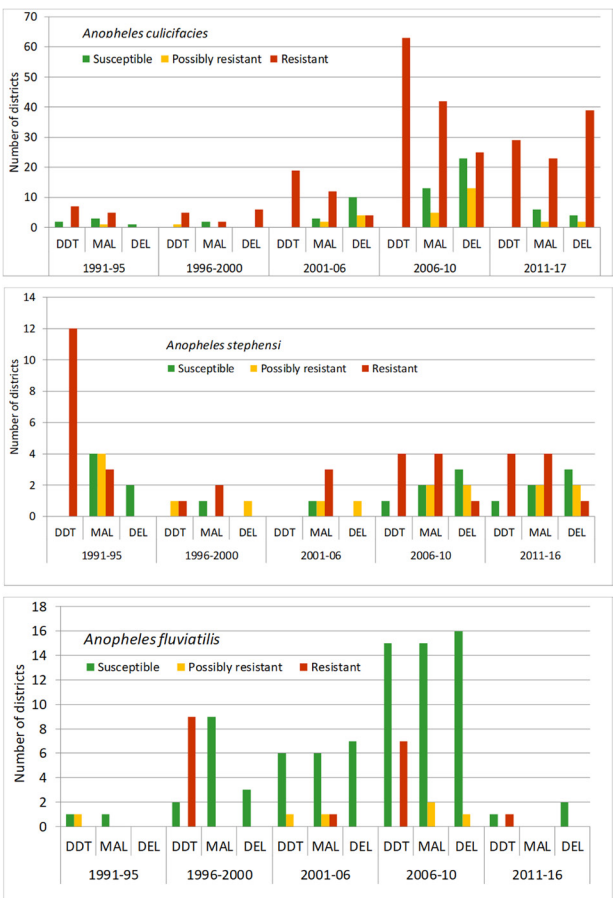

Figure 2: Trends of insecticide resistance against three principal malaria vectors in India to DDT, malathion (MAL) and deltamethrin (DEL) for data based on 1991–2017

considered of lesser/secondary significance (Table 2). The geographical distribution of the principal malaria vectors in different agroclimatic regions of India is given in Figure 1. Among these, *An. culicifacies* contributes to nearly two-third of new malaria cases each year in the rural and peri-urban areas followed by *An. fluviatilis* that contributes ~15% cases occurring in the forested/foothills [15]. *An. minimus* and *An. dirus* (*An. baimaii*) transmit malaria in the hills and foothills of the east and north-eastern states [16]. Besides these, *An. sundaicus* and *An. stephensi* are predominant vector species in the coastal areas of the Andaman and Nicobar Islands and urban India respectively [17]. Insecticide resistance in malaria vectors is wide spread in India, however, response to different insecticides varied across states [18]. Given the set guidelines for monitoring insecticide resistance [19], the summary data on distribution of phenotypic resistance in some of the dominant malaria vectors recorded during 1995–2018 are presented in Table 3 inclusive of some unpublished observations from recent investigations. There has been a gradual increase in the number of districts reporting resistance to DDT, malathion and deltamethrin (Figure 2). In the last two decades, resistance in *An. culicifacies* to pyrethroids has increased significantly which could be attributed to selection pressure possibly due to extensive use of pyrethroid products (LLINs, IRS) in vector control. A summary of the current insecticide susceptibility status of dominant malaria vector species in malaria endemic states of India is given as below:

Anopheles culicifacies (the vector of rural malaria)

An. culicifacies is widely abundant throughout India and has been characterized to be species complex with variable dominance and disease transmission relationships [20]. Data relating to insecticide susceptibility of this species are available from 17 states inclusive of Union Territories [18]. The species is widely resistant to DDT. In an area in Gujarat, persistence to DDT resistance was observed even after 30 years of its withdrawal from the control programme [21]. In the states of Andhra Pradesh, Chhattisgarh, Gujarat, Madhya Pradesh, Maharashtra, Odisha and Telangana; the species is reported to be triple-resistant to DDT, malathion and deltamethrin, and double resistant to DDT and malathion in Haryana and West Bengal. It is recorded to be highly resistant to malathion in Gujarat [22], susceptible in Delhi, Uttar Pradesh, Rajasthan and Tamil Nadu, and variably resistant in other parts of the country. It is reported to be susceptible to deltamethrin in northern states but resistant in central and southern states. In Gujarat and Karnataka, a recent study showed complete susceptibility to bendiocarb (K. Raghavendra, unpublished data). In another study conducted recently in 19 districts of 5 states, *An. culicifacies* was found to be resistant to deltamethrin (K. Raghavendra, unpublishd data).

Anopheles fluviatilis (the vector of malaria in hills and foothills)

The data on insecticide susceptibility of this species is available from 9 states and none from the north-eastern zone [18]. It is resistant to DDT in most of the reporting states but fully susceptible to DDT, malathion and deltamethrin in Odisha and Andhra Pradesh. It is susceptible to malathion in Himachal Pradesh, Uttarakhand, Karnataka and Tamil Nadu, and deltamethrin in Jharkhand, Chhattisgarh and Uttarakhand. In the

state of Maharashtra, it is reported to be possibly resistant to malathion and deltamethrin.

Anopheles baimaii (the vector of forest malaria)

It is reported to be susceptible to DDT and malathion in Assam and Tripura state. No such information is available on its current status of resistance against deltamethrin.

Anopheles minimus (the vector of foothill malaria)

The insecticide susceptibility status of this species is reported from Jharkhand and Odisha from the east zone and states of Assam, Meghalaya and Tripura from the north-east zone in India. The species is reported to be mostly susceptible to DDT, malathion and deltamethrin in these states; but recorded to be resistant to DDT in Odisha.

Anopheles stephensi (the vector of urban malaria)

The species is reported to be triple-resistant to DDT, malathion and deltamethrin in Karnataka, and double resistant to DDT and malathion in Delhi, West Bengal and in three western states of Goa, Gujarat and Rajasthan. It is reported to be susceptible to malathion in Maharashtra and deltamethrin in Delhi, Uttar Pradesh and West Bengal.

Anopheles sundaicus (the vector of coastal malaria)

The species is found in the Andaman and Nicobar Islands and recorded to be resistant to DDT but susceptible to malathion. No such data are available on pyrethroid susceptibility.

Insecticide resistance mechanisms

Insecticide resistance mechanisms include behavioral resistance, reduced penetration, target-site and metabolic resistance (biochemical resistance), of which the latter two are prominent ones [23], although the relative contribution of the four mechanisms is largely unknown. Behavioral resistance is due to repellency or irritability caused by insecticides based on mosquito's innate capability to detect and avoid contact with treated surfaces. For example, deltamethrin cause low repellency than DDT. Certain insects resist insecticidal action by reducing the permeability of insect cuticle or digestive tract linings. Target-site resistance is a common mechanism in mosquitoes against organophosphates, carbamates, organochlorines and pyrethroids involving a specific site in the nervous system [24]. The two major target sites in mosquito species are acetylcholinesterase (AChE) and sodium channels.

The major target site for both organophosphate and carbamate insecticides is synaptic AChE mutation. In Indian malaria vectors there is no evidence of this mutation, known as ace-1R conferring carbamate and organophosphate resistance. A low frequency inhibition has been observed in *An. culicifacies* in the states of Andhra Pradesh, Gujarat, Haryana and Uttar Pradesh [25], as well as in Chhattisgarh in the presence of community wide use of pyrethroid-LLINs [26]. On the contrary, *An. culicifacies* was found to be highly susceptible (mortality 97–100%) to bendiocarb 0.1% (K. Raghavendra, unpublished data). The involvement of ace-1 Rmutation in conferring resistance in *Culex pipiens* [27], *An. gambiae s.l.* and *An. albimanus* populations in heterozygous state has been reported [28-31].

DDT and pyrethroids cause target site insensitivity by mutations that modify the voltage-gated sodium channels [32]. This is through point mutations at amino acid 1014 position resulting in either a leucine residue by phenylalanine (L1014F) [33] or serine (L1014S) [34] often referred to as knockdown resistance (*kdr*) that is insects with these alleles withstand prolonged exposure to insecticides without being knocked-down [35]. In pyrethroid resistant populations of *An. gambiae*, another mutation reported is at 1575Y position within domains of III-IV of voltage-gated sodium channels asparagine to tyrosine [36]. Both the mutations, L1014F and L1014S, have been reported in Indian malaria vectors *An. culicifacies* and *An. stephensi* [37-39].

In a study based in Chhattisgarh state where *An. culicifacies* faced selection pressure against use of pyrethroids for more than a decade, involvement of *kdr* allele in low frequencies in heterozygous condition indicated plausible development of knockdown resistance in the vector population. Here genotyping studies showed an association between *kdr* genotype and deltamethrin phenotype. A low frequency of *kdr* mutations of 1.2–7.4% with heterozygous condition was detected in *An. culicifacies* in the states of Gujarat, Chhattisgarh, Haryana and Rajasthan in India where it exhibited a significant protection against deltamethrin [39].

In insects, metabolic resistance is the most general mechanism involving enzymatic degradation. Three major gene family enzyme groups are responsible for the metabolic resistance to insecticides, namely esterases, Glutathione S-transferases (GST) and cytochrome P450 enzymes. In the wild populations of Indian malaria vectors, resistance to DDT is conferred by involvement of GSTs and elevated levels of α- and β-esterases and monooxygenases. The carboxylesterase mediated resistance mechanism for malathion resistance is demonstrated in *An. culicifacies*.

Insecticide Resistance Management

Policy Options and Technical Support:

The WHO global plan for insecticide resistance management calls upon malaria endemic countries to plan and implement insecticide resistance management strategies by proper and timely monitoring of resistance, managing data effectively, developing innovative vector control tools and approaches, updating knowledge on resistance mechanisms, assessing the impact of interventions, stepping up advocacy and greater investment for human and financial resources [40]. To support countries, guidelines for insecticide

resistance have recently been updated [19]. WHO coordinates supply of resistance test kits, develops insecticide quality standards, and provides technical support and training countries in vector surveillance and control and sound management of pesticides within the context of an integrated vector management [41].

Management Strategies and New Vector Control Products

In India, DDT was introduced for indoor residual spraying on a large-scale for malaria eradication in 1950s. In subsequent years, owing to resistance to DDT in malaria vectors, dieldrin and later malathion were introduced as alternative interventions. Pyrethroids were introduced for malaria vector control in the mid-1990s. Currently, insecticides in three major classes used for vector control include malathion, pyrethroids and DDT. Of the total use of DDT globally, 82% is consumed in India alone for malaria vector control [5]. Additionally, large amounts of insecticides are used for control of agricultural pests that may possibly have impact on development of resistance in disease vectors [42]. Thus, the prolonged use of same class of insecticides in a given area may have resulted in the selection of resistance in malaria vectors.

The intensity of insecticide resistance in malaria vectors in India is of a low-to-moderate level. It is therefore essential that steps are taken to prevent increase in the frequency and the intensity of insecticide resistance in the country to sustain efficacy of given intervention. WHO promotes universal coverage of populations at any risk with LLINs and IRS as core-interventions; the larval source management is recommended as supplementary interventions where larval habitats are few, fixed and accessible.

There is a great concern as to whether LLINs are still effective in the control of malaria in areas with widespread resistance to pyrethroids. A recent multi-country, prospective, observational study including India, however, has reported that LLINs protect people against malaria despite pyrethroid resistance of varying levels, although supplementary vector control tools may need to be combined in high malaria transmission areas [43].

IRS is also the main strategy for vector control in India involving use of pyrethroids, DDT and malathion in different areas. Three of the principal vectors, *An. minimus, An. baimaii* and *An. fluviatilis*, are fully susceptible to all available insecticides in areas with high burden of malaria. However, in areas with *An. culicifacies* or other vectors, IRS with pyrethroids, DDT and malathion have limited role due to multiple insecticide resistance of varying levels. Forvector control, IRS should be undertaken employing effective and quality assured insecticides for optimal outcome [44].

In the Indian context, following are the main intervention approaches in different ecotypes: (i) use of LLINs, IRS with DDT, malathion or pyrethroids, use of larvivorous fish in suitable habitats, and environmental management in specific situations in rural areas, (ii) use of larvicides, larvivorous fish, and environmental management in urban/industrial areas. Space spraying is used occasionally to contain rising density of vector/nuisance causing/biting insect populations.

For effective control of malaria vectors and to delay/stop escalation in insecticide resistance, the following approaches may be considered:

Application of IRS alone: To prolong the life of insecticides, rotation or mixture of insecticides with unrelated mode action could be the potential approach. Rotational use of pyrethroids and malathion may be considered. Use of DDT may be continued in areas where vectors such as *An. fluviatilis*, *An. minimus* and *An. baimaii* are still susceptible but in areas with resistance alternatives to DDT may be considered. New insecticides or formulations recently field tested in India for malaria vector control could serve as alternatives; these include: SumiShield WG[45], Fludora® Fusion WP-SB[7], and Actellic300 CS and K-Othrine Polyzone SC-PE [46]. Use of mixtures with new chemistries, e.g., Fludora® Fusion WP-SB, may be considered.

LLINs alone: LLINs continue to impact on malaria despite presence of pyrethroid resistance in vector populations. LLINs with PBO (synergist) or those containing a non-pyrethroid active ingredient such as Interceptor G2 holds promise when their public health value (epidemiological impact) has been well demonstrated. Universal coverage with LLINs of populations at any risk should be aimed to achieve malaria elimination.

Environmental and larval source management: An integrated disease vector control project in India has amply demonstrated that environmental and biological control methods are effective against malaria vector, *An. stephensi* in rural, urban and industrial areas [47-49]. In these areas, larvivorous fish have a great potential in vector control [50]. Therefore, well

Figure 3: Location of zonal entomological surveillance teams in different malaria endemic states of India

planned environmental management approach can play a significant role in control of *An. stephensi* transmitted malaria in urban and semi-urban areas.

In areas with unknown resistance, the approach should be to conduct susceptibility tests, monitor and map resistance, detect resistance mechanisms, use emerging evidence to inform national resistance management policies, develop or update an insecticide resistance management country plan, and strengthen capacity for entomological surveillance and vector control.

The National Vector-Borne Disease Control Programme in India is the nodal organization for the prevention and control of vector-borne diseases and responsible for monitoring and management of insecticide resistance. The programme is strengthened with a network of 78 zonal malaria offices spread across the country alongside several research laboratories that undertake entomological surveillance and monitoring insecticide resistance (Figure 3) [51]. There is a need for significant enhancement in capacity and human resource for this network for decisive action to defeat the growing menace of insecticide resistance.

New Vector Control Tools

Newer and innovative vector control tools are mandated not only to overcome growing resistance but also invent cheaper, safe and cost-effective alternatives to existing products to protect outreach populations groups in high transmission ecosystems. For a long time, vector control has relied on just four insecticide classes. While LLINs and IRS are currently the two chemical-based core interventions in India, pyrethroids are the only class recommended by WHO for treatment of nets or LLINs. LLINs with new chemistries are required to meet the current needs. In the last decade, a lot of efforts and significant

Table 4. New mosquito adulticides and larvicides for malaria vector control

Product name[1]	Insecticide/synergist	Class[3]	Application
Actellic 300 CS	Pirimiphos-methyl	OP	Indoor residual spraying
K-OthrinePolyzone SC-PE	Deltamethrin	PY	Indoor residual spraying
SumiShield 50 WG	Clothianidin	NN	Indoor residual spraying
Fludora Fusion WP	Clothanidin + deltamethrin	NN + PY	Indoor residual spraying
Tsara Plus (DawaPlus 3.0) LN	Deltamethrin + PBO[2] on roof	PY	Long-lasting insecticidal net
Tsara Boost (Dawa Plus 4.0) LN	Deltamethrin + PBO	PY	Long-lasting insecticidal net
Interceptor G2 LN	Alpha-cypermethrin + Chlorfenapyr	PY+PR	Long-lasting insecticidal net
Olyset Plus LN	Permethrin + PBO	PY	Long-lasting insecticidal net
PermaNet 3.0 LN	Deltamethrin + PBO on roof	PY	Long-lasting insecticidal net
Veeralin LN	Alpha-cypermethrin + PBO	PY	Long-lasting insecticidal net
SumiLarv 2MR	Pyriproxyfen	JH	Larvicide
VectoMax FG	Bti AM65-52 + Bs ABTS-1743	BL	Larvicide

Spinosad 7.48% DT	Spinosad (spinosyns A & D)	SP	Larvicide
Spinosad Monolayer DT	Spinosad (spinosyns A & D)	SP	Larvicide
Spinosad EC	Spinosad (spinosyns A & D)	SP	Larvicide
Spinosad 25 GR	Spinosad (spinosyns A & D)	SP	Larvicide
Mozkill 120 SC	Spinosad	SP	Larvicide
Aquatain AMF	Polydimethysiloxane	MMF	Larvicide

[1] CS, capsule suspension; DT, tablet for direct application; EC, emulsifiable concentrate; FG, fine granules; LN, long-lasting insecticidal nets; MR, matrix release formulation; SC, suspension concentrate; SC-PE, polymer enhanced suspension concentrate; WG, water dispersible granules; WG-SB, water dispersible granules in sealed water-soluble bags; WP, wettable powder; WP-SB, wettable powder in sealed water-soluble bags.

[2] PBO, piperonyl butoxide; [3] BL, bacterial larvicide (Bti, *Bacillus thuringiensis israelensis*; Bs, *Bacillus sphaericus*); C, carbamate; JH, juvenile hormone mimics; MMF, monomolecular film; NN, neonicotinoid; PR, pyrrole; PY, pyrethroid; OC, organochlorine; OP, organophosphate; SP, spinosyns

investments have been made to bring new products for public health utility. These efforts have led to repurposing of insecticides with old chemistries and formulating them into safe and effective vector control products as well as mining for new chemistries to discover novel insecticides.

WHO has recently prequalified certain improved formulations or new IRS products, e.g., K-Othrine Polyzone, Actellic 300 CS, SumiShield 50 WG and Fludora-Fusion WP-SB [7]. The new adulticide and larvicide products recommended by WHO in recent years are summarized in Table 4. Availability of improved packaging for IRS products such as water-soluble sachets will ensure operators' safety while handling these products. Evaluation of newer tools and approaches for vector control is being coordinated through a WHO Vector Control Advisory Group set up in 2013 [52]. Innovative tools under development and evaluation include attractive toxic sugar baits (ATSB), treated clothing, LLINs treated with new compounds, such as pyriproxyfen and chlorfenapyr; spatial repellents, sterile insecticide technique, genetically modified mosquitoes, microbial control with *Wolbachia* and the gene drive technology [53].

To conclude, management of insecticide resistance is essential to prevent reversal of gains achieved in malaria elimination initiatives. Effective insecticides with old or new chemistries must be deployed in such a way that their effective life can be prolonged for their sustainable use. The insecticide resistance management strategies underlie methods to avoid or delay onset of resistance in disease vectors. The strategies include use of insecticides of different classes with different modes of actions and insecticide resistance mechanisms in a structured programmatic way. The strategies mainly include alternation of insecticides, mosaic applications and mixtures. These strategies are work intensive and often not meeting success at desired level. However, recently mixtures in IRS and use of synergist with insecticides in LLINs have shown promise as effective interventions for vector control and insecticide resistance management.

Research and capacity development

Operational research relating to insecticide resistance management include molecular studies on mechanisms, evaluation of new vector control tools and development of resistance management approaches in the field. The Indian Council of Medical Research (ICMR), the premier organization on health research, have taken the task head on providing research inputs to the control programme through network of its institutes spread across the country [54, 55]. Some of the institutes that have core capacity in this regard are: National Institute of Malaria Research, New Delhi (NIMR) and its network of 10 field-based laboratories spread across the country; Vector Control Research Centre (VCRC), Puducherry; Regional Medical Research Center (RMRC) at Bhubaneswar and Dibrugarh; National Institute of Research in Tribal Health (NIRTH), Jabalpur. Of these, NIMR and VCRC are the WHO Collaborating Centre for insecticide testing and integrated vector management respectively. Both centers are in an advance stage of being certified as the Good Laboratory Practice compliant laboratories for field testing of vector control products.

Important actions to manage insecticide resistance include enhancement of entomology capacity and infrastructure, updating the national resistance monitoring and management plan, engaging ICMR laboratories for monitoring resistance and determination of resistance mechanisms. The existing entomological teams, ICMR laboratories, and other institutions should work in coordination for monitoring resistance and data sharing helping formulate informed policy mitigating impending threat of insecticide resistance.

Conclusions

WHO has developed a roadmap to eliminate malaria from most parts of the endemic World by 2030 [56]. Insecticide resistance is a biological threat that is increasing in both geographical scale and intensity threatening malaria elimination efforts. The epidemiological impact of insecticide resistance is not fully known as of now, and scientific opinion differs as to whether increasing resistance will indeed impact malaria. There has been a great momentum in research and modelling in quantifying insecticide resistance and optimal outcome [57]. While trend analyses have shown that during 2010–2016 the pyrethroid resistance frequency in malaria vectors has increased globally, the resistance frequency is comparatively low in India than Africa.

To mitigate the threat of pyrethroid resistance in malaria vector control, the following steps may be considered:

- Step up global efforts to develop new resistance-breaking tools and strengthen surveillance for insecticide resistance through regional initiatives and networks, viz., African Network on Vector Resistance, Asia-Pacific Malaria Elimination Network, Pan African Mosquito Control Association and the like for data sharing and coordinated action.

- The distribution of LLINs should continue to be scaled up ensuring universal coverage of populations at any risk of malaria. While distribution and intensity of resistance is increasing, LLINs continue to be promising technology for personal protection against infective mosquito bites.
- Limited evidence from field-based data does not yet justify a complete switch from pyrethroid-only LLINs to PBO-LLINs in all epidemiological settings, although the evidence is sufficient to justify pilot (exploratory) implementation of PBO-LLINs accompanied by robust evaluation of the impact using both entomological and epidemiological indicators.
- A recent study has confirmed that pyrethroid-LLINs continue to play a significant role in malaria control in the face of emerging insecticide resistance and providing personal protection against malaria across all the study areas [58]. This protection was no different between areas of varying levels of pyrethroid resistance and that in study clusters with higher levels of resistance there was some evidence of loss of community protection using LLINs, but there was no evidence of an increase in malaria incidence associated with higher levels of pyrethroid resistance. Further field studies are required to confirm this evidence in transmission settings with different malaria vectors and levels of pyrethroid resistance.This study has also reported that synergist PBO can increase the efficacy of pyrethroids in LLINs, but in highly resistant mosquito populations, the impact may vary in different regions based on resistance intensity and mechanisms [59]. In Sudan, where vectors were resistant to pyrethroids but susceptible to bendiocarb, addition of IRS with deltamethrin produced no additional protection, whereas adding IRS with bendiocarb in LLIN area reduced malaria incidence by 50% relative to LLINs alone, and that IRS with carbamate in addition to LLINs appeared to slow the emergence of pyrethroid resistance relative to LLINs alone.

One of the foundations of the Global Vector Control Response is to enhance vector surveillance, monitoring and evaluating interventions [60]. As such, there is a need for regular monitoring of insecticide resistance and create a national database on insecticide resistance in malaria vectors. The available datasets provide sparse and incomplete information limiting their usefulness for implementing effective vector management strategies. The main reason for the lack of resistance data sets is the low health system capacity for vector surveillance, inadequate resources and irregular logistic supplies. WHO's efforts to establish a facility for impregnation of insecticide papers in India will help ensure logistic supplies and augment entomological monitoring capacities helping institute appropriate interventions in time and place defeating insecticide resistance.

Disclaimer

The views and opinions expressed in the paper are entirely those of the authors alone and do not necessarily represent the views and policies of their respective organization.

Acronyms	
AChE	acetylcholinesterase
ATSB	attractive toxic sugar baits
BL	bacterial larvicide
BS	Bacillus sphaericus
Bti	Bacillus thuringiensis israelensis
C	Carbamate
CS	capsule suspension
DDT	dichloro-diphenyl-trichloroethane
DEL	deltamethrin
DT	tablet for direct application
EC	emulsifiable concentrate
EW	emulsion oil in water
FG	fine granules
GST	glutathione S-transferases
IRS	indoor residual spray
ICMR	Indian Council of Medical Research
JH	juvenile hormone mimics
kdr	knockdown resistance gene
LLIN/LN	long-lasting insecticidal net
MAL	malathion
MMF	monomolecular film
MR	matrix release formulation
NIMR	National Institute of Malaria Research
NIRTH	National Institute of Research in Tribal Health
NN	neonicotinoid
OC	organochlorine
OP	organophosphate
PBO	piperonyl butoxide
PR	Pyrrole
PY	Pyrethroid
RMRC	Regional Medical Research Centre
SC	suspension concentrate
SC-PE	polymer enhanced suspension concentrate
SP	spinosyns
VCRC	Vector Control Research Centre
WG	water dispersible granules
WHO	World Health Organization
WP	wettable powder
WP-SB	water soluble granules in sealed water-soluble bags
WT	water dispersible tablet

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Chapter 13

Malaria vector control in India: present perspectives and continuing challenges

Chazhoor John Babu*

Ex- Scientist, ICMR – Vector Control Research Centre, Puducherry– 605 006, India (Present address: 56 Kuber CHS, Sector-17, Vashi, Navi Mumbai - 400703)

* Correspondence: babu_chazhoor@hotmail.com

Abstract

Vector control is an integral component of the national malaria control strategy and getting eminence in the renewed efforts towards malaria elimination. However, development of multiple insecticide resistance in *Anopheles culicifacies* and varied response to other dominant mosquito vector species is jeopardizing the control efforts resulting in continued transmission in high-risk states. In rural India, the control programme largely rests on indoor-residual spraying and distribution of pyrethroid-treated netting materials/long-lasting insecticidal nets (LLINs). While the indoor residual spraying operations remained off target population coverage for varied reasons *inter-alia* poor community acceptance, LLINs instead were widely accepted and held operationally feasible in resource-poor settings; population coverage, however, remained miniscule of what is required for blanket coverage. In urban areas, anti-larval interventions including larvicides, larvivorous fish, and civic/building by laws enforcing source reduction methods are in vogue but poorly applied, and programme largely rests on passive case detection and treatment. The onset of pyrethroid resistance and outdoor transmission are viewed as emerging challenges for which newer innovative technologies are mandated that are community-based and sustainable. The march of insecticide resistance seems unstoppable for which bio-environmental control of malaria based Integrated Vector Management (IVM) approach offers long-term solution for species-specific interventions that are ecologically sound and cost-savvy. Given the declining transmission observed in India there is window of opportunity to universalize interventions to realize the goal of malaria elimination in the foreseeable future.

Keywords: Malaria, insecticide resistance, vector control, pyrethroids, outdoor transmission, integrated vector management

Introduction: a historical perspective

India is historically malaria endemic with record number of 75 million cases in pre-DDT era before 1953 and deaths close to a million annually [1]. History is replete with ravages of malaria in the colonial period reporting record number of deaths in World War II [2]. Malarial disease outbreaks were of common occurrence and for its control bio-environmental measures were in vogue including anti-larval operations, personal protection methods and mosquito-proof housing against infective mosquito bites. India holds the distinction for malarial research with epoch-making discovery that malaria is transmitted by mosquito bite way back in 1898 and subsequent research on mosquito fauna and bionomics of disease vectors [3]. The advent of DDT proved panacea with spectacular success stories based on which the National Malaria Control Programme (NMCP) was launched post-independence in 1953 and later converted to National Malaria Eradication Programme (NMEP) in 1958. During 1960s, malaria was observed fast disappearing with 0.1 million cases and deaths no more. It was strongly believed that malaria elimination would be a reality within the available resources under vertical execution of the programme. Henceforth, malarial research was discontinued and given low priority resulting in depletion of human resource and expertise on the subject. Disappearing malaria resulted in all round economic development in the country marked by green revolution and rapid industrialization. Malaria control efforts had relegated, and transmission continued unabated in certain pockets least attended by the programme resulting in development of parasite reservoir and herd immunity.

It was bit too late until insurmountable rise in cases was seen in town/urban mega-metropolitan cities constituting nearly 10% of reporting cases in the country associated with labour migration and creation of urban slums [4]. Fearing spilling over cases in malaria-free territories, Urban Malaria Scheme (UMS) was launched in 1971-1972 in major towns/cities reporting >2 API (two cases per thousand population) mainly based on anti-larval interventions. By then, malaria had diversified in various ecotypes in the country including industrial malaria, forest/tribal malaria, mining malaria, desert malaria, border malaria and the like, and cases were seen rising again both in the rural and urban agglomerations alike [5]. Health systems were not equipped to meet the services for shortages of skilled work force to meet the challenge of growing population pressure. Malarial outbreaks had once again resurfaced with reported increased morbidity and attributable mortality. The programme was failing logistically both for DDT and antimalarials shortages. Worse that DDT and chloroquine, two commonly used interventions against malaria were seen to be less effective owing to insecticide resistance in disease vectors and drug-resistance in parasite [6-8]. The malaria situation had worsened in 1976 with record number of 6.45 million cases, the highest ever resulting in the launch of Modified Plan of Operation (MPO) beginning 1977 making malaria control programme a state subject under Primary Health Care, i.e., from vertical to horizontal mode of operation [9].

India is co-endemic for both *Plasmodium vivax* and *P. falciparum*; the former was the predominant infection (>80%) but beginning 1970s, the proportions of the latter seen rising slowly and steadily each passing year [10, 11]. Realizing the enormity of the problem associated with this deadly parasite, *P. falciparum Containment Programme* (*PfCP*) was launched in 1977 initially in the north-eastern states (believed to be the corridor for drug-resistant malaria) but latter extended to other states contributing most cases to contain the

spread [12]. This programme assisted (in part) by the Swedish International Development Authority (SIDA) was later abandoned in 1988 after 11 years of intensified operations. Malaria transmission was contained to greater extent in 1990s reporting about two million cases a year, but rising trend of *P. falciparum* cases remained unabated presently constituting >50% of total reported cases in the country despite additional inputs from World Bank, Global Fund to fight AIDS, Tuberculosis and malaria (GFATM) and the donors alike [13]. *P. falciparum* presently have grown from mono to multi-resistant to anti-malarial drugs and continues to threaten malaria elimination efforts in the country [14].

Vector Control

Malaria control operations in India rests on two pillars, i.e., anti-vector for containment of vector populations and anti-parasite measures for radical cure of malaria cases [15]. Vector control is an integral component of the National Strategic Plan for Malaria Elimination in India and incurs huge costs annually [16]. There are six dominant mosquito vectors of importance namely *Anopheles culicifacies* (rural malaria), *An. fluviatilis* and *An. minimus* (foothill malaria), *An. stephensi* (urban malaria), *An. baimaii* (forest malaria), and *An. sundaicus* (Island malaria) operating in different geo-epidemiological regions of the country requiring different control strategies specific to the region [17]. All of these except *An. stephensi* are species complexes having distinct species-specific bionomical characteristics and response to residual insecticides [18]. Among wide array of available vector control tools including indoor residual spraying (IRS), insecticide-treated netting materials and anti-larval operations; the application of residual insecticides continues to be major stay in India. Among armamentarium of residual insecticides, the following are presently in operation in the National Malaria Control Programme (Table 1).

Table 1. Insecticide formulation and dosages applied for indoor residual spraying in the national malaria vector control programme in India

S. N.	Insecticide formulation	Quantity required for 10 litres of suspension	Dosage applied per m^2 of active ingredient	Residual effect in weeks	Area covered (m^2) by 10 litres of suspension	Requirement of insecticide in metric tons per million population
1.	DDT 50% WP	1.000 Kg	1 gm	10-12	500	150.00
2.	Malathion 25% WP	2.000 Kg	2 gm	6-8	500	900.00*
3.	Deltamethrin 2.5% WP	0.400 Kg	20 mg	10-12	500	60.00
4.	Cyfluthrin 10% WP	0.125 Kg	25 mg	10-12	500	18.75
5.	Lambdacyhalothrin 10% WP	0.125 Kg	25 mg	10-12	500	18.75
6.	Alphacypermethrin 5% WP	0.250 Kg	25 mg	10-12	500	37.50
7.	Bifenthrin 10% WP	0.125 Kg	25 mg	10-12	500	18.75

*Requirement shown is for three rounds of residual spray of malathion. Source Reference [19].

Residual insecticides for adult vector control

DDT (dichloro-diphenyl-trichloroethane)

DDT, the organochlorine compound once extensively used in the control programme had lost its galore evidenced by emergence of resistance in *An. culicifacies*, the major vector spread throughout the country generating 65% of cases annually [7,8]. The other major vectors including *An. fluviatils*, *An. stephensi*, *An. sundaicus* have developed varied degree of resistance except for *An. minims* and *An. baimaii* (sibling-species of *An. dirus* complex) which continue to be susceptible [20]. The phenomenon of resistance was largely attributed to excessive and irrational use of insecticide and its pilferage in the agricultural sector. DDT even though banned under Stock holm Convention on Persistent Organic Pollutants (POP) in 2001, yet still approved for use in public health exclusively for indoor residual spraying in countries including India with susceptible vector populations and proven evidence of reduced transmission [7]. It is the cheapest available insecticide and remains the choice application for control of *An. minimus* and *An. baimaii* transmitted malaria in north-eastern states of India [21]. Vector populations of these two species continue to be highly susceptible to DDT ever since inception of the control programme in domains of their influence including eastern Indian state of Odisha. What remains critical is the scheduled application of this residual insecticide in relation to rising vector density ensuring blanket coverage of population at any risk of malaria. But in practice, besides belated spray operations, coverage remained below 50% of the target risk population owing to high-refusal rate/poor community acceptance [22]. This insecticide is scheduled for two rounds of IRS (50% wp @ 1 gm per square metre) annually corresponding to peak transmission period of which many a times only one round was applied due to operational constraints resulting in continued transmission [23]. The residual effect of DDT is estimated to last 10-12 weeks mandating second round in continuation providing protection uninterrupted throughout transmission period lasting six months against endophilic and anthropophagic mosquito vectors. Situational analyses of high-risk districts of Assam, northeast India, however, revealed that spray coverage was grossly inadequate and remained far from satisfactory. Many pockets were left unsprayed years together resulting in build-up of high vector density and consequent devastating outbreaks [24]. It is strongly believed that full coverage of target population coupled with good quality spray in scheduled times still can do the magic in keeping vector populations at bay disrupting malaria transmission much due to its intrinsic excito-repellent action [25]. The analogue insecticides to DDT, i.e., hexachlorocyclohexane (HCH) against target species were also rendered inadequate later withdrawn due to cross-resistance in the same class of insecticide.

Malathion

Malathion (25% wp), is an organo-phosphorous compound used as an alternative insecticide in DDT resistant areas. It has different mode of action than DDT by inhibiting cholinesterase, preventing breakdown of the neuro transmitter acetylcholine, resulting in neuromuscular overstimulation and death of the vector. Unlike two rounds of DDT, three rounds of residual sprays of malathion are mandated @2 gm per square metre for its residual toxicity lasting only 6-8 weeks. It is comparatively toxic both for mammalian and non-target beneficial fauna and costlier requiring larger quantities to protect target

population. Among six dominant vector mosquito species, *An. culicifacies* (rural vector) and *An. stephensi* (urban vector), both are resistant to malathion whereas *An. fluviatilis*, *An. minimus*, *An. baimaii* and *An. sundaicus* vector populations are still by and large susceptible to the given diagnostic dosage concentrations [20]. It is strongly believed that the underlying cause of malathion resistance was its large-scale application in rice-agroecosystem supporting heavy breeding of *An. culicifacies* in paddy/fallow fields.

Synthetic Pyrethroids

Synthetic pyrethroids are normally applied in areas resistant to both DDT and malathion. Number of pyrethroid molecules have been approved by the Central Insecticide Board (http://ppqs.gov.in/divisions/cib-rc/registered-products) for use in public health (Table 1). Pyrethroids have rapid knock-down effect and residual efficacy is longer than malathion at given concentrations. These are comparatively expensive than DDT and malathion but are relatively safe with much less mammalian toxicity. Pyrethroids and DDT both have similar mode of action on the mosquito vector by open sodium channels leading to continuous nerve excitation, paralysis and death. Pyrethroid class of insecticides are presently employed both for IRS, space spraying/thermal fogging as well as in mosquito net impregnation/production of long-lasting insecticidal nets (LLINs). A variety of LLINs have been recommended for use in public health by World Health Organization Pesticide Evaluation Scheme (WHOPES) of which PermaNet 2.0 (deltamethrin coated polyester fibre) and Olyset Net (permethrin incorporated polyethylene fibre) have been accorded full recommendation and are currently being used in the control programme [26]. These two types of LLINs have been field-evaluated to be effective against dominant mosquito vector species in reducing vector density and transmission intensities in high-risk states of India and assessed to be operationally feasible [27-29]. These are ready-to-use factory treated nets and retain residual efficacy for 3-4 years (the expected net-serviceable life). The advent of LLINs has proved boon to malaria vector control programme for wider community acceptance and are currently being promoted as key-intervention aiming universal coverage for populations at any risk. Most LLINs employ pyrethroid and there is growing concern of reported resistance to this class of insecticide in malaria endemic countries. However, there is no shred of evidence of decreased protection and LLIN based intervention has been held efficacious for its inherent proprieties of excito-repellent action preventing infective mosquito bites [30].

Anti-larval operations

Anti-larval interventions are largely applied in urban metropolitan cities to check mosquito proliferation by application of larvicides including chemicals, bio-larvicides, insect growth regulators and larvivorous fish in breeding habitats (Table 2).

Table 2. Larvicide formations and dosages for control of mosquito breeding in different habitats

S. N.	Larvicide	Type of larvicide	Commercial formulation	Preparation of spray solution	Requirement per hectare	Frequency of application	Type of breeding habitat
1.	Mosquito Larvicidal Oil (MLO)	Non-insecticidal	100% petroleum product	Full strength	200 litres	Weekly	stagnant water
2.	Temephos (Abate)	Organo-phosphorus	50% EC	2.5 cc in 10 litres of potable water	200 litres	Weekly	clean as well polluted water
3.	*Bacillus thuringiensis var israelensis (Bti)*	Bio-larvicide	5% Wettable Powder (WP) Strain - 164, Serotype H-14	5 Kg in 200 litres of water	200 litres	Fortnightly	clean and non-potable polluted water
4.	*Bacillus thuringiensis var israelensis (Bti)*	Bio-larvicide	5 % WP Strain - ABIL, Serotype H-14 Accession No 01318	7.5 Kg in 200 litres of Water	200 litres	Weekly	clean water
				10 Kg in 200 litres of Water	200 litres		polluted water
5.	*Bacillus thuringiensis var israelensis (12 AS)*	Bio-larvicide	5% Aqueous Suspension	One litre in 200 litres of water	200 litres	Weekly	clean water
				Two litres in 200 litres of water	200 litres		polluted water
6.	Diflubenzuron	Insect growth regulator	20% Wettable Powder (WP)	100 gm (25 mg a.i.) in 100 litres of water	100 litres	Weekly	clean water
				200 gm (25 mg a.i.) in 100 litres of water	100 litres		polluted water
7.	Pyriproxyfen	Insect growth regulator	0.5% granular	Ready to use	2 Kg	3 weeklies	clean water
					4 Kg		polluted water

Source Reference [19]

All these interventions have been approved for vector control operations in the Indian National Control Programme for application in urban settlements. Among these, mosquito larvicidal oil (MLO) is ready to use petroleum emulsion to be applied in stagnant water bodies that kills the mosquito larvae by formation of thin film over water surface cutting off oxygen supply. It is a non-insecticidal method that is nontoxic to plants, animals and human beings. Instead, Temephos (Abate) is an organophosphorus compound that kills the mosquito larva by ingestion or contact and considered suitable for both clean and polluted water bodies. Among others, bio-larvicides (based on mosquitocidal toxins), application of various serotypes of *Bacillus thuringiensis israelensis* (*Bti*) have been evaluated to be successful for control of mosquito breeding particularly *An. stephensi* (the urban vector of malaria) and considered safe for non-target organisms [31]. Insect growth regulators (IGRs), instead inhibits the development of immature stages of the mosquito by interference of chitin synthesis during the moulting or disruption of pupal and adult transformation processes. Most IGRs have extremely low mammalian toxicity and can provide long-term protection ranging from 3-6 months at low dosages [32]. Besides these measures, civic and building bylaws promulgated in big cities to prevent mosquito breeding in house premises

and building construction related projects have been proven success to greater extent [33]. Anti-larval operations also envisage bio-environmental control measures using non-insecticidal methods such as drainage, earth-work reducing breeding resources, application of larvivorous fish, *Poecilia reticulata* (guppy fish) and *Gambusia affinis* (mosquito fish) in water bodies which could well be integrated to supplement all other interventions helping control vector populations substantially [34].

Contingent plans for containment of disease outbreaks/complex emergencies

The national control programme has made adequate provision for additional round of residual spraying of insecticide in the given locality to check mosquito nuisance/rising vector density to contain disease outbreaks/epidemics. To further these containment measures, additional tools are made available that include space- spraying indoors and thermal fogging outdoors to mitigate disease onslaught building confidence in the communities at high risk. The space spraying operations are largely transitory generally conducted coinciding with high mosquito density and timed in relation to crepuscular activity (dusk time) of vector populations providing relief without any residual effect. The insecticides employed for thermal fogging usually include pyrethrum and malathion (Table 3).

Critical appraisal

For vector control in rural India, indoor residual spraying is the major intervention amounting to bulk of investment in control operations annually. Much of the allocated budget (80%) is spent on control of *An. culicifacies* alone, a major vector in the mainland India [1, 7]. Given the wealth of knowledge on disease vectors, malaria transmission continues in many parts of the country for variety of reasons *inter-alia*, (i) lack of community acceptance for IRS being inconvenient, foul smell, and unaware on disease prevention and control; all of which amounted to low coverage (<50%) of target population, (ii) spray coverage remained patchy and poor quality for lack of supervision, (iii) spray schedule was not followed strictly many a times amiss due to financial and operational constraints resulting in inadequate protection [22]. Not to blame the programme per se, many high-risk villages were invariably marooned due to heavy rains and floods restricting access leaving them unprotected months together. All these issues were addressed at least in part by advent of ready-to-use insecticide-treated netting materials/LLINs providing continuous protection for duration of net-serviceable life for at least three years without any need for net re-impregnation [35]. This technology has revolutionized the concept of vector control and held appropriate in many transmission settings globally for its wider community acceptance and sustainability. The bottleneck, however, is the population coverage which remained only a fraction of what is needed in poor-resource countries [36]. Even more critical is monitoring residual efficacy of field-distributed nets against target vector mosquito species in space and time for which extra-provision for replacement of torn nets those rendered unserviceable should be inbuilt in the programme [37]. In urban India, anti-larval operations and civic/construction building by laws are poorly enforced in letter and spirit resulting in continued transmission [38].

Table 3. Insecticides in use for indoor space spray and outdoor thermal fogging in the National Vector Borne Disease Control Programmeof India

S. N.	Name of Insecticide	Class of Insecticide (formulation)	Preparation of spray solution	Equipment used	Remarks
1.	Pyrethrum Extract	Plant Extract (2% extract)	1:19, i.e., 1 part of 2% Pyrethrum Extract in 19 parts of Kerosene (50 ml in 1 litre Kerosene Oil)	Pressurised spray machine or fogging machine	Used for Indoor space Spray
2.	Cyphenothrin	Synthetic pyrethroid (5%EC)	0.5 mg a.i per sq. mt. (20 ml in 1 litre Kerosene Oil)		
3.	Malathion	Organophosphorus (Technical Malathion)	1:19, i.e., 1 part of Malathion Tech in 19 parts of Diesel (50 ml in 1 litre diesel)	Shoulder mounted fogging machine or vehicle mounted thermal fogging machine	Used for outdoor thermal fogging
4.	Cyphenothrin	Synthetic pyrethroid (5% EC)	3.5 g a.i per hectare (7 ml in 1 litre diesel)		

Source Reference [19]

Management of insecticide resistance

To realize the goal of malaria elimination, continued attack on disease vectors is mandated to disrupt transmission and prevent re-establishment in malaria-free territories. Insecticide resistance is imminent in any class of insecticide and to maintain its effectiveness for longer periods or better delay the development and spread of resistance, several strategies have been proposed under Global Plan for Insecticide Resistance Management (GPIRM) by WHO [39]. Some of these include rotation of insecticides (with different mode of action) for residual spraying, combination of interventions, e.g., pyrethroid on nets and different insecticide for residual spraying, mosaic spraying involving different class of insecticides and LLINs encompassing mix of fibres and insecticides plus synergist such as piperonyl butoxide (PBO) to ward off multi-resistant vector species. Mosaic LLINs treated with pyrethroid and a different class of insecticide (non-pyrethroid) were proven efficacious against pyrethroid resistant mosquitoes, thus holds promise in preventing malaria transmission in multi-resistant areas but require validation in different geo-epidemiological/transmission settings [40-43].

There is dire need for newer technologies obviating the need for application of insecticide that are eco-friendly, doable and community-based defeating insecticide resistance [44]. Some of these which are being contemplated include next generation antimalarial mosquito nets arresting the development of parasite in the mosquito host [45, 46], sterile insect release method (SIRM) for population control [47, 48], Wolbachia (endosymbiotic bacteria) based infection for induced infertility/cytoplasmic incompatibility [49-51], and population release of genetically modified mosquitoes (GM) replacing vector populations with those incapable of transmitting pathogen should all be considered in its entirety and put to field-evaluation in India before these are operationalized [52]. Vector genomics have ushered a new era hoping to evolve new tools to disrupt parasite development in the mosquito host replacing vector populations with those of not capable of transmitting the causative parasites [53]. However, it would be long before these methods are put into practice requiring every consideration to prevent adverse consequences, if any.

To counter outdoor transmission, newer tools namely ‘eave tubes’ and ‘attractive toxic

sugar baits (ATBS)' seem to offer sustainable solution for containment of outdoor resting/residual vector populations requiring minimum quantities of insecticide application [54]; these technologies when integrated with other components of IVM can yield rich dividends defeating insecticide resistance. Other than that development of plant-based nano-pesticides [55], and endectocide ivermectin (a molecule that has been used for more than 30 years to control lymphatic filariasis) are still in preliminary stages and require considerable research on efficacy for control of vector populations and environmental considerations for effect on non-target beneficial insect species [56, 57]. Besides these, host of measures including topical repellents, insecticide-treated clothing and spatial/airborne repellents have all been proposed for personal protection but not included and recommended in the national control program for low certainty evidence [58]. Until effective malaria vaccine becomes available, we must rely on rational use of available intervention tools/mix of technologies that are situation-specific and appropriate to keep vector populations at bay.

Future challenges

The development of multiple resistance against available insecticides remain the biggest threat in vector control operations [59, 60]. *An. culicifacies*, the major vector in India is already reported to be resistant to DDT, malathion as well as synthetic pyrethroids [61]. Another challenge that has emerged is the outdoor transmission due to shift in mosquito behaviour in relation to application of residual insecticides indoors avoiding contact with sprayed surfaces/LLINs [62]. Vectors are getting outdoors and establishing extra-domiciliary transmission inflicting heavy morbidity in forest dwellers and mobile population groups leaving them unprotected and untreated for lack of healthcare services in the periphery [63]. The problem is exacerbated along international borders there being no provision for population screening for malaria and treatment, least the protection against the mosquito bites resulting in continued transmission and spread of drug-resistant malaria. Population migration and deforestation resulting in altered ecology require cross-border strengthening between neighbouring countries for sharing data and coordinated vector control operations. It is advocated that besides rational use of current interventions, newer innovative technologies are mandated which are environmentally sound and community-based to overcome outdoor transmission. Entomological capacity (classical taxonomists in particular) is fast depleting in the country which should have been the core-strength of the programme for monitoring and evaluation should be addressed adequately in meeting the programme needs.

Conclusions

Insecticide resistance is growing menace in disease vector control programmes necessitating the need for alternative innovative technologies that are eco-friendly, community-based and sustainable. Insecticide resistance is harsh reality and most disease vectors have grown mono to multi-resistant threatening malaria elimination efforts [60]. Rise in cases in certain malaria endemic countries is largely attributed to pyrethroid resistance calling

for renewed efforts including LLINs incorporating insecticides with dual mode of action [64, 65]. There are not many new molecules in pipeline and rising costs are operationally prohibitive. Population explosion, increased infrastructure and environmental degradation have all created enormous breeding habitats and consequent risk of vector-borne diseases. Taking cognizance of the prevailing scenario, the national control programme has formulated Integrated Vector Management (IVM) approach similar in line of action with WHO Guidelines for Malaria Vector Control built on four pillars [66]. These include: (i) strengthening inter- and intra-sectoral action and collaboration; (ii) engaging and mobilizing communities; (iii) enhancing vector surveillance and monitoring and evaluation of interventions; and (iv) scaling up and integrating tools and approaches [58].

Given the multiplicity of disease vectors and diverse ecology in the Indian context [67], no single approach may suffice to defeat insecticide resistance leaving no option except to exercise IVM encompassing multiple situation-specific interventions/right mix of technologies for species sanitation. The adoption of IVM holds collateral benefits for interventions being common for control not only malaria but also other vector-borne diseases, e.g., dengue, filariasis, Japanese encephalitis. Community participation and compliance, and intersectoral coordination are cardinal for sustainable action and saving operational costs. Even more important is the creation of skilled workforce given the higher attrition rate and declining entomological expertise for which human resource development should be the continuing activity at all echelons of operation. Entomological surveillance and monitoring resistance in disease vectors should be the cornerstone for synchronized action averting outbreaks/re-establishment of transmission in malaria-free territories [68].

To sum up, given the present-day intervention tools including artemisinin-based combination therapy, IRS and LLINs; India has registered significant decline in cases over the past few years [69] and it is time to universalize these interventions to realize the goal of malaria elimination by target date of 2027 [70]. Malaria-free India would be a significant contribution in history of public health in the South-East Asia joining league of countries certified to be malaria-free in the world.

Acronyms	
ATSB	attractive toxic sugar baits
Bti	*Bacillus thuringiensis israelensis*
DDT	dichloro-diphenyl-trichloroethane
GFATM	Global Fund against Aids, Tuberculosis and Malaria
GM	genetically modified mosquitoes
GPIRM	Global Plan for Insecticide Resistance Management
HCH	Hexachlorocyclohexane
IGRs	Insect growth regulators
IRS	Indoor residual spray
IVM	Integrated Vector Management
LLIN	long-lasting insecticidal net
MLO	mosquito larvicidal oil
MPO	Modified Plan of Operation

NMCP	National Malaria Control Programme
NMEP	National Malaria Elimination Programme
PBO	Piperonyl butoxide
PfCP	*Plasmodium falciparumContainment Programme*
POP	Persistent Organic Pollutants
SIRM	Sterile Insect Release Method
SIDA	Swedish International Development Authority
UMS	Urban Malaria Scheme
WP	Wettable Powder
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

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Chapter 14

Integrated disease vector control: a holistic approach targeting malaria

Virendra Kumar Dua[1] and Vas Dev[1*]

[1] Ex - Scientist, ICMR – National Institute of Malaria Research, New Delhi – 110 077, India

[*] Correspondence: mrcassam@hotmail.com

Abstract

Integrated disease vector control was attempted in mission mode through a network of field stations spread across India in different geo-epidemiological conditions. It encompassed multifaceted approach with major focus on field-evaluation of alternative interventions that are environment-friendly, self-sustainable and community-based obviating the need for reliance on insecticides to large extent. A wealth of information on disease vectors and parasite biology was generated that helped formulate species-specific intervention strategies disrupting transmission. Multiple interventional technologies that were put to field evaluation and practice included: (i) insecticide-treated netting materials for vector control, (ii) rapid test kits for on-the-spot diagnosis for malaria treatment, (iii) therapeutic efficacy investigations of new antimalarials for treatment of drug-resistant malaria, (iv) larvivorous fish for control of mosquito breeding, and above all (vi) information, education and communication activities for eliciting community participation in disease prevention and control. All these measures were integrated in the existing healthcare services that resulted in appreciable transmission reduction so much so that India is now envisaging malaria elimination in the foreseeable future by 2027. Communities are better informed, and India stands equipped with host of technologies and skilled human resource to defeat malaria.

Keywords: Malaria, parasite, vector control, community-based interventions, disease surveillance, health education, India

Rationale

India is historically malaria endemic with records of devastating disease outbreaks taking heavy toll on human lives affecting industrial growth and national productivity [1]. For its control, National Malaria Control Programme (NMCP) was launched in 1953 based largely on two main pillars, i.e., disease surveillance for detection and treatment of malaria positive cases with effective chemotherapy and indoor residual spraying of DDT for containment of vector populations. Based on the spectacular success in disease transmission reduction in the following five years, beginning 1958 the programme was converted into National Malaria Eradication Programme (NMEP). In the continuing battle against malaria, during 1960s malaria cases were reduced to less than 0.1 million and deaths no more. Malaria was believed to be disappearing in large tracts of land up until its resurgence in 1976 with record number of over six million cases with reports of upsurge of cases in urban areas [2]. The dominant mosquito vector species had become resistant to DDT and parasite was not responding to chloroquine treatment adequately [3]. Up until now, the programme was executed vertically, but in 1977 the control programme was decentralised under modified plan of operation on shared costs basis between state government and that of the centre [4]. The situation was worsening with rising proportions of *Plasmodium falciparum* infections every passing year marked with high morbidity and malaria-attributable mortality associated with increasing levels of insecticide resistance and emergence and spread of drug-resistant malaria [5, 6]. The control operations were getting cost prohibitive with diminishing returns.

Taking stock of the situation, 'Integrated Disease Vector Control (IDVC)' project was launched in mission mode in 1986 jointly funded by the Ministry of Science & Technology and the Indian Council of Medical Research (ICMR) under the aegis of National Institute of Malaria Research (formerly Malaria Research Centre). The broad mandate of the project was to field test newer interventions which are cost-effective, community-based and self-sustainable for incorporation into the 'National Vector Borne Disease Control Programme (NVBDCP)' saving operational costs. Accordingly, a network of research 'Field Stations' was established across the country in different geo-epidemiological conditions addressing both urban and rural malaria ecotypes (Figure 1). These included Hardwar (Uttarakhand), Jabalpur (Madhya Pradesh), Rourkela (Odisha), Guwahati (Assam), Ranchi (Jharkhand), Raipur (Chhattisgarh), Nadiad (Gujarat), Panjim (Goa), Bengaluru (Karnataka) and Chennai (Tamil Nadu). Each field station had its own mandate for situational analysis and pilot situation-specific alternative intervention strategies in close coordination with the respective state health department for decisive attack on disease vectors. In this context, some of the field stations generated valued information on disease epidemiology, viz., (i) disease distribution and transmission dynamics, monitoring therapeutic efficacy of antimalarial drugs helping upgrade drug-policy for treatment of drug-resistant malaria, (ii) much needed information on vector incrimination, seasonal abundance, and current status of insecticide resistance, (iii) testing species-specific newer interventions which are ecologically sound, doable and self-sustainable, (iv) and above all preparing for the malaria challenge in developing human resource and community awareness on disease prevention and control [7]. Presented below is the success story of one such Field Station based in Assam, the main gate to north-east India resulting in appreciable transmission reduction. The state of Assam had history of recurring disease

outbreaks and for consistently contributing nearly 50% of reported cases in northeast region of India; majority of which were *P. falciparum*.

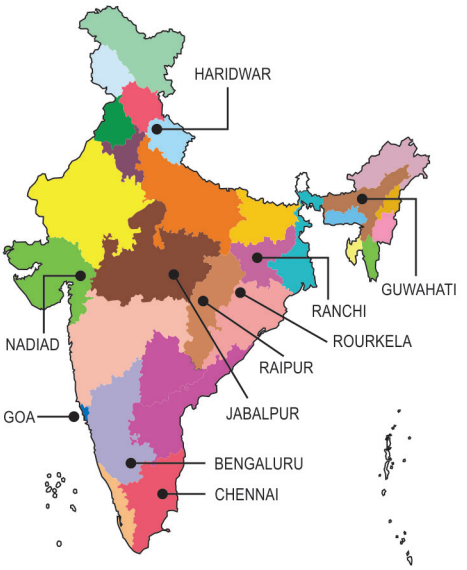


Figure 1: A network of Field Stations of the ICMR - National Institute of Malaria Research (NIMR) located in different physiographic zones of India

The beginning

India has embarked upon malaria elimination foundation for which was laid way back in 1983 with the launching of Integrated Disease Vector Control (IDVC) project in Kheda district of Gujarat. It all began with bio-environmental control interventions in select villages of Nadiad Taluka of Gujarat involving earth work eliminating vector breeding habitats, channelization for reducing water stagnation, introduction of larvivorous fish in water bodies and drains, intensified disease surveillance and above all health education campaigns promoting awareness on disease prevention and control. Based on the resounding success in reducing malarial morbidity and forthcoming community participation [8-10]; beginning 1986, the project was extended in mission mode to field test this technology in varied physiographic regions of the country. The overall objective of this exercise was to evolve sustainable solution encompassing right-mix of technologies to address long standing issue of rising proportions of *P. falciparum* and spread of drug-resistant malaria transmitted by different mosquito vector species across Indian landscape saving operational costs.

Amongst network of 10 different field stations established in the country (Figure 1), the Sonapur Primary Health Centre (PHC) 20 km east of Guwahati (the capital city of Assam) was selected based on specific recommendation of the state government for reporting consistently highest number of *P. falciparum* cases and malaria-attributable deaths. It is a

model health care facility under Primary Health Services dominated by tribal population groups experiencing persistent malaria transmission not responding to conventional interventions in force. It was firmly believed that demonstration of bio-environmental control interventions in this typical transmission setting would help the state formulating unified strategy for large scale implementation. The then most districts of the Assam were highly receptive for malaria transmission and disease outbreaks were common sight characterized by high rise in *P. falciparum* cases and attributable deaths. All death cases were invariably confirmed to be due to this deadly parasite and proportions of which were seen rising unabated. Transmission of the causative parasites by and large was perennial and persistent with seasonal peak during months of rainfall (April – September).

North-eastern region of India shares vast international border with China, Bhutan, Myanmar, and Bangladesh having implications in formulating intervention strategies related to malaria elimination initiatives. These border areas by and large are porous and remote with poor access, population groups in which are often deprived of healthcare services amounting to build up of infectious reservoir/asymptomatic cases. The border areas are prone to fulminating disease outbreaks and source for spread of drug-resistant varieties in peninsular India. There was imperative need to contain malaria in these inter-border areas by alternative interventions which are operationally feasible, cost-effective, community-based and sustainable. In this context, multifaceted approach was put to field test on experimental basis in high-risk villages of Sonapur, a typical foothill PHC bordering Meghalaya with major focus on bio-environmental control interventions keeping application of residual insecticides to bare minimum (Figure 2). Number of interventional technologies that were subject to field evaluation and subsequent implementation in the control programme are enumerated as below.

Figure 2: Components of the Integrated Disease Vector Control (IDVC) strategy for control of malaria transmission. IEC = Information, Education and Communication; ITN = Insecticide Treated Net; LLIN = long-lasting insecticidal net

Understanding vector bionomics and disease transmission relationships

There was paucity of information on current knowledge of mosquito vectors and disease distribution and determinants. Entomological expertise was waning with attrition of skilled workforce and it was considered logical first to generate entomologic information afresh enabling evaluation and implementation of species-specific interventions. Assam receives heavy rainfall (2-3 meters) spanning nearly six months (April - September); and temperatures (22–33^{0}C) and humidity (60–90%) are conducive for most part of the year for mosquito proliferation. Among number of anopheline mosquito species prevalent in the valley, *Anopheles minimus* and *An. baimaii* (sibling-species of the *An. dirus* complex) mosquitoes were recorded to breed and subject to further investigations *de novo* for disease transmission relationships [11]. *An. minimus* which was once presumed to have disappeared in Assam were recorded resting indoors human dwellings throughout the year with peak density corresponding to months of rainfall. *An. minimus* mosquitoes were proven vector by detection of motile sporozoites in salivary glands for all months of the year and held responsible for active transmission [12, 13]. Application of Geographic Information System (GIS) technologies seem to help and hold promise for benefit of the control programme for targeting vector populations in place and time [14]. Instead, *An. baimaii* was seasonal species with records of its prevalence during monsoons/rainy months but could not be incriminated in the study villages. Distribution of *An. baimaii*, however, was patchy and sparse restricted to forest-fringe villages. Other than that *An. fluviatilis* mosquitoes were recorded only during winter months (November – March), populations of which were later identified as hyper-melanic variants of *An. minimus* [15]. Among others, *An. philippinensis/ nivipes* was the commonest species often considered as suspected vector but their role in disease transmission could not be clearly established [16]. These data were considered important to formulate species-specific interventions for effective vector management specific to north-east India.

Malaria parasite load and transmission dynamics

Disease surveillance was an integral component of the integrated strategy to ascertain parasite load and delimit high-risk areas. Accordingly, both active (weekly) and passive (malaria clinic) surveillance were conducted in villages of Sonapur PHC to ascertain true prevalence of malaria, parasite formula and seasonal transmission dynamics (Figure 3). Based on the disease surveillance data, it was evident that malaria was the most prevalent infection affecting all age groups of both sexes inclusive of infants <1 year of age [17]. Cases were recorded in all months with seasonal peak during monsoon season, and average parasite rate varied from 34% in febrile subjects compared to 12% in afebrile villagers [18]. Both *P. falciparum* and *P. vivax* were abundant while a few sporadic cases of *P. malariae* were also recorded; mixed infections (<1%) were few and far [19]. The proportions of *P. falciparum* cases, however, exceeded those of *P. vivax* commencing rainy season in April till onset of winter in October. Conversely, asymptomatic carriers/gametocyte carriers were significantly higher in dry season (October – March) than wet season ($P<0.01$). Overall, transmission intensities were assessed to be low-to-moderate evidenced by entomologic inoculation rate of <1% across malaria endemic districts of the state. Distribution of

malaria, however, was heterogenous with districts sharing inter-state and/or inter-country border being at greater risk requiring prioritization for vector control interventions. Spatial transmission risk was much greater in foothill villages/houses located nearer (<1 km) to perennial streams (the preferred breeding habitat of *An. minimus*), and those located >5 km away from nearest healthcare facility [20]. These data (formerly sparse) enabled understanding of local disease epidemiology and transmission risk factors helping formulate appropriate interventions in time and place for follow up investigations.

Figure 3: Active malaria surveillance to ascertain prevalence of malaria and delimit high-risk villages for prioritizing interventions. Communities in forest-fringe villages were assessed to be at greater risk of malaria evidenced by parasite rate and abundance of *Plasmodium falciparum* cases.

Insecticide-treated netting materials for vector control

In north-east India, vector populations of *An. minimus* were assessed to be highly susceptible to DDT and continues to be the choice insecticide for vector control ever since inception of the control programme dating back to 1953 [13]. Nevertheless, malaria transmission remained uninterrupted attributed to poor community acceptance, recurrent flash-floods and difficult-to-reach areas limiting access to high-risk population groups [21]. Focal disease outbreaks were the common sight and returning with vengeance amidst panic and chaos in the marginalized population groups [22]. There was an imperative need for alternate interventions that are community-based and sustainable. In this context, initial field evaluation of insecticide-treated nets (ITNs) as an alternative intervention in Africa prompted to attempt this technology in Assam (the first time in India) to assess its operational feasibility in Indian geo-epidemiological conditions. Given the bionomical data on disease vectors, village-scale trials were launched in the Sonapur PHC in coordination with the state health department. The study results were promising reporting >70% decline in cases in the beneficiary population groups over two years study period (1988-

1990) and duly accepted by the Technical Advisory Committee (TAC) of the NVBDCP for pilot project in other sister states of north-east under supervision of respective state health departments [23, 24]. This task of knowhow technology was entrusted to NIMR for demonstration, supervision and monitoring, and training of health staffs of all participating north-eastern states on the subject. The national control programme made a provision of one hundred thousand insecticide–treated nets for distribution gratis in high-risk communities of respective states. ITNs were distributed beginning 1995 and for reporting states of Assam, Meghalaya and Arunachal Pradesh for which data were analysed (1995-1996), the results were comparable across states in reducing disease transmission over 70% compared to baseline year data, and above all much needed public responses were overwhelming and forthcoming [25]. The communities clearly opted ITNs against DDT residual spraying and reported collateral benefits for decreased nuisance of insect pest populations [26]. The ITN programme was runaway success based on which distribution programme was extended to other malaria endemic states of Orissa and Madhya Pradesh. The ITNs distribution programme had become order of the day and increased provisions were made under Global Fund to fight Aids, Tuberculosis and Malaria (GFATM) including impregnation of community-owned nets with insecticide. The limitation of this technology, however, was that these nets required treatment manually every six months which was operationally difficult and consequently re-treatment rates remained less than acceptable (<5%). The advent of long-lasting insecticidal nets (LLINs), however, revolutionized the concept of vector control world over. LLINs are ready-to-use factory treated nets that do not require re-treatment for 3 years (for duration of the lifespan of net) obviating the need for periodic re-treatment. A variety of LLINs that are in the offing were put to field evaluation in Assam and other malaria endemic states for operational feasibility in the given ecological conditions against different mosquito vector species [27-29]. These LLINs were assessed to be wash-resistant up to 20 standard washes and proven boon to the control programme for wider community acceptance, convenient to operate and overall low operational costs. Transmission reduction was tangible evidenced by decreasing parasite and entomologicalinoculation rates. Large-scale distribution of LLINs has become the states' agenda as major public heath intervention against malaria prioritizing high-risk population groups living under impoverished conditions.

Rapid diagnostic kits

The development of rapid diagnostic kits (RDKs) in the late 1990s enabling on-the-spot diagnosis was a hallmark development reducing time-lag between blood-smear collection and microscopic test results. This technology is largely based on capture of circulating parasite antigen in the peripheral blood stream. It was mandated to evaluate this technology popularly termed as 'dipsticks' against gold standard method of microscopic results for confirmed diagnosis and parasite species identification in the given field conditions. Accordingly, number of available popular brands of RDKs were subject to field evaluation for their comparative sensitivity and specificity in relation to microscopic results. Majority brands that were based on *P. falciparum* specific histidine-rich protein (Pf-HRP2) antigen capture assay revealed 100% sensitivity and high specificity (>94%) and concluded to be reliable tool to initiate curative therapy [30]. The major drawback, however, was that

Pf-HRP2 based kits continued to show positive results for extended periods up to day 7 post parasite clearance presenting scenario of repeated treatments of the given subject. To overcome this limitation, continued research efforts led to the development of combo-kits (incorporating both *P. falciparum* and pan-malarial antigen), results for which were less than optimal particularly for non-falciparum malaria for sensitivity ranging anywhere between (81-89%). It was imperative that judicious use of these kits would help the control programme instituting early diagnosis and treatment (the core-intervention) in the periphery where microscope facility is non-existent. These kits were assessed to be operationally feasible, easier to operate, store and transport in the field, and have been incorporated in the existing healthcare services for rapid diagnosis and currently widely deployed averting spread of malaria and saving lives.

Monitoring therapeutic efficacy of anti-malarial drugs and drug-policy for treatment

Instituting appropriate drug policy for treatment of malaria is of vital importance for radical cure to disrupt transmission. Ever since inception of the control programme, chloroquine therapy was widely deployed for treatment of both *P. vivax* and *P. falciparum* malaria throughout India. It was in 1973 that drug-resistant *P. falciparum* malaria had surfaced in Karbi Anglong district of Assam and subsequently multiplied and spread to peninsular India and further west wards [5]. *P. falciparum* is fast evolving parasite for rapid multiplication characteristics and ability to invade all ages of RBC. The inoculation rates are much higher compared to *P. vivax* malaria. To arrest and development of drug-resistant varieties, it was of utmost importance to monitor therapeutic efficacy helping institute appropriate drug-policy for radical cure. This task (in part) was trusted with NIMR field stations spread throughout the country for benefit of the control programme. North-east is of strategic significance for sharing vast international border and considered nidus for proliferation of drug-resistant varieties. NIMR field station Sonapur played pivotal role for periodic monitoring therapeutic efficacy for implementation of revised drug-policy right in time and place (Figure 4). *In vivo* follow up investigations revealed that not only the efficacy of chloroquine in the treatment of *P. falciparum* malaria was declining but also some local strains were getting multi-drug resistant [31, 32].

Among therapeutic efficacy studies of alternate treatments, artemisinin derivatives (short-acting) alone [33] and in combination with partner drugs (long-acting) resulted in good treatment success reporting rapid parasite clearance. Artemisinin monotherapy was discontinued as matter of policy and decision was taken for rollout of combination therapy for treatment of every single case of *P. falciparum* throughout India beginning with ASP (artesunate + sulfadoxine-pyrimethamine). Soon enough, declining efficacy of this combination was seen to have surfaced in north-east sector and was replaced by AL (artemether + lumefantrine) restricted to north-east region [34-36]. Newer molecules involving azithromycin alone or in combination with chloroquine were also attempted for treatment of *P. falciparum* and *P. vivax* (relapsing malaria) for adding to the armamentarium in reserve [37, 38]. Periodic monitoring of therapeutic efficacies of anti-malarial drugs have indeed helped the programme in upgrading drug-policy for radical cure of malarial infection preventing spread of drug-resistant varieties [39].

Figure 4: Hospital-based study for monitoring therapeutic efficacy of anti-malarial drugs in high-risk areasresulted in shift of drug-policy for radical cure of *Plasmodium falciparum* malaria. Based on study results chloroquine therapy was replaced by sulfadoxine-pyrimethamine (SP) in 2004 which in turn got replaced by ASP (artesunate +sulfadoxine-pyrimethamine) in 2007 that later got changed over to AL (artemether + lumefantrine) in 2013.

Biological control agents for vector control

Vector control is the main stay for malaria control operations globally. Interventions based on indoor residual spraying are logistically prohibitive requiring huge recurring costs. Given the funding gap, bio-environmental control interventions including application of larvivorous fish and other biological control agents that hold promise particularly in urban/semi urban settings, were revived. Among various options, larvivorous fish *Poecilia reticulata* (guppy fish) and *Gambusia affinis* (mosquito fish) were extensively employed for control of *An. culicifacies*, predominant vector of malaria in south Indian state of Karnataka [40]. Given the resounding success, fish-based intervention was endorsed by the national programme for control of disease vectors across Indian states. Accordingly, larvivorous fish intervention programme was expanded in Assam for control of *An. minimus* vector populations beginning with select malaria endemic districts in collaboration with the state health department [41]. Mother hatcheries were established for mass-scale application in water bodies supporting breeding of mosquito vector as an integral component of integrated strategy (Figure 5). The programme received overwhelming response from communities for perceived benefits in reducing mosquito nuisance and improved hygiene in the surroundings.The fish-based intervention is presently being expanded to other north-eastern states for application in conjunction with other vector control options for being cost-savvy.

Figure 5: Mass-scale distribution of larvivorous fish in malaria endemic districts of Assam; an activity funded by the National Health Mission of Ministry of Health & Family Welfare, Government of Assam.

Technical support to the National Control Programme

Owing to rapid attrition of healthcare professionals, there was dire need for training of new recruits and re-orientation of field staffs for optimal performance. Besides, research inputs to the control programme, NIMR field stations were given the mandate to provide consultancies as well as impart trainings to the state health technicians updating with the latest technologies. Accordingly, refresher courses and workshops were periodically held for benefit of the state healthcare staffs. In addition, services were provided for preparing malaria action plan, coordinating inter-border meetings, identification and situational analyses of high-risk districts for epidemic control preparedness and conducting malaria outbreak investigations [42, 43]. Services were equally indented by other sectors including government (Defense), public (Refineries) and the private sector alike, viz., Tea Gardens and small-scale units for capacity building and combating disease outbreaks [44, 45]. Technical expertise was also provided to the World Health Organization preparing for estimating disease burden and strengthening malaria elimination initiatives for benefit of the South-East Asia regional member countries.

Educating communities & human resource development

A good fraction of communities (>30%) were estimated to be living under impoverished conditions little aware of disease and preventive measures. Thus, educating communities was considered an essential component of the integrated disease vector control strategy for enhanced community compliance and participation. Among allied measures, anti-malaria month (June of each year) corresponding to onset of high transmission season was observed

in full fervor during which information, education and communication (IEC) activities, viz., group meetings, media coverages/TV spots, booklets/leaflets distribution were all undertaken often in close collaboration with district/state health authorities, NGOs, civic societies/faith-based organizations to elicit community responses (Figure 6). All these measures resulted in much needed awareness on disease prevention, behavior change for treatment seeking well in time, mosquito net ownership resulting in substantial disease transmission reduction [25, 46]. In rolling back malaria initiative, these remain continuing educational activities for human resource development, and communities today are better informed and stand equipped to meet the malaria challenge.

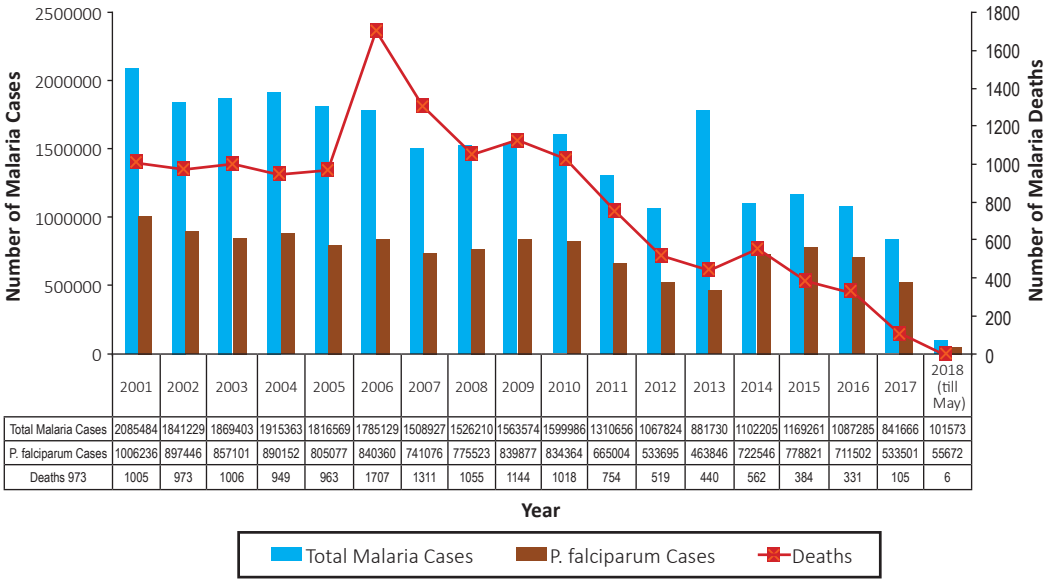
Figure 6: Anti-malaria month: group meetings were routinely held for educating communities on malaria prevention and control in high-risk areas.

The study outcome and way forward

The demonstrated success of the Integrated Disease Vector Control strategy, particularly ITNs/LLINs for vector control, RDKs for diagnosis and improved drug-policy for treatment of drug-resistant malaria resulted in incorporation of these interventions in the national control programme. In addition, number of agencies including tea industry, small scale industrial units, Oil and Natural Gas Commission (ONGC), hydro-electric projects, defense services and Railways were all benefitted in achieving transmission control to greater extent. Disease transmission trends were observed declining every passing year attributed largely to mass-scale distribution of ITNs/LLINs, roll out of artemisinin-based combination therapy and increased awareness on disease prevention and control [46]. Using similar approaches, unprecedented decline in cases (>80%) was reported in eastern coastal state of Odisha (formerly contributing nearly 40% of the total reported cases in the country) compared to baseline year of 2016 [47]. Application of bio-environmental control approaches also resulted in appreciable transmission reduction in Uttarakhand

(industrial malaria) and forest/tribal belts of central Indian state of Madhya Pradesh [48, 49]. The network of field stations spread across the country served as launching pads and strengthened the national control programme substantially providing research inputs in vector biology and control, transmission dynamics, therapeutic efficacy investigations helping upgrade drug-policy, training and re-orientation exercises of state health personnel helping build cadre of skilled workforce, and above all eliciting community participation.

Given the present-day intervention tools, rolling back malaria has become reality [50]. India stands better equipped with additional programme support under National Health Mission (NHM) and Integrated Disease Surveillance Project (IDSP) reporting steady decline of cases and deaths over the preceding few years targeting malaria elimination by 2027 [51, 52] (Figure 7). Under these umbrella programmes, induction of Accredited Social Health Activists (ASHA) for intensified disease surveillance and strengthening entomological component for averting impending disease outbreaks have proven boon to the control/elimination efforts.



	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018 (till May)
Total Malaria Cases	2085484	1841229	1869403	1915363	1816569	1785129	1508927	1526210	1563574	1599986	1310656	1067824	881730	1102205	1169261	1087285	841666	101573
P. falciparum Cases	1006236	897446	857101	890152	805077	840360	741076	775523	839877	834364	665004	533695	463846	722546	778821	711502	533501	55672
Deaths 973	1005	973	1006	949	963	1707	1311	1055	1144	1018	754	519	440	562	384	331	105	6

Figure 7: Malaria transmission trends in India. The cases have consistently declined from 2.08 million to 0.84 million during 2001 to 2017. *Plasmodium falciparum* cases declined from 1.0 to 0.53 million cases during the corresponding period. Malaria-attributable deaths were also seen declining from 1707 in 2006 to 105 in 2017. Source Reference [51].

Up until malaria vaccine become available, there is no single magic bullet which would suffice to kill malaria; instead combination of technologies which are community-based and doable would make the difference in defeating malaria. Newer innovative technologies, viz., attractive toxic sugar baits (ATSB), eave-tubes, nano-synthesized pesticides loaded with microbial and plant-borne compounds, biocontrol agents with little nontarget effects, new adult repellents, oviposition deterrents, even acoustic larvicides, genetically modified mosquitoes (transgenesis) and paratransgenesis (modification of causative parasite in the mosquito host); all hold promise to overcome challenges of outdoor transmission and growing threat of insecticide resistance [53, 54].

Integrated bio-environmental control strategy is being revived world over reducing reliance on use of insecticides in public health [55]. It is firmly believed that these measures when combined with the existing tools would yield rich dividends in accelerating towards malaria elimination. What remains crucial is the sustained political commitment for increased allocation of resources for universal coverage and judicious application of interventions in real time and place ensuring equity in healthcare services [56, 57]. There is no room for complacency at any echelon of operation for emerging challenges of continually evolving drug-resistant malaria and insecticide resistant vectors [58-60]. For achieving malaria elimination in due time, universal coverage of evidence-based interventions holds the key to success; everyone and each one should have access to prevention and affordable treatment [61, 62]. LLINs should be the household commodity for personal protection against infective mosquito bites and treatment access should be the cornerstone for decisive attack on the dare devil as component of the integrated disease control approach rather than exclusive approach [63]. Eliminating malaria in second largest World's population will be a hallmark achievement and a big leap forward in public health.

Acronyms	
AL	artemether + lumefantrine
ASHA	Accredited Social Health Activist
ASP	artesunate + sulfadoxine-pyrimethamine
DDT	dichloro-diphenyl-trichloroethane
Pf-HRP 2	*Plasmodium falciparum* histidine-rich protein 2
GFATM	Global Fund to fight Aids, Tuberculosis and Malaria
ICMR	Indian Council of Medical Research
IDSP	Integrated Disease Surveillance Project
IDVC	Integrated Disease Vector Control
IEC	Information Education Communication
ITN	Insecticide treated net
LLIN	long-lasting insecticidal net
NGO	Non-Governmental Organization
NHM	National Health Mission
NMCP	National Malaria Control Programme
NMEP	National Malaria Elimination Programme
ONGC	Oil & National Gas Commission
PHC	Primary Health Centre
RDK	Rapid diagnostic kit
SP	sulfadoxine-pyrimethamine

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Chapter 15

Paratransgenesis: a novel approach for malaria transmission control

Tapan Kumar Barik[1,2] and Usha Rani Acharya[1,2*]

[1] Post Graduate Department of Zoology, Berhampur University, Berhampur-760007, Odisha, India

[2] Post Graduate Department of Biotechnology, Berhampur University, Berhampur-760007, Odisha, India

*Correspondence:ura_zl@rediffmail.com

Abstract

Malaria is one of the world's deadliest vector-borne disease affecting millions of people globally. Malaria eradication and control in different parts of the globe is accomplished mostly by mosquito population control. However, conventional methods to control mosquito vector population through chemical insecticide treatments are often coupled with adverse impacts on human health, environmental and most strikingly by the development of insecticide resistance strains of mosquito vectors. Therefore, effective eco-friendly alternative strategies are deemed essential. Transgenic technology in this regard promises to be the suitable intervention that allows modification of genome of the living being(s) by addition of foreign DNA to generate the genetically modified forms. However, production and introduction of these transgenic mosquitoes of all the strains of principal mosquito vector(s) in the wild is a herculean task. Of late, microbial endosymbionts of mosquito vectors are drawing a considerable attention in connection to the advancement of a novel technique to control mosquito vectors. Paratransgenesis one such approach which aims to use genetically modified endosymbionts of mosquito vectors that express molecules inside the mosquito host which are lethal to pathogens they transmit. This chapter would focus on the present state of knowledge about paratransgenesis and the feasibility of use of endosymbionts that can block the malaria transmission by preventing the parasite proliferation within the malaria vectors.

Keywords: Paratransgenesis, mosquito, malaria, vector control, endosymbionts, pathogens

Introduction

Malaria is a vector-borne disease caused by single-celled protozoan *Plasmodium* parasite transmitted by female *Anopheles* mosquito widely distributed in tropical and subtropical regions of the world [1]. Malaria cases have been reported in nearly 100 countries with disproportionate share from Africa-south of Sahara (92%) followed that by South-East Asia (5%) and Eastern Mediterranean countries (2%) [2]. In recent years, declining trends in malaria transmission are being reported globally but malaria elimination efforts are being threatened by funding gap, weakening health systems, increased anopheles mosquito resistance to various chemical insecticides, and above all declining the rapeutic efficacy of antimalarial drugs for treatment of *Plasmodium* parasites to combat the disease illness. Insecticide resistance in mosquito vectors is harsh reality and getting operationally difficult yielding diminishing returns [3]. Consequently, given the present-day intervention tools, the acceleration towards elimination during the period from 2015-2017 has stalled; instead rise in cases has been registered in some of the 10 high burden countries in Africa [4]. There is an imperative need for newer interventions to sustain the gains and move ahead with elimination agenda by 2030. Amongst several approaches, despite extensive research in the field of vaccine against malaria, no such operational vaccine is yet made available for universal use. Thus, there is scope for additional tools that are self-sustainable, community-based and doable which could be integrated invector control operations to end malaria transmission for good [5]. Here, we present a comprehensive review of the currently available tools and upcoming novel technologies in defeating insecticide resistance which hold good promise for effective control of mosquito vector populations.

Current malaria vector control strategies

Malaria transmission control in different parts of the globe is accomplished largely by control of mosquito vector population by application of insecticides through indoors residual spraying (IRS) and insecticide-treated mosquito nets (ITMNs)/long-lasting insecticidal nets (LLINs) [6]. Nevertheless, such interventions are getting relatively ineffective due to growing insecticide resistance in mosquito vectors along with adverse impact on environment [7]. Although novel insecticides are being developed for vector control, insecticide resistance in due course of time is imminent. Besides, logistics costs are rising and getting operationally difficult in resource-poor settings. Microbial control of mosquito vectors includes the use of varied and diverse range of microorganisms like viruses, bacteria, fungi, nematode etc [8]. Experiments on Bactoculicide formulations against *An. stephensi* was found effective in all the experimental habitats [9]. A dose of 0.5 g/m2 of Bactoculicide formulation of *Bacillus thuringiensis* were able to control 96-100% mosquito breeding up to five weeks [10]. Laboratory evaluation of *Bacillus sphaericus* liquid and granule formulations showed good larvicidal activity against *Culex quinquefasciatus* [11]. Further, it was noticed that malaria incidence declined substantially when *Bti* and larvivorous fish were used against *An. stephensi* in Goa [12]. In addition to microbial control, other available intervention tools, viz., use of other biological control agent, phytochemicals, and environmental management are not so promising to stand alone. Therefore, alternative methodologies are need of the hour that are effective, environment-

friendly and self-sustainable which could be implemented in the control programme for containment of vector populations disrupting transmission.

Transgenesis in insect disease vectors and challenges

Manipulation of genome of wild mosquito vector populations to suppress their capability to transmit pathogens is a very old scientific dream. Transgenic technology allows modification of genome of living beings by the addition of foreign DNA to produce the genetically modified forms. Considerable efforts have been dedicated for the genetic alteration of mosquito vectors resulting in production of refractory mosquito strains incapable of transmitting pathogen and those carrying a lethal gene [13, 14]. However, generating transgenic mosquitoes of all strains of the principal vector species and their spread in the wild are mammoth tasks the one that difficult to accomplish.

Paratransgenesis: a novel alternative strategy

Insects are the most abundant and varied clade of metazoans [15]. Insects and microbes have co-evolved over several hundred million years and reflect extensive symbiotic associations. The term microbiota refers to the microbial communities that steadily or momentarily colonize in various parts of the insect body. These microbial communities vary from viruses to bacteria, and protists to yeasts. Some of this microbiota is useful to their respective hosts in several ways like nutritional supplementation, acceleration of digestive processes, acceptance of ecological imbalances, protection from parasites/pathogens and preservation and/or development of host-immune system homeostasis [16-21]. Several reviews substantially explained the possible role of microbes in insects as well as usefulness of these microorganisms and their metabolic abilities in biological control of mosquito vector-borne diseases. Some of the basic requirements of symbiotic microorganisms which must be met for paratransgenesis to be accomplished are given in Table 1. 'Symbiotic control' is a new multi-dimensional strategy that refers to the application of symbiotic microbes for the management of insect vector populations in reducing vector competence. Three different strategies presently at the forefront are: (i) disturbance of microbial endosymbionts essential for insect vectors (ii) exploitation of endosymbionts that are able to express anti-pathogen molecules inside body of the host, (iii) addition of endogenous microbes that can influence the life-span as well as vectoral capacity in insect vector populations.

Table 1: The fundamental prerequisites for paratransgenesis*

I	Well established symbiosis relationship among vector populations and microbes
II	Symbiotic microbes that can be cultivated in vitro and all genetic manipulation
III	Effector gene product should not harm the symbiotic microorganisms and vector fitness
IV	Effector gene product should be secreted to assure interaction with the target pathogen
V	Technologies for introducing the engineered endosymbiont into field must be developed

*Source reference [22]

Paratransgenesis, on the other hand is defined as the use of endosymbionts that inhabit naturally in the insect midgut to express antipathogen effector molecules and can spread rapidly among vector populations [23,24]. An appropriate microbial agent for paratransgenesis would have a symbiotic relationship in the form of mutualistic, commensal or parasitic with the insect vector. Such paratransgenic forms could be easily multiplied and manipulated to express the gene(s) without change in the fitness of the microorganism and can be easily delivered in the field populations [25]. Ideally, the genetically altered microorganism would also be established in the surroundings and can be passed on to successive generations with minimal effects on non-target organisms. The initial step in paratransgenesis to recognize proteins that prevent the mosquito vectors from transmitting the pathogen. The genes responsible for production for these proteins are then brought into the endosymbiont so that they can be expressed inside the body of mosquito host. The last step in this approach is to inoculate these genetically engineered microbes in the vector populations in the field. Both metagenomics and meta-transcriptomics are essential techniques to reveal the diversity, genomic composition and potentiating inside and across micro-environments by using the culture-independent techniques. This is further accomplished by targeting 16S (bacteria) and 18S (eukaryotes) rRNA gene sequences and internal transcribed spacers (ITS2) for fungi and whole-metagenomic shotgun sequencing [26, 27].

Paratransgenesis in mosquitoes

Application of endosymbionts for mosquito vector control offers numerous favorable features compared to other control interventions. To begin with, genetic engineering of endosymbionts is much simpler and faster than transgenic mosquito vectors, and similarly engineered or altered endosymbionts can easily be introduced into mosquito vector populations compared to transgenes. For instance, producing engineered endosymbiont in large numbers is much simpler than producing genetically modified mosquitoes. Further, use of engineered endo symbionts can circumvent the genetic barriers of reproductively isolated mosquito species complexes involved in malaria transmission in high malaria endemic areas which normally would have presented a huge barrier to expand the mosquito transgenes. Again, microbes can be cultured in large quantities at low cost and possible inactivation of microbial transgenes is certainly not a major concern in view of the easier logistics of introducing genetically engineered endosymbiont in the surroundings. Finally, regarding the regulatory and ethical formalities for the use of genetically modified microbes in paratransgenesis, already-existing guidelines and regulations on the release of engineered microbes into the environment are fair enough for applications of mosquito endosymbionts in the field. Methodologies have already been developed specific to mosquito vector transformation using engineered endosymbionts for blocking malarial transmission (Figure 1) [22, 28, 29].

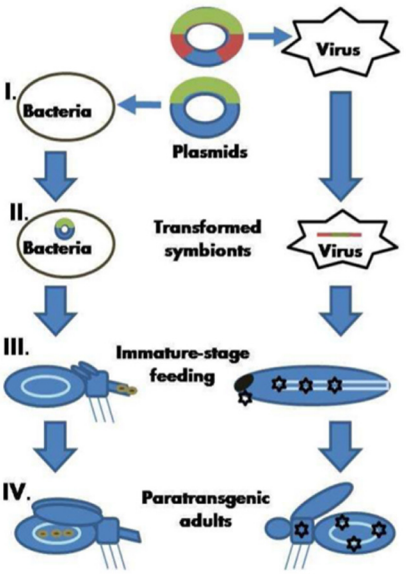

Figure 1: Procedure of insect transformation using engineered endosymbionts. Source Reference [28].

Endosymbionts of malaria vectors and their interactions

Endosymbiosis is a symbiosis where the microbial partner lives inside its host body and represents the closest contact between the interacting organisms [30]. The arthropod host domesticates the endosymbionts for its own welfare by using the capacities that are available in the symbionts but lacking in the host. Insect endosymbionts were classified into two different categories such as primary and secondary. Primary endosymbionts have been associated with their insect hosts obligatorily since millions of years and are essential for the survival of the arthropod. These are usually transmitted vertically found in the specialized cells that provide nutrients to bacteria [31]. On the other hand, secondary endosymbionts are microorganisms that appear to be the resultant of multiple independent infections and their contribution to the welfare of arthropod host may not necessarily be major or essential but are not obligate. These endosymbionts are transmitted vertically, horizontally or through the environment and can be located in the haemocoel of the host [32]. Some of the commonly abundant endosymbionts are described briefly as below:

Bacteria

Besides the native gut microbiota, endosymbiotic bacteria can thrive in other organs such as salivary glands, ovaries and hemolymph. Bacteria colonizing the midgut lumen can potentially alter the intestinal condition that hinder the proliferation of causative parasites by regulating the immune system and expression rate of immunity genes encoding antimicrobial peptides [33-35]. These peptides play pivotal role in the availability of endosymbiotic bacteria and believed to spread the responses towards parasite infections [36,37]. When infection is established, bacterial symbionts modulate vectoral competence

by inhibiting the development of Plasmodium species [38-42]. But it is strikingly different in Gram-negative bacteria which inhibit oocyst formation completely else partially [38, 39]. Anopheline species are reportedly associated with number of bacterial genera, viz., *An. gambiae* (71 genera) followed that by *An. stephensi* (46 genera). Laboratory reared *An. gambiae* and *An. stephensi* are reported to harbor wide range of bacteria, viz., *Asaia, Enterobacter*, *Mycobacterium, Sphingomonas, Serratia* and *Chryseo bacterium* [41 ,43, 44]. Similarly, *An. gambiae* and *An. funestus* were reported to harbor 16 bacterial species of 14 genera [45]. Some of the commonly abundant endosymbiotic agents that have been evaluated for paratransgenesis are described below:

***Asaia*:** Bacteria of the genus *Asaia* have been evaluated as potential tool in paratransgenic vector control in reducing malaria transmission [43, 46]. It has been reported that genetically engineered *Asaia* are capable of colonizing laboratory-bred populations of *An. stephensi* mosquitoes both by vertical and horizontal transmission routes. Boissière et al [47] also reported positive correlation between the presence of *Enterobacteriaceae* bacteria and an infected mosquito blood meal. Functionally, *Asaia bacteria* hold the potential to act as an immuno modulator inside body of the mosquito host by inducing production of antimicrobial peptides that may meddle with the course of infection especially during invasion of epithelial tissues and subsequently salivary glands. *Asaia bacteria* may also act as an up regulator of immunity genes which is considered appropriate for genetic manipulation strategies. Capone et al [48] reported that genetically manipulated *Asaia bacterial* strains were widely spread in the midgut and salivary glands of infected mosquitoes and co-localize with *Plasmodium* parasites by passing genetic barriers among mosquito populations [46]. However, on the contrary Rani et al [44] and Djadidet al [49] did not record these *Asaia bacterial* strains in the field-collected as well as laboratory-reared *An. stephensi* and *An. maculipennis* from Iran and India respectively.

***Serratia*:** *Serratia marcescens* HB3 strain was reportedly isolated from laboratory-reared *An. stephensi* in hibiting *Plasmodium* proliferation by disturbing ookinete invasion through the midgut epithelial cells. Phenotypic variation both at the structural and cellular levels was noticed associating with the capacity to induce resistance against *Plasmodium* invasion [50].

***Pantoea*:** Bacteria of the genus *Pantoea* has been proposed for applications of *paratransgenic* approach [49, 51]. Wang et al [52] developed a *Pantoea agglomerans* strain secreting five different antimalarial proteins in midgut of the mosquito host. They observed that the development of *P. falciparum* and *P. bergheiin An. gambiae* and *An. stephensi* was inhibited up to 98% and 83% respectively.

***Enterobacter*:** Bacterial strains belonging to genus *Enterobacter* isolated from *An. arabiensis* from Zambia can act directly on P. *falciparum* hindering the development of malaria parasite in *An. gambiae*. This impact is due to the production of reactive oxygen species interfering with the development of malaria parasite causing death of the parasite before invading the intestinal epithelium [42].

***Wolbachia*:** It has been reported that *Wolbachia*, a genus of intracellular α-proteo bacteria has an inhibitory effect on *Plasmodium* parasite infection in anopheline mosquitoes. *Wolbachia*

infected *An. gambiae* mosquitoes challenged with *P. falciparum* resulted in significant reduction in production of oocysts. These bacteria were able to spread widely throughout the mosquito host organs except gut and ovaries [53]. Furthermore, Bianet al [54] reported successful establishment of *Wolbachia* infection in laboratory-reared *An. stephensi*. It has also been established that *Wolbachia* wAlbB strain not only exhibited maternal transmission but also cytoplasmic incompatibility in mosquitoes [55].

Fungi

Fungal species have the ability to survive in the environment for months in the form of spores and can infect mosquitoes directly through the cuticle (external envelope of an insect). The potentiality of many fungi belonging to the genera Lagenidium, Coelomomyces, Entomophthora, Culicinomyces, Beauveria, and Metarhizium as mosquito vector control agents are well documented, and few of them have been already been commercialized for use in mosquito abatement programs. Fungi are usually easy to alter genetically, cultivated and mass reared in the endemic environment, and hence used in paratransgenesis. Modified fungus, *Metarhizium anisopliae* can express molecules which are generated affecting the development of *P. falciparum* in An. gambiae [56, 57].

Densovirus

Different viruses that harbor the insect body may also be a potent agent for paratransgenesis for insect control. Mosquito specific denosovirus (MDV) have been isolated from several mosquito vector species including *An. gambiae*. These viruses have the capacity to infect almost all developmental stages of the mosquito [58, 59]. After infection with these viruses, the larvae become malformed, sluggish and curved in shape and die before reaching adult stage. These MDVs vary significantly in their capacity to spread and persist in the environment and pathogenicity across mosquito species. It has been reported that MDVs decrease appreciably the oviposition rate, fecundity and fertility of Aedes mosquitoes. Further, a dose dependent shortening of adult forms has been observed in Aedes mosquito infected with MDVs [60]. However, *An. gambiae* densoviruses were found to be infectious but non-pathogenic to larvae [25]. Bird *et al* [61] described that cytoplasmic polyhedrosis virus in *An. stephensi* surrogating *P. bergeiyoel* is squarely reduced the transmission of malaria parasite. The authors therefore had reasonably speculated that the virus had deleterious effect on the mosquito and the developing parasites. Consequently, a significant fraction of sporozoites exhibited deformed structure or vacuolation. Genetically modified *An. gambiae* densovirus (AgDNV) reported to act as a biological late-life insecticide targeting older adult *An. gambiae* [62]. Ren *et al* [25] demonstrated that *An. gambiae* densonucleosis virus (AgDNV) is readily transmitted between generations in natural populations both vertically and horizontally. Further, it has been reported that recombinant AgDNV is able to exhibit anti-Plasmodium peptides in *An. gambiae* and reportedly enter in large quantities in fat body and ovaries with negligible impact on adult mosquito survival and transcriptome composition making it a best candidate for paratransgenesis [63-66].

Use of effector molecules in endosymbiont for paratransgenesis

Paratransgenic approaches using antipathogen effector molecules to diminish vector competence are dependent on "toolbox of effectors" that antagonizes the pathogen without changing the host's fitness. Such type of effectors molecules has been detectedin the mosquito immune response to pathogens by high-through put peptide screens such as phage-display libraries and even from investigations exploring the components of animal toxins. Wang and Jacobs-Lorena [22] elaborated about the use of various effector molecules having anti-plasmodial activity against malaria vectors. In this connection, the recognition of potent anti-*Plasmodium* effector genes happens to be critical condition for the expression of a refractory mosquito. Usually, the effector molecules should hamper the parasite transmission without arresting the fitness cost to the mosquito. Large number of anti-Plasmodium effector molecules have been identified such as mosquito innate immune peptides (defensins and cecropins), lytic peptides (scorpine, a component of scorpion toxin), synthetic lytic peptides (Shiva-1), peptides that bind to the parasite or parasite-produced factors (enolase–plasminogen interaction peptide or EPIP, antibody single-chain variable fragments or scFvs), and peptides that bind to mosquito receptors blocking uptake of parasites (e.g., salivary gland and midgut peptide 1 or SM1; mutant phospholipase A2 or mPLA2). When different kinds of effector molecules were used in fungi, *Meterhizium anisopliae* to block malaria infection in *An. gambiae*, it was observed that the levels of malaria parasite in mosquito salivary glands were inhibited up to 98% compared to controls [57].

Riehle et al [67] observed that the formation of oocysts of *P. berghei* in *An. stephensi* mosquitoes can be diminished by expression of effector molecules like SM1 and mPLA2 by commensal recombinant *Escherichia coli.* It has been observed that combinations of various anti-Plasmodium effector molecules like cecropin A, SM1, Scorpine, EPIP, scFvs and mPLA2 secreted by transgenic *Pantoea agglomerans* inhibited the growth and development of *P. falciparum* and *P. berghei* in *An. gambiae* and *An. stephensi* mosquito species [52, 67].

Field evaluation of paratransgenic anopheline mosquitoes

Mass release of insects with engineered endosymbiont may possibly disturb ecosystems by shifting established living organisms, disturbing biodiversity and changing balance of microbial communities. Therefore, release of such paratransgenic mosquitoes in the field imposes a continuous risk assessment. A first semi-field pilot study on paratransgenic anopheline mosquito species, *An. stephensi* and *An. gambiae* with an engineered *Asaia* bacterial species expressing the Green Fluorescent Protein (Asaiagfp) was undertaken in large cages in Italy. The goal of this semi-field trial was to explore the potentiality of *Asaia* bacterium in paratransgenesis to control malaria transmission and other such mosquito vector-borne diseases. It was observed that these modified *Asaia* bacteria were capable to spread at high rate in various anopheline mosquitoes exploring horizontal transmission and effectively colonizing in the midguts of mosquito. Further, it was also noticed that vertical and transstadial diffusion mechanisms in *An. gambiae* also favors the use and application of *Asaia* bacteria in paratransgenesis approach for malaria control [68].

Conclusions

New and novel strategies should be included in the current interventions for successful integrated vector control management for control of vector-borne diseases. Paratransgenesis is a new innovative strategy that attempts to minimize the vector competence by manipulation of microbes inside mosquito host [69, 70]. Malaria researchers have been paying attention towards paratransgenesis approach as a novel alternative strategy to conventional transgenic strategies [43]. Paratransgenic-based control technique have an extraordinary potential as endosymbionts that appear not to have fitness load on mosquitoes, and they can spread both by vertical and horizontal transmission routes. Concerning about regulatory and ethical requirements, already-existing regulations for release of modified microbes into the environmental surroundings are good enough for planning and formulating more specific rules and regulations for large scalefield applications of mosquito endosymbionts.

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Chapter 16

Larvivorous fish: repurposing of an old strategy for sustainable malaria vector control – a success story based in Karnataka, South India

Susanta Kumar Ghosh*

Ex-Scientist, ICMR - National Institute of Malaria Research (Field Unit), Bengaluru, Karnataka, India

Correspondence: ghoshnimr@gmail.com

Abstract

Two Poecilid larvivorous fish species, *Poecilia reticulata* (guppy fish) and *Gambusia affinis* (mosquito fish) have been successfully applied for biocontrol of *Anopheles culicifacies*, the major vector of malaria in South Indian state of Karnataka. Both these fish species were found suitable to target mosquito breeding in wells and ponds respectively to contain vector mosquito populations. Large scale introductions of these fish species in respective mosquito breeding habitats resulted in disruption of active transmission in the malaria endemic districts. This technology is community-driven, eco-friendly, and assessed to be sustainable, operationally feasible and cost-savvy. Based on this success story, this technology is being extended to other malaria endemic states under the umbrella of "integrated and inclusive vector management" concept in the Gandhian principle of sustainable growth.

Keywords: malaria, *Anopheles*, larvivorous fish, biological control, mosquito breeding, community participation

The beginning

India has made substantial gains in malaria transmission control over the past few years and targeting elimination by 2027 [1, 2]. Yet with record of nearly half a million cases in 2018, there is a huge challenge and an uphill task to be accomplished within the available resources and strategies [3]. In 2017, the World Health Organization (WHO) reported an estimated 219 million malaria cases and 435,000 related deaths in the world [4]. In the malaria endemic world, amongst 11 high burden countries contributing most cases and deaths, India is also included for sharing 4% of the total disease burden, the rest 10 are all African countries south of Sahara. In India, malaria transmission is heterogenous and diversified

into various ecotypes, viz., rural, urban, forest/tribal, desert malaria and the like. Most Indian states are reporting cases amongst which the eastern and central states of Odisha, Jharkhand and Madhya Pradesh, Chhattisgarh respectively shared almost 74% of the total reported cases in the country [1, 3]. Malaria control operation is primarily dependent on indoor residual spraying of various classes and formulations of insecticides. In certain situations, this strategy encounters rejection from the local communities necessitating alternate methods of vector management. One such challenge was experienced in the sericulture areas of southern Indian state of Karnataka. The basic material for the famous `Mysore Silk' is the mulberry silk fiber produced from the lepidopteran moth (*Bombyx mori*). These moths are cultured in specially designed rooms in most villages in neighbouring districts of Bengaluru. Sericulture is a cottage industry where most of the local farmers are dependent for their livelihood. Malaria was endemic in this area for decades, and any attempt to control with application of insecticides was not possible for collateral damage in killing the silk moths of economic importance.

The inception of bio-environmental control

To address this gigantic challenge, a research station of the ICMR-National Institute of Malaria Research was established in Bengaluru beginning 1992 to field-test alternative interventions that are community-based, eco-friendly and sustainable. At the very outset there was no available alternative strategy with sufficient body of evidence that can be applied at larger scale. The only option that could be revived was larval source management (LSM), the main vector control intervention in the pre-DDT era. In this direction, a mega-project on bio-environmental control of vector management had given a new lease of life launched in the mid-1980s under the stewardship of (Late) Dr. V. P. Sharma, the founder director of the National Institute of Malaria Research (NIMR), the erstwhile Malaria Research Centre. A dozen of research field stations were established in different states of the country with the mandate to evaluate alternative interventions for vector control in different geo-epidemiological settings and help transfer state of art technology to the local state government for incorporation in the healthcare services. Here in Karnataka, LSM method was the only available option that could be applied given the community acceptance and requirements obviating the application of residual insecticides [5]. Given the mandate, a detailed action plan was envisaged with the following objectives: (i) to demonstrate the probable strategy in high malaria endemic areas, (ii) to analyze the village level malaria transmission profile, (iii) presence of probable malaria vector(s) and to ascertain their breeding habitats, (iv) mapping of all water bodies through geographical reconnaissance, (v) training of health personnel for hands on the new intervention strategy, and (vi) eliciting community acceptance and engagement.

Species-specific bionomics and control interventions

Two interesting observations had emerged: (i) there were far more malaria cases in villages surrounded by wells and ponds in comparison to villages located along nearby streams, (ii) there were no malaria cases in the neighboring areas where larvivorous fish *Poecilia*

reticulata (guppy fish) were present. The genetic analysis of two prevalent malaria vector species, i.e., *Anopheles culicifacies* and *An. fluviatilis* revealed some interesting facts as to their sibling species composition and disease transmission relationships. It was sibling species 'A' (proven vector) of *An. culicifacies* that was prevalent in villages surrounded by ponds and wells, whereas species 'B' (poor vector) was abundant in villages nearby streams. Similarly, in these villages it was sibling species 'T' of *An. fluviatilis* which generally do not participate in transmission corroborated by host-bloodmeal analysis [6]. All these background observations explained the distribution of malaria in relation to breeding resources and prevalence of disease vectors. Thus, the role of wells and ponds was established serving as `ecological niche' sustaining vector populations of *An. culicifacies*. These observations clearly guided the planning for application of larvivorous fish in specific-specific habitats for control of *An. culicifacies* breeding. Initially the guppy fish, *P. reticulata* was released in all water bodies, and post one-year introduction these were observed breeding exclusively in wells and virtually absent in ponds. Instead, in the following years the mosquito fish, *Gambusia affinis* was released in all ponds and recorded breeding profusely enough to eliminate mosquito breeding. Large scale application of these species-specific control interventions resulted in transmission disruption with no report of fresh cases in the target villages [3].

Extension of fish-based intervention for vector control

As a follow up of these field-based observations, the WHO under Roll Back Malaria (RBM) initiative as well as WHO country office (India) came forward to support the fish-based intervention for control of disease vectors in areas endemic for malaria, dengue and Japanese Encephalitis (JE) to assess operational feasibility. The results were overwhelming. Both malaria and JE were contained to greater extent with the extensive application of larvivorous fish. Subsequently this strategy was implemented in the entire state of Karnataka resulting huge impact in reducing malaria burden. At present, Mysore district reported zero malaria from 2017 onwards and would be eligible for certification of malaria elimination by 2020 at sub-national level [7]. Overwhelmed by these research findings, Ex-Professor Chris Curtis of London School of Tropical Medicine and Hygiene (UK) visited one of the experimental areas in Arsikere Taluka, district Hassan in 2004 to have first hand information on tangible impact of these two fish species for elimination of mosquito breeding in the ponds and wells. Having satisfied by these field-based observations, Dr Curtis became an ambassador promoting fish-based vector control in fight against malaria and other vector-borne diseases [8].

In between, the fish-based malaria control exercise was audited by the technical wing of Comptroller of Auditor General (CAG) of Government of India for its worthiness, modalities, operational costs and feasibility in the country. Based on their specific recommendations and positive outcome, this strategy was extended to many other malaria endemic states including Assam, Goa, Andhra Pradesh and Maharashtra [9]. It was observed that *P. reticulata* are most suited for well ecosystem having sub-column feeding zone and the capacity to survive in polluted water bodies (Figure 1). On the contrary *G. affinis* is surface feeder and considered ideal for large water body ecosystems, viz., ponds [10]. *Gambusia* have also been used successfully in the mixed edible fish culture related to income generating schemes for benefit of communities [11]. For instance, grass carp

fish (*Ctenopharyngodon idella*) can be added in ponds to clear all the aquatic weeds and thus favoring Gambusia to devour on the mosquito larvae making the pond unfit for mosquito breeding [3]. The cost of this operation was assessed to be very low (US$ 0.02 per capita) compared with other interventions such as DDT (US$ 0.5 per capita) making it an affordable well within the resource-poor settings [12].

Eliciting community participation and social engagement

Community participation is vital to success of the disease prevention and control programs for which there are several methods for eliciting community awareness and engagement. One such exercise was to use folk theatre (*Kalajatha*). This method was assessed to be very effective and penetrative carrying forward the messages of bioenvironmental control of malaria that prompted the local communities to participate in the fish release operation making vector control a community-based intervention. The participation of the local political members also made huge impact reposing confidence in the communities at risk of malaria. Thus, political and social engagements are the key-elements for successful implementation of such programme [13]. Based on this success story, a *taluka* (block) level fish release programme has been formulated involving the local departments seeking intersectoral convergence. This model is presently being followed in Karnataka popularly known as `Karnataka Role Model' (Figure 2), and has been tested to be effective where decisions are taken at the block level and implemented in the right earnest presently functioning under the National Health Mission of Government of Karnataka [14]

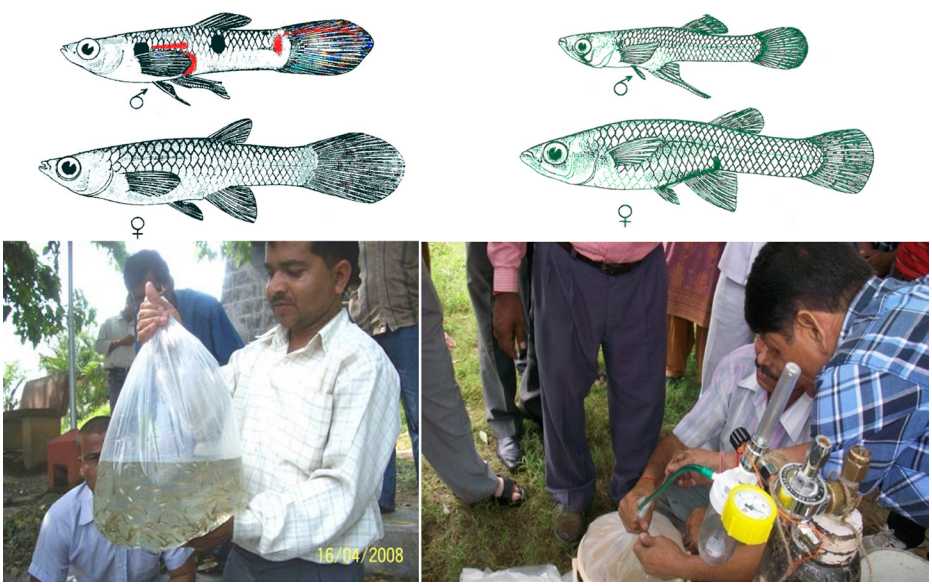

Figure 1: Promoting larvivorous fish for biological control of mosquito breeding: Top left: *Poecilia reticulata* (guppy fish); Top right: *Gambusia affinis* (mosquito fish). Guppy is ideal for polluted water bodies, viz., drains and unused wells; instead Gambusia is good for lakes, streams, ponds and water reservoirs. Both these fish species are viviparous and prolific breeders. Bottom left: Fish collection for mass distribution; Bottom right: Oxygen packaging process for long-distance transportation

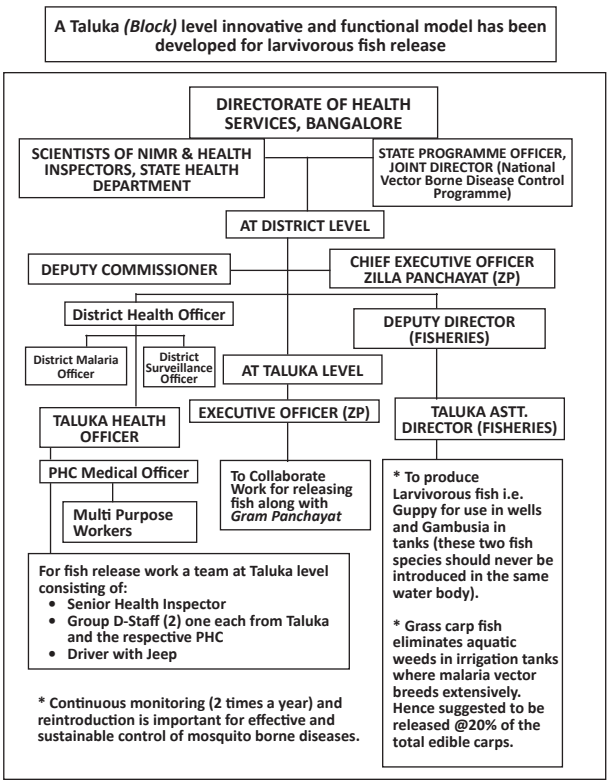

Figure 2: Karnataka role model for promoting larvivorous-fish based intervention for mosquito vector control.

Larvivorous fish fauna diversity and applications

There are about 300 larvivorous fish species in the world. But two of these Poecilid fish species, i.e., guppy fish (*P. reticulata*) and mosquito fish, *Gambusia sp*. are widely used in vector control operations. Guppy fish considered a native of the Caribbean Islands was brought to India in 1908 from England. Similarly, *G. affinis* was brought by Dr BA Rao in 1928 from Italy and released in Lalbagh tank in Bangalore. Gambusia has two subspecies *G. affinis affinis* (western mosquito fish) and *G. affinis holbrooki* (eastern mosquito fish), the former is best suited for hot climate while the later in colder climate regions. It is difficult to identify both the subspecies on morphological parameters. This means that the original stock of *Gambusia* brought to India was mixed populations of both subspecies corroborated by recent report from the Nainital Lake, Uttarakhand [3,15]. Besides these, there are other potential local larvivorous fish species namely *Aphanis sp*. in the Middle East countries, *Nothobranchius sp*. in African countries and *Aplocheilus sp*. in most of the coastal belts [11]. Guppy fish are now routinely used in small water storage tanks in many areas in Karnataka through community action for control of mosquito breeding of dengue mosquito, *Aedes aegypti* and *Ae. albopictus* [16].

Fish-based intervention for vector control was becoming increasingly popular making rounds in the country. To explore their utility to control other mosquito-borne diseases, a special fish-based intervention programme was launched by the Indian Council of Medical Research (New Delhi) for control of vectors of Japanese Encephalitis (JE) in Gorakhpur district of Uttar Pradesh (UP) reporting most case fatalities annually. The main vectors of

JE are member species of the *Culex vishnui* group recorded breeding in wells and rice fields. Release of guppy fish in wells and *Gambusia* in ponds and rice fields made significant impact in containment of the vector populations. Initially this fish was brought to Gorakhpur from mother hatcheries based in Bangalore but did not proliferate to the desired levels for meeting the logistic requirements given the huge water bodies and enormous acreage under paddy cultivation. Subsequently, few fish stocks originating from Nainital Lake were found suitable and recorded breeding profusely in local water bodies, and these were identified to be *Gambusia a. holbrooki* [15,17]. These findings suggest that stocks in northern India are indeed *Gambusia a. holbrooki*, while these are *Gambusia a. affinis* in south India. Similar experiences were observed in Spain in 1920s where all larvivorous fish stocks were *Gambusia a. holbrooki* [18]. Genomic analysis revealed that this fish has the genetic capacity to adapt to the local environments rather quickly [19].

In conclusion

Historically, larvivorous fish has been used in the mosquito control programme worldwide. But its impact has been skeptical for lack of sufficient body of evidence and proper study designs. The meta-analysis also suggested there is no concrete evidence of adverse effect of these fishes on the local environment/ecosystem [20]. Nevertheless, routine monitoring and proper care are some of the important aspects for fish survival and sustained supply which must be incorporated [12]. In Karnataka, fish-based vector control is an integral part of the control programme so much so that in most areas, routine insecticide operations have been withdrawn for there being no fresh cases post-fish release.

The impact of fish-based intervention on mosquito control is a reality and should be promoted in conjunction with other vector control interventions for sustained vector control aiming malaria elimination. In India, this programme should be linked with the pink revolution and the *Swachh Bharat Abhiyan* (clean India movement) for being community-driven and cost-savvy. Time is ripe now to repurpose the fish-based malaria control under the umbrella of `integrated and inclusive vector management' concept in the Gandhian principle of sustainable growth [21,22].

Acronyms	
CAG	Comptroller of Auditor General
DDT	Dichloro-diphenyl-trichloroethane
ICMR	Indian Council of Medical research
JE	Japanese Encephalitis
LSM	Larval Source Management
RBM	Roll Back Malaria
UP	Uttar Pradesh
WHO	World Health Organization

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Chapter 17

Malaria prevention through personal protection: insecticide-treated mosquito nets and allied intervention tools

Surya Kant Sharma*

ICMR – National Institute of Malaria Research, New Delhi – 110 077, India

*Correspondence: suryaksharma@gmail.com

Abstract

Malaria contributes to significant disease burden in the tropical and subtropical regions of the world for which vector control is indispensable to contain its spread. Large scale application of insecticide residual spraying has resulted in widespread resistance in some important disease vectors resulting in continued disease transmission.To overcome the insecticide resistance menace, the advent of insecticide-treated netting materials including long-lasting insecticidal nets (LLINs) has revolutionized the concept of vector control in making judicious use of insecticides and one that is community-based and sustainable for low operational costs. The LLINs are widely accepted and assessed to be operationally feasible in varied malaria endemic settings and advocated as key-intervention tool for universal coverage of population living at any risk of malaria. Included in this chapter is discussion on LLIN technology and allied interventions for personal protection against infective mosquito bites. It is strongly believed that right-mix of technologies as an integral component of integrated disease vector control strategy would result in appreciable transmission reduction making malaria elimination a reality in the foreseeable future.

Keywords: malaria elimination, personal protection methods, insecticide-treated netting materials, long-lasting insecticidal nets, insect repellants, community participation

Introduction

Malaria is a major public health problem contributing to larger proportion of the global disease burden. Over 40% of the World's population in some 100 countries are exposed to varying degrees of malaria risk. In 2017, an estimated 219 million cases of malaria and about 0.5 million deaths occurred worldwide [1]. Most malaria cases were reported in the African countries south of Sahara (92%), South-East Asia (5%) and remaining in the Eastern Mediterranean Region (2%). As many fifteen countries in sub-Saharan Africa including India contributed 80% of the global disease burden. Five countries added up to almost half of all malaria cases worldwide; these included Nigeria (25%), Democratic Republic of the Congo (11%), Mozambique (5%), India (4%) and Uganda (4%). In India, there had been a significant disease transmission reduction and reduced mortality due to malaria in the recent past [2]. During 2000–2015, malaria cases declined from 2.03 to 1.13 million and deaths 932 to 287 registering decline of 44% and 69% in malaria morbidity and mortality respectively. *Plasmodium falciparum* proportions remained, however, static around 50% from 2000 –2013, but had risen significantly from 65.6% in 2014 to 67.1% in 2015 [3]. Despite declining transmission trends, malaria continuing to be a major health problem and amounts to heavy economic burden perpetuating poverty limiting socio-economic development.

The history of malaria control in India dates to the early 1900s when control measures were largely based on antilarval operations executed mainly in major establishments, viz., tea gardens, railways and military cantonment areas. Pyrethrum space spray was introduced during 1930s, and in 1940s several field trials proved the residual efficacy of DDT for malaria vector control. Consequently, DDT was the first-choice insecticide employed for indoor residual spraying during 1950s with demonstrated success [4]. The results achieved by widespread use of DDT in vector control operations were so dramatical that in 1965, less than 0.1 million cases were reported and deaths no more, and malaria eradication seemed to be a reality. However, success achieved during eradication phase was short lived and soon there was resurgence of malaria with record number of >6 million cases in 1970s [5]. The technical reasons for resurgence were attributed largely to development of resistance in malaria vectors to DDT and chloroquine resistance in malaria parasite. Consequently, major epidemics were reported in India (1974-79), Turkey (1976-78) and Brazil (1985-89). Populations of *Anopheles culicifacies*, the primary vector of malaria in the plains of rural India had emerged DDT resistant and contributed 65% of malaria cases reported in the country. Among others, *An. fluviatilis, An. minimus* and *An. dirus* are some of the important vectors responsible for transmission of malaria in foothills valleys and forested/tribal belts [6]. DDT resistance was widespread and replaced with malathion in 1970s, and subsequently synthetic pyrethroids were introduced during 1990s in areas with widespread resistance to both DDT and malathion [7-9].

The renewed attack on disease vectors: multi-pronged approach

Insecticide-treated netting materials: Insecticide resistance was growing menace and had retarded the progress of the disease control efforts. Due to limited arsenal of

insecticides, the only available choice is the vector management by judicious application of chemicals and incorporation of personal protection tools as complementary interventions. This has necessitated the continued need to evaluate newer efficacious alternative intervention strategies that are socially acceptable, sustainable and cost-effective. To overcome insecticide resistance, the World Health Organization called for a renewed attack on malaria through community-based action programme [10]. Towards this objective, insecticide-treated mosquito nets (ITMNs) with synthetic pyrethroids have proven boon to the control program for multiple added advantages [11]. Number of insecticide products (both conventional and long-lasting formulations) that are recommended by the WHO Pesticide Evaluation Scheme (WHOPES) for the treatment of mosquito nets are given in Table 1 [12]. The study outcome evaluating ITMNs was promising in many endemic countries for control of malaria transmitted by different mosquito vector species [13-15]. Similar field trials carried out in states of Odisha and Assam (both combined contributing nearly 50% of total cases in India) demonstrated appreciable transmission reduction resulting in large-scale application of this technology in the national operational control program. Deltamethrin treated nets were found effective against malaria transmitted by *An. minimus* in the north-eastern state of Assam reporting appreciable transmission reduction [16]. Similarly, field trials of nets treated with deltamethrin SC & tablet formulation and lambda-cyhalothrin EC against *An. culicifacies*, and nets treated with cyfluthrin EW against *An. fluviatilis* in mining area of Orissa were also successful in reducing malarial morbidity [17-22]. Even though large-scale application of this technology seemed to offer community-wide protection against malaria but not without operational constraints [23-25]. One of the major operational issue was the necessity to retreat nets periodically at six-monthly intervals for waning residual efficacy being less than optimal. Moreover, repeated washings and erratic dosing of the of field distributed/community-used nets resulted in inconsistent results for reduced bio-efficacy.

The advent of long-lasting insecticidal nets (LLIN) technology ushered a new era of hope for durability, extended residual efficacy and above all overcoming the problem of re-treatment exercises [26]. These nets treated in the manufacturing process with insecticide either incorporated (polyethylene netting) or coated on fiber (polyester netting) are resistant to multiple washes. The biological activity lasts the serviceable life of net itself (3 to 4 years for polyester net, 4-5 years for polyethylene net). A variety of LLINs have been approved and granted either full or interim recommendation by WHOPES for use in public health (Table 2). These LLINs have been extensively evaluated globally in varied transmission settings against different vector species and are proven success for their durability, residual bio-efficacy, wash-resistance and usefulness in reducing malaria-attributable morbidity and mortality [27-39]. Long-lasting Insecticidal nets are assessed to be universally operationally feasible and duly incorporated by the National Vector Borne Diseases Control Programme (NVBDCP) of Government of India [40].

Table 1: Recommended insecticides for the conventional and long-lasting treatment of mosquito nets for malaria vector control by WHO Pesticide Evaluation Scheme (WHOPES). Source Reference [12]

Conventional treatment of nets		
Synthetic Pyrethroids insecticides	**Formulations**	**Dosage (mg/m²)**
Alpha-cypermethrin	Suspended Capsules (10%)	20-40
Cyfluthrin	Emulsion oil in water (5%)	50
Deltamethrin	Suspended capsules (1%); Water dispersible tablet (25%); Water dispersible tablet (25% + binder)[1]	15-25
Etofenprox	Emulsion oil in water (10%)	200
Lambda-cyhalothrin	Capsule suspension concentrate (2.5%)	10-15
Permethrin	Emulsifiable concentrate (10%)	200-500
Long-lasting insecticidal treatment of nets		
Product name	**Product/formulation and dosages**	**Status of WHO recommendation**
ICON® MAXX	Lambda-cyhalothrin (10% CS+ binder) target dose of 50 mg/m2	Interim

[1] K-O TAB 1-2-3®

A meta-regression analysis of over 2000 scholarly articles on LLINs bio-efficacy had shown that there was significant difference in study characteristics and effectiveness, but the overall Odd Ratio (OR) for malaria reduction with LLIN use was 0.44 (95% CI=0.41-0.48, p< 0.01) indicating risk reduction of 56%, whereas conventionally treated nets (ITMN) were slightly less effective with an OR of 0.59 (95% CI=0.57-0.61, p<0.01) [41]. This analysis confirmed that LLINs are significantly more effective than ITMNs preventing malaria. Large scale distribution of LLINs resulted in nearly 80% reduction in cases in eastern state of Odisha making a strong case for roll-out of this intervention across the country (Figure 1) [42]. These findings have practical implications for policy makers in decision making process formulating malaria control strategies.

Figure 1: left: A typical housing structure receptive for malaria transmission in tribal area of Odisha; right: Mass-scale distribution of long-lasting insecticidal nets in high-risk population groups resulted in appreciable transmission reduction across the state

In many countries, indoor residual spraying (IRS) along with long-lasting insecticidal nets is being applied under national malaria control programmes. WHO has recommended four classes of insecticides for IRS and LLINs (Table 3) [43]. The use of LLINs in combination

with non-pyrethroid IRS, though increased the operational costs significantly but it has two distinct advantages. First, the application of a non-pyrethroid on the walls restrict the selection for pyrethroid resistance that may occur as a result of an LLIN in the same habitat and therefore recommended as a tool for insecticide resistance management [44]. Secondly, the combined effect of IRS and LLINs is certainly more effective than either of the interventions alone provided there is an overall increase in population coverage at risk.

Table 2. List of long-lasting insecticidal nets recommended by WHO*

S. N.	Product name	Fabric	Product type	Status of WHO recommen-dation
1.	DawaPlus 2.0	Polyester	Deltamethrin Coated	Interim
2.	DawaPlus 3.0	Polyester (Side panels) Polyethylene (Roof)	Combination of Deltamethrin coated on polyester and Deltamethrin + PBO incorporated into polyethylene	Interim
3.	DawaPlus 4.0	Polyethylene	Deltamethrin +PBO incorporated	Interim
4.	Duranet	Polyethylene	Alpha-cypermethrin incorporated	Full
5.	Interceptor	Polyester	Alpha-cypermethrin coated	Full
6.	Interceptor G2	Polyester	Alpha-cypermethrin and chlorfenapyr coated	Interim
7.	LifeNet	Polypropylene	Deltamethrin incorporated	Interim
8.	Magnet	Polyethylene	Alpha-cypermethrin incorporated	Full
9.	MiraNet	Polyethylene	Alpha-cypermethrin incorporated	Interim
10.	Olyset net	Polyethylene	Permethrin incorporated	Full
11.	Olyset Plus	Polyethylene	Permethrin +PBO incorporated	Interim
12.	Panda Net 2.0	Polyethylene	Deltamethrin incorporated	Interim
13.	PermaNet 2.0	Polyester	Deltamethrin coated	Full
14.	PermaNet 3.0	Polyester (Side panels) Polyethylene (Roof)	Deltamethrin coated on side panels and Deltamethrin + PBO incorporated into roof	Interim
15.	Royal Sentry	Polyethylene	Alpha-cypermethrin incorporated	Full
16.	SafeNet	Polyester	Alpha-cypermethrin coated	Full
17.	Veeralin	Polyethylene	Alpha-cypermethrin +PBO incorporated	Interim
18.	Yahe	Polyester	Deltamethrin coated	Interim
19.	Yorkool	Polyester	Deltamethrin coated	Full

*Source: WHO Pesticide Evaluation Scheme (WHOPES), updated 29 June 2017

Table 3: Different classes of insecticides recommended by WHO for indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs). Source Reference [43]

Insecticide class	ITN/ LLIN	IRS	Molecules available	Residual efficacy in months
Synthetic Pyrethroids	Yes	Yes	6	3-6
Organo-chlorinated hydrocarbons (DDT)	No	Yes	1	6-12
Organo-phosphates	No	Yes	3	2-3
Carbamates*	No	Yes	2	2-6

*Carbamates currently not used in the control programme in India

Noble strategies and approaches are required to address the growing threat of insecticide resistance globally. Long-lasting insecticidal nets incorporating synergist piperonyl-butoxide (PBO) proved to be more effective than nets treated with pyrethroid alone against resistant vector populations. Overall, addition of PBO in long-lasting insecticidal nets resulted in a protective efficacy of 44% post one year, and 33% at the end of the second year versus standard netting [45]. There are more in the offing with new generation LLINs including Interceptor G2 (nets treated with chlorfenapyr + alpha-cypermethrin) and nets incorporating antimalarials inhibiting development of parasite in mosquito host; all of which seem to be promising in defeating insecticide resistance [46-48].

There has been a steady investment over the years in the growth and production of insecticide-treated nets/LLINs for malaria vector control. Between 2015–2017, a total of 624 million insecticide-treated mosquito nets (mostly LLINs) were delivered globally [1]. Majority (89%) of these nets were distributed in sub-Saharan Africa reporting 90% of cases and high mortality due to malaria. This represents a substantial increase from 465 million nets during the previous period 2012–2014. Of these, globally 85% of LLINs were distributed gratis to communities through mass-distribution campaigns, 8% to pregnant women in antenatal care facilities and 4% during immunization programs. Besides insecticide-treated nets, there are several other personal protection tools which are being employed by the communities for protection from vector-borne diseases. A summary of the available vector control tools in relation to mosquito behavior targets are given in Table 4 [49].

Topical repellents: Repellent containing DEET (diethyl toluamide) or Picaridin are recommended for use by persons who are actively engaged in work during the night in the forest/forest-fringe to avoid being bitten by the disease carrying insect pests. In India, a limited community-based trial on DEET-based repellents in conjunction with ITMNs provided significantly protection against malaria [50]. A household randomized trial on DEET-based repellent products also showed protection against *P. falciparum* malaria in a refugee camp in Pakistan [51].

Table 4: Personal protection tools for vector control and their mosquito behaviour targets. Source Reference [49]

Intervention Tool	Mosquito behavior target				Type of protection
	Biting time	Feeding preference	Place of biting	Place of resting	
ITNs/LLINs	Night	Humans	Indoor	Indoor	Personal & Community*
LLINs hammocks and other nets designed to suite outdoor conditions	Evening & Night	Humans Animals	Outdoors	Outdoors	Personal & Community*
Insecticide-treated plastic sheets for temporary shelters	Evening & Night	Humans Animals	Indoors & Outdoors	Outdoors	Personal
Insecticide-treated clothing and topical repellents,	Evening & Night	Humans	Indoors & Outdoors	Indoors & Outdoors	Personal & Community*

*Community protection will be subject to large coverage of the intervention

Air-borne spatial repellents: A variety of products including mosquito coils, vaporizers, herbal products (Citronella oil) and the like are currently marketed more so in urban areas. These devices emit volatile chemicals that repel mosquitoes away from the host reducing human-mosquito contact and are considered advantageous for reducing risk of infective bites by outdoor/early-evening biting mosquitoes or in areas where all other core-interventions are not feasible. Spatial repellents can be of added value alone or in combination with other interventions against insecticide-resistant mosquito populations especially in areas of residual transmission and malaria-free territories, however, these intervention tools presently not included in the control programme for lack of certainty evidence warranting additional investigations.

Insecticidal-treated plastic sheeting (ITPS): The logistic constraints associated with household spraying campaigns has prompted manufacturing of insecticide-treated plastic sheeting and durable wall lining as a long-lasting alternative to indoor residual spraying. The woven polypropylene sheeting is either treated at factory during manufacturing process or these may be manually soaked or sprayed with pyrethroid insecticides. The dosages applied are minimal and quantities required of pyrethroid insecticides, viz., permethrin, cyfluthrin, deltamethrin or lambda-cyhalothrin are miniscule of IRS operations. Capsule suspension (CS) formulations are preferred choice for adhesion to the plastic material. The ITPS is fixed on the walls of the shelter and may also be used to cover entry door(s) and window exits. Mosquitoes that rest on the sheeting are either killed or repelled after brief encounter. The use of treated sheeting has additional advantages of providing privacy and protection from the wind, and the it can be removed and reused elsewhere when the shelter is abandoned. In India, application of ITPS as an interior wall lining has resulted in a reduction of over 70% in malaria incidence [52].

Insecticide-treated durable wall lining (DWL): Durable wall lining is based on LLIN technology where deltamethrin is incorporated into the polymer during yarn manufacturing at factory level. The insecticide migrates to the surface in a controlled and uniform fashion irrespective of surface or wall shape. The walls and roof are covered with DWL that adds aesthetic value to the interior of the rural home thereby encouraging users' compliance. Deltamethrin treated tarpaulin wall linings have been proven efficacious for control of malaria vectors and disease transmission in high-risk areas of Africa and South-East Asia [53, 54].

Long-lasting insecticidal hammock nets (LLIHNs): In many forested areas of the South-East Asia, whereas bed-net alone has not been considered wide enough to disrupt malaria transmission owing to exophilic nature of the main local vector *An. dirus*, IRS is not accepted because of the socio-cultural aspects of the local inhabitants [55]. In such situations, insecticide-treated hammock nets provide added protection in the evening hours when people are yet to retire and in situations where people do not like to use bed-nets, such as ploughing, harvesting, hunting and sleeping in forest. Long-lasting insecticidal hammocks (LLIH) proved very effective in reducing malaria incidence and prevalence in a forested area of central Vietnam [56]. LLIH may prove to be an additional intervention tool in transmission settings such as remote forest settlements more so for forest-goers where conventional control interventions have only modest impact in averting malaria.

Insecticide-treated clothing: Personal clothing may be treated with fast acting pyrethroid such as permethrin to prevent insects from landing/feeding. Permethrin acts both as

repellent as well as residual insecticide which repel or kill mosquito attempting to land or feed. Pyrethroids are widely used in disease vector/pest management programs for their low mammalian toxicity. Treatment of clothing can be done through spraying the permethrin or by dipping in an aqueous emulsion. The recommended application dosage of permethrin for personal cloths is 1.25 g/m^2 (0.125 mg/cm^2). An alternative to permethrin, DEET can also be used @ 20 g/m^2 (2 mg/cm^2). However, permethrin or other synthetic pyrethroids are generally recommended for treating clothing because of their fast action to repel or kill number of biting insects. Pyrethroids are long-lasting, withstand weathering, sunlight and washing, odorless, safe, and cost savvy for infrequent applications [57]. Therefore, insecticide-treated clothing can give effective protection against mosquitoes and other pests such as sandflies, fleas, body lice, biting midges, ticks and mites. Permethrin-treated clothing can retain protective efficacy against target insect vectors for several months despite repeated washings.

Treated bedsheets: Situations/complex emergencies in which communities are forced to sleep outside in cool weather conditions or when mosquito nets are unavailable or impractical may consider using sheets or other fabrics treated with insecticide for protection against insect bites. The treatment is similar to the one used in treating personal clothing. Treated top-sheets, chaddars, blankets and other bedding materials resulted in appreciable transmission reduction in malaria cases (64%) among children in northwest Pakistan, and significantly inhibited blood feeding activity by host-seeking mosquito vectors [58].

Socio-cultural and behavioral aspects in personal protection interventions

The successful implementation of insecticide-treated netting materials for control/prevention of malaria and other vector borne diseases involve socio-cultural and behavioral aspects of the targeted communities in order to achieve maximal compliance and sustainable success of the control program. In areas where such intervention tools are being introduced for the first time, an extensive health education and awareness along with behavior change communication (BCC) campaigns are essential to ensure compliance by the communities. Significant resources and efforts should be made towards community assessments and developing appropriate educative materials for community mobilization [59]. Strong messages which are simple and easy to comprehend taking into consideration the specific needs of target population groups need to be devised and implemented supported through interpersonal communication by community leaders. Public health programmes would be more effective in the long-term by empowering individuals and communities to be more self-reliant in addressing local health issues.

Conclusions

In conclusion, all these interventions singly or in combination can help reduce mosquito vector-human contact and disrupt disease transmission. However, except for IRS, insecticide-treated mosquito nets and long-lasting insecticidal nets (core-interventions), no

other interventions are included in the national control programme for vector control for lack of substantial body of evidence [60]. The basic principle for effective vector control is the universal coverage of population at any risk and ensuring community compliance. It is strongly believed that 'integrated disease vector control approach' encompassing situation-specific interventions will help reduce transmission risk defeating malaria. India is targeting malaria elimination by 2027 for which countdown has begun for achieving coveted goal of malaria-free status amidst host of challenges [61,62]. There is no letup in control efforts and reporting steady decline in disease transmission each passing year, and given the present-day intervention tools, massive infrastructure, strength of skilled health personnel and evidence-based documentation; malaria elimination seems reachable [63].

Acronyms	
BCC	behavior change communication
CS	capsule suspension concentrate
DDT	dichloro-diethyl-trichloroethane
DEET	diethyltoluamide
DWL	insecticide-treated durable wall lining
EC	emulsifiable concentrate
EW	emulsion oil in water
IRS	indoor residual spray
ITMN	Insecticide-treated mosquito net
ITPS	insecticidal-treated plastic sheeting
LLIN	long-lasting insecticidal net
LLIHN	long-lasting insecticidal hammock net
NVBDCP	National Vector Borne Disease Control Programme
PBO	piperonyl-butoxide
WHO	World Health Organization
WHOPES	WHO Pesticide Evaluation Scheme
WT	water dispersible tablet

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Section V

Molecular Taxonomy and Phylogenetics

Chapter 18

Molecular taxonomy of *Anopheles dirus* complex with special reference to phylogenetics of *Anopheles baimaii*, the vector of malaria in north-east India and neighbouring countries

Debojit Kumar Sarma[1], Dibya Ranjan Bhattacharyya[2] and Anil Prakash[1*]

[1] ICMR – National Institute for Research in Environmental Health, Bhopal 462 030, Madhya Pradesh, India

[2] ICMR – Regional Medical Research Centre, Dibrugarh – 786 001, Assam, India

[*] Correspondence: anilprakashin@yahoo.co.in

Abstract

Mosquito species of the *Anopheles dirus* complex are of medical importance for distinct bionomical characteristics related to transmission and spread of drug-resistant malaria. Several member species of the *An. dirus* complex are regarded as primary vectors of human malaria in South-East Asia including north-east India. The Dirus complex comprises of at least seven isomorphic species of mosquitoes with variable vectoral competency. Understanding the taxonomy and phylogenetics of this complex is important for targeting species-specific interventions as member species have different biology, behaviour and disease transmission potential. The taxonomy of the Dirus complex mosquitoes is not univocal as different molecular markers have yielded different phylogenetic relationships. This chapter deals with the molecular taxonomy of member species of *An. dirus* complex, phylogenetic relationships and evolutionary genetics of *An. baimaii*, the dominant malaria vector in north-east India.

Keywords: *Anopheles baimaii*, dirus complex, forest malaria, molecular taxonomy, north-east India, population genetics, phylogenetics

Introduction

Mosquito species of the genus *Anopheles* are of medical importance primarily due to their role in transmission of malaria, a disease of public health significance in tropical countries. The genus *Anopheles* includes 465 formally recognised species and more than 50 are yet to be named under several species' complexes [1]. Approximately, 70 *Anopheles* species have been considered as competent human malaria vectors [2], of which 41 species, encompassing 15 species complexes, are presently regarded as dominant malaria vectors world over [3]. In South-East Asia (SE Asia) alone, 28 *Anopheles* species comprising of 10 species complexes are considered as malaria vectors [4]. Interestingly, most vector species under the genus *Anopheles* have been identified as sibling-species, which are morphologically similar but genetically distinct and reproductively isolated. Therefore, recognition and taxonomy of species complexes is of paramount importance for formulating species-specific interventions for all species within a complex are not equally efficient to transmit malaria helping save operational costs.

Anopheles dirus species complex

Several sibling-species of *An. dirus* complex mosquitoes are regarded as very efficient and important malaria vectors inhabiting the sylvatic environment in the SE Asia region including north-east India (NE India) [5-6]. In India, distribution of Dirus complex mosquitoes ranges from tropical rainforest areas of NE India, Andaman-Nicobar Islands to the Western Ghats in south-western peninsular India [7]. The taxonomy of this mosquito species complex has been resolved and now all member species have Latin names and mapped distribution in SE Asia region [5, 8, 9]. However, recent studies on phylogenetic relationships among member species of the Dirus complex revealed many interesting facts not only on the distribution and phylogenetic position of the species but also the assignment of newer species within this group.

Dirus complex species as malaria vectors in the forest ecosystem of South-East Asia

SE Asia, sharing almost half of the global population at risk of malaria, is facing huge problem of malaria with approximately 1.24 million reported cases annually [10]. Several member species of the Dirus complex and *An. minimus* are considered primary vectors of human malaria in this region. Being a biodiversity hot-spot, approximately 45 percent of the primary forests in the Asia-Pacific region are in SE Asia and much of SE Asia's biodiversity is contained within forests [11]. Member species of the Dirus complex mainly inhabit the forest and forested foothills from India to Taiwan from 30^{O} north parallel to the Malaysian peninsula and thought to transmit malaria in this vast region (Figure 1) [5].

The statement of Rosenberg *et al*., "*the danger from An. dirus s.l. is not only that it is very resistant to control within its habitat but that it is an extraordinarily efficient vector, so long-lived and anthropophilic that only a small population is necessary to maintain high malaria endemicity*", gives a fairly good idea about the vectoral status of member species belonging to the Dirus complex [12]. It is only during the last 50 years that the role of *An. dirus*

complex in transmission of malaria has been elucidated [5]. Due to its biological attributes and close association with the forest eco-system, *An. dirus s.l.* mosquitoes transmit malaria in the whole SE Asia region including NE India and regarded as the primary vector(s) in the region.

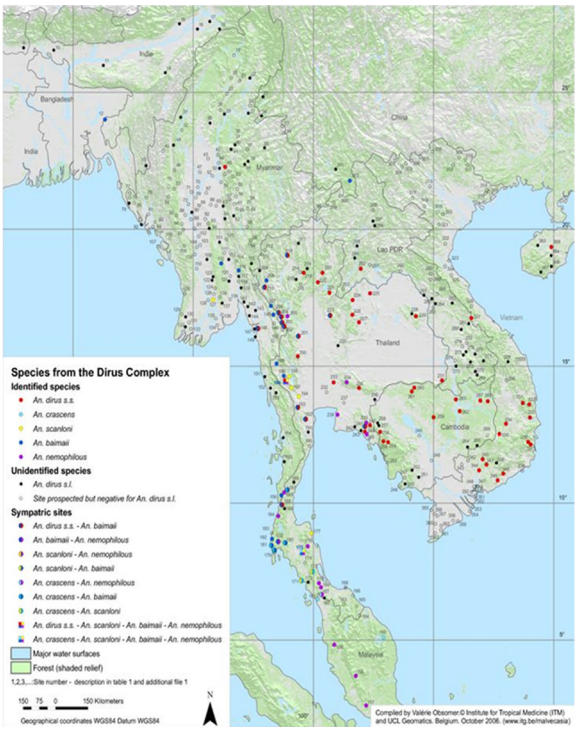

Figure 1: Range of distribution of the *Anopheles dirus* complex mosquitoes in South-East Asia. Source Reference [5]

Both *An. dirus s.s.* (earlier species A of Dirus complex) and *An. baimaii* (earlier species D of Dirus complex) are regarded as malaria vectors in Myanmar, Thailand, China, Indochina and NE India [13-15]. These species were incriminated as vector in Myanmar [16], Thailand [12, 17], Lao PDR [18], Vietnam [19-20], Cambodia [21], Nepal [22], Bhutan [23], Bangladesh [24] and NE India [14, 25-27]. Seasonal sporozoite infection rates of *An. dirus s.l.* varied between locations, the highest being in October (7.8%) in Assam, India [27] and 14% in forested sites of Myanmar [28]. Increased acreage under agriculture and activities such as logging, mining, resettlement of population groups, and military and forest surveillance expose people to high transmission risk worsening the malaria situation in the whole SE Asian region. Rosenberg reported that the influx of the jungle by humans in Bangladesh probably increased the density of *An. dirus s.l.* mosquitoes as it provides hosts for blood feeding and small transitory pools for oviposition [29]. In NE India, the military and para-military forces are being deployed along the strategically important international borders of Bangladesh, Myanmar, China and Bhutan. Most of these border areas are heavily forested and patrolled by defence personnel (often the non-immune population groups) exposing them to infective mosquito bites resulting in high morbidity and attributable mortality [30-31]. It is strongly believed that transmission and spread of chloroquine resistant *P. falciparum* is associated with prevalence of *An. dirus s.l.* [25-26, 32-33].

Taxonomic status of the Dirus complex

The Dirus complex comes under the Leucosphyrus subgroup of Leucosphyrus group of Neomyzomyia series of subgenus *Cellia* of genus *Anopheles* (Diptera: Culicidae) [34] and includes at least 7 isomorphic species [8]. The Leucosphyrus group as such is regarded medically important for as many six species belonging to this group, namely, *An. balabacensis, An. latens, An. leucosphyrus, An. baimaii* (species D of Dirus complex), *An. dirus* (species A of Dirus complex) and *An. sulawesi* are considered very efficient and highly competent vectors of human malaria in the SE Asia [5,8]. Recently, two members of the Leucosphyrus group, *viz., An. latens* (=*leucosphyrus*) and *An. cracens* (species B of Dirus complex) have been incriminated as vectors for transmitting *P. knowlesi* in Malaysia [35-36]. As such the Leucosphyrus group contains 21 species under 3 subgroups, namely, Leucosphyrus subgroup, Hackeri subgroup and Riparis subgroup [8]. Apart from Dirus complex mosquitoes (7 species), the Leucosphyrus subgroup includes the Leucosphyrus complex (4 species), *An. baisasi*, and Con Son form. The Dirus complex consists of seven sibling-species some of which can be identified based on morphological characters of the adult, pupaland larval stages [13]. Based on the cross-mating data, cytogenetics and morphological evidence, *An. takasagoensis* was elevated to species status and separated from both *An. dirus* and *An. balabacensis* [37]. Thereafter, the description of most members of the Dirus Complex was achieved primarily based on cross-mating and cytogenetic studies/polytene chromosome banding patterns [38-43]. The species status of A, B, C and D species was reinforced by population genetic evidence using allozymes [44]. Cytogenetic, allozyme and crossing studies indicated that *An. dirus* A and C are very closely related [38-39, 44]. More recently, molecular techniques such as polymerase chain reaction (PCR) based methods were developed in Thailand using species-specific primers, known as Allele Specific PCR (ASPCR) to differentiate *An. dirus, An. cracens, An. scanloni,* and *An. baimaii* of the Dirus Complex [45-46]. Similarly, a species diagnostic PCR assay was developed based on the fixed differences in the second internal transcribed spacer (ITS2) sequences of the ribosomal DNA (rDNA) to separate populations of *An. dirus* A and *An. dirus* D in China [47]. Walton *et al* [46] observed that ITS2 sequence of Chinese species *An. dirus* D of Xu & Qu [48] is distinct from that of Thailand specimens and thus, it may represent an unrecognized species of the complex.

The Leucosphyrus complex consists of *An. leucosphyrus*, *An. latens, An. introlatus,* and *An. balabacensis*. The Riparis subgroup includes *An. riparis, An. cristatus* and *An. macarthuri.* Recently, Sallum *et al* [49] transferred *An. elegans* to the Dirus complex and renamed the earlier Elegans subgroup as the Hackeri subgroup to reflect the change. Currently, the Hackeri subgroup includes *An. hackeri, An. pujutensis, An. mirans, An. sulawesi,* and *An. recens*. Based on this, the species previously called *An. elegans* from Sri Lanka [50] and Southern India [51] has been renamed as *An. mirans* [49] and is now a part of Hackeri subgroup. Therefore, according to the latest classification and formal naming by Sallum *et al* [8], the Dirus complex includes the following 7 species namely, *An. dirus* Peyton & Harrison (formerly species A), *An. cracens* Sallum & Peyton (formerly species B), *An. scanloni* Sallum & Peyton (formerly species C), *An. baimaii* Sallum & Peyton (formerly species D), *An. elegans* James (formerly species E), *An. nemophilous* Peyton & Ramalingam (formerly species F) and *An. takasagoensis* Morishita (Figure 2).

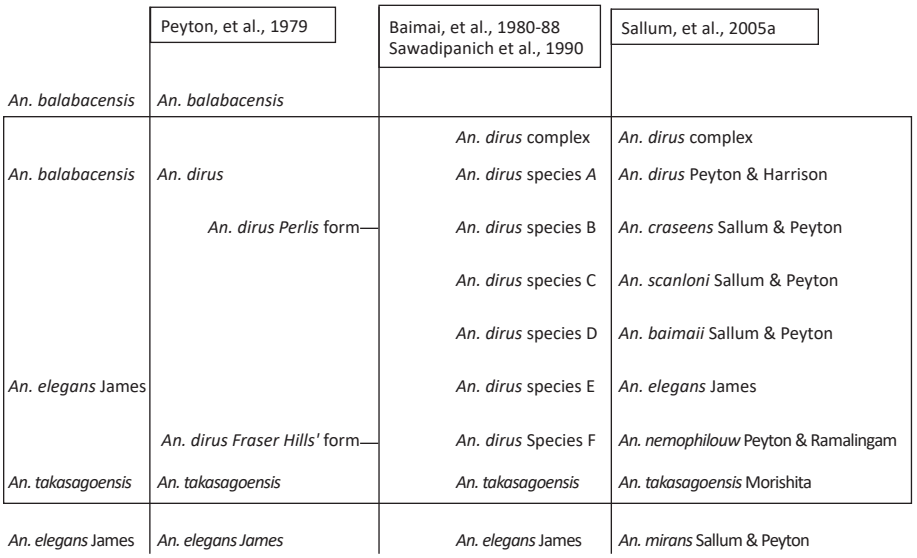

Figure 2: List of sibling-species in the Dirus complex and their taxonomic history. Source Reference [5]

The distribution, bionomical characteristics and vectoral status of the member species of Dirus complex is presented in Table 1.

Molecular taxonomy of the Dirus species complex

For most of the molecular taxonomic and phylogenetic relationship studies in *Anopheles* mosquitoes, both nuclear and mitochondrial markers have been utilized widely. [52]. Of these, markers based on the second Internal Transcribed Spacer (ITS2) region [46, 53-54] and restriction fragment length polymorphism (RFLP) of the ITS2 are sensitive, specific and provide rapid method for molecular confirmation [55-57]. The mitochondrial cytochrome oxidase I (COI) barcode region is another important region used for mosquito systematic studies, however, it lacks resolution as a single marker and needs to be combined with nuclear markers to get higher resolution for systematic and speciation studies in mosquito species groups [52].

Taxonomy based on cytogenetic and cross-mating experiments

The recognition of all member species of the Dirus complex mosquitoes is based on results from cross-mating experiments. The unidirectional F_1 hybrid male sterility, observed between the Bangkok strain (identified as *An. dirus s.s*. by Peyton and Harrison 1979 [13]) and Perlis form strain, was the first evidence that *An. dirus* is a complex consists of two sibling-species [58]. The Bangkok strain was designated as species A and the latter species B [4]. Mitotic karyotype of these two species were described by Baimaii *et al* [59]. Likewise, species C, D, E, F and takasagoensis of the Dirus complex were also identified based on

cytogenetic and fertility data [38-41, 43, 59]. The mitotic karyotypes of all members of *An. dirus* complex and their distribution in SE Asia were illustrated by Baimai [9]. However, this method has several practical disadvantages such as extensive intraspecific variation in the amount and distribution of heterochromatin on the sex-chromosomes requiring good chromosomal preparation and expertise that preclude their large-scale application [60].

Taxonomy based on isoenzyme or allozyme analysis

Isoenzyme analysis is an assay in which the protein molecules are subjected to an electrical field in gel matrix to study heterogeneity between populations for significant variation, if any [61]. The method was described to distinguish member species of the *An. gambiae* complex and a biochemical key was made available for identification [62, 63]. The advantage of the isoenzyme electrophoresis technique over cytogenetic analysis is that it does not require a specific sex or larval stage, however, the specimens need to be fresh or stored in liquid Nitrogen. Although isozymes have been used to differentiate four members (A, B, C, D) of the Dirus complex with some success [44]; use of allozyme analyses has now largely been superseded by DNA-based methods for identification.

Table 1. Bionomical characteristics and disease transmission relationships of sibling-species of the *Anopheles dirus* complex

Anopheles Species	Distri-bution range	Bionomical characteristics					Disease transmission relationships
		Larval habitat	Resting habitat	Feeding habit	Peak biting activity (hrs)	Suscep-tibility status to insecti-cides	
An. dirus	Cambodia, China, Laos, Myanmar, Thailand, Vietnam	Natural containers, potholes, slow running stream, drying streambeds, water pits, rock pools, mud pools etc.,	Outdoor	Highly anthro-pophilic, both indoor and outdoor biter	20:00-23:00	Susceptible	Highly efficient human malaria vector (*P. falciparum* and *P. vivax*)
An. cracens	Southern Thailand, peninsular Malaysia, Perlis, Indonesia and Sumatra	Footprints of elephant and other animals; fresh, stagnant, temporary, clear or turbid water exposed to sun or under partial shade in secondary rain forest situated inboth plains and mountainous areas	Outdoor	Anthropo-philic	19:00-21:00	Susceptible	Vector of simian malaria (*P. cynomolgi*) and *zoonotic malaria* (*P. knowlesi*)
An. scanloni	Myanmar, Thailand	Rock pools, ground pools, seepage springs, vegetated limestone rock pools	Outdoor	Anthropo-philic	18:00-20:00	Susceptible	Vector of human malaria (*P. falciparum* & *P. vivax*) in some areas of Thailand
An. baimaii	Bangla-desh, China, NE India, Myanmar, Thailand	Natural containers, potholes, wells (Mudon, Myanmar), Rock pools, water pits, animal footprints, rock bed ravine, mud pools etc.	Outdoor	Highly anthropo-philic, both indoor and outdoor biter	21:00-03:00	Susceptible	Highly efficient human malaria vector (*P. falciparum* and *P. vivax*)

An. elegans	India (Western Ghats), Sri Lanka	Natural containers, elephant footprints, potholes, muddy pools, spring pools, rock pools, other pools, tree holes etc.	Outdoor	Anthropo-philic	Not known	Not known	Vector of simian malaria (*P. cynomolgi, P. fragile, P. inui*)
An. nemop-hilous	Thai-Malaysia border, peninsular Malaysia, south-eastern, southern and western *Thailand*	Shallow, temporary pools, such as clay soil or rock pools, in stream beds, elephant footprint etc.	Outdoor	Highly zoophilic	18:30-22:00	Not known	Refractory to human malaria
An. takasag-oensis	Foothills and mountainous regions of Central and Southern Taiwan	Potholes, rock pools, drying stream, rock bed ravine etc.,	Outdoor	Highly zoophilic	18:30-22:00	Not known	Refractory to human malaria

Taxonomy using DNA based techniques

Since DNA based techniques are more specific, accurate and generally applicable to any specific developmental stage or to any sex and have less stringent requirements for preservation of materials typically by desiccation or in ethanol; they have replaced other sibling species identification methods. For DNA-based species identification, polymerase chain reaction (PCR) based diagnostic markers, *viz.,* microsatellites, randomly amplified polymorphic DNA (RAPD), restricted fragment length polymorphism (RFLP) and ITS2 of ribosomal DNA (r-DNA ITS2) are presently widely used.

Sequence characterized amplified region (SCAR) markers

Unlike microsatellites, RAPDs have the advantage that they can be applied to any organisms where there is lack of prior molecular information about the genome [64]. For example, RAPD markers have been used successfully to identify species in the *Anopheles albitarsis* complex [65], and like wise to distinguish *An. gambiae* from *An. arabiensis* [66]. This technique was further modified to develop species-specific SCAR markers to identify four sibling-species (A, B, C and D) of *An. dirus* complex [67]. However, low reproducibility of the technique, dominant nature of RAPD alleles and the difficulties in assigning homology to amplified SCAR marker fragments complicate the interpretation of results [68].

rDNA-ITS2 based taxonomy

The species-specific nucleotide differences are exploited successfully in the development of diagnostic PCR assays. These assays are able to distinguish two species differ by as little as only one nucleotide pair by utilizing a specific DNA sequence. Nuclear sequences like the ribosomal DNA (rDNA) are preferred to organelle genomes like mitochondrial DNA (mtDNA) because the latter have the propensity to be transferred across species boundaries by rare interspecific hybridization events or at stable interspecific hybridization zones. Collins and Paskewitz have advocated the use of rDNA to differentiate cryptic species of *Anopheles* [53]. The rDNA is a multicopy gene family that exists as tandem arrays of many

transcriptional units per cell [69], where mutations were spread rapidly and homogenously to all members of the gene family by concerted evolution, even if arrays are located on different chromosomes [70]. Due to this, it is relatively easy to design and use primers that anneal to conserved parts of the ribosomal RNA genes to amplify an intervening variable region [71]. This method is useful in identifying species within the complex regardless of life stage and sex using either extracted DNA or fragments of a specimen. This method, known as allele specific PCR (ASPCR), uses a universal forward primer that anneals to the DNA of all species and a series of species-specific reverse primers [72].

In NE India, *An. baimaii* of the Dirus complex was found almost exclusively prevalent throughout the region except for a focal presence of another unnamed species *An. dirus*`X' in Jatinga Hills (Dima Hasao, Assam) [73]. The rDNA-ITS2 regions of member species of Dirus complex mosquitoes were found varying from 479-786 bp [15, 46]. Interestingly, the unnamed species *An. dirus* X from Assam state (specimen no. DA70) was found genetically close to that of *An. dirus* found in China [48], which was sub sequently named as *An. dirus* species X by Walton *et al* [46]. The pair wise genetic distances between ITS2 sequence of this individual (DA70) with that of *An. dirus* (dirus A), *An. cracens* (dirus B), *An. scanloni* (dirus C), *An. baimaii* (of Thailand and India) (dirus D) and *An. nemophilus* (dirus F) were 0.013, 0.046, 0.015, 0.109 and 0.033 respectively (Table 2) showing a clear distant genetic relationship of DA70 with these species and was observed to be closest to *An. baimaii* of China. On the contrary, ITS2 sequences of the Dirus complex (previously thought as *An. baimaii*) collected in Yunnan province [48] were found substantially distinct than that of *An. baimaii* from Thailand and NE India, possibly representing a different species, which was termed as `species X' by Walton *et al* [46].

Table 2. Pairwise genetic distances of ITS2 region among sibling-species of the Dirus complex mosquitoes

	dirus A	dirus B	dirus C	dirus D	dirus F	dirus Sp. X	dirus DA70
dirus A	0						
dirus B	0.046	0					
dirus C	0.002	0.044	0				
dirus D	0.093	0.113	0.093	0			
dirus F	0.033	0.040	0.033	0.108	0		
dirus Sp. X	0.013	0.046	0.015	0.109	0.033	0	
dirus DA70	0.013	0.046	0.015	0.109	0.033	0.000	0

It was also observed that *An. baimaii* maintains a distant relationship with all other members of the dirus complex mosquitoes, both in India as well as in SE Asia. Similarly, species X of *An. dirus s.l.* was found to be closely related to *An. dirus* (Dirus A). Looking into the distant and basal position of *An. baimaii* in the phylogenetic tree (Figure 3), it can be easily inferred that *An. baimaii* may be the oldest surviving species of the Dirus complex from where all other species diverged.

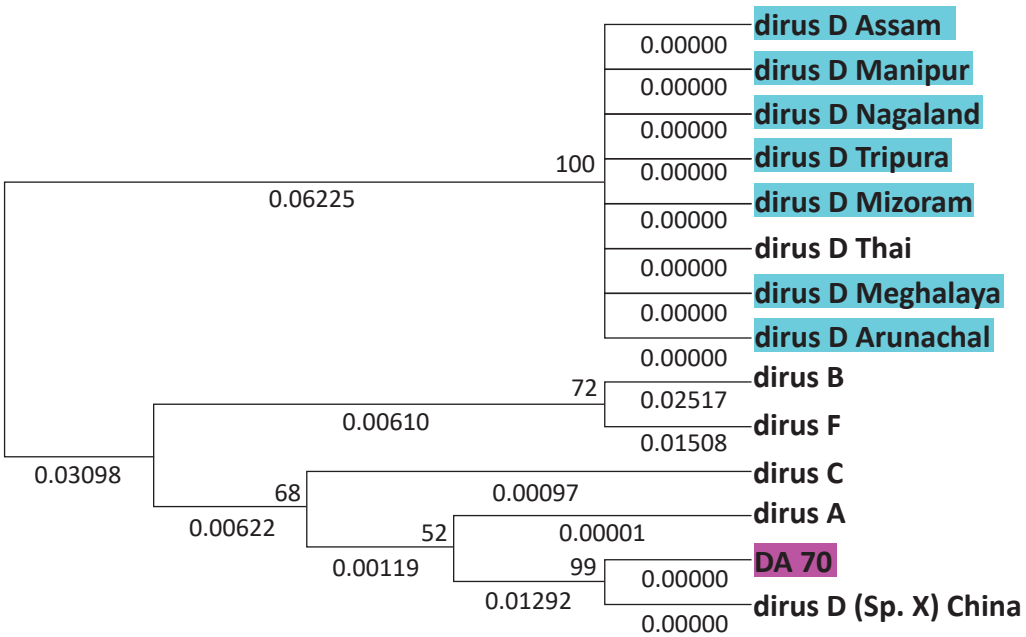

Figure 3: Phylogenetic relationship among the sibling species of Dirus complex mosquitoes based on ITS2 sequences using Neighbour-Joining (NJ) method. Only Boot strap values ≥50% are shown. Note the closer systematic position of DA70 specimen of *Anopheles baimaii* (species X) from Assam (highlighted in red) with *Anopheles dirus* D of China in comparison to *Anopheles baimaii* from various NE states (highlighted in green). Source Reference [73].

Taxonomy based on mtDNA sequences

The taxonomy of *An. dirus s.l.* has been explored by different molecular markers based on mtDNA sequences such as COI, COII, ND5 etc. Using mtDNA COI and ND6 region, Sallum *et al* [74] elucidated the systematic position of members of the *An. dirus* complex mosquitoes within the *An. leucosphyrus* group. It was observed that the Dirus complex mosquitoes formed a monophyletic group and both *An. dirus* and *An. baimaii* are placed together indicating their close relationship [74]. Similar observations were also made by Takeno *et al* [75].

Complete or partial mitochondrial genome of many *Anopheles* species are available in the National Centre for Biotechnology Information (NCBI) and have recently been used for taxonomic studies. The phylogenetic tree constructed using mitochondrial genome indicate a close relationship between *An. epiroticus* and *An. gambiae* complex (BPP: 0.71); *An. dirus* complex and *An. punctulatus* group (BPP: 1.00). Both *An. dirus* and *An. cracens* were found to cluster in a single clade [76-77]. Recently, Sarma *et al* (unpublished observations) sequenced the whole mitogenome of *An. baimaii* from NE India and found that *An. baimaii* is closely related to *An. dirus* than to *An. cracens* (Figure 4).

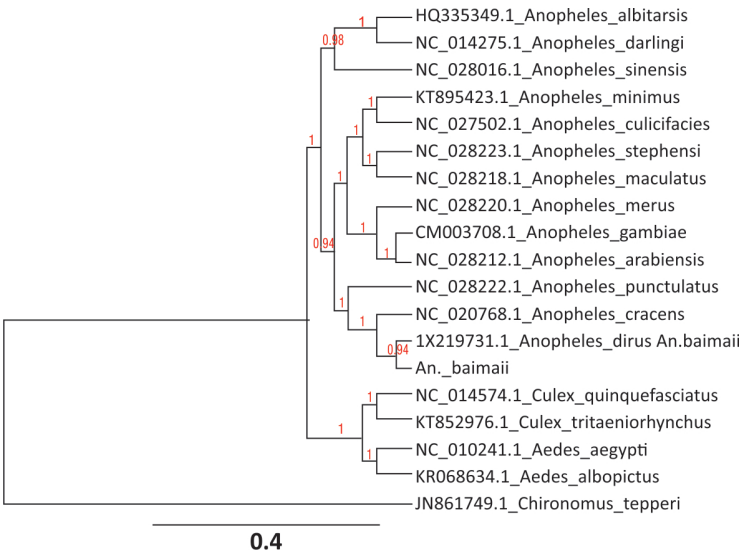

Figure 4: Evolutionary relationships among anopheline species based on variation in whole mitochondrial genome using Bayesian phylogenetic analysis. *Chironomus tepperi* was used as an out group for the analysis. Values (in red colour) above nodes represent Bayasian posterior probability (BPP).

Taxonomy based on rDNA secondary structure

Both rDNA- ITS2 and mtDNA based taxonomy differ in the placement of *An. baimaii* within the Dirus complex, but method based on rDNA-ITS2 secondary structure helped resolve this issue. Addition of rRNA secondary structures was found to improve accuracy of taxonomy of many organisms [78-79]. Using rRNA secondary structure-based phylogeny in Dirus complex, it was observed that *An. baimaii* still holds the basal position of the tree (Figure 5), and it seems that all other member species have evolved from this species (unpublished observations).

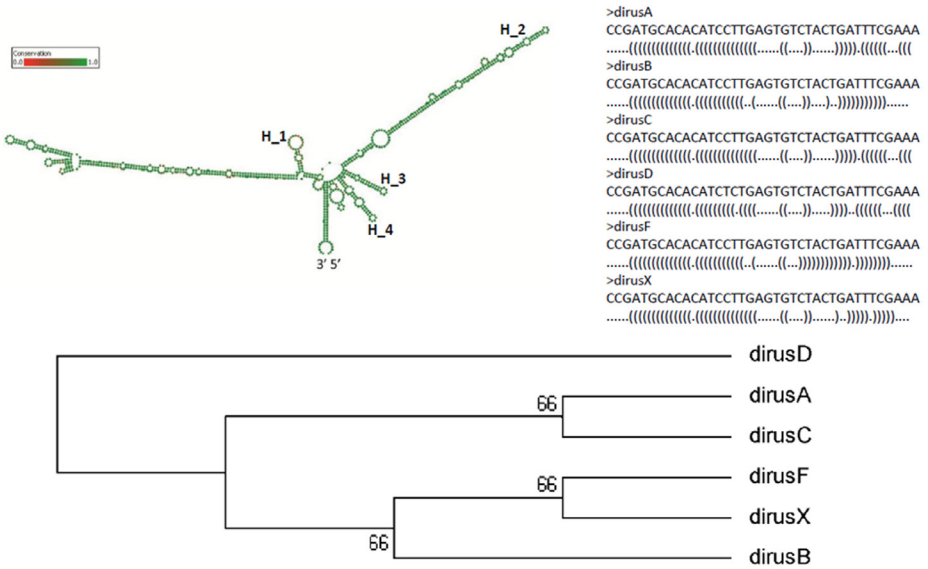

Figure 5: ITS2 rDNA sequence and secondary structure-based phylogeny of Dirus complex mosquitoes. Numerical values in the phylogeny tree indicate boot-strap support values

Choice of markers to study phylogenetic relationships

In population genetics and phylogeographic studies, the relationship between demographic features (such as migration, population size, natural selection, historical events etc.) and the distribution of molecular genetic variants of a species or group of species is investigated from which inferences on biology of the organism can be derived [80]. Thus, by exploring genetic markers with proper rates of molecular change, and therefore suitable signals, information on evolutionary process through the hierarchy of life can be obtained for almost any population. Based on the levels of molecular change which would provide information at different levels of population biology, the genetic markers used for population genetics and phylogeographic study can broadly be divided in to genotypic, genic and gene genealogies. The genotypic markers, such as multiple microsatellite markers, are most commonly used in samples of individuals. In sexual species, these genotypic arrays are reshuffled at each generation, and, therefore, useful for the finest-scale resolution of population processes such as individual identification and tracking, parentage and relatedness of interacting individuals in populations. The genic markers, such as mtDNA and other single-locus markers can be assessed as individual genes and analysed with frequencies and geographical distributions under the effects of drift, selection, gene flow and founder effect. As these properties' changes on larger spatial and temporal scales than genotypic arrays therefore these are regarded as effective markers to study gene flow and population history. In gene genealogies, analysis of mutations in sequences of mtDNA or nuclear markers (single copy nuclear markers) and their evolutionary relations determined by mutation rate, selection and population parameters such as effective population size (*Ne*) and changes in (*Ne*) are derived. These mutations and evolutionary relationships are highly instructive about the long-term evolutionary processes such as phylogeography, speciation and deeper taxonomic phylogenetic reconstruction within as well as among species [81]. The choice of a right marker to address a specific question of evolutionary biology is always important and critical since different markers may give different molecular signals owing to different mutation rates, recombination rates, mode of inheritance and genomic locations, which must be considered for adequate resolution of different evolutionary processes [82-83]. Choice of a proper marker is also central to reduce errors and ambiguities in estimates of population structure and gene flow [84]. Frequently used molecular markers in the field of population genetics and phylogeographic studies of *Anopheles* are reviewed below.

Mitochondrial DNA based markers

mtDNA has been used broadly as a tool for deducing the evolutionary and demographic past of an organism both at population level and species level due to its uniparental (maternal) mode of inheritance, non-recombining nature, high rate of mutation, high copy numbers, nearly neutral fashion of evolution, clock-like evolution and the accessibility of conserved primers and PCR protocols [85-87]. After publication of mitochondrial genome of *An. gambiae* in 1993 [88], both coding NADH dehydrogenase subunit 5 (ND5), cytochrome oxidase subunits I and II (COI and COII) and non-coding (16S and 12S RNAs) regions have been frequently targeted for *Anopheles* phylogenetics [89-90]. However, the utility of mtDNA as a marker in phylogenetic studies has been debated due to the inherent

genetic and biological properties, viz., clonality (maternal inheritance), neutrality, clock-like evolution, introgression and selective sweep of mitochondrial genome [91-93]. Apart from such biological limitations, technical limitations such as illegitimate amplification of *Numts* (nuclear sequence of mitochondrial origin) or mitochondrial pseudogenes in some species can seriously confound phylogenetic studies [94-95]. Despite such problems, mtDNA represents one of the most robust and authenticate tools for detecting population structure and inferring population history [93] and is the most convenient and affordable solution when a new species needs to be genetically explored in the wild [96].

Nuclear DNA based markers

In phylogenetic studies mtDNA is the likely marker of choice to estimate the pattern of population history due to its rapid coalescence time. In contrast, multiple independent loci are needed for robust estimates of demographic variables, and to reduce the process error associated with coalescence [93]. In fact, mtDNA estimates of population size (*Ne*), gene flow (*mNe*), population growth (e.g., mismatch distributions), and divergence times are associated with large confidence intervals [97]. The use of multiple nuclear genes provides a solution to that problem by reducing the coalescent process error.

Various classes of nuclear sequence-based markers are available and being developed for both intraspecific and interspecific phylogeographic study [95,98]. The development of conserved exon - primed - intron - crossing (EPIC) PCR primers to facilitate amplification of low- or single-copy nuclear loci has promoted population genetics studies in past decades [95,99]. Although it is easier to develop conserved PCR primers for mtDNA, the case is very complicated for nuclear DNA except for a limited success achieved with primers amplifying ribosomal ITS region. However, few nuclear markers based on coding regions, introns and SNP were successfully applied to detect population genetic and phylogeographic structure in several *Anopheles* species [100-104]. For Dirus complex mosquitoes, Morgan et al [105] developed 3 nuclear coding markers, *viz*., est6, ninaE and hsp82 to assess the gene flow pattern among three species of the complex.

The primers to amplify nuclear sequence-based markers for intraspecific studies should have the following characteristics: (i) should be evolutionarily well conserved throughout different taxonomic groups, (ii) PCR products should be of reasonable size, (iii) target sequences should be highly variable at the intraspecific level, and (iv) target sequences should be single-copy or low-copy in the nuclear genome.

However, several processes of nuclear DNA evolution hinder the search for such nuclear primers. These include gene duplication or amplification, production of pseudogenes, intron lost, sliding or size change etc. [95]. Nuclear DNA has a lower mutation rate than mtDNA, which minimizes the back and parallel mutations that can reduce phylogenetic resolution in mtDNA data. Hence, in data with low homoplasy like nuclear DNA, even a single fixed difference can provide a statistically significant result at the intraspecific level, regardless of bootstrap support [106]. Although nuclear DNA sequence-based markers have advantages in estimating population demographic processes, issues like recombination, selection (non-neutrality), heterozygosity, insertion/deletion polymorphism, low divergence and polytomy, gene-specific variation in rate and history, PCR and sequencing difficulty are always associated with it and need to be critically assessed to get reliable estimates on

phylogeographic of a species [95]. However, there are several statistical methods (such as four-gamete test) which estimates the minimum number of recombination events for a given nuclear DNA sequence data [107]. Another technical challenge in determination of proper allelic phase can also be handled by both probabilistic and computational (such as using PHASE program) and empirical methods (such as cloning) [108].

Phylogenetic and evolutionary relationships of *Anopheles baimaii* populations

Relationships based on mtDNA sequences

Genetic relationships among populations of Dirus complex mosquitoes from NE India as well as from neighbouring countries of SE Asia have been studied. The first study of population structure of Dirus complex mosquitoes was carried out using enzyme electromorphs [44]. This study based in Thailand suggested complete assortative mating between *An. dirus* species A, B, C, and D, therefore, supported the integrity of each member species. A subsequent study on *An. dirus* A, C and D was carried out using COI comprising populations from 14 sites in Thailand, Myanmar and Bangladesh [109]. Within species FST values showed that the two populations of *An. dirus* C were genetically quite distinct whereas population restructuring was not observed among populations of *An. dirus* A or D. Using hierarchical AMOVA pairwise comparisons, *An. dirus* C was found to be distinct from both *An. dirus* A and *An. dirus* D. Mantel test results suggested no isolation by distance among populations of *An. dirus* A, *An. dirus* D or *An. dirus* A and D combined. A starburst shaped minimum spanning haplotype network, smooth unimodal mismatch distribution curve and negative values of neutrality tests for *An. dirus* A and D suggested a recent population expansion, either demographic or by a selective sweep. A greater genetic diversity was observed for *An. dirus* species D than species A which suggests that population expansion first occurred in species D and subsequently in species A.

Overall, these results were surprising as it was thought previously that *An. dirus* species A and C were very closely related. The genetic similarity between *An. dirus* species A and D was not expected. The most likely explanations were that historical introgression occurred before population expansion when both populations had limited distribution, or that a selective sweep of mtDNA originating from *An. dirus* D passed into *An. dirus* A. Ongoing gene flow between the two species is unlikely due to limited range overlap and unfit hybrids, nevertheless ongoing geneflow could not be ruled out given the data available. In this study homoplasy caused by hyper-variable sites caused some problems in the data analysis.

Later, O'Loughlin *et al* [110] carried out an in-depth population genetics and phylogeographic study on *An. dirus* (species A) and *An. baimaii* (species D) based on mtDNA COI and COII sequences from 21 different populations of mainland SE Asia and reported a more complex population history of the two species. This study included one population of *An. baimaii* from Assam and found that this population bears the highest genetic diversity and it expanded ~ 0.3 MYBP (million years before present) with a stable and oldest population history indicating that this region may possibly be a Pleistocene forest refugium. This study also reported presence of genetic restructuring in *An. baimaii* and in

one population of *An. dirus*. A significant Mantel test in *An. baimaii* populations indicated an isolation-by-distance model of population structure suggesting restricted gene flow, however, estimates of gene flow was not possible due to the signal of expansion. It was also concluded that the population expansion in the two species was not due to human expansion, but rather fits to the Pleistocene climate change.

Looking into the complex topography of NE India with large river basins and several discontinuous hill ranges, it can be hypothesized that there would be a significant genetic structuring within the *An. baimaii* populations in this region. Sarmaet al [111] assessed the relationship among the NE Indian and SE Asian *An. baimaii* populations based on a 750bp fragment of the mitochondrial COII gene. Overall, a high genetic diversity in populations of *An. baimaii* from NE India was observed than populations prevalent in Bangladesh, Myanmar and Thailand (Figure 6). As a matter of fact, the genetic diversity of *An. baimaii* in these SE Asian countries represented a subset of the genetic diversity of *An. baimaii* observed in NE India (Figure 7). A very little genetic differentiation among the populations of *An. baimaii* in NE India was also observed indicating panmixia. However, on a regional scale, the NE Indian populations of *An. baimaii* were found highly differentiated from those of SE Asia (Table 3).

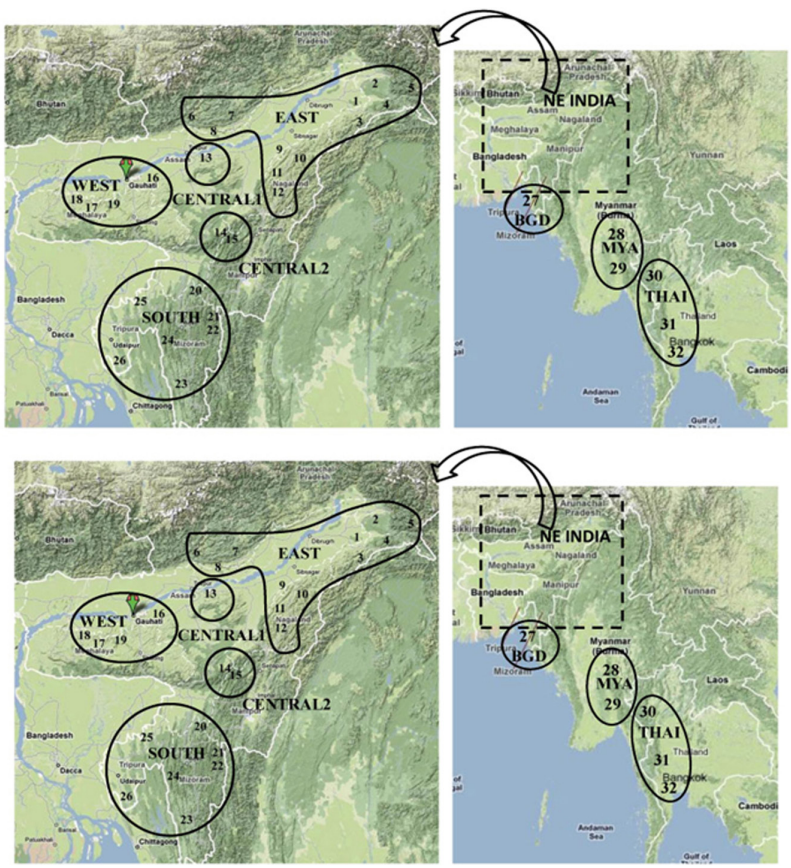

Figure 6: *Anopheles baimaii* collection sites in NE India [the eight population groupings (collection sites 1-32) used for *Anopheles baimaii* are shown in circles]. Source Reference [111]

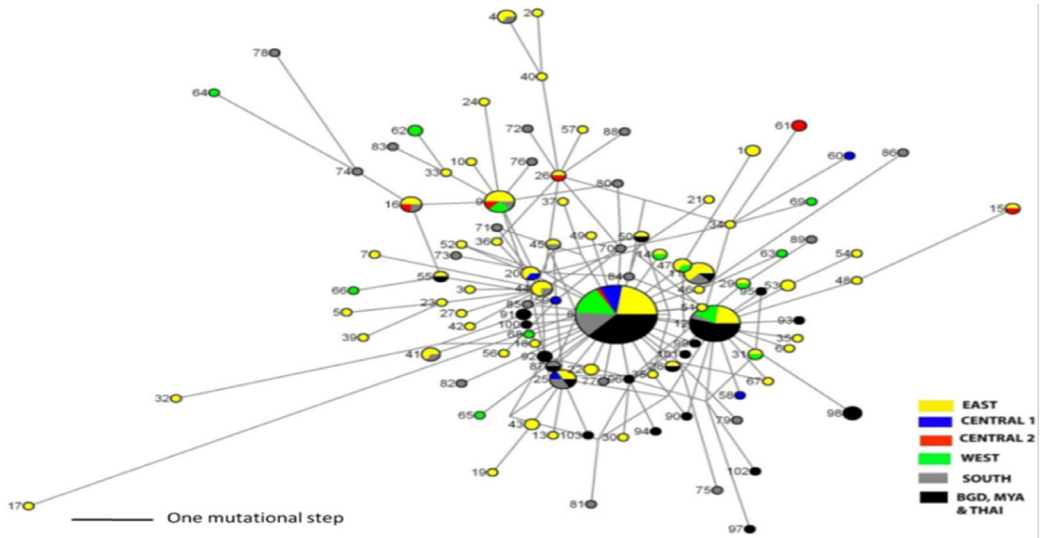

Figure 7: Haplotype network of *Anopheles baimaii* populations (Haplotype network was constructed using Median- Joining algorithm of 103 COII haplotypes of *An. baimaii*). Source Reference [111].

Table 3. Genetic differentiation (FST) among *Anopheles baimaii* populations of NE India and South-East Asia

Population	EAST	CENTRAL1	CENTRAL 2	WEST	SOUTH	BGD	MYA
CENTRAL 1	-0.009						
CENTRAL 2	0.071*	0.175**					
WEST	-0.004	0.013	0.074				
SOUTH	-0.007	-0.010	0.065*	-0.002			
BGD	0.032	0.146**	0.180*	0.026	0.060		
MYA	0.024	0.063*	0.267***	0.031	0.050**	0.014	
THAI	0.055***	0.043	0.351***	0.069***	0.078***	0.071	0.002

Level of significance is indicated by: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$(Central 1, Central 2, West and South are populations from NE India; MYA = Myanmar; THAI= Thailand; BGD = Bangladesh). Source Reference [111].

The mtDNA based phylogenetic studies on *An. dirus* complex mosquitoes established that the genetic history of *An. baimaii* is relatively old and stable compared to the other member species of the complex. The closer relation of *An. baimaii* to *An. dirus* may be due to introgression from *baimaii* to *dirus* as explained by Walton et al [109]. The little genetic differentiation observed among the NE Indian *An. baimaii* populations may be due to the anthropogenic activities related to deforestation and settlement in forest areas, and the Arakan hill ranges may be acting as a barrier to gene flow among NE Indian and SE Asian *An. baimaii* populations (Figure 8) [111].

Figure 8: The North-Western mountain ranges (Arakan range) dividing NE India (higher genetic diversity area) and rest of SE Asia (lower genetic diversity area) Source Reference [111].

Relationships based on nuclear sequences

Nuclear sequence markers such as microsatellite and single copy nuclear DNA have also been used to study the relationships of the Dirus complex mosquitoes. While evidence of population restructuring was found within Dirus species D, two populations of species C showed high differentiation close to the inter-species level suggesting possibility of existence of two different species.The differentiation between Dirus species A and D supported the theory of historical introgression of mtDNA between the two species [112].

Recently, an in-depth phylogeographical study of populations of *An. dirus*, *An. baimaii* and *An. scanloni* from the mainland SE Asia using mitochondrial, microsatellite and nuclear sequence markers revealed that *An. baimaii* had a more confined westerly distribution until it spread eastwards making secondary contact ~62 kilo year ago (kya) with a closely related species *An. dirus* on the Thai-Myanmar border [105]. This resulted in mtDNA introgression from *An. baimaii* to *An. dirus* accompanied by a selective sweep of mtDNA supporting the views of Walton et al[109] and O'loughlinet al [110]. Using highly analytical approaches like coalescent based Isolation with migration analysis (IMa) program [113], a study based on nuclear sequence data revealed a recent divergence [within last 1.5 million year (MY)] for all three species, e.g., 192-877 kya for the divergence of *An. scanloni* from *An. baimaii*; 209 - 932 kya for *An. scanloni* from *An. dirus*, and 163 kya - 1.53 MY for *An. baimaii* from *An. dirus* [114]. This study also included one population of *An. baimaii* from Assam and found that it has substantially higher genetic diversity and significantly differentiated from other *An. baimaii* populations. The divergence of this population of *An.*

baimaii from Assam from that of northern Thailand populations was estimated 117-535 kya during the late Pleistocene period. These findings further supported the observation of O'Loughlinet al [110] proposing NE India as a potential Pleistocene refugial region and strongly pointed out that NE India is the source of origin of all extant populations of *An. baimaii* in SE Asia. Even though bidirectional gene flow was found between *An. baimaii* and *An. dirus* due to secondary contact, speciation between these two species was attributed to prolonged allopatric isolation in separate forest refugium without secondary contact. The recognition of NE India and Indo-Myanmar biodiversity hotspot area as a putative forest refugial region was also postulated based on a phylogenetic study of the Neocellia series of *Anopheles* mosquitoes [114] and a comparative phylogeographic study [115].

Recently, Sarmaet *et al* (unpublished observations) studied the genetic relationship of NE Indian populations of *An. baimaii* collected from 8 diverse locations throughout NE India using 6 nuclear markers and observed that the NE Indian populations have a higher genetic diversity similar to conclusions based on other such investigations [110,115]. The higher genetic diversity, genetic distinctiveness, relatively stable and older demographic histories of *An. baimaii* in NE India in comparison to SE Asia suggests that possibly NE India is the origin of all extant populations of *An. baimaii*, and this region might have acted as a glacial refugial region during the Pleistocene climate change. It was also observed that although the NE Indian *An. baimaii* populations have diverged from that of northern Thailand populations approximately 117-535 kya [115], yet within NE India, a very recent time of population divergence was noticed in *An. baimaii* populations. Using coalescence based IMa2 program, it was estimated that the NE Indian *An. baimaii* populations diverged from their ancestor during last 3200 years only (95% CI 1600-5223 yrs). The present scenario of population divergence fits into our understanding of forest fragmentation and anthropogenic factors in NE India. The NE India has experienced more dry and seasonal climates during the last glacial maximum (~ 18000 years before present), which resulted in increased montane vegetation, grasslands and Savannah and decline of rain forests [116].

Until recently, the NE Indian forests were contiguous with lowland semi-evergreen forests throughout the Brahmaputra valley merging into sub-Himalayan light alluvial semi-evergreen and secondary wet-mixed forests in the hilly regions [117]. However, due to human population expansion, agricultural activities and economic growth, large scale fragmentation of these forests occurred only relatively recently, within the last 150-200 years, [118-119]. Therefore, it can perhaps be considered that the general signal of genetic homogeneity reflects the historical connectivity of *An. baimaii* populations throughout NE India and that the slight signals of genetic differentiation resulting isolation of some populations contemplates recent human mediated forest fragmentation. Human activities like agriculture and pottery were recorded ~3500-4500 years ago in this region [120-121]. The onset of Savannah forest type due to the gradual destruction of tropical rain forests by anthropogenic effects such as agriculture (shifting cultivation), urbanization [122] was evident in NE India ~ 6300-3000 years ago based on pollen records, and this region faced an extensive human activity in the last ~ 540 years before present [123]. It was also viewed that heavy rainfall and dense vegetation in the Western Ghats and NE India probably inhibited early man from colonizing these regions [124], and the fact is further supported by a more recent human colonization, ~ 4000 years before present in NE India [125]. These evidences are in support of a recent time divergence among populations of *An. baimaii* in NE India which is mostly due to the anthropogenic activities.

Implications of phylogenetic study of *Anopheles baimaii* for malaria vector control

The success of vector control depends on the detailed information on gene flow, effective population size, exposure to insecticide and status of insecticide resistance, ecology, bionomics and vectoral capacity of the concerned species. Effective population size (*Ne*) is a central parameter of population structure. The revelation by mtDNA and nuclear DNA based phylogenetic studies of high effective population size of *An. baimaii* along with genetic homogeneity in NE India raises concern on the effectiveness of indoor residual spray, the widely practised current vector control measure under Indian National Vector Borne Disease Control Programme. Observation of panmixia with high gene flow among populations of *An. baimaii* in NE India suggest the possibility of fast spread of insecticide resistance across range of its distribution, if appeared. A high initial (prior to wide spread insecticide use) mosquito-effective population size combined with high gene flow might have precluded regional bottle necks in populations of *An. baimaii* in NE India. Therefore, control of malaria transmitted by *An. baimaii* in NE India requires suitable alternate interventions such as insecticide-treated bed nets.

Conclusions

An. baimaii, a member of the Dirus complex, is a known primary malaria vector in SE Asia including NE India. Although taxonomy of *An. dirus* complex mosquitoes has been studied in-depth and the taxonomic status of all prevalent species ascertained, yet the observation of new or undescribed species (China; Assam, India; Northern Vietnam) phylogenetically close to other species of Dirus complex [73,75] suggests that the taxonomy of *An. dirus* complex mosquitoes needs to be further elucidated. It was also observed that different molecular markers provide different taxonomic position for the mosquito species under the Dirus complex. Based on rDNA-ITS2, *An. baimaii* clearly positioned at the basal position of the phylogeny of Dirus complex mosquitoes. However, based on mtDNA (whole mitochondrial genome or mitochondrial genes), it was observed that both *An. dirus* and *An. baimaii* are mostly at the same taxonomic position. This is possibly due to the mitochondrial introgression from *An. baimaii* to *An. dirus*.

An. baimaii in NE India, based on both mitochondrial DNA [111] and nuclear sequences (unpublished observations), was found to be in panmixia indicating that there is no speciation within the species. However, the presence of another new but distantly related species of the Dirus complex in Assam, NE India is interesting as this species was found to be very distantly related to *An. baimaii* based on rDNA-ITS2 and D3 sequences. No intraspecific differentiation was observed among *An. baimaii* populations in NE India at the rDNA-ITS2 locus, and overall a high genetic diversity in *An. baimaii* in NE India was recorded which was much higher than the diversity of this species recorded in Bangladesh, Myanmar and Thailand. As a matter of fact, the genetic diversity of *An. baimaii* in these countries was a subset of diversity observed in NE India.

Overall, little genetic structuring was observed in NE Indian populations of *An. baimaii* indicating genetic homogeneity throughout NE India. However, at regional scale compared to Bangladesh, Myanmar and Thailand populations; significant structuring

in NE India was evident indicating geographical barriers to gene flow (possibly high mountain ranges) between NE Indian and SE Asian populations of. High genetic diversity, genetic distinctiveness, relatively stable and older demographic history of *An. baimaii* in NE India in comparison to SE Asia suggests that possibly NE India is the origin of all extant populations of *An. baimaii* and might have acted as a glacial refugial region during Pleistocene climate change.

Significant genetic differentiation observed in some populations of *An. baimaii* in NE India must be assessed in terms of their biology, behaviour and attributes to malaria transmission. More analytical approaches such as landscape ecological genetics are likely to provide insights on the finer scale and micro-geographic variations in *An. baimaii* in NE India. Assessment of genetic variations (both temporal and spatialas well as neutral and adaptive) and behavioural heterogeneity combining both genomics and ecological approaches in the target vector species will help understand the vector population dynamics and disease control in NE India.

Acronyms	
AMOVA	Analysis of Molecular Variance
ASPCR	Allele Specific Polymerase Chain Reaction
BPP	Bayesian Posterior Probability
CO I	Cytochrome Oxidase I
CO II	Cytochrome Oxidase II
D3	Domain 3 region of 28S rDNA
EPIC	Exon - Primed - Intron – Crossing
ITS 2	Internal Transcribed Spacer 2
mtDNA	Mitochondrial DNA
MY	Million Years
MYBP	Million Years Before Present
ND 5	NADH dehydrogenase subunit 5
ND 6	NADH dehydrogenase subunit 6
Ne	Effective population size
NE India	North-east India
NCBI	National Centre for Biotechnology Information
numts	Nuclear mitochondrial pseudogenes
PCR	Polymerase Chain Reaction
rDNA	Ribosomal DNA
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restricted Fragment Length Polymorphism
SCAR	Sequence Characterized Amplified Region
SE Asia	South-East Asia
SNP	Single Nucleotide Polymorphism

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Chapter 19

Population genetics and distribution of *Anopheles nivipes* and *Anopheles philippinensis* in north-east India: vectoral capacity and implications in cross-border malaria

Nilanju Pran Sarmah[1], Anil Prakash[2] and Dibya Ranjan Bhattacharyya[1*]

[1] ICMR – Regional Medical Research Centre, Dibrugarh – 786 001, Assam, India

[2] ICMR – National Institute for Research in Environmental Health, Bhopal –462 001, Madhya Pradesh, India

[*] Correspondence: drbhattacharyya@yahoo.com

Abstract

Anopheles philippinensis and *An. nivipes* are closely related species of the Annularis group and regarded as secondary vectors of human malaria in north-east India and adjoining countries of the Southeast Asia. Both these species are recorded to occur in varying proportions, but *An. nivipes* predominates in north-eastern states. Although the identification of these species is unreliable for subtle variations in morphological characters but could be characterized aided by molecular tools unequivocally. Due to the diverse geography of north-east India and presence of natural physical barriers such as hills and mountain ranges, considerable lack of gene flow among populations of the two species with those of the neighbouring countries has been recorded resulting in genetic distinctiveness of *An. nivipes* and *An. philippinensis* populations having implications in cross-border malaria elimination efforts.

Keywords: Malaria, vector bionomics, genetic diversity, transmission control, cross-border malaria, north-east India, Southeast Asia

Introduction

Mosquitoes are ubiquitous in all temperate and tropical regions of the world with high diversity in tropical rainforest ecosystems [1]. Fewer than 200 of 3524 recognized species of mosquitoes are medically important transmitting various causative parasites (http://mosquito-taxonomic-inventory.info/). In many anopheline taxa including the disease vector species, morphological similarities amidst close relatives, e.g., cryptic or sibling species complexes pose operational challenges in traditional taxonomy, phylogenetic inferences and formulating appropriate disease transmission control strategies [2].

Till date, as many 61 mosquito species of genus *Anopheles* are recorded to occur in India. These mosquito species are split into the subgenera *Anopheles* and *Cellia* [3]. Subgenus *Cellia* contains 35 species incorporating all important vectors of human malaria of which many medically important anopheline taxa are complexes or groups of morphologically identical species [4]. At least 12 species of *Anopheles* are implicated in malaria transmission in India, comprising species of the *An. annularis* group, *An. maculatus* group, *An. culicifacies* complex, *An. dirus* complex, *An. fluviatilis* complex, *An. minimus* complex and *An. sundaicus* complex [5]. *An. baimaii* (earlier species D of Dirus complex), *An. fluviatilis s.s.* and *An. minimus* (species A) are regarded principal vectors of human malaria in north-east (NE) India [6].

However, several other species are suspected to be playing some role in transmitting malaria in NE region under local geo-epidemiological conditions. Among these, the Annularis group of mosquito species are considered important in view of occasional records of natural infections in its member species and wide distribution in NE India [7]. Annularis group belongs to Neocellia series of the subgenus *Cellia* of genus *Anopheles* and comprises five recognized species, i.e., *An. annularis*, *An. nivipes*, *An. pallidus*, *An. philippinensis* and *An. schueffneri* [8]. Among these, *An. philippinensis* and *An. nivipes*, the two most important members of Annularis group, are distributed mainly in the eastern and north-eastern and isolated localities of central and north India while generally absent in western and north-western region [9]. However, the vectoral status and geographical distribution of *An. philippinensis* or *An. nivipes* is far from being resolved because of the problems associated with the precise identification of these two species based on morphological characters. The adults of these two species are morphologically identical and can only be identified with conformity either in pupal stage or at the molecular level. In the earlier studies, the distinction between *An. philippinensis* and *An. nivipes* was not made, thus their distribution andvectoral status remained ambiguous until the development of DNA-based identification methods [10-12]. This chapter reviews the distribution, vectoral status and genetic diversity of *An. nivipes* and *An. philippinensis* in NE India helping strengthen cross-border malaria elimination efforts specific to Southeast Asia region.

Taxonomic status of *Anopheles philippinensis* and *Anopheles nivipes*

An. philippinensis and *An. nivipes* are morphologically so close that these were considered synonymous. Reid raised *An. nivipes* to species level and suggested that *An. philippinensis* reported in India and Bangladesh may literally refer to *An. nivipes* [13]. However,

these were confirmed as separate species only after establishment of their reproductive isolation based on cross-breeding experimental hybridization data [14]. Therefore, most of the earlier workers generally referred to *An. philippinensis* in their study results which could have been either of the two species. Nagpal and Sharma [15] were the first to report the existence of *An. nivipes* in NE India based on the wing-based character proposed by Reid [13]. However, the wing character was not found diagnostic to distinguish Indian and Thai populations of *An. philippinensis* from that of *An. nivipes*. It was in 2000 that Subbarao et al [16] confirmed the presence of *An. nivipes* in NE India based on cytotaxonomic methods. Subsequently, presence of both *An. philippinensis* and *An. nivipes* in NE India were reported using diagnostic pupal characters [17,18] as well as allele specific polymerase chain reaction (ASPCR) based method developed by Walton *et al* [10]. This molecular method is able to distinguishall four members of the Annularis group, viz., *An. annularis*, *An. philippinensis*, *An. nivipes* and *An. pallidus* by generating PCR products of diagnostic length of the second Internal Transcribed Spacer 2 (ITS2) of ribosomal DNA (r-DNA), and this technique was successfully used in investigating the distribution and vectoral attributes of *An. philippinensis* and *An. nivipes* populations in the NE states detailed in the following sections [19].

Study sites, topography and climate

The NE region of India comprises a geographical area of 262,230 sq km (101,250 sq mi) sharing 3100 km long international border with China, Bhutan, Myanmar and Bangladesh. Populations of both *An. philippinensis* and *An. nivipes* were subject to genetic analyses collected from all seven NEstates including Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland and Tripura lying between 22004'N and 97025'E (Figure 1). The NE region comprises of the Eastern Himalayas, north-east hills (Patkai-Naga Hills and Lushai Hills) and plains of the Brahmaputra and Barak Valley of Assam. North-east India has basically humid sub-tropical climate marked with torrid and humid summers, harsh monsoons and mild winters. Apart from the west coast of India, NE region has some of the Indian sub-continent's rain forests with diverse flora and fauna.The region receives high rainfall averaging >1000 millimetres in most areas that harbour rich ecosystem, and subject to high seismic activity and seasonal recurrent flash-floods. Most of the NE states have nearly 60% of their geographical area under forest cover supporting diverse fauna and flora and is a part of Indo-Burma biodiversity 'hotspot'. Amongst the 25 hotspots globally, northeast India is the second largest and next only to the Mediterranean basin with a cover of 262,230 km^2 [20].

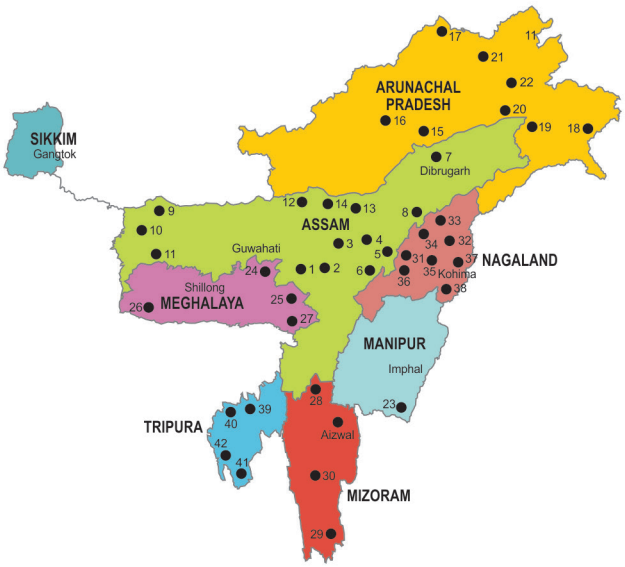

Figure 1: Map of north-eastern states of India showing geographical proximity and mosquito collection sites (1-42).

Mosquito sampling and taxonomy

Host seeking adult female mosquitoes of *An. philippinensis* and *An. nivipes* were collected from the human dwellings in various localities (42 sites) using CDC (Centre for Disease Control) miniature light traps (Figure 1). Attempt was made to have representative collection from all biotopes in the state such as hills, foothills, plains, forest and deforested areas. Mosquito adults thus collected were first identified by available pictorial keys based on morphological distinguishing characters [18] and then subject to confirmation by molecular assays [10, 11]. Morphologically identified *An. nivipes* (n=165) were all confirmed by molecular assays reaching correct identification by both methods [19]. However, among 337 individuals identified as *An. philippinensis*, only 97 confirmed *An. philippinensis* and remaining 240 were *An. nivipes* using Allele Specific Polymerase Chain Reaction (ASPCR) method indicating only 28.7% concordance between morphological and molecular identification techniques. The level of concordance was much lower (13.9%) in *An. philippinensis* possessing Type 3 wing in comparison to those with Type 2 wing (59.8%) [19].

Distribution of *Anopheles nivipes* and *Anopheles philippinensis* in north-east India

Annularis group of mosquitoes have a wide distribution throughout the Oriental region [5,9]. However, *An. schueffneri* is confined to Java and Sumatra while *An. pallidus* seems to be restricted to India, Myanmar and Sri Lanka for lack of well documented report of this species further east [8]. The remaining three species, *An. annularis*, *An. philippinensis*

and *An. nivipes* are widespread in the Oriental region. Among these, *An. nivipes* and its closely related species *An. philippinensis* have been reported to occur in the NE India and incriminated as vector of human malaria in Assam [21, 22] and deltaic West Bengal [23]. The prevalence of *An. philippinensis* was also reported from other Southeast Asian (SE) countries bordering NE India such as Bangladesh [24-26]. In NE region of India, populations of both *An. philippinensis* and *An. nivipes* were subject to confirmation using ASPCR assay [12, 19]. Of total mosquito adults screened for true species status (508), 411 individuals were identified as *An. nivipes* and 97 as *An. philippinensis*. It was evident that *An. nivipes* was the predominant species in Assam (90.5%), Nagaland (83.8%), Meghalaya (60%) and Tripura (70.8%), whereas *An. philippinensis* were more abundant in Mizoram (70.6%) and Arunachal Pradesh (43.3%) (Figure 2).

Distribution of *Anopheles nivipes* and *Anopheles philippinensis* in different ecotypes

Of various ecotypes studied, *An. nivipes* mosquitoes were found almost equally abundant in the plain-forested (45.01%) as well as hilly-forested villages with paddy-fields (38.9%); relative abundance, however, was the least in the deforested villages (1.4%). Instead, *An. philippinensis* was predominant in the hilly-forested villages with paddy-fields (80.2%) and much less in hilly-forested sans paddy fields (11.5%) and plain-forested with paddy fields (8.3%), and not recorded to occur in deforested villages with paddy-fields. Relative abundance of *An. nivipes* (60%) and *An. philippinensis* (63%) was the highest in the altitude range (0–199m) and in (400-599m) respectively. However, >600m msl, both *An. nivipes* and *An. philippinensis* occurred in nearly equal proportions and comprised approximately 16% of total mosquito collection (Table 1).

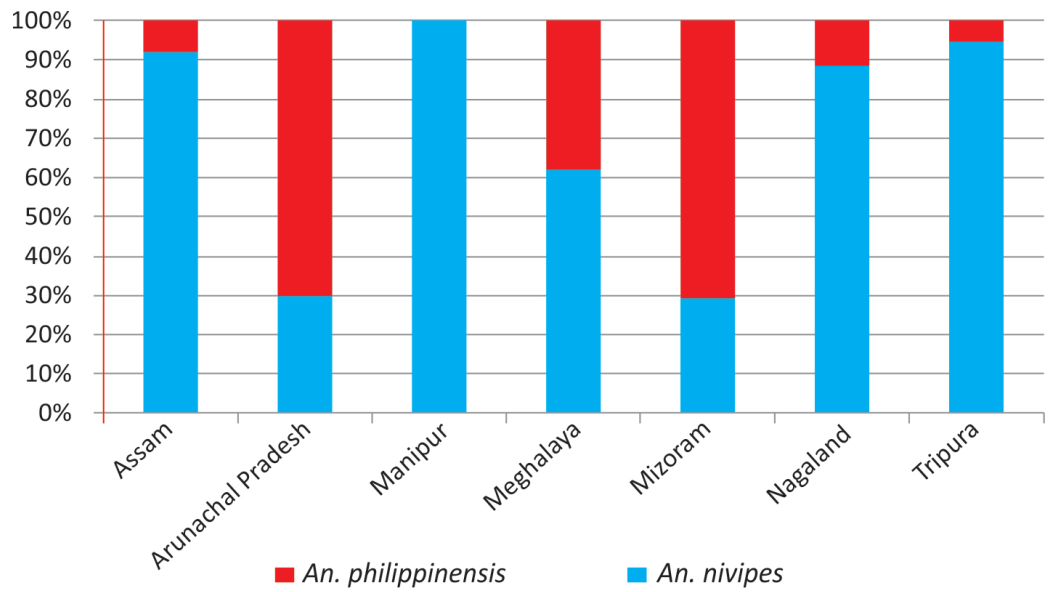

Figure 2: Relative abundance of *Anopheles nivipes* and *Anopheles philippinensis* mosquitoes in north-eastern states of India.

Table 1. Relative abundance of *Anopheles nivipes* and *Anopheles philippinensis* at different altitude ranges above mean sea level

Anopheles mosquito species	Number (%) of mosquitoes collected at different altitude ranges in metres (m)					
	0-199 m	200-399 m	400-599 m	600-799 m	800-999 m	1000-1300 m
An. nivipes	247 (60)	69 (17)	30 (7)	0 (0)	6 (1.5)	59 (14.4)
An. philippinensis	13 (13.4)	8 (8.2)	61 (63)	5 (5)	0 (0)	10 (10.3)

Malarial parasite infectivity in *Anopheles nivipes* and *Anopheles philippinensis*

An. nivipes was first incriminated as a vector of human malaria in north-western Thailand and subsequently in peninsular Thailand using enzyme-linked immunosorbent assays [27, 28]. *An. philippinensis/nivipes* has been regarded as an important vector in the neighbouring Bangladesh [29], Deltaic West Bengal [23], and Assam/Meghalaya [22]. ELISA based study also detected circum-sporozoite-proteins (CSP) of *P. falciparum* and *P. vivax* in *An. philippinensis/nivipes* species collected from NE states of Assam and Arunachal Pradesh [30]. Investigations using molecular tools for species identification and vector incrimination provided obvious confirmation of role of *An. nivipes* in transmitting *P. falciparum* in NE India [7]. Further more, a total of 508 mosquito adult females of *An. nivipes* and *An. philippinensis* collected from NE states were analyzed by nested-PCR methodfor parasite positivity (Table 2). These comprised 411 *An. nivipes* and 97 *An. philippinensis* mosquitoes which were processed for the detection of malaria parasite in the salivary glands of which only two of these mosquitoes were found to be harboring Plasmodium infection. Both were *An. nivipes* including one from the Dimapur district of Nagaland and the other from the Jorhat district of Assam.

Table 2. Detection of Plasmodium parasite in *Anopheles nivipes* and *Anopheles philippinensis* mosquitoes in north-eastern states of India using PCR

State	Study site	Mosquito species	No. mosquitoes processed	No. positive for malarial parasite
Assam	Longnit	*An. nivipes*	46	0
		An. philippinensis	4	0
	Namboor	*An. nivipes*	2	0
	Bagori	*An. nivipes*	2	0
	Kaziranga	*An. nivipes*	2	0l
	Hathidandi	*An. nivipes*	2	0
	Kanchanjuri	*An. nivipes*	3	0
	Saraipung	*An. philippinensis*	1	0
	Titabor	*An. nivipes*	29	1*
		An. philippinensis	1	0
	Kumarikata	*An. nivipes*	101	0
	Tamulpur	*An. nivipes*	30	0
	Amlong	*An. philippinensis*	4	0
	Budong	*An. philippinensis*	1	0
	Boithalangsu	*An. nivipes*	2	0
		An. philippinensis	8	0

Arunachal Pradesh	Bilat	*An. nivipes*	6	0
		An. philippinensis	2	0
	Panbari, Tezu	*An. nivipes*	2	0
		An. philippinensis	3	0
	Chawkham	*An. nivipes*	2	0
		An. philippinensis	4	0
	Jairampur	*An. nivipes*	1	0
		An. philippinensis	14	0
	Wingko	*An. philippinensis*	3	0
Manipur	Churachandpur	*An. nivipes*	6	0
Meghalaya	Burnihut	*An. nivipes*	19	0
	Dawki	*An. nivipes*	5	0
		An. philippinensis	24	0
	Barato	*An. nivipes*	15	0
Mizoram	Rengtelvi	*An. philippinensis*	12	0
	Tlabung	*An. nivipes*	4	0
	Thenzwal	*An. nivipes*	1	0
Nagaland	Aiyonkum	*An. nivipes*	1	1*
	Lower Bhandari	*An. nivipes*	19	0
	Dihoma	*An. nivipes*	4	0
		An. philippinensis	4	0
	Tsemanayau	*An. philippinensis*	2	0
	Kezubacha	*An. nivipes*	15	0
		An. philippinensis	3	0
	Longsavill	*An. nivipes*	1	0
	Jaluki	*An. nivipes*	50	0
		An. philippinensis	6	0
	Meziphema	*An. nivipes*	24	0
Tripura	Ramychera	*An. nivipes*	2	0
	Haptabari	*An. nivipes*	3	0
		An. philippinensis	1	0
	Jaisingh, Sabroom	*An. nivipes*	12	0
Total mosquitoes processed		*An. philippinensis*	97	0
		An. nivipes	411	2

*Positive for *Plasmodium falciparum*

Molecular taxonomy and evolutionary relationships

Anopheline mosquitoes especially the species group or complexes cannot always be distinguished reliably using only morphological characters. Members of such anopheline species differ in biological attributes such as anthropogenicity, exophagy/endophagy, exophily/endophily, longevity and larval habitat preferences. These attributes relate to the vectoral capacity of a species and such data are essential to formulate effective vector control intervention strategies. As such, reliable molecular tools for understanding of intraspecific genetic diversity and population structure are useful in understanding evolutionary relationships having implications in vector control [31]. Frequently used targets in phylogenetic and population genetic studies include both coding regions such as NADH dihydrogenase subunit 5 (ND5) and Cytochrome oxidase sub unit II (COII) of mitochondrial DNA (mtDNA), and non-coding regions such as 16S and 18S RNA genes of ribosomal DNA (rDNA). Both these markers were applied to ascertain genetic affinities between populations of *An. nivipes* and *An. philippinensis* detailed as below.

Ribosomal DNA (rDNA) characterization of *Anopheles nivipes* and *Anopheles philippinensis*

Second internal transcribed spacer (ITS2) and domain 3 (D3) region of rDNA have been used extensively for discrimination of closely related anopheline mosquito taxa using species-specific primers [32]. Variations in length and settled substitutions among rDNA sequences are taken as evidence of evolutionary divergence and help provide phylogenetic information.

Sequencing of the second internal transcribed spacer (ITS2) region was done in 20 mosquito adults of *An. nivipes* and 13 *An. philippinensis* collected from Assam, Arunachal Pradesh, Tripura, Mizoram Meghalaya and Nagaland [19]. The ITS2 length was found to be 348 base pair for *An. nivipes* and 362 base pair for *An. philippinensis* with ~80% sequence identity. The sequences of *An. nivipes* and *An. philippinensis* have been deposited in the Gene Bank.

Analysis of ITS2 sequence variation

Based on the ITS2 sequences, populations of both the species from NE India were found to be genetically similar. The typical ITS2 sequences of *An. nivipes* (accession number-JN654428) and *An. philippinensis* (accession number-JN654436) acquired from our study sites were aligned with sequences of *An. nivipes* from Indian states of Odisha (FJ 159607), Andaman and Nicobar Islands (DQ279442), and Chinese state of Yunnan (EU650426) and Hainan (EU919722) and that of Myanmar (FJ526624); along with the sequences of *An. philippinensis* from Odisha, (FJ159606), Yunnan (GU373719) and Laos (FJ526618) using multiple sequence alignment. *An. nivipes* from Andaman and Nicobar and Odisha differed from those of the north-east by a single base transition of G to T at position 31, whereas this position was heterozygous for sequence of Myanmar. *An. nivipes* from Hainan, China differed from the north-eastern population by a single transition of C to G at position 73. On the other hand, NE Indian sequence of *An. philippinensis* was identical to the those of Laos and Odisha, except that of Yunnan (China) sequence by a transition of G to T at position 285.

A molecular phylogeny was carried out amid the ITS2 sequences of *An. nivipes* and *An. philippinensis* from NE India and bordering countries using Neighbour-joining methods using Kimura 2 parameter distance matrix with 1000 bootstrap replicates (Figure 3). In case of *An. philippinensis*, the sequences from NE India, Odisha and Laos formed a single clade barring the sequence from Yunnan, China showing a bootstrap value of 100. On the other hand, the sequence of *An. nivipes* from NE India was seen to be considerably different at the genetic level from those of Yunnan & Hainan (China), Odisha & Andamans (India) and Myanmar showing significant bootstrap values. Pair wise genetic distances between *An. nivipes* from the NE India and Hainan, Andaman and Nicobar and Odisha recorded were 0.003, 0.007 and 0.007 respectively indicating genetic distinctness of these populations. The genetic distance among *An. philippinensis* from the NE India, Odisha and Laos was nil, while it was 0.003 forYunnan population.

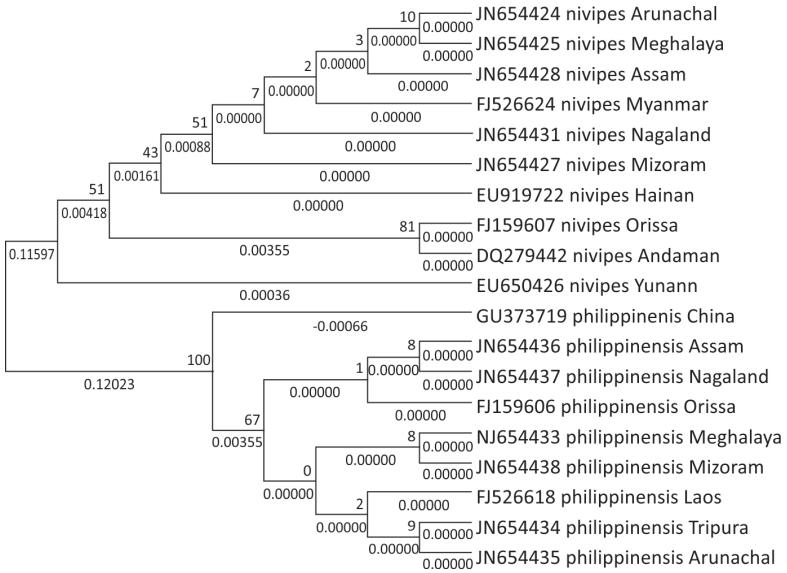

Figure 3: Phylogenetic tree based on the ITS2 sequences of *Anopheles philippinensis* and *Anopheles nivipes*. Numbers below the line and above are branch lengths and percentage bootstrap values respectively. Source Reference [12].

Domain 3 (D3) characterization of ribosomal DNA

The D3 region of rDNA was sequenced in 20 mosquito adults of *An. nivipes* (Assam -4, Arunachal Pradesh-4, Tripura-4, Mizoram-4, Meghalaya-2, Nagaland-2) and 13 adults of *An. philippinensis* (Assam-3 and Tripura-2, Mizoram-2, Nagaland-2, Arunachal Pradesh-2 and Meghalaya-2) [19]. Post determining the 5.8S and 28S borders, D3 length was found to be 386 base pair for *An. annularis*, 385 base pair for *An. nivipes* and 383 base pair for *An. philippinensis* with ~80% homogeneity. Ten sequences of *An. annularis* (JQ364918, JQ364919, JQ364920, JQ364921, JQ364922, JQ364923, JQ364924, JQ364925, JQ364926, and JQ364927), twenty sequences of *An. nivipes* (accession numbers JN979378, JN979379, JN979380, JN979381, JN979382, JN979383,JN979384, JN979385, JN979386, JN979387, JN979388, JN979389, JN979390, JN979391, JN979392, JN979393, JN979394, JN979395, JN979396, JN979397) and fifteen sequences of *An. philippinensis* (accession numbers JN872197, JN872198, JN872199, JN872200, JN872201, JN872202, JN872203, JN872204, JN872205, JN872206, JN872207, JN872208, JN872209, JN872210, JN872211) have been deposited in the Gene Bank.

Analysis of sequence variation in D3 region

GC content of the D3 region was found to be 56.99%, 56.96% and 57.2% for *An. annularis*, *An. nivipes* and *An. philippinensis* respectively. No intra-specific variations were detected in any of the three species from northeast India. The representative D3 sequence of *An. annularis* (JQ364918) obtained in this study was aligned with the sequences of the same species from Indian state of Odisha (DQ483027), Madhya Pradesh (EU570058) and that of Mengla,

China (EU570058). All sequences were identical except that *An. annularis* from Mengla that differed from the NE Indian *An. annularis* by transition of G to T and T to A at positions 340 and 343 respectively. The representative D3 sequence of *An. nivipes* (JN979378) obtained in the study was aligned with sequences of *An. nivipes* from Odisha, (EU366357). Similarly, representative D3 sequence of *An. philippinensis* (JN872207) was aligned with the sequences of *An. philippinensis* from Odisha, India (DQ402059) and Yunnan, China (GU373720). *An. nivipes* from Odisha differed from those of the NE population by insertion of A at position 54, transitions of A to G, and G to T at positions 72 and 111 respectively. *An. philippinensis* from China differed from the NE population by G to A transition at position 216. Similarly, as in case of the ITS2 sequences of these species, a molecular phylogeny was carried out by using the neighbour joining methods of phylogenetic tree construction using Kimura two parameter model with 1000 bootstrap replicates (Figure 4). The representative D3 sequence of *An. nivipes* from NE India differed from the D3 sequence of the same species from Odisha forming a different sister clade. Similar observations were made in the *An. philippinensis* sequence of D3 from NE India and China. The pair wise distance between *An. nivipes* from the NE India and Odisha was 0.003, whereas the pairwise distance between the NE *An. philippinensis* and Hainan was 0.008. The topographical intraspecific variances in ITS2 and D3 region of *An. philippinensis* and *An. nivipes* from NE India with population of other provinces may conceivably be as a result of differential adaptation to ecological settings resulting in naturally differentiated populations due to barriers in gene flow.

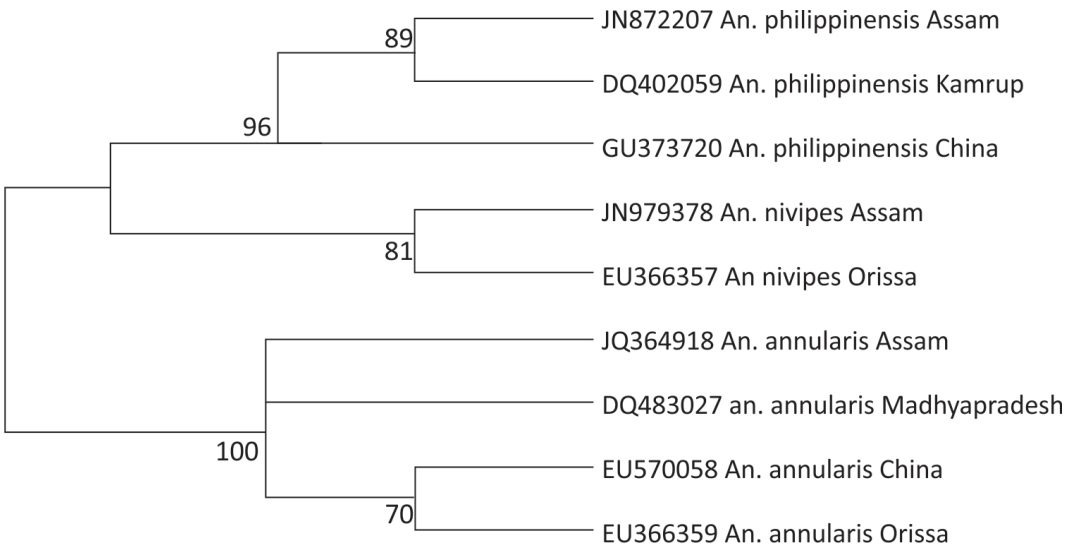

Figure 4: Dendrogram based on D3 sequences of *Anopheles nivipes, Anopheles philippinensis* and *Anopheles annularis* mosquito species.Value above the line are percentage bootstrap values. Source reference [19].

Mitochondrial DNA-based genetic diversity of *Anopheles nivipes* and *Anopheles philippinensis* populations

In population genetic studies mitochondrial DNA (mtDNA) coding regions such as NADH dehydrogenase subunit 5 (ND5) and Cytochrome oxidase subunit II (COII) have been

widely used to infer population history and expansion, and both past and current rates of gene flow as well as to probe whether barriers to dispersal are current or historical [33-35].

A total of 36 sequences were included in the final dataset of ND5 gene of *An. nivipes* for phyllo-geography study. Of these 30 were from NE India (7 from Assam, 4 from Arunachal Pradesh, 6 from Meghalaya, 6 from Nagaland, 2 from Mizoram, 1 from Manipur and 4 from Tripura) and remaining 6 sequences (2 each from Thailand, accession numbers-FJ526714, FJ526713; and Myanmar, accession numbers - FJ526715, FJ526716; and 1 each from Cambodia, accession number - FJ526712) and Laos , accession number - FJ526717, were retrieved from NCBI (www.ncbi.nlm.nih) [19,36]. A total of 21 haplotypes were obtained with a haplotype diversity of 0.9651. As many as 36 polymorphic sites with 17 singleton variable sites and 19 parsimony informative sites were detected. Significant value of pairwise FST (0.12780; $p<0.001$) suggested that the populations of *An. nivipes* from NE India and SE Asia differed distinctly from each other at the molecular level. The NE Indian populations of *An. nivipes* had genetic diversity of 1.0000 +/- 0.0065, and nucleotide diversity of 0.010421 +/-0.006778 and θs- 6.28782.

Similarly, a total of 33 ND5 gene sequences of *An. philippinensis* across the six NE states (5 from Arunachal Pradesh, 6 from Assam, 8 from Meghalaya, 4 from Mizoram, 4 from Nagaland and 6 from Tripura) were compared with 2 sequences of Thailand (accession number-FJ526710 and FJ526709), one sequence each from Laos and Cambodia (FJ526708 and FJ526711) and one from India (accession number-FJ526707) retrieved from NCBI (www.ncbi.nlm.nih) [19]. A total of 13 haplotypes defined 36 sequences with a haplotype diversity of 0.7042. As many as 114 polymorphic sites in the dataset contained 13 parsimony informative sites and 101 singleton variable sites. Pairwise comparison among the sequences of *An. philippinensis* from NE India and SE Asia showed significant value of FST (0.06931; $p<0.01$) between the populations suggesting genetic isolation of two groups of populations.The NE Indian population has genetic diversity of 1.0000 +/- 0.0063, θS-27.308272 and nucleotide diversity of 0.005611 +/-0.004143.

Correspondingly, the total dataset of *An. nivipes* COII sequences contained 33 sequences. Of these, 29 from north-east India (7 from Assam, 5 from Arunachal Pradesh, 5 from Meghalaya, 6 from Nagaland, 2 from Mizoram and 4 from Tripura) and the remaining 4 sequences one each from Cambodia (accession no-FJ526472), Thailand (FJ526473), China (FJ526471) and Myanmar (FJ526475) were retrieved from gene bank (www.ncbi.nlm.nih) [19,36]. In this dataset, 31 haplotypes were found with haplotype diversity of 0.9962. There were 87 polymorphic sites including 20 singleton variable sites and 67 parsimony informative sites. The FST value was (0.04442;$P< 0.01$) which in turn suggests that populations of *An. nivipes* from NE India and SE Asia are genetically different. The genetic diversity, nucleotide diversity and θs estimates among the COII sequences of *An. nivipes* were 1.0000 +/- 0.0071, 0.004045 +/- 0.003257and 12.09148 respectively.

Similarly, a total of 28 sequences of *An. philippinensis* COII gene generated using specimens from seven NE states (5 from Assam, 5 from Arunachal Pradesh, 5 from Meghalaya, 5 from Mizoram, 4 from Nagaland and 4 from Tripura) were added to this dataset of 51 sequences of *An. philippinensis* (Thailand =9, Vietnam =9, Laos=32 and Myanmar=1) retrieved from gene bank (www.ncbi.nlm.nih) for comparison on a regional basis, thus making the final dataset of 79 sequences [19]. The total dataset contained 37 haplotypes with a haplotype diversity of 0.9004 of which 25 originated from the NE Indian populations of *An. philippinensis*. A total

of 421 variable sites observed in the final dataset contained 7 singleton variable sites and 417 parsimony informative sites. Pairwise FST value for the NE Indian population and SE Asian population of *An. philippinensis* was found to be (0.97233; p<0.001) showing genetic differentiation of NE and SE Asian populations of *An. philippinensis*. Genetic diversity, nucleotide diversity and θs estimates of COII gene were found to be 1.0000 +/- 0.0095, 0.047067+/-0.023621 and 11.17497 respectively.

Conclusions

It is concluded that *An. nivipes* can be identified with conformity by both morphological and molecular assays, however, in case of *An. philippinensis*, wing-based morphological identification method is not reliable as evident from the high degree of discordance between morphological and molecular methods of identification. The level of concordance is much lower (13.9%) in *An. philippinensis* possessing Type 3 wing compared to those with Type 2 wings caling/venation pattern (59.8%). Further, it can be concluded that *An. nivipes* is the most prevalent species in NE India and playing some role in transmitting malaria evidenced by the presence of *P. falciparum* parasite antigen using molecular tools. ITS2 and D3 regions of *An. philippinensis* and *An. nivipes* across the six NE states of India did not reveal any intraspecific variation but had diverged from populations of the neighbouring countries. The geographical intraspecific differences in ITS2 and D3 region of *An. philippinensis* and *An. nivipes* from NE India with the specimens of adjoining countries may perhaps due to the impact of differential adaptation to various ecological conditions and gene flow barrier.

The study revealed exceptionally high mitochondrial DNA diversity in *An. philippinensis* and *An. nivipes* populations throughout the NE India. On the other hand, less mtDNA divergence was observed outside the NE India which may be due to demographic bottleneck. This significant bottlenecking of mtDNA of *An. philippinensis* and *An. nivipes* populations from outside NE India resulting in considerable genetic differentiation from NE India to that of Myanmar, Thailand, Laos and Vietnam is indicative of major geographical barriers to gene flow from/into NE India. The considerable divergence between the NE Indian population of both the species and those of Myanmar, Thailand, Laos and Vietnam clearly indicates allopatric fragmentation. For both the species, there is compelling evidence for population expansion in the NE India evidenced by significant negative values of Tajima'D and Fu's Fs statistics (Tajima'D values <0.01 and Fu's Fs <0.001). Significant FST values for both the species have shown considerable population structure reflecting low level of gene flow.

Northeast India is of strategic importance for sharing long international border with Myanmar in the East, China to North and Bangladesh to South, and considered corridor for spread of drug-resistant malaria in India and beyond. Thus, these data are of importance helping formulate appropriate cross-border intervention strategies averting impending disease outbreaks and spread of drug-resistant malaria. Many countries in the SE Asia including India are accelerating towards malaria elimination in the foreseeable future for which targeting interventions against all mosquito vector species including those of minor importance have become relevant to end residual transmission and maintaining malaria-free status post-elimination [37-39].

Acronyms	
ASPCR	Allele Specific Polymerase Chain Reaction
CO II	Cytochrome Oxidase II
CSP	Circum-Sporozoite-Protein
D3	Domain 3 region of 28S rDNA
FST	Fixation index
ITS 2	Internal Transcribed Spacer 2
mtDNA	Mitochondrial DNA
m	metre
msl	mean sea level
ND 5	NADH dehydrogenase subunit 5
NE India	North-East India
NCBI	National Centre for Biotechnology Information
PCR	Polymerase Chain Reaction
rDNA	Ribosomal DNA
SE Asia	South-East Asia

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Executive Summary

From the desk of Editor

Conclusions and Specific Recommendations

The advent of molecular entomology has revolutionized the taxonomy of mosquito vectors and targeting species-specific interventions for effective control saving operational costs. Nevertheless, the multiplicity of disease vectors and ecological succession consequent to massive infrastructure development, population movement, urbanization and deforestation continue to thwart the control efforts.

From the foregoing detailed account of individual mosquito vector species, it is apparent that *Anopheles culicifacies s.l.*, the dominant mosquito vector of human malaria, is not only growing multi-resistant but also spreading and accessing ecological niche of susceptible populations of *An. minimus* and *An. baimaii* (sibling-species of the Dirus Complex), the proficient vectors in north-east India. Populations of the latter two species are depleting attributed to large scale deforestation, changing land cover and population migration unabated across borders. *An. fluviatilis s.l.*, equally an efficient vector in hills and foothill areas, transmits malaria parasites often in conjunction with aforementioned vector species more so in winter months (relay transmitter) calling for reinforcement of interventions for most part of the year uninterrupted.

Among others, *An. sundaicus s.l.*, a brackish water vector, has retracted from its erstwhile distribution in coastal Indian states to Andaman and Nicobar Islands presenting a window of opportunity for reducing population below threshold by strengthening interventions helping disrupt transmission rendering Islands free from malaria. All these vector taxa are species complexes with varied bionomical characteristics and role in disease transmission. Instead, *An. stephensi* is not a species complex but known to comprise only variants and considered to be an invasive species for establishing in growing urbanized settlements and desert ecotype alike well beyond the carrying capacity of healthcare services in urban agglomerations.

Besides these dominant mosquito vectors, secondary vectors including *An. annularis, An. subpictus*, *An. maculatus* and *An. nivipes/philippinensis* are gaining eminence for rising density and increased possibility of human/vector contact permitting residual transmission in the wake of disappearing malaria in most Indian states formerly considered high-risk. For attaining malaria elimination, it has become imperative to target all probable vector species to minimize/preventing re-establishment of transmission in malaria-free territories.

What concerns most is the imminent threat of multi-insecticide resistance and rising operational costs. What more, the altered behaviour of mosquito vectors, i.e., mosquitoes

are getting outdoors from indoor resting avoiding contact with insecticide sprayed surfaces. This new emerging ecotype of outdoor transmission calls for innovative technologies preventing infective mosquito bites more so in forest-fringe/outreach population groups that are often considered to be the infectious reservoirs for continued transmission. To corner these vector populations, entomological surveillance is of utmost importance for instituting appropriate intervention in time and place averting impending disease outbreaks and spread of drug-resistant malaria.

The threat of insecticide resistance looms large globally throttling elimination efforts.To overcome the menace of insecticide resistance, application of innovative technologies integrating various species-specific interventions, viz., mass-scale distribution of insecticide-treated netting materials/long-lasting insecticidal nets (LLINs), and promoting bio-environmental control interventions that are community-based and doable should be universalized benefiting communities at any risk of malaria.

There is gamut of issues for effective vector control, yet skilled human resource is grossly inadequate and inapt to help the control programme on equal footing. Entomology strength is waning and getting scarce globally. For malaria transmission control, even though drug-policy in place (artemisinin derivatives in particular) has played pivotal role in reducing parasite reservoir in endemic communities, yet vector control is of paramount importance to disrupt transmission. Both parasite and mosquito vectors are continually evolving evidenced by emergence of drug-resistant malaria and insecticide resistant strains costing malaria elimination efforts dearly.

The vicious cycle of parasite/vector seems unstoppable, yet Indian National Control Programme has made significant strides in reducing transmission over the past few years by large scale implementation of interventions supported by research-based data. These include rollout of artemisinin-based combination therapy (ACT) for radical cure, mass scale distribution of LLINs for vector control, intensified disease surveillance aided by village level link worker/social activists, training and re-orientation of health personnel, and monitoring and evaluation services aided by research establishments.

Yet the mandate is mammoth to eliminate malaria by 2027 given the host of challenges including huge population at risk (estimated to be close to a billion), multiple mosquito vector species, varied terrain and contextual determinants. India is currently reporting close to half a million cases and is on radar being among 11 high-burden countries for contributing nearly 4% of reported casesin the world, mandating renewed attack to stay on track defeating malaria. Nevertheless, steady decline in cases with many states (Odisha reporting 80% decline) and Union Territories approaching pre-elimination, is certainly a landmark development.

There is renewed hope and optimism to end malaria but miles to go before finish line. To accelerate towards malaria elimination, much more can be achieved by strengthening entomological capacity in keeping vector populations at bay averting thousands of cases and saving many more lives reposing confidence in the high-risk communities. In country led response, the following specific recommendations are made for vector control benefiting the programme helping achieve envious goal of malaria elimination by due date:

1. First and foremost is the building cadre of entomologists in the country to address the local needs at the state/district level for monitoring vector density enabling institute

species-specific interventions well in time and place. Much needed data on distribution of sibling-species of dominant vectors using molecular identification assays is available but nowhere this information has been applied in the control programme for lack of adequate expertise at large.

2. Data on insecticide resistance is patchy and far from complete. Periodic monitoring of insecticide resistance at state level should be the cornerstone helping make the right choice of insecticide for residual sparing. What even more important is the quality control, adherence to spray schedule, and coverage of target population at risk in malaria foci, which continues to be clear neglect.

3. Strengthening interaction with research establishments helping monitor the logistic preparedness and sharing information/technology transfer on recent developments will help boost the control programme. Closer ties with adjoining neighbouring countries need to be translated into action for data sharing and coordinated vector control operations along interborder areas to forbid dissemination of drug-resistant malaria. Rising proportions of drug-resistant *Plasmodium falciparum* (the deadly parasite) is wakeup call to prevent its spread westwards.

4. Among various options for vector control, LLINs have been assessed to be operationally feasible and widely accepted by the communities. There is no shred of evidence for lack of protection against infective bites in LLIN beneficiary population groups in areas with insecticide-resistant vector populations. The distribution of LLINs is presently patchy and should now be upscaled to protect communities at any risk prioritizing vulnerable groups hard hit by this preventable and curable disease.

5. Political commitment for increased allocation of resources for procurement and supplies of LLINs is warranted. The funding gap is too far wide calling for concerted efforts by public and private sectors alike, donors and international funding agencies to ensure sustained supplies not only for mass-scale distribution but also making provisions for net-replacement of those worn-out providing protection uninterrupted (the net-serviceable life is expected to be about three years of continuous use in field conditions by the communities).

6. Given the dwindling populations of some important vectors, viz., *An. minimus, An. baimaii*; it is time to target interventions against all vector species inclusive those of lesser significance, viz, *An. subpictus*, an emerging vector in urban India (formerly disregarded). Others included *An. annularis*, *An. maculatus* and *An. nivipes/philippinensis*; all hold the capacity to resume transmission. These mosquito species are likely to gain eminence post-elimination. There is virtually no data on distribution of their sibling-species and insecticide susceptibility status.

7. To keep pace with growing urban metropolitan cities, civic bylaws should be enforced coupled with increased awareness on disease prevention and control to prevent mosquito breeding in household premises not only of malaria vectors but also dengue (an emerging arbovirus infection). The campaign' Zero Malaria Starts with Me' befits well in the given situation for making communities equal partner along with other stakeholders to defeat malaria.

8. Given the available information on disease vectors, it is time to move away from the notion of 'one-size-fits-all'. It is opportune time for analysis of quality data for better

strategic applications and delivery mechanisms for maximal impact through primary healthcare services. Thus, all other evidence-based interventions, viz., large scale introduction of larvivorous fish and bio-environmental measures reducing vector breeding resources should all be integrated for decisive attack on disease vectors.

9. Lastly, the programme should draw its strength through coordinated response complemented by other sectors such as environment, education and agriculture ensuring utilization of resources maximally towards common goal of living in malaria-free world. Clock is ticking and given the political leadership, country ownership led by research-based data and broad range of stakeholders; together we can accelerate towards finish line sooner than deadline of 2030. Eliminating malaria in India would be big leap forward for the entire Southeast Asia for which investments made in this region would pay rich dividends for equitable socio-economic development in this part of the world.

In summary, malaria parasite may disappear locally (if not globally) but malaria carrying mosquitoes are likely to thrive for generations to come given their innate capacities to evolve and proliferate in varied environments. There should be no let-up in efforts to monitor these tiny daredevils for keeping them at an arm's length even post-elimination preventing re-establishment of transmission in malaria-free territories. As of today, with more than half of the world's population free from malaria, aspirations are high and movement towards eradication is building and building. With continuing decline in malaria transmission intensities globally and available tools for high-quality enhanced access to diagnosis, treatment, vector control and closing financial gap, 'malaria eradication' seems ambitious, achievable and necessary within a generation's time making malaria a thing of the past by 2050.

"No one should die of malaria in this world of information technology. The onus is on the scientific fraternity to innovate and update with the latest interventional technologies in continuing battle against malaria. What is most critical is to reach out the outreach communities and ensure 'universal coverage'for access to prevention and treatment. There is an imperative need to invent newer tools for spearheading species-specific interventions to prevent transmission and maintaining vigil in keeping the vector mosquitoes at bay. Malaria elimination is well within reach; let us make it happen with continuing research endeavours even beyond post-elimination."

Given below are the updated taxonomic molecular tools and bionomical characteristics of the dominant mosquito vectors of human malaria specific to India which should serve as ready reckoner for researchers and stakeholders alike.

Taxonomic tools and bionomical characteristics of the dominant mosquito vectors of human malaria in India

Anopheles species/ taxa	Number of sibling-species identified (species prevalent in India)	Diagnostic tools*	Breeding habitats	Feeding behaviour (peak biting activity)	Seasonal abundance & (resting habitats)	Incrimination status (average sporozoite infectivity rate)	Insecticide susceptibility status	Distribution range
An. culicifacies s.l.	5 (A, B, C, D, E)	Polytene chromosome karyotype/fixed paracentric inversions, PCR based sequencing of rDNA 28S-D3, 28S-D2 domain; ITS2-PCR-RFLP; rDNA ITS2; mt-DNA COII	Rainwater collections, riverine pools, rice fields, seepage water, streams, borrow pits, irrigation channels	Predominantly zoophilic except 'E' (A & B: 22:00 – 23:00; C & D: 18:00 – 21:00; No data for E)	Monsoon species/ wetseason (human-dwellings indoor and cattle sheds)	Incriminated, sporozoite infection rate of A, B, C, D (<1%) & E (>4%)	Resistant to DDT, Malathion & Pyrethroids (in certain pockets)	Throughout rural India
An. fluviatilis s.l.	3 (S, T, U)	Polytene chromosome karyotype/fixed paracentric inversions, PCR based sequencing of rDNA ITS2; 28S rDNA-D3	Seepage water foothill streams, irrigation channels, riverine ecology, terraced rice-fields, shallow wells	Sibling species 'S' - highly anthropophilic (20:00-24:00); T& U – zoophilic	Winter species (sibling species 'S' – human dwellings indoors, foothill ecotype; 'T & U' – cattle sheds, foothill & plain ecotype)	Sibling species 'S' incriminated (3%)	Sibling species 'S' highly susceptible to DDT, Malathion & Pyrethroids	Throughout rural India
An. minimus s.l.	3 (*An. minimus s.s.*)	PCR based sequencing of rDNA ITS2; 28S rDNA-D3 AS-PCR, RFLP-PCR	Perennial foothill seepage water streams	Highly anthropophilic (01:00 – 04:00)	Perennial species (human dwellings indoors)	*An. minimus s.s.* incriminated (3%)	Highly susceptible to DDT, Malathion & Pyrethroids	North-eastern state of Arunachala Pradesh, Assam, Meghalaya, Manipur, Mizoram, Nagaland, Tripura, and Eastern State of Odisha
An. dirus s.l.	8 (*An. baimaii*)	Polytene chromosome karyotype, cross-fertility data, PCR based sequencing rDNA ITS2; SCAR based PCR, ASPCR, RFLP	Jungle water pools, elephant footprints	Highly anthropophilic (23:00 – 03:00)	Monsoon species/ wet season (outdoor resting exophilic species)	*An. baimaii* incriminated (3%)	Highly susceptible to DDT, Malathion & Pyrethroids	North-eastern states of Arunachala Pradesh, Assam, Meghalaya, Manipur, Mizoram, Nagaland, Tripura & Andaman & Nicobar Islands

An. sundaicus s. l.	4 (Species D)	Mitochondrial DNA cytochrome oxidase 1 and cytochrome-b; rDNA ITS2	Sunlit brackish water bodies including swamps, saltwater lagoons, creeks	Predominantly zoophilic except indoor resting-populations (21:00 –2:30)	Both indoors and outdoors	Cytotype D incriminated (<1%)	Highly susceptible to DDT, Malathion & Pyrethroids	Andaman & Nicobar Islands
An. stephensi	Ecological variants (type form, intermediate and *var. mysorensis*)	Egg morphometrics, Y chromosome polymorphism, spiracular index	Domestic containers, building construction sites, overhead water storage tanks, underground cement tanks, desert coolers	Predominantly anthropophilic (22:00 – 24:00)	Monsoon species/ wet season (endophilic)	Type form Incriminated (<2%)	Resistant to DDT, dieldrin. malathion except pyrethroids	Urban metropolitan cities except northeastern states

*rDNA: ribosomal DNA; ASPCR: Allele specific polymerase chain reaction;SCAR: sequence characterized amplified region; ITS2: internal transcribed spacer 2; RFLP: restriction fragment length polymorphism

Vector Biology and Control
An Update for Malaria Elimination Initiative in India

Edited by Vas Dev

Vas Dev is an alumnus of the Panjab University, Chandigarh having graduated with Bachelor's and Master's degree in Zoology (Hons) in 1975 and 1977, respectively. In pursuit for higher education, he moved offshores for the doctorate degree programme in the United States of America on Government of India scholarship for studies abroad and graduated with Ph.D. degree in Vector Biology from the University of Notre Dame in 1983. In continuation, he had his post-doctoral stint at the US Department of Agriculture/Agricultural Research Service (1983-1985) and described polytene chromosomes of the primary screwworm fly, *Cochliomyia hominivorax*, an insect pest of economic importance in the Americas. On his return to India, he worked for the Indian Council of Medical Research (1986-2016) and piloted a research project on 'Epidemiology and Control of Malaria', testing newer interventions in high-risk areas of North-East India. His research efforts culminated in a number of technologies, viz., insecticide-treated netting materials for vector control, monitoring therapeutic efficacy of anti-malarial drugs, upgrading drug-policy for radical cure, alternate diagnostics, and large-scale introduction of larvivorous fish for promoting biological control; all of these interventions have been incorporated in healthcare services and resulted in substantial reduction in disease transmission. Vas Dev has authored more than 150 research publications in indexed journals and co-edited a book, 'Towards Malaria Elimination – A Leap Forward', published in 2018 by IntechOpen, London. He is the recipient of several coveted scholarships, awards and distinctions in his chosen field of research and is currently on the panel of reviewers for several national and international journals and serving active member of the National Academy of Sciences, India.

This Book

This book was commissioned by the National Academy of Sciences, India to spread the current knowledge of the mosquito vectors of human malaria and control options in different geo-epidemiological risk-zones of India. Given the clarion call for malaria elimination by 2027, understanding vector bionomics in the changing ecological context is of paramount importance to formulate species-specific intervention strategies to disrupt transmission of malaria. India is fast accelerating towards the ambitious goal of malaria elimination presenting a window of opportunity for scaling up interventions and developing stronger health systems for universal coverage. This book is of immediate relevance to programme/policy managers and researchers alike, reinforcing continued research on innovative technologies for sustainable control helping end transmission for good in the foreseeable future towards envious goal of a malaria-free world.

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