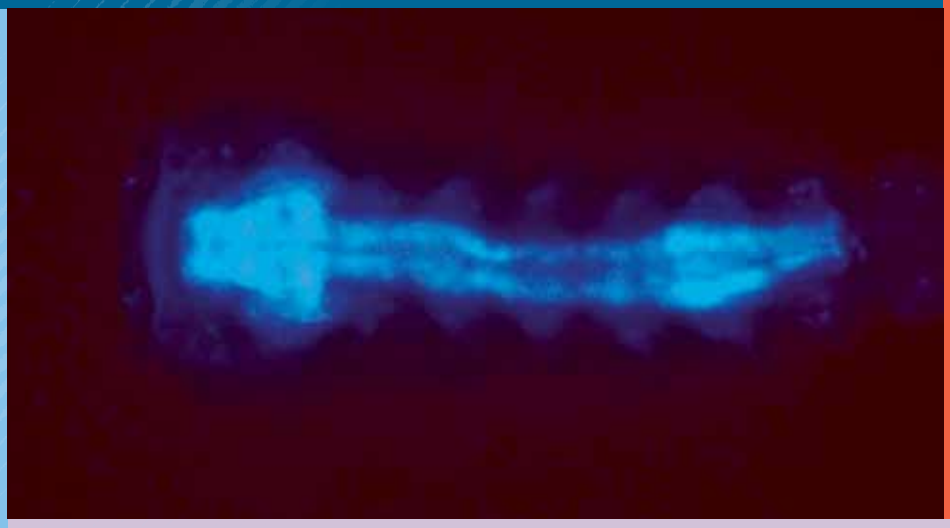
TRAINING MANUAL



Biosafety for human health and the environment in the context of the potential use of genetically modified mosquitoes (GMMs)

A tool for biosafety training based on courses in Africa, Asia and Latin America, 2008–2011

World Health Organization

TDR For research on diseases of poverty
UNICEF • UNDP • World Bank • WHO

WHO Library Cataloguing-in-Publication Data:

Biosafety for human health and the environment in the context of the potential use of genetically modified mosquitoes. Training Manual. A Tool for biosafety training (CD and internet version) based on courses in Africa, Asia and Latin America, 2008-2011 I.World Health Organization.

ISBN 978 92 4 154927 1
Subject headings are available from WHO institutional repository

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Graphic Design: Lisa Schwarb, Lausanne
Printed by the WHO Document Production Services (DUP), Geneva, Switzerland

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ACKNOWLEDGEMENTS

This *Training manual: Biosafety for human health and the environment in the context of the potential use of genetically modified mosquitoes (GMMs)* is based on biosafety training courses on GMMs undertaken in Africa, Asia and Latin America from 2008–2011. The courses were conceived by Yeya Touré, formerly Unit Leader (Vectors, Environment and Society), WHO/TDR, Geneva, Switzerland. Brij Kishore Tyagi (Asia course coordinator and Principal Investigator of the "Biosafety Manual-GMM Project") prepared the first draft and compiled the Manual with invaluable assistance from the late Madama Bouaré (Africa course coordinator), Maria Corena-McLeod and Ivan Velez (Latin America course coordinators), and all those involved in preparing the courses.

Acknowledgements are due to Dr Touré for his constant cooperation and guidance, and to the trainers and facilitators who contributed the manuscripts which form the basis of this Manual including: Adedapo O Adeogun; Georgina D Barnabas; Camilla J Beech; Bharat Char; Jhansi Charles; Azger Dusthacher; Arwa H Elaagip; Kumaresan Ganesan; T Jayalakshmi; P Paul Kumaran; HL Lee; John M Marshall; Judith Mary Hariprakash; Ritesh Mishra; Karthik Nagaraj; WA Nazni; Patricia N Okorie; Srinivas Parimi; N Selvakumar; Dziedzom K de Souza, Brij Kishore Tyagi and Vijay Veer (for authors' contact addresses please see Annex II).

Camilla J Beech (Head of Regulatory Affairs, Oxitec Ltd., Oxfordshire, United Kingdom), and Lee Han Lim (Head, Medical Entomology Unit & WHO Collaborating Centre for Vectors, Institute for Medical Research, Kuala Lumpur, Malaysia) reviewed the first draft of the Manual. Kittayapong Pattamaporn (Director, Center of Excellence for Vectors and Vector-Borne Diseases (CVVD), Faculty of Science, Mahidol University, Salaya, Nakhon Pathom 73170, Thailand) provided academic and scientific support and guidance.

Dr Toure's assistants, Ms Ekua Johnson and Ms Madhavi Jaccard-Sahgal (WHO/TDR, Geneva, Switzerland) and staff at the Centre for Research in Medical Entomology (Madurai, Tamil Nadu, India) provided valuable support during the realization of the project.

Karthigai Selvi, the project Research Assistant, assembled the material for the Manual. Diana Hopkins, external consultant, copy edited it. Lisa Schwarb, Lausanne designed the layout.

ABBREVIATIONS

ACL	Arthropod containment level
AeDNV	*Aedes aegypti* densovirus
AHTEG	Ad Hoc Technical Expert Group of the CBD
AIA	Advance Informed Agreement of the Cartagena Protocol
APVMA	Australian Pesticides and Veterinary Medicines Authority
BSL	Biosafety level
Bt	*Bacillus thuringiensis*
CBD	Convention on Biological Diversity
CFR	Case fatality rate
CHIKV	Chikungunya virus
CI	Cytoplasmic incompatibility
cm	Centimetre
CPB	Cartagena Protocol on Biosafety
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DBT	Department of Biotechnology, India
DDT	Dichlorodiphenyltrichloroethane
DEC	Disease-endemic country
DENV	Dengue virus
DNA	Deoxyribonucleic acid
EFSA	European Food Standards Authority
EU	European Union
F1	First filial generation; the offspring from a parental cross
FAO	Food and Agricultural Organization
FNIH	Foundation for the National Institutes of Health (USA)
G	gram
GEAC	Genetic Engineering Appraisal Committee, India
GM	Genetically modified
GMAC	Genetic Modification Advisory Committee, Malaysia
GMM	Genetically modified mosquito
GMO	Genetically modified organism

GMV	Genetically modified vector
HEG	Homing endonuclease gene
IBC	Institutional Biosafety Committee, India
IIBAT	International Institute of Biotechnology and Toxicology, India
IMR	Institute for Medical Research, Malaysia
IPM	Integrated pest management
IVM	Integrated vector management
JE	Japanese encephalitis
KFD	Kyasanur forest disease
L	litre
LAI	Laboratory-associated infection
LF	Lymphatic filariasis
LMM	Living modified mosquito
LMO	Living modified organism
M	metre
MEC	Monitoring and Evaluation Sub-Committee, India
ml	millimetre
MoEF	Ministry of Environment and Forests, India
MRCU	Mosquito Research and Control Unit, Cayman Islands
mRNA	Messenger RNA
MT	Metric ton
NBB	National Biosafety Board
NGO	Nongovernmental organization
NRE	Ministry of Natural Resources and Environment, Malaysia
NVBDCP	National Vector Borne Disease Control Programme, India
ORF	Open reading frame
PB	PiggyBac
PCR	Polymerase chain reaction
R&D	Research and development
RCGM	Review Committtee on Genetic Manipulation, India
RIDL	Release of insects carrying a Dominant Lethal
RNA	Ribonucleic acid

RNAi	RNA interference
Rs	Rupees India
SIT	Sterile insect technique
SOP	Standard operating procedure
TALEN	Transcription activator-like effect or nucleases
TCTF	Temporary contained trial facility
TDR	Special Programme for Research and Training in Tropical Diseases of the WHO
tTA	Tetracycline-repressible transcriptional activator
UN	United Nations
UNDP	United Nations Development Programme
UT	Union territories, India
VBD	Vector-borne disease
WHO	World Health Organization
wMelPop	Wolbachia strain
WNV	West Nile virus
WTO	World Trade Organization

DEFINITION OF TERMS

The following terms are defined in the context in which they are used in this manual.

Accountability
Accountability ensures that vectors, biological and other materials are controlled and traced as intended, by formally associating the specified materials with the individuals who provide oversight and are held responsible for them.

Arbovirus
A class of viruses transmitted to humans by arthropods such as mosquitoes and ticks.

Bioethics
The study of the ethical and moral implications of biological discoveries, biomedical advances, and their applications as in the fields of genetic engineering and drug research. Bioethics is one of the three components that contribute to a successful biorisk management culture.

Biological laboratory
A facility within which vectors and microorganisms, their components or their derivatives are collected, handled and/or stored. Biological laboratories include clinical laboratories, diagnostic facilities, regional and/or national reference centres, public health laboratories, research centres (academic, pharmaceutical, environmental, etc.), and production facilities (manufacturers of vaccines, pharmaceuticals, large-scale genetically modified organisms – GMOs, etc.) for human, veterinary and agricultural purposes.

Biorisk
The probability or chance that a particular adverse event, accidental infection or unauthorized access, loss, theft, misuse, diversion or intentional release, possibly leading to harm, will occur.

Biorisk assessment
The process to identify acceptable and unacceptable risks embracing biosafety and accidental infection risks, and laboratory biosecurity risks (risks of unauthorized access, loss, theft misuse, diversion or intentional release) and their potential consequences.

Biorisk management
The development of strategies to minimize the likelihood of the occurrence of biorisks. The management of biorisk places responsibility on the facility and its manager (director) to demonstrate that appropriate and valid biorisk reduction (minimization) procedures have been established and are being implemented. A biorisk management committee is established to assist the director to identify, develop and reach biorisk management goals.

Laboratory biosafety
Laboratory biosafety is the containment principles, technologies and practices that are implemented to prevent unintentional exposure to pathogens and toxins, or their accidental release.

Laboratory biosecurity
Laboratory biosecurity describes the protection, control and accountability for valuable biological materials (VBMs, see definition below) within laboratories, in order to prevent their unauthorized access, loss, theft, misuse, diversion or intentional release.

Genetically modified organisms (GMOs)
Organisms whose genetic material has been altered using techniques generally known as "recombinant DNA technology". Recombinant DNA technology is the ability to combine DNA molecules from different sources into one molecule in a test tube. GMOs are often not reproducible in nature, and the term generally does not cover organisms whose genetic composition has been altered by conventional cross breeding or by "mutagenesis" breeding, as these methods predate the discovery (1973) of recombinant DNA techniques.

Genetically modified vectors (GMVs)
Entomological vectors that have undergone the process of genetic modification, rendering them ineffective in carrying pathogenic agents responsible for causing the diseases.

Valuable biological materials (VBMs)
Biological materials that require, according to their owners, users, custodians, caretakers or regulators, administrative oversight, control, accountability, and specific protective and monitoring measures in laboratories to protect their economic and historical (archival) value, and/or the population from their potential to cause harm. VBMs may include pathogens and toxins, as well as non-pathogenic organisms, vaccine strains, foods, GMOs, cell components, genetic elements, and extraterrestrial samples.

Entomology
Branch of zoology dealing with the scientific study of insects, including their taxonomy, morphology, physiology and ecology. Applied aspects of entomology, such as the harmful and beneficial impact of insects on humans, are also studied.

Chapter 1
Introduction

Some of the world's most devastating vector-borne diseases (VBDs) are transmitted to people by blood-sucking arthropods, particularly mosquitoes. Rampant in most tropical and subtropical disease endemic countries (DECs), these VBDs affect billions of people globally and are of serious public health concern.

Population growth, poorly managed urbanization, the greater incursion of human activities into natural ecosystems, and the transition and expansion of the geographical distribution of vectors due to climatic changes have contributed to an unprecented growth in several VBDs, particularly dengue and malaria. This situation has been aggravated by the accidental spread of vectors and pathogens through increased global travel, and the collapse of vector control in public health programmes.

The conventional methods of controlling disease vectors, for example mosquito populations, which involve insecticide fogging, aerosol space spraying, larviciding, indoor residual insecticide spray have proved largely ineffective in reducing vector density. This is principally because mosquitoes have developed resistance to insectides, but also because the insectides are costly and environmentally hazardous.

This crisis has prompted the development of alternative safe methodologies or tools that effectively control diseases such as dengue and malaria. With the successful development of genetically modified (GM) crops and a few GM insects through the sterile insect technique (SIT) to control pests on fruit and agriculture produce, these new tools use genetic engineering to control some disease vectors. Several of them, such as the technology known as Release of Insects carrying a Dominant Lethal (RIDL™) gene against the dengue vector, *Aedes aegypti*, have shown great promise in rapidly reducing the vector population, thereby also reducing disease transmission in endemic areas.

1.1 Scientific innovations in vector control

The genetic engineering of insects or arthropods is not a recent invention in the scientific world. For a long time now, many countries have used it successfully against agricultural pests and disease vectors. Scientists and researchers have started using the same technology against insect vectors of human diseases by either suppressing or replacing vector populations with genetically modified vectors (GMVs), thus making the insect vectors unable to reproduce or transmit pathogens. Curtis proposed the idea of genetically controlling VBDs in 1968,[1] but the idea only became famous following the molecular manipulation of *Drosophila melanogaster* during the 1980s. In the last two decades, researchers around the world have focused on developing genetically modified mosquitoes (GMMs) as an effective strategy to control transmission of VBDs. The strategy has focused either on reducing the overall number of target mosquitoes to levels unable to support pathogen transmission (population suppression), or on introducing a genetic modification that renders the local mosquito population unable to transmit the pathogen (population replacement).

Under the population suppression strategy, SIT was at the forefront of pest insect control with release trials of sterile male mosquitoes being conducted during 1960–1970s. It is based on the principle of massive production, sex separation, sterilization, and subsequent release of large quantities of sterile male mosquitoes into targeted populations where they mate and produce non-viable offspring.[2,3] Although this method was effective against some agricultural pests, it had limited impact on controlling disease vectors because of the high costs involved.[4]

RIDL is a similar approach to SIT but with several improvements in that it offers solutions to issues experienced with SIT such as sex separation and sterilization by irradiation.[5-7] With this approach, GMMs carrying a dominant lethal gene are introduced into the field to mate and pass the gene onto their progeny. As a result, the female progeny die either in pupae or as adults without genetic repressor to survive or they are unable to fly. Thus, the female is unable to act as a vector, mate, seek a host or escape from predators. Laboratory modelling studies and small field trials have demonstrated the success of this approach. However, the scientific world still has to carry out field trials in various regions to independently monitor the impact of this technology on mosquito population suppression, disease reduction and ecosystems.

Another technique in GM technology is RNA interference (**RNAi**)[8] aimed at improving the mosquitoes' natural defence against viruses and suppressing virus replication. Research is also underway into homing endonuclease genes (**HEGs**), site-specific so-called selfish genetic elements[9,10] to examine ways to eliminate: (i) the gene required for disease transmission; (ii) the gene involved in survival and reproduction; and (iii) the sex-determining gene.

Another leading approach under the population replacement strategy is **MEDEA** which is a synthetic selfish genetic element first discovered in a species of flour beetle, *Tribolium castaneum*.[11] MEDEA is able to spread through a population causing the death of all offspring of heterozygous females that do not inherit the allele.

Since 1967, a pathogenic strain of *Wolbachia* (wMelPoP) for vector control has been used. Studies have found that *Wolbachia* act as a natural agent in suppressing disease[12,13] by making the vectors resistant to human pathogens. Some strains of *Wolbachia* can influence fecundity[14] or oogenesis[15] arresting the development of embryos whereas life-shortening strains of *Wolbachia* can dramatically reduce the longevity of adult female mosquitoes.[16]

Apart from these techniques, there are some new strategies in the pipeline such as the use of site- specific DNA lesion, transcription activator-like effector or nucleases (TALENs), and studies related to the microbial midgut of mosquito population. These are still in the initial stages of development and require further study.

Although these techniques are promising and are being accepted in some countries, opposition has been growing against the use of GMMs. It is argued that the reduction of mosquito species will give other species the opportunity to proliferate in the wild and may also pose an unknown ecological risk. Hence, there is a need to assess both the benefits and the risks associated with the release of GMMs on a case-by-case basis, and to develop safety precautions to address the associated social, legal, economic and ethical implications prior to the experimental release of any GMVs.

1.2 Legal frameworks and regulation

The concept of genetically modified organisms (GMOs) has been emphasized and considered by a number of international agencies including:

- United Nations Development Programme (UNDP)
- United Nations Food and Agriculture Organization (FAO)
- Pew Initiative on Food and Biotechnology
- World Bank
- WHO Special Programme for Research and Training in Tropical Diseases (WHO/TDR).

In 1990, the Special Programme for Research and Training in Tropical Diseases (TDR), together with the United Nations Children's Fund (UNICEF)/WHO/UNDP/World Bank, as well as the John D. and Catherine T. MacArthur Foundation and the University of Arizona, organized a meeting in Tucson, Arizona, hosting experts from various regions to discuss the control of VBDs through the genetic modification of mosquitoes.[17]

Since 1991, a series of technical consultations, planning meetings, and capacity building workshops[18–20] in London, Atlanta, Wageningen in 2001 and 2002, and Nairobi in 2004 were organized to address a number of issues concerned with GM insects.[21,22]

In 2009, the first technical consultation on the current status and future development of GMMs for malaria and dengue control was organized by WHO/TDR in collaboration with its partners and the US Foundation for the National Institutes of Health (FNIH) in Geneva.[22] Similar meetings were regularly organized to produce internationally accepted guidelines, principles and frameworks for testing and evaluating GMMs. Additionally, various research programmes on GMMs were also being supported and sponsored by WHO/TDR, the John D. and Catherine T. MacArthur Foundation, the Wellcome Trust, the Burroughs Wellcome Fund, the FNIH, the Bill & Melinda Gates Foundation's Grand Challenges in Global Health (GCGH), and other funding agencies.

As a part of the regulatory framework, the Cartagena Protocol on Biosafety (CPB) to the Convention on Biological Diversity (CBD) came into force in 2003. As of August 2014, there were 167 Parties and 103 signatories to the Convention.[23] The Protocol aims to ensure the safe handling, transport, and use of living modified organisms (LMOs) resulting from biotechnology, and to protect biological diversity and human health from the risks posed by the deliberate release of LMOs into the environment. The Conference of the Parties to the CBD serving as the Meeting of the Parties to the Prototol (COP-MOP) is the governing body of the Protocol and meets biennially. The Fourth meeting of the Conference of the Parties (COP-MOP 4) was held in Bonn, Germany, from 12–16 May 2008, following which guidance documents on risk assessment and risk management of living modified mosquitoes (LMMs) was developed through the joint endeavours of the "Open-ended Online Forum" and the Ad Hoc Technical Expert Group (AHTEG). This guidance was finalized and subsequently revised at the Fifth meeting of the Conference of the Parties (COP-MOP 5) in Nagoya, Japan, from 11–15 October 2010.[24,25] The revised version was presented at COP-MOP 6 in Hyderabad, India, from 1–5 October 2012.[26] This guidance largely focuses on living modified crop plants and risk assessment of LMMs of the *Culicidae* species. It maintains that the risk assessment will vary and different strategies need to be developed on the basis of the specific characteristics of the LMMs.[26]

The Protocol has been widely adopted in developing countries, but not in some countries with extensive experience of GMOs (e.g. Argentina, Australia, Canada and the USA). It requires the Parties to take specific decisions regarding the importation of LMOs for intentional introduction into the environment. At international level, the Protocol regulates open release, and ensures the safe international trade and exchange of GMOs as commodities with the aim of resolving trade disputes in the European Union (EU) market.

Some international organizations, including FAO, WHO and the United States Food and Drug Administration (FDA) have already published guidelines on the safety assessment of GM animals and their derived products. In 2010, the European Food Standards Authority (EFSA) also published a report entitled *Defining environmental risk assessment criteria for genetically modified insects to be placed on the EU market*[27] describing progress in the development of GM insects in terms of what might be placed on the European Union (EU) market in the next decade. It identifies the potential implications and methods to be investigated, and recommends a case-by-case approach for the environmental risk assessment of GM arthropods. Acting on a request from the European Commission, the EFSA developed two guidelines: (i) *Guidance on the risk assessment of food and feed from genetically modified animals and on animal health and welfare aspects;*[28] and (ii) *Guidance on the environmental risk assessment (ERA) of genetically modified animals*, published in 2012 and 2013, respectively.[29] The EFSA will publish a final guidance after considering comments from the public and the outcome of a public consultation undertaken in 2011 and 2012. Although international laws theoretically govern all issues related to GMOs, their effectiveness depends on the existence of national legislation. Some countries, including Brazil, Cayman Islands, Malaysia, Mexico and the USA, which had already undertaken the experimental release of GM insects, found that the slow development of biotechnology in vector control was due to the fact that: regulatory guidelines for permitting open release were scientifically inadequate; information for the public prior to release was virtually nonexistent;[30] and national legislation was either lacking or in the inception stage.

Thus, there is a need to balance the demand for GM tools to control disease with internationally accepted guidelines and legal frameworks for regulating research and development (R&D) related to GM insects. At present, WHO/TDR and the FNIH, in collaboration with many experts worldwide, have developed guidance for assessing the safety and efficacy of GMMs by addressing legal, ethical, social and cultural issues related to the release of GMMs.

1.3 Public engagement

The WHO carried out a trial study of SIT releases for multiple species, including *Aedes aegypti* and *Culex* spp., in Delhi, India, in the 1970s.[31–34] Unfortunately this project was prematurely terminated[35] because it lacked proper consultation and engagement with the government and the public.

Commonwealth Scientific and Industrial Research Organization (CSIRO) carried out an independent social and environmental risk analysis prior to the open-field testing of releases of *Wolbachia*-infected mosquitoes. This Australian government agency concluded that it carried negligible risk to both the environment and human safety.[36] The risk analysis was subsequently reviewed by an international panel of experts and was given to the Australian government with an analysis addressing social concerns. Such social surveys show that the concerns of the public and stakeholders must be thoroughly addressed, and that the public needs to be reassured that any alternative remedies, either biological or genetic, will not harm humans or the environment. These studies have given other countries a standard for public participation and demonstrated that obtaining appropriate consent from the community is one of the ethical requirements to be met prior to the initiation of any research activity in the community.

1.4 Capacity building

A pool of scientists from Africa, Asia and Latin America was selected to respond to the need for capacity building on GMVs in developing countries. The scientists provided training on biosafety risk and management assessments in the use of GMMs for disease control.[37,38] The Asian biosaftey training course (1–3 phases) on "Biosafety assessment for human health and environment using genetically modified vectors" was organized by WHO/TDR and coordinated by the Centre for Research in Medical Entomology (ICMR), Madurai, Tamil Nadu, India, where the recent strategies, progress and other associated issues were discussed. The goal of this course was threefold: (i) to increase Asian researchers' and decision-makers' awareness of issues and challenges such as the ethical, legal and social implications of the development and implementation of this technology; (ii) to ensure the feasibility and safety of GMVs in Asian countries; and (iii) to build capacity in Asia to ensure the safe development and implementation of GM technology.

1.5 Objectives and scope of the Manual

In partnership with international research units, WHO/TDR has undertaken multidisciplinary research into the management of VBDs in parallel with training programmes for multidisciplinary research teams. Following on from the WHO/TDR GMM biosafety courses in Africa, Asia and Latin America during 2008–2011, it was decided to prepare a manual pooling the knowledge and experience of the experts/scientists working in the field of GMOs. As a result, this Manual was compiled using the manuscripts from multidisciplinary experts from the various regions who attended these courses and who are working in the GMO field. The key objective of this Manual is "to provide collective perspectives and experience of the experts excelling in the field of LMOs/GMOs." It does not provide technical guidance on the selection of a specific approach.

1.6 Biosafety courses: objectives, content and outline

1.6.1 Course objectives

The genetic transformation of disease vectors has recently opened a new era in the control of viral and parasitic diseases by significantly reducing the ability of some vectors to transmit pathogens. Currently, there has been progress in the genetic transformation of *Aedes* and *Anopheles* mosquitoes to make them refractory to transmission of dengue virus and *Plasmodium falciparum*, respectively. However, this technology raises concerns not only in the scientific world, but also in the general population with regard to its safety for humans and the environment, handling, feasibility, efficiency, and release of such GMOs. With the central objective of creating a pool of regional scientists well trained in the assessment and management of biosafety related to the implementation of GMVs for the control of VBDs in Asia, the aim of the biosafety training courses was threefold:

i. increase awareness of Asian researchers and decision-makers to issues and challenges such as the ethical, legal and social implications related to the development and implementation of this technology;
ii. ensure the feasibility and safety of GMVs in Asian countries; and
iii. build capacity in Asia for the safe development and implementation of this technology.

The course trained dedicated researchers, vector biologists and decision-makers in vector control and in the assessment and management of biosafety for human health and the environment. The course was also open to local community leaders. Three courses were conducted in each of the African and Asian regions and two in the Latin American region. Close to 150 trainees, belonging to the target group enrolled and were trained over a period of three years (see Tables 1 to 3). These courses have, therefore, helped in the assessment and management of biosafety, and in the setting-up and management of regulatory principles and bodies.

1.6.2 Course content

Table 1. African region course

No.	Name	Title of presentation
1.1	Abdourahamane Sangare	Lessons learnt from the use of genetically modified plants in agriculture: risk and advantages of GMP
1.2		Transgenes: how are GMP made up?
2.1	Amidou Dembele	La convention des nations unies sur la diversité biologique (CDB)
2.2		Protocole de Cartagena sur la prévention des risques biotechnologiques
2.3		Cadre juridique national de Biosécurité (CNB)
2.4		Regime de responsabilite dans l'utilisation des OGMs
2.5		Introduction à la propriété intellectuelle
2.6		La brevetabilite du vivant: (1) bref rappel de l'état de la question; (2) position du droit malien ; et (3) analyse
2.7		Accès aux ressources génétiques et les droits de propriété intellectuelle: le biopiratage
2.8		La procédure malienne de prise de décision relative à la libération dans l'environnement d'un OGM
2.9		Présentation sommaire du droit de la proproriété intellectuelle en relation avec les OVM
2.10		L'accès aux ressources génétiques et l'interface avec le système actuel du droit de la propriété intellectuelle
2.11		Besoin d'amélioration du système de régulation de la biosécurité
2.12		Responsabilité en cas de dommage causé par un OGM
2.13		Présentation sommaire de la réglementation (internationale/ régionale) en matière de biosécurité

Table 1. African region course (continued)

No.	Name	Title of presentation
2.14		Identification de structures et de cadres juridiques relatifs aux insectes génétiquement modifiés
2.15		Mise en place et suivi d'une structure nationale de biosécurité
2.16		Missions of the control structures
2.17		Pouvoirs des structures de contrôle
3.1	John Marshall	Containment issues during planned field cage trials
3.2		Gene drive systems for spreading refractory genes
3.3		Public perspectives to genetically modified organisms in Western nations and Africa
3.4		The Cartagena Protocol and GM mosquitoes
3.5		Ethical issues related to GM mosquitoes
3.6		Gene drive systems and containment
3.7		GM mosquitoes for malaria control: perspectives of people in Mali, West Africa to a transgenic release
4.1	Ken Vernick	Engineering mosquitoes refractory to malaria and dengue fever in the laboratory
4.2		Identification of hazards and risks
4.3		Overview of genetic control methods for insect vectors
5.1	Madama Bouaré	Introduction to biosafety for humans and the environment
6.1	Abdoulaye M. Touré	Overview of disease vector control: issues and challenges
7.1	Samba Diop	Création et gestion d'un Comité national d'ethique: structure et role
7.2		Transparence, participation et communication avec le public
7.3		Course No 2: Implications éthiques et sociales dans l'utilisation des OGM
7.4		Course No 3: Implications éthiques et sociales dans l'utilisation des OGM
8.1	Willy K. Tonui	Risk management
8.2		Introduction to biosafety & biosecurity in laboratories
8.3		Overview on biosafety
9.1	BK Tyagi	Overview of the Asian biosafety training course format, objectives and general logistics
		Medical arthropodology: biosafety risk assessment overview
10.1	Camilla Beech & SS Vasan	Foundations of risk assessment and risk management
10.2		Risk management and development of emergency response plan

Table 2. Asian region course

No.	Name	Title of presentation	
1	BK Tyagi	1st ABTC	Risk assessment for arthropod vectors: GMVs and biosafety issues Medical arthropodology: biosafety risk assessment overview
		2nd ABTC	Overview of the Asian biosafety training course format, objectives and general logistics
		3rd ABTC	Overview of the Asian biosafety training course format, objectives and general logistics
2	SS Vasan	1st ABTC	Transgenic insects: from laboratory to field Innovative control using modified insect vectors Identification of legal frameworks and guidance documents in relation to GM vectors
		2nd ABTC	Transgenic insects: from laboratory to field Innovative control using modified insect vectors
		3rd ABTC	Innovative control using modified insect vectors Identification of legal frameworks and guidance documents in relation to GM vectors
3	Madama Bouaré	1st ABTC	Introduction to biosafety for humans and the environment
		2nd ABTC	Introduction to biosafety for humans and the environment
		3rd ABTC	Introduction to biosafety for humans and the environment
4	Camilla Beech	1st ABTC	Foundations of risk assessment and risk management Risk management and development of emergency response plan Monitoring and environmental impact assessment (Session 20A)
		2nd ABTC	From lab to field and use stepwise Identification of legal frameworks and guidance documents in relation to GM vectors Selecting a field site Cartagena Biosafety Protocol under the Convention on Biological Diversity Communications Sterile insect GM strategies status of RIDL
		3rd ABTC	Identification of legal frameworks and guidance documents in relation to GM vectors Cartagena Biosafety Protocol under the Convention on Biological Diversity Selecting a field site Monitoring and environmental impact assessment Risk management and development of emergency response plan
5	Vijay Veer	1st ABTC	Genetically modified vectors (GMVs), biodefence & bioterrorism
		2nd ABTC	Genetically modified vectors (GMVs), concern- biodefence & bioterrorism
		3rd ABTC	Genetically modified vectors (GMVs), bioterrorism & biodefence

Table 2. Asian region course (continued)

No.	Name	Title of presentation	
6	Selva Kumar	2nd ABTC	Basic safety measures in biological laboratories
		3rd ABTC	Basic safety measures in mycobacteriology laboratories
7	G Kumaresan	2nd ABTC	Molecular biology of transgenesis & heterologous gene expression
		3rd ABTC	Molecular biology of transposon mediated transgenesis strategies
8	Jhansi Charles	2nd ABTC	Biosafety issues in genetically modified organisms Importance of biosafety and medical microbiology – in a practitioner's perspective
		3rd ABTC	Importance of biosafety and medical microbiology – in a practitioner's perspective Packaging and transport of sputum specimens from the districts to the reference laboratory
9	Pattamaporn Kittiyapong	1st ABTC	Best practice guidance for deployment of genetic control methods against mosquito vectors in disease endemic countries
	Pattamaporn Kittiyapong (L Kriangkrai)	2nd ABTC	Best practice guidance for deployment of genetic control methods against mosquito vectors in disease endemic countries
10	P Paul Kumaran	2nd ABTC	Importance of biosafety: ethical Issues
		3rd ABTC	Importance of biosafety: ethical Issues
11	T Jeyalakshmi	2nd ABTC	Biosafety, regulatory and laboratory experience of the International Institute of Biotechnology and Toxicology (IIBAT)
		3rd ABTC	Biosafety, regulatory and laboratory experience of IIBAT
12	S Visalakshi	1st ABTC	Overview of the Cartagena Protocol: PART-1 Regional initiatives under Cartagena Protocol Overview of the Cartagena Protocol: PART-2 Incorporating ethical issues in making biotechnology policy
		2nd ABTC	Overview of the Cartagena Protocol and provisions of biosafety Regional initiatives under Cartagena Protocol Incorporating ethical issues in making biotechnology policy Ethical, socioeconomic, cultural issues in relation to use of GM vectors
13	Dr Lee	1st ABTC	First field release of transgenic *Aedes aegypti*. What needs to be done?
		2nd ABTC	First field release of transgenic *Aedes aegypti*. What needs to be done?
		3rd ABTC	First field release of transgenic *Aedes aegypti*. What needs to be done?

Table 2. Asian region course (continued)

No.	Name	Title of presentation	
14	TS Saraswathy	1st ABTC	Biosafety review process Communication plan
		2nd ABTC	Regulation and coordination required for a first transgenic release?
		3rd ABTC	Ethical, socioeconomic, cultural issues in relation to use of GM vectors Regulation and coordination required for a first transgenic release? Communication plan
15	Sarala Subbarao	2nd ABTC	Review of sterile male techniques in India during the 1960s & 70s
16	Bharat Char	1st ABTC	Biotech crops in India: from lab to reality
17	Ritesh Mishra	3rd ABTC	Effective use of modern biotechnology: GM crops
18	Worachart Sirawaraporn (on behalf of K Pat-tamaporn)	3rd ABTC	Best practice guidance for deployment of genetic control methods against mosquito vectors in disease endemic countries

Table 3. Latin American course

No.	Name	Title of presentation
1.1	Manuel Lluberas	A review of the current vector control methods and strategies from the scientific and practical point of view
1.2		A critical assessment of transgenic vector risks and impact on health and the environment based on previous experiences with conventional vector control programmes
2.1	Manuel Lluberas–Pilar Corena	Hypothetical release exercise 1: from the laboratory to the field
3.1	Ann Kramer	Introduction to GM vectors and relevance to human health
3.2		Why is it important to address biosafety in the context of GM insects of medical importance?
3.3		Introduction to biosafety and biosecurity and their relevance to humans and the environment
3.4		Principles and practices of biosafety and biosecurity under different conditions
4.1	Marcelo Jacobs-Lorena	Introduction to genetic control methods in the laboratory (population suppression, population replacement and paragenesis) and risk assessment considerations
4.2		Challenges for development and implementation of laboratory control methods using GMVs. Infrastructure, equipment and materials
4.3		Introduction to strategies for transgene containment and site selection
4.4		Genetic drive mechanism

Table 3. Latin American course (continued)

No.	Name	Title of presentation
4.5		Hazards and risks associated with handling of GMVs: regulatory and ethical issues
4.6		Transgenesis, paratransgenesis and other modifications of insects
5.1	BK Tyagi	Physical and biological characterization of the release site
5.2		Containment management systems including packaging and transport of GMVs
5.3		Ethical, social and legal implications of transgenic release and implications for cross-border movement of GMVs
5.4		Lessons learnt from Tsunami experience: what needs to be done prior to first transgenic release of *Aedes aegypti*
5.5		Rules, regulations, responsibilities, training research and field personnel
6.1	Rene Gato	Containment levels: facility design and practices
6.2		Conventional insect sterile technique (SIT) versus RIDL-SIT, with examples from public health and agriculture: containment facility design and work practices
7.1	Camilla Beech	MosqGuide
7.2		Overview of the Cartagena Protocol
7.3		Systematic risk assessment for GM vectors
7.4		Environmental risk management
7.5		Regulatory and legislative aspects of GMVs
7.6		Site selection/plan criteria
7.7		Principle of biosafety applied to genetically modified vectors and disease transmission.
8.1	Hervé Bossin	Moving GMVs from the lab to the field. Who is responsible for what in the event of an unintended accident?
9.1	Ivan D Velez	Vector behaviour and infection risk in the context with vector control in Latin America
9.2		Insectary design using biosafety principles
10.1	Elizabeth Hodson	Risk assessment, management and communication
11.1	Anita Villacis and Andre da Silva	Ethical, socioeconomic, cultural (ESC) and other implications of use of GMVs
11.2		Accidents in handling GMVs
12.1	Anita Villacis	Sterilization and disinfection in the laboratory

1.6.3 Course outline

The courses lasted two weeks and involved on average 15 trainees. The following major topics were covered:

(i) basic principles of the genetic manipulation of vectors and their potential impact on humans and the environment;

(ii) ethical, legal and social implications of the use of GMVs;

(iii) identification of potential hazards; assessment and management of risks for humans and the environment; risk/benefit analysis;

(iv) principles and practices for the assessment and management of biosecurity and biosafety in laboratories;

(v) guiding principles for the creation and management of institutional or national biosafety review boards and ethics review committees;

(vi) introduction to the development and application of a biosafety regulatory framework and its related legal principles at national levels for securing the development and use of vector control methods based on genetic modification strategies.

A post-course survey highlighted the fact that the wisdom gained in the three regional courses by representative trainees from all strata of public life on how to apply GMVs in future to control VBDs had advanced immensely. It formed a solid foundation for successfully implementing such a novel tool. The success of any disease control programme is after all based on such knowledge and the extent of advance preparation.

1.7 Conclusion

In the last two decades, the development of the application of molecular biology and genetic engineering in vector control has advanced with funding from international agencies. In parallel, consultations with scientists and various stakeholders have assessed the benefits and risks associated with different strategies in order to prepare standardized risk assessment methodologies, and universal guidelines and regulations encompassing all the issues relating to GMMs.

Although there has been rapid progress in the development of GM insects as an effective control tool, there is still controversy surrounding their use. However, innovative control methods to reduce the disease burden can only be realized when: (i) the scientific community, and national and international regulatory authorities address the issues surrounding the use of GM insects; and (ii) community participation is strengthened to ensure the success and sustainability of the programmes.

The development of vaccines and drugs against mosquito-borne diseases are also in progress. A reduction in VBDs can be reached when control strategies combine the development of genetically engineered (GE) strategies, vaccines and drugs along with modified existing insecticide control methods.

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Chapter 2
Overview of arthropod-borne diseases

Arthropods form the *Phylum Arthropoda* and include insects, spiders, centipedes, shrimp and crayfish. They are the most numerous phylum and account for approximately 80% of all living animal species. Insects belong to six orders of *Phylum Arthropoda* made up of Diptera, Hemiptera, Lepidoptera, Phthiraptera, Siphonaptera and Thysanoptera. However, the species of most interest to public health authorities are those found in the Diptera order comprising Psychodidae (sandflies), Corethrellidae (midges), Culicidae (mosquitoes), Simuliidae (black flies), Ceratopogonidae (biting midges), Tabanidae (horse flies, deer flies), and Anthericidae and Rhagionidae (snipe flies). Their contribution to the environment ranges from being pollinators to pests, and from disease-causing organisms to producers of economically important products. While butterflies, honeybees and silkworms are considered useful to humans, there are many insects harmful to human life, agricultural crops and animals because they transmit etiological agents to vertebrate hosts, called vectors. Pathogens (protozoa, helminthes, bacteria and viruses) transmitted by arthropod vectors, especially mosquitoes, ticks, sand flies, and midges, are some of the most dangerous and unpredictable inflicting heavy loss of life on both humans and livestock in some parts of the world, either directly by biting and sucking blood, or indirectly by transmitting VBDs. The world's most important arboviruses causing human diseases are listed in Table 4.

Table 4. The world's most important arboviruses causing human diseases

Arthropod	Vector	Family	Virus	Disease	Geographical distribution	Distribution in India
Mosquito	*Aedes aegypti*, *Ae. albopictus*	Flaviviridae	Dengue virus 1-4	Dengue	Tropical region	Yes
	Aedes aegypti, *Ae. albopictus*		Yellow fever virus	Yellow fever	Africa and South America	No
	Culex tritaeniorhynchus		Japanese encephalities virus	Japanese encephalitis	Asia, Pacific	Yes
	Culex annulirostris		Murray Valley encephalitis virus	Murray Valley encephalitis	Australia and Papua New Guinea	No
	Culex spp.		Rocio virus	Rocio	South America and Brazil	No
	Culex pipiens and *Culex quinquefasciatus*		St. Louis encephalitis virus	St. Louis encephalitis	North and South America	No
	Culex spp.		West Nile Virus	West Nile	Africa, Asia, Europe and North America	Yes

Table 4. The world's most important arboviruses causing human diseases (continued)

Arthropod	Vector	Family	Virus	Disease	Geographical distribution	Distribution in India
Mosquito	*Culex* spp.	Bunyaviridae	Rift Valley virus	Rift Valley fever	Africa, Middle East	No
	Aedes triseriatus		La Crosse virus	La Crosse encephalitis	North America	No
			California encephalitis virus	California encephalitis	Asia, Europe, North America	No
	Aedes aegypti, Aedes albopictus	*Alphavirus* (Togaviridae)	Chikungunya virus	Chikungunya	Africa and Asia region	Yes
	Culex annulirostris, Ae. vigilax, Ae. camptorhynchus		Ross River virus	Ross River	Australia, South Pacific	No
	Culicidae *Haemagogus* spp.		Mayaro Virus	Mayaro	South America	No
	Anopheles funestus, Anopheles gambiae		O'nyong-nyong virus	O'nyong-nyong fever	Africa	No
	Culex spp.		Sindbis virus	Sindbis	Africa, Australia, Egypt, Israel, Philippines	No
	Aedes notoscriptus		Barmah Forest virus	Barmah Forest	Australia	No
	Culiseta melanura and *Culiseta morsitans*		Eastern equine encephalitis virus	Eastern equine encephalitis	North, Central and South America and the Caribbean	No
	Culex tarssalis		Western equine encephalitis virus	Western equine encephalitis	Americas	No
	Aedes taeniorhynchus		Venezuelan equine encephalitis virus	Venezuelan equine encephalitis	Americas	No
	Anopheles spp.	Plasmodiidae	*Plasmodium spp.*	Malaria	Tropical and subtropical regions	Yes
	Anopheles and *Culex* spp.	Filarioidea	Wuchereria bancrofti, Brugia malayi, and Brugia timori	Lymphatic filariasis	Africa and South East Asia	Yes

Table 4. The world's most important arboviruses causing human diseases (continued)

Arthropod	Vector	Family	Virus	Disease	Geographical distribution	Distribution in India
Tick	*Haemaphysalis spinigera*	Flaviviridae	Kyasanar forest diseases virus	Kyasanar Forest Diseases	Saudi Arabia and South Asia (India)	Yes
	Dermacentor reticulatus, Dermacentor marginatus, Ixodes persulcatus		Omsk haemorrhagic fever virus	Omsk haemorrhagic fever	Western Siberia regions of Kurgen, Novosibirsk, Omsk and Tyumen	No
	Ixodes scapularis, Ixodes ricinus and *Ixodes persulcatus*		Tick-borne encephalitis virus (Russian spring summer encephalitis virus)	Tick-borne disease	Asia and Europe	No
	Hyalomma spp.	Bunyaviridae	Crimean Congo hemorrhagic virus	Congo-Crimean hemorrhagic fever	East and West Africa, Asia, the Balkans, Middle East	Yes
	Ornithodoros spp.	Borrelia	Borrelia bacteria	Tick-borne relapsing fever	Africa, Spain, Saudi Arabia, Asia, Canada and the western USA	No
	Ixodes ricinus (Europe), *Ixodes pacificus* (North America)		Spirochetal bacteria	Lyme disease	North America, Europe	No
	Ixodes holocyclus and *Ixodes tasmani*	Rickettsiaceae	Rickettsia bacteria	Queen land tick typhus	Australia	No
	Dermacentor variabilis		Rickettsia rickettsii	Rocky Mountain spotted fever	USA	No
	Ixodes ricinus		Rickettsia helvetica	Helvetica Spotted fever	Sweden, Switzerland, France	No
	Amblyomma americanum Lone star tick	Ehrlichiaceae	Anaplasma phagocytophilum	Granulocytic anaplasmosis	South-Atlantic South-Central	No
	Dermacentor andersoni, Dermacentor variabilis	Francisellaceae	Francisella tularensis	Tularemia	North America, Europe and Asia	No
	Dermacentor andersoni	Reoviridae (Coltivirus)	Colorado tick fever virus	Colorado tick fever	North America	No
	Ixodes scapularis, Ixodes pacificus	Babesiidae	*Babesia microti*	Babesiosis	North America	No

Table 4. The world's most important arboviruses causing human diseases (continued)

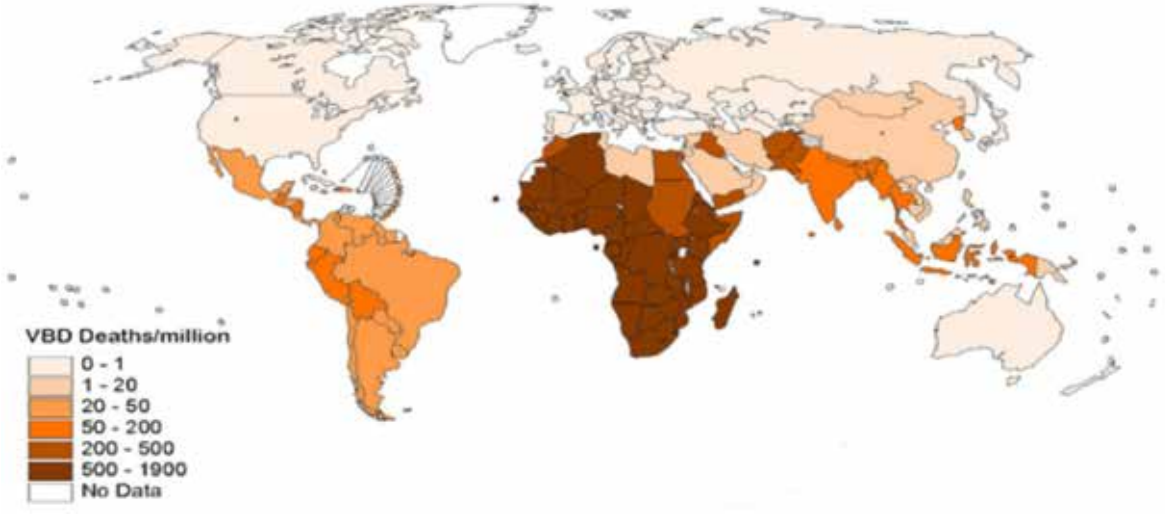
Arthropod	Vector	Family	Virus	Disease	Geographical distribution	Distribution in India
Tick	Brown Dog Tick *(Rhipicephalus sanguineus)*, Rocky Mountain Wood Tick *(Dermacentor andersoni)*, Lone Star Tick *(Amblyomma americanum)*	Coxiellaceae	*Coxiella burnetii*	Q fever	Eastern North America	No
Midge	*Culicoides paraensis*	Bunyaviridae	Oropouche virus	Oropouche	Central and South America	No
Sand Fly	Phlebotomine sand flies	Leishmania	Protozoan parasites	Leishmaniasis	East and North Africa, Europe	No
Black Fly	Black Fly	Onchocercidae	*Onchocerca volvulus*	Onchocerciasis (river blindness)	Africa, Latin America and Yemen	A rare case in India
Tse-tse fly	Glossina/Tse-tse fly	Trypanosoma	*Trypanosoma brucei gambiense, Trypanosoma brucei rhodesiense*	African trypanosomiasis/ sleeping sickness	Africa	No
Triatomine bug	Triatomine/kissing bugs	Trypanosoma	*Trypanosoma cruzi*	American trypanosomiasis (Chagas disease)	Latin America, Canada, European and Western Pacific countries	No
Fleas	Fleas that infest rats	Rickettsiaceae	*Rickettsia typhi*	Murine typhus	Africa, the Mediterranean, Southeast Asia, and the USA	No.
	Rats via fleas	Yersinia	*Yersinia pestis*	Plague	Africa, Asia and South America	No
Lice	*Pediculus humanus corporis*	Rickettsiaceae	*Rickettsia prowazekii*	Epidemic typhus	Africa, America and Asia	No
	Lice	Rickettsiaceae, Spirochaetaceae	*Rickettsia* and *Borrelia*	Epidemic relapsing fever	Ethiopia and Sudan	No
Mites	*Leptotrombidium* spp. (red mites)	Rickettsiaceae	*Orientia tsutsugamushi*	Scrub typhus	AsiaPacific region	Yes

2.1 Resurgence of VBDs globally

The most important challenge to global public health in the 21st century is the growth in VBDs which now account for over 17% of the estimated global burden of infectious diseases, with more than 1 billion cases and 1 million deaths annually.[1,2] Mosquitoes and ticks account for the majority of VBDs transmitted.[3] Today, mosquito-transmitted diseases are present in more than 100 countries worldwide, mainly in tropical and subtropical regions. They pose a major risk to half the world's population (Figure 1).[2] Malaria and dengue are the most prevalent mosquito-borne diseases with more than 3.3 billion and 2.5 billion people at risk for malaria and dengue, respectively. These two VBDs have a severe impact on economic and social development.[2]

Other VBDs such as West Nile virus (WNV) in the Americas, chikungunya and Japanese encephalitis (JE) in Asia and Oceania, and Rift Valley fever in Western and Eastern Africa are also rapidly emerging.[4] The global prevalence of the most important arboviral and mosquito-borne diseases is shown in Table 5. The emergence and re-emergence of arbovirus diseases of serious public health concern are attributed to a number of factors such as the growth in human populations, increased urbanization, the incursion of human activity into new ecosystems, increased global travel, climatic changes, insecticide and drug resistance, and genetic changes in pathogens.[5,6] Although VBDs are an important recent global health issue, the true magnitude of arboviral disease is difficult to quantify and is most likely underrepresented.

Figure 1. Global incidence of deaths from VBDs



Source: 1

2.2 India's public health emergency

India is the second most populous country in the world (75% of the population of the South Asian region live in India),[7] with over 1.2 billion people (2011 census). It is the tenth largest economy, with a gross domestic product (GDP) of US$ 1847.9 billion in 2011. India is drawing world attention not only because of its population explosion and economic liberalization but also because of prevailing and emerging health issues which account for 21% of the world's global burden of disease.[8] In 2005, it was estimated that malaria, dengue and other VBDs accounted for 1.6% of India's total disease burden.[9] Overall, out of 4.2 million disability adjusted life years (DALYs) lost to VBDs, malaria alone was responsible for an estimated 1.85 million DALYs annually in India.[10,11] India's climatic zones range from cold, wet alpine regions to semi-arid regions to the wet tropics, all of which favour the spread of a diverse number of vectors and pathogens of medical importance.[12] More than 130 arboviruses known to cause human diseases belong to one of three virus genera comprising *flavivirus, alphavirus* (Togaviridae) and *bunyavirus*.[13] The evolving epidemiology of major arboviral diseases is discussed in this Manual to illustrate the most important changes in public health concerns since the beginning of the 21st century.

2.3 Flavivirus

Flavivirus is a genus of viruses belonging to the family Flaviviridae which contains 70 recognized viruses including human and animal pathogenic viruses of global importance, of which nearly 50% produces clinical disease in humans.[14] Human flaviviruses are West-Nile virus (WNV), dengue virus (DENV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), yellow fever virus and hepacivirus – hepatitis C virus (HCV). More than 80% of the flaviviruses of importance to humans are transmitted to vertebrates by a bite from infected arthropods (mosquitoes or ticks). Hence, they are classified as arboviruses (arthropod-borne viruses). The flaviviruses can be divided into two clades: one with the vector-borne viruses and the other with no known vector.[15] The vector clade can be subdivided into a mosquito-borne clade and a tick-borne clade.[16] The former has two branches. One branch contains neurotropic viruses, which are spread by the *Culex* species and are predominantly associated with encephalitic disease. The second branch contains the non-neurotropic viruses, often associated with haemorrhagic disease and shock syndrome in humans where the *Aedes* species act as vectors. A tick-borne clade has two major groups: those that form the tick-borne encephalitis virus complex and are mainly associated with encephalitic disease; and those that infect seabirds and their associated ticks, for which no human diseases have been described.

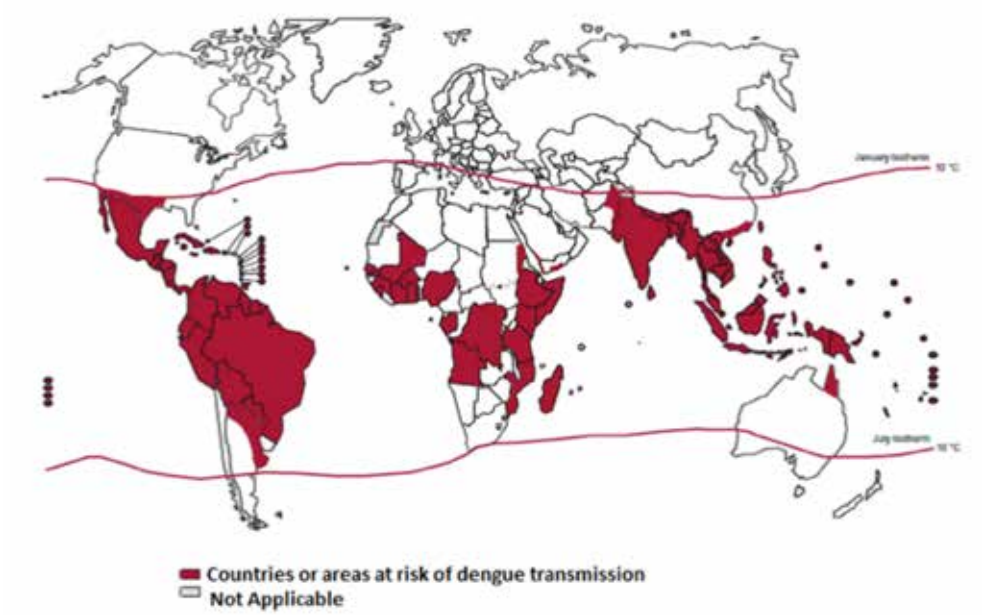
2.3.1 Mosquito-borne flavivirus infections in humans: distribution and health significance

A. Dengue

Dengue is the most important VBD affecting the human population and is likely to be more important than malaria globally in terms of morbidity with more than 70% of people living in Asian Pacific countries at risk of infection. It is endemic in more than 125 countries.[2,17,18] Although the full burden of disease is difficult to estimate, it is present in almost all WHO regions (Figure 2).[19] Dengue viruses (DENVs-1–4) are transmitted from one human host to another by mosquitoes of the *Aedes* genus, principally *Ae. aegypti* and *Ae. albopictus*.[20] A 2013 study estimated that annually there are 390 million dengue infections globally – 96 million apparent infections, and 294 million inapparent infections – which is threefold higher than the WHO's official estimate (Figure 3).[21] In the global burden of apparent dengue infection,

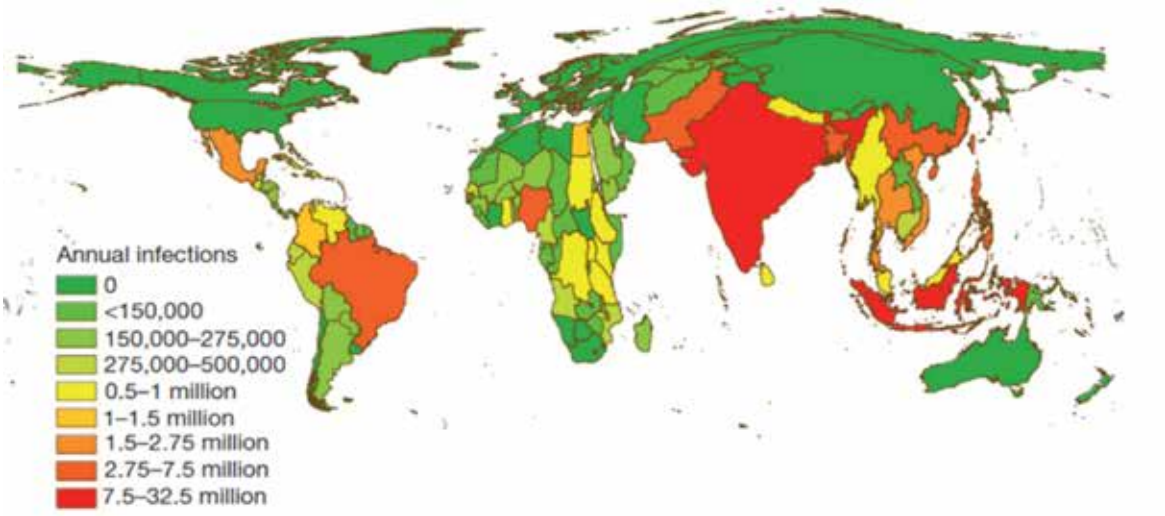
Asia accounts for 70% (67 million infections) of which India contributed 49% of the total disease burden in Asia and 34% (33 million infections) of the disease burden globally.[21] Figure 3 shows how India is contributing significantly to the high dengue burden compared to other continents.[21] Between 1996 and 2012, India reported a total of 197 440 dengue cases and 2049 deaths.[22] An average of 11 614 cases of dengue with a case fatality rate (CFR) of 1.04% was estimated per year for the country. The CFR was 3.3% in 1996, but thereafter it declined to about 1.0% until 2007. The most number of cases was found in northern Indian states (35.11%), followed by those in southern India (34.23%). Maximum dengue mortality was reported in the north (46.66%), followed by the south (22.94%), the west (19.96%), the east (5.95%) and the central region (4.49%). During the last two decades (1991–2012), more states have reported dengue cases with the number increasing from 4–8 by 2001 and to 34 by 2012. The high prevalence, lack of an effective vaccine, and absence of specific treatment make dengue fever a global public health concern.[23] The goal of the global strategy for dengue prevention and control 2012–2020 is to reduce morbidity by at least 25% and mortality by 50% by 2020, and to estimate the real burden of disease by 2015.[24] Many countries have national dengue prevention and control programmes to reduce the vector population but, in India, there is no such programme. However, biological and chemical vector control strategies, one of the components under the Integrated Disease Surveillance Programme (IDSP), are implemented for vector management. In addition, recent inter-country collaboration between the Serum Institute of India and the University of Mahidol in Thailand aims to develop a dengue vaccine, which signals significant changes in policy formulation aimed at controlling and eliminating the disease.[25]

Figure 2. Distribution of countries at risk of dengue transmission, 2011



Source: 19

Figure 3. Estimated burden of dengue by continent, 2010



Source: 21

B. Japanese encephalitis (JE)

JE is widespread over Southeast Asia and the Pacific where 3 billion people are at risk of infection (Figure 4). JE virus is the leading cause of viral encephalitis in Asia accounting for 68 000 clinical cases and 25–30% of CFR annually.[26,27] In 2011, it was estimated that approximately 67 900 cases occur each year in the 24 endemic countries; an incidence of 1.8 per 100 000 overall. It is primarily a disease of children and approximately 51 000 (75%) cases occur among children (0–14 years).[28] CFR in humans ranges from 25–30%, with 22% of patients left with objective neurological deficits and 28% with subnormal intelligence quotients.[29]

JE is transmitted by infective bites of female mosquitoes mainly belonging to the *Culex tritaeniorhynchus*, *Culex vishnui* and *Culex pseudovishnui* group. The disease was first reported in Japan in 1924 and subsequently reported in other Asian countries. In India, the first case was reported in 1955, where its chief vectors are the *Culex vishnui* group (*Cx. tritaeniorhynchus, Cx. vishnui* and *Cx. pseudovishnui*). WHO does not consider JE a neglected tropical disease (NTD) but it is an important neglected disease in India where it is widespread. It is most commonly found in rural rice-growing areas where flooded fields and irrigation systems favour the larval habitats of these vector mosquitoes. Up to 2012, about 17 states/union territories in India had reported cases of JE.[30] Since 2008, the highest number of cases reported was in 2011 (1214) and 2012 (745). It is estimated that the average incidence of cases and deaths annually is approximately 719 and 121, respectively. Eastern and central regions of India are the worst affected. Some effective vaccines (mouse brain-derived JE vaccine) are available on the market in India and, from time to time, the government launches a JE vaccination campaign in endemic districts.

See Table 6 for the annual distribution of dengue and JE mosquito-borne flavivirus infections in humans in India for the period 2007–2012.

Figure 4. Countries at risk of JE based on 2012 data

Source: 27

C. Yellow fever virus

Yellow fever is a viral haemorrhagic disease transmitted by the bite of *Aedes* mosquitoes, which principally affects humans and monkeys. The virus is commonly found in the tropical regions of Africa and the Americas where 900 million people are at risk. It is estimated that annually there are 200 000 cases and 30 000 deaths worldwide.[31] Approximately 90% of infection occurs in Africa. There is no specific treatment for yellow fever but it can be prevented and controlled by mass vaccination campaigns. Although conditions in Asia are favourable for transmission, no cases have so far been reported there.

D. West Nile Virus

WNV infections have commonly been reported in Africa, Asia, Europe and the United States of America (USA). The virus usually causes sub-clinical infection or mild infection in humans and horses although no cases in horses have been documented in India. Various *Culex* species such as *Cx. tritaeniorhynchus*, *Cx. vishnui*, *Cx. bitaeniorhynchus* and *Cx. univittatus*, *Cx. pipiens fatigans* and *Aedes albopictus,* act as potential vectors of WNV. Apart from mosquitoes, other arthropod-borne viruses, such as those found in ardeid birds, also play a possible role in the maintenance of WNV, as has been reported in India. Unlike dengue and JE, no serious epidemic of WNV has been reported in India. Antibodies against WNV were first detected in humans in Mumbai (formerly Bombay) in 1952.[32] WNV neutralizing antibodies (about 20–30%) were detected in human sera collected in Andhra Pradesh, Gujarat, Karnataka, Madhya Pradesh,

Maharashtra, Orissa, Rajasthan and Tamil Nadu. Most virus activity has been reported in southern, central and western India.[33] Prevalence and epidemiological data on WNV in India as a whole have not been documented well enough to assess its overall disease burden but some district- and state-based serological studies report on the prevalence of the virus. A vaccine is currently undergoing laboratory investigation but is not yet available.

2.3.2 Tick-borne flavivirus infections in humans

A. Kyasanur forest disease (KFD)

KFD is a tick-borne viral hemorrhagic fever endemic to South Asia.[34] The disease is caused by a virus belonging to the family *Flaviviridae*. The vector for disease transmission is *Haemaphysalis spinigera*, and, as it name suggests, it is a forest tick. The first KFD case was reported in 1956 in the Kyasanur Forest of Shimoga District, Karnataka State, India, where it was first recognized among monkeys.[35] This was followed by reports of a high incidence of cases among human beings living in the neighbouring forest areas.[35] In 1956, it was detected in four villages and, by 1957, it had spread to 20 villages and had affected more than 70 villages covering four districts near Shimoga district. Although KFD was initially viewed as a rare disease, it is now ranked as a high-risk pathogen requiring Biosafety Level 3 (BSL-3) handling because it has caused numerous infections among field and laboratory personnel.

2.4 Alphavirus (*Togaviridae*)

The *Togaviridae* are a family of viruses, consisting of four genera, such as rubivirus (rubella virus), pestivirus (bovine viral diarrhoea virus, hog cholera virus and border disease virus), arterivirus (equine arteritis virus) and alphavirus. There are currently 29 recognized alphaviruses, of which 11 are pathogenic for humans. All alphaviruses are mosquito-borne and are distributed on all inhabited continents. They can be classified into two types of viruses: encephalitic viruses (e.g. eastern, western, or Venezuelan equine encephalitis viruses) and arthritic viruses (e.g. chikungunya, Ross River, Mayaro, O'nyong-nyong, Semlike forest and Sindbis viruses).[36]

2.5 Mosquito-borne alphavirus infections in humans

Chikungunya

Chikungunya fever (CHIK fever) is caused by chikungunya virus (CHIKV), which is found in Africa, Southeast Asia and India.[37,38] *Aedes albopictus* is the main vector for the transmission of CHIKV infection. CHIKV causes febrile illness similar to dengue virus infection but it primarily affects the peripheral small joints and leads to prolonged arthralgic syndrome. CHIKV was first isolated in the United Republic of Tanzania (formerly Tanganyika) in 1953 during an outbreak of dengue-like illness. It was then repeatedly detected in African and Asian countries. From the 1960s to 2003, frequent outbreaks were reported in Cambodia, India, Indonesia, Malaysia, Myanmar, Pakistan, Thailand and Viet Nam.[39,40] The entry point of CHIKV in India is unknown although outbreaks were reported in 1963 in Kolkatta state, and in 1965 in Tamil Nadu (Chennai) when more than 300 000 people were affected with adverse haemorrhagic complications.[41,42] Between 1973 and the end of 2005, no CHIKV cases were reported and it was thought it had almost disappeared from the Indian subcontinent. However, in 2006, it re-emerged in Karnataka and Andhra Pradesh, southern India. In 2006, approximately 1.11 million people were affected with an infection rate of 4–45%.[43] Table 7 shows the prevalence

of CHIKV, and the incidence of morbidity and mortality of malaria in India between 2007 and 2012. CHIKV has been prevalent since 2006, and has continuously affected a proportion of people with an average of 52 078 cases annually.

Between 2007 and 2012, the highest number of cases was documented in 2008 and 2009, respectively. As with dengue, CHIV infection has become a leading vector-borne disease of public health importance in terms of its high morbidity. Since there is no available vaccine, the only way of preventing the disease is by using protective equipment.

2.6 Other mosquito-borne infectious diseases in humans

A. Malaria

Malaria is a life-threatening disease prevalent in tropical and sub-tropical regions. In 2010, WHO estimated that, globally, there were approximately 219 million cases of malaria (with an uncertainty range of 154–289 million) and an estimated 660 000 deaths (with an uncertainty range of 490 000–836 000). The African region was highly affected with around 174 million cases, followed by South-East Asia with 28 million cases. Approximately 70% (1.75 million) of reported cases in the WHO South-East Asia Region were in India.

Malaria is caused by four parasites: *Plasmodium falciparum*; *Plasmodium vivax*; *Plasmodium malariae*; and *Plasmodium oval*. It is transmitted by the bite of infected female mosquitoes of the genus *Anopheles* (in India, *An. culicifacies* and *An. Fluviatilis* are responsible for the transmission of 60–70% and 15–20% of cases, respectively). Two types of parasites of human malaria – *P. vivax* and *P. falciparum* – are commonly reported in India.

About 80.5% of India's population resides in malaria-endemic areas, with 4.2%, 32.5% and 43.8% living in areas of high, moderate and low risk for malaria, respectively. In 1947, approximately 75 million cases were documented in India resulting in 0.8 million deaths. The government launched a National Malaria Control Programme in 1953. Table 8 shows the decline in the prevalence of malaria in India from 1995 to 2012. The 20th century saw a gradual decline in reported cases but there has still been a high case load of around 1.5 million annually despite India's introduction of a number of modified anti-malaria programmes, malaria eradication projects and anti-malaria drug policies as well as recent progress in clinical and medical research. Northern, central and eastern states have seen the highest incidence of morbidity and mortality, and the southern region has registered the lowest. The Joint Monitoring Mission Report (2007) of the National Vector Borne Disease Control Programme (NVBDCP) revealed that the true burden of malarial disease is unknown because health professionals in the private sector see more than 50% of the cases. These data are not captured by the government's surveillance system, which suggests that the incidence of malaria is underreported.

B. Lymphatic filariasis (LF)

LF is commonly known as elephantiasis. Approximately 90% of infections are caused by *Wuchereria bancrofti*, 9.9% by *Brugia malayi* and the remaining 0.1% by *Brugia timori*. *Cx. quinquefasciatus* is the vector of *W. bancrofti*. WHO estimates[44] that approximately 1.43 billion people are at risk globally, 65% of whom live in the South-East Asia region, 30% in the Africa region and the remainder in other parts of the tropics. Globally, more than 120 million infections are reported at present – with over 40 million patients severely affected. There are 78 million cases in South-East Asia, 40% of which are in India.

Table 5. The global prevalence of world's most important mosquito-borne diseases

Vector	VBD	Parasite/ pathogen	Global distribution	Pop. at risk	People infected	Regions											
						Africa			Americas			South-East Asia			Western Pacific		
						CFR[a]	Cases[b]	Deaths	CFR[a]	Cases[b]	Deaths	CFR[a]	Cases[b]	Deaths	CFR[a]	Cases[b]	Deaths
Anopheles spp.	Malaria	*Plasmodium* spp.	Tropical and subtropical regions – sub-Saharan Africa, Asia and the Americas; 60% of cases occurring in Africa, South of the Sahara	3.6 billion	219 million	0.343	174	596 000	0.109	1.1	1200	0.136	28	38 000	0.23	2	4600
Aedes aegypti, Aedes albopictus	Dengue	*Flavivirus*	Tropical and subtropical countries; 75% of people at risk (1.8 billion) live in the Asia-Pacific region	2.5 billion	50–100 million	–			0.053	1.6	804	0.55	0.36	1959	0.3	0.35	1075
Anopheles and Culex spp.	Lymphatic filariasis	*Wucheria bancrofti*	65% of people at risk reside in the South-East Asia region, 30% in the Africa region and the remainder in other parts of the tropics	1.43 billion	120 million	–	36	–	–	1.2	–	–	78	–	–	3.6	–
Aedes aegypti, Aedes albopictus	Chikungunya	Alphavirus	Africa and Asia regions	5.17 billion	1.67 million	–											

Table 5. The global prevalence of world's most important mosquito-borne diseases (continued)

Vector	VBD	Parasite/ pathogen	Global distribution	Pop. at risk	People infected	Regions											
						Africa			Americas			South-East Asia			Western Pacific		
						CFR[a]	Cases[b]	Deaths	CFR[a]	Cases[b]	Deaths	CFR[a]	Cases[b]	Deaths	CFR[a]	Cases[b]	Deaths
Aedes aegypti, Aedes albopictus	Yellow fever[c]	Flavivirus	Tropical and subtropical areas in Africa and South America, but not Asia; 90% of infections occur in the Africa region.	900 million	0.0026 million[d]	12.5	0.0001	16	46.85	0.0001	52	Nil			Nil		
Culex tritaeniorhynchus	Japanese encephalitis	Flavivirus	Countries of the Asia-Pacific region	3 billion	0.068 million[d]	Nil			Nil			20–30	0.009[d]	2191–2629	20–30	0.002[d]	360–550
Culex pipiens and *Culex quinquefasciatus*	St. Louis encephalitis	Flavivirus	North and South America	900 million	0.0001 million	Nil			0	0.000008	0	Nil			Nil		
Culex spp.	Rift Valley fever	Phlebovirus	Sub-Saharan Africa	–	–	10.74	0.00024[e]	26	Nil			Nil			Nil		
Culex tarsalis	Western equinine encephalitis (WEE)	Alphavirus	USA	–													
Culiseta melanura	Eastern equnine encephalities	Alphavirus	USA	–													
Culex pipiens, Culex tarsalis and *Culex quinquefasciatus*	West Nile virus	Flavivirus	Africa, Central Asia, Europe, the Middle East, North America and South-West Asia	–	–	–			4.51	0.00539[f]	243	–			–		

– : no data.

[a] Case fatality rate.

[b] In millions.

[c] Data for 2004.

[d] Reported cases in 2011.

[e] Data provided by the National Institute for Communicable Diseases (NICD), South Africa, 2011.

[f] Data provided by Centers for Disease Control and Prevention (CDC), USA, 2012.

Source: Data from WHO.

Table 6. Annual distribution of mosquito-borne flavivirus infections in humans (dengue and JE), India, by region and state, 2007–2012

Region	Affected states/ union territories	Dengue cases (C) and deaths (D)														JE cases (C) and deaths (D)											
		2007		2008		2009		2010		2011		2012		Average (2007–12)		2008		2009		2010		2011		2012		Average (2008–12)	
		C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D
North	Chandigarh	99	0	167	0	25	0	221	0	73	0	351	2	156	0	0	0	0	0	0	0	0	0	0	0	0	0
	Delhi	548	1	1312	2	1153	3	6259	8	1131	8	2093	4	2083	4	0	0	0	0	0	0	9	0	0	0	2	0
	Haryana	365	11	1137	9	125	1	866	20	267	3	768	2	588	8	0	0	1	0	1	0	12	3	3	0	3	1
	Himachal Pradesh	0	0	0	0	0	0	3	0	0	0	73	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0
	J & K[a]	0	0	0	0	2	0	0	0	3	0	17	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0
	Punjab	28	0	4349	21	245	1	4012	15	3921	33	770	9	2221	13	0	0	0	0	0	0	0	0	0	0	0	0
	Rajasthan	540	10	682	4	1389	18	1823	9	1072	4	1295	10	1134	9	0	0	0	0	0	0	0	0	0	0	0	0
	Uttrakhand	0	0	20	0	0	0	178	0	454	5	110	2	127	1	10	0	0	0	7	0	0	0	1	0	4	0
Total		1580	22	7667	36	2939	23	13 362	52	6921	53	5477	30	6324	36	10	0	1	0	8	0	21	3	4	0	9	1
South	A & N Islands[b]	0	0	0	0	0	0	25	0	6	0	24	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0
	Andhra Pradesh	587	2	313	2	1190	11	776	3	1209	6	2299	2	1062	4	16	0	35	0	7	5	4	1	3	0	13	1
	Karnataka	230	0	339	3	1764	8	2285	7	405	5	3924	21	1491	7	0	0	7	0	3	0	23	0	1	0	7	0
	Kerala	603	11	733	3	1425	6	2597	17	1304	10	4172	15	1806	10	0	0	0	0	0	0	37	3	2	0	8	1
	Puduchery	274	0	35	0	66	0	96	0	463	3	3506	5	740	1	0	0	0	0	0	0	0	0	0	0	0	0
	Tamil Nadu	707	2	530	3	1072	7	2051	8	2501	9	12 826	66	3281	16	7	0	18	0	11	1	24	3	25	4	17	2
Total		2401	15	1950	11	5517	32	7830	35	5888	33	26 751	109	8390	39	23	0	60	0	21	6	88	7	31	4	45	3

Table 6. Annual distribution of mosquito-borne flavivirus infections in humans (dengue and JE), India, by region and state, 2007–2012 (continued)

Region	Affected states/ union territories	Dengue cases (C) and deaths (D)														JE cases (C) and deaths (D)											
		2007		2008		2009		2010		2011		2012		Average (2007–12)		2008		2009		2010		2011		2012		Average (2008–12)	
		C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D
East	Arunachal Pradesh	0	0	0	0	0	0	0	0	0	0	346	0	58	0	0	0	0	0	0	0	0	0	0	0	0	0
	Assam	0	0	0	0	0	0	237	2	0	0	1058	5	216	1	157	33	218	46	142	40	489	113	463	100	294	66
	Bihar	0	0	1	0	1	0	510	0	21	0	872	3	234	1	0	0	0	0	0	0	145	18	8	0	31	4
	Jharkhand	0	0	0	0	0	0	27	0	36	0	42	0	18	0	0	0	0	0	2	2	101	5	1	0	21	1
	Manipur	51	1	0	0	0	0	7	0	220	0	6	0	47	0	0	0	1	0	45	5	9	0	0	0	11	1
	Meghalaya	0	0	0	0	0	0	1	0	0	0	27	2	5	0	0	0	0	0	0	0	0	0	0	0	0	0
	Mizoram	0	0	0	0	0	0	0	0	0	0	6	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	Nagaland	0	0	0	0	25	0	0	0	3	0	0	0	5	0	0	0	9	2	2	0	29	5	0	0	8	1
	Orissa	4	0	0	0	0	0	29	5	1816	33	2255	6	684	7	0	0	0	0	0	0	0	0	0	0	0	0
	Sikkim	0	0	0	0	0	0	0	0	2	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	Tripura	0	0	0	0	0	0	0	0	0	0	9	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	West Bengal	95	4	1038	7	399	0	805	1	510	0	6456	11	1551	4	41	1	57	5	1	0	101	3	87	13	57	4
Total		**150**	**5**	**1039**	**7**	**425**	**0**	**1616**	**8**	**2608**	**33**	**11 079**	**27**	**2820**	**13**	**198**	**34**	**285**	**53**	**192**	**47**	**874**	**144**	**559**	**113**	**422**	**78**
West	D&N Haveli[c]	0	0	0	0	0	0	46	0	68	0	156	1	45	0	0	0	0	0	0	0	0	0	0	0	0	0
	Daman and Diu Union Territory	0	0	0	0	0	0	0	0	0	0	96	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0
	Goa	36	0	43	0	277	5	242	0	26	0	39	0	111	1	3	0	1	0	9	0	1	0	9	0	5	0
	Gujarat	570	2	1065	2	2461	2	2568	1	1693	9	3067	6	1904	4	0	0	0	0	0	0	0	0	0	0	0	0
	Maharashtra	614	21	743	22	2255	20	1489	5	1138	25	2931	59	1528	25	0	0	4	0	0	0	6	0	3	0	3	0
Total		**1220**	**23**	**1851**	**24**	**4993**	**27**	**4345**	**6**	**2925**	**34**	**6289**	**66**	**3604**	**30**	**3**	**0**	**5**	**0**	**9**	**0**	**7**	**0**	**12**	**0**	**7**	**0**
Central	Chattisgarh	0	0	0	0	26	7	4	0	313	11	45	0	65	3	0	0	0	0	0	0	0	0	0	0	0	0
	Madhya Pradesh	51	2	3	0	1467	5	175	1	50	0	239	6	331	2	0	0	0	0	0	0	0	0	0	0	0	0
	Uttar Pradesh	132	2	51	2	168	2	960	8	155	5	342	4	301	4	193	36	302	50	325	59	224	27	139	23	237	39
Total		**183**	**4**	**54**	**2**	**1661**	**14**	**1139**	**9**	**518**	**16**	**626**	**10**	**697**	**9**	**193**	**36**	**302**	**50**	**325**	**59**	**224**	**27**	**139**	**23**	**237**	**39**
Overall total		**5534**	**69**	**12 561**	**80**	**15 535**	**96**	**28 292**	**110**	**18 860**	**169**	**50 222**	**242**	**21 834**	**128**	**427**	**70**	**653**	**103**	**555**	**112**	**1214**	**181**	**745**	**140**	**719**	**121**

[a] J & K, Jammu and Kashmir State.

[b] A & N Islands, Andaman and Nicobar Islands.

[c] D&N Haveli, Dadra and Nagar Haveli Union Territory.

Source: Data provided by the NVBDCP, India.

Table 7. Prevalence of chikungunya and malaria cases and deaths, by region, states and union territories, India, 2007–2012

Region	Affected states/ UTs[a]	Chikungunya cases							Malaria cases (C) and deaths (D)									
		2007	2008	2009	2010	2011	2012	Average (2007–12)	2009		2010		2011		2012		Average (2009–12)	
									C	D	C	D	C	D	C	D	C	D
North	Chandigarh	0	0	0	0	1	0	0	430	0	351	0	582	0	201	0	391	0
	Delhi	203	14	18	120	110	6	79	169	0	251	0	413	0	382	0	304	0
	Haryana	20	35	2	26	215	9	51	30 168	0	18 921	0	33 401	1	26 819	1	27 327	1
	Himachal Pradesh	0	0	0	0	0	0	0	192	0	210	0	247	0	216	0	216	0
	J & K[b]	0	0	0	0	0	0	0	346	0	802	0	1091	0	864	0	776	0
	Punjab	0	0	0	1	0	1	0	2955	0	3477	0	2693	3	1689	0	2704	1
	Rajasthan	2	3	256	1326	608	172	395	32 709	18	50 963	26	54 294	45	45 809	22	45 944	28
	Uttrakhand	0	0	0	0	18	0	3	1264	0	1672	0	1277	1	1948	0	1540	0
Total		**225**	**52**	**276**	**1473**	**952**	**188**	**528**	**68 233**	**18**	**76 647**	**26**	**93 998**	**50**	**77 928**	**23**	**79 202**	**29**
South	A & N Islands[c]	0	0	0	59	96	256	69	5760	0	2484	0	1918	0	1539	0	2925	0
	Andhra Pradesh	39	5	591	116	99	2827	613	25 152	3	33 393	20	34 949	5	24 699	2	29 548	8
	Karnataka	1705	46 510	41 230	8740	1941	2382	17 085	36 859	0	44 319	11	24 237	0	16 466	0	30 470	3
	Kerala	24 052	24 685	13 349	1708	183	66	10 674	2046	5	2299	7	1993	2	2036	3	2094	4
	Lakshad-weep	5184	0	0	0	0	0	864	8	0	6	0	8	0	9	0	8	0
	Puduchery	0	0	0	11	42	45	16	65	0	175	0	196	1	143	0	145	0
	Tamil Nadu	45	46	5063	4319	4194	5018	3114	14 988	1	17 086	3	22 171	0	18 869	0	18 279	1
Total		**31 025**	**71 246**	**60 233**	**14 953**	**6555**	**10 594**	**32 434**	**84 878**	**9**	**99 762**	**41**	**85 472**	**8**	**63 761**	**5**	**83 468**	**16**

Table 7. Prevalence of chikungunya and malaria cases and deaths, by region, states and union territories, India, 2007–2012 (continued)

Region	Affected states/ UTs[a]	Chikungunya cases							Malaria cases (C) and deaths (D)									
		2007	2008	2009	2010	2011	2012	Average (2007–12)	2009		2010		2011		2012		Average (2009–12)	
									C	D	C	D	C	D	C	D	C	D
East	Arunachal Pradesh	0	0	0	0	0	0	0	22 066	15	17 944	103	13 950	17	8368	15	15 582	38
	Assam	0	0	0	0	0	0	0	91 413	63	68 353	36	47 397	45	29 999	13	59 291	39
	Bihar	0	0	0	0	91	34	21	3255	21	1908	1	2643	0	2605	0	2603	6
	Jharkhand	0	0	0	0	816	86	150	230 683	28	199 842	16	160 653	17	131 476	10	180 664	18
	Manipur	0	0	0	0	0	0	0	1069	1	947	4	714	1	255	0	746	2
	Meghalaya	0	0	0	16	168	0	31	76 759	192	41 642	87	25 143	53	20 834	52	41 095	96
	Mizoram	0	0	0	0	0	0	0	9399	119	15 594	31	8861	30	9883	25	10 934	51
	Nagaland	0	0	0	0	0	0	0	8489	35	4959	14	3363	4	2891	1	4926	14
	Orissa	4065	4676	2306	544	236	129	1993	380 904	198	395 651	247	308 968	99	262 842	79	337 091	156
	Sikkim	0	0	0	0	0	0	0	42	1	49	0	51	0	77	0	55	0
	Tripura	0	0	0	0	0	0	0	24 430	62	23 939	15	14 417	12	11 565	7	18 588	24
	West Bengal	19 138	17 898	5270	20 503	4482	1381	11 445	141 211	74	134 795	47	66 368	19	55 793	30	99 542	43
Total		**23 203**	**22 574**	**7576**	**21 063**	**5793**	**1630**	**13 640**	**989 720**	**809**	**905 623**	**601**	**652 528**	**297**	**536 588**	**232**	**771 115**	**485**
West	D&N Haveli[d]	0	0	0	0	0	100	17	3408	0	5703	0	5150	0	4940	1	4800	0
	Daman and Diu Union Territory	0	0	0	0	0	0	0	97	0	204	0	262	0	186	0	187	0
	Goa	93	52	1839	1429	664	571	775	5056	10	2368	1	1187	3	1714	0	2581	4
	Gujarat	3223	303	1740	1709	1042	1317	1556	45 902	34	66 501	71	89 764	127	76 246	29	69 603	65
	Maharash-tra	1762	853	1594	7431	5113	1544	3050	93 818	227	139 198	200	96 577	118	58 517	96	97 028	160
Total		**5078**	**1208**	**5173**	**10569**	**6819**	**3532**	**5397**	**148 281**	**271**	**213 974**	**272**	**192 940**	**248**	**141 603**	**126**	**174 200**	**229**
Central	Chattisgarh	0	0	0	0	0	0	0	129 397	11	152 209	47	136 899	42	124 006	90	135 628	48
	Madhya Pradesh	0	0	30	113	280	20	74	87 628	26	87 165	31	91 851	109	76 538	43	85 796	52
	Uttar Pradesh	4	11	0	5	3	13	6	55 437	0	64 606	0	56 968	0	47 400	0	56 103	0
Total		**4**	**11**	**30**	**118**	**283**	**33**	**80**	**272 462**	**37**	**303 980**	**78**	**285 718**	**151**	**247 944**	**133**	**277 526**	**100**
Overall total		**59 535**	**95 091**	**73 288**	**48 176**	**20 402**	**15 977**	**52 078**	**1563574**	**1144**	**1599986**	**1018**	**1310656**	**754**	**1067824**	**519**	**1385510**	**856**

[a] Union territories.

[b] J & K, Jammu and Kashmir State.

[c] A & N islands, Andaman and Nicobar Islands.

[d] D&N Haveli, Dadra and Nagar Haveli Union Territory.

Source: Data provided by the NVBDCP, India.

In India, around 600 million people in 250 districts (20 states/union territories) are at risk of LF infection. The *Wuchereria bancrofti* species is responsible for 99.4% of all infections and *Brugia malayi* is responsible for the remaining 0.6%. LF infection is prevalent in all regions except the northern states. To combat LF, the government launched the National Filariasis Control Programme (NFCP) in 1955, and introduced a National Health Policy in 2002 with the aim of eliminating the disease by 2015. A gradual decrease in microfilaria rates has been observed over a number of years. In 2004 and 2011, it was reported to have decreased to 1.24% and 0.34%, respectively. The main control measures were mass administration of diethylcarbamazine, anti-larval measures in urban areas, and indoor residual spraying in rural areas.

Table 8. Decline in malaria morbidity and mortality, India, 1995–2012

Year	Population ('000)	Total malaria cases (million)	Deaths
1995	888 143	2.93	1151
1996	872 906	3.04	1010
1997	884 719	2.66	879
1998	910 884	2.22	664
1999	948 656	2.28	1048
2000	970 275	2.03	932
2001	984 579	2.09	1005
2002	1 013 942	1.84	973
2003	1 027 157	1.87	1006
2004	1 040 939	1.92	949
2005	1 082 882	1.82	963
2006	1 072 713	1.79	1707
2007	1 087 582	1.51	1311
2008	1 119 624	1.53	1055
2009	1 150 113	1.56	1144
2010	1 167 360	1.60	1018
2011	1 194 901	1.31	754
2012	1 211 509	1.06	519

Source: Data provided by the NVBDCP, India.

2.7 Factors involved in VBD emergence

Over the past 60 years, more than 300 infectious diseases have emerged in humans, about a quarter of which are VBDs.[45] The emergence/resurgence of VBDs as a result of the interaction between arthropod vectors, hosts and pathogens (disease triangle) is highly influenced by the effects of geoclimatic changes, anthropogenic, insecticide and drug resistance, genetic changes in pathogens, and natural factors that make the cycles of VBDs highly complex. Since mosquitoes, ticks and sandflies are ectothermic, their life cycles and effect on disease transmission are potentially influenced by minor climatic changes, i.e. temperature, precipitation, humidity, wind, etc. [46,47] Temperature directly affects the life cycles of mosquitoes in terms of increased activity and reproduction. This in turns leads to increased frequency of blood meals, faster digestion of blood, and even faster maturing of the pathogens harboured by mosquitoes.[48] Increased water temperature causes mosquito larvae to grow faster which also increases overall vector capacity.[49] The temperature threshold for human pathogens and their vectors[50] is given in Table 9.

Table 9. Temperature thresholds of some human pathogens and their vectors

Disease	Pathogen			Vector	
	Name	Threshold (°C) min for transmission	Maximum for survival	Name	Lower threshold (°C) for biological activity
Dengue fever	Dengue virus	11.9	Not known	*Aedes* mosquitoes	6–10
Malaria	*Plasmodium falciparum*	16–19	33–39	*Anopheles* mosquitoes	8–10
	Plasmodium vivax	14.5–15	33–39	*Anopheles* mosquitoes	8–10
Chagas disease	*Trypanosoma cruzi*	18	38	Triatomine bugs	2–6 for survival, 20 for biological activity
Schistosomiasis	*Schistosoma spp.*	14.2	>37	Snails (*Bulinus* and others)	5 for biological activity, 25±2 as optimal
Lyme disease	*Borrelia burgdorferi, Anaplasma phagocytophilum, Babesia microti*	ND	ND	*Ixodes* ticks	5–8

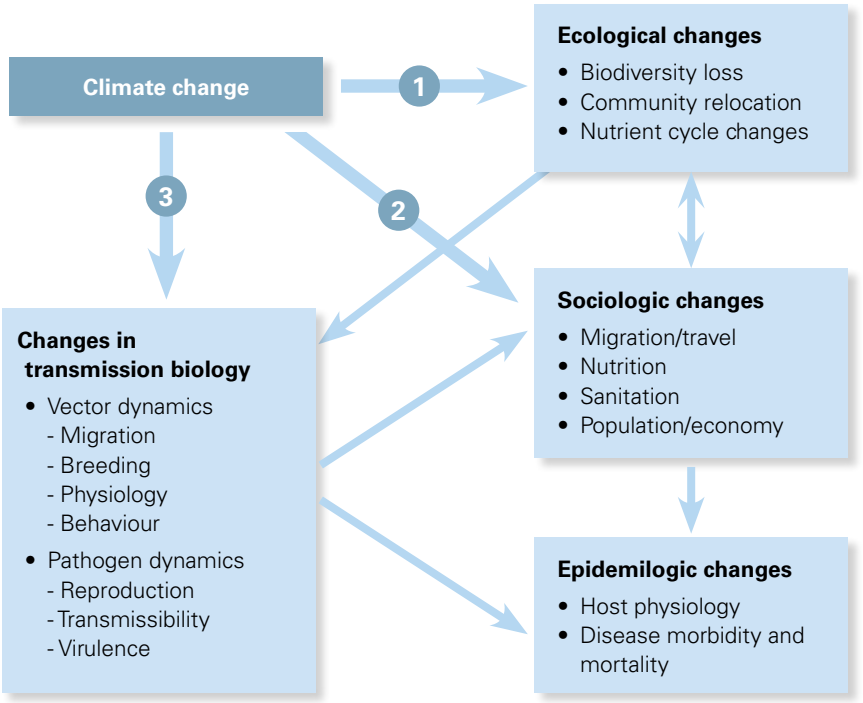
ND: not determined.
Source: 50

The incidence, seasonal transmission and geographical range of VBDs are largely determined and influenced by climatic changes which have been highly pronounced in developing countries in recent decades. Global warming has become an important factor influencing the epidemiological and entomological issues of disease transmission (Figures 5 and 6 on the following pages).

Other factors include:

- man-made ecological niches suitable for breeding
- lifestyle changes
- scanty and irregular water supplies
- urbanization (habitat change)
- globalization with increased trade and transport
- changes in land use
- agricultural and industrial development
- increased air travel
- the movement of vectors by wind and migrating birds
- war and civil unrest
- gaps in public health delivery systems
- poor infrastructure to monitor mosquitoes' breeding sites.

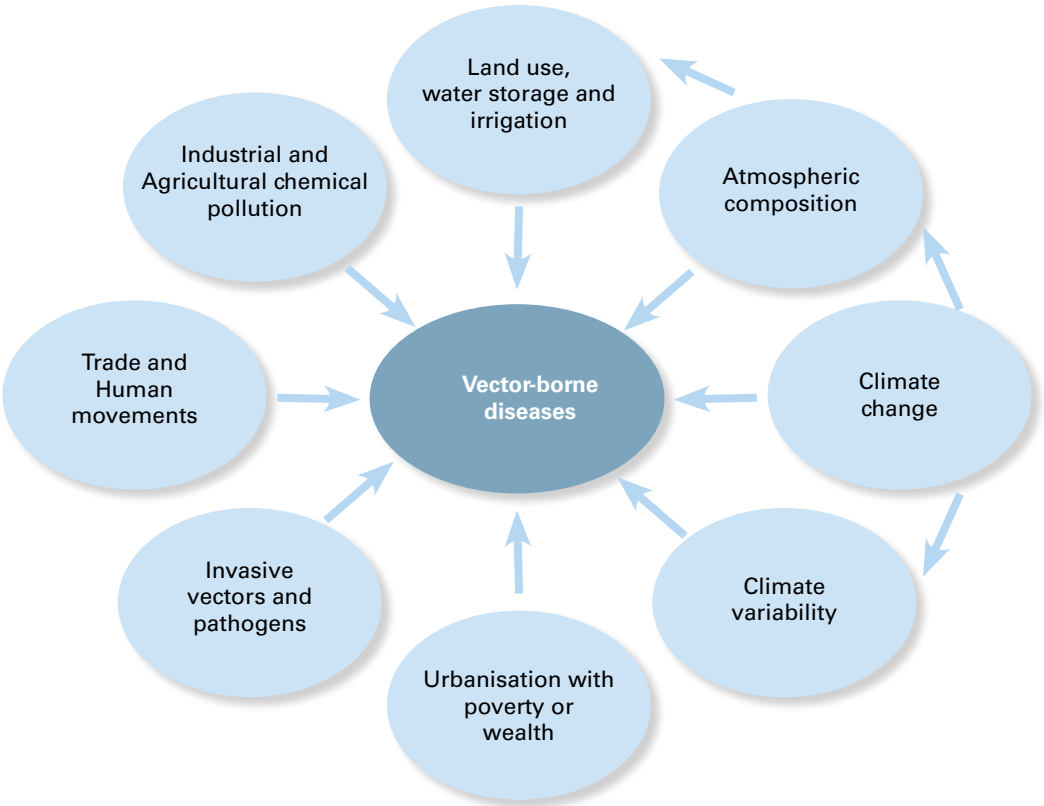
Figure 5. Framework for assessing the impact of climate change on VBDs



Note: Arrows 1–3 show the direct effects of climate change.

Source: 47

Figure 6. Effect of global changes on VBDs



Source: 51

2.8 Current vector control strategies and the need for alternative control mechanisms

There are no effective vaccines or treatments for all VBDs, hence, all interventions have targeted the mosquito instead of the pathogen through the use of insecticides.[52,53] In India, insecticides play a key role in the control and prevention of infectious diseases. The NVBDCP Directorate is mandated to lead the prevention and control programmes against VBDs in India. Since the mosquito is the primary vector transmitting infection in the country, all vector control strategies target mosquito control. Currently, the main insecticide-based strategies India is using belong to the organochloride group (DDT or dichlorodiphenyltrichloroethane), organophosphates (Malathion) and certain synthetic pyrethroid groups (Deltamethrin, Cyfluthrin, Alphacypermethrin, Lambdacyhalothrin and Bifenthrin) for indoor residual spraying, fogging, and aerosol space spraying. Other control approaches include biological methods (anti-larval measures – use of larvivorous fish), personal prophylatics, such as bednets, repellents, insecticide-treated nets or long-lasting insecticide nets, and environmental management and source reduction measures. Although insecticides were effective in bringing disease under control in the initial stages of their application, the current disease burden indicates that these strategies are no longer effective due mainly to: (1) the development of resistance; (2) the fact that widespread and long-term application is not cost effective; and (3) logistical difficulties especially in developing and under-developed countries. Accordingly, an eco-friendly and cost-effective method was urgently warranted.

The period 1950–1960 saw DDT used heavily worldwide both in agricultural and vector control. Globally, DDT is produced in the People's Republic of China, the Democratic People's Republic of Korea and India. The majority of DDT produced in India is used for vector control.[54]

Since 1950, India has been using DDT for IRS. In 2007, approximately 3725 metric tons (MTs) were used globally for vector control, 3188 (85.6%) MTs of which were used by India, the largest consumer of DDT. In 2007, India produced nearly 6344 MTs, which could be the result of increased demand from other countries because, at that time, compared with previous years, India's use of DDT was in decline. Since the 1970s, DDT has been banned in industrialized countries because of vectors' increasing resistance following its intensive use in the agricultural sector as well as national and international pressure to reduce its use because of environmental concerns. In 2001, about 91 countries and European Union (EU) members meeting in Stockholm signed the Stockholm Convention, an international treaty to phase out the use of DDT. India and 30 other countries requested public health exemptions to the treaty so that they could continue using DDT to control malaria. According to WHO's estimates, there are 25 countries still using DDT for vector control including India who is still one of its leading consumers. Six countries are currently using it for vector control and the remainder are long-term users because of its cost effectiveness and efficacy in reducing the burden of disease, despite objections from environmentalists. WHO's committee on malaria has accepted the use of DDT but insists that it should only be used in well defined, high-risk or special-risk situations.

In South-East Asia, resistance to DDT[a,b] is particularly widespread. WHO also estimates that countries in sub-Saharan Africa and India are of greatest concern because of widespread reports of resistance to DDT, and patches of resistance to pyrethroids and organophosphates (Malathion). In some areas, there is resistance to all classes of insecticide with an

[a] DDT is classified as "reasonably anticipated to be a human carcinogen." [b]DDT falls into Group 2B ("possibly carcinogenic to humans") under the IARC Carcinogenicity Classification System.

increased rate of malaria transmission.[55] Multiple resistance to DDT and other insecticides in the major vector *Anopheles culicifacies* is seen in many parts of the region, including India,[56] which has reportedly caused a major decline in the effectiveness of interventions. [57]

DDT and its breakdown product dichlorodiphenyldichloroethylene (DDE) are categorized as "probable" human carcinogens, mainly associated with adverse health outcomes such as breast cancer, diabetes, decreased semen quality, spontaneous abortion, and impaired neurodevelopment in children.[58–61] Data from Brazil, India, Mexico, and South Africa suggest that higher levels of DDT are found in water or soil samples in areas with DDT residual spraying than in areas without spraying.[55,62] Humans are exposed to DDT primarily through food. A nationwide food survey conducted in 2001 in India revealed that about 75% of the food samples had detectable levels of DDT, 10–15% of which had more than the prescribed level. Indian dietary consumption of DDT is also estimated to be amongst the highest in the world.[63] Exposure is linked to human developmental disorders, hormone disruption and reproductive disorders, and has been well documented in animal studies.[60,61] Recent studies have also linked exposure to reduced lactation in nursing mothers[64] and researchers in the USA recently linked DDE levels in American women with increased risk of premature delivery and reduced infant birth weight.[63] Studies in Uttar Pradesh, India, have revealed that DDT levels in the blood of people occupationally exposed to DDT were significantly higher than in those not so exposed.[65] Retired malaria control workers in Costa Rica and India, for example, showed reduced neurobehavioural functions.[66] The researchers estimated that breast-fed children in those areas where DDT had been applied had received more DDT than the safe level recommended by WHO and the FAO.[67] Studies from India also show that the use of DDT in vector control has had serious consequences on the environment since, in those Indian districts with more intensive spraying of DDT, higher concentrations of DDT are found in human breast milk.[68]

The scientific community has agreed that targeting the disease-carrying vector population to reduce vector abundance is the most effective way of controlling disease transmission. Even now, many countries, including India, use mass spraying of insecticides as their principal strategy in controlling these carriers in parallel with biological control and environmental modification. It has yielded substantial progress in bringing down the disease burden but the long-term use of every class of chemical insecticide has led to resistance in most major insect disease vectors. This, together with human-made ecological changes, has led to failure to effectively reduce the burden of VBDs.

In addition, other contributing factors for the escalation of the disease burden in India include: the lack of regular and accurate monitoring of the susceptibility/resistance mechanism; lack of macro-level scientific enquiry into identifying vectoral resistance to the insecticides, and the effects of chemical insecticides on disease reduction, as well as their adverse effects on health and the environment; and the lack of disease reporting coverage in surveillance systems. Resistance to insecticides has led to serious mosquito control problems, contributing to the resurgence of mosquito-borne diseases which directly necessitate the need for alternative, environmentally friendly and chemical-free control methods or the invention of vaccines or drugs. During the last two decades, technological-based genome-modification approaches have been developed for mosquito control either to suppress the target population or to replace it with a pathogen-resistant strain, which have demonstrated some success in areas where they have been implemented.

2.9 Conclusion

Major VBDs, particularly malaria, dengue, JE, African trypanosomiasis, Chagas disease, schistosomiasis and filariasis, are threatening and infecting billions of people throughout the world. Children and the poor are still highly susceptible to infection despite intense efforts at vector control and recent scientific advances. There is a need to rebuild public health vector control programmes either by upgrading conventional methods or finding an innovative approach, or by integrating both to reduce the disease burden and improve people's health and welfare.

The scientific community and policy-makers from developing and developed nations should come together to discuss in a public forum the best possible environmentally friendly ways of addressing this enduring challenge.

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Chapter 3
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3.1 Introduction

Mosquitoes are of such important public health concern because they transmit dangerous diseases, which are prevalent in half the world's population. Reducing contact between man and vectors can control the impact of VBDs. This can be achieved by suppressing the size of the vector population or by replacing it with a non-refractory one. Vector population suppression involves various methods, such as use of insecticides, pathogens, predators, "lure and kill" trapping, environment management, etc. Vector population replacement involves genetic manipulation so that the vectors either reproduce nonviable generations, or become unfit for reproduction or disease transmission. At present, the control of VBDs is mainly carried out through the use of insecticides. But their heavy and prolonged use has led to the issues of resistance and environmental degradation. Thus the search is on for eco-friendly alternative control methods in order to minimize the use of insecticides. Several environmentally friendly methods involving the use of insectivorous fish, biopesticides, pheromones, sterilized males, refractory mosquitoes, endosymbiont, midgut symbionts, etc., are being developed with varing degrees of success.

Genetic modification of vector mosquitoes is one such technology, which is mainly used either to suppress or replace the wild population. These applications include the release of laboratory-reared mosquitoes into the environment to introduce modified genetic traits in the wild population. Genetic modification encompasses multiple approaches that are broadly categorized into two types. The first category includes SIT for population suppression and the second is the gene-drive system for population replacement or manipulation. SIT includes RIDL, *Wolbachia*-mediated cytoplasmic incompatibility (CI), and classical radiation-induced male sterility. Population manipulation technologies include *Medea*-based gene drive, underdominance gene drive, HEG, *Wolbachia*-mediated heritable biocontrol or GM midgut bacteria and transposable elements like *piggyBac* (PB). However, most of them are still in laboratory development.

3.2 History and recent approaches in vector control

Enhancing vectors' natural enemies/predators through genetic manipulation can also be a way of controlling them. This can be achieved by traditional breeding methods such as by developing pesticide-resistant predatory mites to control almond tree mite pests. In California, USA, this method has saved the industry US$22 million per year. In the genomic era, the use of SIT has become the conventional method and has opened up new possibilities in the development of various genetic control strategies for mosquito control. In the FAO's Area-wide Integrated Pest Management (AW-IPM) programme, males are mass reared and, after sterilization, are released into the open. Wild females are unable to produce viable offspring when they mate with them, as their sperm is either inactive or nonexistent. This reduces the size of the pest population or eradicates it. In this way, the new world screwworm was successfully eradicated from central and southern USA. The last reported screwworm infestation in southeastern USA was in Florida in 1959. Cattle farmers in

Central America, Mexico and the USA have also greatly benefited from this approach; in southern Texas alone, livestock increased by 43.7% from 1959 to 1972, and by 37.2% from 1964 to 1972. Similarly, in 1997, the tsetse fly (*Glossina austeni*) was controlled in Zanzibar, the United Republic of Tanzania, the Queensland fruit fly in Western Australia, the melon fly in the Okinawa Islands, Japan, and the medfly in various parts of the world. The SIT campaign has been successfully used for the control of vectors for some decades but release trials with sterile male mosquitoes were conducted during the 1960s and 1970s which relied on the introduction of sterility into the wild female population (Figure 7).[1,2] Sterility can be introduced through various methods: chemosterilants (DNA alkylating agents); gamma irradiation; X-rays; by releasing hybrids; or through modern biotechnological approaches, in which ionising radiation is used as the principal technique for sterilization, although it has been reported to reduce the mating competitiveness of male mosquitoes.

Several trials were undertaken with mosquitoes (*Aedes aegypti, Ae. albopictus, Culex pipiens, Cx. quinquefasciatus, Anopheles albimanus, An. gambiae*), but they failed to achieve long-term control or eradication. In India, SIT was tried against *Ae. aegypti* and *Cx. quinquefasciatus* in 1970. Two research groups are currently trying to develop radiation-based SIT for *Ae. albopictus* and *Ae. arabiensis*. However, it was found that the continuous release of sterilized male mosquitoes leads to population reduction or the complete elimination of mosquitoes under certain circumstances. Also, there are two potential issues associated with this strategy. Firstly, it requires the mass production of insects and males need to be separated from females either manually or mechanically before release because the release of only males is a prerequisite for any SIT programme. A second and potentially larger issue is that the process of sterilization by irradiation causes a dramatic loss of competitive mating ability relative to the wild males. Therefore, the use of SIT for mosquito control is not feasible, mainly due to the mosquitoes' loss of fitness, operational difficulties in mass rearing, sex-separation, irradiation and wide distribution, and the technical difficulty of maintaining the competitiveness of the male mosquitoes. Furthermore, adult mosquitoes are likely to suffer damage during transit and release because they are less robust, and the density-dependent nature of the target mosquito population tends to reduce the cost-effectiveness of SIT. It requires intensive rearing of large numbers of males which is costly and cumbersome as the ratio of released sterile males to wild males is 10:1–100:1. It also requires pest population reduction before release and, above all, requires repeated long-term release.

Various female-killing and sex-sorting genetic systems have been developed, known generically as genetic sexing mechanisms (GSMs) which removes the need for irradiation and relies on the linking of a dominant selectable marker to the male-determining Chromosome.[3] These chromosome aberration–based systems tend to be unstable and reduce the fitness of the insects, making them less effective agents for SIT. In 2000, a new strategy was proposed by Thomas et al.[4] which uses RIDL without irradiation which is similar to SIT but with several improvements. In this approach, a dominant lethal gene is introduced under the control of a female-specific promoter (vitellogenin gene) which produces a protein called a tetracycline-repressible transcriptional activator (tTA) that binds with some of the cell's essential machinery and causes the mosquito to die by disrupting its normal function. The antibiotic-tetracycline (supplementary) binds to the tTA protein and inactivates the expression of the lethal gene in the laboratory (Figure 8). Before the mosquitoes are released into the environment, the repressor (tetracycline) is removed from the system and the lethal gene is expressed, causing the death of all the females and leaving the males to be released to mate with females where they carry and deliver female acting transgenes into the population. First generation (F1) progeny of RIDL males and wild females inherit a dominant female-specific lethal gene; the F1 females die either in pupae or as adults without a genetic repressor (tetracycline) to survive in the environment, thereby reducing the reproductive potential of the wild population. The F1 males are viable and fertile. One approach of RIDL is based on female-specific dominant lethal genetic (fsRIDL) constructs where the

F1 progeny of male mosquitoes die, thereby reducing the vector population. Another approach is transgenes carrying a conditional female-specific late-acting flightless phenotype which reduces the expression of the gene that activates in the flight muscle in female pupae (Figure 9). Flight ability is essential for mating, finding a blood meal and escaping predation. As a result, the female progeny of the released males are unable to fly but male progeny can fly normally in the absence of tetracycline. The flightless mosquitoes are not able to act as vectors, mate, seek hosts and escape from predators. The genetic repressible female-specific lethal genes could provide effective genetic sex separation[4,5] and also allow fluorescence-based sorting by the sex-specific expression of a visible marker, such as a fluorescent protein.[6,7] RIDL is Oxitec's patented technique for GM insects where the OX513A *Ae. aegypti* mosquito used in its experiments contains the red fluorescent marker and conditional lethality trait. This is a self-limiting system as the mosquito progeny will die before they can bite and transmit disease. But males have to be released for a long time before the wild population collapses. More recently, GMMs carrying the flightless-female construct were released on Grand Cayman, in the Cayman Islands. Trial-based experiments were also trailed by Oxitec in Brazil, Cayman Islands, Mexico and Malaysia. A similar experiment is also planned in India, Panama, Sri Lanka, the USA (Florida Keys) and other countries. The most recent trial in Brazil with Oxitec's OX513A mosquito achieved 96% suppression of the dengue mosquito, *Ae. aegypti*, in the village of Mandacarú, north-eastern Brazil.

Compared to SIT and other GM techniques, RIDL is the most advanced technique with respect to its implementation and properties. However, some queries have been raised by the scientific community that need to be addressed through intensive studies and brought into the public domain before mass release into the environment (see Box 1). Although laboratory modelling studies and small field trials have demonstrated the success of this approach, it is based on certain assumptions which have prompted scientists to demand that independent trial field studies be carried out to monitor the impact of this technology on mosquito population suppression, disease reduction as well as on ecosystems in various regions.

Another technique in the GM technology, aiming at improving the natural defence system of the mosquito is RNA interference (RNAi), which has become an important tool in studying the functional genomics of insects and its potential for control. The RNAi pathway is an innate immune pathway of invertebrates, which acts as a gatekeeper/an antiviral immune pathway in mosquitoes that is able to effectively modulate arbovirus replication to allow virus transmission.[14] Consequently, RNAi is potentially a major factor determining the vector competence of mosquitoes for arboviruses[14] and is able to inhibit viral RNA infections. In one method, *Ae. aegypti* mosquitoes were genetically engineered to express inverted-repeat (IR) sequences derived from DENV-2 genomic RNA. Double-stranded RNA formed soon after expressing the IR-RNA in the midgut of female mosquitoes after ingestion of viremic blood that triggers endogenous RNAi pathway against DENV-2 in the mosquito midgut and proved that genetically triggering RNAi pathways exhibit reduced levels of vector competence for DENV-2 (Figure 10).[15] Virus resistance and suppressed replication of DENV-2 genotypes was observed in the initial stage but, over a period of observation, laboratory experiments showed that the transgenic mosquitoes harbouring an antiviral effector gene lost their stability and virus resistance due to genetic changes occurring outside the targeted region.[16] Another approach in RNAi interference was using recombinant *Ae. aegypti* densovirus (AeDNV) vector to induce RNAi in *Ae. albopictus*, in which it was found that recombinant AeDNV caused more serious pathogenic effects than the wild-type virus.[17] Although this system is unique in vector control, there is a need to conduct studies to assess the risks of gene flow, and also to ensure environmental safety since RNAi constructs have fewer limitations associated with recombinant viruses. Another system is under development using site-specific so-called selfish gene element which includes HEGs, group II introns and some site-specific LINE-like transposable elements.[18,19] The HEG is

BOX 1. UNMET KEY ISSUES IN RIDL STRATEGY

1. Uncertainty on the release ratio of GMMs to wild female mosquitoes. Need to ensure that adult GMMs mate with wild females at release site.
2. *Ae. albopictus* is an aggressive biter, re-emerging recently in South-East Asia. It has become as predominant as *Ae. aegypti* and may occupy the vacuum left by the reduced population in the environment.
3. How could the design of the mass release and forecast of the level of population suppression be based on the results of small pre-trial release experiments of GMMs, which have not even undergone independent scientific monitoring and evaluation?
4. Preliminary results from experiments in Brazil suggest that the ratio used in the experiment was 54:1 (GMM: wild female), with an average competitiveness of only 0.03 (3 in 100). As a result of the experiment, the mosquito density in the untreated area increased.[8]
5. Though the reduction of disease-transmitting vector correlates with the reduction of the incidence of disease, a study in Thailand suggests that reducing *Ae. aegypti* abundance from the highest level to a moderate level (insufficient reduction) would increase long-term incidence of dengue haemorrhagic fever (DHF) (> 40%), because of the existence of a complex cross-immunity effect.[9,10] Reducing mosquito populations only has a marginal effect on the incidence of dengue since there are other factors (rainfall, population density and poverty) associated with disease transmission.
6. The survival rate of the offspring of GM males will be high even in the absence or contamination of tetracycline/presence of tTA in the environment which makes this technique less effective.[11,12]
7. Vector population suppression will be complex in the sylvatic cycle of transmission and the involvement of more than one vector in disease transmission.
8. The possibility of the development of resistance against the RIDL approach which renders the technology ineffective.
9. Using conventional methods of control will kill the GM males before they mate and will make the technology less effective. Without the use of conventional methods, there would be a chance of increasing the mosquitoes' density.
10. If sex sorting were ineffective, there is a possibility that releasing GM females with tTA in their salivary glands would create allergic reactions in humans if they were bitten.[13]
11. The fluorescent marker traits would not be reliable since they disappear in hot weather.

a class of selfish or parasitic genes discovered in bacteria. It has recently been discovered that it can be experimentally engineered and used for mosquito control. HEGs encode highly specific endonucleases that recognize and cut a specific DNA sequence. The HEG is engineered and can be inserted into the middle of its own recognition DNA sequence, which disrupts the function of the host gene and protects the chromosomes carrying the HEG from being cut. HEG-induced DNA double-strand breaks (DSBs) activate the recombinational repair system of the cell that will typically repair the broken HEG chromosome, which uses the homologous chromosome carrying the HEG as a template for repair. As a result the HEG is copied to the broken chromosome in a process referred to as 'homing' and a heterozygote will have been converted into a homozygote (Figure 11).[20] There are several ways to use this approach, including: (i) knocking out the gene required for disease transmission and reducing the vector competence of *Ae. gambiae*;[21] (ii) knocking out the

gene involved in survival and reproduction; and (iii) knocking out the sex-determining gene. This approach is expected to be able to reduce/eliminate the population over a period of years following its introduction.

Another technique developed by a research team headed by Professor Scott O'Neill of Monash University, Melbourne, Australia, involves a strain of endosymbiont, *Wolbachia* bacterium, is also closer to large-scale open field trials to replace the population of *Ae. aegypti*. *Wolbachia* was first identified in the ovaries of *Culex* mosquitoes in 1924, but the potential use of *Wolbachia* in the history of insect control in vector population replacement strategy has only been explored since 1967. *Wolbachia* are a group of bacteria, commonly found in reproductive tissues of the arthropod, which is capable of manipulating the reproductive system of the host and, thereby, increasing the number of infected hosts within a population. About 76% of the estimated insect species on earth are infected with *Wolbachia*.[22] *Wolbachia* infection is commonly found in mosquitoes but the main vector for dengue fever (*Ae. aegypti*) and malaria (*Anopheles* spp.) are not inherently infected by *Wolbachia*. Some vector control-based experimental studies have found that *Wolbachia* can act as natural agent in suppressing disease[23,24] by making the vectors virtually resistant to human pathogens and unable to transmit the disease. Some strains of *Wolbachia* can influence fecundity[25] or oogenesis[26] and arrest the development of embryos, whereas life-shortening strains of *Wolbachia* can dramatically reduce the longevity of adult female mosquitoes.[27, 28]

Vector competence is key in measuring the efficieny of a vector's capacity to transmit pathogens. Disease transmission is highly influenced by the age of the mosquito since the pathogen needs to be replicated in various tissues before reaching the salivary glands in order to successfully transmit the pathogen into a human host during subsequent blood feeding. The period of development from pathogen ingestion to potential infectivity within the mosquito is called the extrinsic incubation period (EIP), which lasts around two weeks for both dengue and malaria.[29] Therefore, mosquito survival, and the need to survive longer than the non-feeding period is a critical factor in deciding the vector's capacity for pathogen transmission. Any intervention that targets the mosquitoes' lifespan can largely reduce pathogen transmission. The life-shortening *w*MelPop strain that does not occur naturally in mosquitoes was discovered in *Drosophila melanogaster*, but it has, nevertheless, been proposed to use it as a tool in reducing the longevity of adult female mosquitoes.[27] *w*MelPop is transferred from its natural host (*D. melanogaster*) into the dengue fever vector (*Ae. aegypti*) with the intent of artificially disinfecting it against the DENV transmission, usually by embryonic microinjection of *Wolbachia*-infected cytoplasm or *Wolbachia* purified from infected insect hosts. After microinjection of thousands of *Ae. aegypti* embryos, two stable *w*MelPop-CLA (cell-line adapted) lines with maternal transmission rates of approximately 100% were generated.[28] It was found that this reduced the mosquitoes' adult lifespan by approximately 50%. It also induced CI. Some experiments on the phenotypic effects of *Wolbachia* infection have found that *Ae. aegypti* infected with *Wolbachia* were less able to obtain blood meals in ageing and also observed physiological changes of 'bendy' proboscis phenotypes.[30] It was discovered that wMelPop-CLA infection substantially decreased egg production and the viability of desiccated *Ae. aegypti* eggs over time.[31]

Another strategy is based on incompatible insect technique (IIT) by using wPip(Is) strain to control mosquito populations. In this strategy, male mosquitoes infected by a wPip strain are released and mate with native females, which causes complete CI (embryo mortality–arrested embryonic development in populations). Another possible advantage of the *Wolbachia*-induced trait is based on blocking the pathogen by improving the immune response and increasing resistance to different types of RNA viruses. As a result, the *Wolbachia*-infected mosquitoes are highly capable of reducing the ability of pathogens to replicate. Recent studies have reported that the development of resistance to RNA virus infection, including dengue and chikungunya,[32,33] filarial nematodes and bacteria in *Ae. aegypti* and *Anopheles gambiae* induced with wMelPop *Wolbachia* trait has also been observed to significantly reduce the intensity of *Plasmodium* infection.[33,34]

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Under a dengue elimination programme in Australia, mosquitoes carrying different strains of the *Wolbachia* bacterium (*Wolbachia w*Mel) have been released in recent years in the hope that the *Wolbachia* would be passed on to their progeny where it would act as a vaccine and inhibit its ability to transmit the virus to humans. There are few countries – Brazil, Indonesia, the People's Republic of China and Viet Nam – that have also developed a project based on using the *Wolbachia* method to control dengue. Field trials are currently underway.

In the long history of genetic modification, a new strategy, used by a group of scientists at the Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA, better known as Virginia Tech, was developed recently using site-specific DNA lesion that disrupted the targeted genes which control eye colour in mosquitoes.[35] This genome-editing technique relies on artificial restriction enzymes TALENs which are proteins secreted by *Xanthomonas* bacteria that cut DNA strands at a specific sequence thus editing/modifying the genomes of animal and even human cells. This technique has previously been used to modify the genome of animals and human cells of patients with disease. Crystal structures of these effector proteins can be quickly engineered to bind any DNA sequence when these genetically engineered proteins are introduced into cells and used for genome editing/modification. The same kind of strategy was used on mosquito genome using a newer concept where rather than editing the function of a gene, researchers aimed at disabling a gene of interest by snipping away protein products essential for the production of eye pigment in mosquitoes. A pair of genetically engineered TALEN proteins was injected into pre-blastoderm *Ae. aegypti*[35] embryos by targeting and disrupting the gene coding for eye pigmentation that would be passed down to the next generation. It was found that the eye colour in a large percentage of mosquitoes in the next generation was white instead of the typical black, which confirmed that the genetic code had been wiped out (Figure 12).

Paratransgenesis is another upcoming technique by which mosquitoes can be changed into non-transmitting ones by reintroducing GM bacterium of mosquito origin into their gut after genetic modification. These modified bacteria secrete anti-malarial or anti-disease molecules into the vector's gut and thus inhibiting the development of parasites in the gut and interrupting the disease transmission cycle. It suppresses the vector's competence. Work is ongoing with bacterium *Asaia*, a naturally occurring symbiotic bacterium in the mosquito gut of *An. stephensi*. Genetic transformation of *Wolbachia* to express a particular anti-pathogenic product in its host can also be tried.

This technique has been successfully demonstrated in the Chagas bug (*Rhodnius prolixus*) to control Trypanosomiasis (*Trypanosoma cruzi*) in South America through genetic modification of symbiont, (*Rhodococcus rhodnii*) to release anti-parasitic peptide (*Cecropin A*) in lumen. It is also being tried in other vectors. In this technique, introduction of bacteria into the mosquito is easier than transgene. Genetic manipulation of bacteria is much simpler and faster than in the mosquito. Bacteria can easily be engineered for multiple effecter molecules. It is cost-effective as the production cost of bacteria is less than GMMs. It does not require the release of biting insects, it poses no safety or nuisance problems, there are no serious regulatory and ethical problems, and it is compatible with existing methods.

A recent study in India[36] (Figure 13) looked at the microbial midgut flora community of the common house mosquito, *Culex quinquefasciatus*, which is a vector of filariasis and West Nile encephalitis by examining 16S ribosomal RNA amplicons from culturable microflora. It revealed the presence of 82 bacterial species from 31 bacterial genera in the field-collected mosquitoes. All of these species belong to three phyla, i.e. Proteobacteria, Firmicutes and Actinobacteria. During this study, the midgut flora of various populations both from filariasis endemic and non-filariasis areas were also examined to discover the difference in the microbes so that the role of symbionts in disease transmission could be determined. These efforts led to the discovery of a new species, *Chryseobacterium culicis*[37] in the midgut of wild mosquitoes

from the filariasis endemic area of Raipur, India. However, further detailed studies are required to ascertain the role of this or other bacteria in filarial transmission.

Recent approaches used in mosquito vector control are given in Table 10. There are various techniques based on population suppression or population manipulation, all of which are still in the initial stages of development. They need to be field-tested, independently monitored and brought into the public domain before being mass released into the environment. These techniques require large volumes of mosquitoes to be released into the environment at different intervals to either suppress or replace mosquito populations. It is suggested that vector control management would yield better results by combining the newly invented cost effective and environmentally friendly strategies with traditional interventions.

Table 10. Recent advances in transgene approaches for vector control

Technique	Techno-logy	Method	Year	Strategy	Method description	Pros and cons	Progress status
Population suppres-sion	GM	SIT	1937 pro-posed by Edward Knipling. Intro-duced in 1954 for pest control. 1970-80 used against mos-quito control	Hybrid steril-ity, chemo-sterilization, gamma irradiation and X-rays (ironizing radiation is most widely used strat-egy)	Mass produc-tion, sterilized using radiation, released into targeted popu-lation and mate with female. Their offspring is non-viable	*Irradiation creates some level of somatic damage and that can reduce the quality (viability and competitiveness) of the released arthropods * Incomplete steriliza-tion, reduced mating competitiveness and immigration of mos-quitoes can all reduce the effectiveness of SIT * Producing large num-bers of mosquitoes, sex separation, sterilization, release and distribution are not economically fea-sible for poor countries * Effective in reducing the small/isolated pop-ulation but not effective in reducing high-density populations of insects (mosquitoes)	Effectively deployed against some agricultural pest but has limited im-pact on dis-ease vectors. Currently, no large scale of implemen-tation of SIT against mos-quitoes. But experiments are under-way to adjust radiation doses to en-sure mating fitness

Table 10. Recent advances in transgene approaches for vector control (continued)

Technique	Techno-logy	Method	Year	Strategy	Method description	Pros and cons	Progress status
	GM	Release of insects carrying a dominant lethal mutation	Proposed by Thomas et al. in 2000[4]	Mosquitoes carrying a female–specific auto-dial-genetic system	Replacing irradiation with the insertion of conditional dominant lethal gene. Male carry and deliver female acting transgenes into the population. F1 progeny of RIDL males and wild females inherit a dominant female-specific lethal gene. Without repressor, all females die	*It is possible that sex separation increases cost. *Requires recurrent and inundative or mass-release of mosquitoes * Has the properties of genetic sex separation, female-specific lethal gene and allows genetic marker for sorting * This approach would leave an ecological vacuum and another vector could fill it quickly * Experiments in Brazil show high-release ratio and low mating competitiveness * Lack of studies on the effects of vector suppression on disease reduction. It was reported that rebound effect could make the situation worst * *Ae. aegypti* can be replaced by *Ae. albopictus* when target is focused on the former	Laboratory testing has been carried out successfully in Brazil, the Cayman Islands, Malaysia and Mexico
				Mosquitoes carrying a female-specific flightless phenotype	Lethal reduces the expression of the gene that activates the flight muscle. The daughters of released males are unable to fly in the absence of tetracycline		Field testing

Table 10. Recent advances in transgene approaches for vector control (continued)

Technique	Techno-logy	Method	Year	Strategy	Method description	Pros and cons	Progress status
Population replace-ment/ manipula-tion	GM	RNAi	Discov-ered, used in nem-atode worm *C. elegans* and first pub-lished in 1998 by Andrew Fire and Craig C. Mello;[38] since then it has been used in insect order	GE[a] to express inverted-re-peat (IR) in *Ae. aegypti* and another approach by using recombinant AeDNV	Vector immuni-ty to pathogens	RNAi constructs have fewer limitations associ-ated with recombinant virus	In develop-ment
	GM	HEG[b]	ND	Female fertility targeting HEGs	Targets the sex-determin-ing genes, leading to sex ratio skews	Aimed at population suppression	In develop-ment
				Targets the gene in-volved in de-velopment or trans-mission of pathogens to infect mosquitoes	Aimed at either disrupting the genes that contribute to its vectorial capacity or tar-geting a gene that impairs the mosquito's ability to func-tion as a vector for transmis-sion	Aimed at reducing vector competence	In develop-ment

Technique	Techno-logy	Method	Year	Strategy	Method description	Pros and cons	Progress status
Population replace-ment/ manipula-tion	GM	HEG[b]	ND	Targets the survival/ reproduction gene	Targets the gene involved in survival or reproduction, then the num-ber of mos-quitoes may be reduced (population reduction)	Targets species elimi-nation	In develop-ment
	Non-GMM	*Wol-bachia* (neither the Wol-bachia genome nor the host genome was modi-fied)	Wolba-chia-based control tool was pro-posed as early as 1967 but it was first identi-fied in Culex mosqui-toes in 1924	Influencing fecundity or oogenesis (Wolba-chia-induced CI)[c]	Wolbachia-in-fected male mates with an uninfected fe-male, resulting in karyogamy failure and early developmen-tal arrest of the mosquito embryo	* Environmentally friend-ly and area wide imple-mentation at low cost * Compatible with other insecticide-based control measures * Once it is established in the wild mosquito pop-ulation, there is a high possibility of reduction of pathogen transmission	Field testing
				Reduction in the lifespan of mosqui-toes	Some strains of Wolbachia have lost their ability to replicate with the host cell, and can reduce the longevity of adult female mosquito		Field testing
				Inhibition of pathogen replication in mosquitoes	*Wolbachia* acts like a vaccine in blocking pathogen trans-mission		Field testing

ND: not determined.

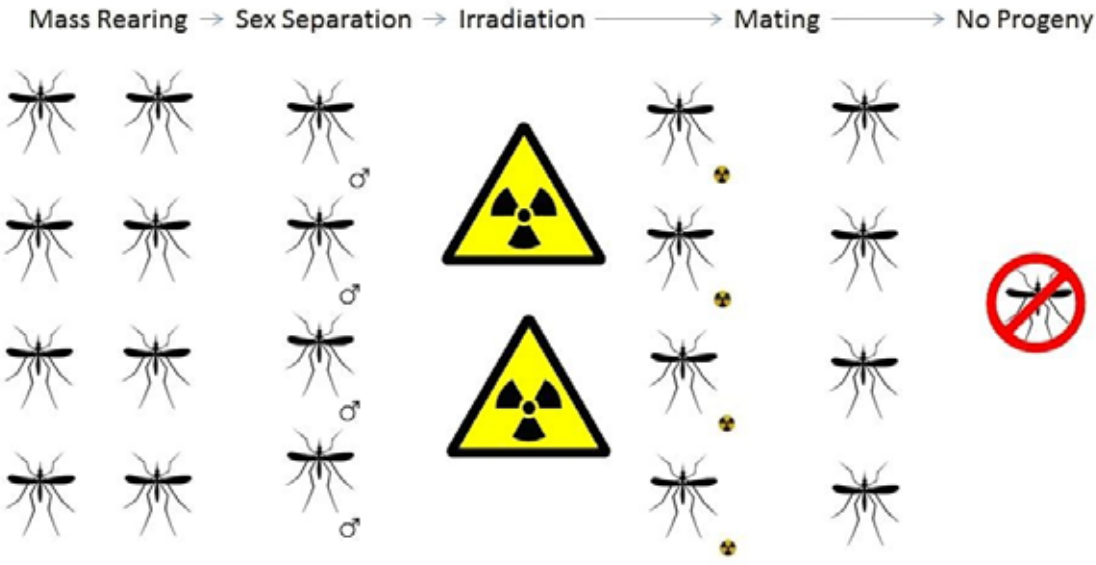
Source: Data compiled by authors.

[a] GE, genetically engineered.

[b] HEG, homing endonuclease gene.

[c] Based on population suppression strategy (SIT).

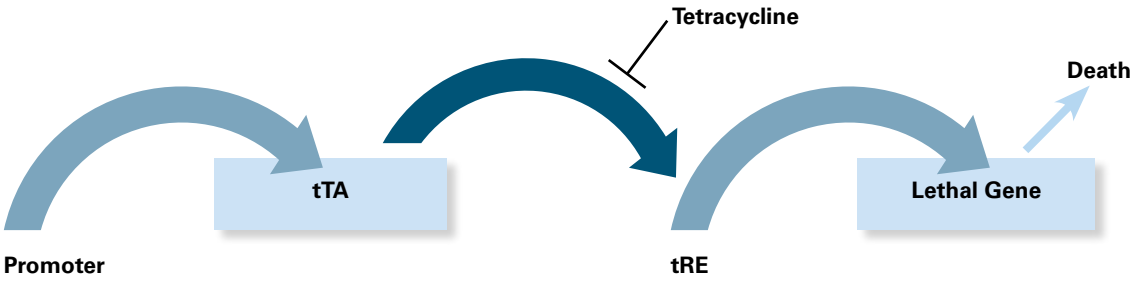
Figure 7. Recent advances in transgene approaches in vector control, conventional SIT schedule



Massive production followed by manual sex separation to ensure that only males are sterilized with irradiation. Subsequent release of large numbers of sterile male mosquitoes into targeted population where they mate with female mosquitoes, which produce non-viable offspring.

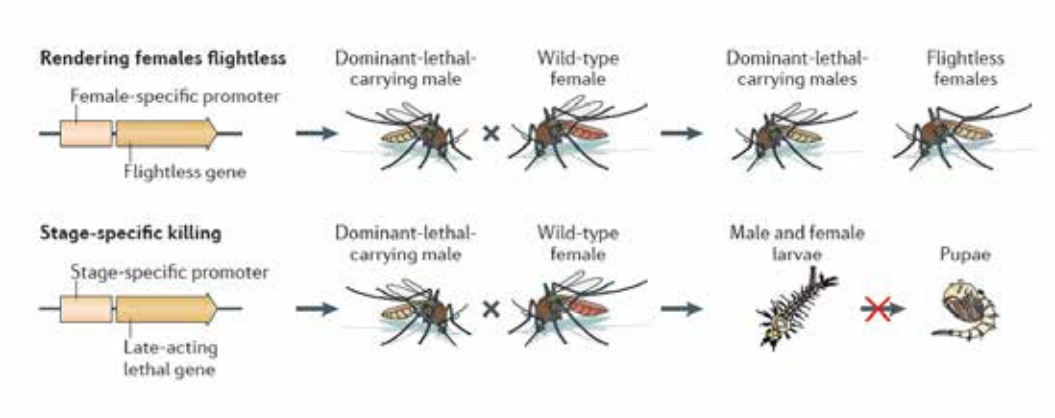
Source: Adapted from 39.

Figure 8. Recent advances in transgene approaches in vector control, tetracycline-repressible lethal system in RIDL



A protein called tTA binds to a specific DNA sequence, tetracycline-response element (tRe) and then activates the expression of a given sequence (the lethal effector gene). However, in the presence of low concentrations of antibiotic-tetracycline (supplementary), the tTA protein does not bind DNA, and so expression of the lethal gene is prevented. *Source:* Adapted from 3.

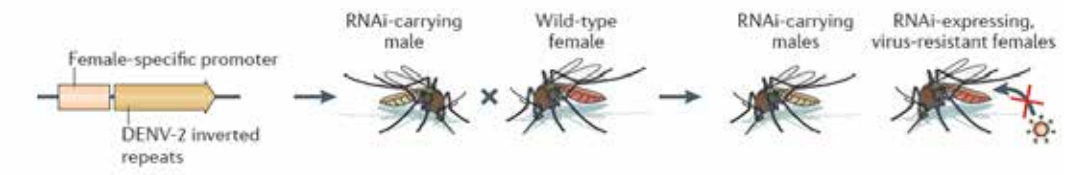
Figure 9. Recent advances in transgene approaches in vector control, RIDL



Males carrying a conditional female-specific late-acting flightless phenotype, which reduces the expression of the gene that activates in the flight muscle of female pupae, mate with wild females and as a result the female offspring are flightless.

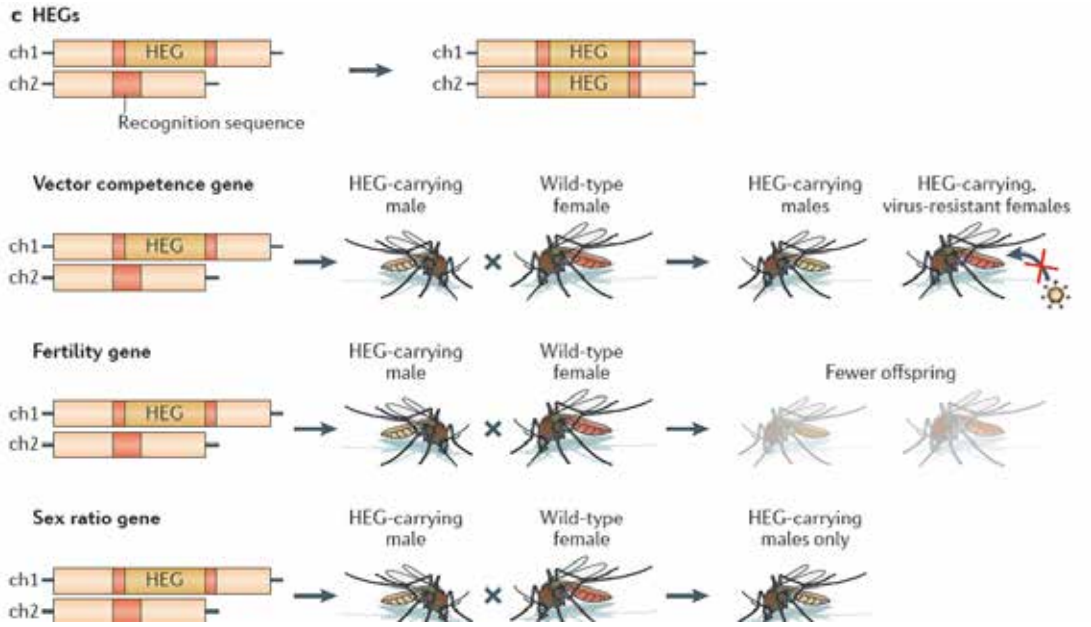
Males carrying a transgene are released in the field, mate with wild-type females, and the resulting offspring die as pupae or adults. *Source:* Adapted from 40.

Figure 10. Recent advances in transgene approaches in vector control, RNA interference



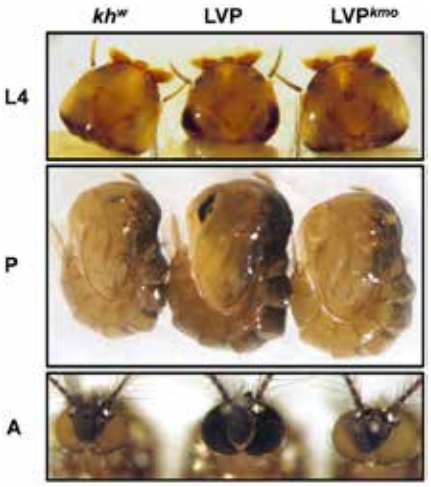
Males carrying a female-acting transgene, designed to express inverted-repeat sequences derived from DENV-2 are released, mate with wild-type females, and the resulting genetically triggered RNAi pathway exhibits a reduced level of vector competence for DENV-2. *Source:* Adapted from 40.

Figure 11. Recent advances in transgene approaches in vector control, HEGs



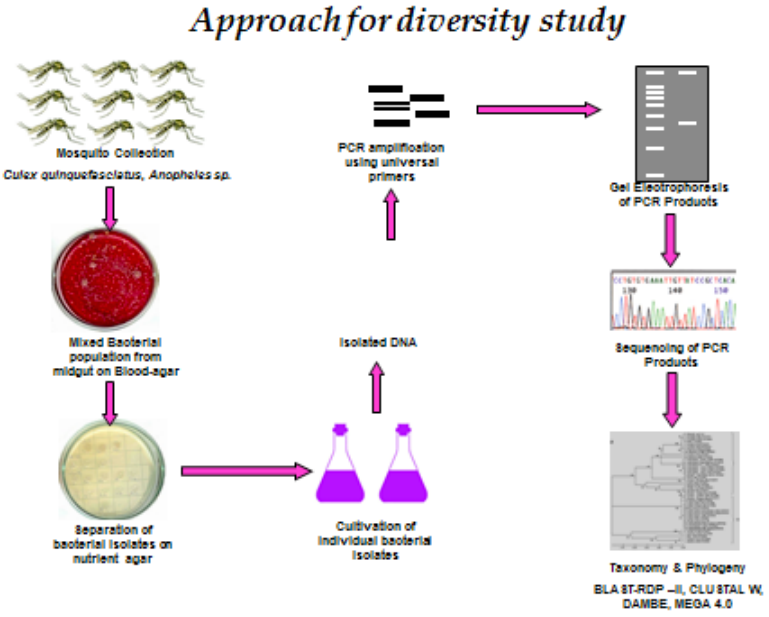
Released males carrying HEGs mate with wild-type females and produce offspring that contain the HEG, which can be designed to target the vector competence gene, fertility gene and sex-ratio gene to reduce vector competence, suppress populations and eliminate species. *Source:* Adapted from 40.

Figure 12. Recent advances in transgene approaches in vector control, TALEN-generated kmo alleles phanocopy khw strain mosquitoes



LVP, kh^{w} and LVP^{kmo} mosquitoes imaged as larvae (L4), pupae (P) and adults (A). *Source:* 35

Figure 13. Recent advances in transgene approaches in vector control, microbial diversity study from midgut of *Culex quinquefasciatus*



Source: Adapted from 36, 37.

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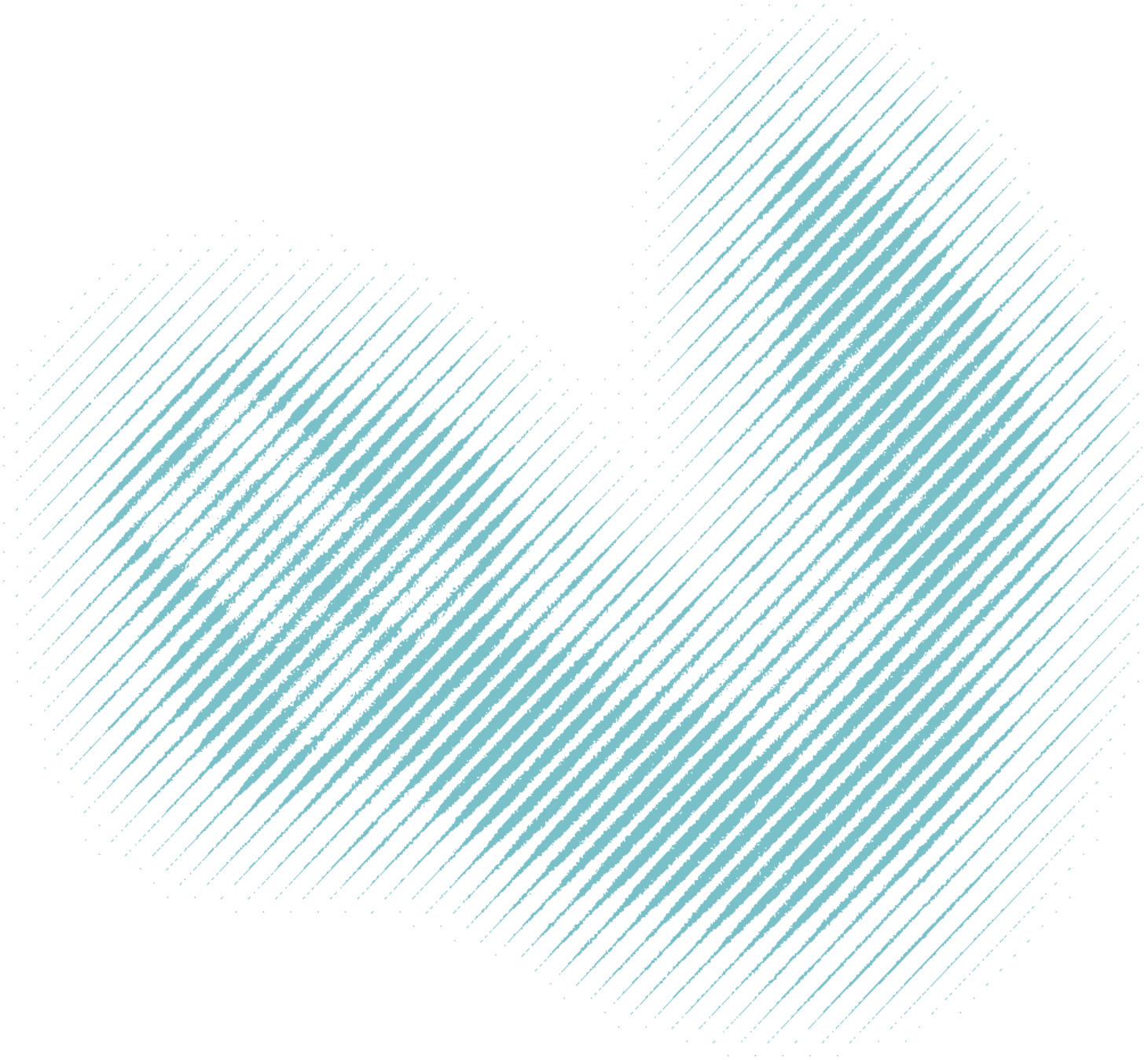

Chapter 4. Molecular genetic research in insects: methods of germ-line transformation and molecular biology of insect transgenesis

4.1 Introduction

Insecta are the largest class of organisms in the Animal Kingdom and host a wide variety of species. Insects play a crucial role in the environment and are associated with human life directly and indirectly. Germ-line transformation approaches have been well established to modify the genetic makeup of insects and to obtain the desired phenotypes. Transgene expression in insects is also extensively used for foreign protein expression. Germ-line transformation technologies led to the development of GM insects, which is the basis for most molecular biology of eukaryotic cells today. The concept of GM insects and their use outside the laboratory raises several potential concerns. This chapter elaborates the basic molecular biological aspects and components of various vectors, methods of germ-line transformation and applications of transgenic insects. It also examines the factors that need to be considered regarding the possible consequences to the ecosystem and alternative gene-manipulation approaches in addressing safety concerns.

4.2 Molecular biology of insect transgenesis

In molecular biology, genetic transformational technologies aim to alter the nucleus of germ cells with the required transgene construct to stablize the transformation. Genetic transformation or transgenesis involves the following steps: (i) design and development of the transgene construct for the aimed transgenesis; (ii) delivery of the transgene construct into the nucleus of germ cells; and (iii) analysis of the integration, inheritance and expression (functionality) of the transgene. The challenging aspect of genetic transformation in insects is the transfer of exogenous DNA into the insect's nucleus in a stable manner. For stable inheritance, the integration of the transferred DNA in the host genome is preferred and is the exclusive option. The major transformation systems which facilitate the integration of foreign DNA are: (i) transposable element-based system; and (ii) FLP/FRT- and Cre/loxP-based recombination systems involving recombinase enzyme.[1]

4.3 Transposon-based vectors for germ-line transformation

Transposons are mobile genetic elements that move from one position to another unique position in the genome. There are three different classes of transposable elements. Class I elements transpose through reverse transcription, and Class II elements transpose by cut and paste transposition mechanism on DNA.[2] Class III elements are also known as miniature inverted-repeat transposable elements (MITES) and are involved in non-replicative translocation to new insertion sites. Class II elements move from one position and integrate with the host genome to another new position. They have very short terminal repeats on either sides of a single open reading frame (ORF), which encodes the transposase protein

responsible for transposition activity. The transposase recognizes the repeat elements in the transposon and the new integration site in the genome, and executes the transposition of DNA sequence from one site to another in the host genome. Class II elements are considered autonomous and often used in germ-line transformation.

As mentioned earlier, transposon consists of two parts: terminal inverted repeats (TIRs) and with the gene coding for transposase. While used in genetic transformation, the transposase ORF is replaced with gene of interest flanked by terminal inverted repeats.[2] Further, for safety purposes, the transposons are made into non-autonomous systems or constructs. One construct carries the gene of interest and a marker gene, flanked by the functional TIR and the construct, carries the ORF for transposase enzyme without TIRs. These two constructs are transferred to germ cells where the transposition of transgene into the host genome occurs.[2] Alternatively, the transgene construct flanked by TIRs is also used separately. In this case transposase protein or messenger RNA (mRNA) is co-injected into the germ cells along with the transgene construct flanked by TIRs.[3]

The most widely used transposon in transgenesisis is PB. It was isolated from the cabbage looper moth *Trichoplusia ni*.[4] The PB transposon system is very stable in transformation since this does not require any specific hosts for transgenesis. Many other transposons like *Hermes, Hobo, Minos* and *MosI* have also been demonstrated to work in the germ-line transformation of insects.[4] The transposon-based gene vectors for insects were derived from different families of insects. *Hermes* was isolated from the housefly, *Musca domestica*, and is a member of the *hAT* family of transposons. *Minos* was isolated from *Drosophila hydei,* which is closely related to *Tc* elements originally discovered in nematodes. *Mariner* was discovered in *Drosophila mauritiana*.[3] The transposon elements originally identified from different and divergent families of insects are found to work across a wide phylogenetic range of organisms from insects to vertebrates.[4] Furthermore, some of the synthetic transposons such as *Sleeping Beauty* were also found to work well in the germ-line transformation of insects.[5]

4.4 Site-specific recombination in transgenesis

Transposition efficiency is found to decrease in transposon-mediated transposition, due to the larger size of the exogenous genetic material to be integrated. To overcome this issue, a site-specific recombination system has been adopted and is capable of allowing the exchange and integration of larger size constructs into the insect genome. Site-specific recombination systems frequently used are FRT/FLP, Cre/Lox, and the attP-attB/phiC31.[1] These recombinases bring out homologous recombinations and the transgenes could, therefore, be delivered to any region in the host genome. The recombineering vectors could be designed with the transgenes flanked by *Lox/att* sites, which aid the homologous recombination. Thus, using these recombinases, the transgenes can be targeted at a specific chromosomal site in the host genome. Most often, the *Lox/att* sites are integrated into the germ line, which acts as a recombinational acceptor site while using the *Cre/Flp* recombination system. Recombinase mediated cassette exchange (RMCE) strategy is used with heterospecific FRT sequences to make recombinational insertions irreversible.[1] This system has been used for the transformation of *Drosophila melanogaster, Aedes aegypti,* and *Ceratitis capitata*, after inserting the *attP* site in the fly genome by transposon-mediated transgenesis, and using *attB* site in the plasmid carrying the transgene.[6] Thus, targeted genomic insertion mediated by recombination allows site-specific targeting of the transgenes in the host genome. Gene transfer vectors also designed with the transgenes flanked with host genome sequences also aid the homologous recombination. This is still a major approach in insect germ-line transformation.[7]

4.5 Viral vectors in the transformation of insects

Viral vectors are effectively used as an expression system. For instance, sinbis RNA virus is highly effective when used as an expression system *in vitro* and *in vivo* conditions.[8] DNA viruses such as the densoviruses have also been used as transducing agents in mosquito larvae.[9] Retrovirus-based vectors which have been used as gene transfer vectors in mammalian cells are also being used in invertebrate systems including several insect species. The following are the different vectors used in insect gene manipulation.

i. Densonucleosis virus-based vectors are only suitable for use in arthropods: These viral vectors are most widely used in mosquitoes to study gene expression when they are integrated into the genome. They have gained greated attention as a gene transfer vector due to their small genome size.[10]

ii. Polydnaviruses are multi-segmented DNA viruses that stably integrate with the chromosomal DNA of the gypsy moth. They are useful in transferring the target genes into the cultures of lepidopteran and coleopteran cell lines.[11] However, since their transducing efficiency is very low they are not frequently used in the genetic engineering of insects.

iii. Sindbis viral vectors: In studying gene expression, Sinbis RNA viruses are particularly specialized for their efficient transducing capacity in mosquitoes. Sinbis viruses are alphaviruses with single stranded RNA genome. These viral vectors are most widely used in yellow fever mosquito (*Ae. aegypti*), the eastern treehole mosquito (*Ae. triseriatus*), the northern house mosquito (*Culex pipiens*), and *Anopheles gambiae*.[12] Alphavirus and Arbovirus Sindbis-based vectors are extensively used to study the gene expression in transgenic mosquitoes, pink bollworms and other insects. Alphavirus Sindbis is more suitable than Arbovirus Sindbis because it can infect the insect hosts cytopathically. Sindbis viruses are used in a wide range of hosts such as mosquitoes, fruit fly, butterflies, beetles and hornworm.[13] The Sindbis virus works as a gene expression vector. Sindbis virus-derived vectors are constructed by inserting the target DNA sequence into the cDNA of the Sindbis viral genome so that the transcription is under the control of the DNA-dependent RNA polymerase promoter.[14] The constructed vector consists of an ORF which contains an antibiotic-resistant gene, a marker gene, a polylinker sequence with multiple restriction sites, and a promoter.[10] The viral genomic sequences are replaced with the gene of interest and other cloning components. Sindbis virus-derived DNA-based expression vectors are also used for heterogenous gene and protein expression in insects. The virus infection can be transmitted orally or by means of an injection. Using a Sindbis viral transducing system containing the gene construct of enhanced green fluorescent protein (EGFP), and specifically by injecting into the larva of pink bollworm, the expression of green fluorescent protein has been observed in all the larva cell types.[8,14]

iv. Retroviral vectors: They are stable and once integrated into the genome, are not capable of self-propagation. They are derived from the Moloney Murine Leukemia Virus (MoMuLV). Retroviral vectors are extensively used in human gene therapy. The larvae are somatically infected using these vectors in *Drosophila melanogaster, Aedes triseriatus, Culex tarsalis*, *Anopheles gambiae*, and *Manduca sexta*.[10] The retroviral vector is composed of packaged enveloped glycoprotein from the vesicular somatitis virus and they bind to the phospholipid components of the host cell membrane. These vectors are highly stable inside the host cells because they lack replicative components. The retrovirus-derived vectors are composed of long terminal repeats and strong promoters, which enable targeted gene expression across species. Retroviral vectors are most frequently used in silkworms and mosquitoes. The retroviral vectors have a number of viral elements, which make the vector efficient in transduction and in integration. The components are: SV40 promoter; long direct terminal repeats (LTR) at both the ends which facilitates integration; three transcriptional units, namely, *gag*, *pol* and *env*; reverse transcriptase ORF; and antibiotic-resistant genes. Ret-

roviral vectors carrying the green fluorescent protein (GFP) construct were used to infect silkworm embryos and the expression of GFP was found in the larval tissues.[8] Retroviral vectors are most often employed as heterologous gene expression systems in insects and, to a lesser extent, for germ-line transformation.[10] The advantages of using the retroviral vectors is their capability of inserting very long transgenes of up to 10–13 kilobases (kbs). Also, inside the host genome, the transgene components are highly stable due to the lack of replicative components. Another striking aspect is their high-transduction efficiency.

v. Densonucleosis virus vectors: Densonucleosis viruses (densoviruses) infect the mosquito species most frequently. Densovirus-derived viral vectors are suitable for insects and, specifically, mosquitoes. They are able to infect mosquitoes with high transformation efficiency during the larval stages.[9] Densoviruses cause some lethal effects to some species of mosquitoes including *Ae. aegypti*.[9] Densoviruses of *Ae. aegypti* are also used as an expression vector in mosquito cells.[9]

vi. Baculovirus-based expression system: Pathogenic to several insects, the baculoviruses or nuclear polyhedrosis viruses (NPVs) have been well exploited as insecticides to control insect pests. The baculoviral expression system was first established in 1985.[15] The foreign DNA is cloned into the baculovirus genome by making use of a transfer vector. The transfer vector is a basic plasmid with 5' and 3' regions of polyhedrin gene, which enables homologous recombination with the wild-type baculoviral DNA in insect cells. The foreign gene is transferred to the viral genome replacing the polyhedrin gene. The advantages of NPV expression systems are: (i) the NPV genome is circular, double stranded and amenable to manipulation; (ii) the rod-shaped capsid of the virus accommodates extra DNA; (iii) the availability of cell-line susceptible to viral infection; (iv) larvae are susceptible, and yield enormous quantities of foreign proteins; (v) larvae are easy to mass rear; and (vi) baculoviruses are species- or genus-specific and so cross-infection is limited.[16] The effective use of recombinant baculoviruses as chemical pesticides in controlling specific insect pests and as an expression system is well established.[17] The production of foreign proteins by a baculovirus expression system has attracted the attention of researchers due to the striking fact that nearly 40–50% of the total cell proteins and insect hemolymph comprise this protein, while used.[18] This expression system is found useful in the production of biologically active proteins including monoclonal antibodies, hormones and interferon.[15,19]

4.6 Gene delivery methods for germ-line transformation

The genetic construct is delivered into the germ-line cells where it is incorporated into the host's genome. Electroporation, biolistics, microinjection, and sperm-mediated transfer are the most widely used methods for the transfer or delivery of exogenous DNA into insect germ cells. Table 11 presents a list of various vectors used in the transformation of different insect species.

i. *Microinjection:* Microinjection remains the most widely used option for the introduction of foreign DNA into insect germ cells.[20] A microinjector is used to deliver the exogenous DNA into the polar plasm of the embryo where germ-line precursor cells will develop. Young embryos are used because the construct has to be delivered in the embryo before the formation of germ-line precursor cells. The embryos that survive the injection process develop into transgenic offspring, which are then back-crossed with a non-engineered parental strain, and the transgenic lines are selected. Microinjection has led to the transformation of a number of insect species spanning the orders *Diptera, Lepidoptera, Coleoptera* and *Hymenoptera*.[21]

ii. *Biolistic transformation:* Biolistics has been used to bombard the exogenous DNA coated with micro carriers into the model organism by means of a gunshot. Baldarelli and Lengyel obtained a single transformed fly using biolistics as a means of delivering a P-element transformation vector, and stated that the method would require some improvements before it would be an effective method for developing transgenic insects. Transformation frequencies using biolistics for microinjected P-elements and PB vectors typically range between 2% and 30% in *D. melanogaster*.[22] While the transformation frequencies attained are generally low, biolistics has the advantage of delivering nucleic acids with much higher throughput than standard microinjection methods.

iii. *Sperm-mediated transgene transfer:* The exogenous DNA is delivered into the sperm by means of electroporation, liposome-DNA complex mediated approach and also by binding monoclonal antibodies with the exogenous DNA, which allows it to enter into the sperm. The DNA complex binds to the sperm's head in the sub-acrosomal region.[23] Once they are bound, the DNA molecules are internalized into the sperm. There are successful cases of sperm-mediated gene transfer in silkworm transgenesis.[24] The fertilization of silkworm eggs involves the entry of a number of sperms which also increases the possible entry of an exogenous genetic construct into the eggs and increases the transformation efficiency. Sperm-mediated transgenesis is also used in domestic cattle (*Bos taurus*), the Australian sheep blowfly (*Lucilia cuprina*), and the honeybee (*Apis mellifera*).[25]

iv. *Electroporation:* Electroporation is another powerful method often used for gene delivery in germ cells. In electroporation, the recombinant DNA construct is injected into the germ or embryoinc or somatic cells.[26] Successful electroporation is achieved by optimizing the parameters such as electrical voltage, number of pulses and their frequency, and buffer conductivity. Electroporation can be performed using three main modes – capacitive discharge, radio-frequency pulses and square-wave pulses.[27] This is less labour intensive than microinjection.

Table 11. Vectors used in the transformation of different insect species

Class of vector	Vector component used in the transformation	Host insect species transformed	Reference/source
Transposons	PB[a] element	Silkworm, fruit fly	6
	Hermes element	Mosquito, butterfly and moth	16
	Homer element	*Drosophila*	16
	PB[a]	*Drosophila* and *Helicoverpa*	28
	Tc1 element	Med fly	28
	PB[a]	Sawfly	16
	PB[a]	Harlequin ladybird	17
	PB[a] and *Hermes*	Red flour beetle	17
	Mariner element	*Drosophila*	29
	PB[a]	Sawfly	16
Viral	Densovirus	*Culex* mosquito	16
	Baculovirus	Silkworm	25
	Retrovirus	Fruit fly	16
	Retrovirus	Tobacco hawkmoth	16
	Retrovirus	Silk moth	25
	Sinbis RNA virus	Yellow fever mosquito	16
	Sinbis RNA virus	Eastern treehole mosquito	16
	Sinbis RNA virus	Northern house mosquito	16
	Densovirus	African malaria mosquito	17

[a] PB, *piggyBac*.

4.7 Applications of transgenesis in insects

In general, insects are modified essentially for four main purposes, namely, to enhance the understanding of insect molecular biology, for protein production, to improve health care, and protect agricultural production. The use of GM insects in laboratories is very common and non-controversial.[12] Apart from routine molecular and cell biological analytical methods of investigation, germ-line transformation is extensively used as a basic approach in insect molecular biology to understand various biological questions in molecular genetics, physiology and developmental biology. Germ-line transformation and whole organism transgenesis approaches are used to modify the genetic make up of organisms to investigate the resultant phenotypes. A range of insect transgenesis has been made for research purposes. One of these is *Drosophila,* the fruit fly, which has always been the model of choice for various studies and has been extensively studied

for almost 100 years.[30] *Drosophlia* has been in used since the days of Thomas Hunt Morgan, to understand the intricacies of genetics, chronobiology, and neurosciences. Germ-line transformations have been used extensively to alter the gene expression in: Noonan syndrome causing PTPN11;[31] beta-amyloid peptide for Alzheimer's disease; polyglutamine repeat proteins for Huntington's disease; and alpha-synuclein for Parkinson's disease.[31] Studies linking the SOD1 gene with the extension of lifespan have also been carried out with transgenic *Drosophila*.[32] Transgenic fruit flies have also been used for chronobiological studies to understand the circadian pattern. Transgenic *Drosophila* is also used as a model for the identification of genes involved in the development of the adult human heart.[33]

Insects have been used as production units or bioreactors for producing a wide range of protein products. For example, the release of large numbers of transgenic insects is intended to suppress the wild pest populations. Transgenic mosquitoes are involved in preventing disease transmission such as dengue and malaria. Silkworm larvae and cocoons are very attractive for recombinant protein production such as cell surface proteins, viral proteins and interferon. Fruit flies, silkworms and a few other insects have been used to produce economically important proteins. *Drosophila* has been modified to produce two antifreeze protein genes from the Atlantic wolffish, *Anarhichas lupus*.[34]

Silkworms have been transformed to produce human serum albumin in their cocoons.[35] Transgenic silkworms have been used for the production of modified silk containing protein molecules to alter the characteristics of the silk, particularly trying to integrate the properties of spider silk with the production capacity of silkworms. An optimized sericin-1 expression system for the large-scale production of recombinant proteins in the middle silk glands of transgenic silkworms has been developed.[36]

Medfly has been engineered to express human growth hormone. The expression of antibody fragments in whole insect larvae of *Trichoplusia ni* has also been accomplished. *Dactylopius confusus*, the cochineal beetle, which produces red dye, has been transformed for both increased yield and altered pigment production.[12] In order to meet the demands of the increasing human population for quality and quantity in food through a safe and sustainable approach, integrated pest management (IPM) methods are being widely adopted. The SIT is a remarkable component in IPM programmes for major agricultural pests. Germ-line transformation can provide significant advances in the current SIT approaches. In this strategy, the sterile male insects are produced by germ-line transformation and released into the environment. They compete with the wild-type males for mating resulting in a remarkable reduction in the population of the insects. This was first introduced to diminish the population of screw-worm flies, the larvae of which are obligatory mammal parasites causing lesions known as myasis. Prospects for vector control through sterilization procedures are based on the successful eradication of the screw-worm fly,[17] which has now been designed for various insects like mosquitoes,[37] fruit flies,[38] melon flies, onion flies, tsetse flies, and so on. The fundamental principle is that due to the introduction of high numbers of sterile males, the target populations become progressively smaller, the sterile male to female ratio increases, sterile mating rates increase, and the target population eventually collapses.[32]

Insects are a threat to human health by acting as vectors for diverse diseases, such as dengue, malaria, sleeping sickness, Lyme disease, plague, etc. Transgenic insects have been developed to control the population of the wild vectors through SITs. The transgenic insects developed over a period for various applications are given in Table 12. *Aedes aegypti* populations are shown to have been controlled using SIT to release of GM sterile males.[37] *Anopheles stephensi, Aedes albopictus, Culex quinquefasciatus* have all been genetically transformed in order to reduce the spread of VBDs.

Table 12. Examples of transgenic insects developed for various applications

Transgenic insects	Application	Strategy	Reference/ source
Drosophila melanogaster	Model organism	Expressing genes for neuro-degenerative diseases	31
Drosophila melanogaster	Protein production	To express antifreeze protein genes from Atlantic wolf fish	34
Aedes aegypti	SIT[a] for controlling the population	Release of sterile males into the environment	37
Anopheles stephensi	Resistant to human malaria parasites	Over expression of *Rel2* gene controls *Plasmodium* infection	39
Anopheles gambiae	Reduce *Plasmodium* infection	Site-specific integration and expression of an anti-malarial gene	40
Aedes albopictus	Control against dengue and chikungunya	PB[b] and site-specific PhiC31 mediated germ-line transformation	41
Culex quinquefasciatus	Inhibit *Plasmodium* development	Expressing transgenes that inhibit *Plasmodium* development	42
Aedes fluviatilis	Avian malaria model	Development of transgenic neotropical mosquito species through germ-line transformation with PB[b] transposable elements	38
Bactrocera tryoni	Sterile insect technique	Germ-line transformation using PB[b] to produce sterile insects	38
Anastrepha ludens	Pest control	Transformation of Mexfly, using PB[b] transposable elements	42
Bicyclus anynana	Model organism	To understand the developmental genetics of colour-pattern formation	43

[a] SIT, sterile insect technique.

[b] PB, *piggyBac*.

4.8 Potential concerns with transgenic insects

The components used to construct a transgene are the factors that first need to be considered when thinking about safety. The gene encodes antibiotic resistance, transposons, recombinase, and other toxic or heterologous genes. The viral components used in vectors need to be considered as potential risks or hazards, and assessed carefully.[44] Although these are merely the possibilities, many alternative methods have also been emerging. Furthermore, the potential risks and hazards are elusive, and need to be demonstrated. Some of the concerns while analysing the possible bio-ecosafety aspects are listed below:

(a) Certain gene expressions in natural conditions may be safe. However, the expression of such genes in unnatural hosts may alter them and raise questions of safety.[45]

(b) Transgenic insects are released in large numbers with the intention of suppressing wild pest populations. Such releases may raise concerns regarding the transport of both living adult modified pests and dead modified larvae or pupae on fruit and vegetables, or other foodstuffs, or in the environment.[44] Thus, transgenic insects may join the food chain.

(c) The release of fertile GM insects may potentially aggravate and augment pre-existing pest problems or create new challenges. It is also possible that GM insects released to control the spread of disease could actually have the unintended consequence of enabling an insect to more effectively spread disease or even carry a human disease that it was not previously able to transmit.[46]

(d) GM insects could have unintended and wide-ranging impacts on the environment and human health due to the complexity of ecosystems and various unknown factors. This also makes risk assessment difficult.

(e) While certain species or populations are diminished by SIT approaches, new insects or diseases may fill the ecological niche left by the insects suppressed or replaced. This could result in new public health or agricultural or ecological problems.

(f) Although there is no evidence of horizontal transfer in transgenic insects to date, there is a possibility that the gene combination engineered into the insects may be transferred into other species through the process of horizontal transfer, causing unintended consequences to the ecosystem.[44]

(g) Novel releases would be impossible to monitor and irreversible.[46]

(h) The risks for workers exposed to transgenic insects also need to be widely assessed.

(i) Evolutionary ecology is balanced in our world. Any alteration or anthropogenic intrusion from outside into the natural environment using technologies could potentially be a human and environmental safety concern.

(j) Cross-border issues have been raised during the release of GMMs in field trials in Brazil, the Cayman Islands and Malaysia.[47] GMMs may spread easily to other countries through a variety of dispersal mechanisms. They may pose risks to biological diversity and human health resulting in adverse safety issues.

By considering the above possible unpredictable risks, the following information needs to be detailed and made available to the public, before planning any environmental release of GMMs: (i) details of the donor and recipient organism; (ii) details of prior safe use; (iii) pathogenicity or infectivity details of the host and related species; (iv) information about the genetic constructs; (v) mode of transformation; (vi) the genetic modification, including phenotypic expression and the stability of phenotype and genotype; and (vii) a description of the methods that could be used to identify the GM insects from their non-GM counterparts. For example, studies should be made and recorded of: (i) the epidemiological factors influencing the transmission of disease in the proposed location; (ii) the survival, multiplication and dissemination of the GM insects; and (iii) the physical, biological, temporal or geographical parameters that limit the potential of the organism. All this information needs to be made available to the concerned authorities and the public.[41]

4.9 Alternative safer molecular components in transformation strategies

To address long-term safety concerns, tremendous improvements in the vectors used in germ-line transformation have been made. A dual vector system was developed for transposon-based gene transfer vectors. With earlier versions, the concern with transposases was that the transposase ORF was also co-delivered into the target cells, which raised the possibility of propagating transposons. In the offspring, the transposon could have been activated at any time, which would have led to many unwanted consequences. This is no longer the case. In order to address safety considerations regarding transposase gene insertions into host genomes, self-inactivating chimeric PB transposase systems have been developed. Improvements to the helper-independent structure were achieved by developing new plasmids in which the PB gene is incorporated. During transposition, the *PB* transposase enzyme recognizes transposon-specific inverted terminal repeat (ITR) sequences located on both ends of the transformation vector and efficiently moves the contents from the original sites and integrates them into the host chromosomal site. In the meantime, the PB transposon is also excised (self-inactivating) and is lost. This characteristic makes PB useful for reversible transgenesis**.** As a consequence, potentially negative effects that may develop by the persistence of an active transposase gene post-transposition are eliminated.[48] The self-inactivating system is an effective alternative in developing the transgenic insects using the transposase-based vectors. Alternatively, transposase genes are not used in many recent strategies. Instead, the transposase mRNA or protein are also co-injected with a transgene construct flanked by terminal inverted repeats.[3] In these cases, there is no possibility for the activation of transposons in the host genome owing to the transgenesis strategy. This procedure increased transgene integration efficiency fivefold compared to conventional pro-nuclear injection or intra-cytoplasmic sperm-mediated transgenesis.[21]

Similarly, self-inactivating retroviral vectors are designed for transgene expression, wherein the vector confers efficient self-inactivation without lowering the vector titer or impairing the expression of the transgene both *in vitro* and *in vivo*.[49] These vectors which are capable of self-inactivation are an effective alternative to the conventional vectors. The SIN vectors are developed by introducing a deletion in the U3 region of the 3'long terminal repeat (LTR) of the DNA which is used to produce the vector RNA. During reverse transcription, this deletion is transferred to the 5'long terminal repeats of the proviral DNA. Since a part of the element is eliminated to abolish the transcriptional activity of the LTR, the production of full-length vector RNA in transduced cells is abolished.[50] The SIN vectors are unable to transcribe full length RNA and they are transcriptionally inactive once they are integrated without lowering the titer or impairing the expression of the transgene. Moreover, viral transductions are carried out only in the laboratory, and again with the help of helper plasmids and packaging cell lines.[51] The retroviral system has been modified by using two plasmids: the helper plasmid and the vector plasmid. The helper plasmid contains the structural proteins for packaging the viral components, but it lacks the packaging sequence (Psi), and hence the transcribed RNA does not incorporate into the recombinant virus. The vector plasmid contains the packaging sequence along with the transgene.[51] The recombinant virus produced will be replicated incompetent since it lacks the structural genes required for replication into a new virus. The packaging is carried out in the laboratory and is never released into the environment. Thus, many self-limiting strategies are designed to remove or inactivate the critical vector components from the genome or organism after performing the required function in the process of transformation. This prevents the persistence of any GM insect with a genome-altering component.

In 2010, the British biotechnology company Oxitec Limited developed and released over 3 million male conditional lethal RIDL mosquitoes over a six-month period in Grand Cayman. An 80% reduction in *Aedes aegypti* was achieved in an area of ~40 acres.[10] The OX513A mosquitoes were used in the trial which carries the LA513 transposon integrated into their genome through a PB helper plasmid.[52] LA513 encodes the tTA.[47] When expressed, the tTA protein binds to the *tetO* operator sequence present in the upstream of *tTA* and drives expression of *tTA* from a nearby minimal promoter, which in turn binds to *tetO*, creating a positive feedback system.[47] High-level expression of *t*TAV is toxic, due to the interaction of the VP16 domain with the transcription factors and this forms a tetracycline-repressible lethal system. When tetracycline is available, it binds to *tTA*, preventing the activator from interacting with *tetO*, thus transgenic mosquitoes can be grown in the presence of the antibiotic in the laboratory, whereas in its absence, transgenic mosquito larvae die in the environment.[47] The resultant transgenic *Aedes* eggs are collected for hatching at a trial site and the smaller male pupae sorted from females. On maturity, they are released into the field, where breeding with wild-type female mosquitoes results in sterile mating.[24] Oxitec's late-acting lethality approach is likely to be more effective at reducing mosquito populations than SIT which uses irradiated insects. A cost-benefit analysis claims that Oxitec's genetic control strategy for *Aedes aegypti* mosquitoes could eliminate dengue rapidly from a human community, at a lower cost than the cost of the disease.[46] Though there is no evidence of horizontal gene transfer between insects and humans due to the consumption of food containing dead transgenic insects or by any other means there is a concern about horizontal gene transfer among transgenic insects. However, recent strategies, such as conditional lethality and the self-inactivating approach are much safer. Since no toxic proteins are produced in the transgenic insects, natural predators would not suffer any harmful effects from the consumption of a modified insect.

4.10 Conclusion

The development of GM insects is an innovative tool to prevent VBDs, control pests and produce recombinant proteins. Despite recent developments in insect transgenesis, the public has a few concerns about its impact on human health and the environment. Research carried out in laboratories, and closed or controlled environments under biosafety regulations is obviously safe, while working within a biosafety framework. This is also true with organisms developed for protein production in controlled environments. With reference to the environmental release, several safe alternative approaches in developing transgenic insects have been optimized and established. Most concerns regarding the current generation of GM insects remain elusive and without a sound evidence base. Hence, they need to be tackled or considered as a policy issue and should be assessed on a case-by-case basis.

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Chapter 5. Risk assessment, risk management and communication protocol, and biosafety considerations

5.1 Introduction

Major ongoing innovations in the control of VBDs involve the use of a variety of recombinant techniques. They can either suppress populations of insects or transform them into less harmful forms. Since the late 2000s, the use of GMVs has moved from theoretical laboratory-based studies to field evaluation and wider deployment. Regulatory frameworks available for the environmental release of GMOs are now being adapted for GM insects, as countries make decisions regarding R&D. The last few years have seen national approvals for open field releases, particularly of GMMs. As a consequence of this activity, authorities are also reviewing their regulatory frameworks and requirements for the field release and deployment of GM insects. Given that the implementation of any new technology could potentially cause human and environmental safety concerns, risk assessment and management planning has to be carried out on a case-by-case basis since the profiles of each GM approach and targeted insect are different.

Science- and technical-based risk analysis, risk management, and the weighing of the potential benefits, are essential elements of the regulatory process and represent a cornerstone for biosafety considerations. Risk analysis should be regarded as a tool providing evidence to support a decision on whether or not to use an intervention. In theory, the release of self-limiting genetically engineered insects should have similar risks, although assessments must be conducted on a case-by-case basis for each insect species and inserted trait combination. The approaches used in the release of beneficial insects and/or SIT programmes can serve as a precedent for countries considering how to regulate genetically engineered insects.[1]

However, to date there has been a preference for regulators to use the genetic engineering legislative approach. There is a need to develop a universal assessment criteria framework for genetic control methods, as has been done for SIT applications of Tephritid fruit flies,[2] so that information is transparent and can easily be exchanged between the countries.

5.2 Phased testing for risk assessment

A phased testing approach is considered an appropriate way to assess risk for new technologies in a wide range of fields including GM crops, chemicals and pharmaceuticals.[3-8] A step-wise approach has also been taken in the evaluation of genetically engineered insects. Phases can include the following, which may or may not always be sequential, depending on facilities, experience and mutual recognition of data:

a) laboratory testing in contained use conditions
b) confined field testing
c) open field release
d) pilot operational evaluation

5.2.1 Laboratory testing

Transformation of, and research into GM insects in contained use (laboratories and quarantine facilities) is widespread and non-controversial, following established guidance for recombinant organisms.[9–13] Containment refers to practices that prevent unplanned or uncontrolled release of organisms into the environment and is likely to encompass physical structures, standard operating procedures (SOPs), working practices, and the use of trained staff. The small sizes, high degree of mobility and, in some cases, long lifespan represent unique challenges in the physical containment of arthropods.

5.2.2 Confined releases

Confined release is often a key step in the phased testing of transgenic arthropods, and contributes to the evaluation of the strains for open release by providing a semi-natural or larger natural environment to conduct experiments. Confined release as defined by the North American Plant Protection Organization (NAPPO) Regional Standards for Phytosanitary Measures (RSPM) No. 27[14] can include physical confinement, but may also include biological, geographical and/or temporal barriers to facilitate confinement. Field cages are often temporary facilities or large insectaries in which research is carried out with arthropod vectors. However, the difference is that if there are GMM escapees from a field cage, the mosquito may become established in the environment, depending on the trait that has been introduced into the mosquito. See Knols et al., Ferguson et al. and Helinski for a review of field cages and protocols for the use of GMMs and SIT mosquitoes.[15–17] A semi-field system and protocol for contained trials of a self-limiting GM *Ae. aegypti* in Mexico is described by Facchinelli et al.,[18] in Malaysia by Lee et al.[19] and Chambers et al.[20] and details a semi-field cage design and the results of experiments with *Wolbachia*-introgressed *Ae. polynesiensis*, the vector for LF in the South Pacific. Confined releases are by their nature limited in scope and scale, and although useful at providing early evaluations and data for future risk assessments, some information on, for example, dispersal, can only be obtained through open field releases.

5.2.3 Open field releases

Releases in the environment play a critical role in the evaluation of GMMs particularly for the assessment of mating competitiveness, longevity and dispersal, and for determining the efficacy of the intervention in an environmental setting typical of the insect species. Releases also facilitate the collection of information for biosafety evaluation. Several open field releases of GMMs have been conducted and published in the scientific literature.[21–25]

5.2.4 Pilot operational study

This phase of study is a pre-operational phase treating a much larger area than in open field releases. There have been two examples of GM insects being released on this scale to date. The first is the pilot phase for the release of GM pink bollworm in the USA, which contained a marker gene to facilitate identification and was irradiated for sterility. Over 15 million GM pink bollworm were released over several thousand acres of cotton in Arizona.[26] The second pilot open release has recently been approved by the Comissão Técnica Nacional de Biossegurança (CTNBio) in Brazil for the release of self-limiting GMMs (*Aedes aegypti* OX513A) in an area of 50 000 people in the north-east of Brazil, although, at the time of writing, this trial has not yet begun. Pilot operational studies may also potentially include epidemiological endpoints alongside entomological ones, as the scale of the trial could accommodate such investigations.[27]

5.3 Field site selection

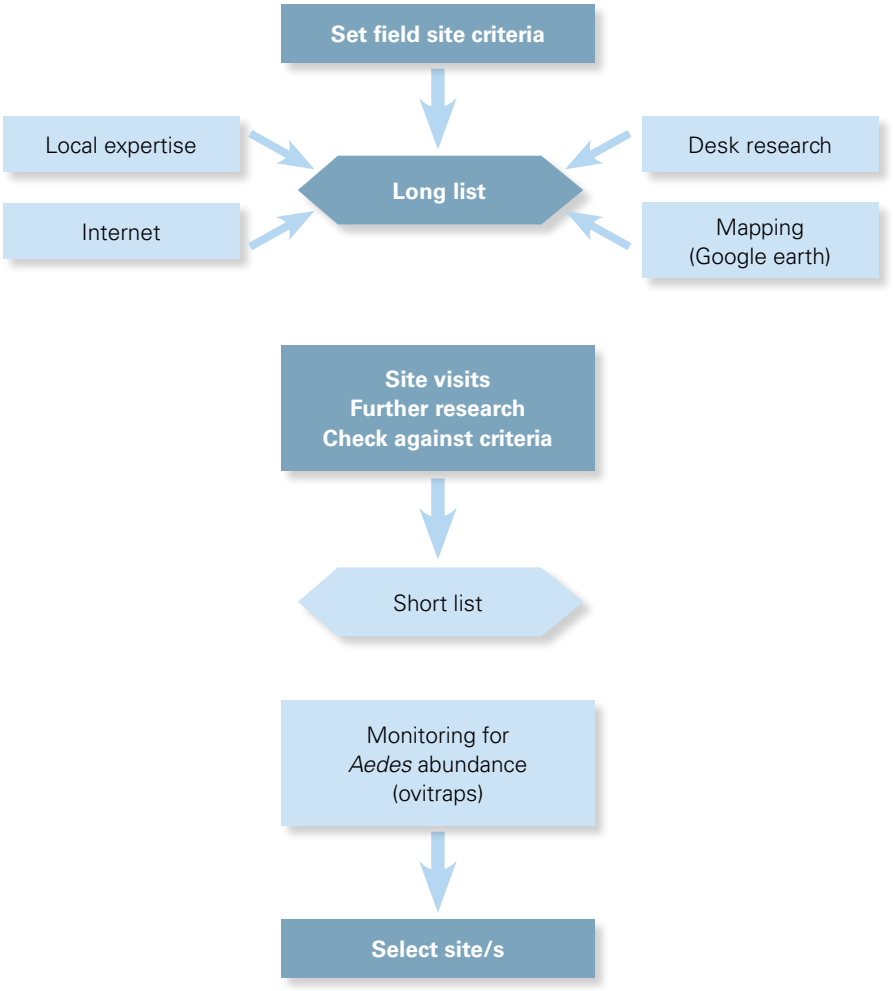
Field site selection criteria are an important consideration and will be largely dependent on trial objectives and design. However, there are three main considerations: (i) scientific/technical requirements; (ii) community engagement and ethical considerations; and (iii) regulatory approval/acceptability. Each of these will need to be tailored to the context of the trial's objectives. Some general issues that need to be considered are given in Box 2.

BOX 2. **LIST OF ISSUES TO BE CONSIDERED DURING FIELD SELECTION**

- Presence of the target insect species at the release site.
- Size, location and ecological stability of the release site/human population size to meet the trial objectives.
- Availability of similar control sites/historical data on the native insect population, and disease incidence at the site
- Resources to deliver trial (human, equipment, logistics including transport, monitoring, data handling).
- Community engagement at the release site and in the wider national and international community.
- Communication strategy – this goes hand in hand with community engagement and should be considered and implemented in advance of any trial.
- Regulatory approval for the release of the vector insect.

The schematic diagram in Figure 14 presents an approach to field site selection. It should be noted that the majority of the initial selection of a long list of potential sites could be approached as a desk–based exercise using existing data and Internet information. Site visits can then be conducted to confirm or discount potential sites. Approaching site selection in this way can be cost effective compared to using personnel to conduct visits to all potential sites.

Figure 14. Schematic design for field site selection



5.4 Components of risk analysis

It is widely agreed that risk analysis consists of the following three major components:

- risk assessment (identification of risks)
- risk management (deployment of plans to manage risks)
- risk communication (exchange of ideas).

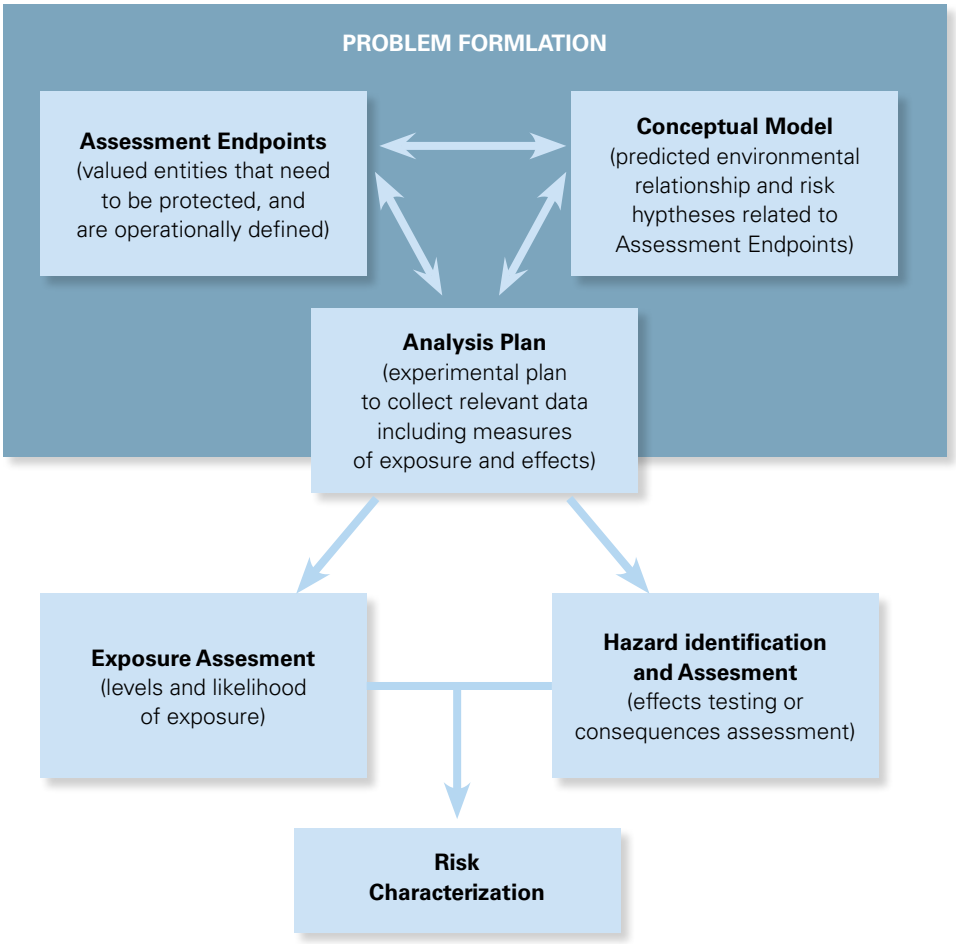
These three components are highly inter-dependent, although the separation of risk assessment and risk management is seen as critical by several regulatory authorities, for example, the Office of the Gene Technology Regulator (OTGR)[28] and the EFSA.[29] A fourth element is problem formulation, which directs the other three steps in the risk analysis. Risk analysis should be regarded as a framework that provides evidence to the decision-makers.

5.5 Problem formulation

The first part of risk analysis is the identification of potential harms by addressing the question: "what is the concern?" Why should these products be analysed for their risk and what are you aiming to protect by that risk analysis? This stage is often known as problem formulation and helps to direct the risk assessment to protect the valued entities. These entities will frequently be different in different environment and may include threatened and endangered species along with those that have cultural significance, or are valued by the public for other attributes. Once it is known what entities need protecting, the risk assessment can be constructed to ensure the risk to those entities is assessed. However, several problems can be encountered at this stage, such as protection goals (i.e. what you are trying to protect from risk), which are often ill defined or vague in the legislation. Some examples of this are the EU Directive 2004/35/EC (Environmental Liability) stating, "any damage representing measurable adverse change in a natural resource" and also the Cartagena Protocol on Biosafety (CPB) which defines harm as "measurable loss or damage… that has adverse impact upon conservation… of biodiversity." It is very difficult from these statements to define what needs protection, and often this is a decision required at policy or governmental level, making it even more difficult for the risk assessor to determine. The skill of the risk assessor lies in the ability to translate these high-level policy statements into specific ecological endpoints that are capable of scientific measurement. However, generally, they can be translated into broad areas for consideration of risk: protection of the environment; protection of human and animal health; and protection of agricultural systems. These may be further broken down into areas such as: the protection of ecosystem services; the protection of threatened and endangered species; and the protection of specific habitats. The increasing granularity and definition of the protection goals allows the prediction of the relationship between measurable endpoints and the potential "stressor", i.e. the GMV, and assists with the development of an assessment plan. The measurable endpoints may be defined by regulation, e.g. the U.S. Department of Agriculture assesses risk of the "stressor" being a plant pest. Data can then be collected to test the hypothesis of the effect of the "stressor" on predicted environmental endpoints.

The use of problem formulation is widespread in risk assessment and Figure 15 shows the scheme proposed by the US Environmental Protection Agency in 1998[30] for chemical risk assessment. This has subsequently been largely adopted for risk assessment of GM crops. [31–34]

Figure 15. Schematic diagram of problem formulation in the context of the risk-assessment process as proposed by the U. S. Environmental Protection Agency, 1998



Source: 30

5.5.1 Risk assessment

Although risk assessment may mean different things to different people depending on their values, it is commonly agreed that it is a formal or rational procedure or process to assess the significance of risk, and to input into informed decision-making. Perhaps more simply put, it is a formal procedure to assess the "probability of something bad happening, multiplied by how serious that is", in order to make a decision whether or not to proceed. Risk assessment can be qualitative, semi-quantitative or quantitative, or a mixture of all three. The choice often depends on the amount and quality of available scientific data, the complexity of the risk under consideration, and the level of uncertainty concerning the potential risk. In some cases, where the scientific knowledge base is limited, there will be scientific uncertainty. A higher degree of scientific uncertainty might point towards a qualitative risk assessment, rather than a quantitative one. Risk assessments for products interacting in biological systems such as GMOs are often qualitative due to the complexity of inputs, limited information in certain areas, the potential for multiple outcomes, and the potential for adverse effects that may only be identified in the long term. In all cases, the aim is to have a repeatable, systematic and structured approach to risk evaluation based on sound scientific evidence.

Codex Alimentarius Commission[35] has defined risk assessment as "a scientifically based process" consisting of the following steps:

i. hazard identification
ii. hazard characterization
iii. exposure assessment
iv. risk characterization

These steps can be considered as a series of simpler questions.

- What might happen? (Hazard identification)
- How might it happen? (Causal pathway to harm)
- Will it be serious if it happens? (Hazard characterization and exposure assessment)
- How likely is it to happen? (Exposure assessment)
- What is the risk? (risk characterization)

In reality, risk assessment is iterative with risk management and risk communication informing the assessment element. Provision should, therefore, be made to allow the risk assessment to be varied in the light of new information. It should also allow the regulator to vary, suspend or revoke the permission to proceed if new information comes to light that negatively alters the risk assessment and the potential risk to human health and/or the environment. The opportunity should also be given to the permitee to initiate remedial action, if appropriate.

Certain activities may become routine and the risks so well known that provision should also be given to reduce the regulatory requirements for these certain activities.

To date several risk assessments have been conducted and published for genetic-based mosquito control methods.[36–40] Others are available on-line.[41,42] Risk assessments are also available for other GM insects.[43–45] Information that might be pertinent to risk assessment of GM mosquitoes is summarized from a wide variety of guidance documents in Box 3.[46–49]

BOX 3. FACTORS FOR CONSIDERATION IN RISK ASSESSMENT OF GMMs

1. The characteristics of the recipient mosquito, including taxonomy, source, geographical distribution, mobility, longevity and reproductive potential.
2. The characteristics of the donor organism(s), prior history of safe use, nature of pathogenicity or infectivity.
3. The characteristics of the genetic construct, vector and mode of transformation.
4. The genetic modification, including phenotypic expression and a description of the genetic construct, stability of phenotype and genotype, and a description of the methods that could be used to identify the GMM from its non-GM counterpart.
5. The agents that might be transmitted and whether the mosquito is or may be infected, along with the ability of the mosquitoes to transmit (one or more) pathogens.
6. The epidemiological factors influencing transmission of disease in the proposed location.
7. Survival, multiplication and dissemination of the GMMs and conditions that might affect these parameters in the receiving environment.
8. Physical, biological, temporal or geographical parameters that limit the potential survival, multiplication and dissemination of the GMMs.

Source: 50

5.5.1.1 Hazard identification

A hazard is an intrinsic property of the organism, which in the context of GMMs should be considered if the genetic modification itself has altered the intrinsic properties of an unmodified comparator mosquito strain. The process of hazard identification consists of envisioning all potential hazards that could occur. An important concept is that although a hazard could theoretically occur, it may not necessarily be harmful if it does not have a negative effect on the protection goal, or the effects are not specified in regulation.

5.5.1.2 Hazard characterization

Once a list of all the potential hazards has been compiled, hazard characterization is the next step. This step looks at the likelihood of the hazard occurring and the magnitude of the effect (consequence) if the hazard was to occur. It is at this stage that it is important to establish if a causal link between the hazard and the outcome can be identified. If not, then this hazard is unlikely to occur and can be assigned as negligible. This framing helps assess what information/data might be required to address the potential risk and whether there is either uncertainty or confidence in the assessment. It also allows certain unrealistic hazards to be set aside. Likelihood and magnitude (consequence) can either be qualitative or quantitative, and can be assigned a confidence statement, i.e. where there are a lot of data and or previous experience, a higher degree of confidence in the assessment may be assigned compared to where data or information are sparse.

5.5.1.3 Exposure assessment

If there is no route of exposure the risk can be regarded as negligible. For example, in the case of GMMs, if a hazard has been identified that is only applicable to female mosquitoes but no females will be present in the programme, then a route of exposure cannot be established and any risk is unlikely.

5.5.1.4 Risk characterization

Risk is measured by a combination of the likelihood that a hazard will result in an adverse outcome and the consequence of that adverse outcome being realized. These elements can then be incorporated into a risk assessment matrix as shown in Table 13 below.

Table 13. Example of risk assessment matrix

Likelihood	Risk estimate			
Highly likely	Low	Moderate	High	High
Likely	Negligible	Low	High	High
Unlikely	Negligible	Low	Moderate	High
Highly unlikely	Negligible	Negligible	Low	Moderate
	MARGINAL	MINOR	INTERMEDIATE	MAJOR
	CONSEQUENCES			

Source: 28

5.5.2 Risk management

Risk management also can follow a step-wise process, similar to risk assessment, with different risk management strategies depending on the phase of evaluation. Risk management has the ability to reduce uncertainty and increase confidence.

5.5.2.1 Laboratory trials

For risk management in the laboratory, the primary strategy is physical containment. This could include the use of cages, contained rooms, levels of physical containment including air curtains, mesh screens on doors and windows as well as waste disposal. Other elements of risk management in the laboratory include the training of staff and their use of SOPs as well as record keeping of insect stocks.

Physical containment and procedures for laboratories holding arthropods have been well described in many documents (the list below is indicative and not exhaustive), and these documents should be consulted for further information.

- American Society Tropical Medicine and Hygiene (ASTMH). Arthropod Containment Levels (ACLs).[51]
- Canadian Food Inspection Agency (2005).[52]
- North American Plant Protection Organization (NAPPO).[53]

- International Sanitary Protection Measures (ISPM).[54]
- World Health Organization (WHO) Laboratory Biosafety Guidelines.[55]
- Australian Guidelines for the Certification of a Physical Containment Level 2 Arthropod Facility.[56]
- MR4 Methods in Anopheles Research.[57]

5.5.2.2 Semi-field or cage trials in the environment and open trials

In semi-natural environments, cage trials or open field trials, physical containment becomes less important and other considerations should be factored into risk management. These could include: the scope and scale of the release; the physical, temporal, geographical or biological barriers in place; the handling and experimental/operational procedures; the training of staff involved; the nature of the introduced traits in the GMMs (self-limiting vs. self-sustaining); vector management practices proposed or already in use (fogging and integrated vector management or IVM), behavioural activities, etc. Risk management may form part of the authorities' conditions for granting the regulatory permit for the trial, as in the field release of self-limiting GMMs in Malaysia.[37] An essential part of the system must be the monitoring of compliance with the regulatory system, ensuring that risks to human health and the environment are monitored, and the risk management plans are carried out according to the provisions of the permit.

One element of a risk management plan that warrants further attention is the preparation of an emergency response document, and this is often a requirement under the release legislation. An emergency response plan allows the applicant to have a pre-meditated procedural document that could be followed in the case of accidental or inadvertent release from containment. Also, this procedure could be followed if adverse effects were noted on human health or the environment during the course of a trial, irrespective of the scope, scale and duration. This emergency response plan should consider and have procedural actions for the following elements.

- Clear accountability for emergency decision-making, where a chain of command is outlined.
- Methods for monitoring and detection, so it will be known if an inadvertent or accidental release has occurred. However, consideration should be given to the sensitivity, reliability and specificity of detection methods, as well as to the duration and frequency of the monitoring proposed. Monitoring is a valuable tool in validating/invalidating the original risk assessment.
- Reporting of accidents/incidents – timeframe, to whom, by whom.
- Methods and procedures for controlling the GMO in case of unexpected spread or to "clean up the affected area"; this could include extended trapping, or the use of insecticides. Any methods considered here should be commensurate with the risks identified in the risk assessment.
- Plans for protecting human health and the environment in the case of an adverse event occurring.
- Communications plan on how to handle media reporting and consideration of any liability issues that might arise.

Risk management may involve decisions that are influenced by socioeconomic factors as well as the political zeitgeist. However, for GMMs, it is valuable to be able to consider the potential risks against alternative strategies for vector control, such as pesticides as well as other interventions such as the availability and reliability of vaccines or other therapeutic interventions, where they exist. This allows the potential risk of the use of GMMs to be balanced with overall programme costs (financial and otherwise) and benefits – or at least reduction in risks – to human health and the environment.

5.5.3 Risk communication

A report by the National Research Council[58] described risk communication as "an iterative process of exchange of information and opinion among individuals, groups, and institutions". It further states that risk communication "involves multiple messages about the nature of risk and other messages, not strictly about risk, that express concerns, opinions, or reactions to risk messages or to legal and institutional arrangements for risk management". Similarly, the Codex Alimentarius Commission has defined risk communication as:

…the interactive exchange of information and opinions throughout the risk analysis process concerning risk, risk-related factors and risk perception among risk assessors, risk managers, consumers, industry, the academic community and other interested parties, including the explanation of risk assessment findings and the basis of risk management decision.[35]

The Scientific Steering Committee of the European Commission[59] has suggested that expressing the results of risk assessment in a user-friendly format such as risk ranking, comparison with alternatives, and using risk benefit analysis would be a useful way of communicating risk.

Risk communication is, therefore, a key component in the acceptance and uptake of novel public health interventions. Early and effective community engagement can help to offset some of the uncertainty and corresponding need for precaution in the introduction of novel technologies,[60] particularly those based on genetic modification, for which GM crops continue to be the subject of entrenched and polarized positions.[61] The use of new drugs and vaccines delivered to individuals represents a counter position, where much effort and harmonization of protocols for ethical consideration has taken place in the area of clinical interventions.[62] GMM interventions need to be able to bridge both these activities as they are being developed ultimately for the improvement of public health, but at the same time are under the legislative frameworks of GMOs.

5.6 Conclusion

The use of GMMs represents a novel and innovative tool to address human VBDs. However, despite rapid advances, there are no widely accepted regulatory or biosafety frameworks that provide guidance on all aspects including risk analysis, although some are currently in development.[46–48,63] This vacuum has sometimes led to criticism of regulatory processes and how developers of the technologies should be proceeding.[1,64,65] It is proposed that such guidance could facilitate the standardization of procedures and the comparability of results and conclusions, allowing robust assessments by decision-makers.[48,49] However, even if such a document were in place, there would still be a requirement for countries to develop national guidance and policies within their own legal frameworks, as well as to build local capacity to safely assess the risk of the environmental use of GM insects. In discussing the principles of risk assessment, management and communication, field site selection, and how they might be applied to GM insects, it is hoped that this chapter represents a step in building that capacity. However, it is likely that risk communications and the risk perception of the public along with the acceptability of such risks, balanced against the potential benefits of sustainable vector control, will ultimately decide the pace of GMM development for the control of VBDs.

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Chapter 6. Field preparation and regulatory needs prior to open release of GMMs[1]

6.1 Background – dengue epidemiological scenario in Malaysia

Dengue is the most rapidly spreading VBD in the world and Malaysia is amongst the worst affected country with more than 38 000 notified cases annually in the past six years. There is no specific treatment for dengue and no vaccine;[1,2] none is expected for at least 10 years. Control of the vector mosquito is therefore the only way to control or prevent dengue. However, current methods such as space spraying (fogging) with insecticides and the application of larvicides are of limited effectiveness. *Aedes* mosquitoes breed in a wide range of small containers, generally rainwater-filled or stored drinking water. It is an impossible logistical challenge to find and treat all of these containers in a tropical country such as Malaysia. The difficulties are further compounded by the relatively low number of mosquitoes needed to sustain the epidemic transmission of dengue, which can be as low as 2–3 adult female mosquitoes emerging each day per 100 people in a locality. Although bednets are effective for malaria control, they are ineffective for dengue as the mosquito bites during the day. Many of the same issues apply to chikungunya, another viral disease transmitted by *Aedes* mosquitoes.

6.2 RIDL® OX513A – a promising biotechnology solution to dengue virus

The Institute for Medical Research (IMR) in Malaysia has therefore refocused its research efforts on the evaluation of new technologies that seem promising to reduce the vector population below the low dengue transmission threshold. One such technology currently under field evaluation by the IMR is RIDL.[3–6] This new biotechnology solution for dengue is based on advances in molecular biology and genetics made at the University of Oxford with Oxitec Limited. As a company, Oxitec is trying to reduce the spread of the DENV by limiting the population of *Aedes aegypti*. This chapter describes the evaluation by IMR of a RIDL strain (RIDL-513A) of the mosquito *Ae. aegypti* and of an experimental fitness study of the same strain by International Institute of Biotechnology and Toxicology (IIBAT), India.

6.3 RIDL strategy

The science behind RIDL is known as 'repressible lethality', i.e. introduction of a specific DNA construct into *Ae. aegypti* eggs through microinjection so that the transformed mosquito is destined to die at larval or pupal stage unless it is provided with a nutritional supplement (tetracycline) in the rearing medium. This supplement represses the lethal gene and hence allows the mosquito larvae to grow normally into adults when they are reared in a laboratory or rearing facility. However,

1. Evaluation of a RIDL strain (RIDL-513A) of the mosquito *Aedes aegypti* by IMR, Malaysia, and experimental study of RIDL strain in IIBAT, India.

these larvae are destined to die in the wild due to the absence of tetracycline, which is an unstable compound in the environment, degrading by several routes including photo-degradation in sunlight with a half-life of under two hours at pH 8.[7] Using the RIDL technology, Oxitec has developed genetically sterile (homozygous) male *Ae. aegypti* mosquitoes, which mate with wild females to produce (heterozygous) offspring that will all die as larvae or pupae in the absence of a food supplement.[6] By releasing large numbers of these sterile male mosquitoes (remembering that male mosquitoes cannot bite) in a sustained manner, *Ae. aegypti* population can be crashed below the disease transmission threshold, and possibly even eradicated within a year from large communities according to research led by Stanford University.[8]

6.4 Attained regulatory consideration and containment requirements in Malaysia

Following the submission of technical and other information, site inspections were conducted by three different committees, as given below.

- IMR's Occupational Safety and Health (OSH) Committee.
- The Biological Safety Sub-Committee.
- The national Genetic Modification Advisory Committee (GMAC), leading to regulatory clearance from the GMAC to proceed with the confined evaluation.

The written consent of the Director General of Health was also obtained, and the Prime Minister's Department was also notified as per the regulations.

The IMR established two state-of-the-art facilities viz. an ACL-2 laboratory and ACL-2 field house for the first two stages of the evaluation. These facilities meet or exceed the level of containment adequate for conducting confined trials in line with the requirements of the above- mentioned regulatory committees. The ACL-2 laboratory was set-up to colonize the RIDL mosquitoes and conduct bionomic studies on them, while the ACL-2 field house was built to conduct mating competitiveness and compatibility studies.

6.5 Phased studies on RIDL *Ae. aegypti* prior to release

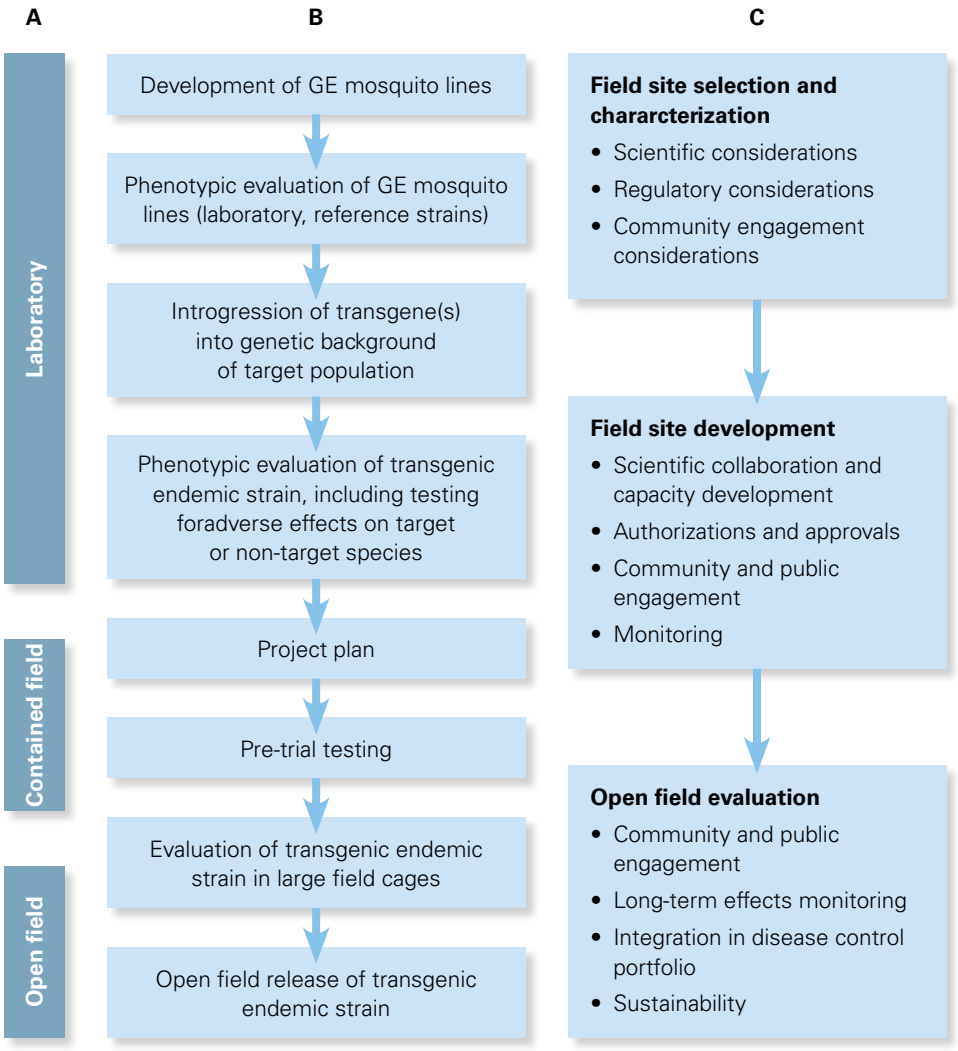
Prior to the open field release of RIDL *Ae. aegypti,* the project was divided into three major phases (Figure 16):

i. Laboratory study and evaluation of the RIDL strain;

ii. Semi-field contained trial to evaluate the mating competitiveness of RIDL *Ae. aegypti*; and

iii. Open field release.

Figure 16. Phased testing prior to release of transgenic insects/vectors



Source: 9

6.5.1 Stage 1: Laboratory study and evaluation of RIDL *Ae. aegypti* in Malaysia

Objectives

The main aim in conducting the laboratory study was to evaluate the following basic characteristics of GMMs.

- Study molecular, genotypic, physiological and behavioural characteristics of GMMs.
- Compare these characteristics to those of wild-type mosquitoes.
- Evaluate the stability of these characteristics over subsequent generations.
- Evaluate the ability of the gene drive system and lethal gene to spread through a mixed population of transgenic and wild-type mosquitoes in the laboratory.

Colonization and rearing

The RIDL strain, called RIDL-513A, was imported and successfully colonized in purpose-built facilities at the IMR. The strain maintained consistent properties throughout the study. Successful colonization of RIDL *Ae. aegypti* was a prerequisite for the bionomic and mating competitiveness studies. The IMR's experience in colonization and rearing of wild-type mosquitoes goes back over 100 years to its establishment as a research outpost for the London School of Hygiene and Tropical Medicine in 1900. In order to master new developments such as transgenic RIDL mosquitoes, the IMR established the necessary ACL-2 facilities, and also conducted an 'Intensive workshop on wild type and genetically sterile *Aedes* mosquitoes' with Oxitec Limited.[10] Oxitec's SOP for rearing RIDL *Ae. aegypti*[11] was obtained and modified to suit local requirements.

Process of colonization, rearing and comparative life history studies on wild-type and transgenic *Ae. aegypti* (L.)

Fifteen single pairs (one male and one female) each of LA513A and of wild-type (WT) mosquitoes were established in small cages (Figure 17). These mosquitoes were termed F0. The females were blood-fed on mice five days after eclosion. For 513A, the larvae were reared in water supplemented with tetracycline to 30 µg/ml. All experiment containers were maintained at 26±1°C with a 12:12 hour (light/dark) photoperiod and 75% relative humidity in the ACL-2 laboratory in the insectarium.

Figure 17. Steps followed in the laboratory







Collected 30 pupae

↓

Individually into 15 ml

↓

Pairing F0 into individual cages

↓

Bloodmeal for 24 hours or until fully engorged

↓

Set ovicup for egg laying

↓

Embryonated eggs air-dried

↓

Hatching eggs until fully engorged

↓

150 1st instars larvae from each parent in single 15 ml vial

↓

Pairing the first 20 adults emerged

↓

Survivorship determined every 24 hours

↓

Bloodmeal for 24 hours or until fully engorged

↓

Daily fecundity and hatchability for 4 consecutive gonothropic cycles

↓

Calculate sterility rate

↓

Record sex ratio for each GC

Results of the laboratory study

No significant differences on morphology and life history traits: Many physical and biological traits of RIDL-513A were measured, relating to all life-cycle stages from egg to adult. There are various traits such as female oviposition/fecundity, egg hatch rate, and developmental rate (number of days as fourth L4, number of days as pupa, time taken to develop from L1 to adult) was assessed. No significant differences were detected between the RIDL-513A strain and a control wild-type strain in any of these traits. The comprehensive tests indicated that RIDL-513A has promise as a new vector control tool, without any adverse effects observed.

No significant differences in survivorship in each life-cycle stage: The number of larvae developing to each successive developmental stage was assessed. There was no significant association between strain and survivorship in any larvae stage (L1–L4), or pupae, or in the number of adults emerging (Chi-squared test, $p>0.05$ for all stages).

No partial life tables and preimaginal development for F1 generation: Eggs were collected from F0 females (i.e. F1 eggs) for three gonotrophic cycles. These were allowed to hatch and develop into adults (under permissive conditions). The following life history and development parameters were measured for each gonotrophic cycle:

- time to oviposition
- egg production
- egg hatch rate (%)
- larval survival to pupa
- pupal survival to adult
- sex ratio of emerging adults.

In general, few noteworthy differences were seen between the wild-type and RIDL-513A strains for these parameters. These exceptions were:

- preoviposition time in first gonotrophic cycle was slightly longer for RIDL-513A (1.78 ± 0.17 days) than for wild type (1.32 ± 0.11 days);
- hatch rate was quite variable, which is not uncommon for *Ae. aegypti*. In these experiments, the hatch rate was higher for RIDL-513A in the first two gonotrophic cycles but lower in the third.

There was no significant difference in adult fecundity between the number of eggs laid by transgenic and wild-type *Ae. aegypti* for all gonotrophic cycles. The sex ratio for adult progeny for each strain across all gonotrophic cycles was similar, and approximately 1:1, as expected.

Post-emergence adult longevity was also assessed. Adult longevity for the Malaysian wild-type strain was longer than for the RIDL strain. For the first 40 days, the percentage of male mortality in the RIDL strain (85%) was higher than in the Malaysian wild strain (60%). Mean adult lifespan for Malaysian wild- strain males and RIDL males was 25.67±6.53 days and 20.00±10.60 days, respectively. This is consistent with data from the Institute Pasteur indicating that the mean lifespan of RIDL-513A is approximately 18% less than that of control wild-type strains.[12] Mortality of RIDL females (15%) was higher than wild females (5%) after 40 days. During this period, mean adult longevity for RIDL females and wild females was 11.33±3.05 days and 8±0.00 days, respectively.

Discussion

This study investigated bionomic and life history parameters of a transgenic mosquito strain carrying a repressible lethal gene, relative to a Malaysian wild-type strain. An earlier study on *Anopheles stephensi* measured whole life-cycle fitness of several transgenic strains by monitoring the change in allele frequency over time in mixed populations of transgenic and wild-strain mosquitoes in the laboratory.[13] Irvin et al.[14] conducted a similar study comparing various bionomic and life-history parameters of four transgenic and one wild-type strain of *Ae. aegypti*. These studies focused on transgenic mosquitoes designed to replace the natural *Ae. aegypti* population and, thereby, to establish stable and persistent populations of transgenic mosquitoes in the wild. In contrast, it was intended to use RIDL strains to suppress the target population in a method analogous to the SIT. Therefore, the transgene is not required or expected to persist in the wild, and different aspects of fitness and performance become significant. Parameters, such as female fecundity, egg hatch, larval development, etc., relate primarily to the ease and efficiency of mass production, whereas adult male performance parameters, such as mating competitiveness, relate to field performance.

The fitness of the transgenic 513A strain and wild strain of *Ae. aegypti* used in this study was not significantly different in several parameters. There was no significant difference in number of eggs laid, larvae hatched and pupae in F1 generations, nor in the number of days in each stage of life (developmental period). The number of larvae, pupae surviving and adult emerged in each stages of life for both strains were not significantly different.

The results obtained were different from previous study by Irvin et al.[14] The transgenic strains they investigated showed significant reduction in many fitness parameters relative to the wild-type strain. In essence, the transgenic strains were severely compromised and uncompetitive with wild type. In contrast, in our study most of the parameters measured showed no significant difference between wild-type and transgenic strains; others (e.g. egg production or hatch rate in different gonotrophic cycles) showed differences but not enough to imply a major difference in fitness between the two strains.

There are several potential reasons for this apparent discrepancy. Firstly, the RIDL-513A strain was pre-selected by Oxitec using some basic experimental determinants of fitness, although detailed bionomic measurements had not been performed. Secondly, and more significantly, Irvin et al.[14] had shown that transgenic lines can reduce fitness, not that this is inevitable. The fact that insertional mutants can be deleterious is obvious; the question is whether strong negative effects are unavoidable. Marelli et al.[15] discussed this question, taking into account also the larger literature from *Drosophila* on insertional mutations, and concluded that a significant proportion of insertional transgenics should have only "modest" (but probably "non-zero") fitness penalties relative to wild type.

A further issue confounding some previous studies of fitness relates to inbreeding. Catteruccia et al.[13] found significant fitness defects in their transgenic *Anopheles stephensi*, but concluded that much or all of this was due to inbreeding. Moreira et al.[16] avoided inbreeding effects by using out-crossed heterozygotes, and found that the fitness penalty differed depending on the expressed gene, i.e. zero for SM1 but significant for PLA2. Prior to transfer to the IMR, Oxitec made considerable efforts to minimize inbreeding effects during the construction of the RIDL-513A strain. Similarly, after transfer to IMR, the strain was kept at a minimum population size of 200 individuals, and egg storage was used to minimize the number of effective generations, both of which to minimize genetic drift and bottlenecking.

It is hardly surprising that some genes might be deleterious when ectopically expressed, especially when they are supposed to cause the death of that organism. However, in the specific case of RIDL-513A, the gene is supposed to be lethal when

de-repressed. Therefore, any significant uncontrolled leakiness in the control of gene expression might reasonably be expected to lead to mortality or loss of fitness. Therefore, there would need to be tight regulation of the gene. The fact that significant reductions in fitness were not observed validates the proposal of Gong et al.[17] that the "positive feedback" design incorporated into RIDL-513A[6] may give superior control over gene expression and minimize adverse fitness effects.

The transgenic strain RIDL-513A was initially constructed using transposon-mediated transformation with a non-autonomous *PB*-based transformation vector.[6] PB has several advantages for this purpose. One is that its insertion and excision is unusually precise for this class of elements, with no evidence of imprecise insertions and excisions during transformation. This effect, well known for *P* elements in *Drosophila*, can lead to the creation of additional deleterious mutations in the course of transformation or remobilization of *P* elements; the advantage of PB in this respect has been noted previously.[18] A second advantage of *PB*, though not specifically related to fitness, is that PB insertions in *Ae. aegypti* are extremely stable, even in the presence of exogenous PB transposase.[19] The single report of an apparently unstable PB insertion in *Aedes* represented an aberrant insertion event with multiple copies of the entire plasmid in an array.[20] Such an array is susceptible to recombination, without the need to invoke a transposase-mediated mechanism.

In summary, it found that for multiple parameters (pre-oviposition period, life-time fecundity, offspring sex ratio and female sterility) the transgenic RIDL-513A strain was not significantly different from the wild strain. This contrasts with previous studies by Cateruccia et al. and Irvin et al.[13,14] These studies reported that transgenically modified mosquitoes especially *Ae. aegypti* and *Anopheles stephensi* were not competitive for all measured parameters compared to the wild strain of these mosquitoes. Instead, sharing the conclusion of Marelli et al. and Moreira et al.[15,16] that transgenic lines can be constructed which have little or no fitness penalty relative to wild type; RIDL-513A seems to be an example of such a line.

Finally, it should be noted that all these experiments were conducted under permissive conditions; RIDL-513A larvae reared in restrictive conditions die as late larvae or pupae. The strain, therefore, has a fitness of approximately zero under restrictive conditions. This is done by design so that the offspring of wild-type mosquitoes mating with RIDL-513A will not survive.

6.5.2 Stage 2: Semi-field contained trial to evaluate the mating competitiveness of RIDL *Ae. aegypti* in Malaysia

Establishment of ACL-2 field house facility at the Medical Entomology Unit, IMR

In 2006, the IMR built a state-of-the-art Temporary Contained Trial Facility (TCTF), to ACL-2 standards (figures 18–22). These experiments, in a purpose-built field house at the IMR, are perhaps the most advanced (near-field) tests of a RIDL mosquito strain so far conducted worldwide. Male mating competitiveness is the most important parameter used by entomologists to determine whether a given strain of GM male mosquito is fit for further evaluation and to understand the major problems with radiation-sterilized mosquitoes. This was carefully measured under conditions that mimicked the natural domestic and peridomestic environment of the mosquito.

Previous mating competitiveness studies were conducted in small cages, e.g. the study by the Institute Pasteur used cardboard cylindrical cages of 0.54 litres (L) or 0.02 cubic feet capacity (diameter 8.5 cm and height 9.5 cm) to house as many as 15 mosquitoes.[21] Such cages are not only an artificial environment, but are also likely to result in chance mating due to lack of space. As *Ae. aegypti* is anthropophilic, the IMR decided to overcome these drawbacks with cages

by evaluating mating competitiveness under semi-field conditions in a field house (i.e. the mosquitoes' natural habitat). Each step (importation, testing in quarantine conditions, testing in a confined field house) was preceded by appropriate regulatory approval from the competent authorities including the GMAC.

Figure 18. TCTF outline

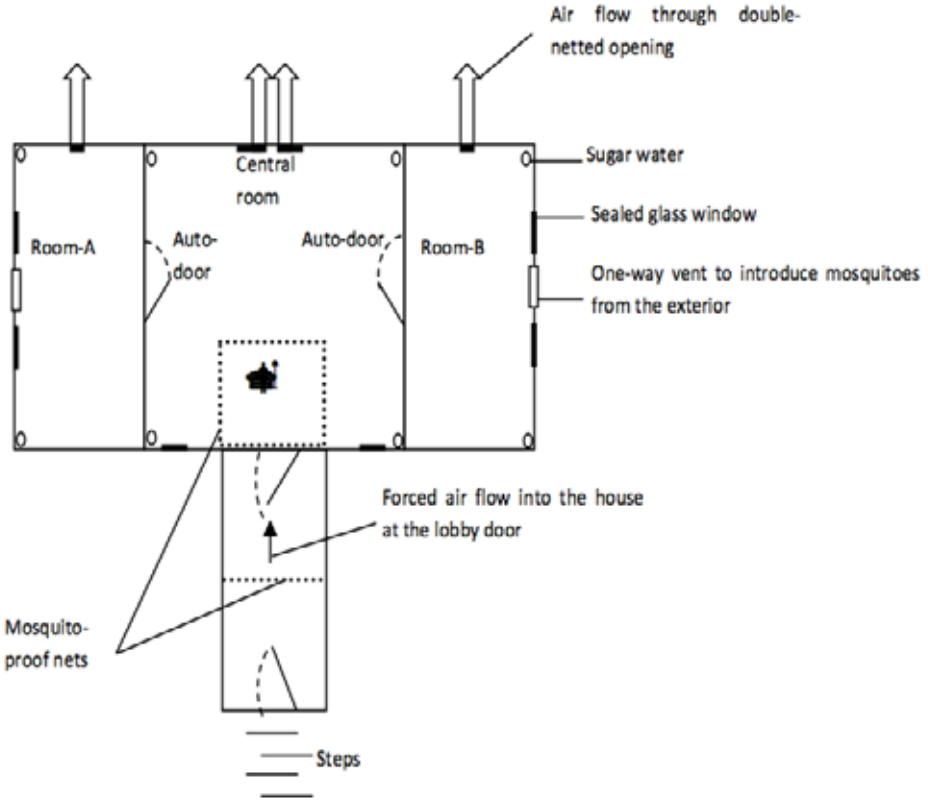


Figure 19. Fully contained TCTF

Note: The TCTF above is a fully contained structure, simulating the living space for a household of 2–4 people in Peninsular Malaysia. It is located within the IMR's compound in Kuala Lumpur.

Figure 20. ACL-2 field house (TCTF): semi-natural setting

Figure 21. TCTF: internal features showing netted chamber (left) and hallway with double doors (right)

Figure 22. TCTF: netted chamber (left) and centreline (right)

Note: The netted chamber has been built symmetrically and the person will sit along the centreline inside the chamber so as to not create any CO_2 gradient that might prejudice the mock run.

Mating competitiveness of RIDL-513A versus wild-type Malaysian strains – purpose and experimental design

The world's first semi-field evaluation of the mating competitiveness of transgenic *Ae. aegypti* was conducted. Previous studies on the fitness of the *Ae. aegypti* RIDL-513A strain (including the one at the Institute Pasteur) were all conducted using the RIDL-513A strain from a Rockefeller background (Rockefeller is a widely-used strain that has been bred in the laboratory for several decades). However, the studies performed at the IMR made use of a Malaysian out-crossed strain of RIDL-513A. This out-crossed strain is likely to be fitter in the field than the one with the Rockefeller background[22] because the latter was selected for laboratory rearing and genetic drift. Mating competitiveness of RIDL males was judged against its closest counterpart: the original, untransformed wild-type strain that would provide a fair comparison. An alternative experimental design was considered in which the wild-type strain would be males and females caught in the wild, rather than a laboratory-reared wild-type strain. However, wild-caught females typically feed and lay eggs poorly in a laboratory environment, which is likely to reduce the quality and quantity of data, and there were also concerns about the infection status of wild-caught females. Therefore, a wild-type laboratory strain of Malaysian origin was used as the comparator, as for the bionomic studies described above.

Mosquito strains

The RIDL-513A strain was originally generated in a Rockefeller strain background. Rockefeller is a laboratory strain, originally of Caribbean origin, colonized in the early 1930s. After several decades, this strain has adapted well to laboratory rearing, but conversely is likely to have lost traits related to field performance. The RIDL-513A insertion was, therefore, introgressed into more recently colonized strains, by backcrossing for at least five generations. In each case, multiple independent homozygotes were generated and pooled to try to minimize inbreeding/genetic bottleneck effects. The three resulting strains are list below.

- RIDL-513A-Mx1: a Mexican strain background provided by the Instituto Nacional de Salud Pública Cuernavaca (INSP), Mexico. Mx1 was generated using more than 20 homozygous female founder parents.
- RIDL-513A-My1a: This strain was generated using a laboratory strain of Malaysian origin, which was provided to Oxitec by the IMR. My1a was generated using 12 homozygous female founder parents.
- RIDL-513A-My1b: As 12 female parents was considered a little lower than ideal for the purposes of minimizing the risk of adverse consequences from genetic bottleneck effects, an additional line was created using My1a and an additional 32 homozygous female founder parents; this was named My1b.

These lines are constructed by mating the homozygous founder females in pools to equivalent founder males.

The RIDL-513A strains used in the mating experiments were supplied by Oxitec Limited, and have been maintained in ACL-2 laboratory in Malaysia since January 2007. All mosquitoes used for mating experiments were screened for fluorescence as pupae using a Nikon SMZ-1000 fluorescence microscope. As the RIDL-513A strain has an Actin5C-DsRed marker,[6] it is visible with the DsRed filter set (excitation 520–550 nm, emission 580 nm+). All pupae were fluorescent, as expected, confirming that they are homozygous. Abnormally large and abnormally small pupae were removed so that all selected male pupae were approximately of the same size. After screening, the RIDL male pupae were placed in an aluminium cage (23 x 23 x 23 cm), and the newly emerged adults were provided with 10% sucrose and 1% vitamin B complex solution (soaked in lint cloth). The adult mosquitoes were examined to ensure that sex separation had been

correctly performed, i.e. there were no females in the male group or vice versa, and that there were no males that were abnormally large or abnormally small.

RIDL *Ae. aegypti* rearing

RIDL *Ae. aegypti* were reared and colonized in an ACL-2 insectarium maintained at 26°C±1°C and 80%±10% humidity with a 12-hour light cycle and constant air flow to prevent temperature gradients. Eggs of RIDL *Ae. aegypti* on filter paper were submerged in a plastic yellow storage tray (25 x 34 x 7 cm). For hatching eggs, 1.7 L of distilled water was mixed with 17 ml of tetracycline solution (final concentration was 3 µg/ml) and 2.5 ml of fish food (Liquifry No. 1, Aquatics-Warehouse, UK) to induce the egg hatching. On the first day after hatching, the larvae were fed approximately 0.15–0.2 g per tray daily with ornamental fish flakes (Tetramin, Aquatics-Warehouse, UK). Normally, larvae pupated after 7–10 days.

Pupae could reliably be sexed by the genital lobe on the end of eighth pupal abdominal segment. Pupae were easily removed from larval trays using 2.5 ml Pasteur pipettes and transferred into plastic cups in a wooden cage (50 x 50 x 50 cm) for the adults to emerge. For quality control, pupae were screened for fluorescence marker using a fluorescence microscope (Nikon SMZ-1000). The RIDL *Ae. aegypti* strain had an Actin5C-DsRed marker,[6] which is visible with the DsRed filter set (excitation 520–550 nm, emission 580 nm+) to determine whether the fluorescence profile is consistent with the strain. Normally, adults emerged 2–3 days later and newly emerged adults were provided with 10% sucrose and 1% vitamin B complex solution soaked in lint cloth.

In order to produce eggs, female RIDL *Ae. aegypti* were fed blood from white mice, which were placed in a special trap overnight. Then, one plastic container 8 cm deep and 13 cm in diameter containing tap water and lined with Whatman No. 1 filter paper was offered for five nights starting two days after the first blood meal. Filter paper was labelled to prevent cross contamination. The filter paper containing eggs were removed after seven days of oviposition and allowed to dry at room temperature.

Wild-type *Ae. aegypti* rearing

Like RIDL strain, in order to obtain uniform condition, the wild-type strain was also reared in the same temperature at 24–26°C and 80 ± 10% humidity in a 12-hour light cycle and constant air flow to help prevent temperature gradients. For eggs hatching purposes, filter paper carrying approximately 1000 eggs were submerged in a plastic tray (29 x 37.5 x 7.5 cm) in 2.55 L of tap water without tetracycline. Three days after hatching, the filter paper was removed to prevent larvae from being stuck and dying due to mashed filter paper. During first and second instar of larvae, they were fed with approximately 1.42 g of liver powder mixture, while at the third instar were fed on a small piece (about 1.17 g) of partially cooked cow's liver. Each tray contained about 3000 larvae. Pupae were removed daily using a plastic pipette and put into a plastic container, which was transferred into a wooden cage (46.5 x 46.5 x 46.5 cm) for emergence. The newly emerged adults were provided with 10% sucrose and 1% vitamin B complex solution (soaked in lint cloth).

Adult females were blood-fed as in transgenic strain and eggs were collected similarly. RIDL and wild- type strains were reared in separate rooms but under similar conditions in order to obtain a cohort of RIDL and wild-type pupae of synchronous age.

Preparing mosquito cohorts for field-house experiments

All mosquitoes used in mating competitiveness experiments were of the same age, namely, four days old. Pupae were collected and sorted by sex under a dissection microscope on day two of pupation. On the day of the experiment, a cage containing 10 wild-type female mosquitoes was prepared for introduction into room C; in addition, two more cages (each containing five RIDL males and five wild-type males) were prepared, one for room A, and the other for room B. Female mosquitoes were introduced first into room C; male mosquitoes were introduced at the same time into rooms A and B through external one-way vents.

All the rooms were brought to optimal temperature and humidity by 08:00, using the air conditioning system. Mosquitoes were introduced into the three rooms at 08:00, half an hour prior to the start of the mating period, giving them sufficient time to get used to the inside of each room. The automatic doors connecting rooms A and B with room C were opened at 08:30 using remote control to initiate the mating competitiveness experiment. During the experiment, temperature and humidity in all three rooms were recorded every 30 minutes using a digital weather station with a Thermo-Hygrometer (Oregon Scientific, USA, Model BAA913 HG). Sucrose solution (10%) was provided in all the rooms as an energy source for the adults.

Adults were allowed to mix and potentially mate for eight hours, from 08:30 to 16:30. Starting from 16:30, all mosquitoes were recaptured starting with the females so as to conclude the experiment as quickly as possible. Mosquitoes were recaptured by aspiration, and then put into separate tubes to prevent them from mating. The time of capture of the last female mosquito and the last male mosquito was recorded. Each repetition of a mating experiment lasted a day: two hours of preparation including half an hour to equilibrate, eight hours of mating, and up to three hours of post-processing including half an hour to catch all 30 mosquitoes. There were at least three repetitions every week, so that each series of mating experiments could be concluded within two months.

Wing-length measurement

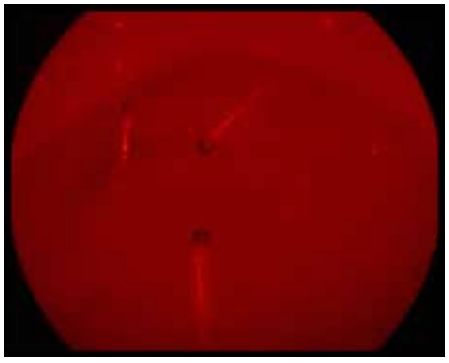
Adult pools consisted of at least 20 similar-sized wild-type adult males, and 10 similar-sized wild-type adult females. Wing length was used as a reliable indicator of adult size.[23,24] Just before the start of the experiment, 10 wild-type males were selected at random to be introduced into the field house. The remaining 10 (or so) wild-type male mosquitoes were sacrificed and their wing length measured (as a proxy measure of the wing length of the males that were used in the field-house experiment). This is reported in Table 14 as the wing length of proxy wild-type males. The 10 wild-type females that were used in the field-house experiments were collected, sacrificed after they had blood-fed and laid eggs (in individual cages), and their wing length measured.

Screening of larvae

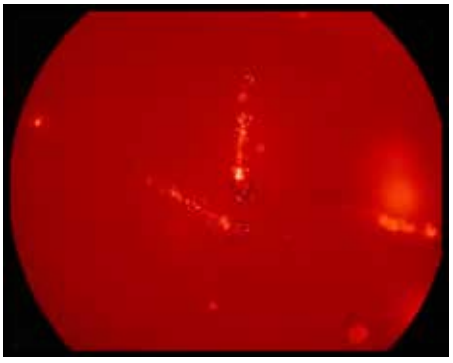
Eggs were collected from each female individually, matured and hatched. The resulting larvae were scored for fluorescence. Since all the females were wild type, fluorescent (heterozygous) F1 larvae indicated that the female mated with a homozygous RIDL-513A male. Absence of fluorescence indicated that the female mated with a wild-type male. As all larvae from a given female will either all be fluorescent or all non-fluorescent (Figure 23), this method of determining parentage is very reliable.

However, in view of the importance of accurate genotyping of progeny in relation to inferred parentage and hence male mating competitiveness, we chose to re-confirm these data by independent molecular analysis. Larvae (or pupae) were killed, preserved in 70% ethanol, and sent to Oxitec's laboratories. These were then analysed by polymerase chain reaction (PCR) for reconfirmation. One pair of primers was used to amplify a genomic fragment of ~450bp; this was a positive control for template DNA quality and quantity. Two construct-specific primer pairs were also used, amplifying bands of 472bp and 379bp, respectively.

Figure 23. Screening of larvae



Fluorescent: fathered by RIDL males.

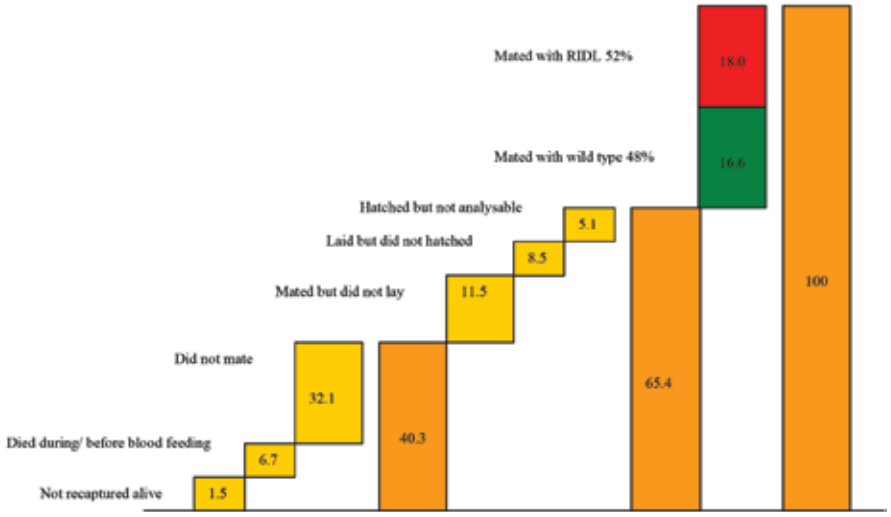


Not flourescent: fathered by wild-type males.

Mating competitiveness of RIDL-513A vs wild-type Malaysian strains

A small fraction (1.5%) of the female mosquitoes died during recapture, and another 6.7% before or during blood feeding (Figure 24); 32.1% of the female mosquitoes did not mate, while 11.5% mated but did not lay. In 8.5% of the cases, the laid eggs did not hatch; in another 5.1% of the cases, the hatched eggs did not give a sufficient number of larvae for unambiguous determination of parentage with statistical confidence. All these observations are normal and expected from the biology of this mosquito. Thus, only a third (34.6%) of the female mosquitoes that were subjected to mating experiments resulted in 'useful data' (Figure 24). The mating experiment was repeated 39 times (each time with 10 females), the pooled useful data constituted 135 binomial experiments (i.e. measurable individual female mating choice) for statistical confidence. About 48% of the females in these useful data mated with wild-type males, and the remaining 52% mated with RIDL-513A males. Statistically, this result is not significantly different from the expected scenario in which 50% of the matings would take place with RIDL males if the latter were as competitive as the wild-type males (Chi-square=0.185, p=0.67). This shows that *Ae. aegypti* RIDL-513A has excellent mating competitiveness under semi-field conditions.

Figure 24. Mating competitiveness of Malaysian (My1) wild-type female mosquitoes with My1 wild-type males and RIDL-513-My1 males



Note: Ten Malaysian (My1) wild-type female mosquitoes were introduced with equal numbers of Malaysian (My1) wild-type males and RIDL-513A-My1 males in a Malaysian background. When repeated 39 times, this constituted 390 female mosquitoes: 34.6% of which was useful data, i.e. 135 binomial experiments, each capable of measuring individual female mating choice; 52% of these were matings with RIDL-513A-My1 males, and the remaining 48% with My1 wild-type males.

If the RIDL-513A-My1 males were equally competitive with wild-type males, females would mate with each type of male in proportion to their numbers. In this experiment, equal numbers of RIDL and wild-type males were used so that approximately 50% of the females would mate with RIDL males and the other 50% with wild-type (My1) males. This is essentially what was observed – the small numerical bias towards RIDL (52:48 rather than 50:50) is not statistically significant. Therefore, we conclude that the RIDL males are fully competitive with the wild-type males in this assay.

Wing-length measurement

Wing-length measurement of the mosquitoes used in the experiment is shown in Table 14. It can be seen that the standard deviations were small for all four wing-length parameters (namely, 'all males', 'proxy RIDL males', 'proxy WT males' and 'all females'), indicating that the field-house experiments were carefully designed so that all males were roughly of the same size, and that all females were roughly of the same size, as intended.

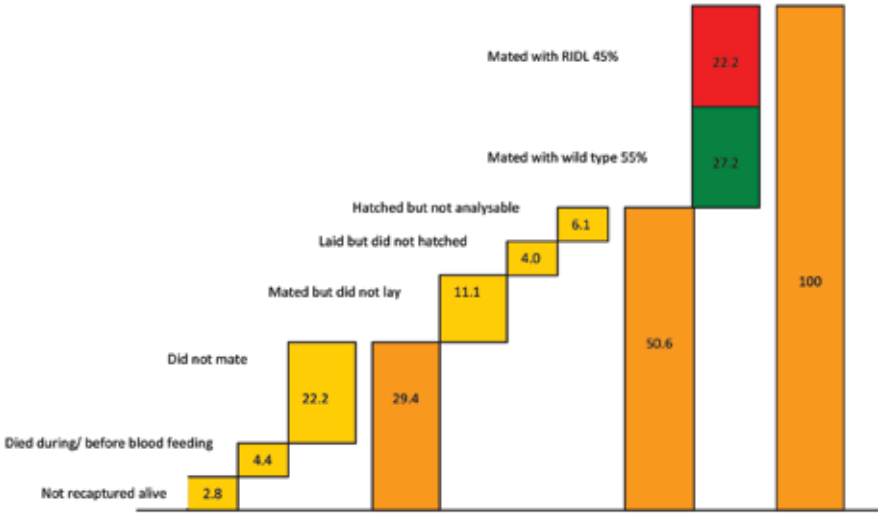
Table 14. Mean ± S.D. of wing length of mosquitoes used in the experiment

Ae. aegypti mosquito strain	Mean ± S.D. (mm)	Sample size
All males (RIDL-513A-My1 and My1 wild type)	2.49 ± 0.20	725
Proxy RIDL males (RIDL-513A-My1)	2.60 ± 0.16	368
Proxy wild-type males (My1)	2.41 ± 0.18	360
All females (My1 wild type)	3.13 ± 0.20	367

Mating compatibility between Malaysian and Mexican strains

From Figure 25, we can see trends which are similar to Figure 24, and that 45% of the Malaysian (My1) wild-type females chose to mate with RIDL-513A-Mx1 males of a Mexican background, while the remaining 55% mated with Malaysian (My1) wild-type males. Statistically, this result is not significantly different from the expected scenario in which 50% of the matings would take place with RIDL males if the latter were as competitive as the wild-type males (Chi-square=0.91, p=0.34).

Figure 25. Mating compatibility between Malaysian (My1) wild-type female mosquitoes with RIDL-513-Mx1 males in a Mexican background



Note: Ten Malaysian (My1) wild-type female mosquitoes were introduced with equal numbers of Malaysian (My1) wild-type males and RIDL-513A-Mx1 males of a Mexican background. When repeated 18 times, this constituted 180 female mosquitoes: 49.4% of this was useful data, i.e. 89 binomial experiments, each capable of measuring individual female mating choice; 45% of these useful data were matings with RIDL-513A-Mx1 males, and the remaining 55% with My1 wild-type males.

Wing length measurement

Again, it can be seen from Table 15 that the standard deviations were small for all four wing-length parameters (namely, 'all males', 'proxy RIDL males', 'proxy WT males' and 'all females'), indicating that the field-house experiments were carefully conducted so that all males were roughly of the same size, and that all females were roughly of the same size, as intended.

Table 15. Mean ± S.D. of wing length of mosquitoes used in the experiment

***Ae. aegypti* mosquito strain**	**Mean ± S.D. (mm)**	**Sample size**
All males (RIDL-513A-Mx1 and My1 wild type)	2.57 ± 0.21	347
Proxy RIDL males (RIDL-513A-Mx1)	2.65 ± 0.21	88
Proxy wild-type males (My1)	2.35 ± 0.18	90
All females (My1 wild type)	3.09 ± 0.21	163

Discussion

A significant and novel aspect of this study was the use of semi-field conditions. As the mating competitiveness was assessed in the TCTF, there was much more space compared to laboratory cages, as well as a more natural mating environment for these urban, highly anthropophilic mosquitoes. The experiment also simulated realistic mating conditions such as the presence of a human host inside a netted chamber to simulate mating, and limiting mating time to a few hours during the diurnal period of peak activity.[25] Substantial improvements were also made in the way the mosquitoes were introduced into the experiments (after allowing them to equilibrate inside the room for half an hour). But the biggest advantage of using a field house comes from the space.

While the Institute Pasteur laboratory cage experiments had up to five females and 10 males in 0.02 cubic feet (0.54 L), the IMR field house had twice as many mosquitoes in 3043 cubic feet (86 178 L) – an 80 000-fold increase in volume per mosquito. The idea is to encourage the males to fly at least a few metres (from rooms A and B towards the host in the ante-room) to find and compete for females, and to also prevent having an unrealistically high mosquito density inside. The increase in volume also gives space for females to fly away from males that they do not want to mate with. In summary, the mating arena is more realistic, and the data therefore more likely to accurately predict field performance, than previous tests of mating competitiveness.

One of the key decisions in the experimental design was the number of mosquitoes to be used. This number is a compromise between having a statistically meaningful number of mated mosquitoes versus realistic population density in a natural mating environment. The field house represents a typical flat in Kuala Lumpur; its dimensions (371 inches by 210 inches) are also comparable to the study by Getis et al.[26] in which the mean house width in Maynas (Iquitos, Peru) was 7±3 metres. It was decided to use 10 female mosquitoes because in highly dengue-endemic areas, it is indeed possible to find 10 female mosquitoes in a typical house.[21] Getis et al.[26] reported up to 11 *Ae. aegypti* (i.e. five to six females) inside a house in Iquitos, Peru. If, at the same time, each female mosquito has the choice to mate with a wild-type male or a RIDL male, then we end up with 10 RIDL males and 10 wild-type males being introduced into

the field house. Therefore, the selected density is at the upper end of what would be found in the wild (10 male and 10 female wild-type mosquitoes), plus 10 RIDL mosquitoes. In the context of a control programme, such RIDL males would indeed be added to the wild population. Keeping the number of mosquitoes per trial so low reduces the amount of useful data obtained from each trial (i.e. the number of mated females recovered that successfully blood-feed, lay and hatch F1 larvae). However, it substantially increases the realism and therefore the predictive value of the experiment. As the mosquito density is roughly one mosquito per 100 cubic feet, the field house is a lot less crowded than the cage experiments (in which several mosquitoes are put inside a cage which is typically one cubic feet). Keeping the number of female mosquitoes low (i.e. 10) also enabled us to recapture them within a short amount of time (typically 15 minutes) in comparison to the total duration (eight hours) of the experiment.

After considerable preliminary experimentation with different parameters (such as time of starting the experiment, period allowed for mating, etc.), it was decided to allow the mosquitoes to mate for a period of eight hours (08:30–16:30) before recapture and analysis. This experimental period includes the main active periods, while allowing the experiment to be run with a new batch of mosquitoes each day. Female mating was adequate in this period, with more than half of the females mating, on average. A human being was present for most of this time, screened behind mosquito-proof mesh to avoid actual contact with the mosquitoes, but providing human odours and carbon dioxide. Preliminary experiments showed that this was more effective in stimulating mating than either caged mice or the absence of any animal or human. The design of the field house allowed for the presence of clothes on hangars in rooms A and B, but it was found that this tended to reduce mating. It appeared that some of the males settled on this clothing in the acclimatization period and then did not fly out and mate. Males appeared to be more mobile when these attractive resting surfaces were not provided.

One feature of the field-house design is that it allows air exchange with the outside environment, and a degree of influence from that environment, e.g. light, humidity, etc. In these semi-field conditions, it is neither practical nor desirable to closely regulate environmental parameters. In practice, relative humidity did not go below 42% or above 68% at any time inside the field house, and temperature variations were relatively modest (between 23°C and 26°C). However, it is crucial that different experimental strains are not differentially exposed to such small environmental fluctuations within the field house, which could then distort the mating results, for example, if the RIDL males were allowed to acclimatize in a room with a different temperature to that in which the wild-type males acclimatized. To avoid such potentially confounding environmental effects, RIDL and wild-type males were mixed in equal numbers prior to their introduction into the field house. Therefore, each of rooms A and B had five RIDL males and five wild-type males during the acclimatization period. This configuration was preferred to releasing males inside one of the rooms and females inside the other in order to have a roughly uniform density of mosquitoes in all three rooms.

Previous studies on the fitness of the *Ae. aegypti* RIDL-513A strain (including the one at the Institut Pasteur) were all conducted using the RIDL-513A strain with a Rockefeller background (Rockefeller is a widely used strain that has been bred in the laboratory for several decades). However, the studies performed at the IMR made use of a Malaysian out-crossed strain of RIDL-513A. This out-crossed strain is likely to be fitter in the field than the one with the Rockefeller background[22] because of the selection for laboratory rearing and genetic drift in the latter. Mating competitiveness of RIDL males was judged against its closest counterpart: the original, untransformed wild-type strain that would provide a fair comparison. An alternative experimental design was considered, in which the wild-type strain would be wild-caught males and females, rather than a laboratory-reared wild-type strain. However, wild-caught females typically feed and lay eggs poorly in a laboratory environment, which is likely to reduce the quality and quantity of data, and there were

also concerns about the infection status of wild-caught females. Therefore, a wild-type laboratory strain of Malaysian origin was used as the comparator.

It can be seen that the standard deviations were small for all four-wing length parameters (namely, 'all males', 'proxy RIDL males', 'proxy WT males' and 'all females'), indicating that the field-house experiments were carefully designed so that all males and females were roughly of the same size, as intended. This was important because each male can mate with as many as eight females,[12] so a few big males of one type could have had a considerable bias on the experiment. It was also important to ensure that all females were of roughly the same size for consistency between different repetitions of the same experiment. The wing length of 'proxy RIDL males' is slightly larger than that of 'proxy WT males', which is not unrealistic because mass-reared male mosquitoes released in a RIDL-SIT programme are likely to be bigger than the males in the wild.

In the competitive mating experiment, if the RIDL-513A-My1 males were equally competitive with wild-type males, females would mate with each type of male in proportion to their numbers. In this experiment, the use of equal numbers of RIDL and wild-type males would result in approximately 50% of the females mating with RIDL males and the other 50% mating with wild-type (My1) males. This is essentially what was observed – the small numerical bias towards RIDL (52:48 rather than 50:50) is not statistically significant. Therefore, we conclude that the RIDL males are fully competitive with the wild- type males in this assay. If this translates to field performance against wild males, it would be a very strong predictor of success of a RIDL-SIT-based control programme against *Ae. aegypti* and dengue.

The experiment of mating compatibility between Malaysian and Mexican strains was conducted to evaluate whether RIDL-513A of a non-Malaysian strain background mate well with Malaysian wild-type female mosquitoes. The question of whether strain background matters is not just of academic interest, it is also useful to decide whether an SIT programme involving RIDL-513A in a given background can be easily extended to another endemic country without the need for out-crossing. As 124 countries are at risk from dengue, it is neither practical nor economical to out-cross RIDL-513A into 124 different local strains, and such a step may also be undesirable in countries where the local strain has developed resistance to insecticides. Moreover, classical SIT has set many precedents of using the same strain background across countries and continents; therefore, mating compatibility is unlikely to be a major issue. Here, we have presented evidence to support this hypothesis by describing mating compatibility between Malaysian wild-type females and RIDL-513A males of a Mexican strain background. Mexican strain background was chosen because it is likely that considerable distance and isolation would lead to more differences between Malaysian and Mexican strains; besides, a Mexican out-crossed strain was also readily available for experimentation. As mentioned earlier, RIDL-513A strain was out-crossed into a Mexican strain background (provided by InstitutoNacional de SaludPública Cuernavaca, Mexico) using more than 20 homozygous female founder parents. By backcrossing for at least five generations, it was ensured that ~97% of the strain had a Mexican background. This strain was subjected to semi-field trials in the field house. The results indicated high mating compatibility between recently colonized Mexican RIDL males and laboratory-reared Malaysian wild-type females. This implies that there are no significant or relevant mating barriers between the Mexican strain and the Malaysian strain and, therefore, indicates that a single RIDL strain could be used over a very wide geographical range, perhaps globally.

6.5.3 Stage 3: Open field release

Prerequisite

The open-release trials can only be performed after a complete risk assessment has been performed and the efficacy of GMMs has been ascertained in contained trials.

Selection of trial site

The selection criteria for a suitable trial site are given below.

- Inhabitation by human beings (as *Ae. aegypti* is highly anthropophilic, i.e. it preferentially bites human beings).
- Modest human population (so that the number of RIDL mosquitoes required for the trial are not too large).
- Modest to high population density (so that the area to be treated is also proportionately modest or small).
- Dominant presence of *Ae. aegypti* in mosquito population surveys.
- History of dengue and/or chikungunya cases.
- Reasonable accessibility.
- Cooperation/support of the local population and health authorities.
- Ecological stability with no special/protected status.
- Geographical isolation from other inhabitations by at least 1 km (as *Ae. aegypti* seldom flies more than 200–400 metres in its lifetime) to prevent reinvasion from untreated areas.
- Presence of one or more additional, comparable sites to act as controls.

The IMR conducted several surveys and preliminary data indicated Bentong in Pahang state fulfilled the requirements as a trial site for open release. Subsequently, baseline surveys were conducted from June 2008 to December 2009.

Baseline studies

Ovitrap surveillance was methodically conducted in selected areas in Bentong, Pahang, Malaysia, in order to identify insular sites with stable *Ae. aegypti* population. Bentong is a district belonging to the state of Pahang. It comprises an area of 183 119 hectares, with 112 900 inhabitants.[27]

Preliminary surveys were conducted in 11 sites of Bentong district, and one location (N03°33' E101°54') was found to be suitable for further study. In 2008, four dengue cases were reported in this area. This site is a geographically isolated suburban residential area covering 29 hectares surrounded by vegetation and greenery. There are approximately 5600 inhabitants living in around 1120 houses, which consist of single-storey terraced houses with a proper concrete storm water drainage system running through the site.

To study the distribution of *Aedes sp*. in the study site, this area was divided into four zones, namely East A, West A, East B and West B. Each zone comprised similar house type – one storey structure with a kitchen, living room, bathroom and two to three bedrooms.

Ovitrap surveillance

The ovitrap described by Lee[28] was used in this surveillance based on the Malaysia Ministry of Health guidelines.[29] An ovitrap (Chevron Phillips, Singapore) consisted of a 300 ml black plastic container with a diameter of 6.5 cm and height of 9.0 cm. Fresh water was added to a level of 5.5 cm and an oviposition paddle (10 x 2.5 x 0.3 cm) made from hardboard was placed in the water with the rough surface upwards in each ovitrap.

Ovitraps were placed indoors and outdoors in randomly selected houses after obtaining informed consent from the house owner. Ovitraps were collected after seven days and immediately transported to the IMR laboratory. The contents of ovitrap were poured individually into labelled and covered plastic containers (15 x 7 x 8.5 cm) together with the paddle. All larvae were counted and identified under compound microscope (Nikon Eclipse E200, Japan) at third and fourth instar using taxonomy keys prepared by IMR.

A total of 1630 ovitraps were placed indoors and outdoors randomly in selected houses from June 2008 to December 2009. During the surveys, 80–100% of ovitraps were recovered after seven days. In total, 29 085 larvae were examined of which 20.00% were *Ae. aegypti*, 76.93% *Aedes albopictus*, 1.21% were other mosquitoes (*Culex* spp. and *Toxorhynchites* spp.), and 1.86% were non-mosquitoes, *Chironomus* spp.

In this study, ovitrap indices of *Ae. aegypti* and *Ae. albopictus* were found to be in the range of 8–47% and 30%–71%, respectively. Overall, the mean number of *Ae. aegypti* larvae per ovitrap was 3.83 ± 2.81 for indoor ovitraps and 4.72 ± 5.28 for outdoor ovitraps. The mean number of *Aedes albopictus* larvae per ovitrap was 10.41 ± 5.93 for ovitraps placed indoors compared to 19.13 ± 9.37 for ovitraps placed outdoors. There was significant difference between the populations of *Ae. albopictus* larvae from indoors and outdoors ovitraps ($p<0.01$), while no such difference ($p>0.05$) was observed for *Ae. aegypti*.

This study demonstrated that *Ae. albopictus* populations were dominant in all four zones compared to *Ae. aegypti* populations both indoors and outdoors. The highest percentage of positive ovitrap with *Ae. aegypti* and *Ae. albopictus* was 60.96% (indoor-West A) and 99.28% (outdoor-East B), respectively. Mixed breeding was found in both indoor and outdoor populations in all study sites ranging from 7.95% to 29.67% and from 5.52% to 44.95%, respectively.

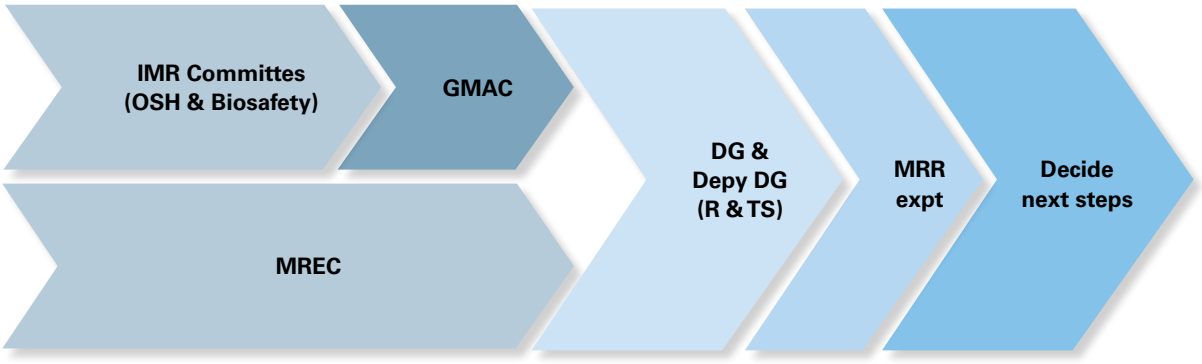
The distribution of *Ae. aegypti* was more abundant in both East A and West A, while the presence of *Ae. albopictus* remained stable in all zones. This site recorded the highest *Ae. aegypti/Ae.albopictus* ratio of 1.00:39.73 for indoors ovitraps and 1.00:36.90 for outdoors ovitraps. These results are likely to aid in the selection of trial sites for the first release of RIDL *Ae. aegypti*.

6.5.4 Preparation for open release of RIDL *Ae. aegypti*[30]

Regulatory affairs and community engagement

Regulatory permission was sought and received in accordance with the Malaysia Biosafety Act (2007).[31] This process included scrutiny from the Institutional Biosafety Committee (IBC) of the IMR in Kuala Lumpur, the Ministry of Health's Medical Research Ethics Committee (MREC) and the GMAC at the Ministry of Natural Resources and Environment (NRE) (Figure 26).[32] The final approving body was the National Biosafety Board (NBB) of the NRE. NBB approved the project on 5 October 2010 [Permit No. JBK (S) 602-1/1/3(29)].

Figure 26. The regulatory process undergone before open release



IMR: Institute for Medical Research.
OSH: occupational safety and health.
MREC: Medical Research Ethics Committee.
GMAC: Genetic Modification Advisory Committee.
DG: Director General.
Depy DG (R&TS): Deputy Director General (Research & Technical Support).
MRR expt: Mark-Release-Recapture experiment.

As part of the NBB's approval process, the intent to conduct limited releases was advertised twice in two national newspapers during August 2010, the *Berita Harian* (in Bahasa Malaysia) and the *New Straits Times* (in English), as part of a 30-day public consultation process. In order to proactively solicit comments from nongovernmental organizations (NGOs) and improve the risk assessment process, the NRE also wrote to nine NGOs, and requested meetings with them including the World Wide Fund for Nature and Third World Network. Taking into account the information and comments received during this consultation, GMAC concluded that the limited field trial would not endanger biological diversity or human, animal or plant health. After considering all inputs, comments and concerns, NBB granted approval for IMR's limited open-release experiment, with specific terms and conditions, in accordance with the Biosafety Act 2007.[31,32]

As part of public engagement prior to open release in the uninhabited site, NRE asked IMR to obtain permission from local government authorities, and to display large multilingual posters in the uninhabited trial site for at least two weeks prior to the date of release. These conditions were met by IMR to the satisfaction of NRE inspectors at the trial site located in an uninhabited area in Bentong district. Permission was also obtained from Pahang state authorities. In addition, IMR also participated in public meetings arranged by the Bentong Municipal Council and the Bentong Malaysian Chinese Association in which information was presented to the local community using visuals and non-technical language (Bahasa Malaysia and Mandarin). These public meetings reinforced the ground-level support for the trials and *The Star* newspaper ("GM mosquito plan gets the thumbs-up", 1 November 2010) reported positive feedback from the local community following its independent survey of Bentong residents. NRE carefully observed the entire implementation and progress of the project and details were made available on the IMR website.[33]

In accordance with the Malaysian Ministry of Health guidelines for dengue control, after the end of the study, the entire site was treated twice by thermal fogging with Resigen™ (Bayer CropScience AG, Leverkusen, Germany) on the 6th January 2011 and the 18th January 2011 by the Vector-borne Disease Control Programme in the Bentong Public Health district. This was a condition of the trial stipulated by NRE. Both strains used in this study were found to be equally susceptible to a range of insecticides including the pyrethroids used in the study site.

Field site

The release site is located in *Hutan Tanah Kerajaan* (*Bukan Hutan Simpanan* or non-reserved government forested land) off Jalan Tentera, which is off the highway known as "Lebuhraya Bentong-Raub", Bentong district, Pahang, Malaysia. It is an uninhabited area on the side of a hill comprising a jungle area (government land), a cleared area and a young rubber plantation (private land). The cleared area is a low bush vegetated area with numerous cut trees and vegetation cover. In accordance with the requirements of the regulatory authorities, posters announcing that a limited trial with transgenic mosquitoes was being conducted were placed downhill (340 m from release point) and uphill (130 m from release point) 22 days in advance of the release and maintained until the end of the study. Prior to release, written informed consent was received from the landowners. The release point (3° 33.92' N, 101° 52.99' E) was in a cleared area approximately 100 m from the rubber plantation. The nearest inhabited areas were >500 m to the northeast and over 1 km to the southeast and southwest of the release point. A weather station was set up in the area to record temperature, humidity, rainfall, and wind direction and strength.

Rearing

Mosquitoes were reared in a dedicated facility (ACL-2) at the IMR, Kuala Lumpur, at 27.5°C (±1°C) and 70% (±10%) humidity. Eggs were hatched under vacuum. Larvae were fed daily with Vipan® fish food (Sera, Heinsberg, Germany). Two strains were used during this experiment, a laboratory strain originating from Jinjang, Kuala Lumpur, which has been reared in IMR since the 1960s (referred to as My1 strain), and an OX513A strain. The OX513A strain was constructed by making a line homozygous for the OX513A insertion after introgressing the insertion from its original Rockefeller strain background[6] into the My1 strain by backcrossing for five generations so that ~97% of the genome of the resulting strain, termed OX513A-My1, would derive from the Jinjang strain. Both strains were reared at the same initial density and feeding protocol.

Sorting

After pupation, larvae and pupae were separated, first mechanically on the basis of size[34,35] and then manually by microscopic examination; 6500 pupae of each strain were allowed to eclose into a cage (38 x 38 x 38 cm); emerged adults were provided with 10% sugar solution supplemented with vitamin B complex solution until their release. Additional sorted pupae were re-examined to assess the sorting efficiency; adults were also visually checked for the presence of females the day before their release. Three days after sorting, the pots containing the pupae were removed from the cages to count dead pupae, live pupae and dead adults remaining in the pots.

Marking and release

Release was conducted three days after pupal sorting when the males were already sexually mature, i.e. 2 ±1 day-old.[36] On the day of release, cages covered with a wet cloth were put in a secure plastic box and taken to the release point.

Cages were transferred to a plastic bag and sprayed with fluorescent powder (DayGlo, Switzer Brothers, Cleveland, OH, USA) following a standardized protocol which had been found not to adversely affect the lifespan or dispersal of wild-type mosquitoes in previous laboratory and field assays: Saturn Yellow (A-17-N) and Red Rocket (A-13-N) powders for the My1 and OX513A-My1 males, respectively.[37] The release was carried out using a simple manual rope remote opening to enable the operators to open the upper part of the cage at a 10 m distance; the operators then left the area rapidly to limit potential bias to the males' dispersal caused by their presence. Cages were then left for 15 minutes before being collected and brought back to the laboratory to count the number of dead and non-released adults.

Recaptures

A network of 45 BG-Sentinel traps[38] was set around the release point. Due to the topography and vegetation of the site, traps could not be set evenly in every direction from the release point. The furthest traps from the release point were 96 m and 328 m uphill and downhill, respectively. Traps were baited using BG-lure (Biogents™) and their positions recorded with GPS. Traps were powered by sealed batteries (12V, 12Ah, JSB 12120), which were changed and charged daily. Nets were collected and replaced daily and all trapped mosquitoes taken to the laboratory for identification. The trapping period was from the time of release (Day 0) until three consecutive days without recaptures (Day 15). The size of a sample of the recaptured mosquitoes was assessed by measuring the distance from the auxiliary incision to the apical margin of the wings, excluding the fringe of scales.[39] Digital images of the wings alongside a micrometer, for purposes of scale, were taken using a Nikon DSFi1 camera and analysed using ImageJ 1.42q.

Monitoring

A network of ovitraps was set in the area weekly. Ovitraps were set at least at 5 m from BG-Sentinel traps to minimize interference with the adult traps. The ovitrap sampling described by Lee[28] was used for surveillance, based on the Malaysian Ministry of Health Guidelines.[29] Forty-four traps were placed in the uninhabited area and 35 were placed in the nearest inhabited places to monitor presence and abundance of wild-mosquito populations, as availability of female *Ae. aegypti* could have an impact on dispersal behaviour. In addition, the ovitrap can be used to monitor dispersal and persistence of the RIDL gene into the environment by checking the eggs for presence of RIDL gene. Ovitraps were brought back to IMR; recovered larvae were identified by species. First and second instar larvae were scored for fluorescence; all larvae were allowed to develop to adult and then genotyped by PCR.

PCR

Genomic DNA was extracted from adults, using the GeneJETDNA purification kit (Fermentas) according to the manufacturer's instructions. PCR was carried out using two primer pairs, and DreamTaq™ polymerase (Fermentas), using a touchdown PCR programme with annealing temperature decreasing by 0.5°C / cycle over 10 cycles, from 55°C to 50°C then 25 cycles with annealing at 50°C. Primers AeA4F2 (CAATCGAAGCGAGGTATCCTCACCC) and AeA4R2 (CTGGGTACATGGTGGTACCACCAGAC) amplified the Actin-4 gene, so acted as a control for DNA quality. Primers WT1 (GAAATCCCCTAGTAAAATTCGCGGAGAAATTC) and IRV1 (CGTCATTTTGACTCACGCGGTCGTTATAGTTC) amplified across the insertion-flanking sequence boundary so would only be positive in insects carrying the OX513A transgene. A positive gDNA control known to amplify with WT1-IRV1 was also included.

6.6 India-IIBAT's experience

The IIBAT is a non-profit institution located in Chennai, Tamil Nadu, India, and is recognized by the Department of Scientific and Industrial research (DSIR). The Institute entered into research collaboration with Oxitec. The scope of the collaboration is limited to carrying out fitness experiments under containment involving Oxitec's RIDL strain of the mosquito *Ae. aegypti* in IIBAT's Arthropod Containment Facility (ACF). It is overseen by an independent Monitoring and Evaluation Sub-committee (MEC) of government experts appointed by the Review Committee on Genetic Manipulation (RCGM). See Chapter 8: Biosafety regulation and legal framework for GMVs.

6.6.1 Visit, suggestion and approval

In connection with the above, IIBAT was inspected by a RCGM sub-committee on 6 October 2007 through the IBC to assess IIBAT's competency to handle the mosquito vector, and the infrastructure housing the GMMs, if imported. It also made suggestions on conducting mock experiments (for release and recapturing) using wild *Aedes*. IIBAT was re-inspected by RCGM after it had submitted the mock/control experiment results.

A live demonstration/experiment was conducted before the RCGM sub-committee's inspection. The committee suggestedto carry out additional containment experiments with adequate measures and submit the results to RCGM. The suggestions were implemented and communicated to the RCGM, following which an import permit was granted to IIBAT to import the GMMs (ref: BT/BS/17/151/2005-PID), which was valid up to 31 July 2009. The RCGM appointed a MEC of independent government experts to oversee the work to be carried out at IIBAT. The MEC met at IIBAT to monitor the progress of the project and suggested the ACL be redesigned.

6.6.2 Sequence of activities carried out by IIBAT to import GM mosquitoes

- IIBAT participated in a workshop at IMR Malaysia – ACL exposure.
- As per the suggestion, IIBAT re-designed the ACL facility with separate larval sections for RIDL and wild type (Figure 27).

Figure 27. ACL laboratory facility – IIBAT

ACL facility

Wild larval section

Ante-room

RIDL larval section

Mating chamber

Double curtain for handling escapees

- RIDL eggs received from Oxitec were allowed to hatch and were maintained in the RIDL section as per the procedure specified by them.
- Culture was maintained in the ACL, which had provisions such as an air curtain with inward air flow, double blinds, mosquito zapper and light attractant so that the chance that there would be escapees was remote.
- Stability of culture was studied through hatchability, wing length and fecundity of the mosquitoes.

6.6.3 Results of the mating experiments study

- Results on various parameters of culture stability revealed that the hatchability varied from 26% to 94% for the first 12 generations (Figure 28).
- During the study on fecundity, 50 females were caged separately after a blood meal. Eggs were collected and the average number of eggs laid by a single female during the first gonadotropic cycle varied from 86 to 108. During this study, the number of females successfully alive during the egg collection varied from 19 to 47 (Figure 29).

Figure 28. Data on hatchability

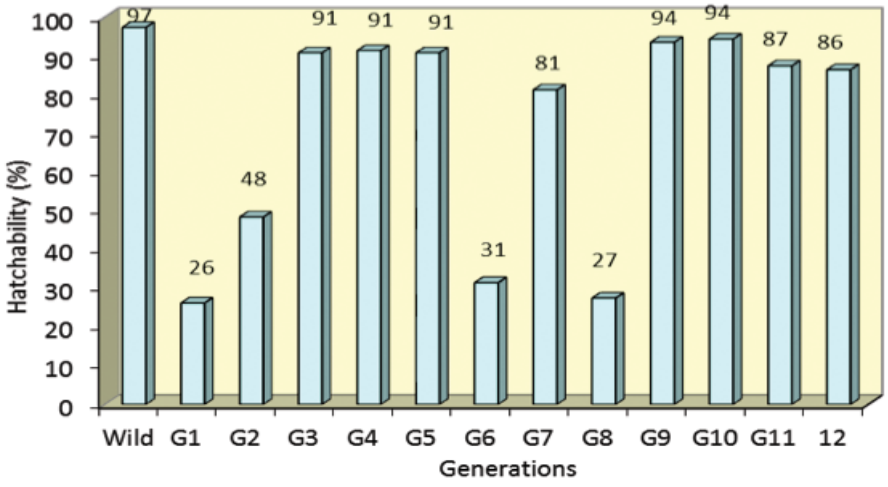
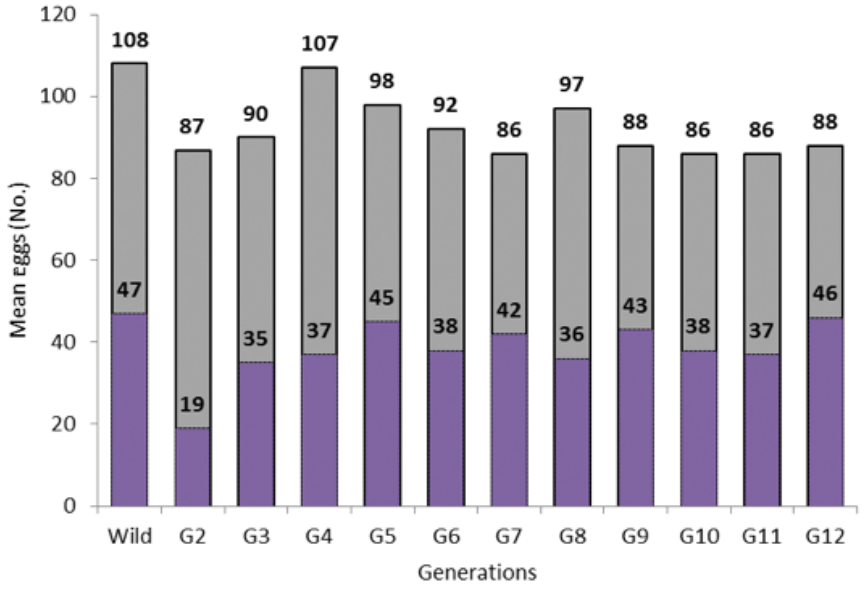


Figure 29. Data on fecundity



- The study on wing length revealed that the wing length for males varied from 2.28 to 2.75 mm. For females it varied from 3.04 to 3.53 mm (Table 16).

Table 16. Data on wing length

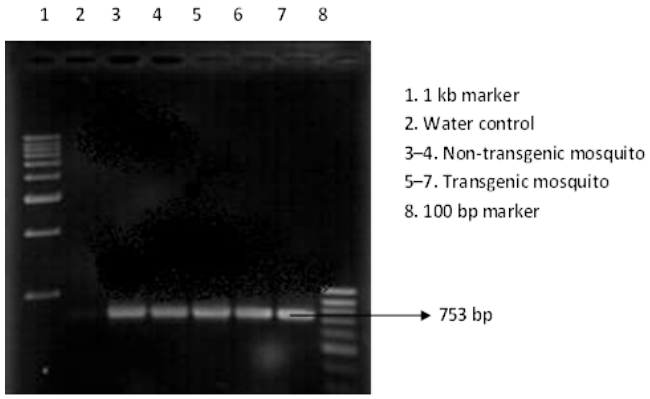
Param-eters	Data	Wild	RIDL generations										
			G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
Female	Wing length* (mm)	3.33	3.26	3.53	3.23	3.05	3.20	3.07	3.05	3.18	3.12	3.04	3.11
	± SD	0.23	0.31	0.14	0.59	0.36	0.34	0.39	0.36	0.17	0.11	0.19	0.22
Male	Wing length* (mm)	2.61	2.51	2.63	2.53	2.42	2.42	2.47	2.50	2.75	2.48	2.28	2.32
	± SD	0.22	0.27	0.10	0.15	0.20	0.21	0.22	0.26	0.49	0.15	0.10	0.09

* Mean of 50 replicates.

- Purity was checked by the Department of Biotechnology, IIBAT using the primers and procedure supplied by Oxitec Ltd. (figures 30 and 31). Once the culture was stabilized, IIBAT was permitted to carry out fitness experiments under containment for a single ratio of 10 wild females: 5 RIDL males: 5 wild males.

Figure 30. PCR condition for ACTIN4 gene and amplication

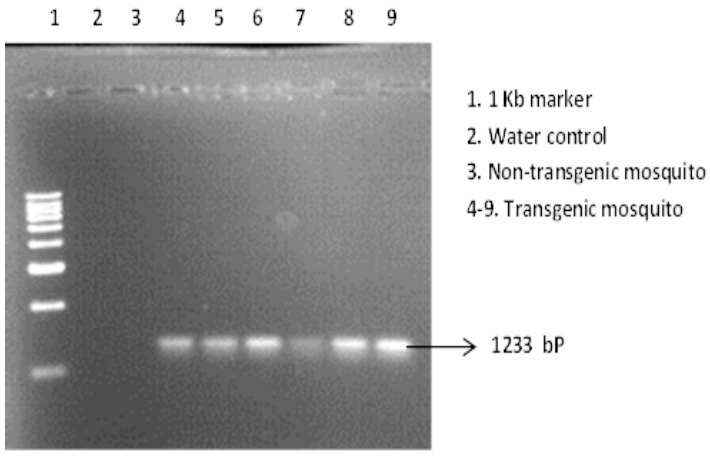
PCR condition	Temperature	Time
AeA4F2 5′ CAATCGAAGCGAGGTATCCTCCTCACCC-3′ AeA4R2 5′ CTGGGTACATGGTGGTACCACCAGAC-3′	– ND	– ND
Initial denaturation	94°C	2 min
Denaturation	94°C	15 sec
Renaturation	55°C	40 sec
Extension	72°C	1 min
Final extension	72°C	7 min
Amplicon length	753 bp	ND
Actin4 primers are used to check the quality of the genomic DNA as they amplify endogenous mosquito Actin4 gene.		
Cycles	10	ND



ND: not determined.

Figure 31. PCR condition for OX513A gene amplification

PCR condition[a]	Temperature	Time
HspdiagR 5′ GCAGATTGTTTAGCTTGTTCAGC-3′ DrosF 5′ ATGAGCAATTAGCATGAACGTT-3′	= N/A	= ND
Initial denaturation	94°C	2 min
Denaturation	94°C	15 min
Renaturation	50°C	40 sec
Extension	72°C	1 min
Final extension	72°C	7 min
Cycles	25	ND
Amplicon length	1233 bp	ND



ND: not determined.

[a] Software used to detect Amplicon length: Vilbert Lourmat- BIOcap and BIOID software.

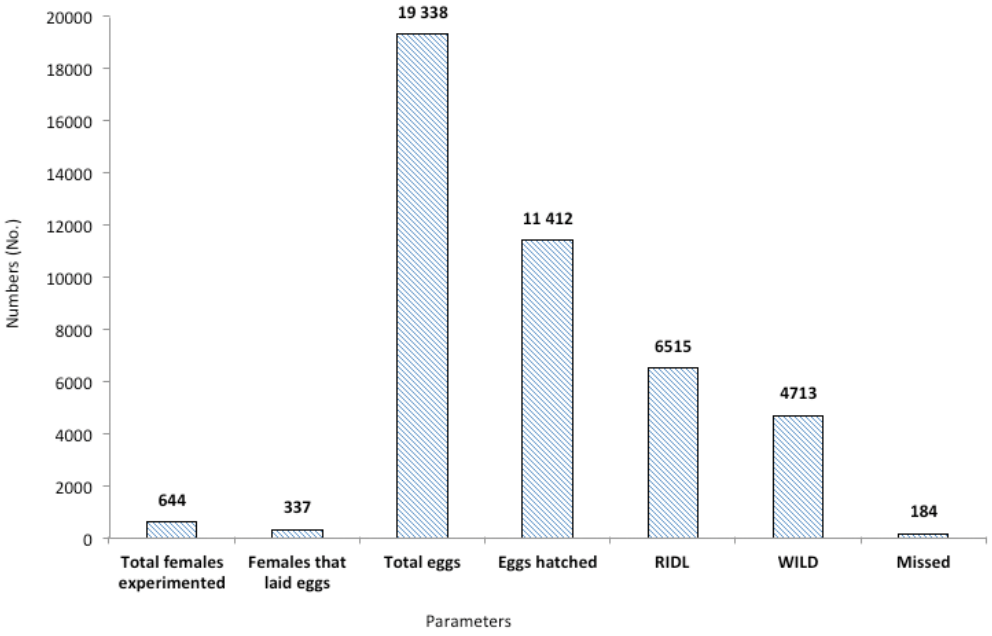
- Mating experiment:

  Mating experiments were carried out under total containment. Initial mating experiments with wild *Ae. aegypti* were conducted and the minimum period for successful mating was identified as 20 minutes between 11:00 to 13:00. Based on this, mating experiments were carried out between 11:00 and 11:20, 12:00 and 12:20, and 13:00 and 13:20.

  After exposure, the females and males were collected separately. Females were blood fed and eggs were collected separately from individual female mosquitoes. Collected eggs were put to hatch and larvae (first instar) were examined for the presence/absence of fluorescence to determine the paternal inheritance. The presence of fluorescence

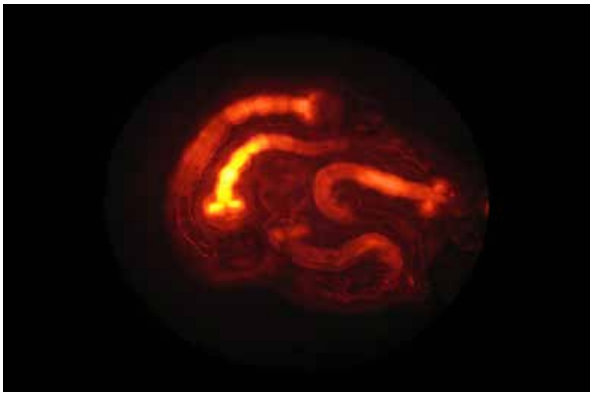
indicated successful mating wth RIDL males and the absence of fluorescence indicated the successful mating with wild males (Figure 32).

Figure 32. Results on mating competitiveness of RIDL strain of mosquito *Ae. aegypti* with wild *Ae. aegypti* under total containment



- About 65 experiments were conducted where 10 wild females were included in each experiment. The test ratio was 10 wild females: 5 RIDL males: 5 wild males.
- About 644 females were successfully collected and found have laid 19 338 eggs. Hatchability was observed to be 59.01% (11 412). When larvae were scored for paternity, 57.09% (6515) were observed to be RIDL and 41.30% (4713) were observed to be wild. While scoring, 184 lavae were missed (1.6%). The experiments were successfully completed for the above-mentioned ratio under the strict vigil of RCGM and the report was submitted to RCGM.
- A representative photograph of RIDL and non-RIDL larvae (first instar) collected after the mating experiment is given in Figure 33. Based on this, scoring for RIDL and non-RIDL was carried out.

Figure 33. Image of RIDL and wild larvae



6.7 Conclusion

Half the world's population is at risk from dengue – the most rapidly spreading VBD with 100 million new infections every year in over 100 countries. As both dengue and chikungunya have no specific medication and no safe vaccine available for public use, we need a new approach to complement traditional vector control methods. Following successful confined semi-field trials of RIDL, the IMR plans to test this promising *Aedes* control technology in the field. After extensive contained studies and regulatory scrutiny, the field release of RIDL *Ae. aegypti* was safely and successfully conducted in Malaysia. The engineered strain showed similar field longevity to an unmodified counterpart, although, in this setting, dispersal was reduced relative to the unmodified strain. The evaluation study conducted by IIBAT found that both RIDL and wild males are competent in identifying wild female under total containment. However, given that it was a single ratio study, it is not possible to reach a concrete conclusion from that experiment. To draw a candid conclusion on the mating competitiveness between the RIDL and wild males under total containment, it is expected that further studies with different ratios of wild females, RIDL males and wild males will be carried out. These data are encouraging for the future testing and implementation of genetic control strategies, and will help guide future field use of this and other engineered strains.

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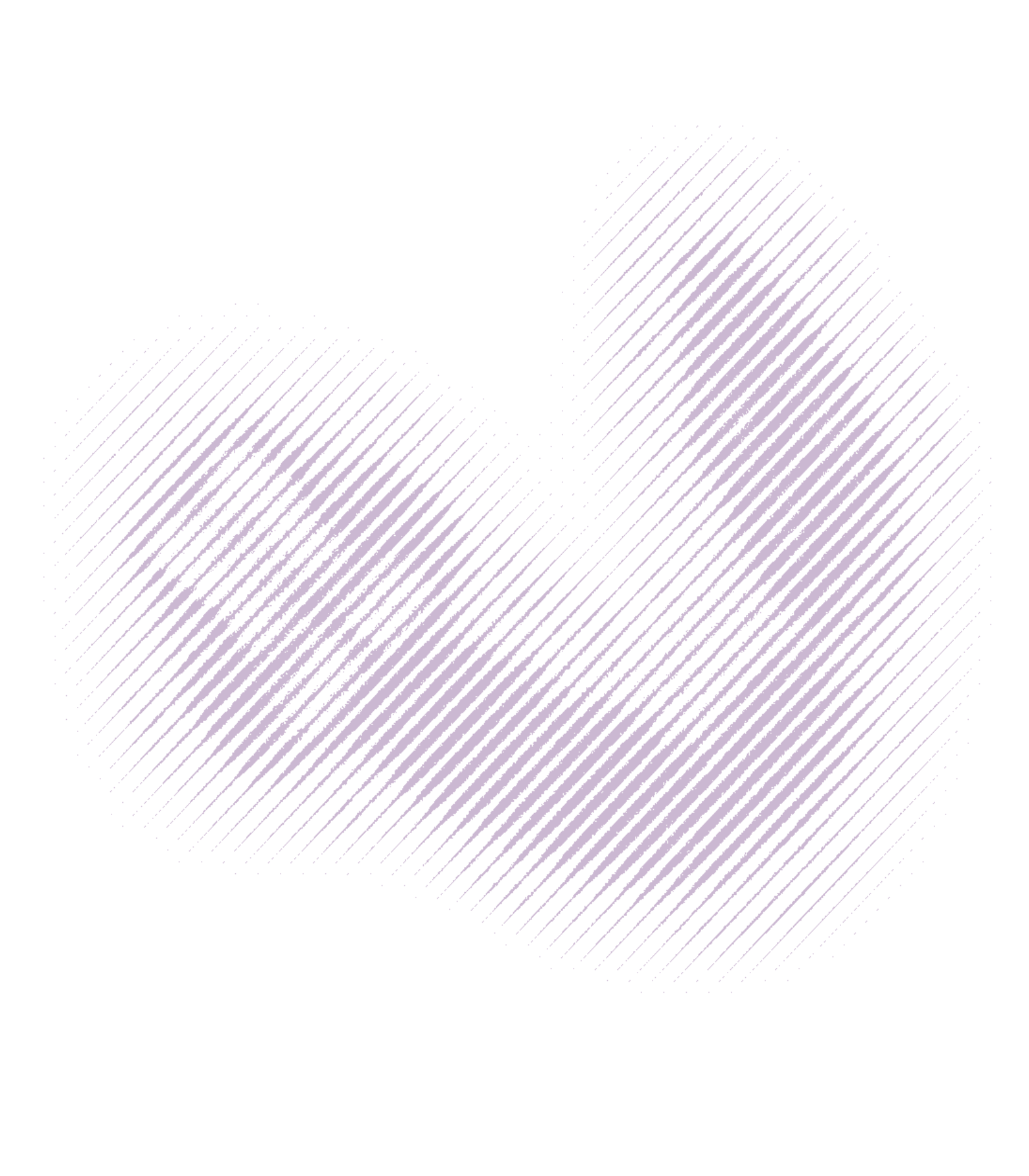

Chapter 7. GM crops in India: projects, history and development

7.1 Introduction

Globally, over 28 countries are growing GM crops of which 18 are so-called 'biotech mega' countries including India with more than 50 000 hectares under biotech crops.[1] Currently, five EU countries have a total area of 129 071 hectares under biotech *Bacillus thuringiensis* (Bt) maize cultivation. The first commercial GM food crop released in the world was a tomato variety 'FlavrSavr' in 1994. It was engineered for its slow-ripening character. GM crops that have been commercialized in the past 20 years include cotton, corn, soybean, canola, rice, squash, tomato, potato, papaya and melon of which soybean, corn, cotton and canola are of major importance.[2] Since their introduction, the area being cultivated with GM crops has increased by an unprecedented 100-fold, from 1.7 million hectares in 1996 to 170 million hectares in 2012, thereby significantly increasing farmers' income by up to US$250 per hectare. Since 1996, biotech crops have contributed to food security, sustainability and enhancement of the environment by increasing crop production valued at US$98.2 billion. They have provided a better environment by saving 473 million kg of pesticides, reducing CO_2 emissions by 23.1 billion kg in 2011 alone, conserving biodiversity by saving 108.7 million hectares of land and by helping to alleviate the poverty of >15.0 million small farmers.[1]

7.2 Food and feed safety assessment

In India, before any biotech food crop can be released into the environment, it has to undergo stringent biosafety tests, including environmental safety testing as well as food safety testing mandated by the regulatory authorities. The Environment Protection Act 1986 and Rules 1989 of the Ministry of Environment and Forests (MoEF) deal with rules and procedures for handling GMOs and hazardous organisms. In July 2001, the MoEF issued a draft notification amending regulations on permissions and the approval of foods. This notification restricts a person from importing, manufacturing, transporting, storing, distributing or selling any food, feed, raw or processed or any food ingredient, additive or product that contains GM material, without the approval of the Genetic Engineering Appraisal Committee (GEAC). Issues for action include the review and control, and monitoring of large-scale use of GMOs in R&D, industrial production, environmental release and experimental field trials.

The extraordinary growth of the Indian biotechnology sector has significant implications for policy in the area of regulation, and two specific reports were commissioned by the Ministry of Agriculture and the MoEF to evaluate the regulatory framework for products of agricultural biotechnology and recombinant pharmaceuticals, respectively. A set of guidelines for the conduct of field trials under regulated plans and SOPs for genetic engineering was approved by the RCGM and GEAC in June 2008. The Guidelines describe the application process and general requirements for confined field trials, and the SOPs cover the transport, storage, management, harvest/termination and post-harvest management during the conduct of the trials. The concerned state departments of agriculture become involved in the process when a genetically engineered plant is ready to be field tested in the state.

7.3 Safety assessment of food derived from GM plant

A systematic evaluation of safety concerns to address GM food safety for human, animal and environmental health will be ensured within a framework for decision-making. It also provides for safety evaluation parameters to be reviewed in future as and when further information becomes available. In 2008, the Indian Council of Medical Research (ICMR) and the Department of Biotechnology (DBT) established *Guidelines for the safety assessment of foods derived from genetically engineered plants and protocols for food and feed safety assessment of GE crops*.[3,4]

List of biosafety studies to be conducted on GM crops:

- Acute Oral Safety Limit Study in Rats and Mice
- Protein Thermal Stability
- Pepsin Digestibility Assay
- Sub-chronic Feeding Study in Rodents
- Livestock Feeding Study
- Compositional Analysis.

Sub-chronic toxicity testing, compositional analysis and a livestock feeding study will also be conducted before Biosafety Research Level II (BRL-II) field trials; however, these studies need RCGM approval before initiation.

7.4 Rationale and historical development of GM crops

7.4.1 Rationale for *Bt* cotton development

Bt cotton was the first GM crop commercialized in India in 2002. Cotton crop in India was limited in its production due to the damage caused by insect pests, diseases and weeds. Cotton is highly susceptible to insects; the larvae of lepidopteran pests are the most important pests impacting successful cotton production. With the introduction of American cottons (*G. hirsutum* and *G. barbadense*) and hybrids, plant protection became an important part of cotton cultivation, as these varieties/hybrids were highly prone to insect pests and diseases.[5] The most important lepidopteran insect pests attacking cotton are American bollworm (*Helicoverpa armigera* Hübner), the Pink bollworm (*Pectinophora gossypiella* Saunders), the spotted bollworm (*Earias vittella* Fb.), spiny bollworm (*Earias insulana* Boisd.) and tobacco caterpillar, *Spodoptera litura* Fb. The total loss due to damage to cotton crop is estimated to be more than Rs. 1200 crores (US$ 200 million).

Chemical control has been the most popular and often the only approach to suppress these insect pests. About 50% of the total insecticides consumed in the country are used for cotton crop. The heavy and indiscriminate use of different insecticides led to an increase in the cost of crop protection (10–16 sprays for bollworm management) and production, environmental pollution and health hazards and eventually, the insect pest, *H. armigera*, developed resistance to insecticides, such as dichlorodiphenyltrichloroethane (DDT), endosulfan, organophosphates, carbamates and synthetic pyrethroids.[6–9] *B. thuringiensis* and *B.t.k.* microbial formulations which were part of traditional integrated pest management (IPM) programmes were found to exhibit a specific effect on the target insect, and to be safe to the non-target organisms.[10] However, their use was limited due to their short residual action, instability in sunlight and surface run-off during wet weather. A study found that the IPM packages for cotton reduced the number of insecticide spray applications and were

accepted by the farming community.[11,12] The large-scale adoption of IPM practices was a challenge due to farmers' lack of knowledge and pest-scouting practices, and the scarcity of resources. Henceforth, the specificity of Bt formulations was further exploited by isolating specific genes that demonstrated efficacy on lepidopteran insect pests.

7.4.2 *Bt* cotton development

In India, work on the development of Bt cotton started in 1995. The first Bt cotton was introduced following collaboration between Maharashtra Hybrid Seeds Company Ltd. (Mahyco), Mumbai, and the Monsanto Company, St. Louis, USA. Bt cotton being a transgenic crop, it required environmental clearance under Rules 7–10 of the rules and procedures notified by the MoEF Notification No. 1066 (E) dated 5.12.1989.

Initially, Bt cotton was developed by transforming the parental cotton cultivar Coker 312. Bt cotton contains the following three genes inserted via genetic engineering techniques.

- The *Cry1Ac* gene, which encodes for an insecticidal protein, Cry1Ac, derived from the common soil microbe *Bacillus thuringiensis* subsp. k*urstaki (B.t.k.)*.
- The *nptII* gene, which encodes the selectable marker enzyme neomycin phosphotransferase II (NPTII), was used to identify transformed cells that contained the Cry1Ac protein. It served no other purpose and has no pesticide properties. The *nptII* gene is derived from the prokaryotic transposon Tn5.[13]
- The *aad* gene which encodes the bacterial selectable marker enzyme 3"(9)-O- aminoglycoside adenyltransferase (AAD) allowed for the selection of bacteria containing the PV-GHBK04 plasmid on media containing spectinomycin or streptomycin. The *aad* gene was isolated from transposon Tn7.[14]

In 2002, Bt cotton (Bollgard®) was approved for release in India. Subsequently, in 2006, Bt cotton expressing two Bt genes, (Bollgard II®, cry1Ac and cry2Ab genes) was approved for the release in the country followed by two other events, cry1Ab+ cry1A gene (GFM Event) by Nath Seeds Ltd., India, and cry1Ac gene (Event 1) by JK Agri Genetics, India.

7.4.3 Biosafety testing

Prior to the deregulation of transgenic insect resistant Bt cotton, it was necessary to produce enough data to demonstrate that it was equivalent to currently grown non-Bt cotton varieties in composition and agronomic performance. Furthermore, evidence was needed to show that the Bt protein expressed by the inserted gene caused no adverse effect when consumed by domestic or wild animals and beneficial insects. The biosafety and environmental issues related to this novel protein were assessed including: molecular characterization of induced gene; biochemical characterization of the expressed protein; estimation of the level of the expressed proteins in cotton, and proteins in cotton products; safety of the expressed proteins to non-target organisms (field evaluations); environmental fate of the Bt protein through soil micorbiota studies; and agronomic, compositional, and food and feed safety evaluation of Bt cotton compared to non-Bt cotton seed. The food and feed safety was evaluated using several animal model systems such as fish, chicken, cow, goat and buffalo, as per the regulatory approvals. Two studies were conducted to investigate any possible changes in allergenicity of endogenous cotton-seed proteins in Bt cotton seeds; Mahyco conducted allergenicity studies with Bt cotton in guinea pigs and in Brown Norway rats.

7.4.4 Adoption of *Bt* cotton

According to recent estimates, in the 2002 cotton season, approximately 7.2 million farmers cultivated Bt cotton on 10.8 million hectares, which is equivalent to 93% of India's total 11.6 million hectares.[1] The acreage has steadily increased from 3.8 million hectares in 2006 to 10.8 million in 2012. In addition to controlling bollworms, Bollgard II® also controls S. *litura* and the Cotton Semi-looper Moth giving higher yields and hence increasing cultivation to 9.7 million hectares during 2012 compared with 0.15 million during the first year of Bollgard II® in 2006. After "a-decade-plus one" years of the launch of Bt cotton in India in 2002, cotton production has more than doubled, making it the second largest producer.

7.4.5 Resistance management in *Bt* cotton

The success and sustainability of Bt technology depends primarily on a viable insect resistance management strategy within the IPM systems which includes: planting of refuge, either structured or unstructured, for producing Bt susceptible moths; a strong resistance monitoring programme; and refuge compliance and technology adoption. The introduction of Bt technology as an integral part of an IPM programme, caused a paradigm shift in cotton pest management programmes by controlling specific caterpillars (bollworm complex and *S. litura*) which reduced the use of broad spectrum insecticides in cotton ecosystems, significantly benefiting biological control organisms.[15]

The conditional approval of the first three Bt cotton hybrids in India was given by GEAC at their 32nd meeting held in New Delhi on 26 March 2002 with the following refuge recommendation: "Every field where Bt cotton is planted shall be fully surrounded by a belt of land called 'refuge' in which the same non-Bt cotton variety shall be sown. The size of the refuge belt should be at least five rows of non-Bt cotton or shall be 20% of total sown area whichever is more."[16] In addition to the structured refuge requirements natural refuge crops, temporal rotation of crops like wheat, pigeon pea or vegetables such as okra, tomato and chilli with Bt cotton will provide a source of "off-season habitats" for pests such as *H. armigera*, *E. vittella* and *S. litura*; and aid in the dilution of resistance.[17] A resistance monitoring programme involves establishing baseline susceptibility of each of the target pest species to the relevant Bt protein present in the transgenic crop. The baseline susceptibility information will help in the estimation of diagnostic concentration or dose for resistance monitoring[18] that ensures 99% or more mortality of susceptible insects in a population. Technology developers such as Mahyco are in regular consultation with scientists from the Indian Council for Agricultural Research (ICAR), including CICR, Nagpur, and state agriculture universities.

With the technology adoption being more than 90% in 2012–13, a major stumbling block has been the growers' compliance with refuge planting. Continued efforts to encourage refuge planting are underway, and may be achieved through focused education programmes, rigorous monitoring and appropriate rewards for compliance. Considering the refuge compliance and faster adoption rates, it is imperative that a strong resistance-monitoring programme monitors and reports on any shifts in the susceptibilities of target insect populations. Eventually, reduction in the bollworm complex with the Bt technology resulted in resurrgence of minor sucking insect pests in cotton. Hence, introgression of Bt genes into the sucking pest tolerant germplasm is the way forward for Bt cotton hybrids to effectively fit into an IPM programme.

7.4.6 *Bt* Brinjal development and biosafety studies

Brinjal or eggplant is one of the most common and popular vegetable crops grown in many regions of India. The area under brinjal cultivation in the country is estimated at 0.55 million hectares with a total production of about 8.4 million tonnes. Brinjal is mainly cultivated on small family farms and is a source of cash income for resource-poor farmers.[19]

This important vegetable crop is extensively damaged by insects, with the brinjal fruit and shoots borer (FSB) causing losses of 50–70% even after repeated insecticide spraying. The affected fruits lose not only their market value, but also their yield. Farmers use large quantities of chemical insecticides singly or in combination to get blemish-free fruits, which fetch premium prices in the market.

7.4.7 Rationale for the development of *Bt* brinjal

As mentioned above, the brinjal fruit and shoot borer (FSB) is the most damaging pest on brinjal crops, and farmers need to spray 25–80 rounds of pesticides during each growing season. Experts estimate that the financial loss to the country because of the 50–70% damage caused by the FSB is equivalent to Rs. 1000 crores (US$ 166.66 million) per annum. As present control methods for FSB involve heavy pesticide spraying on the crops, brinjal produce potentially contains significant amounts of pesticide residues, posing health concerns for consumers as well as farm workers. Bt brinjal, a GM or biotech crop, provides an alternative method for controlling FSB and reducing pesticide application. Field studies with Bt brinjal have demonstrated that farmers can use 70% less insecticide for FSB control and, as a result, 42% less pesticide overall for control of all insect pests. Field studies have shown that this results in an average 116% increase in marketable fruits over hybrids and 166% increase over open-pollinated varieties of brinjal. The higher yield and better quality would result in higher net income for brinjal farmers to the tune of Rs. 16 000–19 000 per acre, which works out at Rs 2000 crore (US$ 333.33 million) to farmers over India as a whole.

7.4.8 Biosafety studies

The biosafety and environmental studies conducted to prove the safety of Bt Brinjal include: molecular characterization of inserted gene; biochemical characterization and estimation of the expressed protein; safety of the expressed proteins to non-target organisms; environmental fate of the Bt protein through soil micorbiota studies; and agronomic, compositional, and food and feed safety evaluation of Bt brinjal compared to non-Bt brinjal.

Germination tests and aggressiveness studies demonstrated that there is no significant difference between Bt brinjal and its non-Bt counterpart. The data suggest that there is no aggressiveness or weediness demonstrated by Bt brinjal plants. The pollen flow trials conducted during 2002 and then in 2008 established a percentage of out-crossing of up to 2.7% (0.14–2.7%). The maximum distance traversed by pollen from Bt brinjal plants was determined to be 20–30 metres based on the Grow Out Test and ELISA.

The effects on non-target insects, beneficial arthropods, soil microbiota were studied in >60 controlled field trials conducted over a period of 4–5 years. Results of these multi-location replicated research trials demonstrated no significant differences between Bt hybrids, the non-Bt counterparts, and the incidence of sucking pests (aphids, jassids, whitefly) and beneficial arthropods (chrysopa, lady-bird beetle, spiders); further indicating that there were no effects on non-target insect pests and beneficial arthropods. It was also clearly demonstrated that there were no differences between Bt and non-Bt plots in respect of soil bacteria and fungal count both at the rhizosphere and the soil beyond the rhizosphere; and no detectable residual Bt protein in the soil was found.

Substantial equivalence studies conducted with Bt and non-Bt brinjal fruits showed no appreciable differences in composition when major components like protein, carbohydrate, oil, calories, ash, nitrogen, crude fibres and moisture contents were analysed. Chemical fingerprinting studies to estimate alkaloids in Bt and non-Bt brinjal fruits were conducted at Indian Institute of Chemical Technology, Hyderabad. The assessments showed no significant differences in chromatographic profiles of Bt and non-Bt brinjal fruits.

Several food and feed safety studies were carried out to evaluate biosafety of Bt Brinjal. Oral toxicity (acute and sub-chronic) studies were done with Sprague Dawley rats at recommended doses, at INTOX Pvt. Ltd., Pune, Maharashtra. These studies proved that the Cry1Ac protein (expressed in Bt brinjal) is non-toxic to the study animal by oral administration and the no-observed-adverse-effect-level (NOAEL) of transgenic Bt brinjal expressing Cry1Ac protein in Sprague Dawley rat, following oral administration for 90 days was found to be more than 1000 mg/kg body weight.

The sub-chronic (90 days) feeding studies conducted using New Zealand white rabbits and goats at Advinus Therapeutics Private Ltd., Bangalore, concluded that, based on the health, growth and physio-pathological parameters analysed during the experiment, there were no significant differences between the groups fed with transgenic Bt brinjal containing *cry1Ac* gene and control non-Bt brinjal fruit.

Bt Brinjal was used as a feed ingredient in studies conducted with fish, broiler chickens and lactating cows. Studies on common carp, *Cyprinus carpio*, were conducted at Central Institute of Fisheries Education, Mumbai, with broiler chickens at Central Avian Research Institute, Izatnagar, and with cows at G.B. Pant Unviversity of agriculture and technology, Pantnagar. These studies found that using Bt brinjal as a feed ingredient did not have an impact on the growth parameters, feed intake, blood biochemical constituents and/or histopathology of the animals. Furthermore, the results were statistically similar between Bt brinjal and non-Bt brinjal-fed groups. The study conducted with lactating cows concluded that the nutritional value of both Bt and non-Bt brinjal fruits were similar in terms of feed intake, milk yield and milk constituents without any adverse effects on the health of lactating crossbred dairy cows.

Several other studies, such as thermal stability, allergenicity, estimation of Bt protein in cooked Bt Brinjal, baseline susceptibility studies, etc., were conducted to demonstrate that Bt brinjal is safe for environment and as food or feed. Also, academic groups have carried out a number of socioeconomic studies indicating farmers' receptiveness to the technology, the potential of Bt brinjal to increase farmers' welfare through reduced insecticide spraying, and increased marketable yields of brinjal.[20–23]

7.5 Conclusion

India's stringent regulatory system is on a par with or more rigorous than those in other countries. The unprecedented adoption of Bt cotton has provided insights into the profound impact new technologies can have on agriculture. The situation with food crops has varied, with the commercial release of Bt brinjal being put on hold, although the same genes have been used in Bt cotton. In the case of Bt brinjal, safety studies have established its equivalence to conventional counterparts, with added beneficial impacts which have led to: a reduction in the number of pesticide applications needed; a higher proportion of marketable yields for the farmer; improved quality products for the consumer; and environmental benefits. Looking to the future, a variety of technologies have been developed that address gaps in conventional breeding. These include herbicide tolerance for combating weeds, nitrogen-use efficiency, drought and salinity tolerance, and virus resistance. A robust and dynamic regulatory system would help to bring these to the farmer and the consumer.

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Chapter 8. The biosafety regulation and legal framework for GMVs

8.1 Introduction

Mosquito-borne diseases, such as dengue and malaria, are a significant threat to the world's population for both morbidity and mortality indicators.[1] Mosquitoes are becoming resistant to existing pesticide chemicals and fewer new pesticides are being introduced due to societal demand for less polluting chemicals, as well as more demanding regulatory burdens for the approval of pesticides worldwide. Consequently, new solutions for mosquito control are urgently sought that are capable of being integrated into vector control programmes and are environmentally sustainable. One such method is the use of the mosquito itself that has been modified through recombinant DNA mechanisms to either suppress the population of the mosquito through breeding with a "sterile" form of the mosquito, or to convert the mosquito itself to one that is less harmful. Most progress to date has been made with the "sterile" or self-limiting mosquitoes for reduction or suppression of the insect population, with open field releases being conducted since 2010 in several countries. This is unlikely to be a standalone tool but be integrated into existing vector control programmes. The use of GM or genetically engineered insects has moved from theoretical laboratory-based studies to field evaluation and wider deployment since the late 2000's. Regulatory frameworks available for environmental release of GMOs are now being adapted for GM insects, as countries make decisions regarding R&D. The last few years have seen national approvals for open field releases, particularly of GMMs. As a consequence of this activity, authorities are also reviewing their regulatory frameworks and requirements for the field release and deployment of GM insects.

8.2 Legal frameworks and regulation

WHO/TDR has been taking a lead in considering the issues raised by the genetic modification of insects that are vectors of human disease since 1991.[2] It has been helping low- and middle-income countries to strengthen their capacity to conduct research and use research evidence when setting policies and making decisions, and by hosting international expert consultations and other forums.[3–7] However, all regulation is based on the laws of the country in which it operates. Even international regulations such as the WHO International Health Regulations (IHR)[8] or the CPB[9] acquire legal force through implementation into national law by the Parties (signatory countries) that have ratified them.[a] The CPB has been widely adopted in many developing countries, although not in some countries with extensive experience of GMOs (e.g. Australia, Argentina, Canada and the USA) and requires the Parties to take specific decisions

a Definition of ratification extracted from the United Nations Treaty Handbook, 2006. Upon ratification, the State becomes legally bound under the treaty. Ratification at the international level, which indicates to the international community a State's commitment to undertake the obligations under a treaty, should not be confused with ratification at the national level, which a State may be required to undertake in accordance with its own constitutional provisions before it expresses consent to be bound internationally. Ratification at the national level is inadequate to establish a State's intention to be legally bound at the international level. The required actions at the international level shall also be undertaken.

according to the CPB regarding the importation of living modified organisms (LMOs) for intentional introduction into the environment. As such, this forms part of the regulatory framework in many countries for the risk assessment and management of engineered organisms, and will be used to assess genetically engineered insects for both open-release and transboundary movement (i.e. movement across national borders). The CPB has recently focused on one specific type of genetically engineered insects: The Ad Hoc Technical Expert Group on Risk Assessment and Risk Management was convened after the 4th Conference of the Parties – Meeting of the Parties (COP-MOP) in 2009, and a sub-working group was formed to develop guidance documents on risk assessment and risk management of LMMs. After a series of online forums and working group meetings, the sub-group report was finalized at the 5th COP-MOP meeting in Nagoya, Japan, in 2010.[10] The Guidance is available online.[11,12] Some commentators have questioned whether this is an appropriate instrument for some releases of genetically engineered insects.[13,14] The EFSA commissioned a report on *Defining environmental risk assessment criteria for genetically modified insects to be placed on the EU market*.[15] This report describes developments in genetically engineered insects with particular reference to what might be released in the EU in the next 10 years, identifies potential adverse effects as well as methodologies that might be used to investigate them. The report concludes that a case-by-case approach to risk assessment of genetically engineered insects is necessary in their environmental risk assessment. It should be noted that in the EU, the term "environment" includes consideration of the potential of adverse effects on human health. The report contributed to a draft *Guidance on the environmental risk assessment of genetically modified animals*, which was disseminated for public consultation between August and September 2012. This document has now been published as a final Guidance document.[16]

In addition to these framework and guidance documents, several countries have made decisions under their own legislative frameworks regarding field cage assessment or open release of genetically engineered insects in the environment including Brazil, the Cayman Islands, Malaysia, Mexico and the USA. The relevant authorities in the countries involved assessed these releases and all were approved prior to release taking place. These trials have been published in the scientific literature (with the exception of Brazil, where the manuscript is in preparation).[17–25]

National ratification and implementation proceeds asynchronously as each country takes time to develop laws, and pass them through national governmental processes. Nonetheless, in the last decade over 140 developing countries have developed or implemented national biosafety frameworks in response to the CPB mainly through the United Nations Environment Programme (UNEP).[26] While reflecting on ways of using genetic engineering or biological tools to control disease, there is a need to balance the demand with internationally accepted guidelines/legal frameworks for regulating R&D related to GM insects. At present, WHO/TDR and FNIH, in collaboration with many experts worldwide, has developed guidance on the "safety, efficacy, regulation and ethical, social and cultural issues" related to the release of GMMs.

8.3 Biosafety and its related regulations with regard to GMOs in India

Among developing countries, India has led the way in the initiation of national biosafety rules for the use of GMOs in 1986 through the enactment of various acts and guidelines prior to the adoption in 1992 of the CBD.[27] India's Environment Protection Act was enacted in 1986 and the Government of India framed and issued Rules and Procedures (Rules) in 1989 for manufacturing and handling hazardous microorganisms/genetically engineered organisms (GEOs) or cells under the Act.[28] Sections 6, 8 and 25 prohibit institutions from handling hazardous substances without fol-

lowing procedures prior to approval. It also empowers central government to lay down rules and regulations regarding procedures and safeguards for handling hazardous substances.[27] Rules 8, 9, 10 and 11 of the Rules of 1989 explain the requirements of prior approval and permission for production and release of GMOs/cells into the environment and even require permission for experimental trials.[28]

Under the Environment Protection Act, a three-tier mechanism has been functioning which comprises the IBSC at each Institute conducting GM research; the RCGM in the Department of Biotechnology, and the GEAC in the MoEF for granting approval, and guiding and monitoring those institutions and industries involved in recombinant DNA work. The biosafety decision-making structure in India is given in Figure 34 which illustrates the structure related to genetic engineering at different levels, and the functions of various committees under each authority and their role in ensuring safety regulations in India. This excludes the involvement of civil society and the community. There are three levels: central, state/district and institutions. Biosafety is strictly governed by regulatory mechanisms with an objective to regulate the modern biotechnology work at different levels so as to protect the environment.

The MoEF and the DBT under the Ministry of Science and Technology are the two departments that the government has authorized to implement the Biosafety Rules 1989, and to periodically draft Recombinant DNA Safety Guidelines.[29] These bodies notify updated rules and regulations on the importation and use of the modified organisms for research purposes, which include the mass release of these organisms in nature. DBT has constituted various committees such as the Recombinant DNA Advisory Committee (RDAC) to recommend appropriate safety regulations in India based on recent developments in biotechnology, a Review Committee on Genetic Manipulation, and a Monitoring and Evaluation Committee to monitor the safety-related aspects of ongoing research projects and activities involving GEOs. The state and district level committees mainly ensure adherence of safety guidelines at the institutional level through periodic visits and assessments of the damage, if any, caused by the release of GMOs.

The authorities at various levels execute the rules and regulations. The authorities monitor the research from the sanctioning of licences for the importation of the modified organisms for research work to the implementation of the projects at all levels. In order to work within the ambit of the biosafety regulatory framework, RDAC recommends safety regulations for the work based on recombinant DNA technology. Research work using modified organisms/DNA recombinant technology are routed through the IBSC, the nodal point of interaction to the higher committee, the RCGM, which is responsible for reviewing all ongoing r-DNA projects involving high-risk category and controlled-field experiments. Apart from reviewing, the Committee decides on the importation of modified organisms for research, and frames guidelines and procedures for generating data on these organisms. The functioning of various research activities with modified organisms involving recombinant DNA technology is monitored at state and district levels by the State Biotechnology Coordination Committee (SBCC) and the District Level Committee (DLC), respectively. Field release, large-scale production and commercial application of the modified organisms are approved and strictly monitored by the GEAC. Thus, under India's stringent regulatory framework biosafety, GMOs and products derived from them have to undergo approval processes starting from the initiation of research right up to the commercialization of the product. The biosafety regulatory mechanism in India approved the commercial release of GM plants and biotech medicines. However, this triggered a debate amongst civil society and industrialists on streamlining biosafety regulations, which has prompted periodic amendments.

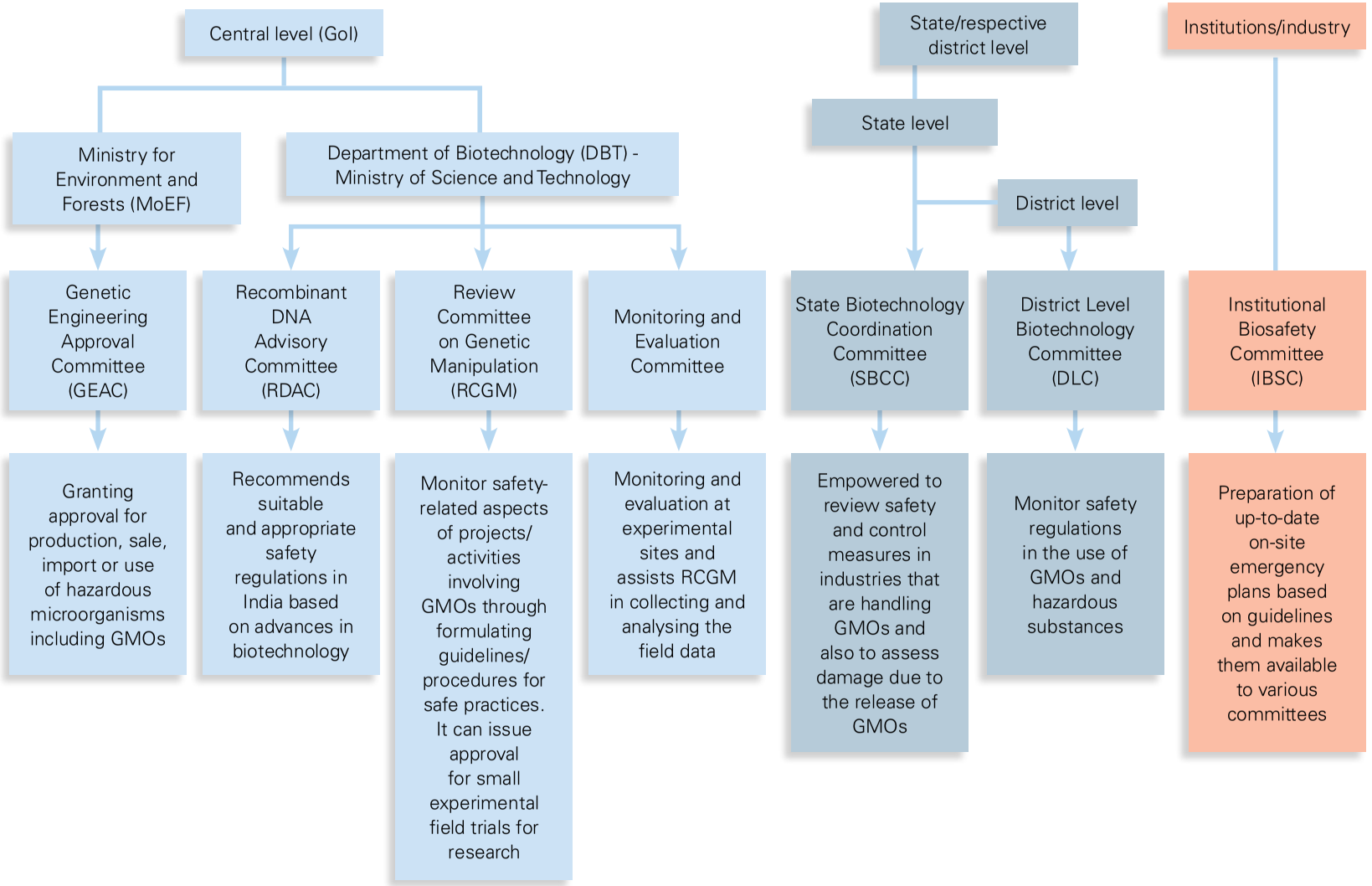
8.4 Biotechnology Regulatory Authority of India Bill, 2013

A bill was introduced in the Lower House of the Indian Parliament on 22 April 2013 to set up the Biotechnology Regulatory Authority of India (BRAI). The statutory independent regulator is to be the nodal agency of the Indian government to ensure the comprehensive safety of organisms and biotech products. It is proposed that BRAI be located under the Ministry of Science and Technology and be responsible for regulating the research, transportation, importation, manufacture and use of organisms and products of modern biotechnology, so as to ensure their safety to human and animal health, and the environment. BRAI would have three regulatory divisions – the first would deal with agriculture, forest and fisheries, the second with human health and veterinary products, and the third with environmental and industrial applications.

8.5 Conclusion

Although international laws govern issues related to GMOs, their effectiveness depends on national legislation. In those countries carrying out experimental releases of GM insects, there is a lack of scientific quality and information to the public prior to release. Therefore, national and international biosafety regulations should be sufficiently stringent in order to ensure there is no harm to the public or to the environment greater than that of the existing mosquito and it's current control measures.

Figure 34. Framework of biosafety decision-making structure based on GMOs in India



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Chapter 9. The cartagena protocol and releases of transgenic mosquitoes

9.1 Introduction

The CBD came into force in 1993 with the aim of preserving the world's biological diversity.[1] Ever since the first LMOs/GMOs (for example, GM foods) were developed, transported and made commercially available, transgenic technology has provoked highly polarized views both within and between nations. Proponents have maintained that GM crops enhance nutrition and will balance future food shortages by increasing food supply; while opponents have cited risks to the environment and human health in addition to economic and legal concerns.[2] This rift is particularly apparent between the USA and the EU. The USA and countries that are heavily dependent on it for trade (e.g. several Latin American countries) are highly supportive of GM crops, whereas the EU and several of its former colonies (e.g. most African countries) are less supportive.[3]

Given these geopolitical differences in support and the potential for GMOs to cross international borders, Parties to the CBD called for a protocol to be developed to ensure "the safe transfer, handling and use" of GMOs. The protocol, which became known as "The Cartagena Protocol on Biosafety" was adopted on 29 January 2000 and entered into force on 23 September 2003.[4] As could be expected, UN Member States did not always agree on a number of issues, e.g. what should be covered by the Protocol, the extent to which the precautionary principle should be applied, and how the Protocol should relate to World Trade Organization (WTO) trade laws.[5] However, following negotiations, the Protocol was finalized and adopted in Montreal, Canada, on 29 January 2000, and opened for signature in Nairobi, Kenya, in 2000. It became legally binding after 90 days and once 50 countries had ratified it, i.e. on 11th September 2003.[4] As of November 2014, the Protocol has been ratified by 167 countries.[6] As an international treaty, it governs the biosafety concerns related to safe transboundary movement, transit, handling and use of GMOs/LMOs in order to protect biological diversity, human health and the environment from the risks posed by the deliberate release of LMOs into the environment.

The terms of the Protocol were primarily concerned with GM crops. However, at the same time, it was intended to apply to all GMOs (referred to as LMOs in the text). GMM technology is moving very quickly and, in recent years, open releases of LMMs have taken place in the Cayman Islands (2009–2010), Malaysia (2010), and Brazil (2011), with further releases being planned in other countries.[7,8] The release by Harris et al. in the Cayman Islands was the biggest (~3.3 million sterile male LMMs).[7] These releases have been of genetically sterile *Aedes aegypti*, the vector of dengue fever, and have demonstrated that large-scale releases of transgenic males can lead to reduced mosquito densities and, by implication, reduced dengue transmission.[7] Another landmark in this context is the recent release of *Wolbachia* to control mosquito-transmitted diseases such as dengue fever and chikungunya. *Wolbachia*, a maternally inherited intracellular bacterium, are of interest because, although not transgenic, the infected mosquitoes display important physiological changes which are inherited from one generation to the next, and are capable of spreading through populations and potentially across international borders.[9] This is a truly remarkable achievement by the scientists concerned.[10-12] The first open field trials of these mosquitoes took place in Queensland, Australia in 2011.[11] The open release was permitted in Australia, which

is not a signatory to the CPB, after the relevant national authorities performed risk assessments in line with the government's draft rules.[13] This chapter describes the application of the Protocol to GMMs and discusses the applicability of the Protocol to recent releases of sterile GMMs and mosquitoes infected with the *Wolbachia* bacterium.[14] Depending on the type of GMM technology being considered, the Protocol is either applicable or requires further consideration. In particular, there are a number of overarching issues regarding GMMs capable of spreading transgenes across national borders that are not covered by the text.[15] A sub-working group assigned by the CBD's AHTEG on Risk Assessment and Risk Management developed a document containing comprehensive risk assessment guidelines for GMMs,[16] but it does not address the overarching issues. In 2010, the CPB was strengthened with the adoption of a new international treaty called the "Nagoya–Kuala Lumpur Supplementary Protocol on Liability and Redress". Several countries are not signatories to the Protocol and, consequently, may not feel obliged to abide by it. There are, however, independent commentators, such as Marshall,[14] who feel that the Cartagena Protocol has weaknesses. They have suggested that it should be addressed prior to an open release of mosquitoes engineered with invasive gene drive systems**.** The chapter concludes with a discussion of the application of the Protocol to additional novel GMM technologies, and efforts that are currently underway to address the deficiencies of the Protocol in its present form.

9.2 Types of GMMs

Several strategies are being considered under two major types of effects, such as population suppression and population replacement, to control VBDs using GMMs which were explained in detail in Chapter 3. Some are well covered by the Protocol, and others require further elaboration.

9.2.1 Population suppression (sterile GMMs)

Strategies that target vector "demography/density" intend to reduce (suppress) the size of the mosquito population and thereby control pathogen transmission. These include methods to reduce the overall numbers of female mosquitoes, which will result in decreased reproduction. The strategy that has received the most interest to date is the release of GM sterile males that, upon mating with wild females, produce unviable offspring, thus resulting in population suppression.[17] This strategy benefits from the fact that male mosquitoes do not bite and so the large numbers of released GM sterile males decrease the disease-transmitting female population in the next generation without transmitting disease themselves. There are actually two variants of this technology – one in which both male and female offspring are rendered unviable by the sterility gene,[18] and another in which sterility is specific to female offspring.[19] The bi-sex lethal strategy has the benefit that transgenes are eliminated from the population within a generation or two; while the female-specific lethal strategy allows the sterility gene and, hence, the population suppressing effect, to persist for a few generations longer. The technology for this strategy has already been developed for *Aedes aegypti*,[18,19] and has been tested in open field trials in Brazil, the Cayman Islands and Malaysia. This technology is well covered by the Cartagena Protocol due to the fact that the transgenes are self-limited in time and space.

9.2.2 Population replacement (self-propagating GMMs)

Another strategy being developed involves the use of a "gene drive system" to spread disease-refractory genes into mosquito populations that target vector competence in order to reduce the inherent ability of individual mosquitoes to transmit a given pathogen.[20] This involves the introduction of engineered DNA and/or the manipulation of endogenous genes in order to inhibit pathogen replication within the mosquitoes, making them refractory to transmission of particular viruses or parasites. Upon release into the environment, these refractory GMMs will be expected to mate and introduce the change into the local mosquito population, "replacing" their ability to spread the targeted pathogen with a reduced or eliminated transmission capability. As a proof of principle for this strategy, a malaria-refractory gene has been engineered in *Anopheles stephensi*, the vector of rodent malaria, which works by preventing the passage of the malaria parasite through the mosquito midgut following ingestion and through the mosquito salivary glands.[21] A dengue-refractory gene has also been engineered in *Ae. aegypti* that takes advantage of the natural antiviral pathway in the mosquito, placing it under the control of a blood-meal specific promoter.[22] Other approaches are also being explored, such as the expression of antibodies that kill malaria parasites within the mosquito,[23] and the discovery of genes that govern disease-refractoriness in natural mosquito populations.[24]

Progress has also been made in developing gene drive systems to spread these genes into mosquito populations. One of the early inspirations for this strategy was the observation that a transposable element known as the *P* element was observed to spread through the worldwide population of *Drosophila melanogaster* in just a few decades simply through biasing inheritance in its favour.[25] This led to the idea that a disease-refractory gene could "hitchhike" such a system. Since then, a synthetic gene drive system known as *Medea* has been engineered in *D. melanogaster*.[26] Progress has been slow at engineering the *Medea* system in *Ae. aegypti*. However, another gene drive system known as a HEG has been engineered in *Anopheles gambiae*, the primary malaria vector in sub-Saharan Africa.[27]

If gene drive systems such as these can be stably linked to disease-refractory genes, then just a few GMMs with these constructs would be capable of propagating transgenes over the entire geographical range of the species. This has far-reaching implications for wide-scale disease control. However, the application of the Cartagena Protocol to this technology is more problematic because the gene drive systems would be capable of spreading transgenes across international borders regardless of whether neighbouring countries were supportive of the technology.

9.3 Application of the Cartagena Protocol to GMMs

9.3.1 Definition of the terms of the Cartagena Protocol

Article 4 of the Cartagena Protocol states that the Protocol applies to, "the transboundary movement, transit, handling and use of all LMOs that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health." There are a few terms in this definition that require definition themselves. An LMO is essentially a GMO that is living and is defined in the Protocol as "any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology" (Article 3). A living organism is defined as "any biological entity capable of transferring or replicating genetic material" (Article 3) and, of particular interest, is the definition of "modern biotechnology," which encompasses "the application of in vitro nucleic acid techniques, including recombinant DNA and direct injection of nucleic acids into cells or organelles… that overcome

natural physiological reproductive or recombination barriers" (Article 3). Finally, "transboundary movements" include movements of LMOs both between Parties of the Protocol and between Parties and non-Parties (Article 3). Taken together, this means that the Protocol applies to the international movement of GMMs provided that at least one of the two countries is a Party to the Protocol.

9.3.2 Protection against an accidental release

The Cartagena Protocol was, in part, written with the intent of protecting developing countries against threats to biosafety due to a lack of resources to conduct their own risk assessment. The Advance Informed Agreement (AIA) procedure – the centrepiece of the Protocol – allows an importing country to demand the exporting country to both conduct a risk assessment and bear the costs of this assessment. During negotiations, however, it was decided that GMOs in transit or destined for contained use were exempt from this procedure (Article 6). Countries with strong biotech industries argued that GMOs in transit and containment pose negligible risks and hence the AIA procedure restricts trade unnecessarily.[5]

This exemption may be acceptable for GM crops and sterile varieties of GMMs; however it begs re-examination for GMMs with invasive gene drive systems. It is likely that such mosquitoes will undergo phased testing, beginning with laboratory studies of their basic characteristics and testing for adverse effects.[28] Following this, contained field cage trials are likely as an intermediate between laboratory studies and an open release. The fact that the AIA procedure does not apply to LMOs destined for contained use means that it may not apply to GMMs destined for field cage trials and, hence, the importing country (most likely a disease-endemic country – DEC - and possibly a developing one) is not entitled to require the exporting country to conduct a risk assessment at its own expense. The significance of this exemption is elevated for GMMs with invasive gene drive systems due to the fact that flying insects are particularly difficult to contain, and breaches of containment are impossible to rule out. In addition to this, once released, GMMs with gene drive systems could spread transgenes on a large scale prior to assessment of risks in that environment. Given the inability of some countries to conduct a sufficiently detailed risk assessment on their own, it has been argued that the Protocol should be adapted so that GMMs in transit or destined for contained use are covered by the AIA procedure.[15]

9.3.3 Movement of GMMs between non-Parties

Another situation where the protections of the Protocol do not apply is for movements of GMMs between non-Parties to the Protocol. Of particular relevance, the USA and several other countries with large biotech industries are not signatories, such as Australia, Argentina and Canada. Additionally, several developing countries with endemic mosquito-borne diseases are yet to sign the Protocol. These include in Africa: Cote d'Ivoire, Equatorial Guinea, Sao Tome and Principe, and Sierra Leone; and in South America: Chile and Uruguay.[6] The concern here is that, for GMMs originating in countries such as Australia and the USA, several DECs are not protected by the Protocol at all. Countries with strong biotech industries have refused to sign the Protocol because they claim it allows environmental precaution to provide an excuse for violation of WTO trade laws.[5] This may be relevant to GM crops, but is completely irrelevant for self-propagating GMMs, highlighting the problem of having a single Protocol that applies collectively to all GMOs.

The severity of these concerns is heightened by the fact that the emergency response and liability measures listed in the Protocol are inadequate for dealing with GMMs with gene drive systems. According to the Protocol, if a GMM travels from one country to another, then the recipient country may ask the country where the release occurred to "dispose, at its own expense," the GMMs "by repatriation or destruction, as appropriate" (Article 27). All Parties to the Protocol are

also required to prevent GMMs from crossing into their borders illegally. These measures are impractical for mosquitoes and many other disease vectors with gene drive systems.

9.3.4 Provision for an intentional release

GMMs with gene drive systems present a quandary for the Cartagena Protocol due to their ability to propagate transgenes across international borders in the absence of an international agreement. As discussed earlier, the Protocol seems to offer inadequate protection against an accidental release of GMMs with gene drive systems; however, its requirements for an intentional release seem very difficult to satisfy. The AIA procedure applies prior to the first environmental release of GMMs in another country (Article 7) and, under this procedure, the importing country may request the exporting country to perform a risk assessment at their own expense (Article 15), part of which is to determine the likelihood of unintentional transboundary movement (Article 16). Furthermore, the Protocol states, "Each Party shall take appropriate measures to prevent unintentional transboundary movements of LMOs" (Article 16). For GMMs with gene drive systems, unintentional movements are very difficult to prevent, which questions the practically of approving an environmental release.

While this is an important restriction for a technology that has not been tested, it would be disappointing to rule out a technology with the potential to control diseases on a global scale. One solution would be a multilateral agreement regarding GMMs with invasive gene drive systems. The Cartagena Protocol has a provision for bilateral, regional and multilateral agreements that are "consistent with the objective of this Protocol" and "do not result in a lower level of protection than that provided for by the Protocol (Article 14)." An agreement on GMMs with gene drive would have to acknowledge that any environmental release is intentionally international.

The problem with a multilateral agreement would be its scale and feasibility. Gene drive systems have the potential to spread through a species wherever they exist in the world. Consequently, an agreement on GMMs with invasive gene drive systems would potentially require the compliance of every country that the vector species inhabits. The possibility of achieving this is questionable given that, in 2002, Zambia rejected GM food aid from the USA during a famine that threatened hundreds of thousands of lives simply because it was genetically modified.[29] However, it is possible that GMMs may be more widely acceptable than GM crops given that their purpose is to improve health without an overt profit motive. In support of this hypothesis, medical applications of genetic engineering (such as insulin-producing GM bacteria) have much higher acceptability ratings than GM crops.[30]

9.4 Application to *Wolbachia*-infected mosquitoes

With the regulatory difficulties associated with releasing GMMs with invasive gene drive systems, one alternative is to escape the definition of "modern biotechnology" and hence the application of the Protocol altogether. The text of the Cartagena Protocol states that it applies strictly to living organisms engineered through the use of in vitro nucleic acid techniques (Article 3). Organisms with gene deletions produced by traditional means are, therefore, exempt as are mosquitoes infected with a non-transgenic strain of the *Wolbachia* bacterium. Some commentators (e.g. Macer[31]) have drawn attention to Article 5 of the Protocol which states that GM organisms considered "pharmaceuticals for humans" are exempt provided they "are addressed by other relevant international agreements or organizations", although the interpretation of GMMs as pharmaceuticals is not widespread.

Ideally, modified mosquitoes should be regulated on the basis of the physiology of their modification rather than the process by which the modification is achieved. However, since the Cartagena Protocol applies specifically to GMOs, then mosquitoes infected with a non-GM strain of *Wolbachia* are exempt from its application.[15] *Ae. aegypti* mosquitoes have recently been stably infected with a natural mutant strain of *Wolbachia* known as *wMelPop*,[32] which provides an interesting case study because, although no genetic modification is involved, the modification leads to a range of physiological effects such as reductions in mosquito lifetime, dengue viral load and biting ability with age.[9] The stability of the *Wolbachia* infection essentially means that an entire bacterial genome has been assimilated into the mosquito. Additionally, its manner of inheritance allows it to propagate into a mosquito population and potentially spread across international borders much like a gene drive system.[9] However, despite all of these factors, there are no international regulations that apply to non-GM *Wolbachia*-infected mosquitoes because they do not satisfy the Cartagena Protocol's definition of "modern biotechnology". It would be unfortunate if a method of modification were chosen first and foremost for its immunity to excessive regulatory requirements, rather than on the basis of its safety and efficacy.

9.5 Implications of recent releases of sterile GM and *Wolbachia*-infected mosquitoes

Research into GM and *Wolbachia*-infected mosquitoes is progressing extremely quickly, with releases of sterile GMMs occurring since 2009, and releases of *Wolbachia*-infected mosquitoes occurring since 2011. The first sterile GMM releases were of GM *Ae. aegypti* mosquitoes carrying RIDL in the Cayman Islands.[7] Since then, releases have occurred in Brazil and Malaysia.[33,34] These allow us to assess the suitability of the Protocol to self-limiting strains of GMMs.

The first *Wolbachia*-infected releases were of *Ae. aegypti* mosquitoes infected with the *wMelPop* strain of *Wolbachia* (henceforth referred to as *wAe. aegypti*).[11] This is a non-transgenic strain of *Wolbachia*,[32] and, hence, does not fall within the remit of the Cartagena Protocol. However, it does have several properties in common with GMMs with gene drive systems and, thus, highlights questions of relevance to GMMs with gene drive systems.

9.6 Releases of sterile GMMs

The strain of GMM released in the Cayman Islands field trial (OX513A) consisted of a repressible sterile phenotype that affects both male and female offspring of transgenic individuals.[18] Large-scale releases of males of this strain are expected to reduce dengue transmission because wild females that mate with transgenic males produce no viable offspring, thus reducing the population size of the disease vector. The Mosquito Research and Control Unit (MRCU) implemented the first Cayman Islands releases with the Oxitec Ltd. strain OX513A in November–December 2009 to assess the competitiveness of transgenic males in the field. This was followed by a large-scale release of transgenic males in May–October 2010 to test for population suppression.[35] Results from these trials suggest an up to 80% reduction in the local Ae. aegypti population ~11 weeks following commencement of the trial which was sustained until the trial ended.[7]

More recently, the Institute for Medical Research (IMR) in Malaysia carried out another open field trial in December 2010–January 2011 using the same OX513A strain, and a large-scale release of the same strain has been taking place in Brazil since February 2011.[34] The Malaysian release took place in an uninhabited area of Bentong to assess the dispersal

and longevity of transgenic males in the field.[33] The Brazilian release took place in the city of Juazeiro, resulting in the release of more than 10 million mosquitoes over the course of the last year.[8] Another self-limiting strain engineered with a female-specific flightless phenotype[19] has undergone successful contained trials and is being evaluated.

As Benedict and Robinson[36] have argued, it is appropriate that the first releases of transgenic mosquitoes were of genetically sterile males. Male mosquitoes do not bite, reducing the risks to human health; and sterile males produce unviable offspring, thus reducing the population of disease-transmitting female mosquitoes in subsequent generations. Furthermore, because the released transgene encodes sterility, it is only expected to persist in the wild for a few generations following a release. This minimizes biosafety issues because, provided that the GMMs are not released at the border between two countries, they will remain confined to the country of release. The Cartagena Protocol then applies to the import of GMMs intended for release into the environment, and requires that Parties make decisions on this import based on a scientifically sound risk assessment (Article 15).

In terms of the sterile GMMs developed by Oxitec Ltd., a risk assessment was conducted for the strain released into the Cayman Islands and Malaysia following a UNDP-sponsored Workshop on the Risk Assessment of Transgenic Insects in Malaysia in November 2008.[37] This Workshop assessed 31 risks of a hypothetical open field release of the bi-sex lethal GMM strain (OX513A), and has been expanded upon by Patil et al.[38] who identified an additional two risks for this strain, and another eight risks for a strain engineered with a repressible female-specific flightless phenotype.[19] All of the potential risks were determined to be of either low or negligible magnitude. One of the most serious concerns was that suppression of *Ae. aegypti* populations could lead to their replacement with *Aedes albopictus* and a consequent increase in chikungunya transmission. However, this risk was considered to be of low magnitude because releases of sterile *Ae. aegypti* are not expected to result in population extinction, and the long-term strategy would be to suppress both *Ae. aegypti* and *Ae. albopictus* populations.[37] This risk assessment satisfies the requirement, mandated by the AIA procedure (Articles 8–10 and 12) that the exporting country perform a risk assessment at its own expense, if requested by the importing country, prior to the first transboundary movement of a GMO intended for release into the environment.

One weakness of the Cartagena Protocol, highlighted by exports of GMM eggs by Oxitec Ltd. to the Cayman Islands (a British Overseas Territory), Malaysia and several other destinations, is that the AIA procedure does not apply to GMOs destined for contained use (Article 7). In almost all cases, GMMs are first exported for careful analysis in laboratory studies and cage trials in the receiving country, and the importing country is not entitled to request the exporting country to perform a risk assessment under these circumstances. Therefore, although risk assessments were performed for the GMM strains developed by Oxitec Ltd.,[37,38] it is not clear that these were required for exports to Brazil, France, India, Malaysia, Singapore, the USA and Viet Nam. Initial containment effectively sidesteps the risk assessment requirement because, once the GMMs have been received by the importing country, the country is free to release them into the environment in accordance with their own national regulations.

Releases of Oxitec Ltd. GMMs by the MRCU in the Cayman Islands also highlight confusion over the applicability of the Cartagena Protocol to transboundary movements between Parties to the Protocol and their overseas territories. As an overseas territory of the United Kingdom, the Cayman Islands are not able to ratify UN protocols independently of the United Kingdom. Parliamentary discussions suggest that overseas territories are not considered to be the same Party as the United Kingdom, but they are encouraged to become a Party to the United Kingdom's instrument of ratification of the Protocol.[39] The Cayman Islands have not done this and are, therefore, considered to be a non-Party. Parliamentary discussions further suggest that the provisions of the Protocol do not apply to transboundary movements of LMMs

from the United Kingdom (a Party) to the Cayman Islands (a non-Party).[39] However, this contradicts Article 24 of the Protocol, which states, "transboundary movements of LMOs between Parties and non-Parties shall be consistent with the objective of this Protocol."

Regardless of the applicability of the Cartagena Protocol, the export of GMMs to the Cayman Islands was subject to a similar European Commission regulation (EC Regulation 1946/2003), which requires that the exporter notify the competent authority in the importing country of the first transboundary movement of a GMO, and to await its consent to proceed.[39] The decision to release the GMMs into the environment is then subject to local legislation. Parties to the Protocol are required to notify the Biosafety Clearing House (BCH) of decisions to import or release GMOs into the environment (Article 20), and Malaysia has done this. However, since the Cayman Islands are a non-Party to the Protocol, they are not required to make such a notification.

The release of GMMs into the environment in the Cayman Islands and Malaysia was greeted with some controversy.[33,40–43] The major criticisms concerned the manner in which information about the trials was disseminated – an issue that is beyond the scope of the Cartagena Protocol. In both cases, the degree of community engagement was questioned, and several groups complained that they had not been given advanced information about the releases. Additionally, for the Cayman Islands, a video was posted on YouTube describing the trial, but it did not mention that the released mosquitoes were GM. Nevertheless, the releases did abide by national regulations in both cases. In the Cayman Islands, the trial abided by a draft biosafety bill that had yet to become law, the MRCU obtained a permit from the Cayman Islands Department of Agriculture, and a risk analysis and environmental impact assessment were carried out.[39] In terms of community engagement, elected political representatives were educated, information about the trial was sent to local newspapers, and flyers were distributed among the local population.[41]

In Malaysia, Oxitec Ltd. and the IMR worked closely with the Malaysian Government in assessing risk factors examined by the Genetic Modifications Advisory Committee.[44] The Natural Resources and Environment Ministry placed advertisements about the trial in local newspapers on 5 and 9 August 2010[45] and nine NGOs were invited to provide feedback during a one-month public feedback period from 5 August to 4 September 2010.[46] The terms of the trial were publicized on the IMR's website for a month, and the IMR put up notices in the trial area three weeks in advance.[45] Permission was obtained from local authorities, and the IMR held two public talks with local people – one in collaboration with the Bentong Municipal Council and another with the Bentong Malaysian Chinese Association.[46] Community engagement requirements for the first release were reduced because the trial was carried out in an uninhabited area. Despite this, negative reactions were encountered, particularly from NGOs and the media. Lessons should be learned from their criticisms, while acknowledging that the first use of a transgenic technology may always be greeted with some opposition.

9.7 Releases of *Wolbachia*-infected mosquitoes

Releases of *Wolbachia*-infected mosquitoes in Queensland, Australia, in January 2011 highlighted questions of relevance to self-propagating varieties of GMMs.[47,48] These were the first open releases of *wAe. aegypti* and were greeted with much less controversy than releases of genetically sterile mosquitoes, probably because the *Wolbachia* infection did not involve genetic modification. This also meant that there were fewer regulatory hurdles to overcome prior to releasing the *wAe. aegypti* mosquitoes into the environment,[49] making them an attractive alternative to GMMs with gene drive systems. Since the Cartagena Protocol does not apply to mosquitoes infected with non-transgenic strains of *Wolbachia*,

the Australian release was subject to national regulations, but was not subject to international regulations regarding subsequent potential transboundary movements.

When the Eliminate Dengue Program at the University of Queensland sought approval to release *wAe. aegypti* into the environment, the situation was described by regulators as a "regulatory no man's land".[50] Both *Wolbachia* and *Ae. aegypti* were already present in Australia, so the release did not constitute the introduction of a new species. Furthermore, the Office of the Gene Technology Regulator ruled that *wAe. aegypti* was not a GMO, and so could not be regulated under the Gene Technology Act. In the end, a submission was presented to the Primary Industries Ministerial Council, following which it was concluded that the Australian Pesticides and Veterinary Medicines Authority (APVMA) provided the most appropriate regulatory framework: *wAe. aegypti* was considered to be a "veterinary chemical product" on the basis that *Wolbachia* is a "substance that is used for application to an animal… as a way of directly or indirectly modifying the physiology of the animal so as to alter its natural development or reproductive capacity."[51] A decision was taken to approve the trial based on the results of a prior risk assessment undertaken by the Commonwealth Scientific and Industrial Research Organisation (CSIRO)[52] and a risk assessment focusing on environmental impact undertaken by the APVMA with support from the Federal Commonwealth Government's Department of Environment, Water, Heritage and the Arts. The trial was monitored by the APVMA.

As pointed out by De Barro et al.[50], the Australian regulatory framework is rigorous. Nevertheless, a major weakness is that, if the trial is successful, *wAe. aegypti* is predicted to spread throughout Australia and possibly beyond, while only Australia was considered in the regulatory approval. The same weakness is reflected in the risk assessment conducted by the CSIRO.[52] Here, a total of 50 risks were identified following community engagement exercises, a workshop and email solicitation with a dengue consultation group. The CSIRO acknowledged that, if successful, "*Wolbachia Ae. aegypti* will be self-sustaining after the inoculative release and… will be driven into the Australia *Ae. aegypti* populations…".[52] However, the possibility that the strain may spread into other countries was not considered.[53] It may be argued that, since the release is judged safe by Australia's rigorous standards, it will also be safe for other countries. However, this assumes that the standards of one country apply to another, which is not necessarily the case. Nevertheless, there are no international regulations that apply to non-transgenic *Wolbachia*-infected mosquitoes, and so, even if a release is expected to have international implications, the decision to release is a national one.

Furthermore, even if *wAe. aegypti* was considered to be a GMO and within the remit of the Cartagena Protocol, the significance of the Protocol would be undermined by the fact that Australia is not a signatory. Like many countries with strong biotech industries, such as Argentina and the USA, Australia is reluctant to sign the Protocol because it may restrict trade, partly because a strong interpretation of the precautionary principle may allow economic protectionism to masquerade as environmental protection.[5] Article 24 states that the Protocol also applies to "transboundary movements of LMOs between Parties and non-Parties", and a release of *wAe.aegypti* in Australia could conceivably spread into any number of Parties to the Protocol (for instance, Papua New Guinea). However, it seems overly optimistic that a non-Party would feel obliged to abide by a Protocol to which it did not agree. The Nagoya-Kuala Lumpar Supplementary Protocol on Liability and Redress[54] applies to "damage resulting from transboundary movements of LMOs", and includes movements originating from non-Parties in its scope (Article 3 of the Supplementary Protocol). Yet, it is unclear whether the liability procedures (Article 12 of the Supplementary Protocol) would provide an adequate incentive to prevent a non-Party from releasing a self-propagating LMO on their own accord. In essence, even if the Protocol did apply to *wAe. aegypti*, the Australian release may still have occurred.

9.8 Application of the Protocol to novel self-propagating GMM technologies

The regulatory approaches that governed the releases of genetically sterile and *Wolbachia*-infected mosquitoes have significant implications for novel varieties of self-propagating GMMs, which are also capable of propagating transgenes across national borders. Of particular concern, the AIA procedure of the Cartagena Protocol is not expected to apply to many imports of self-propagating GMMs because the first mosquitoes will likely undergo cage trials in the receiving country to assess their behaviour in the ambient environment in a location that the species naturally inhabits.[28] Under these circumstances, the importing country will not be entitled to request the exporting country to perform a risk assessment at their own expense, which is a concern for developing countries with a lack of resources to conduct a comprehensive risk assessment of their own.[15] As for the sterile GMMs developed by Oxitec Ltd., it is likely that a risk assessment for self-propagating GMMs will be conducted anyway, either voluntarily or as required by national or regional regulations in the exporting country. However, it is of concern that the Cartagena Protocol does not mandate a risk assessment under the most likely scenario in which a release follows contained study.

Another concern is that, although a release of GMMs with invasive gene drive systems could propagate transgenes over entire continents, the Cartagena Protocol may not be strong enough to prevent some countries from acting unilaterally in a decision to release them. A strict interpretation of the Protocol suggests that a release of GMMs with invasive gene drive systems would require a multilateral agreement between all affected nations – a difficult task considering the potential scale of spread.[15] However, the unilateral decision by a non-Party to the Protocol, in this case Australia, to release *wAe. aegypti* mosquitoes, which are expected to propagate across national borders, highlights the possibility that GMMs with invasive gene drive systems could also be released in the absence of an international agreement. Such a release could occur because: (i) a non-Party may not feel obliged to abide by the terms of a protocol to which it did not agree; (ii) the likelihood of damage to a Party resulting from an unintentional transboundary movement may be considered minimal; and (iii) the liability measures outlined in the Supplementary Protocol may be inadequate to dissuade a non-Party (or even a Party) from conducting a release on their own accord. Several DECs, such as Cote d'Ivoire, Equatorial Guinea, Sao Tome and Principe, and Sierra Leone, have yet to sign the Protocol, in addition to the countries with strong biotech industries, mentioned earlier.

9.9 *Medea* and X-shredders

Issues relating to self-propagating GMMs should be taken seriously, as work is currently ongoing to create *Medea* elements capable of driving disease-refractory genes (e.g. Ito et al.; Franz et al.[21,22]) into mosquito populations in order to render entire vector populations refractory to disease.[26] Work is also ongoing towards the development of an X-shredder which utilizes a HEG to cleave the X chromosome during spermatogenesis,[55] thus creating a bias towards Y-bearing spermatozoa and eventually leading to an all-male population crash.[56] Both systems are capable of spreading across national borders, with the latter strategy inducing a cascade of population crashes in its wake. Interestingly, the goal of creating an X-shredder in *An. gambiae* has led to the intermediate creation of a GM sterile male, which could be used for self-limiting population suppression field trials in sub-Saharan Africa.[55]

9.10 Gene drive systems with thresholds

Finally, work is ongoing towards the creation of gene drive systems that will only spread into a population if they exceed a critical population frequency.[57–59] These systems have three desirable features for biosafety when the goal is local population replacement – accidentally released mosquitoes are unlikely to persist in the wild because they will inevitably be present at sub-threshold levels; mosquitoes released at super-threshold frequencies at an isolated release site are expected to spread transgenes locally while remaining at sub-threshold levels at nearby locations; and transgenes can be eliminated from the release site through a sustained release of wild mosquitoes diluting transgenes to sub-threshold levels. These desirable features are acknowledged in the first guidance document of the Sub-Working Group on LMMs,[16] although a proper ecological assessment will be required on a case-by-case basis when such a release is considered.

9.11 Discussion

Ostera and Gostin[60] have argued for a new international treaty governing the environmental release of genetically or biologically modified disease vectors. A dedicated treaty would certainly be able to address the unique biosafety concerns posed by GM and *Wolbachia*-infected mosquitoes, but any treaty will inevitably be faced with the problem that there will be non-signatories who may choose to release self-propagating modified mosquitoes on their own accord. Given that releases of GMMs have already begun, an adaptation of the Cartagena Protocol that quickly addresses the pressing needs of GMMs provides a better solution, particularly considering that the Protocol already has 167 signatories – a total which has taken more than a decade to achieve.

The exemption from the AIA procedure of GMMs being considered for release following initial laboratory studies and/or cage trials is a major weakness of the Protocol. This issue is important to address prior to the development of GMMs with invasive gene drive systems because their risks are magnified by the ability of transgenes to spread globally; however a risk assessment is not currently mandated under the most likely release scenario. Another weakness is that it may currently be impossible to release GMMs with invasive gene drive systems in a manner that is consistent with the Protocol if one of the countries into which they may spread is fundamentally opposed to GMOs.[15] A mechanism for the independent review of GMOs with international implications for biodiversity and/or human health should be considered in these cases,[60] since a paralytic Protocol is less likely to be upheld. Such a review should consider both the risks and benefits of LMMs,[61] and the promise that GMMs have for reducing the global burden of vector-borne diseases.

It will be difficult to encourage non-Parties to abide by the terms of the Protocol no matter what changes are made. Nevertheless, clarification should be provided to Parties of the Protocol that the Protocol does apply to exports of GMOs to non-Parties. Clarification should also be provided that the Protocol does apply to mosquitoes infected with transgenic strains of *Wolbachia*, if such strains prove useful.

9.12 Conclusion on efforts to address the deficiencies of the Protocol

Further discussions will be required to guide the evolution of the Cartagena Protocol, and lessons learned from recent releases will ensure that it moves in the right direction to address the unique biosafety concerns posed by self-limiting and self-propagating LMMs. For the time being, modifications to the Protocol have taken the form of an additional guidance document that serves as an extension of Annex III of the Protocol on risk assessment, with a specific section on GMMs. The guidance document was written by an AHTEG-assigned Sub-Working Group on LM Mosquitoes. The first draft of the document was presented to the Fifth Meeting of the Conference of the Parties Serving as the Meeting of the Parties to the Cartagena Protocol on Biosafety, which took place in Nagoya, Japan, in October 2010.[16]

This guidance document is an important first step towards incorporating the biosafety issues posed by GMMs into the Cartagena Protocol. It raises a number of important considerations regarding risk assessment that may be largely adequate for releases of sterile and self-limiting GMMs. However, for strategies involving mosquitoes capable of replacing entire populations with disease-incompetent varieties, several issues still need to be resolved. For these strategies, a balance must be sought between the precautionary principle, respect for the sovereignty of states, and the ethical mandate to prevent disease on a global scale. Further discussion is needed to address the international regulatory challenges posed by GMMs in working towards the goal of global VBD control.

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Chapter 10. Importance of biosafety: ethical, legal and social/cultural issues of GMMs

10.1 Introduction

The development of GMMs is pioneering the use of modern molecular approaches for effective vector control. This technological initiative is progressing as open releases of GMMs from laboratory to field take place in many countries with further releases planned in other countries. Although transgenic technology has strong support in the context of science and technology and is perceived as a viable solution for vector control, it also has opponents who are against the use of such technology because of the potential risks to the environment and human health. The reason behind the confusion and controversy about genetic engineering is a failure to consider and deal with ethical and social issues in an organized manner.[1] The success of any scientific and public health endeavour depends on the issues associated with GMMs being addressed in a systematic and scientific manner at an early stage prior to open trial testing in order to improve public good will, cooperation and participation.[1] The public has the right to expect research to comply with ethics and social values in addition to conforming with regulatory requirements and codes of conduct.

The word "ethics" evokes concepts of moral philosophy, and involves systematizing, defending, and recommending concepts of right and wrong. Ethics seek to resolve questions concerned with human morality — such as good and evil, right and wrong, virtue and vice, justice and crime. Geographical variations in ethical values are attributable to the socio-cultural milieu in which humans establish their identity and uniqueness, which imply standards higher and more rigorous than those of civil authority. Regulations, laws and organizational policies dictate standards and procedures with which individuals and organizations must either comply or face sanctions. In contrast to regulatory emphasis on compliance and enforcement, the purpose of ethics can be understood as activity or enquiry to shed light on the correctness or justifiability of conduct. In the context of GMM trials, ethics aims to understand the interests of stakeholders and their various entitlements, rights, other types of claims and obligations, including what actions or activities are required to ensure respect for communities hosting the trials. The success and sustainability of new approaches to control the burden of disease can only be realised if the scientific community, national and international regulatory authorities, and joint forums address the ethical and social challenges, and issues associated with these approaches, and look at ways of strengthening community participation.

10.2 Principles of bioethics and ethics of disease prevention

The basic values and principles of bioethics include: respect for persons/community or autonomy; beneficence or '*do good*'; non-maleficence or '*do no harm*'; and justice or fairness. The Universal Declaration of Human Rights of 1948 states that all human beings possess equal rights of health, the chance to exercise their autonomy and justices of accessing equal resources. The ethical matrix for the principles of bioethics and ethics of disease prevention is presented in Table 17.

Table 17. Principles of bioethics and ethics of disease prevention – ethical matrix

Stakeholders	Well-being	Autonomy	Justice
Technology users	Efficacy, safety and remuneration	Freedom to adopt or not adopt	Fair treatment in trade and law
Affected citizens	Safety and quality of life	Democratic decision-making	Individual and regional justice
Technology providers	Commercial viability and working conditions	Freedom to innovate	Equitable trading system
Environment	Protection of the environment	Biodiversity of biotic populations	Sustainability of the Environment

Source: 2

10.2.1 Principle of respect of persons/community

The principle of respect of persons and community dictates that all people should be given the right to fully exercise their autonomy. Showing respect for people is a system by which interaction with one entity ensures that the other has the freedom to make choices. Thus, it incorporates at least two ethical convictions: firstly, that persons/communities should be treated as autonomous agents (with dignity and integrity, ensuring their privacy and the confidentiality of their data/information), and secondly, that persons/communities with diminished autonomy are entitled to protection.

10.2.2 Principles of beneficence/non-maleficence

These are often understood to cover acts of kindness or charity that go beyond strict obligation. The act of doing well or thinking of the welfare of community is a basic concept in research ethics which states that researchers and research work should focus on improving the welfare of people and applying technology only for the beneficial use of communities.

10.2.3 Principle of justice

Justice is a concept of moral rightness based on the concept of justice, equality and social contract. All peoples and individuals have equality of civil rights before the law, without discrimination. It conceives that equals ought to be treated equally. The person/community ought to receive the benefits or burdens equally. In the Indian context, in addition to the basic values and principles, additional principles are adopted during the conduct of biomedical research on human/community participants as listed below.[3]

- Principle of essentiality
- Principle of voluntariness, informed consent and community agreement
- Principle of non-exploitation
- Principle of privacy and confidentiality
- Principle of precaution and risk minimization
- Principle of professional competence
- Principle of accountability and transparency
- Principle of the maximization of the public interest and of distributive justice
- Principle of institutional arrangements
- Principle of public domain
- Principle of totality of responsibility
- Principle of compliance.

10.3 Principles of public health ethics

Public health is "the science and art of preventing disease, prolonging life and promoting health through the organized efforts and informed choices of society, organizations, public and private, communities and individuals." The focus of public health intervention is to improve health and quality of life through the prevention and treatment of disease and other physical and mental health conditions, through surveillance of cases and health indicators, and through the promotion of healthy behaviours. It is what we as a society, group, or population do collectively – it is an application-oriented procedure to assure conditions in which people can be healthy. It deals with the health of the entire population; it is primarily related to epidemiology, but also to social, environmental, economical and political matters. The focus is directed at populations, communities, and the broader social and environmental influences of health.

The principles of public health ethics, in addition to the basic values and principles, include: (i) the harm principle; (ii) the principle of the use of least restrictive or coercive means; (iii) the reciprocity principle; and (iv) the transparency principle.

The **harm principle**, as set out by John Stuart Mill,[4] is the foundation principle for public health ethics in a democratic society. The principle as stated, "the only purpose for which power can be rightfully exercised over any member of a civilized community, against his will, is to prevent harm to others; his own good, either physical or moral, is not a sufficient warrant."

The **principle of the use of least restrictive or coercive means**, enshrined in the Siracusa principles, recognizes that a variety of means exist to achieve public health ends, and that more coercive methods should be employed only when less coercive methods have failed, provided coercion is absolutely necessary, legitimate, and can be justified legally, with no discrimination in their usage.

The **transparency principle**, as discussed by Habermas,[5] also termed as an "ideal speech situation", refers to the manner and context in which decisions are made; all legitimate stakeholders should be involved in the decision-making

process that is made free of political interference and coercion or the domination of specific interests; the decision is clear and accountable as possible.

The **reciprocity principle**, as discussed by Harris and Holm,[6] holds that the society must be prepared to facilitate individuals and communities in their efforts to discharge their duties.

10.4 Ethics of disease prevention

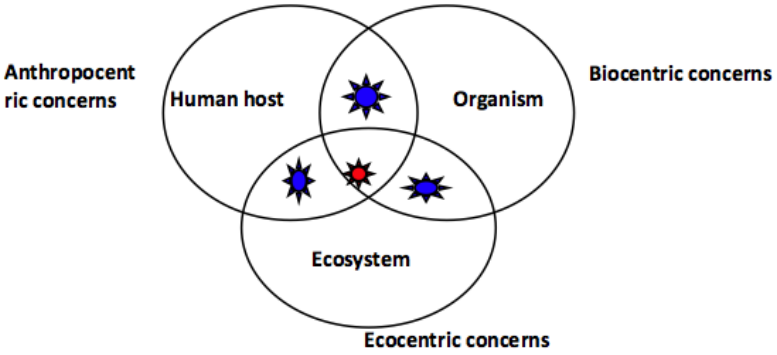
The widely accepted, acknowledged and held ethical principle is that human life is worth saving.[7] The ethical reason behind increasing global and local support for efforts to improve existing and develop new approaches for preventing, diagnosing, treating and controlling diseases is to protect human life and right to health and protection from diseases. The risks associated with implementation of transgenic techniques to human life, damage to the environment and other living organisms should be assessed well in advance of the preparation of the protocol for trial release in order to prepare ethical measures to ensure that there will be no harm to humans and the environment.

As there is great inequality between and within rich and poor nations in the distribution and access to proven benefits, the principle of justice argues that efforts should be made to minimize the variation and everyone should wish for equal opportunity and equal exposure to risk.[7] The principle of beneficence states that the development of science and technology and its provision should be for the welfare of the people and betterment of their health.[8,9] Also, the principle of non-maleficence argues that there should be reasonable caution about the premature use of technology before potential risks are understood. Supporting the principle of '*do no harm*', the Cartagena Protocol on Biosafety, an international, legally binding agreement, advises that no technology with any known risk should be attempted, and that the principals of the benefits and risks are used to assess technology.[10,11] These basic ethical principles will help in decision-making in a range of bioethical dilemmas.

10.5 Ethical concerns associated with GMOs

The application of scientific knowledge has resulted in the development of genetic modification techniques that will better support human life. There are two kinds of ethical arguments against the technological development of GMOs that include extrinsic and intrinsic objections. The intrinsic objections against GMOs include: "playing God" or having a right to make very important decisions that seriously affect other people's lives; crossing natural species boundaries; altering reproduction by nonsexual means; and disrupting the beauty, integrity and balance of Nature. The ethical issues focus on whether, and to what extent, humans may legitimately exercise control over nature. The extrinsic argument emphasizes that GMOs are wrong because the risks outweigh the benefits, while the intrinsic argument states that GMOs are wrong, no matter how great the benefits. The extrinsic objections against GMOs are that they have potential safety risks for humans, organisms, and the environment. This raises ethical issues in terms of anthropocentric, biocentric and ecocentric concerns (Figure 35). Anthropocentric thinking focuses on humans, the biocentric thinking emphasizes the value of individual organisms – whether plant or animal. Ecocentric thinking values the ecosystem as a whole and finds expression in environmental concerns. The reverence for all of life[12] can apply to the whole ecosystem or to every member of it.

Figure 35. Extrinsic concerns – anthropocentric, biocentric and ecocentric concerns



There is a trend for more ecocentric views to be included in recent legislation, with protection of ecosystems. It is not realistic to separate human/nature and social interactions, people and the ecosystem. The basic ethical principles of autonomy, justice, beneficence and non- maleficence can be applied to help decision-making in a range of bioethical dilemmas in medical and environmental ethics. An attempt of prioritize issues was given in Table 18.

Table 18. Ethical priorities in community engagement over genetic methods of vector control

Expected benefits	Negative concerns	Autonomy/justice
1. Prevent human disease. 2. Less health and environmental damage compared to insecticides. 3. Less environmental change compared to civil engineering approaches to vector control. 4. Development of social consensus processes that can be applied to other public policy. 5. Emergence of informed choice and empowerment of individuals leading to greater personal responsibility for health choices. 6. Sites of field trials could be promised to be beneficiaries of more permanent use. 7. Modified mosquitoes would not be killed, so the vector species would remain alive	1. Risk of damage to the environment from ecological changes under ecocentric and/or biocentric views. 2. Possibility of horizontal transfer of the transgene(s) to non-target organisms. 3. Modification of one ecosystem component, altering the *telos* (purpose) of an organism. 4. Indigenous persons place higher value on the unmodified native fauna. 5. Human control of nature. 6. Greater concerns over mobile genetic elements compared to "sterile" vectors. 7. Unforseen consequences on human health. 8. Intellectual property issues.	1. Regulatory systems for oversight need to find proper balance between expected benefits and precaution. 2. Education materials and process, after a two-way development process. 3. Whether consent is required from every individual, including children. 4. Options for those who refuse to be involved, e.g. alternative insecticide protection methods. 5. Inequality in access to the modified mosquitoes. 6. Roles of external persons, e.g. activists, media, NGOs, commercial actors. 7. Payment mechanisms for trials, and insurance for accidents. 8. Sustainability of intervention.

Source: 13

10.6 Animal rights concerns

There are few intrinsic ethical concerns about killing insect pests. Ethical concerns when discussing animals are their capacity to suffer or feel pain. If insects do not feel pain or sense feelings, then the most prevalent ethical approach would argue that there is nothing intrinsically wrong in manipulating them.[14] Followers of the Jain religion in India regularly refrain from killing insects that are human pests; there are still some people who may object to killing mosquitoes. It is not known if manipulating the insects so that they would not be a human pest would be more acceptable than traditional methods of insect control that attempt to eradicate a whole insect population. Teleology is the branch of moral philosophy dealing with the cause and effect of an action, the belief that there is purpose and design in nature, and consequently, with the belief in the existence of a Creator. There are concerns that the ability to alter the *telos* of an animal has profound implications. If one believes that every organism has a purpose, then the telos is an intrinsic concern, and genetic engineering alters the telos or "being-ness" of an organism. However, it is debatable whether changes and control through genetic engineering are significantly different from changes made by humans to animals and plants in farming and modern life. Similar to animal rights concerns, the organism rights concerns emphasizes that genetic engineering alters the *telos* (purpose) of the organism's existence in nature, that humans are manipulating life for human purposes without considering the interests of the organisms as they also suffer pain and other sensations.

10.7 International and national regulatory guidelines and bodies for biosafety

The concept of biosafety refers to the need to protect human health and the environment from the possible adverse effects of the products of modern biotechnology. At the same time, modern biotechnology is recognized as having great potential for the promotion of human well-being, particularly in meeting critical needs for food, agriculture and health care. The general principles of biosafety have always attempted to contain pathogens, with the exception of vaccines. R&D into ways to combat disease and improve health must adhere to internationally accepted legal and ethical principles of risk versus benefit assessment. To ensure the development of appropriate procedures to enhance the safety of biotechnology in reducing all potential threats to biological diversity, taking into account the risks to human health, the process should be prescreened, accepted, and approved for use by an independent IBC. To ensure the protection of dignity, rights, and well-being, and to safeguard the welfare of the human participants, the process should be subjected to a competent review of all the ethical aspects of the project in an objective manner by an independent institutional ethics committee (IEC). The IBC and IEC are useful ways of making decisions that should be transparent and established locally to review research projects. This renders the intrinsic arguments against GMOs unsound, and keeps the valid extrinsic concerns and arguments against GMOs to the bare minimum but with a sound risk-benefit judgement after establishing and putting in place proper risk minimization procedures.

The International Centre for Genetic Engineering and Biotechnology (ICGEB)[15] provides assistance in biosafety training for the development of genetic engineering in many countries. The Cartagena Protocol on Biosafety is an advanced informed agreement procedure on the safe transport, handling and use of LMOs resulting from modern biotechnology that specifically focuses on the transboundary movements of LMOs.

10.8 Consent from the participants involved in trial study

The ethics of human subject research is dominated by the requirement to obtain informed consent from all members of the community who will be participating in a study.[16] The risks may not arise directly from the ability of the vector to carry the target pathogen. Altering the behaviour of blood-feeding insects could have a negative impact on human health. Informed consent requires the provision and dissemination of information on the plans and progress of the project and the consent of any person potentially affected by the release of transgenic insects. Furthermore, project information dissemination, and community awareness and education should be sensitive to local cultural norms. In order to address the issues at different levels, two main committees need to be established: (i) a committee or institutional review board to discuss the ethical issues surrounding the procedures and to review the benefits, risks and scientific merit of the application; and (ii) a local ethics committee (LEC) to consider the issues at a local level.

10.9 Consent from the community and environmental risk

A variety of potential ecological, environmental and health risks are associated with the release of GMOs. Environmental risks can be considered from both anthropocentric- and ecocentric-based approaches. The risks identified include the possibility of the horizontal transfer of the transgene to non-target organisms, and possible disturbance of insect ecology.[17,18] Any risks to the agricultural systems of rural communities also require assessment, as animal diseases transmitted by vectors are important to farming families. In addition, there may also be risks to wild animals in surrounding areas, which those with ecocentric environmental views may consider have more intrinsic rights to be left undisturbed than farm animals.[19] This calls for broad ecological understanding of the impact that goes beyond public health.

10.10 Conclusion

There are a variety of ethical issues concerned with the use of GM insects, most of which can be managed by obtaining the informed consent and participation of communities. Before field release of transgenic insects, researchers must assess all the scientific and social issues associated with GMVs and develop safety precautions to address the potential risks. The scientific and social risks should be minimized through the careful design of the vector system, relevant laboratory experience, and meticulous choice of the site, including consideration of appropriate social and cultural factors.

As society advances its demands for scientific and technological health solutions increase. Prioritizing research needs in an economically challenged situation with adequate regulatory guidelines can result in better holistic development processes. Thus, scientific endeavours to assure a well society will advance if biosafety and bioethics concerns arising out of R&D processes are addressed according to a community's needs and demands.

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Chapter 11. Measuring public attitudes to releases of transgenic mosquitoes for disease control

11.1 Introduction

Since their commercial application a few decades ago, GMOs have been highly controversial with vocal opponents in both developed and developing nations.[1,2] This was particularly apparent in 2002 when Zambia rejected food aid from the USA during a famine on the basis that it was GM.[1] More recently, regarding GMMs, vocal opposition in Malaysia has led to releases of GM *Aedes aegypti* – the mosquito species that transmits dengue fever – being delayed,[3] and the announcement in the Cayman Islands of an open field trial of GMMs was met with controversy.[4]

However, vocal opposition is not always representative. The potential use of GMMs to control dengue fever in Key West, Florida, USA, illustrates this point. In 2011, a town hall meeting on the subject was met with resistance and an online campaign to prevent the intervention.[5] However, two recent surveys of residents in the region found that the majority of respondents actually support the intervention and consider it safer than the use of chemical insecticides.[6,7] In a democratic society, it is essential that we obtain a representative sample of attitudes in order to inform scientific and policy-related decisions. In addition to informing political decisions, surveys of public attitudes lead to information exchange with community members and contribute to how disease control programmes are implemented.[8]

In this chapter, the author describes experience with measuring public attitudes to the use of GMMs in Africa and outlines how descriptive surveys can be used to inform the design of quantitative ones.[9] The chapter is based on a descriptive survey of public attitudes to GMMs for malaria control in Mali, West Africa, with a team of Malian doctors and scientists in 2008 and 2009.[9] Qualitative surveys like this one are useful because they provide a detailed picture of the range of views that a population holds on a certain issue. However, they only provide a crude idea of how common these views are in the population. This information is better provided by quantitative surveys, the design of which is well informed by initial qualitative studies (Table 19). The chapter continues by describing the design of quantitative surveys and the types of questions that a quantitative survey should include.[10] Finally, a summary of surveys of public attitudes to GMMs that have already been conducted,[6,7,9,11] and opportunities for further work preceding a potential transgenic release in Africa are presented.

Table 19. Pros and cons of qualitative vs. quantitative surveys

	Qualitative surveys	Quantitative surveys
Pros	Allow for freedom and spontaneity in responses	Fast and less costly
		Well-suited to group comparisons
	Provide a deep understanding of how respondents think	Allow a large number of respondents to be interviewed
Cons	Time-consuming and costly	Lack the spontaneity and freedom of qualitative surveys
	Only a small number of respondents can be interviewed	

CASE STUDY: A QUALITATIVE SURVEY OF PERSPECTIVES TO GMMS IN MALI

In late 2008 and early 2009, a group of Malian doctors and scientists and Marshall et al.[9] conducted a qualitative survey of public attitudes to GMMs for malaria control. Mali was chosen because it is the site of extensive research on the ecology of malaria vectors of relevance to GMM projects,[12] and because it is home to a range of ethnic groups, including the Bambara, Dogon, Peul, Songhai and Taureg. The team focused on malaria, because it is the most devastating VBD in sub-Saharan Africa; and on mosquito population replacement, because it is generally considered to be a strategy that holds great promise for malaria control.[13] In this strategy, a disease-refractory gene is linked to a gene drive system capable of spreading genes to fixation in one or many populations.[14]

The survey consisted of semi-structured interviews that lasted on average 45 minutes. Semi-structured interviews consist of a set of open questions, for which response options are not provided. They also incorporate a degree of flexibility, allowing new questions to be brought up depending on interviewees' responses. The series of open questions covered perspectives on mosquitoes, nature, heredity, diseases, genetic alteration, and acceptable conditions for a release of GM crops and GMMs for disease control into the environment. Since the majority of the population were subsistence farmers and were familiar with selective breeding, genetic alteration was described as "a faster way to develop more desirable animals, fruits and vegetables, but that this method could lead to unknown consequences for the environment." The full text of the survey is now available.[9]

Sample: For a preliminary descriptive survey, the best sample is a judgement sample, the goal of which is not to be representative, but to obtain as diverse a range of responses as possible. This helps to understand how people think about a topic and to formulate meaningful questions and response options.[15] A sample of 30–40 people is usually sufficient but, as a general rule, sampling should continue until no new ideas are obtained. For the descriptive survey in Mali, a judgement sample was used consisting of 80 people – 30 of various ethnicities in the district of Bamako, 20 predominantly Bambara in ethnicity in the region of Koulikoro, 10 predominantly Dogon in ethnicity in the district of Mopti, and 20 traditional and Western-trained health professionals in Bamako and the region of Mopti. In each group, men and women of a variety of ages and social statures were interviewed, thus satisfying the criteria for a preliminary survey.

Protocol: In each village or suburb, the team visited the local chief and decision-makers and explained the purpose of the survey to them and their desire to obtain a diverse sample. The chief met the elders to discuss the survey, and they

selected the participants together. The chief generally offered to be the first participant, and a young guide escorted the interview team from one participant to the next. Questions were posed by a local Malian (translator) and other members of the interview team posed the follow-up questions. The responses were then translated, transcribed and recorded to check for errors. Participants were offered a confidential setting to respond to the questions, however, most participants appeared comfortable in a common setting with friends and relatives surrounding them. Ethical approval was obtained from the institutional review boards of the Malaria Research and Training Center (Bamako, Mali) and the University of California (Los Angeles, California, USA).

Results: The collective responses of the 80 survey participants provided interesting insights into many issues surrounding the GMM project – from nature and heredity, to conditions for a release of GM corn and mosquitoes. Awareness of the fact that mosquitoes cause malaria was widespread, with 80% of participants citing mosquitoes as at least one main cause of malaria. This is captured by the following statement from a woman in Banambani: "When a mosquito bites, it can then bite another person and carry blood from the first person to the second person. This is how malaria is transmitted." However, a number of other causes were often cited in conjunction with mosquitoes, including mangoes, sugar, oily foods and exposure to the sun or cold weather. Mosquitoes were also singled out as nuisance creatures which can be killed without problem; however one respondent pointed out that insects are a possible object of sorcery referred to as the "korote": "It's a missile, but it's also an insect. They can fire it from one place and it will get you in your home." This highlights the importance of studying cultural symbols and beliefs prior to a transgenic release.

With most of Mali's population being involved to some extent in subsistence farming, understanding of selective breeding and a modest awareness of the controversies surrounding GM crops was useful in explaining the concept of GMMs for malaria control. Selective breeding was stated as being practised on various animals, crops and trees. Most respondents considered heredity to be caused by both blood and God, as captured by the following statement by a man in Koulikoro: "There are two reasons for offspring resembling their parents – first, if you have the same blood then it is expected that you will look alike; and second, this [resemblance] is due to God." Only six of the 80 participants referred to genetics, although several were aware that neighbouring Burkina Faso had recently accepted to release GM cotton into their environment for commercial farming. As a woman in Bamako stated: "The politicians in Burkina Faso made a decision about growing GM cotton without consulting the population."

One of the most illuminating questions regarded perspectives to a hypothetical release of GM corn that promised higher yields due to an engineered insect-resistance trait. The majority of rural respondents requested a trial be performed to confirm the crop's beneficial consequences and lack of negative consequences prior to a large-scale release. Some were very specific about the details of their proposed trial. As one respondent described: "I would choose a different space to culture the new crop, about one to two kilometres away from my farm. I would like this area to have the same area as my farm to provide a good comparison. Afterwards, I would collect the corn from the two farms and would see which produced the better yield." A trial period of one season was suggested and participants pledged to monitor the effects of GM corn on human health during this period.

The concept of a trial was extended to GMMs. Participants were asked to imagine that an organization from a foreign country could provide a GMM capable of reducing malaria in their community, that there were no known negative consequences, but that unknown consequences could not be ruled out. Most participants said that they preferred that the trial be conducted in another village before accepting a release in theirs. However, a few preferred that the first trial be conducted in their village. One man from Mopti stated: "You have to start somewhere… I would like you to

conduct a trial in my village because I would like to be an example for another community." In addition to a successful trial, respondents had a number of additional requirements for a release of GMMs in their community. These included an education campaign, the provision of bednets, and prior approval for the release from a majority of the community. The main concerns were that the project would not work, resulting in a net increase in malaria-transmitting mosquitoes, or that the GMMs would transmit other diseases, such as HIV or a new strain of malaria. These responses are highly informative for technology development, trial design and future quantitative surveys.

11.2 Conducting a quantitative study

Quantitative surveys are, in a sense, complementary to qualitative surveys – they consist predominantly of closed questions, for which a limited set of response options are provided. They are faster and less costly, and therefore allow a large number of respondents to be interviewed. They also provide a good idea of how prevalent certain views are in a population and how these views differ between groups (age, gender, etc.).[16] Their major weakness is that, due to their closed nature, they are not suitable for elucidating the range of views held by a population on a certain issue (Table 20). This is why a successful quantitative survey always precedes a qualitative one. It allows researchers to identify general themes, formulate meaningful questions, and enumerate a near-complete range of responses for the population of interest. The following sections describe how a series of closed questions can be developed beginning with the results of the qualitative survey described earlier. In general, this process will involve the following steps.

1. Identify the population of interest and generate a sample
    a. Identify the full set of individuals whose attitudes you wish to quantify
    b. Draw a sample from this population such that each individual has an equal chance of being selected.

2. Conduct a preliminary qualitative study
    a. Identify themes
    b. Formulate meaningful questions and response options.

3. Design the questionnaire
    a. Write a series of factual, opinion and attitude questions based on the preliminary results.

4. Pilot the questionnaire
    a. Test and improve the questions to ensure that the questions are informative.

5. Conduct the survey

11.2.1 Step 1: Identify the population of interest and generate a sample

The first step in any study is to identify the population of interest. This is the full set of individuals whose attitudes we wish to be quantified, e.g. all Malian citizens 18 years or older living in Mali at the time of the survey. It is not possible to interview our entire population, so we must choose a sample. A completely random sample in which every individual is randomly selected is not feasible in Mali because respondents must be visited individually, and sampling from the entire country would be prohibitively expensive. The best alternative is cluster sampling, which takes advantage of the geographical structure of a population and applies the principle of probability sampling to each stratum sequentially.[17] Attitudes to biotechnology have been measured in this manner in Europe.[18] In Mali, a random sample may be difficult to achieve at the community level because random sampling is inconsistent with the village hierarchy; however, a careful explanation of its purpose may make it acceptable to the chief and elders. The aim should be to achieve as random a sample as possible while being respectful of local cultural etiquette.

In other settings, different sampling methods may be appropriate. In countries where almost everybody owns a telephone or is accessible by mail, random sampling is possible. Attitudes to biotechnology have been measured in this manner by telephone in Japan, New Zealand and the USA[19,20] and by postal questionnaires in Japan[11,21] and Florida.[6] An electoral register or telephone book may be used as a sampling frame. In Florida, USA, a list of addresses was purchased from a survey sampling company.[6] Another option is quota sampling. In this case, a sample can be artificially engineered to have the same demographic qualities as its parent population. In general, the demographic qualities of a sample and parent population can be compared to check for sampling errors.

A number of methods are available for sampling people at the community level, depending on the time and resources available (these methods can also be tested during the piloting phase of the survey). In one method, the coordinates of all households are initially recorded using global positioning system (GPS) units, and a software package is used to determine the optimum route for survey teams to take through the community.[22] Eligible respondents in each household can then be chosen at random, for example by selecting the eligible respondent who most recently celebrated their birthday.[6] A return visit should be arranged if this respondent is not available on the first visit. This method generates a robust random sample; however prior GPS mapping, visiting and re-visiting can be time-consuming. If resources are limited, another standard randomization technique is to begin at the centre of a suburb or village and for each interviewer to choose a direction to work through by spinning a pen on a flat surface. The interviewer then walks in this direction and interviews a person from every *n*th household, where *n* is a random number between two and five chosen at random for each interviewer at the start of the survey. This method is less time-consuming, allowing many more individuals to be sampled in a short time, but is less robust than the GPS method.

Sample size: Finally, the sample size is always a compromise between theoretical requirements (accuracy) and practical considerations (costs). Calculations can be performed to determine the sample size required for an acceptable error. A simple yes/no question with a sample of 3000 people, for example, will produce a standard error of less than 1%. The importance of an accurate sample should be stressed, since a large sample size cannot make up for poor sample design.

11.2.2 Step 2: Conduct a preliminary qualitative study

As already discussed in the section on the Mali case study, a quantitative survey should always be preceded by a preliminary study to understand how people think about a topic. This assists in the design of meaningful questions and response options.[15] Previously, we described how a qualitative survey could serve this role by using a semi-structured interview structure and a judgement sample to obtain a diverse range of responses. Another option is a focus group study. These are group discussions led by a trained moderator who ensures the participation of all group members.[23] For focus studies, group members should be chosen with the goal of obtaining a diverse range of opinions on the topic in question. The advantage of these studies is that they are more efficient at enumerating themes and the range of responses than individual interviews, which can be a lot more time-consuming. However, the two study types are not mutually exclusive, and a combination of the two can be used preceding a quantitative survey design.

In both cases, before conducting the interviews a series of topics should be prepared, but their order may change. The aim is to obtain deep responses on a topic with an emphasis on spontaneity. For the goals of a preliminary study, completeness is not necessary – it is better to obtain rich data on a few topics than superficial data on every topic, since different respondents will provide rich data on different topics.

For the hypothetical survey of Malian citizens 18 years or older living in Mali, the descriptive survey illustrated earlier[9] would serve well as a preliminary study. But it would be good to supplement it by posing the same open questions to communities not covered in the original survey, e.g. communities that are predominantly Peul, Songhai and Taureg in ethnicity and among some of the other six regions of Mali. The same procedure should be followed prior to designing quantitative surveys in other countries. A judgement sample should be chosen and a series of topics prepared depending on national cultural beliefs, the disease of interest and the nature, scale and scope of the applicable GMM strategy.

11.2.3 Step 3: Design the questionnaire

Once peliminary studies have been completed and general themes identified, the first step in the design of a questionnaire is to decide on its aims. The purpose of the questionnaire proposed here is primarily to quantify the proportions of Malians that hold particular views toward GMMs; however a number of secondary hypotheses may also be investigated. For example, what are the social determinants of these attitudes? Are there differences between males and females, parents and non-parents, rural and urban dwellers, or decision-makers and ordinary citizens? Do attitudes correlate with age group, religious affiliation or level of education? Does a better understanding of malaria, heredity or genetic engineering lead to more positive attitudes to GMMs? Do attitudes to GM crops correlate with attitudes to GMMs, and which of these two are viewed more positively? Every question should have a clear reason for being included, and the team should know how it is going to analyse the results.

Questionnaire modules: An example of a quantitative survey designed from the results of the Mali descriptive survey is provided in Marshall et al.[10] This questionnaire is divided into four modules: (i) factual questions on malaria, heredity and GM organisms; (ii) attitude questions on GM crops; (iii) attitude questions on GMMs; and (iv) demographic information. The researchers chose this order based on the internal logic of the inquiry; however piloting can be used to reveal the optimal question order. For a spoken interview, as appropriate in Malian villages, the interviewer has some leeway in reading factual questions in order to offer explanations or correct misunderstandings. Attitude questions, however, must be read precisely due to their strong dependence on question wording.

A preliminary module on GM crops was included because the preliminary study found that this provided a gradual introduction to questions on GMMs. However, pilot studies could be used to determine whether this is necessary. Demographic questions can be placed either at the beginning or end of a questionnaire. If placed at the beginning, they serve as easy warm-up questions. However some surveyors prefer to place them towards the end of a survey so that respondents know what they are linking their information to.[16] For these questions, it is acceptable to probe respondents to ensure the correct information has been obtained. For more details on the questionnaire modules, please refer to Marshall et al.[10]

Factual questions: Questionnaires often begin with factual questions because they offer a straightforward way to warm up into the questioning process. They are followed by a range of response options, of which respondents may choose one or many, as determined during the question design (and informed by the preliminary study). There is some leeway in reading factual questions – for instance, explanations can be offered to correct any misunderstandings – because they are not sensitive to question wording like opinion or attitude-based questions are.

The first module of the quantitative Mali survey consisted of a number of factual questions covering the topics of disease, heredity and GMOs. This is the first module of the questionnaire, and hence acts as a warm up for the interviewees. Three of these questions are shown in Table 20 (Questions 2, 3A and 3B). The questions and response options are closely based on the results of the preliminary study.[9] In the study, most respondents cited God and sharing the same blood as the main reasons why offspring resemble their parents, while a few educated people made reference to genes, as listed in Question 2. Most respondents were familiar with selective breeding, citing its use in raising more desirable pigs, goats, cereal crops and fruit trees, as listed in Question 3B. Note the inclusion of options for "other (please specify)" and/or "don't know" in each of these questions. This is important to ensure that all response options are covered; but should only be used when necessary. Also note the skip pattern for Question 3A which means that Question 3B ("Which animals, vegetables or fruits are selectively bred in your community?") is only asked if selective breeding is practised in the community.

Table 20. Examples of factual questions

No.	Questions and filters	Coding categories		Skip
2	In nature, it is common for offspring to resemble their parents – for example, a daughter may resemble her mother in some ways, and her father in other ways. What do you consider to be the reason for this resemblance? You may choose more than one option.	BLOOD GOD AFFECTION GENES NONE OF THE ABOVE DON'T KNOW OTHER _____ (SPECIFY)	1 2 3 4 5 88 6	
3A	Does your community take advantage of the resemblance between parents and offspring to selectively raise animals, vegetables or fruits with desired characteristics?	YES NO DON'T KNOW	1 2 88	 --> 4A --> 4A

Table 20. Examples of factual questions (continued)

No.	Questions and filters	Coding categories		Skip
3B	Which animals, vegetables or fruits are selectively bred in your community? You may choose more than one option.	GOATS CHICKEN MANGO TREES COWS PIGS ORANGE TREES CEREAL CROPS HORSES DON'T KNOW OTHER _____ (SPECIFY)	1 2 3 4 5 6 7 8 88 9	

Scale questions: These are sometimes referred to as opinion questions and consist of a series of questions with graded response options. The scale can have several levels of intensity, or simply consist of yes/no/don't know options, in which case they are sometimes collectively referred to as list questions. Responses can be added together, weighted by intensity, and used to obtain a crude "score" for each respondent. Factor analysis can then be used to see whether scores correlate with certain covariates (gender, age, number of children, etc.).

Modules two and three of the quantitative Mali survey contain several scale questions covering the requirements, concerns and trusted organizations for a release of GM crops and GMMs. Two of these questions are shown in Table 21 (Questions 9 and 11). As before, the questions and response options are closely based on the results of the preliminary study.[9] In this study, participants were told to imagine that an organization from a foreign country could provide them a GMM that would be able to reduce the burden of malaria in their community, but could have unknown consequences. A large number of respondents wanted to see the results of a trial before accepting a release in their community, most of whom wanted the trial to be conducted in a community other than their own. A number of other requirements were mentioned, such as evidence from laboratory experiments and the provision of bednets with the release.

In Question 9, respondents are asked to rate these requirements on a scale of one (not important) to three (very important). Combining the responses of several people allows us to scale the relative importance of each requirement to see whether different groups of people tend to have different sets of requirements. In Question 11, respondents are asked whether they would accept a release of GMMs in their community if some of these requirements were satisfied. Affirmative responses to the first six options may be added to obtain a crude "acceptability score" for each respondent. Factor analysis may also be used to see whether certain requirements tend to be grouped together.[24] Note the inclusion of "always" and "never" options in Question 11 for completeness.

Table 21. Examples of scale questions

No.	Questions and filters	Coding categories				Skip
9	Here are some concerns that people in Mali have about GMMs. Please read through the list and, for each concern, indicate how much it worries you.	VERY WORRIED	A LITTLE WORRIED	NOT WORRIED	DON'TKNOW	
	(a) GMMs will continue to transmit malaria	1	2	3	8	
	(b) GMMs will be resistant to insecticides	1	2	3	8	
	(c) GMMs will transmit diseases other than malaria	1	2	3	8	
	(d) Accidentally eating GMMs will make me sick	1	2	3	8	
	(e) GMMs will harm the environment	1	2	3	8	
	(f) GMMs will be expensive for the community	1	2	3	8	
	(g) Other _____ (SPECIFY)	1	2	3	8	
11	Under what circumstances would you consider it acceptable to release GMMs in your community? "I would consider it acceptable to release GM mosquitoes in my community…	YES	NO	DON'T KNOW		
	(a) … if the United Nations said they were safe and help reduce the number of malaria cases."	1	2	8		
	(b) … if the Malian government said they were safe and help reduce the number of malaria cases."	1	2	8		
	(c) … if they were approved by a majority of my community."	1	2	8		
	(d) … if I saw the results of a successful trial in a nearby community."	1	2	8		
	(e) … if I saw the results of a successful laboratory experiment."	1	2	8		
	(f) … if it was possible to abort the project."	1	2	8		
	(g) …always. I approve of releasing GM mosquitoes unconditionally."	1	2	8		
	(h) …never. I don't approve of releasing GM mosquitoes under any circumstances."	1	2	8		

CHAPTER 11
Measuring public attitudes to releases of transgenic mosquitoes for disease control

Attitude statements: Attitude statements are single sentences that express a belief or point of view and are phrased so that respondents can either agree or disagree with varying intensity.[16] For example, "It is the duty of doctors to keep people alive for as long as possible: (a) Strongly agree; (b) Agree; (c) Neutral/undecided; (d) Disagree; (e) Strongly disagree." By using a pool of such statements, it is hoped that people can be placed on an attitudinal scale, in relative terms. Half of this pool should be positive and half should be negative, in order to compensate for the tendency of people to give affirmative responses. Before use, attitude statements should be placed in random order. They must also be read precisely, because responses may vary according to their wording.

A series of six attitude statements about GMMs are listed in Table 22. To generate these, six statements were chosen from the preliminary interview transcripts – three favourable and three unfavourable – about GMMs, which were then randomized and assigned a Likert response scale.[25] Here, favourable statements are scored from five (strongly agree) to one (strongly disagree) and unfavourable statements are scored from one (strongly agree) to five (strongly disagree). Total scores can serve as a crude measure of favourability towards GMMs. This can then be compared between groups of respondents and analysed to see how it relates to other variables.

Table 22. Examples of attitude questions

No.	Questions and filters	Coding categories					Skip
12	For each of the following statements regarding GMMs, please indicate to what extent you agree or disagree.	STRONGLY AGREE	AGREE	DISAGREE	STRONGLY DISAGREE	DON'T KNOW	
	(a) A mosquito is a mosquito – modified or unmodified, it will always transmit malaria.	1	2	3	4	8	
	(b) If the United Nations tells me that GMMs will be good for my community, I will believe them.	1	2	3	4	8	
	(c) Malaria is far worse than any negative consequences that the GMMs could have.	1	2	3	4	8	
	(d) If GMMs could have unknown risks, then they shouldn't be released.	1	2	3	4	8	
	(e) Bednets and insecticides have barely reduced the number of malaria cases in Africa. GMMs will not be any different.	1	2	3	4	8	
	(f) We have tried to kill mosquitoes and it hasn't worked. It is better to modify them so they can't transmit diseases.	1	2	3	4	8	

Demographic questions: These are factual questions selected with secondary hypotheses in mind – for example, do attitudes to GMMs correlate with age group, gender or parenthood? Hypothetically, mothers may support a release to protect their children against malaria, or oppose a release to protect their children from the risks of an unknown technology. These are included at the end of the Mali quantitative survey[10] so that respondents know what they are linking their information to;[16] however they could equally be placed at the beginning of the questionnaire as warm-up questions. A few examples of demographic questions from the Mali quantitative survey are provided in Table 23.

Table 23. Examples of demographic questions

No.	Questions and filters	Coding categories		Skip
D1	How old are you?	18–29 30–39 40–49 50–59 60+ DON'T KNOW	1 2 3 4 5 88	
D2	Are you male or female?	MALE FEMALE	1 2	
D3	Do you have any children?	YES NO	1 2	

Finally, it is worth noting that questionnaire design is highly dependent on the results of preliminary qualitative interviews. For surveys in other DECs, it would be necessary to conduct these studies before design begins; however, the internal logic of the inquiry should be relatively general. A general progression from factual questions on disease, heredity and GMOs, to opinion and attitude questions on GMMs, to demographic questions should remain appropriate.

11.2.4 Step 4: Pilot the questionnaire

Questionnaires do not emerge out of the design phase in their final form – they must be tested, improved and tested again, possibly several times over. This process is referred to as "piloting".[16] Every aspect of the questionnaire should be piloted, from the wording of questions to the relative positions of answer categories in a list, to the interview setting, to the amount of space allocated for a "please specify" option. Expert advice can help to point out aspects of a questionnaire that might be problematic, but this is no substitute for actual pilot work. Respondents in a pilot study should be drawn from the population of interest and should be as diverse as possible, essentially forming a judgment sample.

In the Mali quantitative survey, several aspects should be piloted. The questionnaire is quite long, which suggests that respondent fatigue might be a problem. If it is, then which questions should be left out? Are there particular questions that respondents have trouble with? Is the module on GM crops informative and useful, or would the questionnaire be more efficient without it? Do respondents make regular use of "please specify" options, and if so, should more response options be included? Are response options to some questions redundant? Attention should also be paid to contextual effects relating to the order of the questions, for example, do earlier questions on GMMs affect levels of agreement with

subsequent attitude statements? Other questions relate to the method of questionnaire delivery. Does the gender, ethnicity or nationality of the interviewer affect the responses? Do female respondents feel more comfortable with female interviewers? And should the interviewer read out the questions and response options every time, or should literate respondents be allowed to complete the questionnaire in writing? This consideration is important because spoken interviews are more susceptible to social desirability bias, which could disproportionately influence the responses of illiterate respondents.

Some questions require particular attention. For example, a definition of "genetic engineering" is provided early on in the survey. How do responses to questions on GMMs and GM crops change when the definition of genetic engineering is also changed? The wording of the introductory sections to questions on GM crops and GMMs should also be experimented with. In Question 9, is a three-level rating scale optimal, or should more or less levels be provided? Are show cards helpful for these questions? Finally, the attitude statements in Question 12 (Table 22) must go through several stages of testing and improvement. Unfavourable statements can be spotted if respondents quibble, if there are many "don't know" responses, or if the statements are skipped or crossed out. An item analysis can be used to determine which statements are most informative. This is done by calculating the correlation coefficient of each statement with the total statement pool, and keeping the statements with the highest correlation coefficients. It should be checked whether a training session on Likert scales leads to more informative responses. One possibility is to have respondents fill out a brief survey on how much they like the taste of different foods in order to familiarize them with the use of linear scales.

11.3 Discussion

Public consultation is essential prior to field trials of GMMs; however very little data are available on DEC views of GMMs. This chapter has outlined how to conduct a descriptive survey of attitudes to GMMs in Mali, and described the main steps required to conduct a quantitative survey of this technology in a DEC. These steps include: (i) sampling from the population of interest; (ii) conducting a preliminary qualitative study; (iii) designing the questionnaire; and (iv) piloting the questionnaire to enhance its efficacy. The focus has been on the population replacement strategy for malaria as an example;[14] however, the methodology can easily be adapted to other locations, diseases and transgenic strategies. In Brazil and Malaysia, for instance, a release of genetically sterile males is being considered to suppress the local *Ae. aegypti* population – the main vector of dengue fever and chikungunya.[26,27] For surveys in these locations, the questions on disease causation and GMMs would need to be altered accordingly.

For researchers interested in conducting these surveys, a number of caveats should be kept in mind. First, initial views on GMMs may be obtained before field trials have been conducted. Hypothetical questions are known to have poor predictive reliability,[16] which should be kept in mind when interpreting the results of early surveys. Second, describing GMMs requires several essential words, such as "gene" and "genetic engineering," which are difficult to explain in local dialects. It is therefore important to understand what respondents understand by these terms and the context in which they give their responses. Third, in many DECs, including Mali, there are a number of local dialects into which the questionnaire must be translated. Translation leads to subtle changes in meanings and overtones, which should be acknowledged because attitude statements can be very sensitive to these changes.

Sources of bias should be identified and minimized, particularly for opinion and attitude-based questions. Some of the main types of bias in quantitative surveys are non-response bias, interviewer bias, social desirability bias and question-

naire bias. The number of non-respondents is less of a problem than the possibility that non-respondents hold distinct attitudes. If a chief who disapproves of the survey is less likely to approve of GMMs, and this view is reflected in his community, this could lead to bias. Building a rapport with the community and providing incentives can minimize this bias. Interviewer bias can be caused, for example, when interviewers become careless at some point during their repetitive task. This can be managed by providing encouragement, reducing shift lengths, and following up with quality control. Social desirability bias occurs when questions are loaded with prestige. For example, people like to appear knowledgeable so they may claim to have heard of more GMOs than they really have. One solution is to ask questions in an indirect way so that respondents do not know the purpose behind the question. Another solution, used by Cobb[7] in Key West, Florida, USA, is computer-assisted self-interviewing. Here, respondents are given an iPad or similar device to administer the survey themselves, allowing them to provide responses confidentially. Interviewer assistance is available if required. Finally, questionnaire bias can be minimized through good questionnaire design.

Previous surveys of public attitudes to GMMs

Several surveys have been conducted on public attitudes to GMOs in Western nations;[18–21] however, the first one to include a question on people's views of GMMs was a survey in Japan by Masakazu and Macer,[11] and the first survey in a DEC was the case study described here by Marshall et al.[9] Since then, the technology has progressed very quickly, with releases of sterile GMMs having already taken place in the Cayman Islands, Brazil and Malaysia.[4,26,27] Public attitudes were reportedly studied prior to the releases in Brazil and Malaysia; however these studies have not yet been published.

Other studies are scarce and have been limited to researchers specializing in the technology or to citizens in developed countries. A paper by Boete[28] documents the views of people working on malaria and mosquito control, particularly their attitude to public involvement in the research process. This study suggests that more than 90% of researchers working on GMMs welcome interactions with the public. However, only 52% of these researchers were comfortable with their work being submitted for evaluation and prior agreement by the public. Researchers working on other aspects of malaria and vector control were more comfortable with such an evaluation. Boete argues that, since GMMs are one of the more controversial novel vector control strategies, structures should be set in place that encourage better public involvement in debates about the technology.[28]

In the USA, a nationwide poll on attitudes to GMMs conducted in 2012[29] found mixed support for the technology. Most of the respondents were unaware of the technology, and support varied depending on whether the mosquitoes were labelled as "genetically engineered" or "sterile." Furthermore, support was lower if risks were included in the information provided to respondents. This survey was followed by two more detailed surveys in Key West, Florida, USA,[6,7] which could possibly be the first location in the country to conduct open field trials of GMMs.

Interestingly, both awareness and support for the technology was higher in Florida. In a large-scale mail survey, 61% of respondents supported the use of GMMs to control dengue fever in their community (compared to 18% who opposed it), and GMMs were generally considered a safer technology than chemical insecticides.[6] One source of bias in these results was the increased response rate of older, better-educated and wealthier community members (as compared to census data). Cobb suggested including a cell phone sub-sample and an inducement (e.g. a US$500 prize pool) to increase the response rate among other demographics. These results were mirrored in a complementary in-person survey over the same period.[7] However, Cobb suggests that few respondents had thought very deeply about the issue as evidenced by the lack of benefits and risks provided when respondents were prompted to provide these. Consequently, opinions regarding GMMs among this population could be highly malleable.

The work on the assessment of public attitudes to GMMs has to be encouraged in all DECs where these strategies are being considered. The disease of interest, strategy of control and local cultural beliefs may all differ; but the underlying methodology will remain the same. In all cases, new preliminary studies will be required and piloting will be necessary, but it is hoped that the case study discussed here will provide a useful template. These studies will provide useful information on the level of public support for novel genetic strategies in combatting dengue fever, chikungunya and malaria.

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Chapter 12. Disease control using GMVs: where do we stand in Africa after nearly half a century?

12.1 Introduction

Mosquito-borne diseases such as chikungunya, dengue, LF, malaria and WNV continue to pose major health problems throughout the world. Africa has the highest burden of malaria and LF.[1,2] The goal of the WHO's Global Malaria Programme is to reduce the burden of malaria so that it is no longer of public health concern, with the long-term aim of eliminating it in DECs.[3] Initiatives such as Roll Back Malaria, which aimed to halve malaria deaths by 2010, have barely succeeded in reducing them.[4,5] This is particularly so in Africa where the burden of malaria and lymphatic diseases is high and both diseases are transmitted by *Anopheles* or *Culex* vectors, with *Anopheles* mosquitoes being vectors of both diseases in West Africa.[6,7] Mosquito vector control has relied mainly on the use of indoor residual spray and long-lasting insecticidal nets (LLINs).[8–10] Other control methods include larviciding and environmental control. Generally these control methods rely heavily on the use of insecticides and have been faced with several challenges, such as the development and spread of insecticide resistance,[11] as well as limitations in the development of new insecticide molecules with innovative modes of action. In order to achieve elimination, there is an urgent need for alternative approaches,[12] one of which involves the use of GMMs for the control of malaria and dengue.[13–15]

The basic concept of genetic control of VBDs was proposed nearly 50 years ago.[16] Based on laboratory successes in the development of virus- and protozoan-resistant mosquito strains, WHO/TDR identified three research aims that would have to be met before a genetic control strategy could be field tested.[17] These aims are: (i) the development of genetic engineering tools that could be used with malaria vectors; (ii) the identification of effector genes that could block parasite transmission; and (iii) the development of effective methods for driving these effector genes to fixation in natural vector populations. Based on these recommendations several methods of germ-line transformation have been developed and used in at least three species of malaria mosquito vectors.[18–21] Genetic constructs that significantly reduce vector competence in experimental malaria models have also been developed,[22,23] as well as a set of molecular markers that can be used in studies of gene flow and population structure in Anopheline malaria vectors.[24–27] However, population replacement strategies for controlling the transmission of mosquito-borne diseases call for the introgression of anti-pathogen effector genes into vector populations, thereby interrupting transmission.[28–30] It is anticipated that these genes, if present at high enough frequencies, will impede transmission of the target pathogens and result in reduced human morbidity and mortality. Despite the achievements in developing GMMs, the risks and benefits associated with their release will have to be explored.

A variety of initiatives have been underway to assess the biosafety, risk assessment and management as well as the ethical, social and cultural issues related to the release of GMMs for disease control.[31,32] One of these initiatives was the "African training course on biosafety for human health and the environment in relation to potential release of genetically modified

disease vectors", supported by WHO/TDR. During this course, scientists from different African countries were trained and sensitized to future initiatives on GMVs in African countries. Similar training activities have taken place in Latin America and the Asia Pacific region. However, the position of Africa remains particularly challenging.

12.2 Perspectives on GMOs in Africa

Currently, very little information is available on the public's attitudes to GMOs in Africa. The use of GMOs such as GM crops has been received with strong skepticism in both developed and developing nations.[33,34] It may even be more complicated in the case of mosquitoes which are vectors of human diseases. Therefore, public consultation prior to undertaking studies on GMOs is essential. Public consultation results in information exchange with community members and contributes to the development of disease control strategies.[35,36] In the case of GMMs, it may be useful to learn from the controversies that have arisen as a result of the release of other GMMs to increase its public health impact.[36]

Several surveys have been conducted on public attitudes to GMOs in Western nations,[37–40] and at least one of them has asked people their views on GMMs.[41] However, very little data are available from DECs in Africa. To date, only one study in sub-Saharan Africa has been undertaken to assess public sentiment towards GMMs. It revealed that the majority of people were pragmatic towards a release of GMMs, as long as social and cultural issues associated with malaria mosquitoes and genetic engineering could be successfully addressed.[42] Data from a study in Ghana also showed that while many individuals were open to GMMs despite the perceived risks, the decision to accept them was not influenced by education, age, sex or religion.[43] Preliminary results from a recent survey in Nigeria[44] suggest that the majority of Nigerian scientists encourage the use of genetic modification techniques to make mosquitoes incapable of transmitting diseases, but would only support its deployment in Nigeria if their safety concerns were addressed and more scientific evidence provided. Thus, from these three studies, it is apparent that public education and stakeholder consultations are essential in obtaining the public's consent before embarking on any malaria control programme using GMMs. However, more studies are needed to engage communities as they could suggest paths that would make these technologies transparent and more acceptable.[12,36]

12.3 Adequate laboratory facilities and expertise for GMM studies

One thing that is clear is that for GMMs to be implemented in Africa, African communities and scientists want to have ownership of the project (by being part of the development and implementation process) and may not accept the transfer of technologies from the Western world if they have not been involved in their development.[44] However, the most pertinent question is whether we have the technical expertise to undertake such efforts. Another important question that needs to be asked is whether there are adequate research facilities in Africa to handle the R&D needs for GMM studies. Perhaps, this would be the case for a few African countries with research centres of excellence. However, to make the use of GMMs feasible and sustainable in Africa, it is important to build the capacities of scientists and laboratories in the African countries. Capacity building should empower scientists and laboratories with: (i) the ability to rear mosquitoes in numbers large enough for mass release; (ii) an efficient method of modifying large numbers of insects with minimal effects on fitness; (iii) the ability to efficiently sort successfully modified mosquitoes; (iv) an effective method to distribute the modified insects; (v) a quick and efficient method to identify released individuals;[45,46] and (vi) investment in local research towards enhancing the understanding of the mosquito species' genetic structure on which the success of the programme largely depends.[24]

12.4 Cost of development and deployment

Another important question is who will bear the cost of the development and deployment of GMMs in Africa. African countries are faced with many developmental issues. The health systems of the majority of the countries have to fight a myriad of health issues of which mosquito-borne diseases such as malaria and LF are just a few. For the use of GMMs to be sustainable in Africa, financial commitments have to be clear from the onset so that no country is left to manage what it cannot handle on its own.

12.5 Biosafety issues

The issue of biosafety has to be handled effectively and scientific evidence presenting both the benefits as well as the risks in different ecological settings has to be weighed carefully so that the safest option can be chosen. The release of GMMs should only be carried out when it is the most viable option,[15] and in combination of other measures using IVM approaches.

12.6 Regulations for the release of GMMs

In 2001, the African Union drafted a model legal instrument for developing national biosafety legislations. This was endorsed by the African Ministerial Conference on Science and Technology in 2007, and by the African Ministerial Conference on Environment in 2008.[47] Currently several African countries have established or are in the process of establishing national biosafety laws. Regulatory bodies would be needed in Africa before the deployment of GMMs. National and international regulations would also be needed since mosquitoes can spread across several countries.[48] Regional and/or international agreements may also be necessary.[12]

12.7 The African malaria vector – *Anopheles gambiae* Giles

Of all the challenges to malaria control using GMMs in Africa, perhaps the most important is the African malaria vector – the *An. gambiae* Giles. The *An. gambiae* complex is the most important malaria vector complex in the world. Initially, the complex was thought to be just one species. However, studies by Ribbands[49] in West Africa, and Thomson[50] in East Africa provided the initial evidence for the specific distinctive nature of saltwater species of *An. gambiae s.l.* Currently, eight formally named species, not morphologically distinguishable, have been identified.[51] These are: *Anopheles gambiae* s.s Giles (formerly the *An. gambiae* molecular S form), *An. coluzzii* Coetzee & Wilkerson (formerly the *An. gambiae* molecular M form), *An. arabiensis* Patton, *An. quadriannulatus* Theobald, *An. bwambae* White, *An. melas* Theobald, *An. merus* Donitz, and *An. amharicus* Hunt, Wilkerson & Coetzee (formally *An. quadriannulatus* B).[52]

The behaviour of members of *An. gambiae* complex is highly diverse, reportedly feeding indoors (endophagic) or outdoors (exophagic); resting indoors (endophilic) or outdoors (exophilic); with preference for humans (anthropophilic) and/or animals (zoophilic). The larvae are found in a wide variety of habitats including rain pools, hoof prints, rice paddies, mineral springs and saline water. In many areas, the members of the complex are known to be sympatric. In some places, it was reported to not even transmit malaria.[53] On the other hand, some species have been reported as being better

vectors than others.[54,55] Thus, the coexistence and ecological differences among the sibling species of the *An. gambiae* complex pose serious challenges to malaria control in Africa using GMMs. Thus, while germ-line transformations have been developed and used in at least three species of malaria mosquito vectors,[18–21] the *An. gambiae* remains problematic due to its complexity. Consequently, in different settings in sub-Saharan Africa, different and multiple species must be targeted simultaneously for successful control.

In addition to the above, the high levels of malaria transmission encountered in much of sub-Saharan Africa require epidemiological assessments that must confront the multiplicity of the vectors. Due to the sympatric nature of the species with the *An. gambiae*, GMM trials necessitate the selection of sites matched for human demographics and disease patterns, while providing sufficient confinement to satisfy the requirements of risk assessment and trial design. Furthermore, the ecological variations between species demand an intensive study of the mosquito biology and ecology. While population genetic studies on mosquitoes are common,[56] studies on vector ecology in relation to seasonality and distribution of the member species of the *An. gambiae* are needed, especially in light of vector migration as a result of climate change and human migration. Finally, an in-depth understanding of the genetic structure of mosquito populations in most parts of Africa may serve a central role in driving effector genes into natural populations, and have a greater impact on the success of the programme. Some microsatellite loci have provided information on the limitation of gene flow in natural populations of *An. gambiae* mosquitoes in Nigeria[24,57,58] and on the effect of urbanization on the inversion frequencies of *Anopheles* aimed at capturing certain genes in the population, which may play a key role in the success of GMMs.[24]

12.8 Alternatives to GMMs

Despite the challenges associated with GMMs implementation in Africa, there are other methods of vector control that do not require genetic modification (change in genetic make-up or introduction of new genes). One such method is the SIT, which is a species-specific and environmentally sound method of insect vector control that relies on the release of large numbers of sterile insects into a target population. Highly successful, area-wide SIT programmes have eliminated or suppressed a range of major veterinary and agricultural pests around the world.[46,59] Mosquitoes are an ideal choice for SIT and a number of trials to control mosquitoes date back to the 1950s.[60,61] The experience and knowledge gained in past years coupled with advances in transgenic technology has resulted in considerable interest in SIT for malaria vector control. Sterility can be induced through chemosterilants, irradiation or modern biotechnological approaches.[62]

In the last couple of years, there have been two trials in Africa on the use of SIT in malaria vector control. The first, in northern Sudan, was to ascertain the feasibilityof using SIT to control the African malaria vector *An. arabiensis*.[63] The project was initiated in 2004 to develop: (i) innovative ways of mass-rearing large numbers of mosquitoes; (ii) methods to eliminate females so that only sterile males would be released; and (iii) appropriate ways to sterilize male mosquitoes. The project also had a field component to collect and evaluate baseline data in potential field sites where feasibility studies could be carried out in the future.[46] The second study in South Africa compared mating success, fertility and fecundity between a long-established laboratory reared colony and a perennial and geographically isolated population of *An. arabiensis* at Malahlapanga, Kruger National Park, which presented a unique opportunity for assessing the feasibility of SIT as a malaria vector control option.[61] Thus, the SIT may present a more ethically and socially acceptable alternative to GMMs, although it also faces challenges of cost, and laboratory and human capacity, in order to be implemented on a large scale.

12.9 Conclusion

In conclusion, this chapter has focused on some of the challenges and alternative solutions to GMMs in Africa. It is hoped that it will play a part in stimulating the interest of African stakeholders in the prospects and challenges of deploying GMMs in Africa.

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Chapter 13. Arthropod-specific risk assessment and laboratory biosafety

13.1 Introduction

The emerging and re-emerging of infectious diseases, and health emergencies including biorisks and bioterrorism attacks are responsible for keeping the world in a constant state of public health uncertainty. Microbiological laboratories are a unique environment in which a wide range of scientific tests to support diagnosis and disease management are carried out, but they pose a high risk of transmitting infectious diseases to people working in or near them. As part of their research activities, laboratory workers are routinely involved in collecting, identifying, rearing, examining and preserving a diverse range of living arthropods which may be harmful to them and the surrounding communities.

All people who are directly or indirectly connected to a laboratory are at risk of infection. There is danger of infection from infective clinical specimens, laboratory cultures and animal experiments. Also, non-infective exposures such as accidental cuts and other injuries, electric shocks, fire and explosions of gases and solvents, burns from corrosive chemicals, and acute and chronic poisoning from exposure to toxic substances can be harmful if not treated in time.

All people who are directly or indirectly connected to the laboratory are at risk of infection. In order to provide appropriate containment and security of microbiological agents, it is crucial that managers of public and private research facilities, public health clinical and diagnostic laboratories, and animal care facilities regularly evaluate and ensure: (i) the effectiveness of their biosafety programmes; (ii) the proficiency of their workers; (iii) the capability of equipment; and (iv) effective facility management practices. Besides these, individual workers who handle pathogenic microorganisms must understand the containment conditions under which infectious agents can be safely manipulated and secured.

To safeguard and protect laboratory workers, the environment and the public from exposure to infected pathogens, it is mandatory to have clear policies to ensure that risks related to laboratory-associated infection (LAI) will be addressed appropriately, and will ensure the highest quality of life of laboratory workers and the public. In 1984, the USA's Centers for Disease Control and Prevention (CDC) and the National Institute of Health (NIH) published *Biosafety in Microbiological and Biomedical Laboratories* (BMBL).[1] A revised 5th edition was published in 2009. The principles of containment level and risk assessment in the BMBL define four levels of biosafety: Biosafety Level 1 (BSL-1), BSL-2, BSL-3 and BSL-4. They are mainly intended to provide a degree of protection and recommended best practices at different levels of hazard to safeguard laboratory workers and the public. The guidelines for proper specimen collection ensure timely transport, identification and susceptibility testing of microbes and rapid reporting of test results, and facilitate close consultation between the clinician and medical microbiologist. The safer methods for managing and handling infectious materials in the clinical environment, including microbiological laboratories, provide information needed to make final medical decision. The application of information in the BMBL,[1] and the use of appropriate techniques and equipment will help the microbiological and biomedical community prevent personal, laboratory and environmental exposure to potentially infectious agents or biohazards.

13.2 Incidence of LAIs and biorisk globally

13.2.1 LAI due to mycobacteria

LAI refers to all direct or indirect human infections acquired as a result of close contact with pathogenic organisms (GM or not) with clinical/subclinical onset of symptoms in a medical laboratory.[2] A survey of LAIs was started and published in 1898 reporting an infection with *Corynebacterium diphtheria* via mouth pipetting. The largest survey was conducted in 1976 which reported 3921 cases due to 159 different agents out of which 10 were biologic agents (Table 24) belonging to biological risk class 3 (BSL3) for humans/animals, which accounted for more than 50% of the cases.[3]

Laboratory workers are exposed to a variety of pathogenic microorganisms that may put them at risk of infection. Pike and Sulkin (1978)[4] reported 4079 LAIs resulting in 168 deaths between 1930 and 1978. They found 10 shortlisted agents causing observable infections among the laboratory workers.[5] Singh[6] points out that laboratory personnel have a 3–9 times greater chance of acquiring infection than the general population,[7,8] and notes that ascertaining the source of infection is difficult due to potential exposure outside the workplace in addition to the long incubation period before the onset of a disease. A study conducted in the United Kingdom during 1994–1995 reported that tuberculosis and gastrointestinal laboratory infections predominated.[9,10] According to Baron and Miller, the bacteria *Shigella*, followed by *Brucella, Salmonella* and *Staphylococcus aureus* were identified as the main causes of LAIs.[10]

Table 24. Most frequently reported LAIs worldwide

Biologic agent	Biological risk class
Brucella spp.	3
Coxiella burnetii	3
Salmonella typhi	3*
Hepatitis B, C and D viruses	3*
Francisella tularensis	3
Mycobacterium tuberculosis complex	3
Trichophyton mentagrophytes	2
Venezuelan equine encephalitis virus	3
Rickettsia bacteria	3
Chlamydia psittaci (avian)	3

*: class of risk 3 infectious agents that are not airborne pathogens.

Source: 3

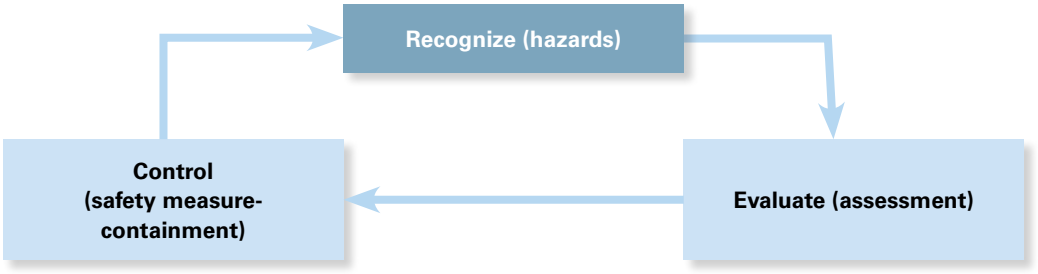
13.2.2 LAIs due to arthropod-borne agents

The LAIs as a result of arthropod-borne agents can happen in the laboratory environment but are rarely reported in the scientific literature. Arthropod-borne bacterial (mostly tularemia and relapsing fever) and viral diseases are considered as primary LAIs, which are mainly transmitted through aerosol. The American Committee of Arthropod-Borne Viruses (ACAV) and Subcommittee on Arboviral Laboratory Safety (SALS) emphasise that arboviral LAIs need more monitoring than other arthropod-borne diseases and reviews laboratory surveys assessing the hazards of working with these viruses. The International Catalogue of Arboviruses has registered 515 viruses, out of which, approximately 25% and 16% of viruses are known to produce naturally occurring human infections and LAIs, respectively.[11] Most commonly reported arboviruses causing LAIs are Kyasanur Forest, Venezuelan equine encephalitis, Rift Valley Fever, chikungunya and yellow fever. There have been case reports of transmission of arbovirus infection through non-vector, health-care associated transmission of DENV including percutaneous transmission via needle-stick injuries, mucocutaneous transmission through a blood splash to the face, vertical transmission, and transmission via bone marrow transplant.[12] Many reports of personnel acquiring incidental infections during manipulation of arboviruses within the laboratory are documented globally.[10,12,13] Human infections with WNV have been reported to occur from needle stick, breastfeeding, blood transfusion, organ transplants, haemodialysis and intrauterine or transplacental routes.[14–16] The transmission of arbovirus infection through non-vectors is very rare but some cases have been reported, which emphasises the importance of considering the possibility of LAIs when making differential diagnosis of mosquito-borne viral infections, particularly in non-endemic areas.

13.3 Biological and arthropod-specific risk assessment and biosafety

Biological and arthropod risk indicates here that there is a probability that harm, injury or disease will occur among laboratorians or the general public because of the accidental release of a competent disease vector and/or associated agents. Biosafety for GMMs can be achieved through a process of risk analysis. This consists of an assessment described in terms of risk concern, risk assessment, risk management and risk communication (Figure 36). Biosafety associated with the development of GMMs focuses on reducing to acceptable levels any potential adverse risks to human health and the environment that might be posed by these technologies.

Figure 36. Risk analysis



CHAPTER 13
Arthropod-specific risk assessment and laboratory biosafety

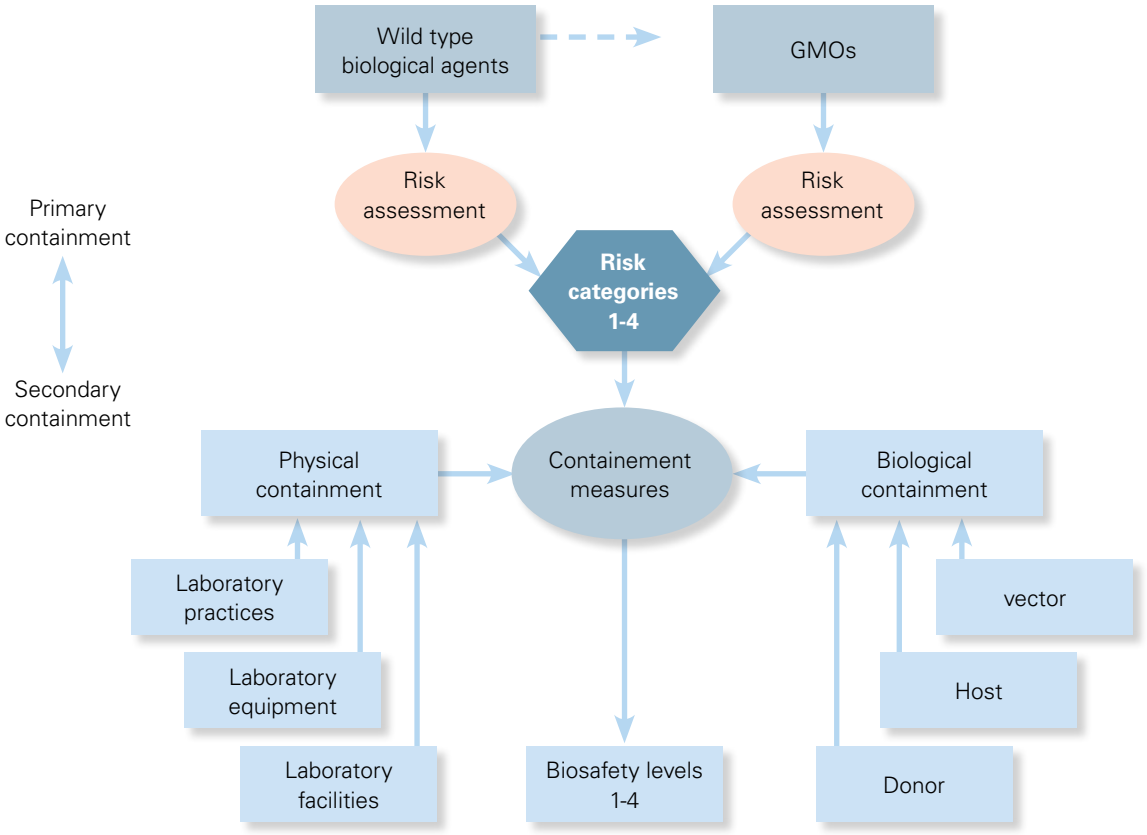
Biosafety promotes the use of safe practices in the handling of vectors and pathogenic microorganims at different levels as given below.

- In the laboratory
- During transportation
- In field investigations
- In manufacturing facilities
- In health-care facilities.

In the context of vector research laboratories, risk assessment considers of two kinds of effects: direct effects such as biting, infestations and myiasis; and indirect morbidity and mortality due to the pathogens transmitted. The steps in conducting risk assessment are as follows, and instructions for conducting risk assessment and containment measures are presented in Figure 37.

- Hazard identification (H)
- Evaluation of likelihood (L)
- Evaluation of consequences (C)
- Estimation of the risk (H X L X C)
- Risk management strategies to control (safety measures, control measures, etc.)
- Determination of overall risk.

Figure 37. Risk assessment and containment measures



Source: 17

13.3.1 Arthropod-specific risk assessment

Arthropod-specific risk assessment is primarily a qualitative judgement that cannot be based on a prescribed algorithm. There are several factors that must be considered in combination: the agents transmitted, whether the arthropod is or may be infected, the mobility and longevity of the arthropod, its reproductive potential, biological containment, and epidemiological factors influencing transmission in the proposed location or region at risk. The risk assessment should be carried out considering different domains as given below. The risk categorization for arthropods was presented in Table 25.

- Arthropods known to be free of specific pathogens
- Arthropods known to contain specific pathogens

- Arthropods may contain specific pathogens
- Arthropods containing unknown infectious agents or of which the status is uncertain
- Vector arthropods containing recombinant DNA molecules
- Mobility and longevity of arthropod
- Reproductive potential
- Epidemiological factors influencing transmission in the proposed location.

Table 25. Risk categorization for arthropods

Low risk	Arthropods that are known to be free of specific pathogens
Moderate risk	Arthropods known to contain a certain pathogen (patterns and efficiency of transmission and severity of disease)
Medium risk	Arthropods infectious status unknown or may contain unknown infectious agents.
High risk	GM arthropods expressing recombinant DNA molecules Phenotypic change Potential impact on wild-type populations if escape Unpredicted genetic change

13.3.2 Arthropod containment levels

When arthropods are used in the laboratory, facilities, trained staff and established practices must be in place to ensure appropriate safety, and the protection of the health and well-being of workers, and the environment. The basic biosafety level for different kinds of laboratories was recommended (Tables 26, 27) in order to reduce or eliminate exposure of laboratory workers and other persons, and the outside environment to potentially hazardous materials. If working with a vector in a particular set of circumstances, certain containment levels may be recommended (Table 28).[18]

Table 26. The standard level of biosafety in the laboratory

Laboratory type	Laboratory practices	Safety equipment	Risk group	Biosafety level (BSL)
Basic teaching, research	Good microbiological techniques	None; open bench work	1	Basic BSL-1
Primary health services; diagnostic services, research	Good microbiological techniques plus protective clothing, biohazard sign	Open bench plus BSC[a] for potential aerosols	2	Basic BSL-2
Special diagnostic services, research	Level 2 plus special clothing, controlled access, directional airflow	BSC[a] and/or other primary devices for all activities	3	Containment BSL-3
Dangerous pathogen units	Level 3 plus airlock entry, shower exit, special waste disposal	Class III BSC[a] or positive pressure suits in conjunction with Class II BSCs[a], double ended autoclave (through the wall), filtered air	4	Maximum containment BSL-4

[a] BSC(s), Biological Safety Cabinet(s).

Table 27. Domain of containment in the laboratory

Containment	Issues
Physical containment	Negative pressure
	Air filtration
	Sewage treatment
Organism containment	BSC,[a] isolators
	Lab gowns
	Gloves
	Respiratory protections
Operating procedures	GLP[b]
	Universal precautions
	Sharps
	Aerosol containments

[a] BSC, Biological Safety Cabinet.

[b] GLP, Good laboratory practices

Table 28. Summary of ACLs

Containment level	ACL-1[a]	ACL-2[a]	ACL-3[a]	ACL-4[a]
Arthropod distribution	Exotic, in viable or transient	Exotic, indigenous and transgenic		
Infection status	Uninfected or infected with non-pathogen	Up to BSL-2[b]	Up to BSL-3[b]	Up to BSL-4[b]
Active VBD cycling	No	Irrelevant		
Practices	ACL-1[a] Standard Arthropod handling practices	ACL-1[a] + more rigorous disposal, signage & limited access	ACL-2[a] with more highly restricted access, training & record-keeping	ACL-3[a] with highly access restriction, extensive training & full isolation
Primary barrier	Species appropriate containers	Species appropriate containers	Escape-proof arthropod containers, glove-boxes, BSC	Escape-proof arthropod containers handled in cabinet or suit laboratory
Secondary barrier	ND	Separated from laboratories, double doors (2), sealed electrical/plumbing opening, breeding containers and harborages minimized	BSL-3[b]	BSL-4[b]

ND: not determined.

[a] ACL, Arthropod containment level. [b] BSL, Biosafety level.

ACL-1 is suitable for work with uninfected arthropod vectors or those infected with a non-pathogen including:

- arthropods that are already present in the geographical region regardless of whether there is active VBD transmission in the locality; and
- exotic arthropods that, upon escape, would be inviable or become only temporarily established in areas not having active VBD transmission.

ACL-2 must be practised if working with exotic and indigenous arthropods infected with BSL-2 agents associated with animal and/or human disease, or that are suspected of being infected with such agents. Uninfected GM arthropod vectors also fall under this level provided the modification has no, or only negative effects on viability, survivorship, host range or vector capacity.

ACL-3 involves practices suitable for work with potential or known vectors that are, or may be infected with, BSL-3 agents associated with human disease. Arthropods that are infected or potentially infected with BSL-3 pathogens may pose an additional hazard if the insectary is located in an area where the species is indigenous, or if alternative suitable vectors are present, as an escaped arthropod may introduce the pathogen into the local population. The different components of containment levels are given in Table 29.

Table 29. Different components of containment levels

	ACL-1[a]	ACL-2[a]	ACL-3[a]
Location of arthropods	Furniture and incubators containing arthropods located away from general traffic to minimize accidental contact.	Arthropods located in dedicated rooms, closets, incubators out of the traffic flow.	Dedicated rooms, wings or suites in incubators located out of the traffic flow in BSL-3[b] areas.
Supply storage	The area maintained to allow detection of escaped arthropods. Material unrelated to arthropod rearing and experimentation (e.g. plants, unused containers, clutter) that provide breeding and refuge sites are minimized.	Designated area and no open shelves. Closed storage room, cabinets with tight-fitting doors or drawers.	Equipment and supplies not required for ongoing work removed from the insectary after appropriate decontamination. If present, located in a designated area and cabinets with tight-fitting doors or drawers.
General arthropod elimination	Accidental sources of arthropods from within the insectary are eliminated by cleaning work surfaces after a spill of materials, including soil or water that might contain viable eggs. Pools of water are mopped up immediately.	Same as ACL-1[a]	In addition materials are autoclaved before disposal. Only persons trained and equipped to work with arthropods and BSL-3[b] agents clean up spills.
Isolation of un-infected arthropods	NA	Spread of agents to uninfected arthropods is prevented by isolating infected material in a separate room	Only arthropods requiring ACL-3[a] procedures are housed in the ACL-3[a] insectary.
Primary container identification and labelling	Arthropods are identified adequately. Labels giving species, strain/origin, date of collection, responsible investigator, etc., are firmly attached to the container. Eggs, pupae, hibernating adults are securely stored.	Same as ACL-1[a]	Same as ACL-1[a]

	ACL-1[a]	ACL-2[a]	ACL-3[a]
Prevention of accidental dispersal on persons or via sewer	Precautions to prevent transport or dissemination of arthropods from the insectary on their persons or via the sewer.	Before leaving the insectary and after handling cultures and infected arthropods, personnel wash their hands, taking care not to disperse viable life stages into the drainage system. If uninfected materials are disposed of via the sewer, all material is destroyed by heat or freezing and preferably by autoclaving or incineration. Air curtains are recommended.	No material is disposed of through the sewer. Uninfected material is disposed of by autoclaving or incineration.
Pest exclusion programme	Programme to prevent the entrance of wild arthropods (e.g. houseflies, cockroaches, spiders) and rodents effectively precludes predation, contamination and possible inadvertent infection.	Same as ACL-1[a]	Same as ACL-1[a]
Escaped arthropod monitoring	Effective arthropod trapping programme is recommended to monitor escape.	Effective arthropod trapping programme to monitor escape. Oviposition traps, ground-level flea traps, oil-filled channels surrounding tick colonies, light traps for mosquitoes, etc. Exotic arthropods → exterior monitoring considered. Records of exterior captures maintained.	Commissioning process of a new facility the physical integrity and security practices tested by a simple release-recapture study. Records of exterior captures are maintained. If even one is missing and cannot be found, the facility is shut down and treated with a pesticide/fumigated.
Source and refugia reduction	Refugia and breeding areas reduced as appropriate. Furniture and racks minimized and can be easily moved to permit cleaning, and location of escaped arthropods.	Equipment in which water is stored or might accumulate (e.g. humidifiers) is screened to prevent arthropod access or contain chemicals to prevent arthropod survival.	Same as ACL-2[a] Individual arthropods are counted and accounted for throughout the experiment. No one enters or leaves the room until all arthropods are accounted for and secured in double taped cages and placed in secondary sealed holding trays.

	ACL-1[a]	ACL-2[a]	ACL-3[a]
Microbiological and medical sharps	Syringes that re-sheath the needle, needle-less systems and other safe devices used. Plastic-ware substituted for glass-ware whenever possible.	Same as ACL-1[a]	Sharps are stringently limited and use is justified only when alternatives are not available.
Arthropod sharps	Needles, probes, dissecting tools and syringes minimized.	These are restricted for use in the insectary if infected materials are used. If needed, only trained and staff conduct the procedures.	Restricted for use in the insectary regardless of infection status of material handled. If needed only trained and certified staff conduct the procedures.
Routine decontamination	NA	Equipment and work surfaces in the insectary routinely decontaminated after actual or potential contact with an infectious agent. Especially after spills and splashes of viable materials (including soil or water that might contain infectious agents or eggs).	Staff are trained to conduct these procedures. SOPs are available for spills and splashes of viable materials (including soil or water that might contain infectious agents or eggs).
Notification and signage	Persons entering the area are aware of the presence of arthropod vectors.	Infected material →biohazard sign. List all species handled Identity of arthropod species, agent(s) known or suspected to be present. Lists name and telephone number of the responsible person(s). Indicate special requirements for entering the insectary (need for immunizations or respirators).	Same ACL-2[a]
Procedure design	–	All procedures carefully designed and performed to minimize the risk of arthropod escape.	ACL-2[a] measures.

	ACL-1[a]	ACL-2[a]	ACL-3[a]
Safety manual	–	Safety manual prepared and adopted. Manual contains: emergency procedures, SOPs, waste disposal and other necessary information.	ACL-2[a] measures.
Training	–	Personnel advised of special hazards and required to follow instructions on practices and procedures. Performance review of employee. Additional training for procedural or policy changes. Records of all training maintained.	Person is trained and certified to conduct work in BSL[b]/ACL-3[a]
Containment during blood-feeding	Arthropods fed on host animals prevented from accidental transfer to host cages. When handling/removing animals after exposure to arthropods, precautions taken to prevent arthropod escaping through screens, covers and by flying.	Containment of arthropods during blood-feeding more stringently assured by special practices and container design.	Strictly assured by special practices and container designs that prevent escape of arthropods.
Accidental release reporting	Insectary director notifies accidental release of vectors.	A release procedure is developed and posted. This includes contacts and immediate mitigating actions. Location, number and type of material is prominently posted until the source is eliminated. Follow up medical evaluation, surveillance and treatment provided as appropriate and written records are maintained.	Same as ACL-2[a]

	ACL-1[a]	ACL-2[a]	ACL-3[a]
Escaped arthropod handling	Escaped arthropods killed or collected and disposed of.	Infected arthropods not killed with bare hands and transferred using filtered mechanical or vacuum aspirators.	Only personnel properly trained and equipped to work with designated arthropods and BSL-3 infectious agents and to recover and/or kill escaped arthropods. If even one is missing and cannot be found, the facility is shut down and treated with a pesticide/fumigated.
Arthropod-specific PPE[c]	PPE is worn as appropriate, e.g. respirators for arthropod-associated allergies, particle masks, head covers.	In addition to ACL-1[a] measures, PPE is used for all activities involving manipulations of infected or potentially infected arthropods.	ACL-2[a] Some facilities have specific PPE[c] requirements.
Location of insectary	The insectary area is separated from areas that are used for general traffic within the building.	The insectary is separated from areas that are open to unrestricted personnel traffic within the building. It is recommended that this be accomplished by at least two self-closing doors that prevent passage of the arthropods. Increased levels of physical isolation are recommended, e.g. separate buildings, wings, suites, etc.	The insectary is strictly separated from areas that are open to unauthorized, untrained personnel within the building by locked doors. These are opened by key locks, proximity readers, card keys, etc. The insectary is strictly separated from areas that are open to unauthorized, untrained personnel within the building by locked doors. These are opened by key locks, proximity readers, card keys, etc.

NA: not applicable.

[a] ACL: Arthropod containment level.

[b] BSL: Biosafety level.

[c] PPE: Personal protective equipment.

13.4 Transportation and transfer of biological agents and arthropod vectors

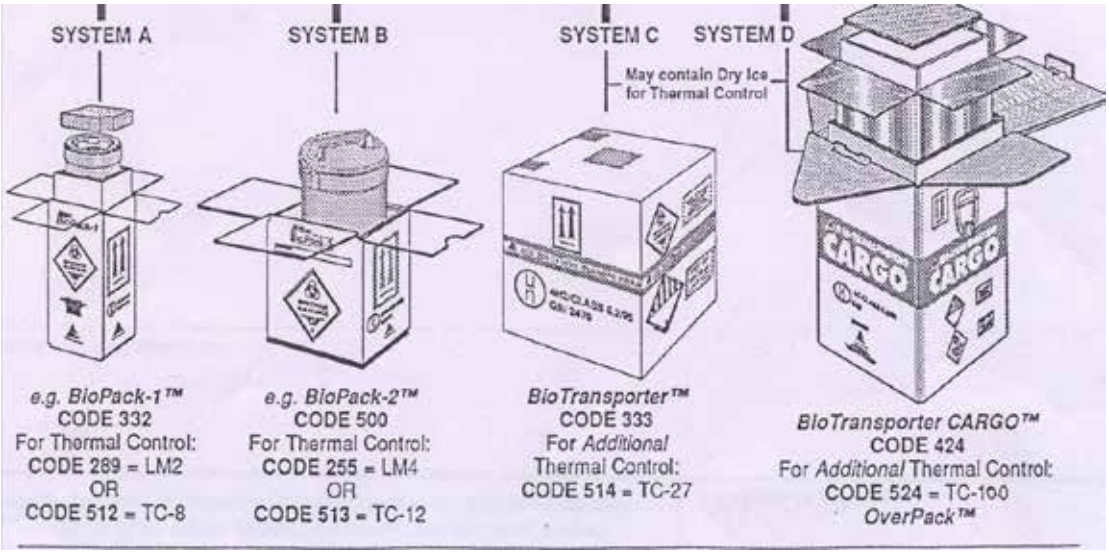
Transportation refers to the packaging and shipping of materials by air, land or sea, generally by a commercial conveyance. Transfer refers to the formal process of exchanging these materials between facilities. Regulations on the transportation of biological agents and live vectors aim to ensure that the public and workers in the transportation chain are protected from exposure to any agent that might be in the package, and that the package prevents the agent or live vector from escaping. Protection is achieved through: (i) the requirements for rigorous packaging that will withstand rough handling and contain all liquid material within the package without leakage to the outside; (ii) appropriate labelling of the package with the biohazard symbol and other labels to alert workers in the transportation chain to the hazardous contents of the package; (iii) the availability of documentation of the hazardous contents of the package should such information be necessary in an emergency situation; and (iv) training of workers in the transportation chain so that they are able to respond appropriately to emergency situations.

- Absolute alcohol→ can be used if identifying by PCR, genotyping, finger printing, etc., drying of specimens from leakages should be avoided.
- Transport-media→ for virus isolation, leakages should be avoided for biosafety.
- Transportation on dry-ice/liquid N2→ for virus isolation.
- Transportation on 4°C→ for bacterial isolations, serological tests, etc. Cold chain should be maintained.
- Dry preservation or pinning of arthropods→ identification purpose. Aerosol should be avoided for biosafety.
- Transportation of live arthropods→ understanding the biological characteristics and suspected incorporation of genome of the agents into the genome of arthropods. Escape should be avoided for biosafety.

13.4.1 Packing

- Tertiary container should be very sturdy and should have the following (Figure 38):
- mailing address
- sender's address
- label – diagnostic specimen/infectious specimen.

Figure 38. Biopack

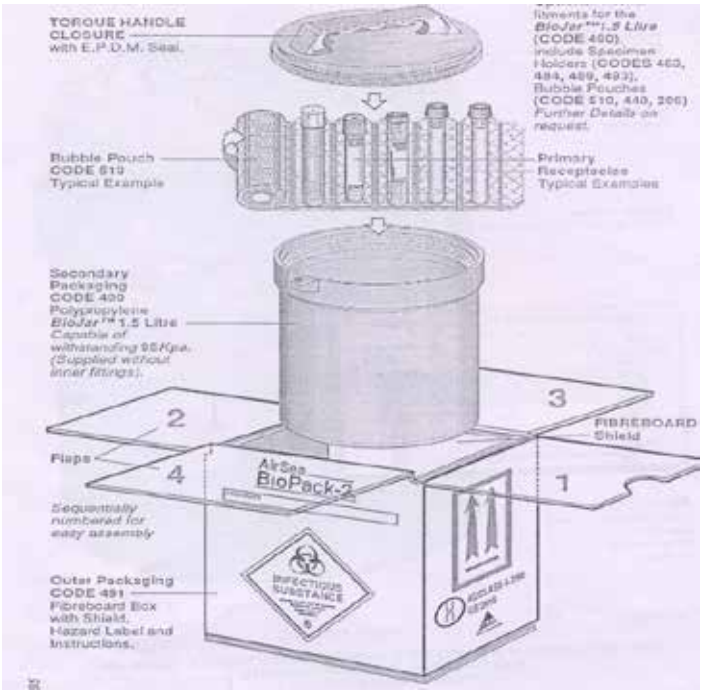


13.4.2 Dispatching precaution

See Figure 39 for dispatching details.

- Always correct label
- Max net weight allowed in passenger aircraft – 50ml
- Cargo aircraft – 4 L.

Figure 39. Dispatching



13.5 Laboratory biosecurity as a complement to laboratory biosafety

Biological materials are materials that require (according to their owners, users, custodians, caretakers or regulators) administrative oversight, control, accountability, and specific protective and monitoring measures in laboratories to protect their economic and historical (archival) value, and/or the population from their potential to cause harm. The general public expects laboratory personnel to act responsibly and not to expose the community to biorisks. It is also expected that they will follow safe working practices (biosafety) that will help keep their work and materials safe and secure (biosecurity), and to follow an ethical code of conduct (bioethics).

Laboratory biosafety is the expression used to describe the containment principles, technologies and practices that are implemented to prevent unintentional exposure to pathogens and toxins, or their accidental release. A comprehensive biosafety culture translates into the understanding and routine application of a set of safe practices, procedures, actions and habits that protect people working with biological materials. Laboratory biosecurity may be addressed through the coordination of administrative, regulatory and physical security procedures and practices implemented in a working environment that utilizes good biosafety practices, and where responsibilities and accountabilities are clearly defined. Biosafety and laboratory biosecurity are complementary. In fact, the implementation of specific biosafety activities already covers some biosecurity aspects.

The systematic use of appropriate biosafety principles and practices reduces the risk of accidental exposure and paves the way for reducing the risks of biological material loss, theft or misuse caused by poor management or poor accountability and protection. Laboratory biosecurity should be built upon a firm foundation of good laboratory biosafety.

13.6 Conclusion

This brief review summarizes the importance of the biosafety of laboratory personnel, the safety issues in the mycobacteriology laboratories, and the ways and means to overcome accidents in the laboratory environment including the prevention of exposure to such health hazards.

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Chapter 14. MosqGuide: role and achievement in deployment of genetic control methods against mosquito vectors

14.1 Introduction

MosqGuide[1] is a project funded by WHO/TDR to develop guidance on the potential deployment of different types of GMMs to control VBDs, specifically dengue and malaria. This guidance is intended to support DECs and other stakeholders in considering the safety and legal/regulatory aspects, as well as ethical, cultural and social issues of such deployment. It was commissioned in 2008 as a three-year project. Using fundamental principles of risk/benefit as a foundation, the MosqGuide project is preparing guidance in the form of a series of modules on best practices for the testing, importing, deploying and monitoring of GMMs designed for the control of malaria and dengue. The modules are aimed at different user groups, including researchers, regulators, public health officials, funding bodies and interested public. Each module will be tested with target audiences, primarily regulators and decision-makers in the DECs, and will also feed into other WHO initiatives, such as the Regional Biosafety Training Centres for GM Vectors. The guidance will also include a module that demonstrates a prototype issues/response model to assist DECs in making an informed choice about whether and under what conditions to deploy specific genetic control methods for mosquito vectors of malaria and dengue.

14.2 Project participants in MosqGuide project

The MosqGuide project, led by the Centre for Environmental Policy at Imperial College, London, has created a network of expertise in vector biology, genetics, disease control, regulation, social science and risk analysis from Brazil, India, Kenya, Mexico, Panama, Thailand and the United Kingdom, (Figure 40; Table 30).

The purpose of the network is to prepare guidance on best practices, peer-reviewed literature, emerging data and related experiences of risk assessment and management. The project itself is not involved in any field release programmes, although partners may be under separate funding.

The project was launched in July 2008 with a network meeting at Imperial College, London, where the parameters of the guidance were specified. The MosqGuide project will address issues surrounding the deployment of GMVs where the mosquito's DNA has been directly modified, but it will not include other potential strategies to control mosquito vectors, such as paratransgenesis. The project will also concentrate efforts on addressing technologies likely to reach field use within 10 years of the project's start date (for implementation up to 2018).[2]

Figure 40. MosqGuide project country network

Table 30. Project members in MosqGuide project network

Project member country	Project member
Brazil	Dr MargarethCapurro Dr Mauro Marrelli University de Sao Paulo
India	Dr Rachel Reuben (Retired)* Centre for Research in Medical Entomology (CRME), India Council of Medical Research.
Kenya	Dr Kenneth Ombongi University of Nairobi
Mexico	Dr Janine Ramsey Instituto Nacional de Salud Publica
Panama	Dr Vicente Bayard The Gorgas Institute
Thailand	Dr Pattamaporn Kittayapong Mahidol University
United Kingdom	Dr Luke Alphey, Oxitec Ltd Camilla Beech, Oxitec Ltd Dr Jon Knight Dr Megan Quinlan Prof John Mumford Imperial College

* Deceased during the course of the project.

14.3 Worldwide GMM guidance and training

- WHO Epidemic and Pandemic Alert and Response (EPR) Biosafety Unit – Laboratory Biosafety
- WHO/TDR BL5 Biosafety Training Centre (Africa)
- WHO/TDR BL5 Biosafety Training Centre (Latin America)
- WHO/TDR BL5 Biosafety Training Centre (Asia)
- WHO/TDR BL5 Genetically Modified Vectors Projects Coordination Committee
- WHO/TDR BL5 Project on Best-Practice Guidance for Deployment of Genetic Control Methods Against Mosquito Vectors in Disease Endemic Countries (MosqGuide)
- UNDP-Sponsored Risk Assessment Workshop Series on Transgenic Insects
- Ethical, Social and Cultural Program for the Grand Challenges in Global Health (GCGH) Initiative – MRC Centre, University of Toronto, Canada
- Regional Standards for Phytosanitary Measures (RSPM) 27 published by the North American Plant Protection Organization (NAPPO)
- Environmental Impact Statement (EIS) prepared and published by the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA/APHIS)
- FNIH/WHO Technical Meeting on GM Vector Control
- Cartagena Biosafety Protocol – Ad Hoc Technical Group on Risk Assessment – guidance for LMMs
- European Food Safety Authority – GMO Panel – Environmental Risk Assessment Criteria

14.4 GM/SIT mosquito facilities

Table 31 shows the countries, status and contact details of the GM/SIT mosquito facilities developed or being development.

Table 31. GM/SIT mosquito facilities

Country and contact	Facility	Status
Malaysia (Dr Lee Han Lim, MOH)	Institute Medical Research field house (*Aedes aegypti*)	Fully constructed and operational
Mexico (J Ramsey, INSP)	CRISP field cages (*Aedes aegypti*)	Soft cages in development Hard cages in development
Italy (A Crisanti, ICL)	InfraVEC field cages and Mass rearing (*Aedes albopictus*)	Construction planned for 2009/2010, funded by EU
Sudan (M Benedict, IAEA)	Mass rearing for SIT (*Anopheles arabiensis*)	Not constructed, but funding agreed
Brazil (A Malavasi, Biofábrica Moscamed)	Mass rearing (*Aedes aegypti*)	Proposed addition to site producing other insects

14.5 Progress on guidance preparation/modules

Guidance will be presented in seven modules as given below, based on main target audiences.

Module 1: Overview of technology options, social and regulatory issues (completed at the Regional Biosafety Training Center in Mali, Africa)

Module 2: Technology research and production phase (final draft completed, currently in the process of evaluation and consultation)

Module 3: Pre-deployment country decisions (started in second year of the project along with Module 4)

Module 4: Post-deployment data handling and environmental monitoring

Module 5: Stakeholder role and community engagement – case study on national decision-making (completed)

Module 6: Coordination, capacity strengthening and curriculum materials (ongoing)

Module 7: Prototype and decision tools (in development).

14.6 Conclusion

WHO is initiating a series of technical and public consultations with its partners/networks including MosqGuide with the aim of helping DECs to prepare for the use of GMMs, and developing a framework for national assessment and approval of genetic modification as a disease vector control tool.

REFERENCES

1. MosqGuide news. http://www.mosqguide.org.uk/ (accessed 11 November 2014).
2. Mumford J, Quinlan MM, Beech C, Alphey L, Bayard V, Capurro ML et al. MosqGuide: a project to develop best practice guidance for the deployment of innovative genetic vector control strategies for malaria and dengue. *AsPac J Mol Biol Biotechnol.* 2009;17:93–5 (http://www.msmbb.org.my/apjmbb/html173/173e.pdf, accessed 11 November 2014).

ANNEXES

Annex I. List of manuscripts/articles contributed by experts from African, Asian and Latin American regions

Serial No.	Authors	Title of the manuscript/article contributed
1	Beech C	The regulation of genetically modified vectors and their risk analysis
2	Jhansi C	Importance of biosafety in medical microbiology and biomedical laboratories within the context of GMMs – in a practitioner's perspective
3	Jayalakshmi T	Biosafety, regulation and laboratory experience of International Institute of Biotechnology and Toxicology
4	Lee HL, Nazni WA	What needs to be done prior to first open release of genetically modified *Aedes aegypti* (L.)?
5	Kumaran PP	Importance of bio-safety: ethical issues – a review
6	Dusthacher A, Selvakumar N	Biosafety in microbacteriology
7	Parimi S, Char B, Mishra R	Regulatory and biosafety aspects of genetically modified crops in India
8	Tyagi BK	Introduction to this Training manual: Biosafety for human health and the environment in the context of the potential use of genetically modified mosquitoes (GMMs) - A tool for biosafety training (CD and Internet versions) based on courses in Africa, Asia and Latin America, 2008–2011
		Arthropods as most suitable genetically modified organisms, with special reference to dengue and malaria as well as other infections of public health importance
		The GMO Project
9	Veer V	Recent advances in genetic modification of disease vectors
10	Barnabas GD, Hariprakash JM, Nagaraj K, Ganesan K	Recent strategies for developing eco-bio-safe transgenic insects
11	de Souza DK, Okorie PN, Adeogun AO, Elaagip AH	Disease control using genetically modified vectors: where do we stand in Africa nearly half a century after the initiation of the idea?
12	Marshall J	Measuring public attitudes to release of transgenic mosquitoes for disease control
13		Applying the Cartagena Protocol to releases of transgenic mosquitoes

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Figure 41. TDR/WHO Regional Biosafety Training Courses on genetically modified vectors in Asia (1, 2), Africa (3) and Latin America (4) (2008-11)

1.

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4.

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ISBN 978 92 4 154927 1