LARVICIDAL ACTIVITY OF NATIVE *Bacillus thuringiensis* ISOLATED FROM SOIL SAMPLES OF NEPAL

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF MICROBIOLOGY

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN MICROBIOLOGY

BY

GANGA GHARTY CHHETRI

JANUARY 2024

LARVICIDAL ACTIVITY OF NATIVE *Bacillus thuringiensis* ISOLATED FROM SOIL SAMPLES OF NEPAL

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF MICROBIOLOGY

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN MICROBIOLOGY

BY

GANGA GHARTY CHHETRI

JANUARY 2024

TRIBHUVAN UNIVERSITY

Institute of Science and Technology

DEAN'S OFFICE

Kirtipur, Kathmandu, Nepal

Reference No.:

Institute of Science & Technology Tribhuvan University Kirtipur 2045

EXAMINERS

The Title of Ph.D. Thesis: "Larvicidal Activity of Native *Bacillus thuringiensis* Isolated from Soil Samples of Nepal"

Name of Candidate: Ganga Gharty Chhetri

Internal Examiner:

Prof. Dr. Anjana Singh
Central Department of Microbiology
Tribhuvan University, NEPAL

External Examiners:

(1) Dr. Narayan Raj Bhattarai
B.P. Koirala Institute of Health Sciences
Dharan, NEPAL

(2) Dr. Pradeep Kumar Srivastava
Ex National Vector Borne Disease Control Program
Ministry of Health & FW
Delhi, INDIA

(3) Dr. Daniel M Parker
University of California
Irvine, USA

May 14, 2024

(Dr. Surendra Kumar Gautam)
Asst. Dean

DECLARATION

The thesis entitled **"LARVICIDAL ACTIVITY OF NATIVE *Bacillus thuringiensis* ISOLATED FROM SOIL SAMPLES OF NEPAL"** which is being submitted to the Central Department of Microbiology, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of Associate. Prof. Dr. Komal Raj Rijal of Central Department of Microbiology, Tribhuvan University and co-supervised by Prof. Dr. Prakash Ghimire of Central Department of Microbiology, Tribhuvan University and Associate Prof. Dr. Ishan Gautam, Natural History Museum, Tribhuvan University.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

.....

Ganga Gharty Chhetri

RECOMMENDATION

This is to recommend that **Ganga Gharty Chhetri** has carried out research entitled **"LARVICIDAL ACTIVITY OF NATIVE *Bacillus thuringiensis* ISOLATED FROM SOIL SAMPLES OF NEPAL"** for the award of Doctor of Philosophy (Ph.D.) in **Microbiology** under our supervision. To our knowledge, this work has not been submitted for any other degree.

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph.D. degree.

Dr. Komal Raj Rijal

Supervisor

(Associate Professor)

Central Department of Microbiology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Dr. Prakash Ghimire

(Co-Supervisor)

Professor

Central Department of Microbiology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

.....

Dr. Ishan Gautam

(Co-Supervisor)

Associate Professor

Natural History Museum

Tribhuvan University

Swayambhu, Kathmandu, Nepal

JANUARY 2024

TRIBHUVAN UNIVERSITY
INSTITUTE OF SCIENCE AND TECHNOLOGY
CENTRAL DEPARTMENT OF MICROBIOLOGY
Kirtipur, Kathmandu, Nepal

Ref.No.:

Date: 02/01/2024

Central Department of Microbiology Tribhuvan University Kirtipur, Kathmandu

LETTER OF APPROVAL

On the recommendation of **Assoc. Prof. Dr. Komal Raj Rijal/ Prof.Dr Prakash Ghimire/ Assoc. Prof. Dr. Ishan Gautam**, this Ph.D. thesis submitted by **Ganga Gharty Chhetri**, entitled **"LARVICIDAL ACTIVITY OF NATIVE *Bacillus thuringiensis* ISOLATED FROM SOIL SAMPLES OF NEPAL"** is forwarded by Central Department Research Committee (CDRC) to the Dean, Institute of Science and Technology, Tribhuvan University.

.....

Dr. Komal Raj Rijal
Associate Professor
Head
Central Department of Microbiology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

ACKNOWLEDGEMENTS

It gives me pleasure to express my sincere gratitude to my respected supervisor Associate Prof. Dr. Komal Raj Rijal and Co-supervisors Prof. Dr. Prakash Ghimire, Central Department of Microbiology, Tribhuvan University, for their constant inspiration, encouragement, continuous support, valuable suggestions and superb guidance during the entire period of this research work.

I would sincerely like to express my respectable appreciation to my Co-supervisor Associate Professor. Dr. Ishan Gautam, Natural History Museum, Tribhuvan University for his encouragement, continuous support, valuable suggestions, providing a suitable site, and valuable suggestions for the field trial and guidance during the entire period of this research work.

I am grateful to Associate Professor Dr. Megha Raj Banjara, Central Department of Microbiology, Tribhuvan University for his valuable suggestion, guidance, and support during the entire period of this research work.

I am grateful to all the faculty members Prof. Dr. Anjana Singh, Prof. Dr. Dwij Raj Bhatta, Prof. Dr. Binod Lekhak, Associate Prof. Dr. Dev Raj Joshi, Associate Prof. Dr. Shyam Prakash Dumre Associate Prof. Dr. Reshma Tuladhar, Dr. Shaila Basnyat, Dr. Supriya Sharma, Mr Sanijb Adhikari, Mr. Nabaraj Adhikari, Mr. Upendra Thapa Shrestha, of Central Department of Microbiology, Tribhuvan University, for their support during my research period.

I am also thankful to those M.Sc. students of the Central Department of Microbiology, who helped me in different ways, in the fieldwork or laboratory work, by handling and counting eggs and larvae of mosquitoes and by providing photographs of my laboratory research works helping me in one or the other way while carrying their thesis work in the same laboratory for the past five years period “Thank you to all”. And I am also thankful to all the Ph.D. students for their encouragement.

I am indebted to the University Grants Commission for providing financial support through Ph.D. grants and Fellowship award (S&T-12), to the Nepal Health Research Council (NHRC) for providing the Provincial Research Grant FY 2079/2080 to carry out the Field trial research work, and to Pasteur Institute, Paris, France for providing the standard reference strain Bti IPS-82.

I am thankful to the Central Department of Microbiology, Tribhuvan University for providing the laboratory facility for the entire research work as well as the study site for the evaluation of the newly formulated bio larvicide *Bt*-14P2A and to the staff of the Department especially to Mr. Ramesh Ghimire, Mr. Madhukar Thapa, Mr. Ramesh Khadka, for their support to carry the research work. Thankful to the store Department of Tribhuvan University for providing the premises as well as the tires to evaluate the efficacy of the bio larvicide.

I am grateful to the people for providing the premises for the installation of containers for oviposition and the evaluation of the bio larvicide *Bt*-14P2A for a longer duration.

I am thankful to my friend Dr. Kshama Parajuli Central Department of Chemistry, Kirtipur, Kathmandu, Nepal for the constant encouragement throughout the research work.

I would like to thank my family members and friends for their encouragement and support in carrying out the research work.

--- .....

Ganga Gharty Chhetri

JANUARY 2024

शोध सार

जलवायु परिवर्तनका कारण लामखुट्टेबाट सर्ने रोगहरूको संक्रमण बढ्दै गईरहेको अवस्था छ । Malaria, Dengue, Japanese encephalitis आदि रोगहरू लामखुट्टेको माध्यमबाट सर्ने भएकाले यसलाई रोगवाहक (vector) भनिन्छ । लामखुट्टेको वृद्धि रोक्ने विधिलाई भेक्टर नियन्त्रण भनिन्छ । भेक्टर नियन्त्रण गर्न धेरै विधिहरू अपनाउन सकिन्छ । भेक्टर नियन्त्रणबाट लामखुट्टेको लार्भाका स्रोतहरूलाई कटौती गरेर वा नष्ट गरी वयस्क लामखुट्टेको वृद्धि रोक्न सकिन्छ । जैविक कीटनाशक जस्तै *Bacillus thuringiensis* var *israelensis* (*Bti*), *Lysinibacillus sphaericus* (*Ls*) जस्ता ब्याक्टेरिया लाई पनि भेक्टर नियन्त्रणका लागि प्रयोग गरिन्छ, जसले पानीमा भएका लार्भा नष्ट गरी वयस्क लामखुट्टेको वृद्धि रोक्ने काम गर्दछ । विभिन्न मुलुकले लामखुट्टे नियन्त्रण गर्न जैविक कीटनाशक *Bt* र *Ls* को प्रयोग गर्ने गरेका छन् । जैविक कीटनाशकलाई विभिन्न प्रकार जस्तै तरल र ठोसकारूपमा उत्पादन गर्ने गरेका छन । यी जीवाणु मानव र अन्य जीवजन्तुका निम्ति हानिकारक हुँदैनन्, तर यिनले उत्पादन गरेका विषालु कीटनाशक क्रिष्टल प्रोटीनले लामखुट्टेको लार्भा नष्ट गरी तिनको स्रोत व्यवस्थापनमा सहयोग पुर्‌याउँदछ । यस अनुसन्धानको उद्देश्य नेपाली माटोका नमुनाबाट *Bt* र *Ls* को खोजी गर्नु र लामखुट्टे नियन्त्रणमा यसको प्रभावकारिता मुल्याङ्कन गर्नु हो । नेपालका विभिन्न वातावरणीय भूभागबाट माटो सङ्कलन गरी फरक फरक प्रविधिद्वारा माटोलाई प्रशोधन गरी *Bt* र *Ls* लाई छुट्याई सुरक्षित गरियो । यी दुई जीवाणुलाई सूक्ष्मदर्शक यन्त्र र coomassie brilliant blue (CBB) का सहायताले तिनीहरूको उत्पादन क्रिष्टल प्रोटिन अवलोकन गरी पहिचान गरिएको थियो । यस बाहेक छुट्याउन वा पहिचान गर्न अन्य प्रविधिहरू पनि अपनाइएको थियो । *Bt* र *Ls* को लार्भानाशक (Larvicidal) परीक्षण र *Bt* को16srRNA gene sequence बाट phylogeny को अध्ययन गरिएको थियो । लार्भानाशक क्षमताको परिक्षणका लागि काठमाडौँका विभिन्न स्थानबाट लार्भा सङ्कलन गरिएको थियो । प्रयोगशालामा पनि *Aedes aegypti* लामखुट्टेका लार्भा पालनपोषण गरिएको थियो । लार्भानाशक क्षमता परीक्षणका लागि विश्व स्वास्थ्य सङ्गठनको मापदण्ड अपनाइएको थियो । परीक्षण गर्दा लार्भालाई *Bt* का दुई प्रकारका उत्पादन जस्तै : ठोस र तरल जैविक लार्भानाशक (Bio-larvicide) खुवाइएको थियो । प्रयोगशाला र बाहिरी वातावरणमा तरल *Bt*-14P2A लार्भानाशकको परीक्षण गरिएको थियो । परीक्षणबाट सकारात्मक र नकारात्मक नियन्त्रणका साथै ५०% र ९०% लार्भालाई मार्न चाहिने मात्रा,

(Lethal concentration dose LC_{50}, LC_{90}) मृत्यू प्रतिशत र प्रभावकारिताको निर्धारण गरिएको थियो । यस अध्ययनका क्रममा ४५४ माटोका नमुना परीक्षण गर्द । गोलाकार क्रिष्टल उत्पादन गर्ने *Bt* माटोमा प्रशस्त (५८.३३%, ८०८/१३८५) भएतापनि केवल चार *Bt* (*Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, *Bt*-3P1B) को गोलाकार क्रिष्टल प्रोटिनले मात्र शत प्रतिशत (100%) लार्भालाई मार्ने क्षमता राख्ने देखाएको थियो । प्रारम्भिक जाँचका क्रममा *Bt*-14P2A ले सङ्कलित लार्भा र प्रयोगशालामा पालनपोषण गरिएका *Aedes aegypti* लामखुट्टेका लार्भा विरुद्ध LC_{50} ३.९८ र LC_{90} २.११ PPM देखाएको थियो । तरल बनाइए जैविक लार्भानाशक *Bt*-14P2A लाई Simulated field trial परीक्षण र खुला स्थलगत परीक्षण (Open field trial) मा प्रयोग गरिएका सबै मात्राहरूले २४ घण्टा भित्र शतप्रतिसत लार्भा नास गरेको थियो । खुला स्थलगत परीक्षणमा सबैभन्दा कम मात्राले १४ दिनका लागि वर्षाको पानी पुन:पूर्ति हुँदा शतप्रतिशत (100%) मृत्युदर देखायो । त्यसैगरी, घरेलु प्रयोजनका निम्ति प्रयोग हुने बाल्टिनहरूमा जैविक लार्भानाशकको छ हप्तासम्म residual effect देखिएको थियो । यसै क्रममा 16srRNA gene sequence को परीक्षणबाट ३ ओटै *Bt* जैविक लार्भानाशक हो भनी प्रमाणित भएको छ । केवल एउटा *Ls* ले *Ae. aegypti* लार्भा विरुद्ध २५% मृत्युदर देखाएको थियो । नेपालको माटोबाट पाँचवटा लार्भानासक व्याक्टेरिया (चार) *Bt* र (एक) *Ls* प्राप्त भएको थियो । तसर्थ, प्राप्त *Bt* द्वारा पानी भण्डारणका क्रममा भाँडामा हुर्किएका लामखुट्टेका लार्भा नष्ट गरी यसलाई नेपालमा लामखुट्टेबाट सर्ने रोगहरूको नियन्त्रणका लागि प्रयोग गर्न सकिन्छ।

मुख्यशब्द: *Aedes aegypti, Bacillus thuringiensis, Lysinibacillus sphaericus*, लार्भानाशक, लामखुट्टे

ABSTRACT

Climate change is exacerbating the spread of mosquito-borne diseases globally. Vector control with *Bacillus thuringiensis* var *israelensis* (*Bti*) and *Lysinibacillus sphaericus* (*Ls*) the biological control agents for larval source management is an effective technique for preventing mosquito-borne diseases by focusing on mosquito larvae in diverse water sources. While these larvicides are used in many other countries, they are not widely available in Nepal for mosquito vector control. This study aims to isolate mosquito larvicidal *Bacillus thuringiensis* (*Bt*) from the soil samples collected in Nepal. It was isolated from the soil sample by the acetate selection method. It was identified by observing crystal protein stained by coomassie brilliant blue and further characterized by larvicidal assay and 16srRNA gene sequencing. The larvae were collected from the fields; and reared in the laboratory for the bioassay. Bioassay was carried out in four replicates with the positive control strain (coded as *Bt*-IPS-82), negative control, and test *Bt*. The mortality percentage was calculated after 24 hours. Formulated larvicide (*Bt*-14P2A) in the form of pellet and liquid suspension were evaluated in simulated and open field trials. The lethal concentration (LC), residual effects, and mortality rates were estimated. *Bt* isolates were isolated from 454 soil samples, with four strains (*Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B) demonstrating 100% larvicidal activity against mosquito larvae during primary screening. *Bt*-14P2A's LC_{50} and LC_{90} against *Ae. aegypti* larvae in pellet form were 3.98 ppm and 25.11 ppm, respectively, whereas in liquid form they were 6.45 ppm and 37.15 ppm respectively. Simulated field testing revealed a residual impact of seven weeks for the liquid formulation, with 100% mortality for six weeks. Open-field experiments also showed efficacy, with very modest concentrations delivering 100% mortality for up to 14 days and residual effects lasting up to six weeks in household containers such as buckets. The obtained larvicidal *Bt* strains were found to be genetically identical to those in GenBank. In addition, 11 *Ls* isolates were isolated from 32 soil samples, one of which demonstrated mild toxicity against *Ae. aegypti* larvae. Finally, the efficient isolation procedure produced *Bt* strains with 100% larvicidal activity against *Ae. aegypti* larvae. These isolates show promise as future strategies for managing larval sources and combating mosquito-borne illnesses in Nepal.

Keywords*:* *Aedes aegypti – Bacillus thuringiensis – Bio-larvicide – Larval Source Management - Lysinibacillus sphaericus*

LIST OF ACRONYMS AND ABBREVIATIONS

Bs	:	*Bacillus sphaericus*
Bt	:	*Bacillus thuringiensis*
Bti	:	*Bacillus thuringiensis* var *israelensis*
CDC	:	Centers for Disease Control and Prevention
CDM	:	Central Department of Microbiology
EDCD	:	Epidemiology Disease Control Division
INTs	:	Insecticide Treated Nets
IRC	:	Institutional Review Community
IRCIOST	:	Institutional Review Community-Institute of Science and Technology
IRS	:	Indoor Residual Spray
ITU	:	International Toxic Unit
IVM	:	Integrated Vector Management
LLINTs		Long Lasting Insecticidal Treated Bed Nets
Ls	:	*Lysinibacillus sphaericus*
LSM	:	Larval Source Management
MBDs	:	Mosquito-Borne diseases
PPM	:	Parts Per Million
SAARC	:	South Asian Association for Regional Cooperation
SC	:	Suspension Concentrate
TU.	:	Tribhuvan University
VBDs	:	Vector-Borne Diseases
VCRC	:	Vector Control Research Centre
WDG	:	Water Dispersible Granule
WHO	:	World Health Organization

LIST OF TABLES

Page No.

Table 1: *Bacillus thuringiensis* and the target insect pest. 11

Table 2: Number of *Bt* obtained from soil samples. 40

Table 3: Shapes of insecticidal crystal proteins (ICPs)..... 41

Table 4: Distribution of isolates obtained from three different regions..... 41

Table 5: Larvicidal activity of native *Bt*. 43

Table 6: Larvicidal activity of *Bt* and control strain *Bti* -IPS-82 against field-collected larvae. 44

Table 7: Larvicidal activity of control and test strain against field-collected larvae. . 46

Table 8: Larvicidal activity of control and test strain against *Ae. aegypti* larvae. 46

Table 9: Mean LC_{50} and LC_{90} of *Bti*-IPS-82 and *Bt*-14P2A against field-collected larvae. 48

Table 10: Mean LC_{50} and LC_{90} of *Bti* and *Bt*-14P2A against *Ae. aegypti* larvae. 49

Table 11: Mean mortality of *Ae. aegypti* larvae caused by dosages within 24 hours. 51

Table 12: The survival and mortality % of *Ae. aegypti* larvae with 6mL/Lt bio-larvicide treatment. 53

Table 13: Effect of 3mL/Lt bio-larvicide on mosquito larvae present in an open field setting in CDM. 54

Table 14: Effect of 3mL/Lt bio-larvicide on mosquito larvae present in the tires of TU. 55

Table 15: Effect of 4mL/Lt bio-larvicide on mosquito larvae present in the tires of TU. 55

Table 16: Effect of 3mL/Lt bio-larvicide on mosquito larvae present in the tires of Kalanki site. 56

Table 17: Effect of bio larvicide on mosquito larvae present in the pre-domestic area of Maharajgunj site.. 57

Table 18: Effect of bio larvicide on mosquito larvae present in the pre-domestic area of the Sifal site. 57

Table 19: Effect of 3mL/Lt bio-larvicide on mosquito larvae in a cornfield Kirtipur site. 58

Table 20: Effect of 2mL/Lt bio-larvicide on mosquito larvae in pre-domestic area kirtipur site. 59

Table 21: Effect of 1mL/Lt bio-larvicide on mosquito larvae in indoor Sitapaila site. 60

LIST OF FIGURES

			Page No.
Figure 1	**:**	Transmission cycle of diseases.	12
Figure 2	**:**	Life cycle of a mosquito.	12
Figure 3	**:**	Comprehensive flow chart of research design.	39
Figure 4	**:**	Sample collection sites.	42
Figure 5	**:**	Concentration of *Bt*-14P2A required to show 100% mortality against field-collected and *Ae. aegypti* larvae.	47
Figure 6	**:**	Phylogenetic tree.	50
Figure 7	**:**	Mean mortality % of *Ae. aegypti* larvae in weeks by simulated field trial.	52
Figure 8	**:**	Simulated field trial of *Bt*-14P2A showing 100% mean mortality in weeks.	53
Figure 9	**:**	Sifal site outdoor.	63
Figure 10	**:**	Sifal site outdoor.	63
Figure 11	**:**	Kirtipur cornfield site.	63
Figure 12	**:**	Imadole site pre-domestic area.	63
Figure 13	**:**	Simulated field trial CDM.	63
Figure 14	**:**	Tribhuvan University dean's office.	63
Figure 15	**:**	Maharajgunj site pre-domestic area.	64
Figure 16	**:**	Kirtipur outdoor.	64
Figure 17	**:**	Larvicidal activity in 4 replicates.	64
Figure 18	**:**	Microscopic observation of *Bt*-14P2A.	64
Figure 19	**:**	Colony morphology of *Bt*-14P2A.	64
Figure 20	**:**	Microscopic observation of *Ls*-10P5B.	64

TABLE OF CONTENTS

Page No.

DECLARATION.....**ii**

RECOMMENDATION..... **iii**

LETTER OF APPROVAL.....**iv**

ACKNOWLEDGEMENTS .....**v**

शोध सार.....**vii**

ABSTRACT.....**ix**

LIST OF ACRONYMS AND ABBREVIATIONS .....**x**

LIST OF TABLES .....**xi**

LIST OF FIGURES ..... **xiii**

TABLE OF CONTENTS .....**xiv**

CHAPTER 1.....**1**

INTRODUCTION.....**1**

1.1 Introduction.....1

1.2 Rationale 5

1.3 Objectives 7

1.3.1 General objectives.....7

1.3.2 Specific objectives 7

CHAPTER 2.....**8**

LITERATURE REVIEW **8**

2.1. Biological control agents 8

2.1.1 *Bacillus thuringiensis* (*Bt*) 8

2.1.2 Insecticidal crystal proteins (ICPs) 10

2.1.3 Mode of action of ICPs.....11

2.2 Mosquito vector 11

2.3 Transmission cycle of the infectious agent by mosquito 12
2.4 Biology of mosquito 13
2.5 Scenario of mosquito-borne diseases in Nepal 14
2.6 Control measures used in Nepal for MBDs 14
2.7 Mosquitocidal *Bacillus thuringiensis* var *israelensis* (*Bti*) 17
2.8 Mosquitocidal *Ls* 18
2.9 Larval source management (LSM) 19
2.10 Characterization of *Bt* 21
2.11 Insect bioassay 22
2.12 Molecular characterization 22
CHAPTER 3 24
MATERIALS AND METHODS 24
3.1 Sample collection and size 24
3.2 Laboratory analysis 24
3.2.1 Isolation of *Bacillus thuringiensis* (*Bt*) 24
3.2.2 Isolation of *Lysinibacillus sphaericus* (*Ls*) 25
3.3 Molecular characterization 25
3.3.1 DNA extraction 25
3.3.2 16srRNA gene analysis 25
3.3.3 16srRNA gene sequence 26
3.3.4 Phylogenetic tree 26
3.4 Bioassay 26
3.4.1 Collection of mosquito larvae 26
3.4.2 Laboratory rearing of field-collected larvae 26
3.4.3 Preliminary screening for larvicidal activity 27
3.4.4 Standard reference strain (control strain) 27
3.4.5 Selective bioassay 27

3.4.6 Confirmation of larvicidal mortality 28
3.4.7 Quantitative bioassay against field-collected larvae 28
3.4.7.1 Preparation of pellet 28
3.4.7.2 Preparation of stock solution 28
3.4.7.3 Preparation of working solution 29
3.4.7.4 Bioassay 29
3.4.7.5 Quantitative bioassay against *Ae. aegypti* larvae 29
3.4.7.6 Collection of larvae 29
3.4.7.7 Identification keys for *Aedes* 30
3.4.7.8 Rearing *Ae. aegypti* in the laboratory 30
3.5 Formulation of *Bt*-14P2A bio larvicide 31
3.6 Field trial 32
3.6.1 Simulated field trial 32
3.6.1.1 Treatment 32
3.6.2 Open field trial in different sites 33
3.6.2.1 Central Department of Microbiology (CDM) 27°40'59.7"N 85°17'15.7"E 33
3.6.2.2 Maharajgunj, Kathmandu site 27°44'17.1"N 85°20'27.8"E 33
3.6.2.3 Sifal, Kathmandu site 27°42'48.0"N 85°20'40.7"E 34
3.6.2.4 Tribhuvan University, Kirtipur, Kathmandu 27°40'46.3"N 85°17'35.6"E 34
3.6.2.5 Panga Charghare, Kirtipur, Kathmandu site A 27°39'44.6"N 85°16'43.4"E 34
3.6.2.6 Panga Charghare, Kirtipur, Kathmandu site B 27°39'44.6"N 85°16'43.4"E 34
3.6.2.7 Cow farm Panga, Kirtipur, Kathmandu site 27°39'44.6"N 85°16'43.4"E 34
3.6.2.8 Open cornfield Kirtipur, Kathmandu site 27°39'44.6"N 85°16'43.4"E34

3.6.2.9 Champadevi, Kirtipur Kathmandu site 27°40'00.2"N 85°15'25.5"E ...34
3.6.2.10 Vegetable farm Kirtipur, Kathmandu site 27°40'00.2"N 85°15'25.5"E ...35
3.6.2.11 Bafal, Kathmandu site 27°42'15.6"N 85°16'54.3"E ...35
3.6.2.12 Sitapaila Kathmandu site 27°42'17.2"N 85°16'42.6"E ...35
3.6.2.13 Kalanki, Kathmandu site 27.6931^0N, 85.3474^0E...35
3.6.2.16 Koteshwor, Kathmandu site 27°40'13.0"N 85°20'54.3"E...35
3.6.2.17 Garbage collection site A and Garbage collection site B at Koteshwor, Kathmandu 27°40'13.0"N 85°20'54.3"E...35
3.6.2.18 Chicken and meat shop in Koteshwor, Kathmandu 27°40'13.0"N 85°20'54.3"E ...35
3.6.2.19 Small cow farm in Koteshwor, Kathmandu 27°40'13.0"N 85°20'54.3"E ...36
3.6.2.14 Gwarko, Lalitpur site 27°40'08.7"N 85°20'02.3"E ...36
3.6.2.15 Imadole, Lalitpur site 27°39'36.8"N 85°20'42.0"E...36
3.6.2.16 Puddles in the UN park 27°41'09.0"N 85°19'34.5"E, Roadside, Sankha park 27°40'44.3"N 85°19'52.3"E, and in Manohara park 27°40'08.6"N 85°21'07.1"E ...36
3.6.3 Containers ...36
3.6.4 Surveillance of mosquito larvae...36
3.6.5 Identification of mosquito...37
3.6.6 Larval density determination ...38
3.6.7 Treatment ...38
3.7 Data analysis ...38
CHAPTER 4 ...40
RESULTS AND DISCUSSION ...40
4.1 Isolation of *Bacillus thuringiensis* ...40
4.2 Larvicidal activity ...43

4.3 Selective bioassay against field-collected larvae 44
4.4 Quantitative bioassay against field-collected and laboratory-reared *Ae. aegypti* larvae 45
4.5 Estimation of the lethal concentration (LC) LC_{50} and LC_{90} 48
4.6 Molecular characterization of larvicidal *Bt* 49
4.7 Simulated field trial 51
4.8 Open field trial 52
4.9 Identification of mosquitoes 61
4.10 Limitation of study 62
CHAPTER 5 64
CONCLUSION AND RECOMMENDATION 64
5.1 Conclusion 64
5.2 Recommendations 65
CHAPTER 6 66
SUMMARY 66
REFERENCES 68
APPENDICES I
Consent Form I
List of Equipment's and Materials II
List of Publications III
16srRNA gene sequence IV

CHAPTER 1

INTRODUCTION

1.1 Introduction

Global warming is increasing the number of infectious mosquito-borne diseases worldwide. The mosquitoes that were considered to be breeding outdoors now prefer to live indoors and breed in clean water used for domestic purposes for instance *Aedes aegypti* and *Anopheles stephensi*. It has become necessary to use alternative measures that are sustainable and nonharmful to the environment as well as to humans in addition to personal protective measures (Nets, Repellents, Coils, etc) to control the mosquito vector. Biological control methods for mosquito-borne diseases especially by using *Bacillus thuringiensis* var *israelensis* (*Bti*) is effective, safe, and sustainable methods compared to other biological control agents. This bacterium produces a protein toxin toxic only to mosquito larvae, when the toxic is ingested by larvae of the mosquito it kills the larvae, thus the bacterium is called larvicidal bacteria. The application of biological control against the larval stages of mosquito vectors avoids chemical pollution of the environment and mitigates the hazardous effect of chemical insecticides against humans. The most widely used biological control agents are *Bti* and *Lysinibacillus sphaericus* (*Ls*) formerly this bacteria was known as *Bacillus sphaericus* (*Bs*) (Derua *et al.,* 2018; Huang *et al*., 2017; WHO, 2011). The advantage of *Bti* and *Ls* is that they destroy larvae of mosquitoes in aquatic habitats, but spare other insect species that may be present in the surroundings. These bacteria produce toxic metabolites, toxic only to mosquito larvae. The mosquito larvae present in aquatic habitats feed on this toxin resulting in the paralysis of larvae and death of the larvae of the mosquito. Thus the bacterial toxin is safe to use in domestic water-holding containers to kill the larvae of the mosquitoes that breed indoors to break the transmission chain of the dengue virus as well as other water sources where the mosquito breeds.

Dengue has been a serious public health concern across the world. The dengue disease is now reported from 100 countries in the WHO regions of Africa, the Americas, the Eastern Mediterranean, South Asia and Southeast Asia, and the Western Pacific (WHO,

2023a). Among them, the Americas, Southeast Asia, and Western Pacific Regions are the most seriously affected, with Asia representing around 70% of the global disease burden (WHO, 2023a). In 2023 and until the beginning of November, over 4.5 million cases and over 4000 dengue-related deaths have been reported from 80 countries/territories globally (European Center for Disease Prevention and Control, 2023).

Dengue outbreaks within Nepal have increased significantly in recent years, according to the Epidemiology and Disease Control Division (EDCD), 2023 dengue situation updated on 23/12/15 from 1 January to 15 December 2023, the number of reported dengue cases was 51243, with the confirmed death of 20, affected district 77 and number of the district with >500 cases were 16 the most affected district was Dharan with 8 death in the monsoon season. There are no specific drugs for dengue treatment mainly focused on treating the pain by using pain killer paracetamol. Dengue vaccine, Dengvaxia (CYD-TDV) discovered in 1997 and Qdenga (TAK-003) discovered in 2022 are commercially available but the dengue vaccine Dengvaxia is given to the person who had dengue once and it is available where the disease is more prevalent (WHO, 2023a).

Dengue is caused by the virus called dengue virus in short (DENV) belonging to the family Flaviviradae and genus *Flavivirus* and is spread by the bites of female mosquitoes of *Ae. aegypti* and *Ae. albopictus,* the two vectors responsible for dengue fever. The occurrence of two vector mosquito species responsible for dengue fever was previously thought to be restricted to the Terai Region of Nepal. However, later, its geographical expansion became more apparent in hilly regions ">2000m" of Nepal (Dhimal *et al*., 2015; Gupta *et al*., 2018). Therefore, the risk is present throughout the country. The expansion of *Aedes* in Nepal is a threat to Zika, chikungunya, and other arboviral infections (Dhimal *et al*., 2018; Poudel, 2022). These two vectors are responsible for more than one arboviral disease like Zika, dengue, chikungunya, yellow fever, etc., and *Ae. albopictus* is one of the major vectors of at least 22 arboviruses (WHO, 2011).

The first case of dengue was reported in 2004 from Chitwan, Nepal (Pandey *et al*., 2004) then in 2006 outbreak with 32 cases was reported (EDCD, 2022a). The continuous increase in dengue cases in Nepal is from 2010 to date (EDCD, 2022b). In 2022, Nepal faced large dengue fever epidemics with 54784 reported cases and 88

deaths (EDCD, 2023). Due to the widespread distribution of vectors, climate change, and lack of immunity against Zika virus (ZIKV) in the native population, there is a high risk of ZIKV transmission in Nepal which may follow the footsteps of chikungunya virus (CHIKV) and DENV which have already invaded Nepal (Dhimal *et al*., 2018).

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are caused by one of four closely related, but antigenically distinct, virus serotypes mainly DENV-1; DENV-2; DENV-3; and DENV-4; that include in family Flaviviridae and *Flavivirus genus*. Dengue viruses are small, spherical-shaped containing single-stranded mRNA as a genome inside the nucleocapsid. All four serotypes have emerged from sylvatic strains and are responsible for causing dengue disease in Nepal the serotypes prevailing in different districts of Nepal are identified by RT-PCR (Malla *et al*., 2008). The uninfected *Ae. aegypti* and *Ae. albopictus* gets infected with the virus while taking the blood meal from the asymptomatic and symptomatic individual as they become the carrier after infection with the dengue virus. *Ae. aegypti* and *Ae. albopictus,* are a day feeder and feed from dawn to dusk during the endemic period. Once the mosquito becomes infected with the dengue virus, the mosquito will remain infectious with the virus for its whole life span. Infected mosquitoes can continue transmitting the dengue virus to new healthy individuals on each blood meal, the life span of a female mosquito is greater than male and in the availability of nutrients, its life span is up to four weeks. DENV remains in an environment during interepidemic periods by vertical transmission.

Ae. aegypti native to Africa, and *Ae. albopictus* indigenous to Asia, were wild species, that have adapted to breed in artificial containers of rural, suburban, and urban human environments. They are introduced to the rest of the nation by global trade and travel (WHO, 2011). The increase in the disease rate is due to the adaptation of *Aedes* spp. to a variety of breeding habitats in indoor and outdoor environments. Throughout Southeast Asia, *Ae. aegypti* lay eggs in domestic and discarded water-holding containers. These include multiple types of containers made of different types of materials like plastic, metals, paper, rubber, wood, etc., found in urban surroundings and its periphery, in households, various construction sites, and industries. The plastic jars, saucers on which flowerpots rest, indoor flower vases, wooden, plastic, and metal drums, discarded tires, plastic and glass bottles, various tin cans, different types of polystyrene containers, plastic cups, discarded watered batteries, broken glass

containers, spirit houses (shrines), drain pipes (WHO, 2011). Which remains unnoticed by humans, and helps mosquito proliferation even in a very small amount of water. The water provides a suitable environment to lay eggs and to hatch the eggs to larvae, pupa, and adults. The *Aedes* eggs can remain dominant and resist desiccation for more than a year due to embryonic desiccation resistance (EDR), it is acquired during embryogenesis through the formation of the serosal cuticle (SC) (Vargas *et al*., 2014; WHO, 2009a, 2011), and its tight attachment to the surface, this peculiar characteristic of the eggs of *Aedes* provides the sustainability of its generation. These tiny eggs are unnoticed in domestic containers too. So, when the next rain floods the discarded containers, flower pots outdoors, or domestic containers when replenished with water, the eggs hatch and continue their life cycle.

There is no vaccine available in Nepal. The appropriate method at present in the context of Nepal is to reduce the disease-carrying mosquitoes in the surrounding environment. Therefore, mosquito vector control is one of the options and the most effective disease control strategy. The main strategies adopted for effective control of vector-borne diseases (VBDs) are disease management with early diagnosis and treatment, community awareness, and vector control (Shrestha *et al*., 2019). Vector control practices followed in Nepal are the distribution of long-lasting insecticidal treated bed nets (LLINTs), indoor residual spraying (IRS), elimination of breeding sources (practice for *Aedes* spp.), and surveillance of disease-transmitting vectors (Shrestha *et al*., 2019). To achieve long-term and sustainable control of VBDs various other integrated approaches are needed (i) environmental management (ii) biological control agents (iii) chemical control (WHO, 2011). New interventions and approaches are required to address the expansion of mosquito vectors.

Bt and *Ls* are a naturally occurring bacterium. They are the member of the genus *Bacillus* ability to form endospores. They are Gram-positive. The most differentiating feature of *Bt* from closely related *Bacillus* species is the production of a crystalline structure or crystal protein during sporulation. The crystal proteins or delta (δ) endotoxins were found to be active against specific insects. Similarly, *Ls* produce toxin at the time of sporulation. It differs from *Bt* morphologically by producing a swollen sporangium at the terminal end.

1.2 Rationale

Mosquito vector expansion from endemic regions to non-endemic regions is due to climatic change, urbanization, globalization, changes in mosquito behavior, etc. These factors are challenging the vector control strategies. The vector control measures like IRS, LLINTs, or Insecticidal treated bed nets (ITNs) practices so far may not be sufficient in the prevention of diseases in the future. For instance, *Ae aegypti* has a different behavior than the other mosquito vectors, it prefers to live in human surroundings, breed in domestic containers like buckets, tubs, flower pots, etc., where rainwater and clean water are stored or collected, flight range of adult mosquito 100-400 meter only, feed on human blood at daylight (day biter), resting habitats indoor, etc., these behavior knowledge gaps among the human or members of the house protect it from the control measures like insecticides coil, liquid vaporizer, insecticidal treated bed nets, etc., used earlier and in present, which is used by the people during the night time and is meant to be highly reliable impactful control measures for the mosquitoes that bite during the night time or in the evening. Thus, the life cycle of *Ae. aegypti* continues indoors in the domestic containers and the dengue virus circulates indoors and in the urban area. The control measure should target the breeding habitats.

Recent year's larval source management (LSM) is focused on managing larval habitats in different ways, to prevent the development of adult mosquitoes (Imbahale *et al*., 2012; Olalubi, 2016; WHO, 2013; Worrall & Fillinger, 2011). LSM can be done by various means one as by using a biological control agent like *Bti* and *Ls*. Larvicidal activity (killing of larvae of mosquitoes) by using *Bt* is a novel work in Nepal. For sustainable dengue mosquito control, it is necessary to reduce the larvae of mosquitoes by using *Bt*, a safer, cost-effective, and environmentally acceptable tool. "Search and destroy campaign" requires the *Bti* as an impactful control measure.

Nepal has yet to use mosquito larvicidal bacteria *Bt* and other biological control agents. No attention is paid to these types of vector control interventions in Nepal. New strategies or more targeted methods are required for combating vector-borne diseases. This proposed work will provide the *Bt* and *Ls* for commercialization and further utilization for mosquito vector control which has not yet been carried out in Nepal. This will open the path for other feasible biological control agents to fight against the mosquito vector. *Bti* is also recommended by the World Health Organization for LSM.

The study aims to isolate the novel mosquito larvicidal bacteria from the soil samples of Nepal using different techniques and identify it by comparing it with the standard strain *Bti*-IPS-82 and to evaluate the larvicidal activity of the formulated bio larvicide *Bt* isolate of Nepal against the *Ae. aegypti* mosquito larvae, reared under laboratory conditions and field-collected mosquito larvae in a laboratory and field trial.

1.3 Objectives

1.3.1 General objectives

The general objective of this research is to characterize and determine the larvicidal activity of *Bt* isolated from soil samples of Nepal against larvae of *Ae. aegypti*

1.3.2 Specific objectives

1. To isolate *Bacillus thuringiensis* from soil samples of different three topographical regions of Nepal
2. To identify the *Bt* isolates by phenotyping (Coomassie Blue Staining (CBB) and various biochemical tests)
3. To confirm the *Bacillus thuringiensis* isolates by phylogenetical analysis of 16srRNA
4. To determine the larvicidal activity of the *Bt* isolates against *Aedes aegypti* larvae.

CHAPTER 2

LITERATURE REVIEW

2.1. Biological control agents

Biological control is a sustainable and efficient alternative to chemical pesticides. Biological agents like insects, fish, bacteria, nematodes, copepods, neem cake, oil, etc., are used to control the mosquito vector (Abagli *et al*., 2019; Chatterjee *et al*., 2023; Okumu *et al*., 2007; WHO, 2011). Biological control agents mainly focus on the reduction of larvae of mosquitoes in aquatic habitats which hinder the life cycle of a mosquito by preventing the development of pupae and adult mosquitoes. These biological control agents are targeted for larval source management (LSM), by the production of the toxin, predatory property, reducing the supply of oxygen to larvae, etc. Predatory properties of proven larvivore fish species are recommended by WHO, (2003) to control different types of mosquito vectors. Various studies have shown the reduction in mosquito larvae in different aquatic habitats by using fish species (Howard *et al*., 2007; Louca *et al*., 2009) similarly, the copepods *M. leuckarti*, and *M. scrassus* are recommended as suitable candidates for biocontrol of *Aedes* vectors in South Asian country, Sri Lanka (Udayanga *et al*., 2019). Among the bacteria, the most extensively used and studied agents *Bacillus thuringiensis* var *israelensis* and *Bacillus sphaericus* produce toxins when the larvae feed on them causing the death of the larvae (WHO, 2011).

2.1.1 *Bacillus thuringiensis* (*Bt*)

A wide array of microbial biocontrol agents such as *Pseudomonas* spp., *Bacillus* spp., *Streptomyces* spp. including *Bt* are regarded as environmentally friendly alternatives of chemical insecticides that have been marketed as commercial products as well (Ronin *et al*., 2019). *Bt* was first discovered by Japanese sericultural engineer Shigetane Ishiwata in 1901 (Ishiwata, 1901). It was isolated from the diseased larvae of *Bombyx mori* (silkworms), called sotto disease, which refers to bacillary paralysis which caused a large number of deaths of the silkworm. Ishiwata named the bacterium *Bacillus sotto* (Milner, 1994). In 1911 Ernst Berliner German Microbiologist isolated *Bt* from the Mediterranean flour moth in Thuringia Germany and named according to the place of

isolation Thuringian to thuringiensis (Berliner, 1911). Later *Bacillus sotto* was also renamed as *Bacillus thuringiensis* var *sotto*. *Bt* is a naturally occurring bacterium, the organism is a Gram-positive, aerobe, endospore producer, and motile, rod-shaped bacterium. *Bt* resembles other *Bacillus* species in morphology and shape as it is one of the members of the *Bacillus cereus* group containing other six species such as *Bacillus cereus*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis* and *Bacillus cytotoxicus* as they share many phenotypic and genotypic properties (Ibrahim *et al*., 2010; Palma *et al*., 2014; Xu *et al*., 2014). It is distinguished from the other *Bacillus* species as it produces a protein inclusion (crystal protein) during sporulation. In 1915 Berliner reported the existence of inclusion bodies "RestKorper" rhomboid-shaped (crystal protein) close to the endospore within the *Bt* sporangium. This feature is mainly used for identification of *Bt* from another *Bacillus* spp. The insecticidal property of the crystal protein was only known later. The discovery history shows they were pathogens of that particular larvae from which *Bt* was isolated. *Bt* Sporine was first commercialized in France in 1938 and used to destruct flour moths. *Bt* as biopesticides were used commercially to control agricultural pests of crops. From then onwards *Bt* has been isolated from different sources and registered as a biopesticide to control the pests of the crops. *Bt* is indigenous to many environments including soil it has been isolated from insects, leaves of plants, drooping of birds, aquatic environment, and from marine sediments (Poopathi *et al*., 2014). Thus, it is obvious that *Bt* is widespread in nature. The organism grows naturally as saprophytes, feeding on dead organic matter, therefore the spores of *Bt* persist in soil and the vegetative growth occurs when the nutrients are available.

The life cycle of the *Bacillus* species has two main stages: the vegetative stage and the sporulation stage. The vegetative stage is characterized by active cell growth, while the sporulation stage is characterized by the cessation of cell growth and spore formation. The sporulation stage brings changes in both the morphology and the biochemistry of the *Bt* cells. At this stage *Bt* synthesis, a unique structure or parasporal crystalline inclusions (crystal protein) known as δ endotoxins. This crystal protein is toxic to different types of insect pests, the crystal protein is called insecticidal crystal protein (ICP).

2.1.2 Insecticidal crystal proteins (ICPs)

These insecticidal crystal proteins or crystalline inclusions or δ endotoxins are synthesized during the stationary phase and accumulate in the ce1l as a crystal inclusion, which is 25% of the dry weight of the sporulated cells is called insecticidal crystal proteins (ICPs). These ICPs are of bipyramidal, cuboid, spherical, ovoid, flat rectangular, or heteromorphic in shape they are found inside the exosporium and readily separated from the spore. Which are highly toxic to a wide variety of important agricultural and health-related insect pests as well as other invertebrates. But in some *Bt* the crystal protein remains attached to the spore and is called as spore attached crystal protein or cap-headed crystal protein (Rampersad & Ammons, 2005; Reyaz *et al*., 2013). ICPs are formulated during sporulation. It is the main phenotypic integrity that separates two closely related bacterial species, *Bt* and *Bacillus cereus* taxonomically (Xu *et al*., 2014). The ICPs show a wide range of specificity for different insect orders: Lepidoptera (butterflies, moths), Diptera (black fly, mosquito), Coleoptera (beetles, weevils), Hemiptera (bedbugs, aphids), Hymenoptera (wasp, bees), Gastropoda (snails, slugs), nematodes, Mallophage (lice, bird lice) and Human cancer cells. Various *Bt* subspecies have been established as biopesticides *Bt* subspecies *kurstaki, tenebrionis,* and *aizawai* with the highest activity against lepidopteran, coleopteran larval species Table 1. ICPs are formed by two types of protein called cry protein and cyt protein (Xu *et al*., 2014), which exert toxicity to a variety of insect pests. The cyt and cry proteins are toxic endotoxin toxic to a variety of insects are solubilized in water and belong to the delta-endotoxin class of bacterial proteins (Palma *et al*., 2014; Schünemann *et al*., 2014). *Bt* also produces insecticidal proteins during the vegetative development phase, which are then secreted into the growth media. These proteins, known as vegetative insecticidal proteins (Vips), have insecticidal activity against lepidoptera, coleoptera, and several homopteran pests (Palma *et al.*, 2014). A less well-characterized secretory protein with no amino acid resemblance to VIP proteins has exhibited insecticidal efficacy against coleopteran pests and is designated a secreted insecticidal protein. (Sip) (Palma *et al*., 2014).

The toxin-producing Genes i.e. *cry* genes, located on plasmids produce endotoxin (Schünemann *et al.*, 2014). Different varieties of *cry* genes are present in the plasmids of the *Bt* bacteria that can be used for further characterization of *cry* genes and the different types of endotoxins with specific activity against different types of pests.

Table 1 below shows that not all *Bt* endotoxins are effective in causing the mortality of the insect pest. Different varieties of *Bt* show toxicity against the different insect pests. Thus, the *cry* gene sequences vary from one *Bt* to another *Bt* strain.

Table 1: *Bacillus thuringiensis* and the target insect pest.

***Bt* variety**	**Target pest**
Bacillus thuringiensis subsp sotto	Silkworm
Bacillus sphaericus	Mosquito larvae
Bacillus thuringiensis subsp *aizawai*	Moth larvae
B. thuringiensis subsp. *Israelensis*	Mosquito and blackflies
B. thuringiensis subsp. *kurstaki*	Lepidopteran larvae *Helicoverpa armigera*, *Spodoptera litura*,
B. thuringiensis subsp. *tenebrionis*	Colarado potato beetle
Bacillus thuringiensis subsp. *Galleriae*	Lepidopteran larvae

Source (Jisha *et al*., 2013).

2.1.3 Mode of action of ICPs

The crystal protein produced by different *Bt* strains is active against insect orders *viz.*, Lepidoptera, Diptera, Hymenoptera, and Coleoptera while the Cyt toxins are toxic to Diptera. (Palma *et al*., 2014). As the *Bt* toxins are specific towards the target pest, safe for humans, and environmentally compatible and sustainable alternative to chemical pesticides *Bt* is used worldwide. After ingestion of *Bt* toxin, the crystals protein first dissolves in the intestinal tract, which ultimately causes an intoxication, which results in immediate paralysis of the GI tract, thus preventing food intake by insects. This paralysis of GI tract is ultimately responsible for the destruction of the intestinal epithelium, which decreases pH in the intestinal tract, thus the spores ingested with the crystal protein germinate and increase the number of bacterial cells in the insect carcass (Palma *et al*., 2014).

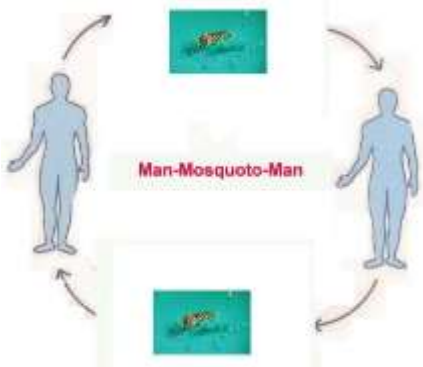
2.2 Mosquito vector

Mosquitoes are insects found everywhere in the world. There are more than 3500 (European Centre for Diseases Prevention and Control, 2022; WHO, 2020a) types of mosquitoes. Included under the phylum Arthropoda. Class Insecta, and into three subfamilies Anophelinae, Culicinae, and Toxorhynchitina. *Anopheles*, *Aedes*, and

Culex are the mosquito genera of public health significance. *Anopheles* spp. are included in the sub-family Anophelinae, *Aedes* spp., and *Culex* spp. in Culicinae. These genera are responsible for transmitting mosquito-borne diseases (European Centre for Diseases Prevention and Control, 2022; WHO, 2020a). According to the Center for Disease Control and Prevention, (2023) mosquito the deadliest animal in the world causes one million deaths of humans per year by transmitting diseases causing agents of malaria, Japanese encephalitis, lymphatics filariasis, dengue, zika, chikungunya, and other arboviral diseases. According to the WHO., (2020b) vector-borne diseases causes more than 17% of diseases, and 700,000 deaths annually. Globally 219 million malaria cases with more than 400,000 deaths and 96 million symptomatic cases of dengue with 40,000 deaths are estimated every year.

2.3 Transmission cycle of the infectious agent by mosquito

The female mosquito spread the pathogens by sucking the blood of an infected person taking those infectious microbes inside the gut and when it bites the healthy person, transferring those pathogens to the healthy individuals. The disease-causing agents are transmitted from one person to another by the mosquito during its blood meal from an infectious person Figure 1. The infectious agent propagates inside the mosquito without causing disease in them. In *Ae. aegypti* and *Ae. albopictus*, dengue virus (DENV) enters along with the blood meal of an infectious individual and multiple in the midgut. Newly formed virion particles circulate to various mosquito organs and get transmitted to the next host in subsequent bites. *Ae. aegypti* and *Ae. albopictus* have intricate immune control to allow DENV production at a sub-pathogenic level (Mukherjee *et al*., 2019).



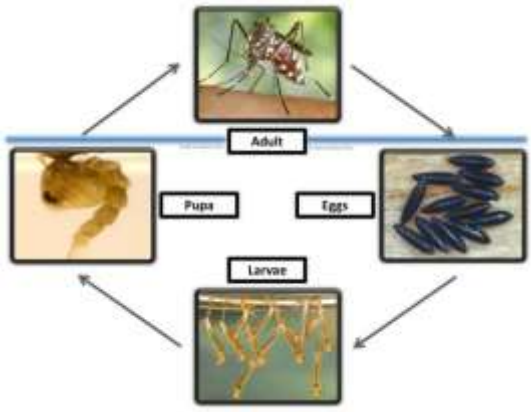


Figure 1: Transmission cycle of diseases. **Figure 2:** Life cycle of a mosquito
Source:https://ncvbdc.mohfw.gov.in/index4.php?lang=1&level=0&linkid=488&lid=3764&theme=Cream

In general, the perpetuation of arboviruses requires vertebrate blood so that arthropods bear infectious viruses and blood during feeding (Ruckert & Ebel, 2018). Transmission of the virus to a new host by an arthropod infected in this manner requires that this arthropod be a competent vector. In public health entomology, the term vector competence refers to the inherent ability of a particular arthropod to transmit a specific type of virus (Ruckert & Ebel, 2018; WHO., 2011). In competence vectors, the virus is acquired during blood feeding, undergoes replication in gut tissue, and disseminated to secondary sites such as salivary glands, and is ultimately released into the arthropods salivary secretions, where it may be entered into the skin during bite and cutaneous vasculature of the host during subsequent biting of the mosquito (Ruckert & Ebel, 2018). Breaking the transmission chain will prevent the spread of infectious agents.

2.4 Biology of mosquito

It is important to know the life cycle of a mosquito Figure 2 to break the transmission chain of mosquito-borne diseases. Four stages are observed in the life cycle of a mosquito (Centers for Disease Control and Prevention, 2016) eggs, larva, pupa, and adult fly. The three stages of mosquito require water for their survival or continuation of their generation. Eggs are laid on the surface or on the wet walls of the water sources. Eggs hatch to larvae only in the presence of water. Larval and pupae survive only in water. The adult fly emerged from the pupa to survive outside the water.

Eggs: Female *Aedes* mosquitoes lay their eggs just above the water line (Centers for Disease Control and Prevention, 2016; Shragai *et al*., 2018), on the other hand *Anopheles* spp. and *Culex* spp. lay eggs on the surface of the water. Eggs are laid by different mosquitoes in different types of aquatic habitats. Different species *Anopheles* and *Culex* can breed in clean and polluted water or waste water. *Aedes* spp. prefer clean water for breeding (WHO, 2009b, 2011) eggs hatch into larvae when they come in contact with water. Depending upon the humidity and the temperature eggs hatch to larvae within 48 hours.

Larvae: Water triggers the eggs to hatch into larvae. Larvae present in water feed on the microorganism and undergo molting three times and develop into pupa.

Pupae: The pupae also live in water. The pupae are more active than larvae but do not feed.

Adults: Pupa eventually transforms into an adult mosquito. Which fly off to mate and repeat the cycle. Male mosquitoes do not take blood meals feed on plant nectar, and their life span is only 6 or 7 days on average. Females with sufficient nutrition can live up to 5 months or longer, with an average life span of about 6 weeks. To produce eggs, the female takes blood meal of animal or human in addition to plant nectar (Vector Disease Control International (VDCI), 2024). It is important to know the breeding habitats and stages of a mosquito for the application of vector control measures. To control mosquito-borne diseases vector control measures should intervene in all the stages of the life cycle of a mosquito for sustainable control of the diseases.

2.5 Scenario of mosquito-borne diseases in Nepal

Major mosquito-borne diseases in Nepal are in the following order in the present scenario dengue, malaria, lymphatic filariasis, and Japanese encephalitis (Nepal Health Research Council, 2020). In Nepal mosquito-borne diseases before 2000 were more prevalent in the Terai region, the expansion of malaria from endemic regions to nonendemic regions like Mugu, Bajura, Baitadi, Humla, etc., is increasing (Dhimal, Gautam, *et al*., 2014; Dhimal, O'hara, *et al*., 2014; Himalayan News service, 2019; Poudel, 2023) indicating threat for other mosquito-borne diseases (MBDs) in future. Earlier Health professionals used to ask "Have you been to the Terai region" if a person go to them for treatment with fever or symptoms similar to mosquito-borne diseases. Mosquito-borne diseases with newer infectious agents are in increasing trends, for instance, dengue in Nepal from 2014 to 2022 the number of cases recorded was 54784, and 88 deaths in 2022 marking the highest number ever recorded in the country with the highest number of cases in Nepal Epidemiology and Disease Control Division (EDCD, 2022). This figure was more than three times the number of cases reported in 2019. Both the mosquito vectors *Ae. aegypti* and *Ae. albopictus* are prevalent in Nepal. Relatively malaria, Japanese encephalitis (JE), and Lymphatic filariasis (LF) are less concerned are significantly reduced compared to dengue, a significant reduction of malaria cases has been observed in the last 10 years (EDCD), 2019).

2.6 Control measures used in Nepal for MBDs

The key interventions to control malaria in Nepal have been the use of insecticides, insecticidal treated nets (INTS) or LLINTs, and environment management of vector breeding sites (EDCD), 2020). Apart from this, early diagnosis and treatment are also

practiced for the reduction of malaria in Nepal. In addition, people use various kinds of personal protective devices, eg., electrical rockets, chemical insecticides, e.g., Mortein, coils, insane, smoke of paper, cardboard, bioactive i.e., phytochemical compounds, e.g., mustard oil, etc. The Ministry of Health and Population (MoPH), with the support of its other partners, especially WHO, which is coordinating the technical assistance, has implemented a strong malaria control program, steadily improving the coverage and quality of indoor residual spraying (IRS), introducing long-lasting insecticide-treated nets, and increasing access to rapid kit for malaria diagnosis (RDTs) and use of drug, artemisinin-based combination therapy (ACTs).

That resulted in Nepal surpassing the Millennium Development Goal #6 by reducing 50% malaria morbidity and mortality rates in 2010 as compared to 2000 (Department of Health Services. Epidemiology and Disease Control Division., 2016). The same control measures followed to control malaria may not be effective in controlling the dengue diseases in Nepal. So, a new strategic plan has to be implemented to address the dengue disease. The number of JE cases in Nepal began to plummet, and not a single JE death has been recorded in Nepal since 2010. Nepal made tremendous progress in JE prevention. Through surveillance, introduction and expansion of JE vaccination, and improved case management, the country has significantly reduced JE morbidity and mortality (PATH, 2016). Mass Drug Administration (MDA) is the primary strategy used by the Lymphatic Filariasis Elimination Program in Nepal to prevent transmission of the disease (WHO., 2023b). Various surveys have been conducted during the past 13 years, including mapping, baseline surveys, follow-up surveys, post-MDA coverage surveys, and transmission assessment surveys (TAS). TAS conducted in 26 districts found that the prevalence of LF infection has greatly reduced, contributing to the prevention of impairments and suffering caused by LF (Government of Nepal, 2022). These different types of interventions each used so far are going to lead to the elimination of JE, LF, and malaria from Nepal in the coming years. As can be seen, different interventions were used to prevent and control MBDs in Nepal. Many Southeast Asian countries are not able to reduce MBDs in the availability of additional interventions. So, to prevent and control dengue diseases additional and new intervention strategies like vaccination, drugs for treatment, and vector control methods are required.

There are various methods to control mosquitoes to prevent the transmission of mosquito-borne diseases. (i) Environmental management (ii) Biological control (iii) Chemical control. These control methods can be applied to control all types of mosquito vectors. Revised national guidelines on dengue prevention, management, and control in Nepal 2019 have focused on the above-mentioned methods to assist program managers, or health workers across the country to successfully prevent dengue in Nepal.

Ae. aegypti is a predominantly urban species due to its adaptive ability to live around humans in a domestic setup. This established *Ae. aegypti* as an efficient dengue vector in urban settings even in its low density. Feed on human blood at daylight (day biter), resting habitats indoors these behavior knowledge gaps among the humans or members of the house protect it from the control measures used by the people.

Impactful control measures that are safe, effective, and sustainable targeting the domestic and pre-domestic *Ae. aegypti* is essential to address the burden of dengue diseases at present and other arboviral diseases in the future. Preventing or reducing dengue virus transmission depends entirely on mosquito vector control or interruption of human-vector contact. Surveillance of resting places indoors or outdoors is mandatory for effective control of *Ae. aegypti*. A study carried out by (Phuyal *et al*., 2022) shows that the knowledge, attitude, and practice of the Nepalese community people of lowland and highland on dengue fever is not sufficient or adequate to prevent and control future dengue epidemics. A similar study carried out by (Paudel *et al*., 2023) among Police personnel showed the level of knowledge and attitude was low but the prevention practice was moderate. So, it is essential to use multiple control measures indoors and outdoors for effective control of dengue disease (Sirisena & Noordeen, 2016). The effective method of targeting *Ae. aegypti* is indoor residual spraying (IRS) an expensive insecticide and technology sprays large quantities of insecticides on every indoor wall and ceiling, but found to be less impactful. Later the identification of the resting behavior of *Ae. aegypti* on the wall below 1.5m and in dark places, spraying at this targeted wall and dark recesses rather than the entire house showed an impactful control of *Ae. aegypti* vector in indoor and it is known as Targeted indoor residual spraying (TIRS) (Facchinelli *et al*., 2023; Seang *et al*., 2023). Capturing resting and flying mosquitoes indoors during a defined period is considered a gold standard for indoor adult *Ae. aegypti* sampling or density or abundance determination to predict the outbreak of dengue disease. *Ae. aegypti* becomes infected after biting infected humans

symptomatic or asymptomatic as humans are the main amplifying host of the virus. The virus infects the mosquito midgut and subsequently spreads to the salivary gland and the virus is transmitted to other humans during subsequent feeding. Thereafter the mosquito remains infective for the rest of its life.

2.7 Mosquitocidal *Bacillus thuringiensis* var *israelensis* (*Bti*)

The important property of the bacteria that was discovered about 56 years ago was the mosquito larvicidal property of *Bt*. It was isolated from the dead mosquito larvae and found to produce toxin toxic only to the mosquito larvae. Later it was identified and designated as *Bti* serotype H-14 (Goldberg & Margalit, 1977; Nicolas, 1992). *Bti* was discovered in 1976 by Goldberg and Margalit from Israel from dead *Culex pipiens* (Goldberg & Margalit, 1977; Margalit, 1990) and later serotyped and identified as H-14 by de Barjac. The first commercial products based on *Bti* were distributed in the United States: Vectobac, Bactimos, and Teknar were produced by Abbott Laboratories, Biochem Ltd., and Sandoz, respectively (Lacey & Orr, 1994). Like other *Bt* varieties, *Bti* produces a parasporal inclusion at the time of sporulation containing the larvicidal toxins, and the spherical toxin is produced by *Bti*. Mosquito larvicidal *Bt* has been isolated from a wide variety of samples from different parts of the country for instance Bello *et al*., (2016) isolated 12 mosquitoes larvicidal *Bt* from the soil sample of Zaria, in which L3 isolated showed 100% mortality of *Ae. aegypti* larvae at 100PPM concentration of spore crystal mixture. El-Kersh *et al*., (2016) isolated 23 mosquitoes' larvicidal *Bt* obtained from different types of samples like soils, dried leaves, animal manure, standing rainwater, insects, snails, sewage water, and fish guts collected from Saudi Arabia against *An. Gambiae*. Lobo *et al*., (2018) isolated 12 mosquitocidal *Bt* from the soil sample of Cerrado of Brazil showed toxicity against *Ae. aegypti* larvae. Similarly from Amazones of Brazil, six mosquitocidal *Bt* against *Ae. aegypti* was isolated by Soares-da-Silva *et al*., (2015).

Laboratory and field trial assays showed the effectiveness of *Bti*-H14 in controlling different types of diseases transmitting mosquito larvae. Laboratory assays against *Ae. aegypti* larvae by using the water dispersible granule (WDG) formulation of *Bti*-H14 manufactured by Abbott Laboratories at the dosages of 3000,6000, and 15000 ITU/liter were effective against *Ae. aegypti* giving a minimum of 42 days of effective killing activity. The residual effect increases as the dosages increase (Lee, YW and Zairi, 2005). In Sub-Saharan Africa malaria remains one of the major health problems, *Bti*-

H14 WDG used in tubs against *Anopheles* spp, all used concentrations successfully killed 100% of all larvae within 24 hours (Dambach *et al*., 2014). Bactivec SC *Bti*-H14, liquid formulation assayed by field trial in natural breeding habitats of *An. stephensi*, *Cx. quinquefasciatus*, and *Ae. aegypti*, in freshwater habitats 1mL/50-liter dosage was found to be effective, whereas in polluted water habitats against *Cx. quinquefasciatus* 5ml/m^2 was found effective (Uragayala *et al*., 2018). Aerial application of Vecto Bac WG containing *Bti* strain AM 65-52 caused a significant reduction in the adult female population throughout the summer in Florida USA (Pruszynski *et al*., 2017).

An interventional, analytical study of *Bti* larviciding with liquid and granular formulation from 2012-2013 was carried out in Bobirwa of Botswana, larviciding reduced larval density by 95% in Bobirwa in 2012 with two cases of malaria, while in 2013 larval density reduction was 81% with 11 cases of malaria in comparison with the non-larvicide Shorobe of Botswana 20 and 70 cases of malaria were detected in the respective years (Obopile *et al*., 2018).

2.8 Mosquitocidal *Ls*

Ls is an endospore-producing Gram-positive aerobic bacterium widely distributed in soil and water. Previously known as *Bs* reclassified as *Ls* because the cell wall of this bacteria contains lysin in the peptidoglycan. It produces a spherical spore at the terminal or subterminal end during sporulation in a swollen sporangium. The *Bs* strain toxic to mosquito larvae was first reported by (Kellen *et al*., 1965). The first reported *Bs* strain active against mosquito larvae was isolated from moribund larvae (Charles *et al*., 1996). Initially, the isolated *Bs* strain showed less toxicity towards the mosquito larvae. So, due to low toxicity, its use in mosquito control would never have been considered, by renewed search exhibited to isolate higher levels of mosquitocidal activity *Bs* stain. Several different strains of *Bs* have been isolated showing different levels of toxicity. *Bs* strains are generally highly active against larvae of *Anopheles* and *Culex* species and are poorly or not toxic to larvae of *Aedes* species (Charles *et al*., 1996). The mosquitocidal property of *Ls* is due to the production of two kinds of toxins crystal toxin or binary toxin (Bin) BinA and BinB and Mosquitocidal toxin (Mtx) Mtx1, Mtx2, Mtx3. Strain-producing both toxins are highly toxic to mosquito larvae. They differ both in their composition and time of synthesis. The crystal toxin is composed of two proteins that are synthesized in equimolar amounts and assembled in crystal structures (Charles *et al*., 1996). The amino acid sequences of these crystal proteins are not similar

to those of any other bacterial toxins, including those produced by *Bti*. Therefore, crystal proteins of *Bs* constitute a separate family of insecticidal toxins.

2.9 Larval source management (LSM)

In recent years LSM has focused on a supplementary approach to manage larval habitats to prevent the development of adult mosquitoes to protect from the bites of infected female mosquitoes. LSM targets the immature larvae, aquatic stages of mosquitoes mainly larvae and pupae, thereby decreasing the abundance of adult vectors. If all potential breeding sites of mosquitoes were cleared, it could be expected that LSM would be the best method for reducing the number of mosquito bites per person per year (Fillinger & Lindsay, 2011; Imbahale *et al*., 2012; Mukabana *et al.*, 2022; Olalubi, 2016; WHO., 2013). LSM mainly focuses on managing different aquatic habitats (water bodies) which are the main source of mosquito larvae, which prevents the completion of development of the immature stages. For LSM it is necessary to carry the entomological surveillance to know the breeding habitats and resting places of mosquitoes**.** Entomological surveillance is defined as the systematic collection, analysis, and interpretation of vector data, or measurement of the abundance of arthropod (mosquito) vectors of human diseases. The purpose of entomological surveillance is to (i) determine the changes in the geographical distribution of vectors, (ii) identification of high dense infestation areas or periods of mosquito, (iii) major habitats, (iv) to detect newly introduced mosquitoes in an area or a locality, (v) for monitoring and evaluation of control programs, (vi) to use the most appropriate vector control tools, (vii) monitor the effectiveness of the tools, (viii) for obtaining vector population overtime and for facilitating appropriate and timely decisions regarding interventions (ix) for monitoring insecticidal resistance or susceptibility etc. (WHO, 2003, 2009b, 2011). The most common practice to predict the infestation is larvae, and pupae surveillance in the houses or premises by inspecting water storage containers or discarded containers. The indices commonly used to record *Ae. aegypti* and *Ae. albopictus* density levels are calculated by using a formula.

The House Index (HI) or *Aedes* mosquito Index: percentage of residents or premises that detect larvae of *Aedes* mosquito. The house index is calculated as the formula mentioned below:

$$\text{HI} = \frac{\text{No. of houses positive for } \textit{Aedes} \text{ mosquito larvae}}{\text{No. of houses screening for } \textit{Aedes} \text{ larvae}} \quad \text{x}100\%$$

2. Container Index (CI): It is the total percentage of containers detected larvae of *Aedes* mosquito.

Cl is calculated as follows:

$$\text{CI} = \frac{\text{No. of positive containers}}{\text{No. of container inspected for } Aedes \text{ larvae}} \quad \text{x } 100\%$$

3. Breteau Index (BI): number of containers that contain *Aedes* mosquito larvae per 100 houses in a specified area.

BI is calculated as follows:

$$\text{BI} = \frac{\text{No. of containers with } Aedes \text{ larvae}}{\text{No. of houses screening for } Aedes \text{ larvae}} \quad \text{x } 100\%$$

These entomological indices are the most widely used indices for monitoring the infestation level, for evaluation of the vector control program, to determine the efficacy of a newly produced larvicide, etc. In the context of Nepal, this indices' critical threshold value has not been determined for dengue outbreak, but the national guidelines have stated to monitor this indices value to determine the infestation level in a particular site (Department of Health Services. Epidemiology and Disease Control Division., 2019). Indices like HI, CI, and BI should be monitored before and after the intervention or vector control measures to evaluate the efficacy of the interventions and control measures. Various types of research studies (Bigio *et al*., 2022; Ferede *et al*., 2018; Getachew *et al*., 2015; Padonou *et al*., 2020; Udayanga *et al*., 2018) show considerable reduction in HI, CI, and BI indices, reduce the number of dengue cases and no death due to dengue was reported after the intervention. To set threshold values longitudinal studies should be carried out (Udayanga *et al*., 2018). However, the threshold value may be different according to the geographical regions.

WHO recommended four different types of larval source management (LSM);

1. Modification of habitat: a permanent change to the environment, e.g. land reclamation;
2. Manipulation of habitat: a displacement activity, e.g. flushing of streams;
3. Use of larviciding: frequent application of various biological or chemical insecticides to water bodies;

4. Use of biological control agents: Use of natural predators in various water bodies.

As the dengue vector likes to live around human surroundings, it has become necessary for every human to become alert and protect themselves from the bite by the intervention of vector management packages to control the vector inside the human inhabitants. Various studies have shown the integrated larval source management measures adopted by the community people in an urban area resulted in the reduction of dengue cases and deaths. For instance, a cluster randomized trial of a package of interventions like putting guppy fish in various household containers, trapping adult mosquitoes, taking off mosquito breeding sites from around the houses, and providing community education about dengue, particularly to school children, resulted in reducing entomological indices (CI, HI, BI) that in turned reduced the dengue cases in Champong Cham provinces Cambodia (Bigio *et al*., 2022). A similar outcome was obtained by Imbahale *et al*., 2012 in western Kenya and by (Ingabire *et al*., 2017) in Rwanda in the reduction of malaria mosquito density in the rice field with an LSM approach by using *Bti*, larvivores fish, and community participation.

2.10 Characterization of *Bt*

Bt is isolated from different sources by using acetate selection methods. The acetate present in the broth media encourages spores of other *Bacillus* spp in the samples to germinate during enrichment but discourages the spores of *Bt* bacteria. When the broth media after overnight incubation is provided with the heat shock the germinated cells are killed due to heat but the spore of *Bt* remains viable and on subculturing the broth in selective media T3 or NA by spread plate technique or pour plate technique *Bt* spore germinate producing isolated colonies within 24-48 hours. From the isolated colonies differential staining techniques like Gram staining, spore staining, and coomassie brilliant blue staining technique were performed. The *Bt* bacteria are identified by observing the unique crystal protein stained by coomassie brilliant blue under a light microscope. Under the light microscope with 100X magnification three distinct morphological structures can be observed the vegetative cell of *Bt* will appear as blue rod-shaped, and the crystal proteins will appear as dark blue in color depending upon the type of *Bt* strain, the shape of the crystal protein is different and the spores appear as empty and light blue in color. In other *Bacillus* species like *B. cereus*, the extract's unique crystal protein structure is absent. By observing the crystal protein, the isolates

are confirmed as *Bt* bacteria present in the sample. But in the case of some *Bt* strain the crystal protein remains attached to the spore the attached crystal protein will be stained dark blue in color and the spore light blue in color they are also referred to as capheaded crystal protein or spore-attached crystal protein. The staining technique especially CBB is the primary identification of *Bt* bacteria. Further, the crystal protein shape and size can be confirmed by using a scanning electron microscope (SEM) along with Image J software to measure the size of the crystal protein.

Classification of subspecies or varieties based on serotyping using H-serovars (flagellar serotyping) resulted in the identification of almost 71 H serotypes and 84 serovars (Wang *et al*., 2013).

2.11 Insect bioassay

Production of insect mass of different stages under laboratory conditions is necessary to evaluate the effectiveness of newly produced insecticides towards the different stages of a particular insect pest. Before commercialization of the insecticides, the efficacy of the compound must be determined in the laboratory conditions as well as by the field trial. Due to the unavailability of the mosquito colony, different types of phytochemicals cannot be evaluated to determine the efficacy for large-scale production as a protective measure like repellent, larvicide, pupicide, or adulticide to control mosquito-borne disease in Nepal. Bioassay are method for determining the entomotoxicity of *Bt* or bioactive against different types of insect pests. Bioassay further characterizes the *Bt* bacteria based on the crystal protein. The *Bt* crystal proteins are found to be toxic to specific insect pests. To determine the specificity of the crystal protein pure cultures of *Bt* are cultured in a defined media to produce the crystal protein and spore. Primary screening it toxicity against insect pests is by insects reared in laboratory conditions and fed with the spore and crystal protein mixture to identify the specificity of the *Bt* isolates. This type of bioassay characterizes the presence of different strains of *Bt*. The insect bioassay helps to obtain the specific species with higher toxicity.

2.12 Molecular characterization

There are various molecular approaches for identifying or detecting the novel gene or similar genes present in the *Bt* isolate obtained from different geographical regions. The 16srRNA gene sequencing is the standard method to identify bacteria and for

taxonomic classification of bacterial species. The 16srRNA gene is universally present in all prokaryotes and has multiple sub-regions namely hypervariable region V1-V9 which can be used for distinct identification of various prokaryotes. Along with the hypervariable region, there are the conserved regions C1-C9 across all prokaryotes (Bukin *et al*., 2019; Johnson *et al*., 2019; Patwardhan *et al*., 2014; Petrosino *et al*., 2009). The 16srRNA gene sequencing is used as a tool to identify bacteria at the species level and assist with differentiating between closely related bacterial species. It was observed that the 16srRNA sequence is mostly used for comparing it with related Genera, gene sequences present in the database for species identification or to distinguish from other related species. In GenBank, the sequences from the different countries are deposited and are compared for identification. The divergence and their evolutionary relatedness among the sequences are not known. The size of the 16srRNA gene sequence length is 1500 basepair (bp). However, the deposited 16srRNA gene sequences in the database are not in this length, the short-length sequences also align with the comparative sequences or query sequence and are identified as a particular organism. Plasmid profiling is used to detect the types of plasmid present in the *Bt*. Types of cry genes. Protein profiling is done to determine the molecular weight of the crystal protein. Whole genome sequencing is done to determine the types of genes and their respective bioactive product. As well as other molecular techniques have been utilized to study the genomic integrity of the *Bt* bacteria.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection and size

Soil (10gm) samples were collected by convenient sampling method from the different geographical regions of Nepal in a Ziplock bag, 5cm below the surface by clearing the decaying leaves and other organic matter (Ammouneh *et al*., 2011). Soil samples were collected from a diverges location as much as possible, forest, fertile land, uncultivated land, nearby ponds, lakes, streams, University premises, parks, etc. The collected soil sample was brought to the laboratory and stored at room temperature before the isolation of native *Bt*. The defined sample size may not provide the chance of isolation of larvicidal *Bt* so, to increase the possibility greater the 300 soil samples were collected. The study was carried out in Kathmandu, Nepal in the Central Department of Microbiology, after obtaining ethical approval from the Nepal Health Research Council. A show description of the study is provided in the Flow chart Figure 3.

3.2 Laboratory analysis

3.2.1 Isolation of *Bacillus thuringiensis* (*Bt*)

Isolation of *Bt* was carried out by the acetate selection method, 1gm soil was added to a 10mL test tube containing 9mL of nutrient broth (NB) acetated with 0.25M sodium acetate and enriched overnight at 28^0C in an incubator. After enrichment, the broth was exposed to heat shock (100^0C) in a water bath for 5 minutes as described by (Travers *et al*., 1987) with slight modification. A spread plate technique was performed for the isolation of *Bt* in nutrient agar (NA) by loading 100µl of serially diluted nutrient broth in a saline solution after heat shock. The plates were incubated at 28^0C for 48 hours in an incubator. The isolated colonies were further sub-cultured to obtain the pure culture in NA. The isolates were identified as *Bt* by observing coomassie brilliant blue stained (0.133% Coomassie Brilliant Blue (CBB) G250 in 50%acetic acid) crystal protein and its shape under the light microscopy, as described by (Rampersad *et al*., 2002) from a 48 hours culture plate. Isolates identified as *Bt* were preserved in a nutrient agar slant with 60% glycerol.

3.2.2 Isolation of *Lysinibacillus sphaericus* (*Ls*)

In the heat shock selection method, 10 grams of soil sample was added to 100 mL of saline solution in a conical flask homogenized manually and placed in a shaker water bath maintained at a temperature of 80^0C for 30 minutes (Suryadi *et al*., 2015). After heat treatment, the suspension was tenfold serially diluted in saline solution and from 10^{-2} and 10^{-3} dilutions, 100 µl of each dilution was spread in nutrient yeast extract salt medium (NYSM) (nutrient agar enriched with 0.5g/L yeast extract, 0.2g/L $MgCl_2$, 0.01g/L $MnCl_2$, and 0.1g/L $CaCl_2$) by spread plate technique (Suryadi *et al*., 2015). The plates were incubated at 28^0C for 48 hours. The isolated colonies were subcultured in NA and preserved in NA agar slant with 60% glycerol at -20^0C. Identification of the isolates was done through their cultural and microscopic characteristics. To study the microscopic characteristics pure culture of the isolates obtained in nutrient agar was stained by three different staining techniques Gram staining, spore staining, and coomassie brilliant blue staining (CBB) staining. Biochemical properties like catalase, oxidase, and substrate hydrolysis tests like starch, gelatin, casein, and Tween 20, hydrolysis tests were performed.

3.3 Molecular characterization

3.3.1 DNA extraction

The larvicidal *Bt* isolates from a fresh NA plate culture were inoculated into 5 mL LB broth and incubated at 28^0C in shaker water for 16 hours. After 16 hours the broth was centrifuged in 1.5mL Eppendorf tube in a bench-top centrifuge at 8000rmp for 10 minutes to obtain the pellet. The DNA was extracted from the pellet by using a Qiagen kit QlAMP DNA Mini Kit (50) Cat.No. 51304 at room temperature as per the manufacturer's instruction.

3.3.2 16srRNA gene analysis

The extracted DNA was used for PCR amplification of the 16srRNA gene by using the universal primers 27 Forward- 5’ *(AGA GTT TGA TCM TGG CTC AG)* 3’ and 1492 Reverse- 5’ *(TAC GGY TAC CTT GTT ACG ACT T*) 3’. The concentration of DNA used for PCR is between 10 and 30ng/microliter. DNA concentration was determined using Nanodrop. The PCR program includes an initial denaturation step of 3min at 94^0C followed by 30 cycles with a denaturation of 1 min, a hybridization of 30s at 45^0C

followed by an extension of 2 min at 72^0C for the primer pair, and finally a final elongation of 10min at 72^0C.

3.3.3 16srRNA gene sequence

The bidirectional sequencing of the PCR product was done by using the primers 785 Forward- 5' *(GGA TTA GAT ACC CTG GTA)* 3' and 907 Reverse- 5' *(CCG TCA ATT CMT TTR AGT TT)* 3'in Macrogen, Seoul Korea.

3.3.4 Phylogenetic tree

The obtained sequences as query sequences were searched against the NCBI nucleotide database (www.ncbi.nlm.nih.gov) by using the BLAST tool for identification and the determination of genetic similarity between the existing larvicidal *Bt* bacteria in the GenBank. Using the MEGA program, a multiple sequence alignment was built using the 16srRNA gene sequences of larvicidal and non-larvicidal *Bt* and sequence from *B. cereus*, *E. coli* and *B. megaterium* as an outgroup to design a boot-strapped neighbor joining phylogenetic tree to establish the relative degree of genetic similarity between the various sequences.

3.4 Bioassay

Larvicidal activity of the identified *Bt* isolates was carried out by (i) preliminary screening against the field-collected larvae, (ii) selective bioassay against field-collected mosquito larvae and laboratory-reared *Ae. aegypti* larvae.

3.4.1 Collection of mosquito larvae

Mosquito larvae were collected from Kathmandu and Lalitpur districts during the period (June 2021-September 2021 and April 2022-September 2022) in 200ml plastic cups by using a dropper or dipper or strainer depending upon the need to obtain larvae from the discarded tires, water store domestic container, puddles, metal drums, flower pots cemented tanks or other water source were larval activity was observed (Medlock *et al.,* 2018). The collected larvae were transferred into a transparent Ziplock plastic bag containing clean water and brought to the laboratory.

3.4.2 Laboratory rearing of field-collected larvae

The collected larvae were placed in enamel trays containing water that was dechlorinated for 2 days for acclimatization before using them for larvicidal activity.

The larvae were fed with the baker's yeast and dog biscuits (Devidas *et al*., 2014). The larvae which turned into pupae were collected and removed from the trays. Only those larvae showing acclimatized healthy and active movement were used for larvicidal activity experiments.

3.4.3 Preliminary screening for larvicidal activity

All the *Bt* isolates producing spherical-shaped crystal proteins were screened by preliminary screening. For preliminary screening, 25 larvae, size range (9mm -14mm) equivalent to 3rd instar to early 4th instars were placed in a plastic cup containing 100mL sterile distilled water. A fresh culture of *Bt* isolates in the NA plate after 48 hours of incubation was taken for the assay. With the help of a standard, sterilized loop, three loopfuls of culture were scooped (≈ 0.03mg (0.00003%)), and added to the cups containing larvae, homogenized gently without harming the larvae. The culture contains a crude mixture of spore, crystal protein, and vegetative cells. The cups were incubated at room temperature for 24 hours the experiment was performed in the months of August and September 2021 and 2022. The average room temperature recorded during the analysis was 24.5^{0}C and relative humidity (RH) was 66% obtained from the mobile app (Room Temperature Thermometer, Morsol Technology). After 24 hours the number of alive larvae was counted. The experiment was repeated if more than 10% of pupa were pupated from the larvae within 24 hours (WHO, 2005). The *Bt* isolate showing larvicidal activity further confirmed by selective bioassay.

3.4.4 Standard reference strain (control strain)

Bacillus thuringiensis var *israelensis* (*Bti*) IPS-82 positive control strain was provided by the Pasteur Institut, Paris, France. The control strain was culture in the laboratory condition in NA. The phenotyping and biotyping of the control strain were performed along with the native strain in the same environmental condition. The larvicidal activity of the control strain was performed along with the native *Bt* isolates.

3.4.5 Selective bioassay

A selective bioassay was performed after the preliminary screening of *Bt* showing 100% mortality against the field-collected larvae. Selective bioassay was performed in four replicates. In a plastic cup, 100mL of sterile distilled water and 25 larvae of 3rd instar to early 4th instar larvae were added to each cup. A fresh culture of native *Bt* showing 100% mortality during the preliminary screening was added by using a

standard and sterilized loop, as described for the preliminary screening. Similarly, for positive control fresh culture of standard strain, *Bti*-IPS-82 was added to four cups as of the test *Bt*. Negative control cups contain 25 larvae and 100mL of sterile distilled water in each four cups. All the cups were placed at room temperature for 24 hours. After 24 hours the number of live larvae was counted (WHO, 2005). The experiment was repeated if more than 10% of pupa were pupated from the larvae in 24 hours in negative control or positive control and also in the test cups.

3.4.6 Confirmation of larvicidal mortality

To confirm that the larval mortality occurred due to feeding of the toxin of the inoculated *Bt* or other contaminations, a single dead larva was removed from the positive control cup and test cup, washed with sterile distilled water, and surface sterilized with 70% ethanol twice after evaporation of the alcohol the larvae was crushed in a test tube with a sterilized glass rod in a 1mL of sterile saline solution. Then 100µl of the saline solution was spread with a sterilized "L" shaped glass rod on the nutrient agar surface and incubated at 28^0C for 48 hours. For conformation test that all spherical crystal protein producing *Bt* are not toxic to the mosquito larvae was done by inoculating the larvicidal positive *Bt* and *Bt*-3P2A (spherical ICPs) 48 hours fresh culture colony in a cup containing 25 larvae and 100mL sterile distilled water as mentioned elsewhere.

3.4.7 Quantitative bioassay against field-collected larvae

3.4.7.1 Preparation of pellet

For the preparation of the pellet, the *Bt*-14P2A and *Bti*-IPS-82 were inoculated in 1000mL of Nutrient broth at 28^0C for 48 hours in a shaker water bath at rpm 90. After 48 hours the broth was poured into a 50 mL Tarson falcon tube and centrifuged 5000rpm for 5 minutes in a Biobase centrifuge. The sediments of both the isolates *Bt*-14P2A and *Bti*-IPS-82 collected from the centrifuge were dried for 24 hours in a hot air oven with a temperature of 30^0C. The dried pellet was stored at -20^0C before analysis.

3.4.7.2 Preparation of stock solution

A stock solution of *Bti*-IPS-82 and *Bt*-14P2A was prepared by dissolving 1g of the dried pellet in a 100mL of sterile distilled water in a 200mL of conical flask. Thus, the concentration of the prepared stock solution is 1% or 10000PPM. The prepared stock

solution was checked for contamination by enumerating 100µl of the tenfold diluted stock solution in MA, MSA, and NA as well an endospores count was performed in NA (WHO, 2005). The prepared stock solution was stored at -20 before use.

3.4.7.3 Preparation of working solution

A working solution was prepared by transferring 10mL of stock solution (10000PPM) in a sterile falcon tube. Then the stock solution was tenfold diluted serially in sterile distilled water to obtain the following concentrations 1000PPM, 100PPM, 10PPM, 1PPM, and 0.1PPM.

3.4.7.4 Bioassay

Plastic cups were labeled with the concentration and the type of larvicide to be added. Six different concentrations (10000PPM, 1000PPM, 100PPM, 10PPM, 1PPM, and 0.1PPM) of *Bti*-IPS-82 and *Bt*-14P2A were analyzed parallel in the same laboratory condition at the same time. Plastic cups were filled with 100mL sterile distilled water. Field-collected acclimatized and healthy 25 late 3^{rd} or early 4^{th} instar larvae were added to the cup by using a strainer. Each concentration of four replicate cups was analyzed. The four replicates of the negative control cup contained sterile distilled water and 25 larvae were also incubated with the test cups. Using a micropipettes 1000µl of the concentration of *Bti*-IPS-82 and *Bt*-14P2A was added to their respective cups. The final concentration of *Bti*-IPS-82 and *Bt*-14P2A in the cups became 100PPM, 10PPM, 1PPM, 0.1PPM, 0.01PPM, and 0.001PPM the cups were incubated at room temperature for 24 hours. After 24 hours mortality of the larvae was determined by counting the live larvae. The numbers of live larvae were counted in each cup. No food was added to the cups. If more than 10% larva pupates the test was repeated (WHO, 2005). The bioassay was performed in a laboratory condition at 28^0C 66% RH. The mortality percentage was calculated and the LC_{50} and LC_{90} dosages were determined.

3.4.7.5 Quantitative bioassay against *Ae. aegypti* larvae

3.4.7.6 Collection of larvae

Larval and pupal *Ae. aegypti* were collected from the discarded tires, considering, discarded tires are the main source of *Aedes* (Gautam *et al*., 2009; Dhimal *et al*., 2015) using dropper, dipper, and strainer in plastic cups from Kathmandu and Lalitpur districts of Nepal during August to September 2021 and 2022 was carried (Medlock *et*

al., 2018). Informed consent from each house owner was obtained prior to sample collection. The larvae and the pupa were separated and transferred to a Zip-lock bag containing clean water and brought to the laboratory. The collected larvae and pupa were transferred to the white enamel tray containing chlorine-free clean water. The pupae were placed in a plastic transparent cup containing a small amount of clean water each plastic cups containing 10-12 pupae and the mouth of the cup were closed by using a muslin cloth. The cups were placed at room temperature until the emergence of adult mosquitoes. Similarly, the larvae in the tray were fed with the dog biscuit and yeast until they pupate. After pupation, they were transferred to the plastic cup until the emergence of adults as described earlier. The adult mosquitoes were aspirated by using an aspirator regularly and identified.

3.4.7.7 Identification keys for *Aedes*

Identification of mosquitoes was done only after the emergence of adults; simple identification keys were followed to identify *Ae. aegypti*. The emerged adult mosquitoes were placed in a test tube by using an aspirator, with the help of a magnifying lens, the following morphological key characteristics were observed for identification of *Ae. aegypti* (a) silvery white markings on legs and abdomen, vertex with erect forked scales not numerous restricted to occiput. (b) Contrasting black and white coloration, clypeus with scale patches, mesepimeron with two well-separated white scale patches. (c) scutum (dorsal part of the thorax) has silver scales in the shape of a lyre (a pair of lateral curved white lines, half-moon shaped) on the back in both males and females or scutum black or brown with a pair of submedian longitudinal white stripes, but without median longitudinal white stripe, or with white lyre shaped marking. Male and female identification keys used, male (i) feathery antenna (ii) palpi longer than the proboscis. Female (i) less hairy antenna (ii) Palpi ¼ to half of the proboscis as described (Rueda, 2004; WHO, 2020a).

3.4.7.8 Rearing *Ae. aegypti* in the laboratory

Preparation of adult-rearing cage

A cubical frame was made from the iron plate size 24 x 24cm. The frame was wrapped with a fine mesh around it. With an opening at one side of the frame with a long fine net to tie a knot for closing the cage (Imam *et al*., 2014; Kauffman *et al*., 2017; Onyido *et al*., 2009).

The identified adult *Ae. aegypti* male and female mosquitoes were placed inside the rearing cage, in a ratio of 1:3 (male: female). The ratio was tried to maintain by regular adding of male and female adult mosquitoes. Inside the cage, the adult mosquitoes were fed with the 10% sucrose. A long cotton plug was dipped in a 10% sucrose and placed in a 100mL beaker in an upright position. A small oviposition was placed inside the insectary. The oviposition contains a small amount of water and at the opening end, a white filter paper was placed on it to lay the eggs by the female. The female was fed with the expired human blood collected from the blood bank (Imam *et al*., 2014).

The blood was fed during the daytime. The blood was heated in a heating gauge at a temperature of 40^0C. Heated blood is placed on a cotton roll with the help of a dropper until the cotton roll gets damped with the blood. The cotton roll was placed above the cage. This process was repeated 5 times or more in a day. The laid eggs were collected in a white filter strip.

The eggs with the strips were cut into pieces and dipped in a white enamel tray containing water and incubated at room temperature until the development of immature. The immature was fed with dog biscuits and baker's yeast after two days the immature were transferred to clean chlorine-free water and fed with the same diets. These larvae when they reach the 3^{rd} instar and early 4^{th} instar were used for larvicidal assay.

For quantitative bioassay of *Ae. aegypti* larvae was performed as described for quantitative bioassay of field-collected larvae. The working solution was prepared from the stock solution prepared to assay the field-collected larvae. The *Ae. aegypti* larvae reared in the laboratory were used to determine the larvicidal activity of native *Bt*. The bioassay was performed in a laboratory condition at 28^0C and 66% RH. The mortality percentage was calculated and the LC_{50} and LC_{90} dosages were determined.

3.5 Formulation of *Bt*-14P2A bio larvicide

Bio larvicide *Bt*-14P2A was prepared in the laboratory in a media (T3). The media was sterilized in an autoclave at 121^oC for 15 minutes. To the sterilized media 2% of the broth containing the growth of *Bt*-14P2A was added and incubated at 28^0C for 72 hours. After 72 hours, liquid bio larvicide was cultured in MacConkey Agar (MA) and Mannitol Salt Agar (MSA) to detect the presence of contamination of the bio larvicide by pathogens like *E. coli* and *S. aureus*.

3.6 Field trial

Two types of field trials were carried out (i) stimulated field trials and (ii) open field trials. The field trial was carried out in Kathmandu and Lalitpur districts during the month of April 2023 to November 2023 in the containers mainly focusing on controlling the dengue vector *Aedes* mosquito larvae in domestic containers like buckets, drums, tubs, jars, etc.

A simulated field trial was carried out on the premises of the Central Department of Microbiology, Tribhuvan University in the shaded place outside the laboratory. To determine the efficacy of *Bt*-14P2A bio larvicide prepared from the native *Bt* isolated from the soil samples of Nepal along with to determine the dosage required to control the mosquito in open field conditions in different environments.

3.6.1 Simulated field trial

Small plastic black pots of capacity 1550 mL were used for the simulated field trial. The plastic containers were filled with dechlorinated water of about 1500mL and felt for acclimatization for 24 hours. Six different concentrations of *Bt*-14P2A with four replicates for each concentration and four replicates for the control, the total number of containers used was 28. After 24 hours 25 larvae were added to each container. The larvae were fed with dog biscuits and baker's yeast and left for acclimatization for four hours under the shade of the roof covered the mouth of the containers with muslin cloth and tied around it necks using a thread (WHO, 2005). The containers were labeled according to the concentration to be added to them.

3.6.1.1 Treatment

After four hours of acclimatization to the containers according to the label dosage of *Bt*-14P2A was added (6mL/Lt, 4mL/Lt, 3ml/Lt, 2mL/Lt, 1.5mL/Lt, and 1mL/Lt) and the containers were closed, felt under the natural environment. After 24 hours the number of alive larvae and pupae were counted in all the containers and tabulated. Thereafter every seventh day new set of 25 larvae was added to all the containers and supplied with a pinch of larval food dog biscuit and bakers' yeast. The larval survival was assessed after 24 hours. The number of alive larvae and pupae were counted and tabulated.

After 24 hours 25 third and early fourth instar larvae were added to each container. The larvae were supplied with a pinch of dog biscuits together with baker's yeast and left for acclimatization for four hours. After four hours dosages (1.5mL/liter, 1mL/liter, 2mL/liter, 3mL/liter, 4mL/liter, and 6mL/liter) were added as labeled and the containers were incubated under the shade of the roof covering the mouth of the containers with muslin cloth and tied around their necks using a thread. The number of alive larvae was assessed after 24 hours in all the containers and tabulated in the case of the four control containers all the alive larvae were removed and discarded. Thereafter every seventh day a new set of 25 larvae was added to all the containers and supplied with a pinch of larval food dog biscuit and bakers' yeast. The larval survival was assessed after 24 hours the process was repeated to obtain the lowest mean mortality of the larvae. The containers were not replenished with water during the study period. The pH and the temperature of the water were noted every week before adding the new batch of larvae by using a pH meter and thermometer. The simulated field trial was continued until the mortality percentage was <50%. The mean mortality percentage was determined by comparing it with the control by using the formula mentioned elsewhere (Derua *et al*., 2022; Ritchie *et al*., 2010; WHO, 2005).

3.6.2 Open field trial in different sites

An open field trial was carried out in different environmental conditions by using different types of containers in different sites in Kathmandu and Lalitpur.

3.6.2.1 Central Department of Microbiology (CDM) 27°40'59.7"N 85°17'15.7"E

Tires, buckets, and jars with a defined amount of chlorine water were installed in the open field of the Department premises as well as in the corner of the corridors for oviposition of the mosquito naturally.

3.6.2.2 Maharajgunj, Kathmandu site 27°44'17.1"N 85°20'27.8"E

In Maharajgunj a residential area was selected and in the pre-domestic area inside the compound wall 12 buckets of capacity 25 liters were installed at a distance of 2 meters in the open field as well as in the shaded places. The area was surrounded by green vegetation.

3.6.2.3 Sifal, Kathmandu site 27°42'48.0"N 85°20'40.7"E

It is a residential area in the pre-domestic area inside the compound wall 12 buckets of capacity 25 liters were installed at a distance greater than 2 meters in the open field. The area is surrounded by a green vegetation.

3.6.2.4 Tribhuvan University, Kirtipur, Kathmandu 27°40'46.3"N 85°17'35.6"E

In the dean's office premises on the open field near the vegetation area lots of tree tires were exposed to collect rainwater that serves as an oviposition for the mosquito present in the jungle area to lay eggs and hatch into larvae.

3.6.2.5 Panga Charghare, Kirtipur, Kathmandu site A 27°39'44.6"N 85°16'43.4"E

In pre-domestic area A, B, and C three buckets in each was installed filled in the open field and under the shaded place. In a small vegetable farm, a bucket was installed in the open field to collect rainwater for oviposition.

3.6.2.6 Panga Charghare, Kirtipur, Kathmandu site B 27°39'44.6"N 85°16'43.4"E

In pre-domestic area a 100-liter capacity, two black drums filled with water with a broken rim was used as a control and test.

3.6.2.7 Cow farm Panga, Kirtipur, Kathmandu site 27°39'44.6"N 85°16'43.4"E

Outside the cow farm two buckets were installed in an open area to collect rainwater for oviposition. In this area the cow farm is surrounded by vegetation.

3.6.2.8 Open cornfield Kirtipur, Kathmandu site 27°39'44.6"N 85°16'43.4"E

Panga, and Champadevi site 1 and Champadevi site 2, Kirtipur,

In the cornfield, buckets were installed at different plots of the cornfield to collect rainwater and for oviposition of the mosquito to lay eggs and to hatch them to larvae.

3.6.2.9 Champadevi, Kirtipur Kathmandu site 27°40'00.2"N 85°15'25.5"E

In Champadevi sites A, B, and C three buckets filled with water were installed in each site indoors of the house on the balcony, veranda and in the terrace. In pre-domestic area of Champadevi sites D, and E three buckets were installed at each site in the open area inside the compound wall. In Champadevi site F tubs six were installed in the pre-domestic area filled with water in the shaded place. At this sites *Bt*-14P2A bio larvicide was also added to a small puddle.

3.6.2.10 Vegetable farm Kirtipur, Kathmandu site 27°40'00.2"N 85°15'25.5"E

In a small vegetable farm three jars were installed in the open field by collecting rainwater for oviposition.

3.6.2.11 Bafal, Kathmandu site 27°42'15.6"N 85°16'54.3"E

Installation of three buckets of capacity 15 liters in the pre-domestic area filled with water and left undisturbed for oviposition. Three as a test bucket.

3.6.2.12 Sitapaila Kathmandu site 27°42'17.2"N 85°16'42.6"E

Installation of four buckets of capacity 25 liters filled with water inside the garage of a house for oviposition two serve as control and two serve as test.

3.6.2.13 Kalanki, Kathmandu site 27.6931^0N, 85.3474^0E

Tire resoling garage where the rainwater is collected in discarded tires in the open area, where larvae and the pupae present were selected as a site for treatment with bio larvicide.

3.6.2.16 Koteshwor, Kathmandu site 27°40'13.0"N 85°20'54.3"E

Indoor six tubs of capacity 15 liters filled with rainwater were installed in the different corners of the house for oviposition and left undisturbed until the development of the larval stage. Three tubs as control and three tubs as a test.

In the pre-domestic area 10 flower pots with bottoms, and three buckets, were placed at different place inside the compound wall of the house in the open field for oviposition.

3.6.2.17 Garbage collection site A and Garbage collection site B at Koteshwor, Kathmandu 27°40'13.0"N 85°20'54.3"E

Garbage collection site A is a control site where one drum made of metal, a small cut can, and jars where rainwater gets collected due to exposure to an open area. Similarly, at site B one drum made of metal was used for treatment.

3.6.2.18 Chicken and meat shop in Koteshwor, Kathmandu 27°40'13.0"N 85°20'54.3"E

Two shops were selected one as a control and the other as a test a bucket was installed in each shop underneath the cutting table by filling them with water for oviposition.

3.6.2.19 Small cow farm in Koteshwor, Kathmandu 27°40'13.0"N 85°20'54.3"E

Plastic drums of 500 liters were used to collect rainwater to feed the cows and for other recreational activities under the roof of the house.

3.6.2.14 Gwarko, Lalitpur site 27°40'08.7"N 85°20'02.3"E

Discarded tires placed in an open area in an automobile workshop were selected as a control site.

3.6.2.15 Imadole, Lalitpur site 27°39'36.8"N 85°20'42.0"E

In the pre-domestic areas, three buckets and one drum filled with water used for the treatment in the shaded place at the entrance of the house. In the vegetable garden, two jar of capacity 20 liters were exposed for oviposition after collection of rainwater.

3.6.2.16 Puddles in the UN park 27°41'09.0"N 85°19'34.5"E, Roadside, Sankha park 27°40'44.3"N 85°19'52.3"E, and in Manohara park 27°40'08.6"N 85°21'07.1"E

Puddles were observed for the larval source and in the positive puddles *Bt*-14P2A larvicide 10-30 mL was added surrounding the puddles and the mortality of the larvae was observed after 24 hours.

3.6.3 Containers

Different types of containers like buckets (1.5-liter, 2-liter, 15-liter, and 25-liter), tubs, black drums (100 liters), metal drums (500 liters), jars made of plastics, etc., were used as an ovipositional site for mosquitoes. Discarded tires of different vehicles trucks, scooters, motorcycles, vans, jeeps, etc., were used for oviposition as well. A measuring cylinder with a capacity of 100mL made of plastic was used to measure the bio larvicide while adding it to the field. Plastic cups and white enamel trays were used for rearing the larvae and pupae for identification after the emergence of adult mosquitoes.

3.6.4 Surveillance of mosquito larvae

Surveillance of mosquitoes in different types of containers was carried out from April to November 2023. Some of the site owner's containers were surveyed for the immature. The immatures were counted and transported to the laboratory for identification. The containers were reinstalled in the same place without any change and observed after a

week for immature, if positive they were counted and treated with the bio larvicide *Bt*-14P2A. In most of the sites buckets of different capacities, tubs, and jars were installed in the open area, in the pre-domestic area, indoors of a house, and in the cornfield with permission. The installed containers in the open field were filled manually with the water for oviposition when there was no rainfall and during the rainfall the containers were filled with the rainwater. In the pre-domestic area and the indoor the containers were filled manually with water for oviposition when they were in the shaded place. In the case of a cornfield, the buckets were installed to collect rainwater for oviposition. After installation of containers, the containers were observed for a week or two for the presence of immature. After 15 days if the containers were negative, they were removed from the place and installed in other places for oviposition. Positive containers were reinstalled in the same place after counting the number of immature present in the containers and the immature were transported to the laboratory for identification. The reinstalled positive containers were observed again in a week or 15 days for immature and the immatures were counted and noted. Then the reinstalled positive containers were treated with *Bt*-14P2A. if they were negative, they were installed in a new place. In the case of tires, 12 tires of the scooter were installed by filling with water in the Central Department of Microbiology premises during the month of April 2023 for oviposition. Total tires 30 (motorcycle, scooter, car, van, etc.,) installed during July at the dean's office of Tribhuvan University premises collected rainwater for oviposition after a week immature were counted and the next week the tires were treated. In the workshop the tires were inspected in the month of August after counting the immatures the tires were treated with *Bt*-14P2A and from some of the tires the immatures were transported to the laboratory for identification.

3.6.5 Identification of mosquito

The immature transported to the laboratory were separated, the pupae were placed in a cup containing water and covered the mouth with the help of a muslin cloth. The larvae were placed in a tray until the development of pupae after pupa formation they were placed in a plastic cup as mentioned above until the emergence of adult mosquitoes. The collected immature from different sites were labeled accordingly for identification. After the emergence of adult mosquitoes, they were identified by observing the major keys.

3.6.6 Larval density determination

The 2^{nd} 3^{rd} and 4^{th} instar larvae present in the containers were counted from the positive containers. The larval density was determined before the treatment (Pretreatment) as well as after the treatment (post-treatment) in both the test containers and control containers. From a small container the larvae were removed by the strainer and counted by using a dropper and placed in a box and replaced into the respective containers after counting the larvae present in the containers.

3.6.7 Treatment

Before the addition of the bio larvicide, the larval density was determined in both the test and the control containers. In the control containers, the bio larvicide was not added. The 72 hours of cultured broth *Bt*-14P2A bio larvicide, five different dosages were added to the containers (1mL/ Lt, 2mL /Lt, 3mL/Lt, 4mL/Lt, and 5mL/Lt). Initially, it was started with 1mL/Lt then followed by other doses. One dose in four replicates. After the addition of bio larvicide to the test containers, the test containers were observed after 24 hours and then the containers were observed every week for immatures in the test and control containers. The immatures in the test containers were counted after the appearance of larvae and compared with the control containers. The counting was performed until the larval density in the test containers was 60 to 80 % mortality of the larvae in comparison with the control containers or the test was terminated after the six-week period.

3.7 Data analysis

The data was entered in Microsoft Excel and analyzed manually and by using Excel tools. Using probit analysis, the mean lethal concentration, LC_{50} and LC_{90} was determined. Reduction of larval on post-treatment days is presented in mortality percentage maintained in a particular week, by graph and tabulated. The percentage reduction in larval densities on post-treatment days was estimated for each replicate of each dose along with the control containers**.** The mortality percentage was calculated by using the formula

$$\text{Mortality (\%)} = (x\text{-}y/x)\ x100$$

Where x = percentage survival in the untreated control and y = percentage survival in the treated sample (WHO, 2005).

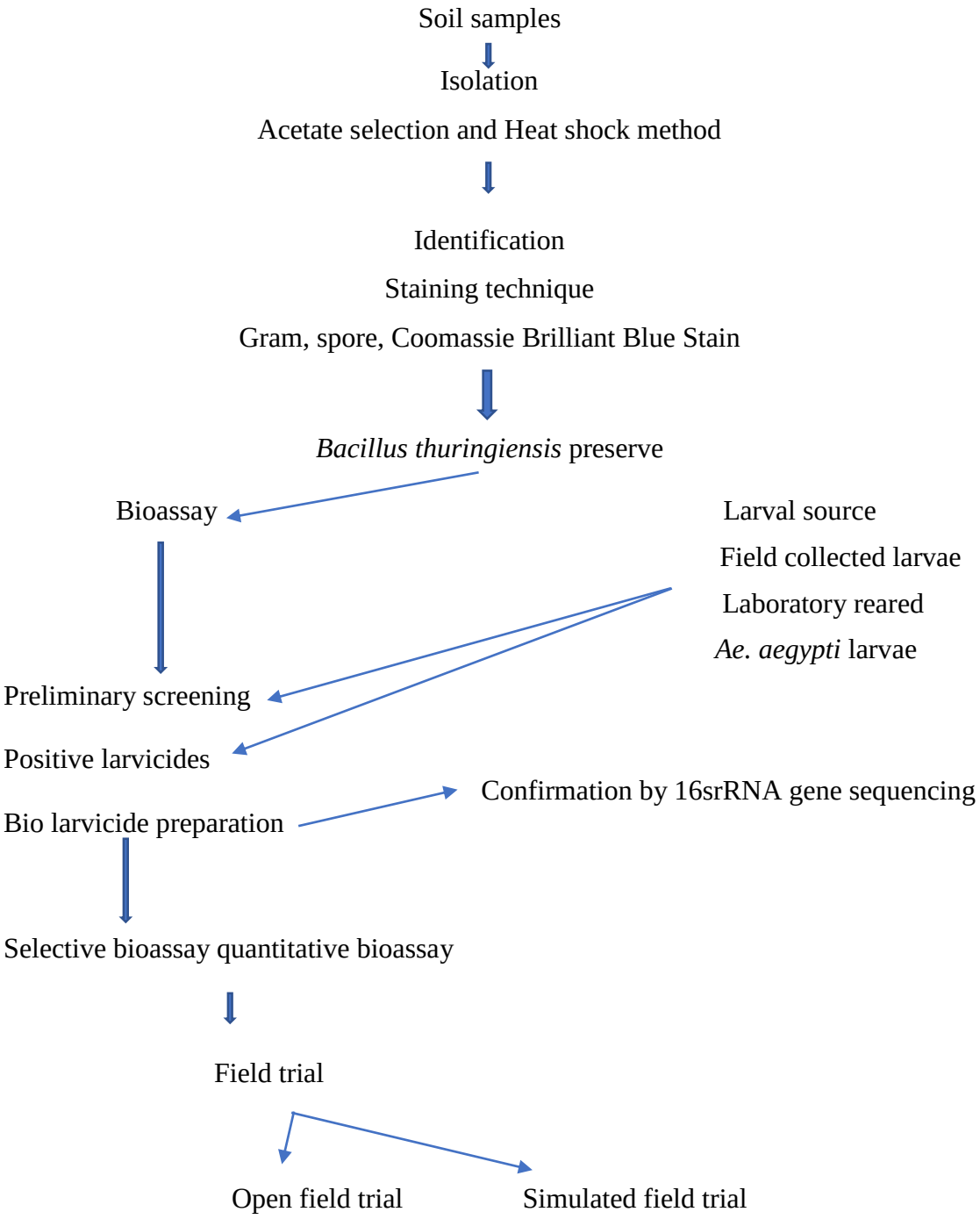


Figure 3. Comprehensive flow chart of research design

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of *Bacillus thuringiensis*

Soil samples 454 collected from different topographical locations of Nepal of different ecosystems resulted in obtaining 1395 *Bt* isolates Table 2 and Figure 4. Identified as *Bt* by observing the unique crystal protein by CBB stain in a light microscope. The *Bt* was distinguished from the other *Bacillus* species by observing the presence of crystal protein stained by CBB stain (Bravo *et al*., 1998; Rampersad *et al*., 2002). Generally, after growth for 48 hours in NA agar, all the *Bacillus* start to sporulate, on staining the isolated colony from the culture plate by CBB stain. In the case of the *Bt* strain, three different types of morphological structure can be observed in a slide, the vegetative cell, the spore, and the crystal protein Figure 18. In the case of other *Bacillus* spp., only two morphological structures can be observed the vegetative cell and the spores. The presence of the extra structure of different shapes confirms it as a *Bt* strain. The extra structure is proteinaceous in nature and known by different names such as cry protein, parasporal body, crystal protein, insecticidal crystal protein, and δ endotoxin (Roh *et al*., 2007) and it is stained by the CBB stain mainly used for staining the protein structure (Rampersad *et al*., 2002).

Table 2: Number of *Bt* obtained from soil samples.

Soil samples	Collection year	No. of *Bt* obtained
25	2020	80
47	2021	152
350	2022	1064
32	2023	99
Total 454		**1395**

Different strains of *Bt*'s crystal protein are very much lethal to a wide variety of important agricultural and human health-related insect pest as well as active against human cancer cells (Palma *et al*., 2014; Roh *et al*., 2007; Zhang *et al*., 2020). Soil samples of Nepal are rich resources of *Bt* from 454 soil samples 1395 *Bt* varieties were isolated. The results are in agreement with the results of (Martin & Travers, 1989). According to (Martin & Travers, 1989) soil samples from Asia were extraordinarily

rich in *Bt*. The native *Bt* isolates produced different shapes of crystal protein Table 3. The dominant insecticidal crystal proteins were the spherical shaped Figure 18. Similar results were obtained by (Apaydin *et al*., 2005; Nair *et al*., 2018; Zothansanga *et al*., 2016). More than three morphologically different *Bt* varieties were obtained from a gram of soil samples.

Table 3: Shapes of insecticidal crystal proteins (ICPs).

Shapes	**No. of *Bt***
Spherical	766 (54.91%)
Bipyramid	13 (0.93%)
Rod shaped	49 (3.51%)
Capheaded	502(35.98%)
Spherical + cap headed	42(3.01%)
Oval	23(1.64%)
Total	**1395**

All the *Bt* isolates showed variation in colony morphology, fried egg type with excess mucous, and irregular in shape were more abundant and present in all soil samples. Similar findings were observed by Apaydin *et al*., (2005); El-Kersh *et al*., (2016) in different types of samples analyzed to isolate *Bt*. All *Bt* isolates were Gram-positive, spore-bearing, but showed variation in the arrangement of spores in a vegetative cell, in the shape of the spore, in cell size, and in the shape of the crystal protein as observed by light microscopy with different staining techniques.

Table 4: Distribution of isolates obtained from three different regions.

Region	**No: of samples**	**No: of *Bt* isolates**
Mountain	4	14
Hill	228	538
Terai	222	843
Total	**454**	**1395**

The media used for isolation of *Bt* from the soil sample was nutrient agar (NA) may be the reason for a smaller number of bipyramid types of crystal protein isolation from the soil sample. Different media and the spore selection method may enhance the isolation of other different types of crystal-producing *Bt* from the soil samples.

The soil samples were collected from three different geographical regions for the isolation of *Bt* Table 4. All the soil samples collected from the different geographical regions were positive for *Bt* in each sample ≥ 3 *Bt* isolates of different morphology were obtained. This proves that the soil samples of Nepal are also enriched with the *Bt* strain and there is the possibility for the isolation of a novel variety of *Bt*. Only four soil samples were collected from the mountainous region so the least number of *Bt* isolates were obtained. To obtain a novel variety it is necessary to explore those mountainous regions in the future.

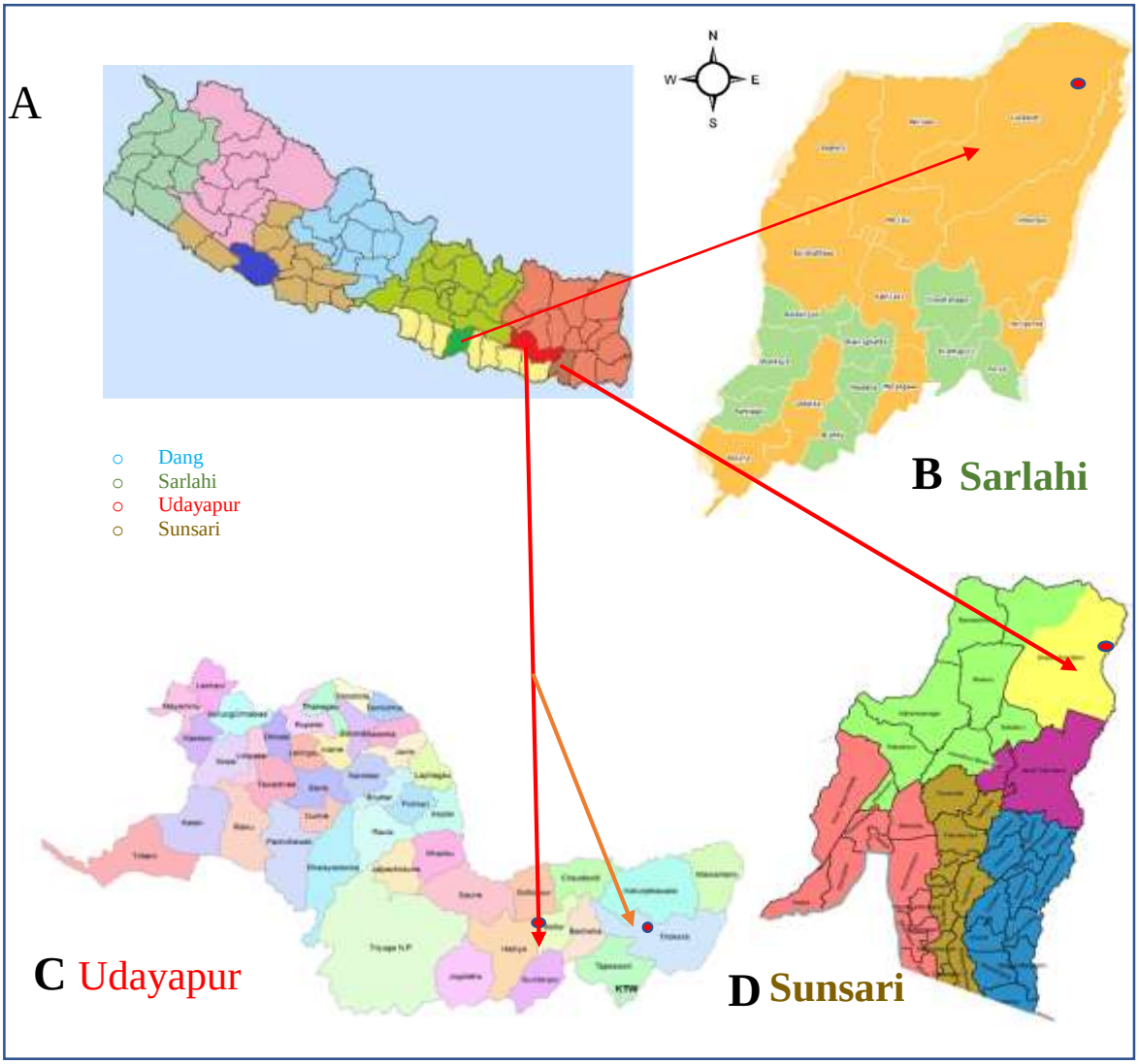


Figure 4. Sample collection sites. A. the color filled district from were the positive isolates were obtained. Dang district soil sample was positive for larvicidal *Lysinibacillus sphaericus.* B Lalbandi, Jabdi of Sarlahi district (27°01'49.5"N 85°37'10.4"E). B Dharan 13, Sunsari district (26°49'53.4"N 87°17'21.2"E). C Beltar (26°50'27.2"N 86°57'10.6"E) and Rampur Thoksila (26°47'37.7"N 87°04'08.9"E) soil samples were positive for larvicidal *Bacillus thuringiensis*.

4.2 Larvicidal activity

On preliminary screening of 808 spherical (766) and spherical + cap headed (42) insecticidal crystal proteins (ICPs) producing *Bt* isolates for larvicidal activity, four isolates coded as *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B producing spherical ICPs showed 100% mortality against the field-collected mosquito larvae Table 5. The larvicidal *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B were among the 766 *Bt* isolates producing spherical-shaped ICPs. The isolates producing both spherical and cap-headed (42) and the 762 spherical ICPs producing native *Bt* isolates lack the mosquito larvicidal property. Based on various literature reviews crystal morphology spherical like is effective against the mosquito larvae, (WHO, 1990, 2005, 2007) about 55.30% (808/1385) isolated were screened. Only four strains of *Bt* with larvicidal activity against field-collected mosquito larvae were discovered. Soares-da-Silva *et al*., (2015) isolated 5 larvicidal *Bt* against *Ae. aegypti* out of 57 *Bt* isolates. El-Kersh *et al*., (2016) isolated 23 larvicidal *Bt* against *An. gambiae* out of 68 *Bt* isolated from different types of samples. Even though a greater number of *Bt* isolates 55.30% (808/1385) producing spherical-shaped crystal protein, the dominant *Bt* of soil samples of different sizes were screened for larvicidal activity, the distribution of mosquito larvicidal *Bt* in Nepal was not found to be ubiquitous. Therefore, the shape of the crystal protein doesn't determine the mosquito larvicidal activity the composition matters the larvicidal property. The isolated larvicidal *Bt* has to be further analyzed for the composition of protein present in it to determine the phylogenetic difference from the existing mosquito larvicidal *Bt* isolates.

Table 5: Larvicidal activity of native *Bt*.

Total isolates	**Larvicidal *Bt* with Positive site**	**Non-Larvicidal *Bt***
808	1 Lalbandi, Sarlahi	804
	1 Dharan Sunsari	
	1 Beltar, Udayapur	
	1 Rampur, Udayapur	
	Total 4 *Bt* larvicide	

4.3 Selective bioassay against field-collected larvae

Selective bioassay proved the isolate's ability to show 100% mortality as the standard strain within 24 hours at 300PPM concentration Figure 17 and Table 6. So, the selective bioassay confirms that *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B are mosquito larvicidal *Bt*. Further confirmation with *Bt*-3P2A also proved *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B as mosquito larvicide *Bt* were as *Bt*-3P2A lack the ability even though it possesses a spherical shaped crystal protein. On analysis of dead larvae after 24 hours by spread plate technique it was found the same morphological colony producing *Bt* were present in the dead larva. Therefore, the death of the larvae is by feeding on the crystal protein of the *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B *Bt* isolate. For bioassay mosquito larvae were collected from the field during the monsoon season.

The colony morphology of the larvicide *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B in NA appear as irregular in shape, size (15mm- 30mm), dry, flat, and pale white Figure 19. The colony character of *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B is different from the colony characters of other spherical crystal protein-producing *Bt* isolates. On biotyping, they were Gram-positive straight rods arranged singly, on spore staining by Schaeffer Fulton stain spores were elliptical in shape and the green spore in the vegetative cell was in subterminal position without bulging. On CBB stain the crystal protein appeared as blue spherical in shape Figure 18. the elliptical spore was faint blue in color and the vegetative cells were dark blue in color.

Table 6: Larvicidal activity of *Bt* and control strain *Bti*-IPS-82 against field-collected larvae.

Isolates	Conc. PPM	Total larvae	Live larvae	Pupa	Adult	Mortality %
Bti-IPS-82	300PPM	25(100) *	0	0	0	100
Bt-14P2A	300PPM	25(100) *	0	0	0	100
Bt-7P1A	300PPM	25(100) *	0	0	0	100
Bt-8P1A	300PPM	25(100) *	0	0	0	100
Bt-3P2B	300PPM	25(100) *	0	0	0	100
Bt-3P2A	300PPM	25(100) *	25(100)	0	0	0
Control	-	25(100) *	25(100)	0	0	0

*Four replicates (each cup contains 25 larvae, four cup 100 larvae). All the four isolates *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, *Bt*-3P2B and *Bt*-3P2A isolated from soil samples of Nepal.

The control strain and the *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B strain were motile. The biochemical properties were catalase and oxidase positive as well as the isolates were able to hydrolyze different substrates like starch, gelatin, casein and Tween20. Selective bioassay performed under the laboratory condition by using the pure culture of the larvicidal *Bt* isolates in NA, containing the mixture of spore, crystal proteins and the vegetative cells, showed 100% mortality of larvae within 24 hours. The 100 (4x25) larvae present in the negative control cup were all alive even in the absence of larval food and were all in the larval stage. The field-collected larvae are a mixture of different species of mosquito larvae, 100% mortality of field-collected larvae means; the four isolates are effective in killing all kinds of mosquito larvae. Similarly, the control strain *Bt*-IPS-82 also showed 100% mortality against the field-collected larvae. Larvicidal activity is due to the production of crystal protein which is more specific and not all types of spherical-shaped crystal protein are toxic to mosquito larvae. Various research studies have shown that the mosquito larvicidal *Bt* will kill all kinds of mosquito larvae and are also toxic to midges (Glare & Callaghan, 1998; WHO, 1999). Therefore, *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B are effective in killing vectors like *Aedes*, *Anopheles*, and *Culex*. So, they can be used as a biological control tool for all mosquito-borne diseases. Likewise, the larvicidal activity of *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B with *Bt*-3P2A spherical crystal-producing *Bt* with different colony morphology, confirms that the crystal protein composition is different between the mosquitocidal *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B.

4.4 Quantitative bioassay against field-collected and laboratory-reared *Ae. aegypti* larvae

A quantitative bioassay was carried out in the laboratory condition during the months of July-August 2022 by using the diluted pellet or sediment in sterile distilled water. Stock solutions prepared in sterile distilled water were free from pathogenic contaminants like *E. coli*, and *S. aureus* after incubation in their respective culture plates at 37^0C for 24 hours. The endospores count of the stock solution of *Bti*-IPS-82 and *Bt*-14P2A was $>10^6$ cfu/mL when analyzed in a general NA at 28^0C temperature.

Table 7: Larvicidal activity of control and test strain against field-collected larvae.

Isolates	Conc PPM	No. of larvae exposed	No. of larvae dead	Mean Mortality
Bti-IPS-82	0.001	300*	0	0
	0.01	300	0	0
	0.1	300	2	0.67
	1.0	300	5	1.67
	10	300	277	92.34
	100	300	300	100
Bt-14P2A	0.001	300	0	0
	0.01	300	0	0
	0.1	300	2	0.67
	1.0	300	10	3.34
	10	300	270	90
	100	300	300	100
Negative	0	300	0	0

*The bioassay was repeated for 3 days with 100 larvae each day.

This indicates the sediment contains viable spores. The average indoor room temperature was 23^0C and relative humidity was 69%. The final concentration of 100PPM present in the cup was effective in causing 100% mortality of field-collected larvae under laboratory conditions and 90% mortality was achieved from 10PPM from *Bt*-14P2A working solution Table 7. Similarly, 100% mortality was observed with a 100PPM concentration of *Bti*-IPS-82 and 92.34% with 10PPM against the field-collected mosquito larvae Table 6. The final concentration of 100PPM of *Bt*-14P2A present in the cup was effective to causing 100% mortality and with 10PPM 95.67% mortality of *Ae.aegypti* larvae under laboratory conditions was observed. Similarly, 99.33% mortality was achieved from 10PPM concentration of *Bti*-IPS-82 and 100% mortality from 100PPM Table 8.

Table 8: Larvicidal activity of control and test strain against *Ae. aegypti* larvae.

Isolates	Conc PPM	No. of larvae exposed	No. of larvae dead	Mean mortality
Bti-IPS-82	0.001	300*	0	0
	0.01	300	0	0
	0.1	300	0	0
	1.0	300	0	0
	10	300	298	99.33
	100	300	300	100
Bt-14P2A	0.001	300	0	0
	0.01	300	0	0
	0.1	300	0	0
	1.0	300	2	0.67
	10	300	287	95.67
	100	300	300	100
Negative	0	300	0	0

*The bioassay was repeated for 3 days with 100 larvae each day.

On plotting a graph of mortality vs concentration in PPM the same type of graph is obtained as in the case of field-collected and laboratory-reared *Ae. aegypti* larvae as shown in Figure 5. A narrow range of concentration required to cause mortality between 10% to 95% lies between 10PPM and 1PPM as shown in Figure 5. As per the graph to cause 100% mortality of larvae concentration greater than 10PPM is required. The field-collected larvae and pupae after the emergence of adults, were identified as *Culex* spp., *Ae. aegypti*, *Ae. albopictus* and *Toxorhynchites* spp. As the healthier and well fed field-collected larvae were used for the bioassay the mortality percentage is not a vast difference in both types of larvae. The quantitative bioassay was carried out by using the pellet prepared of *Bt*-14P2A mosquito larvicidal *Bt* isolate. Other three mosquito larvicidal quantitative assays have not been performed. The quantitative bioassay was carried out along with the standard control stain *Bti*-IPS-82. A similar concentration range was shown to cause mortality between 10% and 95%.

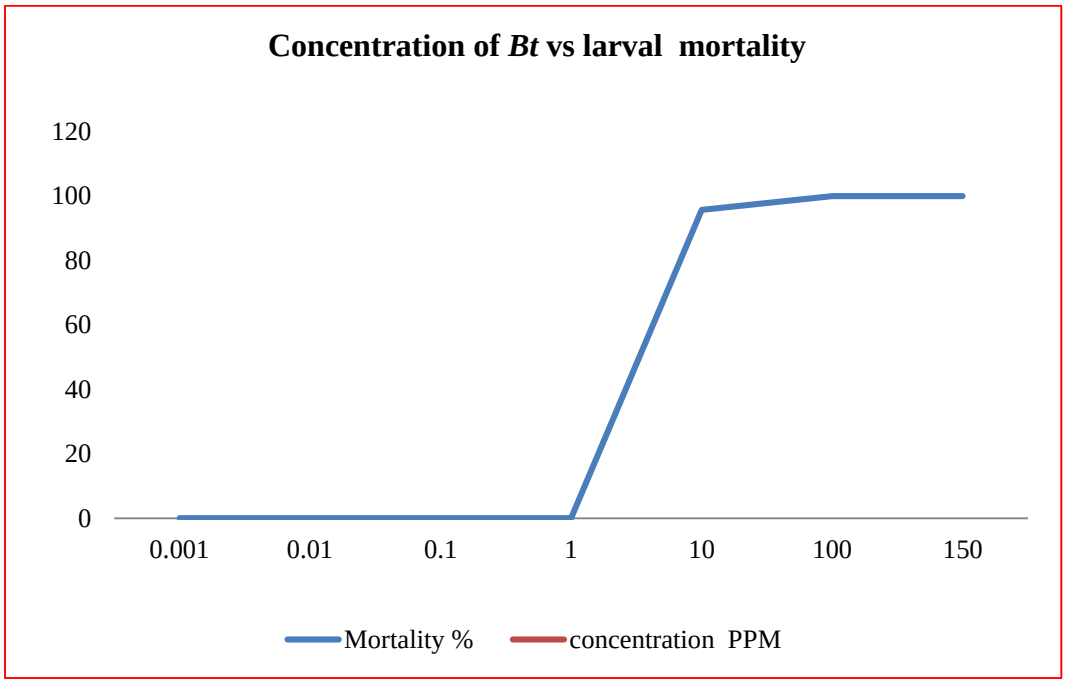


Figure 5: Concentration of *Bt*-14P2A required to show 100% mortality against field-collected and *Ae. aegypti* larvae.

Rearing of *Ae. aegypti* larvae was successfully carried out, in an insectary like cage build temporary in the laboratory. Taking all the necessary precautions to prevent the flying mosquito from escaping from the cup and cage. The identified adult *Ae. aegypti* male and female in the ratio1:3 were placed inside the cage collected from the discarded tires fed with expired blood and 10% sucrose solution from May to August, but not able to produce large quantities of eggs. The blood supply was not continuous may be the

reason for the lower production of eggs. Rearing is important to analyze the resistant development, for the assay of various insecticides. While rearing the larvae from the eggs present in a white strip of paper, within 48 hours eggs hatched into 1st instar larvae. As it was a summer season the room temperature and the humidity present in the room were favorable for rearing. The indoor temperature and humidity of the laboratory room were favorable for rearing the mosquito from May to August. The natural monsoon season of Kathmandu indoor or outdoor provides a favorable condition for the mosquitoes, so the mosquito-borne disease outbreak often is high during the monsoon season.

4.5 Estimation of the lethal concentration (LC) LC_{50} and LC_{90}

The toxicity potential of the *Bt*-14P2A isolated was further determined by estimating LC_{50} and LC_{90} values obtained from the three-mortality evaluation test performed on three different days. The test was performed along with the control strain. The estimation of LC_{50} and LC_{90} was performed after achieving 100% mortality against field-collected larvae. The quantitative bioassay showed that the strain *Bt*-14P2A has more or less similar performance as the control strain *Bti*-IPS-82 against the field-collected larvae Tables 9 and 10.

Table 9: Mean LC_{50} and LC_{90} of *Bti*-IPS-82 and *Bt*-14P2A against field-collected larvae.

Isolates	Conc in PPM	Log 10 concentration	Mortality %	LC_{50}	LC_{90}
Bti -IPS-82	0.001	-3	0		
	0.01	-2	0		
	0.1	-1	0.67	3.99PPM	24.54PPM
	1.0	0	1.67		
	10	1	92.34		
	100	2	100		
Bt-14P2A	0.001	-3	0		
	0.01	-2	0		
	0.1	-1	0.67	3.98PPM	25.11PPM
	1.0	0	3.34		
	10	1	90		
	100	2	100		

However, LC_{50} and LC_{90} values between the two isolates showed a wide variation against larvae. *Bt*-14P2A required to kill 50% of field-collected larvae was 3.98PPM and to cause 90% mortality was 25.11PPM similarly, the control stain *Bti*-IPS-82 required to kill 50% of field-collected larvae was 3.99PPM and to cause 90% mortality

was 24.54PPM Table 9. The lethal concentration dose required to kill 50% of *Ae. aegypti* larvae by *Bt*-14P2A was 6.45PPM and to kill 90% of the larvae was 37.15. Similarly, the lethal concentration dose required to kill 50% of *Ae. aegypti* larvae by *Bti*-IPS-82 was 10.23 PPM and to kill 90% of the larvae was 60.25 PPM Table 10. On comparing the lethal concentration dose to kill 50% and 90% of the field-collected larvae and *Ae. aegypti* larvae by the *Bt*-14P2A and by *Bti*-IPS-82 the concentration required was almost similar so the isolated *Bt*-14P2A must be similar to *Bti.*

Table 10: Mean LC_{50} and LC_{90} of *Bti*-IPS-82 and *Bt*-14P2A against *Ae. aegypti* larvae.

Isolates	**Conc in PPM**	**Log 10 concentration**	**Mortality %**	**LC_{50}**	**LC_{90}**
Bti-IPS-82	0.001	-3	0	10.23PPM	60.25PPM
	0.01	-2	0		
	0.1	-1	0		
	1.0	0	0		
	10	1	99.33		
	100	2	100		
Bt-14P2A	0.001	-3	0		
	0.01	-2	0	6.45PPM	37.15PPM
	0.1	-1	0		
	1.0	0	0.67		
	10	1	95.67		
	100	2	100		

4.6 Molecular characterization of larvicidal *Bt*

The 16srRNA gene sequence of *Bt*-14P2A, *Bt*-7P1A and *Bt*-8P1A larvicidal *Bt* isolates were of length 1502bp, 1483bp, 1527bp. *Bt*-14P2A, and *Bt*-7P1A showed 99.93% identity and *Bt*-8P1A showed 99.73% identity towards *Bacillus thuringiensis* ATCC 10792 of Accession Number CP021061.1with Expected value of 0.0. As well as showed 99% identity towards the *Bti* strain AM 65-52 the WHO-recommended strain to control mosquito vectors in aquatic habitats. Thus, from the 16srRNA gene sequencing results the isolates were identified as *Bt* as the sequence similarity is greater than 97% as well as they are similar to the biological control agent for mosquito larvae as their sequence is 99% similarity with the *Bti* deposited in the Gen Bank of NCBI database. So, the three isolates are identified as *Bti.*

The colorful graphical summary results of nBlast of *Bti* sequences of the database sequence aligned to the 3 (*Bt*-14P2A, *Bt*-7P1A, and *Bt*-8P1A) query sequence. The alignment is coded ranging from black to red as indicated in the color label at the top.

The hits are colored according to the obtained alignment scores. The query sequences appear as a grey horizontal bar at the top. The scale below shows the bp present in the query sequence from 1 to 1500 bp. Below the scale, the horizontal red bars in rows represent the hits of the 21 *Bti* sequences deposited in the GenBank of NCBI. The hits with the highest score are found. The color coding within the graphic is generated by the statistics of each hit. As indicated by the key at the top of the graphic hits with the highest score are red. In this blast search, all the hits are red bars, indicating the highest score for alignment.

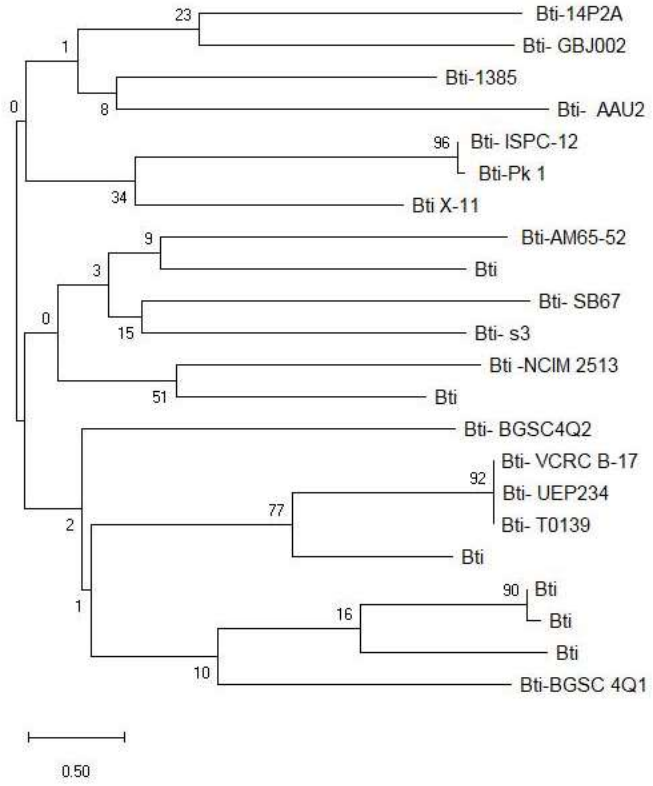


Figure 6: Phylogenetic tree. Neighbor-joining phylogenetic tree based on the 16srRNAgene sequences showing the relationship of 3 *Bt* isolates with its close relative using MEGA software**.** Bar 0.50 substitution per nucleotide substitution.

Further comparison of 16srRNA gene sequences of *Bt*-14P2A, with *Bti* sequences available in GenBank by phylogenetic tree Figure 6 confirmed them as *Bti*. The larvicidal isolates of different countries are presented in the phylogenetic tree. Nodes were supported by 1000 bootstraps. The 16srRNA gene sequence of *Bt*-14P2A was submitted to GenBank of NCBI and the accession number was obtained Accession No. PP499315. The analytical sensitivity of the 16srRNA gene by PCR in the case of

positive culture bacteria it is 89.9% and in case of negative culture bacteria it is 19-42% (Satilms *et al*., 2019).

Ls formerly known as *Bacillus sphaericus* it is renamed as *Ls* due to the presence of lysine in the cell wall of this bacteria. During this study, 11 *Ls* isolates were isolated from 32 soil samples, one of which demonstrated mild toxicity against *Ae. aegypti* larvae. Only one *Ls* showed 25% mortality against *Ae. aegypti* larvae. It was coded as *Ls*-10P5B. *Bacillus sphaericus* Neide first discovered in 1965 was isolated from moribund 4th instar larvae collected near Fresno, California (Kellen *et al*., 1965). It produces a characteristic spherical spore located at the terminal end of the swollen sporangium Figure 20. The spore contains the attached toxin. They all are Gram-positive endospore-producing, saprophytic bacteria. Mosquitocidal *Ls* bacteria are also recommended by the World Health Organization as a bio larvicide for the larval source management of mosquitoes especially for controlling the *Culex* species of mosquito.

4.7 Simulated field trial

Simulated field trial by using liquid formulated *Bt*-14P2A Figure 13. On screening 1000µl of the 72-hour cultured broth, bio larvicide it was found to cause 100% mortality of *Ae. aegypti* larvae within four hours. The bio larvicide was free from the pathogens like *E. coli* and *S. aureus.* To evaluate the efficacy and to determine the residual period of the bio larvicide six (1.5mL/liter, 1mL/liter, 2mL/liter, 3mL/liter, 4mL/liter, and 6mL/liter) different doses were evaluated by simulated field trial all the six doses were able to cause 100% mean mortality within 24 hours Table 11 and Figure 8.

Table 11: Mean Mortality of *Ae. aegypti* larvae caused by dosages within 24 hours.

Dosage	Larvae exposed in control/Test	No. of alive larvae		Mean Mortality %
		Control	Test	
1mL/Lt	100/100*	100	0	100
1.5mL/Lt	100/100	100	0	100
2mL/Lt	100/100	100	0	100
3mL/Lt	100/100	100	0	100
4mL/Lt	100/100	100	0	100
6mL/Lt	100/100	100	0	100

*Four replicates for control and test so the total larvae in four replicates was 100.

A higher dose of 6mL/Lt was found to show a residual effect for seven weeks (49 days) Table 12 and Figure 8. Mean mortality of 100% was shown for the six-week period by a 6mL/Lt dose of *Bt*-14P2A bio larvicide Figures 7 and 8 followed by a decline after the sixth week in mortality percentage. Therefore, the 6mL/liter dosage of bio larvicide

is effective in controlling *Ae. aegypti* larvae in plastic containers used for storing water as it shows the residual effect for a longer duration Table 12. Two dosages 3mL/Lt, and 4mL/Lt, showed residual effects for greater than one month, and mean mortality of 100% was maintained for a five weeks Figure 7.

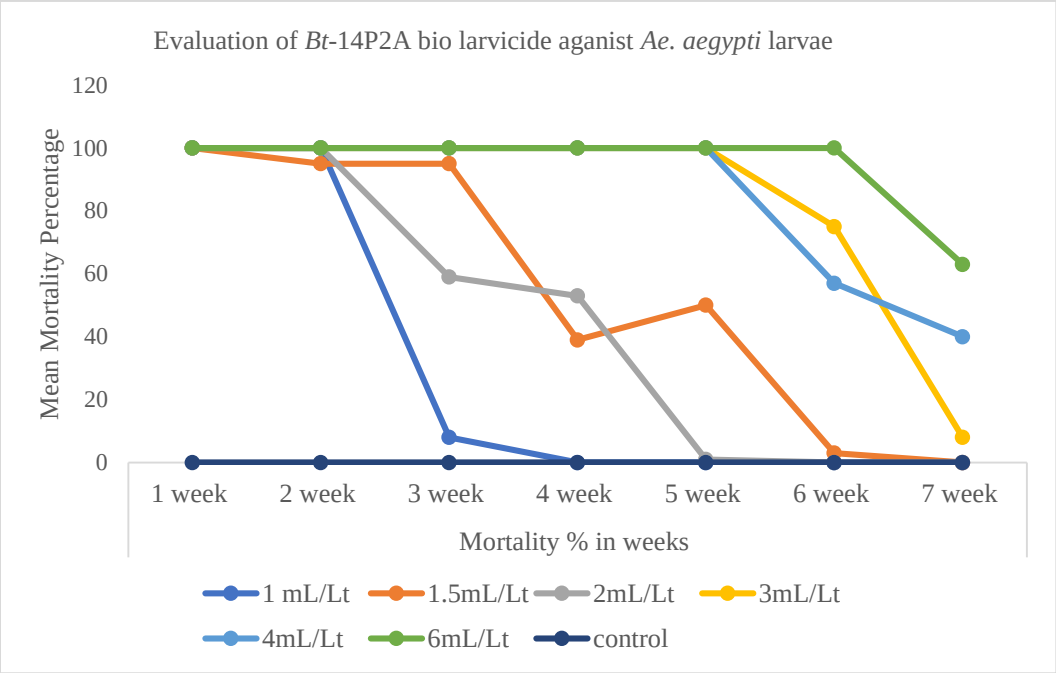


Figure 7: Mean mortality % of *Ae. aegypti* larvae in weeks by simulated field trial.

Even the lowest dose 1mL/liter, 1.5mL/liter, and 2mL/liter showed a residual effect for 2 weeks with 100% mortality. However, the mortality rate declined after fifth week of post-treatment Figure 7. The study was terminated when the mortality % was less than 60%. During the study period in the four replicates of the negative control, the new batch of larvae were all live making the survival percentage of control 100%. The average pH of the water was 8.2 and the average temperature of the water was found to be 25.6^{0}C. The larvae were hatched from the eggs within 48 hours. After three days the healthier larvae were used for the bioassay.

4.8 Open field trial

On a survey of the owner's water storage container all the observed 12 containers were positive for immature larva and pupa and were all outside in the open field made of plastic and metals.

In CDM the indoor containers jars were negative even though placed for one month. But the tires, buckets, and jars kept outside in the open field were all positive. All the containers were installed by adding chlorine-free water.

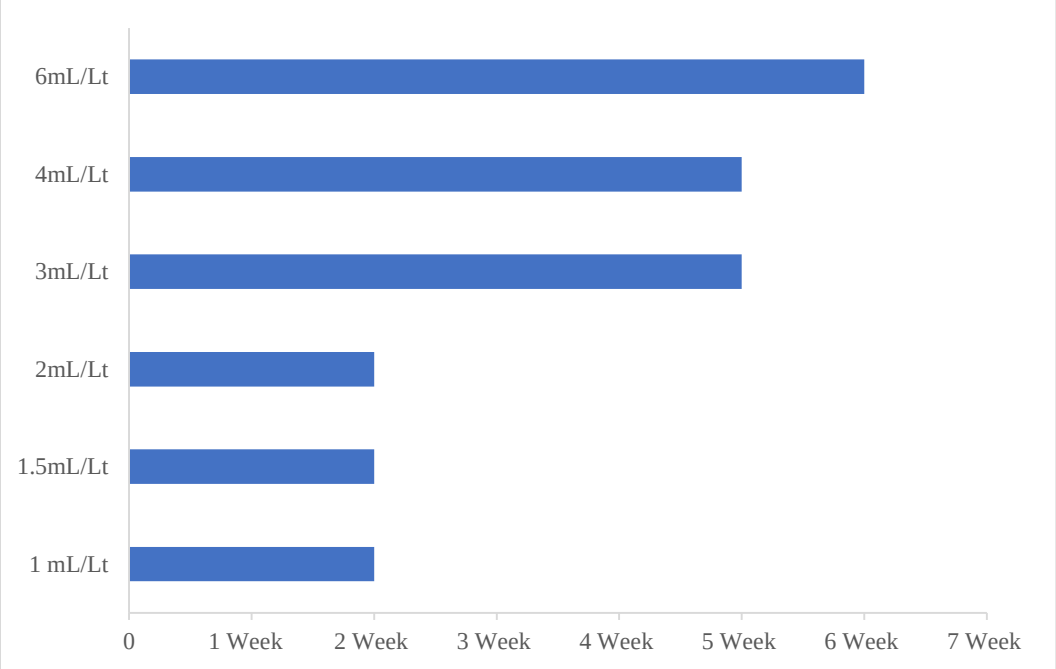


Figure 8: Simulated field trial of *Bt*-14P2A showing 100% mean mortality in weeks.

In the case of tires two liters of water was added to each, in buckets 2.5 liters of water was added and to the jars 15 liters of water was added as it was installed in April month.

Table 12: The survival and mortality % of *Ae. aegypti* larvae with 6mL/Lt bio-larvicide treatment

Product	**Post-treatment weeks**	**Alive larvae after 24 hours in post-treatment**	
		Mean Survival %	**Mean Mortality %**
Test Product	1	0	100
Bt-14P2A	2	0	100
Bio larvicide	3	0	100
	4	0	100
	5	0	100
	6	0	100
	7	37	63
Control	1	100	0
Pots without	2	100	0
Bio larvicide	3	100	0
Bt-14P2A	4	100	0
	5	100	0
	6	100	0
	7	100	0

After the installment of the container's appearance larval activity was observed after two to three weeks.

Larval density was determined during pre-treatment two times at a two-week interval. The mean larval density in tires was 21 and 22.83 in two weeks. In buckets 6.75 and 9.5, and Jars 24.5 and 23.25. As it was not a rainy season the water in the containers decreased due to heat. The water level was maintained as mentioned above before treatment. In bucket residual effect with 85% mortality for three weeks and 100% mortality for two weeks was observed. In tires, the residual effect was seen with 87% mortality in the fourth week. Three weeks of 100% mortality was observed. In jars, the residual effect was seen with 89.25% mortality in the fourth week. Up to three weeks, 100% mortality was observed in Table 13.

Table 13: Effect of 3mL/Lt bio larvicide on mosquito larvae present in an open field setting in CDM.

Larval densities in post-treatment weeks									
Week	**Tires**			**Jar**			**Buckets**		
	Control	**Test**	**Mortality**	**Control**	**Test**	**Mortality**	**Control**	**Test**	**Mortality**
24hrs.	22.83	0	100%	24.5	0	100	9.5	0	100
1		0	100%		0	100		0	100
2		0	100%		0	100		0	100
3		0	100%		0	100	14.5	2.16	85.52
4	22	2.83	87.13%	23.25	2.5	89.25	16.5	11.8	28.30
5	20.16	7.16	64.48%	24	18.2	23.96			

Tribhuvan University, Kirtipur site

The total tires exposed were 30 in an open field, the backyard of the dean's office in a small jungle area with large number of trees, tires were bigger with a capacity of 4-5 liters of water. The tires were exposed to collect rainwater that serves as an oviposition for the mosquito present in the jungle area to lay eggs and hatch into larvae Figure 14. The tires were exposed on 4th June 2023 the rainwater was collected as it rained that week, and all the tires were positive on 29th June. The larval density was determined and the types of mosquitoes present were identified. The 3mL/liter dose was effective with residual effect for four weeks with 87.31% mortality of larvae Table 14. The second dose 4 mL/Lt was added to the same 30 tires by determining the density of larva present in 30 tires Table 15.

Table 14: Effect of 3mL/Lt bio larvicide on mosquito larvae present in the tires of TU.

	Larval densities in tires during post-treatment weeks		
Weeks	**Control**	**Test**	**Mortality %**
24hrs	185.93	0	100
1	-	0	100
2	173.67	3.87	97.77
3	219.93	19.53	91.09
4	212.8	27	87.31
5	133.93	113.93	14.93

The control and test tires were the same as before. The mean initial larvae density in control and test tires were 180.2, and 150.47 respectively. The second dose was added without cleaning the tires. The dose was 4mL/liter for each tire exposed to the same place in the TU. premises. The dose was found to be effective for a 9week period with 78.47% mortality of larvae Table 15.

Table 15: Effect of 4mL/Lt bio larvicide on mosquito larvae present in the tires of TU.

	Larval densities in tires during post-treatment weeks 12 August-30 October		
Weeks	**Control**	**Test**	**Mortality%**
24hrs	180.2	0	100
1		0	100
2		0	100
3	179.46	2.47	98.62
4	192.8	7.07	96.33
5	204.6	12.47	93.90
6	201.87	18.8	90.68
7	186.93	27.27	85.41
8	184.6	30.8	83.32
9	181.73	39.13	78.47

Kalanki and Gwarko site

The larval density at Kalanki and Gwarko automobile workshops was 469.16 and 359 respectively. The treatment with 4mL/liter at the Kalanki site showed 100% mortality after 24 hours and up to one week at this site Table 16, on the second week some of the tires were sold which were under treatment so could not report the residual effect at this site. The treatment was done in the month of July. During this period the automobile workshop owner was warned by the local authorities to manage the tires to prevent the multiplication of mosquitoes. So, the tires were removed from the place some were sold and some were transported to other places. So, could not determine the residual effect

of the bio larvicide used at this site. However, the 100% mortality within 24 hours will reduce the transmission of mosquito-borne diseases. The black color of the tires is more attractive for the *Aedes* spp. to lay eggs. Management of tires in a shaded place or the addition of bio larvicide will prevent the multiplication of dengue vectors in the surroundings

Table 16: Effect of 3mL/Lt bio larvicide on mosquito larvae present in the tires of the Kalanki site.

Larval densities in tires during post-treatment weeks			
Weeks	**Control site Gwarko**	**Test site Kalanki**	**Mortality %**
24 hours	359	0	100
1 week		0	100
2nd week		Tires lost	

Imadole site

On installation of three buckets only two buckets were positive for larvae, buckets placed in the shaded place of the veranda were positive within five days of installation and the other bucket placed in the open area in the pre-domestic area was negative Figure 12. The larval density was determined in the buckets as 253 and 187. Considering one bucket as a control and the other as a test 4mL/liter bio larvicide was added and the residual effect lasted for eight weeks with 92% mortality of the larvae without replenishment of water. The level of water decreased as the buckets were placed in the shaded place where the rain water didn't dilute the bio larvicide.

Maharajgunj site

In the Maharajgunj site only eight buckets were positive after five days of installation other four buckets were negative Figure 15. The larval density was determined during pretreatment and treated with 2mL/liter, 3mL/liter, 4mL/liter, and 5mL/liter at different times from April to November 2023. The average larval density in the bucket containers was 291.5 on this site. All the dosages were effective to show 100% mortality of the larvae for a three weeks Table 17.

Table 17: Effect of bio larvicide on mosquito larvae present in the pre-domestic area of Maharajgunj site.

Larval densities in buckets during post-treatment weeks												
weeks	**2mL/liter May-June**			**3mL/liter June- August**			**4mL/literAugust -September**			**5mL/liter Sept- October**		
	Control	**Test**	**Mortality%**	**Control**	**Test**	**Mortality%**	**Control**	**Test**	**Mortality**	**Control**	**Test**	**Mortality**
24	291.5	0	100	320.67	0	100	292.5	0	100	290.17	0	100
1		0	100		0	100		0	100		0	100
2		0	100		0	100		0	100		0	100
3		0	100	303	0.33	99.89	326	4	98.77		0	100
4	344.33	11.33	96.71	324.67	4.67	98.56	334.33	22	93.42		0	100
5	315.67	29.33	90.71	321.33	57.67	82.05	310.67	68.67	77.90	338	5.33	98.42
6	348.67	78.33	77.53	321.67	104	67.67				335.33	14.33	95.73

Sifal site

In this pre-domestic area, 8/12 buckets of capacity 25 liters were positive after five days of installation, considering four as positive and four as tests different dose was added at different periods, and observed that 5mL/liter was effective with 93.15% mortality of larvae for six-week period Table 18. The larval density was determined during pretreatment and treated with 2mL/liter, 3mL/liter, 4mL/liter, and 5mL/liter at different times from April to November 2023. All the dosages were effective to show 100% mortality of the larvae for two weeks Figure 9 and 10.

Table 18: Table 18: Effect of bio larvicide on mosquito larvae present in the pre-domestic area of the Sifal site.

Larval densities during post-treatment weeks												
weeks	**1mL/liter May-June**			**3mL/liter June- August**			**4mL/liter August-Sept**			**5mL/liter Sept- Nov**		
	Control	**Test**	**Mortality%**	**Control**	**Test**	**Mortality%**	**Control**	**Test**	**Mortality**	**Control**	**Test**	**Mortality**
24	195.88	0	100	190.12	0	100	218.25	0	100	254.13	0	100
1	295	1.5	99.49		0	100		0	100		0	100
2	246	25.25	89.74		0	100		0	100		0	100
3	278	70.75	74.55	221.25	2	99.09	305.75	1.25	99.59		0	100
4				228.75	12	94.75	331	8	97.58	277.5	1.75	99.37
5				255	73,75	71.08	306.5	15.25	95.02	278.75	9	96.77
6							324.25	23.5	92.75	270	18.5	93.15

Panga Charghare site A

In pre-domestic area of Panga Charghare sites A, B, and C rainwater was collected in 12 buckets of capacity 15 liters were installed only two buckets were positive at site A after one week other ten buckets were removed from the area. Buckets were in the open field where rainwater replenished the buckets. Considering one bucket as a test and the other as a control 2mL/liters bio larvicide was added to the test container. The residual

effect was observed for five weeks period with 55.66% mortality of the larva. In the pre-domestic area of the Panga site A four buckets of capacity 15 liters filled with rainwater were placed in the shaded place the four buckets were positive within seven days considering two as control and two as test 2mL/liters bio larvicide was added to the test buckets without replenishment with water. The residual effect was observed for a six-week period with 81.16% mortality of the larva.

Panga Charghare site B

In pre-domestic area a 100-liter capacity two black broken drums filled with rainwater the water holding capacity in the drum is about 50-80 liters Figure 16. Consideration one as control and the other as test, 4mL/liter bio larvicide was added to the test drum. The dosage was effective with 85% mortality with residual effect for four weeks period with replenished with rainwater.

Cow farm Panga site

The two buckets of capacity 15 liters installed near the cow farm in the open field were positive within five days the larval density was 183 and 169, after collection of rainwater, one bucket served as control and the other as a test. The residual effect remained in the bucket for four weeks with 80.48% mortality of larvae.

Open cornfield Kirtipur site

Panga, (cornfield 1) Figure 11. Champadevi Site 1 (cornfield 2) and Champadevi Site 2 (cornfield 3) Kirtipur. In the corn field, 3mL/liter bio larvicide was effective to showing residual effect with >80% mortality of larvae in the cornfields for a period of four weeks Table 19.

Table 19: Effect of 3mL/Lt bio-larvicide on mosquito larvae in a cornfield Kirtipur site.

	Larval densities in post-treatment weeks in cornfield								
Week	**Cornfield 1**			**Cornfield 2**			**Cornfield 3**		
	Control	**Test**	**Mortality**	**Control**	**Test**	**Mortality**	**Control**	**Test**	**Mortality**
24hrs	52.67	0	100%	52.67	0	100	52.67	0	100
1		0	100%		0	100		0	100
2		0	100%		0	100		0	100
3		0	100%		0	100		0	100
4	184.83	8	95.67 %	184.83	3.33	98.20%	184.83	3.33	98.20
5	212.17	30	85.86%	212.17	12.33	94.19%	212.17	22.83	89.24
6	224.42	72	67.92%	224.42	28.67	87.22	224.42	60.83	72.89

Champadevi site

Installation of three buckets of capacity 15 liters in each pre-domestic area of the houses in Champadevi site D and E for 15 days all were negative. Installation of three buckets of capacity 15 liters in each indoor of houses in Champadevi site A, B, and C in the Terrace, Balcony and in Veranda filled with water all the buckets were negative for 15 days in the month of May 2023. In pre-domestic area of Champadevi site F six tubs were installed at each site in the open area inside the compound wall two tubs were positive within five days as the surrounding was enriched with vegetable crops. The larval densities in the tubs were 160 and 156. Considering one as a control and the other as test 2mL/liter bio larvicide was added to one of the tubs. The residual effect was seen for six six-week period with 73.33% mortality of the larvae Table 20.

Table 20: Effect of 2mL/Lt bio-larvicide on mosquito larvae in pre-domestic area Kirtipur site.

Larval densities in pre-domestic area in tubs during post-treatment weeks			
Weeks	**Control tubs**	**Test tubs**	**Mortality%**
24hrs	156	0	100%
1		0	100%
2		0	100%
3	179	12	93.30%
4	204	26	87.25%
5	192	35	81.77%
6	180	48	73.33%

Vegetable farm

In a small vegetable farm, five jars of capacity 20 liters were installed three Jars were installed in the open field by collecting rainwater for oviposition in the Champadevi site, and two jars were in the vegetable farm of Imadole. All jars were positive for larvae after seven days the larval density was 245 and 187 at the Imadole site and 67, 59, and 73 at the Champadevi site. Considering two as control and three as test jar of the Champadevi site. Bio larvicide 4mL/liter was added to the test jar. The residual effect remained in the water for five weeks with 70.35% mortality of the larvae.

Bafal site

Installation of three buckets in the pre-domestic area filled with water and left undisturbed for oviposition. Only one bucket turned positive after 15 days of installation but could not serve as an oviposition for the next 15 days. So, it was

removed from that site. Other buckets were negative even for one-month installation. In this pre-domestic area due to the algal bloom in the buckets may be the reason for negative larval activity.

Sitapaila site

Inside the garage of a house, four buckets of capacity 25 liters filled with 25 liters of water kept at four corners were positive in a week where a single car and scooter were parked. The larval density was determined twice for the confirmation of oviposition, the density in each was 21, 19, 12, and 17 during the second count. The two buckets as control and two as test. To the test bucket 1mL/liter of bio larvicide was added. The residual effect of 79.17% was observed for five weeks Table 21. without replenishment with water.

Table 21: Effect of 1mL/Lt bio-larvicide on mosquito larvae in indoor Sitapaila site.

Larval densities in buckets during post-treatment weeks			
week	**Control bucket**	**Test bucket**	**Mortality %**
24hrs	20	0	100
1		0	100
2		0	100
3		0	100
4		0	100
5	24	5	79.17

Koteshwor site

The 10 flower pots in the pre-domestic area were negative for the whole summer season. Indoor six tubs of capacity 15 liters were all positive considering three as control and three as tests the mean larval density in the tubs was 156. Bio-larvicide 1mL/liter was added to the three tubs, and the residual effect with 80% mortality was observed for six weeks without replenishment with water. Tubs are mainly used to store rainwater during the rainy season as they are wide mouth containers large surface area is provided to lay eggs by the *Aedes* spp. they are not covered no appropriate lid is provided while purchasing.

Chicken and meat shop in Koteshwor site

The two buckets were positive within a week underneath the table. The larval density was 34 and 26. To the test bucket 1mL/liter was added the residual effect remained with 84.72 % mortality for five weeks without replenishment with water. In most of the

chicken and meat shops, proper hygienic condition is not maintained as well and live birds are kept for meat, the flow of consumers and the butcher provide the blood source for multiple female *Aedes* spp. in the shop.

Puddles in the UN park, Roadside, Sankha park, and Manohara park

Not all the observed puddles were positive. May be due to the sun's heat or due to the algal bloom or due to high organic waste the puddles are negative. The water-containing ponds in the Sankha park were all negative during the month of August 2023 due to heat and algal bloom. In the four positive puddles, *Bt*-14P2A larvicide was effective in causing 100% mortality of the larvae in 24 hours. The residual effect cannot be reported as they dried out faster within 2-3 days when there was no rain due to sun heat as well as the roadside cleaning activity carried out by the municipality prevented further accumulation of rainwater, riding of vehicles flashed water from the puddles. Inside the UN park total of six puddles were present only one was positive 100% mortality was observed for 3 days but the water dried after 3 days. In the puddles that were near to the park in the shaded place no larval activity was observed even though the green vegetation was present may be the temperature was lower which prevented the larval growth.

Overall in all types of plastic containers, the residual effect of bio larvicide was observed for a minimum of four weeks without disturbing the containers and without diluting the bio larvicide therefore the bio larvicide can be used in domestic containers used to store water to prevent the multiplication of mosquito vector in indoor and outdoor surroundings.

4.9 Identification of mosquitoes

In different sites different mosquitoes were present, in the indoor containers *Ae. aegypti* and *Culex* spp., in the tires and other containers of CDM and dean TU premises *Ae. aegypti, Ae. albopictus, Toxorhynchites* spp., and other unidentified *Aedes* spp. were also present. *Ae. albopictus* were predominate in the park (Camargo *et al*., 2021). In this study also in different vegetation sites, the predominant mosquito was *Ae. albopictus*. In the Kalanki site tires *Ae. aegypti*, *Ae. albopictus*, and *Toxorhynchites* spp., were present. In this site, a small cornfield was seen during the study period. In all the cornfield *Toxorhynchites, Ae. aegypti,* and *Ae. albopictus* was present as a dominant mosquito. In the pre-domestic area where vegetation was present *Ae.*

albopictus, Ae. aegypti and *Culex* spp. were present mainly in Maharajgunj site bamboo herbs were present in the public space that supported the proliferation of *Ae. albopictus*. Gwarko workshop tires *Ae. aegypti* and *Culex* spp. were found. In the vegetable farm of Kirtipur *Ae. aegypti* and *Toxorhynchites* were present in the vegetable farm of Imadole *Ae. aegypti* and *Ae. albopictus* were found. Garbage containers *Ae. aegypti* and *Culex* were present. Cow farm *Ae. aegypti* and *Culex* spp. were found. During this study period surveillance of larvae was done in the installed containers placed in different sites and the dominant mosquito was *Aedes* spp. The cornfield plantation in the domestic premises during the monsoon season increases the density of *Ae. albopictus*.

4.10 Limitation of study

Exploration of soil samples from a unique ecosystem or other types of samples like dead insects, water, organic waste, phyllosphere, rhizosphere, etc., was not performed during the study, which would help to isolate a greater number and other different varieties of native *Bt*. Only one larvicidal *Bt* strain i.e. *Bt*-14P2A was formulated in the form of liquid for field trial. Comparison of all mosquitocidal *Bt* strains efficacy and residual effect in the laboratory or field trial was not carried out during the study due to time constraints as well and a well-developed insectary was not available for rearing all kinds of mosquito vectors in the research lab where the research was carried out. The isolated larvicidal *Bt* has to be further assayed against laboratory-reared larvae of different mosquitoes to evaluate its efficacy against natural habitats. Evaluation of its effectiveness should be evaluated in different parts of Nepal before including it in the national vector control program. *Ls* was isolated as a contamination during the study period at first. Due to its unique microscopic morphology, it was further studied and found to be *Ls.* Different samples and a greater number of samples were not analyzed for the isolation of *Ls*. Whole genome sequencing (WGS) and 16srRNA sequencing of all the bacterial isolates were not performed during the study. Different molecular characterization techniques were not performed. Standard strain of *Ls* was not available for comparison of its effectiveness in the laboratory condition.

Figure 9. Sifal site outdoor

Figure 10. Sifal site outdoor

Figure 11. Kirtipur cornfield site

Figure 12. Imadole site pre-domestic area

Figure 13. Simulated field trial CDM

Figure 14. Tribhuvan University dean's office

Figure 15. Maharajgunj site pre-domestic area

Figure 16. Kirtipur outdoor

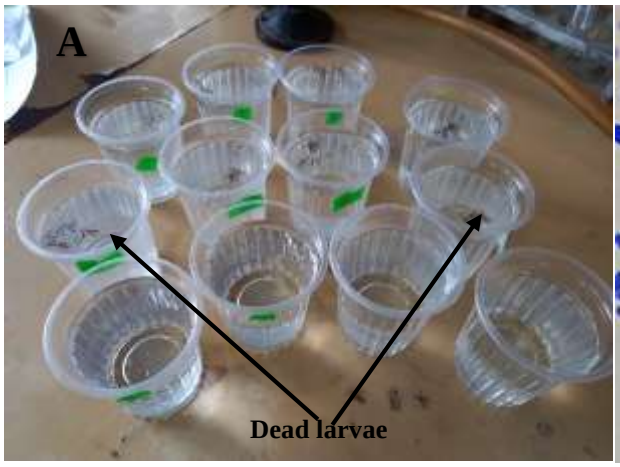


Figure 17. Larvicidal activity in 4 replicates

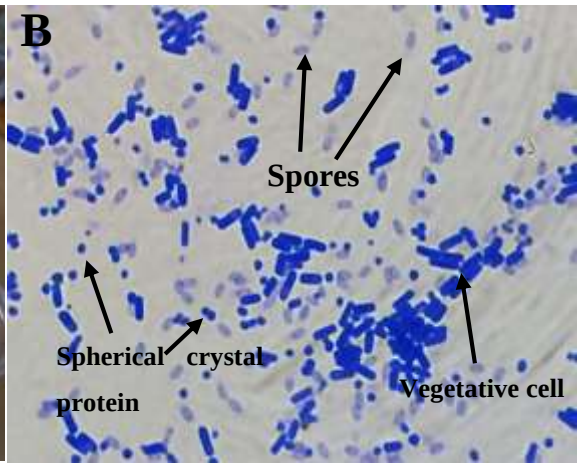


Figure 18. Microscopic observation of *Bt*-14P2A

Figure 19. Colony morphology of *Bt*-14P2A

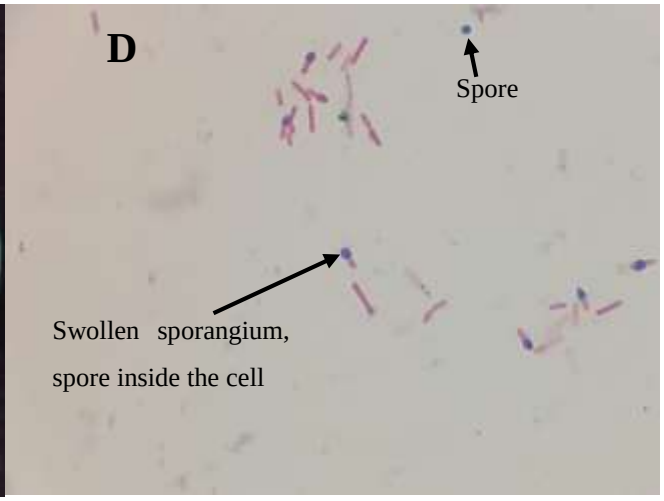


Figure 20. Microscopic observation of *Ls*-10P5B

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Two important mosquito larvicidal indigenous bacteria *Bt* and *Ls* were isolated from the soil sample of Nepal. *Bt* showed 100% toxicity and *Ls* showed mild toxicity against *Ae. aegypti* larvae under laboratory conditions. Larvicidal *Bt* isolated from the soil samples of Nepal is effective in killing all types of mosquito larvae during the field trial. The morphological characteristics of mosquito larvicidal *Bt* were similar to the standard control strain *Bti*-IPS-82, in the colony morphology in the NA agar plate, the size of the cell, the shape of the crystal protein, and the elliptical spore. The 16srRNA gene sequence also showed 97% similarity with the *Bti* strains available in the GenBank of NCBI. *Bt*-7P1A is in the same cluster with the WHO-recommended strain *Bti*-AM65-52 isolated from France and it is commercialized. *Bt*-14P2A is close to the Indian strain *Bti*-ISPC-12 and *Bt*-8P1A is closer to the Srilank strain *Bti*-SB67. The *Bt*-14P2A strain is as effective as *Bti*-IPS-82 in killing the mosquito larvae.

The biological control agent *Bt* isolated from the soil sample of Nepal coded as *Bt*-14P2A was formulated as a liquid formulation as *Bt*-14P2A bio larvicide in the laboratory condition was found to be effective to killing the mosquito larvae in the indoor and outdoor domestic water containers as well as in discarded tires. The bio larvicide was effective against *Ae. aegypti, Ae. albopictus, Culex* spp., and *Toxorhynchites* spp. found in the different environments during the field trial. The residual effect remains longer in the clean water and in the shaded places when the water is free of organic content or other types of debris.

5.2 Recommendations

Nepal currently lacks biological control agents as part of its vector control program. The isolated mosquito bio larvicide *Bt*-14P2A was effective controlling *Ae. aegypti* larvae in the laboratory and in field trial. Thus, it can be incorporated into the vector control program in Nepal could be a potential option for reducing the mosquito population and limiting the spread of mosquito-borne diseases specially to prevent the spread of dengue disease. "Search and destroy campaign" started by the government its effectiveness will be enhanced by including the mosquito larvicidal *Bt* to control the dengue vector in the domestic containers.

Mosquito larvicidal *Bt*-14P2A will be effective if included in the IVM program to control mosquito vectors.

Commercialization of bio larvicide in different forms, liquid formulations, cake, and granules should be made available in the market. Which can be purchased by the consumer to add to the water storage containers regularly to prevent the indoor multiplication of *Ae. aegypti*.

In automobile workshops or in parks, or puddles during the monsoon season addition or spraying of >5ml/Lt bio larvicide at regular intervals will prevent the development of adult mosquitoes. People should made aware of the *Bt* and application at regular interval to prevent the development of adult mosquitoes in the surroundings. From this study *Bt* is effective in domestic water containers stored for different domestic purposes.

However, it's important to conduct further studies to evaluate the safety and effectiveness of using *Bti* in different breeding habitats and at different geographical areas of Nepal on a large scale. Further, molecular studies of these larvicidal *Bt* have to be carried out to know the integrity of the genes responsible for producing different types of bioactive compounds, the novelty of the strains, etc.

CHAPTER 6

SUMMARY

Increasing dengue diseases in the urban area of Nepal should be addressed by additional techniques and tools to prevent dengue and other types of arboviral diseases. Larval source management (LSM) is also an effective method to control the deadliest mosquitoes. LSM is an approach to manage larval habitats to prevent the development of adult mosquitoes mainly targeting the larvae and pupae of mosquitoes, thereby curbing the density of adult vectors. Different techniques and tools can be used for LSM for example *Bti* and *Ls* are tools to prevent the development of adult mosquitoes by killing the larval mosquitoes present in different water sources. *Bt* and *Ls* produce toxins toxic only to mosquito larvae when these toxins are added to the water, the larvae feed on them and die. Various nations formulated in different forms isolated from different sources implemented to control mosquitoes. They are nontoxic for humans and other living organisms. In Nepal, unawareness and unavailability of these isolates made us inaccessible to these tools to control mosquito vectors. To address the increasing trend of mosquito-borne diseases in Nepal these bacteria should be included in the vector control toolbox. The research aims to isolate larvicidal *Bt* from a soil sample of Nepal and to evaluate its effectiveness in controlling mosquitoes. From the soil samples *Bt* was isolated by acetate selection method and identified by observing crystal protein by coomassie brilliant blue in a light microscope and characterized by larvicidal assay and 16srRNA gene sequencing, all the spherical-shaped ICPs producing *Bt* were screened for larvicidal activity. For larvicidal activity, larvae were collected from the field and laboratory-reared *Ae. aegypti* were assayed by feeding *Bt* toxin according to the World Health Organization protocol. Further only one positive isolated *Bt*-14P2A formulated in the form of pellet as well as in the form of liquid suspension (bio larvicide) was evaluated in the laboratory condition and by field trial with positive and negative control in replicates. The lethal concentration (LC), residual effect, and mortality percentage were calculated. Different crystal proteins producing *Bt* 1395 were obtained from 454 soil samples. Only four *Bt* isolates coded as *Bt*-14P2A, *Bt*-7P1A, *Bt*-8P1A, and *Bt*-3P2B producing spherical ICPs showed 100% mortality against mosquito larvae during primary screening. The LC required against the field-

collected and laboratory-reared *Ae. aegypti* mosquito larvae by *Bt*-14P2A in the form of the pellet was LC_{50} 3.98 Parts Per Million (PPM), LC_{90} 25.11 PPM and LC_{50} 6.45 PPM, LC_{90} 37.15 PPM respectively. The liquid formulated bio larvicide *Bt*-14P2A was evaluated by simulated field trial, higher concentration of 6ml/Lt was found to show a residual effect for seven weeks (49 days) by maintaining a mean mortality of 100% for a six-week period. All the concentrations used in the simulated field trial and open field trial were able to cause 100% mortality of the larvae within 24 hours. Molecular characterization showed that the larvicidal *Bt* isolates *Bt*-14P2A, *Bt*-7P1A, and *Bt*-8P1A are similar and identical to *Bti* deposited in the GenBank. On analysis of 32 soil samples 11 *Ls* were obtained. Only one *Ls* showed 25% mortality against *Ae. aegypti* larvae. Five mosquito larvicidal indigenous bacteria four *Bt* and one *Ls* were isolated from the soil samples of Nepal and *Bt*-14P2A effectively killed the field-collected mixture of larvae and the dengue vector larvae under laboratory conditions and in field trials. Thus, it can serve as a future tool for LSM to control mosquito-borne diseases in Nepal.

REFERENCES

Abagli, A. Z., Alavo, T. B. C., Perez-Pacheco, R., & Platzer, E. G. (2019). Efficacy of the Mermithid Nematode, Romanomermis Iyengari, for the Biocontrol of *Anopheles gambiae*, the Major Malaria Vector in Sub-Saharan Africa. *Parasites and Vectors*, **12**(1): 6–13. https://doi.org/10.1186/s13071-019-3508-6

Amit Roy, S. R. (2014). Molecular Markers in Phylogenetic Studies-A Review. *Journal of Phylogenetics & Evolutionary Biology*, **02**(02): https://doi.org/10.4172/2329-9002.1000131

Ammouneh, H., Harba, M., Idris, E., & Makee, H. (2011). Isolation and Characterization of Native *Bacillus thuringiensis* Isolates from Syrian Soil and Testing of their Insecticidal Activities Against Some Insect Pests. *Turkish Journal of Agriculture and Forestry*, **35**(4): 421–431. https://doi.org/10.3906/tar-1007-1117

Apaydin, O., Yenidunya, A. F., Harsa, S., & and Gunes, H. (2005). Isolation and Characterization of *Bacillus thuringiensis* Strains from Different Grain Habitats in Turkey. *World Journal of Microbiology and Biotechnology*, **21**: 285–292. https://doi.org/10.1007/s11274-004-3633-y

Bello, A., Machido, D. A., & Inabo, H. I. (2016). Evaluation of the Larvicidal Activity of *Bacillus thuringiensis* Isolated from Soil Samples against *Aedes aegypti*. *American Journal of Microbiology and Immunology*, **1**(2): 26–31.

Berliner, E. (1911). Die "Schlaffsucht" der Mehlmottenraupe.Z. *Gesamte Getreidewes*, **3**: 63–70.

Bigio, J., Braack, L., Chea, T., Set, S., Suon, S., Echaubard, P., Hustedt, J., Debackere, M., Ramirez, B., Prasetyo, D. B., Bunleng, S., Wharton-Smith, A., & Hii, J. (2022). Entomological Outcomes of Cluster-Randomised, Community-Driven Dengue Vector-Suppression Interventions in Kampong Cham province, Cambodia. *PLoS Neglected Tropical Diseases*, **16**(1): 1–18. https://doi.org/10.1371/JOURNAL.PNTD.0010028

Bravo, A., Sarabia, S., Lopez, L., Ontiveros, H., Abarca, C., Ortiz, A., Ortiz, M., Lina, L., Villalobos, F. J., Peña, G., Nuñez-Valdez, M. E., Soberón, M., & Quintero, R. (1998). Characterization of cry Genes in a Mexican *Bacillus thuringiensis*

Strain Collection. *Applied and Environmental Microbiology*, **64**(12): 4965–4972.

Bukin, Y. S., Galachyants, Y. P., Morozov, I. V., Bukin, S. V., Zakharenko, A. S., & Zemskaya, T. I. (2019). The effect of 16s rRNA Region Choice on Bacterial Community Metabarcoding Results. *Scientific Data*, ***6*:** 1–14. https://doi.org/10.1038/sdata.2019.7

Camargo, C., Alfonso-Parra, C., Díaz, S., Rincon, D. F., Ramírez-Sánchez, L. F., Agudelo, J., Barrientos, L. M., Villa-Arias, S., & Avila, F. W. (2021). Spatial and Temporal Population Dynamics of Male and Female *Aedes albopictus* at a Local Scale in Medellín, Colombia. *Parasites and Vectors*, **14(**1): 1–15. https://doi.org/10.1186/s13071-021-04806-2

Center for Disease Control and Prevention. (2023). *Fighting the Worlds Deadliest Animal*. Global Health. https://www.cdc.gov/globalhealth/stories/2019/world-deadliest-animal.html#:~:text=Spreading diseases like malaria%2C dengue,other creature in the world.

Centers for Disease Control and Prevention. (2016). Mosquito life cycle. *Centers for Disease Control and Prevention*, **2**.

Charles, J. F., Nielsen-LeRoux, C., & Delécluse, A. (1996). *Bacillus sphaericus* Toxins: Molecular Biology and Mode of Action. *Annual Review of Entomology.*, **41**(1): 451–472. https://doi.org/10.1146/annurev.en.41.010196.002315

Chatterjee, S., Bag, S., Biswal, D., Sarkar Paria, D., Bandyopadhyay, R., Sarkar, B., Mandal, A., & Dangar, T. K. (2023). Neem-Based Products as Potential Eco-friendly Mosquito Control Agents Over Conventional Eco-toxic Chemical Pesticides-A review. *Acta Tropica*, **240(**December2022): 106858. https://doi.org/10.1016/j.actatropica.2023.106858

Dambach, P., Louis, V. R., Kaiser, A., Ouedraogo, S., Sié, A., Sauerborn, R., & Becker, N. (2014). Efficacy of *Bacillus thuringiensis* var. i*sraelensis* Against Malaria Mosquitoes in Northwestern Burkina Faso. *Parasites and Vectors*, **7(**1): 1–8. https://doi.org/10.1186/1756-3305-7-371

Department of Health Services. Epidemiology and Disease Control Division. (2016).

Nepal Malaria Strategic Plan 2014-2025.

Department of Health Services. Epidemiology and Disease Control Division. (2019). *National Guidelines on Prevention, Management and Control of Dengue in Nepal*.

Derua, Y. A., Kahindi, S. C., Mosha, F. W., Kweka, E. J., Atieli, H. E., Wang, X., Zhou, G., Lee, M. C., Githeko, A. K., & Yan, G. (2018). Microbial Larvicides for Mosquito Control: Impact of Long Lasting Formulations of *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* on Non-target Organisms in Western Kenya Highlands. *Ecology and Evolution*, **8**(15): 7563–7573. https://doi.org/10.1002/ece3.4250

Derua, Y. A., Tungu, P. K., Malima, R. C., Mwingira, V., Kimambo, A. G., Batengana, B. M., Machafuko, P., Sambu, E. Z., Mgaya, Y. D., & Kisinza, W. N. (2022). Laboratory and Semi- field Evaluation of the Efficacy of *Bacillus thuringiensis* var. *israelensis* (Bactivec ®) and *Bacillus sphaericus* (Griselesf ®) for Control of Mosquito Vectors in Northeastern Tanzania. *Current Research in Parasitology & Vector-Borne Diseases*, **2(**April):100089. https://doi.org/10.1016/j.crpvbd.2022.100089

Devidas, P. C., Pandit, B. H., Vitthalrao, P. S., Gerrard, S. R., Narayanan, S., Tofalo, R., & Vela, A. I. (2014). Evaluation of Different Culture Media for Improvement in Bioinsecticides Production by Indigenous *Bacillus thuringiensis* and their Application against Larvae of *Aedes aegypti*. *The Scientific World Journal*. https://doi.org/10.1155/2014/273030

Dhimal, M., Ahrens, B., & Kuch, U. (2015). Climate Change and Spatiotemporal Distributions of Vector-borne Diseases in Nepal - A systematic synthesis of literature. *PLoS ONE*, **10**(6): 1–31. https://doi.org/10.1371/journal.pone.0129869

Dhimal, M., Dahal, S., Dhimal, M. L., Mishra, S. R., Karki, K. B., Aryal, K. K., Haque, U., Kabir, M. I., Guin, P., Butt, A. M., Harapan, H., Liu, Q. Y., Chu, C., Montag, D., Groneberg, D. A., Pandey, B. D., Kuch, U., & Müller, R. (2018). Threats of Zika Virus Transmission for Asia and its Hindu-Kush Himalayan region. *Infectious Diseases of Poverty*, **7**(40): 1–7. https://doi.org/10.1186/s40249-018-0426-3

Dhimal, M., Gautam, I., Kreß, A., Müller, R., & Kuch, U. (2014). Spatio-Temporal Distribution of Dengue and Lymphatic Filariasis Vectors along an Altitudinal Transect in Central Nepal. *PLoS Neglected Tropical Diseases*, **8**(7): https://doi.org/10.1371/journal.pntd.0003035

Dhimal, M., O'hara, R. B., Karki, R., Thakur, G. D., Kuch, U., & Ahrens, B. (2014). Spatio-Temporal Distribution of Malaria and its Association with Climatic Factors and Vector-Control Interventions in Two High-Risk Districts of Nepal. *Malaria Journal*, **13**(457): 1–14. https://doi.org/10.1186/1475-2875-13-457

El-Kersh, T. A., Ahmed, A. M., Al-Sheikh, Y. A., Tripet, F., Ibrahim, M. S., & Metwalli, A. A. M. (2016). Isolation and Characterization of Native *Bacillus thuringiensis* Strains from Saudi Arabia with Enhanced Larvicidal Toxicity Against the Mosquito Vector *Anopheles gambiae* (s.l.). *Parasites and Vectors*, **9**(1): 1–14. https://doi.org/10.1186/s13071-016-1922-6

Epidemiology and Disease Control Division (EDCD). (2023). *Situation Report on Dengue in Nepal-2023*. Ministry of Health and Population, Department of Health Services.

Epidemiology and Disease Control Division (EDCD). (2019). *Malaria Update* **975**(9).

Epidemiology and Disease Control Division (EDCD). (2020). *National Guidelines on Intergrated Vector Management*. *June*, 1–140.

Epidemiology and Disease Control Division (EDCD). (2022a). *Dengue information by provinces at national as of 30th Nov , 2022 Dengue reporting : Top Ten Districts as of 30th* **2022**(2).

Epidemiology and Disease Control Division (EDCD). (2022b). *Situation update of Dengue 2022 (As of 4 Sept , 2022)* **2022** (1).

Epidemiology and Disease Control Division (EDCD). (2023). *Situation update of Dengue* **2023**(2023).

European Center for Disease prevention and Control. (2023). 12-Month Dengue Virus Disease Case Notification Rate per100000 Population November 2022-October2023. In *European Center for Disease Prevention and control*.

European Centre for Diseases Prevention and Control. (2022). *'Reverse' identification*

key for mosquito species.

Facchinelli, L., Alsharif, B., & Jones, J. D. (2023). Mapping *Aedes aegypti* Indoor Resting Behavior Reveals a Preference Vulnerable to Householder-Led Vector Control. *PNAS Nexus*, **2**(7): 1–9. https://doi.org/10.1093/pnasnexus/pgad226

Ferede, G., Tiruneh, M., Abate, E., Kassa, W. J., Wondimeneh, Y., Damtie, D., & Tessema, B. (2018). *Distribution and larval breeding habitats of Aedes mosquito species in residential areas of northwest Ethiopia*. 1–7.

Fillinger, U., & Lindsay, S. W. (2011). Larval Source Management for Malaria Control in Africa: Myths and reality. *Malaria Journal*, **10(**1): 353. https://doi.org/10.1186/1475-2875-10-353

Getachew, D., Tekie, H., Gebre-michael, T., Balkew, M., & Mesfin, A. (2015). *Breeding Sites of Aedes aegypti : Potential Dengue Vectors in Dire Dawa , East Ethiopia*. **2015**: https://doi.org/10.1155/2015/706276

Glare, T. R., & Callaghan, M. O. (1998). *Environmental and health impacts of Bacillus thuringiensis israelensis*.

Goldberg, L. J., & Margalit, J. (1977). A Bacterial Spore Demonstrating Rapid Larvicidal Activity Against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univeritattus*, *Aedes aegypti*, and *Culex pipiens*. *Mosquito News*, **37**(3): 355–358.

Government of Nepal. (2022). *Lymphatic Filariasis Elimination*. Ministry of Health and Population. https://mohp.gov.np/program/lymphatic-filariasis-elimination/en

Gupta, B. P., Tuladhar, R., Kurmi, R., & Manandhar, K. Das. (2018). Dengue Periodic Outbreaks and Epidemiological Trends in Nepal. *Annals of Clinical Microbiology and Antimicrobials*, **17**(1):1–6. https://doi.org/10.1186/s12941-018-0258-9

Himalayan News service. (2019). Malaria in Hilly, Mountaneous regions. *The Himalayan*.

Howard, A. F. V., Zhou, G., & Omlin, F. X. (2007). Malaria Mosquito Control Using Edible Fish in Western Kenya: Preliminary findings of a controlled study. *BMC Public Health*, **7**: 1–6. https://doi.org/10.1186/1471-2458-7-199

Huang, Y. J. S., Higgs, S., & Vanlandingham, D. L. (2017). Biological Control Strategies for Mosquito Vectors of Arboviruses. *Insects*, **8**(1): 1–25. https://doi.org/10.3390/insects8010021

Ibrahim, M. A., Griko, N., Junker, M., & Bulla, L. A. (2010). A Genomics and Proteomics Perspective. *Bioengineered Bugs*. https://doi.org/10.4161/bbug.1.1.10519

Imam, H., Sofi, G., Zarnigar, & Aziz, S. (2014). The Basic Rules and Methods of Mosquito Rearing (*Aedes aegypti*). *Tropical Parasitology*, **4**(1): 53. https://doi.org/10.4103/2229-5070.129167

Imbahale, S. S., Githeko, A., Mukabana, W. R., & Takken, W. (2012). Integrated Mosquito Larval Source Management Reduces Larval Numbers in Two Highland Villages in Western Kenya. *BMC Public Health*, **12**(1): 1. https://doi.org/10.1186/1471-2458-12-362

Ingabire, C. M., Hakizimana, E., Rulisa, A., Kateera, F., Van Den Borne, B., Muvunyi, C. M., Mutesa, L., Van Vugt, M., Koenraadt, C. J. M., Takken, W., & Alaii, J. (2017). Community-Based Biological Control of Malaria Mosquitoes Using *Bacillus thuringiensis var. israelensis* (Bti) in Rwanda: Community Awareness, Acceptance and Participation. *Malaria Journal*, **16(**1): 1–13. https://doi.org/10.1186/s12936-017-2046-y

Ishiwata, S. (1901). On a kind of severe flacherie (sotto disease). *Dainihan Sanshi Kaiho*, **114**: 1–5.

Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., Leopold, S. R., Hanson, B. M., Agresta, H. O., Gerstein, M., Sodergren, E., & Weinstock, G. M. (2019). Evaluation of 16S rRNA Gene Sequencing for Species and Strain-level Microbiome Analysis. *Nature Communications*, **10**(1): 1–11. https://doi.org/10.1038/s41467-019-13036-1

Kauffman, E., Payne, A., Franke, M. A., Schmid, M. A., Harris, E., & Kramer, L. D. (2017). Rearing of *culex* spp and *Aedes* spp. Mosquitoes. *Bio Protoc*, **7(**17). https://doi.org/10.21769/BioProtoc.2542.Rearing

Kellen, W. R., Clark, T. B., Lindegren, J. E., Ho, B. C., Rogoff, M. H., & Singer, S. (1965). *Bacillus sphaericus* Neide as a Pathogen of Mosquitoes. *Journal of*

Invertebrate Pathology, **7**(4): 442–448. https://doi.org/10.1016/0022-2011(65)90120-5

Lacey, L. A., & Orr, B. K. (1994). *Bacillus thuringiensis* Serovariety *israelensis* and *Bacillus sphaericus* for Mosquito Control. *American Journal of Tropical Medicine and Hygiene*, **50**(6 SUPPL.): 97–115. https://doi.org/10.4269/ajtmh.1994.50.97

Lee, YW and Zairi, J. (2005). Laboratory Evaluation of *Bacillus thuringiensis* H-14 against *Aedes aegypti*. *Tropical Biomsdicine*, **22**(1): 5–10.

Lobo, K. dos S., Soares-da-Silva, J., Silva, M. C. da, Tadei, W. P., Polanczyk, R. A., & Pinheiro, V. C. S. (2018). Isolation and Molecular Characterization of *Bacillus thuringiensis* Found in Soils of the Cerrado Region of Brazil, and their Toxicity to *Aedes aegypti* larvae. *Revista Brasileira de Entomologia*, **62**(1): 5–12. https://doi.org/10.1016/j.rbe.2017.11.004

Louca, V., Lucas, M. C., Green, C., Majambere, S., Fifllinger, U., & Lindsay, S. W. (2009). Role of Fish as Predators of Mosquito Larvae on the Flood Plain of the Gambia River. *J Med Entomol*, **46**(3): 546–556. https://doi.org/10.1016/j.earlhumdev.2006.05.022

Malla, S., Thakur, G. D., Shrestha, S. K., Banjeree, M. K., Thapa, L. B., Gongal, G., Gibbons, R. V. et al., (2008). Identification of All Dengue Serotypes in Nepal. *Emerging Infectious Diseases*, *14*(10), 1669-1670. https://doi.org/10.3201/eid1410.080432.

Margalit, J. (1990). Discovery of *Bacillus thuringiensis israelensis*. In *Bacterial Control of Mosquitoes & Black Flies*. Rutgers University Press. https://doi.org/10.1007/978-94-011-5967-8_1

Martin, P. A. W., & Travers, R. S. (1989). Worldwide Abundance and Distribution of *Bacillus thuringiensis* Isolates. *Applied and Environmental Microbiology*, **55**(10): 2437–2442. https://doi.org/10.1128/aem.55.10.2437-2442.1989

Medlock, J. M., Alten, B., Versteirt, V., & Schaffner, F. (2018). *Field sampling methods for mosquitoes, sandflies, biting midges and ticks. VectorNet project 2014-2018*. https://doi.org/10.2903/sp.efsa.2018.EN-1435

Milner, R. j. (1994). History of *Bacillus thuringiensis*. *Agriculture Ecosystem and*

Environment, **49**(1): 9–13.

Mukabana, W. R., Welter, G., Ohr, P., Tingitana, L., Makame, M. H., Ali, A. S., & Knols, B. G. J. (2022). Drones for Area-Wide Larval Source Management of Malaria Mosquitoes. *Drones*, **6**(7). https://doi.org/10.3390/drones6070180

Mukherjee, D., Das, S., Begum, F., Mal, S., & Ray, U. (2019). The Mosquito Immune System and the Life of Dengue Virus: What we know and do not know. *Pathogens*, **8**(2). https://doi.org/10.3390/pathogens8020077

Nair, K., Al-Thani, R., Al-Thani, D., Al-Yafei, F., Ahmed, T., & Jaoua, S. (2018). Diversity of *Bacillus thuringiensis* Strains from Qatar as Shown by Crystal Morphology, δ-Endotoxins and Cry gene content. *Frontiers in Microbiology*, **9**(APR). https://doi.org/10.3389/fmicb.2018.00708

Nepal Health Research council. (2020). *A Call for Actions for Controlling and Eliminating Emerging and Re-emerging Vector-borne Diseases in Nepal*.

Nicolas, L. (1992). Bacteriological Control of Mosquitoes and Blackflies: Present Aspects and Perspective of Research. Brazilian Agricultural Research, Brasilia, **27**, 37–46.

Obopile, M., Segoea, G., Waniwa, K., Ntebela, D. S., Moakofhi, K., Motlaleng, M., Mosweunyane, T., Edwards, J. K., Namboze, J., Butt, W., Manzi, M., Takarinda, K. C., & Owiti, P. (2018). Did Microbial Larviciding Contribute to a Reduction in Malaria Cases in Eastern Botswana in 2012–2013? *Public Health Action*, **8**(1): S50–S54. https://doi.org/10.5588/pha.17.0012

Okumu, F. O., Knols, B. G. J., & Fillinger, U. (2007). Larvicidal Effects of a Neem (*Azadirachta indica*) Oil Formulation on the Malaria Vector *Anopheles gambiae*. *Malaria Journal*, **6**(February). https://doi.org/10.1186/1475-2875-6-63

Olalubi, O. A. (2016). Promoting Larval Source Management as a Vital Supplemental Addendum and More Likely Cost-Effective Approach for Malaria Vector Control in Nigeria. *Journal of Prevention and Infection Control*, **02**(02): 1–6. https://doi.org/10.21767/2471-9668.100015

Onyido, A. E., Ozumba, N. ., Ezike, V. I., Ikpeze, O. O., & Nwankwo, A. C. (2009). A New Method of Rearing and Maintenance of Mosquito Colony in the

Laboratory. *African Journal of Bioscience*, **2**(1): 16–19.

Padonou, G. G., Ossè, R., Salako, A. S., Aikpon, R., Sovi, A., Kpanou, C., Sagbohan, H., Akadiri, Y., Lamine, B. M., & Akogbeto, M. C. (2020). Entomological Assessment of the Risk of Dengue Outbreak in Abomey-Calavi Commune, Benin. *Tropical Medicine and Health*, **48**(1): https://doi.org/10.1186/s41182-020-00207-w

Palma, L., Muñoz, D., Berry, C., Murillo, J., Caballero, P., & Caballero, P. (2014). *Bacillus thuringiensis* Toxins: An Overview of Their Biocidal Activity. *Toxins*, **6**(12): 3296–3325. https://doi.org/10.3390/toxins6123296

Pandey, B. D., Rai, S. ., Morita, K., And, & Kurane, I. (2004). First Case of Dengue Virus Infection in Nepal. *Nepal Medical College Journal*, **6**(2): 157–159.

PATH. (2016). *Combatting Japanese Encephalitis in Nepal: A Public Health Success Story*.

Paudel, D., Kakchapati, S., Lageju, N., Karki, S., Dhungana, J., Regmi, S., Chudal, D., & Sharma, R. P. (2023). Factors Influencing the Knowledge, Attitude, and Practices of Police Personnel Toward Dengue Fever in Kathmandu, Nepal. *Journal of Occupational Health*, **65**(1): 1–12. https://doi.org/10.1002/1348-9585.12421

Petrosino, J. F., Highlander, S., Luna, R. A., Gibbs, R. A., & Versalovic, J. (2009). Metagenomic Pyrosequencing and Microbial Identification. *Clinical Chemistry*, **55**(5): 856–866. https://doi.org/10.1373/clinchem.2008.107565

Phuyal, P., Kramer, I. M., Kuch, U., Magdeburg, A., Groneberg, D. A., Lamichhane Dhimal, M., Montag, D., Harapan, H., Wouters, E., Jha, A. K., Dhimal, M., & Müller, R. (2022). The knowledge, Attitude and Practice of Community People on Dengue Fever in Central Nepal: A Cross-Sectional Study. *BMC Infectious Diseases*, **22**(1): 1–18. https://doi.org/10.1186/s12879-022-07404-4

Poopathi, S., Thirugnanasambantham, K., Mani, C., Ragul, K., & Sundarapandian, S. M. (2014). Isolation of Mosquitocidal Bacteria (*Bacillus thuringiensis*, *B.sphaericus* and *B.cereus*) from Excreta of Arid Birds. *Indian Journal of Experimental Biology*, **52**(7): 739–747.

Poudel, A. (2022). As Nepal's dengue fatalities hit 45, risk grows of zika, chikungunya

outbreaks. *The Kathmandu Post*.

Poudel, A. (2023). Malaria End in Doubt with 7 Local Cases. *The Kathmandu Post*.

Pruszynski, C. A., Hribar, L. J., Mickle, R., & Leal, A. L. (2017). A large Scale Biorational Approach Using *Bacillus thuringiensis israeliensis* (Strain AM65-52) for Managing *Aedes aegypti* Populations to Prevent Dengue, Chikungunya and Zika transmission. *PLoS ONE*, **12**(2): 296–310. https://doi.org/10.1371/journal.pone.0170079

Rampersad, J., & Ammons, D. (2005). A *Bacillus thuringiensis* Isolation Method Utilizing a Novel Stain, Low Selection and High Throughput Produced Atypical Results. *BMC Microbiology*, **5**:1–11. https://doi.org/10.1186/1471-2180-5-52

Rampersad, J., Khan, A., & Ammons, D. (2002). Usefulness of Staining Parasporal Bodies When Screening for *Bacillus thuringiensis. Journal of Invertebrate Pathology*, **79**(3): 203–204. https://doi.org/10.1016/S0022-2011(02)00018-6

Reyaz, A. L., Shahanaj, I., Gunapriya, L., Nancy, D., Karthik, C., & Arulselvi, P. I. (2013). Plasmid profiling of Indigenous *Bacillus thuringiensis* Isolated from Tamil Nadu and Kashmir. *JOPR: Journal of Pharmacy Research*, **6**(3): 325–330. https://doi.org/10.1016/j.jopr.2013.03.007

Ritchie, S. A., Rapley, L. P., & Benjamin, S. (2010). *Bacillus thuringiensis* var. *israelensis* (Bti) Provides Residual Control of *Aedes aegypti* in Small Containers. *American Journal of Tropical Medicine and Hygiene*, **82**(6): 1053–1059. https://doi.org/10.4269/ajtmh.2010.09-0603

Roh, Y. J., Choi, J. A. E. Y., Li, M. S., Jin, B. R. A. E., & Je, Y. H. O. (2007). *Bacillus thuringiensis* as a Specific, Safe and Effective tool for Insect Pest. *J.Microbiol. Biotechnol.*, **17**(4): 547–559.

Ruckert, C., & Ebel, G. (2018). How Do Virus-Mosquito Interactions Lead to Viral Emergence? *Trends Parasitol*, **34**(4): 310–321. https://doi.org/10.1016/j.pt.2017.12.004.

Rueda, L. M. (2004). *Pictorial Keys for the identification of mosquitoes (Diptera: Cluicidae) associated with Dengue Virus Transmission Zootaxa 589*. Magnolia Press.

Satilms, S., Toprak, NU., Llgin, C., and Soyletir, G. Evaluation of direct 16SrRNA

PCR from clinical samples for bacterial detection in normally sterile body sites. *The Journal of Infection in Developing Countries*, **13**(11): doi:10.3855/jidc.11732

Schünemann, R., Knaak, N., & Fiuza, L. M. (2014). Mode of Action and Specificity of *Bacillus thuringiensis* Toxins in the Control of Caterpillars and Stink Bugs in Soybean Culture. *ISRN Microbiology*, **2014**: 1–12. https://doi.org/10.1155/2014/135675

Seang, C., Hanboonsong, Y., Muenworn, V., Rocklöv, J., & Haque, U. (2023). Indoor Resting Behavior of *Aedes aegypti* (Diptera : Culicidae) in Northeastern Thailand. *Parasites & Vectors*, 1–15. https://doi.org/10.1186/s13071-023-05746-9

Shragai, T., Mader, E., & Harrington, L. (2018). *Egg identification guide for Aedes albopictus in the Northeast, USA current methods of Ovitrap surveillance why do egg identification ?* (July). Cornell University.

Shrestha, S. B., Pyakurel, U. R., Khanal, M., Upadhyay, M., Na-Bangchang, K., & Muhamad, P. (2019). Epidemiological Situations and Control Strategies of Vector-Borne Diseases in Nepal During 1998–2016. *Journal of Health Research*, **33**(6): 478–493. https://doi.org/10.1108/JHR-10-2018-0124

Sirisena, P., & Noordeen, F. (2016). Dengue Control in Sri Lanka – Challenges and Prospects for Improving Current Strategies. *Sri Lankan Journal of Infectious Diseases*, **6**(1): 2–16. https://doi.org/10.1093/trstmh/tru131

Soares-da-Silva, J., Pinheiro, V. C. S., Litaiff-Abreu, E., Polanczyk, R. A., & Tadei, W. P. (2015). Isolation of *Bacillus thuringiensis* from the State of Amazonas, in Brazil, and Screening Against *Aedes aegypti* (Diptera, Culicidae). *Revista Brasileira de Entomologia*, **59(**1): 1–6. https://doi.org/10.1016/j.rbe.2015.02.001

Suryadi, B. F., Yanuwiadi, B., Ardyati, T., & Suharjono. (2015). Isolation of *Bacillus sphaericus* from Lombok Island, Indonesia, and their Toxicity Against *Anopheles aconitus. International Journal of Microbiology*, **2015**: 1–6. https://doi.org/10.1155/2015/854709

Travers, R., Martin, P. A. ., & Reichelderfer, C. F. (1987). Selective Process for

Efficient Isolation of Soil *Bacillus*. *Applied and Environmental Microbiology*, **53**(6): 1263–1266.

Udayanga, L., Gunathilaka, N., Iqbal, M. C. M., Najim, M. M. M., Pahalagedara, K., & Abeyewickreme, W. (2018). Empirical Optimization of Risk Thresholds for Dengue: An approach Towards Entomological Management of *Aedes* Mosquitoes Based on Larval Indices in the Kandy District of Sri Lanka. *Parasites and Vectors*, **11**(1): 1–12. https://doi.org/10.1186/s13071-018-2961-y

Udayanga, L., Ranathunge, T., Iqbal, M. C. M., Abeyewickreme, W., & Hapugoda, M. (2019). Predatory Efficacy of Five Locally Available Copepods on *Aedes* Larvae Under Laboratory Settings: An approach towards bio-control of dengue in Sri Lanka. *PLoS ONE*, **14**(5): 1–14. https://doi.org/10.1371/journal.pone.0216140

Uragayala, S., Kamaraju, R., Tiwari, S., Ghosh, S. K. and, & Neena, V. (2018). Field Testing & Evaluation of the Efficacy & Duration of Effectiveness of a Biolarvicide, Bactivec® SC (*Bacillus thuringiensis* var. *israelensis* SH-14) in Bengaluru, India Sreehari. *Indian Journal of Medical Research*, **147**: 299–307. https://doi.org/10.4103/ijmr.IJMR

Vargas, HCM., Farnesi, LC., Martins, AJ., Valle, D., & Rezende GL. (2014) Serosal cuticle formation and distinct degrees of desiccation resistance in embryos of the mosquito vectors *Aedes aegypti*, *Anopheles aquasalis* and *Culex quinquefasciatus*. *Journal of Insect Physiology*, **62**: 54-60. https://doi:10.1016/j.jinsphys.2014.02.001

Vector Disease Control International (VDCI). (2024). *Understanding the Life Cycle of Mosquito*. Vector Disease Control International Mosquito Management. https://www.vdci.net/mosquito-biology-101-life-cycle/

World Health Organization. (2003). The Use of Fish for Mosquito Control. In *World Health Organiszation Regional office for the eastern Mediterranean Cairo*. https://doi.org/10.1001/jama.1925.02660480077032

World Health Organization. (2023a). *Dengue and severe dengue*. https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue

World Health Organization. (2023b). *Nepal Successfully Completes Mass Drug Administration Campaign aganist Lymphatic Filariasis in 15 Districts*. World Health Organiszation Regional Office South East Asia. https://www.who.int/nepal/news/detail/15-04-2023-nepal-successfully-completes-mass-drug-administration-campaign-against-lymphatic-filariasis-in-15-districts#:~:text=MDA is the primary strategy,risk populations with preventive chemotherapy.

World Health Organization. (1990). *Guidelines for production of* Bacillus thuringiensis *H-14 and* Bacillus sphaericus.

World Health Organization. (1999). *Environmental Health Criteria 217:Microbial Pest Control Agent: Bacillus thuringiensis*.

World Health Organization. (2003). Guidelines for Dengue Surveillance and Mosquito Control. In *World Health Organization, Regional Office for the Western Pacific Manila*.

World Health Organization. (2005). *Guidelines for Laboratory and Field Testing of Mosquito Larvicides*. World Health Organization .

World Health Organization. (2007). *WHO Specification and Evaluations for Public Health Pesticides: Bacillus thuringiensis subspecies israelensis strain AM65-52*.

World Health Organization. (2009a). *Bacillus thuringiensis israelensis (Bti) in drinking-water Background document for development of WHO Guidelines for Drinking-water Quality*.

World Health Organization. (2009b). Dengue: Guidelines for Diagnosis, Treatment, Prevention and control. In *World Health Organization*. https://doi.org/10.1176/pn.41.1.0029b

World Health Organization. (2011). Comprehensive Guidelines for Prevention and Control of Dengue and Dengue Haemorrhagic Fever. In *World Health Organization, Regional Publication SEARO*. https://doi.org/10.1017/CBO9781107415324.004

World Health Organization. (2013). *Larval source management: A supplementary measure for malaria control: an operational manual*. World Health

Organization. https://doi.org/10.1564/v25_feb_13

World Health Organization. (2020a). *Pictorial identification key of important disease vectors in the WHO South-East Asia Region*.

World Health Organization. (2020b). *Vector Borne diseases*. World Health Organiszation. https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases

Worrall, E., & Fillinger, U. (2011). Large-Scale Use of Mosquito Larval Source Management for Malaria Control in Africa: A cost analysis. *Malaria Journal*, **10**: 1–21. https://doi.org/10.1186/1475-2875-10-338

Xu, C., Bi-Cheng, W., Yu, Z., & Sun, M. (2014). Structural Insights into *Bacillus thuringiensis* Cry, Cyt and Parasporin Toxins. *Toxins*, ***6***: 2732–2770. https://doi.org/10.3390/toxins6092732

Zhang, J., Liu, Y., Liu, R., Liu, X., Zhang, B., & Zhu, J. (2020). Complete Genome Sequence of *Bacillus thuringiensis* Serovar *coreanensis* ST7 with Toxicity to Human Cancer Cells. *Advances in Microbiology*, **10(**12): 673–680. https://doi.org/10.4236/aim.2020.1012048

Zothansanga, R., Senthilkumar, N., & Gurusubramanian, G. (2016). Diversity and Toxicity of *Bacillus thuringiensis* from Shifting Cultivation (Jhum) Habitat. *Biocontrol Science*, **21**(2): 99–111. https://doi.org/10.4265/bio.21.99

APPENDICES

Consent Form

I …… agree to provide space or containers indoor and outdoor of my house for the research study entitled as **"Field evaluation of *Bacillus thuringiensis nepalensis* (Btn) native isolate of Nepal against mosquito larvae in Kathmandu"**, conducted by Ganga GC (Gharty Chhetri) she has explained about the research study, and provided me the contact details of her mobile No. 9841204803. Working place, Central Department of Microbiology, T.U, Kirtipur, Kathmandu, Nepal. I foresee no risk and discomfort about the research study. If I sense any risk or discomfort. I can ask her to remove the containers from the surroundings at any time. I have had the opportunity to ask questions about this research study and I have received satisfactory answers. I understand the method of this study.

I consent to: Provide the indoor and outdoor surrounding of my house to place the containers with water to trap mosquitoes present in the surroundings and to add bio-larvicide to kill the mosquito larvae, and to observed the effectiveness of the larvicide.

Name of Participant:

Signature

Date

Name of Principal Investigator: Ganga G.C (Gharty Chhetri)

Signature:

Date:

List of Equipment's and Materials

Conical Flask, Measuring cylinder, Petri plates, Plastic bottles, Plastic cups, Buckets, Tubs, Tires, Drums, Strainer, Dropper, White Enamel tray, Auto clave, Shaker water bath, Hand lens, Micropipette, Tips, and Flower pots.

Media

T3 Media, Mannitol salt agar, MacConkey agar, Nutrient agar, Nutrient broth, LB broth

Composition of Media

Nutrient Agar: Peptone 5g/l, HM peptone 1.5g/l. yeast extract 1.5g/l. NaCl 5g/l, Agar 15g/l

Nutrient Broth: Peptone 5g/l, HM peptone 1.5g/l. yeast extract 1.5g/l. NaCl 5g/l

Luria Bertani broth (LB broth): Tryptone 10g/l, yeast extract 5g/l, NaCl 10g/l

T3 Media: Tryptone 3g/L, Tryptose type I 2g/L, yeast extract 1.5g/L, Sodium Phosphate 0.05M, Manganese chloride 0.005g/L.

Mannitol salt agar

Pancreatic Digest of casein 5.0gm/L, Peptic digest of animal tissue 5.0gm/L, Beef extract 1.0gm/L, Sodium chloride 75.0gm/L, D-Mannitol 10.0gm/L, Phenol red 0.025gm/L, Agar 15gm/L and Distilled water 1L.

MacConkey Agar

Peptone 17Gm/L, Proteose Peptone 3gm/L, Lactose monohydrate 10gm/L, bile salts 1.5gm/L, Sodium Chloride 5gm/L, Neutral red 0.03gm/L, Crystal violet 0.001gm/L, Agar 13.5gm/L and Distilled water 1L.

List of Publications

1. **Morphological Features of Mosquito Larvicidal *Bacillus thuringiensis* isolated from soil samples of Nepal**
    a. Ganga GC, Megha Raj Banjara, Ishan Gautam, Prakash Ghimire, Komal Raj Rijal
    b. Tribhuvan University Journal, Vol. 38, No. 2: 1-14, December 2023
    c. https://www.nepjol.info/index.php/TUJ/article/view/60744
2. **Efficacy of native Bacillus thuringiensis against mosquito vector in Nepal**
    a. Ganga GC, Kshama Parajuli, Megha Raj Banjara, Ishan Gautam, Prakash Ghimire, Komal Raj Rijal
    b. Journal Nepal Health Research Council
    c. http://www.jnhrc.com.np/index.php/jnhrc/article/view/4742
3. **Phylogeny of a Mosquitocidal *Bacillus thuringiensis* var *israelensis***
    a. Ganga GC, Megha Raj Banjara, Ishan Gautam, Prakash Ghimire, Komal Raj Rijal, Tribhuvan University Journal of Microbiology
    b. https://www.nepjol.info/index.php/tujm/article/view/60653

16srRNA gene sequence

- **Bti-14P2A** H230623-R01_C21_1A_907R 1 1288

ACCGGTTCGGGTCTTCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCAC

TAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGA

CTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTG

TCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCT

CTACGCATTTCACCGCTACACATGGAATTCCACTTTCCTCTTCTGCACTC

AAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCAC

ATCAGACTTAAGAAACCACCTGCGCGCGCTTTACGCCCAATAATTCCGGA

TAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCG

TGGCTTTCTGGTTAGGTACCGTCAAGGTGCCAGCTTATTCAACTAGCACT

TGTTCTTCCCTAACAACAGAGTTTTACGACCCGAAAGCCTTCATCACTCA

CGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTG

CTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATC

ACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCAC

CAACTAGCTAATGCGACGCGGGTCCATCCATAAGTGACAGCCGAAGCCGC

CTTTCAATTTCGAACCATGCAGTTCAAAATGTTATCCGGTATTAGCCCCG

GTTTCCCGGAGTTATCCCAGTCTTATGGGCAGGTTACCCACGTGTTACTC

ACCCGTCCGCCGCTAACTTCTTGAGAGCAAGCTCTCAATCCATTCGCTCG

ACTTGCATGTATTAGGCACGCCGCCAGCGTTCATCCTGAGCAGAGTTCTC

AAATTCAAAAACCCCCTTCCCCCCCTTCTTTTCGGCTTCCGTTTGCCCCC

CCGTCTTCCGTGGCTGGTGGGGCGTGCTTTGCGGTGCCTTCTGGTTGGGT

TCCGCCTTCGTGGCCGGTTAGTCAACTGGCACTTGTTCTTCCCTAACACC

GGACTTTACGAATCGGAAGCCTTCATCACTCACGCGACGTGCTCCGTCAG

ACTTCATACTATGCGGAAAATTCCAAATGCTACTTCCGTATGAACCTGGG

TAAGGGGCTAAGGCCATGGTGGCCCATCCACCTTCCAAGTCGGCTTGACA

ACGTTGCCTTGGGGAAGGGGTATCGATGCGAAAATACAACAGAGGGAGGG

AGTTCACCCAAAAGGACCACAAAACAACAGTCGTTTTT

- **Bti-14P2A** H230623-R01_A21_1A_785F 1 918

TGGGGTCGATGAGTGCTAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAA

GTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAAC

TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT

TCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCC

TAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTT

GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA

ACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGC

CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCT

TATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAA

GACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTA

GGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAG

CATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACAC

CACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCA

GCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTACAAGGGGGAA

CACCAAAAGAGGGGGGGGGGCGGGGGGGGGGGGGGGGGGTGCGGGGGGG

GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG

GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGCGGGGGGGGGCGG

GGGGGGGGGGGGCCCCCGCGGGGCCGGGGGGGGGCGCCCCCGCGGCGCGG

CCCGCGGGGCGCGCGGGC

Tribhuvan University Journal
Vol. 38, No. 2: 1-14, December 2023
Research Directorate, Tribhuvan University (TU),
Kathmandu, Nepal
DOI: https://doi.org/10.3126/tuj.v38i2.60744

MORPHOLOGICAL FEATURES OF MOSQUITO LARVICIDAL *BACILLUS THURINGIENSIS* ISOLATED FROM SOIL SAMPLES OF NEPAL

Ganga GC[1, 2*], Megha Raj Banjara, Ishan Gautam[1, 3], Prakash Ghimire[1], Komal Raj Rijal[1]

[1]Central Department of Microbiology, TU, Kathmandu
[2]Trichandra Multiple Campus, TU, Kathmandu
[3]Natural History Museum, TU, Kathmandu
**Corresponding Author: gangagc2@gmail.com*

Received date: 2 Mar. 2023- Accepted date: 25 Nov. 2023

ABSTRACT

Anopheles, Culex, and *Aedes* spp are the main vectors for transmitting malaria, Japanese encephalitis, lymphatics filariasis, dengue, zika, chikungunya, etc. Mosquito-borne diseases are increasing especially dengue in Nepal. Additional tools are required to decrease the disease rate. Biological control tools like *Gambusia* fish, Cyclopods, *Bacillus thuringiensis,* etc., have not been adopted and are unavailable in Nepal. The research aims to isolate mosquitocidal *Bacillus thuringiensis* a biological control tool from the soil samples of Nepal.

Bacillus thuringiensis (Bt) was isolated from the soil samples of Nepal by the acetate selection method. The larvicidal bioassay of Bt was studied against the field-collected mosquito larvae. The isolate showing larvicidal activity was coded as *Bacillus thuringiensis* -14P2A (Bt-14P2A). The growth pattern of the positive control strain *Bacillus thuringiensis* var *israelensis* (Bti-IPS-82) and the test strain were studied in different types of culture media. The microscopic morphology was studied by using different staining techniques.

Bt-14P2A and Bti -IPS-82 caused 100% mortality of field-collected larvae. Both the strains showed the same type of colony morphology in a standard culture media and uniform turbidity with sedimentation type of growth pattern in broth media. The strains were Gram-positive single rod-shaped of size 1.2X4.8µm in both negatively stained slides and Gram-stained slides. A slight difference in the endospore location was terminal in Bti-IPS-82 and subterminal in Bt-14P2A.

The newly isolated Bt-14P2A differs from Bti-IPS-82 in arrangement and endospore location but harbors the same morphological characteristic and larvicidal activity as the positive control strain.

Keywords: bacillus thuringiensis, endospore, larvicidal, Nepal, subterminal

INTRODUCTION

Climate change and an increase in vector-borne diseases (VBDs) are challenging the vector control strategies followed to date. New interventional tools should be introduced and an integrated vector management (IVM) program must be implemented to reduce VBDs in Nepal. The main strategies adopted for effective control of vector-borne diseases are disease management with early diagnosis and treatment, community awareness, and vector control (Shrestha *et al.,* 2019). Vector control practices followed in Nepal are the distribution of long-lasting insecticidal treated bed nets (LLINTs), Indoor residual spraying, elimination of breeding sources (practice only for *Aedes* spp), and surveillance of disease-transmitting vectors (Shrestha *et al.,* 2019). To achieve long-term and sustainable control of VBDs various other tools or techniques must be integrated, like environmental management (modification, manipulation of environment) (ii) Biological control agents (Fish, Bacteria, Cyclopods, etc.) (iii) chemical control (chemical insecticide) (World Health Organization, 2011), along with the conventionally used tools and techniques. More and newer intervention tools and approaches are the requirements for IVM to address the expansion of mosquito vectors.

Bacillus thuringiensis is a Gram-positive rod-shaped endospore-producing bacterium during sporulation it produces a crystal protein. The bacteria are widely distributed in various natural resources. Bt strains produce a wide variety of crystal proteins active against diverse insect pests (Palma *et al.,* 2014). Various studies on Bt strains of Nepal and their molecular characterization showed that diverse Bt strains are present in the soil samples of Nepal (Limbu *et al.,* 2020; Parajuli *et al.,* 2015; Sharma, 2017; Sijapati *et al.,* 1970). The Bt isolates from the soil sample showed toxicity against the agricultural pest *Galleria mellonella* (Limbu *et al.,* 2020). Similarly, bipyramid-shaped crystal protein-producing Bt isolates obtained from the soil samples of Nepal showed larvicidal activity against the pest *Pieris brassicae nepalensis* and *Helicoverpa armigera Hubner* (Rana *et al.,* 2002). Mosquito larvicidal Bt is used for larval source reduction in the aquatic habitat (World Health Organization, 2013). Reduction of larval

sources prevents the development of adult mosquitoes which reduces the transmission of mosquito-borne diseases. Larvicidal Bt when added to the breeding habitat of mosquitoes the larvae feed on the toxin produced by the Bt. Ingestion of toxins causes the death of larvae.

Since the discovery of *Bacillus thuringiensis* var *israelensis* (Bti) in 1976, extensive research has proven its efficacy in controlling mosquitoes (Boisvert, 2005). Larvicidal Bt has been isolated in various parts of the world formulated in different substrates and available in different forms (liquid, solid formulation) for controlling mosquito vectors (*Aedes* spp, *Anopheles* spp, *Culex* spp). For instance, Bti H-14 liquid formulation effectively reduced *Aedes* spp larval density in household containers in Denpasar, Bali (Purnama & Kardiwinata, 2021). Bti H-14, water dispersible granule, formulation was effective in the field for at least 35 days with more than 80% reduction in *Aedes* larvae (Lee & Zairi, 2006). Bti C4P1 solid formulation developed by Far –Manguinhos –Fiocruz, Rio de Janeiro, Brazil, evaluated against *Aedes aegypti* under stimulated field conditions showed 40-54 days residual activity in containers placed in a shade (Melo-Santos *et al.,* 2001). Efficacy of Bti (Bactivec) and *Bacillus sphaericus* (Griselest) to control *Anopheles gambiae* complex, *Culex quinquefasciatus,* and *Aedes aegypti* larvae under semi-field conditions in northeastern Tanzania, Bti provided 91-100% larval mortality within 24 hours whereas *Bacillus sphaericus* resulted in 98-100% larval mortality within the same timeframe (Derua *et al.,* 2022).

In Nepal application of larvicidal Bt is not yet practiced may be due to its unavailability. Even though the standard operating procedure has been developed by the Department of Health Services Nepal, to control dengue vectors by using Bti (Epidemiology and Disease Control Division, 2022). So, the research aims to isolate larvicidal Bt from the soil samples of Nepal. The main focus of the research is to isolate potent bacteria *Bacillus thuringiensis* from the soil sample as a biological control agent to control mosquito vectors as a new intervention for future implementation in vector control programs. Biological control agents like *Bacillus thuringiensis, Bacillus sphaericus,* etc. can be integrated with vector control strategies. Using biological control agents prevents the pollution caused by the chemical larvicide. Prevent bioaccumulation and biomagnifications of chemicals.

METHODS AND MATERIALS

Standard Strain *Bacillus Thuringiensis* Var *Israelensis* (Bti)

The standard reference strain *Bacillus thuringiensis* var *israelensis* (Bti)-IPS-82 was provided by the Pasteur Institute, Paris, France. The control strain was cultured in the laboratory condition in nutrient agar (NA) (Peptone 5g/L, HM peptone 1.5g/L. yeast extract 1.5g/L. NaCl 5g/L Agar 15g/L).

Bacillus Thuringiensis Isolation

Bacillus thuringiensis was isolated from the soil samples of Nepal. The soil samples were collected from seven provinces of Nepal in a ziplock bag. The sample was transported to the Central Department of Microbiology, T.U, Kirtipur, Kathmandu for isolation of Bt. The acetate selection method was performed to isolate the Bt strain (Travers *et al.,* 1987). The isolates were identified as Bt by observing Coomassie brilliant blue stained (0.133% Coomassie Brilliant Blue (CBB) G250 in 50%acetic acid) (Rampersad *et al.,* 2002) crystal protein and its shape in light microscopy from a 48 hours nutrient agar culture plate.

Growth Characteristics of Bti-IPS-82 and Bt-14P2A

The growth pattern of Bti-IPS-82 and Bt-14P2A was studied by inoculating a single colony in the following broth media, Luria Bertani broth (LB broth) (Tryptone 10g/L, yeast extract 5g/L, NaCl 10g/L.), Nutrient broth (NB broth) (Peptone 5g/L, HM peptone 1.5g/L, yeast extract 1.5g/L, NaCl 5g/L.), and in T3 broth (Tryptone 3g/L, Tryptose type I 2g/L, yeast extract 1.5g/L, Sodium Phosphate 0.05M, Manganese chloride 0.005g/L.) from a pure culture plate, and incubated for 48 hours at 28^0C in an incubator to observe the growth characteristic of the organism in broth media as well as to confirm larvicidal activity. The organism was inoculated by performing quadrant streaking to obtain an isolated colony in the nutrient agar (NA) to observe the colony morphology of the organism and also in T3 agar (Tryptone 3g/L, Tryptose type I 2g/L, yeast extract 1.5g/L, Sodium Phosphate 0.05M, Manganese chloride 0.005g/L Agar 15g/L) to confirm whether it stimulate sporulation (Bello *et al.,* 2016; Martin & Travers, 1989). Agar plates were incubated at 28^0C for 48 hours in an incubator. The sporulating property was confirmed after 48 hours by performing Coomassie Brilliant Blue staining and by spore staining from the colony present in T3 and NA agar plates as well as from their broth

culture tubes of Bti-IPS-82 and Bt-14P2A. Triplicate slides were prepared by smearing from the three isolated colonies present in the agar plate of both strains and from the broth culture and observed in a 100X objective lens of a light microscope.

Microscopic Characteristics of Bti-IPS-82 and Bt-14P2A

To observe the microscopic characteristic of Bti-IPS-82 and Bt-14P2A the organism was cultured in a basal media NA for 24 to 48 hours. From the isolated colony Gram staining (crystal violet, Gram's iodine, decolorizing agent {alcohol} and safranin) and Negative staining (an acidic dye, Nigrosin) were performed. Spore staining (Malachite green, decolorizer {water} and safranin) and CBB staining (0.133% Coomassie Brilliant Blue (CBB) G250 in 50%acetic acid) were performed from a 48 hours culture plate. The slides were prepared in triplicate from three isolated colonies.

Size Determination of Bti-IPS-82 and Bt-14P2A

The vegetative cell size was determined from the negatively stained slide and Gram stained slide prepared from a 24 hour NA culture plate. For the size determination, the ocular micrometer was calibrated with the stage micrometer in a 100X objective lens of a well magnifying light microscope Olympus Japan model CHD available in the Central Department of Microbiology. The number of ocular divisions that coincided within the vegetative cell was counted, five different mature vegetative cells were measured and the average size of the vegetative cell was calculated.

Calibration of Ocular Micrometer

One ocular division = (No. of stage micrometer division / No. of ocular division coincided with the stage division) X10µm (Centers for Disease Control and Prevention, 2015).

Larval Source

The immature (Larva, Pupa) was collected from the discarded tires using a strainer and placed in a plastic cup with the help of a dropper and transferred to a transparent ziplock bag containing clean water and transported to the laboratory. In the laboratory, the immatures were placed in a white enamel tray containing fresh water and fed with a pinch of dog biscuit and baker's yeast (nine: one) mixture made to a fine powder. The pupae were collected from the tray and placed in a cup until the emergence of adults and identified.

Larvicidal Activity

The isolated Bt strains producing spherical crystal protein were screened for larvicidal activity by inoculating 3 loopfuls of 48 hours cultures of Bt strain in a cup containing 10 mosquito larvae (third and fourth instar larvae), and 100 millilitre (ml) of distilled water. The mosquito larvae were not provided with larval food during the bioassay (Bello *et al.,* 2016; Lakxmy *et al.,* 2011). The cup was incubated at room temperature for 24 hours. Mortality of larvae was observed after 24 hours. The Bt strain was further subcultured in NA for preservation and in three different tubes containing NB (Peptone 5g/L, HM peptone 1.5g/L. yeast extract 1.5g/L. NaCl 5g/L.), LB, and in T3 and incubated for 48 hours in an incubator. After 48 hours the broth was screened for larvicidal activity by inoculating 1000 microliter of the broth in a cup containing 10 larvae, and 100 ml distilled water. Similarly, the negative control cup containing only 10 larvae with 100 ml distilled water, and the positive control cup containing 10 larvae, 100 ml distilled water, and 1000 microliter of the standard Bti-IPS-82 cultured in NB broth was added and incubated along with the test cups for further confirmation of its larvicidal activity.

RESULTS AND DISCUSSION

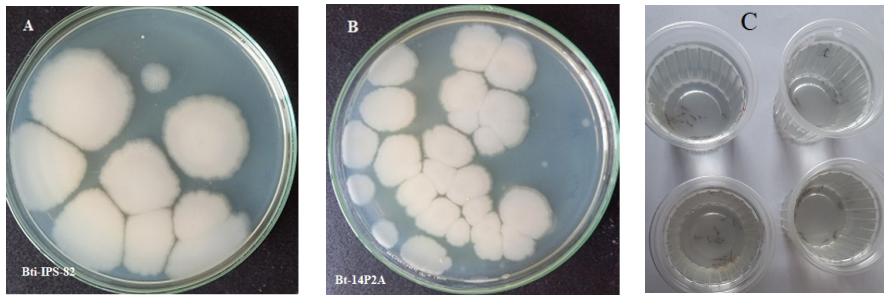
On analysis of 300 soil samples collected from seven provinces of Nepal, 1000 Bt isolates were obtained. The Bt was distinguished from the other *Bacillus* species by observing the presence of crystal protein stained by CBB stain (Bravo *et al.,* 1998; Rampersad *et al.,* 2002). Generally, after growth for 48 hours in NA agar, all the *Bacillus* start to sporulate, on staining the isolated colony from the culture plate by CBB stain. In the case of the Bt strain, three different types of morphological structure can be observed in a slide, the vegetative cell, the spore, and crystal protein. In the case of other *Bacillus* spp, only two morphological structures can be observed the vegetative cell and, the spores. The presence of the extra structure of different shapes confirms it as a Bt strain the structure is the protein and it is stained by the CBB stain mainly used for staining the protein structure (Rampersad *et al.,* 2002). From the 1000 isolates, the majority of the isolates produced spherical shape crystal protein 55%, followed by cap-headed or spore attached 44%, and only two isolates produced bipyramid crystal protein 0.2%. The result is consistence with (El-Kersh *et al.,* 2016) On screening for larvicidal activity against the field-collected mosquito larvae, only one Bt coded as Bt-14P2A showed larvicidal activity. Various study has shown only the presence of a few isolates of larvicidal Bt from

soil samples for instance (Lobo *et al.,* 2018) obtained 3 larvicidal Bt from 300 Bt isolates of soil samples. So, the larvicidal assay distinguishes the mosquito larvicidal Bt from the other Bt strains as the crystal proteins are specific to a target pest (Bravo *et al.,* 1998). As the composition of the crystal protein varies from one species to another so, this characteristic is also an important characteristic to distinguish among the Bt strain (Xu *et al.,* 2014). The larvicidal Bt strain was isolated from the soil sample of Province 2 of Nepal. The addition of 1000 microliters culture broth of LB, NB, and T3 to each cup containing 10 larvae showed 100% mortality of larvae within 24 hours (Figure 1C). So, all three types of broth substrate encourage the growth of the larvicidal Bt, and production of toxin. This confirms the isolates Bt strains as a mosquito larvicidal Bt. The larvicidal activity was not only shown by the organism after growth in the solid agar media but also in the liquid broth containing the crystal protein toxin to mosquito larvae. This confirms the isolate as a mosquito larvicide. The pupae after emergence to adulthood were identified as *Aedes aegypti*, *Culex*, and *Aedes albopictus.* Thus Bt-14P2A is effective in controlling *Culex* and *Aedes* spp.

Bacillus thuringiensis var *israelensis* as a positive control strain and Bt-14P2A as a test strain, both the strains showed the same type of colony morphology after 48 hours of incubation at 28^0C in a solid agar (NA) media, the standard media to study the colony characteristic of bacteria (Table 1). A similar type of growth pattern of the Bt-14P2A and positive control in the culture media indicates the presence of toxic crystal protein required for larvicidal activity. Observation of similar colony morphology would also help to isolate numerous larvicidal Bt from different sample sources in the future (Figure 1A, 1B). In the LB, NA, and in T3 broth uniform turbidity with sedimentation type of growth pattern was observed in both strains. The growth pattern in the broth media will help in the formulation of Bt. Turbid growth indicates a uniform distribution of the organism and the crystal protein in the broth or liquid formulation which will get easily dispersed while used as a bio-pesticidal agent or mosquito larvicidal agent in aquatic habitat. After 48 hours of incubation in the T3, NB, and LB broths visible turbidity was observed. The triplicate slides observation proved that T3 media was an excellent spore stimulating media as the 3 slides contained spore and the crystal only (Figure 2) no vegetative cells were observed so the result states within 48 hours all the vegetative cells sporulated than in NA.

Figure 1

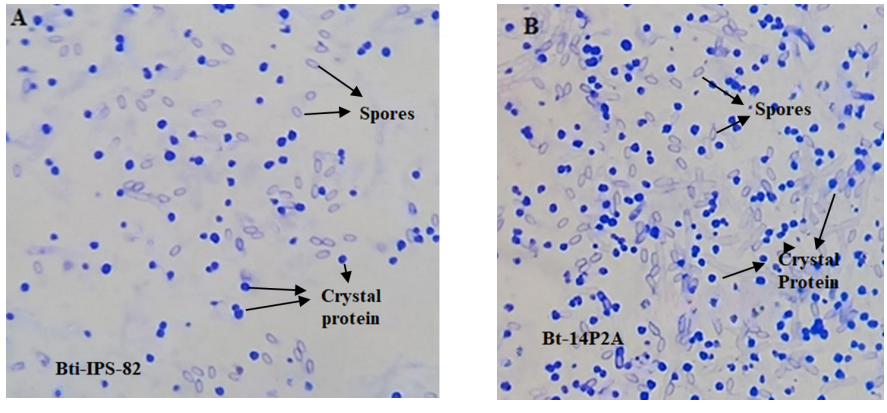
A and B Isolated colonies in nutrient agar plate after 48 hours incubation A. Bti-IPS-82 B. Bt-14P2A. C. Larvicidal bioassay Bti-IPS-82 and Bt-14P2A in a plastic Cup.



In the case of larvicidal or entomopathogenic Bt faster sporulation results in the accumulation of crystal protein in the substrate therefore the spore and crystal protein mixture increase the efficacy of the formulation. The results conclude T3 composition should be used for the formulation of the bio-pesticides. However, in the case of strains isolated in the NA plate, all three morphological structures (vegetative cell, spore, sporulating cell, and crystal protein) can be observed in a slide preparation even after 48 hours of incubation.

Figure 2

CBB stain crystal protein and the spore from T3 agar plate after 48 hours. Crystal proteins are stained as dark blue irregular or spherical like in appearance and the spores are elliptical empty slightly blue in color.



The morphological characteristic colony, and microscopic morphological characteristics were studied by following the guidelines (WHO Environmental Health Criteria 217, 1999). Microscopic morphological observation of a Gram stain slide showed the organisms are Gram-positive single rod-shaped no spore and sporulating stage was observed in a 24 hours NA culture plate of both the strains.

Table 1

Growth Characteristics on Solid Agar (NA) and in LB Broth

Isolates	Shape of the colony	Size	Color	Margin	Consistency	Elevation	Opacity	LB
Bti-IPS-82	Irregular	>80mm	Dirty white	Undulate	Dry	Flat	Opaque	Sediment and turbid
Bt-14P2A	Irregular	>80mm	Dirty white	Undulate	Dry	Flat	Opaque	Sediment and turbid

The average size was found to be 1.2X4.8µm in both strains. The same measurement was obtained in a negatively stained slide as well as in Gram stained slide of both strains.

On observation of a Gram stain slide or a negative stain slide different sizes of vegetative cells were present in the slide prepared (Figure 3) from a 24 hours culture plate this may be due to the division of the cell. Rod shaped bacterial cells divide or multiply by transverse binary fission so the cells that have recently divided are in smaller size than the cells that have attained maturity. The matured cell size was measured and tabulated. Thus, the Bt-14P2A size is the same as the control Bti-IPS-82. One of the important characteristics of Bt is they all produce endospores. The spore inside the vegetative cell is an endospore. Spore staining of both strains showed a slight difference in the location and arrangement of the spore inside the vegetative cell. In the case of Bti-IPS-82, the endospore location is terminal the spore is attached to the lateral side wall of the vegetative cell, and a short constriction is observed between the fore spore cell and the mother cell (Figure 3B and 3C).

Table 2

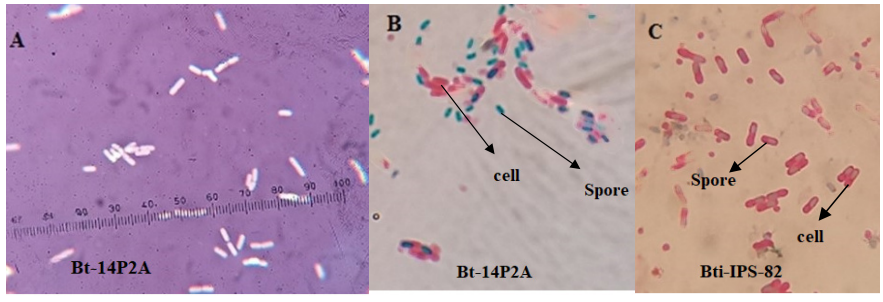
Size of the Larvicidal Bt Cells After 24 Hours by Gram Stain and Negative Stain

Isolates	Negative stain		Gram stain		Spore stain	
	Width	Length	Width	Length	Shape of spore	Location of spore
Bti-IPS-82	1.2µm	4.8µm	1.2µm	4.8µm	Elliptical	Terminal
Bt-14P2A	1.2µm	4.8µm	1.2µm	4.8µm	Elliptical	Terminal

Whereas in the case of Bt-14P2A, the spore location is sub-terminal and it is centralized in the vegetative cell (Figure 3B). However, the shape of the spore is the same elliptical (Table 2) in both strains.

Figure 3

A. Negative staining of Bt-4P2A transparent vegetative cells is seen in a bluish background. B. Spore staining of Bt-14P2A, green ellipticals are liberated spores and red stained vegetative cells. C. Spore staining of Bti-IPS-82, green elliptical spores inside the vegetative cell, and red stained vegetative cells.



Limitations of the study were a well-developed mosquito rearing facility was not available to rear mosquitoes of different genera to produce a large number of uniform larvae for the larvicidal bioassay. For larval sources in Kathmandu, has to wait for the summer season to collect larvae from the field. To study the morphology of the crystal protein by SEM (Scanning Electron Microscope) is not available in Nepal and the sample has to be sent to other Laboratories. The isolated Bt gene has to be further analyzed.

CONCLUSION

The morphological characteristics of Bt-14P2A were similar to the standard control strain Bti-IPS-82, like the colony morphology in

the NA agar plate, the size of the cell, the shape of the crystal protein, and the elliptical spore. The Bt-14P2A strain is as effective as Bti-IPS-82 in killing the mosquito larvae but differs in arrangement and location of spore. Bt-14P2A will be effective in controlling mosquito-borne diseases as it has shown the mortality of dengue vectors and *Culex* mosquito larvae during the study. The isolate Bt-14P2A produces the toxic crystal protein in all three types of broth culture substrate as well as in the agar plate. The method followed for isolation of Bt from soil samples and identification by CBB was a reproducible and reliable method.

ACKNOWLEDGEMENT

The author would like to acknowledge the University Grants Commission for providing the financial support (S&T- 12) to carry out the research work. The author is grateful to the Pasteur Institute, Paris, France for providing the standard reference strain Bti-IPS-82. The author is thankful to the Central Department of Microbiology for providing the laboratory facilities.

REFERENCES

Bello, A., Machido, D. A. & Inabo, H. I. (2016). Evaluation of the larvicidal activity of Bacillus thuringiensis isolated from soil samples against Aedes aegypti. *American Journal of Microbiology and Immunology*, *1*(2), 26-31. http://www.aiscience.org/journal/ajmi

Boisvert, M. (2005). Utilization of Bacillus thuringiensis var. israelensis (Bti)-Based Formulations for the Biological Control of Mosquitoes in Canada. *6th Pacific Rim Conference on the Biotechnology of Bacillus Thuringiensis and Its Environmental Impact, Victoria BC 2005*, 87-93.

Bravo, A., Sarabia, S., Lopez, L., Ontiveros, H., Abarca, C., Ortiz, A., Ortiz, M., Lina, L., Villalobos, F. J., Peña, G., Nuñez-Valdez, M. E., Soberón, M. & Quintero, R. (1998). Characterization of cry genes in a Mexican Bacillus thuringiensis strain collection. *Applied and Environmental Microbiology*, *64*(12), 4965-4972.

Centers for Disease Control and Prevention. (2015). *Calibration of the ocular micrometer*. https://www.cdc.gov/labtraining.

Derua, Y. A., Tungu, P. K., Malima, R. C., Mwingira, V., Kimambo, A. G., Batengana, B. M., Machafuko, P., Sambu, E. Z., Mgaya, Y.

D. & Kisinza, W. N. (2022). Current Research in Parasitology & Vector-Borne Diseases Laboratory and semi- field evaluation of the efficacy of Bacillus thuringiensis var. israelensis (Bactivec ®) and Bacillus sphaericus (Griselesf ®) for control of mosquito vectors in northeastern Tanzania. *Current Research in Parasitology & Vector-Borne Diseases*, *2*(April), 100089. https://doi.org/10.1016/j.crpvbd.2022.100089

El-Kersh, T. A., Ahmed, A. M., Al-Sheikh, Y. A., Tripet, F., Ibrahim, M. S. & Metwalli, A. A. M. (2016). Isolation and characterization of native Bacillus thuringiensis strains from Saudi Arabia with enhanced larvicidal toxicity against the mosquito vector Anopheles gambiae (s.l.). *Parasites and Vectors*, *9*(1), 1-14. https://doi.org/10.1186/s13071-016-1922-6

Epidemiology and Disease Control Division. (2022, October 16). *SoP on BTI for Dengue Control*. edcd.gov.np/resource-detail/sop-on-bti-for-dengue-control

Lakxmy, A. P., Xavier, R., Josephine, C. M. R., Lee, Y. W., Marimuthu, K., Kathiresan, S., & Sreeramanan, S. (2011). Mosquitocidal activity of a native Bacillus thuringiensis isolate Bt ReX02 from Gunung Jerai Forest, Malaysia against Culex quinquefasciatus and Aedes albopictus. *European Review for Medical and Pharmacological Sciences*, *15*, 149–155.

Lee, Y. W., & Zairi, J. (2006). Field evaluation of Bacillus thuringiensis H-14 against Aedes mosquitoes. *Tropical Biomedicine*, *23*(1), 37-44.

Limbu, J., Shrestha, B. K., Shakya, J., Khatri, S. B., & Khanal, H. (2020). Isolation of Bacillus thuringiensis and its Insecticidal Effect against Galleria mellonella. *Himalayan Journal of Science and Technology*, *3-4*, 96-102. https://doi.org/10.3126/hijost.v4i0.33918

Lobo, K. dos S., Soares-da-Silva, J., Silva, M. C. da, Tadei, W. P., Polanczyk, R. A. & Pinheiro, V. C. S. (2018). Isolation and molecular characterization of Bacillus thuringiensis found in soils of the Cerrado region of Brazil, and their toxicity to Aedes aegypti larvae. *Revista Brasileira de Entomologia*, *62*(1), 5-12. https://doi.org/10.1016/j.rbe.2017.11.004

Martin, P. A. W. & Travers, R. S. (1989). Worldwide Abundance and Distribution of Bacillus thuringiensis Isolates. *Applied and Environmental Microbiology*, *55*(10), 2437-2442.

Melo-santos, M. A. V. De, Sanches, E. G., Jesus, F. J. De & Regis, L. (2001). Evaluation of a New Tablet Formulation Based on Bacillus thuringiensis sorovar. israelensis for Larvicidal Control of Aedes aegypti. *Mem Inst Oswaldo Cruz, Rio de Janeiro*, *96*, 4-5.

Palma, L., Muñoz, D., Berry, C., Murillo, J., Caballero, P. & Caballero, P. (2014). Bacillus thuringiensis toxins: An overview of their biocidal activity. *Toxins*, *6*(12), 3296-3325. https://doi.org/10.3390/toxins6123296

Parajuli, P., Rana, N., Shrestha, S., Maharjan, J., Rana, P., & Sijapati, J. (2015). Isolation and characterization of Bacillus thuringiensis strains containing cryI genes from soil samples of Nepal. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences*, *17*(3), 587-592.

Purnama, S. G. & Kardiwinata, P. (2021). Efficacy Bacillus thuringiensis var. israelensis serotype H-14 (Bti H-14) for control Aedes spp. density in Denpasar, Bali. *International Journal of Community Medicine and Public Health*, *8*(9), 4197-4203.

Rampersad, J., Khan, A. & Ammons, D. (2002). Usefulness of staining parasporal bodies when screening for Bacillus thuringiensis. *Journal of Invertebrate Pathology*, *79*(3), 203-204. https://doi.org/10.1016/S0022-2011(02)00018-6

Rana, P., Sijapati, J., Rana, N. & Regmi, C. (2002). Distribution of Bacillus thuringiensis in the Soils of Nepal and Their Bioassay. *Nepal Journal of Science and Technology*, *4*, 27–31.

Sharma, P. (2017). *Isolation of Bacillus thuringiensis from different soil samples and production of eco-friendly insecticidal biopesticide*. Nepal Academy of Science and Technology, Khumaltar, Lalitpur, Nepal.

Shrestha, S. B., Pyakurel, U. R., Khanal, M., Upadhyay, M., Na-Bangchang, K. & Muhamad, P. (2019). Epidemiological situations and control strategies of vector-borne diseases in Nepal during 1998–2016. *Journal of Health Research*, *33*(6), 478-493. https://doi.org/10.1108/JHR-10-2018-0124

Sijapati, J., Rana, N., Rana, P. & Shrestha, S. (1970). Optimization of RAPD-PCR Conditions for the Study of Genetic Diversity in Nepalese Isolates of Bacillus thuringiensis Berliner. *Nepal Journal of Science and Technology*, *9*, 91-97. https://doi.org/10.3126/njst.v9i0.3171

Travers, R., Martin, P. A. W. & Reichelderfer, C. F. (1987). Selective Process for Efficient Isolation of Soil Bacillus. *Applied and Environmental Microbiology*, *53*(6), 1263-1266.

WHO Environmental Health Criteria 217. (1999). *Microbial pest control agent Bacillus thuringiensis.* World Health Organization.

World Health Organization. (2011). Comprehensive Guidelines for Prevention and Control of Dengue and Dengue Haemorrhagic Fever. In *World Health Organization, Regional Publication SEARO*. https://doi.org/10.1017/CBO9781107415324.004

World Health Organization. (2013). *Larval source management: A supplementary measure for malaria control: an operational manual*. World Health Organization. https://doi.org/10.1564/v25_feb_13

Xu, C., Bi-Cheng, W., Yu, Z. & Sun, M. (2014). Structural Insights into Bacillus thuringiensis Cry, Cyt and Parasporin Toxins. *Toxins*, *6*, 2732-2770. https://doi.org/10.3390/toxins6092732

Efficacy of Native *Bacillus thuringiensis* against Mosquito Vector

Ganga GC, [1, 2] Kshama Parajuli,[3] Ishan Gautam,[4] Megha Raj Banjara,[1] Prakash Ghimire,[1] Komal Raj Rijal[1]

[1]Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal, [2]Trichandra Multiple Campus, Ghanta Ghar, Kathmandu, Nepal,[3] Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal, [4]Natural History Museum, Tribhuvan University, Swayambhu, Kathmandu, Nepal.

ABSTRACT

Background: Larval source management is an effective measure to control mosquito-borne diseases. Bacillus thuringiensis produces specific insecticidal crystal proteins toxic to mosquito larvae. In many parts of the South East Asian region, Bacillus thuringiensis is used for larval source management. In Nepal, larvicidal Bacillus thuringiensis is not available. The study aims to isolate larvicidal Bacillus thuringiensis from soil samples of Nepal to control mosquitoes.

Methods: Native Bacillus thuringiensis was obtained from soil samples by the acetate selection method. It was identified by observing crystal protein with Coomassie Brilliant Blue stain in a light microscope. The mosquito larvae were collected from different breeding habitats. A preliminary bioassay was performed by inoculating three loopful of 48 hours culture of spherical crystal protein producing Bacillus thuringiensis in a plastic cup containing 25 larvae and 100 ml of sterile distilled water. The cup was incubated at room temperature for 24 hours to observe the mortality of larvae. Further selective bioassay was performed with the isolate which showed 100% mortality, as described above in four replicates along with the negative and positive control.

Results: Out of 1385 Bacillus thuringiensis obtained from 454 soil samples, 766 (55.30%) were spherical crystal protein producers, among them, a single strain (14P2A) showed 100% mortality against mosquito larvae. The lethal concentration doses required to kill 50% and 90% of the larval population were 32.35 and 46.77 Parts per million respectively.

Conclusions: The native Bacillus thuringiensis produces the crystal protein effective in killing mosquito larvae. The native Bacillus thuringiensis should be included as a tool to control mosquito-borne diseases in Nepal.

Keywords: *Bacillus thuringiensis*; mosquito; Nepal; tool.

INTRODUCTION

Mosquito-borne diseases (MBDs) have expanded from endemic regions to nonendemic regions due to climate change, urbanization, globalization, changes in mosquito behavior, etc. [1-4] Newer interventions are required to address the emerging situation of diseases. In recent years, larval source management (LSM) has been focused as a supplementary approach to manage larval habitats to prevent the development of adult mosquitoes. [5-7]

Bacillus thuringiensis subspp *israelensis* is recommended by WHO for LSM. [8] Nepal lacks *Bacillus thuringiensis* (Bt) and other biological control agents. No attention is paid to these types of vector control interventions in Nepal. For sustainable vector control, it is essential to focus on larval source reduction by using Bt which is a safer, cost-effective, and environmentally acceptable tool.

The research aimed to isolate and identify Bt from the soil samples of Nepal, and to screen the isolates for larvicidal activity against field collected mosquito larvae

Correspondence: Dr Komal Raj Rijal, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal. Email: komal.rijal@cdmi.tu.edu.np. Phone: +9779849002253.

by preliminary and selective bioassay and comparing its toxicity with the standard strain.

METHODS

This study was carried out in the laboratory of Nepal from 2020 to 2022. The research target was to isolate mosquito larvicidal *Bacillus thuringiensis* (Bt) from the soil samples of Nepal. To test the larvicidal activity of the isolated Bt, mosquito larvae were collected from the Kathmandu and Lalitpur districts of Nepal from different types of breeding habitats. Ethical approval was granted by Nepal Health Research Council to carry out the research work (Regd.No. 79/2020).

Soil sample (10 gram) was collected randomly from the seven Provinces of Nepal in a Ziplock bags, five centimetre below the surface by clearing the decaying leaves and other organic matter. [9] Soil samples were collected from a diverse location as possible, forest, fertile land, uncultivated land, nearby ponds, lakes, stream, university premises, parks, etc. [10] Collected soil sample was brought to the laboratory and stored at room temperature before isolation of native Bt.

Isolation of *Bacillus thuringiensis* (Bt) was carried out by the acetate selection method, one gram soil was added to a 10 ml test tube containing nine millilitres of nutrient broth (NB) supplemented with 0.25M sodium acetate. It was incubated overnight at 28^{0}C in an incubator. After enrichment, the broth was exposed to heat shock (100^{0}C) in a water bath for five minutes with slight modification. [11] A spread plate technique was performed for the isolation of Bt in nutrient agar (NA) by loading 100µl of serially diluted nutrient broth in a saline solution after heat shock. The plates were incubated at 28^{0}C for 48 hours in an incubator. The isolated colonies were further subcultured to obtain the pure culture in NA.

The isolates were identified as Bt from a 48 hours culture plate by observing Coomassie brilliant blue stained (0.133% Coomassie Brilliant Blue (CBB) G250 in 50%acetic acid) crystal protein and its shape in light microscope. [12] Biotyping like Gram staining, spore staining, catalase, oxidase, motility test, starch hydrolysis, gelatin hydrolysis, casein hydrolysis, and Tween 20 hydrolysis were performed and the results were recorded after 48 hours. Isolates identified as Bt were preserved in a nutrient agar slant with 60% glycerol.

Mosquito larvae were collected from different breeding habitats in Kathmandu and Lalitpur districts during 10 months period (June - September 2021 and April - September 2022) using dropper and dipper. [13-15] The collected larvae were transferred into a transparent plastic bag with water and brought to the laboratory. Thereafter placed in a tray containing distilled water for two days for acclimatization before using them for larvicidal activity. Meanwhile, larvae were fed with the baker's yeast and dog biscuits. [16] The larvae preceded to pupae were collected and removed from the tray.

For preliminary screening, 25 larvae, size range (9mm -14mm) equivalent to third instar to early fourth instar were placed in a plastic cup containing 100 ml sterile distilled water. [17] Only the acclimatized healthy and active movement showing larvae were used for the larvicidal activity. Fresh culture of Bt isolates in a nutrient agar plate after 48 hours of incubation was taken for the assay. With the help of a standard, sterilized loop, three loopful of culture were scooped (≈ 0.03milligram or 300 Parts per million (ppm)) and added to the cups containing larvae, homogenized gently without harming them. The culture contained a crude mixture of spores, crystal proteins, and vegetative cells. [10,18] The cups were incubated at room temperature for 24 hours the experiment was performed in August and September 2021 and 2022. The average room temperature during the analysis was 24.5^{0}C and a humidity of 66% was recorded from the mobile app (Room Temperature Thermometer, Morsol Technology). After 24 hours the numbers of dead, morbid, and live larvae were counted. The experiment was repeated when more than 10% of pupae were pupated from the larvae within 24 hours. The Bt isolate showing larvicidal activity was further confirmed by selective bioassay. [10]

Standard reference strain *Bacillus thuringiensis* var *israelensis* (Bti) IPS-82 was purchased from Pasteur Institut, Paris, France. The control strain was cultured in the laboratory condition in NA. The phenotyping and biotyping of the standard strain were performed along with the native strain in the same environmental condition. The larvicidal activity of the control strain was performed along with the native Bt isolates.

A selective bioassay was performed after the preliminary screening of Bt showing 100% mortality against the field collected larvae. Selective bioassay was performed in four replicates. In a plastic cup, 100 ml of sterile distilled water and 25 larvae of third instar to early fourth instar larvae were added to each cup. Fresh culture of native Bt showing 100% mortality during the preliminary screening was added by using a standard and sterilized loop, as described for the preliminary

screening. Similarly, for positive control fresh culture of standard strain Bti IPS-82 was added to four cups as of the test Bt. Negative control cups contain 25 larvae and 100 ml of sterile distilled water in every four cups. All the cups were placed at room temperature for 24 hours. After 24 hours the numbers of live larvae were counted. [17] The experiment was repeated if more than 10% of pupae were pupated from the larvae in 24 hours period in negative control or positive control and also in the test cups. The mortality percentage was calculated by using the formula

Mortality (%) = ((X -Y)/X) x100

Where X = percentage survival in the untreated control and Y = percentage survival in the treated sample. [17]

To confirm that the larval death was due to feeding of the toxin of the inoculated Bt or other contaminations. The single dead larvae were removed from the positive control cup and test cup, washed with sterile distilled water, and surface sterilized with 70% ethanol twice after evaporation of the alcohol the larva was crushed in a test tube with a sterilized glass rod in a one millilitre of sterile saline solution. Then 100 µl of the saline solution was spread with a sterilized "L" shaped glass rod on the nutrient agar surface and incubated at 28^{0}C for 48 hours.

For confirmation test that all spherical crystal proteins producing Bt is not toxic to the mosquito larvae was done by inoculating 14P2A and 3P2A (spherical ICPs producing Bt) 48 hours fresh culture colony in a cup containing 25 larvae and 100 ml sterile distilled water as mentioned elsewhere.

Lethal concentration doses required to kill 50% and 90% of the larval population was determined by culturing 14P2A in NB broth incubated for 72 hours in a shaker water bath at 28^{0}C. One percent working solution was prepared from the broth in a sterile distilled water. Following microliter (1000, 500, 400, 300, 200, and100) of the working solution was added to the cups containing 25 larvae in four replicates as described elsewhere and by using probit analysis the LC_{50} and LC_{90} value was determined.

Results were presented in tables, and figures where applicable. Bioassay was performed in four replicates and the mean mortality percentage was calculated as described by WHO [17] manually. LC_{50} and LC_{90} was determined by using Probit analysis. The mortality due to the production of crystal protein was confirmed by comparing it with the standard strain Bti-IPS-82 and the untreated control group. All the experiments were carried out in a room temperature with a photoperiod of 14:10 (L: D) at an average temperature of 24.5^{0}C and 66% relative humidity.

RESULTS

Altogether 1385 Bt isolates were obtained from 454 soil samples collected from different ecosystems such as forests, uncultivated land, university area, etc covering seven provinces of Nepal. On average each soil sample contains greater than three morphologically different types of Bt isolates. The isolates which produced additional structure (crystal protein) apart from the spore and vegetative cell, observed by CBB stain in a light microscope were identified as Bt isolates. The absence of the additional structure in the isolates was assumed as other types of *Bacillus* and not included in the study.

The native Bt isolates produced different shapes of crystal protein (Table 1). The dominant Insecticidal crystal proteins (ICPs) were spherically shaped.

Table 1. Shapes of Insecticidal Crystal Proteins (ICPs).

Shapes	No. of Bt
Spherical	766 (55.30%)
Bipyramid	3 (0.21%)
Rod shaped	49 (3.53%)
Cap headed	502(36.24%)
Spherical + cap headed	42(3.03%)
Oval	23(1.66%)
Total	1385

On preliminary screening of 808 Insecticidal Crystal Proteins (ICPs) producing Bt isolates for larvicidal activity (spherical (766) and spherical + cap headed (42)), only one isolate coded as 14P2A, producing spherical ICPs showed 100% mortality against mosquito larvae (Figure 1). The native larvicidal Bt 14P2A was among the 766 Bt isolates producing spherical shaped ICPs. The Bt isolates producing both spherical and cap headed (42) and the 765 spherical ICPs producing native Bt isolates lack the mosquito larvicidal property.

Selective bioassay proved the isolate's ability to show 100% mortality as the standard strain within 24 hours at 300 ppm (Parts per million) concentration (Figure 1 and Table 2). So, the selective bioassay confirms that 14P2A is a mosquito larvicidal *Bacillus thuringiensis*. Further

confirmation with 3P2A also proved 14P2A as mosquito larvicide Bt were as 3P2A lack the ability even though it possesses a spherical shaped crystal protein.

Table 2. Larvicidal activity of native Bt and the reference strain Bti (IPS-82) against field collected larvae.

Isolates	Conc. PPM	Total larvae	Live larvae	Dead larvae	Pupa	Adult	Mortality %
Bti (IPS-82)	300PPM	25(100) *	0	25(100)	0	0	100
Bt(14P2A)	300PPM	25(100) *	0	25(100)	0	0	100
3P2A	300PPM	25(100) *	25(100)	0	0	0	0
Control	-	25(100) *	25(100)	0	0	0	0

*Four replicates (each cup contains 25 larvae, four cup 100 larvae).

On analysis of dead larvae after 24 hours by spread plate technique it was found the same colony characteristic producing Bt were present in the dead larva. Therefore, the death of the larvae is by feeding on the crystal protein of the14P2A Bt isolate.

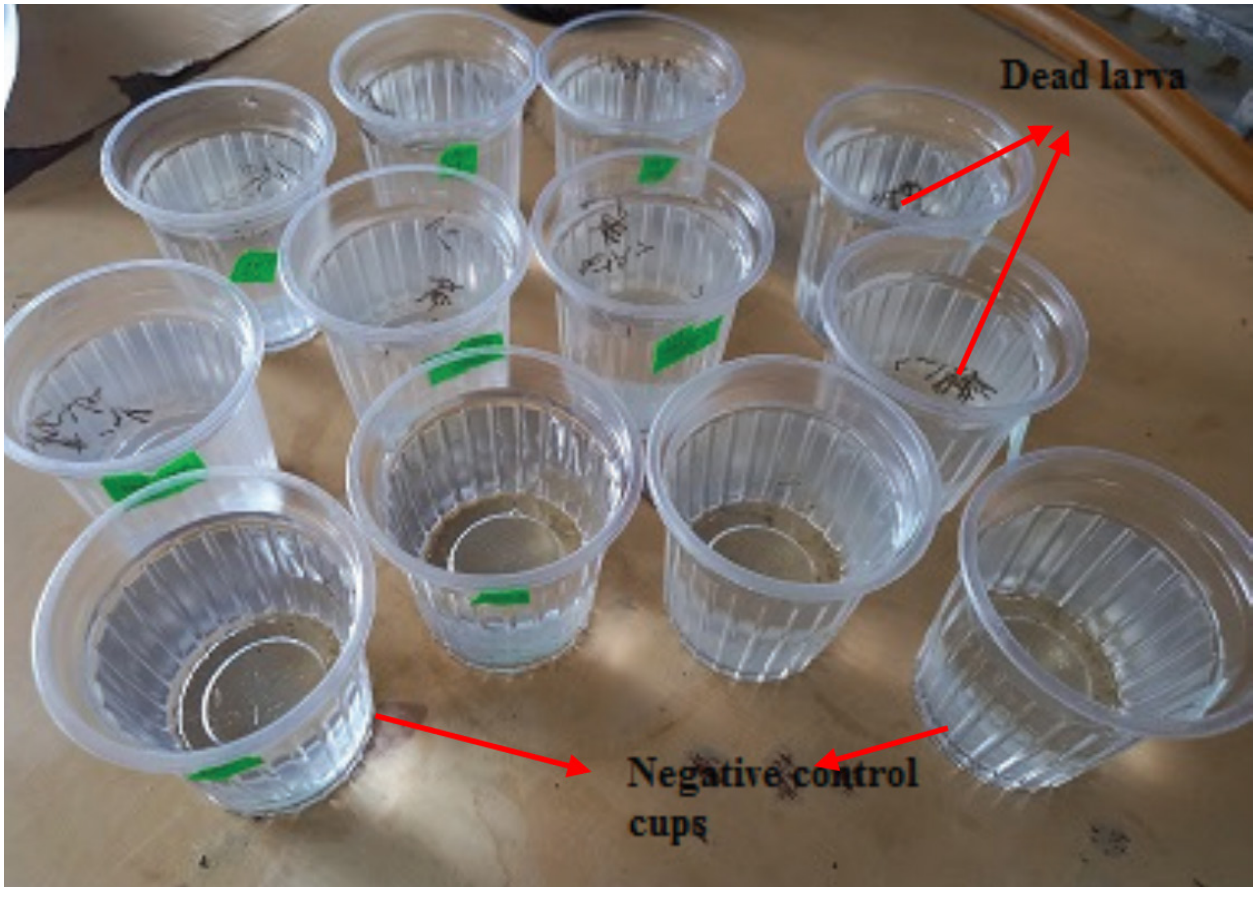


Figure 1.Selective bioassay. Four replicates of control strain, native strain and negative control.

The lethal concentration dose required to kill 50% and 90% of the larval population was found to be LC_{50} 32.35ppm and LC_{90} 46.77ppm against the field collected mosquito larvae. The colony morphology of the larvicide 14P2A in NA appears irregular in shape, and size (15mm- 30mm), dry, flat, and pale white (Figure 2D). The colony character of 14P2A is different from the colony characters of other spherical crystal protein producing Bt isolates. On biotyping it is Gram positive straight rod arranged singly (Figure 2A), on spore staining by Schaeffer Fulton stain, the spores were

elliptical in shape and the green spore in the vegetative cell is in sub terminal position without bulging (Figure 2B). On CBB stain the crystal protein appeared as blue spherical like in shape (Figure 2C) the elliptical spore was faint blue in color and the vegetative cells were dark blue in color.

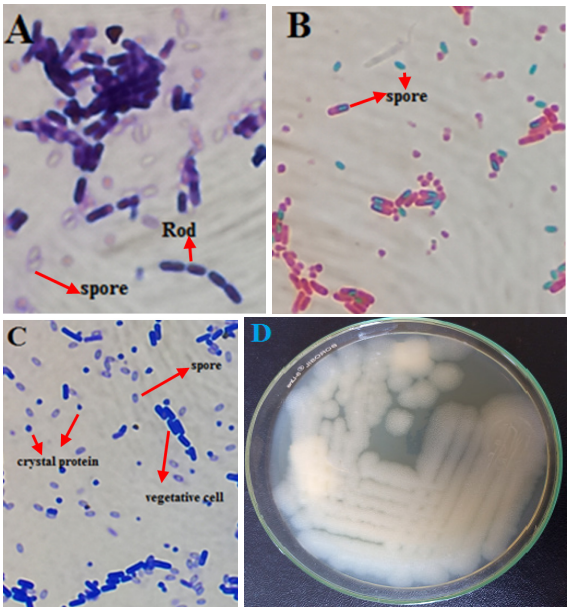


Figure 2.A.Gram staining, purple rod shaped bacteria and unstained elliptical spores. B.Spore staining, green elliptical spores, Red vegetative cells, inside the red vegetative cell the green spore is present at the sub terminal end of the vegetative cell and C.CBB staining of the isolate 14P2A, the crystal protein is dark somewhat spherical in shape. D. Culture plate of 14P2A in NA.

The control strain and the 14P2A were motile. The biochemical properties were catalase and oxidase positive as well as both of the isolates were able to hydrolyze different substrates like starch, gelatin, casein, and Tween20.

DISCUSSION

Soil samples are rich resources of Bt, we detected 1385 Bt strains from 454 soil samples. The result is in agreement with the result of Martin and Travers. [9] This research result also indicates that Nepal soil is rich in Bt biodiversity as different shapes of crystal protein producing Bt has been found. More than three morphologically different Bt varieties were obtained from a gram of soil sample. All the Bt isolates showed variation in colony morphology, fried egg type with excess mucous, and irregular shape were dominant in soil. All Bt isolates were Gram positive, spore bearing, but showed variation in the location of spores in a vegetative cell, in the shape of the spore, in cell size, and in the shape of the crystal protein as observed by light microscope with different staining techniques. [10,19,20] With the assumption of spherical crystal morphology of Bt, its specificity against the mosquito larvae. [8] Bt isolates producing spherical shaped crystal proteins of different sizes were screened for larvicidal activity. The spherical shaped crystal protein producing Bt were more dominant than the other types of crystal protein producer. [10,19,20] But the distribution of mosquito larvicidal Bt in Nepal was not found to be ubiquitous as only one (0.12%) mosquito larvicidal Bt coded as 14P2A was isolated from 454 soil samples. Relatively few strains of Bt with larvicidal activity against insects of public health importance have been discovered. Soares-da-silva et al 2015, [21] isolated five larvicidal Bt against *Ae. aegypti* out of 57 Bt. According to El-Kersh et al 2016, [10] 23 native Bt isolates were identified as larvicidal in the preliminary test against *An. gambiae*. Selective bioassay results also proved that the isolate 14P2A produces the crystal protein toxic to the mosquito larvae within 24 hours. Approximately 300 ppm of the spore, crystal, and vegetative cell mixture is enabled to show 100% mortality of larvae in the laboratory condition. The 100 larvae presented in the negative control cup were all found alive even in the absence of larval food and sustained in the larval stage. The field collected larvae were a mixture of different species of mosquito larvae. Mortality (100%) of field collected larvae showed its effectiveness (14P2A) in killing all kinds of mosquito larvae. Similarly, the control strain IPS-82 also showed 100% mortality against the field collected larvae.

Larvicidal activity evaluated in the present study may be due to the production of crystal protein which was more specific and not all types of spherical shaped crystal protein contain toxicity against the mosquito larvae. Various studies showed that the mosquito larvicidal Bt will kill all kinds of mosquito larvae and are also toxic to midges. [22-24] Therefore native larvicidal Bt 14P2A can be effective against larvae of *Aedes*, *Anopheles*, and *Culex* furthering, its efficiency as an environmentally friendly microbial control options of mosquito-borne diseases. Likewise, the larvicidal activity of 14P2A with 3P2A spherical crystal producing Bt with different colony morphology confirmed that the crystal protein composition was different in both of the isolates even though the shape of the ICPs was the same. ICPs of 14P2A are toxic whereas 3P2A ICPs are not toxic after 24 hours, the cup inoculated with 14P2A showed mortality of larvae as the cup with 3P2A contains alive larvae as in

negative control. On analysis of dead larvae by spread plate method in nutrient agar plate showed the organism present inside it was 14P2A. Because of nontoxic nature of Bt to humans. LSM by using Bt outdoor and indoor at regular intervals, in the water storing containers will not be hazardous. Furthermore, *Aedes* species are adapted to breed indoors. LSM by Bt can be an effective control measure, when by applying it into water holding containers for drinking, recreational and other purposes. [25] Considering the growth of 14P2A in NB broth as a bio larvicide, from the one percentage working solution of the bio larvicide, the lethal concentration doses required to kill 50% and 90% of the larval population was LC_{50} 32.35 ppm and LC_{90} 46.77 ppm against the field collected mosquito larvae.

Through the exploration of different samples and unique habitat such as dead insects, water, organic waste, phyllosphere, rhizosphere etc, more mosquito larvicidal Bt can be obtained which not only help to isolate a greater number of Bt but also their different native varieties leading to adopt more possibilities for prevention and control of mosquito-borne diseases in Nepal. In addition, the isolation of larvicidal activity led to further assay against laboratory reared different species of larval mosquitoes and to evaluate its efficacy in natural habitats as well.

CONCLUSIONS

Native *Bacillus thuringiensis* has been found to be effective in killing mosquito larvae. Nepal currently lacks biological control agents as part of its vector control program. Incorporating native Bt into the vector control program in Nepal could be a potential option for reducing the mosquito population and limiting the spread of mosquito-borne diseases. However, it's important to conduct further studies to evaluate the safety and effectiveness of using Bt in this context, including its impact on non-target species and the potential development of resistance in mosquito populations over time.

Overall, the use of native Bt as a biological control agent could be a useful addition to the toolbox for controlling mosquito-borne diseases in Nepal, but more research and evaluation is needed before its widespread implementation in vector control programs.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the University Grants Commission, Nepal for providing the financial support (S&T-12) to carry the research work. The authors are grateful to Pasteur institute, Paris, France for providing the standard reference strain Bti IPS-82 and to the Central Department of Microbiology, TU, Kirtipur for laboratory facility.

REFERENCES

1. Dhimal M, Thakur AK, Shrestha SM, Banerjee MK, Bhattarai L. Environmental conditions associated with vector of dengue and corrective actions for its prevention in Nepal. First ed. Kathmandu: Nepal Health Research Council; 2009. 1-27 p. Available from: https://nhrc.gov.np/publication/

2. Milali MP, Sikulu-Lord MT, Govella NJ. Bites before and after bedtime can carry a high risk of human malaria infection. Malar J. 2017;16(91).[Download PDF]

3. Dhimal M, Ahrens B, Kuch U. Climate change and spatiotemporal distributions of vector-borne diseases in Nepal - A systematic synthesis of literature. PLoS One. 2015;10(6). doi: https://doi.org/10.1371/journal.pone.0129869

4. Dhimal M, Gautam I, Kreß A, Müller R, Kuch U. Spatio-temporal distribution of dengue and lymphatic filariasis vectors along an altitudinal transect in central Nepal. PLoS Negl Trop Dis. 2014;8(7). doi: https://doi.org/10.1371/journal.pntd.0003035

5. Jambulingam P, Pillai MKK, Gunaasekaran K, Sathyanarayana K, Sadanandane C, Sharma RS, et al. Revised common protocol for uniform evaluation of public health pesticides including bio-larvicides for use in vector control. India: National Vector Borne diseases control programme; 2014. 1-144p. [Download PDF]

6. Olalubi OA, Chinwe GK. Promoting larval source management as a vital supplemental addendum and more likely cost-effective approach for malaria vector control in Nigeria. J Prev Infect Control. 2016;02(02).[Article]

7. Fillinger U, Lindsay SW. Larval source management for malaria control in Africa: Myths and reality. Malar J. 2011;10(353). [Article]

8. World Health Organization. WHO specifications and evaluations for public health pesticides: *Bacillus*

thuringiensis subspecies *israelensis* strain AM65-52. 2012. World Health Organization. Available from:https://extranet.who.int/pqweb/sites/default/files/vcp-documents/WHOVC-SP_Bti_strain_AM65-52_2012.pdf

9. Martin PAW, Travers RS. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl Environ Microbiol. 1989;55(10). [PMC203101]

10. El-Kersh TA, Ahmed AM, Al-Sheikh YA, Tripet F, Ibrahim MS, Metwalli AAM. Isolation and characterization of native *Bacillus thuringiensis* strains from Saudi Arabia with enhanced larvicidal toxicity against the mosquito vector *Anopheles gambiae* (s.l.). Parasites and Vectors. 2016;9(1). [Article]

11. Travers R, Martin PA, Reichelderfer CF. Selective process for efficient isolation of soil *Bacillus*. Appl Environ Microbiol. 1987;53(6).[PMC203852]

12. Rampersad J, Khan A, Ammons D. Usefulness of staining parasporal bodies when screening for *Bacillus thuringiensis*. J Invertebr Pathol. 2002;79(3). [Article]

13. Medlock JM, Alten B, Versteirt V, Schaffner F. Field sampling methods for mosquitoes, sandflies, biting midges and ticks. VectorNet project 2014-2018. 2018.[Article]

14. Garjito TA, Susanti L, Mujiyono M, Prihatin MT, Susilo D, Nugroho SS, et al. Assessment of mosquito collection methods for dengue surveillance. Front Med. 2021;8(685926).[PMC8219211]

15. World Health Organization. Guidelines for dengue surveillance and mosquito control. World Health Organization, Regional Office for the Western Pacific Manila. 2003. https://apps.who.int/iris/bitstream/handle/10665/206987/9290610689_eng.pdf?sequence=1&isAllowed=y

16. Devidas PC, Pandit BH, Vitthalrao PS. Evaluation of different culture media for improvement in bioinsecticides production by indigenous *Bacillus thuringiensis* and their application against larvae of *Aedes aegypti*. Sci World J. 2014;2014. [Article]

17. World Health Organization. Guidelines for Laboratory and Field Testing of Mosquito Larvicides. World Health Organization. 2005.[Article]

18. Rabinovitch L, Cavados CFG, Chaves JQ, Coutinho CJPCA, Zahner V et al . A new strain of *Bacillus thuringiensis* serovar *israelensis* very active against blackfly larvae. Mem Inst Oswaldo Cruz. 1999;94(5).[Article]

19. Ralte Z, Nachimuthu S, Guruswami G. Diversity and toxicity of *Bacillus thuringiensis* from shifting cultivation (jhum) habitat. Biocontrol Sci. 2016;21(2). [Article]

20. Apaydin O, Yenidunya AF, Harsa S, Gunes H. Isolation and characterization of *Bacillus thuringiensis* strains from different grain habitats in Turkey. World J Microbiol Biotechnol. 2005;21.[Article]

21. Soares-da-Silva J, Pinheiro VCS, Litaiff-Abreu E, Polanczyk RA, Tadei WP. Isolation of *Bacillus thuringiensis* from the state of Amazonas, in Brazil, and screening against *Aedes aegypti* (Diptera, Culicidae). Rev Bras Entomol. 2015;59(1).[Article]

22. World Health Organization, International programme on chemical safety. Microbial Pest Control Agent: *Bacillus thuringiensis*. World Health Organization.1999. Available from: https://apps.who.int/iris/handle/10665/42242

23. Silva-Filha MHNL, Romao TP, Rezende TMT, Carvalho KDS, Gouveia de Menezes HS, Alexandre doNascimento N, et al. Bacterial toxins active against mosquitoes : Mode of action and resistance. Toxins. 2021;13(523).[PMC8402332]

24. Glare TR, Callaghan MO. Environmental and health impacts of *Bacillus thuringiensis israelensis*. Lincoln; Biocontrol and biodiversity, Grassland division, Ag Research;1-58p. 1998.[Download PDF]

25. World Health Organization. *Bacillus thuringiensis israelensis* (Bti) in drinking-water Background document for development of WHO Guidelines for drinking-water quality. World Health Organization.2009. Available from: https://cdn.who.int/media/docs/default-source/wash-documents/wash-chemicals/bacillus-thuringiensis-background.pdf?sfvrsn=ab9fd812_4

Oral Presentation Attended

"Biotechnology for Environmental and Sustainable Technologies"
(BEST)

ICBSN 2022

CERTIFICATE
OF PRESENTATION

THIS IS TO CERTIFY THAT PROF./DR./MR./MS.

Ganga Gharti Chhetri

A Professor/Scientist/Academician/Researcher/Entrepreneur/Scholar/Student from

Tribhuvan University

for Keynote/ Plenary/ invited / Oral/ poster presentation during the International conference by Biotechnology Society of Nepal (ICBSN-2022) organized by the Biotechnology Society of Nepal (BSN), Co-organized by Nepal Agriculture Research Council (NARC), in association with the Ministry of Education, Science and Technology (MoEST) & Nepal Academy of Science and Technology (NAST), Nepal held from Dec.17-19, 2022, in Hotel Manang, Thamel, Kathmandu, Nepal.

Nabin Munankarmi
Chairperson
ICBSN-2022
KATHMANDU, NEPAL

Dr. Vasudev Zambare
Convener
ICBSN-2022
KATHMANDU, NEPAL

Dr. Bhuvan Gauchan
Convener
ICBSN-2022
KATHMANDU, NEPAL

Bhubaneswor Yadav
Chief Guest

Nepal Academy of Science and Technology (NAST)

CERTIFICATE OF PARTICIPATION

Awarded to

Ganga Gharty Chhetri

for Presentation in Oral / ~~Poster~~ / ~~Participation~~ in the

9th National Conference on Science and Technology

June 26-28,2022 (Asar 12-14, 2079)

Khumaltar, Lalitpur, Nepal

Ms. Luna Vajra
Chief, Promotion Division

Prof. Dr. Mahesh K. Adhikari
Secretary

Dr. Sunil Babu Shrestha
Vice Chancellor

Awards

1. **Best Research Paper Award -2023**

2. **NICHOD**: Winner of NICHOD, A nationwide competition for students to present their research findings in three minutes. Organizing partners NAYAN, PURC, GPAST, and AFU. 17 September 2023, Chitwan, Nepal.