IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF INSECTICIDE RESISTANT/SUSCEPTIBLE DENGUE VECTOR PREVALENT IN EASTERN PUNJAB, INDIA

A Thesis

Submitted in partial fulfillment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

IN

ZOOLOGY

By

SEEMA DEVI

41500056

Supervised By

Dr. LOVLEEN

Associate Professor

LOVELY PROFESSIONAL UNIVERSITY

PUNJAB

2022

DECLARATION

I hereby declare that the thesis entitled **"IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF INSECTICIDE RESISTANT/SUSCEPTIBLE DENGUE VECTOR PREVALENT IN EASTERN PUNJAB, INDIA"** was carried out by me for the degree of Doctor of Philosophy in "**ZOOLOGY** and submitted to the Lovely Professional University under the guidance of **"Dr. Lovleen, Associate Professor, Department of Zoology, School of Bioengineering and Biosciences, Lovely Professional University, Punjab.**

The interpretation put forth are based on my reading and understanding of original texts and no earlier published document has resemblance with my work to be identified as under act of plagiarism. Wherever I have borrowed material from other sources, I have diligently acknowledged the source of the borrowed material.

For the present thesis, which I am submitting to university does not contain any work for the award of any other degree at any other university.

Research Scholar,

Date:- **Regd. No.41500056**

CERTIFICATE

This is to certify that Ms. Seema Devi (41500056) has completed the thesis entitled**"IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF INSECTICIDE RESISTANT/ SUSCEPTIBLE DENGUE VECTOR PREVALENT IN EASTERN PUNJAB, INDIA".** It is a bonafide record of her original work carried out under my guidance, supervision and submitted to **Lovely Professional University, Punjab** in partial fulfillment for the award of the degree of "**DOCTOR OF PHILOSOPHY**" in **"ZOOLOGY".** No part of this report has ever been submitted for any other degree at any university.

This research work is fit for submission and the partial fulfillment of the conditions for the award of Doctor of Philosophy in Zoology.

Signature of Supervisor**:**

Date:-

Dr. Lovleen,
Associate Professor,
Department of Zoology,
School of Bioengineering and Biosciences,
Lovely Professional University, Punjab,

ABSTRACT

Dengue is a vector borne disease which causes serious illness and mortality throughout the world. Predominate vectors for the transmission of Dengue virus, are *Aedes aegypti* (*Ae.aegypti*), *Aedes albopictus* (*Ae.albopictus*) and *Aedes vittatus* (*Ae.vittatus*). Till date there is no drug and vaccine available against Dengue therefore exclusive prevention of disease depends on vector control. The specific control measures can be practiced by three approaches including mechanical, biological and chemical. Mechanical control is eradication of possible breeding sites of the mosquitoes, which includes removal of stagnant water from discarded tins, coolers, bottles and other non-essential materials. Further, proper covering of long-lasting water vessels in indoor and outdoor areas such as over-head tank, plastic tank, etc. can improve the efficiency of mosquitoes breeding sites eradication. Biological control includes the involvement of the living organisms against the concerned disease vector such as bacterial larvicide crystalline protein of *Bacillus thuringiensis var. israeliensis* (Bti) whereas chemical control approaches emphasis on application of different chemicals such as VGSC gene mutations inducer pyrethroids, anti-acetyl cholinesterase organophosphate, chitin synthesis inhibitor benzoylureas and mimics of natural hormone pyriproxyfen. Biological and chemical control is practiced under specific guidelines of National Vector Borne Disease Control Programme (NVBDCP) Delhi, India. Presently, pyrethroids and organophosphate are the mostly applied for concerned vector eradication. In pyrethroids, Pyrethrum is mainly used to control adult mosquito whereas in organophosphate, Temephos is predominantly used to control the larvae throughout the world.

Long term excessive application of only one specific larvicide and insecticide eventually results in development of insecticide resistance in *Aedes.* This resistance mainly occurs due to modifications in the target sites. The most common target site mutation is the knockdown mutation (kdr) on the para voltage-gated sodium channel gene (VGSC). Pyrethroid, keep VGSC in its open conformation, resulting in repetitive pulses. Furthermore, this prompts nerve cells to generate repeated discharges, consequently bring about paralysis and demise of pests. Organophosphate inhibits the normal functionality of acetyl cholinesterase (AChE). Consequently, acetylcholine neurotransmitter persists in the synaptic cleft, resulting in the exacerbation of nerve impulse transmission and causes death of insects. For better controlling,

understanding the prevalence and seasonal variation of Dengue vectors in rural as well as urban areas of different districts of Punjab. Further, due to drastic increase in Dengue cases from last few years in Punjab, there is an urgent need to assess: the insecticide resistance/susceptibility status and mutations in insecticide resistant genes, which include acetyl cholinesterase (*Ace-1*) and voltage gated sodium channel (knock down resistance -Kdr) in *Aedes* prevalent in Punjab. Therefore, the aim of present study is to assess Dengue vector prevalence, identification, preferential breeding habitats, comparison of Stegomyia indices (HI, CI, BI and PI) of *Aedes* mosquito breeding in different seasons**,** furthermore their correlations with the Dengue cases, identification on the basis of male and female hypopygium structure, to determine the susceptibility status of *Ae. aegypti* to temephos and pyrethroid as well as to study mutation in insecticide resistant genes, which include acetylcholinesterase (*Ace-1*) and voltage gated sodium channel (knock down resistance -Kdr) in predominant species.

To achieve the specific targets various methods such as entomological survey to assess prevalence of Dengue vector, genitalia study through permanent slides, larval bioassay and adult bioassay for insecticide resistance/susceptibility status and polymerase chain reactions for detection of mutations in associated genetic elements had been considered. The study was carried out in four districts of state Punjab *viz.* S.A.S Nagar (Mohali), Patiala, Ludhiana and Fatehgarh Sahib (F.G.Sahib).

For execution of present research work entomological surveys have been performed in pre-monsoon, monsoon and post-monsoon seasons. Further, all kinds of domestic and peri-domestic breeding habitats were also considered for *Aedes* immature stages. Larvae/pupae were collected from selected areas and kept in insectary, under control condition of temperature 27 ± 1^0C and 70% humidity. Rearing of larvae/pupae had been carried out; afterwards imagoes were identified by following pictorial taxonomic keys of Haung, 1979; Rueda, 2004 and Tyagi, *et al*., 2012. The *Stegomyia* indices were also calculated, analysed and compared for urban and rural areas in different seasons. After carrying out this parameter three different species of *Aedes viz. Aedes aegypti, Aedes albopictus* and *Aedes vittatus* were reported in all selected districts. *Aedes aegypti* was observed in urban and rural area of all selected districts. Except rural area of F.G.Sahib, *Aedes albopictus* was also reported in all selected districts of Punjab. *Aedes vittatus* was recorded only from district Mohali during monsoon. All the *Stegomyia* indices were significantly high during monsoon

and post monsoon period as compared to pre-monsoon. Correlation of *Stegomyia* indices with recorded Dengue cases in the selected areas had been scrutinized as significantly positive. The highest rate of positivity for *Aedes* mosquito larvae were recorded in artificial containers *i.e.* earthen pots, desert coolers followed by tyres, discarded material, plastic containers and refrigerator trays.

Study of genitalia was conducted for proper species identification. On the basis of hypopygium, male and female *Aedes aegypti* was also identified. As it is also a morphological marker in the species identification. It was observed that in male *Aedes aegypti* paraproct of hypopygium has a well-developed ventral arm. This feature is absent in all other oriental species of *Aedes*. In *Aedes albopictus* the hypopygium consists of spoon shaped style with appendage arising near tip and the crown of the paraproct has series of hairs arising from the single plane. In *Aedes vittatus* hypopygium is distinct from all other species in the form of style which is much enlarged at the extremity and carries preapical curved appendages. In female genitalia no such conspicuous differences were observed.

Third parameter includes larval and adult bioassay. In which the WHO standard larval test kits were utilized to detect Temephos susceptibility status. Each bioassay was performed separately to determine the LC_{50} and LC_{90} by preparing various concentrations of Temephos *viz* 0.005mg/l, 0.025mg/l, 0.050mg/l, 0.100mg/l, 0.125mg/land 0.187mg/l. The dose mortality data was analyzed by log-probit method of Finney and sub-lethal concentrations for 50% and 90% mortality were calculated by using the software SPSS. To assess the impact of Pyrethrum resistance in adult *Aedes aegypti* Pyrethrum had been applied by hand foggers and spray pumps. Pyrethrum solution was prepared, by adding 50 ml technical grade of Pyrethrum with 950 ml kerosene oil as per World Health Organization (WHO) recommendation. Counting of knocked-down and dead females 1 hour post-treatment (1hKD) was conducted to calculate knockdown effect in each trail.

Larval bioassay test revealed that larvae of *Aedes aegypti* collected from district Patiala (84% mortality) were ‘probable resistance’ against Temephos, whereas from the other three districts *viz.* SAS Nagar (20%), Ludhiana (40%) and Fatehgarh Sahib (68%) exhibited ‘resistance’ against Temephos. The results of the adult susceptibility test with Pyrethrum fogging revealed that *Aedes aegypti* was resistant in districts F.G Sahib with 88% mortality, while specimen from the district Ludhiana

exhibited probable resistance with 92% mortality. Specimens from rest two districts namely SAS Nagar and Patiala indicated complete susceptibility 100% mortality. The adult susceptibility test with Pyrethrum mist revealed ‘resistance’ in two districts *viz* Ludhiana with 84% mortality and Patiala with 88% mortality while ‘probable resistance’ in specimens from the district of Fatehgarh Sahib with 92% mortality. Specimens from districts SAS Nagar exhibited complete susceptibility with 100% mortality.

To deal with observed ‘resistance’ or ‘probable resistance’ against Temephos and Pyrethrum in selected districts, few other controls including Bti, Malathion and Deltamethrin had been evaluated on larvae and adult of *Aedes aegypti.* Further, larval bioassay tests were also conducted with another bio-larvicide *i.e.* Bti on healthy 3rd/early 4th instars larvae of *Aedes aegypti* at WHO recommended diagnostic concentrations of Bti (0.00375gm/100ml). CDC (Centers of Disease Control and Prevention) bottle bioassays were applied to assess insecticide susceptibility of adult *Aedes aegypti* against Deltamethrin and Malathion. After carrying out this parameter Bti exhibited complete susceptibility with 100% mortality in all the selected districts. CDC bottle bioassay test in all study areas exhibited complete susceptibility with 100% mortality towards Deltamethrin and Malathion.

For molecular analysis, polymerase chain reaction (PCR) method was performed for the detection of mutations in *Ace-1* gene and VGSC (knock down resistance -Kdr) gene of *Aedes aegypti*. The sequences of selected genes were studied for most common mutation due to organophosphate in *Aedes aegypti viz.* G119S but no such substitution had been observed in any of the sequences. Moreover, no indels (insertions or deletions) were detected in these sequences for *Ace-1* gene. The sequences were also analysed for most common mutation due to pyrethroid in *Aedes aegypti viz.* V1016G. Known polymorphisms in VGSC gene were not recorded but one synonymous and non-synonymous mutation was detected in *Aedes aegypti* in all selected districts.

Present research work is the detailed study on Dengue vector identification, prevalence, preferential breeding habitats furthermore their correlations with the Dengue cases in Punjab. Obtained data indicates that *Aedes aegypti* and *Aedes albopictus* are present in both urban and rural areas. In addition to, *Aedes vittatus* is

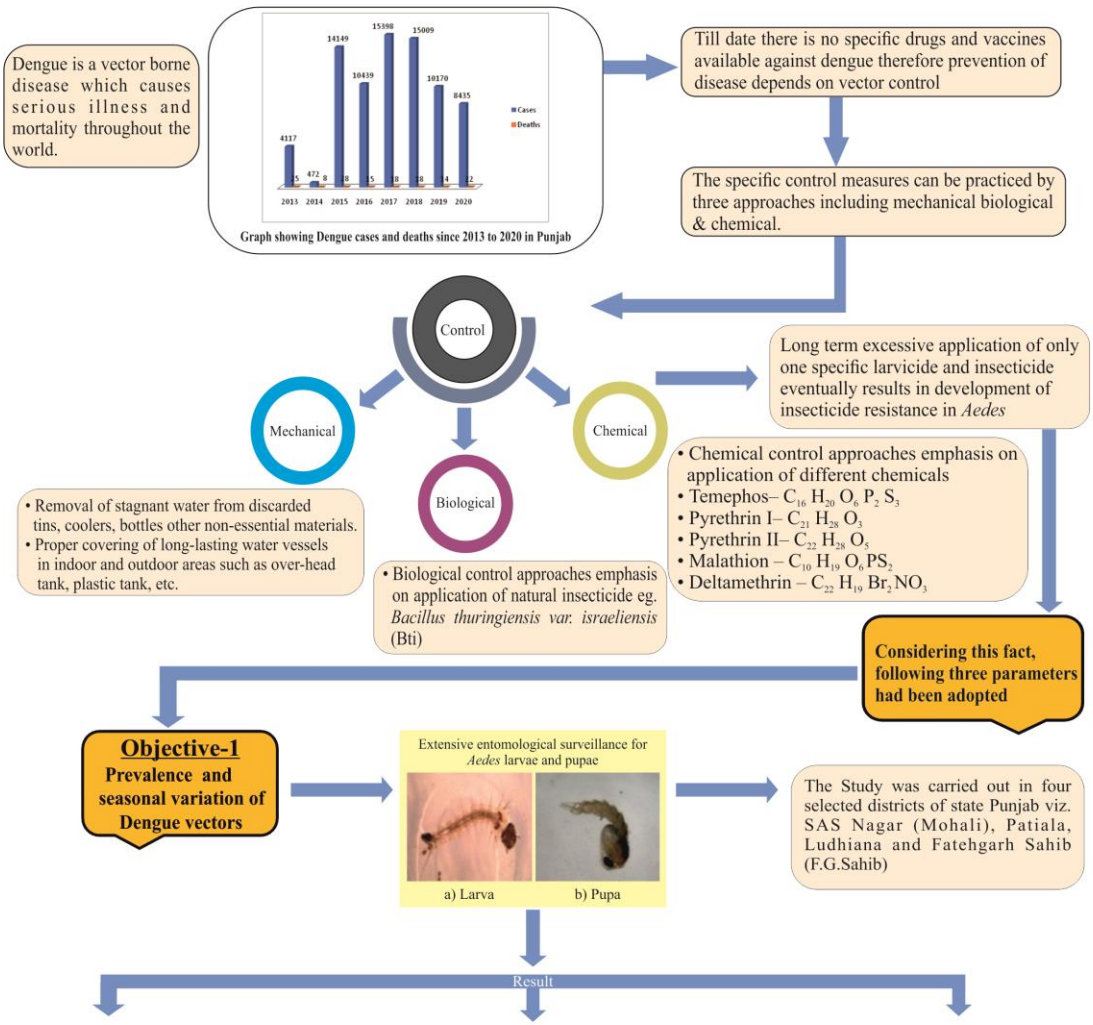
present only in urban area, of the state thereby demonstrating wide range distribution of these vectors. Due to lack of awareness among the human population results in the creation of man-made breeding habitats for *Aedes* proliferation. Such breeding habitats can be control by removal of stagnant from coolers, mud pots, plastic containers as well as other water-holding containers in and around the householdsand proper disposal of discarded tires so as to achieve sustainable Dengue vector control.

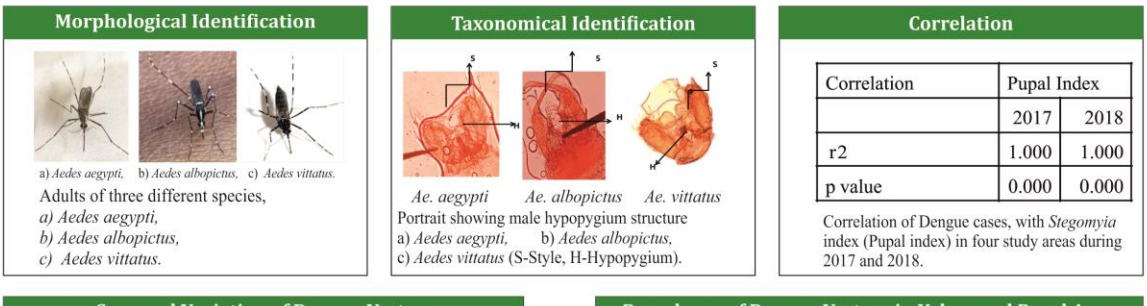
Overall conclusion from the present study indicates that larvae of *Aedes aegypti* in three districts of Punjab *viz*: SAS Nagar, Ludhiana and Fatehgarh Sahib are resistance to Temephos whereas adults of *Aedes aegypti* are reported as moderately resistant to Pyrethrum in districts Patiala, Ludhiana and F.G.Sahib. All the resistant populations are susceptible to Bti, Malathion and Deltamethrin. As no change in *Ace-1* had been detected in *Aedes aegypti*, hence observed resistance against Temephos could be through the modulation of the metabolic pathway. Similarly, no nucleotide change had been observed in VGSC gene of *Aedes aegypti* in all selected districts. Therefore, for the detection of increasing resistance in adult *Aedes aegypti*, further, exploration of polymorphisms in the various domains of the VGSC gene should be analysed with appropriate methodology.

Key words:

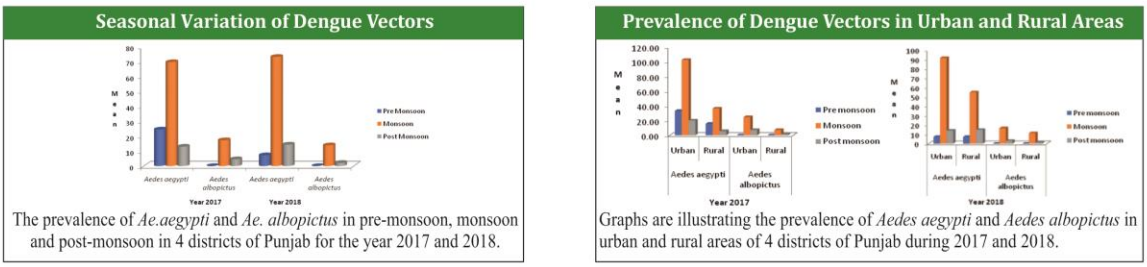
Aedes, Dengue cases, *Stegomyia* indices, Vector, Insecticide Susceptibility and Resistance Status, Temephos, Pyrethrum, *Ace-1* Gene, VGSCGene.

Graphical abstract





Correlation	Pupal Index	
	2017	2018
r2	1.000	1.000
p value	0.000	0.000



Objective-2
Insecticide Resistance/Susceptibility status of larvicide and adulticideused for control of *Aedes*

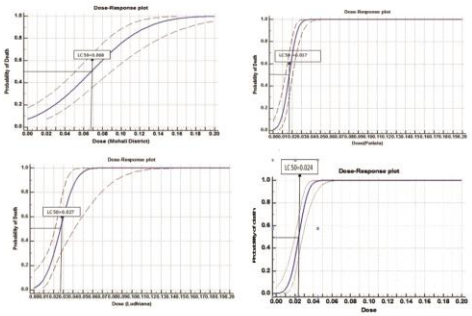
Lab reared F1 generation of larvae and adults of *Aedes aegypti*

Larval Bioassay

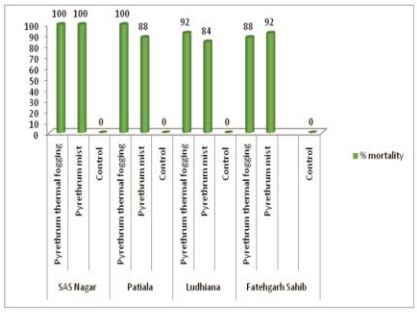
Larval Bioassay by using Temephos & Bti

Adult Bioassay by using Pyrethrum, Deltamethrin & Malathion

Adult Bioassay

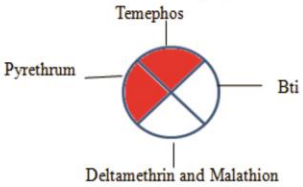


Graph specifying LC_{50} dose response plot of district Mohali, Patiala Ludhiana & Fatehgarh Sahib



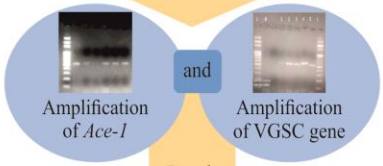
Susceptibility status of *Aedes aegypti* adult against Pyrethrum fogging and spray in 4 selected districts of Punjab.

Objective-3
Mutations in insecticide resistant gene *Ace-1* and VSGC gene and correlation with observed insecticide susceptibility status



Susceptiblity
Confirmed/Probable Resistance

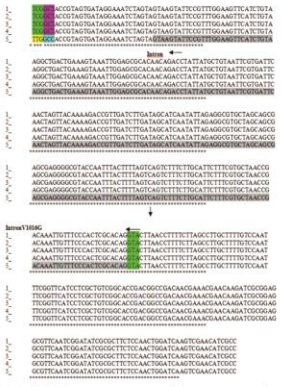
PCR for detection of mutations in *Ace-1* and VGSC (Kdr) gene



Result

No, Substitution observed in any of the sequences of *Ace-1* and VGSC (Kdr) gene

Multiple sequence alignment of *Ace-1* sequences obtained from four different districts with the susceptible Rockfellar strain (AJ621915.1) by using Clustal W.



Multiple sequence alignment of VGSC sequences obtained from four different districts.

Conclusion

1. Obtained data indicates that *Aedes aegypti* and *Aedes albopictus* are present in both urban and rural areas of selected study districts. In addition to, *Aedes vittatus* is present only in urban area, of the state thereby demonstrating wide range distribution of these vectors.
2. Larvae of *Aedes aegypti* in three districts of Punjab viz: SAS Nagar, Ludhiana and Fatehgarh Sahib are resistance to Temephos whereas adults of *Aedes aegypti* are reported as moderately resistant to Pyrethrum in districts Patiala, Ludhiana and F.G.Sahib. All the resistant populations are susceptible to Bti, Malathion and Deltamethrin.
3. As no change in *Ace-1* had been detected in *Aedes aegypti,* hence observed resistance against Temephos could be through the modulation of the metabolic pathway. Similarly, no nucleotide change had been observed in VGSC gene of *Aedes aegypti* in all selected districts. Therefore, for the detection of increasing resistance in adult *Aedes aegypti,* further, exploration of polymorphisms in the various domains of the VGSC gene should be analysed with appropriate methodology.

ACKNOWLEDGEMENTS

First and foremost I would like to thank the almighty God for giving me the strength and patience to work through all these years so that today I can stand proudly with my head held high and whose blessing have made me today what I am.

I wish to express my deepest indebtedness to my respected guide **Dr. Lovleen**, Associate Professor for her generosity and adept knowledge. Without her appropriate suggestions and guidance this difficult study could not be completed. She has supported me in every possible way since the beginning of my research work.

I would also like to express my appreciation to **Dr. Gagandeep Singh Grover,** State Programme Officer, NVBDCP, Punjab for being a great support, allowing me to conduct the experiments regarding my thesis and for providing me his useful guidance. It is great honour to work under his supervision.

I would also like to acknowledge Director, **Dr. Neeraj Dhingra** and Additional Director, **Dr. Kalpna Baruha,** National Vector Borne Disease Control Programme Delhi for providing me permission for use of historical data of Dengue in India.

I would like to thank **Dr. Taruna Kaura,** Scientist C, Post Graduate institute of Medical Education and Research (PGIMER) for her friendly assistant, constant encouragement and above all, her moral support. Her valuable guidance and insightful suggestions have been a crucial formative influence on the present study. I thank her for all her in puts during my presentation and for teaching me molecular technique. I also want to thank Mr. Rahul Rawat for technical support during my work.

I am thankful to **Prof. Neeta Raj Sharma,** Head of School and **Prof. Joydeep Dutta, Professor,** Head of Department of Zoology and Botany, School of Bioengineering and Biosciences, Lovely Professional University, Punjab for their valuable suggestions during annual seminars and change of the titles.

I owe a deep sense of gratitude to **Dr. Rabi Sankar Pandit,** Entomologist, Integrated Disease Surveillance Programme, Directorate of Public Health, Odisha for his prompt inspiration at every stage of my research work. His timely suggestions and guidance enabled me to pave my path successfully and complete my thesis.

No research is possible without infrastructure and requisite material and resources. For this, I extend thanks to **Dr. Suryakant Sharma**, Incharge of National Institute of Malaria Resaerch (NIMR), Dhakoli Punjab for providing me with the requisite institutional facilities through my research tenure and for providing scientific comment for paper writing. It is my sincere duty to express my thanks to **Dr. Jaspreet Kaur,** Scientist B (NIMR) for their valuable guidance and suggestions. I am also thankful to technical staff members of NIMR Mr. Dyal and Mr. Negi for their technical support.

I also express my sincere gratitude my parents **Mr. Sukhpal** and **Mrs. Nachhatro Devi**. They have taught me patience and perseverance. I am thankful for their love and motivation through my difficult time.

Last but not the least, I must acknowledge with tremendous and sincere thanks to my lovely husband **Mr. Mintu Ram**. Through his unfailing support and encouragement, patience and unwavering belief in me, I have been able to achieve this goal and completed this long journey. He has supported me financially, emotionally and spiritually. I appreciate my little girl **Aaliya Sharma** and my son **Manav Sharma** for abiding my ignorance and patience they showed during my research work. Words would never say how grateful I am to both of you. I consider myself the luckiest in the world have such a lovely family, standing beside me with their love and unconditional support.

My heartfelt regards goes to my father in law **Mr. Ved Parkash** and mother in law **Mrs. Neelam Kumari** for their moral support.

Thank you for valuing me.

Research Scholar,

Seema Devi.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AchE	Acetylcholine esterase
Ae.	*Aedes*
AL	Anti-Larval
AS-PCR	Allel Specific-Polymerase Chain Reaction
BAR	Biological Activity Ratio
BI	Breteau Index
BP	Breeding Percentage
Bti	*Bacillus thuringiensis israeliensis*
CA	Carbamates
CDC	The Centre for Disease Control and Prevention
CHIKV	Chikungunya Virus
CI	Container Index
DDT	Dichlorodiphenyltrichloroethane
DENV	Dengue Virus
DF	Dengue Fever
DHF	Dengue Hemorrhagic Fever
DNA	Deoxyribonucleic acid
DPX	Dibutylphthalate Polystyrene Xylene
DSS	Dengue Shock Syndrome
EPA	Environmental Protection Act
F.G.Sahib	Fatehgarh Sahib
HI	House Index
IEC	Information Education and Communication
IFA	Immune Fluroscence Assay
IRS	Indoor Residual Spray
Kdr	Knock Down Resistance
KDT	Knock Down Time
KOH	Potassium Hydroxide
Lab.	Laboratory
LC	Lethal concentration

LD	Lethal Dose
MBD	Mosquito Borne Disease
MFO	Mixed Function Oxidase
No.	Number
NVBDCP	National Vector Borne Disease Control Programme
OC	Organochlorates
OI	Ovitrap Index
OP	Organophosphate
PCR	Polymerase Chain Reaction
PDI	Post Day Infection
PGIMER	Post Graduate Institute of Medical Reasearch
PI	Pupal Index
PMHD	Per Man Hour Density
PPM	Part Per Million
PY	Pyrethroids
RNA	Ribonucleic acid
RP	Reference Population
RR	Resistance Ratio
SD	Standard Deviation
UMS	Urban Malaria Scheme
USEPA	U.S. Environmental Protection Agency
UV	Ultra violet
VGSC	Voltage Gated Sodium Channel
WHO	World Health Organization
YFV	Yellow Fever Virus
ZIKAV	Zika Virus

CHAPTER-I

INTRODUCTION

Arthropods, the most variant metazoans, comprise approximately a million representatives which form 80% of living creatures on the earth (Laroche, *et al*., 2017; Stork, 2018; Barnes, 2021). The specific phylum covers differential groups such as mosquitoes, tse-tse flies, ticks, mites, blackflies, phlebotomine sandflies, biting midges, horseflies and stable flies, which possess divergent features such as jointed legs, segmented bodies and chitinous exoskeleton and additionally are vectors of various diseases (Russell, 2012; Duvallet, 2018; Barnes, 2021). Among all, predominant disease vectors are confined to genera including *Aedes, Anopheles* and *Culex.* These species are responsible for causing infection such as Yellow Fever, Dengue, West Nile Fever, Chikungunya, Dirofilariasis, Malaria, Western equine encephalitis, Eastern Equine Encephalitis, Saint Louis Encephalitis, Barmah Forest Fever, Ross River Fever, La Crosse Encephalitis, Rift Valley Fever, Zika Fever, Filariasis, Japanese Encephalitis etc.to human and other animals (Calzolari, 2016; Lemine, *et al*., 2017; Saldana, *et al*., 2017; Tandina, *et al*., 2018; Dahmana and Mediannikov, 2020).

Mosquitoes are widely distributed around the world due to their adaptability to divergent environmental conditions (Foster, *et al.,* 2002; Tandina, *et al*., 2018; Couper, *et al.,* 2020). The concerned Dipteran animals, are devastating vector, responsible for transmission of various pathogenic microorganisms such as parasite, viruses and bacteria, which ultimately result in 300 million cases and one million deaths annually worldwide (Christophides and Crisanti, 2013; Kalita, *et al.,* 2014; Charrel, *et al*., 2018; Couper, *et al.,* 2020).

Culicidae, the mosquitoes' family possesses about 3500 species and subspecies, in more than 40 genera of mosquitoes, worldwide (Elbers, *et al*., 2015; Lemine, *et al*., 2017). In the specific family, important genera include *Aedes*, *Anopheles* and *Culex.* The genus *Aedes* has the highest species diversity comprising 70 subgenera involving 927 species whereas *Anopheles* has seven subgenera along with approximately 500 identified species and *Culex* has over 25 subgenera comprises 763 species. These species are responsible for causing various health problems (Lemine, *et al*., 2017).

Mosquito-borne diseases (MBDs) are major public health risks, world wide (Getachew, *et al.,* 2015; Diouf and Nour, 2017). These diseases (MBDs) cause significant morbidity and mortality globally. The specific mosquito-borne diseases are

responsible for more than 16% of the approximate worldwide load of all contagious diseases, mainly due to Malaria and Dengue Fever (National Academies Press (US), 2008; Sharma, *et al.*, 2017; Omodior, *et al.*, 2018).

Chinese medical encyclopedia of disease symptoms and remedies from Chin Dynasty (265-420 AD) is the first record of Dengue whereby it was mentioned as "water poison" and was connected with flying insects associated with water (Gubler, 1998; Gupta, *et al.*, 2012; Ferreira-de-Lima and Lima-Camara, 2018). *Aedes* mosquito, as a vector of Dengue Fever, was confirmed in 1906 and Dengue was the second illness succeeding Yellow Fever that had been considered to be caused by a virus. First Dengue Hemorrhagic Fever was recorded in Philippines in 1953 and subsequently in 1981 in South America (Gubler, 2011; Vaddadi, *et al.*, 2015). Besides Dengue, *Aedes* mosquitoes also spread mosquito-borne diseases which include Chikungunya, Yellow Fever (Vontas, *et al.*, 2012) and recently Zika virus (Mubbashir, *et al.*, 2018).

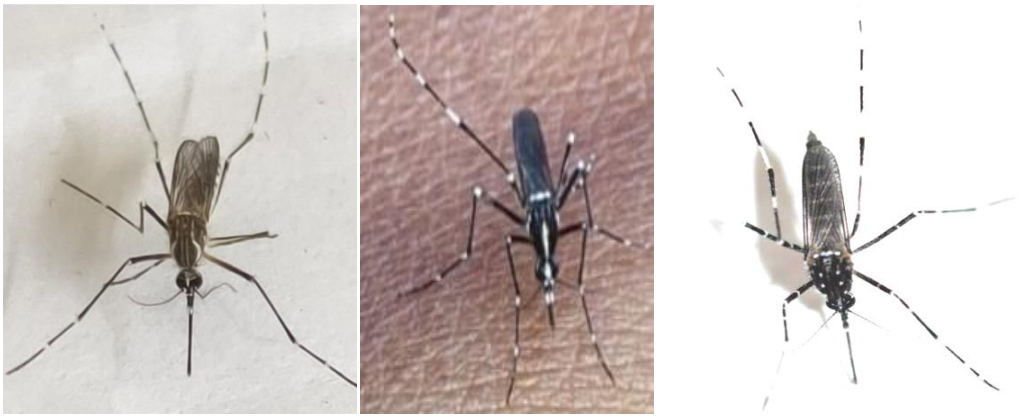
1.1. Vectors of Dengue

For the transmission of Dengue virus in India, two principal vectors are *Aedes aegypti* and *Aedes albopictus* (Gubler, 1998; Mutheneni, *et al.*, 2017; Medeiros, *et al.*, 2018). *Aedes aegypti* is reported all over the world (S. Africa, America and Southeast Asia). Originally, *Aedes albopictus* was considered a native species in Southeast Asia and subsequently, spread to other parts of the world (Vontas, *et al*., 2012). Dengue is also transmitted by third species *i.e Aedes vittatus* and it is spread in Asia, Africa and the Mediterranean region of Europe (Sudeep and Shil, 2017)

1.1.1. *Aedes aegypti*:-The specific mosquito is the competent vector for arboviruses, as this species mainly prefers to feed on human beings, often bites and grows vicinity to the household. *Aedes aegypti* prefers to breed in artificial vessels which are utilized for storage of water in urban areas (Fig 1). It breeds mainly indoors, in fresh water and any kind of neglected cups or jugs (Simard, *et al*., 2005; Saleem, *et al.*, 2014; Arslan, *et al.*, 2015; Ryan, *et al.*, 2019). In urban areas, people are dependent to accumulate the water in receptacles because of limited hours of water supply and these water storage containers create breeding sites for *Aedes aegypti* (Philbert and Ljumba, 2013). These mosquitoes lay their eggs in manmade aqua storage

receptacles, discarded bottles and flower pots that are detected in and around households (Ranson, *et al*., 2010; Scott and Morrison, 2010).

Important breeding sites of *Aedes aegypti* are cemented tanks, mud pots, indoor and outdoor human habitations (Preechaporn, *et al*., 2006; Vikram, *et al*., 2015). The female *Aedes aegypti* prefers to reside indoor, bite humans during daylight hours and generally flies at about 100-500m (Honorio, *et al*., 2003; Getachew, *et al.*, 2015). Its peak biting time is at the crack of dawn and before dark in the evening (Gubler, 1998; Kraemer, *et al*., 2015). *Aedes aegypti* is believed to be an urban mosquito and favors densely populated areas. But, it also breeds in rural areas and Dengue cases are also being reported from rural areas. Unplanned urbanization, accumulation of non-biodegradable and insufficient vector control measures, have produced suitable settings for Dengue virus and its mosquito vectors to expand in various tropical developing countries including India (El-Badry, *et al*., 2010; Chepkorir, *et al*., 2014; Das, *et al*., 2014; Kumar, *et al.*, 2014).



a). *Aedes aegypti* **b). *Aedes albopictus*** **c).*Aedes vittatus***

Fig 1*:*-Adults of three different species, a) *Aedes aegypti,* b) *Aedes albopictus,* c) *Aedes vittatus.*

1.1.2. *Aedes albopictus* (Asian tiger mosquito)**:-**The concerned vector is another predominant species that transmits Dengue, Chikungunya and Zika. *Aedes albopictus* is reported to be adaptable in ecologically diverse conditions like suburban, rural, residential and agricultural habitats (Fig 1). *Aedes albopictus* is detected as a carrier of Den virus and acts as secondary vectors (Waldock, *et al*., 2013; Medeiros, *et al.*,

2018; Ryan, *et al.,* 2019). It breeds in both natural and man- made containers. Larvae of *Aedes albopictus* are usually observed in water holding containers such as tree holes, coconut shells, fruit peels, aqua receptacles, unused tyres and vehicle parts, hence, are prevalent breeding sites. It feeds exclusively on humans in outdoor conditions and also rest in outdoor areas. It feeds on humans, a wide range of mammals and birds in day time, especially in gardens and parks around human habitation. *Aedes albopictus* can fly up to 400-600m (Preechaporn, *et al*., 2006; Higa, 2011; Dhiman, *et al*., 2014; Li, *et al*., 2014).

Aedes aegypti and *Aedes albopictus* prefer to lay eggs few millimeters above the water level in containers (natural and artificial) (Shragai, *et al.,* 2019). Both species are also sympatric species and coexist in the same habitation in both urban and rural areas (Chen, *et al.,* 2006; Hashim, *et al.,* 2018). Whenever both species try to exist together in the same ecological environment, species replacement or dislocation appears. *Aedes albopictus* is dominated over *Aedes aegypti* and it has displaced *Aedes aegypti* in the North of America, because of interspecies competition for food, habitat and environment (Hashim, *et al.,* 2018; Shragai, *et al.,* 2019). Replacement of *Aedes albopictus* by *Aedes aegypti* in the outer region of towns of India is also reported (Kalra, *et al*., 1997; Kamgang, *et al*., 2013; Shragai, *et al.,* 2019).

1.1.3. *Aedes vittatus*:-The third species that plays an important role in maintenance and transmission of Dengue, Chikungunya, Yellow Fever and Zika is *Aedes vittatus* (Fig 1). It is also detected throughout tropical Asia, Africa and the Mediterranean region of Europe. *Aedes vittatus* is commonly observed in forest areas and predominates in rocky areas. It bites wide range of vertebrate hosts and is highly anthropophilic in nature. During the rainy season, breeding is mainly found in natural habitats like tree holes and rock areas. In the absence of natural habitat, it can breed in domestic areas also especially in household water holding containers (Sudeep and Shil, 2017).

1.2. Identification of Species

It is extremely important to know the particular species of mosquito which spreads vector borne diseases so as to customize the species specific strategy to control the spread, to correctly calculate the risk and exposure of the disease (Jourdain, *et al*., 2018). For identification of individual vector species, on the basis of their

morphological character is the only conventional gold standard method. Different mosquito species have different morphological characters, which are used in various taxonomic keys like Harrison and Scanlon (1975), Rattanarithikul (2007), to recognize individual species (Rueda, 2004; Chan, *et al*., 2014). It is equally important to identify the accurate species that is involved in transmitting a pathogen so that appropriate understanding of the mechanisms could be performed which governs any biological system. For morphological identification, the most conspicuous feature *i.e.* genital features of male and female mosquitoes can be used with more reliability. This is the modified ninth abdominal segment in mosquitoes which varies significantly amid the various genera. Therefore, it facilitates in the correct identification of the sibling and new species of mosquitoes (Yadav, *et al.,* 2014). Using these techniques, there can be developed a more reliable and standardized identification system for mosquito species detected in Punjab. Male genitalia of *Aedes albopictus* varies greatly from *Aedes aegypti* in the lateral projection of tergum and in shape and size of median and lateral projection (Hartberg, *et al.,* 1973; Kaur, 2014).

1.3. Transmission of Disease

Dengue viruses spread to human beings when they get bitten *via* infected female *Aedes aegypti* and *Aedes albopictus* (Baskera, *et al*., 2013). Dengue is the most significant vector-borne viral infection in various parts of the world (Murray, *et al.,* 2013; Bhat, *et al.,* 2014). Dengue virus belongs to the Flaviviridae family (World Health Organization, 2009). This Virus has four major serotypes *viz*. DEN (1-4) and these viruses have a single-stranded, positive-sense RNA genome, nearly 11 kb in length (Gupta, *et al*., 2015; Vikram, *et al.,* 2015; Paixao, *et al*., 2018; Shrivastava, *et al*., 2018).

Dengue virus in humans can produce a variety of diseases, ranging from mild flu like syndrome or asymptomatic infection to the most severe clinical symptom form of the disease, Dengue Hemorrhagic Fever (DHF) (Abbasi, *et al.,* 2016). Dengue Fever (DF) may progress into Dengue Hemorrhagic Fever which leads to hemorrhages and Dengue Shock Syndrome (DSS) may cause leakage of plasma (Ooi, *et al.,* 2006; WHO, 2009; Baskera, *et al*., 2013). Dengue virus infection shows different clinical presentations, therefore final diagnosis depends on laboratory confirmation. The

clinical condition of the Dengue patient is usually self-limiting and no antiviral therapy is presently available (Hasan, *et al.,* 2016).

1.4. National Status of Dengue

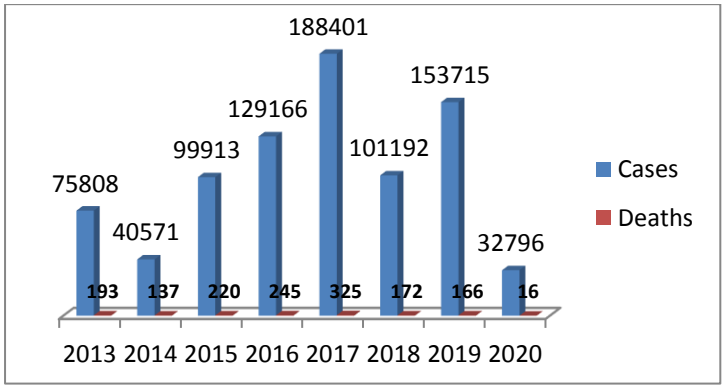
Dengue infections are reported from more than 100 countries of the world including India, it is also endemic in India for more than two centuries. First confirmed Dengue Fever (DF) was reported in 1946 (Gupta, *et al.*, 2006; Bhat, *et al*., 2014; Gupta and Ballani, 2014; Ganeshkumar, *et al*., 2018). About 18 years later, Dengue Fever outbreak was first time clinically proved in Calcutta, Tamil Nadu, Andhra Pradesh, Orissa and West Bengal in 1963-1964. The disease expanded in north-eastern part of India, thereafter reached Delhi and Uttar Pradesh (UP) during 1967-1968, respectively. Simultaneously, Dengue started to spread widely in the southwards and eventually, all the states were intricate with substantial outbreak. Subsequently, autochthonous/hyper endemic prevalence has occurred country-wide (Prakash, *et al*., 2015; Yadav, *et al*., 2015; Hasan, *et al*., 2016).

In India, the initial outbreak of Dengue Hemorrhagic Fever was recorded in 1996, which consists of areas around Lucknow and Delhi. Further, it was expanded throughout the country (Gupta, *et al.,* 2012) and thereafter, the number of Dengue cases was crucially increased in India since 2001. Before 2000s, Dengue was indigenous in a few states/union territories of the south including Tamil Nadu, Karnataka, Pondicherry, Maharashtra and northern states comprising Punjab, Rajasthan, Delhi, Haryana and Chandigarh. Lately, it has been disseminated to many states, including the union territories (Mutheneni, *et al.,* 2017). In India, new expansion of Dengue cases are related to rise in urbanization, unplanned growth of cities, mushrooming of urban slums, unsafe water storage practices. Now, it has become the most important community health concern at tropical and sub-tropical countries (Getachew, *et al.,* 2015; Khan, *et al*., 2015; Arora, *et al*., 2017).

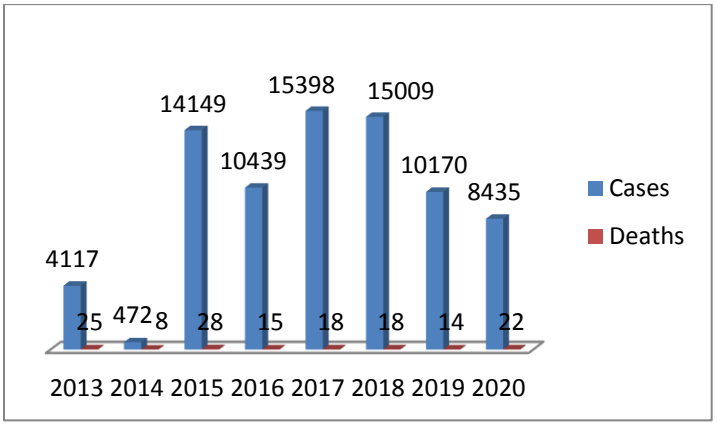
In India, National Vector Borne Disease Control Programme (NVBDCP) Delhi had recorded gradual rise in the number of Dengue cases along with deaths during 2015, 2016 and 2017 with respective value of Dengue cases (deaths) as 99913(220), 129166(245) and 188401(325) (Fig 2) and subsequently Dengue cases were decreased

to 101192(172) in 2018. The number of Dengue cases had also dramatically spread to many new locations in Delhi, West Bengal and Punjab, where it remained an important public health concern. The State of Punjab has been considered highly endemic for Dengue Fever (Fig 2) as over the last 6-7 years, large numbers of confirmed Dengue cases have been recorded (http://nvbdcp.gov.in/den-cd.html).

Dengue transmission is increasing due to the rapid increase in human population, environmental, social changes and lack of knowledge amid people, all leading to increased breeding of vector mosquitoes (Haider and Turner, 2015; Radhika, *et al*., 2019). Disease was restricted to metropolitan areas a few decades earlier and now it is being reported from semi-urban and rural areas (Fig 3) (Chakravarti, *et al*., 2012; Kakkar, 2012).



a). Dengue situation in India



b). Dengue situation in Punjab

Fig 2:-Graph showing Dengue cases and deaths since 2013 to 2020 in India and Punjab, a) Dengue situation in India, b) Dengue situation in Punjab.

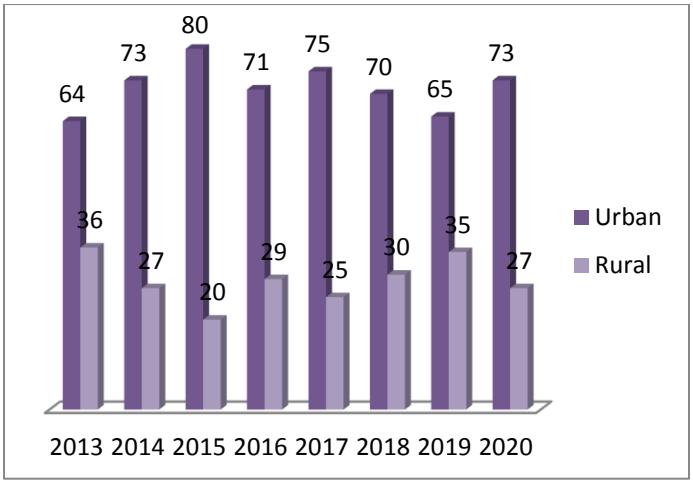


Fig 3:-Graph illustrating proportion of Dengue cases in urban and rural areas of Punjab from 2013-2020.

1.5. Worldwide Burden of Disease

Above 100 nations are known to be endemic for Dengue and the most cases have been recorded from the Western Pacific regions, America and South-East Asia (World Health Organization, 2009). It has a huge disease burden worldwide, as it is estimated that there are 96 million disease cases, with more than 390 million infections every year (Omodior, *et al.,* 2018). Over 2.5 billion people reside in disease endemic countries (Grisales, *et al*., 2013; Matysiak and Roess, 2017).

1.6. Entomological Surveillance and Importance

Surveillance of vectors is used to ascertain various targets such as alternation in the geographical areas, vector density and for calculation of population of species with time. Because these parameters can be utilized easily for taking decisions in regard to interventions (Baskera, *et al*., 2013; World Health Organization, 2016). The specific surveillance is based on different larval indices. These larval indices are recorded to be helpful because signals of their plenty are connected to the occurrence of Dengue Fever and DHF. House Index (HI), Container Index (CI) and Breteau index (BI) are the most commonly used indices for vector surveillance (Siregar, *et al.,* 2018). In India, there is an upsurge in the entomological indices HI, CI, and BI of *Aedes aegypti* from July to October and thereafter, these indices show declining trend. These indices remain very high during the months of August

and September (Ahmed, *et al.,* 2019). These indices are helpful in detection of the occurrence or nonoccurrence of *Aedes* mosquito larvae either in each container or in an individual house. These indices can not only determine the presence of *Aedes* mosquitoes but also have the ability to forecast the possible risk of arboviral infections. It can also be used to set up suitable interference for the control of these infections (Ferede, *et al.,* 2018). To determine Dengue transmission risk, Pupal index (PI) is also a very important evaluator as mortality of pupae is the lowest compared with mortality of larvae. So, this index can be the best guide in control of Dengue (Jimenez-Alejo, *et al*., 2017).

1.7. Methods of Vector Control

There are no specific drugs and vaccines against Dengue therefore, the only available method for prevention of disease depends on vector control (Achee, *et al*., 2015; Bellinato, *et al*., 2016; Bharati, *et al.,* 2018). *Aedes* mosquitoes are larger hosts for Dengue viruses and these mosquitoes show transovarial transmission (means horizontally as well as vertically). *Aedes* species carry viruses from eggs to adults without any harmful effects (Ferreira-de-Lima and Lima-Camara, 2018; Sanchez-Vargas, *et al*., 2018). Community participation and education campaigns are recommended as the best method to control breeding of mosquitoes. Because community can reduce breeding of mosquitoes by removal of water from unnecessary sources of stagnant water, coupled with covering of long-lasting vessels, water sources in domestic and peri-domestic areas (Poupardin, *et al*., 2014; Arslan, *et al*.,2015).

Control of vectors is mostly depends on the multiple methods *i.e.,* to reduce the mosquito density and it can be done by three approaches biological, mechanical and chemical. Basis of mechanical control is eradication or appropriate shielding of possible breeding sites. Biological and chemical control of Dengue is practiced under the specific guidelines of WHO. Biological control includes the involvement of the living organisms against the concerned disease vector such as bacterial larvicide crystalline protein of *Bacillus thuringiensis var. israeliensis* (Bti). Whereas chemical control approaches emphasis on application of different chemicals such as VGSC gene mutations inducer pyrethroids, anti-acetyl

cholinesterase organophosphate, chitin synthesis inhibitor benzoylureas and mimics of natural hormone pyriproxyfen because of their effective, quick activity against insects, their low mammalian toxicity and their degradability in the environment (Bellinato, *et al*., 2016; Ngoagouni, *et al.*, 2016; Plernsub, *et al*., 2016; Bharati, *et al.*, 2018).

The National Vector Borne Disease Control Programme (NVBDCP) Delhi also approved the use of specific insecticides for vector management, like Temephos synthetic chemical (50% EC), Bti (WP) as bacterial larvicide, synthetic pyrethroids for indoor residual spray (IRS), Deltamethrin 2.5% WP for soaking of bed nets and Malathion 25% WP for ultra-low volume (ULV) spray (Srivastava and Dhariwal, 2016).

1.8. Chemical Control: Larvicide and its Mode of Action

Aedes mosquito management is commonly based on anti-larval operation, space spraying of insecticides Pyrethrum and Malathion fogging against adults. Since 1980, Temephos, an anti-acetyl cholinesterase organophosphate synthetic chemical is being used under the vector control programme (Singh, *et al.*, 2014; Chatterjee, *et al.,* 2018). Everywhere Temephos is the best tool due to various points, because of its ease and its application is very easy in various ways depending upon the site and rate of application needed, selective killing of mosquito larvae. The effect of Temephos is more enduring than traditional oil application technique. It can be sprinkled by hand or by injection or spray pumps and in the household water storage containers of various capacities. Its sand granules can be sprayed with a calibrated spoon to achieve an unvarying dosage of 1ppm (Fig 4). It is commercially available in various forms, namely; dilute solutions, emulsifiable concentrates, dusts and granules, including slow release formulations. As per NVBDCP National guidelines, 1ppm Temephos (2.5 ml Temephos in 10 liters of water) is being used in Punjab for the control of immature forms of *Aedes aegypti.* Temephos as a larvicide has been considered worldwide as a keystone in Dengue vector control (George, *et al*., 2015).

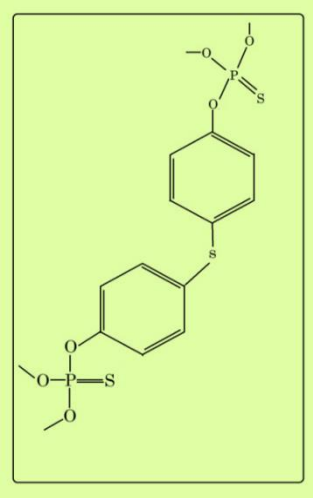


Fig 4:-Picture depicting chemical structure of larvicide Temephos

Temephos act by ingestion or contact and it inhibit the normal functioning of acetyl cholinesterase (AChE), an enzyme which results, acetylcholine neurotransmitter continue in the synaptic cleft, leading to the exacerbation of nerve impulse transmission and causes death of insect (Bellinato, *et al.*,2016).

Urban Malaria Scheme (UMS) the second recommended bacterial larvicide is *Bacillus thuringiensis varisraelensis* (Bti). Bti is used against mosquito larvae due to various reasons such as potency, its quick killing effect as well as fine toxicological profile (Singh, *et al.,* 2014; Chatterjee, *et al.,* 2018). Bti is ingested by larvae; it dissolves in insect gut and becomes active by the formation of spores (sporulation). Later, crystalline protein forms around these spores which release insecticidal toxins (Cry and Cyt families). The modes of action of these toxins are different as a result both disrupt the osmotic balance of the midgut cells of the mosquito larvae and cause the death of larvae (Marcombe, *et al*., 2018).

Malathion is used for indoor residual spray under NVBDCP for control of vector borne diseases in Punjab. It is an acetylcholinesterase inhibitor, when contact with the target organism phosphoester group is strongly bound to the active site of cholinesterase enzyme irreversibly. As a result, it deactivates the enzyme which generates rapid build-up of acetylcholine in the synapse and causes death of insects (Colovic, *et al.,* 2013).

1.9. Chemical Control: Adulticide and its Mode of Action

National Vector Borne Disease Control Programme (NVBDCP) Government of India, Delhi, recommends the use of Pyrethrum 2% Extract (pyrethroid) against adult Dengue vectors. Pyrethrum is used for indoor spray/fogging at low application rates. It has no bioaccumulation, relatively short persistence in the environment, and low mammalian toxicity. From 1945 to the early 1970 Pyrethrum was the only botanical insecticide which was used extensively for household and industrial spray in urban and rural areas for mosquitoes control (Fig 5). Doses of insecticide are used in NVBDCP Punjab as recommended by NVBDCP Delhi *i.e.,*1part of Pyrethrum 2% extract (as an insecticide) is diluted in 19 part kerosene oil against mosquitoes (Table 1.2). This natural insecticide is produced from the dried flower powder of *Chrysanthemum cinerariefolium* (Fig 6**).** During outbreak situation space spray is used to target the adult *Aedes* mosquitoes but in an integrated approach it can be used at the same time to target both larvae and adult stages (Ban, *et al.,* 2010; Dhiman, *et al*., 2014**).** Pyrethroids resistance is a matter of concern because there is resurgence of Dengue and other arboviruses globally as this is the primary insecticide family used against adult mosquitoes. Pan American Health Organization had almost eradicated *Aedes aegypti* in 1950s and 60s by killing both stages, *i.e.* adults and larvae of the mosquito in household habitation with insecticide and by eradication of breeding sites (Leslie, 1994; Dusfou, *et al.,* 2011; Ishak, *et al.,* 2017).

Pyrethroids mainly attack the nervous system of pests; keep their voltage gated sodium channel in its open confirmation by disrupting their sodium and potassium ion exchange process. Furthermore, this prompts nerve cells to generate repeated discharges consequently bring about paralysis and demise (Guglielmone, *et al*., 2001).

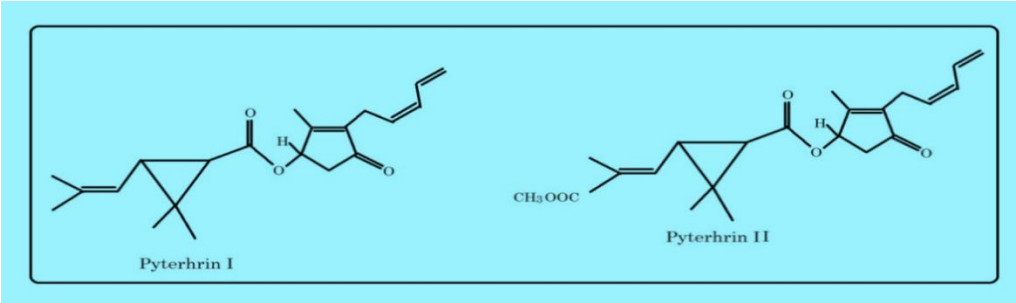


Fig 5:-Click specifying chemical structure of adulticide Pyrethrum

Fig 6:-Potrait showing flower of *Tanacetum cinerariifolium and Chrysanthemum cinerariefolium* (Kharar Railway station, District Mohali Punjab and sector-20 Chandigarh).

1.10. Development of Resistance: A Major Concern

Dengue vector can be controlled by the use of proper dose, drug and selection of insecticide. Contrarily, inappropriate application time and dosage of insecticide can develop vector resistance. For all insecticides a standard dose of diagnosis is fixed by WHO in which if application of insecticide leads to<80% death rate of test insects then that condition is classified as resistance of insecticide and the insecticide cannot be utilized for (Caffery and Nauen, 2011) vector control (Corbel and N'Guessan, 2013; Hasmiwati, *et al*., 2018).

On the contrary, prolonged exposure to insecticides has led many vector control programs to be under threat due to the development of insecticide resistance in *Aedes aegypti* and *Aedes albopictus*. Thus, the insect vectors are under selection pressure in order to develop the resistance, which is one of the major hurdles in achieving successful curbing of vectors. Insecticide resistance in *Aedes albopictus* have been reported in earlier few studies as well as resistance to multiple insecticides (e.g. pyrethroids and organophosphates) in *Aedes aegypti* has been recorded in several regions of the world (Kamgang, *et al.*, 2011; Ngoagouni, *et al.*, 2016).

Temephos (organophosphate) affects nerve synapses through the neuro-transmitter acetylcholinesterase in mosquitoes. It causes changes on the acetylcholinesterase gene (*Ace-1*) in various vector species which are medically important like *Anopheles gambiae, Culex pipiens* and *Culex tritaeniorhynchus*, etc.

Mutation of wild type glycine to serine occurs when the most known *Ace-1* replacement of amino acid at position 119 takes place in mosquitoes. Resistance of organophosphate, Temephos can also take place due to raising levels of esterase enzymes. These enzymes can act to hide out the larvicides, by reducing the amount of active insecticide that reaches the target site. Secondly, these enzymes also raise the rate of turnover of insecticide by alternation of amino acid in the coding sequences of more esterase (Kamgang, *et al*., 2011; Grisales, *et al*., 2013; Poupardin, *et al*., 2014; Aguirre-Obando, *et al.,* 2016).

Pyrethrum and Deltamethrin target the voltage-gated sodium channels (VGSC) and cause slowdown in the closure of VGSC of the nerve cells of insects, succeeding in repeated and increased nerve firings. Because of the loss of motor coordination and paralysis, this hyper-excitation causes the death of the insect (Pyrethrins General Fact Sheet, 2014). This type of resistance is known as knockdown resistance. Due to this structural change in the insecticide target site, the affinity for the insecticide can be lowered. In *Aedes aegypti* many (Knock down) Kdr mutations have been identified, associated with resistance in pyrethroid; V1016G and F1534C in two segments of the vgsc gene, respectively. Instead, in Southeast Asian and Latin American populations mutation at distinct positions of *Aedes aegypti* have been noticed (Faucon, *et al.,* 2015; Kushwah, *et al*., 2015; Li, *et al*., 2015; Kawada, *et al.,* 2016).

In India, recently Dengue has spread dramatically and has been reported from all over the country (Bhat, *et al*., 2014). Dengue remains a major public health problem in Punjab. During 1996, an outbreak of Dengue with 720 confirmed cases and 19 deaths was reported from Ludhiana district (Gill, *et al*.,1997) and during 2011, out of 812 reported cases 399 confirmed Dengue cases were reported from district Shri Muktsar Sahib in the state (Lata, *et al*., 2012).

On other hand NVBDCP depends on only two out of four main insecticides classes available for use in public health: pyrethroids and organophosphates which are also used in Punjab State under Urban Malaria Scheme (UMS) for the control of *Aedes.* However, in tropical areas the development of resistance to these two chemical classes is a major problem in controlling arthropods (Marcombe, *et al.,* 2011). Punjab State is highly endemic for Dengue Fever and there has been a steady increase in confirmed cases of Dengue for the last five years. During 2016 there were 10439

reported cases and 15 deaths and during 2017, 15398 cases and 18 deaths were recorded in the State of Punjab (NVBDCP website http://nvbdcp.gov.in/den-cd.html). Keeping in view the public health, a rising cases of Dengue in Punjab, a detailed study was undertaken entitled as "Identification and Molecular Characterization of Insecticide Resistant/Susceptible Dengue Vector Prevalent in Eastern Punjab, India". The present study deals with to determine Dengue vector prevalence, identification, preferential breeding habitats, comparison of *Stegomyia* indices of *Aedes* in different seasons**,** furthermore their correlations with the Dengue cases in both rural and urban area of four districts, identification on the basis of male and female hypopygium structure and to determine the susceptibility status of *Ae. aegypti* to temephos and pyrethroid as well as to study mutation in insecticide resistant genes, which include acetylcholinesterase (*Ace-1*) and voltage-gated sodium channel (knock down resistance -Kdr) in predominant species in four districts of Punjab. This is the first detailed study in Punjab and also indicates the existence of *Ae.aegypti* and *Ae.albopictus* in both urban and rural areas and presence of *Aedes vittatus* only in urban areas of the state, thereby showing wide distribution of this vector. Distinct breeding habitats recognized in the study should be subjected to selected intervention such as source depletion in order to achieve effective control of Dengue cases. Periodically monitoring and updating of the susceptibility status of Dengue vectors put forward another possible vector control plan for successful disease management and the clarification of the mechanisms of resistance in insecticide may further help in the plan of appropriate resistance control plan to extend the efficacy of the existing insecticide-based control instruments. The outcome of the research work can assist health officials to utilize suitable insecticide in every administrative district to avoid the beginning of resistance and to use appropriate insecticide to obtain utmost results via space spraying and larviciding undertaken in urban and rural areas during different seasons.

CHAPTER – II

REVIEW OF LITERATURE

Dengue is a rapidly growing global public health problem with an approximate 10,000 demise as well as more than 100 million symptomatic diseases, annually in more than 100 nations (Messina, *et al.*, 2019). It is estimated that 50% of the world community recently live in regions which are environmentally suitable for the spread of Dengue (WHO, 2016; Messina, *et al.*, 2019). Transmission of Dengue virus to human is caused by the bite of infected female *Aedes* that flourishes especially in tropical and sub-tropical regions worldwide (Akiner, *et al.*, 2016; WHO, 2016).

There are three major types of Dengue Fever, namely classical Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS). Dengue virus (DENV) belongs to family flavivirdae and is represented by four serotypes (1-4), which are prevalent in various parts of the country since 1956 (Pandya, 1982; WHO, 2011). Yergolkar (2017) also observed that Dengue virus has four antigenically associated serotypes (DENV-1 to 4). In the Philippines the first committed outbreak of DHF was reported during 1953-54 whereas in Thailand, the first case of Dengue had been reported in 1958 (WHO, 2011).

Khan, *et al*., 2022 reported that the maximum number of dengue cases was associated with the predominant co-circulation of DENV-2 and DENV-3 serotypes. A rise in cases was observed in the month of October. Males were more affected by the disease than female. Procurable scientific literature demonstrates that dengue has become a major public health problem worldwide because of the complexity of serologically distinct serotypes. Consequently, concurrent dengue infections cause serious concerns. But due to the lack of resonable diagnostic and screening assays with high sensitivity, these concurrent dengue infections are poorly understood with respect to their occurrence, their clinical presentations, as well as their implications (Sirisena, *et al*., 2021).

Yuan, *et al*., 2022 discussed that total 64 factors, including population and virus characteristics, clinical symptoms and signs, cytokines, chemokines and laboratory biomarkers. Out of these 34 factors were observed to be significantly different between Dengue Fever (DF) and Severe Dengue (SD). In addition, 9 factors were positively associated with SD and these factors can be used as biomarkers for the identification of SD. These factors will be helpful for a prompt diagnosis and early effective treatment for those at greatest risk. Wang, *et al.*, 2020 explained that Plasma

leakages are the major pathophysiological indication that differentiates DHF from DF. Severe plasma leakage can result in a hypovolemic shock. Several factors are speculated to impact the disease presentation and severity. Virus virulence, preexisting dengue antibodies, immune dysregulation, lipid change and host genetic susceptibility are factors reported to be correlated with the development of DHF.

Gupta, *et al*., 2012 discussed that the initial epidemic of clinical Dengue-like illness occurred in Madras, India (1780) and the first virologically proven epidemic of Dengue Fever (DF) was noticed in Calcutta and Eastern Coast of India in 1963-1964. Cecilia in 2014 explained that since then many epidemics have been recorded from all over the nation.

Rise of these arboviruses are related with vector, host, and environmental factors such as temperature and rainfall worldwide. Additionally, there are also various other factors responsible for affecting arboviral disease emergence which include increase of appropriate vector habitations, viral patrimonial variation, human behavior, poor hygienic services, trade transportation. Un-availability of any commercial anti-Dengue vaccine or drug makes the conditions more suitable for rapid emergence and spread of these specific arbo-viruses (Kamal, *et al*., 2018).

Since 1990s, Dengue epidemics have become very periodic in India, mostly in metropolitan areas and have rapidly extended toward new areas, like in north east (Arunachal Pradesh and Mizoram). Further according to the authors, dengue has also extended in south east (Orissa) where anciently it was not present. Before 1999-2000, Dengue was indigenous only in a few Southern States; comprising Tamil Nadu, Karnataka, Maharashtra and union territories Pondicherry. Northern States were including Haryana, Punjab, Rajasthan, union territories Delhi and Chandigarh. Subsequently, in India the number of Dengue cases has gradually increased since 2001(Chakravarti, *et al*., 2012).

Dengue is transmitted primarily by *Aedes aegypti* (Yellow Fever mosquito). It prefers to live in urban and suburban areas, which are closely associated with human dwelling. *Aedes aegypti* is day biter but peak biting time is either in the early morning or in the late afternoon. It is highly anthropophilic in nature and to complete its single gonotrophic cycle it takes multiple blood meals and infects multiple people. Female

mosquitoes deposit their eggs in man-made as well as natural aqua receptacles just above the water surface such as tires cans, and jars. Peak densities of *Aedes* mosquitoes are observed in monsoon season due to more availability of water which is directly related to rise in the Dengue cases (Back and Lundkvist, 2013; Ngugi, *et al.*, 2017; Reinhold, *et al.,* 2018). Ibarra, *et al.,* in 2013 explained that adult density of *Aedes aegypti* is also positively connected to the areas that has high relative humidity and more flora (Back and Lundkvist, 2013; Ngugi, *et al.*, 2017; Reinhold, *et al.,* 2018).

Asian tiger mosquito (*Aedes albopictus*) serves as a secondary vector for human-endemic DENV. It breeds outdoors especially in tree-holes and adapts easily to semi-urban, forest and rural areas. In these areas, there is substantial accessibility of natural containers for egg laying. *Aedes albopictus* prefers to feed on humans in outdoor such as lawns, parks and shrubs around houses during daytime and various other animals in proportion to their relative abundance in the environment (Higa, 2011; Young, *et al.*, 2017; Andrade, *et al*., 2019).

Aedes vittatus is highly anthropophilic in nature, prefers to breed in natural habitat *i.e.* rock holes and can also breed in diverse macro as well as micro-habitats (Sudeep and Shil, 2017).

A comprehensive review, on different aspects such as origin of disease vectors, breeding site preferences of vectors, prevalence of vectors, seasonal variation of *Aedes* species, vectors propensity to transmit dengue, correlation between Dengue cases and larval indices, vector control measures, resistance status at national and international level and different genes responsible for resistance have been described in detail as below:-

2.1. Origin of Disease Vectors

Yellow Fever mosquito was originally endemic to West Africa, Mediterranean region, Asia, South Pacific (Powell, *et al.,* 2018). Afterwards *Aedes aegypti* has been entered and established globally due to development and anthropogenic activities (Brown, *et al*., 2014; Kraemer, *et al.,* 2015).

Asian tiger mosquito was autochthonous to Indian Ocean islands, Western Pacific and South eastern Asia (Delatte, *et al.,* 2009; Izri, *et al.,* 2011). Afterwards, *Aedes*

albopictus expanded its scale to Africa, Europe, Americas due to anthropogenic activities and active movement consequently caused public threats (Ponlawat, and Harrington, 2005; Delatte, *et al.*, 2009).

Aedes vittatus is spread in Asia, Africa and the Mediterranean region of Europe (Sudeep and Shil, 2017).

2.2. Breeding Sites Preferences of vectors

Phong and Nam, 1999, reported that *Aedes aegypti* and *Aedes albopictus* preferred to breed in water-filled drums, jars, concrete tanks, discarded objects and aquariums. Further, they concluded that larval prevalence reached up to a significant level in the rainy season. Thavara, *et al.,* 2002 concluded that both species of DHF vectors, *Aedes aegypti* and *Aedes albopictus* were predominant on the island, Thailand. They further analyzed that the breeding place of *Aedes aegypti* was earthen jars in both indoor and outdoor dwellings and maximum breeding was observed in concrete water storage tanks. Major breeding sites of *Ae.albopictus* were coconut husks and coconut floral spathes in outdoors. Further, Simard, *et al.,* 2005 studied that the most common artificial and natural breeding sites preferred by *Aedes* were discarded tires, rejected tins, plastic containers, unused vehicles parts, brick holes, drop leaves, tree holes and rock pools in Cameroon, Central Africa. Kumar, *et al.,* 2014 reported that breeding of *Aedes species* were exhibited maximum in tires sequenced by coconut shells and plastic containers in Thiruvananthapuram India. Further, Guo, *et al.,* 2016 explained that *Aedes aegypti* favored to breed indoor but at some places breeding was also detected outdoor, conversely *Aedes albopictus* preferred to breed outdoor dwelling but breeding was also observed indoor at few sites. Mukhtar, *et al.,* 2018 reported that maximum breeding of *Aedes aegypti* was noticed in water tanks, room coolers followed by used tires and cities garbage in all the seasons in Rawalpindi, Punjab Province, Pakistan. Mahmud, *et al*., 2018 put lime light on the most dominant/preferred container by *Aedes species* and these containers were plastic receptacles. Man-made containers were the major breeding habitation in Dengue outbreaks in Cheras district, Kuala Lumpur. Similarly Meena and Choudhary in 2019 explained that the most common breeding sites for *Aedes* were used tires followed by metal pots and mud pots in southern Rajasthan.

2.3. Prevalence of vectors

Sharma, *et al.,* (2015) concluded that high density of adult *Aedes albopictus* was observed in Goa India. Reegan, *et al.,* 2018, studied that during indoors and outdoors entomological survey in 20 localities of Bengaluru city, *Aedes aegypti* was more abundant than *Aedes albopictus* in all the surveyed areas. Furthermore, Ferede, *et al*., 2018 concluded that *Aedes aegypti* was the most prevalent species, second prevalent species was *Aedes vittatus* and third species was *Culex* in Metema and Humera, Ethiopia. Similarly Abilio, *et al*., (2018) reported that *Aedes aegypti* was detected in every sampled district, while *Aedes albopictus* was noticed only in one district out of 32 districts in Mozambique.

2.4. Seasonal variation of *Aedes* species

Rainfall is among the important abiotic factors that govern the transmission of mosquito-borne disease. Rainfall not only lowers the temperature and increases the humidity, but also make other environmental condition suitable for mosquitoes breeding. The prevalence of Dengue vectors are not only varies from place to place but also seasonally.

All the larval indices (CI, HI and BI) of Dengue vector were highly connected to all abiotic factors. Dengue risk was also significantly associated with high temperature and higher humidity and higher rainfall (Nasir, *et al*., 2017; Pham, *et al*., 2011; Tuladhar, *et al.,* 2019). There are few studies where it was reported about the distribution of *Aedes* during pre-monsoon and post-monsoon. Ravikumar, *et al.,* (2013) discussed that density of *Ae.aegypti* and *Ae.albopictus* was the highest recorded during pre-monsoon and the post-monsoon season in Nilgiri Hills of Western Ghats, Tamil Nadu. Additionally, Baruah and Dutta (2013) conducted studies on seasonal prevalence of *Aedes aegypti* in urban and industrial areas in Assam. Both the mentioned areas had high percentages for *Aedes aegypti* with higher abundance in the post monsoon season (October) after heavy rainfall.

There are some studies which reported about prevalence of *Aedes* during monsoon as Wongkoon, *et al.,* 2013 concluded that breeding of *Aedes* species larvae per household were observed maximum in the rainy season than in the winter and summer seasons in Thailand. Moreover, *Aedes aegypti* larvae per household were

predominated than *Ae.albopictus* larvae in monsoon season. Furthermore, Camara, *et al.,* in 2016 discussed that monsoon season was the most favorable season for *Ae.aegypti* survivorship and development in Brazil.

2.5. Vectors propensity to transmit dengue

Procurable scientific literature witnesses that certain species of genus *Aedes,* transmit arbovirus to human as Diagne, *et al.,* 2014 confirmed that *Aedes aegypti* and *Aedes vittatus* act as vectors for Chikungunya virus (CHIKV) transmission. Further, they conclusively remarked that *Aedes vittatus* is comparatively potential carrier for concerned virus, than *Aedes aegypti*. Chouin, *et al*., in 2016 speculated that *Ae.aegypti* and *Ae.albopictus* possess the equal potential for pathogen transmission. *Aedes aegypti* had been identified as predominant vector for Yellow Fever, Zika, Chikungunya and Dengue viruses (Agramonte, *et al.,* 2017).

Yellow Fever, Dengue, Chikungunya and Zika virus are transmitted by *Aedes vittatus* (Sudeepand Shil, 2017) and *Aedes aegypti* (Souza-Netoab, *et al.,* 2019) while *Aedes albopictus* can transmit Dengue, Chikungunya and Zika virus but not transmit Yellow Fever (Nejati, 2017; Tsujimoto, *et al*., 2017).

These arboviruses maintain and amplify their cycles in the environment by three transmission process 1.Horizontal transmission, 2.Vertical transmission, 3.Venereal transmission (Marchi, *et al*., 2018).

After the bite of infected *Aedes*, the DENV is mostly transmitted to humans. This process is called horizontal transmission (Ferreira-de-Lima and Lima-Camara, 2018). Transmission of some arboviruses from the infected female mosquitoes to their offspring, during oviposition is known as vertical transmission (Clements, 2012; Lequime, *et al.,* 2016). Moreover, during copulation, transfer of virus from a vertically infected male to directly female is called venereal transmission (Marchi, *et al.,* 2018).

2.6. Correlation between Dengue Cases and Larval Indices

There are three major larval index like House indices (HI), Container Indices (CI) and Breteau indices (BI) for measuring Dengue vectors. The Breteau Index is generally considered the best commonly used indices as it combines houses and containers.

Rahman, *et al*., in 1995 explained through the retrospective Dengue/DHF data that there is strong relationship between the Dengue cases and the *Aedes* larval index. Authors also noticed similarity between the densities of *Ae.aegypti* in relation to various Dengue/Dengue Hemorrhagic outbreaks in India. On other hand larval index above 20 per cent of *Aedes aegypti* appears to be related to a high risk of transmission. Sanchez, *et al*., 2006 also noticed that BI can be used as best predictor for Dengue transmission. On the contrary, Bowman, *et al*., 2014 concluded that, single value of Breteau index is not reliable tool for assessing Dengue transmission. They also suggested that adult sampling should be done on daily basis and better knowledge of vectors ecology is required. Udayanga, *et al*., in 2018 described that threshold values recommended for adult *Ae.aegypti* along with cut-off values for pupal index (PI) could be suggested as indicators for decision making in vector control efforts in Sri Lanka.

2.7. Vector Control Measures

As there are no effective drugs for Dengue, therefore the most important method to curb and prevent the Dengue is to curb population of vectors (Gunther, *et al.,* 2011; Capeding, *et al*., 2014). Karunamoorthi and Sabesan (2013) analyzed that the meaning of vector control is action of any type, conducted in opposition to diseases vectors and planned to curb their potential to spread illness.

In case of Dengue, regular vector control generally consists of source reduction approaches, including use of chemicals against the larvae and adult via spray and space-spraying respectively. However, regulated and vector control attempts on the basis of insecticides are usually deficient in effectiveness and requires a community-based vector control plan that involves environmental management (Ault, 1994; Castro, *et al*., 2012). Moreover, community participation and education campaigns are the dynamic process for *Aedes aegypti* control and education campaigns can be conducted by Health workers during door to door surveys (Perez, *et al*., 2007; Poupardin, *et al*., 2014; Arslan, *et al*., 2015). Zahir, *et al.,* 2016 studied the function of involvement of people in intercept of Dengue Fever in Pakistan and the author noticed that operation for curbing had an important relationship with organization of community to eliminate Dengue mosquitoes.

Beside public participation distinct classes of insecticides, *viz* organophosphates, organochlorines, carbamates and synthetic pyrethroids have been in service since last 1-5 decades in vector control programmes globally (Ranson, *et al.*, 2010; Uragayala, *et al*., 2015).

There is only one class of insecticides *i.e.* organophophates (temephos, fenthion) which are approved against larval control because organophophates has high effectiveness and low persistence in the nature (WHO, 2013). Temephos, an organophosphate larvicide, has been most widely used against mosquito larvae control for many years. It has also been incorporated in the record of World Health Organization (WHO) because it is very appropriate as well as non toxic insecticide which can be used even in potable water for curb of the most mosquito vectors. Organophosphate has very low toxicity and it is also unexpected to give out serious difficulties for public (WHO, 2006). Abai, *et al*., in 2016 studied that over usage of organophosphates in the agriculture sector, as larvicide against mosquitoes, so it is possible to develop resistance in some vectors.

The second types of insecticides are organic insecticides like rotenone, azadirachtin, abamectin and spinosad. These insecticides restrain all biological, chemical and mineral materials and some of them are commercially available, e.g., neem, spinosad, Pyrethrum, abamectin, *Bacillus thuringiensis israeliensis* (Bti), rotenone, cinnamon, essential oil products, pepper and garlic (Mossa, *et al*., 2018).

Bacillus thuringiensisisraelensis (Bti) is also a natural insecticide, having peculiar bacterium that can give rise to several active synthetic compounds. These compounds are used in organic farming and public health sectors as commercial biolarvicide against larvae and to curb pests. Bti is used extensively because it is very safe for public and environment friendly larvicide. Bti is toxic because during the sporulation of the bacterium *viz*:-*Bacil-lusthuringiensis israeliensis,* it produces harmful crystal (Tetreau, *et al*., 2013; Mossa, *et al.*, 2018).

Pyrethrum is a mixture of natural chemical compounds which are obtained from extract of dried *Chrysanthemum cinerariaefolium* flowers. Its extract is made up of more than five distinct compounds which have insecticidal capability of killing insects. Due to the presence of active compounds in the *Chrysanthemum* flower, extracts are named as pyrethrins (Hodgson, 2012; Fradin, 2019). In addition to this,

pyrethrins are recorded as pesticides and near about 2000 trade products are available globally. These are neurotoxicants such as other pyrethroid pesticides (Mossa, *et al.*, 2018).

Pyrethroids and pyrethrins are used to curb adult mosquitoes because bothdegrade quickly in the sunlight compared to other insecticide or microbial degrades. But, pyrethroids are reviewed; poison the nerve fibers and break down more slowly in the daylight than pyrethrins. It is used in agriculture to control insects due to its effectiveness (Gile, *et al.*, 2013). Afterwards, to inhibit degradation of pyrethroids, these can be amalgamated with some other active ingredients, like piperonylbutoxide, and prevents the insect's system from detoxifying the pyrethroid, making it more effective. Slow-down makes the chemical product stay for a long period in nature and need less recurrent doses to destroy insects (Cuervo-Parra, *et al.*, 2016).

Pyrethroids have very fast knock-down results. It mainly attacks the nervous system of pests by disrupting their sodium channels of nerve membrane; consequently cause the disruption of ions transport and transmission of impulses between nerve cells (Bisset, 2002). Furthermore, this prompts nerve cells to generate repeated discharges consequently bring about paralysis and demise (Guglielmone, *et al*., 2001).

Malathion, an organophosphate compound, extensively applies for farming, household, and public health purposes, especially to increase the production of food and to control infectious vectors throughout the world. Chemical formula of Malathion is $C_{10}H_{19}O_6PS_2$ and it is also low persistent in the environment. Malathion attachs with acetylcholinesterase enzyme (AChE) at nerve endings all over the bodies of pests. Major role of AChE is the synaptic transference of nerve impulses. Inhibition of enzyme brings about blockage to signal transmission which further leads to inebriation displayed by restlessness, hyper excitability, convulsions, paralysis, and death (Tchounwou, *et al*., 2015).

Deltamethrin, a synthetic pyrethroid, was initially reported in 1974. Specific chemical is soluble in alcohol, acetone and lipids. Naturally, this chemical occurred in white and light brownish yellow crystals powdery form. Pyrethroids are more than 2200 times extra poisons to insects compared to higher animals therefore insects have very smaller structure; body temperature is low and sensitive sodium channels. It affects

the nervous system of mosquitoes by toxic activity hence extend the opening of voltage-gated sodium channels (VGSC). These VGSC results in membrane depolarization of neurons, repetitive discharges and synaptic disturbances leading to hyper excitatory symptoms of poisoning in insects. This pyrethroid also influences the function of the chloride and calcium channels of the neuron (Wakeling, *et al*., 2012; Field, *et al.,* 2017; Chrustek, *et al*., 2018).

Insecticides are supposed to exist as an essential component of Dengue control for the forthcoming time (Townson, *et al.,* 2005). Pyrethroids have been used worldwide to control mosquitoes and identify them because of their more toxicity toward chosen pests and side effects on humans are very less. But, due to continued use of insecticides in the field, several populations of mosquitoes have exhibited resistance to pyrethroids (Rodriguez, *et al*., 2001; Lin, *et al*., 2003).

2.8. Insecticide Resistance Status at National and International Level

In 1946, DDT was the first insecticide which was introduced for control of mosquitoes. Initial resistance to DDT was detected in *Aedes* in 1947 (Knobler, *et al*., 2003). Bansal and Singh (2003) studied seasonal prevalence and carried out insecticide susceptibility tests against the adults of *Aedes aegypti*, in three barren and three fertile districts of Rajasthan. *Aedes aegypti* was the most prevalent species. Research work was performed against insecticides DDT and dieldrin in both rural and metropolitan regions. Resistance was detected against both insecticides in both barren and fertile regions while an intermediate resistance had been reported from metropolitan areas toward DDT and dieldrin. It was more noticeable in rural areas than urban areas. Tikar, *et al.,* (2008) studied that *Aedes aegypti* collected from few metropolitan areas of Union territory, southern and northern state such as Chennai, Delhi, Coimbatore, Mumbai and Jodhpur were susceptible to organophosphates, on contrary DDT resistance was observed at very low level. Both species were resistant to insecticide DDT, in Koderma (Jharkhand) however, susceptible to malathion, permethrin, deltamethrin, lambda-cyhalothrin and cyfluthrin. Larvae of both species were susceptible to malathion, temephos and fenthion (Singh, *et al*., 2011).

Das, *et al*., 2011 studied the resistance status of the Dengue vectortowards various insecticides in Jharkhand state, India, using the WHO standard susceptibility test kits.

Aedes aegypti mosquitoes were DDT resistant while susceptible to organophosphate and pyrethroids. He further discussed that larvae of *Aedes aegypti* species were susceptible to temephos in Jharkhand state. In Venezuela, Alvarez, *et al.*, in 2013 monitored the populations of *Aedes aegypti* from four districts in regard to insecticides resistance against pyrethroids and organophosphates along with enzymes connected to metabolic resistance mechanisms during 2008 and 2010. Bottle bioassay was performed and after 24 hours mortality was recorded to find the lethal concentration. *Aedes aegypti* exhibited low resistance to malathion and high resistance to deltamethrin with noticeable raised expression of enzymes *viz.* glutathione-S-tranferases and mixed-function oxidases.

Reviewed by Singh, *et al.*, (2013) in different areas of India, the larvae of *Aedes aegypti* were showing susceptibility towards synthetic pyrethriods but resistance to DDT and dieldrin. While, *Aedes aegypti* exhibited resistance to DDT in Kolkata, Gujarat, Pondicherry and Maharashtra but was fully susceptible to other conventional insecticides (malathion and deltamethrin). Conversely, the larvae were DDT tolerant in Goa State and susceptible to organophosphates but resistance to dieldrin was recorded. In Uttar Pradesh, larvae of *Aedes aegypti* were DDT resistant, susceptible to pyrethroids while malathion revealed tolerance up to some extent. Singh, *et al.*, 2014 evaluated susceptibility status of *An.stephensi* against larvicide temephos in Delhi. Mortality of *Aedes aegypti* larvae was recorded which ranged from 64.88% to 98.22% from different localities. Larvae of *Aedes aegypti* were depicting varied susceptible status to temephos such as highly susceptible in one area, tolerant in two areas and development of resistance against in five areas of Delhi respectively.

A similar study was carried out in Delhi, India where a Dengue vector, *Aedes aegypti* had been reported DDT and pyrethroids resistant while susceptible to organophosphates. Delhi and Kerala populations also exhibited signs of initiation of resistance to pyrethroid in *Aedes albopictus* because it was also reported from nearby country Pakistan and Sri Lanka (Das, *et al*., 2014). A study by Azzez has demonstrated resistance to DDT in adults of *Aedes aegypti* in Jharia, Bihar, India during the year 1967. In Tezpur military area of Assam, wild collected *Aedes*

albopictus was DDT resistant and susceptible to temephos, malathion and deltamethrin (Dhiman, *et al*., 2014).

Yadav, *et al*., (2015) *Stegomyia* larval and adult susceptibility bioassay was carried out to discover various lethal concentration and resistance ratios against Temephos and deltamethrin, malathion and DDT respectively in Assam India. *Aedes albopictus* of Sotia was resistant to temephos whereas *Aedes aegypti* of Borgong, Kusumtola and Serajuli revealed high temephos resistance. Outcomes from each study area indicated that both Dengue vectors were completely resistant toward DDT. Whereas deltamethrin and malathion were highly susceptible except for *Aedes albopictus* at one study area where it revealed low level malathion resistance. KDT values for both species exhibited a high level of knock-down DDT resistance.

Brown (1986) discussed that resistance to insecticides was common in *Aedes aegypti*. In the Caribbean, resistance to DDT was developed in 1955. Organophosphate resistance was also widespread in the region (Santo-Domingo) Mekuria, 1991 Rodriguez, 2001 and pyrethroid resistance had been reported in Puerto Rico (Hemingway 1989), Dominican Republic, Mekuria 1991, British Virgin Islands, Wirth 1999, Cuba Rodriguez 2001 and Martinique Brengues, 2003.

Harris, *et al.*, in 2010 explained that *Aedes aegypti* was highly DDT and pyrethroid resistant in Grand Cayman. Increased levels of enzyme glutathione transferase, cytochrome P450 and esterase was also noticed. Author also reported that DDT and pyrethroid resistance was associated with F1534C mutation.

Lima, *et al.,* (2011) explored that temephos and cypermethrin resistance was recorded in Barbalha and Crato whereas Juazeiro do Norte population observed susceptibility against cypermethrin but low level temephos resistance in Brazil. Two Kdr mutations were observed *viz* Ile1011Met, Val1016Ile and formal mutation was examined in all populations; later mutation was detected in Crato and Juazeiro do Norte.

Grisales, *et al.,* in 2013 studied that temephos was resistant in Colombia. Mortality rates were greatly reduced after application of larvicide *i.e* three forth reduced in fortnights and half reduced in one month respectively from Cucuta population. Author also reported that target site resistance was not connected with biochemical assays or due to *Ace-1* gene. Temephos resistance was mainly occurred due to metabolic

mechanisms and enzyme carboxylesterase inhibitor, cytochrome P450 oxidases, DEF, notably CYP6F3, CYP6M11 and CYP6N12 were associated with metabolic mechanism.

First time in 2011, mutation connected to kdr was recorded in *Aedes albopictus* thereafter various confirmations were examined in the distinct areas such as USA, Brazil, Mediterranean Countries, China and India of the world (Auteri, *et al.,* 2018).

Hamid, *et al.,* (2018) reported that development of insecticide resistance due to prolonged use of the same insecticides for control purposes. Malaysia, Mexico, Brazil, Colombia, Thailand, Grand Cayman, India and China worldwide, these different countries have been observed to be developing resistance to commonly used insecticides in *Aedes aegypti*. Temephos resistance has also been recorded from the country Malaysia and America in *Aedes aegypti*.

So, it is very essential for managing well-planned vector curbs to monitor resistance of synthetic pyrethroids periodically (Kushwah, *et al.,* 2015).

2.9. Different Genes Responsible for Resistance

Muthusamy and Shivakumar in 2015 detected that the larvae of *Aedes aegypti* in Namakkal (NKL) districts of Tamil Nadu were reported resistant to temephos whereas in other districts moderate susceptibility towards insecticides was observed. Further, biochemical tests revealed that activity for enzymes jumped up which was proved by insensitivity of acetylcholinesterase and mutation in G119S gene was noticed with high frequency. These raised activities of enzymes and *Ace-1* mutation were related to Temephos resistance.

Kushwah, *et al.,* in 2015 studied sensitivity status against synthetic pyrethroids and presence of knockdown resistance (kdr) mutations in *Aedes albopictus* in four states and one Union territory of India. Among five populations of *Aedes albopictus* resistance was observed against DDT. Developing resistance against synthetic pyrethroids was recorded in *Aedes albopictus* in Kerala and Delhi populations. On the contrary, populations from Assam, Haryana and Uttrakhand were susceptible for DDT and Kdr mutations were not noticed in any insecticide resistant individuals.

Bharati, (2018) worked in West Bengal, India on susceptibility status of insecticide on *Aedes aegypti* and presence of mutation were also observed. It was recorded that the huge amount of screened populations had developed resistance to different insecticides. Deltamethrin followed by lambdacyhalothrin whereas lambdacyhalothrin followed by malathion, propoxur and least resistance observed against permethrin and DDT. Moderate temephos resistance was reported in one population. Raised activity of enzyme carboxylesterases and cytochrome P450s was evident for the cause of resistance.

Study was explored by Soni, *et al.,* (2018) where high resistance to DDT was recorded in both species and least resistance was noticed against pyrethroid and malathion in State Assam and Arunachal Pradesh. Both species had exhibited V1016G heterozygous mutations in three districts namely Dibrugarh Sivasagar and Tinsukia. Likewise, both species had exhibited F1534C heterozygous mutations also. But *Aedes aegypti* exhibited mutation in Tezpur, Tinsukia and Sivasagar district while *Aedes albopictus* revealed mutation in these three districts Dibrugarh, Tinsukia, and Sivasagar.

Aedes aegypti was noticed to be susceptible towards organophosphate, malathion and adulticide, Pyrethroids and DDT resistance was very high. Polymorphisms of high rate were also recorded in the voltage gated sodium channel gene in West Bengal. DDT resistance was observed to be connected with double mutant V1016G + F1534C. Aquatic stages have also revealed low to moderate levels of Temephos resistance (Saha, *et al.,* 2019).

Moderate resistant was also noticed in the wild *Aedes aegypti* against temephos and spinosad but 100% susceptibility was observed towards bacterial larvicides *Bacillus thuringiensis israelensis* (Bti) and synthetic pyrethroids (Marcombe, *et al.,* 2018)**.** Hasmiwati and Supargiyono in 2018 explained that in West Sumatra, Indonesia, *Aedes* populations had point mutations in the VGSC gene which were related to S989P and V1016G substitutions in amino acids. The authors also observed through kdr genotyping that populations of *Aedes aegypti* were malathion and permethrin resistant. A study of the peri urban area of Indonesia indicated that *Aedes aegypti* was highly resistant towards permethrin and bendiocarb. Furthermore, through allele specific PCR, it was also analysed that *Aedes aegypti* had point mutation in V1016G

gene which had notable connection with permethrin resistance in Banjarmasin city Kalimantan, Indonesia (Hamid, *et al*., 2018). In New Mexico USA, the majority of the populations of *Aedes aegypti* were exhibited resistant to pyrethroids, permethrin and deltamethrin. During screening, through Kdr genotyping it was detected that the population of *Aedes aegypti* had kdr mutations which had a significant relationship between F1534C gene and resistance in pyrethroid (Kandel, *et al*., 2019).

Pinto, *et al*., 2019 conducted a study on different classes of insecticides in *Aedes aegypti* from the districts of Chosica, Punchana and Piurain Peru. DDT resistance was reported in all three districts and pyrethroids resistance was reported in two areas except Chosica. Malathion resistance was also observed in only one area *i.e* Chosica. 1016I and 1534C alleles were observed in two areas *i.e* Punchana and Piura, whereas 1534C allele was exhibited that was related to mutation in *Aedes aegypti* in Corsica. Hasmiwati, (2018) investigated the *Aedes aegypti* resistance status and identified point mutation in Padang city. Among five populations, *Aedes aegypti* were temephos resistant in two areas whereas in three areas, temephos susceptibility was recorded. *Ace-1* genotyping revealed that *Ace-1* was 495 bp in length. No mutation was observed at T506T site. Three alleles were observed in T506T sites, comprising two mutant alleles, TA, AA and a wild type allele TT and study indicated that two populations of *Aedes aegypti* were noticed to be temephos resistant, three populations were susceptible. A new mutation was reported as substitution (ACT>ACA) in the T506T site.

After reviewing these papers it was noticed that only two studies were conducted on *Aedes* species by Gill, *et al.,* 1997 and Lata, *et al*., 2012 in Punjab. Dengue cases are increasing continuously from the last 5-6 years and no detailed study has been conducted on identification and molecular characterization of insecticide resistant/ susceptible Dengue vector prevalent in eastern Punjab, India. Therefore to bring out efficient and continuous *Aedes* control measures there is a necessity to find out the prevalence, resistant/susceptibility status of Dengue vector to commonly used insecticides and genes responsible for resistance in Punjab. Regular observation of resistance is also required for selecting the most effective adulticide and larvicide for *Aedes* control in Punjab.

CHAPTER-III

HYPOTHESIS

Morbidity in Punjab is equally prevalent in both poor and socioeconomically better communities of the state, due to Dengue Fever. The high incidence of the disease per year is the adverse outcome of rapid unplanned urbanization and varied social practices such as water storage practices and inappropriate disposal of solid waste materials in and around house/premises. Such practices are resulting in the creation of more favorable breeding habitats for *Aedes* mosquitoes. Although there are many methods to control *Aedes* population such as space sprays, thermal and ultra-low volume fogging by applying insecticides and effective larvicides but still Dengue cases persist. Therefore, the choice of potent insecticidal agents (larvicide and adulticide) is very important for vector control measures. Due to regular spray of Temephos and Pyrethrum in both Dengue and Malaria vector control programs by the Health Department and Municipal Corporation of India, Temephos and Pyrethrum resistance in mosquito population might have been developed. Considering this fact, the target of present research work is to detect genetic elements responsible for the same.

CHAPTER-IV

OBJECTIVES OF THE STUDY

The brief objectives of the present research work are:-

1. To study the prevalence (identification and preferential breeding habitats, comparison of *Stegomyia* indices of *Aedes* in different seasons) of Dengue vectors (along with their correlations with the Dengue cases) in rural and urban area of different districts Mohali (Balongi), Ludhiana (Mundian Kalan), Patiala (Jhill) and Fategarh Sahib (Chuni Kalan) of Punjab. Male/female genitalia of mosquito for identification of species.

2. To study the insecticide susceptibility/resistance status of larvicides (Temephos) and adulticide (Pyrethrum) for the control of most abundant species.

3. To study mutation in insecticide resistant genes, which include acetylcholinesterase (*Ace-1*) and voltage gated sodium channel (knock down resistance -Kdr) in predominant species.

CHAPTER-V

MATERIAL AND METHODS

Aedes aegypti had been selected to execute specific research work as it was detected as the most abundant species in selected study areas. To assess prevalence of Dengue vectors, identification of species, insecticide resistance/susceptibility status and mutation in associated genetic elements, various parameters including entomological surveys, study of genitalia for species identification, larval bioassay, adult bioassay and polymerase chain reactions based amplification of *Ace -1*and Kdr mutation had been executed.

Test organism:-*Aedes* (*Ae.*) is a holometabolous insect with four main developmental stages, including egg, larva, pupa and adult in its life cycle. *Ae.aegypti*, *Aedes albopictus* and *Aedes vittatus* belonging to family Culicidae of order Diptera were considered as experimental models.

Brief Bionomics of *Aedes aegypti, Aedes albopictus* and *Aedes vittatus*:-

Description of *Aedes aegypti*:-Size of *Aedes aegypti* is small to medium about 5-7 millimeters. It is black colored mosquito with contrasting silver white line on the head, abdomen, scutum and legs. Adult *Ae.aegypti* possesses violin/lyre shape white scales on the dorsal surface of its thorax (Muktar, *et al*., 2016).

Description of *Aedes albopictus*:-Size of *Aedes albopictus* is small to medium *i.e*., more than 3.0 to near about 10.0 millimeters. *Aedes albopictus* possesses black scutum with white dorso-central strip on the thorax. The proboscis of both male and female is dark in color. Simple identifying feature of *Aedes albopictus* is prominent black shiny scales and on the tarsi and palpus distinct silvery white scales are present (Hawley, 1988; Foster and Walker 2002; Rios and Maruniak, 2004).

Description of *Aedes vittatus*:-It consists of three pairs of small round silvery white spots on the scutum. Other characteristic features include, dark tibiae with white spots, presence of white band on the base of tibiae, white bands on the tarsomeres 1–4, fully white fifth tarsomere etc (Sudeep and Shil 2017).

To execute the present research work following parameters has been adopted and briefly described below:

1. **Prevalence of Dengue vector**: - Larvae/ pupae were collected from selected areas and kept in insectary, under control condition of temperature 27±1^{0}C and 70% humidity (Costa, *et al*., 2010). Rearing of larvae/pupae had been carried out, afterwards imagoes were identified by following pictorial taxonomic keys of Haung, 1979; Rueda, 2004 and Tyagi, *et al*., 2012.

2. **Study of Genitalia for Species Identification**: -In this parameter methodology adopted by Yadav, *et al*., 2014 was followed for identification of species. Male and female mosquitoes were identified on the basis of hypopygium as it is also a morphological marker in the species identification.

3. **Assessment of Insecticide Resistance/Susceptibility Status*:* -** Both larval and adult bioassays were conducted as per guidelines of World Health Organization to determine insecticide resistance and susceptibility status of *Aedes aegypti* against larvicides Temephos, Bti and insecticides Pyrethrum 2% extract, Deltamethrin 2.5% WP and Malathion 25% WP respectively (World Health Organization, 2005; WHO, 2016).

4. **Detection of Target Site Resistance**:-Two genes including acetyl-cholinesterase (*Ace-1*) and voltage gated sodium channel (Kdr) were selected as per mode of action of specific larvicide Temephos and adulticide Pyrethrum respectively. For this *Ace-1* gene and VGSC gene were amplified from susceptible/resistant and control larvae and adults.

Elaborative Description of Executed Research Work is as Following:-

Study area, Survey design and Period:-Study was carried out in four districts of Punjab, India**.** Punjab is situated in the northwest part of India and bounded by Jammu and Kashmir in north, Himachal Pradesh to the northeast, Haryana to the southeast and Rajasthan to the southwest. Punjab is also bordered by Pakistan in the west. It has an area of 50,362 km^2 and is situated at the latitude 31.14° North and longitude 75.34° east. Maximum area of this state consists of fertile plains, alluvial plains with many rivers and an extensive canal system for irrigation.

In the present study, cross sectional entomological surveys were conducted in 4 districts of Punjab. On the basis of previous three year retrospective data of Dengue

cases, these include S.A.S Nagar (Mohali), Patiala, Ludhiana and Fatehgarh Sahib (F.G.Sahib) (Table 1, Fig.7**)**. The surveys were done in indoor and outdoor areas during pre-monsoon (May-June), monsoon (July-September) and post-monsoon (October-December), for three years from 2017 to 2020. In Punjab, dengue cases mostly started appearing from the month of May onwards. Furthermore, breeding habitats are also positive for *Aedes* immatures during these months. Therefore, the study was done for the stated duration.

Table 1:- Location of four selected districts of Punjab specifying their latitude and longitude

Name of the districts	Latitude	Longitude	Localities inspected
S.A.S Nagar	30.7046 °N	76.7179˚E	Metropolitan and rural area of S.A.S Nagar
Patiala	30.3398˚N	76.3869˚E	Metropolitan and rural area of Patiala
Ludhiana	30.9010˚N	75.857˚E	Metropolitan and rural area of Ludhiana
Fatehgarh Sahib	30.6435˚N	76.3970˚E	Metropolitan and rural area of FatehgarhSahib

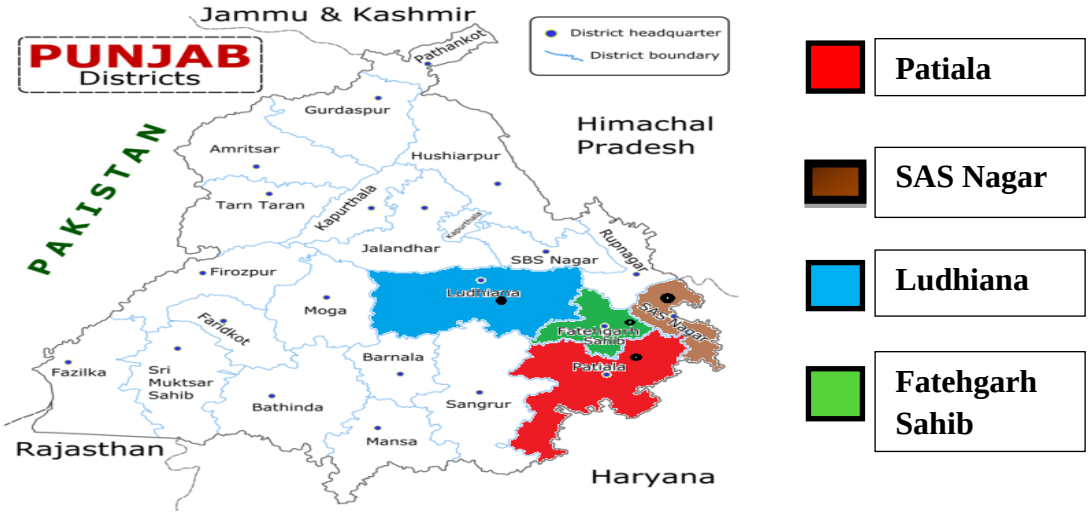


Fig 7:-Punjab map showing the location of sampling of four populations in districts of Punjab. Point 1 blue colored (Ludhiana urban and Village Koomkalan), point 2 brown colored (SAS Nagar urban and Village Balongi), point 3 green colored (Fatehgarh Sahib Urban and Village Chuni Kalan), point 4 red colored (Patiala urban and Village Jhill) to study the prevalence of Dengue vectors.

5.1. Collection, Rearing and Identification of Larvae

5.1.1. Larval Collection:-Door to door entomological survey was conducted in the selected districts. Each and every type of indoor and outdoor breeding habitats was inspected in distinct areas of SAS Nagar, Patiala Ludhiana and F.G.Sahib, to collect the *Aedes* larvae and pupae by following the dipper method (Getachew, *et al*., 2015). The habitations in the present study were chosen by a systematic random sampling technique. From all the study areas, the first house was randomly included in the study. A vessel containing any amount of water was regarded as a wet vessel; the wet vessel holding any amount of immatures (larvae, pupae or both) was regarded as a positive container. The larvae were collected with the help of distinct collecting techniques such as dipper, ladle depend on the kind and size of the breeding source. The collected larvae or pupae were placed in plastic vessels tagged with the code of home identification, area, sources of breeding and date of collection (WHO, 2009; Bhat, *et al.*, 2014).

5.1.2. Rearing and Identification of Mosquitoes:-The specimens were brought to the lab. at Department of Medical Parasitology, Post-Graduate Institute of Medical Education and Research (PGIMER) Chandigarh**.** Afterwards individual immature specimens were kept in separate rearing trays, for every vessel types in the insectaries and were fed daily on larval food yeast and dog biscuit prepared at a ratio of 3:1 (Munhenga, *et al.*, 2016; Ong and Jaal, 2018). Then pupae were transferred to individual adult cages for emergence. Identification of adult mosquitoes had been carried out by following pictorial taxonomic keys of Rueda, 2004 and Tyagi, *et al.*, 2012. Subsequently, adults were fed on 10% sucrose solution through soaked cotton pads placed either on petri-dishes or on top of the mosquito nets (Gunathilaka, *et al*., 2019).

Calculation and analysis of larval survey data was done by *Stegomyia* indices *viz* House Indices (HI), Container Indices (CI), Breteau Indices (BI), Pupal Indices (PI), Pupae per Container (PPC) and Pupae per Positive Container (PPPC) according to standard methods (Arduino, 2014).

Stegomyia indices were calculated on the basis of following mathematical formulae (No. = Number):-

House Indices (HI) = $\frac{\text{No. of houses infested}}{\text{Total households inspected}} \times 100$

Container Indices (CI) = $\frac{\text{No. of positive containers infested}}{\text{Total containers inspected}} \times 100$

Breateau Indices (BI) = $\frac{\text{No. of containers infested}}{\text{Total household inspected.}} \times 100$

Pupal Indices (PI) = $\frac{\text{No. of pupae collected}}{\text{Total households inspected}} \times 100$

5.2. Preparation of Hypopygium Slides

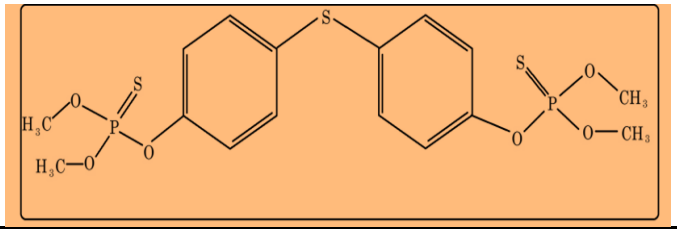
On the basis of morphological characteristics, separation of male and female mosquitoes was carried out. Then for half an hour mosquitoes were kept in a wrapped jar with some damp wool. Last two or more segments of the abdomen were then cut off under the dissecting microscope. Then, for 15 minutes, samples were kept in 90% alcohol so as to prevent the hypopygium from floating during later stages of preparation. The hypopygium was shifted to a test tube consisting of 10% potassium hydroxide (KOH) afterwards, slowly heated over a Bunsen burner up to nearly the boiling point. Then, it was cleaned with two or more drops of distilled water for 3-4 minutes, thereafter hypopygium was mounted in DPX medium. Compound microscope with 40X magnification was used to examine the slides (Yadav, *et al*., 2014).

5.3. Protocol for Insecticides Susceptibility/Resistance Assessment

Selection of Insecticides:-For the present research work, two pesticides Temephos and Pyrethrum have been selected. Temephos is an organophosphate, (Table 2) while Pyrethrum belongs to biodegradable pyrethrins class (Table 3). Temephos and Pyrethrum are broad spectrum pesticides which are used for the control of a wide variety of pests. Pyrethrum has no bioaccumulation, relatively short

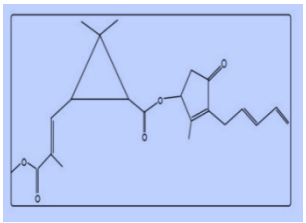
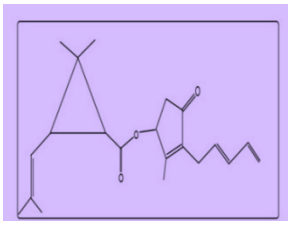
persistence in the environment, and low mammalian toxicity. Pyrethrum is used for indoor spray/fogging at low application rates while Temephos is also very effective, it has quick activity against pests, low mammalian toxicity and degradability in the environment. Some of the more important physico-chemical properties with other relevant details of two pesticides are summarized as follows.

Table 2:-Physicochemical properties of larvicide Temephos which is used against the larvae of *Aedes* species.

Sr. no.	Chemical Compound	Temephos
1	Chemical Structure	
2	IUPAC Name	O,O,O′,O′-Tetramethyl O,O′-sulfanediylbis (1,4-phenylene) diphosphorothioate
3	Chemical Formula	$C_{16}H_{20}O_6P_2S_3$
4	Molecular Mass (g/mol)	466.5
5	Melting Point(^{O}C)	30.0-30.5^{0} C
6	Solubility inWater	30 µg/l at 25 °C

(World Health Organization, 2009)

Table 3:-Physicochemical characteristics of adulticide Pyrethrum 2% extract which is used to control adult mosquito.

Sr.no.	Chemical Compound	Pyrethrin I	Pyrethrin II
1	Chemical Structure		
2	Chemical Formula	$C_{21}H_{28}O_3$	$C_{22}H_{28}O_5$
3	Molecular Mass(g/mol)	328.4	372.4
4	Boiling Point (^{O} C)	170	200
5	Solubility in Water	0.35 mg/l	125.6 mg/l

(Todd, 2003; El-Wakeil, 2013)

5.3.1. Larval Bioassay:-The larval bioassay had been performed at different exposure limits including WHO recommended diagnostic concentrations of Temephos (0.02mg/L) and lethal concentration *viz*: 0.005mg/l, 0.025mg/l and 0.125mg/l. Working solution of Temephos was prepared in a bowl 500 ml (12.7*10.2*12.7 cm) then 25 healthy larvae of 3rd early and 4th instars of *Aedes aegypti* were transferred and exposed to diagnostic concentrations Temephos larvicide. Experiments were performed in set of triplicates at room temperature 27±1˚C and 70% humidity. Similarly, parallel controls were kept in pesticide free distilled water. Further, larval bioassay was performed to ascertain the lethal dose of Temephos for 50% (LC_{50}) and 90% (LC_{90}) by preparing several doses of Temephos *viz*: 0.005mg/l, 0.025mg/l, 0.050mg/l, 0.100mg/l, 0.125mg/l, and 0.187mg/l. Thereafter, as per WHO procedure three replicates for every concentration and the control were used (Fig 8). After 24 hours of continuous exposure, the larval mortality for each concentration and control was recorded. Abbott's formula was used to find out corrected mortality whenever required and log-probit method of Finney was used to analyze the dose mortality data. Software SPSS (Statistical Product and Services Solutions) was used to calculate lethal concentrations for 50% and 90% mortality (Singh, *et al.,* 2014). The larval resistance ratio (RR) was calculated by dividing the LC_{50} and LC_{90} of the field population by the LC_{50} and LC_{90} obtained for susceptible colony respectively.

0.005mg/l **0.125mg/l** **Control** **0.025mg/l**

Fig 8:-Click specifying WHO standard kit of Temephos with different concentrations 0.005mg/l, 0.025mg/l, 0.125mg/l and control which had been used for larval bioassay.

5.3.2. Adult Bioassay Performed through Fogging and Hand spray:-Pyrethrum solution was prepared, by adding 50 ml technical grade of Pyrethrum with 950 ml kerosene oil as per World Health Organization recommendation (Fig 9). For indoor spray, a hand-held spray pump (Ganesh pump) and for outdoor fogging hand fogging machine was used (Abramides, *et al.,* 2011; Paredes-Esquivel, *et al.,* 2016). F_1 progeny of *Aedes aegypti* was used for adult bioassays (Marcombe, *et al*., 2009) (Fig

10). 25-25 adult female mosquitoes of F_1 progeny were transferred to the three cages (1 feet * 1 feet) covered with a mosquito net (1 mm mesh). These cages containing 25 mosquitoes were placed at each test room individually. Then formulation was diluted as 1:19 and applied using a hand fogger as well as hand held spray pump at room temperature of 27±1 ºC and 70% humidity. Counting of knocked-down and dead females 1 hour post-treatment (1hKD) was done to calculate knockdown effect in each trial. Thereafter, all mosquitoes from a specific cage were captured with the help of an aspirator tube and stored for further molecular analysis. The susceptibility and resistance status of the mosquitoes was categorized according to the WHO guideline as when mortality was > 97% indicated susceptibility, resistance confirmed when ≤ 80% and probable/suspected resistance when mortality rate is between 80-97% (Ayorinde ,*et al.*,2015;WHO, 2016).

Fig 9:-Picture presenting adulticide Pyrethrum 2% extract which is used to control adult mosquitoes.

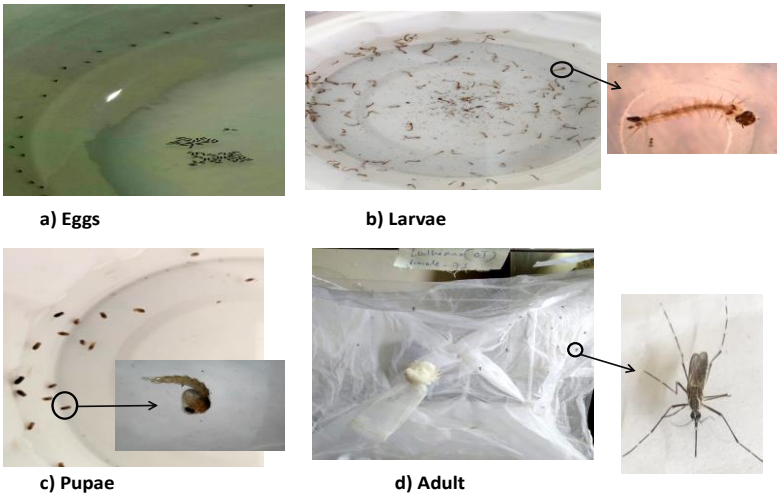


Fig 10:-Photograph depicting four stages (eggs, larvae, pupae and adults) life cycle of *Aedes aegypti.*

5.4. Method for Target Site Resistance in Selected Genetic Elements

Protocol for the amplification of *Ace-1* and VGSC gene:-The processing and *in vitro* amplification of DNA was carried out through the following steps:-

A) DNA extraction
B) PCR amplification using specific primers
C) Agarose gel electrophoresis of PCR products
D) Sequencing of PCR products and sequence submission to Gen bank
E) Data analysis by using Clustal W programme to find out insertions, deletions and transition and transversion.

5.4.1. DNA Extraction Protocol:-Genomic DNA extraction was carried out from pooled larvae of female *Aedes aegypti* of each batch *viz* Temephos resistant, susceptible and control. On other hand, DNA was extracted from whole mosquitoes of each batch *viz* Pyrethrum resistant, susceptible and control by using Qiagen blood and tissue kit following manufacturer's instruction with modification as following:-

1. Genomic DNA extraction was done from pooled larvae (n=4) or whole mosquitoes (n=1) by grinding their tissues with a micro pestle in an extraction buffer (200µl ATL buffer and 20µl Proteinase K).
2. Reaction mixture was incubated for 3-4 hrs at 56°C.
3. 200µl ethanol was added and it was amalgamated properly through vortexing.
4. DNeasy mini spin column was used to pipet the mixture and centrifugation was done for 1 min at 8000 rpm. Discard the flow through and collecting tube.
5. In new 2 ml collection tube, the spin column was placed and added AW1, 500µl buffer. Afterwards, centrifugation was done at 8000 rpm for 1 minute. Waste flow through and collecting tube was discarded.
6. In a new 2 ml collection tube, the spin column was placed and added AW2, 500µl buffer. Afterwards centrifugation was done at 14000 rpm for 3 minute. Then again waste flow through and collecting tube was discarded.
7. The spin column was transferred to the micro centrifuge tube of 1.5 ml or 2ml.
8. In the centre of the spin column 40µl AE for pool larvae and 25µl AE for adults were added and incubation was done for 1 minute at room temperature.

9. Centrifugation was done at 8000 rpm for 3 minutes.
10. Storage of DNA was carried out at -20°C.

5.4.2. a) PCR Amplification of *Ace-1* Gene using Specific Primers:-Amplification of genomic DNA was done for *Ace-1* gene by using primers (Hasmiwati, *et al*., 2018) as mentioned in (Table 4). 15µl DNA cocktail (Table 5) was prepared and PCR reaction was performed with initial step of denaturation at 94º C for 5 min, followed by 35 cycles of amplification at 94º C for 1 min, 52º C for 1min, and 72ºC for1min and final extension at 72º C for 10 mins.

Table 4:-Sequence of forward and reverse primers for detection of *Ace-1* gene

Sr. No	Primer	5'-3' direction
1	Forward Primer	(5'-CGATAACGAATGGGGAACG-3').
2	Reverse Primer	(5'-TCAGAGGCTCACCGAACACA-3').

Table 5:- Details of reaction mixture for PCR

Sr. No.	Concentration	Reagent
1	7.6 µl	H_2O
2	1.5 µl	Buffer 10X
3	1.5µl	$MgCl_2$,10mm
4	1.2 µl	dNTP 2mm
5	0.2 µl	*Taq* polymerase
6	1 µl	DNA
7	1 µl	Forward primer (5pm)
8	1 µl	Reverse primer(5pm)
Total	15 µl	

5.4.2. b) PCR Amplification of VGSC Gene using Specific Primers:-For amplification of VGSC gene, genomic DNA was amplified using the primers. 15µl DNA cocktail was prepared (Table 5) and PCR reaction was performed with initial step of denaturation at 94º C for 5 min, followed by 35 cycles of amplification at 94º C for 1 min, 60º C for 1min, and 72ºC for 1min and final extension at 72º C for 10 mins. The following primers were designed *viz.*FP:-ATTGTATGCTTGTGGGTGAC and RP:-AACTGAGATGATTGTGCTGC to amplify a fragment of domain II in the sodium channel gene (GenBank Accession No.- NC_035109) 536bp for the detection of V1016G, A1007G, I1011Mmutation in the VGSC gene of *Ae. aegypti.*

5.4.3. Agarose Gel Electrophoresis of PCR Products: - 0.8% agarose for checking integrity of extracted DNA: - Dissolve 0.2g of agarose in 25 ml of IX TAE. Heated to boil until the whole of Agarose gel completely dissolved. 2% agarose for checking PCR amplified products - Dissolved 0.5g of agarose in 25 ml of IX TAE. Heated to boil until the whole of the agarose gel completely dissolves.

5.4.4. Sequencing of PCR Products and Sequence Submission to GenBank:-The PCR amplified products of both selected genes were purified and got sequenced from M/S Agrigenome Pvt. Limited, Bangalore, India. The sequences obtained were submitted to the gen bank to obtain the accession numbers.

5.4.5. Data Analysis by Using Clustal W Program to find out Transition and Transversion:-This is a computer program that aids to align nucleotide sequences in such a manner as to increase the number of bases that match by establishing gaps or spaces into one or the other sequence. It gives biologically important multiple sequence alignments of distinct sequences and calculates the best match for the selected sequences. Arrange them in such an order that similarities and differences in the nucleotide sequences can be seen. In fact, Clustal W also works a pairwise comparison of each sequence first and afterwards initiates multiple alignments with the pair of sequences which is most similar for which the sequences are added one by one to the alignment based on the similarities to the starting pair.

This work has been carried out additionally:

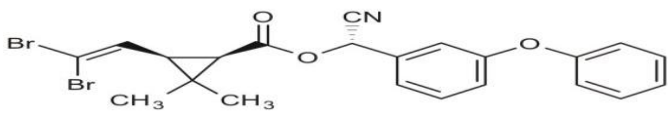
5.5. Procedure for Larvicide Susceptibility/Resistance with Bti

When larvae of *Ae.aegypti* from three districts *viz.*, Mohali (SAS Nagar) Ludhiana and Fatehgarh Sahib exhibited resistance against Temephos, then this species was also tested against bio-larvicide Bti. Working solution of Bti was prepared in a 500 ml bowl containing 100 ml water with 0.00375 gram Bti powder in order to get the final diagnostic concentrations. Then 25 healthy larvae of 3rd early and 4th instars of *Aedes aegypti* were transferred to 500 ml bowl and exposed to WHO recommended dose (0.00375gm/100ml) of Bti larvicide. Experiments were performed in set of triplicates at room temperature 27±1°C and 70% humidity. Similarly, parallel controls were kept in pesticide free distilled water. After continuous exposure of 24 hrs, the larval mortality for each concentration and control was recorded (Kamgang, *et al*., 2011).

5.6. CDC Bottle Bioassay of *Aedes aegypti* with Deltamethrin and Malathion

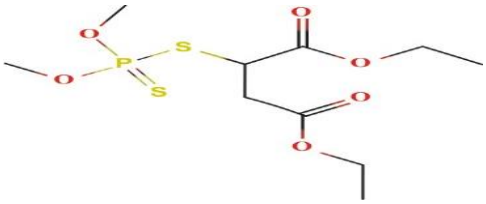
Adults of *Ae.aegypti* from three districts *viz.* Patiala, Ludhiana and Fatehgarh Sahib exhibited resistance against Pyrethrum then this species was also tested against adulticide Deltamethrin 2.5% WP and Malathion 25% WP (Table 6 and 7). To assess insecticide susceptibility/resistance by CDC bottle bioassays, the adult *Aedes aegypti* obtained from the F_1 progeny was used (Fig.10). Procedure given by Brogdon & McAllister, 1998 was followed. Insecticides Deltamethrin 2.5% WP was purchased as technical grade material from {M/s Hafed India (The Haryana State cooperative supply and marketing federation limited) and Malathion 25 % WP was also purchased as technical grade from {M/s Coromandel International Limited Telangana, India}. To perform the experiments 250 ml glass bottles were utilized. 4mg Deltamethrin mixed in 10 ml acetone were applied to the bottle. Similarly, 2mg Malathion mixed in 10ml acetone were applied to the bottle. For each insecticide 2 bottles and one untreated was prepared for a control experiment (acetone only). After preparation of bottles, fifteen female mosquitoes of F_1 progeny were transferred into the glass bottles. If the mosquito could not stand or fly then mortality was regarded to have occurred. Counts of the numbers of affected mosquitoes were made at , 5, 10, 15, and every 15 min thereafter until all mosquitoes were dead or two hours had elapsed (Brogdon & McAllister, 2010; McAllister, *et al.*, 2012).

Table 6:-Physicochemical features of Deltamethrin 2.5% that is used against adult mosquitoes.

Sr. No.	**Chemical Compound**	**Deltamethrin**
1	Chemical Structure	
2	IUPAC Name	[(*S*)-cyano-(3-phenoxyphenyl)methyl] (1*R*,3*R*)-3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropane-1-carboxylate
3	Molecular Formula	$C_{22}H_{19}Br_2NO_3$
4	Molecular Weight	505.2 g/mol
5	Solubility (water)	0.002-0.0002 mg/L.
6	Melting Point	98 - 101 °C

(Deltamethrin technical fact sheet, 2011; https://pubchem.ncbi. nlm.nih. gov/ compound/Deltamethrin)

Table 7:-Physicochemical properties of Malathion 25% WP for the control of adult mosquitoes.

1	Malathion	
2	IUPAC Name	diethyl 2-[(dimethoxyphosphinothioyl)sulfanyl] butanedioate
3	Molecular Formula	$C_{10}H_{19}O_6PS_2$
4	Molecular Weight	330.4 g/mol
5	Melting Point	2.9 °C
6	Solubility (water)	145 mg/L

(https://pubchem.ncbi.nlm.nih. gov/compound /Malathion; Malathion technical fact sheet, 2011)

CHAPTER- VI

RESULTS AND DISCUSSION

Out of 22 revenue districts 4 districts of state Punjab had been selected. These districts were: SAS Nagar (Balongi), Ludhiana (Mundian Kalan), Patiala (Jhill) and Fatehgarh Sahib (Chuni Kalan). For specific research work various parameters including entomological surveys, study of genitalia for species identification, larval bioassay, adult bioassay and polymerase chain reactions based amplification of *Ace-1* and Kdr mutation had been executed.

6.1. Analysis of Entomological Surveys

Cross sectional entomological surveys were carried out in 4 districts from where Dengue cases were reported. The surveys had been executed in indoor and outdoor areas during pre-monsoon (May-June), monsoon (July-September) and post-monsoon (October-December) during 2017 and 2018 to find out the favored sources of breeding and Dengue vectors distribution in eastern Punjab.

6.1.1. *Aedes* mosquito Larval Indices and Risk of Dengue Transmission

Larval densities and their preference towards breeding sites had been surveyed in different localities of all the selected study areas (Fig.11, 12). The observations were recorded in pre-monsoon (May-June) during monsoon (July-September) and in post-monsoon (October-December) period in year 2017 and 2018.

During an entomological survey in 2017 in urban areas, 1123 houses were screened and 2220 domestic containers were examined out of which 307 (13.82%) were detected as sites for *Aedes* breeding. Out of these 307 (13.82%) containers which were having *Aedes* breeding, 82.22% had been carrying *Aedes aegypti* and 17.77% were reported with *Aedes albopictus.* Similarly in rural areas, 1087 houses were screened and 1948 domestic containers were examined out of which 217 (11.13%) were detected for *Aedes* breeding. Out of these 217 (11.13%) scanned containers, 90.27% were observed as favourable sites for *Aedes aegypti* and 9.72% for *Aedes albopictus*.

During an entomological survey in 2018, in urban areas, 1026 houses were screened and 1969 domestic containers were examined out of which 251 (12.74%) were detected as sites for *Aedes* breeding. Out of these 251 (12.74%) containers which were observed as habitat for *Aedes*, 82% were identified as *Aedes aegypti*, 14.18% showed *Aedes albopictus* and 3.8% observed *Aedes vittatus*. In rural areas, 1026 houses were screened and 1882 domestic containers were examined out of which 180 (9.56%) were detected for *Aedes* breeding. Out of these 180 (9.56%)

scanned containers, were identified as favourable sites for 85.47% *Aedes aegypti* and 14.53 % for *Aedes albopictus*.

Fig 11:-Image presenting water filled man-made containers of *Aedes* breeding habitats, which were identified during larval surveys in different districts of Punjab in 2017, a) Desert coolers, b) Earthen pot, c) Plastic containers, d) Discarded tires, e) Refrigerator tray, f) Sand bucket.

Fig 12: -Picture depicting water filled artificial containers of *Aedes* breeding habitats which were identified during larval survey in different districts of Punjab in 2017, a) Discarded material, b) Cemented tank, c) Money plant bottles, d) Stagnant water around Fountain.

a). Cemented tank b). Flower pot c). Plastic containers

d). Discarded material e). Tree hole f). Desert cooler

Fig 13:-Photograph showing water filled natural and man-made containers of *Aedes* breeding habitats, which were identified during larval surveys in different districts of Punjab in 2018, a) Cemented tank, b) Flower pot, c) Plastic containers, d) Discarded tin, e) Tree hole, f) Desert cooler.

All the larval indices *viz.* HI, CI, BI, and PI for *Aedes* mosquito larvae from the study areas were calculated in 2017 and 2018 (Table 8 and 9) in both urban and rural areas. All the indices were analysed and detected to be high in urban as compared to rural area in both years *i.e*. HI: 24.58 and 19.41; CI: 13.83 and 11.14; BI: 27.34 and 19.96 and PI: 81.66 and 51.33 in urban and rural areas respectively in 2017. During 2018 these larval indices were analysed and detected to be high in urban as compared to rural area *i.e*. HI: 22.81 and16.86; CI: 12.75 and 9.56; BI: 24.46 and 17.54 and PI: 77 and 52.24 urban and rural areas respectively.

Table 8:- Total number of houses and containers infested with *Aedes* mosquito breeding in urban and rural areas of 4 districts of Punjab in 2017-2018.

Name of district	Total Houses 2017		Total Houses 2018		Positive Houses 2017		Positive Houses 2018		Total Containers 2017		Total Containers 2018		Positive container 2017		Positive container 2018	
	U	R	U	R	U	R	U	R	U	R	U	R	U	R	U	R
SAS Nagar	292	284	257	258	110	71	78	53	534	547	518	537	114	74	84	59
Patiala	274	247	250	234	61	47	65	42	518	416	501	447	73	47	73	43
Ludhiana	275	293	260	284	68	62	50	45	579	572	490	447	75	62	52	45
F.G .Sahib	282	263	259	250	37	31	41	33	589	413	460	451	45	34	42	33
Total	1123	1087	1026	1026	276	211	234	173	2220	1948	1969	1882	307	217	251	180

Table 9:- Larval and Pupal indices of *Aedes species* mosquito breeding in urban and rural areas of 4 selected areas of Punjab in 2017-2018.

Name of district	HI 2017		HI 2018		CI 2017		CI 2018		BI 2017		BI 2018		PI 2017		PI 2018	
	U	R	U	R	U	R	U	R	U	R	U	R	U	R	U	R
SAS Nagar	37.67	25	30.35	20.54	21.34	13.52	16.22	10.99	39.04	26.05	32.68	22.87	101	68.66	99.3	50.19
Patiala	22.26	19.02	26.00	17.95	14.09	11.29	14.57	9.62	26.64	19.02	29.20	18.38	98.9	40.89	101	64.95
Ludhiana	24.72	21.16	19.23	15.85	12.95	10.83	10.61	10.07	27.27	21.16	20.00	15.85	71.27	41.63	63.1	43.66
F.G .Sahib	13.12	11.78	15.83	13.20	7.64	8.23	9.13	7.32	15.95	12.92	16.22	13.20	54.6	43.72	44.8	36.4
Total	24.58	19.41	22.81	16.86	13.83	11.14	12.75	9.56	27.34	19.96	24.46	17.54	81.66	51.33	77	52.24

These larval indices were also analysed by using ANOVA test to calculate mean ± SD of all 4 study areas. The mean ± SD of entomological indices for the year 2017 was significantly higher for district SAS Nagar than Patiala, Ludhiana and F. G. Sahib (Table 10) whereas the mean ± SD of entomological indices for the year 2018 was significantly higher for district Patiala than SAS Nagar (Mohali), Ludhiana and F. G. Sahib in 2018 (Table 10). Comparison of Dengue cases with the larval indices of 2017 as well as 2018 was also analyzed by using spearman's correlation test which highlighted positive correlation with PI and Dengue cases (Table 11**).** The analysis of seasonal variations in HI, CI, BI as well as PI (larval and pupal indices) during pre-monsoon, monsoon and post-monsoon period were calculated by using wilcoxon-sign test, which revealed that *Aedes* breeding was significantly high during monsoon as compared to pre and post monsoon ($p < 0.025$, 0.025, 0.025 as well as 0.017) in 2017 in addition ($p < 0.012$, 0.027, 0.012 and 0.028) during 2018 respectively (Table 12and 13). It is clearly indicated that rain water collection in containers supports maximum breeding of *Aedes* species during the monsoon.

Table 10:- Entomological indices of *Aedes species* mosquito breeding in 4 surveyed districts of Punjab during the year 2017 and 2018.

Name of districts	HI		CI		BI		PI		No. of Dengue cases	
	Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD			
	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
SAS Nagar	30.21 ± 19.63	21.35 ± 11.81	16.15 ± 9.67	11.23 ± 5.78	31.31 ± 20.27	23.10 ± 14.37	30.63± 23.65	71.86 ± 54.45	2472	1079
Patiala	20.65 ± 15.29	23.89 ± 17.88	12.22 ± 10.73	12.33 ± 8.22	22.93 ± 18.51	26.10 ± 19.28	23.25 ± 22.97	78.77 ± 72.66	2434	2332
Ludhiana	21.64 ± 16.59	16.93 ± 14.42	10.99 ± 8.33	9.08 ± 7.10	22.77 ± 18.08	17.30 ± 15.06	19.88 ± 18.10	51.22 ± 54.47	1083	489
F.G Sahib	12.43 ± 8.53	14.59 ± 6.80	8.22 ± 5.05	8.24 ± 3.58	14.39 ± 9.56	14.75 ± 6.93	16.81 ± 11.46	40.22 ± 29.49	222	334

Table 11:- Correlation of Dengue cases, with *Stegomyia* index (Pupal index) in four study areas during 2017 and 2018.

Correlation	Pupal Index	
	2017	2018
r2	1.000	1.000
p value	0.000	0.000

Table 12:- Comparison of *Stegomyia* indices (HI and CI) of *Aedes* mosquito breeding in different seasons in year 2017-2018.

Season	HI	p value	HI	p value	CI	p value	CI	p value
	Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD	
	2017		2018		2017		2018	
Pre-monsoon	18.52 ±3.24		12.75 ± 5.94		9.93 ± 2.79		6.24 ± 2.35	
Monsoon	34.93 ±14.83	0.025	30.66 ±10.79	0.012	19.33 ±6.16	0.025	15.85 ±4.40	0.027
Post monsoon	9.53 ±8.06	0.025	12.17 ± 4.73	0.012	5.34 ±4.24	0.017	7.90 ±2.68	0.889

Table 13:- Illustration of *Stegomyia* indices (BI and PI) of *Aedes* mosquito breeding in pre-monsoon, monsoon and post-monsoon in year 2017 -2018.

Season	BI	p value	BI	p value	PI	p value	PI	p value
	Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD	
	2017		2018		2017		2018	
Pre-monsoon	19.15 ± 4.12		12.75 ± 5.94		46.58 ± 18.52		53.63 ± 37.97	
Monsoon	38.69 ±15.83	0.025	33.19 ±12.72	0.012	111.94 ±46.75	0.017	338.06 ±135.74	0.028
Post monsoon	9.69 ±7.98	0.025	12.62 ±5.16	0.012	26.13 ±23.67	0.069	92.46 ±37.21	0.069

6.1.2. *Aedes* mosquito Potential Larval Breeding Habitats for the Year 2017 and 2018

Out of 4168 different types of water-holding containers 2220 from urban and 1948 from rural areas were inspected respectively. During the survey, 307 containers in urban (13.82%) and 217 in rural areas (11.13%) were observed as favourable sites for *Aedes* larvae (Fig. 13). The kind of water-carrying receptacles in the research work with the highest rate of positivity for *Aedes* mosquito larvae were noticed in artificial containers *i.e.* earthen pots (20.90%; 18.08%), desert coolers (14.82%; 12.5%) followed by discarded material, plastic containers and refrigerator trays (Table 14). *Ae.aegypti* breeding was detected maximum in desert coolers (35.61%), plastic containers (13.20%), earthen pots (12.48%), water tanks (6.34%) and refrigerator trays (4.91 %). The common breeding habitats for *Ae.aegypti* and *Ae.albopictus*

collectively was also noticed in earthen pots (12.48%- *aegypti* and 9.21%- *albopictus*), discarded material (2.76% -*aegypti* and 2.45 % -*albopictus*), discarded tyres (2.76% -*aegypti* and 1.74% - *albopictus)* and other miscellaneous material (5.11%- *aegypti* and 2.25 % -*albopictus*).

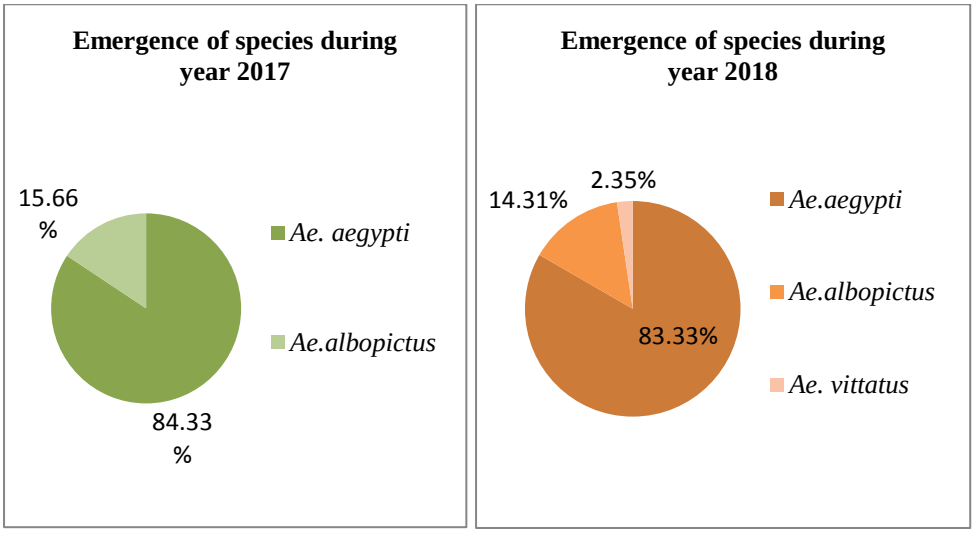
During 2018, overall 3851 different types of water-holding containers 1969 from urban and 1882 from rural areas were examined respectively. During the survey (Fig. 13) 251 containers in urban (12.74%) and 180 in rural areas (9.56%) were detected as sites for *Aedes* larvae. The kind of water-carrying receptacles in the research work with the highest rate of positivity for *Aedes* mosquito larvae were observed to be in artificial containers *i.e.* desert coolers (20.57%; 15.01%), earthen pots (15.87%; 13.66%) followed by other miscellaneous, discarded materials, tyres, refrigerator tray and plastic containers (Table 14). *Ae.aegypti* breeding was noticed maximum in desert coolers (41.27%), earthen pots (15.10%), plastic containers (6.48%), refrigerator trays (6.26 %) water tanks (1.78%) and garden (0.223%). The common breeding habitats for *Ae.aegypti* and *Ae.albopictus* collectively was also noticed in earthen pots (15.10%- *aegypti* and 8.16%- *albopictus*), discarded material (3.80% -*aegypti* and 2.34 % -*albopictus*), discarded tyres (2.23% -*aegypti* and 1.67% - *albopictus)* and other miscellaneous material (5.92%- *aegypti* and 2.125 % -*albopictus*).

Table 14:-Different types of containers inspected for *Aedes* immatures from four surveyed sites of Punjab during the year 2017-2018.

Container type	No of container examined		No of Positive containers		No of container examined		No of Positive containers		Container positivity		Container positivity	
	U	U	U	U	R	R	R	R	U	U	R	R
	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
Desert coolers	735	593	109	122	632	526	79	79	14.829	20.57336	12.5	15.01901
Plastic containers(all type)	342	283	44	21	311	302	25	14	12.865	7.42	8.038	4.635762
Water tank(underground and over- head)	201	151	20	8	218	206	15	3	9.95	5.298013	6.88	1.456311
Earthen pot(water vessel)	330	315	69	50	293	278	53	38	20.909	15.87302	18.09	13.66906
Discarded material	100	84	13	7	88	85	11	8	13	8.333333	12.5	9.411765
Refrigerator tray	159	260	18	18	117	215	10	16	11.32	6.923077	8.547	7.44186
Tyres	131	90	11	10	132	112	10	4	8.396	11.11111	7.575	3.571429
Other miscellaneous	198	158	16	13	157	150	14	17	8.08	8.227848	8.917	11.33333
Garden (tree holes)	24	35	7	2	0	7	0	1	29.166	5.714286	0	14.28571

6.1.3. *Aedes* mosquito Species and their Seasonal Variation for the Year 2017 and 2018

After the rearing of larvae and pupae, two species of *Aedes* (*Ae*) *viz. Ae.aegypti* and *Ae.albopictus* were identified in selected study districts of Punjab during the year 2017 respectively. The adult emergence rate was detected 66.23% in 2017. The percentage of species from emerged adults was calculated from both urban and rural area *i.e*. *Ae.aegypti* (84.33%, 824/977) was reported from all selected districts, *Ae.albopictus* (15.66%, 153/977) was also noticed from 4 districts except rural area of F.G Sahib of Punjab in 2017 (Fig 14). After the rearing of immature, three species of *Aedes viz. Ae.aegypti, Ae.albopictus* and *Ae.vittatus* were identified in all the study districts of Punjab in 2018 respectively. The adult emergence rate was observed 67.57% in 2018. The percentage of species from emerged adults was calculated from both urban and rural area *i.e Ae.aegypti* (83.33%, 745/894) was detected from all the 4 selected districts, *Ae.albopictus* (14.31%, 128/894) observed from 4 districts and *Ae.vittatus* (2.35%, 21/894) observed only from district SAS Nagar (Fig 14).



a). Emergence of two species in 2017 b). Emergence of three species in 2018

Fig 14:-Emergence rate of *Aedes aegypti*, *Aedes albopictus* and *Aedes vittatus* in urban and rural areas of 4 districts of Punjab during 2017 and 2018, a) Emergence of two species in 2017, b) Emergence of three species in 2018

The seasonal trend was analysed and detected to be high in monsoon as compared to pre and post monsoon period in both urban as well as rural areas in 2017. On

comparison of breeding of both species in urban and rural areas by Man-whitney test (descriptive analysis), it was observed that *Ae.aegypti* number was significantly higher than *Ae.albopictus*. The breeding of *Ae.aegypti* was observed to be on the higher side in both urban and rural areas (urban- mean 102.75 & SD ± 39.63; rural- mean 36.50 & SD ± 10.28 and p value = 0.021) in monsoon (69.63 ± 44.41 with p value = 0.017) as compared to pre-monsoon and post-monsoon collections (Fig 15 and 16). *Ae.albopictus* was observed to be significantly high in urban areas as compared to rural areas (urban- mean 24.75 & SD ± 8.73 and rural- mean 7.33& SD ± 2.52 with p value = 0.034) respectively (Fig 16).

Their seasonal trend was analysed and detected to be high in monsoon and post-monsoon as compared to pre monsoon period in both urban as well as rural areas in 2018. It was also observed that *Ae.aegypti* number was significantly higher than *Ae.albopictus*. The breeding of *Ae.aegypti* was equally high in both urban and rural areas (urban- mean 91.50 & SD ± 35.37 rural- mean 54.75 & SD ± 21.76) in monsoon (73.13 ± 33.54 with p value = 0.012) and post monsoon (14.38 ± 8.33 with p value = 0.012) as compared to pre monsoon collections (Fig 15 and 17). However, *Ae.albopictus* were observed to be significantly high in urban as compared to rural area with (mean- 16.50 & SD ± 5.07 and mean 11.25 & SD ± 4.27) respectively in monsoon (13.88 ± 5.17 with p value = 0.012) and post monsoon (2.13 ± 1.96 with p value = 0.012) as compared to pre monsoon collections (Fig 17).

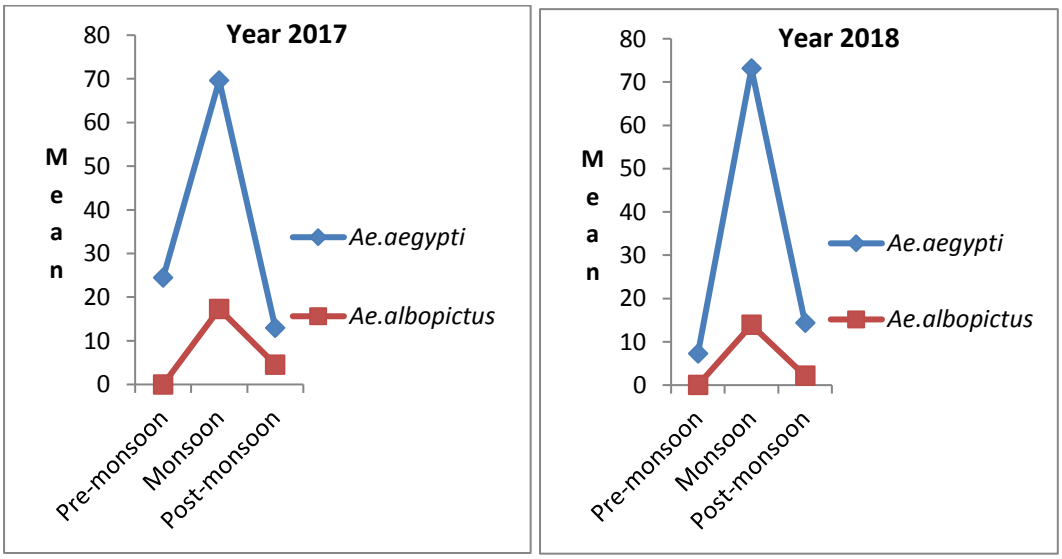


Fig 15:-The prevalence of *Ae.aegypti* and *Ae.albopictus* in pre-monsoon, monsoon and post-monsoon in 4 districts of Punjab for the year 2017 and 2018.

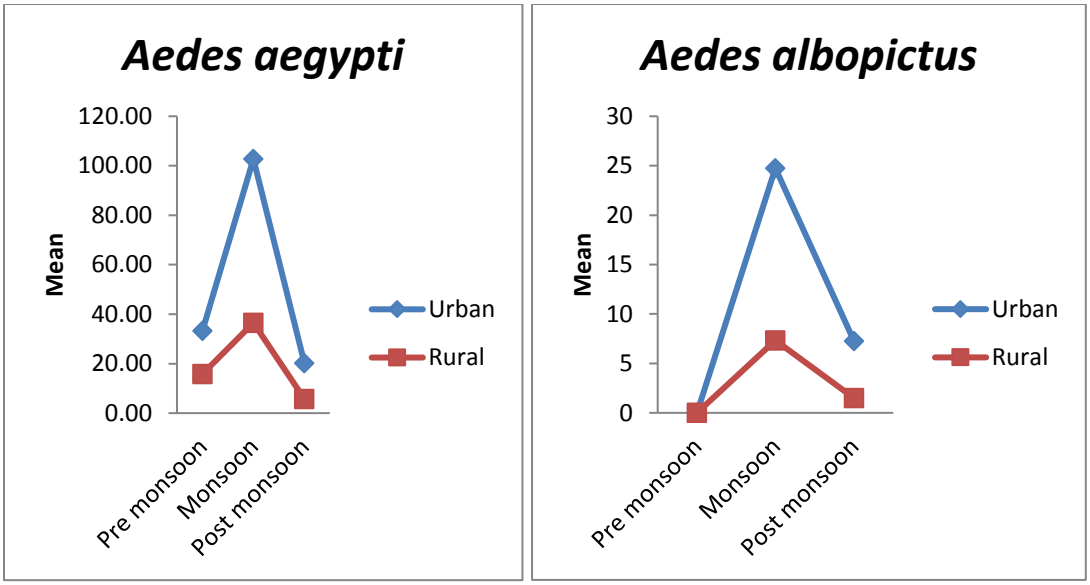


Fig 16:-Graphs are illustrating the prevalence of *Aedes aegypti* and *Aedes albopictus* in urban and rural areas of 4 districts of Punjab during 2017.

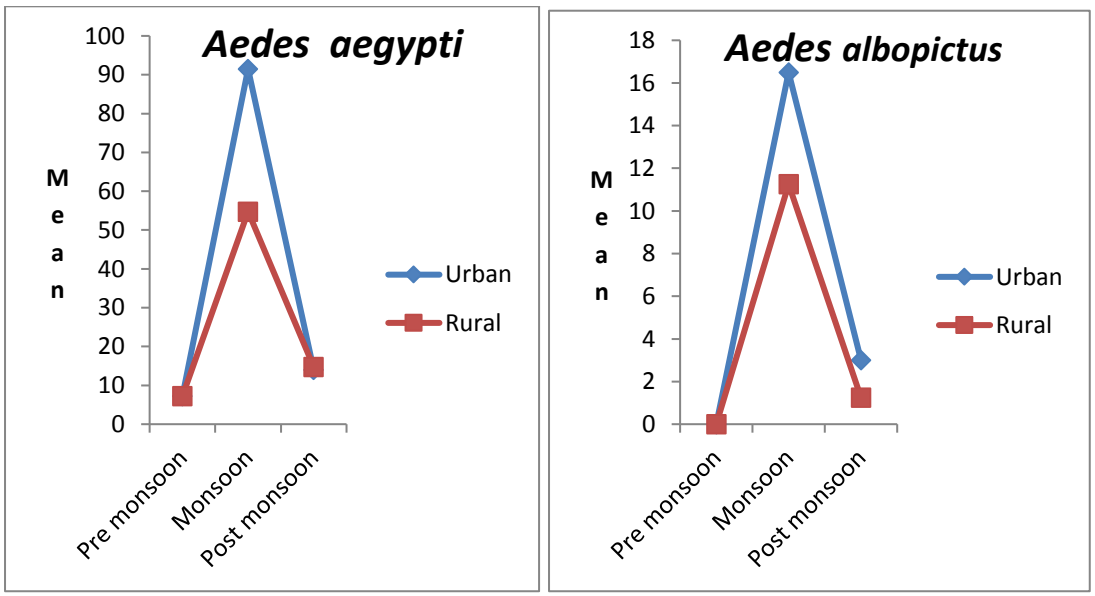
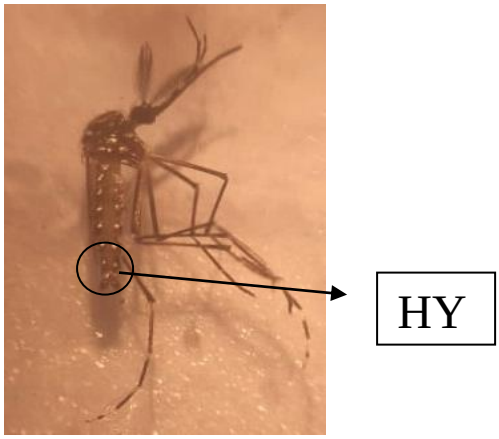


Fig 17:-Prevalence of *Aedes aegypti* and *Aedes albopictus* in urban and rural areas of 4 selected sites of Punjab during 2018.

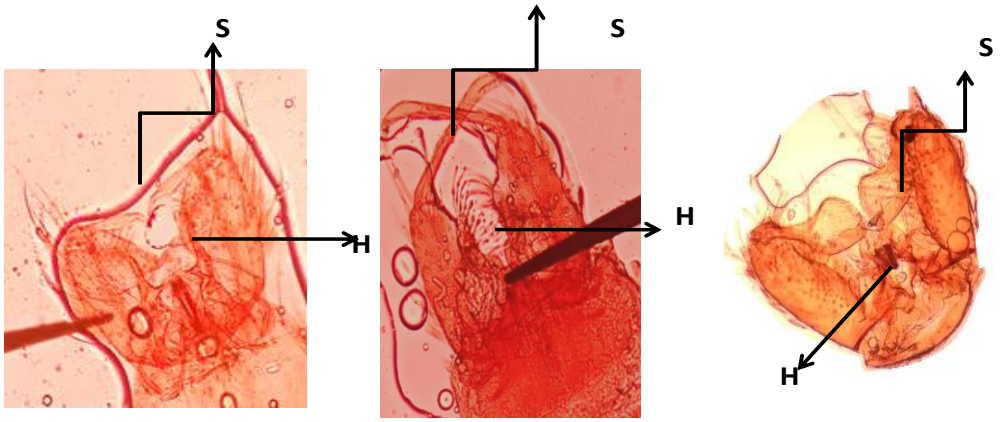
6.2. Species Identification on the Basis of Male and Female Hypopygium Structure

During entomological surveillance in 2017 only two species of *Aedes* were reported but in 2018 three different species of *Aedes* were observed *viz. Aedes aegypti*, *Aedes albopictus and Aedes vittatus* (Fig. 18). Thereafter, for their correct identification and

confirmation of species, along with morphological characteristics, the slides hypopygium of all the three species were also prepared.



a). Male mosquito terminal abdominal segment constituting hypopygium (HY)



b). *Ae. aegypti* **c). *Ae. albopictus*** **d). *Ae. vittatus***

Fig 18:-Portrait showing, a) male mosquito terminal abdominal segment constituting hypopygium (HY), b) illustration of male hypopygium of *Aedes aegypti*, c) male hypopygium of *Aedes albopictus*, d) male hypopygium of *Aedes vittatus*(S-Style, H-Hypopygium).

It was observed that in *Aedes aegypti* paraproct of hypopygium (Fig.18.b) has a well-developed ventral arm. This unusual feature is absent in all other Oriental species of *Aedes*. In *Aedes albopictus* (Fig.18.c), the hypopygium consists of spoon shaped style with appendage arising near tip and the crown of the paraproct has series of hairs arising from the single plane. In *Aedes vittatus* (Fig.18.d), hypopygium is distinct from all other species in the form of style which is much enlarged at the extremity and carries preapical curved appendages.

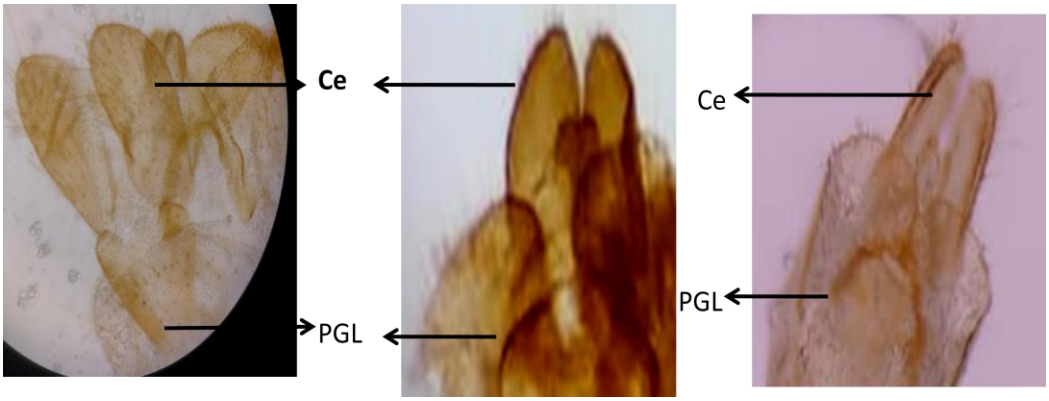


Fig 19:-Image presenting, a) female genitalia of *Aedes aegypti,* b) female genitalia of *Aedes albopictus,* c) female genitalia of *Aedes vittatus* (Ce-Cerci, PGL-Post Genital lobe).

In female genitalia no such conspicuous difference was observed. In all three species, postgenital lobe and a pair of cerci were present with short and long setae (Fig 19).

6.3. Detection of Susceptibility/Resistance Status

For larval testing, lab. reared larvae of F_1 generation of *Aedes aegypti* were exposed. Larvae of *Aedes* mosquitoes were tested against larvicide Temephos to assess their susceptibility and resistance status. The susceptibility and resistance status of the mosquitoes was categorized according to the WHO guideline as when mortality was $> 97\%$ indicated susceptibility, resistance confirmed when $\leq$ 80% and probable/suspected resistance when mortality rate is between 80-97% (Ayorinde ,*et al.*,2015; WHO, 2016).

Table 15:- Insecticide susceptibility status of the larvae of *Aedes aegypti* against diagnostic concentration (0.020mg/l) of Temephos in four selected districts of Punjab.

Name of district	No. of larvae exposed		No. of Larvae dead		Average mortality	Susceptibility status
	Test	Control	Test	Control		
SAS Nagar	25	20	5	0	20 %	Resistance
Patiala	25	20	21	1	84 %	Probable resistance
Ludhiana	25	20	10	1	40 %	Resistance
Fatehgarh Sahib	25	20	17	1	68 %	Resistance

6.3.1. Larval Bioassay with Diagnostic Concentration of Temephos

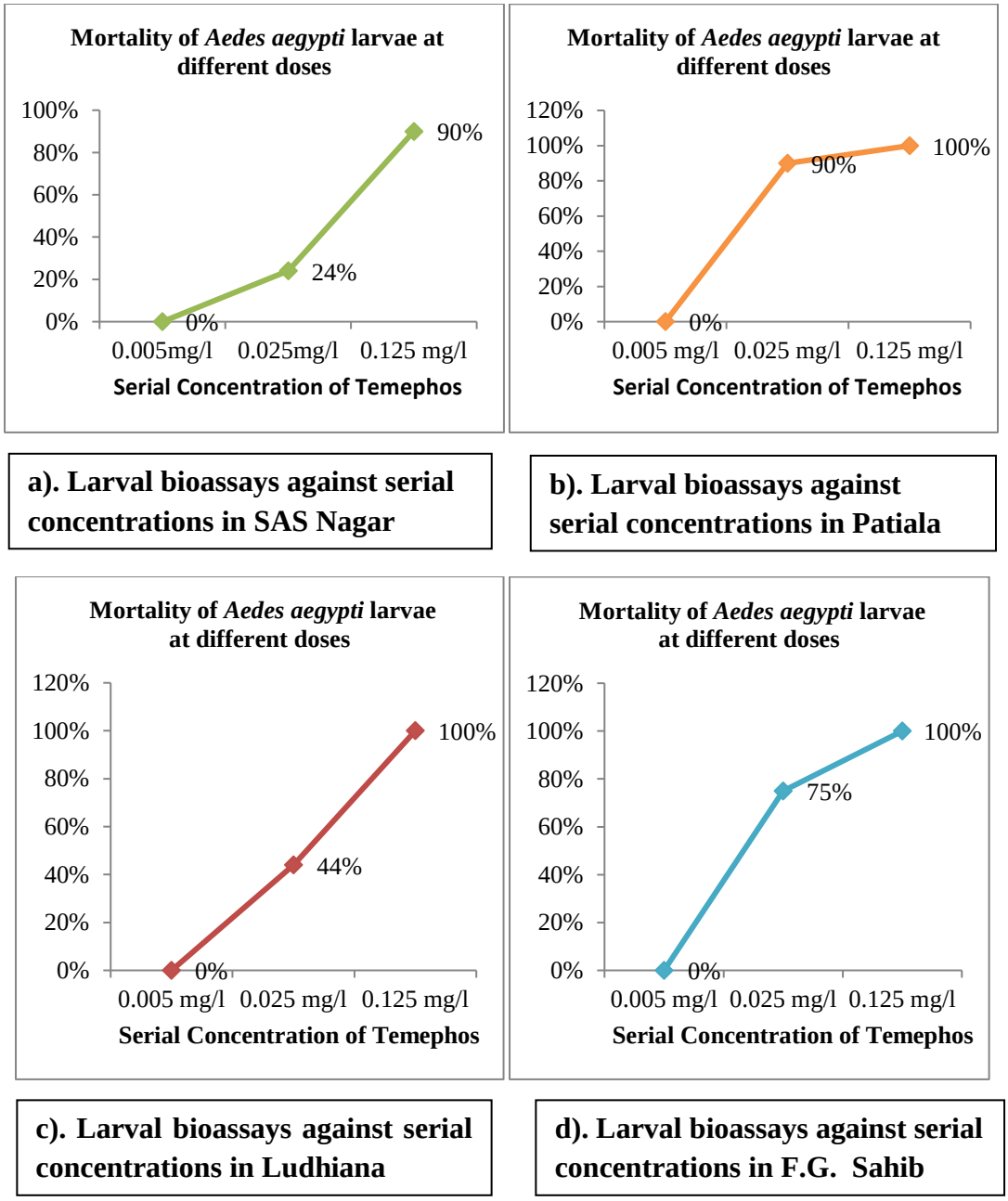
Table 15 indicates the larval susceptibility status of *Ae.aegypti* collected from selected districts of the state (Fig. 20), in accordance with WHO recommended diagnostic concentration of Temephos (0.02 ppm). Distinct mortality was reported among larvae in four districts such as in SAS Nagar 20%, Patiala 84%, Ludhiana 40% and in Fatehgarh Sahib 68%. Larvae of *Ae.aegypti* collected from Patiala were only 'probable resistant' to Temephos, while from the other three districts *viz.* SAS Nagar, Ludhianaand Fatehgarh Sahib, exhibited'resistance' against Temephos.

a). Testing of larvae from district Patiala

b).Testing of larvae from districts Ludhiana and SAS Nagar

c). Testing of larvae from districts Fatehgarh Sahib

Fig 20:-Susceptibility/resistance testing of larvae from four districts SAS Nagar, Patiala, Ludhiana and Fatehgarh Sahib with the help of WHO standard test kit of Temephos at different concentrations and control, a) Testing of larvae from district Patiala, b) Testing of larvae from districts SAS Nagar and Ludhiana, c) Testing of larvae from districts Fatehgarh Sahib.



a). Larval bioassays against serial concentrations in SAS Nagar

b). Larval bioassays against serial concentrations in Patiala

c). Larval bioassays against serial concentrations in Ludhiana

d). Larval bioassays against serial concentrations in F.G. Sahib

Fig 21:-Graph specifying results of larval bioassays against serial concentrations of Temephos in four districts of Punjab, a) Larval bioassays against serial concentrations in SAS nagar, b) Larval bioassay against serial concentration in Patiala, c) Larval bioassays against serial concentrations in Ludhiana, d) Larval bioassays against serial concentrations in F.G. Sahib.

6.3.2. Larval Bioassay with Serial Concentration of Temephos

Larval bioassays with serial concentrations of Temephos (0.005mg/l, 0.025mg/l and 0.125mg/l) were performed for four selected districts and the results are shown in

Figure 21. The results indicatesthat no mortality of larvae was observed on 0.005mg/l dosages afterwards experiment was performed at dose 0.025mg/l. Distinct mortality was detected in four districts such as in SAS Nagar 24%, Patiala 90%, Ludhiana 44% and in Fatehgarh Sahib 75%. At this dose (0.025mg/l) of Temephos in three districts SAS Nagar, Ludhiana and Fatehgarh Sahib 'resistance' was observed while in district Patiala 'probable resistance' was detected (WHO, 2016). Thereafter, experiment was repeated in triplicate at 5 times higher dose *i.e.* 0.125 mg/l. The mortality rate was (90%) SAS Nagar, (100%) Patiala, (100%) Ludhiana and (100%) Fatehgarh Sahib. So, at higher dose (0.125 mg/l) 90-100% mortality was recorded in all the selected areas.

Table 16:- Lethal concentrations of Temephos for 50% (LC_{50}) and 90% (LC_{90}) mortality of *Aedes aegypti* in districts SAS Nagar, Patiala, Ludhiana and Fatehgarh Sahib of Punjab.

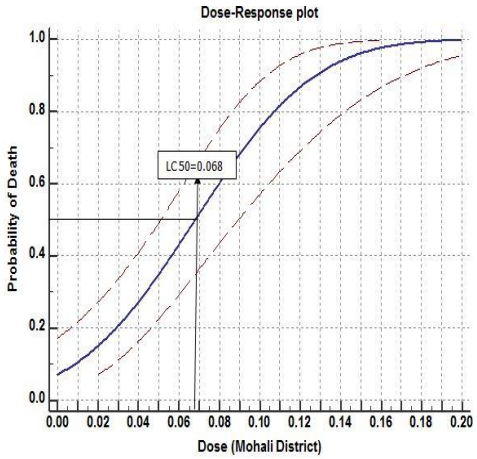
Name of district	Confidence limit (LC_{50})	Confidence limit (LC_{90})
SAS Nagar	0.068 (0.051-0.089) mg/l	0.13 (0.10-0.17) mg/l
Patiala	0.017 (0.013-0.020) mg/l	0.026 (0.022-0.032) mg/l
Ludhiana	0.027 (0.021-0.041) mg/l	0.043 (0.033-0.083) mg/l
Fatehgarh Sahib	0.024(0.020-0.028) mg/l	0.034 (0.030-0.043) mg/l

The lethal concentrations of Temephos for 50% (LC_{50}) and 90% (LC_{90}) in selected districts for mortality of *Ae.aegypti* larvae are shown in (Table 16 and Fig. 22). The *Ae.aegypti* larvae were least Temephos susceptible in district SAS Nagar.

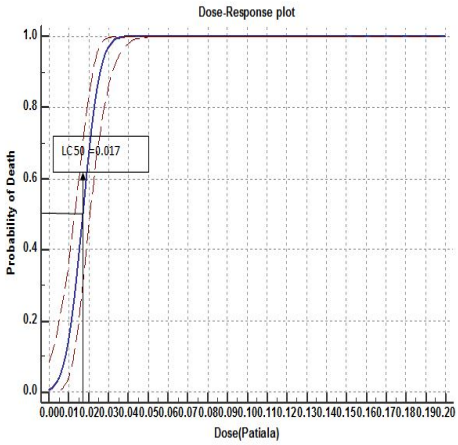
Table 17:- Temephos sensitivity of *Aedes aegypti* larvae and resistant ratio (RR) in selected population.

Sr. No	Name of districts	LC_{50}	LC_{90}	RR ratio LC_{50}	RR ratio LC_{90}	P value	X2 (Cox and Snell R2)
1	SAS Nagar	0.068	0.13	1.09	1.428	< 0.0001	0.4458
2	Patiala	0.017	0.026	0.274	0.285	< 0.0001	0.6134
3	Ludhiana	0.027	0.043	0.435	0.472	< 0.0001	0.5582
4	Fatehgarh Sahib	0.024	0.034	0.387	0.373	< 0.0001	0.5797

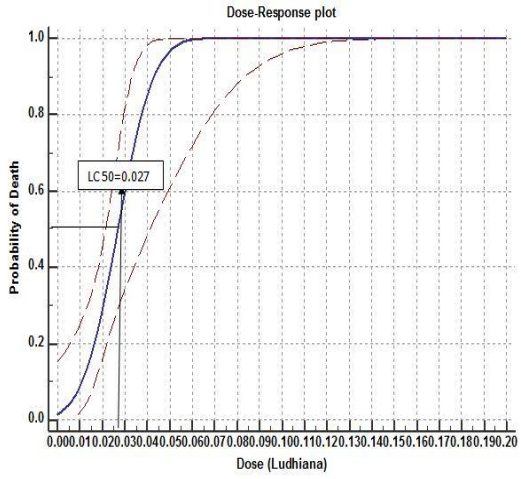
The results of LC_{50} obtained from the larval bioassays of *Aedes aegypti* from different districts when compared with the already known susceptible population (Rockfeller strain with LC_{50} = 0.062) it was observed that calculated LC_{50} of three districts *i.e.*, Patiala (0.017), Ludhiana (0.027) and Fatehgarh Sahib (0.024) was lesser whereas LC_{50} of SAS Nagar (0.068) was margin-ably higher than Rockfeller strain (Table 17 and Fig.22)**.**



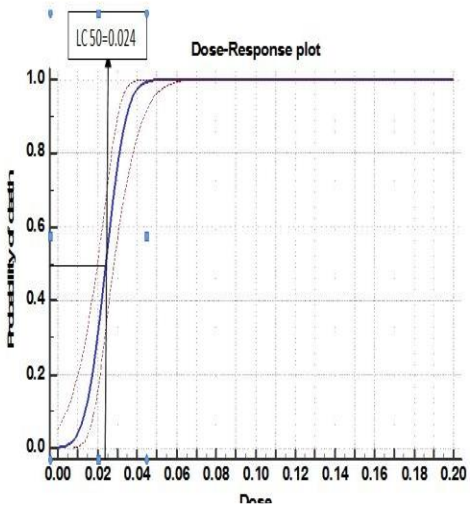
a). LC50 of Temephos for district SAS Nagar-0.068



b). LC50 of Temephos for district Patiala-0.017`



c). LC50 of Temephos for district Ludhiana -0.027



d). LC50 of Temephos for district F.G. Sahib-0.024

Fig 22: -Graph specifying LC_{50} dose response plot of four districts a) LC_{50} of Temephos for district SAS Nagar-0.068, b) LC_{50} of Temephos for district Patiala-0.017, c) LC_{50} of Temephos for district Ludhiana -0.027, d) LC_{50} of Temephos for district F.G. Sahib-0.024.

6.3.3. Adult Bioassay

For adult bioassay testing, lab. reared adults of F_1 generation of *Aedes aegypti* were exposed against insecticide Pyrethrum and each test were performed in triplicate to assess their suceptibility and resistance status as given below.:-

Insecticide susceptibility tests were performed on laboratory reared (Fig. 23) *Aedes aegypti* of F_1 adult mosquitoes against fogging and mist formulation of Pyrethrum in 6 phase SAS Nagar (Hostel room of State institute of health and family welfare). The results of the adult susceptibility test with Pyrethrum fogging revealed that *Aedes aegypti* was resistant in districts F.G Sahib with 88% mortality, while specimen from the district Ludhiana exhibited probable resistance with 92% mortality. Specimens from rest two districts namely SAS Nagar and Patiala exhibited complete susceptibility with 100% mortality. The adult susceptibility test with Pyrethrum mist exhibited 'resistance' in two districts *viz* Ludhiana with 84% mortality and Patiala with 88% mortality while 'probable resistance'was observed in specimens from the district of Fatehgarh Sahib with 92% mortality. Specimens from district SAS Nagar revealed complete susceptibility with 100% mortality (Table 18).

a). Spray of Pyrethrum mist with Ganesh pump **b). Fogging of Pyrethrum with fogging machine** **c). Control**

Fig 23:-Picture showing susceptibility/resistance profile of adults *Aedes aegypti* from district Patiala with the help of Pyrethrum 2% extract by two methods a) Spray of Pyrethrum mist with Ganesh pump, b) Fogging of Pyrethrum with fogging machine, c) Control.

Table 18:- Susceptibility status of *Aedes aegypti* adult against Pyrethrum fogging and spray in 4 selected districts of Punjab.

Name of district	Type of test	No. of mosquitoes exposed	No. of mosquitoes knockdown/ dead after 30 min	No. of mosquitoes knockdown/ dead after 60 min	No. of mosquitoes dead after 24 hrs.	% mortality	Susceptibility status
SAS Nagar	Pyrethrum thermal Fogging	25	7	25	25	100	Susceptible
	Pyrethrum mist	25	6	13	25	100	Susceptible
	Control	25	0	0	0	0	
Patiala	Pyrethrum thermal Fogging	25	14	25	25	100	Susceptible
	Pyrethrum mist	25	0	22	22	88	Resistance
	Control	25	0	0	0	0	
Ludhiana	Pyrethrum thermal Fogging	25	23	23	23	92	Probable Resistance
	Pyrethrum mist	25	12	21	21	84	Resistance
	Control	25	0	0	0	0	
Fatehgarh Sahib	Pyrethrum thermal Fogging	25	22	22	22	88	Resistance
	Pyrethrum mist	25	23	23	23	92	Probable Resistance
	Control	25	0	0	0	0	

6.4. Analysis of *Ace-1* mutation and Kdr mutation

After performing the larval and adult bioassay experiments of most abundant species, then mutation analysis in insecticide resistant genes, which include acetylcholinesterase (*Ace-1*) and voltage gated sodium channel (knock down resistance -Kdr) in predominant species was carried out.

6.4.1. Detection of Target Site Resistance (*Ace-1* mutation): -Genomic DNA was extracted from Temephos resistance and control larvae from each location site. The 480 bp fragment of the *Ace-1* gene was amplified (corresponding to nucleotides 1288–1708 of AChE ORF) from the four populations and were analysed. The sequences were studied for the most common mutation due to organophosphate exposure in mosquitoes *viz.* G119S. But no such substitution has been observed in any

of the sequences. Moreover, no indels (insertions or deletions) were detected in these sequences (GenBank accession Number: **SAS Nagar MT993470, Patiala MW380118, Ludhiana MW690113 and F.G. Sahib MW380117**) when compared to the reference sequence of *Aedes aegypti* Rockefeller strain partial *Ace-1* gene for acetylcholinesterase (***AJ621915.*1**) as shown (Fig.24 and 25) Additionally, no amino acid polymorphic site was detected within these sequences.

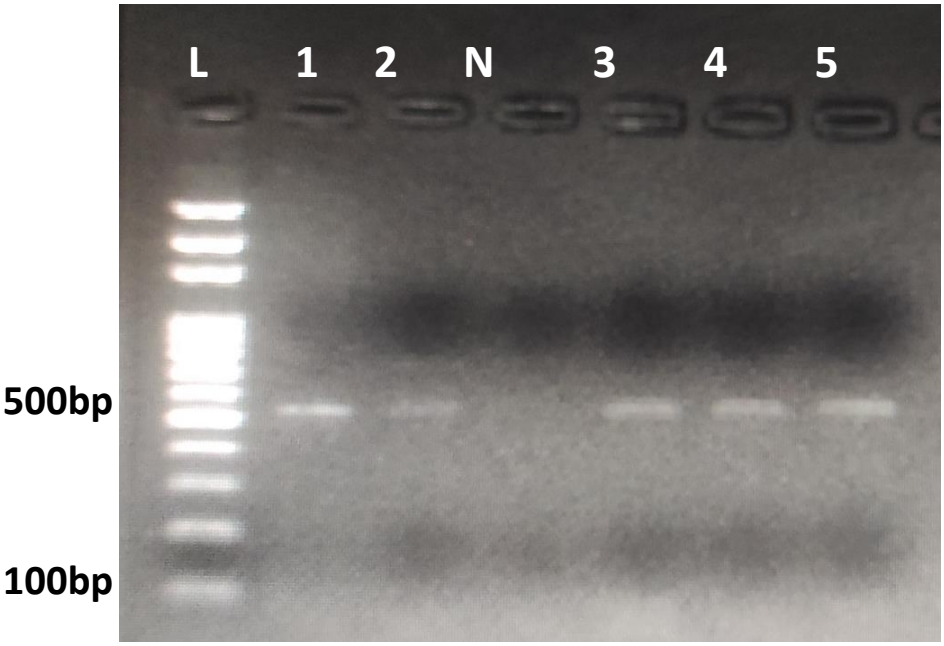


Fig 24:-Gel-run illustrating amplification of *Ace-1* gene from susceptible and resistant larvae of *Ae.aegypti* (L-gene rule (100bp), 1-SAS Nagar, 2-Fatehgarh Sahib, 3-Ludhiana, 4-Patiala, N-negative control).

CLUSTAL 2.1 multiple sequence alignment

```
RF_       GGACATCCTAACCGGCAGTAATACGGAGGAAGGTTATTACTTCATAATATACTA
5_        GGACATCCTAACCGGCAGTAATACGGAGGAAGGTTATTACTTCATAATATACTA
2_        GGACATCCTAACCGGCAGTAATACGGAGGAAGGTTATTACTTCATAATATACTA
4_        GGACATCCTAACCGGCAGTAATACGGAGGAAGGTTATTACTTCATAATATACTA
3_        GGACATCCTAACCGGCAGTAATACGGAGGAAGGTTATTACTTCATAATATACTA
          **************************************************

RF_       CTTGACTGAACTATTGCGGAAAGAGGAGGGTGTCACAGTTTCACGGGAGGAGTTCTTGCA
5_        CTTGACTGAACTATTGCGGAAAGAGGAGGGTGTCACAGTTTCACGGGAGGAGTTCTTGCA
2_        CTTGACTGAACTATTGCGGAAAGAGGAGGGTGTCACAGTTTCACGGGAGGAGTTCTTGCA
4_        CTTGACTGAACTATTGCGGAAAGAGGAGGGTGTCACAGTTTCACGGGAGGAGTTCTTGCA
3_        CTTGACTGAACTATTGCGGAAAGAGGAGGGTGTCACAGTTTCACGGGAGGAGTTCTTGCA
          ************************************************************

RF_       GGCCGTTAGAGAACTGAATCCTTACGTGAACGGAGCCGCGAGGCAGGCTATCGTGTTCGA
5_        GGCCGTTAGAGAACTGAATCCTTACGTGAACGGAGCCGCGAGGCAGGCTATCGTGTTCGA
2_        GGCCGTTAGAGAACTGAATCCTTACGTGAACGGAGCCGCGAGGCAGGCTATCGTGTTCGA
4_        GGCCGTTAGAGAACTGAATCCTTACGTGAACGGAGCCGCGAGGCAGGCTATCGTGTTCGA
3_        GGCCGTTAGAGAACTGAATCCTTACGTGAACGGAGCCGCGAGGCAGGCTATCGTGTTCGA

          ************************************************************
```

```
RF_       GTACACCGACTGGACTGAACCGGAAAATCCCAACAGCAATCGGGATGCATTGGACAAAAT
5_        GTACACCGACTGGACTGAACCGGAAAATCCCAACAGCAATCGGGATGCATTGGACAAAAT
2_        GTACACCGACTGGACTGAACCGGAAAATCCCAACAGCAATCGGGATGCATTGGACAAAAT
4_        GTACACCGACTGGACTGAACCGGAAAATCCCAACAGCAATCGGGATGCATTGGACAAAAT
3_        GTACACCGACTGGACTGAACCGGAAAATCCCAACAGCAATCGGGATGCATTGGACAAAAT
          ************************************************************
RF_       GGTCGGAGATTATCACTTCACGTGTAATGTGAATGAGTTTGCCCAGCGATATGCAGAAGA
5_        GGTCGGAGATTATCACTTCACGTGTAATGTGAATGAGTTTGCCCAGCGATATGCAGAAGA
2_        GGTCGGAGATTATCACTTCACGTGTAATGTGAATGAGTTTGCCCAGCGATATGCAGAAGA
4_        GGTCGGAGATTATCACTTCACGTGTAATGTGAATGAGTTTGCCCAGCGATATGCAGAAGA
3_        GGTCGGAGATTATCACTTCACGTGTAATGTGAATGAGTTTGCCCAGCGATATGCAGAAGA
s         ************************************************************

RF_       AGGCAACAATGTGTACATGTATCTGTACACTCATAGAAGCAAAGGTAACCCCTGGCCACG
5_        AGGCAACAATGTGTACATGTATCTGTACACTCATAGAAGCAAAGGTAACCCCTGGCCACG
2_        AGGCAACAATGTGTACATGTATCTGTACACTCATAGAAGCAAAGGTAACCCCTGGCCACG
4_        AGGCAACAATGTGTACATGTATCTGTACACTCATAGAAGCAAAGGTAACCCCTGGCCACG
3_        AGGCAACAATGTGTACATGTATCTGTACACTCATAGAAGCAAAGGTAACCCCTGGCCACG
          ************************************************************

RF_       GTGGACCGGTGTGATGCATGGTGACGAGATCAATTATGTGTTCGGTGAGCCTCTGAA
5_        GTGGACCGGTGTGATGCATGGTGACGAGATCAATTATGTGTTCGGTGAGCCTCTGAA
2_        GTGGACCGGTGTGATGCATGGTGACGAGATCAATTATGTGTTCGGTGAGCCTCTGAA
4_        GTGGACCGGTGTGATGCATGGTGACGAGATCAATTATGTGTTCGGTGAGCCTCTGAA
3_        GTGGACCGGTGTGATGCATGGTGACGAGATCAATTATGTGTTCGGTGAGCCTCTGAA

          ************************************************************
```

Fig 25:-Multiple sequence alignment of *Ace-1* sequences obtained from four different districts with the susceptible Rockfellar strain (AJ621915.1) by using Clustal W.

6.4.2. Knockdown Resistance Genotyping (Kdr mutation):- Genomic DNA was extracted from Pyrethrum resistant and control individual female mosquitoes from each location site. PCR for sequencing of VGSC gene domain II was carried out by using specific primers to amplify 500bp for the detection of V1016G mutation.

The 500bp fragment of the VGSC gene was amplified from resistant and susceptible individuals from all the 4 districts. The sequences were studied for the most common mutation due to pyrethroids in mosquitoes *viz.* A1007G, I1011M and V1016G. But no such substitution has been observed in any of the sequences. Moreover, no indels (insertions or deletions) were detected in these sequences (GenBank accession Number:-**SAS Nagar MZ392029, Patiala MW654198, Ludhiana MW690112 and F.G. Sahib MW602461**) when compared to the reference sequence (LC485541) of *Aedes aegypti* for V1016G as shown (Fig 27). In addition, only one synonymous mutation A1007A (GCC-GCT) and one non synonymous mutation L1006S (TTG- TCG) in domain II was recorded from the sequences of resistant *Ae.aegypti* mosquitoes from all districts (Fig 27).

PCR for Sequencing of VGSC Gene:-

The individuals from all the districts in study area were amplified to study V1016G mutation in amplified 500bp band (Fig.26).

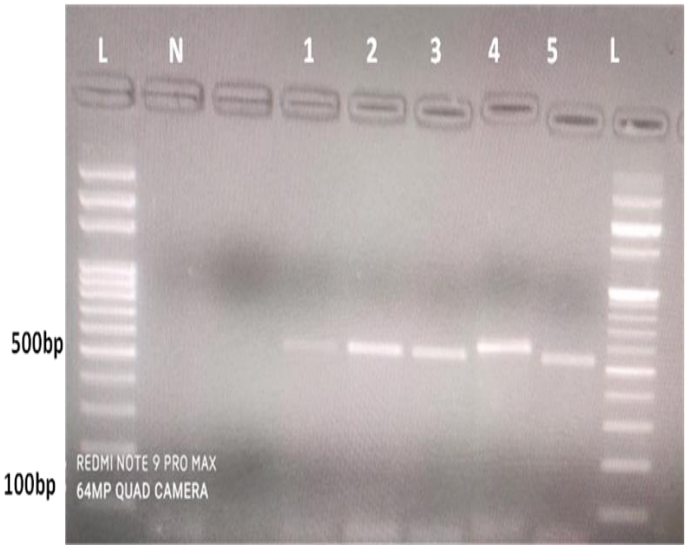


Fig 26:-Picture presenting amplification of VGSC gene domain II from susceptible and resistant adults for pyrethroid of *Ae.aegypti* (L-gene rule (100bp), 1-SAS Nagar, 2-Fatehgarh Sahib, 3-Ludhiana, 4-Patiala, N-negative control).

CLUSTAL 2.1 multiple sequence alignment

```
1_      TCGGCTACCGTAGTGATAGGAAATCTAGTAGTAAGTATTCCGTTTGGAAGTTCATCTGTA
2_      TCGGCTACCGTAGTGATAGGAAATCTAGTAGTAAGTATTCCGTTTGGAAGTTCATCTGTA
3_      TCGGCTACCGTAGTGATAGGAAATCTAGTAGTAAGTATTCCGTTTGGAAGTTCATCTGTA
4_      TCGGCTACCGTAGTGATAGGAAATCTAGTAGTAAGTATTCCGTTTGGAAGTTCATCTGTA
5_      TTGGCCACCGTAGTGATAGGAAATCTAGTAGTAAGTATTCCGTTTGGAAGTTCATCTGTA
        * *** **************************************************

                                   Intron ←
1_      AGGCTGACTGAAAGTAAATTGGAGCGCACAACAGACCTATTATGCTGTAATTCGTGATTC
2_      AGGCTGACTGAAAGTAAATTGGAGCGCACAACAGACCTATTATGCTGTAATTCGTGATTC
3_      AGGCTGACTGAAAGTAAATTGGAGCGCACAACAGACCTATTATGCTGTAATTCGTGATTC
4_      AGGCTGACTGAAAGTAAATTGGAGCGCACAACAGACCTATTATGCTGTAATTCGTGATTC
5_      AGGCTGACTGAAAGTAAATTGGAGCGCACAACAGACCTATTATGCTGTAATTCGTGATTC
        ************************************************************

1_      AACTAGTTACAAAAGACCGTTGATCTTGATAGCATCAATATTAGAGGCGTGCTAGCAGCG
2_      AACTAGTTACAAAAGACCGTTGATCTTGATAGCATCAATATTAGAGGCGTGCTAGCAGCG
3_      AACTAGTTACAAAAGACCGTTGATCTTGATAGCATCAATATTAGAGGCGTGCTAGCAGCG
4_      AACTAGTTACAAAAGACCGTTGATCTTGATAGCATCAATATTAGAGGCGTGCTAGCAGCG
5_      AACTAGTTACAAAAGACCGTTGATCTTGATAGCATCAATATTAGAGGCGTGCTAGCAGCG
        ************************************************************

1_      AGCGAGGGGCGTACCAATTTACTTTTAGTCAGTCTTTCTTGCATTCTTTCGTGCTAACCG
2_      AGCGAGGGGCGTACCAATTTACTTTTAGTCAGTCTTTCTTGCATTCTTTCGTGCTAACCG
3_      AGCGAGGGGCGTACCAATTTACTTTTAGTCAGTCTTTCTTGCATTCTTTCGTGCTAACCG
4_      AGCGAGGGGCGTACCAATTTACTTTTAGTCAGTCTTTCTTGCATTCTTTCGTGCTAACCG
5_      AGCGAGGGGCGTACCAATTTACTTTTAGTCAGTCTTTCTTGCATTCTTTCGTGCTAACCG
        ************************************************************
                                                        ↓
```

Fig 27:-Multiple sequence alignment of VGSC sequences obtained from four different districts.

The presence of non-synonymous mutations in the concerned gene position indicated that the *Aedes* mosquitoes have progressively started to develop resistance capability towards pyrethroid. More such studies should be planned by screening large populations with various domains of VGSC to rule out the role of this mutation in pyrethroid resistance.

After completing these experiments it was observed that larvae of *Ae.aegypti* from three districts *viz.* SAS Nagar, Ludhiana and Fatehgarh Sahib exhibited probable resistance against Temephos. Adults of *Ae.aegypti* from three districts *viz.* SAS Nagar, Ludhiana and Fatehgarh Sahib exhibited 'probable resistance' against Pyrethrum. Then to find out the most effective chemical this work has been carried out additionally:-

6.5. Interpretation of Susceptibility/Resistance Status with Bti

Larvae of *Aedes aegypti* were exposed to the diagnostic dosages of Bti, it exhibited 100% mortality indicating that the species was 100% susceptible to this larvicide (Table 19 and Fig. 28).

Table 19:- Test showing tolerance of *Ae.aegypti* larvae in four study sites against Bti.

Districts name	mortality	Diagnostic conc.	Control
SAS Nagar	100%	0.00375mg/l	All live
Patiala	100%	0.00375mg/l	All live
Ludhiana	100%	0.00375mg/l	All live
Fatehgarh Sahib	100%	0.00375mg/l	All live

a). Testing of larvae from district Patiala and Ludhiana.

b). Testing of larvae from district SAS Nagar and Fatehgarh Sahib.

Fig 28:-Photograph presenting susceptibility/resistance testing of larvae from four districts with the help of larvicide Bti (WP) at diagnostic concentration and control, a) Testing of larvae from district Patiala and Ludhiana, b) Testing of larvae from district SAS Nagar and Fatehgarh Sahib.

6.6. Assessment of Susceptibility/Resistance Status with Deltamethrin and Malathion

Laboratary reared *Aedes aegypti* of F_1 generation adults were exposed for bottles bioassay to the diagnostic dosages of Malathion and Deltamethrin, it exhibited 100% mortality indicating that the species was susceptible to these insecticides (Table 20, Fig. 29) and these insecticides are highly effective against mosquitoes.

Fig 29:-Potrait illustrates adult bottle bioassay by using Malathion 25% WP, Deltamethrin 2.5% WP in all selected districts.

Table 20:-Susceptibility status of *Aedes aegypti* against insecticide Malathion 25% WP and Deltamethrin 2.5% WP.

Name of Districts	**Chemical of test**	**No. of mosquitoes exposed**	**No. of mosquitoes knockdown/dead** dead after 30 min.	**No.of mosquitoes knockdown/dead** dead after 60 min.	**No. of mosquitoes dead after 24 hrs.**	**% mortality**	**Susceptibility status**
SAS Nagar	Malathion	15	15	15	15	100	Susceptible
	Deltamethrin	15	15	15	15	100	Susceptible
	Control	15	0	0	0	0	--
Patiala	Malathion	15	14	15	15	100	Susceptible
	Deltamethrin	15	14	15	15	100	Susceptible
	Control	15	0	0	0	0	
Ludhiana	Malathion	15	7	15	15	100	Susceptible
	Deltamethrin	15	15	15	15	100	Susceptible
	Control	15	0	0	0	0	
Fatehgarh Sahib	Malathion	15	14	15	15	100	Susceptible
	Deltamethrin	15	15	15	15	100	Susceptible
	Control	15	0	0	0		

Dengue Fever has globally emerged as one of the major vector borne viral disease. More than one-third (2.5 billion) of the world's population is living in areas, at risk of Dengue transmission because the incidence of the disease has increased during the last 5 decades and it influence more than 100 million people every year

(Guzman, *et al.,* 2010; Messina, *et al.,* 2019). Indian population has also faced cyclic outbreak of Dengue during the years. Dengue is the most common cause of hospitalization and mortality amid children in the country (Das, *et al.,* 2017). Denguevectors are spreading in areas which were earlier free from the disease in India (Dev, *et al.*, 2014; Dev, *et al*., 2015).

After obtaining above results, it revealed that in Punjab morbidity is equally prevalent in both poor and socioeconomically better communities of the state, due to Dengue Fever. The high incidence of the disease per year is the adverse outcome of rapid unplanned urbanization and varied social practices such as water storage practices and inappropriate disposal of solid waste materials in and around house/premises. Such practices are resulting in the creation of more favorable breeding habitats for *Aedes* mosquitoes. There are many methods to control *Aedes* population such as space sprays, thermal and ultra-low volume fogging by applying insecticides and effective larvicides. The choice of potent insecticidal agents (larvicide and adulticide) is very important for vector control measures. The present study has focused on morphological identification of Dengue vectors, preferential breeding habitats, their correlations with the Dengue cases and molecular characterization of insecticide resistant/susceptible Dengue vector prevalent in Eastern Punjab, India.

Present research work estimated the entomological indices in high, medium and low Dengue risk areas of Punjab during different seasons and their correlations with the Dengue cases reported from the study area. The *Stegomyia* indices are significantly high during monsoon season and suggest a high risk of Dengue transmission as well as also positively correlate with the recorded Dengue cases in the state. These results are comply with previous studies where monsoon positively correlates with Dengue cases (Ferede, *et al*., 2018; Bisht, *et al*., 2019; Tuladhar, *et al.,* 2019). The reason could be during the monsoon season, there is possibility of formation of multiple wet containers due to accumulation of water which allows the vector for multiple sites for oviposition. Beside, larvae multiplies very aggressively in various water storage containers density of larval stages of vector results in higher values for vector indices and vector populations compared to pre-monsoon and post-

monsoon (Kumari, *et al*., 2016; Tuladhar, *et al*., 2019).Thus, rapid activities are necessary to tackle the load of emerging arboviral diseases.

During the current study it was observed that artificial containers such as earthen pots/mud pots, desert coolers and discarded tyres act as conspicuous habitats for *Aedes*. This could be due to the presence of multiple artificial containers that are detected near households. These containers can collect the rainwater for a longer duration, and making these objects appropriate for mosquito oviposition and completion of aquatic life cycle. The other reason could be once the room coolers which are usually kept near the windows of upper floors of schools, offices, households and kept till its reuse in next season. It is place at inaccessible areas so desert coolers act as a potential breeding container for *Aedes aegypti* larvae from its mother foci which spread to secondary foci (Bohra and Andrianasolo, 2001; Patel, *et al.,* 2020).The present results are in confirmation with the earlier studies where earthen/mud pots, desert coolers and tires were the prominent habitats for *Aedes* breeding (Kumari, *et al*., 2011; Singh, *et al*., 2013; Ferdousi, *et al.*, 2015; Patel, *et al.*, 2020). The above findings may be relevant as a focused strategy for vector control measures where such type of water holding receptacles would be targeted to minimize the vector density. In that perspective, source reduction would have the better influence to control disease transmission.

In the resent exploration, three species of *Aedes* were observed *viz Aedes aegypti, Aedes albopictus* and *Aedes vittatus* but *Ae.aegypti* was detected as primary species followed by *Ae.albopictus* and *Ae.vittatus.* Present observation are also in conformity with earlier studies (Tewari, *et al.,* 2004; Ravikumar, *et al.,* 2013; Bhat, *et al*., 2014; Kumari, *et al.,* 2016). For this specific observation reasons could be rapid urbanization in rural areas and introduction of piped water supply leading to water storage practices resulting in shift of breeding of *Ae.aegypti* in rural areas too. These factors have influenced the dispersal pattern of distinct species of *Aedes* mosquitoes because both species like *Ae.albopictus* and *Ae.vittatus* modify their breeding in artificial containers in metropolitan areas of Punjab.

Aedes vittatus has been reported in the SAS Nagar thereby suggesting a high risk of arbovirus transmission. It is also a competent vector of arboviruses and it feeds on human and other animals, due to this broad feeding behavior may limit its vectorial

competency. Therefore, it becomes imperative to check the breeding potential of these vectors in order to control the disease outbreaks. The breeding vessels recognized should be targeted to suitable control measures, like reduction of sources via the elimination of water-holding vessels around living and working areas.

In the present research work, *Aedes aegypti* has been reported as a predominant species responsible for Dengue virus transmission. Similarly, *Ae.albopictus* was also recorded in low proportions from the study areas. The numbers of both species, *Ae.aegypti* and *Ae.albopictus* were higher during monsoon and post-monsoon period than pre-monsoon periods. These observations are in accordance with previous studies in various parts of India (Sharma, *et al.,* 2005; Kumari, *et al.,* 2011; Kumar, *et al.,* 2013; Kanhekar, *et al.,* 2016). The possible reason behind this could be that the rainfall leads to mushrooming of breeding sites which cause higher values for vector indices and vector populations compared to pre-monsoon (Tuladhar, *et al.,* 2019).

Moreover, the present study also revealed that *Ae.aegypti* was high in both rural and urban areas whereas *Ae.albopictus* was abundant in urban than rural areas. The present study is consistent with previous studies (Dev, *et al*., 2014; Tedjou, *et al*., 2019). In Punjab the reason could be that with the growth of cities and reduction of wood area, *Aedes aegypti* is apparently accessing the semi urban and other metropolitan sites in the state. Therefore *Aedes aegypti* has competitively replaced *Aedes albopictus*, as evident through the abundance in both city and rural areas (Dev, *et al*., 2014).

Ae.albopictus known to be a rural species and prefers to breed outdoors (Li, *et al.,* 2014), but in the present study, it was detected to be more abundant in urban areas. It clearly indicates a shift in the breeding preferences from outdoors to peri/intra-domestic containers in urban areas. It may be due to depletion of its preferential breeding places from rural areas of Punjab. Observation from current study reveals that urbanization has substantially contributed to rise in density of such species which successively poses potential threat of Dengue transmission in urban areas. In another study, it has been reported that *Ae.albopictus* has higher blood feeding rate in urban areas as compared to rural areas, more likely due to more host availability (Tsuda, *et al.,* 2006).

Present exploration is the first assessment to identify the existence of *Aedes* mosquitoes and their favored breeding habitats in Punjab. There are only few study reports on the number of Dengue cases from few districts but that lack data on the Dengue vector prevalence and breeding characteristics (Paul, *et al*., 2017; Kaur, *et al*., 2018). Lata, *et al*., 2012 studied the epidemiological and entomological aspect of Dengue in district Shri Muktsar Sahib of Punjab. During their entomological surveillance, the majority of cemented tanks, plastic containers, mud pots and flower vase were reported as breeding sources of *Aedes*. Further they reported all three larval indices for *Aedes* breeding wereobserved high and *Aedes aegypti* was recorded as principal vector for Dengue Fever. They concluded the possible reason behind this was uncovered water containers. Lesser supply of water for short duration and all vessels in households were filled with water permanently due to which breeding of adult mosquitoes was flourishing. Gill, *et al.*, 1997 reported an outbreak of Dengue in Ludhiana district of the state in 1996. Both epidemiological and entomological surveillance were carried out for the investigation of outbreak related factors. Entomological surveillance revealed high house index and container indices in different localities of the city. Breeding was mostly in desert coolers, tin/plastic containers, earthen pots and plastic buckets. Mosquito adults *Ae.aegypti* were collected from rooms and water receptacles.

In India, very few studies have been conducted on the comprehensive analysis of male and female genitalia of *Aedes* species. In the present research work, an effort has been made to understand anatomical differences in the male and female genitalia of *Aedes* species detected in the study areas. The specific structure male hypopygium connected to last three abdominal segment differ greatly among distinct species of *Aedes i.e. Aedes aegypti, Aedes albopictus* and *Aedes vittatus* because anatomical differences of mosquitoes species are more prominent in male hypopygium**.** The anatomical feature “male genitalia” remained the most reliable character for identification upto species level as it is less exposed to harm/destruction. As such, even in damaged specimens it can withstand as a key anatomical tool. Among several species of mosquito anatomical differences are very distinguished in male hypopygium as compared to larval and female characters. Barraud, 1934, who extensively surveyed different areas of India and also provided information related to genitalic features, while illustrating species details. These studies are in agreement of present observation where male and female genitalia characters were reported. This is

the first study where male and female genitalia of *Aedes vittatus* was studied (Kaur, 2014; Shipali and Kirti, 2014; Yadav, *et al.*, 2014).

Present exploration further indicated that *Ae.aegypti* susceptibility/resistance against larvicide Temephos, Bti, Malathion, Deltamethrin and Pyrethrum 2% extract as adulticide respectively as well as mutation in *Ace-1* gene and *VGSC* gene were also recorded from selected areas. Temephos and Bti are the most commonly and continuously used larvicide in almost all districts of Punjab. In fact, Temephos is the only larvicide which can be used in potable water containers as recommended by WHO (WHO, 2006). Due to regular spray of Temephos in both Dengue and Malaria vector control programs by the Health Department and Municipal Corporation of India, Temephos resistance in mosquito population is being developed (Bharati and Saha, 2018). Temephos resistance in *Ae.aegypti* has been recorded in Asia and South America but full susceptibility of *Ae.aegypti* larvae to Temephos have been observed in Cameroon and Gabon**.** Till date, both species of *Aedes* in African countries are susceptible to Temephos and Bti (Kamgang, *et al*., 2011). There are two main types of resistance mechanisms in *Aedes* mosquitoes:-1.Target-site mutation, 2.Metabolic resistance. The most common target site mutation is the knockdown mutation (kdr) on the para voltage gated sodium channel gene (VGSC). Pyrethroids keep VGSC in its open conformation, resulting in repetitive pulses. Furthermore, this prompts nerve cells to generate repeated discharges consequently bring about paralysis and demise. 2. Temephos, an organophosphate larvicide it act by ingestion or contact and it inhibit the normal functioning of acetylcholinesterase (AChE), an enzyme as a consequence, acetylcholine neurotransmitter persists in the synaptic cleft, resulting in the exacerbation of nerve impulse transmission and causes death of insects (Bellinato, *et al*., 2016; Auteri, *et al.,* 2018; Hidajat, *et al*., 2020; Moyes, *et al*., 2021).

The results obtained in the present study revealed that as per susceptibility criteria (WHO, 2016) larvae of *Ae.aegypti* collected from Patiala were probable resistant to Temephos, while other three districts *viz.* Fatehgarh Sahib, SAS Nagar and Ludhiana exhibited resistance against Temephos at diagnostic concentration. Susceptibility tests with larvicide Bti exhibited 100% mortality. These results are in agreement with previous studies where Temephos resistance in *Ae.aegypti* has been recorded earlier (Araujo, *et al.,* 2013; Suter, *et al.,* 2017; Goindin, *et al.,* 2017; Su, *et al.,* 2019).

Present study also revealed that *Aedes* samples collected from all four study areas did not exhibit *Ace -1* mutation and no amino acid changes on the *Ace-1* gene were detected in Temephos resistant mosquitoes. These observations are in conformity with previous studies where mosquitoes were resistant to Temephos but no alternation in amino acid on this gene was observed (Grisales, *et al.*, 2013; Muthusamy and Shivakumar, 2015; Valle, *et al.,* 2019). Resistance status observed among populations was due to intense use of Temephos from the last two decades under Urban Malaria Scheme (UMS) against *Ae.aegypti*. Under this scheme, larvicide Temephos-50% EC is used in these localities. For the curb of mosquito breeding sites Temephoshas been used in all 22 districts of Punjab for many years. This prolonged exposure might be the possible reason behind noticed probable resistance amid the current population of *Ae.aegypti* in district Fatehgarh Sahib, SAS Nagar and Ludhiana. So, the method of application of Temephos should be observed carefully or more related studies on Temephos resistance are required**.** Resistance to Temephos was affected by synaptic enzyme acetylcholinesterase coded by *Ace-1* gene in mosquitoes. Even though mutations on the gene encoding *Ace-1* have been related to Temephos resistance observed in few species of *Culex* and *Anopheles* but in *Ae.aegypti* this mutation has not yet been observed. Lack of *Ace-1* mutations in *Ae.aegypti* in Punjab could be because many of the common mutations, like G119S, are not happen spontaneously; on contrary resistance level may be associated with increased activity of enzymes mixed function oxidases and glutathione-S-transferases, esterases.

On other hand, Pyrethroids are used for the control of mosquitoes (World Health Organization approved) which are highly effective against insects and mammalian toxicity is very low (Rehman, *et al*., 2014). The vast use of insecticides for the curb of vectors has increased problems regarding the development of insecticide resistance, bad impact on the environment and public health (Kushwah, *et al*., 2015). Appearance and distribution of resistance to pyrethroids in *Aedes* mosquitoes is a global concern for the control of vector borne diseases and large environmental alternation of resistance to pyrethroids has been observed. Normally, in Asia and Africa, lesser resistance was observed whereas a higher grade resistance was noticed in Caribbean, Mexico, and South America mosquitoes (Smith, *et al.,* 2016).

In the present study it had been observed that the adult susceptibility test with Pyrethrum fogging revealed that *Ae.aegypti* was observed to be resistant in districts *viz* F.G Sahib with 88% mortality, while specimen from the district Ludhiana revealed probable resistance with 92% mortality whereas remaining two districts namely SAS Nagar and Patiala exhibited 100% mortality. The adult susceptibility test with Pyrethrum mist revealed resistance in two districts *viz* Ludhiana with 84% mortality and Patiala with 88% mortality while probable resistance in specimen from the district of Fatehgarh Sahib with 92% mortality. 100% mortality was observed in districts SAS Nagar. CDC bottle bioassay test in all the selected districts detected 100% mortality towards Deltamethrin and Malathion (WHO, 2016). While the obtained mortality rates from district Ludhiana, Patiala and F.G Sahib were below the WHO suggested 90% mortality rate for resistance. So, the results recommended that the population of *Aedes aegypti* were probable resistant to Pyrethrum in the study localities but 100% susceptible to Deltamethrin and Malathion. These results are in agreement with previous studies where resistance to Pyrethrum but susceptible to Malathion and Deltamethrin in *Ae.aegypti* has been previously reported (Thanispong, *et al*., 2008; Kamgang, *et al*., 2011; Marcombe, *et al*., 2011; Yadav, *et al*., 2015; Amelia-Zep, *et al*., 2018; Pinto, *et al*., 2019). In many endemic countries, developments of resistance in mosquitoes have developed due to application of insecticides in agriculture and public health over the last 50 years. Resistance to Pyrethrum might have occurred gradually because of continued application in public health programmes for several years. Beside this factor, fogging by the local bodies in Dengue affected areas for many years is also resulting in developing resistance against Pyrethrum.

When V1016G domain II was amplified and sequenced, already known V1016G was not observed but L1006S (GCC-GCT: - synonymous mutation and TTG- TCG: - non synonymous mutation) in domain II was observed in both susceptible and resistant mosquitoes. Resistance of *Ae.aegypti* to Pyrethrum may represent the major issue for Punjab India, so extensive investigations on pyrethroid resistance in VGSC domain III and VGSC domain IV are needed to explore the function of present mutation in resistance of pyrethroids. As in the current study VGSC gene mutation was studied at codon V1016G for domain II. The mutation of G119S was also not detected in selected districts. Due to absence of *Ace-1* gene mutations in *Ae.aegypti*, hence, this resistance to the Temephos is most likely to occur

through metabolic mechanisms (Hidajat, *et al*., 2020). Further, extensive research work should be planned on Temephos resistance related to metabolic mechanisms.

Another important finding from study that V1016G gene point mutation revealed no correlation with *Aedes aegypti* towards Pyrethrum resistance in all four selected areas. These findings are in consistent with previous studies where *Ae.aegypti* populations was resistant to permethrin but no VGSC gene mutation was detected (Kushwah, *et al*., 2015; Amelia-Yep, *et al*., 2018; Hamid, *et al.*, 2018; Soni, *et al.*, 2018). Resistance of *Aedes aegypti* against insecticide is expanding into sparsely populated sites. Consequently, there is a requirement of more surveillance so as to slow down development of resistance in *Aedes aegypti*. Routine supervision is needed for effective vector control management as the appearance of probable resistance in Punjab and simultaneously how to reduce such Dengue outbreak so the general community should become aware about Dengue. For reduction of vector population health authority has to consider other insecticides such as Malathion and Deltamethrin in their control Programme.

CHAPTER-VII

CONCLUSION

Dengue Fever has appeared as a major mosquito borne viral disease in the world. After carrying out present research execution it has been concluded that *Aedes aegypti* and *Aedes albopictus* act as Dengue vectors in both urban and rural areas whereas *Aedes vittatus* is present only in urban areas of the State of Punjab, thereby demonstrating wide range distribution of these vectors. The breeding habitats for proliferation of *Aedes* are usually man-made creations resulting from lack of awareness among the population. Such breeding habitats can be controlled effectively by removal of the stagnant water from coolers, mud pots, plastic containers and other water-holding containers in and around the household and proper disposal of discarded tires so as to achieve Dengue vector control. Since there are no vaccines or drugs available for treatment of Dengue, therefore exclusive prevention of disease depends on vector control. To achieve this entomological surveillance before and after control of vector is necessary to confirm the efficacy of the control measures undertaken. In addition to this, routine entomological surveillance is required for the planning and implementation of an effective vector control program.

Furthermore from specific research exploration it had been scrutinized that distinct mortality among the larvae was detected in four selected districts at Temephos dose 0.025mg/l. Therefore, it had been detected that larvae of *Aedes aegypti* in three districts of Punjab *viz.* SAS Nagar, Ludhiana and Fatehgarh Sahib were resistant to Temephos while in district Patiala 'probable resistance' was detected**.** The *Ae.aegypti* larvae were least Temephos susceptible in district SAS Nagar. Resistance might have developed over time due to continuous exposure to Temephos under Urban Malaria Scheme (UMS) since 1980. *Aedes aegypti* resistant to Temephos was observed to be 100% susceptible to Bti (0.00375mg/ml) at diagnostic concentration.

On other hand, different lethality amid the adults was also observed in four study districts at WHO recommended Pyrethrum dose. It revealed that adults of *Aedes aegypti* were reported as moderately resistant to Pyrethrum in districts Patiala, Ludhiana and F.G.Sahib. Adults were least pyrethrum susceptible in district Ludhiana and specimens from district SAS Nagar revealed complete susceptibility with 100% mortality. This resistance might have been occurred gradually because continue application of adulticide in Public Health Programmes. Beside this factor, fogging by the local bodies, in Dengue affected areas for many years has also resulted in

developing resistance against Pyrethrum. *Aedes aegypti* resistant to Pyrethrum was noticed to be 100% susceptible to Malathion (0.2mg/ml) and Deltamethrin (0.4mg/ml) at diagnostic concentration. These insecticides are highly effective against mosquitoes.

Genomic analysis indicated that no change in *Ace-1* of *Aedes aegypti* had been detected hence observed resistance against Temephos could be through the modulation of the metabolic pathway. Similarly, no nucleotide change had been observed in VGSC gene of *Aedes aegypti* in all selected districts. Therefore, for the detection of increasing resistance in adult *Aedes aegypti,* further, exploration of polymorphisms in the various domains of the VGSC gene should be analysed with appropriate methodology.

Overall conclusion drawn from concerned research exploration depicted that obtained data will serve as a baseline for the control of Dengue vectors. It also emphasise the importance of entomological surveillance which should be conducted at potential sites and the fact should be used to predict the chances of upcoming outbreaks of Dengue Fever. The generally-utilized larval index (house, container and breteau) are helpful for ascertaining common spread, cyclic variation and major habitats of *Aedes* larvae.

Furthermore, with regards to disease control, the lack of community coordination in prevention of mosquito breeding was felt as a major hurdle. There is a need to engage the community through targeted campaigns for better participation of the masses. Government and people should work hand in glove for better collaboration to reduce vector breeding thus resulting in Dengue control. Rigid regulations in source reduction should be implemented to reduce the breeding sites of the *Aedes* mosquitoes. For prevention of an impending outbreak and spread of resistance in near future, it is important to explore time-to-time larval and adult bioassay to understand and manage insecticide resistance. Further research work should be carried out to explore the metabolic resistance mechanism and target site resistance to identify mutations that are responsible for Temephos and Pyrethrum resistance.

BIBLIOGRAPHY

Abai, M.R., Hanafi-Bojd, A.A., Vatandoost, H. (2016). Laboratory Evaluation of Temephos against *Anopheles stephensi* and *Culex pipiens* Larvae in Iran. *Journal of arthropod-borne diseases*,**10(4),** 510–518.

Abbasi, A., Abbas, K., Arooj, S., Habib, N., Aziz, W., and Ashaq, A. (2016). Dengue Fever: A Statistical Analysis Regarding Awareness about Dengue among University Students in Azad Kashmir. *Journal of Healthcare Communications*, **2**, 2472-1654.

Abilio, A.P., Abudasse, G., Kampango, A., Candrinho, B., Sitoi, S., Luciano, J., Tembisse, D., Sibindy, S., Almeida, A.P.G.D., Garcia, G.A., David, M.R., Freitas, R.M.D., Gudo, E.S. (2018). Distribution and breeding sites of *Aedes aegypti* and *Aedes albopictus* in 32 urban/peri-urban districts of Mozambique: implication for assessing the risk of arbovirus outbreaks. *PLoS neglected tropical Diseases***, 12(9**), e0006692.

Abramides, G.C., Roiz, D., Guitart, R., Quintana, S., Guerrero, I., and Giménez, N. (2011). Effectiveness of a multiple intervention strategy for the control of the tiger mosquito (*Aedes albopictus*) in Spain.*Transaction of the Royal Society Tropical Medicine Hygiene,* **105(5)**, 281–288.

Achee, N.L., Gould, F., Perkins, T.A., Reiner, R.C.J., Morrison, A.C., Ritchie, SA, Gubler, D.J., Teyssou, R., Scott T.W. (2015). A Critical Assessment of Vector Control for Dengue Prevention. *PLoS neglected tropical Diseases,* **9(5),** e0003655.

Agramonte, N.M., Bloomquist, J.R., Bernier, U.R. (2017). Pyrethroid resistance alters the blood-feeding behavior in Puerto Rican *Aedes aegypti* mosquitoes exposed to treated fabric. *PLoS neglected tropical Diseases*, **11(9),** e0005954.

Aguirre-Obando, O. A., Pietrobon, A. J., Bona, A.C.D. and Navarro-Silva, M.A. (2016).Contrasting patterns of insecticide resistanc and knockdown resistance (kdr) in *Aedes aegypti* populations from Jacarezinho (Brazil) after a Dengue Outbreak. *Revista Brasileira de Entomologia*, **60(1)**, 94–100.

Ahmed, R.M.H., Hassan, S.M. (2019). Seasonal indices of *Aedes aegypti* (Diptera: Culicidae) in an urban area of Kassala City, Sudan, 2014-2015. *European Academic Research,* **1(10),** 5933 – 5948.

Akiner, M.M., Demirci, B., Babuadze, G., Robert, V., Schaffner, F. (2016). Correction: Spread of the Invasive Mosquitoes *Aedes aegypti* and *Aedes albopictus* in the Black Sea Region Increases Risk of Chikungunya, Dengue, and Zika Outbreaks in Europe. *PLoS neglected tropical diseases*, **10(4)**, e0004764.

Alvarez, L.C., Ponce, G., Oviedo, M., Lopez, B.E., Flores, A. (2013). Resistance to Malathion and Deltamethrin in *Aedes aegypti* (Diptera: Culicidae) From Western Venezuela. *J. Med. Entomol*, **50(5),** 1031-1039.

Amelia-Yap, Z. H., Chen, C. D., Sofian-Azirun, M., & Low, V. L. (2018). Pyrethroid resistance in the Dengue vector *Aedes aegypti* in Southeast Asia: present situation and prospects for management. *Parasites & vectors*, **11(1),** 332. https://doi.org/10.1186/s13071-018-2899-0.

Andrade, P.D.S., Urbinatti, P.R., Coelho, R.D.R., Almeida, R.M.M.S., Ribeiro, S.S., Lima-Camara T.N.d., (2019). Parity and gonotrophic discordance of females of *Aedes aegypti*and *Aedes albopictus* (Diptera: Culicidae) in the city of São Paulo SP, Brazil. *Journal of Vector Ecology*, **44(2)**, 233-240.

Araujo, A.P.d., Paiva, M.H.S., Cabral, A.M., Cavalcanti, A.E.H.D., Pessoa, L.F. F, Diniz, D.F.A., Helvecio, E., Silva, E.V.G.D., Silva, N.M., Anastácio, D.B., Pontes, C., Nunes, V., Souza, M.D.F.M., Magalhães, F.J.R., Santos, M.A.V.D. M. and Ayres, C.F.J. (2019). Screening *Aedes aegypti* (Diptera: Culicidae) Populations from Pernambuco, Brazil for Resistance to Temephos, Diflubenzuron, and Cypermethrin and Characterization of Potential Resistance Mechanisms. *Journal of Insect Science,* **19(3),** 12-16.

Arduino, M.D.B. (2014). Assessment of *Aedes aegypti* Pupal Productivity during the Dengue Vector Control Program in a Costal Urban Centre of Sao Paulo State, Brazil. *Journal of Insects*, 1-9.

Arora, P., Arora, M., Sharma, V., Kotwal, A. (2017). Dengue: awareness, preventive practices and water storage behavior in an urban community of Delhi. *International Journal of Community Medicine Public Health*, **4(12)**, 4460-4465.

Arslan, A., Uzair, M., Mushtaq, S., Zakki, A.B., Hammad, M., Bhatti, A. (2015). Comparison of susceptibility status of laboratory and field population of *Aedes aegypti* against Temephos in Rawalpindi.*Journal of Entomology and Zoology Studies*, **3(4)**, 374-378.

Ault, S.K. (1994). Environmental management: a re-emerging vector control strategy. The *American Journal Tropical Medicine Hygiene*, **50(6)**, 35– 49.

Auteri, M., La Russa, F., Blanda, V. and Torina, A. (2018). Insecticide Resistance Associated with kdr Mutations in *Aedes albopictus*: An Update on Worldwide Evidences. *Biomed Res Int*, **3098575**.doi: 10.1155/2018/3098575.

Ayorinde, A., Oboh, B., Oduola, A., Otubanjo, O. (2015). The Insecticide Susceptibility Status of *Aedes aegypti* (Diptera: Culicidae) in Farm and Nonfarm Sites of Lagos State, Nigeria. *Journal of Insect Science*, **15(1),** 75.

Back, A.T. and Lundkvist, A. (2013). Dengue viruses-an overview. *Infect Ecol Epidemiol*.**3,** 19839.

Balakrishnan, N., Katyal, R., Mittal, V., Chauhan, L.S. (2015). Prevalence of *Aedes aegypti* - The vector of Dengue/ Chikungunya fevers in Bangalore City, Urban and Kolar districts of Karnataka state. *J Commun Dis*, **47(4),** 19-23.

Ban, D., Sladonja, B., Luki, M., Luki, I., Lušeti, V., Ganic, K.K. and Žnidar, D. (2010). Comparison of pyrethrins extraction methods efficiencies. *African Journal of Biotechnology*, **9 (18)**, 2702-2708.

Bansal, S.K., Singh, K.V. (2003). Insecticide susceptibility status of *Aedes aegypti* to DDT and dieldrin in desert and non-desert parts of Rajasthan. *Journal of Environmental Biology*, **24(1),** 113-116.

Barnes, R.D. (2021). "Arthropod". Encyclopedia Britannica.https://www.britannica.com/animal/arthropod.

Barraud, P.J. (1934). The fauna of British India, including Ceylon and Burma.Diptera vol. V. Family Culicidae, Tribes Megarhinini & Culicini Taylor and Francis, London.**463**, 8.

Baruah, S. and Dutta, P. (2013). Seasonal prevalence of *Aedes aegypti* in urban and industrial areas of Dibrugarh district, Assam. *Tropical Biomedicine*, **30(434),** 43.

Baskera, P., Kannanb, P., Porkaipandianab, T. R., Saravananab, S., Sridharanab, S., Kadhiresanab, M. (2013). Study on Entomological Surveillance and its Significance during a Dengue Outbreak in the District of Tirunelveli in Tamil Nadu, India. *Osong Public Health and Research*, **4(3),** 152-158.

Bellinato, D.F., Viana-Medeiros, P.F., Araújo, S.C., Martins, A.J., Lima, J.B.P. and Valle, D. (2016). Resistance Status to the Insecticides Temephos, Deltamethrin, and Diflubenzuron in Brazilian *Aedes aegypti* Populations. *BioMed research international*, **8603263,** 12.

Bharati, M. and Saha, D. (2018). Multiple insecticide resistance mechanisms in primary Dengue vector, *Aedes aegypti* (Linn.) from Dengue endemic districts of sub-Himalayan West Bengal, India. *PLoS One*, **13(9),** e0203207. .

Bhaskar, R.B., George, B. (2010). Breeding patterns of *Aedes (Stegomyia) albopictus* in periurban areas of Calicut, Kerala, India. *Southeast Asian Journal of Tropical Medicine Public Health*, **41(3)**, 536– 40.

Bhat, M.A., and Krishnamoorthy, K. (2014). Entomological investigation and distribution of *Aedes* mosquitoes in Tirunelveli, Tamil Nadu, India. *International Journal of Current Microbiology Application Sciences*, **3(10)**, 253- 60.

Bisht, B., Kumari, R., Nagpal, B.N., Singh, H., Gupta, S.K., Bansal, A.K. and Tuli, N.R. (2019). Influence of environmental factors on Dengue Fever in Delhi. *International Journal of Mosquito Research*, **6(2),** 11-18.

Bisset, J. (2002). Uso Correcto de Insecticidas: Control de la resistencia. *Revista Cubana de Medicina Tropical*, **54(3),** 202–219.

Bohra, A. and Andrianasolo, H. (2001). Application of GIS in Modeling of Dengue Risk Based on Sociocultural Data: Case of Jalore, Rajasthan, India. *Dengue Bulletin*, **25(1)**, 92.

Bowman, L.R.,Runge-Ranzinger, S. and McCall, P. J. (2014). Assessing the Relationship between Vector Indices and Dengue Transmission: a systematic review of the evidence. *PLoS Neglected Tropical Diseases,* **8(5),** e2848.

Brogdon, W. G. (1998). Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay.

Brogdon, W., Chan, A. (2010). Guideline for evaluating insecticide resistance in vectors using the CDC bottle bioassay. *Centers for Disease Control and Prevention, USA: CDCAtlanta*.

Brown, A.W. (1986). Insecticide resistance in mosquitoes: a pragmatic review. *Journal of the American Mosquito Control Association*, **2(2)**, 123–140.

Brown, J.E., Evans, B.R., Zheng, W., Obas, V., Barrera-Martinez, L., Egizi, A., Zhao, H., Caccone, A. and Powell, J.R. (2014). Human impacts have shaped historical and recent evolution in *Aedes aegypti*, the Dengue and Yellow Fever mosquito. *Evolution,* **68(2),** 514–525.

Caffery, M.C. and Nauen, R. (2011). Prevention and management of Insecticide Resistance in vector of Public health importance. *IRAC*, **2**, 11-14.

Calzolari, M. (2016). Mosquito-borne diseases in Europe: an emerging public health threat. *Reports in Parasitology***, 5**, 1-12.

Camara, D.C.P., Codeço, C.T., Juliano, S.A., Lounibos, L. P., Riback, T. I. S., Pereira, G.R., Honorio, N.A. (2016). Seasonal Differences in Density but Similar Competitive Impact of *Aedes albopictus* (Skuse) on *Aedes aegypti* (L.) in Rio de Janeiro, Brazil. *PLoS One*, **11(6),** e0157120.

Capeding, M.R., Tran, N.H., Hadinegoro, S.R.S, Ismail, H.I.H.J.M., Chotpitayasunondh, T., Chua, M.N., Luong ,C.Q., Rusmil, K., Wirawan, D.N., Nallusamy,R., Pitisuttithum, P., Thisyakorn, U., Yoon, I.K., Vliet, D.V.D, Langevin, E., Laot, T., Hutagalung, Y., Frago, C., Boaz,M., Wartel,

T.A., Tornieporth, N.G., Saville, M., Bouckenooghe, A. (2014). Clinical efficacy and safety of a novel tetravalent Dengue vaccine in healthy children in Asia: A Phase 3, randomized, observer-masked, placebo-controlled trial. *The Lancet*, **384(9951),** 1358–1365.

Caraballo, H. and King, K. (2014). Emergency Department Management of Mosquito-Borne Illness: Malaria, Dengue, and West Nile Virus. *Emergency Medicine Practice*, **16(5), 1-23.**

Castro, M., Sanchez, L., Perez, D., Carbonell, N., Lefèvre, P., Vanlerberghe, V., Stuyft, P.V.D. (2012). A community empowerment strategy embedded in a routine Dengue vector control programmea cluster randomised controlled trial. *Transactions of Royal Society Tropical Medicine and Hygiene*, **106(5)**, 315–21.

Cecilia, D. (2014).Current status of Dengue and Chikungunya in India.WHO *South East Asia Journal of Public Health***, 3(1)**, 22.

Chakravarti, A., Arora, R., and Luxemburger, C. (2012). Fifty years of Dengue in India. *Transactions of the Royal Society of Tropical Medicine Hygiene*, **106(5)**, 273–282.

Chan, A., Chiang, L.P., Hapuarachchi, H.C., Tan, C.H., Pang, S.C., Lee, R., Lee, K.S., Ng, L.C. and Lam-Phua, S.G. (2014). DNA barcoding: complementing morphological identification of mosquito species in Singapore Chan. *Parasites & Vectors***, 7(1),** 1-12.

Charrel, R. N., Berenger, J.M., Laroche, M., Ayhan, N., Bitam, D.P. and Parola, P. (2018). Neglected vector-borne bacterial diseases and arboviruses in the Mediterranean area. *New Microbe and New Infect*, **26**, 31–36.

Chatterjee, M., Ballav, S., Maji, A.K., Basu, N., Sarkar, B.C., Saha, P. (2018). Polymorphisms in voltage-gated sodium channel gene and susceptibility of *Aedes albopictus* to insecticides in three districts of northern West Bengal, India. *PLoS Neglected Tropical Diseases*, **12(1),** e0006192.

Chen, C.D., Nazni, W.A., Lee, H.L., Seleena, B., Mohd Masri, S., Chiang, Y.F. and Sofian-Azirun, M. (2006). Mixed breeding of *Aedes aegypti* (L.) and *Aedes albopictus* Skuse in four Dengue endemic areas in Kuala Lumpur and Selangor, Malaysia.*Tropical biomedicine*, **23(2),** 224–227.

Chepkorir, E., Lutomiah, J., Mulwa, F., Mutisya, J., Limbaso, K., Orindi, B. and Sans, R. (2014).Vector competence of *Aedes aegypti* population from Kilifi and Nairobi for Dengue 2 virus and the influence of temperature. *Parasite and Vectors*, **7(1),** 1-8.

Chouin-Carneiro, T., Vega-Rua, A., Vazeille, M., Yebakima, A., Girod, R., Goindin, D., Dupont-Rouzeyrol, M., Lourenço-de-Oliveira, R., Failloux, A.B. (2016). Differential Susceptibilities of *Aedes aegypti* and *Aedes albopictus* from the Americas to Zika Virus. *PLoS Neglected Tropical Diseases*, **10(3**), e0004543.

Christophides, G.K. and Crisanti, A. (2013). Vector and vector-borne disease research: need for coherence, vision and strategic planning. *Pathog Glob Health*, **107(8),** 385–386.

Chrustek, A., Iganska-Iwan, H., Dziembowska, I., Bogusiewicz, J., Wróblewski, M., Cwynar, A. and Dorota, O.S. (2018). Current Research on the Safety of Pyrethroids Used as Insecticides. *Medicina*, **54(4),** 61.

Clements, A.N. (2012). Arboviruses: Characteristics and concepts. In: Clements AN, editor. The biology of mosquitoes: Transmission of viruses and interactions with bacteria. Wallingford: CAB International, 125.

Colovic, M.B., Krstic, D.Z., Lazarevic-Pasti, T.D., Bondzic, A.M., Vasic, V.M. (2013). Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. *Current neuropharmacology*, **11(3**), 315–335.

Corbel, V. and N'Guessan, R. (2013). Distribution, Mechanisms, Impact and Management of Insecticide Resistance in Malaria Vectors: A Pragmatic Review. *In Anopheles Mosquitoes-New insights into malaria vectors.* IntechOpen.http://dx.doi.org/10.5772/56117.

Costa, E.A.P.D.A., Santos, E.M.D.M., Correia, J.C. and Albuquerque, C.M.R.D. (2010). Impact of small variations in temperature and humidity on the reproductive activity and survival of *Aedes aegypti* (Diptera, Culicidae). *Revista Brasileira de Entomologia*, **54(3),** 488–493.

Couper, L., Farner, J., Caldwell, J., Childs, M., Harris, M., Kirk, D., Nova, N., Shocket, M., Skinner, E., Uricchio, L., Exposito-Alonso, M., and Mordecai, E. (2020). How will mosquitoes adapt to climate change? *Authorea Preprints*. DOI:10.22541/au.160589900.06282166/v1.

Cuervo-Parra, J. A., Cortés, T. R., and Ramirez-Lepe, M. (2016). Mosquito-Borne Diseases, Pesticides Used for Mosquito Control, and Development of Resistance to Insecticides. *Rijeka: InTechOpen*, 111-34.

Dahmana, H., and Mediannikov, O. (2020). Mosquito-Borne Diseases Emergence/Resurgence and how to Effectively Control Biologically.*Pathogens***, 9(4)**, 310.

Das, M., Gopalakrishnan, R., Kumar, D., Gayan, J., Baruah, I., Veer, V., and Dutta, P. (2014). Spatiotemporal distribution of Dengue vectors & identification of high risk zones in district Sonitpur, Assam, India. *Indian Journal of Medical Research,* **40(2),** 278–284.

Das, M.K., Singh, R.K., Lalc, R.K., &. Dhiman, R.C. (2011). Susceptibility of *Aedes aegypti* to insecticides in Ranchi city, Jharkhand state, India. *Dengue Bulletin*, **35,194.**

Das, S., Sarfraz, A., Jaiswal, N., Das, P. (2017). Impediments of reporting Dengue cases in India. *Journal of Infection and Public Health*, **10(5),** 494-498.

Delatte, H., Gimonneau, G., Triboire, A., Fontenille, D. (2009). Influence of temperature on immature development, survival, longevity, fecundity, and gonotrophic cycles of *Aedes albopictus*, vector of Chikungunya and Dengue in the Indian Ocean. *Journal of medical entomology*, **46(1),** 33–41.

Deltamethrin technical fact sheet npic national pesticide information center, (2011). npic@ace.orst.edu.

Dev V, Mahanta N, Baruah, B.K. (2015). Dengue, an emerging infection in Assam, Northeast India. *Tropical biomedicine,* **32 (4),** 796-799.

Dev, V., Khound, K. and Tewari, G.G., (2014). Dengue vectors in urban and suburban Assam, India: entomological observations. *WHO South-East Asia Journal of Public Health*, **3(1),** 51–59.

Dhiman, S., Rabha, B., Yadav, K., Baruah, I. and Veer, V. (2014). Insecticide susceptibility and Dengue vector status of wild *Stegomyia albopicta* in a strategically important area of Assam India. *Parasite and vector*, **7(1)**, 1-5.

Diagne, C.T., Faye, O., Guerbois, M., Knight, R., Diallo, D., Faye, O., Ba, Y., Dia, I., Weaver, S.C., Sall, A.A. and Diallo, M. (2014). Vector Competence of *Aedes aegypti* and *Aedes vittatus* (Diptera: Culicidae) from Senegal and Cape Verde Archipelago for West African Lineages of Chikungunya Virus. *The American Journal of Tropical Medicine and Hygiene*, **91(3),** 635–641.

Diouf, K. andNour, N.M. (2017). Mosquito-Borne Diseases as a Global Health Problem: Implications for Pregnancy and Travel. *Obstetrical Gynecological Survey*, **72(5),** 309-318.

Dusfou, I., Thalmensy, V., Gaborit, P., Issaly, J., Carinci, R. and Girod, R. (2011). Multiple Insecticide resistance in *Aedes aegypti* population compromises the effectiveness of Dengue vector control in French Guiana. *Memorias. Do Instituto. Oswaldo Cruz,* **106,** 346-352.

Duvallet, G., Vincent, R., (2018). Skin and Arthropod Vectors.

El-Badry, A.A. and Al-Ali, K.H. (2010). Prevalence and Seasonal Distribution of Dengue Mosquito, *Aedes aegypti* (Diptera: Culicidae) in Al-Madinah Al-Munawwarah, Saudi Arabia. *Journal of Entomology,* **7(2),** *80-88.*

Elbers, A.R.W., Koenraadt, C.J.M. and Meiswinkel, R. (2015). Mosquitoes and Culicoides biting midges: vector range and the influence of climate change. *Rev. Sci. Tech,* **34 (1),** 123-137.

El-Wakeil, N. (2013). Botanical Pesticide and their mode of action. *Gesunde Pflanzen,* **65**, 125-149.

Faucon, F., Dusfour, I., Gaude, T., Navratil, V., Boyer, F., Chandre, F., Sirisopa, P.,Thanispong, K., WJuntarajumnong, W., Poupardin, R., Chareonviriyaphap,

T., Girod, R., Corbel, V., Reynaud, S. and David, J.P. (2015). Identifying genomic changes associated with insecticide resistance in the Dengue mosquito *Aedes aegypti* by deep targeted sequencing. *Genome Research*, **25**, 1347-1359.

Ferdousi, F., Yoshimatsu, S., Ma, E., Sohel, N., Wagatsuma, Y. (2015). Identification of Essential Containers for *Aedes* Larval Breeding to Control Dengue in Dhaka, Bangladesh. *Tropical Medicine and Health*, **43(4**), 253-264.

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. *Epidemiology and Health*, **40**, e2018015.

Ferreira-de-Lima, V. H. and Lima-Camara, T.N.L. (2018). Natural vertical transmission of Dengue virus in *Aedes aegypti* and *Aedes albopictus:* a systematic review. *Parasites & Vectors*, **11**(1), 77.

Field, L.M., Davies, T.G., O'reilly, A.O., Williamson, M.S., Wallace, B.A. (2017). Voltage-gated sodium channels as targets for pyrethroid insecticides. *European Biophysics Journal*, **46(7)**, 675-679.

Foster, W.A. and Walker, E.D. (2002). Mosquitoes (Culicidae) Medical and Veterinary Entomology.

Fradin, M. S. (2019). Travel Medicine (Fourth Edition) Science direct, *Insect Protection.*

Ganeshkumar, P., Murhekar, M.V., Poornima, V., Saravanakumar, V., Sukumaran, K., Anandaselvasankar, A., John, D., Mehendale, S.M. (2018). Dengue infection in India: A systematic review and meta-analysis. *PLoS Neglected Tropical Disease*, **12(7),** e0006618.

George, L., Lenhart, A., Toledo, J., Lazaro, A., Han, W.W., Velayudhan, R., Ranzinger, S.R. and Horstick, O. (2015). Community-Effectiveness of Temephos for Dengue Vector Control: A Systematic Literature Review. *PLoS Neglected Tropical Diseases*, **9(9),** e0004006.

Getachew, D., Tekie, H., Gebre-Michael, T., Balkew, M. and Mesfin, A. (2015). Breeding Sites of *Aedes aegypti*: Potential Dengue Vectors in Dire Dawa, East Ethiopia. *Interdisciplinary Perspectives on Infectious Diseases*, **706276**, 8.

Giddings, M. M., Magara, Y., & Ohanian, E. (2009).Temephos in Drinking-water: Use for Vector Control in Drinking-water Sources and Containers. 1-7. WHO. URL: http://www. who.int/water_sanitation_health/dwq/chemicals/Temephos. pdf.

Gile, S., Johns, A. and Thai, V. (2013). Integrated Pest Management for Mosquito Control in Massachusetts (thesis) Massachusetts: Worcester Polytechnic Institute.

Gill, K.S., Bora, D., Bhardwaj, M., Bandyopadhyay, S., Kumar, K. and Katyal, R. (1997). Dengue Outbreak in Ludhiana (Punjab), India, 1996. *Dengue Bull*, ***21,*** 47–51.

Goindin, D., Delannay, C., Gelasse, A. Ramdini, C., Gaude, T., Faucon, F. and Fouque, F. (2017). Levels of insecticide resistance to Deltamethrin, Malathion, and Temephos, and associated mechanisms in *Aedes aegypti* mosquitoes from the Guadeloupe and Saint Martin islands (French West Indies). *Infectious Disease Poverty*, **6(1),** 38.

Grisales, N., Popuardin, R., Gomez, S., Fonesca-Gonalez, I., Ranson, H. and Lenhart, A. (2013). Temephos Resistance in *Aedes aegypti* in Colombia Compromises Dengue Vector Control. *PLoS Neglected Tropical Disease*, **7(9),** e2438.

Gubler, D. (2011). Dengue, urbanization and globalization: The unholy trinity of the 21st century. *Int J Infect Dis*, **16,** 1-2.

Gubler, D.J. (1998). Dengue and Dengue Hemorrhagic Fever.*Clinical microbiology reviews.***11(3)**, 480-96.

Guglielmone, A., Castelli, M., Volpogni, M., Medus, P., Anziani, O. and Mangold, A. (2001). Comparación de la concentración letal 50 de diazinón y cipermetrina para Haematobia irritans (Diptera: Muscidae) entre áreas de producción de leche o carne de Santa Fe y Entre Ríos, Argentina. *Revista de Medicina Veterinaria*, **82,** 209–211.

Gunathilaka, N., Upulika, H., Udayanga, L., Amarasinghe, D. (2019). Effect of Larval Nutritional Regimes on Morphometry and Vectorial Capacity of *Aedes aegypti* for Dengue Transmission. *Bio Med Research International,* **3607342**, 11.

Gunther, V.J., Putnak, R., Eckels, K.H., Mammen, M.P., Scherer, J.M., Lyons, A., Sztein, M.B. and Sun, W. (2011). A human challenge model for Dengue infection reveals a possible protective role for sustained interferon gamma levels during the acute phase of illness. *Vaccine*, **29(22),** 3895–3904.

Guo, Y.H., Lai, S.J., Huang, Q., Ren, D.S., Zou, J.H., Liu, Q.Y., Zhang H.Q., (2016). Coexistence of *Aedes aegypti* and *Aedes albopictus* in Jinghong City, Yunnan Province: A Survey of *Aedes aegypti* Invasion. *J Trop Dis,* **4(5),**1-6.

Gupta, B.P., Singh, S., Kurmi, R., Malla, R., Sreekumar, E. and Manandhar, K. D. (2015). Re-emergence of Dengue virus serotype 2 strains in the 2013 outbreak in Nepal. *Indian Journal of Medical Research,* **142(1),**1–6.

Gupta, E. and Ballani, N. (2014). Current perspectives on the spread of Dengue in India. *Infection and Drug Resistance*, **7**, 337–342.

Gupta, E., Dar, L., Kapoor, G. and Broor, S. (2006). The changing epidemiology of Dengue in Delhi, India. *Virology Journal,* **3(1)**, 92.

Gupta, N., Srivastava, S., Jain, A. and Chaturvedi, U. (2012). Dengue in India.*Indian Journal of Medical Research,* **136(3),** 373–390.

Guzman, M., Halstead, S. and Artsob, H. (2010). Dengue: a continuing global threat. *Nat Rev Microbiol;* **8**, 7–16.

Haider, M. and Turner J. (2015). Variables that May Affect the Transmission of Dengue- A Case Study for Health Management in Asia. *Topics in Public Health,***77**.

Hamid, P.H., Ninditya, V.I., Prastowo, J., Haryanto, A., Taubert, A. and Hermosilla, C. (2018). Current Status of *Aedes aegypti* Insecticide Resistance Development from Banjarmasin, Kalimantan, Indonesia. *BioMed Research International,* **1735358**, 7.

Harris, A. F., Rajatileka, S. and Ranson, H. (2010). Pyrethroid resistance in *Aedes aegypti* from Grand Cayman. *The American Journal of Tropical Medicine and Hygiene*, **83(2)**, 277–284.

Hartberg, W.K. and Craig, J.R.G.B. (1973).Gene controlled morphological differences in male genitalia of *Aedes aegypti* and *Aedes Mascarensis. Reprinted from mosquito news,* **33(2),**206-14.

Hasan, S., Jamdar, S.F., Alalowi, M., Ageel, A. and Beaiji, A.S.M., (2016). Dengue virus: A global human threat: Review of literature. *Journal of International Society of Preventive Community Dentistry,* **6(1),** 1–6.

Hashim, N.A., Ahmad, A.H., Talib, A., Athaillah, F. and Krishnan, K.T. (2018). Co-breeding Association of *Aedes albopictus* (Skuse) and *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) in Relation to Location and Container Size. *Tropical Life Sciences Research,* **29(1),** 213–227.

Hasmiwati, H. and Supargiyono, S. (2018). Genotyping of kdr allele in insecticide resistant-*Aedes aegypti* populations from West Sumatra, Indonesia. *BiodiversitasJournal of Biological Diversity*, **19(2)**, 552-558

Hasmiwati, Rusjdi, S.R. and Nofita, E. (2018). Detection of Ace-1 gene with insecticides resistance in *Aedes aegypti* populations from DHF-endemic areas in Padang, Indonesia. *Biodiversitas Journal of Biological Diversity,* **19(1),** 31-36.

Hawley, W.A. (1988). The biology of *Aedes albopictus. Journal of the American Mosquito Control Association*, **1**, 1-39.

Hidajat, M.C., Dharmana, E., Prihatin, M.T., Ambargarjito, T., (2020). Molecular Resistance Status of *Aedes aegypti* to the Organophosphate and Pyrethroid Insecticides in Central Sulawesi and East Nusa Tenggara Provinces, Indonesia. *In 5th Universitas Ahmad Dahlan Public Health Conference (UPHEC 2019),* **24,** 122-127**.**

Higa, Y. (2011). Dengue vectors and their spatial distribution. *Tropical Medicine and Health*, **39(4)**, 17-27.

Hodgson, E. (2012). Progress in Molecular Biology and Translational Science, Toxicology and Human Environments Summary and Conclusions. *Toxicology and Human Environments,* **112**, 417-421.

Honorio, N.A., Silva, W.C., Leite, P.J., Gonçalves, J.M., Lounibos, L.P. and Lourenço, O.R. (2003). Dispersal of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in an Urban Endemic Dengue Area in the State of Rio de Janeiro, Brazil. *Memórias do Instituto Oswaldo Cruz,* **98(2),** 191-198.

https://pubchem.ncbi.nlm.nih.gov/compound/Deltamethrin.

https://pubchem.ncbi.nlm.nih.gov/compound/Malathion.

Huang, M.Y. (1979). The subgenus *Stegomyia* of *Aedes* in the Oriental region with keys to the species (Diptera: Culicidae). Contribution of the American Entomological Institute.**15 (6)**, 1 –79.

Ishak, I.H., Kamgang, B., Ibrahim, S.S., Riveron, J.M., Irving, H. and Wondji, C.S. (2017). Pyrethroid resistance in Malaysian populations of Dengue Vectors *Aedes aegypti* is mediated by Cyp9 Family of Cytochrome P450 Genes. *PLoS Neglected Tropical Disease*, **11(1)**, e0005302.

Izri, A., Bitam, I. and Charrel, R.N. (2011). First entomological documentation of *Aedes* (*Stegomyia*) *albopictus* (Skuse, 1894) in Algeria.*Clinlical Microbiology Infection,* **17(7),** 1116–1118.

Jimenez-Alejo, A., Morales-Pérez, A., Nava-Aguilera, E., Flores-Moreno, M., Apreza-Aguilar, S., Carranza-Alcaraz, W., Guzmán, A.J.C., Salas, I.F., Ledogar, R.J., Cockcroft, A. and Andersson, N. (2017). Pupal productivity in rainy and dry seasons: findings from the impact survey of a randomised controlled trial of Dengue prevention in Guerrero, Mexico. *BMC Public Health*, **17(1)**, 71-78.

Jourdain, F., Picard, M., Sulesco, T., Haddad, N., Harrat, Z., Sawalha, S.S., Günay, F., Kanani, K., Shaibi, T., Akhramenko, D., Sarih, M., Velo, E., Paronyan, L., Pajovic, I., Faraj, C., Sikharulidze, I., Putkaradze, D., Maric, J., Bosevska, G., Janceska, E., Bouattour, A., Hamidi, A., Sherifi, K., Alten, B., Petrić, D. and Robert, V. (2018). Identification of mosquitoes (Diptera: Culicidae): an

external quality assessment of medical entomology laboratories in the Medi Lab Secure Network. *Parasites and Vectors*, **11(1),** 553.

Kakkar, M. (2012). Dengue Fever is massively under-reported in India, hampering our response. *BMJ*, **345,** e8574.

Kalita, J.C., Deka, A.C. and Misra, K. (2014). Mosquito vector survey in Guwahati city of Assam, India. *Journal of entomology and nematology*, **6(3),** 42-50.

Kalra, N.L, Kaul, S.M. and Rastogi, R.M. (1997). Prevalence of *Aedes aegypti* and *Aedes albopictus*–Vectors of Dengue and Dengue Haemorrhagic Fever in North, North-East and Central India. *Dengue Bulletin*, **21**, 84-92.

Kamal, M., Kenawy, M.A., Rady, M.H., Khaled, A.S. and Samy, A.M. (2018). Mapping the global potential distributions of two arboviral vectors *Aedes aegypti* and *Ae. albopictus* under changing climate. *PLoS ONE,* **13(12),** e0210122.

Kamgang, B., Marcombe, S., Chandre, F., Nchoutpouen, E., Nwane, P., Etang, J., Corbel V. and Paupy, C. (2011). Insecticide susceptibility of *Aedes aegypti* and *Aedes albopictus* in Central Africa. *Parasites & Vectors*, **4(1)**, 79.

Kamgang, B., Ngoagouni, C., Nakoune, E., Paupy, C. and Kazanji, M. (2013). Temporal Patterns of abundance of *Aedes aegypti* and *Aedes albopictus* and mitochondrial DNA analysis of *Aedes albopictus* in the Central African., Republic. *PLoS Neglected Tropical Disease*, **7(12)**, e2590.

Kandel, Y., Vulcan, J., Rodriguez, S. D., Moore, E., Chung, H.N., Mitra, S., Cordova, J.J., Martinez, K.J.L., Moon, A.S., Kulkarni, A., Ettestad, P., Melman, S., Xu, J., Buenemann, M., Hanley, K.A. and Hansen, I.A. (2019). Widespread insecticide resistance in *Aedes aegypti* L. from New Mexico, U.S.A. *PLoS One,* **14(2),** e0212693.

Kanhekar, L.J., Ravikumar, R., Meena, R.K., Sharma, R.S., Sharma, A.K. and Enkatesh, S.V. (2016). Population dynamics of *Aedes aegypti* and *Aedes albopictus* in Jagdalpur city, Bastar district of Chhattisgarh state, India. *International Journal of Mosquito Research*, **3(6),** 40-4.

Karunamoorthi, K. and Sabesan, S. (2013). Insecticide Resistance in Insect Vectors of Disease with Special Reference to Mosquitoes: A Potential Threat to Global Public Health, *Health Scope,* **2(1),** 4-18.

Kaur, M., Singh, K., Sidhu, S.K., Devi, P., Kaur, M., Soneja, S. and Singh, N. (2018). Coinfection of Chikungunya and Dengue viruses: A serological study from North Western region of Punjab, India. *Journal of Laboratory Physicians,* **10(04),** 443-447.

Kaur, S. (2014). Variation in the male genitalia of *Aedes albopictus* from Chandigarh and its surrounding areas. *International Journal of Mosquito Research,* **1(3),** 55-60.

Kawada, H., Higa, Y., Futami, K., Muranami, Y., Kawashima, E., Osei, J.H.N., Sakyi, K.Y., Dadzie, S., Souza, D.K., Appawu, M., Ohta, N., Suzuki, T. and Minakawa, N. (2016). Discovery of Point Mutations in the Voltage-Gated Sodium Channel from African *Aedes aegypti* Populations: Potential phylogenetic reasons for gene Introgression. *PLoS Neglected Tropical Disease,* **10(6),** e0004780.

Khan, V., Zala, D.B. and Srivastava, H.C. (2015). Entomological indicators during transmission season of Dengue in Silvassa (India). *Journal of Parasitc Disease,* **39(2),** 342–344.

Khan, J., Adil, M., Wang, G., Tsheten, T., Zhang, D., Pan, W., Khan, M.A., Rehman, I.U., Zheng, X., Wu, Z., Wu, Y. (2022). **A** cross-sectional study to assess the epidemiological situation and associated risk factors of dengue fever; knowledge, attitudes, and practices about dengue prevention in Khyber Pakhtunkhwa Province, Pakistan. *Front Public Health*, **29(10),** 923277. doi: 10.3389/fpubh.2022.923277.

Knobler, S.L., Lemon, S.M. and Najafi, M. (2003). Institute of Medicine (US) Forum on Emerging Infections; Washington (DC): National Academies Press (US).

Kraemer, M.U., Sinka, M.E., Duda, K.A., Mylne, A.Q., Shearer, F.M., Barker, C.M., Moore, C.G., Carvalho,R.G., Coelho, G.E., Bortel, W.V., Hendrickx, G.,Schaffner, F., Elyazar, I.R.F., Teng, H.J.,Brady,O.J., Messina, J. P., Pigott, D.M.,Scott,T.W.,Smith, D.L., Wint, G.R. W., Golding, N. and Hay, S.I.

(2015). The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae.albopictus*. *eLife,* **4**, e08347.

Kumar, K., Sharma, A.K., Sarkar, M., Chauhan, A. and Sharma, R. (2014). Surveillance of *Aedes aegypti* (L.) Mosquitoes in Mumbai International Seaport (India) to Monitor Potential Global Health Risks. *Journal of Insects,* **951015**, 5.

Kumari, R., Kumar, K. and Chauhan, L.S. (2011). First Dengue virus detection in *Aedes albopictus* from Delhi, India: its breeding ecology and role in Dengue transmission.*Tropical Medicine & International Health,* **16(8),** 949-54.

Kumari, R., Patel, S., Kanhekar, L.J., Sharma, S. N., Chharang, N., Visth, B. and Venkatesh, S. (2016). Entomological Surveillance and Warning for Dengue Outbreaks in Alwar City, Rajasthan, India. *Journal of Communicable Disease,* **48(4),** 29-35.

Kumari, R., Sharma, R.S., Kumar, K., Singh, P., Krishnan, S., Dashc, A.P., and Chauhana, L.S. (2013). Mapping of Dengue vectors and Dengue virus activity in Delhi during 2011–2012.*Dengue Bulletin*, **37**, 87.

Kushwah, R.B.S., Mallick, P.K., Ravikumar, H., Dev, V., Kapoor, N., Adakl, T. and Singh, O.P. (2015). Status of DDT and pyrethroid resistance in Indian *Aedes albopictus* and absence of knockdown resistance (*kdr*) mutation. *Journal of Vector Borne Disease,* **52(1)**, 95–98.

Laroche, M., Bérenger, J.M., Delaunay, P., Charrel, R., Pradines, B., Berger, F., Ranque, S., Bitam, I., Davoust, B., Raoult, D. and Parola, P. (2017). Medical Entomology: A Reemerging Field of Research to Better Understand Vector-Borne Infectious Diseases. *Clinical Infectious Diseases,* **65(1),** 30–38.

Lata, S., Singh, G. and Dubey, S. (2012). An Epidemiological Investigation of Dengue Outbreak in Shri Muktsar Sahib District, Punjab, India. *International Journal of Advance Research, Ideas and Innovations in Technology,* **3(**1).

Lemine, A.M.M., Lemrabott, M.A.O., Ebou, M.H., Lekweiry, K.M.,Salem ,M.S.O.A., Brahim, K.O., Moukah, M.O., Bouraya, I.N.O., Brengues, C.,

Trape, J.F, Basco, L., Bogreau, H., Simard, F., Faye, O. and Boukhary, A.O.M.S. (2017). Mosquitoes (Diptera: Culicidae) in Mauritania: a review of their biodiversity, distribution and medical importance. *Parasites & Vectors,* **10(1)**, 35.

Leong, C.S., Vythilingam, I., Liew, J.W.K, Wong, M.L., Sulaiman, W., Yusoff, W. and Lau, Y.L. (2019). Enzymatic and molecular characterization of insecticide resistance mechanisms in field populations of *Aedes aegypti* from Selangor, Malaysia. *Parasites & Vectors*, **12(1)**, 236.

Lequime, S., Paul, R.E. and Lambrechts, L. (2016). Determinants of arbovirus vertical transmission in mosquitoes. *PLoS Pathog,* **12(5),** e1005548.

Leslie, A.R. (1994). Handbook of integrated pest management for turf and ornamental.541-557.

Li, C-X., Kaufman, P.E., Xue, R-D., Zhao, M-H., Wang, G., Yan, T., Guo, X-X., Zhang, Y-M., Dong, Y-D., Xing, D., Zhang, H.D.and Zhao, T.Y. (2015). Relationship between insecticide resistance and *kdr* mutations in the Dengue vector *Aedes aegypti* in Southern China. *Parasites & Vectors*, **8(1)**, 325.

Li, Y., Kamara, F., Zhou, G., Puthiyakunnon, S., Li, C., Liu, Y., Zohu, Y., Yao, L., Yan, G. and Chen, X.G. (2014). Urbanization Increases *Aedes albopictus* Larval Habitats and Accelerates Mosquito Development and Survivorship. *PLoS.neglected tropical disease*, **8(11)**, e3301.

Lima, E.P., Paiva, M.H.S., Araújo, A.P., Silva, É.V.G., Silva, U.M., Oliveira, L.N. , Santana, A.E.G., Barbosa, C.N., Neto, C.C.D.P., Goulart, M.O., Wilding, C.S., Ayres, C. F.J. and Melo Santos, M.A.V.D. (2011). Insecticide resistance in *Aedes aegypti* populations from Ceará, Brazil. *Parasites & Vectors***, 4(1),** 5.

Lin, Y.H., Teng, H.J., Ho, C.M., Pai, H.H., Wu, S.C. and Hsu, E.L. (2003). Insecticide resistance in *Aedes aegypti* during Dengue epidemics in Taiwan. *FormosanEntomol,* **23**, 263–74.

Mahmud, M.A.F., Mutalip, M.H., Aliza, N. and Shahar, L.H. (2018). Study on key *Aedes* spp breeding containers in Dengue outbreak localities in Cheras district, Kuala Lumpur. *International Journal of Mosquito Research,* **5(2),** 23-30.

Malathion technical fact sheet npic national pesticide information center, (2011). npic@ace.orst.edu .

Marchi, S., Trombetta, C.M. and Montomoli, E. (2018). Emerging and Re-emerging Arboviral Diseases as a Global Health Problem. *Public Health; Majumder, MAA, Ed.; IntechOpen: London*, UK, 25-46.DOI: 10.5772/intechopen.77382.

Marcombe, S., Chonephetsarath, S.,Thammavong, P. and Brey, P.T. (2018). Alternative insecticides for larval control of the Dengue vector *Aedes aegypti* in Lao PDR: insecticide resistance and semi-field trial study. *Parasite and Vectors,* **11(1)**, 616.

Marcombe, S., Darriet, F., Tolosa, M., Agnew, P., Duchon, S., Etienne, M., Tcha, M.M.Y., Chandre F., Corbel, V. and Yébakima, A. (2011). Pyrethroid Resistance Reduces the Efficacy of Space Sprays for Dengue Control on the Island of Martinique (Caribbean). *PLoS Neglected Tropical Disease,* **5(6),** e1202.

Marcombe, S., Poupardin, R., Darriet, F., Reynaud, S., Bonnet, J., Strode, C., Brengues, C.,Yébakima, A.,Ranson, H., Corbel,V. and David, J.P. (2009). Exploring the molecular basis of insecticide resistance in the Dengue vector *Aedes aegypti*: a case study in Martinique Island (French West Indies). *BMC Genomics*, **10(1)**, 1-14.

Matysiak, A. and Roess, A. (2017). Interrelationship between Climatic, Ecologic, Social, and Cultural Determinants Affecting Dengue emergence and transmission in Puerto Rico and Their Implications for Zika Response Review. *Journal of Tropical Medicine*, **8947067,** 14.

Medeiros, A.S., Costa, D.M.P., Branco, M.S.D., Sousa, D.M.C., Monteiro, J.D., Galvão, S.P.M., Azevedo, P.R.M., Fernandes, J.V., Jeronimo, S.M.B. and Araújo, J. M.G. (2018). Dengue virus in *Aedes aegypti* and *Aedes albopictus*

in urban areas in the state of Rio Grande do Norte, Brazil: Importance of virological and entomological surveillance. *PLoS One*, **13(3),** e0194108.

Meena, R.A. and Choudhary N.L., (2019). Container breeding preference of *Aedes albopictus* in urban environment. *International Journal of Mosquito Research*, **6(5),** 44-47.

Messina, J.P., Brady, O.J., Golding, N., Kraemer, M.U.G., Wint, G.R.W., Ray, S.E., Pigott, D.M., Shearer, F.M., Johnson, K., Earl, L., Marczak, L.B., Shirude, S., Weaver,N.D., Gilbert, M., Velayudhan, R, Jones, P., Jaenisch, T, Scott, T.W., Robert, C., Reiner, J. and Hay, S.I. (2019).The current and future global distribution and population at risk of Dengue. *Nature Microbiology*, **4(9),** 1508–1515.

Mossa, A.T.H., Mohafrash, S.M.M. and Chandrasekaran, N. (2018). Safety of Natural Insecticides: Toxic Effects on Experimental Animals. *Biomed Research International*, **4308054**, 17.

Moyes, C.L., Vontas, J., Martins, A.J., Ng, L.C., Koou, S.Y. and Weetman, D. (2021). Correction: Contemporary status of insecticide resistance in the major *Aedes* vectors of arboviruses infecting humans. *PLoS Neglected Tropical Diseases*, **15(1),** e0009084.

Mubbashir, H., Munir, S., Kashif, R., Nawaz, H.B., Abdul, B. and Baharullah, K. (2018). Characterization of Dengue virus in *Aedes aegypti* and *Aedes albopictus* spp. of mosquitoes: A study in Khyber Pakhtunkhwa, Pakistan. *Molecular Biology Research Commun*ications, **7(2),** 77–82.

Mukhtar, M.U., Han, Q., Liao, C., Haq, F., Arslan, A., Bhatti, A. (2018). Seasonal Distribution and Container Preference Ratio of the Dengue Fever Vector (*Aedes aegypti*, Diptera: Culicidae) in Rawalpindi, Pakistan. *Journal of Medical Entomol*ogy, **55(4),** 1011-1015.

Muktar, Y., Tamerat N. and Shewafera, A. (2016). *Aedes aegypti* as a Vector of Flavivirus. *J Trop Dis*, **4(223),** 2.

Munhenga, G., Brooke, B.D., Gilles, J.R.,Slabbert, K., Kemp, A., Dandalo,

L.C., Wood, O.R., Lobb, L.N., Govender, D., Renke, M. and Koekemoer, L.L. (2016). Mating competitiveness of sterile genetic sexing strain males (GAMA) under laboratory and semi-field conditions: Steps towards the use of the Sterile Insect Technique to control the major malaria vector *Anopheles arabiensis* in South Africa. *Parasites and Vectors*, **9(1)**, 1-12.

Murray, N.E.A., Quam, M.B. and Smith, A.W. (2013). Epidemiology of Dengue: past, present and future prospects. *Clinical Epidemiol*ogy, **5,** 299–309.

Mutheneni, S.R., Morse, A. P, Caminade, C. and Upadhyayula, S.M. (2017). Dengue burden in India: recent trends and importance of climatic parameters. *Emerg Microbes Infect.* **6(8),** e70.

Muthusamy, R. and Shivakumar, M.S. (2015). Susceptibility status of *Aedes aegypti* (L.) (Diptera: Culicidae) to Temephos from three districts of Tamil Nadu, India.*Journal of Vector Borne Disease*, **52(2),** 159–165.

Nasir, S., Jabeen, F., Abbas, S., Nasir, I. and Debboun, M. (2017). Effect of Climatic Conditions and Water Bodies on Population Dynamics of the Dengue Vector, *Aedes aegypti* (Diptera: Culicidae). *Journal of Arthropod-Borne Disease*, **11(1),** 50–59.

National Academies Press (US). (2008). Institute of Medicine (US) Forum on Microbial Threats. Washington (DC).

National vector borne disease control programme (NVBDCP).website http://nvbdcp.gov.in/den-cd.html.

Nejati, J., Bueno-Marí, R. Collantes, F., Hanafi-Bojd, A. A., Vatandoost, H., Charrahy, Z. and Sedaghat, M.M. (2017). Potential risk Areas of *Aedes albopictus* in South-Eastern Iran: a vector of Dengue Fever, Zika, and Chikungunya. *Frontiers in Microbiol*ogy, **8**, 1660.

Ngoagouni, C., Kamgang, B., Brengues, C., Yahouedo, G., Paupy, C., Nakouné, E., Kazanji, M. and Chandre, F. (2016). Susceptibility profile and metabolic mechanisms involved in *Aedes aegypti* and *Aedes albopictus* resistant to DDT

and Deltamethrin in the Central African Republic. *Parasites & Vectors*, **9(1),** 1-13.

Ngugi, H.N., Mutuku, F. M., Ndenga, B.A., Musunzaji, P.S., Mbakaya, O.O., Aswani, P., Irungu, L.W., Mukoko, D.A., Vulule, J., Kitron, U. and Labeaud, A. D. (2017). Characterization and productivity profiles of *Aedes aegypti* (L.) breeding habitats across rural and urban landscapes in western and coastal Kenya. *Parasites & Vectors*, **10(1), 1-12.**

Omodior, O., Luetke, M.C. and Nelson, E.J. (2018). Mosquito-borne infectious disease, risk-perceptions and personal protective behavior among U.S. international travelers. *Preventive Medicine Reports*, **12**, 336–342.

Ong, S.Q. and Jaal, Z. (2018). Larval Age and Nutrition Affect the Susceptibility of Culex quinquefasciatus (Diptera: Culicidae) to Temephos. *Journal of Insect Science*, **18(2),** 38.

Ooi, E.E., Goh, K.T. and Gubler, D.J. (2006). Dengue prevention and 35 years of vector control in Singapore. *Emerging Infectious Disease*, **12(6),** 887–893.

Paixao, E.S., Teixeira, M.G. and Rodrigues, L.C. (2018). Zika, Chikungunya and Dengue: the causes and threats of new and re-emerging arboviral diseases. *BMJ Global Health*, **3(1),** e000530.

Pandya, G. (1982). Prevalence of Dengue infections in India. *Defence Sci J*, **32**(**4)**, 359–370.

Paredes-Esquivel, C., Lenhart, A., Rio D.R., Leza, M.M., Estrugo, M., Chalco, E. Casanova, W. and Miranda, M.Á. (2016). The impact of indoor residual spraying of Deltamethrin on Dengue vector populations in the Peruvian Amazon. *Acta Tropica*, **154**, 139–144.

Patel, S., Singh, P., Prakash, V., Sharma, A.K., Dhan, S., Singh, R. and Singh, S.K. (2020). A Study on the influence of climatic factors on the preferential breeding places of *Aedes,* the Dengue vector, in Delhi, India. *International Journal of Mosquito Research,* **7(4),** 95-104.

Paul, R., Oberoi, L., Singh, K. and Devi, P.A. (2017). Comprehensive study of Dengue Fever patients admitted to a tertiary care hospital in Amritsar. *Asian Pacific Journal of Health Science,* **4(4)**, 100-3.

Perez, D., Lefèvre, P., Sánchez, L., Sánchez, L.M., Boelaert, M., Kourí, G. andStuyft, P.V.D. (2007). Community participation in *Aedes aegypti* control: a sociological perspective on five years of research in the health area ‘‘26 de Julio’’, Havana, Cuba. *Tropical Medicine & International Health,* **12(5),** 664-672.

Pham, H.V., Huong, T.M.D., Phan, T.T.T. and Minh, N.N.T. (2011). Ecological factors associated with Dengue Fever in a central highlands Province, Vietnam. *BMC Infectious Diseases,* **11(1),** 172.

Philbert, A. and Ljumba, J.N. (2013). Preferred breeding habitats of *Aedes aegypti* mosquito and its public health implications in Dares Salaam, Tanzania. *Environmental Research and management,* **4(10),** 344-351.

Phong, T.V. and Nam, V.S. (1999). Key breeding Sites of Dengue Vectors in Hanoi, Vietnam, 1994-1997. *Dengue Bulletin,* **23,** 67-72.

Pinto, J., Palomino, M., Mendoza-Uribe, L., Sinti, C., Kelly, A. L.and Audrey, L. (2019). Susceptibility to insecticides and resistance mechanisms in three populations of *Aedes aegypti* from Peru. *Parasites & Vectors,* **12(1)**, 494.

Plernsub, S., Saingamsook, J., Yanola, J., Lumjuan, N., Tippawangkosol, P., Sukontanson, K., Walton, C. and Somboon, P. (2016).Additive effect of knockdown resistance mutations, S989P, V1016G and F1534C in a heterozygous genotype conferring pyrethroid resistance in *Aedes aegypti* in Thailand. *Parasite and Vectors,* **9(1)**, 417.

Ponlawat, A. and Harrington, L.C. (2005). Blood Feeding Patterns of *Aedes aegypti* and *Aedes albopictus* in Thailand. *Journal of Medical Entomology*, **42(5),** 844–849,

Poupardin, R., Srisukontarat, W., Yunta, C. and Ranson, H. (2014). Identification of Carboxylesterase Genes Implicated in Temephos Resistance in the Dengue Vector *Aedes aegypti*. *PLoS Neglected Tropical Disease,* **8(3),** e2743.

Powell, J.R., Gloria-Soria, A. and Kotsakiozi, P. (2018). Recent History of *Aedes aegypti:* Vector Genomics and Epidemiology Records. *Bioscience*, **68(11)**, 854-860.

Prakash, O.,Singh, D.D., Mishra, G., Prakash, S., Singh, A., Gupta, S., Singh, J., Khan, D.N., Jain, P., Vishal, A., Pandey, M.K. and Jain, A. (2015). Observation on Dengue cases from a virus diagnostic laboratory of a tertiary care hospital in North India. *The Indian journal of medical research,* **142 (1),** 7-11.

Preechaporn, W., Jaroensutasinee, M. and Jaroensutasinee, K. (2006). The larval ecology of *Aedes aegypti* and *Aedes albopictus* in three topographical areas of Southern Thailand. *Dengue Bulletin,* **30,** 204-213.

Pyrethrins General Fact Sheet, (2014). National Pesticide Information Center.http://npic.orst.edu/factsheets/pyrethrins.

Radhika, N.M.L**,** Gunathilaka, N., Udayanga, L., Kasturiratne, A. and Abeyewickreme, W. (2019). Level of Awareness of Dengue Disease among School Children in Gampaha District, Sri Lanka, and Effect of School-Based Health Education Programmes on improving knowledge and practices, *BioMed research international,* **3092073**, 8.

Rahman, S.J., Saba, J., Sharma, R.S. and Verghese, T. (1995). Relevance of the*Aedes* Larval/House Index in predicting outbreaks of Dengue/Dengue Haemorrhagic Fever. *Dengue Newsletter*, **19**, 5-8.

Ranson, H., Burhani, J., Lumjuan, N. and Black, W.C. (2010). Insecticide resistance in Dengue vectors.*Trop.IKA,* **1**, 1.

Ravikumar, R., Reegan, A.D., Chandrasekar, P.and Kumar, S.C. (2013). Distribution of Dengue Vectors during Pre- and Post-Monsoon Seasons in Higher Attitudes of Nilgiri Hills of Western Ghats, India. *Journal of Insects*,**627304**, 5.

Reegan, A.D., Kumarand, K.R. and Devanand, L. (2018). Prevalence of Dengue vectors in Bengaluru city, India: An entomological analysis. *International Journal of Mosquito Research,* **5(1),** 101- 105.

Rehman, H., Aziz, A.T., Saggu, S., Abbas, Z.K., Mohan, A. and Ansari, A.A. (2014). Systematic review on pyrethroid toxicity with special reference to Deltamethrin. *Journal of entomology and zoology studies*, **2(6),** 60–70.

Reinhold, J.M., Lazzari, C.R. and Lahondère, C. (2018). Effects of the Environmental Temperature on *Aedes aegypti* and *Aedes albopictus* Mosquitoes: a Review. *Insects*, **9(4),** 158.

Reuben, R. (1978). A report on mosquitoes collected in the Krishna-Godavari delta, Andhra Pradesh. *Indian J Med Res*, **68**, 603-609.

Rios, L. and Maruniak, J.E., (2004).University of Florida, EENY-319.

Rodriguez, M.M., Fernandez, J.B.D.M., Lauzan, L. and Soca, A. (2001). Detection of insecticide resistance in *Aedes aegypti* (Diptera: Culicidae) from Cuba and Venezuela. *Journal of medical entomology*, **38(5)**, 623–628.

Roop, K., Priya, S., Sunita, P., Mujib, M., Kanhekar, L.J. and Venkatesh, S., (2016). Way Forward for Seasonal Planning of Vector Control *of Aedes aegypti* and *Aedes albopictus* in a Highly Dengue Endemic Area in India. *Austin Journal of Infectious Diseases*, **3(1),** 1022.

Rueda, L.M. (2004). Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated with Dengue Virus Transmission. *Zootaxa*, ***589***, 1–60.

Russell, R.C. (2012). Medical Microbiology (Eighteenth Edition),

Ryan, S.J., Carlson, C.J., Mordecai, E.A. and Johnso, L.R. (2019). Global expansion and redistribution of *Aedes*-borne virus transmission risk with climate change. *PLoS neglected tropical diseases*, **13(3),** e0007213.

Saha, P., Chatterjee, M., Ballav, S., Chowdhury, A., Basu, N. and Maji, A.K. (2019). Prevalence of kdr mutations and insecticide susceptibility among natural population of *Aedes aegypti* in West Bengal. *PLoS One*, **14(4),** e0215541.

Saldana, M.A., Hegde, S. and Hughes, G.L. (2017). Microbial control of arthropod-borne disease. *Memórias do Instituto Oswaldo Cruz*, **112(2),** 81–93.

Saleem, M., Ghouse, G., Hussain, D., Saleem, H.M. and Abbas, M. (2014). Distribution of Dengue vector during pre and post monsoon season in three districts of Punjab Pakistan. *Journal of Mosquito Research*, **4(1),** 16.

Sanchez, L., Vanlerberghe, V., Alfonso, L., Marquetti, M.C., Guzman, MG., Bisset, J. andVan Der Stuyft, P. (2006). *Aedes aegypti* larval Indices and risk for Dengue epidemic. *Emerging infectious diseases*, **12(5),** 800–806.

Sanchez-Vargas, I., Harrington, L.C., Doty, J.B., Black, W.C. and Olson, K.E. (2018). Demonstration of efficient vertical and venereal transmission of Dengue virus type-2 in a genetically diverse laboratory strain of *Aedes aegypti*. *PLoS neglected tropical diseases,* **12(8),** e0006754.

Scott, T.W. and Morrison, A.C. (2010). Vector dynamics and transmission of Dengue virus: Implications for Dengue surveillance and prevention strategies: vector dynamics and Dengue prevention. *Dengue virus***, 338,**115–128.

Sharma, A., Gupta, V. and Khandelwal, A. (2017).The knowledge, attitude and practices regarding commonly occurring mosquito borne diseases among people in catering area of urban health and training centre. *Int J Community Med Public Health*, **4(8),** 2864-2869.

Shipali.and Kirti, J.S. (2014). Description of male and female genitalia of Anopheles (cellia) culicifacies giles with the aid of scanning electron microscope. *International Journal of Pure and Applied Zoology*,**2(4)**, 348-350.

Sharma, A.K., Kumar, K., and Singh, S., and Sharma, A. K. (2015). Entomological Surveillance for the vector of Yellow Fever/Dengue/Chikungunya in and around ports of Goa, India. *International Journal of Pure and Applied Zoology*, **3(3),** 204-209.

Sharma, R.S., Kaul, S.M. and Sokhay, J. (2005). Seasonal fluctuations of Dengue Fever vector, *Aedes aegypti* (Diptera: Culicidae) in Delhi, India. *Southeast Asian J Trop Med Public Health*, **36(1)**, 186-90.

Shragai, T., Harrington, L., Alfonso-Parra, C. and Avila, F. (2019).Oviposition site attraction of *Aedes albopictus* to sites with conspecific and heterospecific

larvae during an ongoing invasion in Medellín, Colombia. *Parasites and Vectors*, **12(1)**, 1-10.

Shrivastava, S., Tiraki, D., Diwan, A., Lalwani, S.K., Modak, M., Mishra, A.C. and Arankalle, V.A. (2018). Co-circulation of all the four Dengue virus serotypes and detection of a novel clade of DENV-4 (genotype I) virus in Pune, India during 2016 season. *PLoS One*, **13(2),** e0192672.

Simard, F., Nchoutpouen, E., Toto, J.C. and Fontenille, D.N. (2005). Geographical distribution and breeding site preference of *Aedes albopictus* and *Aedes aegypti* in Camroon. *Journal of medical entomology*, **42(5)**, 726-731.

Singh, R.K., Dhiman, R.C., Mittal, P.K. and Dua, V.K. (2011). Status of Dengue vectors against various insecticides in Koderma (Jharkhand) India: *Journal of vector borne diseases*, **40(2)**, 116-118.

Singh, R.K., Haq, S., Kumar, G., Mittal, P.K and Dhiman, R.C. (2013). Insecticide-susceptibility status of Dengue vectors *Aedes aegypti* and *Aedes albopictus* in India: a review. *Dengue Bulletin*, **37**, 177-191.

Singh, R.K., Mittal, P.K., Kumar, G. and Dhiman, R.C. (2014). Insecticide susceptibility status of *Aedes aegypti* and *Anopheles stephensi* larvae against Temephos in Delhi, India. *International Journal of Mosquito Research*, **1(3)**, 69-73.

Siregar, F.A. and Makmur, T. (2018). Survey on *Aedes* mosquito density and pattern distribution of *Aedes aegypti* and *Aedes albopictus* in high and low incidence districts in north sumatera province. *In IOP Conference Series: Earth Environ. Sci*, **130 (1),** 012018.IOP Publishing.

Sirisena, P.D.N.N., Shakuntala, M., Sharma, C., Jain, J., Sujatha, S., (2021). Concurrent dengue infections: Epidemiology & clinical implications. *Indian Journal of Medical Research*, **154(5)**, 669-679.

Smith, L.B., Kasai, S. and Scott, J.G. (2016). Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus*: Important mosquito vectors of human diseases. *Pesticide biochemistry and physiology*, **133**, 1–12.

Soni, M., Bhattacharya, C., Sharma, J. and Dutta, P. (2018). Bioassay and molecular study for detection of insecticide resistance Dengue causing mosquito vectors. *Indian journal of medical microbiology,* **36(3)**, 435-438.

Souza-Neto, J. A., Powell, J. R. and Bonizzoni, M. (2019). *Aedes aegypti* vector competence studies: A review.*Infection, genetics and evolution,* **67**, 191-209.

Srivastava, P.K. and Dhariwal, A.C. (2016). Compendium on entomological surveillance and vector control in India.*National Vector Borne Disease Control Programme,* 67.

Stewart Ibarra, A.M., Ryan, S.J., Beltrán, E., Mejía, R., Silva, M. and Muñoz, Á. (2013). Dengue Vector Dynamics (*Aedes aegypti*) Influenced by Climate and Social Factors in Ecuador: Implications for Targeted Control. *PLoS ONE*, **8(11**), e78263.

Stork, N.E. (2018). How Many Species of Insects and Other Terrestrial arthropods are there on Earth? *Annual review of entomology*, **63**, 31–45.

Su, X., Guo, Y., Deng, J., Xu, J., Zhou, G., Zhou, T. and Chen, X. G. (2019). Fast emerging insecticide resistance in *Aedes albopictus* in Guangzhou, China: Alarm to the Dengue epidemic. *PLoS neglected tropical diseases,* **13(9),** e0007665.

Sudeep, A.B. and Shil, P. (2017).*Aedes vittatus* (Bigot) mosquito: An emerging threat to public health. *Journal of vector borne diseases,* **54(4),** 295-300.

Suter, T., Crespo, M.M., de Oliveira, T.S.A., de Melo-Santos, M.A.V., de Oliveira, C. M.F., and Silva-Filha, M.H.N.L. (2017).Insecticide susceptibility of *Aedes albopictus* and *Ae.aegypti* from Brazil and the Swiss-Italian border region.*Parasites Vectors,* **10(1), 1-11.**

Tandina, F., Doumbo, O., Yaro, A.S., Traoré, S.F., Parola, P. and Robert, V. (2018). Mosquitoes (Diptera: Culicidae) and mosquito-borne diseases in Mali, West Africa. *Parasites & Vectors*, **11(1),** 1-12.

Tchounwou, P.B., Patlolla, A.K., Yedjou, C.G. and Moore, P.D. (2015). Environmental Exposure and Health Effects Associated with Malathion Toxicity.*Toxicity and Hazard of Agrochemicals*, **51**, 2145-9.

Tedjou, A.N., Kamgang, B., Yougang, A.P., Njiokou, F, Wondji, C.S. (2019). Update on the geographical distribution and prevalence of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae), two major arbovirus vectors in Cameroon. *PLoS neglected tropical diseases*, **13(3),** e0007137.

Tetreau, G., Patil, C.D., Chandor-Proust, A., Salunke, B.K., Patil, S.V. and Despres, L. (2013). Production of the bio insecticide Bacillus thuringiensis subsp. israelensis with Deltamethrin increases toxicity towards mosquito larvae. *Letters inapplied microbiology*, **57(2)**, 151-156.

Tewari, S.C., Thenmozhi, V., Katholi, C.R., Manavalan, R., Munirathinam, A. and Gajanana, A. (2004).Dengue vector prevalence and virus infection in a rural area in south India.*Tropical Medicine & International Health*, **9(4)**, 499-507.

Thanispong, K., Sathantriphop, S. and Chareonviriyaphap, T. (2008). Insecticide resistance of *Aedes aegypti* and *Culex quinquefasciatus* in Thailand. *Journal of Pesticide Science*, **33(4),** 351–356.

Thavara, U., Tawatsin, A., Chansang, C., Kong-ngamsuk, W., Paosriwong, S., Boon-Long, J., Rongsriyam, Y. and Komalamisra, N. (2002). Larval occurrence, oviposition behavior and biting activity of potential mosquito vectors of Dengue on Samui Island, Thailand. *Journal of Vector Ecology*, **26(2),** 172-80.

Tikar, S.N., Mendki, M.J., Chandel, K., Parashar, B. D. and Prakash, S. (2008). Susceptibility of immature stages of *Aedes* (*Stegomyia) aegypti;* vector of Dengue and Chikungunya to insecticides from India.*Parasitology Research*, **102(5),** 907-913.

Todd, G.D., Wohlers, D. and Citra, M. (2003). Toxicological Profile for pyrethrins and pyrethroids. *Department of health and human Services Public Health Service Agency for Toxic Substances and Disease Registry.Atlanta, GA*.1-328.

Townson, H., Nathan, M. B., Zaim, M., Guillet, P., Manga, L., Bos, R., & Kindhauser, M. (2005). Exploiting the potential of vector control for disease prevention. *Bulletin of the World Health Organization,* **83,** 942-947.

Tsuda, Y, Suwonkerd, W, Chawprom, S, Prajakwong, S. and Takagi, M. (2006). Different spatial distribution of *Aedes aegypti* and *Aedes albopictus* along an urban-rural gradient and the relating environmental factors examined in three villages in northern Thailand. *Journal of the American Mosquito Control Association,* **22(2),** 222-228.

Tsujimoto, H., Hanley, K.A., Sundararajan, A., Devitt, N.P., Schilkey, F.D. and Hansen, I.A. (2017). Dengue virus serotype 2 infections alters midgut and carcass gene expression in the Asian tiger mosquito, *Aedes albopictus*. *PloS One*, **12(2)**, e0171345.

Tuladhar, R., Singh, A., Banjara, M.R.,Gautam, I., Dhimal, M., Varma, A. and Choudhary, D.K. (2019). Effect of meteorological factors on the seasonal prevalence of Dengue vectors in upland hilly and lowland Terai regions of Nepal. *Parasites and Vectors,* **12(1),** 1-15.

Tyagi, B.K., Munirathinam, A., Krishnamoorthy, R. and Venkatesh, A. (2012). A field-based handbook of identification keys to mosquitoes of public health importance in India. *Centre for Research in Medical Entomology, Madurai, India,* **1**, 25-27.

Udayanga, L., Gunathilaka, N., Iqbal, M.C.M., Iqbal, M.C.M., Najim, M.M.M., Pahalagedara, K. and 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.

Uragayala, S., Verma, V., Natarajan, E., Velamuri, P.S. and Kamaraju, R. (2015). Adulticidal & larvicidal efficacy of three neonicotinoids against insecticide

susceptible & resistant mosquito strains. *The Indian journal of medical research,* **142 (1),** 64-70.

Vaddadi, S. and Vaddadi, R.S. (2015). Dengue Fever: A review article. *J. Evol. Med. Dent. Sci*, **4**, 5048-5058.

Valle, D., Bellinato, D.F., Viana-Medeiros, P.F., Lima, J.B.P. and Martins, A.D.J. (2019). Resistance to Temephos and Deltamethrin in *Aedes aegypti* from Brazil between 1985 and 2017. *Memórias do Instituto Oswaldo Cruz*, **114**.

Vijayakumar, K., Kumar, T.S., Nujum, Z.T., Umarul, F. and Kuriakose, A. (2014).A study on container breeding mosquitoes with special reference to *Aedes (Stegomyia) aegypti* and *Aedes albopictus* in Thiruvananthapuram district, India. *Journal of vector borne diseases*, **51(1),** 27-32.

Vikram, K., Nagpal, B.N., Pande, V., Srivastava, A., Gupta, S.K., Anushrita, Singh, V.P., Singh, H., Saxena, R., Tuli, N.R., Yadav, N.K., Paul, R., Valecha, N. and Telle, O. (2015). Comparison of *Ae. aegypti* breeding in localities of different socio-economic groups of Delhi, India. *International Journal of Mosquito Research*, **2 (2),** 83-88.

Vontas, J., Kioulos, E., Pavlidi, N., Morou, E., Della Torre, A. and Ranson, H. (2012). Insecticide resistance in the major Dengue vectors *Aedes albopictus* and *Aedes aegypti. Pesticide Biochemistry and Physiology*, **104(2),** 126-131.

Wakeling, E.N., Neal, A.P. and Atchison W.D. (2012). Pyrethroids and Their Effects on ion channel. *Pesticides—advances in chemical and botanical pesticides. Rijeka, Croatia: InTech,* 39-66.

Waldock, J., Chandra, N.L., Lelieveld, J., Proestos, Y., Michael, E., Christophides, G. and Parham, P.E. (2013). The role of environmental variables on *Aedes albopictus* biology and Chikungunya epidemiology. *Pathogens and global health,* **107(5),** 224–241.

Wang, W.H., Urbina, A.N., Chang, M.R., Assavalapsakul, W., Lu, P.L., Chen, Y.H., Wang, S.F. (2020) Dengue hemorrhagic fever - A systemic literature review of current perspectives on pathogenesis, prevention and control. *J Microbiol Immunol Infect,* **53(6),** 963-978.

WHO (2013). Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Geneva: World Health Organization.

Wongkoon, S., Jaroensutasinee, M. and Jaroensutasinee, K. (2013). Distribution, seasonal variation &Dengue transmission prediction in Sisaket, Thailand. *The Indian journal of medical research,***138(3),** 347–353.

World Health Organization, (2006).Guidelines for prevention and control of Dengue Fever and Dengue Hemorrhagic Fever.

World Health Organization, (2011).Comprehensive Guidelines for Prevention and Control of Dengue and Dengue Hemorrhagic Fever.

World Health Organization, (2016).Entomological surveillance for *Aedes* spp. in the context of Zika virus Interim guidance for entomologist (No. WHO/ZIKV/VC/16.2). World Health Organization.

World Health Organization, (2016). Test Procedures for insecticide resistance monitoring in malaria vector mosquitoes.

World Health Organization, Special Programme for Research, Training in Tropical Diseases, World Health Organization. Department of Control of Neglected Tropical Diseases, World Health Organization.Epidemic & Pandemic Alert. (2009). Dengue: guidelines for diagnosis, treatment, prevention and control: new edition. World Health Organization.

World Health Organization, (1981). Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides (No. WHO/VBC/81.807). World Health Organization.

World Health Organization, (2005). Guidelines for laboratory and field testing of mosquito larvicides.Communicable disease control, prevention and eradication, WHO pesticide evaluation scheme. WHO, Geneva. WHO/CDS/WHOPES/GCDPP/1.3.

World Health Organization, (2013). Larval source management: a supplementary malaria vector control measure: an operational manual.

World Health Organization, (2016). Monitoring and managing insecticide resistance in *Aedes* mosquito populations: Interim guidance for entomologists.

World Health Orgnization Geneva, (1990). International Programme on Chemical safety Environmental Health Criteria 97 Deltamethrin.

Yadav, K., Sarkar, P.K. and Veer, V. (2014).Utility of male mosquito hypopygium in species identification Tezpur, Assam. *International Journal of Mosquito Research*, **1(4),** 50-54.

Yadav, K.K., Bora, A., Datta, S., Chandel, K., Gogoi, H.K., Prasad, G.B.K.S. and Veer, V. (2015). Molecular characterization of midgut microbiota of *Aedes albopictus* and *Aedes aegypti* from Arunachal Pradesh, India.*Parasites and Vectors*, **8(1),** 1-8.

Yergolkar, P.N., Cherian, S.S., Jadhav, S., Raut, C.G. and Mourya, D.T. (2017). Genetic characterization of Dengue virus types 1 and 2 in India, with emphasis on the viruses circulating in Karnataka. *The Indian journal of medical research*, **146(5)**, 662.

Young, K.I., Mundis, S., Widen, S.G., Wood, T.G.,Tesh, R.B., Cardosa, J., Vasilakis, N.,Perera, D. and Hanley, K.A. (2017). Abundance and distribution of sylvatic Dengue virus vectors in three different lands cover types in Sarawak, Malaysian Borneo. *Parasites & Vectors*, **10(1),** 1-14.

Yuan, K., Chen, Y., Zhong, M., Lin, Y., Liu, L. (2022). Risk and predictive factors for severe dengue infection: A systematic review and meta-analysis. *PLoS ONE*, **17(4),** e0267186.

Zahir, A., Ullah, A., Shah, M. and Mussawar, A. (2016). Community participation, Dengue Fever prevention and control practices in Swat, Pakistan. *International Journal of MCH* and AIDS, **5(1),** 39–45.

PUBLICATIONS

J Vector Borne Dis 57, June 2020, pp. 176-181

Prevalence of dengue vectors, larval breeding habitats, Stegomyia indices and their correlation with dengue cases in urban and rural areas of Punjab, India

Seema Devi[1*], Taruna Kaura[2*], Jaspreet Kaur[2*], Lovleen[3], Jaspreet Takkar[4], S.K. Sharma[2], G.S. Grover[1]

[1]*National Vector Borne Disease Control Programme, 34-A Chandigarh, Punjab-160022, India;* [2]*National Institute of Malaria Research, Dhakoli, S.A.S. Nagar, Punjab -160104, India;* [3]*Department of Zoology, Lovely Professional University, Jalandhar, Punjab;* [4]*Gian Sagar Medical College, Banur, Punjab, India.*

**Equal contribution*

ABSTRACT

Background & objectives: The state of Punjab, India is highly endemic for dengue fever as high number of confirmed dengue cases have been reported since 2013. A better understanding of vectors distribution and their seasonal variation is necessary to control the disease. Therefore, the present study was conducted in both rural and urban areas of 11 out of 22 districts of Punjab to highlight seasonal prevalence of *Aedes* vector mosquitoes.

Methods: Entomological surveys were carried out in different seasons and all kinds of indoor and outdoor breeding habitats were examined and *Aedes* immatures were collected. The *Stegomyia* indices were calculated and compared from urban and rural areas in different seasons.

Results: Both vectors of dengue, i.e. *Aedes aegypti* and *Ae. albopictus* were recorded to be prevalent. *Ae. aegypti* mosquitoes were observed in all districts surveyed while *Ae. albopictus* were found only in seven districts of Punjab. The *Stegomyia* indices were significantly high during monsoon as compared to pre- and post- monsoon periods. Occurrence of dengue cases were found to be correlated with the *Stegomyia* indices.

Interpretation & conclusion: This is the first detailed study of prevalence of dengue mosquito vectors in Punjab showing the presence of *Ae. aegypti* and *Ae. albopictus* in both urban and rural areas of the state, thereby demonstrating wide distribution of this vector. Different breeding habitats identified in the study should be subjected to targeted intervention such as source reduction in order to achieve effective control of dengue cases.

Key words *Aedes aegypti*, *Aedes albopictus*, breeding habitats, dengue cases, *Stegomyia* indices, Punjab, India

INTRODUCTION

Dengue fever is one of the major mosquito-borne viral infections in tropical and sub-tropical regions and is considered as the leading cause of illness and death[1-2]. It is the most common and widespread arboviral infection in the world today with significant morbidity and mortality. The incidence of the disease has increased over the last 50 years with 2.5 billion population living in areas at risk where dengue is endemic affecting 100 million people each year[3-4], and has emerged a major public health concern at international level[5]. Dengue infection has been known to be endemic in India for over two centuries[6]. Increasing urbanization, unplanned growth of cities, mushrooming of urban slums, unsafe water storage practices have contributed to rise of dengue. The mosquitoes *Aedes aegypti* and *Aedes albopictus*, the established vectors of dengue and chikungunya are widespread throughout the tropical, subtropical and temperate areas of the world. Primarily, *Ae. aegypti* is reported to be native to Africa and now known to spread to tropical and sub-tropical areas in six continents[7]. *Ae. albopictus* mosquito has spread from Asia (where it originated), to Africa, North and South America, Europe, the Caribbean and also some parts of Pacific islands[8]. *Ae. albopictus* has been known to be carrier of DEN virus and has been considered as a secondary vector of dengue. It is reported to be adaptable in ecologically diverse conditions[9]. Both species are found in a wide array of water receptacles, including both artificial and natural containers which hold clean water. *Ae. aegypti* preferentially breeds in artificial containers while *Ae. albopictus* have a preference for natural water receptacles found outdoors.

Since there is no specific vaccine and/or treatment available for dengue[10], for vector control it has become imperative to generate data on vector density, population at infection risk and the sensitivity of mosquitoes to the insecticides[11-12]. Monitoring of dengue vector population in each region, such as the *Stegomyia* indices [Container

Table 1. Reported dengue cases in Punjab (2012–2017)

Year	Total dengue cases	Rural	Urban
2012	770	209	561
2013	4117	974	3049
2014	472	126	346
2015	14,149	2736	11,413
2016	10,439	3007	7432
2017	15,398	3816	11,582

Index (CI), House Index (HI), Breteau Index (BI) and Pupal Index (PI)] have become indispensable for entomologist to plan disease surveillance and control programmes. In India, dengue has recently dramatically spread and has been reported from all over the country including Punjab where it remains a major public health problem[1]. During 1996, an outbreak of dengue with 720 confirmed cases and 19 deaths were reported from Ludhiana district in the state[13]. In 2015, the state has reported 14,149 confirmed dengue cases and there has been a steady increase in confirmed cases of dengue in the last five years[14] (Table 1). The current study was designed to assess the prevalence of dengue vector in relation to *Stegomyia* indices during different seasons in urban and rural areas along with their correlation with dengue cases in 11 districts of Punjab. Based on the results, appropriate control measures can be planned to contain rise of dengue cases in the state.

MATERIAL AND METHODS

Description of study area

The state of Punjab is located in north western India (latitude 30° 4' North and longitude 75° 5' East) and has an area of 50,362 km^2. It is bounded on the west by Pakistan, north by Jammu and Kashmir, northeast by Himachal Pradesh and south by Haryana and Rajasthan. Most of the area in this state comprises of fertile plains, alluvial plain with three rivers and an extensive canal system for irrigation.

Selection of study area

The state of Punjab comprises of 22 revenue districts of which 11 districts were selected and based upon the retrospective analysis of last 5 years' (2012–2016) dengue cases, stratification was done into high, medium, and low risk areas. A cut off value of <250, >250, and >500 dengue cases was used to stratify the districts into low, medium and high-risk areas respectively. These districts were - Ludhiana, Patiala, Mohali, Amritsar (high risk); Hoshiarpur, Jalandhar, Fatehgarh Sahib, Bathinda (medium risk); Sangrur, Shaheed Bhagat Singh Nagar (S.B.S) and Muktsar (low risk).

Study/survey design and period

Cross-sectional entomological surveys were carried out in localities of 11 districts from where dengue cases were reported. The surveys were carried out in houses and peri-domestic areas during pre-monsoon (May–June), monsoon (July–September) and post-monsoon (October–December) seasons in 2017 to determine the preferred breeding sources and distribution of dengue vectors.

Entomological/ Aedes infestation survey

A house-to-house entomological survey was carried out in the study areas. The houses included in the study were selected by a systematic random sampling technique. From all the study areas, the first house was randomly included in the study. Thereafter, every 30th house was inspected for mosquito breeding in potential breeding habitats. All kinds of indoor and outdoor breeding habitats were examined to collect the *Aedes* immatures by following the dipping method[15]. A container containing any amount of water was considered as wet container and the wet container containing any number of immatures (larvae, pupae or both) was considered as positive container. The larvae were collected by using ladle, dipper, pipette depending upon the type and size of breeding source. The collected immatures were kept in plastic containers labelled coded for type of breeding source, locality code, house and date of collection[16].

Rearing and identification of mosquitoes

Mosquito larvae were reared in trays for each container type in laboratory and pupae were transferred to individual adult cages for emergence. The number of adults emerged out of total number of larvae were recorded and adult emergence rate was calculated. Every day the emerged adult mosquitoes were morphologically identified to species level by following the pictorial taxonomic keys[17-18].

Data analysis

The classical *Stegomyia* indices were calculated by following the guidelines of World Health Organisation (WHO)[19]. Based on estimated indices, we classified the areas/sites in terms of epidemic risk levels for DEN as low, medium or high with reference to established epidemic thresholds[19].

Statistical analysis

Significance of difference in *Stegomyia* indices in

different dengue risk areas and during different seasons in urban and rural areas were compared using ANOVA. Pearson's correlation test was applied to compare the dengue cases reported in the study area during 2017 with *Stegomyia* indices. The analysis was carried out using the SPSS version 16.0 software package.

RESULTS

Relative abundance of Aedes mosquito species and breeding habitats

Overall, 12,412 different types of water-holding containers, 6525 from urban and 5887 from rural areas were inspected during the survey, of which 628 in urban (9.6%) and 428 in rural areas (7.2%) were found to be infested with *Aedes* mosquito larvae. Some of the common breeding habitats found positive for *Aedes* larvae during surveys are shown in Fig. 1. The type of water-holding container in the study with the highest rate of positivity for *Aedes* mosquito larvae were found to be artificial containers, i.e., earthen pots (14.1%), followed by desert cooler (10.5%), discarded tyres (6.8%), refrigerator trays (6.7%), plastic containers (6.4%), discarded material (6.2%), water tank (5.3%) and miscellaneous (5.1%) in decreasing order. *Ae. aegypti* was found to be the predominant species and constituted 88.5% of all the emerged adults from all 11 study districts. In comparison, the proportion of *Ae. albopictus* was only 11.5% in 7 districts of Punjab. *Ae. aegypti* breeding was highest in desert coolers, plastic containers, earthen pots, refrigerator trays and tyres, whereas the habitats in which both *Ae. aegypti* and *Ae. albopictus* were found co-breeding were earthen pots (*Ae. aegypti* -16.5%; *Ae. albopictus* - 6.5%), discarded material (*Ae. aegypti* - 3.4%; *Ae. albopictus* - 2.3%), discarded tyres (*Ae. aegypti* - 3.2%; *Ae. albopictus* - 2.2%) and other miscellaneous material (Ae. *aegypti* - 4.0%: *Ae. albopictus* - 1.6%).

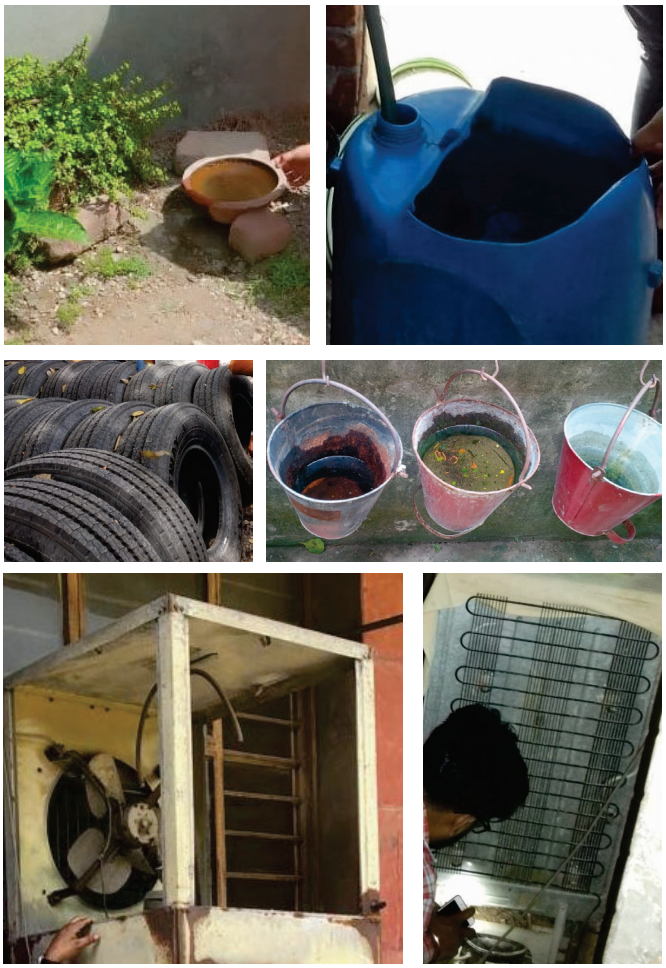


Fig. 1: Breeding habitats of *Aedes aegypti* and *Aedes albopictus* in study area in the state of Punjab, India. [Upper left: an earthen pot containing water kept for birds; upper right: a water storage plastic container; middle left: discarded tyres; middle right: fire buckets; lower left: desert cooler; lower right: refrigerator tray].

A higher dengue vector density was recorded during monsoon when compared with pre- monsoon and post-monsoon period. *Ae. aegypti* was found to be significantly high in both urban and rural areas whereas *Ae. albopictus* was found to be significantly high in urban as compared to rural areas (Table 2).

Stegomyia indices and correlation with dengue cases

A total of 3054 and 2828 houses were searched from urban and rural areas, respectively, for *Aedes* breeding. Out of the total houses surveyed, 565 houses from urban and 390 houses from rural areas were found positive for *Aedes* breeding. *Stegomyia* indices (House index, Container index, Breteau index and Pupal index) for *Aedes* mosquito larvae from the study areas were calculated and all indices were found to be higher in urban as compared to rural areas (Table 3) indicating high breeding potential of *Aedes* in urban areas. Moreover, the difference in *Stego-*

Table 2. The prevalence of *Aedes aegypti* and *Aedes albopictus* adult females in urban and rural areas in study districts of Punjab in year 2017

Season	Ecotype	*Ae. Aegypti*	*Ae. albopictus*
		Mean ± SD	Mean ± SD
Pre-monsoon	Urban	16.6 ± 14.7	0.0 ± 0.0
	Rural	10.2± 9.5	0.0 ± 0.0
Monsoon	Urban	71.7 ± 38.5	15.4 ± 18.9
	Rural	38.8 ± 18.8	2.0 ± 3.6
Post-Monsoon	Urban	16.7± 7.4	7.8 ± 15.3
	Rural	9.2 ± 7.7	0.3 ± 0.6

Table 3. *Stegomyia* indices of *Aedes* mosquito breeding in urban and rural areas along with number of dengue cases in study districts of Punjab during 2017

Risk Status for Dengue	Name of district*	No. of dengue cases, 2017	HI		CI		BI		PI	
			U	R	U	R	U	R	U	R
High	Mohali	2472	37.67	25.00	21.34	13.52	39.04	26.05	101.02	68.66
	Patiala	2434	22.26	19.02	14.09	11.29	26.64	19.02	98.9	40.89
	Ludhiana	1083	24.72	21.16	12.95	10.83	27.27	21.16	71.27	41.63
	Amritsar	222	16.07	9.62	8.24	4.70	19.64	11.11	41.78	30.74
Medium	Bathinda	557	16.9	9.81	9.49	5.33	19.71	10.94	48.59	34.71
	Hoshiarpur	1280	18.81	18.8	8.98	8.88	20.29	18.8	67.15	60.00
	Jalandhar	455	18.05	13.10	8.56	5.42	20.21	`13.10	44.76	39.30
	F.G.Sahib	789	13.07	11.78	7.64	8.23	15.9	12.92	54.41	43.72
Low	Sangrur	627	14.74	7.26	6.86	3.65	14.74	8.11	39.56	21.36
	S.B.S.Nagar	250	11.4	9.23	6.39	7.96	14.06	18.07	31.17	36.53
	Muktsar	348	8.36	3.86	3.96	1.59	8.72	3.86	33.09	17.16

*The districts were stratified into high, medium and low risk status for dengue based on the retrospective data of five years (2012–2016). However, during 2017 dengue cases were increased in Hoshiarpur and Sangrur districts and decreased in Amritsar district.

House index (HI)= number of positive premises per 100 houses inspected; Container index (CI)= number of containers infested with larvae and/or pupae per 100 containers inspected; breteau index (BI)= number of positive containers per 100 houses inspected; pupal index (PI): number of pupae per 100 houses searched.

myia indices between high, medium and low dengue risk areas were found to be significant ($P<0.001$). The analysis of seasonal variations in *Stegomyia* indices during pre-monsoon, monsoon and post-monsoon periods showed that breeding was found to be significantly high during monsoon as compared to pre- and post-monsoon ($P<0.001$), indicating that rain water collection in containers support maximum breeding of *Aedes* species during the monsoon (Table 4). The Pearson correlation analysis between dengue cases for data based in 2017 and *Stegomyia* indices in both urban and rural areas (Table 5) was found to be positively correlated ($P<0.005$). The correlation was much more significant with Breteau Index and Pupal Index as compared to House Index and Container Index (Table 5).

Table 4. Comparison of *Stegomyia* indices of *Aedes* mosquito breeding in different seasons

Season		HI	CI	BI	PI
		p value	p value	p value	p value
Pre-monsoon	Monsoon	.0001**	.0001**	.0001**	.0001**
	Post-monsoon	.016*	.030*	.021*	.427^{ns}
Monsoon	Pre-monsoon	.0001**	.0001**	.0001**	.0001**
	Post-monsoon	.0001**	.0001**	.0001**	.0001**
Post-monsoon	Pre-monsoon	.016*	.030*	.021*	.427^{ns}
	Monsoon	.0001**	.0001**	.0001**	.0001**

**- Highly significant; *- Significant, ns- non significant by using ANOVA test

Table 5. Correlation of dengue cases# with *Stegomyia* indices for data based on 2017*

Correlation	HI	BI	CI	PI
r2	0.839	0.881	0.809	0.925
p value	0.001	0.000	0.003	0.000

*Total no. of dengue cases in study districts during 2017: 10517

#: Pearson correlation was calculated based upon the number of cases of each district along with their *stegomyia* indices.

DISCUSSION

Dengue fever has emerged as the most important mosquito-borne viral disease and its vectors are spreading in areas hitherto free from the disease in India[20-21]. In Punjab, dengue fever puts a high load of morbidity not only on the poor but also among socio-economically better communities of the state. The high incidence of the disease every year is regarded as a negative reward of the rapid urbanization and changed social behaviour such as water storage practices and improper disposal of solid waste materials in and around house dwellings thus resulting in the creation of more favourable breeding habitats for *Aedes* mosquitoes. The present study has focused on the identification of different type of dengue vectors and their preferential breeding habitats in some dengue endemic districts of Punjab so that targeted interventions may be planned to curb the disease. The study has also determined the *Stegomyia* indices during different seasons in high, medium and low dengue risk areas and worked out their correlations with the dengue cases reported from the study area. The *Stegomyia* indices were observed to be significantly high during monsoon period and suggest high-risk of dengue transmission and positive correlation with the recorded dengue cases in the state. This study showed that *Aedes* mosquitoes seemed to preferentially

breed in earthen pots followed by desert coolers and discarded tyres. The possible reason behind this is that artificial containers were abundantly located close to human habitation and they can also hold water for extended periods of time making these suitable breeding habitats for mosquitoes[22-24]. The present results are in conformity with the earlier studies where earthen/mud pots, desert coolers and tyres were reported to be the prominent habitats for *Aedes* breeding[25-26]. These findings may enable a more focused approach to vector control in which specific types of water-holding containers would be targeted. In such an approach, source reduction would have the greatest impact to control disease transmission.

In this study, the dominant *Aedes* mosquito species was found to be *Ae. aegypti* which appeared to be playing a major role in dengue virus transmission; further studies are underway to isolate DEN virus from different *Aedes* species to ascertain their role in disease transmission. Similarly, *Ae. albopictus* was also recorded in low proportions from the study areas but its role in DEN virus transmission is yet to be established in Punjab. There is a need to identify circulating dengue virus strains and incriminating vectors specific to an area for formulating intervention strategies[27]. The relative abundance of both species, *Ae. aegypti* and *Ae. albopictus*, was higher during monsoon period than pre-monsoon and post-monsoon periods in agreement with previous studies in different parts of India[28-29]. Another important finding from the present study shows that *Ae. aegypti* was found to be high in both rural as well as urban areas whereas *Ae. albopictus* was abundant in urban than rural areas. The underlying reasons could be rapid urbanization in rural areas and introduction of piped water supply leading to water storage practices resulting in shift of breeding of *Ae. aegypti* in rural areas too. *Ae. albopictus*, which is believed to be predominantly a rural species and prefers to breed outdoors, was found more in abundance in urban areas, which indicates a shift in the breeding preferences from outdoors to peri/intradomestic containers in urban areas. However, it was suggested that urbanization has substantially resulted in its increased density which in turn is potentially increasing its vectorial capacity in urban areas[30]. In another study, it was found that *Ae. albopictus* has higher blood feeding rate in urban areas compared to rural areas attributed to more host availability[31].

This study was the first attempt to characterise the presence of *Aedes* mosquitoes and their preferred breeding habitats in Punjab. There are only few study reports on number of dengue and chikungunya cases from few districts but that lacked data on the dengue vector prevalence and breeding characteristics [4, 32-33]. An outbreak of dengue was reported in Ludhiana district of the state in 1996. Both epidemiological and entomological surveillance was carried out for the investigation of outbreak-related factors. Entomological surveillance revealed high House index and Container indices in different localities of the city. Breeding was mostly in desert coolers, tin/plastic containers, earthen pots and plastic buckets. Mosquito adults *Ae. aegypti* were collected from rooms and water receptacles[13].

The present results are consistent with the number of dengue cases indicating the potential of spreading dengue viral infection. Further investigations on arboviral transmission by *Aedes* mosquitoes are warranted. The study further showed that entomological indices were directly correlated with the number of reported dengue cases in the study area. The districts were stratified into high, medium and low risk status for dengue based on the retrospective data of five years before the start of the study. However, during 2017, dengue cases increased substantially in Hoshiarpur and Sangrur districts and decreased in Amritsar district, thereby indicating that the stratification of districts on the basis of dengue cases in the past years does not hold well because of year-to-year variations in the cases. Despite this limitation, this study provides the baseline data on the presence of the arbovirus vector *Aedes* mosquitoes in the state of Punjab.

CONCLUSION

Lack of awareness results in the creation of man-made breeding habitats for *Aedes* proliferation. Such breeding habitats should be subjected to targeted interventions such as source reduction with the removal of water-holding containers around household premises and proper disposal of discarded tyres so as to achieve sustainable dengue vector control.

Conflict of interest: None

REFERENCES

1. Bhat MA, Krishnamoorthy K. Entomological investigation and distribution of *Aedes* mosquitoes in Tirunelveli, Tamil Nadu, India. *Int J Curr Microbiol App Sci* 2014; *3*: 253–9.
2. Mutheneni SR, Morse AP, Caminade C, Upadhyayula SM. Dengue burden in India: recent trends and importance of climatic parameters. *Emerg Microbes & Infec* 2017; *6*: e70.
3. Guzman MG and Kouri G. Dengue: an update. *Lancet Infect Dis* 2002; *2*: 33–42.
4. Paul R, Oberoi L, Singh K, Devi P. A comprehensive study of dengue fever patients admitted to a tertiary care hospital in Amritsar. *Asian Pac J Health Sci* 2017; *4*: 100–3.
5. Balakrishnan N, Katyal R, Mittal V, Chauhan LS. Prevalence of

Aedes aegypti - The vector of Dengue/ Chikungunya fevers in Bangalore City, Urban and Kolar districts of Karnataka state. *J Commun Dis* 2015; *47*: 19–23.
6. Gupta E, Dar L, Kapoor G, Broor S. The changing epidemiology of dengue in Delhi, India. *Virol J* 2006; *3*: 92.
7. Powell JR. Mosquito-Borne Human Viral Diseases: Why *Aedes aegypti*? *Am J Trop Med Hyg* 2018; *98*: 1563–5.
8. Benedict MQ, Levine RS, Hawley WA, Lounibos LP. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. *Vector Borne Zoonotic Dis* 2007; *7*: 76–85.
9. Medeiros AS, Costa DMP, Branco MSD, Sousa DMC, Monteiro JD, Galvão SPM, *et al*. Dengue virus in *Aedes aegypti* and *Aedes albopictus* in urban areas in the state of Rio Grande do Norte, Brazil: Importance of virological and entomological surveillance. *PLoS One* 2018; *13*: e0194108.
10. Baly A, Toledo ME, Boelaert M, Reyes A, Vanlerberghe V, Ceballos E, et al. Cost effectiveness of *Aedes aegypti* control programmes: participatory versus vertical. *Trans R Soc Trop Med Hyg* 2007; *101*: 578–86.
11. Madeira NG, Macharelli CA, Carvalho LR. Variation of the Oviposition Preferences of *Aedes aegypti* in Function of Substratum and Humidity. *Mem Inst Oswaldo Cruz* 2002; *97*: 415–20.
12. Vikram K, Nagpal BN, Pande V, Srivastava A, Gupta SK, Singh A et al. Comparison of *Ae. aegypti* breeding in localities of different socio-economic groups of Delhi, India. *Int J Mosq Res* 2015; *2*: 83–8.
13. Gill KS, Bora D, Bhardwaj M, Bandyopadhyay S, Kumar K, Katyal R. Dengue Outbreak in Ludhiana (Punjab), India, 1996. *Dengue Bull* 1997; *21*: 47–51.
14. National vector borne disease control programme (NVBDCP). Dengue/DHF situation in India. Dengue Cases and Deaths in the Country since 2015. 2019; Available from: https://nvbdcp.gov.in/index4.php?lang=1&level=0&linkid=431&lid=3715 (Accessed on 2 January 2020)
15. Reuben R. A report on mosquitoes collected in the Krishna Godavari delta, Andhra Pradesh. *Indian J Med Res* 1978; *68*: 603–9.
16. World Health Organization. Dengue guidelines for diagnosis, treatment, prevention and control: new edition. World Health Organization. 2009; Available from: https://apps.who.int/iris/handle/10665/44188 (Accessed on 2 January 2020)
17. Rueda LM. Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated with Dengue Virus Transmission. *Zootaxa* 2004; *589*: 1–60.
18. Tyagi BK, Munirathinam A, Venkatesh A. A catalogue of Indian mosquitoes. *Int J Mosq Res* 2015; *2*: 50–97.
19. WHO. Guidelines for Dengue surveillance and mosquito control (2 Ed.), Regional Office of the Western Pacific, Manila, 2003.
20. Dev V, Khound K, Tewari GG (2014). Dengue vectors in urban and suburban Assam, India: entomological observations. WHO South-East Asia J Public Health; 3(1): 51–59.
21. Dev V, Mahanta N, Baruah BK (2015). Dengue, an emerging infection in Assam, Northeast India. Tropical Biomedicine 32 (4): 796–799.
22. Yee DA, Kneitel JM, Juliano SA. Environmental correlates of abundances of mosquito species and stages in discarded vehicle tires. *J Med Entomol* 2010; *47*: 53–62.
23. Saleeza SNR, Norma-Rashid Y, Sofian-Azirun M. Mosquitoes larval breeding habitat in urban and suburban areas, Peninsular Malaysia. *Int J Biol Vet Agri Food Eng* 2011; *5*: 81–5.
24. Wilson JJ, Sevarkodiyone SP. Spatial and temporal distribution of mosquitoes (Culicidae) in Virudhunagar district, Tamil Nadu, South India. *Int J Mosq Res* 2014; *1*: 4–9.
25. Sharma SK, Padhan K, Rath Y, Subbarao SK. Observations on the breeding habitat of *Aedes* species in the steel township, Rourkela. *J Commun Dis* 2001; *33*: 28–35.
26. Bhaskar RB, George B. Breeding patterns of *Aedes (Stegomyia) albopictus* in periurban areas of Calicut, Kerala, India. *Southeast Asian J Trop Med Public Health* 2010; *41*: 536– 40.
27. Dutta P, Khan SA, Chetry S, Dev V, Sharma, K. C, Mahnata J (2015). First evidence of dengue virus infection in wild caught mosquitoes during an outbreak in Assam, Northeast India. J Vector Borne Dis 52: 293–298.
28. Kumar RR, Daniel Reegan A Chandrasekar P, Kumar CS. Distribution of Dengue Vectors during Pre- and Post-Monsoon Seasons in Higher Attitudes of Nilgiri Hills of Western Ghats, India. *J Insects* 2013; *2*: 1–5.
29. Kanhekar LJ, Ravikumar R, Meena RK, Sharma RS, Sharma AK, Enkatesh SV. Population dynamics of *Aedes aegypti* and *Aedes albopictus* in Jagdalpur city, Bastar district of Chhattisgarh state, India. *Int J Mosq Res* 2016; *3*: 40–4.
30. Li Y, Kamara F, Zhou G , Puthiyakunnon S, Li C, Liu Y, *et al*. Urbanization increases Aedes albopictus larval habitats and accelerates mosquito development and survivorship. *PLOS Neglect Trop Dis* 2014; *8*: e3301.
31. Tsuda Y, Suwonkerd W, Chawprom S, Prajakwong S, Takagi M. Different spatial distribution of *Aedes aegypti* and *Aedes albopictus* along an urban-rural gradient and the relating environmental factors examined in three villages in northern Thailand. *J Am Mosq Control Assoc* 2006; *22*: 222–8.
32. Kaur M, Singh K, Sidhu SK, Devi P, Kaur M, Soneja S, *et al*. Coinfection of chikungunya and dengue viruses: A serological study from North Western region of Punjab, India. *J Lab Physicians* 2018; *10*: 443–7.
33. Lata S, Singh G, Dubey S. An Epidemiological Investigation of Dengue Outbreak in Shri Muktsar Sahib District, Punjab, India. *IJARIT* 2012; *3*: 153–8.

Correspondence to: Dr Gagandeep Singh Grover, Department of Health & Family Welfare, Parivar Kalyan Bhavan, Sector 34-A, Chandigarh India
E-mail: dr.gagangrover@gmail.com

Submitted: November 4 2019 *Accepted in revised form:* 7 January 2020