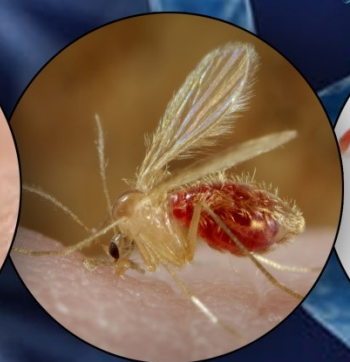
Application of Molecular Techniques in Vector-borne Disease Research






E- WORKSHOP ON APPLICATION OF MOLECULAR TECHNIQUES IN VECTOR-BORNE DISEASES RESEARCH

Organized by the Board of Study in Zoological Sciences

Postgraduate Institute of Science

University of Peradeniya

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Vector-borne diseases are a major reason for many global disease outbreaks that significantly affect human health and the nation's economy. Implementing effective responses to reduce the burden of these diseases is crucial. The application of molecular tools has been embraced by many scientists as these techniques have enabled them to address many unanswered questions related to both vectors and pathogens. Molecular-based research has directed investigators to develop more precise vector and pathogen identification methods, increasing the efficiency of existing vector control strategies and developing new, more environmentally friendly vector control approaches. Therefore, this e-workshop aims to provide the participants with an overview of vector-borne diseases that are currently important in Sri Lanka and acquire knowledge on molecular techniques required to carry out research and diagnosis of such diseases. Further, it also provides knowledge and skills in quantitative analysis and handling of molecular data.

On successful completion of the workshop, participants will be able to gain detailed knowledge on vector-borne diseases in Sri Lanka and the world, molecular taxonomy involved in vector and pathogen identification, molecular detection of specific infectious agents, determining the molecular basis of many types of insecticide resistance, and application of bioinformatics in vector-borne disease research.

The encouragement and guidance given by Prof. H.M.T.G.A. Pitawala, Director, PGIS, is gratefully acknowledged. I am thankful to the members of the Organizing Committee, who have generously contributed to this event. Special gratitude goes to Prof. W.A.I.P. Karunaratne, the Chairperson of the Board of Zoological Science, for the support rendered.

INTRODUCTION TO VECTOR-BORNE DISEASES IN SRI LANKA

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1. The global significance of vector-borne diseases

Vector-borne diseases (VBDs) are among the most important global public health problems. They are associated with a significant economic burden, primarily in tropical and subtropical regions accounting for more than 17% of all infectious diseases, causing over 700,000 deaths annually. The VBDs are transmitted by hematophagous arthropods, mostly mosquitoes, ticks, sand flies, and triatomine bugs. These diseases are caused by parasites, bacteria or viruses. The most deadly disease, malaria, is caused by the parasite species *Plasmodium* transmitted by anopheline mosquitoes. It causes an estimated 219 million cases globally and results in more than 400,000 deaths every year, mostly among children under five years. Dengue is the most prevalent viral infection transmitted by *Aedes* mosquitoes. More than 3.9 billion people from 129 countries are at risk of contracting dengue, with an estimated 96 million symptomatic cases and an estimated 40,000 deaths every year. Other viral diseases transmitted by mosquitoes include chikungunya fever, Zika virus fever, yellow fever, West Nile fever, and Japanese encephalitis. Although mosquitoes are responsible for most VBDs worldwide, ticks transmit most infections in the United States, the most common being Lyme disease and rickettsioses, caused by bacteria.

2. Current status of four major VBDs: malaria, dengue, leishmaniasis and rickettsioses in Sri Lanka

For many decades, malaria has been a significant VBD in Sri Lanka. However, after achieving elimination status in 2016, it is no longer considered a major public health concern. Sri Lanka is now in the phase of prevention of re-introduction of malaria as imported malaria remains a challenge to the resurgence of the disease. Imported cases have been reported due to *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*; however, in 2016, the first imported case of *Plasmodium knowlesi*, the fifth species of human malaria parasite, was reported in Sri Lanka in an army officer who returned from Malaysia. *Plasmodium knowlesi* is a non-human primate origin species. Efforts to remain malaria-free through epidemiological investigation together with integrated and cost-effective vector control and entomological surveillance should continue to prevent re-introduction.

Currently, dengue is a significant health concern in the country. In 2019, Sri Lanka was placed eighth worldwide and fifth in Asia among the countries reporting the highest number of cases. Sri Lanka faced a massive dengue epidemic in 2017, the largest outbreak in the country during the last three decades with 186,101 cases, and over 320 deaths. Although the epidemic was controlled by intense measures bringing the numbers down to 51,659cases and 58 deaths in 2018, the cases rose again in 2019 reaching 102,746 and 90 deaths, indicating the re-emergence of an outbreak. Deaths were mainly due to delay in the hospitalization of severe dengue patients. The mortality of dengue

hemorrhagic fever is 2–5% if detected early and treated promptly but is high as 20% if left untreated. A downward trend has been observed during 2020 and 2021 (up to December), reporting 31,162 and 17,490 cases, respectively. Close to half of the cases reports are from the Western Province.

For decades leishmaniasis in Sri Lanka was considered an exotic disease. It is a neglected tropical VBD, and according to the Ministry of Health, cutaneous leishmaniasis (CL) is now recognized as an endemic disease in Sri Lanka. Up until the early 1990's only a few imported cases of cutaneous leishmaniasis were noted. The first locally transmitted case of CL was reported in 1992 from Ambalanthota, and the second case in 1995 in Mahiyangana. The number of cases has increased since then. Molecular studies showed that cutaneous leishmaniasis in Sri Lanka is caused by the *Leishmania donovani*, a usually visceralizing and the most virulent species parasites elsewhere, including India. Further research confirmed that the local parasite *L. donovani* exists in multiple variants causing cutaneous, muco-cutaneous and visceral leishmaniasis. The first case of muco-cutaneous leishmaniasis was reported in 2005. For an accurate diagnosis, using a two-staged clinical algorithm and conventional PCRs for efficiency is important. The probable vector of the Sri Lankan leishmaniasis parasite is the *Phlebotomus argentipes glaucus* sandfly, which demonstrates zoophilic behaviour and differs from the *Phlebotomus* species of sandflies found in southern India. The continuous upsurge of the disease transmission in Sri Lanka may hamper the regional effort to eliminate visceral leishmaniasis in South Asia and call for local interventions.

Tick-borne rickettsioses are emerging infections in Sri Lanka, and little is known about the prevalence, distribution, vectors, reservoirs and infectious agents. The first record of rickettsia came from the central hills reporting Spotted Fever Group Rickettsia (SFGR), scrub typhus (*Orientia tsutsugamushi*) and murine typhus (*Rickettsia typhi*). Later, lowland Western Province showed the predominance of *O. tsutsugamushi* infection and then from southern Sri Lanka scrub typhus, SFGR, Q fever, murine typhus, and *Rickettsia felis* infections. Moreover, in 2009 a case of travel-acquired SFG rickettsiosis in an Australian visiting Sri Lanka was reported. The disease burden due to SFGR is far more significant than that of the other rickettsial infections in Sri Lanka. Some rickettsioses with low pathogenicity cause only a few cases. Since they are being discovered recently and there are no proper specific diagnostic protocols to identify, the exact agent and their impact are unknown. Nevertheless, they contribute to the increased incidence. The extent to which infections persist in the host animal, the relative importance of different hosts for tick maintenance by different stages of ticks, and the means and efficiency of transfer of rickettsiae from an infected to an uninfected tick are all uncertain and are still open questions today.

3. Challenges for control

Most VBDs are emerging or re-emerging in new regions after a significant decline in incidence. Social and demographic factors such as human population growth, urbanization, globalization, trade exchange, travel and close interactions with livestock have significantly been linked with the emergence or re-emergence of VBDs. The ongoing evolution of pathogens, proliferation of reservoir populations, and antimicrobial drug are also principal exacerbating forces for the emergence and re-emergence of VBDs. Even though many important VBDs are becoming better controlled, our success in preventing the new appearing and resurging VBDs that may happen in the future seems uncertain.

4. Reservoirs and effect of biodiversity on zoonotic cycles maintaining reservoirs

Most VBDs of humans are spillovers from non-human animals via zoonotic transmission. These VBDs exist in complex zoonotic cycles involving a variety of birds, rodents, and other vertebrate hosts. Factors determining the emergence of zoonotic VBDs and continuing circulation in sylvatic cycles are not understood completely. Identifying potential sylvatic reservoirs and characterizing the macro-ecological traits common to known wildlife hosts are important to predict the risk of sylvatic transmission among wildlife and identify regions that could be vulnerable to outbreaks. Pristine natural areas with high biodiversity were earlier seen as likely sources of new zoonotic pathogens, suggesting that biodiversity could negatively impact human health. However, recent studies show biodiversity has been recognized as potentially benefiting human health by reducing the transmission of some pathogens that have already established themselves in human populations. Some taxa are much more likely to be zoonotic hosts than others are, and that these animals often proliferate in human-dominated landscapes, increasing the likelihood of spillover. However, these zoonotic reservoir hosts are less abundant in less-disturbed areas, and non-reservoirs predominate. Thus, biodiversity loss appears to increase the risk of human exposure to both new and established zoonotic pathogens. This new synthesis of the effects of biodiversity on zoonotic diseases presents an opportunity to articulate the next generation of research questions that can inform management and policy. Future studies should focus on collecting and analyzing data on the diversity, abundance, and capacity to transmit the taxa that share zoonotic pathogens with humans. Restoration of biodiversity is an important frontier in managing zoonotic disease risk.

5. Effect of climate change on vector-borne diseases

Climate change affects the appearance, resurgence and transmission dynamics of VBDs and impacts are likely to worsen. It could affect the range and population of pathogens, host and vectors, transmission season, etc. Reliable surveillance for diseases that are most likely to emerge is required. As vectors do better in a warmer world, the intersection of how meteorological variables affect the incidence, transmission-season duration and spread of VBDs and scenario-based modeling of the effects of future climate change can aid long-term planning for the prevention and control of VBDs.

6. One Health Approach

The interactions among pathogens, hosts, and the environment play a key role in the emergence or re-emergence of VBDs. The One Health concept must be integrated into the struggle against emerging VBDs of zoonotic origin. The information about the reservoir hosts maintaining the natural cycle of most VBDs and the vector/s responsible for transmitting the disease is largely unknown. Some VBDs like SFGR are being discovered recently; therefore, there are no proper specific diagnostic protocols to identify the exact agent, but suspected cases are treated with broad-scale antibiotics. To predict and prevent future epidemics, researchers should also focus on how these metrics change in response to human impacts on the environment and how human behaviours can mitigate these effects.

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MOSQUITO CONTROL IN SRI LANKA: IS TRANSGENESIS A WAY FORWARD?

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1. Background

The prevalence of mosquito-borne diseases in Sri Lanka is attributed to conducive tropical environmental conditions, including rainfall, temperature, and relative humidity. Mosquito-borne diseases continue to be of public health concern globally. Although, Sri Lanka has been declared free of malaria, and filariasis, dengue, remains a serious public health problem in the country. A dramatic increase in the reported cases of dengue fever (DF) and dengue hemorrhagic fever (DHF) in Sri Lanka has been observed in recent years (Malavigae et al., 2021). An epidemic of chikungunya was reported in year 2006-2007 (Surendran et al., 2007). Currently, there are no vaccines or specific drugs available for dengue, and the control of the principal mosquito is the only option to control the transmission of the disease.

2. Mosquito vector control

Vector control measures in Sri Lanka focus on adult and larval control. The control mainly relies on indoor residual spraying (IRS) of houses with synthetic pyrethroids such as lambda-cyhalothrin and deltamethrin. The application of these residual insecticides varies from district to district (Perera et al., 2008). Another adult control measure is the use of bed-nets impregnated with insecticides which are usually distributed among families living in endemic areas. Active source reduction and thermal fogging using Pesguard (a formulation containing knock-down and killing agents) are employed to control dengue vectors. Immature stages of vector populations are controlled chemically by applying the larvicide (e.g. temephos) in selected localities and biologically using larvivorous fish (e.g. *Poecelia reticulata*). Various other control measures have also been either tested or being recommended to prevent man-vector contact. Emphasis has also been laid on applying self-protection methods such as bednets and mosquito repellents in the form of smoke, oil, and chemicals (http://www.malariacampaign.gov.lk; http://www.dengue.health.gov.lk).

3. Genetically modified mosquitoes for vector control

The failure to control mosquito-borne diseases has been attributed to (i) the lack of effective vaccines, (ii) ineffective vector control programmes, (iii) the development of drug resistance in pathogens, and (iv) the development of insecticide resistance in vector populations (Beaty, 2005). These drawbacks have justified using genetically modified mosquitoes (GMM) to control the disease transmission (Scot et al., 2002).

Two different endpoints are targeted in GMM: (i) reducing vector populations and (ii) replacing vector populations with strains that are refractory to pathogen development and transmission. However, the release of large numbers of female transgenic mosquitoes capable of becoming a biting nuisance is an issue and researchers have developed techniques to select and release only male transgenic mosquitoes for field

release. However, the sterile male technique has not proved popular for controlling mosquito vector populations because of the presence of several different vector species or sibling species transmitting the same pathogen in many endemic areas, the fitness disadvantage shown by irradiated males in the wild, and the facilities needed for irradiating large numbers of male mosquitoes.

In this regard, releasing mosquito vectors carrying dominant lethal (RIDL) gene appears to be a more promising approach in vector control for reducing vector population (Alphey et al., 2008). Only males are released into the environment to mate with wild females so that the subsequent female progeny die in their immature stage or are flightless.

A different approach utilizes *Wolbachia* species, a group of obligate intracellular maternally inherited bacteria for mosquito vector control. *Wolbachia* interferes with host reproduction and are transmitted vertically. *Wolbachia* causes cytoplasmic incompatibility where when a male carrying the bacteria mate with a female carrying no *Wolbachia* or a different strain of *Wolbachia*, the resultant embryos die. , the reproductive modification resulting from *Wolbachia* infection has been used to adapt two strategies to control insect vectors. They are: (i) population suppression (similar to the SIT) and (ii) population replacement (genetically modified *Wolbachia* are used as a vehicle to drive desirable phenotypes (gene drive) into natural vector populations. Studies have shown that *Wolbachia* infection of vector mosquitoes results in reduced infection of dengue, chikungunya, and yellow fever viruses to the infected mosquitoes (Kamtchum-Tatuene et al., 2017; Yen et al., 2020). Recently, various gene drive technologies, mainly CRISPR/Cas9, are used to replace vector populations as non-vector populations to control the transmission of mosquito-borne diseases (Macias et al., 2017).

4. Releasing GMM under Sri Lankan Context

The dengue vector mosquitoes *Aedes aegypti* and *Ae. albopictus* are widespread in the country and their larvae are in similar containers, although *Ae. aegypti* is more common in areas of higher population density (Surendran et al., 2020). The replacement or suppression of one vector species may aid in propagating another potential vector species, which may continue the transmission momentum even more vigorously. Therefore, extensive studies are to be carried out to establish population biology, gene flow, population genetics, entomological inoculation rate and pathogen transmission (Scott et al., 2002) before releasing only a single genetically modified vector species in areas where other potential species are present.

Using GMM as a control measure requires serious consideration of ethical, legal, ecological, and social aspects. Asian countries like Malaysia, India and Thailand are ready to incorporate GMM technology in vector control programmes. Sri Lanka has never previously been involved in an GMM programme. However, being a small island nation, Sri Lanka has the advantage of releasing GMM without cross-border issues.

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MOLECULAR TAXONOMY

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1. **What is Taxonomy?**
   It is the practice and science of categorization or classification.

2. **Methods/ Approaches**
   - Morphology
   - Molecular

3. **Molecular approach**
   - A gene is the basic physical and functional unit of heredity
   - Organisms are made up of genes that shape the characters
   - Each gene is unique
   - Their evolution is also unique

4. **Species v/s. gene tree**
   - Each gene tree reflects a unique story linked to species history but often significantly differs from it. So, there is no need for a gene tree to always reflects the true evolutionary history of a species.
   - Orthologs are homologous genes in different species that diverged from a single ancestral gene after a speciation event
   - paralogs are homologous genes that originate from the intragenomic duplication of an ancestral gene.

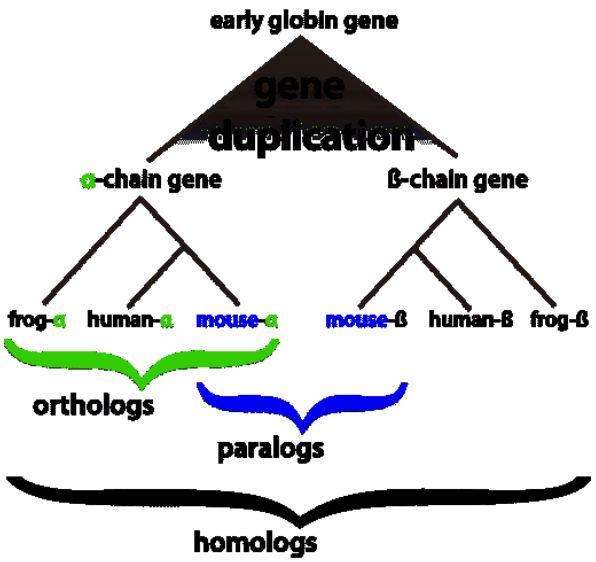

Figure 1. Orthologs vs Paralogs illustrated by the evolution of globin genes

5. Tree building

5.1. DNA data

The DNA sequence data provides valuable information about the genetic structure of an organism. DNA sequence consists of only four letters (nitrogen bases) which in different combination write the life story of an organism. DNA sequences are obtained through sequencing a piece or whole gene or an entire genome. Polymerase Chain Reaction is a method where a target piece of DNA can be obtained. The sequencing can be done using various methods. A DNA sequence is obtained from organisms in Abi format

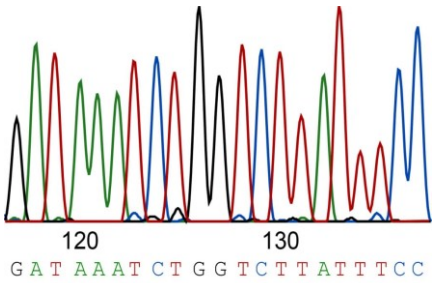

Figure 2. An example chromatogram

5.2. Fasta format: Text file

The sequence can be conveniently stored in a format called Fasta (a simple text format). Fasta files are used in many sequence analysis tools.

```
>NG_008679.1:5001-38170 Homo sapiens paired box 6 (PAX6)
ACCCTCTTTTCTTATCATTGACATTTAAACTCTGGGGCAGGTCCTCGCGTAGAACGCGGCTGTCAGATCT
GCCACTTCCCCTGCCGAGCGGCGGTGAGAAGTGTGGGGAACCGGCGCTGCCAGGCTCACCTGCCTCCCCGC
CCTCCGCTCCCAGGTAACCGCCCGGGCTCCGGCCCCGGCCCGGCTCGGGGCCCGCGGGGCCTCTCCGCTG
CCAGCGACTGCTGTCCCCAAATCAAAGCCCGCCCCAAGTGGCCCCGGGGCTTGATTTTTGCTTTTAAAAG
GAGGCATACAAAGATGGAAGCGAGTTACTGAGGGAGGGATAGGAAGGGGGGTGGAGGAGGGACTTGTCTT
TGCCGAGTGTGCTCTTCTGCAAAAGTAGCAAAATGTTCCACTCCTAAGAGTGGACTTCCAGTCCGGCCCT
GAGCTGGGAGTAGGGGGCGGGAGTCTGCTGCTGCTGTCTGCTAAAGCCACTCGCGACCGCGAAAAATGCA
GGAGGTGGGGACGCACTTTGCATCCAGACCTCCTCTGCATCGCAGTTCACGACATCCACGCTTGGGAAAG
TCCGTACCCGCGCCTGGAGCGCTTAAAGACACCCTGCCGCGGGTCGGGCGAGGTGCAGCAGAAGTTTCCC
GCGGTTGCAAAGTGCAGATGGCTGGACCGCAACAAAGTCTAGAGATGGGGTTCGTTTCTCAGAAAGACGC
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Figure 3. An example fasta file

5.3. Alignment

Various tools can be used to align sequences. Ex.: Clustal W / Muscle

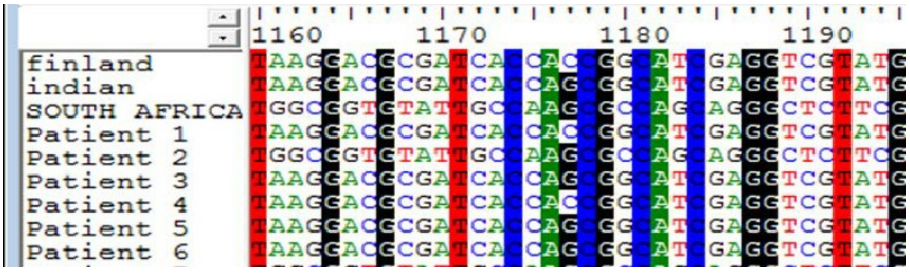

Figure 4. An example alignment of DNA sequence

Alignment is just like comparing different sequences for the similarity.

5.4. Tree building

Phylogenetic tree building is done based on comparing sequences for the similarity and the pattern of nucleotide substitutions using mathematical models.

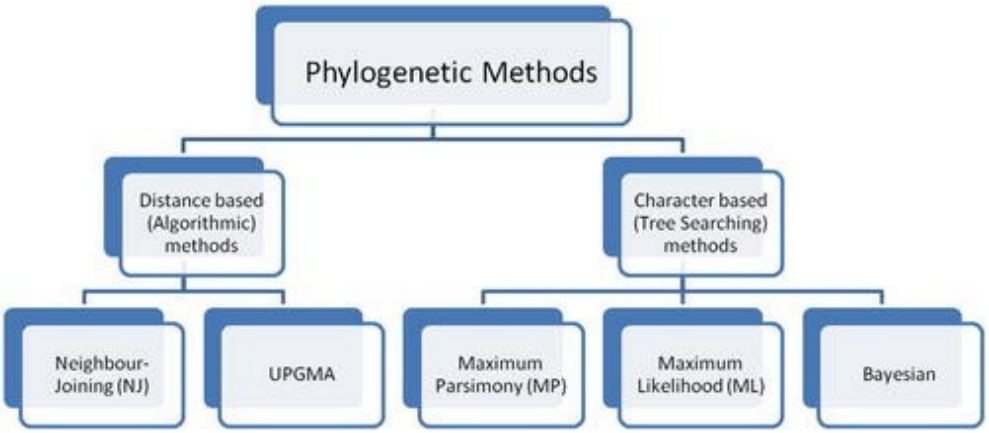

Figure 5. Different models and methods used in phylogenetic tree construction

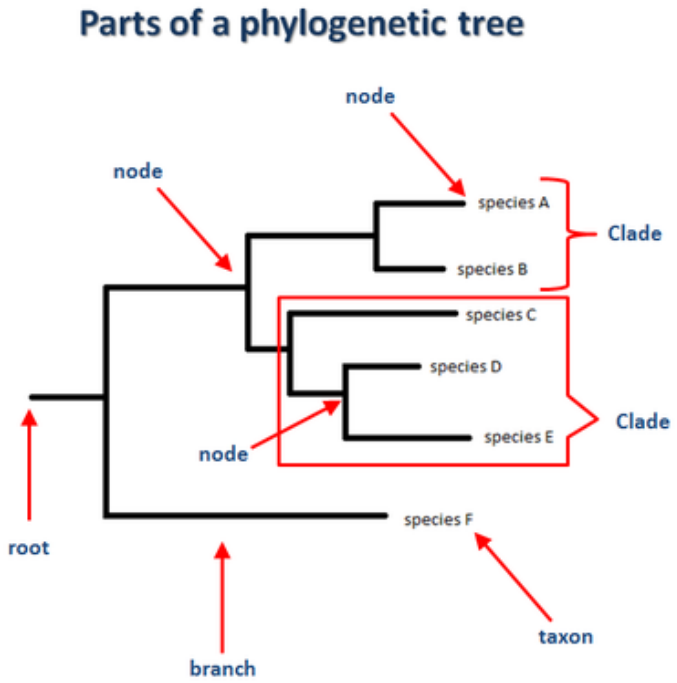

Figure 6. An example phylogenetic tree

6. Numerical taxonomy

Numerical taxonomy is a classification system in biological systematics which deals with the grouping by numerical methods of taxonomic units based on their character states. It aims to create a taxonomy using numeric algorithms like cluster analysis rather than using subjective evaluation of their properties. The concept was first developed by Robert R. Sokal and Peter H. A. Sneath in 1963 and later elaborated by the same authors. They divided the field into phenetics in which classifications are formed based on the patterns of overall similarities and cladistics in which classifications are based on the branching patterns of the estimated evolutionary history of the taxa.

	Characters									
Taxa	1	2	3	4	5	6	7	8	9	10
A	+	+	+	+	+	+	+	+	+	-
B	+	+	+	-	-	+	+	+	-	-
C	+	+	+	+	-	+	+	+	-	+
D	+	+	-	-	-	+	-	-	-	-
E	+	+	-	-	-	-	+	-	-	-

Figure 7. A matrix created to assess the phylogeny using numerical data

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MOLECULAR DETECTION OF THE MECHANISMS OF INSECTICIDE RESISTANCE IN VECTORS

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1. Introduction

Vector control has become the major strategy to control diseases transmitted by insect and acarine vectors such as mosquitoes, ticks and mites. Vector control programmes largely depend on synthetic insecticides, especially during disease outbreaks. The development of resistance against commonly used insecticides is a major threat in control strategies in a situation where not many alternatives are available to combat insect populations. Therefore, research on insecticide resistance, molecular mechanisms that underlie the resistance, and rational resistance management are paramount in controlling the development and spread of insecticide resistance in vector populations.

2. Commonly used insecticides and their target sites

Commonly used synthetic insecticides can be divided into four major groups; organophosphates, carbamates, pyrethroids and organochlorines. All these insecticides attack the nervous system of the insect. The target site for organophosphates and carbamates is the insect acetylcholinesterase which hydrolyzes the neurotransmitter acetylcholine on the post-synaptic nerve membrane. Inhibition of acetylcholinesterase by the insecticides results in continuous stimulation of post-synaptic nerve membrane leading to insect's death. For pyrethroids and a group of organochlorines (DDT + its analogues), the target site is Na^+ channel regulatory proteins of the nerve membrane. The binding of these insecticides to Na^+ channel regulatory proteins causes persistent activation (opening) of the sodium channels by delaying the normal voltage-dependent mechanism of inactivation (closing). The persistent opening hinder the active outside pumping of sodium ions leading to the continuous firing of the nerves causing the ultimate coma and death of the insect. For the rest of the organochlorines (cyclodienes), the target site is γ-aminobutyric acid (GABA) receptors which regulate Cl^- conductance through the nerve membrane. Binding to GABA receptors will also cause continuous firing of the nervous system leading to the insect's death.

Insects develop resistance against these insecticides through two major mechanisms; Metabolic Resistance (changes in insect enzyme systems for rapid detoxification of insecticides) and Target-site Insensitivity (alterations of the target sites to prevent their binding to insecticides) (Karunaratne et al., 2018).

3. Mechanisms of insecticide resistance

3.1. Increased detoxification by metabolic enzymes

Three major groups of enzymes *i.e.* esterases (also known as carboxylesterases), glutathione S-transferases (GSTs) and monooxygenases (also known as oxidases or cytochrome P450s) are known to be involved in insecticide detoxification in the insect

body. Basically, esterases can resist organophosphates, carbamates, and pyrethroids rich with ester-bonds. GSTs can mediate resistance to organochlorines, organophosphates and pyrethroids. Monooxygenases act against all classes of insecticides (Li *et al.,* 2007). Enhancement of enzyme activity in resistant insects compared to susceptible insects can be due to a) quantitative changes and/or b) qualitative changes of the enzyme in resistant insects. Increased quantities (qualitative changes) of metabolic enzymes are produced in resistant insects due to gene amplification or increased upregulation of the gene. Increased catalytic centre activity (qualitative changes) is achieved through coding sequence mutations.

3.1.1. Esterases

Esterase-based resistance mechanism has been studied extensively at the biochemical and molecular levels in *Culex quinquefasciatus* mosquitoes. Broad-spectrum organophosphate resistance is conferred by increased esterases in more than 80% of resistant *Culex* worldwide. Esterases act by rapid binding and slow turning over of the insecticide. They sequester rather than rapidly metabolize the insecticide. Therefore, to keep the system effective, large quantities are required (Hemingway and Karunaratne, 1998). It was found that the esterase gene is amplified up to 80 copies in resistant insects for the increased production of esterases.

In order to detect the presence of this mechanism in an insect population, crude homogenates of individual insects can be tested with an ester substrate such as naphthyl acetate or para-nitrophenyl acetate, and the esterase activity can be measured as an end-point assay or as a kinetic assay using a spectrophotometer. By doing protein assays for each respective crude homogenate, esterase activity can be normalized and obtained per mg. of the insect (see sections 4.1. and 4.4.). Esterase activity profiles for each population can be prepared by testing about 200 insects per population. Elevation of esterases can also be detected by running the crude homogenate on native polyacrylamide gels. In *Cx. quinquefasciatus,* the most common elevated esterase phenotype involves two enzyme types based on their preferences for α-or β-naphthyl acetate and their mobility on the gels.

Increased esterase activity may not always be a result of gene amplification. In contrast to the situation in *Culex,* a number of *Anopheles* species have a non-elevated esterase mechanism that confers resistance, specifically to malathion, through increased rates of metabolism (Karunaratne and Hemingway, 2000). These enzymes are not found in large quantities in resistant insects compared to susceptible individuals but have a higher catalytic centre activity than the susceptible enzyme. To detect the presence of this mechanism, insect crude homogenate can be incubated with malathion for 2 hours and the mixture can then be extracted in chloroform and run on thin-layer chromatography to visualize spots of malathion and its metabolites (see section 4.6.).

3.1.2. Glutathione S-transferases

Resistance to insecticides can be provided by GSTs through several different pathways: O-dealkylation or O-dearylation conjugation for organophosphates, dehydrochlorination and GSH conjugation for organochlorines, detoxification of lipid peroxidation products and passive sequestration for pyrethroids (Che-Mendoza *et al.,* 2009). Biochemical assays can be conducted to detect GST enzyme levels in insect populations (see section 4.2.).

3.1.3. Monooxygenases

Monooxygenases are a complex family of enzymes that bind to molecular oxygen and receive electrons from NADPH to introduce an oxygen molecule into the substrate. Increased monooxygenase levels can also be detected in insect populations by biochemical assays (see section 4.3.).

The genome sequence of the major dengue vector, *Aedes aegypti*, reveals an abundance of detoxification genes belonging to esterases, GSTs and mooxygenases. In order to identify detoxification genes associated with resistance to insecticides, microarrays containing unique oligonucleotide probes for these genes have been constructed and their expression level in insecticide-resistant and susceptible strains have been compared. Several candidate genes have been identified, with the majority belonging to two gene families, the CYP9P450s and the Epsilon GSTs. This '*Ae. aegypti* Detox Chip' can facilitate the implementation of insecticide resistance management strategies (Strode et al., 2008).

3.2. Target site insensitivity through alteration of target sites

Insects acquire target site insensitivity mainly through non-silent point mutations within structural genes. However, only a limited number of changes can decrease insecticide sensitivity without disrupting the normal physiological functions of the target site (Karunaratne et al., 2018). Therefore, the number of possible amino acid substitutions is very limited. Hence, identical resistance-associated mutations are commonly found across highly diverged taxa. Since the gene mutations alter the binding affinities of target sites to different insecticides depending on the molecular structure of the insecticide, altered target sites do not mediate the same level of resistance to all the insecticides belonging to a particular group.

3.2.1. Insensitive acetylcholinesterases

Since acetylcholinesterase is an enzyme, its insensitivity to insecticides can be tested by a biochemical assay. Insect crude homogenate can be incubated with carbamate and the percentage of inhibition of the enzyme can be tested with a substrate. Acetylcholinesterase activity of the inhibited fraction can be expressed as a percentage of the activity shown by the uninhibited crude homogenate to decide on the percentage insensitivity of acetylcholinesterase to the insecticide (see section 4.5.).

3.2.2. Insensitive Sodium Channel regulatory proteins and Insensitive GABA receptors

The *`kdr'* (*`knock-down resistance'*) type gene mutations at *VGSC* (voltage-gated sodium channel) genes prevent the binding of DDT and pyrethroids to their target site. To date, several resistance-associated *`kdr'* mutations have been identified in pyrethroid and DDT resistant insect populations. To detect kdr type mutations, specific fragments of the VGSC gene (especially in the domains II, III, and IV) can be amplified by PCR and sequence. Specific primers for these PCR reactions can be found in the literature (Nugapola et al., 2020).

The *`Rdl' (`resistance to dieldrin locus') type gene mutations at* GABA receptor genes prevent the binding of cyclodienes (a group of organochlorines) to GABA receptors. Since the use of organochlorines has been discontinued in many countries due to environmental concerns, investigations on GABA mutations has very little importance.

4. Biochemical assays

Biochemical assays can be carried out according to the procedures outlined by WHO (1998). From each population, >200 mosquitoes are individually subjected to total protein, esterase, GST, monooxygenase and acetylcholinesterase assays in three replicates. Each mosquito is homogenized in 200 µL ice-cold distilled water. An aliquot of 100 µL is taken for AChE assay and the rest is centrifuged at 13,000 rpm for two minutes. The supernatant is used for esterase, GST, monooxygenase and protein assays (WHO, 1998).

4.1. Esterase assay

10 µL of each homogenate supernatant is mixed with 200 µL of PNPA working solution (1 mM ρ-nitrophenyl acetate in 50 mM sodium phosphate buffer pH 7.4) in a microtitre plate well. 10 µL of distilled water mixed with 200 µL of working PNPA solution can be used as the control. The plate is read at 405 nm continuously for 2 min as a kinetic assay.

4.2. Glutathione S-transferase assay

Ten microliters of each homogenate supernatant is mixed with 200 µl of 10.5 mM reduced glutathione (GSH) in 100 mM phosphate buffer and 63 mM 1-chloro 2,4-dinitrobenzene (CDNB) in methanol. 10 µL of distilled water and 200 µL of the GSH/CDNB working solution can be used as the control. The plate is read at 340 nm continuously for 5 minutes as a kinetic assay.

4.3. Monooxygenase assay

2 µL of homogenate supernatant is mixed with 80 µL of 0.625 M potassium phosphate buffer (pH 7.2) in microtitre plate well. 200 µL of the working solution of tetramethylbenzidine and 25 µL of 3% hydrogen peroxide are added to each replicate. For the control, distilled water instead of the crude homogenate can be used. The mixture is left for 2 hours at room temperature and the absorbance is read at 650 nm as an endpoint assay.

4.4. Protein assay

In a microtitre plate well, 10 µL of the homogenate is mixed with 300 µL of Bio-Rad solution (prepared according to the manufactures instructions). Absorbance is read at 570 nm after a 5 min incubation at room temperature.

4.5. Acetylcholinesterase assay

Two replicates of crude insect homogenate (2 × 25 µL) are placed in separate wells of a microtitre plate. Then 145 µL of 1% Triton X-100 in 0.1 M sodium phosphate buffer (pH 7.8) and 10 µL of dithiobis-2,nitrobenzoic acid (DTNB) in phosphate buffer (pH 7.0) are added to each well. Twenty-five microliters (25 µL) of acetylthiocholine iodide (ASChI) and propoxur solution is added to one replicate and 25 µL of 0.01M ASChI alone is added to the other replicate. For the control, 2x25 µL of distilled water instead of the crude homogenate can be used. Plates are read continuously at 405 nm for 5 minutes.

4.6. Detection of malathion carboxylesterase activity

Resistance caused by a malathion carboxylesterase mechanism results in increased production rates of the monoacid and/or diacid of malathion in the resistant insects. For each detection experiment, 25–50 adult mosquitoes are homogenized in 1 ml of Tris buffer at a concentration of 25 mmol/l (pH 7.5) and centrifuged at 13 000 g for 5 min. The supernatants are adjusted to equal protein concentrations and incubated with malathion at a concentration of 300 mmol/l for 2 h at room temperature. The samples are then extracted with three volumes of chloroform and blown to dryness under a stream of air. The extract is re-suspended in 20 ml of chloroform and loaded onto a silica gel thin-layer chromatography (TLC) plate. The plate is eluted with a mobile phase consisting of n-hexane: diethyl ether (1:3), then spray with a 0.5% (w/v) solution of 2,6-dibromoquinone 4- chlorimide in cyclohexane and leave at 100 ^{o}C for 2 h to visualize spots of malathion and its metabolites. Distilled water or boiled insect homogenate is incubated with the same concentration of malathion as a negative control.

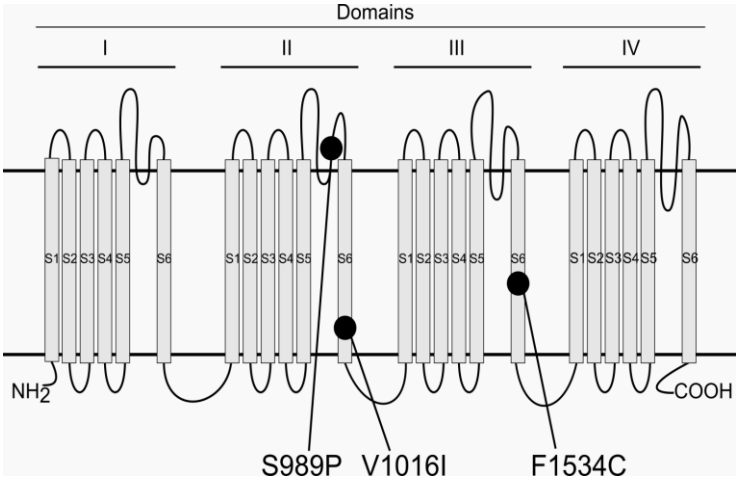

Figure. Positions of the resistant associated kdr mutations in the voltage-gated sodium channel gene of Aedes aegypti mosquitoes from Sri Lanka (Nugapola et al., 2020)

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STATISTICAL PERSPECTIVE ON PHYLOGENETIC TREES

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1. Phylogenetics

Phylogenetics is the scientific study of phylogeny, which pertains to the evolutionary history of a taxonomic group of organisms. Thus, phylogenetics is mainly concerned with the relationships of an organism to other organisms according to evolutionary similarities and differences. It allows us to compare specific characteristics of the species under the natural assumption "similar species are genetically close".

2. Phylogenetic Tree

The phylogenetic tree is a diagram that shows the evolutionary relationships among various biological species (Warnow, 2014). A phylogenetic tree could be constructed based on the physical or genetic characteristics of the species of interest. This reflects the evolutionary history and shows how closely organisms are related, just like your family tree, which began a long time ago. An example of a phylogenetic tree is shown in the following figure, which denotes how various species A, B, C, D and E evolved since their share a common ancestor.

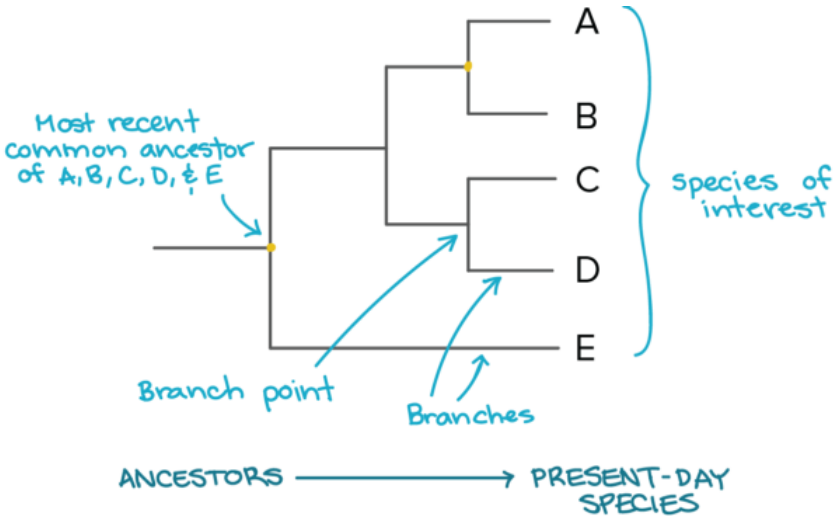


Figure 1. Phylogenetic Tree for the species A, B, C, D and E (Image source: Khan Academy)

Most phylogenetic trees are rooted, meaning they share a common ancestor (could be unknown). The branches (tips or edges) of the tree represent the species under comparison, and these branches meet at points called branch points (nodes) that represent a common ancestor. The branch point that denotes the most recent common ancestor for all the species is

called the root of the tree. Phylogenetic trees can be rooted or unrooted. A rooted tree is a tree in which one of the branch points is the root, and thus the direction of ancestral relationships is evident. An unrooted tree shows how close the species are and has no predefined root, resulting in no hierarchy.

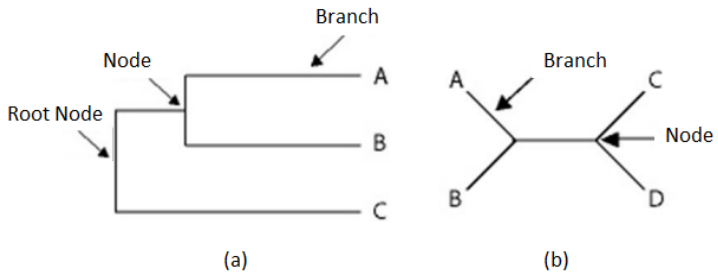


Figure 2. (a) Rooted Tree, (b) Unrooted Tree

3. Building a Phylogenetic Tree

A phylogenetic tree can be constructed by comparing and analyzing many characteristics of the species (Paradis, 2012). These characteristics can include the physical appearance (external or internal), behaviours, DNA and protein sequences, etc. Based on two types of data gathered from species can the two approaches of tree construction can be listed as follows:

1. Distance-Based Method
2. Character-Based Method

4. Distance- Based Method

The second approach of tree construction is based on distance information on the relative closeness of species under consideration. These distances are used to estimate both the tree structure and its branch lengths which usually represent the genetic change. A phylogenetic tree could be obtained by aggregating/amalgamating the most closely related observations or splitting/distributing the most distant sets of observations. The following section lists down several possible distance matrices depending on the method used to compute them.

4.1. Calculating the Distances

From a statistical perspective, a distance can be viewed as a geometric distance between two observations. But in evolutionary biology, a distance is an estimate of the divergence between two units which is usually measured in quantity of evolutionary change. For example, the alignment score of the two DNA sequences could be considered as a distance measure that discriminates the two species. Based on the nature of the data there are several ways to define the distance between two observations.

Once the measures of the distance between the pairs of species are defined, the ultimate target is to find the phylogenetic tree that arranges the species such that similar species appear as close as possible. The following figure shows an example of a phylogenetic tree constructed based on distance measures.

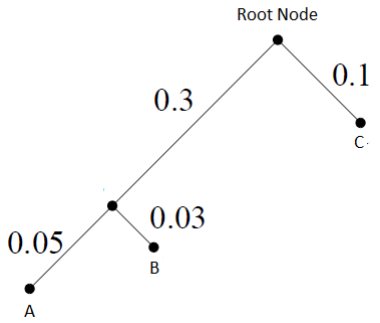


Figure 3. Distance-based Phylogenetic Tree. E.g. The total distance from A to B is 0.05+0.03 = 0.08.

4.2. Tree Construction

The Unweighted Pair Group Method with Arithmetic mean (UPGMA) and Neighbor-Joining (NJ) are two widely used algorithms in phylogenetic tree construction. UP- GMA assumes equal rates of evolution so that branch tips stay at the same level. The NJ allows for unequal rates of evolution, so that branch lengths are proportional (see figure 4).

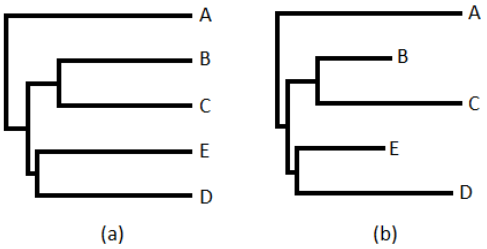


Figure 4. Tree resulted from (a) UPGMA (b) NJ

5. Character-Based Method

Distance-based methods are efficient and less computationally intensive; however, one of the major drawbacks is that the actual characters of the species are discarded once the distance among species is derived. Character-based approaches examine the characters of the sequences (DNA or Protein) and try to find the tree that best explains the observed sequences. Since the approach is based on the number of character changes in the observed sequences, the best phylogenetic tree is the one that requires the fewest evolutionary changes or maximizes a pre-specified target function. For example, the Parsimony score, which defines the total cost of all mutations in a tree is required to explain the observed differences. It depends on the structure of the tree (known as tree topology), the locations/branches assigned to each of the species, as well as the inferred DNA sequences for each of the common ancestors.

However, tracking the optimal solution for such a situation is difficult as there could be a large number of candidate trees. The trivial solution to this problem is to consider all possible trees and calculate the target function for each one. When this approach is closely analyzed we can see that the number of possible trees grows in a super-exponential manner when the number of species under consideration increases. For example, if the number of species is 20 than the number of possible rooted tree structures is approximately 10^{21}. This is much higher for an unrooted tree.

The researchers have further divided this problem into two: The large Parsimony problem and the Small Parsimony problem—the former attempts to find the structure/topology that gives the best score. The latter tries to find the minimal number of changes required when the species under consideration are already assigned to a predefined tree structure.

5.1. Maximum Likelihood Method

Maximum likelihood is considered as a modern-day estimation method in statistics (Cho, 2012). Researchers have widely applied this estimation method to phylogenetic problems since it is based on a simple principle. It evaluates a hypothesis about evolutionary history in terms of the probability that observed data comes from the predefined model. Hence a model, i.e. a phylogenetic tree, with a higher probability of describing the observed state is preferred than a model with a lower probability. Hence the method searches for the tree with the highest probability or likelihood. Thus, it is a very computationally intensive method and extremely slower than the other methods. The following example demonstrates the calculation of the likelihood for the given DNA information for four species and the tree topology.

5.2. Example: Likelihood Evaluation

Assume that alignment of the nucleotide sequences for three species are as follows:

Species S_1 A G C T C A

Species S_2 A G T T G A

Species S_3 A G C C G A

Let's evaluate the likelihood of the below given rooted tree (see figure 5a) represented by the nucleotides of site 3 from left in the three species sequence. To calculate the likelihood L(3) for site 3, all the possible scenarios by which the nucleotides present at the branch tips of the tree could have evolved need to be considered. Therefore, the likelihood for a site is the sum of the probabilities of every possible reconstruction of ancestral states at nodes N_1 and N_2, given some model of base substitution.

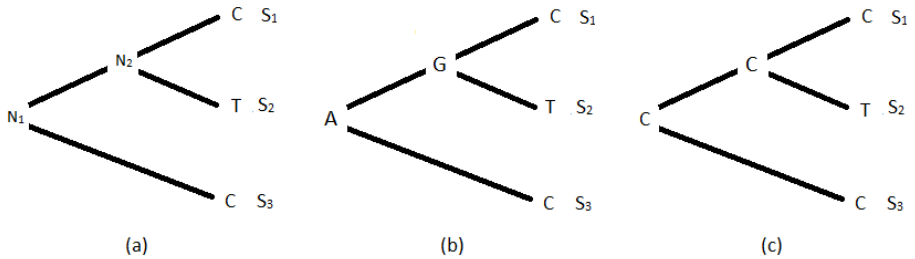
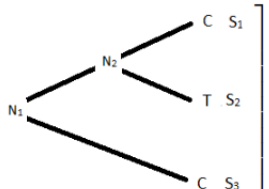


Figure 5 (a): Predetermined tree topology with unknown nucleotides at nodes N_1 and N_2, (b) and (c): Two realizations for nodes N_1 and N_2 out of 16 possibilities

The corresponding likelihood L(3) can be calculated as follows:

$$L(3) = \sum_{N_1,N_2 \in \{A,G,C,T\}} \left[\right.$$


If the nucleotide substitution information is given the above equations could be simplified as follows.

$$L(3) = \sum_{N_1 \in \{A,G,C,T\}} \sum_{N_2 \in \{A,G,C,T\}} P(N_1) \cdot P_{N_1 \to N_2} \cdot P_{N_1 \to C} \cdot P_{N_2 \to C} \cdot P_{N_2 \to T}$$

In an instance, if the lengths of the branches indicate the transition time, then the respective probabilities could be used in the above equation. Here, $P_{i \to j}$ denotes the probability that state i will transform into state j. Similarly, the likelihood for the complete tree with the consideration of the full sequence of three species is given by

$$L(T) = L(1) \times L(2) \times L(3) \times (4) \times L(5) \times L(6) = \prod_{i=1}^{6} L(i)$$

When the above procedure is then repeated for all possible phylogenetic tree topologies, the tree with the highest probability is the tree with the highest maximum likelihood. The product of the individual site likelihoods are extremely small numbers; the sum of the log-likelihoods is considered in calculations.

5.3. Nucleotide Substitution Information

Primarily, the nucleotide substitution information gives the probability of change from one nucleotide to any other nucleotide. In addition, these models are also used to calculate distances between pairs of sequences. Scientists have come up with different substitution models, such as Jukes and Cantor 1969 (JC69), Kimura 1980 (K80), Felsenstein 1981 (F81) and Tamura 1992 (T92), to name a few. The choice of the substitution model depends on the several factors, such as the number of parameters and the transition rates of the nucleotides. Moreover, recent studies suggest that there is still room for improving the commonly used substitution models (Kaehler et al., 2014).

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LABORATORY DIAGNOSIS OF RODENT-BORNE ZOONOSES: LEPTOSPIROSIS IN SRI LANKA

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1. The Global Status of Leptospirosis

Leptospirosis is a rodent-borne zoonotic disease caused by the bacterial species of genus *Leptospira* with a wide geographic distribution. Southeast Asia, Oceania, the Indian subcontinent, the Caribbean and Latin America are considered endemic to the disease (Evangelista and Coburn, 2010). The most important determinants of infection in these regions include high rainfall, climatic changes, flooding, poor levels of sanitation and a high number of reservoir hosts (Cachay and Vinetz, 2005). Some of the recently reported outbreaks in both endemic and non-endemic countries were directly related to recreational settings (Stern *et al*., 2010; Mutoh *et al*., 2017). In the Asia-Pacific region, leptospirosis is considered a waterborne disease; recent outbreaks in Indonesia (2003), India (2005), Sri Lanka (2008) and the Philippines (2009) occurred after major urban flooding. The disease is also seen in rural areas, where the potential risk factors are agricultural activities, such as farming and animal husbandry. The outcome of the infection changes with the amount of adaptation of the serovar type to the infected mammalian host, and it is probably due to direct pathogen effects and host immune response.

2. Leptospirosis in Sri Lanka

Leptospirosis is one of the notifiable diseases with a high burden in Sri Lanka. Leptospirosis in a human was first described in Sri Lanka in 1953 (Rajasuriya *et al*., 1959; Nityananda and Harvey., 1971). The first *Leptospira* isolation was reported in 1959 from the blood of a patient in Colombo and then from the kidney of a sewer rat trapped in the vicinity of that patient's home (Nityananda, 1962). The epidemic that broke out of the disease was reported in 2008 in which the number of human leptospirosis cases reached 7423 from 2198 in 2007, indicating a threefold rise (Epidemiology Unit, Ministry of Health). According to Sri Lankan Ministry of Health notification data, leptospirosis is common in nine of twenty-five districts. All leptospirosis endemic districts are within wet zones and have an annual rainfall above 2,000 mm.

Several studies have been carried out targeting humans and domesticate animals to identify the exposure and carrier status of leptospirosis. There were publications on human leptospirosis in Sri Lanka (Nwafor-Okoli et al., 2012; Koizumi *et al.,* 2009). Previous domestic animal studies show that a considerable percentage of domestic dairy cattle had anti-leptospiral antibodies and were circulating an array of leptospiral serogroups - i.e., Sejroe, Grippotyphosa and Pomona (Nityananda and Harvey, 1971). Rats, mice, other rodents, cattle, buffalos and domesticated elephants are important carrier reservoir animals of leptospirosis in Sri Lanka (Agampodi *et al*., 2010; Athapattu *et al*., 2019; Gamage *et al*., 2011; Gamage *et al*., 2014).

3. Classification of *Leptospira*

Leptospires are seen under darkfield illumination in liquid or semisolid media as thin, helical, motile spirochetes often hooked at one or both ends. The length of *Leptospira* cells varies from 10 – 20 μm (Holt, 1978; Carleton *et al*., 1979). A vast range of animals, including mammals, amphibians, and reptiles, can act as reservoirs or carriers of this disease. Disease transmission happens when they are exposed directly to the urine or tissue of carrier mammals or indirectly through the contaminated environment (Dolhnikoff *et al*., 2007). The pathogen enters the host animals through mucus membranes or the damaged skin, and the leptospireamic phase initiates. Then they colonize in the proximal renal tubules of the carrier animal's kidney (Bharti *et al*., 2003) and are excreted in the urine for months or years in the case of chronic carriers. Infections vary from asymptomatic to severe, but no pathognomonic clinical signs.

3.1. Genomic classification

Leptospires are classified under the family Leptospiraceae (Hovind-Hougen, 1979). Species of genus *Leptospira* forms two clades, namely pathogens and saprophytes. Two clades were divided into four subclades, namely P1, P2, S1, and S2. P1 pathogenic subclade includes 17 leptospira species that cause leptospirosis in humans and animals (Vincent *et al*., 2019).

3.2. Serological classification

The classification was carried out by a serological cross agglutination absorption test. The carbohydrate structural differences in the moiety of Lipopolysaccharide (LPS) is the determinant for the large degree of antigenic variation observed among serovars. Currently, there are over 300 pathogenic serovars belonging to 25 serogroups. Major and unique antigens are currently being defined by raising monoclonal antibodies and these may be applied to the identification of certain specific serovars (Mgode *et al*., 2015).

4. Diagnosis of Leptospirosis

4.1. Microscopic visualization

After being properly stained, Leptospires may be observed in clinical samples by dark-field microscopy or by immunofluorescence or light microscopy. About 10^4 leptospires/ml are needed to be visible under dark-field illumination. Body fluids such as blood, urine, CSF, and dialysate fluid have been used to observe under a dark-field microscope but with very low sensitivity and specificity. Immunofluorescence staining (Hodges and Ekdahl, 1973), immunoperoxidase staining (Terpstra *et al*., 1983), histopathological stains along with immunohistochemical methods are being used to visualize leptospires both in human and animal samples. (Bolin *et al*., 1989, Zaki *et al*., 1996).

4.2. Antigen detection

Leptospiral antigen detection can have more sensitivity and specificity over the dark-field microscopic method. Studies show that antigen detection methods like radioimmunoassay

(RIA) and enzyme-linked immunosorbent assay (ELISA) could detect 10^4 to 10^5 leptospires/ ml (Adler *et al*., 1982). In a recent study done using the urine of cattle infected with serovar hardjo, an immunomagnetic antigen-capture combined with fluoro-immunoassay was able to detect as few as 10^2 leptospires/ml (Yan *et al*., 1998).

4.3. Serological methods to detect antibodies

Serology can be used to detect the anti-leptospiral antibodies, which are detectable approximately five to seven days after the onset of symptoms for the diagnosis of most leptospirosis cases.

Microscopic agglutination test (MAT)

This is the gold standard reference method for serological diagnosis of acute leptospirosis with paired serum samples collected in the acute phase and convulsion phase. In MAT, patient sera are incubated to react with known live antigen cultures of leptospiral serovars. After incubation, the agglutination in the serum-antigen mixtures is observed under the dark-field microscope, and the titres are determined compared to control. The panel of cultures used should include serovars representative of all serogroups and all serovars found locally with the age of 4 to 7 days. Complete MAT includes qualitative and quantitative methods as described in WHO guidelines (WHO, 2003). The endpoint of the quantitative MAT is the highest dilution of serum at which 50% or less than 50% free leptospires per field compared to the control (Faine, 1982). There is a previous study that has been carried out to optimize a MAT culture panel suitable for use in hyperendemic, low resource settings in Sri Lanka (Jayasundara *et al*., 2021a).

Interpretation of the MAT is usually becoming complicated, especially in acute-phase sera because of the high degree of cross-reaction between different serogroups, for the confirmation of diagnosis, a fourfold or greater rise in titre between paired sera is required. If symptoms of leptospirosis are present, an interval of 3 to 5 days may be enough to detect rising titres. According to the case definition by CDC, to define a probable case with leptospirosis should have a MAT titre of $\geq$ 200. A higher cut-off titre like $\geq$800 is needed to indicate the presence of disease in areas where the disease is endemic (Faine, 1988).

MAT is used for epidemiological serosurveys with sera from any animal species. MAT titre $\geq$100 can be used for a past infection (Faine, 1982). Only about the serogroups circulating within a population can be drawn from MAT results and implies nothing about the serovars responsible for the infections. (Everard and Everard, 1993).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA to detect anti-leptospiral antibodies is based on the utilization of either whole-cell antigens or recombinant antigens. Whole-Cell Leptospira-based assays are the widely used methods for laboratory diagnosis. Several commercial ELISA kits have been developed using whole-cell antigens. This conventional method has a considerable level of sensitivity (33% to 67% in the acute phase and 84% to 100% in the convalescent phase) and specificity as high as 88% to 100%, even though it is time-consuming (Merien *et al*., 1992). Still, some studies report that whole-cell *Leptospira* assays have low specificity (Blacksell *et al*., 2006, Vijayachari *et al*., 2002).

Recombinant protein-based tests, which are more specific and reproducible, have been developed to mediate the problem of the low specificity of whole-cell *Leptospira* assays. Recently hundreds of genes encoding surface-exposed lipoproteins and outer membrane proteins have been used for the rapid diagnosis of leptospirosis. Out of them LipL32, LipL41 and LigA are commonly used to produce recombinant antigens which are present only in pathogenic *Leptospira* species with high conservation across a broad range of pathogenic serovars (Chen *et al*., 2013). In Sri Lanka, a previous study has been shown specific and sensitive results for the in-house IgG and IgM ELISA methods developed using mixed antigens collected from *Leptospira biflexa* serovar Pattoc culture (Niloofa *et al*., 2021)

4.4. Molecular detection methods

PCR methods

PCR-based assays are widely used for the detection of pathogenic leptospires in humans and animals because of their greater sensitivity and ability to provide an early diagnosis in the period before antibodies become detectable (Adler and de la Pen˜a Moctezuma, 2010). Several conventional (including nested PCR) and real-time PCRs have been developed to detect leptospiral genes. With the DNA extraction protocols, PCR technique has become more accurate and shown to detect as few as 5 to 10 leptospires/ml in blood (Adler *et al.*, 2011). Another study using 16S rRNA primers claimed to detect 10 cells of leptospires/ml in urine, CSF and blood when combined with DNA hybridization technique (Ellis *et al*., 1986a).

Multiplex PCR technique (Koizumi and Watanabe, 2003) which uses more than one primer pair in a single reaction, allows the distinguishing of pathogenic and non-pathogenic species. The nested PCR technique, which requires two rounds of PCR reaction with outer primer and inner primer pairs, was developed to increase sensitivity and specificity in pathogen detection. Recently, real-time quantitative PCR has significantly emerged in the diagnosis of leptospirosis. This assay is rapid, sensitive and quantifies the amount of DNA in the clinical samples. Most of the real-time PCR techniques involve using double-stranded DNA intercalating dye, such as SYBR Green I or the use of fluorescent labelled probes such as Taqman (Ko *et al.*, 2009). *LipL32, lipL41, rrs, secY, adk, icdA, flaB, gyrB, ompL1, ligA* and *ligB* genes are used to develop both conventional and real-time PCR. Since 2009, several studies have been carried out to detect different stages of leptospirosis in animals targeting specific portion of *flaB* gene only present in pathogenic leptospires in Sri Lanka (Athapattu *et al*., 2019, Gamage *et al.,* 2011, Gamage *et al*., 2012; Gamage *et al.,* 2014).

Next-Generation Sequencing (NGS) and Multi Locus Sequence Typing (MLST) have been carried out to detect leptospira species infected in human patients with suspected clinical history reported in 15 districts out of 25 in Sri Lanka. According to the results, four pathogenic leptospira species and 12 novel clonal leptospira groups were detected using 25 isolated cultures from humans (Jayasundara *et al.,* 2021b). Another study in Sri Lanka has been revealed novel genotypes of Leptospira spp from 25 leptospira isolated from humans in MRI and three isolates collected from rodents using the same NGS and MLST techniques (Karunanayeke *et al*., 2020). Furthermore, DNA metabarcoding has been used to identify leptospirosis eco epidemiology using environmental samples collected from two agro-ecological zones in Sri Lanka (Gamage *et al*., 2020).

Loop-mediated isothermal amplification (LAMP)

This method amplifies a target DNA sequence under isothermal conditions approximately in one hour with high specificity and efficiency. The LAMP reaction requires a DNA polymerase with strand displacement activity and a set of specially designed primers, termed inner and outer primers, which improve specificity. The end result after the amplification of DNA can be detected by unaided-eye as fluorescence or as turbidity, without using electrophoresis gels (Notomi *et al*., 2000). LAMP methods targeting *lipL41* and *rrs* genes have been developed for the detection of pathogenic leptospires (Lin *et al*., 2009; Koizumi *et al*., 2012). The sensitivity of this method ranges, having a detection limit of 2-100 leptospires per reaction mixture (Sonthayanon *et al*., 2011; Koizumi *et al*., 2012).

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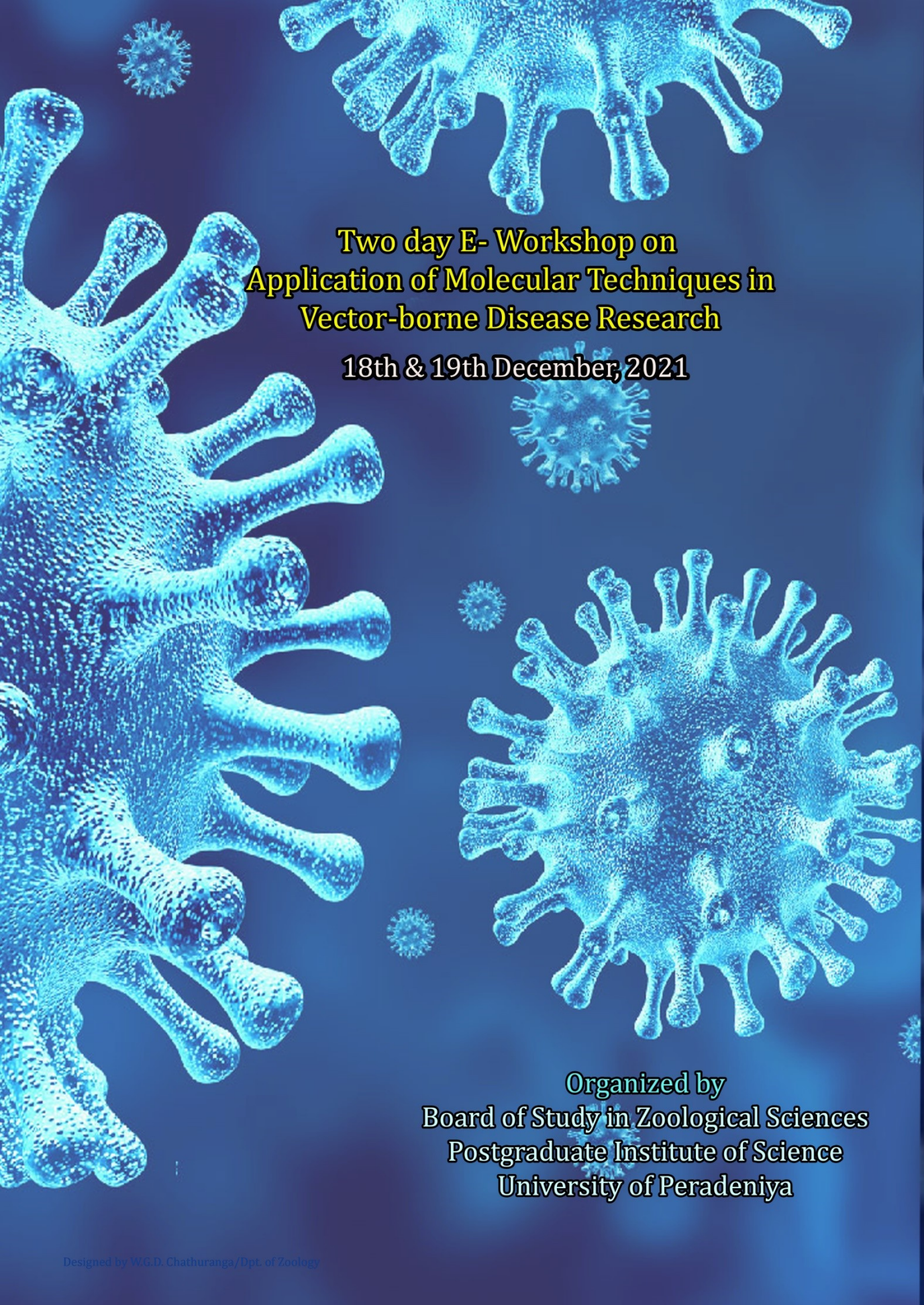

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