

TRAILBLAZER

in Molecular Life Sciences



Felicitating
Vidya Jyothi Emeritus Professor
Eric Karunananayake

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A Walk along the Pathway of a Trailblazer

A few of us for whom Vidya Jyothi Emeritus Professor Eric Karunananayake had been a guiding light got together early this year to discuss the best way to celebrate his 80th birthday falling on 6th December 2021. Times were unprecedented; covid-19 pandemic was disrupting many scheduled activities; we felt that uncertainty of gathering together to celebrate will hang in the air for many months to come. Thus we decided to put on record how Professor Karunananayake guided and motivated us to reach the stars. His former postgraduate students from the Colombo Medical Faculty, other Universities and Research Institutes were invited to contribute. His research students at the Institute of Biochemistry, Molecular Biology and Biotechnology (IBMBB) were naturally on the bandwagon.

Sweden played a major role to support him with training himself and other personnel in his teams in Molecular Biology; substantial grants for research; a soft loan to the country for building IBMBB. His collaborators from Sweden travelled long distances to be at IBMBB events such as its inauguration, felicitation of Professor Karunananayake on his retirement and conferences organized. Extending an invitation to them was natural, they very generously accepted our request to contribute to the Felicitation Volume.

What you are going to read in the essays to follow will tell you about personal interactions, scientific developments and how each of the authors cherished Professor Karunananayake's friendship or guidance. Many individuals from his "Guru" in Molecular Biology, Professor Ulf Pettersson from Sweden onwards to his first PhD student, Professor Jayantha Welihinda to current students have put these on record with much affection. I do not wish to outline them here and dilute the joy of reading the original. There were a few who could not be

contacted or could not contribute due to various reasons. Thus work on rubber and papaya; antiepileptic drugs and the role of chemical constituents of tea on caffeine metabolism etc. are missing from this Volume, while a few other gaps have been filled by other authors. Contents of the essays give evidence for the title chosen by us for this Volume.

Let me now give you a brief introduction to this Trailblazer in Molecular Life Sciences.

Eric Karunananayake was the eldest of six children born to an Ayurvedic Physician and his wife, from Gampaha, a town at a distance of about 28 kilometers from Colombo. His primary education was at the village school, then he moved to St Joseph's College, Colombo for secondary education. He entered the University of Ceylon in 1963 and graduated with a special degree in Chemistry in 1967. After a short stay in the Department of Chemistry of the Peradeniya Campus (now University of Peradeniya) as an Assistant Lecturer, he was appointed as Biochemist to the Bandaranaike Memorial Ayurveda Research Institute (BMARI) under the Ministry of Health in 1968. Three years later, on a Colombo-Plan scholarship, he proceeded to the United Kingdom for postgraduate studies.

He obtained Masters in Biochemistry in 1972, becoming the first in the batch and PhD in Biochemistry in 1975, from the Imperial College, University of London. Upon his return to Sri Lanka, Dr. Karunananayake continued to serve the BMARI and later the Medical Research Institute, Colombo. However he was destined to serve the wider Academia. His academic career at the Faculty of Medicine, University of Colombo commenced in 1980, and with successive promotions he was appointed to the Chair of Biochemistry in 1987 and promoted to Senior Professor in 1994. From late 1980s to early 2000s, Department bustling with research under his leadership was the envy of many.

He founded the Institute of Biochemistry, Molecular Biology and Biotechnology of the University of Colombo in 2004 with assistance from Sweden; became its Founder Director and retired in 2007.

This Felicitation Volume is truly a team work. Full credit is due to the team, if there are unintentional errors or omissions, I wish to extend my apologies.

It is my duty to put on record our appreciation to everyone involved. All the authors from Sweden, Professors Ulf Pettersson, Malin Åkerblom, Marie Allen and Erik Bongcam-Rudloff for time and effort despite their busy schedules; all the Sri Lankan authors for the dedication and cooperation; members of the Felicitation Volume Committee for their ideas and inputs and all those who personally financed the publication need to be gratefully acknowledged. So are the printers for a job well done. Many more are to be acknowledged in connection with the launch of this unique Felicitation Volume.

Kamani H. Tennekoon
Editor

December 2021

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Eric – a Sri Lankan Crusader

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It brings me great pleasure to share a few thoughts about my encounters with my great friend and colleague Eric Karunananayake over the years.

It all started in 1982. I had received a telephone call from Rune Liminga, a person who at the time was unknown to me. He was hosting two visitors from Sri Lanka - a certain Professor Balasubramaniam (Bala) and a scientist named Eric Karunananayake. Bala and Eric were interested in setting up a laboratory in Colombo, Sri Lanka, focusing on emerging gene technologies. Together with Rune, they came to my office at the Biomedical Center in Uppsala the following day. There I was briefed about Bala's ambitious plans for a new laboratory and we discussed whether it would be possible to use my laboratory as a base for training Sri Lankan scientists. Rune, who was in charge of the International Science Program in Uppsala (ISP) shared Bala's visions and offered funds from his organization to provide means for the Sri Lankan scientists to stay in Uppsala during their training periods. I was very impressed by their visions and we all agreed to make them a reality.

The reason why Rune approached me was that, at the time, my laboratory in Uppsala was the only laboratory in Sweden, which mastered gene technologic tools, such as molecular cloning and DNA and cDNA sequencing. My own PhD training was in Protein Biochemistry under the mentorship of the late Lennart Philipson who later became the director of the EMBO Laboratory in Heidelberg and subsequently founding director of the Skirball Institute in New

¹ Professor Emeritus

York. After an 18-month postdoctoral fellowship in Cold Spring Harbor Laboratory under the leadership of Dr. James Watson, I was converted from a protein chemist to a molecular biologist. After returning to Uppsala in 1973, I decided to set up my own research group using molecular technologies to dissect the DNA genome of human adenoviruses. Shortly after my return, I was invited to a symposium in Stockholm that was organized by United Nations Industrial Development Organization (UNIDO). During the symposium colleagues from prominent European Laboratories presented visions about how gene technology could solve health problems in the developing world. A focus was on the potential use of the technology for vaccine production. The lectures were very inspiring and I decided that from that moment on research with particular importance for the developing world would be included in my future research programs. UNIDO offered me to become a consultant, which strengthened my contacts with research in countries outside Europe. At the time, UNIDO was hoping that an International Gene Technology laboratory, serving the third world, would be created in Sweden. A funding proposal was put forward to the Swedish Government, which unfortunately turned it down. Trieste won the bid for this laboratory, now called the International Centre for Genetic Engineering and Biotechnology (ICGEB).

The visit from Bala, Rune and Eric was thus very timely as I was eager to welcome students from the developing world. In 1984, the Vice Chancellor of the University of Colombo, Professor Stanley Wijesundera selected two Sri Lankans, Eric Karunananayake and Jayantha Welihinda for training in Uppsala. They arrived in Uppsala in the beginning of February 1985. The Swedish winter offered them a harsh welcome and I imagine that it was particularly shocking for Eric who was accompanied by his wife Padmini. Bala had informed his Vice Chancellor, Stanley Wijesundera about his visions of a

Gene Technology laboratory and hope was growing that Sweden would become more involved in the creation of a Gene Technology Institute in Sri Lanka. Sweden is a big contributor of funds to developing countries. In fact, 1% of the GNP is set aside for this purpose and the funding is managed by the Swedish Agency for Research Cooperation with Developing Countries (SAREC). Stanley came to Sweden in 1985. I remember vividly a discussion I had with Rune and him in my office. He was an unforgettable person. A few days later, the three of us visited the main office of SAREC in Stockholm. A proposal for continued funding was well received by SAREC but they wanted a slow start. After a one-year-training period in Uppsala, Eric returned to Colombo filled with plans for a future laboratory and had numerous meetings with Stanley on the subject. Two rooms encompassing about 70 square meters were set aside for Eric's research and an agreement was reached about an MSc/PhD training program in Biochemistry, Molecular Biology and Gene Technology. The first regional course with 12 students was launched in 1987, including both foreign students and local participants. Eric asked me whether some personnel support could be mobilized in Uppsala. Uppsala gladly accepted: myself as a lecturer, two graduate students and one laboratory engineer traveled to Sri Lanka to provide support for the course. The visit was a delightful and memorable experience for all of us!

One request from the Vice Chancellor was that Eric should start research programs that would benefit Sri Lankan health care and industry. Eric's choice was to focus on diagnostics of parasite infections based on DNA technology. As filariasis was a major health problem in Sri Lanka at the time, it became the first target. While in Uppsala Eric joined one of my research groups that was engaged in malaria research. This group was a spinoff of my UNIDO engagements. It focused on the malaria genome and my graduate students had created a so-called genome library of DNA from the parasite. Among the DNA

clones, we searched for sequences, which are repeated many times in the genome. Malaria DNA like human DNA is rich in sequences of this kind. One isolated clone contained sequences, which were repeated thousands of times. Based on this, a simple diagnostic test was created. Blood from patients with suspected malaria infections was spotted on filter paper and radioactively labeled repetitive DNA was hybridized to the filter. The test turned out to be both highly sensitive and specific for malaria diagnostics (Franzen et al., 1984). Eric's idea was to use a similar approach to establish filarial diagnostics – a project that met with success.

Altogether, a handful of scientists came from Sri Lanka to Uppsala for training. They were without exception skilled and dedicated researchers who fitted very well with the atmosphere of my laboratory and were very much appreciated by my collaborators. After a time Eric's operations were self-sufficient. His laboratory, in spite of its minimal space, became productive and MSc and PhD students were trained and joined his group. My own contacts with the laboratory became more sporadic. Without my involvement Eric, first together with Rune Liminga and later with his successor Malin Åkerblom, were furiously fighting to reach Eric's ultimate goal, a new building and institute for research on Biochemistry, Molecular Biology and Biotechnology in Colombo. After protracted discussions with SAREC and other authorities, the institute became a reality. What Eric and his colleagues had achieved in his 70 square meter laboratory provided justification for a new building, financed by a soft loan from INEC, the investment division of SAREC. The building, located in a prime spot in Colombo was opened in 2004 and the inauguration of the building took place on April 28, 2004. It was an unforgettable delight for me to attend the event and celebrate the realization of Eric's vision!

Eric is to be commemorated for a large number of achievements, including but not limited to setting up the first functional Molecular Biology laboratory in Sri Lanka, establishing an MSc/PhD program in Gene Technology and his tireless efforts to convince authorities that Sri Lanka needed a new institute and to secure its funding. The design of the laboratories and their instrumentation and furnishing are all the result of Eric's visionary thinking. Thank you Eric for what you have achieved and for being such a wonderful friend and excellent host!

A memorable part of my Sri Lankan collaboration was my encounters with some exceptional personalities - including but not limited to Eric himself. Bala, for example, was a highly dedicated, energetic and charismatic person. Regrettably, my meeting with him in 1982 turned out to be my last one. For political reasons, Bala had to move to the University of Jaffna and was then unable to participate in the program. Stanley Wijesundera was another unforgettable person with strong visions about the future of science in Sri Lanka. We became good friends and one encounter remains vividly in my memory. During a visit to Colombo, I was invited to his home where I met his wife and one of his sons, who was a trained cook who prepared the meal for the evening. Regrettably, this was the last time I saw Stanley as he was assassinated in 1989. He is very much missed. In more recent times, I have had the pleasure to get to know Kamani Tennekoon who spent 3 months in the Rudbeck Laboratory in Uppsala while I was director of the institute. Kamani who is a highly accomplished person with a sharp mind has become a close friend. She kindly invited me back to Colombo in 2014 for the symposium "Frontiers in Molecular Life Sciences". Moreover, in May 2021, it was my pleasure to serve as an external examiner of Eric's and Kamani's student Vahinipriya Manoharan whose high quality work and deep understanding of science impressed me.

My interactions with both Eric and Kamani have brought me several times to beautiful Sri Lanka, always staying in the legendary Galle Face Hotel. I have fallen in love with the country and enjoyed every minute of my stays there, carrying with me the great memories of a splendid collaboration.

Congratulations, Eric!

What is the next step?

The IBMBB is now a well-equipped Molecular Biology facility. From my recent discussion during the thesis defense of Dr. Manoharan, I learnt that tools for so-called massive parallel sequencing are missing. It seems urgent that this ability is attained in the near future. DNA sequencing has become inexpensive and a cost of 1000 US dollar per human genome is within reach. I understand that expertise in Bioinformatics is being developed within another Swedish collaboration. Another recommendation is a deepening of the cooperation between IBMBB and clinicians to form a partnership in molecular diagnostics, which is likely to become an important branch of clinical medicine, particularly in oncology. A hitherto focus at the IBMBB on DNA technology has been rewarding. Worldwide there is a change in focus from nucleic acids to proteins and their modifications. Proteomic insights will be necessary to advance our understanding of how the human body is constructed. Below I take the liberty to discuss some aspects of Proteomics with focus on areas where scientists in my home country have made significant contributions.

Proteomics – a Swedish saga

Today the primary structures of more than 20,000 human proteins are known. It is therefore difficult to imagine that one hundred years ago, it was unknown how proteins are constructed. One theory, proposed in 1902, was that proteins are linear polymers of amino acids linked by peptide bonds. However, many scientists believed that such molecules would be unstable in solution and a common belief was that proteins are colloids or assemblies of small molecules. Theodor (“The”) Svedberg, Professor of Chemistry in Uppsala, presented the correct solution. Proteins are well-defined molecular entities (and not colloidal mixtures). This was shown by analytical ultracentrifugation (Van Holde and Hansen, 1998). An elderly colleague of mine told me about the critical experiment: Svedberg had received a sample of hemoglobin from his friend Robin Fahreus, a Professor of Pathology in Uppsala. The ultracentrifuge was running over night and it was great excitement when the two observed that hemoglobin sediments as a molecule with a distinct molecular weight. The construction of an ultracentrifuge, i.e., an instrument which spins at 30 000 rpm or more was a remarkable achievement. Driven by an oil turbine, the necessary speed was reached but the rotors posed problems. Many of them exploded in the first runs. Ultracentrifuges quickly became standard tools in Biochemistry and Molecular Biology laboratories, particularly useful for purification and studies of viruses. Svedberg received the Nobel Prize in Chemistry in 1926 and lived until 1971. I remember seeing him walking around in the Chemistry building of Uppsala University.

Svedberg laid the foundation for the science of protein separation. He attracted many young gifted scientists to his laboratory both from Sweden and abroad, one was Arne Tiselius who became a research assistant in Svedberg's laboratory in 1925 and obtained his doctoral degree in 1930. Whereas

Svedberg used sedimentation for separation and isolation of proteins, Tiselius employed the insight that proteins carry different charges. Separation based on charge ought then to be a way to fractionate and define proteins. His thesis had the title “On the moving-boundary method of studying the electrophoresis of proteins”. Electrophoresis quickly became a key technology that further enabled the characterization of proteins. An important breakthrough was Tiselius’ separation of blood plasma by electrophoresis (Tiselius, 1937). Plasma was shown to contain four major groups of proteins, the main being albumin which was known from studies by others. The three additional groups of proteins were designated alpha-, beta- and gamma-globulins, names that are still in use today. Tiselius received the Nobel Prize in Chemistry in 1948. He became the first Professor of Biochemistry in Uppsala and created a “dream team” of very talented biochemists who further advanced protein separation technologies.

One was Jerker Porath who started a career in Organic Chemistry but switched to Biochemistry and moved to Arne Tiselius' department. He developed methods for zone electrophoresis and ion exchange chromatography and he received his PhD in 1957. Porath’s studies made electrophoresis into a useful technology in both academia and industry. The separation method for which Porath is best known is gel filtration, which he developed together with Per Flodin (Porath, 1997). The latter worked with dextran research at the pharmaceutical company Pharmacia. In 1957, Porath and Flodin discovered that columns filled with dextran gel could be used as "molecular sieves" to separate biomolecules by size. Their results were published in Nature in 1959, and a short time thereafter, Pharmacia introduced the product Sephadex (Separation Pharmacia Dextran). Porath is also one of the founders of affinity chromatography (Axén et al., 1967; Porath et al., 1975). Together with Axén he developed methods by which proteins can be attached

to polymers. The key is cyanogen bromide which reacts with the hydroxyl groups on agarose or Sephadex. Because of its simplicity and mild pH conditions, cyanogen bromide activation became the golden standard for preparing affinity gels. Later in life, Porath developed Immobilized Metal Ion Affinity Chromatography (IMAC). This technique works by allowing proteins with an affinity for metal ions to be retained in a column containing immobilized metal ions. It is an important tool in modern Biotechnology for purification of proteins with affinity tails like runs of histidines. Porath died in 2016. While he never received a Nobel Prize for his discoveries, their importance has been paramount to the advancement of Biochemistry and Biotechnology.

Stellan Hjerten also studied under Tiselius. His thesis, entitled “Free Zone Electrophoresis” was a remarkable achievement. He used a rotating glass tube, which maintained the protein zones in the tube with great precision (Hjertén, 1990). It is a forerunner to capillary electrophoresis, which became a key component in the widely used sequencing machines that brought the human genome project to completion. It is interesting to note that the equipment that Hjerten used in his thesis was constructed in a mechanical workshop that belonged to the Chemistry department. This workshop also manufactured Svedberg’s and Tiselius’ Nobel prize winning devices. Much of Hjertén’s early research focused on the development of chromatographic beds, agarose and polyacrylamide gels, and he was one of the inventors of polyacrylamide gel electrophoresis (Hjertén et al., 1965). He also developed agarose beads for separation of proteins by gel filtration (Hjertén, 1990), which allow for separation of proteins of greater molecular weight as compared to Sephadex.

Another student of Tiselius was Harry Rilbe (Svensson), who invented isoelectric focusing. A column with so-called ampholytes form a pH gradient

and proteins will layer themselves according their isoelectric points. In combination with polyacrylamide gel electrophoresis, isoelectric focusing became a widely used technology for 2-dimensional separation of proteins. It is still used in many laboratories. Lastly, Per Ake Albertsson, another of Tiselius' talented students, developed a technology that allowed partition of cell particles and macromolecules in polymer two-phase systems (Albertsson, 1970).

Protein detection technologies

The study of proteins is now emerging as the new frontier for understanding real-time Human Biology. There is an ongoing hunt for protein markers with pathophysiological importance. These may have a profound impact on improving future healthcare. Progress has, however, been hampered by the lack of technologies that can provide reliable specificity, high throughput, good precision, and high sensitivity.

A key method for the detection of proteins is the radioimmunoassay, (RIA). RIA was first described by Yalow and Berson in 1960. The technique indicates whether a specific antigen or antibody is present in the sample. Yalow shared the Nobel Prize with Roger Guillemin, and Andrew Schally in 1977. Classically, to perform a radioimmunoassay, a known quantity of an antigen is made radioactive, frequently by labeling it with gamma-radioactive isotopes of iodine, attached to tyrosine. The radiolabeled antigen is then mixed with antibody for that antigen. Then, a sample of serum from a patient containing an unknown quantity of that same antigen is added. The antigen in the serum will then compete with the radiolabeled antigen for antibody binding sites. The bound antigens are then separated and the radioactivity is measured.

A disadvantage with the RIA is that it requires a cumbersome separation step. The solution to the problem came from Uppsala and Leif Wide. Wide came to Uppsala in 1960 from Stockholm in company with Professor Carl Gemzell, a well-known expert in human reproduction. Wide's first breakthrough came already in 1960 while he was a PhD student, when he published and patented an entirely new pregnancy test (Wide and Gemzell, 1960). Up to then, pregnancy tests were biological: Urine from women to be tested for pregnancy was injected into animals, most commonly female mice or rabbits. The diagnosis required sacrifice of the animals and examination of their reproductive organs. Wide's test is based on the knowledge that pregnant women secrete human chorionic gonadotropin (hCG) into their urine. Wide made a simple immune assay by lyophilizing antibodies to hCG together with stabilized sheep erythrocytes, coated with hCG, in test ampoules with a round bottom. When urine from pregnant women is added, hCG blocks the antibodies, and the erythrocytes form a ring instead of a mat on the bottom. The pregnancy test was licensed to the pharmaceutical company Organon. It was the golden standard for pregnancy determination for 25 years. After completing his work on hCG, Wide started a collaboration with Axén and Porath. The latter had invented a method for binding of proteins to solid phases like Sephadex in 1966. Wide designed a test called RIST (radioimmunosorbent test), which is similar to the RIA, the difference being that one reagent; the antibody is immobilized on beads of Sephadex (Wide, 1969). The ligand to be measured is then added in a radioactive form together with the sample. The radiolabeled ligand will then compete with unlabeled ligand in the sample. After washing of the beads, the amount of radioactivity is measured that will be inversely proportional to the amount of ligand in the sample. An improvement of the RIST followed. It is designated the "Sandwich test". In a classical sandwich test, unlabeled polyclonal antibody against the

ligand to be measured is immobilized on Sephadex. Patient serum to be tested for the ligand is then added and subsequently radiolabeled antibody of the same kind as the previously mentioned is added. As the antibody is polyclonal, it will bind to several epitopes on the ligand. The radiolabeled antibody will bind to epitopes, which are not bound by the membrane bound antibodies. The sandwich test is ultra-sensitive and more than 100 times more sensitive than RIST. The reason is that the background in the assay is very low due to the requirement for dual binding events.

The sandwich assay led to a major medical discovery. Wide's idea was that the double specificity and high sensitivity of the sandwich assay could be applied as a new allergy test by detection of antibodies of the gamma-E-globulin class to different allergens. Kimishige Ishizaka, Denver, Colorado, had then recently discovered the gamma-E-globulin as a class of antibodies, which recognizes allergens, resulting in allergic reactions. While Wide was doing his experiments, two colleagues, Hans Bennich and Gunnar Johansson, had isolated a myeloma protein that was different from all known immunoglobulins, designated IgND (Johansson, 2016). Wide obtained purified myeloma protein from Bennich and antibodies to the protein. It was used to design a RIST for detection of IgND in serum. Blood from 62 individuals was tested and since all samples contained low but clearly detectable levels the postulated IgND must be a ubiquitous immunoglobulin. One person was unique; her serum contained a 15 times higher level of the protein. Interestingly, she was a sufferer of asthma. Further studies of cohorts with allergic patients established a link between increased levels of IgND and allergy. Next step was to design an assay that would identify the allergens that cause the disease. To this end Wide immobilized various allergens (peanuts, dust, animal hair, etc.) on a solid phase. Serum samples from allergic and non-allergic individuals were analyzed with the solid phase coupled allergens.

Radioactive antibodies to the IgND were used to detect the presence of allergen-antibodies related to the IgND on the solid phase. The test was designated the Radio-Allergo-immunoSorbent Test (RAST) (Figure 1) and became a great success. Wide, Bennich and Johansson licensed the allergy test to Pharmacia. It became a worldwide success and Pharmacia established a separate company, Pharmacia Diagnostics, later called Phadia, which at present is owned by Thermo Fisher Scientific Inc... still being a world leader in allergy testing.

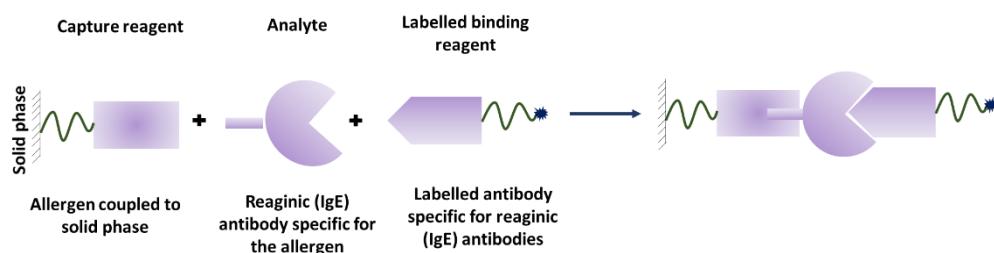


Figure 1. The Radio-Allergo-immunoSorbent Test (RAST)

Enhanced sensitivity

As radioactivity poses a potential health threat, a safer alternative was sought. A suitable alternative to radioimmunoassay would be an assay, which would allow enzymatic amplification of the signal. A key invention was the enzyme-linked immunosorbent assay (ELISA) as was first described by Engvall and Perlman (1972) who worked at Stockholm University. The assay uses a solid-phase type of enzyme immunoassay to detect the presence of a ligand (commonly a protein) in a liquid sample using antibodies directed against the protein to be measured. In the simplest form, antigens from the sample to be tested are attached to a surface. Then, a matching antibody is

applied over the surface so it can bind the antigen. This antibody is linked to an enzyme and then unbound antibodies are removed by washing. In the final step, a substance containing the enzyme's substrate is added. If there was binding the subsequent reaction produces a detectable signal, most commonly a color change. It is still a technology that is in wide use both in scientific and diagnostic laboratories.

Converting protein assays to DNA assays

After returning from postdoctoral studies at California, Institute of Technology, Ulf Landegren joined my laboratory in 1989. His mentor abroad was Leroy Hood and together they had developed a novel technology for detection of point mutations, designated the oligonucleotide ligation assay (OLA). The basis for OLA is that DNA ligase catalyzes the ligation of the 3' end of a DNA fragment to the 5' end of a directly adjacent DNA fragment. This mechanism can be used to interrogate point mutations by hybridizing two probes directly over the SNP polymorphic site (Landegren et al., 1998). Ligation will only occur if the probes are identical to the target DNA. If there is, a mutation in the target genome where the oligonucleotides lie adjacent, ligation will fail. There are many ways by which absence and presence of ligation can be scored. With appropriate sequences and tags on the oligonucleotides, high-throughput data can be generated from the ligated products.

Back in Sweden Landegren continued work with technologies that involve DNA ligation. An invention that followed was the padlock probes. If the ends of an oligonucleotide are complementary to sequences, which lie adjacent on the target DNA, an open circle will be formed spontaneously (Figure 2). If there is perfect complementary at the junction, a closed circle will be formed

after ligation. The padlock probes have many advantages based on their capacity to form circles, the most important being that they can be multiplied by rolling circle amplification (RCA) (Banér et al., 1998). A DNA polymerase encoded by bacteriophage Phi29 DNA replicates circular molecules almost endlessly. There is no termination, as the growing chain will displace the newly synthesized DNA from the circle. Thus, when, for instance, padlock probes are used for mutation detection, extremely long repetitive chains are formed. They form bundles, which are so large that they are detectable in a light microscope and can be counted in a cell sorter. Another very useful property of the padlock probes is that there is plenty of space between the two terminal stretches that are required for target recognition. Thus, various “barcodes” can be included, opening possibilities for multiplexing. Unlike the polymerase chain reaction, RCA is conducted at room temperature.

Although we are rapidly gaining information about the composition of the human proteome we know little about the “sociology” of the proteins. Few of them act alone, but rather in concert with one or more protein partners. Methods allowing investigation of protein partnerships on a large and general scale were missing until Landegren and his colleagues presented the *in situ* Proximity Ligation Assay (PLA) which identifies physical closeness of proteins (Figure 2) (Fredriksson et al., 2002; Söderberg et al., 2006). It is based on the principle that, if two proteins interact and the distance between them is within 16 nm, the interaction can be detected by antibodies against the proteins. In a typical assay, primary antibodies, which have been raised in different species (rabbit and mice, for instance) target the proteins of interest. Secondary antibodies directed against the constant regions of the different primary antibodies, the so-called PLA probes, bind to the primary antibodies. Each PLA probe has a short oligonucleotide attached to it. If the PLA probes are in proximity i.e., if the two proteins of interest are in proximity, the

oligonucleotides can participate in rolling circle DNA synthesis after addition of two oligonucleotides and ligase (Figure 2). The DNA synthesis reaction results in several-hundredfold amplification of the DNA circle. Finally, fluorescent-labeled complementary oligonucleotide probes are added which bind to the amplified DNA. The resulting fluorescence is visible as a distinct bright spot when viewed with a fluorescence microscope. An advantage of PLA is that it overcomes the general problem of cross-reactivity in protein detection by affinity binders, as the assay requires multiple binding events for a positive outcome.

A great future hope for humankind is that diseases can be diagnosed at an early stage when cure is possible. There are great expectations that so-called biomarkers will be identified in the near future, i.e., components in blood and other biological fluids that will signal the presence of a serious illness. The results obtained so far are meager but the expectations are still great. When biomarkers do become available, hypersensitive technology will be required as they are likely to be present in minute concentrations. Landegren's Proximity Extension Assay (PEA) technology takes affinity-based protein measurements to a new level. The protocol for PEA involves the following; a polyclonal antibody is prepared against the protein of interest. The antiserum is divided in two portions each of which is conjugated with oligonucleotides that are different but partially complementary. Both antibody preparations are incubated with the target protein. Since most proteins contain multiple epitopes both DNA-linked antibodies will attach to the proteins. This will bring the partially complementary oligonucleotides into proximity and will hybridize before being extended by a DNA polymerase (Figure 2). The newly created piece of DNA is then ready for readout by qPCR or Next Generation Sequencing after amplification. Different antibody pairs are used for different proteins and as barcodes can be introduced into the antibody-attached

oligonucleotides, the assay allows for multiplexing (Petrera, 2021). Traditional immunoassays do not lend themselves well to multiplexing since cross-reactive binding of antibodies contribute to the signal readout. This problem escalates exponentially with the degree of multiplexing. In contrast, the DNA-based readout of PEA circumvents this by requiring both dual recognition of correctly matched probes. The method is highly scalable and has an exceptional specificity. Extremely small sample volumes are needed to measure large numbers of proteins simultaneously, which is important when precious samples are in limited supply, such as in studies using human samples from clinical cohorts or biobank material.

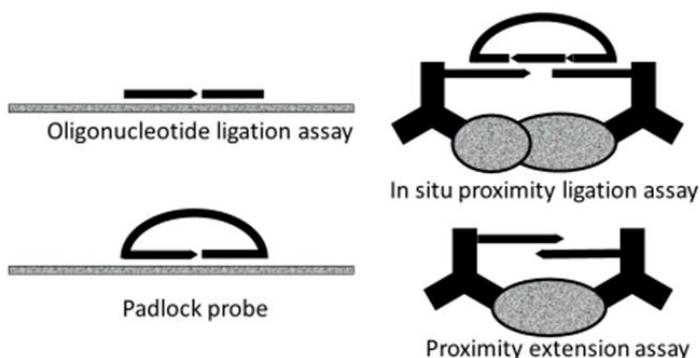


Figure 2. Four different ligation assays

Recently, Zhao, Landegren and colleagues (unpublished) developed a clever test for detection and quantification of antibodies against SARS-CoV-2, based on a modification of the PEA (Figure 3). A minimal portion of a drop of blood, spotted on filter paper, is incubated with recombinant protein, derived from the SARS-CoV-2 capsid. The recombinant protein has been conjugated with two different, partially complementary oligonucleotides. Since the antibodies have two arms, which both can bind the recombinant protein some 50% of them will bind proteins with partially complementary oligonucleotides. After addition of a DNA polymerase, the DNA extension products are quantified by

real-time PCR as a sensitive measure of the titer of antigen-specific antibodies in the sample.

There is an excellent opportunity for IBM-BB to establish this technology and provide services for antibody detection utilizing this simple test. The estimated cost of the reagents for the assay is less than 5 US dollars.



Figure 3. The two arms of an antibody from an immune patient bind two identical SARS-CoV-2 protein fragments, conjugated, with distinct, partially complementary oligonucleotides. A DNA polymerase extends the oligonucleotides to create amplicons that reflect the presence of antibodies in a sample.

A Swedish mega project

In 2003 Mathias Uhlén, a Professor of Microbiology at the Royal Technical Institute in Stockholm, and Fredrik Pontén, a Professor of Pathology at the Rudbeck Laboratory in Uppsala, received a donation of 50 MSEK for the start of a mega project, the aim of which was to study all 20 000 proteins that are encoded by the human genome. The strategy involved the expression of recombinant proteins that represent fragments of more or less all proteins in

the human body. This was a very bold undertaking, still ongoing, which has turned out to be a huge success. More than 95% of all proteins that are predicted from the human genome sequence have been identified. The technical challenges involve both the antigen production and the subsequent generation and characterization of the antibodies. A streamlined approach for affinity purification of the antibodies was developed to generate monospecific antibodies. These are used for immune-histochemical analysis to study protein expression and sub-cellular localization of individual proteins in normal tissues as well as in common cancers and other forms of diseased tissues. The result is a comprehensive, antibody-based protein atlas and certified pathologists provide a knowledge base (Uhlén et al., 2015; 2017). A freely available interactive resource is presented in the Human Protein Atlas portal (www.proteinatlas.org), offering the possibility to analyze tissue profiles for specific protein classes. Comprehensive lists of proteins expressed at elevated levels in the different tissues are compiled to show the localization of the proteins in the sub-compartments of each tissue and organ down to the single-cell level. It is an overwhelming achievement.

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To Build a Research Institute – a Swedish Perspective

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Eric had a dream, a dream he nurtured for many years. A dream about a modern laboratory for Gene Technology and Molecular Biology. I am sure the dream started already in 1985, when Eric was trained in Molecular Biology in the laboratory of Professor Ulf Pettersson. The dream was a follow-up of the vision of Professor Balasubramaniam, who foresaw the need for local knowledge of Molecular Biology in a low-income country. Professor Balasubramaniam together with Professor Stanley Wijesundera, then Vice Chancellor, and Professor of Biochemistry of the University of Colombo had succeeded to persuade ISP³ to support a group working on Gene Technology in Sri Lanka.

Eric was given the task to lead the group when Professor Balasubramaniam left for Jaffna, and by the end of 1986 a small laboratory had been set up. Research in Molecular Biology is not for free, and the support that ISP could give was not sufficient. Eric, together with my predecessor late Professor Rune Liminga and others, struggled to convince SAREC⁴ of the need for and possibility of Molecular Biology in Sri Lanka. Eventually SAREC gave support needed to start up and pursue research on a basic level and the support was handled by ISP.

When I first visited the group 13 years later, in 1998, I could hardly enter. The lab was full of activity and crowded with eager students. A fume cupboard in

² Former Director- ISP Chemistry Programme

³ International Science Programme at Uppsala University, Sweden

⁴ Swedish Agency for Research Cooperation

the pass way to the lab. Eric's desk in a corner in the pass way. In the lab the students could hardly pass one of them who was pregnant. The need for more space was obvious. I got impressed by the students' eagerness, by the way Eric talked science and by his strong wish to strengthen his country through spreading knowledge in molecular biology tools and procedures. I was prepared to try to facilitate his dream to come true.

Colombo University had earlier been invited to apply for international support for an institute of Biotechnology. Eric had written a proposal and designed an institute building according to his visions, and consulting engineers had fine-tuned the drawings. Eventually the support did not materialise. At this point SAREC⁵ already supported his research on a basic level. Now in 1999 Eric brushed up the proposal and SAREC was approached with this heavier task. These were exciting days. Eric visited Sweden several times to discuss plans and to visit SAREC. He also met my husband, Professor in Molecular Biology, and I enjoyed listening to their learned discussions. My husband understood Eric's struggles. He had himself struggled years earlier to convince the Swedish Agricultural University that research in Molecular Biology would be important for agriculture.

SAREC's response to the proposal was positive. Eric's dedication and seriousness was convincing. The group's research had been successful. Contributing were also the trust shown by Professor Ulf Pettersson who wrote a supporting letter, and by my husband.

SAREC continued to support the research including the education of MSc and PhD students. They were prepared to support the expensive equipment needed to step up the level of research. However, SAREC did not have the mandate

⁵ At this time incorporated with Sida, Swedish International Development Cooperation Agency

to support infrastructure such as buildings. So “our” man at SAREC, Per-Einar Tröften, approached INEC⁶. There, supporting research was not heard of. We got an appointment with Mrs Elisabeth Eklund, who listened carefully to Eric’s vision. She eventually managed to convince INEC that an investment in research and higher education in this area would lead to economic benefits for the country. The support would be in the form of a soft loan to the Sri Lankan government, which was willing to accept the loan.

The lab building was expected to meet international standards and specifications needed for a Molecular Biology laboratory also regarding matters such as ventilation, finish and safety. Sweden had a long experience in building advanced laboratories, and in the autumn 2000 three key persons in engineering and architecture visited Sweden together with Eric to gather ideas. They had already worked further on Eric’s first drawings for the building and brought a three-dimensional wooden model about 40x40 cm and four stories high. One could also see laboratories and an auditorium when taking off the roof. The model was fragile, so they had to bring it aboard the airplanes as hand luggage – it must have been rather inconvenient to sit the long journey with it on the lap.

We paid in-depth visits to four laboratory buildings, including two institutes which were dedicated to Gene Technology and Molecular Biology and had recently been inaugurated. The interested quartet was well received and we got in-depth visits to various laboratory rooms, up in the attic to study ventilation, down in the basement to study plumbing, and clean rooms and safety arrangements. We were guided by those responsible for running the buildings, and discussions were vivid and helpful. So were discussions with

⁶ Department for Infrastructure and Economic Cooperation, Sida

an architect with broad experience from laboratories in tropical countries, who was appointed by Sida.

Back in Sri Lanka there were frequent drawings, re-drawings and discussions between architects, engineers and Eric. Biddings from constructors had to be scrutinized and handled. Et cetera et cetera. It was really a special day when the foundation stone was ceremonially laid by the Swedish Chargée d’Affaires Mrs Anne-Marie Fallenius and the Vice Chancellor of the University of Colombo, on August 1, 2002.

At the time when the architects and the engineer visited Sweden in 2000, there was a big laboratory exhibition for scientific equipment and laboratory furnishings, which we visited. Here contacts were made with producers of laboratory furniture. The quality of laboratory furniture is vital for the special demands of Molecular Biology, and the Swedish experience from making such furniture was crucial. One of the producers was genuinely interested in the challenge to provide a country far off with another climate with a dedicated furnishing.

As the building grew in size, Eric’s planning continued with furnishing each laboratory. Eric visualised every laboratory room and its purpose. He wrote lists of furniture and fitting needed, with quantity and dimensions for each room. One list I have contained exact dimensions and placement of 49 wall shelves, 178 lab tables, 68 lab chairs, 76 cupboards, 10 sinks, 12 dish trolleys, fume cupboards, laminar flow benches, CO₂ incubator, emergency shower, eye washers, etc.

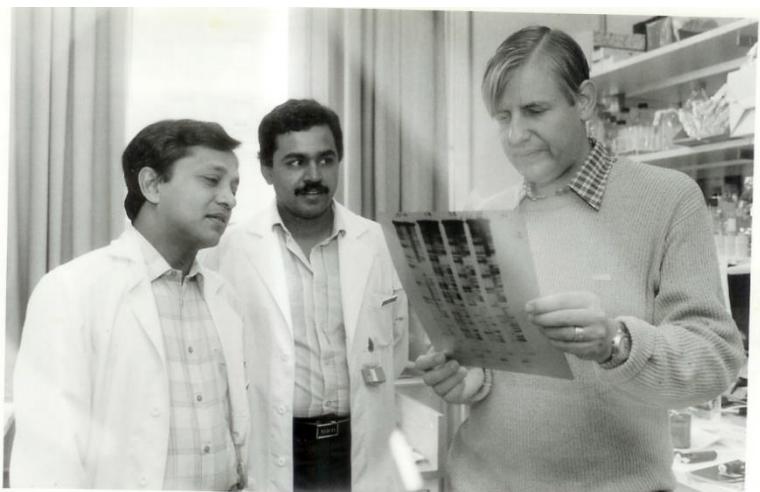
At ISP, Dr Linnea Sjöblom skilfully cared for all formalities around the transfer of laboratory furniture and fittings to Sri Lanka. The shipment left Sweden on the day for the Nobel Prize, December 10, and arrived in January 2004. We were happy but a bit worried. Would the furniture stand the

shipment and the humidity? It did, and when it was eventually released from the Colombo harbour, it was installed by two Swedish engineers together with local ones, so they could later be in charge of the maintenance. Of course, Eric was following the installation tightly, and kept me informed to satisfy my curiosity. The release from the harbour had been delayed – would the installation be completed before the inauguration? It was: the very last adjustments were made by the director of the company the day before.

After skilful work by engineers and builders, the Institute of Biochemistry, Molecular Biology and Biotechnology, IBMBB, could be ceremonially inaugurated on April 28, 2004 by Professor Pettersson and Mrs Fallenius. It was a smiling Eric who welcomed all invited guests on the inauguration day April 28, 2004, to take a tour in the new institute for future science to the benefit of his country.

It was a joy to work with Eric and very interesting. He was a visionary and had many and sound ideas. He was open to discussions and new solutions where the proposed ones failed. I do not know from where Eric got all his energy and time, having his brain and hands in almost every matter. He was also Professor at his department, pursued research, taught and supervised. And there was not only planning and re-planning, but a lot of bureaucracy and arguing, both on the Sri Lankan and the Swedish sides, with endless letters written. We frequently sent each other emails concerning planning and administration, sometimes several per day. But it was always pleasant to receive his messages. I am grateful to have had the privilege to get a glimpse of Eric's visionary mind and to take part in these endeavours.

Professor Karunananayake (left) during his training at BMC, University of Uppsala in 1985 with Professor Pettersson (right)



**First Gene Technology Course held in Sri Lanka-1987
Some trainees and trainers**

Standing: 2nd from left - Professor Eric Karunananayake; 3rd from left- Professor Ulf Pettersson; 1st from right - N V Chandrasekharan; 3rd from right - Jayantha Welihinda; Middle row: 1st from left - Sulochana Wijesundera; 3rd from right - Kithmini Siridewa



Model of IBMBB (Institute of Biochemistry Molecular Biology and Biotechnology) taken to Sweden in 2000



Laying the Foundation Stone for IBMBB – 1st August 2002



Institute of Biochemistry Molecular Biology and Biotechnology



Opening Ceremony of the IBMBB - 28th April 2004
Professor Ulf Petterson and Mrs. Anne-Marie Fallenius,
Swedish Chargée d’Affaires unveiling the plaque





**Professor Karunananayake
with Swedish dignitaries on
the eve of the Opening
Ceremony of IBMBB
28th April 2004**

From left to right:
Mrs. Anne-Marie Fallenius
Late Professor Rune Liminga
Professor Eric Karunananayake
Professor Ulf Pettersson
Professor Malin Åkerblom

Catching Up

Professor Pettersson and Professor Karunananayake in deep conversation before the inauguration of the “International Conference on Frontiers in Molecular Life Sciences” in 2014, held to mark 10th Anniversary of IBMBB.
Professor Åkerblom (left) and Professor Allen (right) in the front row





New collaborations

Professor Marie Allen
-3rd from left with
Professor
Karunananayake during
her visit to IBMBB in
2005

First workshop in Bioinformatics – January 2007

Dr. Erik Bongcam-Rudloff (7th from left back row) and Ms. Hanna Strandberg (2nd from left middle row) from the Linnea Centre of Bioinformatics, University of Uppsala, with Professor Karunananayake (5th from left back row) and the participants



Development of DNA Probes for the Detection of Filarial Parasites

*NV Chandrasekharan
Department of Chemistry, Faculty of Science
University of Colombo*

It is indeed a pleasure to contribute to this felicitation volume to commemorate Professor Karunananayake who was my teacher and mentor at the University of Colombo. My association with Professor Karunananayake began in the early 1980s as a graduate student when I enrolled for an MSc in Biochemistry at the Faculty of Medicine, University of Colombo. He conducted several courses in the MSc programme and I went on to carry out my MSc research project under his supervision. It was around this time that he was able to secure funding from the International Program in the Chemical Sciences (IPICS) of the University of Uppsala, Sweden and subsequently the Swedish Agency for Research and Economic Cooperation (SAREC) with developing countries to establish the first ever Molecular Biology laboratory in the country capable of carrying out molecular cloning experiments (rDNA technology). The laboratory was established at the Department of Biochemistry, Faculty of Medicine, University of Colombo.

After completing the MSc, I joined the Department of Biochemistry and enrolled to read for a PhD under the joint supervision of Professor Karunananayake and Professor Ulf Pettersson, Department of Medical Genetics, Biomedical Center, University of Uppsala, Sweden. As filariasis was a significant health problem in the country at that time it was identified as an area for investigation and initially research in the lab was focused on the study of these filarial parasites. Several of my colleagues including Professor

Sulochana Wijesundera and Professor Kithmini Siridewa too were graduate students of Professor Karunananayake. We worked together in a team with the focus of improving and developing new and alternative diagnostic methods for the detection of filarial parasites.

Filariasis

Filariasis is a group of infectious diseases caused by parasitic nematodes. These filarial nematodes cause disease not only in humans but in animals too. Control programs on filariasis are usually directed against both the parasite and the vector, with the aim of reducing parasite carriers (host) and infected vector populations. The ultimate objective being the complete elimination of parasite reservoirs. For a successful vector control program, it was necessary to have a thorough understanding of the distribution and dynamics of the disease in a targeted population (WHO, 1987). Hence in planning and implementing a filariasis control/eradication program, it is necessary to gather detailed and accurate data from epidemiological surveys. Collection of the above epidemiological data, monitoring of control programs, long term surveillance, and diagnosis of individual infections for effective treatment, require, both field and laboratory investigations. Field and laboratory investigations would invariably entail the sensitive and specific detection, identification and quantitation of different stages of the parasite both in the host and vector populations.

Detection of the parasite for a definite diagnosis of filarial infection is made by demonstration of either the adult worm or microfilariae in the host. Usually, the demonstration of microfilariae in blood films is the most feasible and practical method employed for epidemiological surveys and the diagnosis of individual filarial infections. These methods have several disadvantages and

include the likelihood of missing low-level infections, and species identification of larvae (if necessary) is not always possible. Another important aspect in the control of filariasis is the determination of infectivity rates of mosquito vectors that include estimation of the proportion of vectors infected, their developmental stage and location, and the number of larvae in a single vector. For detection and identification of various larval stages of the filarial parasite in the vector population, the mosquitoes are usually dissected under a binocular dissecting microscope and identified. These methods have a number of limitations and disadvantages, when used to monitor filariasis control programs and do not give accurate estimates of the intensity of transmission for number of reasons (Dissanayake and Piessens, 1992). In many regions of the globe, human and animal filarial parasites share a common vector and their larval stages are morphologically indistinguishable. Many filarial parasites, when exposed to antifilarial drugs, have an abnormal development in the vector making larval identification extremely difficult (Cartel et al., 1990; Cupp et al., 1986). Difficulties are also encountered sometimes in the identification of the vector species. In many endemic regions, specimens collected in the field have to be transported long distances to central laboratories. In such cases the vectors have to be preserved prior to dissection. Detection and identification of larvae in vectors which are not alive is often difficult, if not impossible as they tend to lose their morphological integrity on storage.

Of the different methods available, microscopy has traditionally been the most used method for the diagnosis of filariasis and the detection of larvae in vectors. Though it is comparatively cheap, it has its limitations. The method which requires training, experience, and skill to perform is labor intensive and time consuming. This is especially true when a large number

of samples are to be analyzed and therefore is not entirely suitable for use in large scale epidemiological surveys. Therefore there was an urgent need for the development of alternative technologies to detect filarial parasites. The advent of recombinant DNA technology contributed immensely to the development of novel diagnostic strategies for the detection of pathogens including the use of DNA probes in nucleic acid hybridization assays (Barker, 1990; Pereira, 1986; Tenover, 1988).

The methods which reach to the genomic core of the organism offered considerable advantages over conventional approaches for detecting pathogens (Edberg, 1985; Kulski, 1985; Matthews and Kricka, 1988; Minson and Darby, 1982; Norval and Bingham, 1987; Pereira, 1986; Pettersson and Hyypia, 1985; Tenover, 1988; WHO, 1986; Wolcott, 1992). The use of DNA probes in nucleic acid hybridization assays encompassed many areas, used extensively in many research applications such as detection of genetic diseases, prenatal diagnosis, forensic science, plant breeding etc. Considerable effort was also expended for the development of DNA probes for the detection of a wide variety of pathogenic organisms for clinical diagnosis, including viruses (Flores et al., 1983), bacteria (Hill et al., 1985; Moseley et al., 1982; Totten et al., 1983) and eukaryotic parasites (Barker and Butcher, Barker et al., 1986; 1983; Gonzales et al., 1984; McReynolds et al., 1986). As a diagnostic strategy the use of DNA probes in nucleic acid hybridization assays offered significant advantages such as convenience of use, low cost, sensitivity, specificity, accuracy, high predictive value, and rapid turnaround times making them attractive alternatives to conventional approaches used to detect infectious organisms. Furthermore, because of the rapid development of methodologies including improvements and modifications to existing methods, the use of DNA probes in nucleic

acid hybridization assays was not just confined to a laboratory exercise but was used extensively in field studies.

In this context, several alternate methods using DNA probes for the diagnosis of filariasis were developed, many of them focused on filarial parasites of medical importance. A majority of the methods using DNA probes utilized cloned repetitive DNA in hybridization assays for the detection of microfilariae in the host, and developing and infective larvae in the arthropod vector. Because of their abundance, repetitive sequences are more sensitive as probes than sequences of low copy number. Due to significant advantages of DNA based methods (DNA probes) for detection of filarial parasites over traditional methods, Professor Karunananayake as the principle investigator decided to develop DNA probes for three filarial parasites prevalent in Sri Lanka viz *Wuchereria bancrofti*, *Setaria digitata* and *Dirofilaria repens*. *Wuchereria bancrofti* is a filarial parasite that infects humans and *Setaria digitata* and *Dirofilaria repens* were filarial parasites infecting cattle and dogs, respectively. What follows is a brief description of the work carried out by the team and its achievements.

Wuchereria bancrofti

Lymphatic filariasis is caused by three species, *W. bancrofti*, *Brugia malayi* and *Brugia timori*. Around 893 million people in 49 countries are currently at risk of the disease. *W. bancrofti* alone accounts for more than 90% of the global burden of lymphatic filariasis. Unlike many other infectious diseases, filarial infections are not directly fatal but the suffering and disability they cause lead to a severely compromised quality of life. Furthermore, lymphatic filariasis can have a tremendous socioeconomic impact especially in the third world countries with poor financial resources. Though at present the disease is almost

eliminated in Sri Lanka, around two decades ago the number of persons at risk of developing lymphatic filariasis in the country was around 11.0 million.

In order to develop a DNA probe for the detection of *W. bancrofti* using a nucleic acid hybridization assay it was necessary to isolate from its genome a nucleic acid sequence that was specific to *W. bancrofti*. Furthermore, a repetitive DNA sequence was preferable to single copy sequences as the former would produce stronger hybridization signals increasing the sensitivity of hybridization assays. This required the construction of a DNA library of *W. bancrofti*. Therefore, blood from an infected donor was obtained and processed to isolate microfilariae (mf) using a 5.0 µM filter. DNA was extracted from the isolated mf and further purified by Hoechst dye CsCl centrifugation to remove any residual human DNA. A library was then constructed in the vector λ EMBL3 and screened using ³²P-labelled *W. bancrofti* genomic DNA to isolate strongly hybridizing clones which contain repetitive sequences. Several strongly hybridizing clones were isolated. Of these, a clone containing ~ 16 kb insert was selected and subcloned. A subclone in pUC18 designated pWb12 which gave strong hybridization signals with *W. bancrofti* DNA was selected for further analysis. The specificity, sensitivity and the copy number of the subclone were determined using dot blot hybridization assays. Southern blot analysis revealed it to be an interspersed repetitive sequence and had a moderate copy number of 450-700. The repetitive sequence did not hybridize to DNA from any of the mosquito vectors, other filarial parasites or host DNA indicating it to be specific for *W. bancrofti*. It was capable of detecting as little as 300pg of *W. bancrofti* DNA (Figure 1), a single microfilaria and a single L3 larvae (Figure 2) in a hybridization assay making it an ideal and excellent probe for the detection of *W. bancrofti* in both the host and the vector (Siridewa et al., 1994).

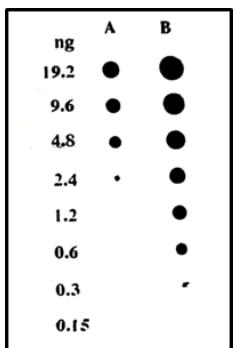


Figure 1.

Autoradiogram of a dot blot of dilutions of *W. bancrofti* genomic DNA probed with ^{32}P -labelled pWB6 (lane A) pWB12 (lane B)

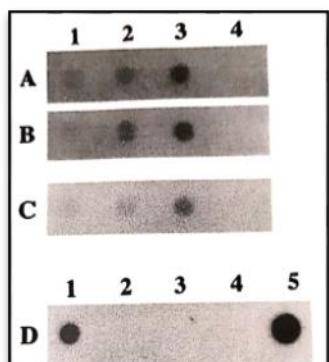


Figure 2.

Autoradiogram of a dot blot of DNA extracted from larvae/ microfilariae probed with ^{32}P -labelled pWB12. **A)** *W. bancrofti* L₃ larvae (rows 1, 2 & 3) and human tissue (row 4). **B)** Same as A except that one *Culex quinquefasciatus* mosquito (vector) was included in each *W. bancrofti* DNA extraction. **C)** DNA from *W. bancrofti* microfilariae (rows 1, 2 & 3) and human tissue (row 4). **D)** DNA from 5 *W. bancrofti* L₃ larvae (row1), *D. repens* (row 2), *S. digitata* (row 3). Controls included human DNA (row 4) and pWB12 (row 5).

[Figures 1 and 2 were reproduced from American Journal of Tropical Medicine and Hygiene. Volume 51. Authors: Siridewa K, Karunananayake EH, Chandrasekharan NV, Abeyewickreme W, Franzen L, Aslund L, Pettersson U. Cloning and characterization of a repetitive DNA sequence specific for *Wuchereria bancrofti*. Pages: 495-500. Copyright (1994) with permission from American Journal of Tropical Medicine and Hygiene]

A highlight of the above work was when the ~16 kb λ clone was evaluated along with other DNA probes developed by other laboratories worldwide at a workshop on DNA diagnostics and filariasis and symposium on filariasis and onchocerciasis sponsored by the UNDP/World Bank/ WHO special program for Research and Training in Tropical diseases (TDR) and New England Biolabs Inc held in Jakarta, Indonesia in 1989. Overall results indicated the probe developed by us to be the most promising of all the probes tested (UNDP/WORLD BANK/WHO, 1989).

Due to the disadvantages in using radioactive material which included their cost, short shelf life and health hazards involved, work was initiated to develop non-radioactive based rapid hybridizations assays. A simple, rapid chemiluminescent based oligonucleotide hybridization assay was subsequently developed for *W. bancrofti* (Gunawardene, 1999). This was preceded by the development of a PCR assay based on the above cloned sequence, pWB 12 (Siridewa et al., 1996).

Setaria digitata

Another parasite of interest was *Setaria digitata*, a filarial worm infecting cattle and buffaloes. A high incidence of filarial infection in cattle due to *S. digitata* has been reported (Gunawardena, 1991). Filariasis due to *S. digitata* in the natural hosts (cattle and buffaloes) does not cause an apparent pathogenic effect and hence is usually not considered to be serious. However, the transmission of infective larvae from mosquitoes to unnatural hosts such as sheep, horses and goats can result in the occurrence of a serious nervous disease commonly referred to as cerebrospinal nematodiasis (CSN) earlier known as lumbar paralysis or “kumri”. The infective larvae of *S. digitata* in unnatural hosts do not develop to complete sexual maturity and hence do not produce microfilariae, instead they behave in a yet improperly understood manner by being strangely attracted to invade and damage the nervous tissue. The invasion of the brain or spinal cord by the wandering, developing immature worm inflicts traumatic damage with ensuing inflammation (Shoho, 1953) resulting in neurological signs such as motor weakness, incoordination, ataxia and complete paralysis. Attempts to introduce goat and sheep farming in Mahaweli development area at that time, was greatly hampered due the occurrence of CSN (Gunewardene,

1991). Since nucleic acid based detection methods using DNA probes had significant advantages over conventional methods such as microscopy in the detection of filarial parasites, work was initiated to develop a DNA probe for the detection of *S. digitata* in cattle and buffalo which act as a reservoir for transmission to abnormal hosts such as sheep and goats and also to detect larvae in the mosquito vector.

To isolate a repetitive sequence from its genome, genomic DNA of *S. digitata* was extracted from adult worms found in the peritoneal cavity of cattle sacrificed at abattoirs. A library was constructed in the vector λ EMBL-3 and screened for repetitive sequences as described earlier for *W. bancrofti*. A strongly hybridizing recombinant clone designated EMBL-3Sd 41 containing an insert of ~14kb was isolated. The insert of the clone was cleaved with Sau3A1 and the resulting fragments cloned in pUC18 and screened for clones carrying repetitive sequences by colony hybridization. A recombinant clone designated pSD7 that gave strong hybridization signals was selected for further study. *S. digitata* genomic DNA was cleaved with various restriction enzymes, Southern blotted and probed with labelled pSD7. Strongly hybridizing bands around 200 – 700 bp were observed in DNA cleaved with Msp1. These fragments were cloned in pBS and screened for repetitive sequences using labelled genomic DNA by colony hybridization. A strongly hybridizing clone designated pSdM2 with an insert of 214 bp was selected for further study. Both clones pSd7 and pSdM2 were dideoxy sequenced and the sequences were found to display a high degree of homology (85%). Southern blot analysis revealed the cloned sequence to be a tandem repeat present in about 10,000 copies in the genome.

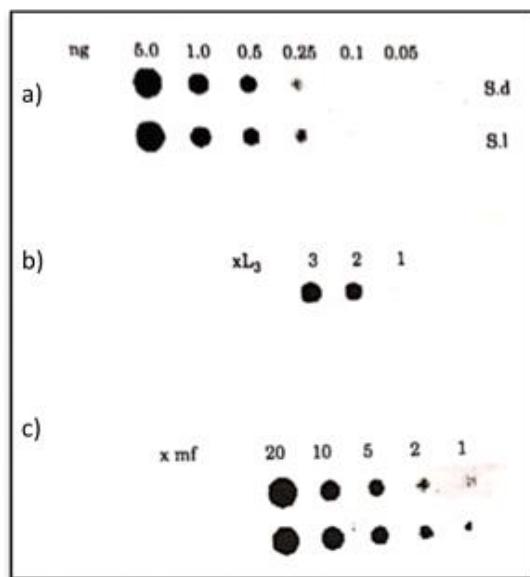


Figure 3.

- (a) Autoradiogram of a dot blot containing dilutions of *S. digitata* (S.d) and *Setaria labiato-papillosa* (S.l) genomic DNA probed with ³²P- Labelled pSdM2.
- (b) Autoradiogram of a dot blot of DNA extracted separately from 1, 2 and 3 L₃ larvae of *S. digitata* and probed with ³²P- labelled pSdM2.
- (c) Autoradiogram of a dot blot of DNA extracted from dilutions of cattle blood containing microfilariae and probed with ³²P- labelled pSdM2.

[This Figure was published in the British Veterinary Journal Volume 152: Authors: Wijesundera WSS, Chandrasekharan NV, Karunananayake EH, Dharmasena SP. Development of a DNA diagnostic probe to detect *Setaria digitata*: The causative parasite of cerebrospinal nematodiasis in goats, sheep, and horses. Pages 561-571. Copyright Elsevier, (1996). Reproduced here with permission from the publisher]

The specificity, sensitivity and the copy number of the sub-clone (pSdM2) was determined using dot blot hybridization assays. It did not hybridize to DNA from any of the mosquito vectors, other filarial parasites or host DNA indicating it to be specific for *S. digitata*. It could detect as little as 100 pg of *S. digitata* DNA, a single microfilaria and a single L₃ larvae (Figure 3), in a hybridization assay making it an ideal and excellent probe for the detection of *S. digitata* in both the host and the vector. Results of the work were published in the British Veterinary Journal (Wijesundera et al., 1996) and it was selected

as the best paper in the British Veterinary Journal for that year. A sensitive PCR assay for *S. digitata* was then developed based on the above cloned sequences (Wijesundara et al., 1999).

Dirofilaria repens

The genus *Dirofilaria* consists of filarial worms infecting dogs, cats, and other animals (Dissanaike, 1971). Dirofilariasis or canine filariasis is caused mainly by two species *Dirofilaria immitis* and *Dirofilaria repens*. In Sri Lanka, canine filariasis is caused by at least three species: (i) *Dirofilaria (Nochtiella) repens* (ii) *Brugia ceylonensis* and (iii) *Dipetalonema* species (Seneviratna, 1966). *D. repens* is the most widely prevalent of the three species. Preliminary studies indicated that the disease was prevalent island wide with an incidence as high as 70% in certain localities (Perera, 1956; Seneviratna, 1966). The *D. repens* adult worm lives in the subcutaneous tissues of dogs and the microfilariae released by the adult female worm eventually finds its way into the circulatory system. The disease in dogs, though not serious, can cause pruritus, dermatitis, anemia, exhaustion, fatigue and enlargement of the liver and spleen. Occasionally, enlargement of the scrotum and lymph nodes have also been observed. Hydrocele, keratitis, opacity of the anterior chamber, conjunctivitis, weakness of hind limbs and skin abscesses have been observed in a few cases (Perera, 1956; Seneviratna, 1966). *D. repens* is a zoonotic filarial worm. Humans can be infected following the bite of mosquito vectors carrying the infective larvae of *D. repens*. Accidental transmission of this parasite to man in some regions is facilitated by the high incidence of *D. repens* infection in dogs and the presence of appropriate vectors. Since man is an unnatural host, the worms rarely develop to sexual maturity. Even if they do, microfilaremia has never been observed in humans.

(Dissanaike, 1971). Human infections caused by *D. repens* results in diverse clinical manifestations as they can lodge in the subcutaneous tissues virtually in any part of the body (Dissanaike, 1971; 1979; 1993; Jariya and Sucharit, 1983). Subcutaneous infections usually appear as nodules or lumps (Dissanaike, 1971) which usually localize after treatment with DEC (diethylcarbamazine).

Diagnosis is usually made by identifying the worm in the cystic swelling or nodular mass after surgical removal or biopsy (Dissanaike, 1979). In the majority of cases, specific identification of the worm is extremely difficult as many of the worms are immature or adolescent females; some have been detected only in tissue sections (Dissanaike, 1971). Cases have been reported from various regions of the country and include patients between 6 months to 65 years of age having variable clinical manifestations. The worm has been recovered from the conjunctiva, eyelids and various subcutaneous tissues (Dissanaike, 1993).

As discussed above, due to the advantages of using DNA probes in nucleic acid hybridization assays for the detection of filarial parasites, work was initiated to develop a DNA probe for *D. repens*. Infected dogs in the Colombo Municipal Council area were euthanized and adult *Dirofilaria* worms were obtained from the subcutaneous tissues by dissection. DNA was extracted and purified using CsCl centrifugation and cleaved with Sau3A1. Using the resulting fragments, a genomic library was constructed in pUC 18 and transformed in *E. coli*. To isolate recombinant clones containing repetitive sequences the library was screened with labelled genomic DNA of *D. repens*, and strongly hybridizing clones isolated, a clone designated pDSAu 81 selected, sequenced and analyzed. To visually determine the presence of any repetitive sequences, genomic DNA was also cleaved with different restriction enzymes and size fractionated on an agarose gel and visualized. A band of high intensity was observed in the lane

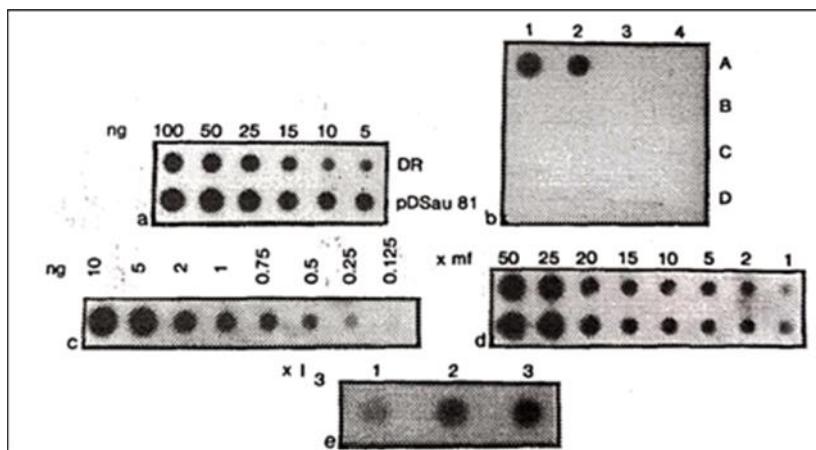


Figure 4.

(a) Autoradiogram of dot blot containing dilutions of *D. repens* (DR) and pDSau 81 DNA probed with ^{32}P -labeled insert of pDSau 81 (IpS).

(b) Autoradiogram of a dot blot containing DNA (0.5 μg) of the host, other filarial parasites, and mosquito vectors, blotted and probed with ^{32}P -labeled insert of pDSau 81. 1A, *D. repens* (10 ng); 2A, *D. repens* (5 ng); 3A, *Canis familiaris*; 4A, *Dirofilaria immitis*; 1 B, *Brugia malayi*; 2B, *Brugia buckleyi*; 3B, *Setaria digitata*; 4B, *Setaria labiato-papillosa*; 1C, *Wuchereria bancrofti*; 2C, *Anopheles tessalatus*; 3C, *Anopheles culicifacies*; 4C, *Anopheles vagus*; 1D, *Culex quinquefasciatus*; 2D, *Armigeres subalbatus*; 3D, *Aedes togoi*; 4D, *Aedes aegypti*.

(c) Autoradiogram of a dot blot containing dilutions of *D. repens* DNA probed with ^{32}P -labeled pDSau 81.

(d) Autoradiogram of a dot blot of DNA extracted from dilutions of microfilariae probed with ^{32}P -labeled pDSau 81.

(e) Autoradiogram of a dot blot of DNA of varying numbers of L₃ larvae probed with ^{32}P -labeled pDSau 81.

[Reprinted from Experimental Parasitology. Volume 78. Authors: Chandrasekharan NV, Karunananayake EK, Franzen L, Abeyewickreme W, Pettersson U. *Dirofilaria repens*: Cloning and characterization of a repeated DNA sequence for the diagnosis dirofilariasis in dogs, *Canis familiaris*. Pages 279-286. Copyright (1994) with permission from Elsevier]

containing genomic DNA cleaved with Rsa1. This 350 bp band was electroeluted and cloned in pBS. Of several recombinant clones one clone pDRsa1 was sequenced and analyzed. The sequence of the inserts of clones pDSau81 and pDRsa1 were found to contain 176 bp repetitive elements displaying a high degree of homology. Some elements appeared to have diverged significantly. The repeats appeared to be present in about 15 000 copies and constituted 3% of the genome. When used in dot blot hybridization assays pDSau81 did not hybridize to DNA from any of the mosquito vectors, other filarial parasites or host DNA indicating it to be specific for *D. repens*. It was capable of detecting as little as 250 pg of *D. repens* DNA, a single microfilariae and a single L3 larva in the host and the vector (Figure 4) making it an ideal and excellent probe for the detection of *D. repens* in both the host and the vector. The repetitive sequence could also aid in the identification of adult worms found as lumps/nodules in human clinical specimens. Since publishing these results (Chandrasekharan et al., 1994) several other investigators developed sensitive and specific PCR assays based on the cloned sequence.

The work carried out by us was very challenging at that time as we were the only laboratory in Sri Lanka carrying out molecular cloning work. Professor Karunanayake inspired and supported us in numerous ways by providing whatever needed. He also encouraged us and emphasized the importance of carrying out quality work with the aim of publishing in high impact journals. In the initial stages, we were also ably supported by Professor Ulf Pettersson, a collaborator who took a very special interest in our work. Some of us were very fortunate to have got the opportunity to carry out a part of our work in his laboratory in Sweden. His graduate students at that time, Lena Franzen and Lena Aslund, and technical officers Maria Rydaker and Elsy Johnsson spent their valuable time training us in the latest techniques in Molecular

Biology and took good care of us when we were in Sweden. They also visited our laboratory to carry out workshops and training programs. We are very grateful to all of them! Unstinted support was also extended to Professor Karunananayake and his graduate students by the late Professor Rune Liminga, Director International Program in the Chemical Sciences, University of Uppsala, Sweden (IPICS) at that time. He took a personal interest in ensuring the setting up of a fully functional Molecular Biology laboratory in Sri Lanka and helped in equipping the laboratory with instruments / reagents etc. needed for research and also in the training of personnel.

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Isolation and characterization of genes from parasitic nematodes

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In the early nineteen nineties, when I joined the Molecular Biology Unit or the MBU as it was fondly known, it was a thriving research laboratory with several research students working under the able mentorship of Professor Eric Karunananayake, and very much the envy of those who were not!

One of the key areas that the MBU focused on was the investigation of parasitic nematodes, namely *Wuchereria bancrofti*, *Setaria digitata* and *Dirofilaria repens*, funded by the SAREC grant. After several years of focus on characterization of species-specific molecular probes, the laboratory was ready to embark on the isolation and analysis of genes from these parasitic nematodes. These parasitic nematodes have a complex life cycle in which they migrate between different vertebrate host and an arthropod vector (Figure 1). At the time, there was barely any molecular level information known about these parasites, and the understanding of gene structure and function was expected to shed light on their life cycles and host-parasitic relationships, opening avenues for further investigation and providing targets for therapeutic interventions. Several previously isolated genes from the free-living nematode, *Caenorhabditis elegans* had been kindly provided by Professor John Sulston, who later went on to share the Nobel prize in 2001 with Sydney Brenner, for their work on *C. elegans*. I had the task of isolating and characterizing the heat shock protein 70 (Hsp70) gene from *S. digitata* for my PhD project. *S. digitata*, one could say, was the laboratory pet, the ‘lucky worm’, a source of publications and PhDs to several in the MBU.

The genus Setaria

The genus *Setaria* include several parasitic nematodes which infect a variety of livestock animals. Among them, *S. digitata*, *S. labiato-papillosa*, *S. marshalli* and *S. cervi* have been reported in Sri Lanka (Senevirathne, 1955). In the preceding chapter Professor Chandrasekharan has already described characteristics and importance of *S. digitata*.

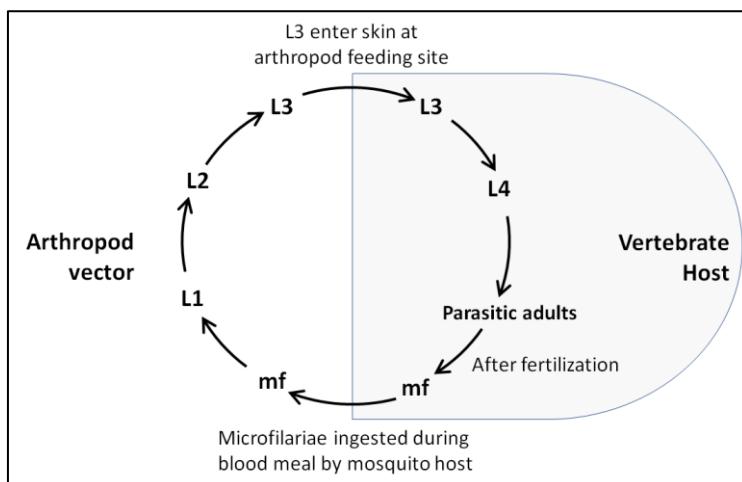


Figure 1. Schematic of the life cycle of parasitic nematodes. L1-L3; larval stages; mf, microfilaria.

The heat shock proteins in parasitic nematodes

The HSP genes are subject to complex regulatory mechanisms, reflecting their multitude of functions. Those constitutively expressed likely function as molecular chaperones, assisting with protein folding. Some are strictly stress-induced, while others, thought to be involved in parasite differentiation, appeared to be developmentally regulated (Neuman et al., 1993; Newport et al., 1988). Therefore, the notion that the 'heat shock' response observed in parasitic nematodes during migration between hosts, may represent a

developmental response, rather than a true heat shock response, was entertained by scientists at the time. Differentiation of certain parasites coincides with the transmission between different hosts, an event frequently associated with the expression of HSPs (Neuman et al., 1993, Tsuji et al., 1997; Weiss et al., 1998).

Heat shock proteins (HSPs) were expected to play an important role in the life cycle of *S. digitata* (and in other filarial nematodes) as they migrated between their homeothermic vertebrate hosts and the poikilothermic mosquito vector. The inevitable temperature shift from ~23-25°C to 37°C, experienced by the parasite was believed to induce expression of some HSPs, possibly crucial for its adaptation and survival within the host environment, even if the expression of some of these genes were to occur as part of a developmentally regulated program.

Based on their apparent molecular mass several HSP families have been identified, namely HSP100, HSP90, HSP70, HSP60 and the small HSPs. Of these, the HSP70s are among the most conserved and prominent members, associated with a plethora of seemingly unrelated functions from protein biogenesis to lysosomal degradation and parasite differentiation to autoregulation of the heat shock response.

HSP70 genes in S. digitata

Eukaryotic genomes frequently encode multiple *hsp70* genes, forming complex multigene families, which may be differentially regulated. They exhibit significant sequence conservation across the phylogenetic spectrum. The *hsp70* multigene family of *C. elegans* is thought to comprise 9-12 members; some are developmentally regulated (Snutch et al., 1988). The

cDNA clone of one of these *hsp70* genes was used to probe a genomic library of *S. digitata*.

The report of the isolation and subsequent sequence characterization of the *S. digitata hsp70-2* gene (Jayasena et al., 1999) holds a privileged position in the local molecular biology research arena as the first complete sequence of a gene to be carried out and published from a Sri Lankan laboratory. The entire gene was manually sequenced by the Sanger dideoxy chain termination method using [α^{35} S]dATP to label the primers. A primer-walking technique was used to obtain the complete sequence of the clones.

Screening of the genomic library of *S. digitata* constructed in the Lambda replacement vector EMBL-3, led to the isolation of a ~13.4 kb recombinant clone. The clone contained two *hsp70* genes in head to tail arrangement, with ~1 kb intergenic region. The 5' region of one gene, (designated *hsp70-1*) was absent in the isolated clone, while the second gene (designated *hsp70-2*) was complete (Jayasena et al., 1999). These results hint at the possibility that these two genes are part of a cluster of *hsp70* genes. Subsequent experiments (Figure 2), indicated the presence of 4-5 *hsp70* genes which appeared to be clustered (Jayasena et al., 1999) similar to observations in other nematodes. *C. elegans* is reported to possess an *hsp70* multigene family with at least nine genes (Heschl and Baillie, 1990). In *S. digitata*, *hsp70-2* gene appeared to be constitutively expressed and some *hsp 70* genes did not seem to be expressed in adult worms.

Sequence analysis

The *S. digitata hsp70-2* gene sequence (NCBI accession No. AF079360), along with its flanking regions spanned ~3.9kb and was observed to be AT-rich (CG content 41.8%), which was characteristic of nematode genomes

(Hammond and Bianco 1992). The coding sequences were also AT-rich (GC content 44.6%). A putative transcription start site was present 30 bp downstream of the 1st T of the TATA box which occurred as ‘TATAAA’ in this gene. Additionally, two CAAT boxes as well as three heat shock elements were identified between the TATA box and the CAAT boxes.

Our observations suggested that the gene may either contain an intron in the 5' UTR or that it may be *trans*-spliced during processing. However further experiments did not provide conclusive evidence on *trans*-splicing of the *hsp70-2* gene (Jayasena, 1999). Polyadenylation (polyA) signal was located in the 3' UTR region of *hsp70-1* gene, but for the *hsp70-2* gene polyA signal appeared to lie further downstream to the 315 base pairs sequenced in the 3' UTR region.

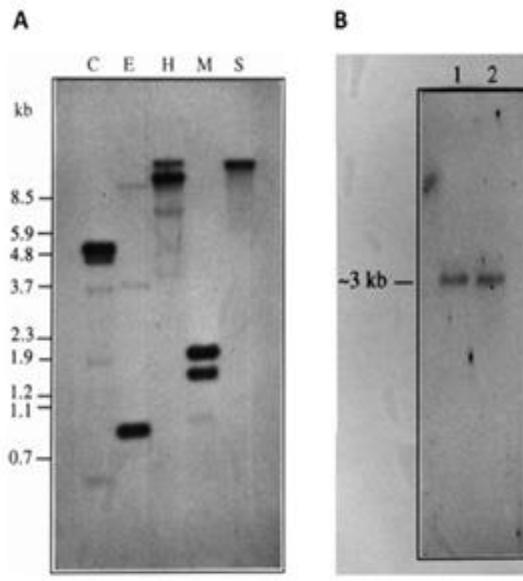


Figure 2. Southern blot (A) and Northern blot (B) analysis of *S. digitata* *hsp70* genes
 A. Genomic DNA cleaved with *Cla*I (C), *Eco*RI (E), *Hind*III (H), *Msp*I (M) and *Sal*II (S) probed with ~1kb fragment of *hsp70-2* gene. B. mRNA from adult *S. digitata* worms prior to (1) and after (2) heat shock treatment at 42°C for 30 minutes probed with a mixture of the ~1 kb fragment as well as another ~1.7 kb fragment of *hsp70-2* gene.

The available sequences for *hsp70-1* gene showed that introns 5, 6 and 7 were conserved with those of *hsp70-2* gene, in both the amino acid position as well

as the phase in which they interrupted the reading frame (Figure 4) (Jayasena, 1999).

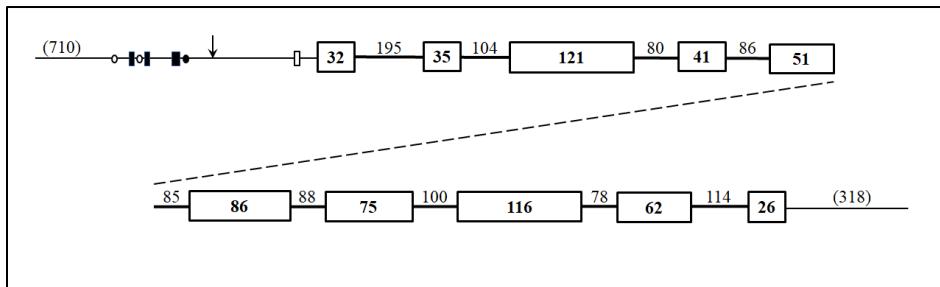


Figure 3. A graphical representation of the organization of the *S. digitata hsp70-2* gene.

Unfilled boxes represent exons and the numbers of amino acids are indicated within them. Introns are represented by thick lines and flanking regions are represented by thin lines. Numbers above the lines denote size in nucleotides.

↓ Transcription start site ■ Heat shock elements □ putative splice site in 5'UTR

- TATA box
- putative CAAT box element

(Adapted from Jayasena SMT, 1999)

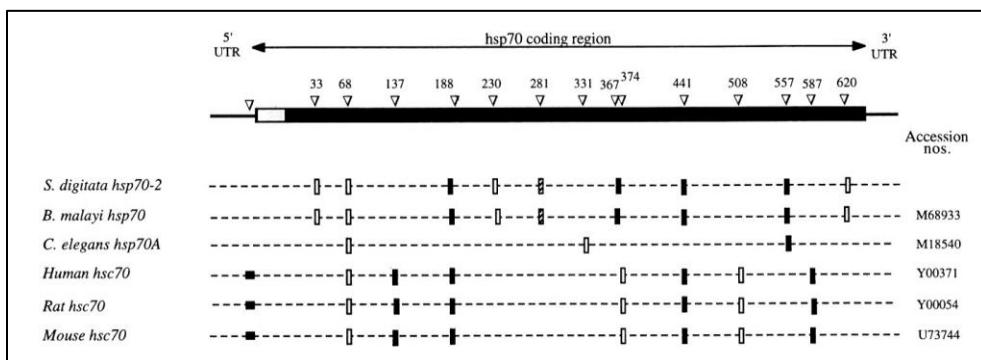


Figure 4. Comparison of the distribution of introns in selected heat shock protein genes. The intron positions are presented schematically on a hypothetical *hsp70* gene at the top of the figure. The black bar represents the coding region. The signal sequence of the ER-bound Hsp70s is indicated as a white bar. Intron positions are characterized by codon numbers given in relation to the deduced *S. digitata hsp70-2*

sequence. Intron sites within individual genes are indicated by; shaded squares, introns in 5' untranslated region (UTR); open rectangles, introns interrupting the reading frame in phase 1; hatched rectangles, introns in phase 2; shaded rectangles, introns directly following a codon (phase 3). Data catalogued from Biosoft® database release 102 1997.

Amino acid sequence of *hsp70-2* gene

The *hsp70-2* gene encoded a protein of 645 amino acids having 97.4% amino acid identity with *Brugia malayi* Hsp70 (Rothstein and Rajan, 1991) and was also highly similar to available partial Hsp70 amino acid sequences of other parasitic nematodes (Jayasena et al., 1999).

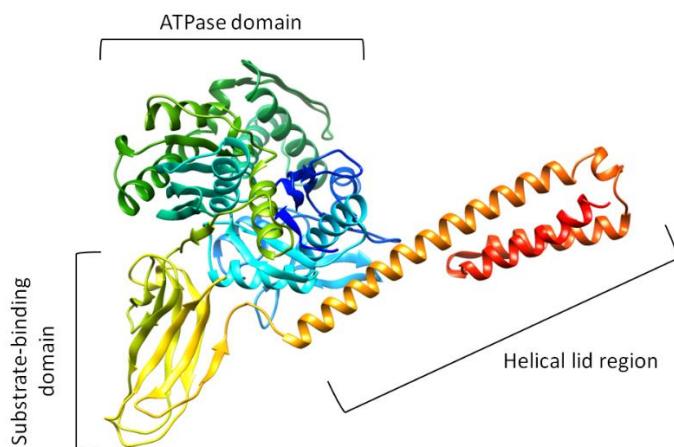


Figure 5. Predicted 3D structure for the *S. digitata* Hsp70-2 protein. The 3D structure was built by homology modelling (SWISS-MODEL) using *Chestomium thermophilum* Hsp70 as template. [Qmean0.34, 50% sequence identity and 92% query cover]. Structure validation was carried out using ERRAT (92.59), Verify3D (96.55%) and PROCHECK (93.7% residues in most favorable regions). Three commonly observed regions in Hsp70 protein sequences were putatively identified in the predicted model. (Madushika Perera is acknowledged for deriving the model).

The deduced amino acid sequences of both Hsp70-1 and Hsp70-2 carried the terminal peptide motif EEVD indicating their localization to the cytoplasm

(Boorstein et al., 1994; Gupta and Singh, 1994). Three repeats of the GGMP tetrapeptide motif was identified in the carboxyl terminal, a sequence that has been found to be conserved among Hsp70s bearing closer evolutionary and functional relationships (Sheppard et al., 1989). The predicted 3D structure of the derived amino acid sequence is represented in Figure 5.

Actin genes of nematodes

Actin is a ubiquitous protein conserved in all known eukaryotes. Often encoded by a multigene family and are spatially and temporally regulated. Several isoforms of actin have been identified, and they fall into two general classes; the cytoplasmic actin and the muscle actin (Rubenstein 1990). Although a vast majority of the actins are highly conserved, some divergent forms (actin-like proteins) have been reported from several organisms (Clark and Meyer, 1992; Lees-Miller et al., 1992; Schwon and Martin, 1992).

Investigating actin in filarial nematodes was important particularly due to the discovery that they were able to evoke an antigenic response (Sritharan and Piessens, 1997). The antigenic epitope on the related filarial parasite *B. malayi* was observed to be the amino terminal of the protein, having the greatest variability. N-termini of actins are post-translationally modified by removal of one or two amino acid residues followed by acetylation of the new terminus (Pollard and Cooper, 1986)

Actin gene of Setaria digitata

The complete actin gene of *S. digitata* was isolated from a genomic library using a partial sequence of a mouse α -actin gene as the probe

(Chandrasekharan, 1994). The ~2.5 kb DNA fragment contained the gene and its flanking regions (NCBI accession no. AF079359).

A putative transcription start point was identified 230 bases upstream of the initiator methionine. A putative TATA box and CAAT boxes were also identified further upstream (Figure 6). The 3'UTR revealed a putative polyadenylation signal, AATAAA, 374 bp downstream of the stop codon.

The coding region was interrupted by 5 introns, ranging in size from 81-194 bp and northern blot analysis of RNA isolated from adult worms, revealed a single molecule at ~1.3 kb. The coding regions predicted a 376 amino acid protein, which was similar to other actins with high degree of homology to previously cloned actin sequences. Isolated *S. digitata* actin closely resembled vertebrate cytoplasmic γ -actin (Rubenstein, 1990). However, typical of most lower-eukaryotes (Kindle and Firtle, 1978; Nellen and Gallwitz, 1982; Ng and Abelson, 1980), the *S. digitata* actin demonstrated some characteristic amino acid conservations found in muscle actin as well.

```
1  aagcttgcta gcaatgagaa ttctcgaaat gatatccctg accactataat aagcctcaga
   ↓
61  atgcaccccta gttccctcac tttcattccg cttagcagcc ttcatgttgc agcttagactt
121 aaagtgttgc ctgtagaagt ggcaactttt ggttgcttag cttcaggaac taatttactg
181 actcatcaga ttgcaaataat tagattgaac taaattgatc cttagtcaaataat tttttgtttc
241 ttatcaattt tatatttaaa ttgagtaaaa ttgcaggta cccgcccaca gctttaaagt
301 cgtaaata
```

Figure 6. The 5'UTR region of the *S. digitata* actin gene. A putative transcription start point is indicated in bold and indicated by an arrow. A putative TATA box is highlighted in bold. A CAAT box is underlined in bold (*Sequence from NCBI accession no. AF079359, Chandrasekharan, 1994*).

Wuchereria bancrofti

W. bancrofti and lymphatic filariasis are described adequately in the preceding chapter. As the microfilariae appear in the infected person's blood at night, volunteers were recruited through a collaboration with the Anti-Filaria Campaign for night blood sampling to obtain microfilariae (mf) for our studies. Two eminent Sri Lankan entomologists (namely Mrs. Nalini Jayasekara and late Dr. Kingsley Kalpage) and Dr. Gamini Karunananayake of the National Blood Bank also assisted this program. On such nights, the MBU remained a busy and lively place. Volunteers brought there were selected for either bleeding for mf or for feeding the mosquito vectors maintained in the laboratory for parallel ongoing studies on the larval form (L3). The volunteers opting for mosquito feeding found themselves with arms exposed to the biting of hungry mosquitoes for a significant length of time, where he couldn't (obviously) slap at them! Those selected for bleeding generally had high microfilaremia ($>1000\text{mf/ml}$) which was estimated through microscopic observation at the time of bleeding. All volunteers were compensated with warm milk tea or 'Nestomalt' drink and rest (after bleeding).

The Actin gene of W. bancrofti

The *W. bancrofti* actin gene was isolated from a genomic library, based on sequence similarity with the previously isolated actin gene of *S. digitata*. The isolated clone contained the entire gene including the 5' and 3' flanking regions. Gene was AT rich (G+C content of 45.18% within the coding regions, overall G+C content of 37.2%) and found to exist as a single copy (Figure 7) (Casinader et al., 2000).

The TATA box (TATAAA) as well as a putative transcription initiation site was identified in the 5' UTR region. The common natural variant of the

polyadenylation signal, ATTAAA (Wickens, 1990) was identified 178 bp from the stop codon, and the motif ATTTA, known to regulate mRNA stability further downstream at 252 bp of the stop codon. The coding region was interrupted by four introns ranging in size from 109-193 bp (Figure 8).

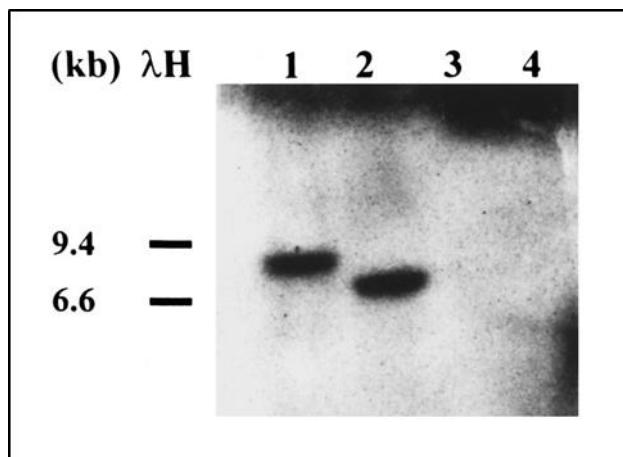


Figure 7. Southern blot analysis of *W. bancrofti* actin gene.

W. bancrofti genomic DNA cleaved with *Hind*III (lane 1) and *Eco*RI (lane 2) and human genomic DNA cleaved with *Hind*III (lane 3) and *Eco*RI (lane 4) and probed with ³²P-labelled *W. bancrofti* actin gene sequences. Lambda DNA cleaved with *Hind*III is used as a molecular weight marker.

The *W. bancrofti* actin gene encoded a protein of 376 amino acids (NCBI accession no. AAF25819.1) which at the time of analysis showed the highest homology (98%) to the free living nematode *Plectus acuminatus*. However, a recent search revealed 100% homology with a *B. malayi* actin protein sequence (NCBI accession no. XP_001894819.1) as well as some others submitted later. Another 678 amino acid protein identified as *W. bancrofti* actin (NCBI Accession No. EJW83248.1) bearing sequence similarity with an ‘actin family’ sequence from *Loa loa* and *B. malayi* among others has been reported. Thus *W. bancrofti* likely harbors multiple actin genes, with little

sequence similarity to each other. This may explain the results obtained in the previous investigations in the MBU.

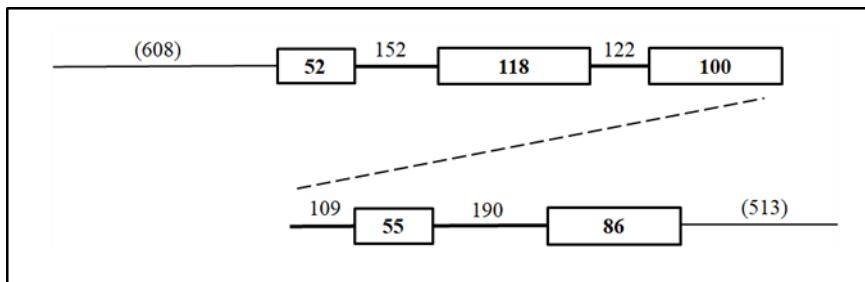


Figure 8. A graphical representation of the organization of the *W. bancrofti* actin gene. Unfilled boxes represent exons and the numbers of amino acids are indicated within them. Introns are represented by thick lines and flanking regions are represented by thin lines. Numbers above the lines denote size in nucleotides.

Trans-splicing in Nematodes

Many lower eukaryotes and nematodes have an unusual form of processing during mRNA maturation known as trans-splicing (Murphy et al., 1986, Rajkovic et al., 1990), where a small ‘spliced leader (SL)’ sequence form a small RNA molecule (known as spliced leader RNA; SL-RNA) is acquired by the mRNA. It is ubiquitously present in nematodes, both free-living and parasitic (Rajkovic et al., 1990). Operons trans-splicing of a short ‘spliced leader’ RNA into the 5’ end of the pre-mRNA via an inter-molecular splicing event is a common feature observed in eukaryotes (Lasda and Bluementhal, 2011). Yang et al., 2017 showed that addition of the spliced leader increases the efficiency of translation in *C. elegans*, providing a role for this phenomenon. Further, this SL sequence essentially provides the 5’CAP for the mRNAs situated downstream of the first gene, facilitating their translation. In

C. elegans, ~84% of genes are found to be trans-spliced (Allen et al., 2011), via one of two functionally distinct splice-leaders. SL-1 is trans-spliced into either monocistronic genes or the first gene in polycistronic mRNA (Krause and Hirsh 1987), while SL-2 is added onto downstream genes in operons, which would otherwise not be translated (Spieth et al., 1993).

Trans-splicing in *W. bancrofti*

As trans-splicing is absent in the host, *trans*-splicing in *W. bancrofti* could be a potential target for control and eradication of lymphatic filariasis. Screening of a *W. bancrofti* genomic DNA library with *C. elegans* 22 nucleotide SL sequence led to the identification of clones which contained three spliced leader RNA genes (SLG1, SLG2 and SLG3) in *W. bancrofti*, isolated along with two 5S-RNA genes (5SR2 and 5SR3) as shown in Figure 9 (Dassanayake et al., 2001).

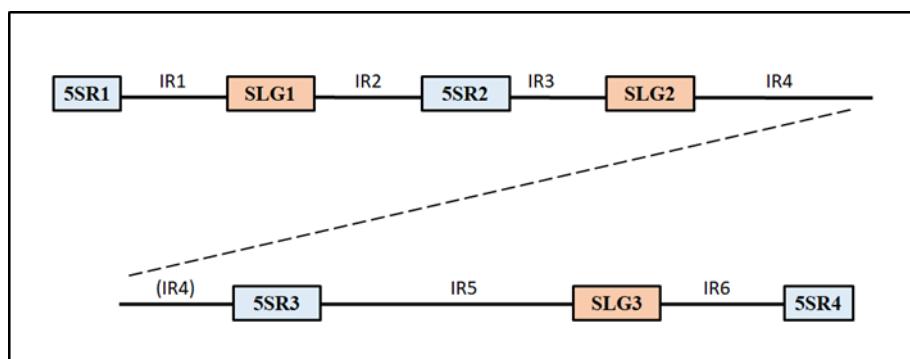


Figure 9. Schematic representation of the sequenced region of the *W. bancrofti* splice leader RNA genes (SLG1 to SLG3) and 5R-rRNA genes (5SR1 – 5SR4). The intergenic regions are numbered consecutively from IR1-IR4. [Only partial sequences of 5SR1 and 5SR4 were available].

The sequences of the splice leader genes SLG1, SLG2 and SLG3 were identical, except that the SLG2 had an additional nucleotide at position 43 (Dassanayake et al., 2001). The ribosomal RNA genes demonstrated high sequence similarity (98%) apart from an unusual 26 nucleotide deletion in the 5SR2 gene.

Conclusions

The initial studies on the characterization of selected genes being investigated in the MBU at the time, led the way for better understanding of these parasitic nematodes. The importance of the knowledge gained in *S. digitata* in the application for human lymphatic filariasis has been reviewed by Perumal et al., 2015. The genome of *S. digitata* has been sequenced and the draft genome was published recently (Senanayake et al., 2020). This, together with the mitochondrial genome sequence (Liu et al., 2017), opens the door to a plethora of investigations in the areas of functional analyses, identification of parasite specific genes etc., leading to a better understanding of these parasites. The application of siRNA -mediated gene silencing in this context (Somarathne et al., 2018 a, b) opens an avenue for the investigation of gene function in these parasites.

Copyright acknowledgement:

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Figure 7 - reprinted from International Journal of Parasitology, Volume: 30, Authors: Casinader Saverimuttu JK, Karunananayake EH, Chandrasekharan NV. Jayasena SMT. Molecular characterization of the actin gene of the filarial parasite *Wuchereria bancrofti*. Pages: 119-12., Copyright (2000), with permission from Elsevier

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Three Other Success Stories of Filariasis Research

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Professor Karunananayake's is a life that illuminates many, this Felicitation Volume in his honour is brought out, as students and colleagues, young and old, spread across the country's leading academic and research institutions. I owe a debt of gratitude to the editor, Professor Kamani Tennekoon for inviting me to put together some of the great work carried out by Professor Karunananayake and his students and colleagues during the period 2000 - 2013 when it was also an era of transition. With the establishment of the IBMBB, in the year 2004, Professor Karunananayake became its founder director. Then on retirement, being an Emeritus Professor, he happily reverted to full academic duties on a voluntary basis and brought his research to the next level showing his desire to complete the tasks undertaken. While there were many different research activities during this period, in this essay I describe some of the advancements made in the filariasis research during 2000 to 2013. As described in the preceding chapters, lymphatic filariasis (LF) is mainly caused by the filarial parasite *Wuchereria bancrofti* while its main vector is the mosquitos of the species *Culex quinquefasciatus*.

From preliminary antigen explorations to a sensitive dot ELISA test for the diagnosis of LF

Detection of microfilaria in night blood samples had long been the standard method of diagnosing filariasis. However, it had been problematic not only in Sri Lanka but also in other endemic countries owing to lack of public compliance and the lack of staff-dedication. Recognizing the urgent need for

developing a cost effective but accurate and sensitive LF diagnostic method, Professor Karunananayake initiated preliminary studies with the other members of his research group i.e. PhD student Kumudini Casinader Saverimuttu, late Professor M.M. Ismail, then Professor of Parasitology, Faculty of Medicine, Colombo and Swedish collaborators Professors K Berzins and P Perlmann from the Department of Immunology, Stockholm University. The group explored *W. bancrofti* antigens and identified an array of mf antigens by immunoblot screening with immune sera of infected individuals; among the antigens detected, only the 14 kDa and 42 kDa antigens were consistently recognized by the immune sera (Saverimuttu et al., 2000). Further experiments carried out using immune sera developed in rabbit by dosing intact parasites as well as isolated antigens revealed that the 42 kDa antigen was shared by both mf and L3 larvae of *W. bancrofti*, therefore it was identified as a potential vaccine candidate. Interestingly, the 14 kDa antigen was specific for mf and therefore it was proposed for the development of a routine diagnostic method to detect active infection. Moreover, another 132 kDa antigen was recognized only by the sera of patients having tropical pulmonary eosinophilia (TPE), a clinical manifestation of LF, therefore it could be used in the diagnosis of TPE (Saverimuttu et al., 2000).

Further developments were however hampered partly by the observation that the individuals in the endemic areas possess low levels of serum antibodies to surface antigens of mf, probably due to filarial defense mechanisms. Therefore exploration of circulating antigens from both mf and adult worms was of significant importance for improving sensitivity of antibody detection tests as well as for developing sensitive antigen detecting tests. Further the limited access to adult *W. bancrofti* parasites that live in deep human lymphatic system of infected individuals led Professor Karunananayake to look for a readily available model organism that is similar to *W. bancrofti*. Our protagonist had

already been exploring cattle filarial parasite *Setaria digitata* as a potential model for this purpose.

Fresh studies were launched, this time with his then MPhil student Mahendra Wickremayake (currently at the Faculty of Science, University of Colombo) and Professor Sriyani Ekanayake (now Emeritus Professor of Parasitology, Faculty of Medical Sciences, University of Sri Jayewardenepura). Immunoblotting of gel-separated cuticle extracts with sera of asymptomatic microfilaraemic individuals and of non-endemic controls revealed that the two antigens of adult *S. digitata* (with molecular weights of 52 and 130 kDa) were recognized by asymptomatic microfilaremic individuals but not by non-endemic individuals (Wickremayake et al., 2001). These 52 kDa and 130 kDa adult parasite derived serum antigens were also found in the serum of asymptomatic microfilaraemic individuals with bancroftian filariasis (Wickremayake et al., 2001). Based on these findings a dot-ELISA test for the detection of *W. bancrofti* microfilariae was developed using 130 kDa and 52 kDa antigens. Both antigens detected microfilaremic asymptomatic individuals with 10 ng of antigen per spot; the endemic normals gave a positive reaction only at 40 ng antigen per spot; non-endemic normals were negative showing no reaction. Therefore, the test could differentiate the endemic normals from the microfilaraemic asymptomatic individuals providing a cost effective, convenient and reliable test method for routine use in the diagnosis and surveillance of LF (Wickremayake et al., 2001). Study was mainly conducted at the Department of Biochemistry and Molecular Biology, Faculty of Medicine.

A sensitive and specific polymerase chain reaction based method for the detection of the vector mosquito

Accurate identification of *Culex quinquefasciatus* (Diptera: Culicidae), the mosquito vector that transmit filarial parasites plays a key role in controlling the disease transmission. Together with research students Usha Hettiaratchi (currently a Professor in Biochemistry, University of Sri Jayewardenepura), DHN Munasinghe (currently a Professor in Zoology, University of Ruhuna), Dr. NV Chandrasekharan (who authored the first essay on filariasis in this Volume), and Mrs. Nalini Jayasekera, formerly Entomologist at the Medical Research Institute, Professor Karunananayake focused on DNA based detection of *C. quinquefasciatus*. Mosquito colonies were raised in the small insectory at the Animal House of the Colombo Medial Faculty. *C. quinquefasciatus* genomic DNA library was constructed and screened for repetitive DNA. A 693 bp A-T rich genomic repetitive DNA sequence identified for the first time was cloned and completely sequenced. Southern blot analysis revealed that the cloned fragment has approximately 30,000 copies interspersed within the genome (Hettiaratchi et al., 2000). High copy number indicated the possibility of developing a highly sensitive PCR detection method and in fact the probe based detection was sensitive enough to detect picogram quantities of mosquito DNA, albeit the probe could not differentiate *C. quinquefasciatus*, from other mosquito species as it hybridized with DNA from species of *Culex*, *Mansonia* and *Anopheles*. Thus they searched for PCR primers for the specific amplification of *C. quinquefasciatus* DNA. Unique *C. quinquefasciatus* specific regions of the cloned DNA fragment were identified and a PCR based assay was optimized for the detection of *C. quinquefasciatus* taking the advantage of high copy number. Interestingly the new method was not only very sensitive and specific to *C. quinquefasciatus* but was also capable of

detecting DNA from all the stages of *C. quinquefasciatus* (Hettiaratchi et al., 2000), thus making it a valuable tool in the fight against LF.

Newly characterized nematode specific genes; a fertile background for drug and vaccine development

As described in the preceding essays absence of a drug that kills the adult worm is a major impediment in the global filariasis elimination program. Hence, identification of new and potent drug targets against filariasis is vital. Several genes of the cattle filarial parasite have been sequenced and characterized by Professor Karunananayake's team. Previous studies on *HSP70* and actin genes have been detailed by Professor Jayasena in this Volume. A young academic from Jaffna, Dr. A. Murugananthan (currently senior lecturer in Parasitology, Faculty of Medicine, University of Jaffna) carried out further work supervised by Professor Karunananayake and Professor Tennekoon at the IBMBB. A cDNA library of adult filarial parasite *S. digitata* has been constructed and several clones were sequenced. A clone containing a 661bp mRNA transcript of the alkali myosin light chain protein (MLC) was identified and further screening of the genomic DNA library of *S. digitata* identified the clone containing the gene that code for MLC (Murugananthan et al., 2010). Southern hybridization studies suggested that this gene is a single-copy gene. Both the gene sequence and the MLC have been characterized *in silico*. The gene was AT rich (65%) found to be absent of TATA box but had a potential CAAT box and seven possible transcription factor binding elements at the 5' end. General features of a gene such as GT-AG splicing rule, 3' polyadenylation signals were present along with three intronic regions (Murugananthan et al., 2010) (Figure 1).

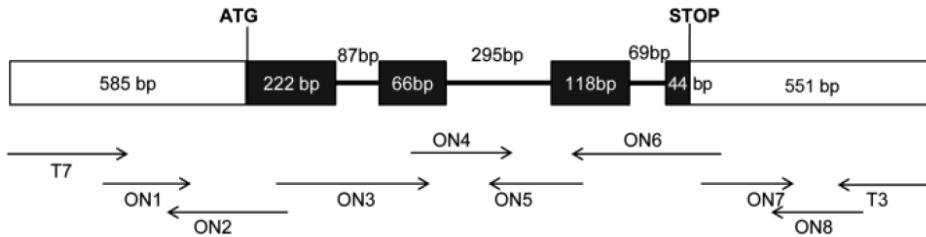


Fig: 3. The structure of the alkali myosin light chain gene of *S. digitata*: Boxed regions represent exons. Protein coding regions are shaded. Arrows indicate sequencing strategy used. ON= primers used for sequencing (Reproduced from Murugananthan et al., 2010 with permission from the Journal)

Further analysis of MCL *in silico* revealed the presence of cytoplasmic localization signals and characteristic calcium-binding domain proteins. *S. digitata* MCL amino acid sequence was very similar to MCL of other filariae with over 80% sequence similarity to *Caenorhabditis briggsae* MCL sequence. However, a low level of sequence conservation with human sequences was observed. It is localized in the parasite's cuticle providing means of selective therapeutic targeting of MCL. Further studies on antigenicity and other aspects are needed to validate MCL as a vaccine target.

The cDNA library constructed and maintained at the IBMBB, according to Professor Karunananayake's own words is a "gold mine" and it was proved and continues to be a real "gold mine". This is because the cDNA library still provides real transcripts for wet lab research although the current research is guided by the sequenced genome of *S. digitata*. One such study carried out by then PhD student Wasana Rodrigo under the supervision of Professor Ranil Dassanayake of the Department of Chemistry, Faculty of Science, University of Colombo and Professor Karunananayake and Professor Jagath Weerasena of IBMBB, extensively characterized a previously unidentified parasitic nematode-specific growth-factor-like protein in *S. digitata* (SDUP) expressed ubiquitously in all stages of the filarial life cycle. The SDUP was 204 amino

acid long globular protein rich in beta sheets and had a molecular weight of 22.8 kDa (Rodrigo et al., 2009). Southern hybridization revealed SDUP to be a single-copy gene. Homologous counterparts of SDUP was found in human filarial parasites including *W. bancrofti*, where the sequences shared 79% similarity (Rodrigo et al., 2009). SDUP was later expressed in different expression system (Rodrigo et al., 2013; 2014). Further GFP fusion studies and immunohistochemical staining demonstrated that SDUP is localized in the nucleus of the longitudinal muscle layers and the endodermis, thus indicating a possible function in locomotion. SDUP was later validated as a potential drug target that cause developmental deformities and motility changes in the larval developmental stages when silenced by siRNA based methods (Somarathne et al., 2018).

Conclusions and future directions

While the development of dot-ELISA method for the diagnosis of LF and PCR based method for detecting the vector provide direct applications, newly characterized nematode specific proteins and drug targets open a way forward for structure based drug and vaccine discovery. Determination of accurate tertiary structures of these proteins will aid in accelerating structure based drug discovery against LF. Research Projects mentioned in this chapter produced two MPhil degree and two PhD degree holders as a part of Professor Karunananayake's effort to human resource development in the field.

The work described above were supported by Sida/SAREC (PI: Professor Karunananayake) and two Grants from National Science Foundation of Sri Lanka (PI: Professor Karunananayake and Professor Ranil Dassanayake). Collection of *S. digitata* worms was facilitated by Professor RPVJ Rajapakse

of the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya.

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Setaria digitata: A Model Organism in the Quest for Novel Antifilarial Drugs

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In writing this essay to felicitate Vidya Jyothi Emeritus Professor Eric Karunananayake, I would like to begin by recalling some of his key contributions and his unique personality as a mentor for myself and for many others. Professor Karunananayake's contribution for Biochemistry dates back to his early work on absorption and metabolism of streptozotocin, (a DNA alkylating agent toxic to insulin producing pancreatic beta cells) at Imperial College, London (Karunananayake et al., 1974). After joining the University of Colombo, he initiated the first ever Molecular Biology experiments in the island. Since then, his courageous academic journey has been the key to the development of laboratory infrastructure and human resource pertaining to Molecular Biology in the island. His zeal also led to the planning from scratch and building up the Institute of Biochemistry, Molecular Biology and Biotechnology (IBMBB), University of Colombo in 2004 which has now become a hub of education and research in a broad spectra of related fields including Biochemistry, Molecular Biology, Immunology, Medicinal Chemistry and Bioinformatics. Professor Karunananayake is a believer and always shared his personal experience in hurdles and successes of his own academic carrier. At personal level, not only he mentored and encouraged the scientifically curious young generations but also made their perseverance in overcoming hurdles and pushing onto success. Professor Karunananayake is undoubtedly the eminent pioneer in the field of Molecular Biology and Gene Technology in Sri Lanka.

Foremost among Professor Karunananayake's many innovative research startups is the timely recognition and use of the cattle filarial worm as a model organism in finding diagnostic methods and cures for the diseases caused by filarial parasites including human lymphatic filariasis (LF) and cerebrospinal nematodiasis in sheep and goat husbandry. Preceding chapters in this volume comprehensively recount the successes in using molecular biological tools to study filariasis, in an era where filarial parasites had been a significant burden for both human wellbeing and nations livestock productions. Primary aim of this chapter is to describe Professor Karunananayake's most recent work of using cattle filarial parasite *Setaria digitata* in finding novel antifilarial drugs as well as specific drug targets that can be used for designing novel antifilarial drugs.

Current drugs used against human lymphatic filariasis only eliminate the larval stages of the causal agents *Wucheraria bancrofti* and *Brugia malayi*, posing a major obstacle in eliminating LF globally. Hence, in the fight against LF, there is an urgent need to identify novel antifilarial compounds with adulticidal activity. In this chapter, I provide information on my experimental findings during my tenure as an MSc student (2010-2012) where I was involved in the characterization of selected cDNA clones of *S. digitata* and as a doctoral student (2013- 2019) where I isolated antifilarial compounds from medicinal plants and elucidated the mechanisms of their action. Both my MSc and PhD research projects were supervised by Professor Karunananayake and carried out at the IBMBB.

cDNA library of *S. digitata*: A gold mine in search for antifilarial drug targets

Marking a milestone, the genome of *S. digitata* has now been sequenced (Senanayake et al., 2020). During the course of development to present levels, cDNA library preparation, sequencing and expression studies led to identification of many important full length proteins of *S. digitata* most of which has been characterized by different researchers under the guidance of Professor Karunananayake. Characterization of a parasitic nematode specific growth factor like protein has been described elsewhere in this Volume (Rodrigo et al., 2014). Other proteins that have been identified are Bax inhibitor like protein (Mendis et al., 2013) and putative cleavage stimulation factor-64 kDa subunit protein (Nagaratnam et al., 2014). Adding to this, I sequenced and characterized the filarial ortholog of Yip1p which is a potential drug target in curbing host parasite interaction (Senathilake et al., 2012).

Human filarial parasites survive in the deep lymphatic system. These parasites overcome the host immune and oxidative reactions by secreting different effectors. Many of these molecules mature and are carried through intracellular vesicular traffic before being secreted. Curbing this pathway would provide an attractive way of designing new antifilarial drugs. One such target is the filarial ortholog of yeast Yip1p. Yip superfamily proteins are a group of multi-span transmembrane proteins that play an essential role in ER to Golgi transport (Matern et al., 2000; Yang et al., 1998). The activation of membrane by Rab family of small regulatory GTPases is the key event in the initiation of downstream vesicle docking (Bonifacino and Glick, 2004). Yip1p interacts preferentially with the GDP-bound form of two Rab proteins (Ypt1p and Ypt3p) which act in ER to Golgi and intra-Golgi traffic, respectively (Grosshans et al., 2006) making it an essential component in the said event.

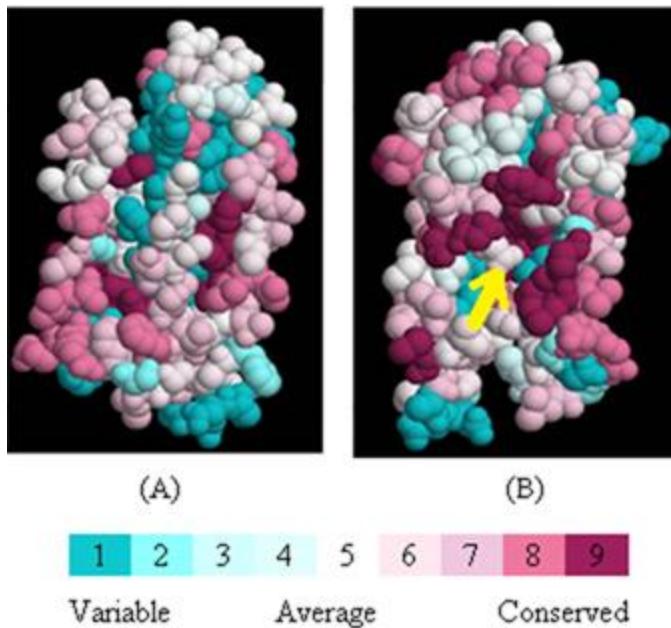


Figure 1. Sequence conservation mapped to 3D model of *S. digitata* Yip1p. A and B are views from either side of the same model. Arrow represents the conserved binding pocket within the membrane spanning region.

By aligning orthologs from Ortho MCL database, a highly conserved N terminal motif was identified in the less conserved highly disordered cytoplasmic domain. Short double helix domain specific to all the orthologs of eukaryotic YIPF5, YIPF4 and YIPF7 were identified in addition to yip1 super family specific signature motifs DLAGP and GY (Shakoori et al., 2003). Filarial sequence showed only 36.6 % sequence similarity to its human counterpart Yipf5 over its entire length of 251 amino acids suggesting the importance of exploring filarial Yip1p as a potential drug and vaccine target. The membrane protein was modeled with relatively good accuracy for binding pocket characterization. By mapping the sequence conservation to three dimensional models, a potential protein interaction interface located in the

membrane spanning region was proposed which can be used as a drug binding pocket (Figure 1).

Endemic medicinal plants in the search of novel antifilarial drugs

Less than 10 % of the world's biodiversity has been evaluated for potential biological activity, many more useful natural lead compounds are still uncovered (Nisha et al., 2002). Plants have a long association with human kind with profound biological and economic impact. From ancient times, plants and plant derived natural products (secondary metabolites) have been successful sources of potential drug leads (Kliks et al., 1985). Plants inspired the traditional healing methods practiced by many civilizations and cultures against LF. My doctoral studies were focused on the discovery of antifilarial properties of selected Sri Lankan endemic and medicinal plants. Initial screening of 12 endemic or medicinal plants provided strong evidence that the Rhizomes of *Curcuma zedoaria* and winged seeds of *Dipterocarpus zeylanicus* possess potent antifilarial properties.

Accordingly, *Curcuma zedoaria* and *Dipterocarpus zeylanicus* were selected for bioactivity guided fractionation and isolation of antifilarial compounds. My doctoral study gave the first ever scientific evidence of the antifilarial activity, molecular and biochemical mechanism of action of different extracts and isolated antifilarial principals of *C. zedoaria* and *D. zeylanicus*.

Curcuma zedoaria (*White turmeric*)

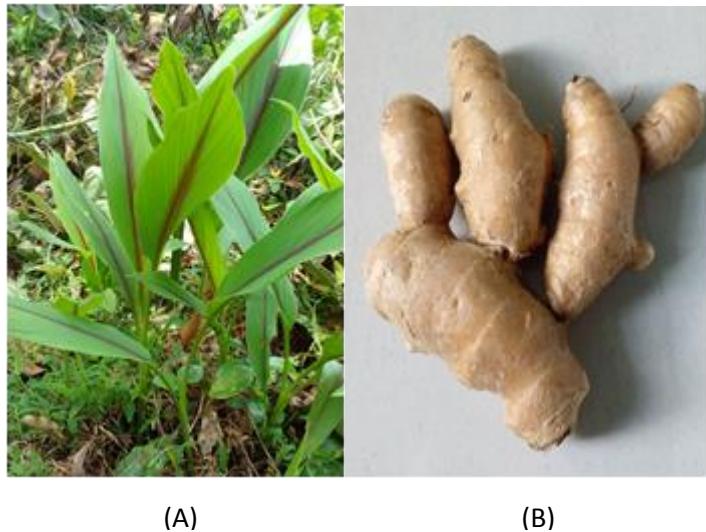


Figure 2. *Curcuma zedoaria* plant (A) and the rhizomes (B)

C. zedoaria Rosc (Zingiberaceae), commonly known as white turmeric (Sinhala: Haran Kaha, Tamil: Poolankilangu) is a perennial rhizomatous herb native to Sri Lanka, India and Bangladesh (Figure 2). Essential oil and nonpolar fractions of *C. zedoaria* are reported to be rich in sesquiterpenes and contain many other biologically active compounds (Kaushik et al., 2011a). Polar fractions mainly consist of flavonoids (Kaushik et al., 2011a). It is a widely cultivated herb and also used as a spice in other Asian countries. Young rhizome of *C. zedoaria* is used to prepare a vegetable soup in Asian countries and leaves are used in flavoring food (Kaushik et al., 2011a). Different parts of *C. zedoaria* are used in traditional medicine in the treatment of cancer, dyspepsia, stomachache and as a carminative. Moreover, it is known to possess antibacterial and anti-inflammatory properties (Banisalam et al., 2011; Kaushik et al., 2011b; Sirirugsa et al., 1998). Traditional use of the rhizome pastes (as a topical application) of *C. zedoaria* by some Sri Lankan traditional

practitioners in the treatment of lymphatic filariasis, encouraged us to investigate the efficacy of *C. zedoaria* rhizome extracts on filarial worms.

Antifilarial efficacy of Curcuma zedoaria

The hexane and chloroform extracts of *C. zedoaria* rhizomes exhibited antifilarial activity against adult parasites and microfilaria of *Setaria digitata* (Senathilake et al., 2016). Data obtained from worm motility assay in combination with viability assay demonstrated a clear dose dependent reduction of viability and motility of both macro and microfilariae upon exposure to the hexane and chloroform extracts. Both extracts showed less toxicity against human peripheral blood mononuclear cells (PBMC) with high selectivity index implying a potential therapeutic window against lymphatic filariasis.

Antifilarial compound isolated from *C. zedoaria* was identified as furanodienone (Figure 3) and exerted potent adulticidal activity ($IC_{50} = 5.21 \pm 0.96 \mu M$) and strong antifilarial activity on microfilaria ($IC_{50} = 3.47 \pm 0.68 \mu M$) (Senathilake, 2019). The structure of the isolated compound was elucidated using the 1H and ^{13}C NMR spectroscopic techniques. Isolation of compounds using NMR spectroscopy was carried out at the Hussain Ebrahim Jamal (HEJ) Research Institute of Chemistry, University of Karachi, Pakistan under the supervision of Dr. Achyut Adhikari (presently Associate Professor at the Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal).

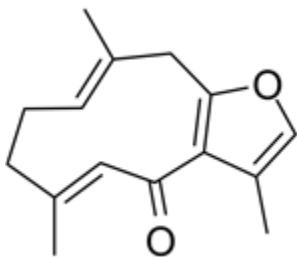


Figure 3:
Structure of furanodienone

Dipterocarpus zeylanicus

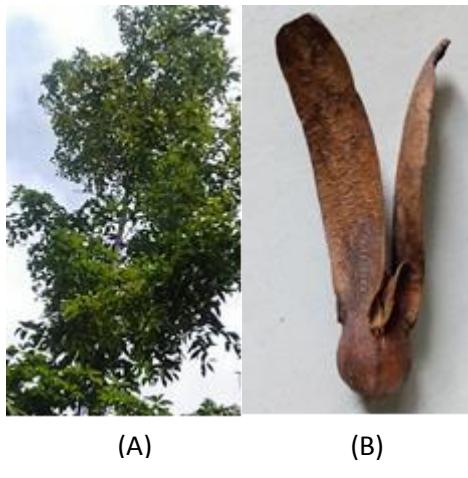


Figure 4. *Dipterocarpus zeylanicus* tree (A) and winged seeds (B)

Dipterocarpus zeylanicus (Sinhala: Hora, Tamil: Challani) is a giant evergreen, endemic and a dominant tree in lowland wet zone of Sri Lanka (Figure 4). Chemical composition of *D. zeylanicus* is not well-known, in fact only a few compounds have been characterized to date (Aslam et al., 2015; Bandaranayake et al., 1975). However, different species of the genus *Dipterocarpus* is reported to be rich in flavonoids, triterpenes and sesquiterpenes.

Although *D. zeylanicus* is primarily used as a timber tree, gum resin and hart wood parts are used in traditional medicine to treat chronic ulcers, fever, sinuses, and fistulae (Appanah et al., 1998; Aslam et al., 2015). *D. zeylanicus* is a poorly investigated medicinal plant for potential antifilarial properties. The bark of *Dipterocarpus* species are used by some veterinary medical practitioners to augment appetite in cattle (Phon, 2000). This observation led us to hypothesize a possible anthelmintic activity of the plant.

Antifilarial efficacy of D. zeylanicus

Methanol extract of winged seeds of *D. zeylanicus* was suspended in distilled water and partitioned sequentially to hexane, dichloromethane and ethyl acetate. Ethyl acetate fraction showed the highest antifilarial activity and the best selectivity index (Senathilake et al., 2017). Therefore, it was chosen for carrying out further studies and isolation of active compounds.

Two antifilarial saponins were isolated from the ethyl acetate fraction of *D. zeylanicus* and identified as oleanolic acid 3-O- β -D- glucopyranoside (Figure 5A) and oleanolic acid 3-O- α -L-arabinopyranoside (Figure 5B) using ^1H and ^{13}C NMR spectroscopy. Both compounds exerted strong antifilarial activity with IC₅₀ values of $20.54 \pm 1.05 \mu\text{M}$ and $29.02 \pm 1.04 \mu\text{M}$ against adult parasites, respectively. They exhibited potent antifilarial activity for microfilaria with IC₅₀ values of 19.71 ± 1.04 and $25.99 \pm 1.03 \mu\text{M}$ respectively. However, these compounds were toxic to PBMC. Acid hydrolysis of both compounds yielded oleanolic acid (Figure 5C) which was less toxic to normal human cells used in the study. Oleanolic acid exerted similar antifilarial activity against adult worms and microfilaria with IC₅₀ values of $38.40 \pm 1.05 \mu\text{M}$ and $35.63 \pm 1.02 \mu\text{M}$ respectively with less toxicity to PBMC (Senathilake et al., 2017). Two other compounds namely betulinic acid and 3β -hydroxyolean-12-en-29-oic

acid were also isolated from active fractions but these did not show antifilarial activity.

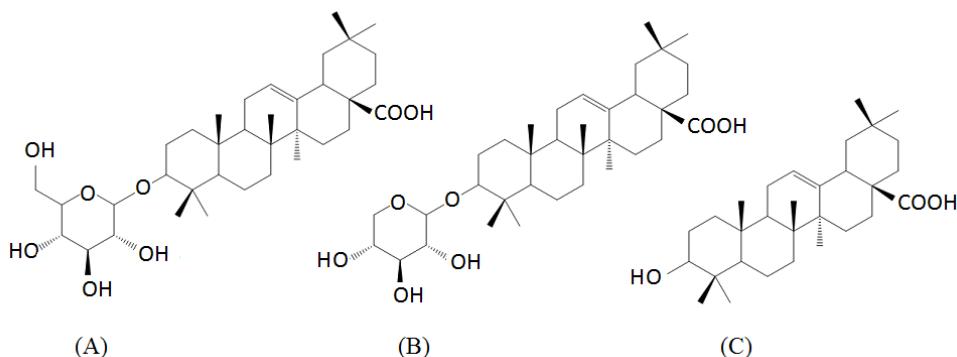


Figure 5. Structures of oleanolic acid 3-O- β -D- glucopyranoside (A) and oleanolic acid 3-O- α -L-arabinopyranoside (B) and oleanolic acid (C)

Apoptotic effects of plant extracts of C. zedoaria and D. zeylanicus

Characteristic features of oxidative stress and apoptosis were studied in parasites treated with each antifilarial plant extract using Hoechst 33342 staining and TUNEL assay. All antifilarial plant extracts exhibited condensation of nuclear DNA and apoptotic body formation in intrauterine developmental stages. Elevated levels of oxidative stress parameters, glutathione S transferase (GST), superoxide dismutase (SOD) and reactive oxygen species (ROS) and reduced glutathione (GSH) were observed in parasites treated with said plant extracts (Senathilake et al., 2016; 2017).

Apoptotic effects of antifilarial compounds furanodienone and oleanolic acid

The compound furanodienone exerted potent antifilarial effects possibly through paralysis followed by oxidative stress mediated apoptosis (Senathilake, 2019). Oleanolic acid induced apoptosis in filarial parasites

through induction of oxidative stress (Senathilake et al., 2017). Tissue damage in adult parasites induced by these antifilarial compounds was clearly visualized by hematoxylin and eosin staining. Oxidative stress parameters (GST, GSH, SOD and ROS) in parasites were modulated by aforementioned antifilarial compounds.

Conclusions and future directions

We have reported the antifilarial activity of oleanolic acid and furanodienone for the first time. This also becomes the first study that shows evidence for oxidative stress and apoptotic effects of oleanolic acid and furanodienone on filarial worms. In addition, it reveals the presence of compounds oleanolic acid and oleanolic acid 3-O- α -L-Arabinopyranoside in *D. zeylanicus*. Interestingly, these findings provide scientific proof of the traditional awareness in using *C. zedoaria* in the treatment of human lymphatic filariasis and identification of *D. zeylanicus* as a good source of antifilarial compounds.

Professor Karunananayake's guidance was paramount in successfully completing my doctoral studies. I also acknowledge funding from Ministry of Higher Education; co-supervisors of the PhD, Professor Kamani Tennekoon and Professor ED De Silva (then at the Department of Chemistry, University of Colombo) and guidance and training by Dr. Sameera Samarakoon which facilitated my success.

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My First Encounter with Science in Sri Lanka and the Beginning of a Rewarding Friendship

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My first encounter with Sri Lanka and Professor Eric Karunananayake started with a request I got from the International Science Programme (ISP) to visit Colombo University to discuss the details on how to implement a bioinformatics platform in connection with a new computer cluster that Sweden had donated to the University of Colombo.

At that time, I was representing the Bioinformatics field at the UPPMAX (Uppsala Multidisciplinary Center for Advanced Computational Science), Uppsala, Sweden. Bioinformatics was at that time not considered one of the big players in the High CPU (Central Processing Unit) Computing club. In 2006 Next Generation Sequencing (NGS) has not appeared yet and the needs from the Life Science area were very modest compared to the calculations made by projects in Physics, Astronomy, Weather forecast, aerodynamics of airplanes, and Chemistry to mention a few.

The landscape changed a few years later when NGS started to be used by many researchers to solve important questions in Life Sciences that were not possible to be investigated before. Today researchers from other fields of sciences are asking to restrict the usage of common advanced computer platforms for Bioinformatics as bioinformaticians use much CPU power and storage. It is also worth mentioning that the first NGS technologies were created in Uppsala by a company called PyroSequencing based on research made at KTH in Stockholm and Uppsala University in Uppsala.

When I arrived in Sri Lanka, I was received by Professors Eric Karunananayake and Kamani Tennekoon. I was later introduced to the staff of Institute of Biochemistry, Molecular Biology and Biotechnology (IBMBB) and to the staff of the School of Computing that had their offices close to the IBMBB. The meeting was very fruitful, and we agreed that Computer Sciences and Life science researchers should start working together on the Bioinformatics arena as bioinformatics is a multidisciplinary field that contain elements of mathematics, computer sciences and biology/medicine.

During the second day of my visit Prof Karunananayake introduced me to his research on Filariasis and to the model organism that he was working on, the nematode *Setaria digitata*. Eric was very detailed when explaining the development stages of the disease and I was shown pictures of patients suffering this terrible disease. I also got the information that the parasite is transmitted by mosquito bites.

After this second very intensive and educational day I went back the fantastic hotel that was booked for me, Hotel Galle Face. The hotel was a colonial style hotel with beautiful rooms and incredibly beautiful surroundings facing to the Indian Ocean. Well in my bed I started to slumber down when suddenly I felt a mosquito bite in my neck. I jumped up and put the lights on to see where the mosquito or the mosquitos were hiding. In my mind I could see all the images from filariasis patients, and I was convinced that I was now infected. Prof Karunananayake also had informed me that the treatment for filariasis was very painful. I could not sleep for the whole night, and I was incredibly tired the morning after when we started our next conversations on how to continue a Swedish Sri Lanka collaboration. I must tell now that I was not infected and have never been after several visits to the wonderful Sri Lanka. But I will never forget that first night at Hotel Galle Face.

The discussions continued and we agreed that IBMBB and Sri Lanka should join the European Molecular Biology network (EMBnet). EMBnet is an international bioinformatics network that does not have the term bioinformatics in its name because it was founded in 1988 at a time when the term Bioinformatics was not widely used. Professor Karunananayake understood very early that bioinformatics was a research field that was needed to accelerate research in the Life Sciences field and he was very supportive of this initiative to join EMBnet.

Sri Lanka joined EMBnet in 2007 and had participated since then on many activities of the network, including preparation of Education materials, participating in international conferences, organizing training courses in Sri Lanka, and publishing on the network's Journal. It is also worth mentioning that the front page of the EMBnet journal have had a few pictures taken during my visits to Sri Lanka.

Eric's vision to use the nematode *Setaria Digitata* as a model to develop new drugs against nematode infections continued with an application made by SLU and IBMBB to the Swedish Research Councils call "Swedish Links". The proposal was granted and allowed us to strengthen the collaboration and the common activities. As a PhD student to work with this project we got Mr. Kanchana Senanayake and he had to work on all aspects of the project together with colleagues in Sweden and Sri Lanka.

When the Swedish links project started between IBMBB and SLU, Kanchana attended SLUs advanced courses in Bioinformatics and started the sequencing of the *Setaria* genome. The work went well, the *Setaria* Genome was published in 2020 and the genomic sequence data was deposited at the European Nucleotide Archive (ENA).

The resulting genome assembly and annotation was considered by the “Worm base” database to be of good quality and want to incorporate the Genome to their nematode database and in that way make it available to parasitology researchers all over the world.

The interesting job only starts with the sequencing and assembly of a genome are made and this is when the hardest and most tedious part starts, the annotation of the genome. This is usually done with automatic workflows but to be of good quality the data needs to be manually curated. For that purpose, Eric helped us together with Kamani, Kanchana and other staff members to organize a series of bioinformatics training at IBMBB. Several students and researchers from both Uppsala University and SLU participated as teachers and they are all incredibly happy to have had the opportunity not only to visit Sri Lanka but also to be able to learn more about Sri Lankan people and to meet the researchers at Colombo University.

The training reached a higher level with the start of the Master’s programme in Bioinformatics at Colombo University in 2012. Bioinformatics is today what Molecular Biology was in the 80-ties 90-ties, and a scientific discipline that needs to be part of the analytic arsenal of all researchers in the Life Science community. We at SLU are happy in teaching on that programme when required. EMBnet members from other countries also participate as teachers and examiners in the IBMBB’s Master’s programme.

The research work is still ongoing, and we are together identifying new drug target candidates against parasitic nematodes. There is still much to be done and I am very hopeful that the collaboration will continue allowing me and members of my group and the EMBnet community to visit the wonderful Sri Lanka and embark in new exciting research adventures.

I want with this short text salute and thank Professor Eric Karunananayake for the opportunities he gave me and colleagues from my university to work together on such exciting field and in line with Eric's research visions.

My warmest Congratulations, Eric!

Inspired by a Visionary: Discovering My Niche

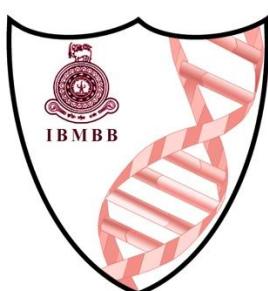
Kanchana S Senanayake

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It is indeed a great honor to write an article for this Felicitation Volume for Professor Eric Karunananayake, truly a trailblazer in the field of Molecular Life Sciences in Sri Lanka. A great mentor as well as a role model for any young and aspiring scientist in any field, not just in Molecular Biology. All throughout this volume there are scientific reports and personal counts from various individuals who were students, collaborators or colleagues in academia that Professor Karunananayake has associated with, showcasing his contribution to science and human resource development in Molecular Biology.

Being a part of the administration of the IBMBB since its inception in 2004, I can shed the light in a different angle in the sense that Professor Karunananayake was not just an excellent scientist but also an exemplary leader and an administrator. I met Professor Karunananayake in the latter part of 2003 when I was working at the University of Colombo, School of Computing (UCSC) as a Project Assistant, when he came to get assistance in designing the logo for the IBMBB. Mr. Harsha Wijayawardane who was the consultant in charge of the Software Development Lab at the UCSC, introduced me to Professor Karunananayake and instructed me to design the Logo and develop the Website of the IBMBB. From that day onwards till Professor Karunananayake retired as the Founder Director of the IBMBB in 2006, I saw an epitomizing leader with a black or white attitude in decision making that made him a great administrator and a perfect role model for me personally at the early stages of my professional career. From the beginning with regards to setting up the IT

infrastructure at IBMBB, Professor Karunananayake was crystal clear in what he wanted to be materialized inside the IBMBB and was very supportive in every step of the way. For a Biochemist/Molecular Biologist he was surprisingly enthusiastic about Computer Science and Information Technology related developments. It was fascinating to hear him voicing his opinion strongly about Bioinformatics and to witness his determination to start research and teaching at IBMBB. Reflecting upon Professor Karunananayake's remarkable journey, starting as a Biochemist and subsequently learning Molecular Biology and then later on setting such high standards in research made me realize that anything is possible if one is determined. The knowledge gained in Bioscience from my graduate studies at University of Colombo and skills developed in Information Technology while working at UCSC enabled me to see what Professor Karunananayake had envisioned with regards to the role of Bioinformatics in future Life Science research. This led me to start my PhD studies in Bioinformatics of which I had no intentions at the beginning of my career at UCSC as a Software Developer/Graphic Designer.



The logo of IBMBB

The field of Bioinformatics generally started as a bunch of isolated databases in laboratories, which was filled with DNA data from individual genomic projects accessible only for scientists that were involved on those respective

projects. Throughout the years these databases were opened to the public via internet and expanded to include RNA data, proteomic data, protein expression and pathway data. Since early 70's where it is considered as the era of the birth of Bioinformatics (Ouzounis and Valencia, 2003) the scientific community has been engaged in sequencing genomes and developing various tools and algorithms to analyze and interpret the vast amount of data generated from these sequencing projects to gain insights to different biological processes within organisms and their evolutionary linkages to other organisms.

With the emergence of Next Generation Sequencing (NGS) technologies, the sequence databases are overwhelmed with genomic and proteomic data from diverse organisms generated from large number of genome projects. The massively parallel nature of NGS techniques that churns out thousands of reads that needs to be put together to form the full genome of an organism to study its biology, presents a big challenge or almost an impossible task for a Molecular Biologist in the context of manual assembly. This is where Bioinformatics possesses the solution in automated assembly and downstream analysis of assembled genomes to assist the Molecular Biologist in further studying the Biology of the organism in question. Professor Karunananayake was the cornerstone in initiating teaching and research in Bioinformatics at IBMBB, realizing its potential and identifying the need for human resources as well as the infrastructure in future research at the IBMBB. At the onset of the journey from 2004 to 2006, IBMBB had few Bioinformaticians visiting from various parts of the world and conducting short workshops at the introductory level in Bioinformatics. But there were no signs of long-term collaborative intent from these visitors until Professor Erik Bongcam-Rudloff walked in to IBMBB in 2006, whom we immediately recognized as a potential long-term collaborator and was an assured Bioinformatician. He was working

at the Linnaeus Center for Bioinformatics, University of Uppsala, Sweden as an Associate Professor and was also the Chairman of European Molecular Biology Network (EMBNet). He convinced us to join EMBNet, which is a world-wide organization comprised with Bioinformatics educators, researchers and professionals mainly from European and South American regions. In 2007 upon the successful proposal made to the EMBNet by Professor Kamani Tennekoon at the AGM held in Malaga, Spain, IBMBB was unanimously accepted to EMBNet as the National node of Sri Lanka. To date this has been a major step in initiating and formulating research and teaching at the IBMBB in Bioinformatics reaping the benefits of collaborative research and teaching offered by EMBNet. Professor Erik Bongcam-Rudloff's arrival is partly owing to the long-standing relationship with University of Uppsala, Sweden established by Professor Eric Karunananayake.

My first glimpse of the level of Bioinformatics research and education in the context of the world was at the EMBNet AGM 2008 in Martina Franca, Italy. It was an intense experience for me personally, to meet researchers and teachers from other countries who were engaged in Bioinformatics research for more than 20 years and me coming from a country that is trying to take its first steps. After that eye-opening experience, I was given the opportunity to spend two months in Professor Erik Bongcam-Rudloff's lab at the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden where I got trained in Grid Applications and Tools in Bioinformatics (this was before my PhD training there). During this period, I also participated in an R and Bioconductor workshop organized by the CSC, Finland which is the Finnish National Node of EMBNet. Both of these trainings were entirely funded by the SAREC Grant awarded to Professor Karunananayake. I participated in numerous workshops and short term training courses organized annually by EMBNet, which allowed me to further my knowledge and keep with new

trends in the field. This has now culminated in the sequencing of the cattle filarial nematode, *Setaria digitata* and I had the opportunity to begin my doctoral studies in Bioinformatics under the guidance of Professor Erik Bongcam-Rudloff and Professor Eric Karunananayake as the first doctoral student in Bioinformatics at the IBMBB.

IBMBB, the brain child of Professor Karunananayake will surely be a beacon of light attracting young scientists in Sri Lanka interested in high quality Life Science research and education including Bioinformatics in years to come. This volume stands as proof of attained success by Professor Eric Karunananayake in the field of Molecular Life Sciences in Sri Lanka. Thank you Sir...

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Deciphering the Genome of *Setaria digitata* in Search of Potential Drug Targets

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Professor Eric Karunananayake led research on *Setaria digitata* for few decades and some studies are mentioned in this Volume by several authors. We had the opportunity to sequence the genome of *S. digitata* that resulted in the assembly and annotation of its complete genome as well as searching for potential drugs for *Wuchereria bancrofti*, the nematode that causes filariasis in humans, by studying the sequenced genome of *S. digitata*.

S. digitata is a parasitic nematode found living inside the peritoneal cavity of cattle. Although nonpathogenic under normal circumstances, there have been instances reported where larval stages have been found in the cerebrospinal cavity of cattle causing fatal paralysis (Tung et al., 2003). Transmission of infective larvae to abnormal hosts such as horses, sheep and goats can cause a serious and often fatal neuropathological disorder, cerebrospinal setariosis, consequent to erratic migration of infective larvae to the brain and spinal cord of the host (Bazargani et al., 2008; Kaur et al., 2015; Mohanty et al., 2000; Shin et al., 2017; Tung et al., 2003). Several genes of *S. digitata* have been characterized with the aim of identifying possible drug targets in *W. bancrofti* under Professor Karunananayake's leadership and some have been described elsewhere in this Volume by Professor Sharmila Jayasena and Dr. Kanishka Senathilake.

Setaria digitata as a model organism to study W. bancrofti infections

Although *W. bancrofti* is the major causative organism for lymphatic filariasis, very little is known about the molecular biology, biochemistry and immune mechanisms of this parasite. This is mainly because of the difficulty in procuring adult worms since they live in human lymph vessels and the impossibility of culturing adult worms in laboratory conditions for research *in vitro*. Therefore, the necessity of a model organism to study the biology of *W. bancrofti* is arisen. *S. digitata* closely resembles *W. bancrofti* with respect to morphology and histology (Decruze and Raj, 1990) as well as antigenic properties (Dissanayake and Ismail, 1980). For this reason, *S. digitata* has been used as a model organism for the study of *W. bancrofti* in view of developing a vaccine against lymphatic filariasis (Madathiparambil et al., 2011; Perumal et al., 2016).

Sequencing, Assembly and Annotation of the Setaria digitata Genome

Genomic DNA was extracted from adult worms of *S. digitata* and sequenced using the Illumina MiSeq platform at the National Veterinary Institute in Uppsala, Sweden. The adult worms were washed thoroughly to remove any contaminants from cattle tissue and microbial residues before the Genome Sequencing was carried out.

Four Gb of DNA sequence data was generated from Illumina sequencing, made up of 14,370,809 paired-end reads with a maximum read length of 301 bp and a minimum read length of 35 bp and a high raw sequence coverage of 44x. The raw reads were checked for adapter sequences and then trimmed at Q30 as part of the quality control process. These sequence reads were then checked for bacterial contamination using RAMBO-K v1.21 (Tausch et al., 2015) against the Refseq bacterial genomes to filter out any contamination.

The decontaminated sequence reads were assembled using SPAdes v3.6 (Bankevich et al., 2012) de-novo assembly tool. The resulting draft assembly was then corrected for any local misassemblies and small INDELs. The completed genome assembly consisted of 8,974 scaffolds which is 89.88Mb in length, with L50 of 882 bp and an overall GC content of 31.7%. Using the k-mer counts generated from the sequencing data, it was estimated that the draft assembly covered over 87% of the total genome size. The size as well as the GC content of the *S. digitata* genome were similar to *Loa loa* and *Brugia malayi* which cause filariasis in humans. Moreover, the number of predicted genes estimated among those filarial nematode genomes was comparable. Additional genomic characteristics and assembly comparisons with other published nematode genomes can be found in Senanayake et al., 2020.

The genome annotation using MAKER2 automated gene annotation tool (Holt and Yandell, 2011) identified 20,568 protein coding genes in the assembly. Annotation completeness was then assessed using CEGMA v2.5 (Parra, Bradnam and Korf, 2007) and BUSCO v2.0 (Simão et al., 2015) gene sets. BUSCO uses 982 conserved genes as opposed to CEGMA that uses 248 genes for the analysis pipeline to assess the completeness of the annotation. This analysis showed a high level of completeness, where CEGMA showed 91.5% and BUSCO 85.5% completeness (Senanayake et al., 2020).

Comparative Genomics and Quality Assessment

In Computer Science the phrase “Garbage in, Garbage Out” (GIGO) is stressed in the sense that, even though you have adhered to all the standards and norms with respect to the algorithms and logic, if the input is erroneous or biased the output will not be valid. Hence validation of the techniques as well as the tools used in this pipeline were evaluated by way of Comparative

Genomics using well defined and established databases and genome sequences as references for quality assessment of the assembled genome as well as the annotations.

Protein sequences of the predicted genes were searched against the Swiss-Prot and TrEMBL databases (release 2019_07) using BlastP program (e-value_10_5) to assess the quality of the gene model. Based on Swiss-Prot which is a database of curated annotations, putative function was assigned to 6,009 protein-coding genes, whereas 11,541 of the total genes were found in the TrEMBL database. Furthermore, these predicted genes were searched against KEGG and Pfam databases. Out of the 20,568 predicted genes, 8,560 (41.6%) genes were found to have well defined protein domains in PFAM database; KEGG terms were assigned to 4,315 genes (20%) of which 375 had orthologs linked to KEGG biological pathways.

Comparative genomics was used to further assess the quality of the assembled genome. Functional annotation and classification, was done as well as orthologous genes were also probed in the genome. These results were then compared with *L. loa*, *B. malayi*, and *W. bancrofti* genomes, which are published genomes of nematodes causing filariasis in humans. It was revealed that 8,369 (40%) of *S. digitata* protein-coding genes were orthologous among the genomes, of which 4,643 genes were single-copy orthologs. *S. digitata* shared 7,493 genes with *L. loa*, 7,070 with *W. bancrofti*, and 6,612 with *B. malayi*. In total, 5,087 genes were shared among the four nematodes. In addition, the functional classification of protein-coding genes classified 9,768 genes into different COGs (Cluster of Orthologs Groups) (Figure 1 A). Protein length correlation analysis of single-copy orthologs showed a high level of correlation between the species (Figure 1 B-D). *S. digitata* showed high level of correlation ($R=0.9$) with *L. loa*, *W. bancrofti*, and *B. malayi*. Strong

correlations with the protein-coding genes of *W. bancrofti* and *L. loa* indicate coverage of protein-coding sequences identified in *S. digitata* is of considerably higher quality. Thus, genomic comparisons will greatly facilitate the identification of genes involved in development and potential drug targets.

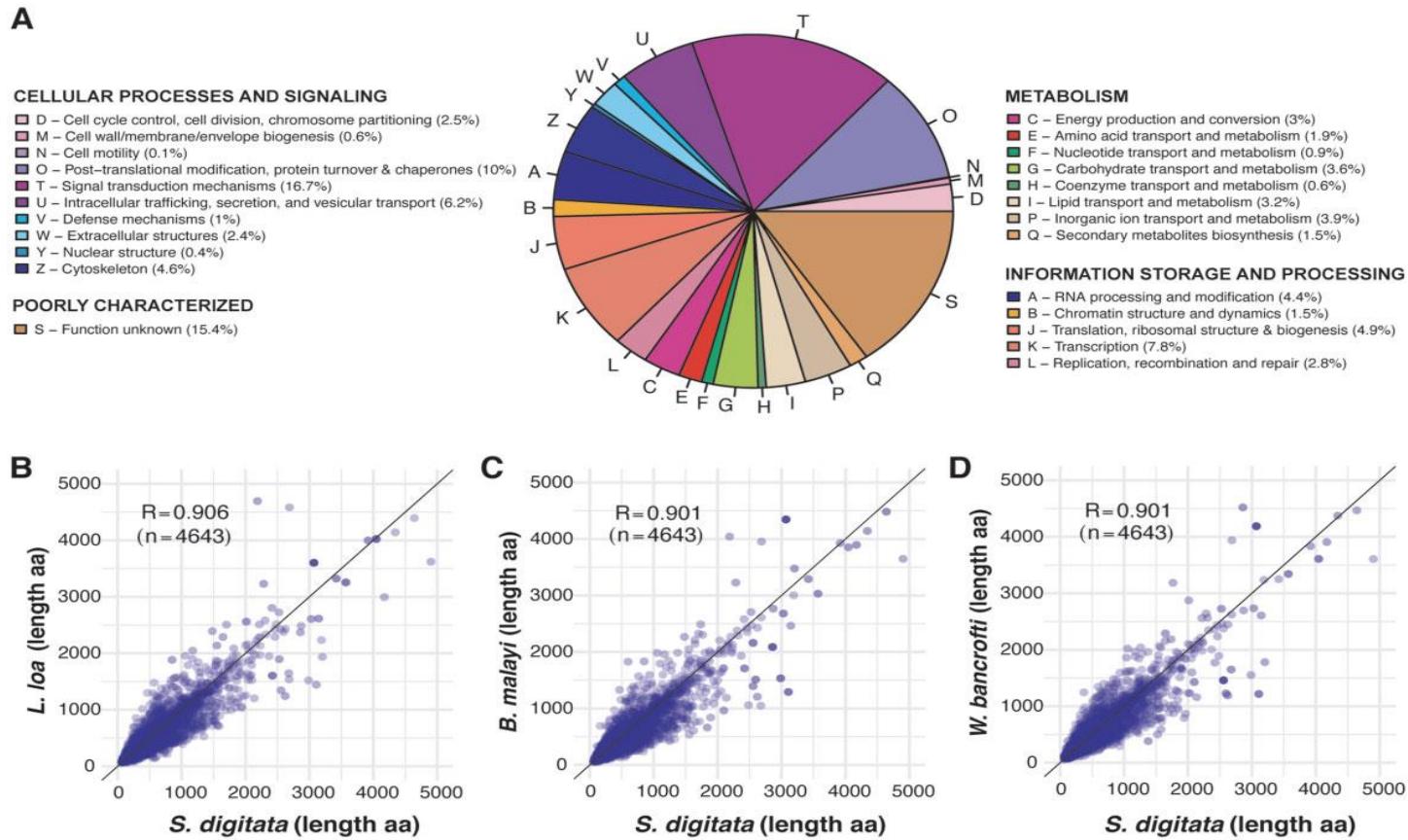


Figure 1: Classification of protein-coding genes in COG functional groups (A); Correlation of *S. digitata* proteins length with *L. loa* (B), *B. malayi* (C), *W. bancrofti* (D), Pearson's correlation coefficients are calculated and shown on each plot.

Phylogenetic Analysis

Phylogenetic tree was constructed with 40 genomes downloaded from NCBI and WormBase version WS250 (Harris et al., 2010) and the *S. digitata* genome assembly created in this study to examine the evolution of filarial parasite in the context of other nematodes (Figure 2). The tree was based on “complete” BUSCO genes across the genomes of all 41 nematodes using the Arthropoda database provided by BUSCO. The tree was similar to 12S rDNA-based tree published earlier (Yatawara et al., 2007).

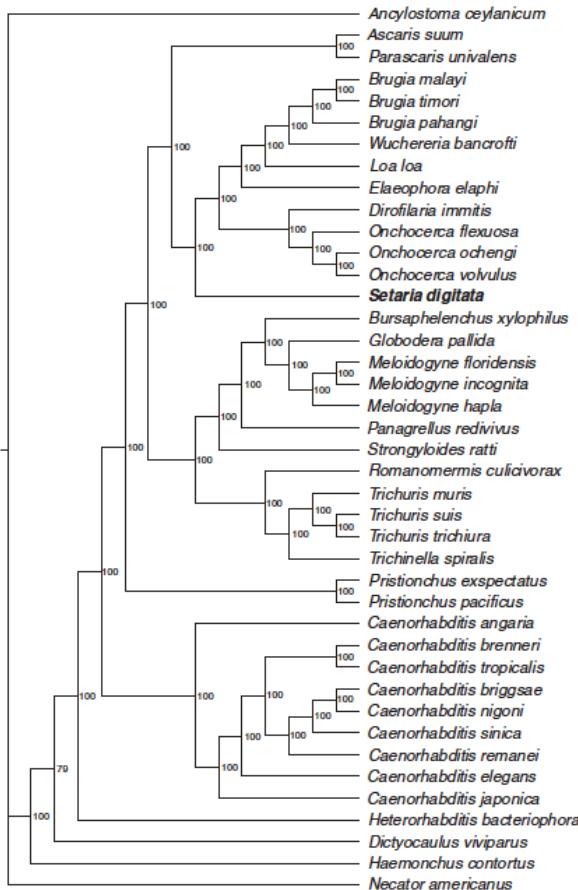


Figure 2: Phylogenetic analysis of the nematode genomes. Consensus tree between 41 nematode genomes, which includes the current *S. digitata* assembly.

Conclusions and Future Directions

Since the draft genome of the *W. bancrofti* is already publicly available (Small et al., 2019), the *S. digitata* genome will greatly facilitate comparative studies at the genetic level between the two nematodes and allow identification of genes that can be used as drug targets, which can then be tested in the laboratory due to their similarities in morphology. Although filariasis has been eliminated in Sri Lanka, the genome studies of *S. digitata* as a model organism will reveal insights into other nematode genomes and could lead to drugs for other diseases caused by nematodes both in humans and animals. Furthermore, availability of the *S. digitata* genome will enable drug development and vaccine production to eliminate or control *S. digitata* infections in abnormal hosts which cause serious economic loss in places where sheep and goat farming is a common livelihood (Bazargani et al., 2008; Nakano et al., 2007; Tung et al., 2003)

Currently the transcriptome of *S. digitata* has been assembled and integrated into a local installation of Webapollo which also includes the automated annotation results generated in this study. Manual curation of the predicted genes is currently under way to produce the complete genome of the *S. digitata* which will lead to identification of genes that can be used as drug targets for *W. bancrofti* and ultimately be tested in the laboratory using *S. digitata* worms.

To our knowledge, this is the first comprehensive genome sequencing project initiated for *S. digitata* or for any other parasitic organism in Sri Lanka, where it traverses the complete pipeline from DNA and RNA sequencing to ultimately finding drug targets using Bioinformatics methods within a single genome project. This would not have been possible if not for the hard work and dedication of Professor Karunananayake in setting up the infrastructure for research, the IBMBB. Carrying on with the paradigms set by Professor

Karunanayake in forming research collaborations with Swedish research institutions and funding agencies as well as high quality of research, this project managed to secure funds from Swedish Research Links Program, awarded by the Swedish Research Council and ably supported by the SLU Bioinformatics Infrastructure (SLUBI), Uppsala, Sweden in providing High Performance Computing facilities for the analysis. The gallantry work described in this article was a collaborative effort of enthusiastic young researchers from EB-R's team at SLUBI namely Dr. Adnan Niazi, Dr. Jonas Söderberg, Aleksei Pölajev, Dr. Maja Malmberg and scientist's from IBMBB, namely Professor Eric Karunanayake, Professor Kamani Tennekoon and Dr. Sameera R Samarakoon.

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The Emerging Sun on the Horizon: Birth of Molecular Entomology in Sri Lanka

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The early 90s mark a memorable era for molecular entomologists in India and Sri Lanka. Malaria, which had been haunting the world for decades, was rising again in epidemic proportion in Sri Lanka and most of the academics and researchers here started many studies related to malaria vectors and parasites. During that time, it was estimated that the global incidence of malaria was app. 300-500 million clinical cases with 1.5 to 3 million malaria deaths per year. Following the same trend, the recorded incidence in Sri Lanka too rose sharply from 38,500 cases in 1982 (of which 1500 were *P. falciparum* infections) to 676,000 (183,000 were *P. falciparum* infections) in 1987. By 1989, the case load declined to 259,000. In 1990, there was an increase in the incidence again to 287,000 followed by a sharp rise in 1991 with 400,000 recorded cases. However, the percentage of *P. falciparum* infections decreased from 25% in 1989 to 19% in 1991. The most alarming feature however was the appearance and spread of chloroquine resistant strains of *P. falciparum*.

Malaria still remains one of the world's most serious public health problems. In 2019, there was an estimated 229 million cases of malaria worldwide and the estimated number of malaria deaths was 409,000. Sri Lanka achieved malaria pre-elimination and elimination status in 2008 and 2012, respectively, and was certified "malaria-free" by the World Health Organization in 2016. I strongly believe that this feat is due to the research carried out by scientists in the country on the malaria parasite and the vector, drugs introduced and administered at the correct time, and clinical management of the disease by

the doctors and health authorities. An indigenous parasite population is no more now.

It is a known fact that *Anopheles culicifacies* is the major malaria vector in Sri Lanka and in the Indian subcontinent. However, the major malaria vectors such as *Anopheles gambiae* in Africa, *An. culicifacies* in South-Asia and *Anopheles maculipennis* in Europe and *Anopheles dirus* in South East Asia are taxonomically categorized as species complexes with many sibling species. Sibling species are morphologically more or less similar and most importantly they are reproductively isolated. In a species complex, sibling species show varying degrees of transmission potential of malaria. Thus, they are named as major vector, minor vector (potential), poor vector and non-vector. They cannot be identified using conventional taxonomic keys. Therefore, sibling species in a taxon need to be identified using either cytogenetic, biochemical or DNA based methods or a combination of these.

The pioneer work based on cytogenetics for the identification of sibling species of *An. culicifacies* conducted by Dr. Salara Subbarao and co-workers at the malaria research center in India revealed four sibling species which they designated provisionally as A, B, C and D. *An. culicifacies* sp. A was the major vector of malaria in the Northern, Central and Southern parts of India, and species C in the Eastern and Western parts, while species D was restricted to central parts of India. Species B on the other hand, was widespread across the Indian subcontinent, but was considered a poor vector or non-vector of malaria in India. Identifying and studying sibling species of this malaria vector was of utmost importance due to their differential distribution, behavior including resting and blood feeding, resistance to insecticides and most importantly transmission potential. Even though, Green and Miles, 1980 found sibling

species B in Sri Lanka using cytogenetic methods, sibling species status of *An. culicifacies* in Sri Lanka was not clear at the time.

Three eminent scientists Professor Eric Karunananayake, late Dr. Maya B. Gunasekera and Mr. W. Abeyewickrema (now retired Professor from the University of Kelaniya) decided to take the bull by its horns. One evening in the office of Professor Eric Karunananayake, these three scientists were in a deep discussion on identifying the major malaria vector in Sri Lanka using DNA based methods, a concept new to our country at the time. They had a very clear idea about the research in question and the methods to follow. But the big question was “who is going to do this?” Mr. Abeyewickrema instantaneously said “Nissanka will do it”. I was in the other room waiting for them to call me in. Professor Karunananayake asked a few things regarding my academic background and asked me one important question “Are you planning to go abroad for your post graduate studies?” My firm reply was “No”, to which he then replied “I need only your commitment for this study and I will provide you everything”. It felt like I had won a lottery!

The following day, 24th April of 1991, I started my research work at the Molecular Biology Unit laboratory, at the Faculty of Medicine, University of Colombo. I was assigned the first work bench of that lab which was close to the front door and next to Ms. K. Siridewa (now Professor Siridewa and Head of the Department of Biochemistry and Molecular Biology) who was one of the two PhD students in the lab when I joined; the other was Mr. N.V. Chandrasekharan (now a Professor in the Department of Chemistry). Next, Ms. Sulochana Wijesundara (now a retired Professor) and Ms. Sharmila de Silva (now Professor S. Jayasena) joined the lab, followed by others. The lab was fully equipped with all the necessary equipment for Molecular Biology work. Although there were very good labs in UK, this lab was par excellent

than those I worked at the Liverpool School of Tropical Medicine and Manchester University, UK. More importantly, the consumables were readily available, a feat we struggle to accomplish even in the most developed labs to date; I still can't imagine how Professor Karunananayake managed to provide all the necessary consumables on time. That is why I was able to finish my PhD in 3 years (from 1992 to 1995: registered for the PhD in 1992), while it was a nightmare for the majority who were doing PhDs in this country at the time.

My research was basically developing DNA probes for the identification of sibling species of *An. culicifacies* in Sri Lanka. I was fortunate to get cytogenetically identified sibling species A, B and C mosquitoes (5 each) as control samples provided generously by Dr. Subbarao. At first, I developed three DNA libraries of A, B and C and screened the libraries with total *culicifacies* DNA to isolate repetitive DNA fragments. Specificity and sensitivity studies were carried out and I was able to develop three DNA probes designated as Rp36, Rp217 and Rp234 to distinguish sibling species A from B and C. Characterization of these three repetitive fragments were done by sequencing. Rp36 and Rp217 repetitive fragments were completely sequenced while, Rp 234 was only partially sequenced. The sequence of Rp36 is devoid of any internal repeats. However, analysis of Rp217 revealed the presence of two related tandemly repeating core sequences of 13 and 16bp. This was the first ever DNA based study for *An. culicifacies* and we were able to publish our findings in the Bulletin of Entomological Research in 1994 (Gunasekara et al., 1994), a highly reputed Journal in the field of Entomology.

Furthermore, I screened wild caught *An. culicifacies* mosquitoes (n=1100) using these probes to see the sibling species status in the country and found that *An. culicifacies* A, the major vector in India, is absent in Sri Lanka, a

finding validated by successive studies (De Silva et al., 1996). In my study, I firmly indicated that sibling species B in Sri Lanka is different from that in India, and that species B is the major malaria vector in Sri Lanka even though it is recorded as a poor vector in India. Initially, P³² labelled DNA probes (Rp36, Rp217 and Rp234) were used to screen wild caught mosquitoes. However, this was impractical and unreliable when screening large numbers of wild caught mosquitoes and the need for a better field kit arose. In response to this, I developed non-radioactively labelled synthetic oligonucleotide probes and a squash blot method for field use. Biotin labelled Rp36, Rp217 and Rp234 probes were prepared instead of radio-labelled probes, squash blot technique was used (for the first time in the lab) for this assay instead of the lengthy procedure comprising DNA extraction and dot blot technique carried out earlier. In addition to DNA based work, studies on vector bionomics were also carried out in Gomadiyagala, a village in the North-Western Province for a period of 3 years. The distribution of *An. culicifacies* B was unimodal, peaking around the North-East monsoon / post monsoon periods during November to April. The density of this sibling species increased during the cool, rainy seasons (De Silva et al., 1998a, b). I was able to establish a forced mating colony at the University of Sri Jayewardenepura from the mosquitoes collected from Gomadiyagala and they were identified as sibling species B. This was the only laboratory colony for this important vector and was used for vector incrimination and parasite isolation studies at that time (De Silva et al., 1993). The training and experience gained from the field work helped me to guide and supervise my students who did undergraduate and post graduate research projects on malaria vector related studies in years to come. In 1995, I submitted my PhD titled “Studies on identification of *An. culicifacies*, Giles, sibling species complex using DNA based techniques, and associated field investigations”.

Professor Karunananayake is very well recognized and distinguished as an academic and scientist in Mother Sri Lanka, as witnessed by the many awards and accolades received by him including the National Award “Vidya Jyothi” and DSc *Honoris Causa* from University of Sri Jayewardenepura and University of Colombo to name a few. Professor Karunananayake was a far sighted academic and a good administrator who took decisions at the right time without fear. Although his research interest was in Biochemistry and Molecular Biology, he initiated many research fields in this country including molecular entomology. A commendable quality of his was that he gave us the freedom to share our knowledge with peers and, also learn other techniques from colleagues. This provided the opportunity for me to get hands on experience in techniques like northern blotting and western blotting, to which I would not have been exposed to otherwise. I was quite amused and also scared, during lab meetings when our research progress was evaluated. Professor Karunananayake was very tough and always focused on the research progress. He solved many research problems we faced and gave quick solutions for logistical issues as well. Lab meetings were open to everybody and we were able to share our thoughts and new research plans were discussed. That is how PCR based work was incorporated into my research and I was fortunate enough to do PCR amplification way back in 1994. The four-year period at the Molecular Biology Unit was a fascinating and memorable time in my life because of the friendly working atmosphere. We did not feel exhausted regardless of the long hours at the lab. Everybody helped each other and I resolutely appreciated the advice, support, encouragement given by my good friend Professor NV Chandrasekharan. We all got together for morning and evening tea-time at the Department; It was a time to relieve our mental stress by making jokes and conversing with colleagues like Dr. Jeevathayaparan, Professor Mathew and Professor Welihinda to name a few

from the Biochemistry lab. A striking note for the friendly atmosphere was that Professor Karunananayake sometimes came to the lab late in the evening and had a chat with us before leaving the department at the end of the day; he was one of the few academics at the Faculty who stayed till 6.00 pm in the evening.

After finishing the PhD I was awarded a research fellowship by the World Health Organization to do a post-doctoral fellowship at the Liverpool School of Tropical Medicine. I also accepted the post of Senior Lecturer at the Department of Zoology, University of Sri Jayewardenepura in 1996. The dilemma of sibling species B being a major vector in Sri Lanka and a poor or non-vector in India was resolved by one of my PhD students (I was a co supervisor of his research) by using mitotic karyotyping method. For the first time in Sri Lanka, he identified sibling species B and E by analyzing mitotic Y chromosomes. Species B shows acrocentric whereas species E shows metacentric (Surendran et al., 2000) positions. He also found that sibling species E is the major vector of malaria and species B indeed is a poor vector. Further studies related to insecticide resistance, fecundity and age structure of the two sibling species revealed a clear difference in the bionomics of the two sibling species (Surendran et al., 2006). We received President's awards for the SCI publication in 2000 and 2006 for this work (now Dr. Surendran is a Professor in Zoology in the University of Jaffna).

Although I was able to secure a grant awarded by the National Science Foundation to continue my work, I did not have a laboratory at the Department of Zoology. Professor Karunananayake allowed me to continue research initially at the Molecular Biology Unit, and then at the IBMBB, where my first MPhil student finished her study on “Analysis of the genetic variations of the *An.*

culicifacies and *Anopheles subpictus* complexes in Sri Lanka using DNA based techniques”.

Professor Karunananayake was a good mentor, and always stressed on the importance of establishing a laboratory in my department and developing the field in this country. With limited facilities and funds given for young researchers it was an uphill task for me. However, I was able to secure another grant from the National Research Council of Sri Lanka which helped me to establish a small laboratory at the Department and produce the first Population Genetics PhD in this country in 2016. Being awarded a President’s Award in 2016 for the publication in SCI journal, attests for the high quality of this study (Harischandra et al., 2016). The second PhD in this field from our laboratory also secured a President’s Award in 2021 (Fernando et al., 2018). Furthermore, I have produced two MPhils as the main supervisor and five PhDs and three MPhils so far as co supervisor, to name a few achievements in my career that resulted from the training and guidance I received from Professor Karunananayake. Following in the footsteps of my mentor, I started the Genetics and Molecular Biology unit at the Faculty of Applied Sciences, University of Sri Jayewardenepura in 2017, to offer Genetics and Molecular Biology as a subject in the undergraduate degree programme.

Professor Karunananayake was a supervisor, mentor, and pathfinder to many of us who read for PhDs and MPhils at the Molecular Biology Unit and IBMBB. I was fortunate to have had the opportunity to be one of his post-graduate students and am eternally grateful for all the guidance he provided during the training. I pray for a healthy long life to Professor Karunananayake, to witness the many contributions of his students to the scientific and academic development in Sri Lanka.

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Validating Traditional Practices Using Tools of Modern Science: Evaluating Plants with Hypoglycaemic Activity

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Professor Eric Karunananayake's interest in scientific evaluation of Ayurveda Medicine may have had early seedling being the eldest son of an Ayurveda Physician, and further fostered when appointed as the Biochemist to the Bandaranaike Memorial Ayurvedic Research Institute (BMARI) in 1968 upon graduation. However inadequate laboratory facilities at BMARI did not permit serious scientific validation of Ayurveda Medicine there, but he was able to publish a review paper in "Ayurveda Pradeepika" and initiate establishment of laboratories at BMARI. Nevertheless making use of his training in Chemistry he researched on the salt intake of Sri Lankans (Mahadeva and Karunananayake, 1970) and on the fluoride content in black tea (Karunananayake et al., 1972) under the guidance of Dr. K Mahadeva, the latter in collaboration with Dr. R. L. Wickramasinghe at the Tea Research Institute.

In order to scientifically validate the use of different plants as a treatment for diabetes mellitus in the Ayurveda systems of medicine in Sri Lanka, Professor Karunananayake obtained a small research grant of Rs. 75,000.00 from NARESA (predecessor to National Science Foundation) in 1981 after he joined the Department of Biochemistry at the Colombo Medical Faculty. He states that the commencement of research had to be delayed due to inadequate animal house facilities (Karunananayake, Two Decades of Capacity Building). Later International Foundation for Science (IFS) Stockholm also supported these studies. Three most commonly used plants, namely *Salacia reticulata*

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Weight (Family, Celastraceae; Sinhala, Kothala himbutu), *Aegle marmelos* Linn. (Family, Rutaceae; Sinhala, Beli) and *Momordica charantia* Linn. (Family, Cucurbitaceae; Sinhala, Karawila) were initially evaluated. Hot water extracts of dried powdered roots of *S. reticulata* and *A. marmelos*, and fresh fruit juice of *M. charantia* were tested on Sprague Dawley rats to assess their effect on fasting blood glucose and on oral glucose tolerance. While all three preparations exerted significant effects, the most profound effect on reducing fasting blood glucose and improving oral glucose tolerance was shown by *M. charantia* fruit juice (Karunananayake et al., 1984).

Subsequently *M. charantia* and *A. marmelos* were chosen for further studies. Jayantha Welihinda started his PhD research on *M. charantia* (Welihinda, 1985), while *A. marmelos* was further studied by an MSc student (Sinnadorai, 1984). Jayantha has outlined his work (Welihinda and Karunananayake, 1986; Welihinda et al., 1982; 1986) in the essay authored by him. Story of *S. reticulata* has a rather sad ending for Sri Lanka as researchers from Japan obtained patents for antidiabetic extracts of *S. reticulata*. Professor Veranja Karunaratne writing to the NSF journal elaborates on this as follows. “The claims of these patents have been made despite the practice of using *S. reticulata* in the treatment of high blood-sugar, which is part of the traditional knowledge of Sri Lanka; and furthermore its efficacy had been scientifically demonstrated through work carried out by Karunananayake et al., 1984 prior to the approval of these patents” (Karunaratne, 2013). Had there been adequate research funding and infrastructure in Sri Lanka perhaps the story would have been different.

With the major research focus being changed to Molecular Biology in mid nineteen eighties, Professor Karunananayake could not devote much time and effort on this line of research. Jayantha also had taken onto Molecular Biology.

Nevertheless Professor Karunananayake was very keen to further elucidate the mechanism of hypoglycaemic action of *M. charantia*. Thus the next phase of research on hypoglycemic plants was initiated as an MSc project of S. Jeevathayaparan which then progressed to a PhD (Jeevathayaparan, 1988; 1999). My self and Professor Saroj Jayasinghe from the Department of Clinical Medicine were part of the team and provided additional support through our research grants⁸. Professor Saroj Jayasinghe together with his predecessor, Professor Rezvi Sheriff had previously collaborated on clinical evaluation of *M. charantia* (Welihinda et al., 1982).

These studies used a diabetic animal model developed using a favourite molecule of Professor Karunananayake. The molecule was none other than “streptozotocin” which he himself researched on for doctoral studies at the Imperial College (Karunananayake et al., 1976). Despite the Nobel Laureate, Sir Ernst Chain, FRS, who was the Professor of Chemistry and group leader at the Imperial College warning young Eric Karunananayake that the project he is embarking on is difficult and there is no guarantee of results, the latter not only completed his PhD successfully but also had seven publications in leading journals arising from the doctoral studies detailing its chemical synthesis, absorption, metabolism and tissue distribution. Streptozotocin specifically destroys pancreatic beta cells and it is this property which was exploited to develop animal models of diabetes.

Professor Karunananayake used streptozotocin to induce diabetes in Sprague Dawley rats in Colombo in late nineteen eighties, and tested the effect of *M. charantia* fruit juice in this model. Dose of streptozotocin used (i.e. 50 mg/kg body weight) appeared to have induced full blown diabetes mellitus as the

⁸Natural Resources and Energy Authority of Sri Lanka (present NSF) and Third World Academy of Sciences

animals had a glycosylated haemoglobin level exceeding 6% (Karunananayake et al., 1990). Interestingly, glycosylated haemoglobin was measured using a method established in house (Karunananayake and Chandrasekharan, 1985), at a time when its measurement had not permeated the clinical management of diabetes in Sri Lanka. While *M. charantia* fruit juice improved glucose tolerance in normal healthy animals, it had no significant effect in the diabetic model further suggesting requirement of pancreatic beta cells for *M. charantia* to exert its hypoglycaemic effect (Karunananayake et al., 1990). In subsequent studies a graded dose of streptozotocin (10, 20, 30 and 40 mg/kg body weight) was used to induce diabetes of graded severity. As expected *M. charantia* was effective in improving glucose tolerance, reduced glycosylated haemoglobin and increased plasma insulin levels when given to animals who developed milder form of the disease but not effective when the disease was severe (Jeevathayaparan et al., 1995). Furthermore, when compared with commonly used oral hypoglycaemic drug tolbutamide, *M. charantia* was effective when diabetes was induced with 10 or 20 mg /kg streptozotocin while tolbutamide was effective only in the former group. Neither *M. charantia*, nor tolbutamide was effective in improving glucose tolerance when diabetes was induced with 30 or 40 mg/kg streptozotocin.

In order to further elucidate the underlying mechanism of hypoglycaemic activity of *M. charantia*, its effect on the fate of C¹⁴ labelled glucose was studied. *M. charantia* significantly reduced intestinal glucose absorption and increased incorporation of glucose in the liver and adipose tissue. Glucose incorporation into glycogen, lipid and protein in the liver was also increased (Jeevathayaparan, 1999; Jeevathayaparan et al., 1991; 1997). Though active fractions were identified, the active principle could not be isolated as a pure compound during these studies as it was found to be a mixture of two isomers. During the course of these studies possible hepatotoxicity of *M. charantia* fruit

juice and the seed extract was evaluated and an enzyme inducing effect was apparent with increased levels of serum γ -glutamyl transferase and alkaline phosphatase in the absence of consistent significant changes in liver histology (Tennekoon et al., 1994).

Overall results from the studies carried out under the direction of Professor Karunananayake suggest that *M. charantia* is effective in the treatment of Type 2 diabetes mellitus but not in Type 1 diabetes mellitus. Professor Karunananayake's initiative on setting up scientific evaluation of medicinal plants used in the traditional and Ayurveda systems of Medicine opened a plethora of opportunities for many other established researchers and novices to explore our untapped treasures in the plant kingdom in the search for novel drugs. Several other groups also began studying medicinal plants with hypoglycaemic activity once Professor Karunananayake set the ball rolling. Elsewhere in this volume Professor Thabrew and three young scientists who obtained their doctorates during the last decade, shed further light on medicinal plant research which benefitted from collaboration with or guidance/supervision by Professor Karunananayake, and laboratory facilities that he established.

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Landscaping Molecular Biology in Sri Lanka

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Having established the discipline of Molecular Biology for the first time in Sri Lanka at the Department of Biochemistry, Faculty of Medicine, University of Colombo, Professor Karunananayake lent more than a helping hand to established researchers and novices to strengthen and develop their own research programmes in Molecular Biology and related fields. A few authors have detailed some of these collaborations elsewhere in this Volume. This essay is an attempt to recapitulate collaborations that have been included as well as to outline those not recorded elsewhere in this Volume.

Collaborations with Research Institutes

Coconut Research Institute

A collaboration was established with the Coconut Research Institute (CRI), with one of its senior scientists, Dr. J.M.D.T. Everad, initiating molecular biology studies on the coconut germplasm in Professor Karunananayake's laboratory. Dr. Everad submitted a very valuable report to the Board of Governors of CRI titled "Evaluation of the extent of genomic variation in the coconut palm using Random Amplified Polymorphic markers" in 1999, based on the work carried out; a very valuable source to direct coconut breeding

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programmes in the country. Nilanthie Dassanayake's doctoral studies that followed are described in her contribution to this volume.

Rubber Research Institute

Rubber, *Hevea brasiliensis* is a crop of immense economic importance to Sri Lanka. A collaboration began with the Rubber Research Institute, Sri Lanka when Dr. Thilak Attanayake who returned to Sri Lanka with a PhD from Birmingham began molecular studies on rubber. He is now Professor of Biotechnology at the Wayamba University. Kalaivani Vivehananthan, who later joined the Wayamba University also carried out research on rubber for her MSc under the supervision of Dr. Attanayake and Professor Karunanayake. Priyanthi Silva, a scientist from RRI, carried out her doctoral studies on the causative organism of leaf fall disease in rubber, *Corynespora cassiicola*. RRI still continues to collaborate with IBMBB.

Tea Research Institute

A major collaborative programme was developed with the Tea Research Institute (TRI) following discussions by TRI scientists with Professor Karunanayake. K M Mewan who was the first doctoral student to work on the tea genomics in Sri Lanka had lucidly described Professor Karunanayake's invaluable role in the essay titled "Bud plus Two - Blended with DNA Technology" to which I would gladly refer the reader for details. Before embarking on Molecular Biology and also concurrently while developing Molecular Biology, Professor Karunanayake had collaborated with the TRI, initially investigating fluoride content in tea in early 1970s and investigating the effect of tea on caffeine metabolism in 1980s.

National Aquatic Research Agency

Professor Karunananayake extended his laboratory facilities to train scientists from NARA in Molecular Biology and was an advisor for building laboratory facilities there.

Collaborations with the Health Sector

Other than various studies related to filariasis, cancer genetics, reproduction and development where collaborations were established with Anti Filaria Campaign, National Cancer Institute and Castle Street Hospital for Women respectively as outlined elsewhere in this Volume, Professor Karunananayake established a collaboration with National Hospital Sri Lanka (then known as General Hospital). The collaboration was with the consultant neurologist Dr. J.B. Peiris to determine the level of anti-epileptic drugs in patient samples. Professor Karunananayake established HPLC techniques for this purpose for the first time in Sri Lanka. Project was funded by Dutch Ministry for Overseas Development in 1981 in the pre Molecular Biology era. Assay facilities extended to all the hospitals in Sri Lanka improved the management of epileptic patients; a MD trainee in Paediatrics based her research project on this work.

Collaborations with / support to Higher Education Sector

Other Universities

Many Universities benefitted from capacity building in human resources in Molecular Biology that Professor Karunananayake implemented. Through the SAREC and IPICS Grants and collaborative Grants from other funding

sources he has provided academics with sound research training to University of Jaffna (Dr. A. Murugananthan in Parasitology and Dr. T. Eswaramohan in Zoology); Eastern University (Dr. Jayanthini Muhunthan in Biochemistry), Wayamba University (Dr. K.M. Mewan in Biotechnology and Dr. S. Jeevathayaparan in Biochemistry); University of Sri Jayewardenapura (Professor Nissanka de Silva in Zoology and Professor Nilanthie Dassanayake in Botany), University of Peradeniya (Dr. Chandima Ariyaratne in Botany). Subsequently Nissanka's research students worked at the Department of Biochemistry and Molecular Biology and later at the IBMBB, until he built his laboratory at USJ.

Department of Biochemistry, University of Jaffna (UJ) worked closely with Professor Karunanayake by virtue of Professor K. Balasubramanium, first Professor of Biochemistry, UJ having been a senior colleague in Colombo and his successor Professor Vasanthy Arsaratnam being an MSc graduate of the Department of Biochemistry, Faculty of Medicine, UoC. Professor Balasubramanium's initial role in developing Molecular Biology in Sri Lanka has been highlighted in this Volume. With Professor Karunanayake's group and Professors Balasubramanium. Arsaratnam group being recipients of Swedish support there were many mutually beneficial activities and lasting friendships forged which rose above ethnic conflicts. In 2005, our Northern colleagues honoured Professor Karunanayake with the Professor K Balasubramanium Gold Medal for Excellence in Research in appreciation of his services to Science.

Award of Doctor of Science *Honoris causa* to Professor Karunanayake by USJ showcase how much Professor Karunanayake's contribution in training their academics and supporting their career advancement was appreciated by the University Authorities. To get a glimpse of Professor Karunanayake's

collaboration with University of Ruhuna, I will direct the reader to the account by Professor Ira Thabrew in this Volume.

Faculty of Science, University of Colombo

Several collaborative research programmes leading to Doctoral or Masters Degrees as well as facilities for undergraduate research projects were provided by Professor Karunananayake through his research funds to the Faculty of Science, University of Colombo.

Late Dr. Maya Gunasekara (Department of Chemistry, UoC who founded Genetech) began her molecular biological research in Professor Karunananayake's laboratory at the Faculty of Medicine following an introduction by Professor Tissa Vitharana, then Director, Medical Research Institute. Like many others, she was left high and dry on return to Sri Lanka armed with a PhD from UK until she was accepted to Professor Karunananayake's laboratory. The work she began on the malaria vector was continued as doctoral studies by young Nissanka de Silva from USJ; the story unfolds elsewhere in this volume. Collaborations with late Professor Ravi Wijesundera and Professor T.S. Tirimanne from the Department of Plant Sciences resulted in four PhDs in Plant Molecular Biology, these were fully or partially supported by Professor Karunananayake's research funds.

Professor Ranil Dassanayake (Department of Chemistry) who trained in Molecular Biology under the wings of Professor Karunananayake when he was a junior academic benefitted from brand new laboratories at the IBMBB to train his PhD student, Wasana Rodrigo. Professor Preethi Udagama (Department of Zoology and Environmental Science) similarly benefitted as her PhD students carried out their research at the IBMBB until she established her own labs.

Several other students of the Faculty of Science, both postgraduate and undergraduate have made use of the facilities developed by Professor Karunananayake for their research under the supervision of other IBMBB staff and still continue to do so.

Other Departments of the Faculty of Medicine, UoC

Fourteen Academic Departments existed in the Faculty of Medicine when Professor Karunananayake was Professor of Biochemistry there. I could count eight of these Departments having academics collaborating with/ supported or supervised by Professor Karunananayake. Molecular genetics studies on Duchenne muscular dystrophy with Professor Rohan Jayasekara (Department of Anatomy); research on medicinal plants and reproductive endocrinology/molecular biology with myself (Department of Physiology); filarial research with late Professor MM Ismail and Dr. W. Abeyewickrema (Department of Parasitology); providing training in Molecular Biology to Professor Jennifer Perera's group (Department of Microbiology), collaborations on toxicity studies and cancer genetics with Professors L.R. Amarasekara and Preethika Angunawela (Department of Pathology); extending laboratory facilities and co-supervision of doctoral studies to Professor Rohini Fernandopulle (Department of Pharmacology); work on hypoglycaemic plants with Professors Rezvi Sheriff and Saroj Jayasinghe (Department of Medicine); helping to establish laboratory facilities and serum uric acid measurement with Professor Harsha Seneviratne (Department of Obstetrics and Gynaecology) are some of the successful collaborations that I can recall. Collaboration with the Department of Pathology in cancer genetics continued at the IBMBB with Professor Angunawela being a co-investigator and co-supervisor for several PhD and MSc students; further new

collaborations by academics of the IBMBB with the Faculty of Medicine was made possible because of the facilities Professor Karunananayake established.

The imprint that Professor Karunananayake left on many molding them to be good scientists is incomparable. Facilities he established were used by several other doctoral students registered elsewhere to accomplish components of their studies. Hundreds of young scientists were trained at the MSc level, which was a stepping stone to doctoral studies locally as well as overseas for many. Besides those mentioned in this essay he endowed the Department of Biochemistry and Molecular Biology and the IBMBB with more than a dozen of highly trained scientists and academics. They recount their experiences elsewhere in this Volume. Professor Karunananayake reached out to many entities beyond his Department and the Faculty. Creation of the IBMBB enabled forging much closer associations with such entities beyond Academia, and every student who entered the IBMBB be they from North or South, Academia or Research Institutes, within or outside University of Colombo, rich or poor, employed or unemployed were welcomed equally to the IBMBB family and afforded same opportunities. His legacy stands tall in the University of Colombo in the physical form of IBMBB and all over the country in scientists and academics he had endowed mother Lanka with.

Investigating Breast Cancer genes, *BRCA1* and *BRCA2* in Sri Lankan women

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Vidya Jyothi Emeritus Professor Eric Karunananayake is undoubtedly the pioneer of Molecular Biology in Sri Lanka. His efforts to establish, improve and advance this field is incomparable. He contributed his knowledge for ground-breaking research into the field of molecular diagnostics in Sri Lanka, as he led the first ever molecular genetics study on breast cancer. He initiated cancer research by selecting the two main breast cancer susceptibility genes, BRCA 1 and 2 (*BRCA 1 and 2*) at a time when there was hardly any molecular genetic testing for cancer in Sri Lanka.

Initiation of breast cancer genetic screening in Sri Lanka

Breast cancer has a complicated aetiology with multifactorial risk factor involvement (Martin et al., 2001). Worldwide, it is the commonest malignancy among women. There were 2.3 million women diagnosed with breast cancer, representing 11.7% of all cancer cases and 685,000 deaths globally in 2020 (Sung et al., 2021), becoming a primary cause of death in women. In Sri Lanka, women are diagnosed with breast cancer at a median age of 50 years and accounts for 23.9% of all cancers in women (National Cancer Incidence and Mortality Data, Sri Lanka 2015). Though the incidence rate of breast cancer is high in the developed countries, the mortality rate is comparatively higher in the developing countries due to lack of resources for preventive/genetic screening for early detection delaying effective treatment.

About 60% of deaths due to breast cancer occur in developing countries (da Costa Vieira et al., 2017).

About 5 to 10% of all breast cancers have a genetic involvement, while the remaining 90 to 95% are due to non-genetic factors (Martin et al., 2000). Family history of the disease is one of the main risk factors for breast cancer. Apparently healthy individuals with first degree relatives (a mother, sister or daughter) diagnosed with breast cancer are known as at-risk individuals who have a higher chance of developing breast cancer later (Breastcancer.org).

Marie-Clare King's laboratory provided evidence for a highly penetrant, autosomal dominant susceptibility allele for breast cancer in 1988 (Newman et al., 1988). Pathogenic variants of *BRCA1* and *BRCA2* genes are now considered as the most potent candidates for this. Presence of pathogenic variants in either of these genes confers a 40-80% risk for breast cancer during the individual's lifetime. Furthermore, risk for developing breast cancer by the age 70 years is 40%-87% for *BRCA1* and 27%-84% for *BRCA2* pathogenic mutations (Fackenthal and Olopade, 2007, Kuchenbaecker et al., 2017). Pathogenic variants of these two genes also confer an increased risk of ovarian cancer (*BRCA1*: 63%; and *BRCA2*: 27%) (Easton et al., 1995, Ford et al., 1998).

Identifying the importance of screening *BRCA1* and *BRCA2* genes in breast cancer patients and in at-risk individuals, Professor Karunananayake diversified his research interests to encompass cancer molecular genetics. He established a multidisciplinary research team at the Faculty of Medicine by establishing a strong collaboration with the National Cancer Institute (NCI) and the programme was reactivated in 2003. Dr. Yasantha Ariyaratne (Consultant Clinical Oncologist), then Director/NCI, warmly welcomed the research team to access patients and facilities, while Dr. Indrani Amarasinghe, Consultant

Oncological Surgeon functioned as a very supportive co-investigator. The active collaboration Professor Karunananayake initiated with the NCI, continues to date by successors on either side. Professor Kamani Tennekoon (then at the Faculty of Medicine) and Professor Preethika Angunawela (Department of Pathology, Faculty of Medicine) continued as co-investigators. Four doctoral studies (described in this volume) and several Master of Science studies benefitted from this collaboration. In this essay I summarize the doctoral studies of Wasanthi De Silva on *BRCA1* (De Silva, 2009; De Silva et al., 2008) and my doctoral studies and subsequent work on *BRCA2* and some on *BRCA1* (De Silva, 2012; De Silva et al., 2011; 2014; 2017). Both doctoral studies were supported by Sida/Secretariat for Research Cooperation (SAREC) Grant for Molecular Biology and Biotechnology (PI: Professor Karunananayake). Professor Karunananayake functioned as the principal supervisor for Wasanthi and as co-supervisor for me having handed over the responsibility of the cancer research programme to Professor Tennekoon upon his retirement.

Identification of the BRCA genes

BRCA genes are main breast cancer susceptibility genes found mutated mainly in familial breast cancer patients with high penetrance. They are well established tumour suppressor genes, specialized for DNA damage repair function. The proteins coded by *BRCA* genes participate in a common pathway to enable homologous recombination and thereby maintain genomic integrity (Powell and Kachnic, 2003). However, the deficiency of one *BRCA* protein cannot be compensated by the other, hence lack of function of either *BRCA1* or *BRCA2* is adequate to cause cancer.

BRCA1 was discovered in 1990 by Professor Mary-Claire King and her team at UC Berkeley through genetic linkage analysis of early-onset breast cancer

families (Hall et al., 1990). Miki et al., 1994 described the identification of strong candidate gene for the breast and ovarian cancer susceptibility known as *BRCA1* by positional cloning studies.

BRCA1 gene is located on the long arm (q) of chromosome 17 and consists of 24 exons. *BRCA1* protein is a 190 kDa nuclear phosphoprotein consisting of 1863 amino acids. The C-terminus of *BRCA1* protein contains an amino-acid sequence motif, called BRCT domain which is recognized by many DNA repair proteins such as Abraxas/CCDC98, BACH1/BRIP1/FANCI, CtIP, *BRCA2* and UHRF1 (Billing et al., 2018). Furthermore, the N-terminus of the protein contains a domain called ring-finger domain involved in protein–protein interactions and in protein ubiquitination (Powell and Kachnic, 2003).

After the linkage analysis on 22 families with multiple cases of early-onset breast cancer, the *BRCA2* gene was mapped on to chromosome 13. *BRCA2* became the second strong candidate for breast cancer susceptibility. The discovery of the gene was reported in 1995 by the research team led by Professor Mike Stratton (Wooster et al., 1995). This gene is located on chromosome 13 (13q12) and consists of 26 exons. *BRCA2* protein is a 385 kDa nuclear protein of 3418 amino acids. Exon 11 of *BRCA2* gene is very large in size and encodes eight conserved motifs termed BRC repeats where each motif is approximately 70 amino acids in length (Chen et al., 1999). Six of the BRC repeats mediate binding of the repair protein RAD51 to the *BRCA2* protein (Boulton, 2006). Furthermore, the C- terminus of *BRCA2* contains a distinct RAD51 binding domain, and phosphorylation of this domain by cyclin-dependent kinases appears to alter the interaction with RAD51 (Nathanson et al., 2001).

BRCA 1 and *BRCA2* proteins work in a common pathway of genome protection but at different stages in the DNA damage response and in DNA

repair, hence function of both proteins are needed to prevent cancer. BRCA2 interacts with BRCA1, indicating the involvement of BRCA2 in recombination-mediated repair of double stranded breaks and the maintenance of chromosome integrity. BRCA2 protein also interacts with single-stranded DNA through oligonucleotide binding (OB) domains (Tarsounas et al., 2004). In addition, BRCA2 interacts with BRCAF35 (BRCA2-associated factor 35) a structural DNA binding protein with specificity for cruciform DNA. BRCA2 protein interacts with several proteins in the function of DNA repair, transcription, and cell cycle in response to DNA damage (Marmorstein et al., 2001).

Hereditary Breast and Ovarian Cancer Syndrome (HBOC)

Hereditary breast and ovarian cancer syndrome (HBOC) is an adult-onset, cancer predisposition syndrome. HBOC is characterized by a high risk of breast and ovarian cancers, and an increased risk of other cancers such as male breast cancer, prostate cancer, pancreatic cancer, and melanoma. *BRCA1* and *BRCA2* pathogenic variants are more frequent in HBOC.

In HBOC, *BRCA1* and *BRCA2* pathogenic variants are inherited via the germline and there is a strong family history with close relatives affected with breast, ovarian, or other related cancers. Cancer is usually premenopausal, multiple related cancers (occurrence of breast and ovarian cancer in a single individual) can be seen, often the cancer subtype is triple-negative (lacks oestrogen, progesterone and HER2 receptors). Presence of male breast cancer and Ashkenazi Jewish ancestry are some other characteristic features in the family history (De Silva et al., 2019, Petrucelli et al., 2016).

Characterizing BRCA1 and BRCA2 mutations in Sri Lankan women

Molecular genetics studies were carried out to characterize sequence variants of both *BRCA1* and *BRCA2* and to identify their pathogenicity in a cohort of patients with breast cancer (with and without a family history), at risk individuals and healthy controls. Initial screening was carried out using single strand conformation polymorphism (SSCP) and samples suspected to carry nucleotide variants were then subjected to in house Sanger sequencing to identify the variants. Since both *BRCA1* and *BRCA2* are large genes, a number of primers were used for PCR amplifications. Besides overlapping primers were used to cover large exons. For *BRCA1*, exon 11, a hotspot area was directly sequenced without prior screening by SSCP. Results from *BRCA1* analysis were published in BMC Cancer in 2008 (De Silva et al., 2008). This became the first report on *BRCA1* mutations and polymorphisms in the Sri Lankan context. The study reported 19 sequence variants in *BRCA1* gene including two novel deleterious frame-shift mutations (one in exon 11 and the other in exon 21), one novel missense possibly pathogenic mutation (in exon 11), one reported possibly pathogenic intronic variant, three novel intronic variants, ten reported polymorphisms and two reported intronic variants (De Silva et al., 2008). Novel deleterious frame-shift mutations created premature stop codons thus truncating the *BRCA1* protein rendering it ineffective in DNA repair for cancer prevention. Of these, the mutation in exon 21 occurred in a woman who had many family members across three generations on the maternal side afflicted with breast and other cancers (Figure 1). Majority of the living female members in the three generations were genotyped and two individuals (one sister, and a second degree relative) were found to harbour the same mutation, though unaffected by the disease at the time.

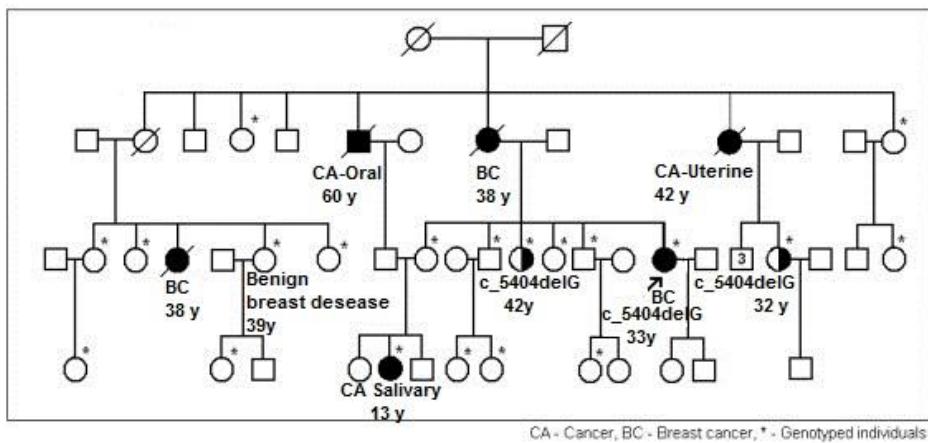


Figure 1. Pedigree of family of the patient carrying *BRCA1*/exon 21 mutation. Age of onset of the disease for members affected with breast cancer (BC), other cancers and benign breast disease indicated. This image was adapted from De Silva et al, 2008 under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by/2.0/>)

I began studies on *BRCA2* in 2008 with the same cohort used for *BRCA1* studies, however with a lesser number of individuals studied in each group (Numbers studied: familial breast cancer (N=55); sporadic breast cancer (N=54) and at-risk individuals N=20). When the findings were published in Experimental and Therapeutic Medicine in 2011, it became the first report on *BRCA2* mutations and polymorphisms in Sri Lankan breast cancer patients. We found twenty-three sequence variants in *BRCA2* comprising of two novel pathogenic frame-shift mutations (both in exon 11), two novel possibly pathogenic mutations (one each in exon 10 and 11), one novel intronic variant, five novel polymorphisms, a previously reported pathogenic intronic variant and twelve previously reported polymorphisms. The pathogenic variants were found in exons 10 and 11 of *BRCA2* gene implying that these two exons can

be targeted in genetic screening as hotspot regions in the Sri Lankan population. The prevalence of clearly pathogenic *BRCA2* mutations was 11% and possibly pathogenic *BRCA2* mutations was 12.73% (De Silva et al., 2011). In contrast the prevalence of *BRCA1* clearly pathogenic and possibly pathogenic mutations was 6.25% in our cohort. Thus, *BRCA2* mutations appear to be more frequent in Sri Lankan familial breast cancer patients. Our findings provided an important direction for genetic screening where one can initially screen for *BRCA2* mutations and direct only those not harbouring pathogenic mutations for subsequent *BRCA1* screening rather than screening both genes simultaneously, thus reducing the cost. Figure 2 illustrates the number of each type of variants/ mutations found in *BRCA1* gene after screening a total of 130 patients (N = 66 with a family history of breast cancer and N = 64 sporadic breast cancer), 70 at-risk individuals and in *BRCA2* gene after screening total of 109 patients (N=55 with a family history of breast cancer and N=54 sporadic breast cancer) and 20 at-risk individuals.

BRCA1 and BRCA2 large rearrangements

We then explored the association of *BRCA1/BRCA2* large genomic rearrangements with breast cancer. Such large rearrangements can be seen when there is a strong family history of breast/ovarian cancer. These are reported in patients negative for *BRCA1/BRCA2* mutations and most are confined to specific families as founder mutations. Large genomic rearrangements cannot be detected by conventional methods. Therefore, multiplex ligation-dependent probe amplification (MLPA) method was used. *BRCA1/BRCA2* rearrangements were not identified in the tested cohort, indicating that *BRCA1* and *BRCA2* large genomic rearrangements are unlikely to significantly contribute to breast cancer in Sri Lanka (De Silva et al., 2014).

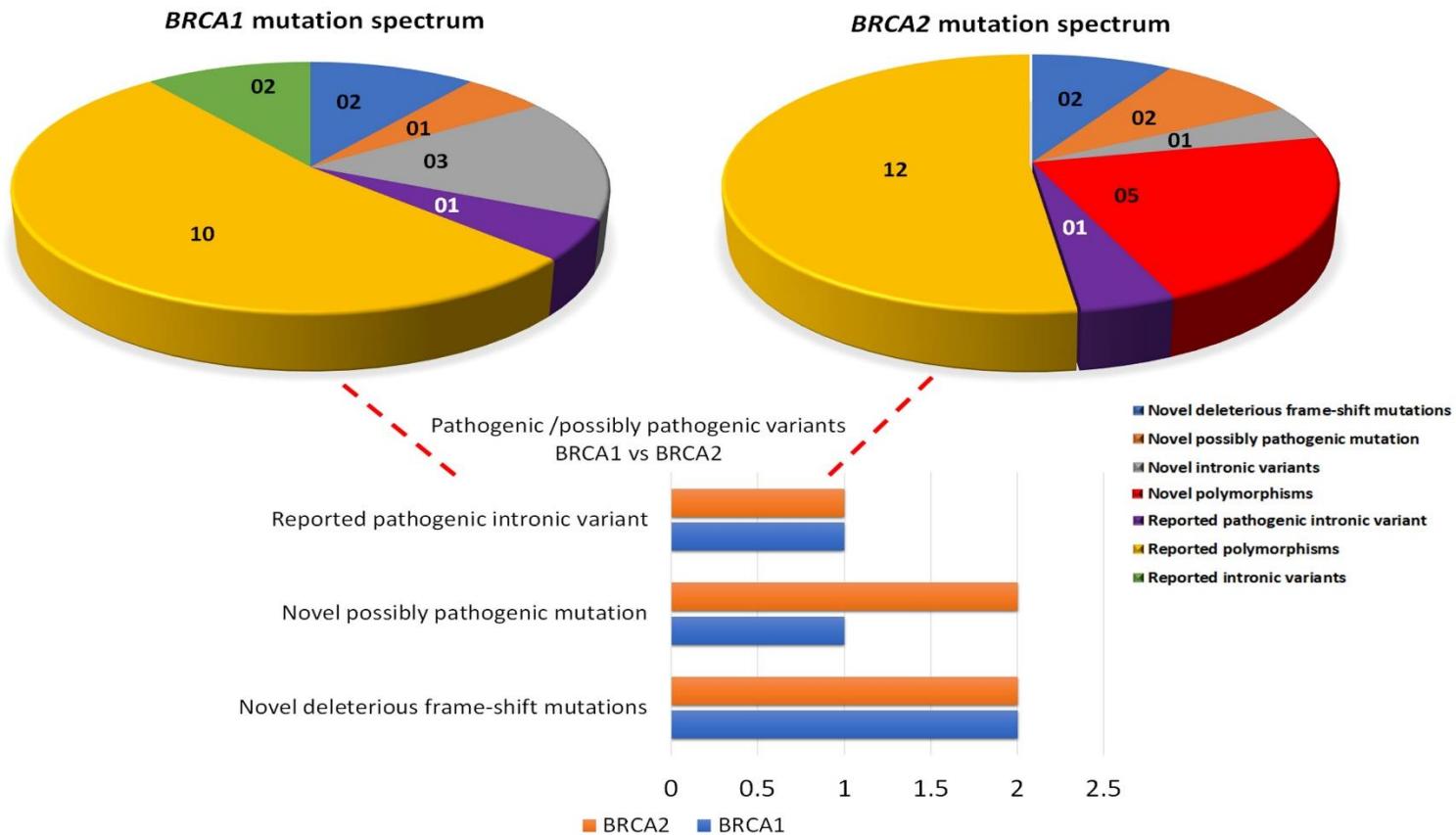


Figure 2. *BRCA 1* and *BRCA 2* mutation spectra in the studied cohort of breast cancer patients in Sri Lanka. Numbers indicate the number of different variants/mutations observed within each category.

I had the opportunity to travel overseas to attend several international conferences and present our findings, which were well appreciated by the participants. My experience is a vindication to the legacy of Professor Karunananayake who as a great teacher, always encouraged foreign exposure to learn what the outside world does in our chosen areas.

BRCA mutations in young breast cancer patients

Guidance of Professor Karunananayake during my doctoral studies, was immensely useful to expand breast cancer research. Initiation of further studies was facilitated via securing an Academic position in 2013. I was very fortunate to continue my career in the same place where I gained valued knowledge through my doctoral studies from two eminent supervisors.

Early onset breast cancer diagnosed among young women in the age range of 15 to 40 years is becoming common. It accounts for about 14% of all young cancer incidences and 7% of all breast cancer occurrences. This presents at an advanced stage comprising of most aggressive subtypes with poor prognosis compared to late onset disease (Keegan et al., 2013). Strong family history of breast cancer is the main risk factor, the risk is further increased by 2.09 fold when there is a relative diagnosed with breast cancer before 30 years (Anders et al., 2009). The five-year survival rate is worse with a rate of 72% if the onset is between 25 to 29 years, while for late onset disease at the age of 45 to 80 years, the rate stands at 84-86%. Women diagnosed with early onset breast cancer with a strong family history have a greater chance of having *BRCA1* or *BRCA2* mutations. Most lethal subtype identified among such women is the triple negative breast

cancer, which harbor *BRCA1* mutations frequently (Foulkes et al., 2010). If a patient with unilateral breast cancer carries *BRCA1/BRCA2* pathogenic variants along with a strong family history, there is a 16–35% risk of developing second primary cancer in the opposite breast (Mavaddat et al., 2013; Narod, 2014). Early onset breast cancer creates a significant burden for the local economy by decreasing productive young labor strength, increasing financial cost of disease management, and so on, thereby having an adverse socio-economic impact on the nation, to which a strong solution is needed (De Silva et al., 2019).

Based on our previous experience on *BRCA2* studies in Sri Lanka, we deduced that exon 10 and 11 of *BRCA2* are hotspots for mutations. Exon 11 of *BRCA2* is an extremely mutable site. A study on comprehensive spectrum of *BRCA1* and *BRCA2* deleterious variants in Asian countries reported 265 pathogenic variants in *BRCA2*, of which 125 were on exon 11 with a rate of 47% (Kwong et al., 2016). We studied 48 young familial breast cancer patients with mean age at diagnosis of 34.5 ± 6.20 years. We were able to find 36 sequence variants including seven pathogenic and two likely pathogenic variants, two intronic variants, two variants of uncertain significance and 23 benign variants. Seven pathogenic variants comprised of three novel frameshift variants, one novel and two reported nonsense variants, and one novel missense variant. Most remarkably six out of the seven pathogenic variants, resulted in stop codons giving rise to truncated *BRCA2* proteins, where normal function of the protein would be eliminated (De Silva et al., 2017). The study was supported by National Science Foundation (NSF) Competitive Research Grant scheme in 2014.

Evaluating breast cancer risk at an early stage is very important for women with a strong history of breast cancer, especially with affected first degree

relatives. IBMBB initiated *BRCA1* and *BRCA2* genetic testing for breast cancer patients and their at-risk individuals referred by the respective oncologist/ onco-surgeon. Though such testing is valuable for Sri Lanka along with genetic counselling, most of the patients cannot afford the cost of the testing and there is no national programme to cover expenses incurred. Thus we could offer this service only to a very small number of patients and at-risk individuals.

Under the very valuable guidance of Professor Karunananayake we were able to document the mutation spectrum of *BRCA1* and *BRCA2* genes in Sri Lankan breast cancer patients. These studies were then expanded to investigate early onset breast cancer. Dr. Chrishani Rodrigo describes her work on obesity related biomarkers in sporadic breast cancer while Dr. Vahinipriya Manoharan describes work on *TP53* in cancer in subsequent essays. Cancer biomarker research continues to date at the IBMBB, as generations of students, patients and the health sector reap the benefits of Professor Karunananayake's vision and dedication.

Copyright Acknowledgement: The Figure 1 was adapted from *De Silva W, Karunananayake EH, Tennekoon KH, et al. Novel sequence variants and a high frequency of recurrent polymorphisms in BRCA1 gene in Sri Lankan breast cancer patients and at-risk individuals. BMC Cancer. 2008; 8: 214.* The publisher of this article is BioMed Central which is a part of Springer Nature. This is an open access article distributed under the terms of the Creative Commons Attribution 2.0 International License (<http://creativecommons.org/licenses/by/2.0/>)

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Sporadic Breast Cancer: Can Obesity Related Biomarkers Predict Its Occurrence?

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“My DNA makes me, me and your DNA makes you, you”

The above phrase may look simple but those are the words of a humble yet a genius Scholar, Scientist and an Academic in Sri Lanka, which inspired me to learn Molecular Biology about 15 years ago. He is none other than the father of Molecular Biology in Sri Lanka, Founder Director of the IBMBB, Vidya Jyothi, Emeritus Professor Eric Hamilton Karunananayake. An encounter with him in the latter part of year 2003, at the Colombo Medical Faculty, to get advice on my postgraduate studies, was an honor and these were the words he gifted me with, when leaving his office. It was an eye opener to read and understand the depth of those words which ultimately illustrated the ample pathways left open to study. I'm forever grateful for his advice to start the MSc in Molecular Life Sciences with the first batch at the IBMBB rather than going abroad for further studies.

The charisma I saw in Professor Karunananayake was that he never dropped a word about his achievements, but filled our heads as students to think out of the box and drove our attention to admire difficulties he faced and taught us how hard work and determination made his dreams come true. He poured Biochemistry not as a theoretical subject but as a revelation to discover. Looking back to MSc days, these inspirational qualities of Professor Karunananayake made each of my fellow students to discover the work of this exceptional Sri Lankan Scholar ‘Prof. Eric’ rather than that

of Watson and Crick. Sir's lectures and pile of articles made me to read on cancer, built an inner passion for the subject in me. He was well aware of the importance of multidisciplinary research and his range of collaborators and collaborating Institutes is a testimony to that. I benefited from the collaboration he established with oncologists, oncological surgeons and pathologists for research on cancer more than a decade before I embarked on my PhD studies.

Breast cancer

Breast cancer, has remained unchanged up to date as the most commonly diagnosed cancer and second leading cause of cancer death in women worldwide (Sung et al., 2021). I wrote the same when I began my MSc research and sadly it still remains true. It is the most common cancer among females in Sri Lanka. According to registry data, its incidence is rising, and approximately 3,000 new cases are diagnosed each year (National cancer incidence and mortality data, 2015). Even though Sri Lanka reported a lower risk of breast cancer compared with many developed countries, the rapid rise in the incidence has caused serious public health concerns necessitating sustainable preventive strategies in the community and to modulate possible preventive and therapeutic measures.

Hereditary breast cancer research at the IBMBB has been described in the previous chapter. Main focus of this essay is on the work I did for my doctoral thesis on sporadic breast cancer.

Broadening the wings of breast cancer research at the IBMBB towards Sporadic breast cancer

Common cancers are frequently demarcated into ‘hereditary’ ('familial') or ‘sporadic’ ('non-hereditary') types (Roukos et al., 2007). Only about 5-10% of all breast cancer cases are familial where an involvement of genetic factors is present and the majority accounts for sporadic breast cancers (Martin and Weber, 2000). Sporadic breast cancers result from a serial stepwise accumulation of acquired and uncorrected somatic mutations, without any germline mutation playing a role. Professor Karunananayake and Professor Kamani Tennekoon as the principal supervisor gave me the substantial opportunity to study on biomarkers related to sporadic breast cancer for my doctoral research at the IBMBB. Professor Karunananayake's initiative in establishing sequencing facilities and his support to establish sensitive hormone and growth factor assays at the IBMBB facilitated my studies.

Obesity has been identified as a major risk factor for breast cancer (Cleary and Grossmann, 2009; Pierobon and Frankenfeld, 2013). The obesity-breast cancer link has been explained using several mechanisms, including high levels of circulating leptin and disrupted insulin/insulin-like growth factor (IGF) signaling (Argolo et al., 2018). In Sri Lanka, the prevalence of overweight and obesity is increasing rapidly in the population and it is widely accepted that this is due to unhealthy dietary habits.

Evaluation of components of the leptin and IGF systems, and visfatin (nicotinamide phosphoribosyl transferase) in sporadic breast cancer

Leptin, visfatin and IGF-1 are implicated in breast cancer risk but studies accounting for bioavailability of leptin and IGF-1 are sparse. Reports on

the association of leptin gene (*LEP*) and leptin receptor gene (*LEPR*) polymorphisms with breast cancer are also inconsistent. Only a very few studies have examined the biochemical and genetic variables concomitantly in the same cohort. For the first time in the Sri Lankan population, the possible association of leptin, soluble leptin receptor (SLR), visfatin, IGF-1 and IGF binding protein-3 (IGFBP-3) and selected polymorphisms in leptin gene (-2548 G/A) and leptin receptor gene (Q223R; K109R) with sporadic breast cancer was studied in my doctoral research which started in November 2012, supported by a grant from the National Research Council (NRC) awarded to Professor Tennekoon. Furthermore, these have not been studied simultaneously in any native South Asian population in relation to breast cancer.

In the study, a matched pair analysis was carried out to ascertain whether plasma leptin, SLR, free leptin index (FLI), serum visfatin, IGF-1, IGFBP-3, free IGF-1 index (FII) and selected *LEP* and *LEPR* polymorphisms are risk factors for sporadic breast cancer. Newly diagnosed sporadic breast cancer patients (N=100) were matched for age, body mass index (BMI), menopausal status and ethnicity with healthy controls. Analysis was limited to 80 matched pairs of Sinhalese of which 42 were premenopausal (Rodrigo et al., 2017).

Leptin, soluble leptin receptor and breast cancer

Leptin gene also known as ‘*OB* gene’ is located on chromosome 7 (Schwartz et al., 1996). The product of the *OB* gene, leptin is an adipocyte-secreted hormone, first discovered by Friedman and colleagues in 1994 (Zhang et al., 1994). Leptin is a protein with a molecular weight of 16 kDa,

comprising 167 amino acids. It is mainly, but not exclusively, produced by white adipose tissue in humans (Maffei et al., 1995).

In human, leptin levels are proportionate to fat mass and play a role in mammary gland development and lactation (Neville et al., 2002). Leptin exerts a greater proliferative effect in breast cancer cells than in normal mammary epithelial cells promoting more aggressive and metastatically potent tumor cells (Cirillo et al., 2008). Leptin exerts its effects through plasma membrane receptors. These exist in several isoforms and among these the SLR binds to circulating leptin reducing its bioavailability. Hence both higher leptin levels and lower SLR levels cause a status of higher leptin action (Wallace, 2000). Both leptin and its receptor are over-expressed in breast cancer, especially in higher grade tumors (Sultana et al., 2017), but are absent in normal breast epithelial tissue (Garofalo and Surmacz, 2006). Thus, the leptin-LEPR system may participate in tumorigenesis and increase the risk of cancer particularly in obese persons having high serum leptin (Guo et al., 2012).

In our study, patients had a significantly higher leptin level than the healthy controls irrespective of the menopausal status, but the difference was statistically significant only in the postmenopausal group ($P=0.0105$). As we have matched the patients to controls for BMI (± 1) the effect of BMI on leptin level is minimized. To further account for the effect of BMI, we normalized leptin levels to BMI. Even after normalization, the leptin/BMI ratio remained significantly higher in breast cancer patients than in the controls. Since the association between plasma leptin levels and breast cancer risk remained after eliminating the effect of BMI, this indicates that there is an adiposity independent positive association of leptin with breast cancer. Higher leptin/BMI in patients suggests a higher leptin secretion by

their adipocytes compared to the controls. When data were stratified by menopausal status a significantly higher leptin/BMI ratio was seen only in postmenopausal women indicating that adipocyte secretion of leptin is higher in them (Rodrigo et al., 2017).

As mentioned before, the bioavailability of leptin is modulated by SLR. Even though many research groups have studied the risk of breast cancer in relation to leptin levels, the focus on SLR is scarce. In our study the serum SLR levels were significantly higher in the controls than in the breast cancer patients irrespective of the menopausal status (Rodrigo et al., 2017). One way of characterizing the balance between leptin and SLR is the free leptin index, which is determined by calculating the ratio between the concentrations of leptin and SLR (Kratzsch et al., 2002). FLI is considered to better reflect leptin activity and bioavailability (Owecki et al., 2010). Results of our study showed a higher FLI in breast cancer patients in the overall analysis as well as when stratified according to menopausal state as leptin levels were higher and SLR levels were lower in patients when compared with their matched controls (Rodrigo et al., 2017). Thus, in breast cancer patients not only the circulating levels of leptin were higher but most of these would have been available at the tissue level for biological activities due to lower SLR levels. Communicating these results at the 129th Anniversary International Medical Congress of the Sri Lanka Medical Association, made a milestone of winning the award “Daphne Attygalle Prize for the best paper in Cancer” in the year 2016.

Correlation of selected leptin and leptin receptor genotypes and sporadic breast cancer

Several *LEP* and *LEPR* gene polymorphisms have been studied in breast cancer patients in different populations. Of these, *LEP* promoter -2548 G/A (rs7799039) and *LEPR* Q223R/Gln223Arg A/G (rs1137101) and K109R/Lys109Arg A/G (rs1137100) polymorphisms were reported to be associated with an increased risk and poor prognosis of breast cancer in some studies (Liu et al., 2007; Mahmoudi et al., 2015). We also investigated possible association of these SNPs with breast cancer. The frequency of alleles and genotypes for both patients and controls were calculated based on the results of SNP analysis.

The *LEP* -2548G/A SNP is a G to A transition at nucleotide position -2548 upstream of the ATG start site in the *LEP* gene 5' promoter region implicated in transcription (Mammès et al., 1998). It is thought to influence gene expression of leptin and the leptin secretion by adipose tissue (Hoffstedt et al., 2002) and is found to be associated with increased BMI (Li et al., 1999; Mammès et al., 2000). In our study a higher frequency of the variant allele A was seen in both the patients and the controls, compared to the G allele. However this polymorphism had no significant association with sporadic breast cancer (Rodrigo et al., 2017).

In the Q223R polymorphism in *LEPR* gene, an A to G transition at nucleotide position 668 from the start codon converts a glutamine to an arginine at codon 223 (Gotoda et al., 1997). This glutamine to arginine substitution occurs within the first of two putative leptin-binding regions and may be associated with impaired LEPR signaling capacity (Yiannakouris et al., 2001). If the G allele (arginine/R) is associated with impaired LEPR signaling then it should be protective against breast cancer,

whereas the A allele (glutamine/Q) should predispose to breast cancer. We observed a higher frequency of the wild type allele A in both patients and controls compared to the G allele and this was not affected by the menopausal status (Rodrigo et al., 2017). Though AA genotype and AG genotype together occurred more frequently among patients suggesting an effect of the wild type A allele on breast cancer risk, *LEPR* Q223R polymorphism showed no significant association with sporadic breast cancer (Rodrigo et al., 2017).

K109R SNP is a transition of an adenine (A) to a guanine (G) in codon 109 (AAG to AGG) at nucleotide 326 in exon 4. This causes a conservative change that changing the amino acid lysine to arginine (Gotoda et al., 1997). Whether this alteration affects the functionality of the receptor is not evident. Numerous studies have shown that the *LEPR* K109R polymorphism of the *LEPR* gene is associated with breast cancer. G allele frequency was significantly higher among patients compared to controls (0.300 vs 0.037) in our cohort, but in multivariate analysis which accounted for other variables tested, it increased breast cancer risk only in postmenopausal women (Rodrigo et al., 2017).

Thus we have been able to replicate in a cohort of Sinhalese women findings of some authors but not of the others on the association of selected SNPs of *LEP* and *LEPR* genes with sporadic breast cancer. It is plausible that there are other factors that modulate the association of *LEP* and *LEPR* SNPs with sporadic breast cancer and some of these may differ between ethnicities.

The association of serum visfatin with sporadic breast cancer

An association of visfatin, a major enzyme involved in the synthesis of NAD⁺ molecules, with breast cancer has been reported but the results have been contradictory and inconclusive (Assiri et al., 2015; Bi and Che, 2010). In our study visfatin levels in the patients were found to be significantly higher than in the controls irrespective of the menopausal status, but the effect disappeared when other variables such as leptin, SLR, leptin normalized to BMI, FLI and LEPR K109R polymorphism were taken into account in the multivariate analysis (Rodrigo et al., 2017). Therefore visfatin has a limited value as a predictive biomarker of sporadic breast cancer.

The association of IGF-1 and IGFBP-3 with sporadic breast cancer

IGF-1 is expressed by both normal and malignant breast cells (Macias and Hinck, 2012). IGF-1 stimulates mitosis and inhibits apoptosis and an association between circulating IGF-1 and breast cancer risk is also observed (Gunter et al., 2009; Samani et al., 2007). Further, high levels of IGF-1 and/or low levels of IGFBP-3 have generally been associated with an increased risk of breast cancer with adverse outcomes (Godwin et al., 2002; Key et al., 2010). In our study IGF-1 levels were significantly higher in patients than in controls when the whole group was considered (Rodrigo, 2018) as well as when data were stratified by menopausal status. IGFBP-3 levels were almost similar in patients and controls with slightly lower levels in premenopausal patients. The free IGF-1 index (IGF-1/IGFBP-3 ratio) indicates the freely available IGF-1 as around 99% of IGF-1 circulates bound to IGF binding proteins, with most bound to IGFBP-3.

Free IGF-1 index was significantly higher in patients than in the controls irrespective of the menopausal status. Although the free IGF-1 index is a much better index of bioavailability of IGF-1 level (Johansson et al., 2004), only a few studies have investigated this.

Although most studies have investigated the association of one or a few variables with breast cancer, studies which looked concomitantly at circulating leptin, SLR, visfatin, IGF-1 and IGFBP-3 levels and *LEP* or *LEPR* SNPs were very much limited. Thus, our matched pairs case-control study on Sri-Lankan patients with sporadic breast cancer, was carried out for the first time to see the association between biochemical variables (leptin, SLR, visfatin, IGF-1 and IGFBP-3), related indices (leptin normalized to BMI, free leptin index, free IGF-1 index) and leptin and leptin receptor genotypes (i.e. SNPs of the polymorphic regions -2548 G/A in *LEP* gene and Q223R, K109R in *LEPR* gene) with sporadic breast cancer. Using several models for multivariate analysis we demonstrated that SLR and free leptin index have the potential to be tested as predictive biomarkers in both premenopausal and postmenopausal women while *LEPR* K109R polymorphism and free IGFI index could be additional biomarkers for postmenopausal and premenopausal breast cancer respectively (Rodrigo, 2018; Rodrigo et al., 2017). Further studies on other ethnicities in Sri Lanka and prospective community studies may help to develop some of these as predictive biomarkers for sporadic breast cancer in future.

This research would not have been successful without the enormous knowledge, guidance and support given by Professor Karunananayake and Professor Tennekoon. Also, an immense support was given by the co-investigators Dr. Indrani Amarasinghe and Dr. Kanishka De Silva,

Oncological Surgeons at the National Cancer Institute (Apeksha Hospital), Maharagama by providing clinical expertise in the recruitment and supervising the acquisition of clinical data of patients. Further Dr. Geethanjali Senanayake, Consultant Radiologist supported the radiological assessment of the controls. Professor Kumudu Wijewardena and Dr. Ananda Wijayasiri, from the Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka provided the facility and advice for multivariate analyses. The collective guidance and interest of all of them gave the opportunity to present the research work both locally and internationally.

Recalling the excited face of Professor Karunananayake, when he was sharing a life story about his days in 1985, in a laboratory far away from Sri Lanka is memorable. With so much enthusiasm he mentioned how excited he was in a cozy corner of a lab in Uppsala, Sweden to read the hot topics of newly released articles on *TP53*, a tumour suppressor gene which play a major role in cancer. And images that drew in his mind at that point were so strong as it diversified his research to cancer, and his only desire was to establish postgraduate training at MSc/PhD level and to initiate a research program. I'm sure he never thought he was actually laying the future for youngsters, toddlers (like me back then), babies yet to be born to carry out research relevant to Sri Lanka using the tools of Molecular Biology and Gene Technology. Professor Eric Karunananayake's is an ideal example of dedicated, unselfish academic, whose main intention still is to serve the nation. This combination of qualities hasn't given him a luxurious life but ultimately brought home a similar lab, though small in scale, the IBMBB as in Uppsala, Sweden. IBMBB is equipped with top quality avant-garde laboratories not second to a laboratory in a developed

country. I am glad to have witnessed along with Dr. Meran Keshawa Ediriweera in Bangalore, India, how Professor Karunananayake's selfless contribution for generations in Sri Lanka was recognized and garlanded by the international scientific community on his 75th birthday by awarding the "World Academy of Science Award for Building Science Institutions in Central and South Asia".

I, as a person and we, as a nation owe Professor Eric Karunananayake for all his sacrifices, hard work and harbor of knowledge rendered. Thank you very much Professor Eric for being a guiding star to reach my success in postgraduate studies.

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The Guardian Angel of the Genome: *TP53*

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The phrase “The Guardian Angel of the Genome” took my attention when I was in the second semester of my MSc Molecular Life Sciences class at the IBMBB. Professor Karunananayake was giving us lectures in the cancer module and introduced the ‘tumour protein gene *TP53*’ using this phrase. He was an inspirer not just for me, but for all my batch mates too. We were proud that the pioneer of Molecular Biology in Sri Lanka was teaching us. Professor Karunananayake taught us in a way not just to give us the subject content, but to inspire and induce our curiosities which led me to fall into the magnetic world of Molecular Biology. The phrase and the way he expressed the importance of *TP53* led me to search more on that during my course work. When we were given the research topics in our last semester, I was really happy my favourite gene was among them and I immediately chose that without any second thought. I was lucky enough to continue research on *TP53* for my PhD after successful completion of the MSc and Sir allowed me to register as his PhD student which is a great honour for me. In this essay, I provide information on my experimental findings with *TP53/p53* during my tenure (2015-2020) as a doctoral student at the IBMBB. I will be referring to the gene as *TP53* and protein as p53 as per nomenclature often used by others.

Discovery of p53

p53 was discovered in 1979 by Professor Sir David Lane as a 53 kDa host protein bound to T antigen of simian virus 40 in virally transformed cells. The gene was initially classified as an oncogene. Subsequent work soon led to a series of important landmark findings that wild type p53, suppresses growth and oncogenic transformation in cell culture, and that inactivating *TP53* mutations are common in human tumours. p53 is the first natural ‘tumour suppressor’ found within human cells (Kastenhuber and Lowe, 2017).

TP53 gene and p53 protein

TP53 is a tumour suppressor gene located in the short arm of human chromosome 17, spanning 20 kilo base pairs comprising 11 exons and 10 introns. The p53 tumour suppressor protein is a DNA binding phosphoprotein made up of 393 amino acids and functions as a gatekeeper of the cellular fate. Under normal conditions, p53 has a low affinity for its specific target DNA sequences. Under genotoxic stress, it adopts the active form with a higher binding affinity and initiates cell cycle arrest, senescence or apoptosis through transactivation of p53 target genes. It forms a homo tetramer when it is functionally active (Okorokov et al., 2006). Each p53 protein contains transcription activation domain (1–67), proline rich region (67–98), core domain (98–303), nuclear localization signal-containing region (303–323), oligomerization domain (323–363) and C-terminal basic domain (363–393).

Functions of p53

p53 lies at the heart of stress response pathway and prevents the generation of genetically altered cells in the body, which are potentially malignant. It has a low affinity for its specific DNA sequence in the latent form but attains a higher affinity when cells are under stress such as DNA damage, oncogene activation, telomere attrition, hypoxia and loss of normal growth and survival signals. Active p53 controls the cell cycle arrest, senescence, DNA repair, apoptosis or inhibition of angiogenesis via pathways involving transactivation of target genes. These stress signals can be encountered at any stage in cells which undergo tumorigenesis from initiation of tumour to invasion and metastasis. Therefore, p53 plays a role in preventing tumour growth at several stages in the malignancy process mainly by two responses, a) by cell cycle arrest, which may be irreversible or temporary which allow cells to repair the damage before a further round of replication b) by apoptosis which permanently remove the cells from the organisms (Okorokov et al., 2006; Ozaki and Nakagawara, 2011; Ryan et al., 2001).

p53 and p21

Active p53 stimulates transcription of the gene *CDKN1A* that encodes a Cdk inhibitor protein called p21. When DNA is damaged, various protein kinases are recruited to the site of damage to initiate a signaling pathway that causes cell-cycle arrest. The first kinase at the damage site is either ATM (Ataxia telangiectasia mutated) or ATR (Ataxia telangiectasia and Rad3 related) protein, depending on the type of damage. Additional protein kinases, called Checkpoint kinase 1 (Chkl) and Checkpoint kinase 2

(Chk2), are then recruited and activated, resulting in the phosphorylation of the gene regulatory protein p53. Phosphorylated p53 stimulates transcription of the gene that encodes the CKI protein p21. The p21 binds and inactivates G1/S-Cdk and S-Cdk complexes, arresting the cell in G1 (Alberts et al., 2007; Bullock and Fersht, 2001).

p53 and MDM2

Even though p53 is constantly transcribed in all cells of the body, p53 proteins do not accumulate in cells under normal conditions as it is rapidly degraded by the proteasomes. This degradation is regulated by an E3 ubiquitin ligase called MDM2. Interaction of p53 with the MDM2 ubiquitilates the protein and target it for the degradation by the proteasomes. The interaction between p53 and MDM2 is finely regulated by multiple covalent modifications of p53 such as phosphorylation, sumoylation, acetylation, methylation and its competition with other proteins such as p14ARF. Modifications weaken the interaction of MDM2 with p53, thus suppressing the degradation of p53 and leading to the accumulation of p53 in the cell (Almazov et al., 2007).

TP53/ p53 as a molecular marker

Efficacy of cancer therapeutic approaches is influenced by the functional status of the p53 tumour suppressor protein (Kandioler-Eckersberger et al., 2000). Thus, identification of TP53 mutation status prior to administration of therapy can predict potential effectiveness of the treatment and influence treatment selection. Furthermore, the *TP53* mutation spectrum provides information on tumour origin, cause of mutation, aetiology,

molecular pathogenesis, prediction of patient survival and chances of recurrence (Greenblatt et al., 1994).

Work on TP53/p53 at the IBMBB

Mutational analysis of *TP53* is not routinely performed for cancer management, more so in resource poor settings including Sri Lanka; as screening the entire *TP53* gene is costly. But identification of hotspots to screen such regions in the *TP53* gene before administration of therapy is attainable in the Sri Lankan context.

Before I joined the IBMBB, several previous attempts were made to find possible mutations in the *TP53* gene in breast cancer patients targeting possible germline mutations in the exons 5 to 8, the hot spot regions. (Gunaratne, 2006; Gnanarajah, 2007; Vivekanandarajah et al., 2006). However, no germline mutations in the tested exons were detected in any of these studies. Although about 50% of cancers are said to carry *TP53* mutations, most are somatic in origin while germline mutations are rare. Thus the need to analyze tumour tissue to identify possible *TP53* mutations was emphasized, and the area was open for preliminary studies as a Master of Science Research Project. Therefore, during my Master of Science research project, I focused on optimizing DNA extraction from paraffin embedded tissue with the ultimate goal of using extracted DNA for *TP53* somatic mutation analysis. Though optimizing the extraction of DNA from FFPE (formalin fixed paraffin embedded) tissue was successful, screening the entire *TP53* gene was challenging as FFPE DNA is highly fragmented (Manoharan, 2014).

Therefore, my PhD study focused on characterizing mutation spectrum of *TP53* in Sri Lankan cancer patients by sequencing DNA obtained from fresh tumour tissue. Head and neck cancer (HNC) and breast cancer (BC) were selected for this study as the prevalence of these two cancers is high in Sri Lankan males and females respectively (National cancer incidence and mortality data, 2015). Colorectal cancer (CRC) was selected as it is reported to have a higher prevalence of *TP53* mutations. Surgically excised tumour tissues from patients with head and neck cancer (N=44), breast cancer (N=30) and colorectal cancer (N=21) were used for *TP53* mutation analysis. An equal number of healthy controls were screened for mutational status of *TP53* using their blood samples. A subset of the cancer tissue samples was analysed for protein expression of p53 using immuno-histochemical analysis and compared with the mutational status of *TP53* of the respective samples. Further, this project also aimed to study the expression of downstream targets of p53 such as, p21 and MDM2 and to compare those with the immuno-detection of p53.

Total of 47 sequence variants of the *TP53* gene were found including 13 novel variants. Out of the 47 variants, 24 were pathogenic while 2 were likely pathogenic variants. These pathogenic and likely – pathogenic variants were observed only in patients and not in any of the healthy controls. Pathogenic variants were detected in 14 head and neck cancer, 9 breast cancer and 7 colorectal cancer tumour samples. All these pathogenic and likely pathogenic variants were located between exons 4 to 8 of *TP53*. There were 2 variants with uncertain significance, 18 likely benign variants and 1 benign variant found both in patients and healthy control cohorts. Out of the 13 novel variants, 5 were pathogenic, one was of uncertain significance and 7 were likely benign (Manoharan et al., 2019; 2020). All

the exonic variants found in this study were deposited in the NCBI database (Accession number series SCV001450475.1 - SCV001450504.1)

The 5 novel pathogenic variants detected are tabulated in Table 1 (Manoharan et al, 2019; 2020). There were some pathogenic variants frequently found in the studied sample cohort than the others. c.637C>T was detected in all three types of cancers and c.524G>A was found in head and neck and colorectal cancer patients. c.733G>A was found in 2 colorectal cancer patients whereas c.743G>A was detected in 3 breast cancer patients (Manoharan et al., 2019; 2020).

Table 1. Pathogenic novel variants of *TP53/p53* detected during the study described

No.	HGVS Nomenclature		Exon	Mutation Type	Cancer
	cDNA	Protein			
1	c.298delC	p.Gln100Argfs*23	4	Frameshift	HNC
2	c.626_637delGAA ACACTTTTC	p.Asn210_Arg213del	6	In-frame	HNC
3	c.848_849delGC	p.Arg283Hisfs*22	8	Frameshift	BC
4	c.851_855delCAG AG	p.Thr284Argfs*20	8	Frameshift	BC
5	c.431_433delAGC	p.Gln144del	5	In-frame	CRC

HNC – Head and Neck Cancer, BC – Breast Cancer, CRC – Colorectal Cancer.

The most common type of mutations observed is the missense variants and was detected in total of 25 patients. Nonsense and frameshift mutations were detected in 4 patients each and the in-frame mutations were detected in 2 patients (Figure 1).

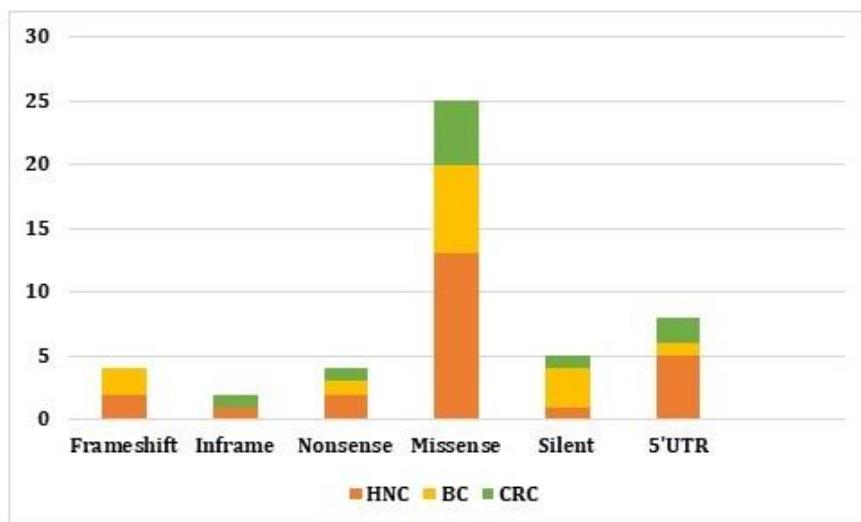


Figure 1. Distribution of different types of mutation among several cancers
HNC: Head and Neck Cancer; BC: Breast Cancer; CRC: Colorectal Cancer

Pathogenic missense variants showed strong positive signal (Pattern A) for p53 immunohistochemistry, whereas truncated proteins showed complete absence of positive signals (Pattern C). However, wild-type *TP53* showed either rare positive cells (Pattern B) or complete absence of positive signals (Pattern C) regardless of the type of the cancer (Figure 2). There was no direct correlation observed between the expression of p21 and MDM2 with the expression of p53 in all head and neck cancers, breast cancers and colorectal cancers (Manoharan et al., 2019; 2020).

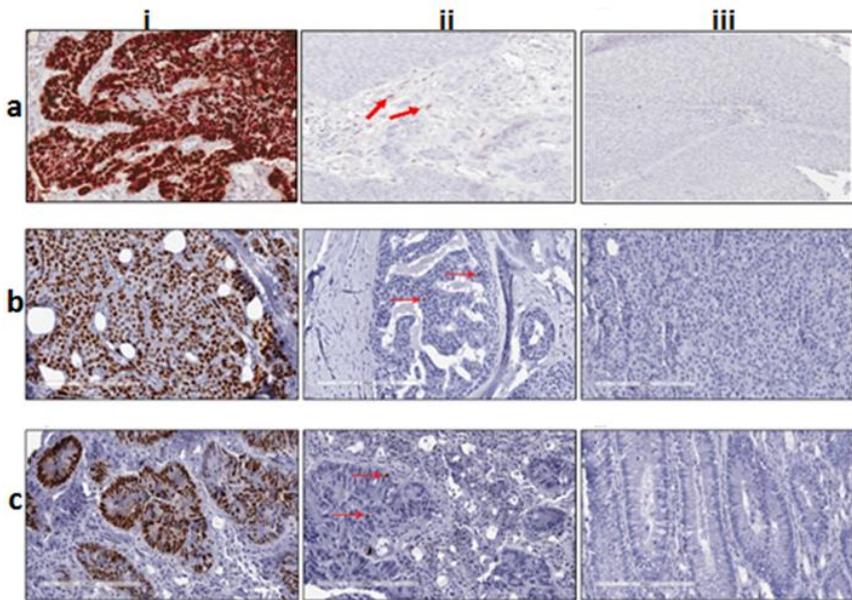


Figure 2. IHC characterization in head and neck (a) breast (b) colorectal (c) cancer tissues

(i) Pattern A with widespread IHC positive nuclei (ii) Pattern B with rare positive single tumour nuclei (Few positive cells are indicated by red arrows) (iii) Pattern C with no IHC positive nuclei.

This image was adapted from Manoharan, 2020.

Conclusions and future directions

This study established the mutation spectrum of *TP53* in a cohort of Sri Lankans with respect to head and neck, breast and colorectal cancers. The prevalence rates of pathogenic somatic *TP53* variant were 31.8%, 30% and 33.33% in the studied head and neck cancer, breast cancer and colorectal cancer cohorts respectively.

Sanger sequencing has several drawbacks in detecting gene mutations due to its restricted sensitivity, especially when it comes to the detection of variants in tumour samples where tumours are highly heterogeneous and

often mixed with normal tissue. Thus, somatic variant analysis using next-generation sequencing which can detect low frequency variants in a sample should be attempted in future studies.

IHC analysis of randomly selected 24 HNC, 13 BC and 14 CRC samples concluded the fact that, all the missense mutations are IHC positive with pattern-A, whereas nonsense and frameshift mutations are immuno-negative (pattern-C). Although, most of the wild type p53 were immuno-negative, there were few cases which were IHC positive with pattern-B. Thus, the present study indicates IHC analysis alone is inadequate to detect underlying *TP53* mutations as the nonsense and frameshift mutations are not distinguished from the wild type. If one relies only on IHC for detection, those who carry such mutations may be considered as wild type and treatment that require presence of the wild type protein may be erroneously administered.

My doctoral work was co-supervised by Dr. Sumadee De Silva and Professor Kamani Tennekoon from the IBMBB, Professor Preethika Angunawela from the Department of Pathology, Faculty of Medicine and Professor John Lunec from the Biosciences Institute, Newcastle University Cancer Centre, Faculty of Medical Sciences, Newcastle University, UK. This study was funded by National Research Council, Sri Lanka (Grant number 15–33 awarded to SDeS). Dr. Kanishka De Silva and his senior registrars from the National Cancer Institute, Maharagama provided clinical expertise, guided in recruitment of study participants and sample collection. Professor Preethika Angunawela supervised histopathological characterization of tumour tissue sample and her staff assisted in paraffin block making. Professor John Lunec provided expertise in immuno-

histochemical studies during my stay in his laboratory supported by the Commonwealth Scholarship Commission of the UK.

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Eric –A True Friend and Visionary

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I have known Eric for over four decades as an academic colleague.

In April, 1976 I had to accompany my husband to Nigeria when he took up an appointment as the chief resident engineer of a consultancy company. I had left for Nigeria by the time Eric joined the then Department of Biochemistry (now Department of Biochemistry and Molecular Biology), at the Faculty of Medicine where I had served as an Academic for a short time. Our paths crossed again when I returned to Sri Lanka and served as the Professor of Biochemistry first at the North Colombo Medical College (NCMC) and subsequently at the newly established Faculty of Medicine, University of Ruhuna.

I first heard about Eric's interest in medicinal plants from two colleagues at the NCMC (Gowry Sinnadorai and Techla Perera) who had worked as post graduate students in the Colombo Medical Faculty under Eric's supervision. I was most interested to hear about his attempts to scientifically validate the therapeutic benefits of medicinal plants used in Sri Lanka for the treatment of diabetic patients because I wanted to do the same with plants and other traditional remedies used for therapy of liver disease by medicinal practitioners in Sri Lanka. Besides the association with Eric as a Fellow Professor of Biochemistry, which led to interactions related to teaching and examinations of medical students, I collaborated

with him on research into hypoglycaemic plants while at the Ruhuna Medical Faculty in Karapitiya. Findings of these Investigations confirmed that plants such as *Ficus benghalensis*, *Artocarpus heterophyllus* and *Asterocantha longifolia* had high antidiabetic activity and rationalized their traditional uses. More close interactions developed once he established the IBMBB.

Although it was possible from preliminary studies conducted at Ruhuna Medical Faculty by the post-graduate student Rohan Fernando (who after completing the MPhil degree obtained his PhD from Japan and subsequently became employed as an Assistant Professor by an American University), to scientifically establish the therapeutic potentials of several plants used in traditional medical practice for management of diabetic patients, I realized that further studies were definitely needed to establish their mechanisms of action at a molecular level before any attempts could be made to develop active compounds identified during these studies, into drugs that could be used by clinicians for diabetic therapy. This became a possibility only after I joined the IBMBB.

When Eric heard that I was retiring from the University of Kelaniya in October 2007, he invited me to join the IBMBB as a Visiting Professor. I was very happy to accept this invitation as I would be able to continue with my academic career even after official retirement from the University. I felt that it would be more interesting to teach postgraduates. On my first day at the IBMBB, I was touched by the warm welcome extended by Eric and other members of the Institute.

Events that led to establishment of the IBMBB have been well articulated elsewhere in this volume. I would like to highlight some of my experiences

working within the IBMBB. To do that, I have adopted some content from my book titled “I faced it all and I stood tall”.

The working environment at the IBMBB was a complete contrast to what I had experienced in other Sri Lankan Universities. Lectures could be delivered in air conditioned comfort, and research could be carried out in the laboratories that had been built to International standards, and equipped with state of the art instruments. Besides lecturing in the program “MSc in Molecular Life Sciences” (initially teaching protein metabolism and later expanding to carbohydrate and lipid metabolism on Eric’s request) I also continued my research. Thus molecular studies on an anticancer herbal decoction were completed, as part of Prasanna Galhena’s doctoral studies (now a Professor in the Department of Biochemistry and Clinical Chemistry, Faculty of Medicine, University of Kelaniya and currently the Director, IBMBB). This was followed by doctoral studies of Sameera Samarakoon and Anuka Mendis. Sameera’s studies were supported by a Research Fellowship that I secured from the National Science Foundation while at the IBMBB. Sameera was also able to obtain a sample of Hep G2 cells for his research from India via the link I had established with the Sri-Ramachandra Medical College, Chennai, through the India- Sri Lanka Foundation, while being employed at the Ragama Medical Faculty. A second such a Fellowship and the Drug Leads from Medicinal Plants grant from Ministry of Health for which I was a co-investigator supported Anuka’s doctoral studies. Besides I was able to co-supervise several other PhD students and supervise several MSc students at the IBMBB, almost all in the field of anticancer plants and herbal remedies. Many publications arose from the research carried out.

In addition to carrying out teaching and research activities, I was able to help Eric and later the subsequent Directors in the management of IBMBB by serving its Board of Management to date and the Academic Committee and Research and Higher Degree Committee until last year. Looking back on the past few years, I have never regretted the decision to accept the invitation extended by Eric to assist the IBMBB. One fact that has really impressed me about the IBMBB was the camaraderie that existed not only between academic and non-academic staff, but also among the students. The students have been ever willing to assist their peers, as well as academic and non-academic staff whenever a need is perceived.

Eric's dedication and strong motivation in establishing the IBMBB, allowed many of us to reap the benefits either as teachers, scientists, students or other staff. It was a great pleasure to work with Eric, who I found was a great visionary with many sound ideas to develop the IBMBB to International standards. He was able to achieve his vision due to the fact that he was open to advice from others and also because of the cooperation extended to him by all the academic and non-academic staff of the Institute. Thank you Eric for enabling me to join the IBMBB family and also extending the freedom to develop an understanding of the mechanisms of action of anticancer herbal remedies (single plants and polyherbal medicines).

Anticancer Medicinal Plants and Cancer Stem Cell Research: A New Era of Anticancer Drug Screening Research in Sri Lanka

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Having a substantial collection of leaders in different disciplines is essential for the development of a nation. Emeritus Professor Eric Karunananayake is recognized as one such leader who dedicated his life for the development of Molecular Life Sciences in Sri Lanka. When I met him for the first time at the IBMBB in 2006, his personality as a scientist inspired me to be a future scientist. I was fortunate to have Professor Karunananayake as my MSc research project supervisor and to receive an invaluable supervision and guidance from him throughout the project.

He trained me in all the Molecular Biological techniques available at IBMBB at the time. Further, how he emphasized to me the value of scientific reading is very interesting. He asked me to sit in the IBMBB library and collect all the journal articles relevant to my MSc and advised me not to leave the library for any other purpose until the task is completed. The motivation he gave to practice scientific reading is the main stem of my current success as a scientist. During my MSc, Professor Karunananayake always encouraged me to re-install some machines (HPLC and real time PCR) and establish new technologies at the IBMBB. This chapter describes research conducted by me at the IBMBB during the last 12 years with the goodwill of Professor Karunananayake. Further direct and

indirect incredible service he has done for the development of the Country will also be highlighted in the chapter.

Anticancer drug screening and development

Cancer is recognized as a major global health issue and the second leading cause of death in most of the countries (Cui et al., 2020). A study conducted by Bray et al., 2018 estimated 18.1 million new cases and about 9.6 million cancer deaths worldwide for 2018. Further the same group indicated that more than 70 % of cancer related deaths occur in low and middle income countries. Lung cancer is recognized as the most commonly diagnosed cancer in the world followed by breast cancer among females, colorectal and prostate cancers. In Sri Lanka, 29,604 new cancer cases were reported in 2020 and the overall cancer incidences have increased by 100% during last 25 years. Breast cancer and cancers in lips and oral cavity have been reported as the most common cancers in Sri Lanka (Jayarajah et al., 2021; WHO, 2020). Consumption of tobacco and alcohol, obesity, insufficient physical activity, exposure to radiation, and bad dietary habits are associated with the risk of development of human cancers. Cancer occurs as a result of the alterations in the expression of collection of genes (Kunnumakkara et al., 2019).

Cancer therapies such as radiotherapy, chemotherapy and hormonotherapy have not completely reduced the number of deaths from cancer. Further, these treatments produce some unpleasant side effects. Patients on long term therapy develop resistance to the existing drugs. The potential of using natural products as anticancer agents was recognized in the 1950's by the US National Cancer Institute and has since made major

contributions to the discovery of new, clinically useful compounds with anticancer activity (Cragg et al., 2005).

When I started my PhD work in 2009 at the IBMBB under the supervision of Professor Ira Thabrew, Professor Kamani Tennekoon and Professor Dilip de Silva, the contribution of Sri Lankan scientists to anticancer drug screening and development was shallow. I started my PhD work with the aim of developing anticancer drug screening research in Sri Lanka.

During my PhD work, in addition to the support and guidance given by my supervisors, Professor Karunananayake played a key role by motivating me to isolate anti-hepatocarcinogenic compounds from a poly-herbal decoction comprised of *Nigella sativa* seeds, *Hemidesmus indicus* roots and *Smilax glabra* rhizomes, a traditional treatment used in cancer therapy. Standardization of herbal drugs helps to maintain the reproducible bioactivity and correct authentication of the formulation throughout the overall treatment process. In my PhD studies, the above decoction was standardized by evaluating organoleptic characters, physicochemical properties, qualitative and quantitative analysis of chemical constituents, High-Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC) (Samarakoon et al., 2010).

Effects of the decoction on apoptosis in human hepatocellular carcinoma (HepG2) cells were confirmed by (a) light and fluorescent microscopic observation of cell morphology (b) DNA fragmentation analysis (c) estimating caspase 3 and caspase 9 activities and (d) RT-PCR and western blot analysis. The mRNA and protein expression of tumor suppressor p53, cell cycle regulator p21, and pro-apoptotic Bax was up regulated in a dose and time dependent manner, while MDM2 (a negative regulator of p53),

anti apoptotic Bcl-2, and inhibitor of nuclear factor kappa B IKK- α were down regulated in a dose and time dependent manner as assessed by RT-PCR and western blot analysis. Decoction did not affect mRNA and protein expression of the nuclear factor kappa B (p50 and p65) in HepG2 cells. Immunohistochemical analysis further confirmed a dose dependent regulation of above genes in HepG2 cells (Samarakoon et al., 2012a, Samarakoon et al., 2012b). Finally, I was able to isolate kalopanaxsaponins I, a triterpenoidal saponin, from the above decoction as the first anticancer compound to be isolated at the IBMBB. Still I can remember, receiving some opinion from Professor Karunananayake regarding the above compound isolation process in addition to guidance from my supervisors.

Anticancer properties of Mangrove plants

While I was completing my PhD, Professor Karunananayake and Professor Tennekoon encouraged me to apply for a competitive research grant. As a result of this motivation, I secured a competitive research grant from the International Foundation for Science (IFS), Sweden to screen mangroves grown in Sri Lanka for their anticancer properties. Findings of this project were reported in five research papers in international peer reviewed journals. The mangroves in Sri Lanka comprise of 22 true species predominantly seen in the amphibious mangrove ecosystem. In Sri Lanka, mangroves are commonly found in Puttalam and Kalpitiya areas associated with estuaries. Since most of these mangrove plants had not been studied for their chemical, pharmacological and toxicological properties and also had not been screened for possible anticarcinogenic compounds, our investigation was designed to study 15 mangrove plants found in Sri Lanka

for anticancer activity and to characterize anticancer natural compound (s) structurally and functionally.

In this research, leaves and stems of 15 mangrove plants were collected separately and sequentially extracted into different solvent systems. Preliminary, cytotoxic potentials of the above extracts were tested in two cancerous (hepatocellular carcinoma-HepG2, breast adenocarcinoma MCF-7) cell lines using the Sulphorhodamine (SRB) assay. According to the results of the cytotoxicity screening, out of the 15 plants, nine were identified to have at least one extract having potential cytotoxic properties (Samarakoon et al., 2016a). Based on the results of preliminary cytotoxic screening, leaves of *Scyphiphora hydrophyllacea* were selected for bioactivity guided isolation of anticancer compounds. All together four compounds (ursolic acid, oleanolic acid, eichlerianic acid, hopenone I) were isolated from *S. hydrophyllacea* leaves for the first time and their chemical structures were elucidated using NMR spectroscopy. Out of the four isolated compounds three (ursolic acid, eichlerianic acid, hopenone I) compounds exerted time and dose dependent cytotoxic effects in both breast and liver cancer cells and eichlerianic acid and hopenone I, induced apoptosis in both cell lines tested (Samarakoon et al., 2016b, Samarakoon et al., 2018).

Two other studies were carried out with the aim of strengthening and providing supportive evidence for anticancer effect of hexane and chloroform extracts of *S. hydrophyllacea* leaves and methanol extract of *Phoenix paludosa* Roxb leaves. The hexane and chloroform extracts of *S. hydrophyllacea* leaves showed dose and time dependent cytotoxic effects. Morphological changes observed under fluorescence microscope showed signs of apoptosis; a significant increase in caspase 3 and 9 levels were

also observed in response to the extracts. The mRNA expression of p53 and Bax were up-regulated by low doses of hexane and chloroform extracts. Highest antioxidant activity was observed in the methanol extract. GC-MS profiles identified 24 and 4 major compounds in the hexane and chloroform extracts, respectively. These included some known anticancer compounds such as lupeol (Samarakoon et al., 2017). The results obtained from the second study indicated that the methanol leaf extract of *P. paludosa* contains potent antioxidant and cytotoxic activities against breast, renal and kidney cancer cells (Samarakoon et al., 2016c).

In silico drug screening and cancer stem cell research in Sri Lanka

Drug screening and development is a complex and time consuming process (Lin et al., 2020). With the advancement of computational chemistry, drug discovery for several diseases benefited dramatically by reducing the time and cost of drug development (Bakheet et al., 2009). Several Bioinformatics tools used in *in silico* drug screening methods further help to predict drug target proteins associated with common diseases including cancer (Lin et al., 2020).

Just after my PhD, I wanted to establish new technologies at the IBMBB with the continuous motivation of Professor Karunananayake and Professor Tennekoon. In 2013, I designed an MSc project for a Bioinformatics master student, Karthika Mayan at the IBMBB as the initial step in establishing *in silico* drug screening methods. It was on *in silico* screening of a small natural compound library for cancer stem cell targeted anticancer activity (Mayan, 2014).

The cancer stem cells (CSCs) or tumor-initiating cells (TICs), a small subset of cells, are responsible for the initiation, extensive proliferation and metastasis of a cancer. CSCs have the capacity to generate a heterogeneous population of cells in a tumor. These cells are highly resistant to conventional chemotherapy and radiotherapy (Atashzar et al., 2020). From a clinical standpoint, the CSC hypothesis provides a compelling explanation for cancer patients who often relapse after treatment (Atashzar et al., 2020). Wnt, Hedgehog, and Notch signaling pathways are recognized as three key evolutionary conserved CSC signaling pathways. Aberrantly activated Wnt, Hedgehog, and Notch signaling pathways are implicated in the modulation of CSCs. Karthika found that a natural compound, gedunin, from the neem tree (*Azadirachta indica*), may act as a useful inhibitor against the Wnt signaling pathway and has the potential to become a natural anticancer stem cell drug (Mayan et al., 2016). We have now taken the *in silico* screening to the next level at the IBMBB by incorporating this technology to screen large compound libraries in our current research on anticancer drug discovery.

As I mentioned previously, the continuous motivation given by Professor Karunananayake helped me to obtain a competitive research grant from the National Research Council (NRC) to screen selected endemic plants for anticancer stem cell activity in 2014. This was the first grant I obtained to establish *in vitro* CSC research at the IBMBB. Ms. Umapriyatharshani Rajagopalan registered as my first PhD student at the IBMBB in 2014 under the above grant. We established a manual isolation method for breast CSCs for the first time in Sri Lanka at the IBMBB. Breast CSCs were isolated from two breast cancer cell lines [MCF-7 (ER-positive) and MDA-MB-231(triple-negative)] and isolated cells were used for CSCs

based research activities. Among the ten endemic plants screened for their anti-breast CSC properties, hexane and chloroform extracts of *Garcinia zeylanica* bark exhibited highest antiproliferative effects on bCSCs/MCF-7 and bCSCs/MDA-MB-231 cells. Garcinol and nigrolineaxanthone-E (from the hexane extract), gerontroxanthone-I and 14-deoxygarcinol (from the chloroform extract) were isolated from the *G. zeylanica* bark using bioactivity guided fractionation. All the isolated compounds demonstrated antiproliferative effects and suppressed mammosphere formation efficiency and alkaline phosphatase activity in bCSCs/MDA-MB-231; and the effects were time and dose dependent. Among the active compounds, garcinol showed the highest antiproliferative effects with less cytotoxic effects on MCF-10A, normal mammary epithelial cells compared to paclitaxel, a currently used anticancer drug. Further, garcinol induced caspase-dependent (caspase 3/7) early and late apoptosis. *In-silico* analysis of drug likeness of the compounds predicted that all four compounds are potential drug leads.

Analysis of human CSC-pathway targeted PCR array revealed remarkable regulation of genes related to breast cancer disease (CSC markers, cell proliferation, self-renewal, pluripotency, asymmetric division, cell migration and metastasis, cancer therapeutic targets) and CSC signal transduction pathways (Notch and Stat/NF-Kb) in bCSCs/MDA-MB-231, treated with 6 μ M of garcinol. Moreover, garcinol exhibited strong potential to become an effective lead molecule specifically affecting growth of bCSCs by modulating the genes involved in Notch and Stat/NF-Kb signal transduction pathways.

Gedunin, recognized as a possible drug lead targeting CSCs during the *in silico* study conducted by Mayan et al., 2016 was also tested *in-vitro*. Its

anti-CSC properties were evaluated in a CSC model known as human embryonal carcinoma (NTERA-2) cells. Gedunin enhanced apoptosis in NTERA-2 cells by regulating few key genes associated with induction of apoptosis (Tharmarajah et al., 2017).

Use of Nanotechnology in anticancer drug research

I had a habit to discuss new research ideas with Professor Karunanayake and Professor Tennekoon when they have free time at the IBMBB. One day, during such a discussion, I informed them about Professor Needra Karunaratne's Nanotechnology based research at the University of Peradeniya. Following my discussions to initiate Nanotechnology based research at the IBMBB, Professor Karunanayake recommended Professor Needra as a good collaborator for my research. To date three MSc students have worked in the field of nano assisted drug delivery systems under my and Professor Needra's supervision.

First such study was conducted by Daniel Nwokw, a recipient of President's scholarship from Nigeria to follow the MSc in Molecular Life Sciences at the IBMBB. The study revealed that the nano-encapsulated liposomal gedunin and chitosan nano-encapsulation can inhibit the growth of human non-small-cell lung cancer (NCI-H292) cells by inducing apoptosis (Nwokwu, 2015; Nwokwu et al., 2017a; 2017b). Another study was conducted by Mohan Sing Rana, also a President's scholarship holder from Bhutan. He developed a new liposomal nano-carrier for co-delivery of gedunin and p-glycoprotein siRNA [siRNA coated liposomal gedunin (Lipo-Ged-siRNA)] to improve the antiproliferative activity of gedunin. In this study siRNA was used to block the translation of p-glycoprotein (P-

gp) which is located in CSC membrane. Characteristics of prepared Lipo-Ged-siRNA demonstrated promising effects. Anticancer stem cell properties of gedunin was drastically increased; expression of P-gp was reduced following exposure to Lipo-Ged-siRNA. Further, Lipo-Ged-siRNA affected the expression of *Bax*, *p53*, *ABCB1*, *Cyclin D1*, and *survivin* genes in bCSCs (Rana, 2016).

The compound garcinol, identified as a potential CSC inhibitor during Uma's PhD studies, was selected for another nanotechnology based study. Amal Hulangamuwa developed a novel nano carrier comprising of garcinol, hyaluronic acid (HA), and poly (lactic-co-glycolic acid) (PLGA) to specifically target breast cancer stem cells (bCSCs) grown under hypoxic conditions. Further, this study confirmed pro-apoptotic effects of the above prepared nano-carrier containing garcinol. *In vitro* anticancer effects of garcinol was improved by the nano formulation and it mediated significant down regulation of hypoxia-inducing factors and notch pathway-related genes in breast CSCs (Hulangamuwa, 2017; Hulangamuwa et al., 2021).

CRISPR Cas 9 in to the IBMBB

CRISPR becomes an indispensable biological tool and Cas 9 enzyme has revolutionized different disciplines of life sciences (Adli, 2018). CRISPR Cas 9 genome editing tool has strengthened the ability of changing DNA and RNA sequences in living cells (Pickar-Oliver and Gersbach, 2019). The scientists who discovered the above technology were awarded the Nobel Prize for Chemistry in 2020. In 2016, I wanted to use this technology to make a transgenic cell line which can be used to screen cancer stem cells targeted small molecules. One day, after lunch, Professor

Karunananayake and Professor Tennekoon were in a discussion in the office room of Professor Tennekoon. I also joined their discussion and mentioned about my idea to use CRISPR Cas 9 technology for my research work. Same evening, Professor Karunananayake called me to his office room and gave a review article on CRISPR Cas 9 technology. That incident further encouraged me to initiate CRISPR Cas 9 application for the first time at the IBMBB in 2016. This episode occurred 9 years after Professor Karunananayake's official retirement from the IBMBB and demonstrates how he continued to be up to date, and encourage and support junior scientists.

One of my MSc students, Nirmal Perera developed a reporter gene assay creating a transgenic cell line to search for possible inhibitors of Wnt/beta-catenin pathway in cancer stem cells by external effectors. In this development, an expression cassette containing a minimal promoter, a gene for nano-luciferase and a separate gene for green fluorescent protein was constructed. T-cell factor/lymphocyte enhancer factor binding element repeats were cloned adjacent to the minimal promoter of the expression cassette. This expression cassette containing T-cell factor/lymphocyte enhancer factor binding element repeats was knocked-in to adeno-associated virus 1 integration site of HEK293 cells by using CRISPR Cas 9 (Perera, 2017). Amalie Jayawickrama, also an MSc student developed another reporter gene assay to screen small molecules which can inhibit Hedgehog (Hh) signaling pathway, a key regulatory pathway of cancer stem cells (Jayawickrama, 2019). In both studies, prepared reporter gene cassettes were transferred to human embryonic kidney (HEK293) and human embryonic kidney 293T (HEK293T) cells, respectively by using CRISPR Cas 9 technology.

Concluding remarks

From 2007 to the present, I was able to establish cancer stem cell research in Sri Lanka, escalate anticancer drug discovery and initiate different technologies for research at the IBMBB with the guidance and opinion provided by Professor Karunananayake. Further, the association I had with Professor Karunananayake helped to create a new era of anticancer drug research in Sri Lanka by establishing and strengthening different technologies at the IBMBB.

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Medicinal Plant *Mangifera zeylanica*: Traditions of Yesterday and Drug Leads of Tomorrow

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Professor Eric Hamilton Karunananayake has made a remarkable contribution to the nation by leading the establishment of the IBMBB which has become a national asset. The IBMBB serves as one of the research hubs in the country by developing human capacity in the fields of Biochemistry, Molecular Biology, Biotechnology, Bioinformatics and Immunology with research training at postgraduate levels. Apart from Professor Karunananayake's contributions to the fields of Biochemistry, Molecular Biology and Biotechnology, he has worked in the field of Medicinal Chemistry. The paper he co-authored: Isolation of mangiferin from the bark of *Mangifera zeylanica* is the first publication from Sri Lanka with the keyword *Mangifera zeylanica* published in the journal Phytochemistry (Herath and Karunananayake, 1970). His initial research findings related to *M. zeylanica* gave me a rational approach to start my doctoral studies with the medicinal plant *M. zeylanica*. Also, as an MSc student (2010-2012), I was fortunate to attend Professor Karunananayake's lectures in Biochemistry and Molecular Biology at the IBMBB which provided me with in depth knowledge to embark on doctoral studies. In this chapter, I provide information on my experimental findings with *M. zeylanica* during my tenure (2012-2016) as a doctoral student at the IBMBB.

Medicinal plants and traditional medicine in Sri Lanka

For ages, plants have offered man with all his requirements in terms of food, shelter, clothing, fertilizers, medicines and fragrances (Gurib-Fakim, 2006). Plants have provided the foundation of traditional and complementary medicine systems which have been in existence for more than five thousand years and still last to supply human with new strategies (Bussmann and Sharon, 2006; Gurib-Fakim, 2006). Most of the plant based remedies rely on empirical findings of thousands of years and communications passed from one generation to another (Bussmann and Sharon, 2006; Gurib-Fakim, 2006). The vast majority of people in the developing countries use medicinal plants and other materials for their healthcare (Jamshidi-Kia et al., 2018). People who utilize medicinal plants based therapies may not fully understand the scientific actions/mechanisms implicated in their medicines (Gurib-Fakim, 2006). However, through personal experiences and communications they are aware that some plant based therapies can give meaningful results when used at clinical/ therapeutic doses (Alves and Rosa, 2007).

Compared with olden days, as we are now equipped with modern chemistries, knowledge about the function of the human body and diseases at the molecular level, we have a great opportunity to understand the miracle healing abilities of plant based remedies and to get an idea about multi-functional secondary metabolites present in plants. Medicinal plants possess a wide range of structurally different compounds which may function individually or in synergy to act against many diseases (Atanasov et al., 2015). It is interesting to note that traditional medicine advances the likelihood of interactions of drugs with pharmacological targets through maximizing synergistic effects or by balancing potential efficacies of

chemically complex plant derived compounds (Atanasov et al., 2015). Today, in most countries, people use western (allopathic) and traditional medicines together in complementary schedules. However, in some acute cases traditional medicines have proven to be erroneous, highlighting the importance of allopathic medicine (Gurib-Fakim, 2006).

Sri Lankan traditional medicine system is one of the oldest and diverse medicine systems (Arsecularatne et al., 1985; Karunananayake et al., 1984). In Sri Lanka, among some traditional healers or physicians, knowledge and wisdom have been delivered from one generation to another generation, which physicians had to memorize and recite. Some generations have preserved the knowledge and wisdom by writing in ola leaf books. Medicinal plants (different parts or whole plant) have formed the basis of Sri Lankan traditional medicine system (Gunawardana and Jayasuriya, 2019; Kankanamalage et al., 2014). It has been reported that there are about 3771 flowering plant species found in Sri Lanka. Of them, nearly 1430 plant species are considered to possess medicinal properties and, out of these, 174 plant species are endemic (Gunawardana and Jayasuriya, 2019; Kankanamalage et al., 2014).

The medicinal plant *M. zeylanica* and its traditional uses

M. zeylanica is a plant endemic to Sri Lanka which also belongs to the same family as *M. indica* (Dassanayake and Fosberg, 1983). It is commonly referred as ‘Etamba’. *Mangifera zeylanica* is a large evergreen tree mostly found in dry zones. It grows to a height of 10-35 m. Trees are branched from a stout trunk. Leaves are 7-13 cm long, dark green and shiny. Fruits are edible and they are 3-5 cm long (Dassanayake and

Fosberg, 1983) (Figure 1). The ripe fruits are yellow in color. Bark of *M. zeylanica* has been used in the traditional Sri Lankan medicine to treat some conditions such as vomiting, fever, uterine ailments, bladder diseases, diarrhea, oral diseases, hiccups, gastro intestinal diseases, fractures and cancers (Ashton et al., 1997; Ayurvedic medicinal plants in Sri Lanka; Batugal et al., 2004).



Figure 1. *M. zeylanica* tree (A), bark (B) and fruits and leaves (C)

Giving a scientific validation for M. zeylanica

Prior to my doctoral studies, Dr. Sameera R Samarakoon, currently a senior lecturer at the IBMBB, and Dr. Anuka Mendis, now founder of the Suaroma therapy oils, had initiated pre-clinical investigations with *M. zeylanica* crude extracts under the guidance of Professor Ira Thabrew (Mendis, 2012). They first strengthened the process of scientific validation of *M. zeylanica*'s use against cancer in the Sri Lankan traditional medicinal system by investigating the effects of the total methanolic extract of *M. zeylanica* bark on the proliferation of liver cancer cells *in-vitro*. According to their findings, the methanolic extract of *M. zeylanica* bark showed strong growth inhibitory effects on liver cancer cells (Mendis, 2012).

Furthermore, the methanolic extract induced apoptosis in liver cancer cells. These experimental findings and Professor Karunananayake's initial work with *M. zeylanica* provided me a strong direction to start my doctoral studies with the medicinal plant *M. zeylanica* under the supervision of Professor Kamani Tennekoon, Professor Ira Thabrew and Professor Dilip de Silva (then at the Department of Chemistry, University of Colombo). My principal supervisor, Professor Tennekoon received a grant from the National Research Council (NRC) of Sri Lanka to support my doctoral studies. In August 2012, I began my doctoral studies with the support and guidance of Dr. Sameera Samarakoon. He helped me to learn chromatographic and animal cell culture techniques required for my project. I remember Professor Karunananayake watching my chromatographic separations with concern in the second floor of the IBMBB, where his office was located. He had a strong interest to monitor my findings with *M. zeylanica* throughout my project.

Anticancer potential of M. zeylanica bark extracts

To obtain a clear anticancer profile of *M. zeylanica* bark, it was first extracted to four organic solvents (hexane, chloroform, ethyl acetate and methanol) sequentially. Of the four extracts, the hexane extract of *M. zeylanica* bark demonstrated strong antiproliferative effects in breast (oestrogen receptor positive MCF-7 breast cancer cells and triple negative MDA-MB-231 breast cancer cells) and ovarian cancer (SKOV-3) cells, with less cytotoxicity to normal mammary epithelial (MCF-10A) cells (Ediriweera et al., 2016a). Furthermore, the apoptotic potential of the hexane extract of *M. zeylanica* in breast and ovarian cancer cells was confirmed with fluorescence microscopy and gene expression studies

using real-time polymerase chain reactions (real-time PCR). The hexane extract of *M. zeylanica* up-regulated pro-apoptotic *Bcl-2* and tumor suppressor *p53* genes and down-regulated antiapoptotic *Survivin* (Ediriweera et al., 2016a). Gas Chromatography Mass Spectrometry (GC-MS) analysis of the hexane extract of *M. zeylanica* bark and some cytotoxic fractions of the hexane extract identified several anticancer compounds and unknown lipophilic compounds that led to isolation of anticancer compounds from the bark hexane extract.

***M. zeylanica* fruit peel exerted anti-cancer potential**

We reported, for the first time, that the fruit peel of Sri Lankan endemic mango exerts antiproliferative effects in MCF-7 breast cancer cells through an oxidative stress related mechanism (Ediriweera et al., 2017a). The chloroform extract of *M. zeylanica* fruit peel obtained following sequential solvent extractions showed promising anti-cancer effects in breast cancer cells indicating that the *M. zeylanica* peel possesses secondary metabolites with anti-cancer potential. The organic extracts of fruit flesh did not show inhibitory effects on breast cancer cell proliferation. MCF-7 breast cancer cells exposed to the peel chloroform extract showed signs of apoptosis (Ediriweera et al., 2017a). Furthermore, peel chloroform extract increased reactive oxygen species (ROS), malondialdehyde (MDA), glutathione-S-transferase (GST) and super oxide dismutase (SOD) levels, implying that the peel chloroform extract can activate an oxidative stress related mechanism in MCF-7 cells (Ediriweera et al., 2017a). GC-MS analysis of the peel chloroform extract identified several anticancer compounds such as α -tocopherol, linoleic acid and beta-sitosterol (Ediriweera et al., 2017a). Our findings showed that the anticancer potential of *M. zeylanica* is not

limited to the bark and provided a future direction to isolate anticancer compounds present in the fruit peel.

Novel anti-cancer compounds from the bark of *M. zeylanica*

Initial studies carried out with *M. zeylanica* bark extracts gave a strong indication for the presence of novel anticancer compounds in the bark extracts. A new resorcinolic lipid (5-((8Z, 11Z, 14Z)-hexatriaconta-8, 11, 14-trienyl) benzene-1, 3-diol) with 36 carbons in the alkyl side chain was isolated from the bark hexane extract of *M. zeylanica* (Ediriweera et al., 2017b) (Figure 2). The structure of the isolated compound was elucidated using ¹H and ¹³C NMR spectrometric techniques. To obtain NMR spectra of isolated compounds, I had to visit the Hussain Ebrahim Jamal (HEJ) Research Institute of Chemistry, University of Karachi, Pakistan. It's then Director, Professor Iqbal Choudhary, who was well known to Professor Karunananayake very kindly accepted me to HEJ. NRC financially supported my visit. At the HEJ, I worked under the immediate supervision of Dr. Achyut Adhikari, who is now at the Tribhuvan University, Kirtipur, Nepal. The isolated resorcinolic lipid showed potent cytotoxic effects in breast and ovarian cancer cells and induced apoptosis. In addition, it was found to induce oxidative stress in MCF-7 breast cancer cells by increasing the levels of ROS, GST and declining GSH levels dose-dependently (Ediriweera et al., 2017b).

From the chloroform extract of the *M. zeylanica* bark two interesting new compounds, chloromangiferamide and bromomangiferic acid (Figure 2), along with two known compounds, quercetin and catechin were isolated (Ediriweera et al., 2016b). As the names indicate, compounds

chloromangiferamide and bromomangiferic acid contain halogens, chlorine and bromine atoms, respectively (Figure 2). Although we found halogenated compounds in the bark of *M. zeylanica* for the first time, a chlorinated compound (3-chloro-N,2-dimethyl-N-(2-phenylethyl) propanamide) had already been isolated from the bark of *M. indica*, which supported our findings (Singh et al., 2015). Chloromangiferamide demonstrated interesting experimental findings. Chloromangiferamide exerted breast cancer cell line specific cytotoxicity. Interestingly, it only killed triple negative breast cancer cells (MDA-MB-231), but not oestrogen receptor positive MCF-7 cells with no cytotoxicity to normal mammary epithelial cells (MCF-10A).

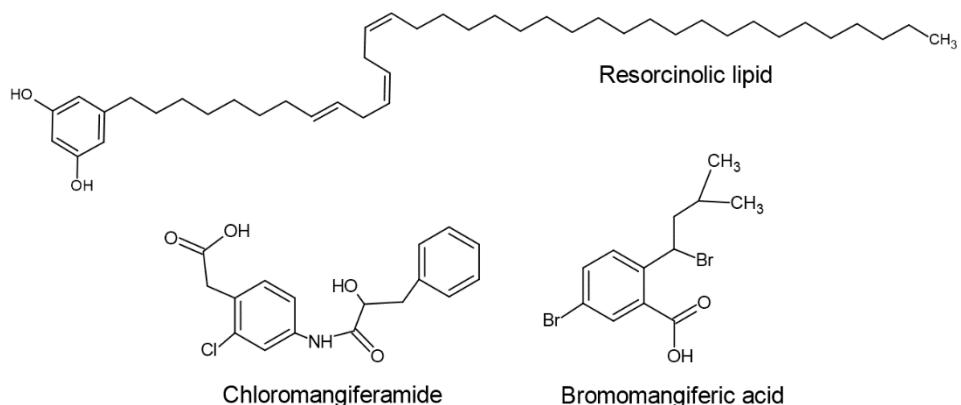


Figure 2. The chemical structures of compounds isolated from the bark of *M. zeylanica*. (Ediriweera, 2017)

To investigate the possible anti-cancer mechanisms of chloromangiferamide in MDA-MB-231 cells, real-time PCR arrays for cancer drug targets were employed. Chloromangiferamide was found to inhibit the expression of aberrantly expressed major cancer drug target genes related to cell cycle (*CDK1*, *CDK2*, *CDK4*, *CDK5*, *CDK7*, *CDK8*,

CDK9, MDM2 and MDM4, tyrosine kinases (*AURKA, AURKB, AURKC, PLK1, PLK2* and *PRKCA*), histone deacetylases (*HDAC1-8* and *11*), PI-3 kinases and phosphatases (*PIK3C3, PIK3CA* and *PIK3C2A*), topoisomerases (*TOP2A* and *TOP2B*) and transcription factors (*ATF2, IRF5, HIF1A* and *NFKB1*), indicating chloromangiferamide as a promising drug lead to target most of the aberrantly expressed cancer genes (Ediriweera et al., 2016b) (Figure 3). Bromomangiferic acid was not cytotoxic to cancer cells.

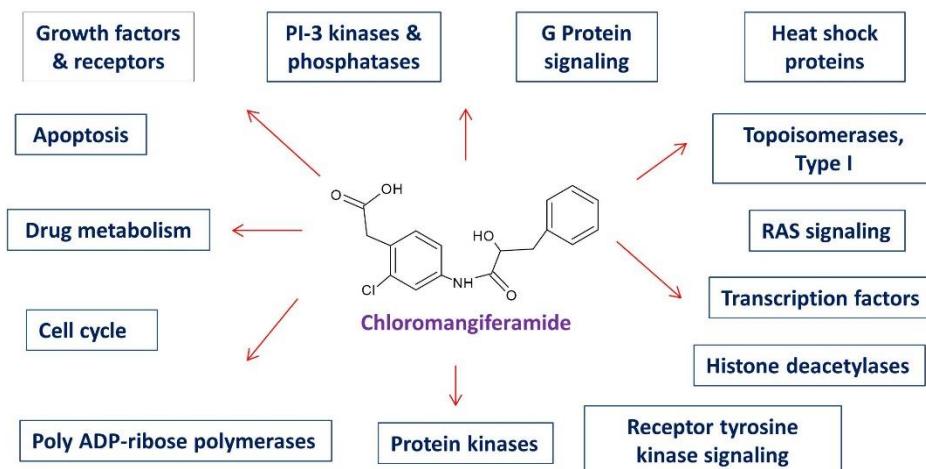


Figure 3. Cancer drug targets of chloromangiferamide identified using real-time PCR array

Chloromangiferamide shows structural similarities with clinically used and clinically tested drugs

Vismodegib is an FDA approved drug for the treatment of locally advanced or metastatic basal cell carcinoma. It is an antagonist of the smoothened receptor (SMO) involved in the hedgehog signaling pathway (Dlugosz et al., 2012). Vismodegib shows some structural similarities with

chloromangiferamide (Figure 4). Some clinically tested histone deacetylase inhibitors (HDACi) such as chidamide, entinostat, mocetinostat, tacedinaline and 4SC202 show structural similarities with chloromangiferamide (Ediriweera et al., 2019) (Figure 4). The results of the PCR array also showed that chloromangiferamide can inhibit the expression of different histone deacetylases (HDACs). The chemical structure of potent and selective GPR139 agonist JNJ 63533054 displays similarities with chloromangiferamide (Shoblock et al., 2019) (Figure 4). This interesting information gives a strong direction to investigate the detailed *in-vitro* and *in-vivo* efficacies and different pharmacological properties of chloromangiferamide and structural derivatives of chloromangiferamide, and to develop them as clinically useful drugs.

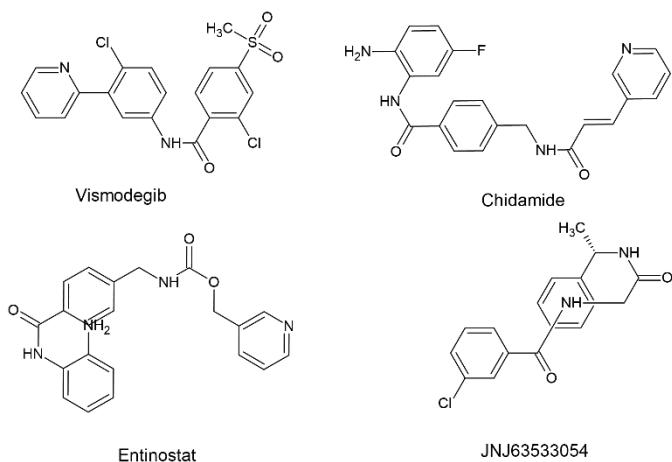


Figure 4. Some clinically used and clinically tested drugs (vismodegib, chidamide, entinostat and JNJ 63533054) show structural similarities with chloromangiferamide

Conclusions and future directions

The hexane and chloroform extracts of *M. zeylanica* bark demonstrated cytotoxic and apoptotic potential in breast and ovarian cancer cells, thereby providing a strong scientific validation for its use in the Sri Lankan traditional medicine system. The chloroform extract of *M. zeylanica* fruit peel also demonstrated antiproliferative effects in breast cancer cells through an oxidative stress associated mechanism, providing a rationale to use *M. zeylanica* fruit peel as a cheap dietary source with anticancer properties.

The isolated resorcinolic lipid showed potent cytotoxic effects in breast and ovarian cancer cells through inducing oxidative stress. Some resorcinolic lipids have been reported to exert anticancer effects through alteration of the lipid bilayer architecture of the cell membranes of cancer cells (Ediriweera et al., 2020). Therefore, future studies are warranted to investigate cancer cell membrane targeting properties of the isolated resorcinolic lipid. Chloromangiferamide showed specific anticancer effects in triple negative breast cancer cells and it regulated expression of genes related to apoptosis, drug metabolism, cell cycle, receptor tyrosine kinase signaling, protein kinases, histone deacetylases, growth factors and receptors, topoisomerases, PI-3 kinases and phosphatases. Chloromangiferamide and its structural derivatives will be more appropriate for clinical assessments as a selective anticancer compound for triple negative breast cancer. Already the anticancer drug discovery group at the IBMBB are exploring structural derivatives of chloromangiferamide.

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The Potential and Benefits of International and Interdisciplinary Collaborations

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Scientific work has often been restricted to one particular research area, where researchers have investigated specific problems using methods typical for their specific field of research. However, the recent technological developments and novel advanced laboratory equipment open up for international and interdisciplinary collaborations to explore questions that have remained unanswered for many years. Rapid technological advancements have been seen in many areas, and in particular within Genetics and Molecular Biology. This accelerating exploration of human genomes has resulted in the discovery of many new molecular markers as natural polymorphisms, variants correlated with diseases or externally visible characteristics (ECV). These markers can be used to identify individuals, diagnose a disease or predict the appearance (e.g. eye and hair color) of a person that left a biological trace in non-suspect cases. An interesting combination of interdisciplinary research is achieved when this novel genetic information is utilized within humanities and cultural heritage studies. For example, archaeological and historical investigations explore the human past by analysis of physical remains, artefacts or written documents.

For some questions, however, historical, anthropological or archaeological data might not give a conclusive answer. Such investigations often involve identity, familial relationships or migration and may be answered by

genetic analysis. Are the remains found in a mass grave after conflicts belonging to related individuals? Are the individuals from an archaeological excavation from Europe, Asia or are they admixed with other populations, or originate from more distant parts of the world? Is a specific tomb the actual burial place of a famous historical person whose remains have been sought for centuries? This can be investigated by novel and improved DNA analysis techniques, which allow DNA typing, even from highly degraded and very old samples. Collaboration between archaeologists, historians and geneticists, has solved some intriguing mysteries from the past that would not have been possible without joint efforts.

Moreover, using the same technologies and methods may help the police and law enforcement to provide investigative leads by predicting a perpetrator's appearance by analyzing markers for externally visible characteristics (ECV) in non-suspect cases. In forensic cases with suspects, a DNA analysis will provide crucial technical evidence for the following court hearings based on nuclear, mitochondrial or Y-chromosomal markers. International and interdisciplinary research will at its best constitute a link between the knowledge and experience of experts from different fields of science and thereby allow a deeper exploration of the subject. Geneticists have methodologies and tools to deliver genetic information even from ancient and historical samples, but the results must be interpreted in historical and archaeological contexts. Similarly, the geneticists can provide DNA profiles and the actors in the judiciary system put these into the case context for a holistic evaluation of the evidentiary value. An example demonstrating the importance of this type of collaboration is the identification of the putative remains of the famous

astronomer Nicolaus Copernicus, which will be described here.

The mystery of Copernicus remains and other historical questions

The search for the remains of the famous astronomer Copernicus has been going on for many years and even Napoleon was involved in the efforts. The search was mainly directed towards the Frombork Cathedral in Poland, where the remains were buried in 1543 according to historical files. Here, a big problem arose because there are over 100 unnamed tombs in the Cathedral, and the location of the Copernicus grave among these was unknown. Nevertheless, since Copernicus served as a canon in the church, an assumption was made that the grave could be located near the St. Cross Altar, which was considered to be “his own” altar. Indeed, during excavations, several skeletons were found in this specific place in 2005. One of the skeletons was from an older man with a characteristic wound above his right eye. The wound indicated that it might have been Copernicus’ remains that had been found because it is known that the astronomer had a wound above his eye and that he died at the age of 70 years. This conclusion was based on morphological studies, but more information was sought to confirm the remains’ authenticity.

DNA analyses have been commonly used for individual identification for decades, and it has become possible to perform DNA typing even from old and highly degraded samples. The putative remains of Copernicus seemed to be well preserved, which was holding promise for a successful analysis. Apart from high quality and quantity of DNA, a reference material for comparison of maternal or paternal lines is crucial for a genetic identification. Unfortunately, a search for any biological material from

Copernicus relatives failed, but hope was reawakened when it was proposed to search for reference material in Uppsala, Sweden. The library of Uppsala University, Carolina Rediviva, has a total of 45 books from Copernicus, which were taken as war booty during the Swedish invasion of Poland in 1662 from the Polish Chapter Library in Frombork. Although representing a war booty, considering the complications and vicissitudes of Polish history, this valuable book collection might not have survived to the present day if kept in Poland. One of the books was highly interesting, the astronomical reference book, *Calendarium Romanum Magnum*, written by Johannes Stoeffler. This calendar was used by Copernicus for many years, and has been exhibited in the Museum Gustavianum of Uppsala University for a long time. With the idea that Copernicus was the principal user of the book, it might be possible to find biological traces from him inside the book. Indeed, after a thorough examination of the book, several shed hairs were found in the midsection of several different book lookups. DNA was then extracted from these hairs to serve as a possible reference material for the remains found in the Polish Cathedral. To avoid contamination, the extraction of DNA and further genetic analyses were performed in a cleanroom, specially designed and used solely for sensitive DNA investigations. Furthermore, each hair was extracted separately to avoid cross-sample contamination.

To compensate for the challenges seen with aged DNA (lacking intact DNA strands) mitochondrial DNA (mtDNA) is often used, rather than nuclear DNA markers present in only two copies per cell. Mitochondrial DNA exists in up to a thousand copies in each cell, allowing analysis despite limited, damaged and degraded DNA. Two parts of the mitochondrial genome are routinely used for human individual

identification, the hypervariable region I (HVI) and II (HVII). As only limited amounts and degraded DNA was expected from the hairs sampled from *Calendarium Romanum Magnum* (if they belonged to Copernicus), mtDNA was chosen to be analyzed. Both longer and shorter fragments of the HVI and HVII regions were analyzed, and a better success was achieved in amplifying shorter fragments, suggesting a high degree of degradation of the DNA. Out of nine collected hairs, four resulted in three different mtDNA profiles (haplotypes). Two of the profiles differed from each other and from the remains found in Frombork (bones and teeth were analyzed). However, one profile, found independently in two of the hairs, was identical to the DNA profile in the remains. With these intriguing results, we investigated how common or rare the matching mtDNA profile (or haplotype) is in the contemporary European population. We used the EMPOP database, a compilation of mitochondrial DNA profiles from different human populations worldwide. The matching haplotype was observed among 4 out of 3 830 individuals in the West Eurasian population. Standing alone, the value of the DNA evidence, in this case, is relatively low, but together with historical and archaeological data, it constitutes a valuable piece of additional evidence in the identification of Copernicus' remains.

The international and interdisciplinary approach with the cooperation of researchers from many different fields in Sweden and Poland was thus crucial to get all the pieces of the puzzle put together to identify the remains of Nicolaus Copernicus. Without the historians and archaeologists long-lasting and extensive investigations, it would have been impossible to find the location of Copernicus' grave. Moreover, without the reference material from the hairs in the book in the library for comparison and the

idea to search in Copernicus books, it would not have been possible to genetically support the authenticity of the remains of Copernicus being those found in Frombork. The knowledge and experience of experts from different disciplines were thus invaluable for the identification of Copernicus. For several years, we have been collaborating with crime scene investigators, forensic pathologists, archaeologists, osteologists, historians, chemists and other specialists from different disciplines by exchanging knowledge and skills. A significant benefit of the collaborations is that a broad spectrum of data, knowledge, methods and ideas from different areas of science is merged. As geneticists, we appreciate that we can take part in solving forensic cases, and historical mysteries from the past, and it is evident that without the knowledge of the crime scene investigators, prosecutors, historians and archaeologists we can only obtain partial information. We have new tools and technologies that allow us to retrieve genetic information, but our data needs to be interpreted, explained and complemented by forensics, criminal investigations, historical and archaeological facts and conclusions drawn.

Another interdisciplinary research project that we have worked with is the identification of the European patron saint, St. Birgitta (1303-1373), who died in Rome in 1373. However, on her request, the remains were taken to Sweden in 1374 and the bones were placed in a relic casket in Vadstena Abbey. In 1489, the remains of Saint Birgitta's daughter Katarina (1331-1381) were also placed in the shrine. Today, two skulls and 23 other human bones are kept in the relic shrine. A third skull has been reported to be present in the shrine until 1645, when it was stolen. An anthropological and archaeological investigation performed by the archaeologists Bygdén, Gejvall and Hjortsjö in 1952 concluded that both of the remaining skulls

were female. One of the relics is from an individual who died at the age of 60-70 (St. Birgitta?), and the other is from a person who died at the age of 50-55 (Katarina?).

To support or exclude a maternal relationship, mitochondrial DNA, which is maternally inherited, was used in the genetic investigation. The analysis of the hypervariable mtDNA regions showed several DNA differences between the two individuals. Thus, a maternal lineage relationship hypothesis could be rejected. Moreover, the efficiency of amplifying mitochondrial DNA indicated a varying level of DNA degradation between the two samples, possibly due to different ages. Radiocarbon dating revealed a difference of at least 200 years between the two relics, and the dating results disagree with the period when St. Birgitta and her daughter lived. None of the skulls is thus from St. Birgitta or Katarina. Mitochondrial DNA can also be used to study ancient evolution, migration and contemporary populations.

Mitochondrial DNA among major contemporary ethnicities and an aboriginal Vedda population analyzed in Sri Lanka

Like in the two studies above, we have experienced many benefits in our collaborations with researchers at the IBMBB, University of Colombo in Sri Lanka. Our collaboration has involved visits in Sweden and Sri Lanka, courses, conferences, meetings with archaeologists, lectures for the leaders within the law enforcement in Sri Lanka, visits to the Tsunami affected regions in southern Sri Lanka, laboratory sessions for master students at IBMBB and much more. It has been a true pleasure to collaborate with Professor Eric Karunananayake, Professor Kamani Tennekoon and Dr. Ruwandi Ranasinghe on this wide range of rewarding activities. In

particular, it has been highly interesting to participate in the research in which mtDNA among major contemporary ethnicities and aboriginal Vedda population in Sri Lanka was analyzed. This study was a part of Dr. Ruwandi Ranasinghe's doctoral studies and was also published in a well-known forensic science journal with a large spread within the community focusing on mtDNA in forensic and ancient DNA research.

This study and further collaborations have been highly encouraged and supported by Professor Eric Karunananayake with his vision of developing an interdisciplinary and international research environment including analysis of mitochondrial DNA in Sri Lanka. To reach this goal, a specialized clean-room facility was built at IBMBB. This allows analysis of the highly sensitive and contamination prone mtDNA to be performed according to international standards and recommendations. With the special laboratory working, Eric initiated discussions with law enforcement, forensic experts, archaeologists and other collaborators that complements the biologists and geneticists at IBMBB. With all this in place, Eric has contributed to the development of these research areas in Sri Lanka in an impressive way.

Our projects have shown that it is highly important to collaborate. As the mentioned examples have shown, we needed geneticists, evolutionary biologists, historians, archaeologists, anthropologists, osteologists and radiocarbon-dating specialists to confirm or refute the actual identity of the putative remains of Copernicus, St. Birgitta and to investigate the origin of the Vedda population in Sri Lanka. Thus, many scientists from multiple disciplines worked together to solve these mysteries from the past. All these experts had a common interest in achieving an in-depth reconstruction of history, and the full potential of cross-disciplinary

research was shown. In particular, Professor Eric Karunananayake, has realized the potential of international and interdisciplinary collaborations.

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Insight into the Maternal Genetic Diversity of Sri Lankans Including Aboriginal Vedda population

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Professor Eric Karunananayake who is being felicitated in this volume was a pioneer not only in Molecular Biology but in several sub-specialties within the field of Molecular Biology. One such area was the study of mitochondrial DNA which is maternally inherited. When the IBMBB was built he ensured a dedicated space for sensitive DNA typing laboratory to carry out highly sensitive mitochondrial DNA analysis.

Genetic changes that evolved in the human genome has created diversities among populations as well as many ethnicities. Population studies based on various markers among Sri Lankan ethnicities are still in progress. However, there are still no solid conclusions on the origin of major ethnic groups including ethnicities with the oldest history in the country as well as aboriginal Vedda population. In order to shed some light on the maternal lineages of Sri Lankans, my Doctoral study was designed with the guidance of Professor Eric Karunananayake and Professor Kamani Tennekoon to analyze the mitochondrial haplotypes (a set of single-nucleotide polymorphisms on a particular DNA sequence) and haplogroups (a group of similar haplotypes that share a common ancestor) in contemporary ethnicities and Vedda populations in Sri Lanka. Preliminary studies supported by Sida-SAREC were extended to Doctoral studies supported by National Research Council, Sri Lanka. Professor Karunananayake extended his contribution as a co-supervisor and mentor throughout the Doctoral study. As a leading Molecular Biological Institute,

the IBMBB has carried out mitochondrial DNA related population studies since 2007. Major findings were reported in my doctoral thesis and published as a peer-reviewed publication in the Japanese Journal of Legal Medicine (Ranasinghe et al., 2015; Ranasinghe, 2016).

Introduction

Understanding of the modern human dispersal over geographical areas is based on many factors such as geological factors, genealogical information from several genetic systems and loci, and factors from physical and cultural anthropology. The geographical factor fluctuations, such as changes in sea levels due to glaciation, extreme weather conditions and food habits may indirectly affect the movements of the populations via different migration paths (e.g., from Asia to Americas via the Bering Strait land bridge). The first evidence of modern humans arises in the Sub Saharan region of Africa 100 to 200 thousand years ago. Afterwards they seem to have left Africa to the Levant (Mediterranean region between Turkey and Egypt) about 100 thousand years ago, then dispersed towards East Asia, Europe and Australia, and occasionally to America (Ingman, 2001; 2003; Salas et al., 2002; Stringer, 2002).

Geographic location of Sri Lanka in the Indian Ocean has led to vast admixture of cultural and genetic factors for more than 2,000 years. Moreover, continuous population migration dynamics of the country enrich the genetic diversity further. Although there is no authentic evidence to prove the chronicles or historical records describing early human inhabitants, there is firm archaeological evidence of human settlements in Sri Lanka with skeletal remains dated nearly 37,000 years

before present (ybp). There is archaeological evidence of prehistoric settlements in Sri Lanka dated as far back as 125,000 ybp. Out of Africa dispersal of the modern human towards Asia is thought to have taken one of two roots, either a Northern route or a Southern coastal route now named the beachcomber route. Many recent studies tend to support the beachcomber route as the first wave of migration out of Africa (Armitage et al., 2011; Macaulay et al., 2005; Mellars et al., 2013; Oppenheimer, 2012; Petraglia et al., 2010). If the modern human migrated towards Australia along the Indian coastline, some of them gravitating towards island Sri Lanka and settling in its more conductive environment even at that time is highly plausible. Supporting evidence for the single southern coastal route of migration out of Africa stems from the findings on mitochondrial DNA evolution (Soares et al., 2009).

Geological location and population admixture of Sri Lanka

Sri Lanka is an island denoted as “Pearl of the Indian Ocean” positioned at $37^{\circ} 00' N$ in latitude and $127^{\circ} 30' E$ in longitude of the world map. The geographic position of this island at the center of the Indian Ocean and the geological connection with the Indian subcontinent via Adam’s Bridge from Mannar in Sri Lanka to Pamban Island in South India place it in a unique position in both maritime activities and population interventions.

Sri Lanka is presently inhabited by several ethnic groups namely, Sinhalese (74.9%), Sri Lankan Tamils (11.2%), Muslims (Sri Lankan Moors - 9.2%), Indian Tamils (4.2%), Malays (0.2%), Burghers (0.2%), indigenous Vedda people (0.1%) and a few others including Sri Lankan Chetty and Bharatha (Census, 2012). The well accepted historical sources

state that the Sinhalese originated from the North Indian migrants about 2,600 years ago, while Sri Lankan Tamil ethnicity originated from invading South Indians. The two ethnicities are linguistically different with Sinhala spoken by Sinhalese belonging to Indo-Aryan linguistic group and Tamil spoken by Sri Lankan Tamils belonging to the Dravidian linguistic group, thus further consolidating Northern and Southern Indian influence respectively. However, the cultural and developmental history of the oldest inhabitants in the country before the arrival of Prince Vijaya from North India is still controversial. Other evidence indicate that ancient King Ravana of Ramayana fame spoke Sanskrit and that he belonged to a Gothra (tribe) known as Yakka, thus suggesting that Sri Lanka was populated by cultured people (not man-eating demons as suggested by some) long before the arrival of Prince Vijaya from Northern India. History beyond the early Indian migration should therefore be considered to obtain a balanced account of peopling of Sri Lanka. It is plausible that Sinhalese and Sri Lankan Tamils are admixtures of Indian visitors and indigenous people.

Sri Lankan Tamils who inhabit Eastern and Northern provinces have a longer settlement in the country compared to the Indian Tamils in the Central province. However, there is no strong evidence of medieval times relating to Sri Lankan Tamil chronicles describing early Tamil settlement of the country. Furthermore, there were many random female migrations as well as diplomatic movements from the Indian sub-continent to Sri Lanka over many centuries (Geiger, 1912; Law, 1959). Indian Tamils (Hill country Tamils or Up-country Tamils) are mainly descendants of South Indians brought by the British rulers from Tamil Nadu during the 19th and 20th centuries as estate workers.

Another major ethnic group studied was the Vedda population. Traditionally considered as the most indigenous population in Sri Lanka, they currently reside in villages dispersed in the interior highland region and along the eastern coast. Recently, at least some Vedda groups have emerged out of isolation and have started interacting, including marrying, with neighbouring populations. Consequently, a lot of the present-day Veddas speak Sinhalese or Tamil fluently, in addition to the Vedda language (Hussein, 2015). However, the genetic origin of the Veddas remains unresolved. According to the Mahavamsa, they are descendants of son and daughter of Prince Vijaya and Kuveni (6th- 5th BCE). A few studies in the past have investigated the genetics of the Vedda people to relate them to worldwide and other ethnic Sri Lankan populations using both Y-chromosome and mitochondrial markers (Harihara et al., 1988; Illeperuma et al., 2010; Ranaweera et al., 2014).

Muslims (Sri Lankan Moors) came to Sri Lanka mainly as traders centuries ago (mainly in the 7th/8th centuries) whereas Malays have arrived from South-East Asia, in the 17th and 18th centuries when the Dutch ruled part of the country. Muslims are the third largest ethnic community as well as the second-largest minority. At the time of 1946 Census, there were two types of Muslims in Sri Lanka, Indian Muslims (Sammankarars) and Sri Lankan Muslims (Sonahars) also called “Moors”. Indian Muslims mainly arrived in the country from India for trading purposes and had their trading near the coastal towns. However, with time there were intermarriages, or they immersed with the Sri Lankan Muslim communities. Sri Lankan Muslims are also traders thought to have originated in Arabia and Persian Gulf. Malay communities in Sri Lanka initially came from different corners of the Indonesian archipelago, Malaysia, Java, Borneo, Moluccas

and Goa. Malay communities in modern-day Malaysia and the Indonesian archipelago comprise of people of many ethnic backgrounds.

Polymorphisms and polymorphic variance of the Hypervariable regions (HV) I and II in Sri Lankans

Human mitochondria contain 1100 bp length region known as D-loop representing around 7% of the mt genome. It is the only significant non-coding portion of the human mt genome located between genes for tRNA^{Pro} and tRNA^{Phe} and extends between nucleotide positions 16024 and 576 (Lutz et al., 2000). This ‘D-loop’ contains more polymorphic sites and hence generally recognized hypervariable regions (HVI and HVII) are identified. During my doctoral studies, the HVI and HVII regions of the mitochondrial genomes were amplified with sequence-specific primers and sequenced in an automated DNA sequencer (Ranasinghe et al., 2015). Sequence polymorphisms were annotated with reference to the rCRS (Anderson et al., 1981; Andrews et al., 1999). Most of the polymorphisms (mostly single nucleotide polymorphisms) were observed in the HVI region. Transitions were the most common (86.76%) while transversions (7.6%) and Indels (5.64%) occurred less frequently (Ranasinghe, 2016).

Interpretation of haplogroups using control region (HVI and HVII) sequences

Haplogroups were classified based on the polymorphisms using several applications (Ranasinghe et al., 2015). Major haplogroups observed comprised of all the out of African clades including macro- haplogroups M and N (Figure 1).

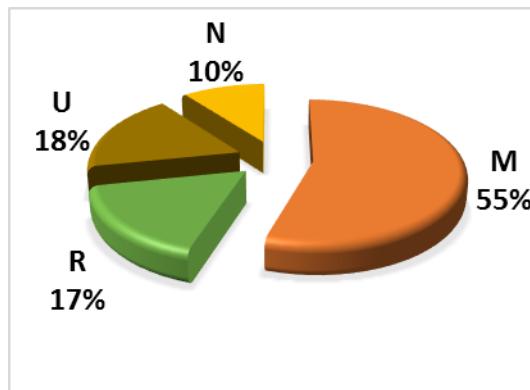


Figure 1. Distribution of major haplogroups among Sri Lankans
(based on HVI & II Sequences)

Distribution of South Asian (Indian), West Eurasian, East Asian, South-East Asian and South Pacific haplogroups among the six ethnic groups observed are shown in Figure 2. When phylogeography of the haplogroups was considered, the majority belonged to South Asian (Indian) haplogroups (M, R31, R5, R6, R7, R30/R8, U2a and U2b) while the second highest was the West Eurasian affinity (H, HV, N1, T, U1, U5, U7, U8, W and X). A higher prevalence of West Eurasian haplogroups was observed among Sinhalese, Sri Lankan Tamils and Vedda populations. G3a1'2 and D4a East Asian sub-haplogroups were present in Vedda population. G3a1 considered as one of the sub-clades that belonged to the period of post-LGM warming is more prevalent among Tibetans and has *de novo* origins within Tibetans (Zhao et al., 2009). The presence of South Pacific haplogroups (P & S) indicate the possible presence of haplogroups left behind by human migration along the Southern coastal line to the Pacific or a back migration of such haplogroups through South-East Asian Islands to Sri Lanka.

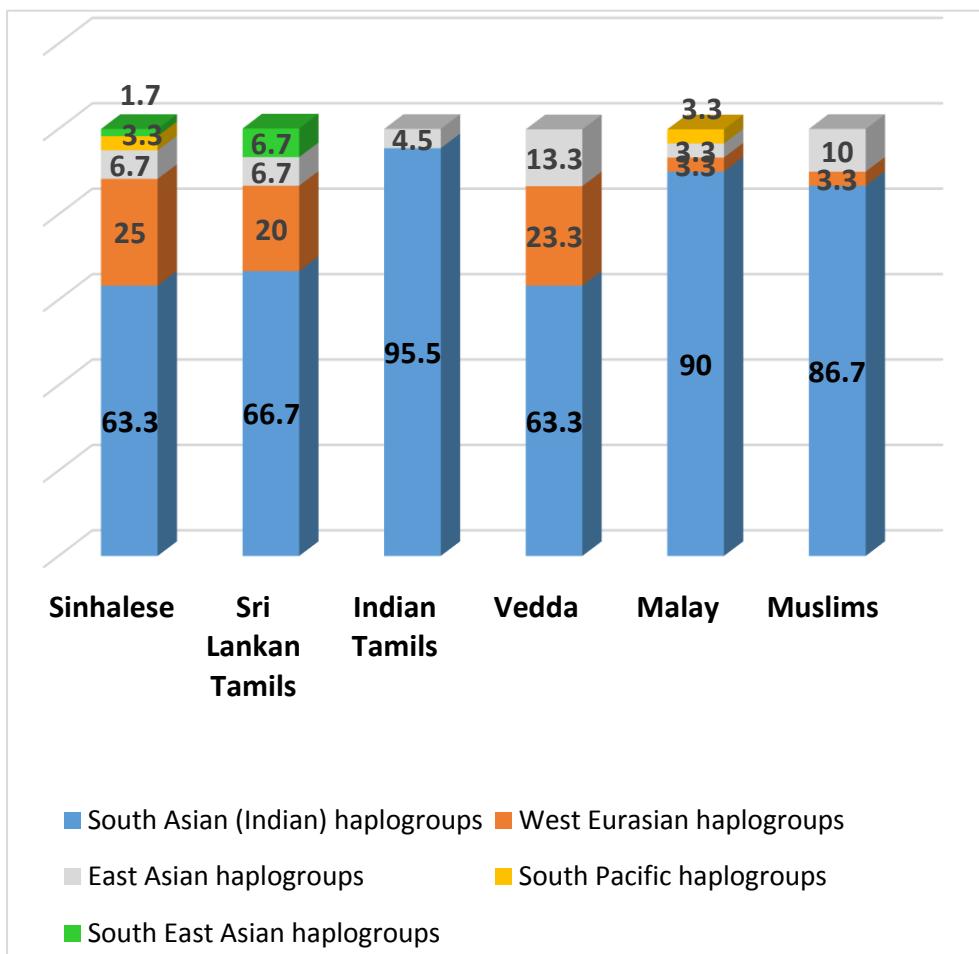


Figure 2. Prevalence (%) of phylogeographical mitochondrial haplogroups among six ethnic groups in Sri Lanka

Estimation of parameters of population variance

When further analyses were carried out (estimation of parameters of population variance), a higher diversity in mtDNA was found among Sri Lankan Tamils and Muslims while the lowest diversity was seen among

Vedda people (Ranasinghe, 2016). Furthermore, Vedda population showed non-significant p values for a few tests along with the lowest diversity indices. Both these signatures further explain the recent population expansion or growth and endogamous nature of the present day Vedda population in Sri Lanka.

Phylogenetic analysis of six ethnicities

Analyses to determine the relationship of maternal lineage among the groups studied (using an unrooted Neighbor-Joining tree and UPGMA tree created based on the population pairwise distances (F_{ST}) obtained from Arlequin Software) clearly indicated the deviation of Vedda population from the rest of the Sri Lankan ethnicities (Ranasinghe et al., 2015). Furthermore, Indian Tamils also formed a cluster separate from Sri Lankan Tamils.

Haplogroup R was found to be more diverse than haplogroup U (analysed using network-based study) and influence of Indian R and U haplogroups on Sri Lankan ethnicities was clearly evident (Ranasinghe, 2016).

Multivariate data analysis

A Principal Component Analysis (PCA) map (a tool used to visualize relationships between categories) constructed based on haplogroup frequencies of the ethnic groups in the present study is shown in Figure 3. Here the Vedda population and Indian Tamils clustered separately while Sinhalese, Muslims, Malays and Sri Lankan Tamils grouped in close proximity to each other than to the Vedda population or to Indian Tamils.

This isolated nature of the Vedda population in the PCA maps was further confirmed by the other genetic diversity indices. Furthermore, it was clearly shown that Muslims and Malays were relatively closer to each other than to Sinhalese. Indian Tamils clustered as a separate group while Sri Lankan Tamils clustered closer to Sinhalese. Furthermore, out of the two Tamils groups, Sri Lankan Tamils clustered relatively closer to Malay and Muslim groups.

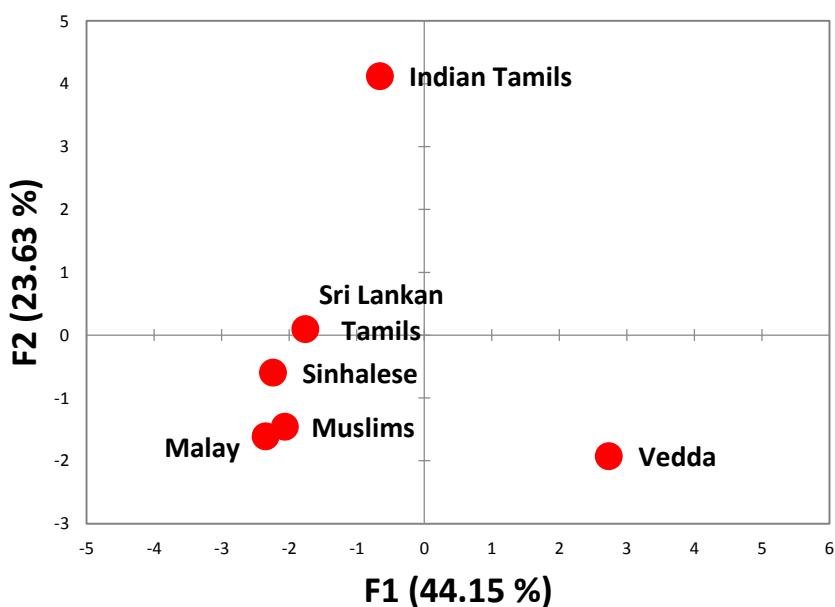


Figure 3. Principal Component Analysis (PCA) map generated for the study samples based on the haplogroup frequencies

Detection of haplogroups by PCR_RFLP

Haplogroup assignment based on the HV I and HV II sequences using bioinformatics tools was further confirmed with haplogroup defining coding region mutations using RFLP and sequencing. Both methods gave comparable results for M and N haplogroup distribution among the ethnic groups. The M haplogroups were commonly observed among Indian Tamils, the recent migrants from South India, whereas haplogroup N and its sub-groups (R and U) were more common among Vedda inhabitants.

In conclusion, there was a vast difference in haplogroup distribution among the ethnicities studied. Vedda population showed the N macro-haplogroup as the most common haplogroup while M macro-haplogroup was the predominant among the rest of the ethnicities. While all ethnic groups showed South Asian region (Indian) affiliated haplogroups, the West Eurasian haplogroups were more prevalent among ethnicities with an older historical settlement in the country (Sinhalese and Sri Lankan Tamils) as well as in aboriginal Vedda population. All analyses showed the maternal inheritance pattern of Vedda population to be different from the contemporary ethnic groups. This observation may further support the historical view of Vedda as descendants of early inhabitants. Furthermore, deeper analysis highlighted the influence of Indian mitochondrial sequences among Sri Lankans.

Future perspectives

Three different systems namely, autosomal chromosome analysis, paternally inherited Y chromosome analysis and maternally inherited mtDNA analysis are regularly used in studying variations of the genetic

pool. Although several studies in Sri Lanka have reported variations in Sri Lankan ethnic groups using these analyses, they do not have adequate resolution to assign present-day gene pool that may be informative enough to shed light on nuclear, maternal and paternal lineages and thus on peopling of Sri Lanka. None of the previous investigations has attempted studying Human Genome-wide Associations (GWAS) (nuclear genome sequencing and/or Single Nucleotide polymorphisms, SNPs) and Next-Generation Sequencing of the full mitochondrial genome. Whole-genome sequencing of human mitochondrial DNA molecule has been made possible by the improvement in speed and accuracy in sequencing techniques. Our current approach includes generation of complete mitochondrial and nuclear DNA sequences and Human Genome-wide associations (GWAS) from present-day Vedda and other major ethnic groups of Sri Lanka which will provide an opportunity for us to test the so-called ‘Southern coastal’ route for the out-of-Africa migration and determine whether the Veddas could have been part of this initial wave that also included the Aborigines of Australia.

I was fortunate to get direction for my studies by the world-renowned scientists, Professor Marie Allen from the Uppsala University, Sweden and Dr. K Thangaraj from the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India. Professor Marie, a long-standing collaborator of Professor Karunananayake played a vital role as a co-supervisor of my doctoral studies. Dr. Thangaraj is a pioneer researcher on mitochondrion genetics in the South Asian region and with the guidance of Professor Eric, I was able to visit his lab at the CCMB, Hyderabad twice. Those visits provided ample training and exposure to international research culture, enabled me to transfer that knowledge to accelerate mitochondrial research

at the IBMBB and develop new collaborations with Indian scientists. Research culture that I had adopted while working at the CCMB lab always helped me to guide the research students working with me.

The memories on Eric Sir's enthusiasm towards the success of mitochondrial genome-based research cannot be expressed fully in this short essay. It was a great pleasure for me to represent Sri Lanka at the Commonwealth Science Conference, Bangalore along with Professor Karunanayake and a few other Sri Lankan delegates. Throughout the conference, I had seen the great personality of this giant and humble human being. Not only that, he also facilitated my participation in the international conference of the 6th EMPOP and 9th Y-User workshop, Brussels, through his research funds.

Finally, it gives a great pleasure to acknowledge the guidance, support and help of Vidya Jyothi Emeritus Professor Eric Karunanayake, co-supervisor of my PhD, for his indispensable role in my doctoral research. His enthusiasm towards human mitochondrial DNA research was infectious. His guidance made it possible for me to overcome many obstacles during my research. As his student, I always wish to carry his mission and vision on furthering the discipline of Molecular Biology and its aligned fields in Sri Lanka. In order to fulfill that I have now embarked on ancient DNA analysis, another area that he was very keen to develop.

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Molecular Determinants of Birth Size: Insulin-Like Growth Factor-I (IGF-I), its Gene and Imprinted Genes *IGF-II* and *H19*

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I met Professor Eric Karunananayake, an eminent personality first in 2009, at the IBMBB. The foundation he laid to establish Molecular Life Sciences in Sri Lanka needs to be highly appreciated. On this journey, IBMBB was the station he built up with his own courage, to produce human resources enriched in the fields of Biochemistry, Molecular Biology and Biotechnology for Sri Lanka. Had I not met this personality in my life, today I may not be in a position to contribute to this type of a volume. I was very fortunate to carry out my PhD under the supervision of Professor Karunananayake. He was a pillar of strength for my academic career and always guided, encouraged and motivated me to do scientific research. Even though his initial training was in Biochemistry and he held the Chair of Biochemistry, initially at the Faculty of Medicine, and then at the IBMBB; his research encompassed many areas in the disciplines of Biochemistry, Medicinal Chemistry and Molecular Biology. In this essay, I provide information on genetic studies on birth size carried out by myself and Dr. Jayanthiny Muhunthan for doctoral studies (Hewage, 2017, Jayanthiny, 2012).

Low birth weight

Birth weight is one of the most important indicators of the health status of a newborn and a strong predictor of both neonatal mortality and morbidity. The World Health Organization (WHO) defines Low Birth Weight (LBW) as birth weight of a live born infant of less than 2,500 g (5 pounds 8 ounces) regardless of gestational age, with the measurement taken within the first hours of life, before significant postnatal weight loss has occurred. Either preterm birth (born at less than 37 weeks of gestation) or being small for gestational age or a combination of both can result in low birth weight (Odendaal et al., 2003). It has been reported that more than 95% of the LBW babies are born in developing countries (Jammeh et al., 2011).

A systematic analysis reported that 20.5 million newborns (14.6%) born globally in 2015 suffered from low birthweight (Blencowe et al., 2019). Such babies are more likely to die during their first month of life. Those who survive have issues such as increased susceptibility to infections and failure to thrive in infancy; adult-onset non-communicable diseases manifest in later life. Hence, one of the six specified global nutrition targets endorsed by the World Health Assembly in 2012 is to achieve a 30% reduction in LBW between 2012 and 2025. It is now estimated that the annual reduction in LBW will have to more than double to achieve this target (Blencowe et al., 2019).

Highest prevalence of low birthweight was reported in South Asia in 2015 (Figure 1). Eastern and Southern Africa, and West and Central Africa also reported a higher prevalence in LBW (Blencowe et al., 2019). Population-level prevalence of LBW in Sri Lanka is 16.9% at present; higher rates are seen in the estate sector (28.4%) compared with the rural (16.6%) and

urban (13.6%) areas (Abeywickrama et al., 2020). Maternal factors such as age below 20 and over 35 years, heavy manual labour, low education and poor nutrition, and low income were reported to be associated with LBW in a Sri Lankan cohort (Pallewatta, 1998). Other factors contributing to low birth weight include pregnancy related morbidities: multiple pregnancies, pregnancy induced hypertension, premature rupture of membranes, placental abnormalities and gestational age below 37 weeks; pre-existing medical conditions such as anemia, malaria, poor nutrition and inadequate antenatal care (Blencowe et al., 2019; Siza, 2008; Ugwuja et al., 2011). Tayie and Lartey, 2008 reported that seeking antenatal care before 12 weeks of pregnancy reduced the risk of having a LBW infant by three folds; multivitamin and mineral supplementation of mothers for more than 5 months improved birth weight.

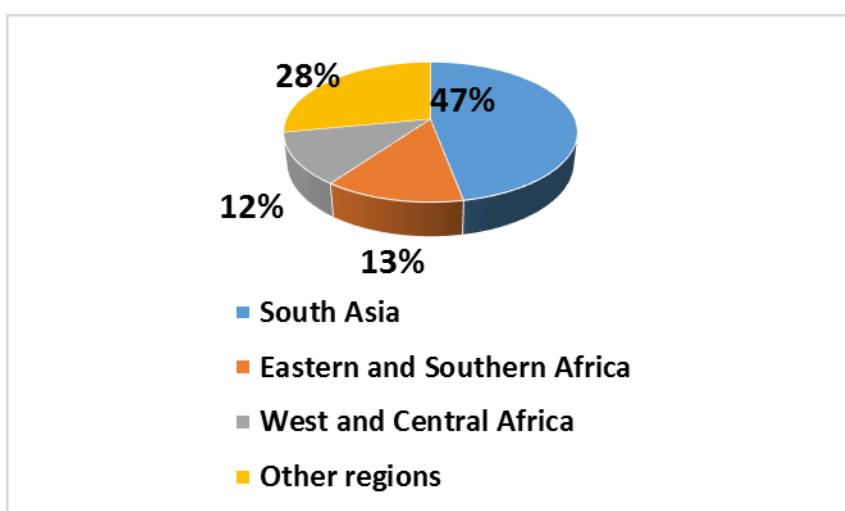


Figure 1. Global distribution of prevalence of low birthweight in 2015 (adopted from Blencowe et al., 2019)

Birth size

Birth size is assessed using parameters such as birth weight, crown-heel length, head circumference, chest circumference and by calculating the ponderal index. A number of factors including parity, gestational length, and adult size and birth weight of the mother influence one's birth size (Ong et al., 2002). Ounsted et al., 1988 reported the possibility of a stronger relationship between the birth weight of the mother and that of the offspring, particularly in low birth weight. This association appears to be complex with influences from *in-utero* programming of foetal metabolism; epigenetic or genetic effects and parity. Maternal blood glucose levels also affect birth weight as glucose transfer to the foetus is increased in diabetic mothers leading to cell hyperplasia, increased insulin secretion and greater foetal adiposity (Hattersley et al., 1998).

Genetics of birth size

Heritability of birth weight was initially estimated to range from 30 to 70% in studies on monozygous and dizygous twins, suggesting the importance of genetic factors (Magnus, 1984). Total heritability for birth weight was later reported to be 42% to 45%, with a slightly stronger maternal versus paternal component (Clausson et al., 2000; Svensson et al., 2006). Besides foetal genes, those regulating the maternal uterine environment may determine size at birth. It is plausible that the variations in the maternally inherited mitochondrial DNA may also influence size at birth (Dunger et al., 2007).

Genetic factors may exist that both reduce foetal growth and increase susceptibility to disease (Basso et al., 2006). Ounsted et al., 1988 who first

reported a strong association between birth weight of the offspring and maternal birth weight suggested that restraint of foetal growth may be inherited through the maternal line. Both restrained foetal growth and risk of preeclampsia are more common in the first pregnancy, hence these two factors are thought to be related (Dunger et al., 2007; Ong et al., 2002). Women who themselves have a lower birth weight are at increased risk of preeclampsia (Kim et al., 2009).

Genomic imprinting where the allele inherited from one parent (i.e. from the mother or the father) is silenced; allowing expression only from the remaining allele (i.e.: inherited from the other parent) has also been implicated in foetal growth restraint (Reik and Walter, 2001, Dunger et al., 2007). Imprinted genes such as insulin-like growth factor-II gene (*IGF-II*) and *H19* have been recognized to play an important role in growth and development of the placenta (Constancia et al., 2002; Zhang and Tycko, 1992). A healthy placenta is an essential requirement for foetal growth and development.

Insulin-like growth factors (IGFs), H19 and birth size

Insulin-like growth factors (IGFs) are molecules with amino acid sequence similarities to insulin. They promote cell proliferation and prevent apoptosis. Components of the IGF system, namely, IGF-I, IGF-II; their receptors IGF-IR, IGF-IIR; and IGF binding proteins have been implicated in foetal growth. Many studies had shown a positive association of cord blood IGF-I and IGF-II levels with birth weight (Lassarre et al., 1991; Giudice et al., 1995; Verhaeghe et al., 1993). Bioavailability of IGFs at the target tissue is limited by the binding proteins (IGFBPs) that sequester

IGFs. Of several IGFBPs in the circulation, IGFBP-1, mainly produced at the foetal and maternal interface has shown a negative correlation with birth weight (Verhaeghe et al., 1993).

Partial deletion of the *IGF-I* gene is associated with severe growth restriction (Baker et al., 1993; Woods et al., 1996). Though loss of function mutations leading to growth restriction have not been identified, transcription of *IGF-I* is thought to be affected by polymorphisms including dinucleotide repeats present in its gene. Two cytosine-adenine (CA) repeats, one in the promoter region, and the other located 3' to the gene; and a cytosine-thymine (CT) repeat located in intron 2 are among the polymorphisms that have been investigated in other populations in relation to foetal growth with conflicting results (Frayling et al., 2002; Kinoshita et al., 2007; Rosen et al., 1998; Vassen et al., 2002).

The maternal allele of *IGF-II* is imprinted in the foetus, meaning that the maternally transmitted allele is silenced. Foetal growth restriction of Silver-Russell Syndrome and foetal overgrowth of Beckwith-Wiedemann Syndrome are associated with depressed versus elevated levels of *IGF-II* (Gicquel et al., 2005; Weksberg et al., 2003).

H19, a non-coding RNA gene (i.e.: its RNA is not translated to a protein), initially reported to be involved in tumor suppression (Brannan et al., 1990; Hao et al., 1993), is growth inhibitory and functionally antagonistic to *IGF-II* (Gabory et al., 2009). Petry et al., 2005 reported an association of a common polymorphisms of *H19* with birth size in a Caucasian population.

The growth and development of the placenta is critical for foetal growth and development. Imprinted genes may play an essential role in the

placental development and foetal growth (Zhang and Tycko, 1992). *IGF-II* and its reciprocally imprinted gene *H19*, have been reported to exert important regulatory function during the development of the placenta and embryo (Constancia et al., 2002).

Genetic studies on birth size at IBMBB

The concept to initiate genetic polymorphism studies on *IGF-I*, *IGF-II* and *H19* associated with birth size came from two eminent Professors at the IBMBB. They are none other than two pioneers in the field of Molecular Life Sciences in Sri Lanka, Professor Eric Karunananayake and Professor Kamani Tennekoon. Professor Karunananayake had previously co-supervised (principal supervisor: Professor Kamani Tennekoon) MPhil studies of Dr. Arjuna Pathmaperuma, a probationary lecturer in the Department of Physiology and Poopalapillai Jayanthiny, an MSc student in the Department of Biochemistry and Molecular Biology at the Faculty of Medicine in the field of IGF research (Jayanthiny, 2006; Pathmaperuma, 2006). Both these studies focused on measuring the protein levels in either maternal or cord blood and all Jayanthiny's and part of Arjuna's lab work had been carried out at the brand new laboratories of the IBMBB.

Association of maternal and cord blood levels of IGF-I, IGFBP-1 and dinucleotide polymorphisms of IGF-I with birth size

Following MSc studies, Jayanthiny (now Dr. Jayanthiny Muhunthan, Senior Lecturer in Biochemistry, Faculty of Health-Care Sciences, Eastern University) embarked on her doctoral studies to look at the association of dinucleotide repeat polymorphisms in the *IGF-I* gene with birth size. By that time there were no studies on the association of dinucleotide repeats

with birth size for Sri Lankans or native South Asians. Jayanthiny's was the first genetic study on the IGF system in Sri Lanka. She was supported by a National Research Council Grant awarded to Professor Tennekoon. Professor Karunananayake functioned as the co-supervisor. A succession of pre-interns assisted as clinical research assistants to recruit mothers, collect relevant anthropometric and clinical data and blood samples from mother-new born pairs at the Castle Street Hospital for Women (CSHW). Several consultant obstetricians namely Drs. Lakshman Senanayake, A. P. de S. Wijesundera and J. M. Kumarasiri (all now retired from the Government service) actively collaborated, while then Director CSHW, Dr. Wimal Karandagoda strongly supported the study. My studies on two genes, *IGF-II* and *H19* that followed are described later in this essay. Jayanthiny also studied *IGF-II* and *H19* in a subset of mother-newborn pairs and her findings were also included in my analysis.

Jayanthiny's studies were carried out on two hundred mother-new born pairs. The majority (N=177) were Sinhalese. All mothers and newborns were healthy. Pregnancies were first or the second and, naturally conceived. IGF-I and IGFBP-1 were measured by ELISA in maternal and cord blood samples. Dinucleotide repeat polymorphisms in the mothers and the newborns were analysed using fluorescence based genotyping on the MegaBACE 1000 DNA sequencer. As expected, maternal and cord blood IGF-I levels positively affected birth size, while IGFBP-1 which limits bioavailability of IGF-I at the tissues had a negative effect (Jayanthiny, 2012; Jayanthiny et al., 2011).

Most frequently seen alleles for the two CA repeats in the study cohort differed from the wild type reported in Caucasians. Wild type allele highly prevalent in Caucasians for the CT repeat was seen in our cohort, but at a

somewhat lower frequency. Of the three repeats studied, only the intron 2-CT repeat showed a significant consistent association with birth size. Two 189-bp alleles of the intron 2-CT repeat in the mother or, one or two alleles in the newborn was associated with a larger birth size while the opposite occurred when two copies of the 191-bp allele was present. Promoter CA and intron 2-CT repeats were associated with cord blood IGF-levels, while these had no effect on maternal IGF-I levels (Jayanthiny et al., 2011).

Association of IGF-II and H19 polymorphisms with birth weight

Genomic imprinting was a favorite area taught by Professor Karunananayake for the MSc students at the IBMBB. When I began my doctoral studies in 2010 as a continuation of Jayanthiny's work, I was fortunate to be thrown into the fascinating world of imprinted genes. My task was to investigate possible association of selected polymorphisms in *H19* and *IGF-II* genes with birth size. Until then, there were no studies in Sri Lanka or other South Asian population living in their native country that described association of *IGF-II* or *H19* genotypes with birth size except what Jayanthiny carried out on a smaller cohort during her PhD studies (Jayanthiny, 2012).

Three common polymorphisms in the *H19* gene (rs2067051 A/G, rs2839703 A/G and rs217727 C/T) and one polymorphism in the *IGF-II* gene (*IGF-II* ApaI) were selected for the study. *H19* polymorphisms were studied in 173 mother-new born pairs and *IGF-II* polymorphism in 155 mother new-born pairs and the majority were from the cohort used for the *IGF-I* study.

The mother-newborn pairs were genotyped for the polymorphisms using PCR-RFLP. Allele and genotype frequencies were determined and their

associations with birth size (birth weight, length, head circumference, ponderal index) were studied. All polymorphism were confirmed by subjecting representative samples to primer extension based SNP analysis in house using ABI PRISM SNAPSHOT Multiplex reagents and ABI 3500DX genetic analyzer.

H19 rs217727 polymorphism showed a significant association with birth size. But *H19* rs2067051, rs2839703 and *IGF-II* ApaI polymorphism did not exert any significant effect on birth size (Hewage, 2017, Hewage et al., 2015). Wild type allele of some *H19* polymorphisms and *IGF-II* ApaI were different from what was reported for other populations (Roth et al., 2002).

Maternal TT genotype for *H19* rs217727 polymorphism was associated with a higher birth weight, crown-heel length, head circumferences and ponderal index than CC genotype. These differences were statistically significant for birth weight and ponderal index but not for crown-heel length (Hewage et al., 2015). Ponderal index, a ratio of birth weight to length, has been used as a marker of leanness or fatness in newborns. Petry et al., 2005 observed that newborn TT genotype for *H19* rs217727 polymorphism is associated with a higher birth weight, crown-heel length and head circumference than the CC genotype in Caucasians in the United Kingdom, but in their study, TT genotype had a lower ponderal index than the CC genotype.

In our analysis, we accounted for the effect of *IGF-I*/intron 2 dinucleotide repeat polymorphism on birth size previously reported for the same cohort and found that the maternal *H19* rs217727TT genotype and *IGF-I*/intron 2 CT repeat 189 allele, independently modulate birth weight. When tested in two different models, both the maternal and newborn alleles of *IGF-*

I/intron 2 CT repeat exerted independent effects with a greater effect being exerted by the newborn allele (Hewage et al., 2015).

Independent association of *IGF-I* and *H19* polymorphisms on birth weight may arise due to different growth regulating mechanisms deployed by these two genes. *IGF-I* gene codes for IGF-I protein, whereas *H19* gene codes for a non-coding regulatory RNA that suppresses translation of IGF-II RNA (Hewage et al., 2015). Both *IGF-I* and *H19* polymorphisms identified to be associated with birth size in our studies have the potential to be developed as markers to identify women likely to have low birth weight babies, permitting possible interventions to improve birthweight.

The work described above was financially supported from Sida/SAREC Grant for Molecular Biology (Principal investigator-Professor Karunananayake) and National Research Council (Principal investigator-Professor Tennekoon). My heartfelt thanks are to Professor Karunananayake who motivated me and changed my mindset to initiate doctoral studies at the IBMBB. Also as my supervisor, the guidance and support that he gave me is highly appreciated.

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Professor Eric Karunananayake and the Link #1 in My PPD¹⁰

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I consider it my obligatory duty to pen a few words for the Felicitation Volume of Professor Eric Karunananayake who reaches the 80th milestone in his life. The following essay describes the evolution of my association with Professor Eric Karunananayake, starting in a personal note moving into the professional/ academic sphere.

When I was a medical student, one Professor of Surgery proudly exclaimed “I have been doing surgery even before you were a spermatogonia”. If I were to modify this statement, indeed Professor Eric Karunananayake had known me even before I was a sperm! Boys are born with spermatogonia, the primitive germ cells which after puberty, start to form mature sperm through spermatogenesis a process that takes about 74 days. Thus Prof. Eric had known at least a half of “me” six decades ago by the virtue of my father being a batch mate at Peradeniya and his “best man”. My earliest memories of Professor Eric Karunananayake are meeting him at family gatherings where I used to address him with a suffix to his first name that befitted the close personal relationship.

On entering the Colombo Medical Faculty as a medical student, I abandoned using that suffix instead started addressing Professor Eric Karunananayake as “Sir” and continues to do so to date. During the first year

¹⁰ Personal and professional development

in the medical faculty, we had to routinely migrate back and forth from the Anatomy lecture hall to the Physiology lecture hall. The Physiology lecture hall was in the 3rd floor of the same building in which the ground floor was occupied by the department of Biochemistry. Usually close to lunch time, Prof. Eric had the habit of standing at the entrance to the Biochemistry department while the great exodus was in full swing. Sight of myself in the midst of wandering medical students sometimes prompted Sir to summon me to inquire how I was progressing in the faculty leaving myself to answer a flurry of questions from inquisitive batch mates! But later on it was clear that occasional “summons” I got was a reflection of the genuine interest in student welfare. Professor Karunananayake would always lend a helping hand to a student in need.

Professor Karunananayake taught several generations of medical students from 1980 to 2004 and I was fortunate enough to learn Biochemistry from Sir in the year 1999. Although it was at the turn of the millennium, the computers and power point were yet to be part and parcel of university teaching. Like all the lecturers, Sir used the overhead projector (OHP) with lecture notes written on transparencies. His lectures on carbohydrate metabolism were elaborate and his vast knowledge was clearly evident through the delivery of lectures, though the content was occasionally indigestible to naïve first year medical students! Professor Karunananayake used to say more than what was displayed in the OHP, much to the dismay of the official lecture note writers in the batch. Professor Karunananayake recommended the “Textbook of Biochemistry with Clinical correlations” by Thomas Devlin, a voluminous book most students thought unnecessary to refer. But I realized the value of this particular textbook since it was useful even in my final year.

Years passed, I crossed the Francis road to exit the “block” and endured the perils of being a medical student. I’m eternally grateful to Sir for his intervention when I contracted pulmonary tuberculosis in my final year. Sir personally spoke to a Professor in the Medicine department who eventually treated me to the point of recovery. I vividly remember visiting Sir at his faculty office accompanied by my father to express our gratitude. He looked at me, smiled and said “you look much better now”. Sir who was in high spirits that day told with a sparkle in his eyes about “the institute that is complete” and that soon he would move there as the Director.

In January 2005 I received a call from Professor Karunananayake probably he been informed by my father that I was home bound and “jobless” after completing the Final MBBS. Sir offered me a job as a clinical research assistant at the IBMBB. Thus Professor Eric Karunananayake became my first “Boss”. The following week, I visited the newly inaugurated IBMBB to meet Sir and Professor Kamani Tennekoon. My job description included assisting Professor Tennekoon in her research, specifically to visit Castle street hospital for women and Cancer Institute Maharagama to recruit patients, collect clinical data and samples.

During those formative years, the institute had a diverse group of researchers and Professor Karunananayake was the bellwether in every conceivable way. He was very particular about the cleanliness in the labs and one day he found that first floor labs were in an unsatisfactory state. Immediately, occupants of the lab were summoned and Sir expressed his displeasure in a way that shook everybody to the core! Following day a “shramadana” was held to clean up the labs. The need to keep the lab space

clean innately sunk into me and later-on I realized the importance of it while working in laboratories in the US and the UK.

It was at the IBMBB that I ventured into quality scientific research under the guidance of Sir and Professor Tennekoon while acquiring many skills viz. performing ELISAs, DNA extraction from blood samples, PCR etc. Now I take the liberty to describe a study on the association between leptin and neonatal birth size conducted towards the latter part of my tenure at the IBMBB.

In humans, leptin is produced and secreted predominantly from adipose tissue. Leptin is known to regulate not only satiety and bodyweight but many other functions, including vascular function, bone and cartilage growth, immune response as well as the normal physiology of the reproductive system.

The proposition that leptin is an important player in human reproduction is strengthened considering that anorexia and obesity modify the onset of puberty in opposite ways while congenital leptin deficiency is associated with subfertility. Thus leptin was the first adipokine claimed to be the ‘missing link’ between fat and reproduction.

Human pregnancy is characterized by dramatic changes in maternal endocrine milieu mediated mainly by the placenta that functions as an endocrine organ. Leptin is produced by the placental tissue and evidence is mounting that leptin is involved in the regulation of placental formation and its function with leptin receptors being detected in trophoblast cells. Leptin regulates differentiation of trophoblast cells, acts as a mitogen in syncytiotrophoblast and stimulates secretion of other placental hormones.

A positive correlation of leptin with birth size initially supported a role in the regulation of intrauterine growth (Koistinen et al., 1997). Observed similar levels when cord blood leptin is normalized to the fat content in appropriate, small and large for gestational age babies, indicate that cord blood leptin reflects adiposity rather than being a regulator of foetal growth (Mouzon et al., 2006). This is further supported by normal birth weight seen in congenital leptin deficiency (Strobel et al., 1998). Foetal endocrine functions, including leptin secretion, are adversely affected by pregnancy complications. Pregnancy induced hypertension (PIH) and gestational diabetes mellitus (GDM) are characterized by microvascular changes in the placenta contributing to placental hypoxia (Jirkovska et al., 2002, Soleymanlou et al., 2005). Placental hypoxia is known to augment leptin secretion. Higher placental and maternal blood levels of leptin have been reported in PIH associated with intrauterine growth retardation compared to normal pregnancies (Mouzon et al., 2006). Umbilical cord blood and placental levels but not maternal blood levels of leptin are increased in GDM (Jirkovska et al., 2002).

In the study, 48 newborns of normal pregnancies (N=18), PIH (N=16) and GDM (N=14) were considered. Cord blood samples were collected and newborn anthropometric indices recorded at delivery. Leptin concentrations were measured using an enzyme immunoassay which revealed that cord blood leptin levels were significantly different between the 3 groups and the difference resulted mainly from higher levels in GDM than in PIH.

The above mentioned study culminated in my first research publication (Silva et al., 2008) that helped me in good stead in 2008 in the application

to an academic position in the department of Physiology at the Faculty of Medical Sciences, University of Sri Jayewardenepura.

In 2010, I returned back to the IBMBB, to conduct cell culture experiments as part of my PhD. The state of the art facilities instituted at the IBMBB due to the visionary efforts of Professor Eric Karunananayake, enabled the establishment of primary human endometrial stromal cell cultures and completion of my PhD in 2012.

Mark Twain in his essay “The turning point in my life” writes “...It means the change in my life’s course which introduced what must be regarded by me as the most *important* condition of my career. But it also implies – without intention, perhaps - that that turning-point itself was the creator of the new condition. This gives it too much distinction, too much prominence, too much credit. It is only the *last* link in a very long chain of turning-points commissioned to produce the cardinal result;...” However in the same essay Twain identifies being morbidly ill with measles at 12 and a half years of age “.... was a turning-point of my life. (Link number one.)”

I never hesitate to acknowledge the role of Professor Eric Karunananayake in inducing me to enter the academic field. Not only did Sir taught myself basics of Biochemistry, he laid the foundation for me to embark on scientific research. Having the rare opportunity of serving as the first clinical research assistant at IBMBB was the link #1 in my professional career as an academic. In this Felicitation Volume many others have recounted the contribution of Professor Eric Karunananayake and the IBMBB in defining the turning points in their lives. Professor Eric Karunananayake has changed the lives of many and in the process has

accrued enough merit to be blessed with long life and good health according to the faith he truly believes.

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Hormones to Genes-Physiology to Molecular Life Sciences: A Collaboration over Three Decades

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My association with Eric is almost 35 years now, encompassing 87.5% of my academic career! Had I not met him and had he not extended the laboratory facilities that he established both in the Faculty of Medicine and in the IBMBB, I may have ended up a glorified teacher with no research to my credit. When I was a child, I inquired from my father how does one get an “Acharya Upadiya” (term for the doctoral degree in Sinhala) after reading a newspaper article written by a Doctor of Philosophy. When he explained that a person has to discover something original to get a PhD, it deeply etched in my mind; my interest in high school was more on becoming a researcher than a medical graduate. Back in the Department of Physiology, armed with a PhD from the University of Sheffield, I would not have been able to realize my passion for research on my own; despite its very conducive environment for a peaceful existence and a very satisfying teaching career.

Plants with Galactagogic Activity

There were several research areas that I collaborated with Eric before moving to IBMBB. The very first was in evaluating plants purported to increase milk production. This was a project that I abandoned when I

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secured a Commonwealth Scholarship for PhD studies in the United Kingdom. The concept was developed initially following my introduction to late Professor Ranjit Roy Chaudhury, a world renowned Indian Professor of Pharmacology (a former Dean of the Postgraduate Institute of Medical Education and Research in Chandigarh) who served as a Consultant to the WHO office in Colombo at the time. Late Professor K N Seneviratne, my mentor and then Professor of Physiology introduced me to Professor Roy Chaudhury as I was very keen to pursue my doctoral studies in Reproductive Physiology. My first taste of scientific writing was acquired by writing a chapter on “Plants as Galactagogues” with Professor Roy Chaudhury (Roy Chaudhury and Tennekoon, 1983) for a volume on Advances in International Maternal and Child Health edited by the famous duo Jelliffe and Jelliffe. I had planned to do an MPhil study evaluating such plants from Sri Lanka with an initial research grant of Rs. 3000.00 from the University of Colombo! Late Professor K Jayasena, then Professor of Pharmacology at the University of Peradeniya had trained me in his team working on identifying possible fertility regulating agents from plants. When Eric realized that I was idling on the research side, he advised me to reactivate this until I found adequate funds to support studies in my pet areas. We evaluated several plants for galactagogic activity, but none of the preparations administered showed a significant effect when tested in a rat model *in vivo* (Tennekoon et al., 1987; 1992).

Effect of age on male reproductive hormones

The work on galactagogic plants was actually to keep in touch with research. During the same time I also got involved in research on plants with hypoglycaemic activity led by Eric as outlined elsewhere in this

Volume. I needed more substantial work that would involve measurement of hormones, my favourite area at the time. Serendipitously, Eric had installed the two major equipment I needed; a gamma counter and a scintillation counter. A visiting WHO team was highly impressed by the laboratory facilities Eric had developed; agreed to give me a small grant; we published the first paper from the Faculty of Medicine where hormone assays were done in house (Tennekoon and Karunananayake, 1993). Follicle stimulating hormone (FSH) and luteinizing hormone (LH) produced by the anterior pituitary regulate gonadal function-ovarian function in females and testicular function in males. It was well known then that in women FSH levels begin to increase several years before menopause and luteinizing hormone follow suit later (Metcalf and Livesey, 1985; Lenton et al., 1988). Studies on age related changes in reproductive hormones in men was inconclusive at the time (Harman and Tsitouras, 1980; Sparrow et al., 1980).

We carried out a cross sectional study on “presumably” fertile men (all had fathered children within the wedlock, hence were considered fertile; but it was a presumption as we did not either confirm they were the biological fathers or assess any other fertility indicators; either would have been unethical) aged 21-85 years. A differential age related increase in FSH and LH was observed, FSH significantly increasing from 6th decade onwards, and LH from 7th decade onwards. Testosterone level significantly decreased from 6th decade onwards. Though this was a simple study carried out with a small grant, the paper published in the International Journal of Fertility, with the title being highlighted on the cover page is still being cited, the most recent being this year, nearly three decades after its

publication. A shorter paper on the effect of age on prolactin was published in the Ceylon Medical Journal (Tennekoon and Karunananayake, 1994).

Lactational Amenorrhoea

Largely guided by late Professor T. Varagunam, who was a close friend of late Professor K N Seneviratne, and Responsible Officer for Latin America in the Special Program in Human Reproduction (HRP)/WHO, I succeeded in securing a substantial research grant from HRP. Eric and Professor Harsha Seneviratne-then a Senior Lecturer, Department of Obstetrics and Gynaecology-were the co-investigators. Lactational amenorrhoea (absence of menstruation during breast feeding) contributes substantially to space and reduce conceptions, hence considered as a main natural contraceptive especially in the developing countries. There were doubts that improved maternal nutrition will lead to an early return of ovarian function during lactation, negating benefits of breast feeding on population control. This would impact population growth in the developing world as the maternal nutrition improves. Besides there were many other determinants of lactational amenorrhoea including breast feeding frequency, total duration of breast feeding per 24 h etc. (Diaz et al., 1988; Gray et al., 1990; Kennedy, 1990). We set out to investigate the effect of supplementing the maternal diet with skim milk on the duration of lactational amenorrhoea using a matched pairs design.

Healthy breast feeding women were recruited from the postpartum ward of the Professorial Unit of Obstetrics at the De Soysa Hospital for Women, Colombo; they were visited weekly in their homes by field investigators to collect breast feeding records, other relevant information and urine

samples; and seen at a follow up clinic every four weeks to assess general health of the mother and the baby, record infant weight and collect blood samples from the mother. The gamma counter and the scintillating counter in Eric's laboratory were in full use again to measure serum prolactin and urinary metabolites of oestrogen and progesterone in the study participants. Our work demonstrated that maternal nutritional supplementation did not negate the contraceptive benefits of lactation when the breast feeding frequency was not compromised. In fact the duration of full breast feeding was lengthened in the supplemented group (Tennekoon et al., 1996). The main publication that appeared in the American Journal of Nutrition accompanied by an editorial was well recognized by international experts active in the field. As the project was progressing, I was appointed as a member of the Task Force on Natural Methods for Fertility Regulation of HRP/WHO. Kandy Society of Medicine chose this work to award me its Health Research Prize in 2000. Successful completion of this project using laboratory facilities Eric established and maintained, enabled me to achieve International and National recognition.

Lactational amenorrhoea and leptin

Eric being always the first to read latest discoveries, one day passed onto me the paper describing discovery of leptin asking me to have a look. It was intriguing and useful for my teaching in Physiology as one of the topics I taught at the time was regulation of food intake; now a long sought chemical messenger had been found. When further reading hinted a role for leptin in reproduction, I wanted to see whether leptin had a role in lactational amenorrhoea. I was investigating lactational amenorrhoea and

its determinants in well-nourished and undernourished women at the time; had transitioned to enzyme immunoassays following my training at the WHO Collaborating Centre for Immunoassay at the Hammersmith Hospital, London. A young academic from another University was reading for her PhD under my supervision and samples were readily available for analyzing leptin. Leptin levels were measured in Eric's laboratory as that required the use of radioimmunoassay. We demonstrated that leptin is unlikely to be a major determinant of early resumption of menstruation in well-nourished women. While leptin showed a significant correlation with body mass index (BMI) as expected, it was BMI but not leptin which negatively correlated with the duration of lactational amenorrhoea (Tennekoon et al., 2005).

Effect of leptin on endometrial function

I was retrained in the United Kingdom in Cell Biology and Molecular Biology techniques supported by a WHO/Rockefeller Career Development Fellowship in Reproductive Molecular Biology. This enabled me to expand my research in reproduction. In collaboration with Eric, I set up cell culture facilities initially at the Faculty of Medicine and later at the IBMBB. A probationary lecturer from the Department of Zoology, University of Jaffna was our PhD student for the project in which we looked at the effect of leptin on the rat endometrium, using a primary cell culture established in house. Leptin increased secretion of prolactin from rat endometrial cells, but not that of IGF-1. (Eswaramohan, 2008; Tennekoon et al., 2007). Thampoe Eshwaramohan, the PhD student went back to his Department in Jaffna upon successful completion of the degree

and was instrumental in developing several research programs there; he continues to be a very active academic in Jaffna.

With the SAREC supported research program being translocated to brand new laboratories at the newly established IBMBB in 2004, I too had to move my collaborative projects to IBMBB for two reasons. I had relied on some equipment available in Eric's laboratory, and the nature of the experiments meant that experiments had to be moved *in toto* to IBMBB where the equipment were relocated. I had become the co-grantee of the SAREC project by then and had to take some responsibility for research administration and supervision of the students. Eric needed a senior academic to support him at the IBMBB. Thus I took my accumulated sabbatical leave and temporarily moved to IBMBB in 2005.

My collaborative research in the areas that were close to my heart, namely Reproduction, Growth and Development continued at the IBMBB. Some of the work on molecular determinants of birthweight has been described by Sudeshini and preliminary work on leptin and birth weight by Nalinda in preceding essays. There were further studies investigating cord blood leptin levels in a larger cohort of newborns, and on the role of leptin and its genotypes, and leptin receptor genotypes in pregnancy induced hypertension.

Insulin-like growth factors and leptin: association with birth weight

Although there had been several studies examining the association of leptin with birthweight, studies that took into account soluble leptin receptor (SLR) which sequesters leptin and reduce its bioavailability were rare. We thus measured cord blood leptin, SLR, insulin like growth factor-

1 (IGF-1) and insulin like growth factor binding protein 1 (IGFBP 1) in a cohort of healthy newborns from healthy full term pregnancies. Our results demonstrated that leptin positively affected fetal growth while IGFBP 1 exerted a negative effect, but leptin and IGFBP 1 acted independently (Tennekoon et al., 2014).

Leptin in pregnancy induced hypertension

Placenta is a major source of leptin in pregnancy (Masuzaki et al., 1997). Identification of placenta specific upstream enhancer indicated that regulation of placental leptin differs from that in the adipocytes (Bi et al., 1997). Underlying pathology of preeclampsia (PE) / pregnancy induced hypertension (PIH); a pregnancy disorder associated with hypertension and other systemic manifestations in the mother and fetal growth restriction; includes abnormal placentation (Roberts and Lain, 2012). The ensuing ischemia of the placental bed leads to hypoxia. Several studies had demonstrated an association of PE/PIH with elevated leptin levels and reduced soluble leptin receptor levels (McCarthy et al., 1999; Muy-rivera et al., 2005).

Among the factors that stimulate placental leptin secretion, hypoxia appears to play a considerable role (Ambrosoni et al., 2002). *LEP* gene transcription was also reported to be enhanced by a polymorphism in the 5' region, -2548 G/A (Hoffstedt et al., 2002). We extended our collaboration with the Castle Street Hospital for Women to initiate a study on leptin in PE/PIH. Our cross sectional study on 62 patients with PE/PIH and 63 controls of normotensive pregnant women demonstrated that the AA genotype of the -2548 G/A polymorphism was associated with

PE/PIH (Sugathadasa et al., 2010). We also observed significantly higher circulating leptin levels and lower soluble leptin receptor levels in PE/PIH. While we could not conclude whether leptin receptor gene polymorphism, *LEPR* c.668A>G is associated with PE/PIH, presence of one or two G alleles at this locus (i.e.: heterozygosity or homozygosity to the G allele) was associated with higher leptin levels in PE/PIH when compared with normotensive controls (Tennekoon et al., 2012). Leptin when normalized to BMI to account for the fat mass, also showed the same pattern.

Cancer

In late 1990's when Eric wanted to investigate breast cancer genes, I gladly agreed to become a co-investigator. For various reasons initial attempts had to be abandoned and the program reactivated in 2003. Three women scientists who worked in cancer genetics/biomarker projects from 2003 onwards; Sumadee, Chrishani and Vahini have eloquently described the work done on cancer. Hence I will not bore the reader with a repetition here.

I discovered that there were many other interesting areas to explore beyond my pet areas. My three months stay at the Rudbeck Laboratory in 2005, exposed me to advance molecular biological technologies, strengthening the training I received in Sheffield. I was in particular interested in mitochondrial DNA and gene expression studies. Denis Noble, cardiovascular physiologist from Oxford, author of the book, "The Music of Life: Biology Beyond the Genome" (2006), said that the "Blue Print of Life-DNA", which he considered the book of life with chromosomes being its chapters, is useless, unless it is read-translated to proteins. Perhaps

being a physiologist to start with I gravitated towards functions of the genes as well. The gene expression studies were largely used in relation to anticancer drug discovery which Sameera and Meran had elaborated in this volume. Recent discoveries have endowed many important roles for non-protein coding DNA; so after all the book has other uses without being read!

Mitochondrial DNA

Ruwandi has described elsewhere in this volume the mtDNA work we carried out; Eric was very eager to develop mtDNA work at the IBMBB; Marie Allen exposed me to relevant technologies in Sweden. A larger audience had been fascinated by our findings; the Professor Stanley Wijesundera Memorial Lecture in 2015 at the IBMBB and the Professor K. Balasubramanium Gold Medal Lecture in 2016 for the Jaffna Science Society were delivered by me based on our mtDNA work. Professor Ulf Pettersson has outlined in his essay, the role of these two eminent academics in setting up Molecular Biology in Sri Lanka, thus it was indeed a privilege to be able to honour them, with our work. Both taught me Biochemistry in the Medical Faculty; Professor Wijesundera in addition was my personal tutor when I was an undergraduate, and the Vice-Chancellor when I joined the Academic cadre.

Anticancer drug discovery, antifilarial drug discovery and the filarial genome

Our work on anticancer drug discovery, antifilarial drug discovery and filarial genome have been eloquently described in this volume by those who are / were in the thickest of these.

My research activities would not have seen the expansion that it has today without the catalyst, Professor Eric Karunananayake. Once a senior colleague having come to know of my collaboration with Eric, told me that I should do research on my own, produce single author publications (he was mostly doing that) and certainly should not collaborate with “non-medical” people. Fortunately, I was not foolish enough to heed that advice, perhaps more so, because of my inclination to science than to medicine.

Research administration

Although I had received training in research, I was a novice to research grant administration. Intricacies of handling research grants, different funding agencies and finance and administrative staff of the Faculty were all learnt from Eric on the job. His stalwart technical officer, Mr. C.S.P Abeysinghe, who also very efficiently handled coordination of clearing equipment and furniture received for the IBMBB from Sweden; their installation; looked after many aspects of the IBMBB from the inauguration onwards to make sure laboratories, the lift, the air conditioning and the generator were running in order; to mention a few; was of great help.

University administration

Eric was also the Head of the Animal House at the Faculty of Medicine, when I began collaborating. As his work load was increasing I was asked to take over this responsibility. This was a voluntary job in which two technical officers and four workers, as well as a continuous supply of food for the small animals held there had to be managed in addition to ensuring that the facilities are provided to various researchers. This initiated me to University Administration and served well when I had to take over as the Head of the Department of Physiology. I can still remember meeting the Vice Chancellor with Eric to request some additional funds to tile the inner walls (few feet from the ground to facilitate cleaning) of the new building of the Animal House which was then being constructed. Then Vice Chancellor, late Professor Stanley Wijesundera called the Bursar (Chief Financial Officer), Mr. Madanayake to the discussion; after listening to our justification, the Bursar replied “if tiling is needed we should find funds from somewhere and give”. Such officers were rare then, and even more so now.

Having graduated from Head/Animal House to Head/Department of Physiology, the next task was to take over the leadership of the IBMBB from Eric on his retirement. Not even in my wildest dream had I thought, that I will leave Physiology and plant myself at the IBMBB for the rest of my working life. Nurturing the Institute he built; keeping the IBMBB family together and taking it forward were my tasks to accomplish. These were made easy by Eric’s continuous support and interest in the welfare of the IBMBB and the IBMBB family; his commitment to produce quality postgraduates; and the commitment and motivation of the majority of the staff and students to achieve its Founder’s Vision. Continued support and

guidance of his Swedish colleagues, late Professor Rune Liminga, Professors Malin Åkerblom and Ulf Pettersson, as well as the new collaborators, Professors Marie Allen and Erik Bongcam-Rudloff was a great asset.

I am very glad to have had this opportunity to put on record how this “Trailblazer” facilitated my career advancement. Thank you Eric for guiding me and being a pillar of strength at difficult times.

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Development of a DNA Probe and a PCR Assay to Detect *Rhizoctonia solani*

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It is indeed a privilege to felicitate Professor Eric Karunananayake on completing eighty years of age in December 2021. I got to know him first, when I started reading for my PhD in 1996. Though, I registered as a postgraduate student at the Faculty of Science, University of Colombo, I had the golden opportunity given by Professor Karunananayake to conduct the Molecular Biology component of my studies at the Department of Biochemistry and Molecular biology at the Faculty of Medicine where he had established a well-equipped Molecular Biology unit (MBU) under Swedish Agency for Research Cooperation with Developing Countries (SAREC) grant awarded to him. On receiving an invitation to contribute to this Felicitation Volume, I was delighted at the honor of being chosen and decided to present the work carried out for my PhD on the “Development of a DNA probe and a PCR assay to detect causative organism of sheath blight disease of rice”.

Sheath blight disease of rice is one of the diseases causing severe loss of yield in rice production worldwide. The disease is identified by characteristic symptom of the appearance of oval or ellipsoidal greenish gray lesions of 1-3 cm length initially on leaf sheath just above the soil or water level. Under favorable environmental conditions the lesions may extend to the upper part of the sheaths or leaves and ultimately spread to neighboring tillers too. In 1980s, Wickramasinghe and Mithrasena (1989)

reported that sheath blight was the most prominent and widely distributed disease of rice in low country wet zone of Sri Lanka. The disease is caused by the fungus *Rhizoctonia solani*. Not only in rice, *R. solani* is the causative organism for many plant diseases including damping off of soybean (Harikrishnan and Yang, 2007), black scurf of potato (Vikee and Singh, 2021), root rot of sugar beet (Skonieczek et al., 2016), brown patch of turf grass (Traquair and Smith, 1981).

Rhizoctonia solani Kühn [teleomorph (sexual state) -Thanatephorus cucumeris (Frank) Donk] is a soil-borne fungus belonging to the phylum Basidiomycota, subphylum Agaricomycotina. Currently, 14 anastomosis groups (AGs 1 to 13 and AG-BI) of the fungus have been identified. Genetically similar isolates that readily undergo hyphal fusion and cytoplasmic and nuclei exchange are considered as same AG while isolates that do not show hyphal contact, hyphal fusion and nuclear exchange are considered as different AGs. Therefore, *R. solani* is considered as a species complex.

In my PhD study, it was possible to isolate 10 different *R. solani* isolates (Figure 1) using soil samples collected from low country wet zone of Sri Lanka. The fungi were isolated from soil by beet seed (*Beta vulgaris* L.) colonization method described by Papavzas et al., 1975. As morphological, physiological and virulence studies revealed a considerable variation among the *R. solani* isolates, genetic variation was also tested using Random Amplified Polymorphic DNA (RAPD) markers. Though, RAPD is now considered as an obsolete marker for assessing genetic variability due to non-specificity, non- repeatability etc., it was the most commonly used method during 1990s. RAPD comparison of isolates followed by cluster analysis showed those *R. solani* isolates are divided into two

genetically different clusters with considerable genetic distances among each different isolate, thereby showing possibility of adaptation to environmental factors as well as to the host resistance against the pathogen.

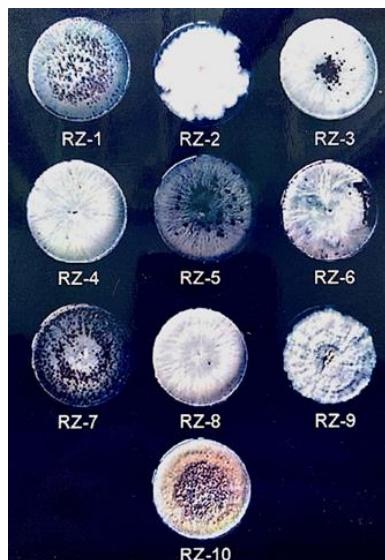


Figure 1.
Different isolates of *R. solani*

Traditional identification of fungi is based mostly on vegetative characters and microscopic examinations which need isolation and culturing of the fungus. Though, the early detection of the pathogen is important in effective control of the associated diseases, traditional identification methods are time consuming and laborious. Further, conventional methods cannot be used to detect low levels of infection as well as low levels of the pathogen present in natural habitats. The conventional methods are hence, not practical and uneconomical when analyzing a large number of samples in epidemiological surveys. Therefore the objective of my PhD study was to develop a DNA based rapid detection method/s for specific and sensitive detection of *R. solani*. As DNA based detection methods, DNA probes and

PCR were the most well established and commonly used methods at that time, it was decided to develop a DNA probe and a PCR assay to detect the fungus.

DNA probe is a stretch of labelled single-stranded DNA molecule used to detect the presence of complementary target nucleic acid sequences by hybridization. A detection probe should be sensitive enough to detect very little amount of DNA, that means little amount of fungal cells are enough when detecting non isolated pathogens from infected plant material or from surrounding soil. The sensitivity is further increased if targeted DNA is having a high copy number within the genome. Therefore, repetitive DNA elements in genomes are considered as a good candidate to develop a DNA probe. DNA repeats or repetitive DNA refers to DNA sequences which are present as more than one copy (usually multiple copies) in a genome. Repetitive DNA may be families of related but individually distinct sequences which show sufficient homology to cross hybridize. These are basically of two types, tandem repeats which are repeated one after the other and interspersed repeats that are dispersed throughout the genome.

A genomic DNA library was constructed by partially digesting *R. solani* genomic DNA with the restriction enzyme *Sau3A*1 and cloning in Bacteriophage λ vector followed by amplification in *E.coli* strain XL1Blue MRF. Thereafter, the library was screened with ^{32}P labeled genomic DNA of *R. solani* as the probe to target clones having high signals (high copy number sequences). I found a clone with the highest signal from the library (named RZ10), and it was *in vivo* excised to a phagemid designated as pRZ10. Phagemids are circular bacterial DNA with both plasmid and bacteriophage properties by having replication origins from

bacteriophages as well as from plasmids and behaves as a plasmid in *E.coli* host cells. Therefore replication and downstream applications are easy to perform. The clone was further characterized by restriction enzyme digestions and Southern blotting of *R. Solani* DNA using the clone pRZ10 as the DNA probe. The results revealed that the cloned sequence is approximately 1.5 kb in size and that was a part of approximately a 4.4 kb interspersed repetitive region (Figure 2) within the genome with the potential to use as a DNA probe for sensitive detection of *R. solani*. When cloned sequence was labeled with ^{32}P and used as a DNA probe to hybridize with *R. solani* genomic DNA in a dot blot (Figure 3), the sensitivity of the probe indicated that the detection is capable down to 0.1 ng of genomic DNA. Further, the probe was tested for specificity, and DNA from other soil fungi isolated from soil and plant material from the same habitat as *R. solani* did not show any cross hybridization with the probe (Weerasena et al., 2004).

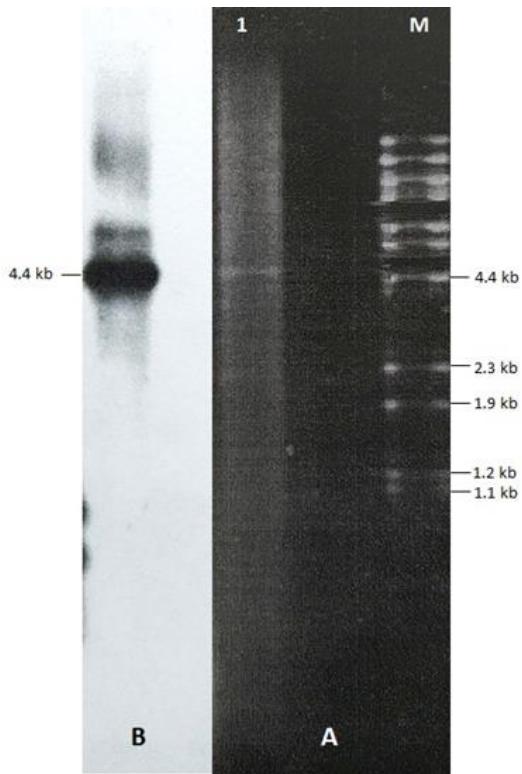


Figure 2.
A-Electrophoresis
image of *Eco* R1
restriction enzyme
digested *R. solani*
genomic DNA (1)
DNA size marker (M)
B -Southern blot
image of *Eco* R1
digested *R. solani*
DNA hybridized with
pRZ10 clone as the
DNA probe

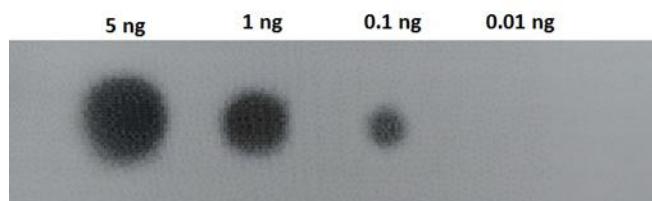


Figure 3. Dot blot showing the sensitivity of pRZ10 as the
DNA probe in detecting *R. solani* DNA

The cloned repetitive sequence of pRZ10 was then sequenced by Sanger's Dideoxy termination sequencing. At that time the whole sequencing process was carried out manually using ^{35}S labeled nucleotides and that was a tedious task to read even a small stretch of a DNA sequence by

polyacrylamide gel electrophoresis followed by autoradiography. Fortunately, Professor Karunananayake was able to facilitate our sequencing work by establishing automated DNA sequencing lab at the department under his SAREC research grant. That was the first automated DNA sequencing facility in Sri Lanka. I was fortunate enough to learn automated DNA sequencing in this facility and sequenced the repetitive DNA sequence of 1550 bp (Accession No: AY309968.1). A PCR assay was developed by designing a set of primers to amplify ~500 bp amplicon (Figure 4) from the sequence. The PCR assay was capable of detecting as little as 10 pg of genomic DNA. A publication (Weerasena et al., 2004) based on the work was awarded the Presidential Award for Scientific Research in year 2010.

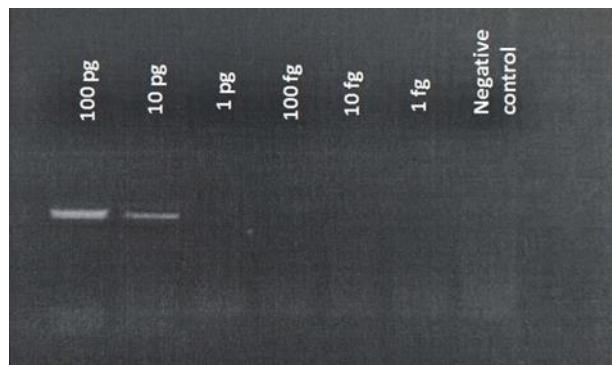


Figure 4: Sensitivity of the PCR assay using primers designed from the cloned repetitive sequence.

I was privileged to work with Professor Karunananayake, an amazing person, researcher and academic who led the research group at the Molecular Biology Unit (MBU). He provided me with guidance and mentoring, needed to succeed in my PhD program. I deeply appreciate how he

supported us with day and night working culture in our laboratory in continuing experiments. He managed to provide, all required state of the art instruments, consumables, chemicals, enzymes etc. available for everyone in the laboratory without limitations.

His relentless effort in introducing and promoting Molecular Biology in Sri Lanka made him successful in negotiating of a soft loan of 15 million Swedish Kroners in year 2000 from Sida, to establish the Institute of Biochemistry Molecular Biology and Biotechnology (a Centre of Excellence) at the University of Colombo, which has become a national asset and world renowned as IBMBB.

After the establishment of IBMBB, I also had an opportunity to join the IBMBB academic staff under the Directorship of Professor Karunanayake who became the founder Director of IBMBB. At that time he encouraged us as young researchers to apply for new research grants and continue the research culture he developed during his tenure as the research leader of the SAREC grant. I was able to secure my first research grant from the National Research Council (NRC) as the principal investigator and thereafter, several other grants from National Science Foundation and NRC as a co-investigator. Under my first grant I was able to establish Fluorescence based Amplified Fragment Length Polymorphism (FAFLP) technique in our laboratory, and genetic differentiation of rice germplasm in Sri Lanka including traditional rice, new improved rice varieties and wild rice species was carried out using FAFLP. Dr. Gowri Rajkumar, Senior Lecturer at the Department of Botany, University of Jaffna, completed her PhD under my supervision supported by my first research grant. Further, research projects in different fields including molecular biology of filariasis, plant diseases, mycology, molecular markers etc.

were carried out and I could produce ten PhDs and three MPhils as a co-supervisor and complete twenty eight MSc projects under my supervision/co-supervision. I hope to fulfill Professor Karunananayake's wishes as one of his followers and wish him excellent health and long life to see his vision and mission become a success.

Copyright acknowledgement: Figures 2, 3 and 4 have been adopted/reprinted from Mycological Research, 108, Weerasena OVDSJ, Chandrasekharan NV, Wijesundera RLC, Karunananayake EH, Development of a DNA probe and a PCR based diagnostic assay for *Rhizoctonia solani* using a repetitive DNA sequence cloned from a Sri Lankan isolate, 649-653, Copyright (2004), with permission from Elsevier.

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Molecular-marker Revealed Genetic Diversity of Coconut (*Cocos nucifera* L.) in Sri Lanka

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The work under discussion refers to the study on molecular marker-based coconut diversity assessment carried out at the Department of Biochemistry, Faculty of Medicine, University of Colombo in pursuance of my PhD under the supervision of Dr. JMDT Everard from CRI, Professor Eric Karunananayake and Professor HG Nandadasa (then Professor of Botany, University of Sri Jayewardenepura) during 1999-2002. The research was supported by the Swedish Agency of Research Cooperation with Developing Countries (SAREC) grant, awarded to Professor EH Karunananayake for capacity building in Biotechnology. Despite being the grantee, Professor Karunananayake was the live wire of the project, who ensured its successful completion with his able guidance and close supervision during the study which has been a great inspiration for my career.

Coconut, *Cocos nucifera* L., the most renowned tree crop in the world in respect of human food security and poverty alleviation plays a significant role in the economic, cultural and social wellbeing of people over 80 coconut growing countries. It is indeed the livelihood of many people living in tropical countries including Sri Lanka as the second most important calorie provider and as a commodity of multitude of uses in addition to being a main source of foreign exchange.

World scenario on coconut production clearly underscores the serious challenge Sri Lanka is facing as the 4th highest coconut producing country but trading only a marginal proportion in the world market. Sri Lanka being a small country can no longer expand the land under coconut to increase the production and thus has to depend on increasing productivity and quality of products to remain in the international market. As for all agricultural crops, for coconut too development of genetically improved planting material has been considered as the most important strategy for increasing its productivity by the Coconut Research Institute (CRI) from its very outset in late 1920's.

The coconut hybridization programs are aimed at searching hybrid vigor for traits such as early flowering and bearing, production of more nuts, having more copra per nut and showing adaptability to a range of agro-climatic conditions. Most coconut hybrids developed to date possess varying characters such as higher number of nuts, bigger nuts, higher copra, tolerance to leaf wilt disease, and resistance to environmental stress.

The coconut hybrids produced in the world so far fall into two categories, intra-varietal and inter varietal. The coconut breeding program at CRI over 10 decades of work has made way for releasing seven (CRI60, CRI65, SRISL98, Kapruwana, Kapsuwaya, Kapsetha, and CRISL20) improved cultivars to the industry. However, with regard to these improved cultivars there is a huge gap between the productivity in experimental plots and actual farmers' fields. In general *Tall x Tall* hybrids are better adapted to rain-fed farmer conditions than *Dwarf x Tall* hybrids. Yet they too require a certain level of management such as application of fertilizer, free of competition from old palms and noxious weeds and evenly distributed rainfall to live up to their potential. In contrast, *Dwarf x Tall* hybrids are

sensitive to change in weather rapidly showing symptoms of drought in dry weather in spite of the fact that they still yield above pure tall coconuts. Therefore, the biggest challenge faced by the coconut breeders in current circumstances is to develop varieties, which are friendlier to low input farming systems.

In order to develop new cultivars with a better adaptability to grower conditions, breeders require a wide collection of germplasm constituting genetically diverse accessions to breed and test for hybrid vigor in important economic traits. This is not an easy task in coconut with conventional breeding methods. Although the CRI has achieved a remarkable success over the years, yet there are many gaps in breeding research that remain unsolved mainly because of various constraints breeders face, some of which are common to all perennial crops such as time and space required for breeding trials and some are specific to coconut such as lack of a viable method for vegetative propagation.

Systematic collection of coconut germplasm in Sri Lanka began in 1984 (Wickramaratna, 1984) and *ex-situ* field gene banks have been established since then with a wider representation of commercial ‘tall’ (SLT ecotypes) and distinctive coconut phenotypes. Understanding the true genetic variation of these accessions has been an important requirement for effective management and utilization of these coconut germplasm in the country.

Characterization of coconut germplasm by morphological descriptors alone has failed to elucidate the true genetic relationships of the *ex-situ* conserved accessions. Molecular marker-based characterization in this regard was believed as a more accurate option because their expression is

not masked by the environment. The study under discussion was a successful attempt made towards assessing the genetic relatedness of coconuts in the country by using, then widely applied three molecular marker techniques, Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLP) and Simple Sequence Repeat Polymorphisms (SSRP).

Among the approximately 100 *ex-situ* conserved coconut germplasm accessions in field gene banks of the CRI, 43 accessions, comprising 19 distinct phenotypes (7 tall (*typica*), 9 dwarf (*nana*) and 3 thembili (*aurantiaca*) forms), 17 Sri Lanka tall ecotypes and 7 San Ramon tall-like ecotypes had been assessed (Dasanayaka et al., 2003; Dasanayaka et al., 2005; Dasanayaka et al., 2009).

Twenty operon primers selected from 100 primers, eight *Eco*RI and *Mse*I selective primer combinations and 17 pairs of coconut specific microsatellite primers were used for detection of RAPDs, AFLPs and SSRPs, respectively. All three maker types revealed the genetic structure of coconuts in the country more or less in the similar manner unveiling the important kinship in par with accepted theories of evolution and dissemination of coconuts worldwide.

In addition to the clear elucidation of genetic relationships of coconut in Sri Lanka the results of the study also led to several implications on effective conservation and breeding of coconuts in Sri Lanka. Close clustering of SLT coconuts from the rest, irrespective of tall or dwarf morphotypes clearly signified SLT as a heterozygous group sharing a narrow genetic base. Therefore, the method practiced to collect SLT germplasm from different geographical locations for conservation in large

blocks of land with 60 or more palms/accession appeared somewhat futile. A single countrywide collection of SLT with small samples from different locations looked a better option for forming a core of SLT coconuts with minimum duplication (Dasanayaka et al., 2009). A molecular marker assessment across populations in different geographical locations prior to collecting germplasm for conservation therefore, is a more efficient strategy for identifying unique populations with specific relevance for breeding.

As revealed by this study the entire genetic diversity of coconut in Sri Lanka is confined within the widely grown commercial ‘tall’, more ornamental type, ‘dwarf’ and the solitary collection from the Clovis estate, San Ramon, which resemble Pacific Tall Coconuts by its appearance. SLT has very little opportunities for further genetic improvement by selecting within itself. First released coconut cultivar, CRIC60, which is a selection of SLT has failed to demonstrate a significant yield increase over unselected SLT. Early studies of CRI have revealed hybrid vigor by combining SLT, dwarf and San Ramon in varying combinations for economic traits such as early flowering/bearing, nut yield and kernel weight. However, this investigation emphasizes the dearth of genetic diversity in Sri Lanka for extensive use in breeding suggesting the option of enriching country’s genetic base by obtaining exotic germplasm. It is interesting to note that Coconut Research Institute has already taken appropriate action by bringing in 22 exotic accessions of exotic germplasm from India, Papua New Guinea and Côte d’Ivoire (Ivory Coast) for *ex-situ* conservation and broadening the genetic base for accelerating the coconut hybridization program.

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‘Bud plus Two’ blended with DNA Technology

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Tea (*Camellia sinensis* L.) is the world’s second most consumed and most popular non-alcoholic beverage. It is brewed by steeping tender leaves of the perennial (Figure 1). Tea believed to have been discovered ‘accidentally’ in 2737 BC by the Chinese emperor, gradually gained a world-wide acceptance as a ‘healthy beverage’ and its’ cultivation spread into various geographical regions in an increasing rate.



Figure 1. (A). Tea plant under natural growing condition (B). A tea bush under cultivation condition (C.). Two tender tea shoots (Top) - a shoot from the tea cultivar TRI 2023 (Below) - a shoot from the tea cultivar TRI 2043

Traditionally, Sri Lanka is well-known as the world leading superior quality black tea producer, and is the 4th largest producer and the 3rd largest exporter (Anon, 2020). Sri Lanka, with its ever popular brand ‘Ceylon Tea’, plays a predominant role in the global tea market. Being one of the

leading foreign exchange generators, sustainable development of tea industry is one of the major areas of concern in the agricultural sector of Sri Lanka.

Commercial cultivation of tea commenced in Sri Lanka in mid-1800's, the 1st seed stock introduced in 1824-1833 era was mainly of 'China type' (*C. sinensis*), followed by 'Indo-China type' (*C. sinensis var. assamica*) imported from Northeast India. Subsequent seedling tea populations (old seedling tea populations- OSTs) were derived by natural hybridization of these two populations. Seeds from a single parent designated as ASM 4/10 imported from Assam, India in 1937 were also used extensively to develop presently recommended improved tea cultivars. Though other 'Indo-Chinese types', were later introduced, their utilization in breeding program was very limited. Therefore it could be claimed that the majority of the recommended 'TRI cultivars' were selections made directly or indirectly from the single parental line ASM 4/10 (Richards, 1965).

The tea cultivar together with several other factors plays a key role in processing of a tea cup with high quality characteristics, thus development of improved tea cultivars is crucial. The Tea Research Institute, Sri Lanka (TRISL) used conventional approaches to accomplish this, but tea being a highly heterogeneous, predominantly out-breeding woody perennial, genetic improvement solely based on conventional methods is tedious and time consuming requiring 20-25 years to release a new cultivar. With the advent of DNA marker technologies in late 1980s and 90s, most countries were successfully integrating these tools into the conventional breeding programs of economically important crops. TRISL was also desperately looking for alternatives. Despite TRISL being very keen to integrate novel DNA marker tools into their tea breeding program, the available resources,

especially potent human capital with state of the art facilities were not there in the country. Time was completely different from present day scenarios as the scholars with adequate knowledge, appropriate exposure and hands-on experience were very rare. On top of that, finding such an eminent scientist generous enough to share this new knowledge, willingness to train people, extend funds to capacity building in the subject was not a reality.

Emeritus Professor ‘Vidya Jyothi’ Eric H. Karunanayake, the founder of IBMBB was the pioneer who made unforgettable and tireless efforts to fill this huge vacuum. He had a great vision and a very sound, straightforward and outcome oriented mission to build capacity in Biotechnology and Molecular Biology discipline in the country within a short period. In addition to the medically related areas, he had identified and prioritized capacity building in Molecular Biology in several areas extremely important to country’s economy such as fisheries, rice, papaya, coconut, rubber, and tea. He extended his supporting hands to country’s leading research organizations such as National Aquatic Resources Agency (NARA), Coconut Research Institute (CRI), Rubber Research Institute (RRI), TRISL to incorporate DNA technology to their Research and Development Programmes.

Thus, in 1998, I was fortunate enough to join his team as a junior scientist from the TRISL to initiate research work on ‘Application of DNA Markers in Tea’ for the first time in Sri Lanka. Since then, our team, Dr. JMDT Everard (Former Deputy Director Research, CRI), Dr. AC Liyanage (Former Senior Research Scientist, TRISL), Professor TLS Tirimanne (Department of Plant Sciences, UoC), Dr. ISB Abeysinghe (Former Director, TRISL) and Professor J. Weerasena (IBMBB) with the leadership, generous funding and infrastructure support of Professor

Karunananayake was able to carry out a number of novel breakthroughs in DNA marker based research on tea as described below.

DNA marker based studies on Sri Lankan tea

Tea breeding refers to the genetic improvement of the tea plant as required by the grower/ consumer concern, by means of manipulating its genetic constitution by selection and /or by hybridization (the main two tools of classical breeding). Identification of sources of genetic variability (Banerjee, 1992) and accurate identification of parents based on true genetic differences is paramount in breeding of crops, especially perennials such as tea (*Camellia sinensis* L). Therefore, genetic characterization and identification of genetic diversity of tea germplasm is fundamental for prioritization of parents for generating heterosis by hybridization. Accurate characterization of tea germplasm followed by scientifically based conservation of tea cultivars could also be considered as the first step to ensure sustainable tea production.

For years, genetic characterization of tea cultivars available in Sri Lanka has been restricted to morphological descriptors or phenotypic characters showing a continuous variation and a high degree of plasticity. Comparison by morphology alone could often lead to inaccurate conclusions and is insufficient to identify diverse cultivars. Until the year 2000, there were no published reports on systematic assessment of genetic variability in tea in Sri Lanka. Comparisons based on morphology and isozymes (Gunasekare et al., 2001; Liyanage et al., 1999; Wickramaratne, 1981) did not reveal much information as sufficient number of polymorphic markers could not be detected.

Rapid development of DNA marker technology offered the breeders a number of powerful new tools to allow crop improvement to proceed at a very rapid and higher scale. In 1990s, globally, several DNA marker studies had been carried out especially in the area of genetic characterization of tea. Randomly Amplified Polymorphic DNAs-RAPDs (Chen et al., 2005; Tanaka and Yamaguchi 1996; Wachira et al., 1995; Wright et al., 1996), Amplified Fragment Length Polymorphisms-AFLP (Paul et al., 1997), and Restriction Fragment Length Polymorphisms-RFLP (Matsumoto et al., 1994) techniques had been widely used in a number of countries.

First DNA marker based study on tea was conducted to screen a selected set of germplasm accessions using RAPD technique. Study aimed to develop a molecular-marker based criterion for germplasm characterization and a considerable genetic diversity (genetic distances ranging between 0.17-0.58) was observed. Several diverse tea accessions that could be very effective in generating heterosis in the breeding programmes were identified; the dendrogram confirmed most of their origins; the sub clusters within the main clusters conformed nicely with their parentages and historical pedigree records (Mewan et al., 2000; 2001a; 2001 b; 2005). With the highly appreciable intervention of Professor Karunananayake and financial support from SAREC and CARP, study was successfully completed; this was evaluated as ‘Grade A-Excellent and above expectation’ by the National Committee on Research Programs and Projects (NCRPP). As a beginner I was really fortunate to be exposed and trained in this ‘new technology’ under the remarkable guidance and support of Professor Eric Karunananayake, the veteran in the Molecular Life Sciences in Sri Lanka.

SAREC grant supported my PhD studies at the IBMBB under the supervision of Professor Karunananayake, Dr. Everard and Dr. Tirimanne. Our aim was to apply molecular marker technology for efficient conservation and utilization of tea genetic resources and to enhance precision and speed of conventional cultivar improvement program through Marker Assisted Breeding (MAB). This being the first ever in detail molecular biology based study on tea in Sri Lanka, a couple of milestones were achieved; first report on isolation and characterization of tea SSR and development of a comprehensive set of EST and genomic SSR primers for tea; first report on construction of frame-work map of tea together with DNA marker based characterization of tea genetic recourses using RAPD, AFLP, and SSRP.

When AFLP became the choice for DNA marker based characterization of tea world-wide (Huang et al., 2004; Mishra and Sen-Mandi 2004; Paul et al., 1997), we too incorporated this highly reproducible, sensitive and technically demanding DNA marker system, and carried-out the first AFLP based study for a set of tea cultivars selected from the *ex-situ* germplasm of TRISL. Genetic diversity of the studied cultivars-a selected set of recommended and non-recommended tea cultivars-was not that high (Mewan, 2011). It clearly reflected the importance of using more powerful DNA marker system informative enough to study closely related individuals, where microsatellites or Simple Sequence Repeat (SSR) have proven its power for the same (Mewan et al., 2012).

During early 2000s, due to its high abundance, power and informative nature, microsatellites or SSRs became one of the most popular sources of genetic markers with a great potential in genetic analysis, mapping and marker assisted breeding in tea. However, limited availability of sufficient

number of SSR marker for tea was an issue to overcome. Thus we focused on ‘Isolation and characterization of genomic and EST (expressed sequence tag) SSR markers for tea’. For isolation of EST-SSRs, expressed sequence tag (EST) sequences were characterized from cDNA libraries of tea cultivars, TRI 2043 and DT 1, whereas for genomic SSR, sequences characterized from two genomic libraries contracted from cultivar TRI2023 enriched for two repeat motifs (CA/GT)_n and (CTT/GAA)_n were used.

A total 305 (157 perfect and 148 imperfect) genomic-SSR and 777 (385 perfect and 392 imperfect) EST-SSR primer pairs were constructed. A higher percentage of genomic SSRs were more informative than the EST-SSRs. Subsequently, SSRs developed were effectively used to construct the genetic map of tea, genetic characterization of cultivated (recommended and OSTS) and non-tea types, and development of a DNA marker for identification of blister blight disease resistance in tea.

Construction of detailed genetic linkage maps is a fundamental first step towards application of marker assisted breeding (MAB) in cultivar improvement programs. These increase precision and speed up breeding processes by eliminating need for subsequent labour and time intensive large scale field screening trials. Although a practical genetic map is not available for tea, the potential of marker assisted selection is clear and strong. Thus, an attempt was made to construct a genetic linkage map of tea. F₁ full-sib population was developed according to a two-way pseudo-testcross strategy (Mewan et al., 2007). A total of 148 F₁ genotypes derived from a cross between TRI 2043 × TRI 2023 was genotyped with 190 primers. Two parental maps, *TRI 2023 parental map* and *TRI 2043 parental map* were constructed using microsatellite loci obtained from

both EST- and genomic- SSRs. These became the first microsatellite maps of tea.

The TRI 2023 map covered 1,227 cM of the genome with a marker density of 6.8 cM per marker; TRI 2043 parental map covered 1,018 cM of the genome with a marker density of 7.0 cM per marker; a substantial improvement over the existing RAPD based map (Tanaka, 1996), RAPD and RFLP map (Hackett et al., 2000) and AFLP map (Huang et al., 2005), especially with regard to marker density. Mapping of SSR loci, especially the EST-SSRs, will be of great value for comparative mapping with related species. Mapped microsatellite loci will also be of value for their practical application in marker-assisted selection. Tea is a clonally propagated plant. Parents and all progenies of the mapping population are maintained clonally and planted in Sri Lanka for field evaluation. This will facilitate further improvement in the mapping effort and enable QTL mapping studies over multiple environments.

The existing map is being further improved by introgression of economically important traits into the map (QTL mapping) and linked markers could be used for direct selection of the trait/s at the early stages using a simple PCR based assay without large scale field assessments. Further improvements of the map towards QTL mapping and Marker-Assisted-Selection are in progress.

Marker-Assisted-Selection (MAS) is a very important application of DNA technology. It is perceived as highly advantageous to aid the conventional breeding; to shorten the breeding period; for higher accuracy in identification of genes prior to their expression; introgression of targeted desirable genes. Molecular marker technology is currently used for the tea

plant, but more for genetic diversity, deriving population structures and unravelling domestication history *etc.* than for MAS. Molecular markers provide a wide range of applications in plant genetics and breeding. SSR markers have been widely applied in many crop species including tea for diversity analysis, genome map construction, QTL analysis, and in MAS (Wang et al., 2016). Even though initial development of SSRs for the species of concern is costly and technically demanding, once developed its potential as a marker is remarkable due to the unique characteristics such as co-dominant inheritance, multi-allelic nature, relative abundance, and reproducibility.



Figure 2. Blister blight infected tea leaf with blisters

Blister blight (BB) leaf disease of tea (Figure 2), caused by the fungus *Exobasidium vexans*, results in 25–30% crop loss annually. BB is presently controlled by Cu based fungicides, but genetic resistance is the most viable option. MAS is vital to expedite breeding programmes and also for better accuracy in gene identification. RAPD technique was first tested to identify a potential marker to distinguish between BB resistant and susceptible tea cultivars (Mewan et al., 2002); importance of a mapping population as well as an informative DNA marker system was identified.

Both field and laboratory experiments were initiated in 2003 to identify a DNA marker for early selection of blister blight resistance to facilitate MAS and breeding using an F1 population segregating for BB. The population was genotyped using a selected set of genomic and EST-SSR loci and alleles detected by fragment analysis. A diagnostic marker identified was validated in a panel of 64 tea cultivars, resulting in the amplification of 12 alleles at EST-SSR073. The EST-SSR073 allele sequences matched with *Camellia sinensis* photosystem-I reaction center subunit-II. This recorded a milestone in MAS in tea.

With the training received during my doctoral studies, I was able to subsequently extend studies to all recommended tea cultivars, old seedling tea populations (OSTs)-a tea genetic resource with great deal of genetic variation and un-tapped potential and to non-tea types (*camellia spp.*). Initial studies based on micro-satellite/ SSR markers were followed by comprehensive studies based on both genomic and EST-SSR markers on recommended cultivars (Karunarathna et al., 2018a; 2018b). Tissa Karunarathna's work which formed his PhD studies was carried out at the IBMBB. Information generated from the above studies would be of paramount importance to the tea breeders as a platform to direct their breeding strategies towards further improvement of tea cultivars in a sustainable manner.

Finally, it is a great honour to note here, that the outcomes of above research activities resulted in commendations at international and national fora with several awards including "Best Researcher Award-2007" 3rd International Conference on O-CHA (tea) Culture and Science (ICOS 2007), University of Shizuoka, Japan; Hiran Tillekeratne Memorial Award- 2011 from the University Grants Commission, Sri Lanka (for

‘Outstanding Postgraduate Research - 2007 to 2009 in Natural Sciences’); SLAAS Postgraduate Research Award 2012. The same project was also selected for National Awards for Excellence in Agricultural Research-2012 by CARP and is in the final evaluation stage.

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Molecular Diagnosis of Phytoplasma Diseases in Sri Lankan Crops

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A discussion on phytoplasma diseases in Sri Lankan crops is highly appropriate in a volume, felicitating the eminent scientist and scholar, Professor EH Karunananayake who through his leadership and utmost dedication established an enabling environment and high standards for research excellence in the fields of molecular plant analysis and pathogen diagnosis in the country. I had the fortune to read for an MPhil Degree in this facility (2004 – 2007) under the supervision of Professor Karunananayake and Dr. JMDT Everard (Deputy Director, Coconut Research Institute of Sri Lanka) in the area of molecular diagnosis of possible phytoplasma association in certain local crop plant disorders.

Phytoplasmas represent a large group of obligate, prokaryotic, plant-pathogens in the class Mollicutes of the phylum Tenericute. Phytoplasma are wall less and pleomorphic with size variations from 200 to 800 nm and small genomes of about 680–1600 kb. The group multiplies in isotonic niches of plant phloem tissues. Recalcitrant biological properties, inability to culture axenically, non-transmissibility by manual inoculation and high vector specificity restrict studying the group by traditional techniques, however the recent molecular studies have revealed interesting features of phytoplasmas. Phytoplasmas are classified within the single provisional genus ‘*Candidatus Phytoplasma*’ based primarily on the analysis of 16S rDNA sequence. Interestingly, the phylogenetic groups or sub clades

correspond to clusters established by restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR)-amplified rDNA (Seemüller et al., 1998). Thereby phytoplasma subclades or the corresponding PCR-RFLP based 16Sr groups represent at least one distinct species under the provisional taxonomic status ‘Candidatus’ (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004) and these species are associated with over 600 diverse plant diseases (Bertaccini et al., 2014).

Recent research developments shed light on the phytoplasma disease development mechanisms (MacLean et al., 2014). Proteinaceous effectors or virulence proteins released during phytoplasma infection modulate development processes in hosts and thereby suppress host defense responses. Phytoplasma effector induced reprogramming of plant development processes results in a wide range of symptoms including general symptoms such as stunting, yellowing, and specific symptoms such as witches’ broom (development of numerous tiny shoot branches with small leaves), phyllody (formation of leaf-like tissues instead of flowers), virescence (greening of floral organs), proliferation (growth of shoots from floral organs), purple top (reddening of leaves and stems), and phloem necrosis. For example during the development of phyllody, phytoplasma effector protein, SAP54 interacts with a subset of MADS-domain transcription factors in the host. SAP54 folds into a structure that mimic the K-domain, a protein–protein interaction domain of MADS domain proteins, thereby, SAP54 destines the MADS-domain TFs for degradation *via* the ubiquitin/26S proteasome pathway resulting in floral defects. Infected plants produce leaf-like flowers that are attractive to the insect vector thereby phytoplasma infection convert their hosts into sterile

‘zombie’ plants, to facilitate vector-borne transmission and propagation of phytoplasma.

Despite devastating damage caused on crops worldwide, still there is no effective method to control phytoplasma diseases. Thus, early detection and removal of infected plants are critical to prevent the spread of such diseases. In this background, the research generated at Professor Karunanayake’s lab revealing definite association of phytoplasma with economically harmful diseases in locally important crops has been a significant achievement as well as a pioneering effort in studies on local phytoplasma diseases. Our studies revealed a wide diversity whereby phytoplasma that belongs to three distinct phylogenetic subgroups causing distinct symptomologies in the host plant species were diagnosed, including Sugarcane White Leaf (SCGS/SCWL) a decline agent, Brinjal Little Leaf (BLL) a proliferating agent and Sesame Phyllody, a virescence agent. These are described in the three case studies below. This work developed facilities, skills and technical capacity for molecular diagnosis of phytoplasma to expedite and expand local R&D activities in phytoplasma, which is a quarantine pathogen in many crops. Decades after these initial reports the volume of information on molecular diagnosis of phytoplasma associated with local crop diseases has increased (Supplementary Table 1).¹²

¹² Available in the electronic version

Case studies on phytoplasma associated diseases in sugarcane, brinjal and sesame

The systematic study undertaken by the author under the guidance of Professor Karunananayake in 2004 examined causative pathogens of three detrimental and widespread diseases in sugarcane (*Saccharum officinarum* L.), brinjal (*Solanum melanzina* L), and sesame (*Sesamum indicum* L) in Sri Lanka based on PCR techniques and DNA sequencing. The outcome of the work was disseminated through scientific publications (Ariyarathna, 2007; Ariyarathna et al., 2004; 2006; 2007) and public seminar for larger impact (Seminar on Phytoplasma disease affecting plants, 17th December 2007, IBM&BB).

Sugarcane cultivation in Sri Lanka is severely affected by a destructive disease locally known as “white leaf”. The disease prevails in all agro-ecologies where sugarcane is grown, spreading the inoculum fast and causing unrecoverable yield losses. Infected stands show extensive tillering, chlorosis and general stunting (Figure 1-A). Depending on the stage of disease development, striped or mottled leaves or entirely white leaves become visible. When the disease progresses a number of slender tillers appear. Stands with profusely proliferated shoots with completely chlorotic or pale yellow color narrow leaves like grassy shoot symptoms appear sporadically. Numerous, slender chlorotic tillers, premature proliferation of auxiliary buds, severe stunting, and sterility are the characteristic symptoms of ratoon crops. Further cane becomes non-millable. However, disease symptoms in the field grown stands are highly variable and the symptom expression is highly environment dependent. Symptoms appear less during the wet season and become conspicuous with the increase in temperature and the onset of drought. Masking of symptoms

and occasional recovery of symptoms has also been recorded here and then.

Brinjal is an important vegetable crop in Sri Lanka affected by a disorder known as “little leaf” which is not considered as a major cause of total brinjal crop loss although severe infections can cause 100% yield loss in epidemics (Rao and Kumar, 2017). The disease is characterized by abnormally large number of branches, roots and leaves, general stunting, chlorosis, malformed leaves that are extremely reduced in size and short petioles. Leaves become narrow, soft, smooth and yellowish in colour and young leaves become smaller in size. Internodes are shortened. Floral buds are deformed and plants become sterile causing total harvest loss (Figure 1-B).

In sesame, phyllody is the most economically harmful disease in the country, causing total yield loss in severe infections. Major symptoms observed in diseased plants are phyllody, extensive growth of axillary shoots, shortening of inter-nodes, and leaf chlorosis (Figure-1-C).

We have attempted to examine the possible association of above mentioned sugarcane, brinjal and sesame disorders with phytoplasma by screening disease sugarcane samples collected from Udawalawa and Siyambalanduwa, disease brinjal samples from Horticulture Research & Development Institute (HORDI), Gannoruwa and disease sesame samples from Udawalawa. Despite the wide range of symptoms observed in the field samples, positive PCR amplification of phytoplasma DNA from all samples and absence of PCR amplification in any of the non-symptomatic samples confirmed association of phytoplasma with the 'white leaf' disease in sugarcane, 'little leaf' disease in brinjal and phyllody disease in sesame.

The sugarcane white leaf disease samples have been further studied based on PCR-RFLP profiles of rDNA, using *RsaI*, *Sau3AI*, *MspI*, *HpaII* or *KpnI* and DNA sequence analysis of 16S rRNA spacer region (NCBI accession number: EU030439) and the results concluded association of one or possible mixed infection with SCWL and SCGS phytoplasma with the symptoms. SCGS and SCWL are phylogenetically closely related (99%) phytoplasma in the 16SrXI taxonomic group (Sdoodee 2001; Wongkaew et al., 1997). However, recent studies reexamining the strains by multi-locus genotyping using *leuS*, *SecA* genes in addition to 16S rRNA spacer region confirmed that the two strains are identical for these genes and therefore potentially represent the same phytoplasma (Abeysinghe et al., 2016).

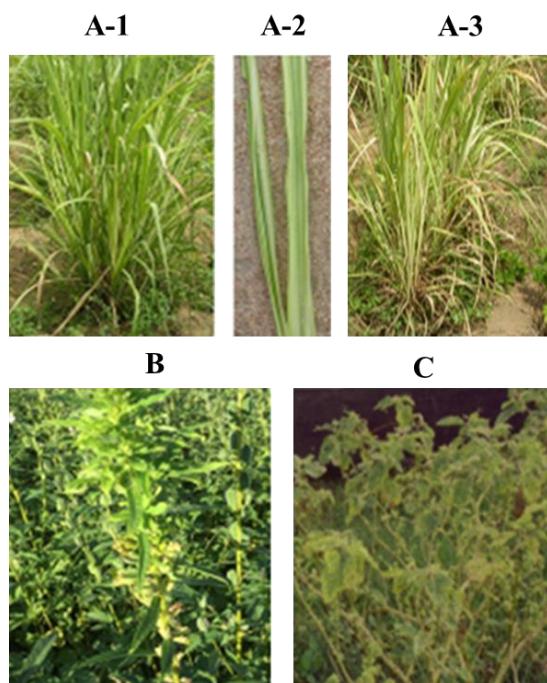


Figure 1. Symptoms of phytoplasma diseases in sugarcane (A), brinjal (B) and sesame (C) observed in the field 294

Concluding remarks

The three case studies that broadly captured genetic diversity of phytoplasma and their diverse pathogenic capacity in sugarcane, brinjal and sesame accelerated development of preventive measures to curtail spread of some of these diseases and contributed significantly to revitalize interests in studies on phytoplasma diseases in local crops and vectors. However, intermittent detection and reports on phytoplasma diseases in new crops or new regions indicates continuous spread of the pathogen and the associated vectors. In the absence of effective control methods phytoplasma diseases are economically and environmentally costly. With the adverse effects of climate change there is an increasing trend in pests and diseases on crops. Rigorous quarantine measures and cost effective disease management, administered through crop and region specific integrated disease management strategies can reduce losses. Priorities for future research should therefore focus on effective and low cost pathogen diagnosis, production of clean planting material, vector control, and development of resistant crop varieties.

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Metabolomics of the Sri Lankan Tea Germplasm Reveals Biomarkers of Black Tea Quality

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“Reverse Time - Pleasant Cognitive Recollections” (RT-PCR) of My Association with Prof. Karunananayake in the Department of Biochemistry:

Reflections of Professor Karunananayake’s First PhD Student

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***Department of Biochemistry and Molecular Biology,
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It is indeed a privilege and an honour to be able to pen down my pleasant memories of the time Professor Eric Karunananayake and myself spent at the Biochemistry department of the Medical Faculty of the University of Colombo.

Time has unforgivingly taken its’ toll and my memory is hazy regarding some details. However, the main events have not yielded to the merciless assault of time.

The veil of time has obscured the exact date on which I met Professor (then Dr.) Eric Karunananayake at the Department of Biochemistry, but it was somewhere in the early eighties that he joined the Colombo Medical Faculty leaving his position as Biochemist at the Medical Research Institute. I remember Dr. Eric, with a head of black hair, unlike the snow white head he now sports, occupying the room occupied by Prof. Stanley Wijesundera, who moved over to College House after being appointed the Vice-Chancellor of the University of Colombo.

Yours truly, a young hot blooded buck, was occupying the room just behind his, but connected by a narrow corridor at the back. My company

¹³ 1977-2012

at that time was a group of equally hot blooded young junior lecturers who could have got into any conceivable trouble before one could say “Jack the Ripper” !!!!!. They were, as far as my memory goes, Guy Dabare, Sunil Epa Seneviratne, Nalini Wickramasinghe, K. Wimalasena (now a Professor in a University in USA), Sarath Sirimanne (now crossed the great divide into the great beyond after returning back to the Department from USA, where he did his PhD and worked as an Academic), Deepal Mathew (who later became a Professor & head of department) and Preethi Kannangara, who I think was a research student, but was like one of us. Guy and Sunil after sometime reverted back to their medical careers. The seniors at that time were, Professor Balasubramanium, Professor Cissy Canakaratna, Dr. Sunethra Atukorale and Dr. Ponmani Gunawardena. Prof Arthur Abraham Hoover had just retired by that time.

Dr. Eric (at that time) used to have his lunch in his room and we used to have ours in my room. Our lunch time was hilarious. Within a short time we realized that Dr. Erics’ lunch should be booooooring and decided to invite him to join us. He did, and from then on it was a great association about which I am about to pen my memories. In those early days the department was more inclined towards teaching than research. Prof. Balasubramanium had a few research projects including work on alpha galactosidase from coconut (on which Deepal Mathew’s PhD was based) going at the time. Eventually Dr. Eric formed a second area of research, namely research on medicinal plants. By and by Dr. Eric and I started work on Karawila (*Momordica charantia*) and that is how I became Dr. Erics’ first research student in the faculty way back somewhere in early eighties. In addition Dr. Eric worked on a few other areas of Biochemistry; measurement of antiepileptic drugs in collaboration with the Consultant

Neurologist Dr. J. B. Peiris and collaborators from Netherlands, and on the effect of chemical constituents of tea on caffeine metabolism. The latter formed the basis of the PhD studies of Preethi Soysa who has now retired having been a Professor in the Department.

That was a memorable time working under sub optimal conditions, equipment as well as chemical wise. I remember going around Dr. Erics' car to the MRI to get some anhydrous Analar glucose to draw a standard curve !!!!. The research arm that began that way grew in leaps and bounds eventually branching into Molecular Biology and the rest is history.

The research project Dr. Eric initiated was on the hypoglycaemic activity of *Momordica charantia* ("Karawila"). I was Dr. Erics' first research student reading for a PhD. We first established the hypoglycaemic activity of the crude extract of the fruit of *Momordica charantia* on fasting rats. Then we proceeded to show the same activity on induced diabetic rats. Having done this, we proceeded to test the extract on diabetic patients and once again showed the efficacy of the fruit extract. Subsequent *in vitro* studies established the increased uptake of glucose by extra hepatic tissue in response to the Karawila fruit extract.

Having completed this work I proceeded to the University of Uppsala, Sweden under the sponsorship of the then "International Seminars" of Sweden. I was attached to the Department of Medical Cell Biology and worked with Prof. Bo Hellman continuing *in vitro* studies on the effect of the *M. charantia* extract on isolated pancreatic islets of ob/ob mice. The work showed that the extract induced the islets to secrete insulin when challenged with the extract. The time I spent in the laboratories of

Professor Bo Hellman was very pleasant and remains permanently etched in my memory.

Now let me dwell into the more interesting episodes connected with life in the Department of Biochemistry. Life in the department was absolutely joyful with no one competing with the other for anything. Anybody's problem was everybody's problem and any opportunity to laugh at somebody's mishap was always grasped with glee!!!! The threat of an embarrassing exposure followed by a hearty laugh at the expense of the unfortunate colleague was always looming!!!! What we had there at the time was one united and happy Biochemistry family.

Once in a while the department staff and their families used to get-together for dinner, fellowship, and of course, substantial amounts of booze. The location was usually at Cissy's residence on account of the convenient location down Rosemead place. When I think back, I now realize the value of such fellowship. It was that which maintained the healthy and happy relationship between members of the staff. There was no room for misunderstandings. Any issue that bothered someone was quickly dispersed because many things happen inadvertently with no intended malice. The fellowship helped to understand and realize that there was nothing underhand. This fellowship and the lack of any desire to overtake the other, made us a united and happy pack.

In the mid1980s there was a decision to establish Molecular Biology in the department and Dr. Eric and I were packed off to the Uppsala University, Sweden and we ended up in the laboratory of Professor Ulf Pettersson. Our families also accompanied us and that was the time my wife and Dr. Eric's wife Padmini became great pals.

The time we spent in Sweden was wonderful, the beautiful summers and the horrific winters where we were chilled to the bone. We were housed in the flats in Flogstavagen, situated at the end of the bus routes No 1 and 2. This made it very convenient for us to travel to the BMC. Almost every weekend was reserved for shopping for food from one supermarket to the other with the wives and window shopping thrown in was an experience. The wives were in their element in the supermarkets and they knew exactly where the cheaper (“extrapis”) chicken and ice cream was!!!! Of course, the wives buy the cut price food in fair bulk and what needed to be frozen is packed into plastic boxes and bags, attached to a string and tossed out of the window during winter. This is because the temperature inside the domestic refrigerator was minus five while the temperature outside the window was minus twenty!!!!

On our return to Sri Lanka we set about setting up the first Molecular Biology & Gene Technology laboratory where it is still located. A group of scientists arrived in Sri Lanka to help us establish the Molecular Biology laboratory at the Faculty of Medicine in Colombo. It was a very pleasant time working with those scientists whom we already knew through our stay in Professor Ulf Pettersson’s lab.

Around that time more joined as research students. They were Kithmini who is currently head of department of Biochemistry, Sulochana Wijesundera and Preethi Jayawardena (both retired recently as Professors), “Charky” Chandrasekaran (now in the Science Faculty), Jeevathayaparan, (who later joined the staff and later left for Malaysia after securing a good position), Dharmasena (now in a University in USA), Sharmila (currently in the faculty staff) and a few more, all of whom were no second to the rest whose names were mentioned before. Other essays in this volume describe

the Molecular Biology work in detail. In the meantime Professor Sunethra Atukorale was building up another branch of research-Nutrition-and she too had research students.

The JVP insurrection days were also memorable on the negative side. Uncertainty was the order of the day. But one day remains in my mind and that was the day the JVP students union was preparing to bring the body of Thrimawitharana, an undergraduate who died under unfortunate circumstances, to the Faculty. That afternoon only a few staff members were in the faculty. I remember Dr. Eric, Dr. Abeyewickrema from Parasitology and myself being there. Things were not too good and some were trying to draw petrol from the Faculty vans parked. I remember Dr. Eric and myself watching with concern if they would try to draw petrol from Dr. Eric's Ford Escort. Just then a high powered military group headed by Wijaya Wimalaratne (I don't know what his rank was at that time) came to the faculty and wanted a discussion. We sent word to Prof. Nandadasa Kodagoda our Dean at the time and he arrived. The few of us who were there met the Military officers in the Forensic department for security discussions. Those times were not pleasant at all.

Yet we all overcame the difficulties to continue teaching generations of medical students, training postgraduates in Biochemistry and Molecular Biology and provide a significant research output from the Department.

Road to Peter Mac: Personal Reflections of the First PhD student of IBMBB

Wasanthi De Silva
Molecular Diagnostic Pathology Department
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My journey in Molecular Biology started as a post graduate student at the Faculty of Medicine, University of Colombo (in 2003) and the IBMBB, University of Colombo (2004-2008) under the guidance and supervision of Professor Eric Karunananayake. My PhD research project initiated in 2004 was “Characterization of sequence variants in the breast cancer susceptibility gene 1 (*BRCA1*) in selected Sri Lankan breast cancer patients and at-risk individuals”.

Professor Karunananayake’s immense knowledge in Molecular Biology was invaluable to me during my PhD studies. With his unstinted support, I successfully completed an international publication in BMC cancer under the title, “Novel sequence variants and a high frequency of recurrent polymorphisms in *BRCA1* gene in Sri Lankan breast cancer patients and at-risk individuals” (De Silva et al., 2008).

Professor Karunananayake is the founder Director of IBMBB. During my time there, I witnessed his immense commitment, hard work and untiring efforts in creating IBMBB, building it up from its very foundations with the help of Swedish grants from Sida and the International Program in Chemical Sciences (IPICS), University of Uppsala, Sweden. Countless were the obstacles and constraints thrown his way during those initial years. But he always found a way to overcome all adversity, successfully

securing grants for research studies and making IBMBB an esteemed institution in Sri Lanka.

As a member of the pioneering batch of PhD students at IBMBB, I was extremely fortunate to have had the opportunity to carry out my research work in the brand-new labs. I am even more fortunate indeed, to be the first student to walk out of IBMBB with completed PhD qualification under my belt. I also had the privilege of delivering the inaugural student representative felicitation speech for Professor Karunananayake at the ceremony held in 2007 on his retirement.

Today, I am a senior medical scientist in the premier Cancer institute in Australia, the Peter MacCallum Cancer Centre (Peter Mac) located in Melbourne. Peter Mac is part of the VCCC (Victorian Comprehensive Cancer Centre), a multi-site, multidisciplinary partnership between 10 leading medical research, academic and clinical institutions. I have been working at Peter Mac's Molecular Pathology laboratory since 2008. This laboratory is the premier clinical cancer molecular diagnostic genomics service in Australia, providing a range of NATA accredited testing to clinicians and researchers at Peter Mac as well as other cancer centers and hospitals throughout the Asia Pacific region. The facility offers molecular services on multiple high-throughput test platforms including preparation of RNA, DNA, ctDNA and protein from a large number of samples for molecular assays, massive parallel next generation sequencing, gene expression and quantitative PCR assays.

The solid foundation I received from IBMBB and the guidance I received from Professor Karunananayake has been invaluable to me in this journey.

I wish him all the very best in life, good health, happiness and long life from the bottom of my heart.

Eric Sir: a Leader, a Mentor, a Guide - Who always has a Friendly Smile

Personal reflections of some “home grown” post-doctoral and doctoral students of the IBM&B

As students of IBM&B, we can all testify to the pleasant and cheerful ambience at IBM&B. From the very first day we visited the institute, to a day filled with work or even when one of us realized that our experiment wasn't a success, there was just something in the air that stopped us from giving up. There was a sense of belonging, an awareness on what we did mattered at IBM&B.

This friendly, disciplined yet approachable nature and the strong sense of professionalism was what Prof. Karunananayake envisioned. His simple gestures - “good morning”, “how are you?” and his smile to any of us characterized this vision, which now resonates and embedded among us all at IBM&B.

As students who initially joined the IBM&B to complete a Master of Science degree in Molecular Life Sciences, we all continued to stay back to pursue our research careers at the IBM&B. That itself is a strong testament to the world-class research, facilities and more importantly the wonderful team working at the institute under the guidance and leadership of Prof. Karunananayake, the founder of IBM&B – who we respectfully call ‘Sir’ – a title that he greatly deserves.

One thing we all remember clearly is that Sir always highlighted the importance of developing not just our knowledge on science and facts – but also our professional skills as scientists of the future. The values of

hard work, gratitude, honesty and integrity were always at the core of Sir's teaching and is reflected even today in the values of all those who have qualified at IBMBB. Sir's teaching style was so unique that we, as students, wanted to go out and find more information and read more on the subject – Sir taught us to be life-long, independent learners.

We are forever more grateful to our dearest Eric Sir, for gifting us and our nation a glorious institute that has an international reputation for science and research. Thank you Sir for playing a strong and influential role in guiding generations of biochemists and molecular biologists to be true professionals in their careers. We are ever so grateful to you and will strive always work hard and uphold these values throughout our own careers.

We are truly blessed to have studied and grown within your leadership and guidance.

*Anuka Mendis (PhD) - CEO and Head of Research and Development,
Suaroma Oils*

*Tharmini Pirabaanandan (PhD) - Senior Scientist – Genelabs Medical
Pvt Ltd*

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Joanne T. Kotelawala – PhD candidate – IBMBB

Amali Fernando –PhD candidate-IBMBB

Anjana Welikala –PhD candidate-IBMBB

Priceless Lessons Learnt from Professor Eric Karunananayake as Young Researchers

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As an academic, Professor Eric Hamilton Karunananayake has achieved many things that very few can aspire. In addition to the great service Professor Karunananayake has rendered to our country, there are many valuable lessons that academics/researchers can learn from his career. We feel that, by following those valuable lessons, academics/researchers can develop their skills and achieve professional success. This concluding chapter presents some valuable lessons we learnt from Professor Karunananayake as post graduate students at the Institute of Biochemistry, Molecular Biology and Biotechnology (IBMBB), University of Colombo.

Be a regular reader

The most valuable lesson we learnt from Professor Karunananayake is the importance of reading. He used to say that reading is a must if you want to be a good scientist. When we were students at the IBMBB, we still remember how he entered the lecture room with files full of research articles. All in the class showed worried faces on seeing his file with research papers. Those days we did not have a strong desire to read

research papers. The research papers he used to bring were complex discoveries in Biochemistry and Molecular Biology. It should take at least 2-3 months to read them properly! In the following class, he used to ask questions from the paper he distributed. But, most of the students failed to give proper answers. Although we may not have realized the importance of the advice he gave us at that time, as we progressed in this field, the value of the impetus he gave us to practice reading became priceless. Research papers that Professor Karunananayake asked us to read were the first valuable research papers that some of us read in our lives. Now we understand that reading sits at the heart research and improves writing skills, communication, vocabulary, imagination, creativity, helps you to discover the rest of the scientific world and supports you to build a strong self-image.

Do not go unnoticed, having a close bond with teachers and supervisors makes a good researcher

Professor Karunananayake always highlighted the value of a close association between students and teachers/supervisors. We remember him stating that students interested in learning and understanding a theory, subject, a study or research problem should always liaise with their teachers or supervisors. Whether group discussions, through class presentations or after class sessions, students who question on research problems become more familiar with the content, highlighting their efforts, dedication and enthusiasm in learning scientific research. Professor Karunananayake noticed his students ‘progress regularly. He always motivated his students to meet him following long experiments although the experiment results are not promising. We can still recall that some

students came out of his office with grinning faces, while some came out with upset faces. In our opinion, his regular observations made most of his students to work enthusiastically and fueled their desire to learn. Also, through regular observations, he must have gauged the research and learning potential of his students and perceived about their personality, which were essential elements in professional relationships. We believe that, by following this habit in one's career, young academics and students can make a strong bond with each other and develop mutual understanding. Through teacher-student liaisons, young students and researchers are more likely to receive the informative and supportive guidance most teachers are willing to offer.

Keep an updated research note book

A lab note keeps all research findings, failures from experiments and data on optimizations. A lab note helps one to maintain reproducibility in the not so uncommon situations where previous protocols or methods of data analysis cannot be remembered. Also, a clear and concise lab note helps lab mates to find what a particular researcher has already done when they want to conduct similar experiments. In addition, if one is preparing a manuscript months after experiments have been completed or months after graduation, a meticulously kept lab notebook is a must to get all the details about experimental procedures and results. Notably, the lab note will be required to show that a researcher has done something for the first time when he/she wants to claim intellectual rights and, sometimes, when data fabrication or falsification accusations are made (this can happen to honest researchers with integrity when jealous elements want to discredit them),

meticulously kept lab note becomes the savior. Professor Karunananayake often explained about the benefits of having a clear lab note. An updated research note book is a must for Professor Karunananayake's students. He always emphasized that an updated research note book is a reflection of the quality of ones 'scientific work and good laboratory practice, and the backbone of all scientific research work that an individual has carried out. Few of us had the rare opportunity to see some of his impressive laboratory notes he had maintained as a PhD student at the Imperial College, London. This was nearly four decades after his PhD and a few years after his official retirement from the University!! We say "official" because he continued to teach and supervise students at the IBMBB for more than a decade after retirement.

Keep laboratories and work places clean

Professor Karunananayake was very strict on laboratory cleanliness. During his tenure as the Founder Director of the IBMBB, every morning he used to do "rounds" walking in the floors of the institute to observe the cleanliness of the laboratories and the work benches. He always wanted to keep the work benches, balance pans, instruments, laminar flow cabinets, fume cupboards, pipettes, glassware and refrigerators clean and organized at the institute. He used say that the cleanliness in the laboratories is a joint responsibility of all the students working in the lab. Although we did not understand this properly during our days as students, we clearly understood the value of his advice he when we were working in laboratories in foreign countries. Keeping your workplace clean gives you self-satisfaction as well as self-encouragement. In some instances, poor cleanliness can even

affect experimental results. Furthermore, when other people observe, clean work bench gives a positive impression about the researchers, the organization or the University they represent. Sometimes, we have noticed that project supervisors get the blame because their students were not keeping the workplaces clean. Professor Karunananayake himself appreciated we practicing the above lesson learnt from him, more so when the same was inculcated in our postgraduate students at the IBMBB.

Integrity in research

He was a keen observer of the integrity of his students' research work. Some of us remember, at an informal discussion, he recalled how he maintained research integrity when he was working as a PhD student at the Imperial College. He mentioned that the integrity in research allows others to develop confidence and trust in one's published work. He nicely explained, with examples, the importance of trust when working in this field and the adversities that can arise when trust is broken. In his opinion, when trust is broken, it is very difficult to get back to where it was before. He also explained how it affects a person's career with the loss of integrity. We would like to add a few more comments to his remarks. In our opinion, the research integrity symbolizes the profile of individual students/researchers or scientists and the Institution. For individual scientists, research integrity has a moral dimension. If a scientist can maintain an untarnished research integrity, he can be a proud scientist on being a highly moral and ethical person even if he or she does not have a longlist of publications. When a person conducts responsible research and maintains honesty, that person can enjoy science! Research integrity impacts upon Institutes and Universities in a quite different way. By

maintaining high standards in research integrity, a University or a Research Institute can be transformed into an exceptional place with an operational environment replete with excellence, reliability and legitimacy. Currently, the recognition we are receiving in the scientific community may be due to the overall training we received from Professor Karunananayake and especially the value of research integrity imbibed in us.

Do not compare yourself with other researchers

This lesson few of us learnt from him is also one of the best lessons that we really appreciate. Although two researchers may have similar goals, aspirations, and interests, the paths that two researchers take and the subjects or areas in which they both do research should not be the same. In essence, what one experience as a researcher should be special and one should not imitate others. A researcher should not compare his or her progress with that of other researchers. A researcher should set his/her own goals and objectives and achieve them step by step without copying others. We all have different goals and objectives. Once goals and objectives are clearly identified a researcher needs to focus on working within a right time frame and plan.

Professor Karunananayake used to say that a researcher needs to create a research lifestyle that makes him/her happy always. Such was his passion for research. He was always the first to read latest breakthroughs and come up with new ideas. Without the qualities that Professor Karunananayake was stressing us to develop, he would not have climbed to greater heights that he reached in scientific research. He set his goals high and showed us the path. Few of us were very fortunate to witness Professor Karunananayake

being recognized at The World Academy of Sciences (TWAS) with an award for building science institutions in Central and South Asia on 6th of December 2016.

Recognition from the World Academy of Sciences -2016



Professor Karunananayake receiving the award from the World Academy of Sciences (TWAS) for building science institutions in Central and South Asia on 6th December 2016 in Bangalore, India

Supplementary Table 1.

Molecular diagnosis of Phytoplasma diseases reported on local crops

Disease	Phytoplasma strain	Disease symptoms	RFLP	GenBank Accession No.	Putative vectors
Sugarcane white leaf Host: Sugarcane (<i>Saccharum officinarum L.</i>) (Ariyarathna et al., 2007; Dayasena et al., 2021; Kumarasinghe and Jones, 2001)	sugarcane white leaf	White line that runs vertically parallel to veins. Eventually cover the entire leaf causing total cholorosis. Stunted stalks and the absence of side shoots, failure to set millable cane. Yield loss 100%	16SrXI-B	JF754450 KU751803 KU751785 KU751804 MN174860 GQ121046 MT872412 MT862368 MT860722 MT860721 MT860714 MT811813 MT811811 MT811809 MT811046 MN186659 KT364325 KT364324 KT364323 KT719319	Deltocephalus menoni (Hemiptera: Cicadellidae, Deltocephalinae) (Chanchala et al., 2014)

Brinjal Little leaf	Brinjal little leaf	Produce numerous, small, leaves on very short petioles. Stem internodes are short, and plants are stunted and bushy due to proliferation of axillary buds. Excessive root branching is common.	Information on local isolates not available	Information on local isolates not available	Information on local vectors not available
Host : Brinjal (<i>Solanum melongena</i> <i>L.</i>) (Ariyrathna et al., 2004)		Flowers formed are leaf-like and sterile. Yield losses can approach 100 percent			
Sesame Phyllody Host: Sesame (<i>Sesamum indicum</i> L) (Ariyrathna et al., 2006)	Forms clusters of leaves and a malformed flowers at the tip of branches resulting in “witches broom” symptoms. In severe infection, flowers were replaced by short abnormal eaves closely	Information on local isolates not available	Information on local isolates not available	Information on local isolates not available	Information on local vectors not available

arranged on a stem.

Cause 100% yield loss.

Weligama coconut leaf wilt	'Candidatus Phytoplasma oryzae'	Flattening and downward bending of leaflets resulting in a ribbed or flaccid appearance.	16SrXI GQ121047 KT369133 KT369127	EU635503 KT719306 KT719305 KT719304 KT719303 KT719302	Homopteran species, <i>Goniagnathus (T.) punctifer</i> , <i>Recilia dorsalis</i> Motschulsky, <i>Kolla ceylonica</i> (Melichar), <i>Idioscopus clypealis</i> (Lethierry), <i>Proutista moesta</i> (Westwood), <i>Proutista</i> sp., <i>Nisia nervosa</i> (Motschulsky) and an unknown hemipteran species
Host: Coconut (<i>Cocos nucifera</i> L.) (Perera et al., 2012, Perera et al., 2010; Wijesekara et al., 2008)	Symptoms start in younger fronds and becomes more prominent in fully opened. Crowns appear dark green. The intense yellowing of lower whorls of fronds is more prominent after a rainy season. The tips of				<i>Stephanitis typica</i> (Distant) carried the

fronds become twisted or break and hang down. Leaflets dry starting from margins and fronds curl down ward giving a ragged appearance to the crown. Flaccidity of leaflets is evident on seedlings younger than 3 years of age, whereas yellowing is always seen only in older palms. With the reduction in the number of fronds, the crown becomes smaller and the trunk begins to taper. Female flower production declines and the palm becomes unproductive. WCLWD

wilt phytoplasms based on PCR using universal phytoplasma primers.

(Kumara et al., 2015)

		predispose the palms to other disease conditions such as rot disease caused by fungi.			
Papaya dieback Host: papaya (<i>Carica papaya</i> L.) (Abeyasinghe et al., 2014)	Ca. Phytoplasma asteris	Yellowing of the upper young leaves that progressively evolved to tip necrosis in the tender leaves, followed by drying of the upper leaves and eventual death of the whole plant. Discoloration of the vascular tissues, and abnormal growth fruit shapes and premature fruit fall	16SrI	KJ027722	Information not available

Areca Yellow Leaf	APYL-SriL	Foliar yellowing of areca palms, the most conspicuous symptom, began from the inner whorl and spread to the outer parts of the crown. Chlorosis was observed on almost all leaves in the whorl.	16SrXIV	KM978910 KT719315 KT719314 KT719313 KT719312 KT719311	Information on local vectors not available
Host: Areca Palm (<i>Areca catechu L.</i>) (Kanatiwela et al., 2015)					

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TRAILBLAZER

in Molecular Life Sciences

Felicitating
Vidya Jyothi Emeritus Professor
Eric Karunananayake

In the Cambridge dictionary, a trailblazer is defined as the “first person to do something or go somewhere, who shows that it is also possible for other people”. This publication is not a mere compendium of essays written by collaborators, colleagues and students. It serves as a testimony to the trailblazing contribution of Prof. Eric Karunananayake towards the nurture of Molecular Life Sciences in Sri Lanka.

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