"Mosquito Larvae Borne Viruses from the Central Travancore Region of Kerala: Isolation and Characterization"

Thesis Submitted to

Cochin University of Science and Technology

In partial fulfillments of the requirements for the award of degree of

Doctor of Philosophy

in

Environmental Biotechnology

Under the Faculty of Environmental Studies

By

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Certificate

This is to certify that the research work presented in the thesis entitled "**Mosquito Larvae Borne Viruses from the Central Travancore Region of Kerala: Isolation and Characterization"** is based on the authentic record of the original work done by Mr. Prem Gopinath (Reg.No.3235) under my guidance at School of Environmental Studies, Cochin University of Science and Technology in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy and that no part of the work has previously formed the basis for the award of any degree, diploma or associateship, fellowship or any other similar title or recognition.

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Declaration

I do hereby declare that the work presented in the thesis entitled "**Mosquito Larvae Borne Viruses from the Central Travancore Region of Kerala: Isolation and Characterization"**is based on the authentic record of the original work done by me under the guidance of Dr. I.S Bright Singh, Professor, School of Environmental Studies, Cochin University of Science and Technology, Cochin- 682 022 and that no part of this work has been previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar or recognition.

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March, 2014

Acknowledgements

It gives me immense pleasure to acknowledge and honour all those people who have helped me in one way or another in accomplishing this piece of work successfully and I humbly dedicate this page to all those who have helped me in exploring the expanses of knowledge.

I take immense pleasure to express my sincere thanks and deep sense of gratitude to my supervising guide Dr. I.S. Bright Singh for his heartfelt support, constant encouragement, enthusiasm, unconditional support and exemplary guidance throughout the period of my research. His guidance coupled with motivation and constant quest and passion for research helped me accomplish a scientific quest. I'm deeply grateful to him for providing me with all necessary facilities as and when required. His passion and zeal for scientific excellence has provided me with exceptional inspiration as well as enrichment for my growth as a student as well as researcher. I am really short of words to express my sincere appreciation for his patience and tolerance to me throughout this period. His understanding, encouragement and personal guidance have provided me with a good basis for the present thesis.

I offer my profound gratitude to Professor Xavier de lamballaerie, Professor, Faculte de Medicine, Marseille, France for his untiring support and guidance throughout the period of my study.

I would like to extend my indebtedness for the support and help rendered by Dr. N. Pradeep Kumar, Scientist-in -Charge, Vector Control Research Centre(VCRC), Kottayam field station for providing me with valuable suggestions, encouragement and supervising me in both laboratory and field work throughout the period of my work. A special and sincere acknowledgement to the technical staffs at VCRC, Mr. Ajithlal, Ms. Abida Suresh and Ms. Jessu Mathew for their constant encouragement and support.

I thank Dr. E. Sreekumar, Scientist, Molecular Virology, Rajiv Gandhi Center for Biotechnology, Trivandrum and to my dear and humble friend Mr. Anoop M. for rendering me with a whole hearted support with his valuable suggestions and by providing me with all cell lines when required.

It is with immense pleasure I would like to thank Dr. K.C. John (late), Former Director, School of Applied Life Sciences, Mahatma Gandhi University for his vision and entomological expertise which shaped the concept of the thesis.

I am deeply indebted to Dr. Rosamma Philip, Assistant professor, Department of Marine Biology, Microbiology and Biochemistry, Cochin University for her untiring support in helping me sort out the issues and for her vision in shaping the title of the thesis. I'm very much thankful to the friends at Marine Biology, Ms. Sruthy, Ms. Parvathy and the senior friends Ms. Jimly Jacob, Ms. Ramya, Mr. Naveen and Mr. Anilkumar for all the support, love and motivation in all my efforts.

I humbly acknowledge the help rendered by Dr. Swapna Antony, DST-INSPIRE faculty, National Center for Aquatic Animal Health for her affection, love, friendship and support showered on me.

I'm thankful to Dr. A. Mohandas, Emeritus Professor, National Center for Aquatic Animal Health for his valuable suggestions and motivation.

I offer my gratitude to Director, School of Environmental Studies, Dr.Ammini Joseph and all the faculties Dr. Harindranathan Nair, Dr. V. Sivanandan Achari, Dr. Rajathy, Dr. Suguna Yesodharan, Mr. M. Anand for all suggestions and support rendered to me throughout the period of research.

I sincerely acknowledge the faculties of National Centre for Aquatic Animal Health, Cochin University, Dr. Valsamma chechi for her encouragement, unconditional support, valuable suggestions and motivating advices, willingness to help each time I approached and above all a sisterly approach which I always treasure and Dr. Sajeevan T.P, for his enthusiasm and support to accomplish the piece of work.

Special thanks to the Technical Officers at School of Environmental Studies Dr. B. Sathyanathan and Dr. Rajalekshmi Subramaniam and also acknowledge the support of all the non-teaching staffs of the School of Environmental studies.

I remember with gratitude the untiring support and encouragement offered to me by the seniors Dr. Sreedharan who deserves special mention by being a good brother and providing me with all support, Dr. Seena Jose whose help in tissue culture practices moulded me a lot, Dr. Somnath Pai, for his constant encouragement and suggestions, Dr. Rejish Kumar and Dr. Sudheer N.S, for their constant support and encouragement. A special thanks to my Senior Dr. Ranjith Kanjur for valuable suggestions and help offered to me right from the beginning of my doctoral research.

I would always remember the moral support and love showered upon me by my friends Dr. Haseeb, Ms. Rose Mary Jose, Dr. Jayesh Puthumana, Dr. Divya Jose, Ms. Ammu Thomas, Ms. Riya George, Ms. Vrinda, Ms. Remya R. Nair, Ms. Jisha, Ms. Sanyo Sabu, Ms. Asha, Ms. Preena, Dr. Surekha, and Ms. Sunitha Poulose and my juniors Mr. Linu, Mr. Boobal, Mr. Anoop, Ms. Dhaneesha and Ms. Lekshmi.

I'm very much thankful to Dr. Haseeb for his support in statistics and all other assistance, Ms. Vrinda for her support in Molecular Biology works and Dr. Gopalakrishnan for his constant support and encouragement.

I express a special thanks to Ms. Deepa G.D. and Ms. Sareen Sarah John for their motivation, support and encouragement. I owe my gratitude to Ms. Sreelakshmi, who took pains to help me in the earlier stages of my work.

I am thankful to my colleagues at School of Environmental Studies, Mr. Dipson P.T. (late), Mr. Rojith, Ms. Deepa Nair and Mr. Abesh Reghuvaran, Mr. Rakesh and Mr. Amar for their friendship and support.

Special thanks to Mr. Syamkumar and Ms. Sherly Roy for their ever blooming friendship and affection. I acknowledge the timely help offered by Ms. Jeena and Mr. Khora, Department of Biotechnology, Cochin University of Science and Technology for helping me with ultracentrifugation studies.

A special thanks to Mr. Amar, GIS Lab, School of Environmental Studies, Cochin University, for helping me with the mapping of sampling points using the Geoinformatic tools.

I would always tender the moral support and love showered to me by my friends Mr. Anit, Mr. Prabhakaran, Mr. Jabir, Mr. Phiros, Mr. Krishna Mohan, Mr. Jyothish and Mr. Nithin.

I thank Mr. Soman chettan for the help rendered. I also cherish the help and friendship provided by the technical staffs Ms. Surya Sugunan and Ms. Blessy Jose from time to time. I thank all the supporting staffs Ms. Parisa and Ms. Kusumam chechi for their whole hearted support.

I would like to extend my gratitude to Cochin University of Science and Technology for providing me with the facility to conduct research as well as awarding me with the University Research Fellowships and DST-PURSE fellowship.

My joy knew no bounds in expressing the heartfelt thanks to my beloved father who sore the patience in spite of his lonely life to accomplish my task and my late mother Dr. V. Mahila whose inspiration helped me a lot in shaping a doctoral thesis. The tender notes of love and untiring support from my beloved wife Ms. Anumol helped me to overcome all the impediments and move ahead to achieve my goals. I hardly find words to jot down a word of appraisal for her. There is no limit of my gratitude for God's biggest blessing on me- my dear son Akshai Surya, whose presence in my life could lighten the harder times in my life.

Above all this piece of work has been accomplished with his blessings and powers that work within me and also the people behind my life for inspiring, guiding and accompanying me through the thick and thin. I bow before you all with a sense of gratitude…

Prem Gopinath

Contents

Abbreviations

APHA	American Public Health Association
BGM	Buffalo Green Monkey
CAR	Coxsackie Adenovirus Receptor
CAV	Coxsackie B Virus
CBV	Coxsackie A Virus
CDC	Center for Disease Control
CHIKV	Chikungunya Virus
CMV	Cytomegalovirus
COI	Cytochrome Oxidase I
OMRV	Omono River Virus
CPE	Cytopathic Effects
DENV	Dengue Virus
DMSO	Dimethyl Sulfoxide
EDTA	Ethylene Diamine Tetra Acetate
EEEV	Eastern Equine Encephalitis Virus
EID	Emerging Infectious Diseases
EPA	Environment Protection Agency
EV	Enteric Virus
FBS	Foetal Bovine Serum
FMDV	Foot and Mouth Disease Virus
HAV	Hepatitis A Virus
HEV	Human Enteric Virus
HIV	Human Immunodeficiency Virus
HRV	Human Rota Virus
HSV	*Herpes Simplex Virus*
ICC-PCR	Integrated Cell Culture PCR
ICTV	International Committee on Taxonomy of Viruses
ID_{50}	Infectious Dose 50
IFA	Immunofluorescence Assay
IRES	Internal Ribosomal Entry Site
LAC	La Crosse virus

LAMP	Loop Mediated Isothermal Amplification
LAMV	Lammi Virus
LD_{50}	Lethal Dose 50
LLIN	Long Lasting Insecticidal Nets
MEGA	Molecular Evolutionary Genetics Analysis
MOI	Multiplicity of Infection
NADH	Nicotine Adenine Dehydrogenase
NADPH	Nicotine Adenine Phosphae Dehydrogenase
NASBA	Nucleic Acid Sequence Based Amplification
NCCS	National Centre for Cell Science
NOUV	Nounane Virus
ORF	Open Reading Frame
PV	Polio Virus
RSV	*Respiratory Syncytial Virus*
RT-snPCR	Reverse Transcriptase Semi Nested PCR
RV	Rhino Virus
SARS	Severe Acute Respiratory Syndrome Virus
SINV	Sindbis Virus
$TCID_{50}$	Tissue Culture Infectious Dose 50
TCVA	Total Culturable Virus Assay
TEM	Transmission Electron Microscope
UTR	Untranslated Region
VEEV	Venezuelan Equine Encephalitis Virus
vRNA	viral RNA
VZV	Varicella Zoster Virus
WHO	World Health Organization
WNV	West Nile Virus

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Chapter 1

General Introduction

1.1 Introduction

In recent years, the issue of emerging and re-emerging infectious diseases specifically related to viruses, has become an increasingly important area of concern in public health. It is of utmost significance to anticipate future epidemics by accumulating knowledge through appropriate research and by monitoring their emergence using indicators from different sources. Most of the emerging and re-emerging pathogens in humans originate from known zoonosis. These pathogens have been engaged in long standing and highly successful interactions within their hosts since their origins are exquisitely adapted to host parasitism (Devaux, 2012). Emerging Infectious Diseases

(EIDs) belong to a nosological entity whose nature is proved to be infectious, regardless of the pathogen, or only suspected in case of novelty and until the agent is identified. It is understood that the identification of a "new" pathogen is compatible with a previously undisclosed pre-existing one. An EID can affect all types of eukaryotic organisms. The EID's do generally have high social impact and economic consequences. An EID is obviously unusual; it is surrounded by uncertainty and anxiety, real or perceived, as to its evolutionary potential, its impact on health and the ability of leaders and stakeholders to control the phenomenon (Devaux, 2012). Just to cite some of the major epidemics to evaluate the situation are Marburg virus epidemic in 1967, Lassa virus in 1969, Rotavirus in 1973, Ebola virus in 1976, Hantaan virus in 1977, influenza A/H5N1 virus in 1997, SARS-Coronavirus in 2003, Influenza A/H1N1 virus in 2009 (Devaux, 2012). Each year, globally about 8, 00,000 people die of malaria and 20, 000 of Dengue. While malaria, a human disease caused by a mosquito-borne infectious parasite of the genus *Plasmodium*, is the most important vector-borne disease because of its global distribution, the number of people affected (more than 220 million people) and the large number of casualties (about 80, 0000 deaths/year), the majority of the vector-borne viruses are clearly the most known numerous insect-borne pathogens.

In the event of expansion of global trade, the global economy continues to improve and the political liberalization promotes changes toward democracy and enhanced world peace. Accordingly, the inherent risks due to pathogens moving from one country to another through the global trade have expanded as well. Serious gaps remain and the threat of pathogens moving to new areas or emerging in new ways has been found to be considerably increasing (Brown, 2010). The huge volumes of people, goods, and services

moving from one country to another, termed as the global express, is an ever-expanding and increasingly complicated conduit supplying the least expensive options for a myriad of products to the widest possible market. Global commerce is a major contributor to improved livelihoods and the emergence of a huge middle class in developing Nations all over the world. An unintended result is that although the world is prospering through the unprecedented expansion of the global express, this same route to prosperity also provides a plethora of possibilities for pathogens to find new places and hosts. Over the last two decades, the world has averaged at least one new extensively emerging disease every year. Each node in this transport chain can be considered as a source of disease emergence. *Aedes albopictus*, the Asian ‘‘tiger mosquito,’’ was imported into the USA as an unexpected passenger in a shipment of used tyres from northern Asia that arrived in Houston in 1985. Within 10 years, the insect population had spread across to 678 countries in 25 states, with distribution following interstate highways (Moore and Mitchell, 1997). One of the vectors, *Ae. albopictus* is known to be capable of vectoring about 22 arboviruses (Brown, 2010).

An emerging disease may be caused by the disturbance of the ecosystem (Gubler, 1998). The exploitation of nature by man, with consequences such as deforestation, and the practice of hunting, may have kept mankind into contact with most of the pathogens (Devaux, 2010). Emerging infectious pathogens have significantly increased over the past few decades, prompting the need for extensive rapid outbreak detection and report (Devaux, 2012). As the global human population continues to expand, there is an increased chance for human contact with natural virus vectors, such as arthropods, birds, rodents, and in recent years we have seen the emergence of a number of new virus diseases, as

well as a resurgence of other diseases that were formerly known to be controlled. Some instances involve the invasion by humans of a new ecological niche which brings them into closer contact with the vector (Mahy, 2004). Now, dengue annually infects an estimated 50 million to 100 million people worldwide (WHO, 1999), many of whom are exposed to two or more co-circulating serotypes of DENGUE (hyperendemicity), resulting in frequent large-scale epidemics and more frequent severe disease (Gubler, 1998).

Human enteroviruses (HEV) are disseminated worldwide and can be transmitted by both faecal-oral and respiratory routes (Pallansch and Roos, 2007).All the identified human pathogenic viruses that pose significant public health risk in water environments are transmitted via the faecal- oral route (Griffin *et al.,* 2003) frequently causing asymptomatic or mild infections resulting in large numbers of progeny produced that are shed in the faeces. The virus particles are very stable under a wide range of environmental conditions (Keswick *et al.,* 1982; Piirainen *et al.,* 1998; Olivieri *et al.,* 1999). Furthermore, enteric viruses are frequently resistant to most employed disinfection methods (Baggi and Peduzzi, 2000; Dongdem *et al.,* 2009). It is imperative to consider human enteric viruses in water quality studies not only because of their incidence as causal agents for diarrhoeal diseases, especially because they can cause illnesses at low viral loads survive in the environment for long periods of time and tolerate changing environmental conditions (Skraber *et al.,* 2004;Espinosa *et al.,* 2008).It is estimated that 17–25% of the world population do not have access to safe water (United Nations Population Fund, 2001; World Bank, 2001) and 88% of deaths from diarrhoea are caused by contaminated water environments (World Health Organization, 2004). The viruses in various types of environmental water samples such as raw sewage

(da Silva *et al.*, 2007; Formiga-Cruz *et al.*, 2005; Harwood *et al.*, 2005; Lodder and de Roda Husman, 2005), treated sewage (da Silva *et al.*, 2007; Harwood *et al.*, 2005; Lodder and de Roda Husman, 2005), river water (Lee *et al.*, 2005; Lodder and de Roda Husman, 2005), seawater (Griffin *et al.*, 1999), and tapwater (Haramoto *et al.*, 2004; Grabow *et al.*, 2001; Lee *et al.*, 2005), could be effectively characterized by the recent metagenomic approaches. These studies have been conducted mainly in developed countries, while there are very few studies available on detection of enteric viruses in water samples in developing countries (De Paula *et al.*, 2007; El-Senousy *et al.*, 2007;Miagostovich *et al.*, 2008; Queiroz *et al.*, 2001; Vaidya *et al.*, 2002; Villar *et al.*, 2007).

Despite the rapid progress in water and wastewater treatment technologies, waterborne diseases still have public health and socioeconomic implications in both the developed and developing world. According to the World Health Organization (WHO), every year there are approximately 2.2 million deaths occur related to unsafe water, sanitation and hygiene, and millions more suffer multiple episodes of non-fatal diarrhoea (WHO, 2008). Viruses are a major cause of waterborne and water-related diseases. The health impact of these viruses varies; some waterborne viruses cause gastroenteritis, respiratory infections, conjunctivitis, hepatitis, aseptic meningitis, encephalitis and paralysis. Moreover, the infective dose can be very low, for instance it has been estimated that approximately 10-100 virions of norovirus are capable of causing human infection (Lindesmith *et al.*, 2003). Thus even the presence of a few viral particles poses a threat to public health. Sometimes the role of viruses in waterborne and water-related diseases is difficult to assess. This could be due to the lack of data, difficulties to define the source of infections,

escape the detection methods (Grabow, 2007). Given the onset and duration of symptoms, it is thought that viruses are the cause of disease in many of the outbreaks associated with agents of unknown etiology (Melnick *et al.,* 1984). Most emerging viruses are zoonotic, with animals forming the natural reservoir of new viruses. Spontaneous mutations and recombinations may also play an important role in the emergence of new human pathogens. Viruses in most genera of the *Picornaviridae* family have been shown to infect several mammalian species and zoonotic transmissions have been described (Brown *et al.,* 1973). It is also likely that many other animal species harbor yet undiscovered picornaviruses. Some of these unknown animal picornaviruses may evolve to infect and cause disease in humans. The presence of Enteric Viruses (EV) has been frequently detected in both raw and treated wastewater (Payment *et al.,* 2001), as well as in sludge.

The major obstacle to the routine detection of enteric viruses from environmental waters using PCR is the presence of amplification inhibitors. Virus detection requires the collection of large volumes of water in order to increase the likelihood of detecting the expected low quantities of virus present in the environment (Metcalf *et al.,* 1995). Typically, volumes of at least 100 litres of source water are filtered through charged microporous filters. Adsorbed viruses are eluted from the filter with beef extract and concentrated by organic flocculation resulting in a final concentrate thousands of times smaller than the original sample (American Public Health Association, 1995). While these procedures concentrate viruses, they also concentrate organic and inorganic compounds naturally present in the environment. Organic compounds such as humic acid (Abbaszadegan *et al.,* 1993; Kreader, 1996; Shieh *et al.,* 1995; Schwab *et al.,* 1993) fulvic acid

(Kreader, 1996), tannic acid (Kreader, 1996), proteins (Schwab *et al.*, 1993), polysaccharides (Shieh *et al.*, 1995; Fang *et al.*, 1992), glycoproteins (Schwab *et al.*, 1993), and inorganic compounds such as metals (Shieh *et al.*, 1995) have been reported to interfere with the enzymes used in PCR. The analysis of viruses in environmental water samples is a complex process that can be divided into two main steps: sample concentration and virus detection. Different methods have been used for the enrichment of viruses from environmental water samples such as adsorption or elution protocols (electronegative membrane, electropositive membrane, electronegative cartridges, electropositive cartridges, glass wool and glass powder), entrapment ultrafilltration (alginate membranes, single membranes and hollow fibres), ultracentrifugation and hydroextraction (Wyn-Jones and Sellwood, 2001). The use of positively charged membranes has been shown to be more efficient compared to negatively charged filters for analyzing coastal water (Katayama *et al.*, 2002). Viruses are typically detected in contaminated waters by concentrating virus particles from large volumes of water on positively charged filters (Fout *et al.*, 1996).

Arthropod-borne viruses (arboviruses) belong to different families and genera of viruses and are spread exclusively through mosquitoes and ticks (Calisher *et al.*, 1994). The diseases of arboviruses range in severity from mild flu-like illness and fever to fatal encephalitis and meningitis (Alatoom *et al.*, 2009). Arboviruses such as WNV have a wide range of temperatures in which they must replicate in order to be propagated within both vertebrate and invertebrate hosts. An arbovirus such as dengue that replicates in primates is incapable of replicating at temperatures above 39°C. This could be an

important factor in the inability of these viruses to utilize birds (that have higher body temperatures) as vertebrate hosts (Kuno, 1997). Certain arboviruses, particularly those of the California (CAL) serogroups, are notable among those arboviruses that may be transmitted transovarially and venereally by their arthropod vectors, as well as by the usual vector-host-vector cycle. Also, arboviruses can occasionally be transmitted through arthropod cycle mechanically involving arthropods (Calisher, 1994).

Zoonotic diseases are infections naturally transmitted between vertebrate animals and people. An estimated 75% of emerging infectious diseases are zoonotic pathogens of viral origin and include Human Immunodeficiency Virus (HIV), Severe Acute Respiratory Syndrome (SARS), Ebola and Monkey pox virus (Chomel *et al.,* 2007). Many zoonoses are indirectly transferred from animal host to humans by blood feeding arthropod vectors such as ticks, flies, and mosquitoes. Arboviruses circulate among wild animals and cause disease after spill over transmission to humans and domestic animals, which are incidental or dead end hosts (Weaver and Reisen, 2009). Global warming also has the potential to increase the distribution of vectors and to enhance transmission potential in temperate climates by elongating transmission seasons, increasing host–vector contact by shortening vector gonotrophic cycles, and shortening extrinsic incubation times. However, the greatest risk of human disease comes from the ability of some arboviruses to adapt to anthropologic urban or peridomestic transmission cycles involving highly efficient and anthropophilic vectors, *Ae. aegypti* and *Ae. albopictus*, are enzootic with peridomestic cycles involving urban avian and Culex populations. In an era of emerging and resurging infectious diseases,

understanding the ecological interactions that underpin the understanding of pathogen activity is critical (Russell and Kay, 2004).

1.2 Picornaviridae

The family Picornaviridae is one of the largest medically and economically important families of human and animal viral pathogens (Ehrenfeld *et al.,* 2010). The family consists of 285 different picornavirus types that form 29 species classified in eight established and five proposed genera (Stanway *et al.,* 2005; Knowles *et al.,* 2010). Six of these genera include viruses infecting both humans and other animals which include Enterovirus, Parechovirus, Hepatovirus, Cardiovirus, Kobuvirus and Cosavirus. Picornaviruses are small, icosahedral positive-sense single-stranded RNA viruses, causing a variety of diseases ranging from benign to fatal (Melnick, 1983). The best known picornavirus pathogens are enteroviruses including poliovirus (PV) and human rhinovirus (HRV), foot-and-mouth disease virus (FMDV), and hepatitis A virus (HAV). There are, however, several other picornaviruses that also cause outbreaks and serious diseases (Zhang *et al.,* 2009; Sapkal *et al.,* 2009; Dussart *et al.,* 2005). The number of picornaviruses is steadily growing through the identification of viruses that escaped detection in the past as well as emerging viruses (Zoll *et al.,* 2009; Brown *et al.,* 2009; Holtz *et al.,* 2008; Kapoor *et al.,* 2008).

These are one of the oldest and most diversified virus families and represent a non-enveloped, single, positive-stranded RNA genome packed in an approximately 30 nm icosahedric capsid. This family includes many important human and animal pathogens, such as poliovirus, hepatitis A virus, and foot-and-mouth-disease virus. Picornavirus genomes vary in size between

7 and 8.8 kb in length and share a conserved organization throughout the family. The 5' end of the genome is covalently linked to the small viral protein VPg (Flanegan *et al.,* 1977; Lee *et al.,* 1977). It contains a long 5'untranslated region (5'UTR) composed of cis-acting structural elements, a 5'terminal domain involved in replication (e.g. the poliovirus ''clover-leaf'') (Toyoda *et al.,* 2007) and an internal ribosomal entry site (IRES) necessary for translation. The IRES is shared by all picornaviruses but presents different secondary structures that mainly correspond to four different types (Belsham, 2009; Fernandez-Miragall *et al.,* 2009). The 3'UTR contains a pseudo knot involved in regulating replication (Jacobson *et al.,* 1993) and ends with a poly A tail that is heterogeneous in length (Yogo and Wimmer, 1972). After binding to its cognate receptor and entering the cell, the virus uncoats and the positive-sense RNA genome is released into the cell cytoplasm (Tuthill *et al.,* 2010). After synthesis of the viral proteins, the genome is replicated in complexes associated with cytoplasmic membranes (Caliguiri and Tamm, 1969). Both human rhino- and enteroviruses are characterized by high levels of genetic variability, as exemplified by the existence of over 250 different serotypes and the recent discovery of new enterovirus genotypes and the Rhinovirus C species. Despite their common genomic features, rhinoviruses are restricted to the respiratory tract, whereas the vast majority of enteroviruses infect the gastrointestinal tract and can spread to other organs, such as the heart or the central nervous system (Tapparel *et al.,* 2013).

1.3 Enteroviruses

Enteroviruses are responsible for a wide variety of diseases and represent a major public health hazard (Melnick, 1996) although their infections may often remain mild or asymptomatic (Melnick, 1996). Amongst the reported illnesses,

the Poliomyelitis-like disease, encephalitis and meningitis occur sporadically but not uncommonly as a result of CAV infections whereas more typical manifestations of Coxsackie A and B viruses are herpangina and myocarditis, respectively (Modlin, 1997a). The division of Coxsackie viruses into A and B subgroups was mainly based on lesions observed in newborn mice: Coxsackie A viruses affect skeletal muscle, while Coxsackie B viruses cause pathological changes in several tissues, including the central nervous system, pancreas, liver and brown fat. Members of the species B, including all Echoviruses (E) and Coxsackie B viruses (CBVs) are the most common cause of aseptic meningitis and outbreaks (e.g. E13 and E30) (Leonardi *et al.,* 1993; Mullins *et al.,* 2004; Oberste *et al.,*1999a; Savolainen *et al.,* 2001). In addition, members of species A such as EV71 and Coxsackie A virus (CAV) 16 are responsible for large outbreaks of hand, foot and mouth disease associated with severe neurological complications. Although most infections with human enteroviruses are asymptomatic or result in mild infection, human enteroviruses can cause diverse clinical syndromes ranging from minor febrile illness to severe and potentially fatal diseases, such as acute hemorrhagic conjunctivitis, aseptic meningo-encephalitis, and acute flaccid paralysis (Palacios and Oberste, 2005). Human enteroviruses are among the most common viruses infecting humans and can cause diverse clinical syndromes ranging from minor febrile illness to severe and potentially fatal diseases. The enteroviruses are pH stable and survive passage through the stomach to the lower intestinal tract, where replication takes place (Melnick, 1990).

Enterovirus virions are non-enveloped and contain a single positive-sense RNA genome of about 7500 nucleotides (nt) with a single open-reading frame (ORF) flanked by an untranslated regions which encodes a single

polyprotein. Co- and post-translational proteolytic processing gives rise to three precursors (P1–P3) secondarily cleaved in to four structural proteins (VP1–VP4), which are assembled into the virion, and non-structural proteins, which are expressed in infected cells (Pallansch and Roos, 2001). Historically, human enteroviruses have been classified into echoviruses (EV), Coxsackie virus groups A and B (CV-A and CV-B), and polioviruses (PV), according to their phenotypic and pathogenic properties. Based on phylogenetic analysis EVs are classified into four species: EV-A to -D (Hyypia *et al.,* 1997; King *et al.,* 2000). Distinguishing enterovirus (EV)-related illnesses from those due to bacteria or non-enteroviruses is important for prognostic, therapeutic, and epidemiological purposes (Kuan, 1997). The classification of enteroviruses (Committee on the Enteroviruses, 1957; Melnick *et* al., 1974), according to recent approaches, is based on both molecular and biological data (Hyppia, 1997; Poyry, 1996). According to them, Coxsackie A and B viruses are distributed into three of the four Human Enterovirus species, namely HEV-A, HEV-B and HEV-C (King *et al.,* 2000). In the case of enteroviruses and rhinoviruses the most N-terminal segment P1, contains four capsid proteins: VP4, VP2, VP3 and VP1. The P2 and P3 segments include the non-structural proteins (2Apro, 2B, 2C, 3A, 3B (VPg), 3Cpro and 3Dpol) that are involved in RNA replication and in polyproteins autocatalytic processing (Bolanaki *et al.,* 2005). Human enteroviruses include over 100 serotypes and can be divided into four species using molecular typing (Yozwiak *et al.,* 2010).

With the exceptions of polioviruses and enterovirus 71 (EV71), which frequently cause neurological complications, human enteroviruses usually cause self-limited infections in children (Lee and Chang, 2010). Therefore,

early detection and serotyping of enteroviruses infections are critical in clinical management and disease surveillance. The traditional standard methods for detection and serotyping of enterovirus infections are virus isolation and immunofluorescence assay (IFA), which are time-consuming and labor-intensive (Tsao *et al.,* 2010). Molecular tests for the detection of human enteroviruses in clinical specimens usually target highly conserved sites in the 5'untranslated region (5'UTR) (Romero *et al.,* 1999). Due to low virus titers in clinical specimens, several reverse transcription (RT)-nested or RT-seminested PCR (RT-snPCR) have been developed to further increase its sensitivity and specificity (Nix *et al.,* 2004; Thoelen *et al.,* 2003). The ORF encompasses four capsid proteins (VP4, VP2, VP3, and VP1) and seven non-structural proteins, such as viral proteases and RNA dependent polymerase (2A-2C, and 3A-3Dpol).

The major drawback of using human enteric viruses as indicators is that, the presence of human enteric viruses in different potable water systems has not yet been successfully documented. Because of this knowledge gap, it is impossible to understand the potential infectious hazards of our environment to cause human diseases (Raoult, 2009). The enteric viruses most commonly found in human stool belong to more than 140 types, of which adenovirus, norovirus genotype I and II, rotavirus (RV), enterovirus (EV), and hepatitis A virus (HAV) are those most often detected in the environment. Adenoviruses are very commonly found in water environments (Sedmak *et al.,* 2005) and have already been proposed as viral indicators (Hundesa *et al.,* 2006).

1.4 Diagnosis

Human pathogenic enteric viruses can be introduced into natural aquatic environments through human activities such as leaking sewage and septic systems, urban runoff, and agricultural runoff. Although few epidemiologically proven examples of waterborne transmission exist (Craun *et al.,* 2002), and not all human pathogenic enteric viruses are transmitted in water, many such viruses can survive for long periods of time in water. The viruses can be transported in the environment through contaminated or insufficiently treated tap water, and private wells (Lee and Kim, 2002; Lipp *et al.,* 2002). The type-specific diagnosis of enteroviral infections conventionally relies on virus isolation in cell cultures followed by identification of the serotype by neutralization with mixed, hyperimmune pools (Melnick *et al.,* 1973). After isolation in cell culture, typing of human enteroviruses consists of identifying the serotype by seroneutralization. Because of their labour intensiveness, neutralization methods have been replaced by molecular tools in many laboratories. Indeed, a correlation between serotype and genotype for the P1 region of the genome has been demonstrated (Oberste *et al.,* 1999b). Therefore, several methods have been developed in order to determine partly the sequences encoding the VP1 protein (Bailly *et al.,* 2000; Caro *et al.,* 2001; Iturriza-Gomara*et al.,* 2006; Nix *et al.,* 2006; Norder*et al.,* 2001; Thoelen *et al.,* 2003) which plays a key role in the constitution of the viral neutralization antigenic sites. In the genomic region VP1 there is an absolute genotype serotype correlation, which is used for molecular characterization of enteroviruses, determining the serotype (Oberste*et al.,* 1999a). Methods targeting other capsid-encoding regions, such as VP2 or VP4, have also been developed (Ishiko *et al.,* 2002; Nasri *et al.,* 2007). These methods were shown

to be suitable for the purpose of determining the serotypes of field or clinical strains. However, the determination of the serotype appears insufficient for surveillance or research purpose, particularly for studying the molecular epidemiology and evolution of human enteroviruses. The standard diagnostic method includes virus isolation in cell culture followed by neutralization typing. The procedure is labor intensive, and time-consuming. In order to enhance virus typing, molecular methods targeting different regions of the genome have been widely used (Kim *et al.,* 1997). Partial or complete sequences of the VP1 region have been analyzed and show that this part of the viral genome has an excellent genetic correlation with the HEV serotypes identified using antigenic methods (Oberste *et al.,* 1999). Therefore, this region is an ideal target for enteroviruses genotyping.

Basically cell culture system is the gold standard to examine the infectivity of the isolated viruses. There are some enteric viruses which are easy to propagate (enteroviruses), others are difficult to propagate (rotavirus and hepatitis A virus), and for some there are no cell lines available for propagation (human noroviruses) (Straub *et al.,* 2007). Indeed, no single cell line is able to propagate all viruses even within the same virus group. For instance, among the EV group, which includes Polioviruses, Coxsackieviruses, Echoviruses, and other numbered serotypes of EV, there is much similarity in terms of structure and surface antigens; however, there is no single cell line that allows proliferation of all EV strains (Leland and Ginocchio, 2007). ICC-PCR has been first proposed as an alternative method for detection of waterborne enteric viruses in environmental samples by Murrin and Slade (1997). Currently, a wide range of analytical methods are available for virus detection in environmental water samples such as enzyme linked immunosorbent

assay (ELISA), polymerase chain reaction (PCR), reverse-transcription PCR (RT-PCR), real-time PCR, real-time RT-PCR, nucleic acid sequence based amplification (NASBA), real time NASBA, loop-mediated isothermal amplification (LAMP) and reverse transcription LAMP; more details are reviewed in Mattison and Bidawid (2009). The standard method for the detection of enteroviruses as mandated by the Environmental Protection Agency (EPA) involves the use of the Total Culturable Virus Assay,the principle of which, relies on the availability of cell strains that are susceptible to and support the replication of human enteric viruses.

1.5 Diseases Caused

Enteric viruses are considered as one of the major causes of gastrointestinal illnesses such as diarrhea and gastroenteritis in humans. In addition, they may also be associated with respiratory infections, conjunctivitis, and hepatitis. Since even low infectious doses can lead to elevated public health risks, it is important to continue efforts to develop and improve detection methods for infectious enteric viruses (Fong and Lipp, 2005). Although enteroviruses mostly cause asymptomatic infections, they also give rise to major, potentially fatal human diseases, such as acute hemorrhagic conjunctivitis, aseptic meningo-encephalitis, and acute flaccid paralysis (Palacios and Oberste, 2005). Human enteroviruses (HEV) are considered as one of the major causes of central nervous system infections in pediatrics (Silva *et al.,* 2008). However, enteroviruses can also cause a wide variety of clinical illnesses, including acute hemorrhagic conjunctivitis, aseptic meningitis, undifferentiated rash, acute flaccid paralysis, myocarditis, and neonatal sepsis-like disease (Pallansch and Roos, 2001). The group B coxsackieviruses, comprising six serotypes (B1 to B6), have been implicated in a variety of diseases including

pancreatitis, insulin-dependent diabetes mellitus, myocarditis, myositis, and aseptic meningitis (Pallansch, 1997). The group B viruses induce a spastic paralysis with focal degeneration of skeletal muscle, cyanosis and death. Death generally occurs in animals that develop encephalitis, myopericarditis and acinar pancreatitis. Common methods for the detection of human enteric viruses are cell culture-based plaque assays and PCR-based assays, including integrated cell culture-polymerase chain reaction (ICC-PCR), and total culturable virus assay (TCVA) (Lee *et al.,* 2008; Lee and Jeong, 2004). Observation of cytopathic effect (CPE) is a standard method for the detection of infectious viruses in aquatic environmental samples. Infectious viruses, such as polioviruses, can spread to neighboring cells and cause visible CPEs on the cell monolayer. However, this cell culture method does not detect non-cytopathic viruses. Recently, various PCR formats have been developed as sensitive tests for the detection and quantification of enteric viruses. These include nested PCR (Formiga-Cruz *et al.,* 2005) and real time quantitative PCR (Costafreda *et al.,* 2006). Enterovirus infections are frequent in all age groups. In addition to acute infections, they have been associated with chronic diseases such as cardiomyopathies and type 1 diabetes. They cause usually mild symptoms but are also a significant cause of more severe diseases such as myocarditis, meningitis, encephalitis, systemic infections in newborns and paralysis (e.g. polio myelitis). Enterovirus infections may also play a role in chronic diseases such as dilated cardiomyopathy, chronic fatigue syndrome (Chia, 2005) and type 1 diabetes (Hyoty and Taylor, 2002).

In general, enterovirus infections go unnoticed, or symptoms are just flu like, and therefore not recognized as such. Occasionally, infection leads to severe neurological disorders or other diseases, e.g. meningitis, encephalitis, pleurodynia,

myocarditis, conjunctivitis or severe systemic infections in neonates (Melnick, 1996). Enteroviruses are also thought to have a pathogenetic relationship to dilated cardiomyopathy (Kitaura, 1981; Bowles *et al.,* 1986; Kandolf *et al.,* 1987; Jin *et al.,* 1990) juvenile diabetes mellitus (King *et al.,* 1983; Frisk *et al.,* 1992) and chronic fatigue syndrome (Cunningham *et al.,* 1990).

1.6 Virus Entry and Replication

Attachment of virus to its cellular receptors is a pre-requisite for viral replication, and different viruses can use various cell surface structures as receptors. It has been shown by several studies that Coxsackievirus group B (CBV) can use the Coxsackie-Adenovirus Receptor (CAR) (Lonberg-Holm *et al.,* 1976; Agrez *et al.,* 1997; Bergelson *et al.,* 1997a, b). The coxsackie–adenovirus receptor (CAR) is the primary cellular receptor for Group B coxsackieviruses (CVBs) and required for the development of CVB myocarditis (Bergelson *et al.,* 1995; Shafren *et al.,* 1995; Coyne and Bergelson, 2005). CAR is also a high-affinity receptor for adenoviruses, mediating viral attachment (Roelvink *et al.,* 1998; Martino *et al.,* 2000). All picornaviruses contain a genetically encoded 3'poly (A) tail, which is essential for virus replication. Both viral RNA translation and RNA replication depend on the presence of a poly (A) tail. The enteroviral 3'poly (A) tail is partly included into the stem-loop structure of domains. Based on chemical and enzymatic digestion experiments, (Pilipenko *et al.,* 1992) suggested that the poly(A) tail can form a putative helix by base pairing with a tract of four to five uridine residues located just upstream of the stop codons.

The RNA is translated into a single large polyprotein, which is processed by virus-encoded proteases to yield the individual structural capsid proteins

and the non-structural P2 and P3 region proteins, including the viral encoded RNA-dependent RNA polymerase (RdRp) 3Dpol and some relatively stable processing intermediates (2BC, 3AB, and 3CDpro) performing activities in viral RNA replication distinct from their final cleavage products (reviewed by Wimmer *et al.,* 1993). A small peptide (VPg), the product of gene 3B, is covalently attached by its single tyrosine to the 5'side of the plus-stranded RNA genome. The key steps in the viral life cycle, as potential targets for antiviral compounds, are virus adsorption, uncoating, RNA translation, polyprotein cleavage, RNA replication, and particle assembly. The majority of currently available antiviral compounds against picornaviruses are directed against either the virions to block early stages of infection or the 3C protease to block genome expression (De Palma *et al.,* 2008a, b, c).

1.7 Enteroviruses Evolution

Two distinct mechanisms play an important role in enteroviral evolution: mutation and recombination (Oprisan *et al.,* 2002; Santti *et al.,* 1999). Enteroviral RNA replication is characterized by a high mutation rate due to the lack of proofreading activity of the viral RNA dependent RNA polymerase. Enteroviruses possess a positive single-stranded RNA genome subjected to high mutation rates and frequent genetic recombination events (Agol, 1997; Chevaliez, *et al.,* 2004; Lindberg, *et al.,* 2003; Lukashev, *et al.,* 2003; Lukashev, *et al.,* 2004 and 2005, Mirand, *et al.,* 2007; Oberste, *et al.,* 2004, 2004a and 2004b; Oprisan, *et al.,* 2002; Santti, *et al.,* 1999; Savolainen, *et al.,* 2001; Simmonds, *et al.,* 2006) and thereby display a great diversity (Brown, *et al.,* 1999;Mulders, *et al.,* 2000; Poyry, *et al.,* 1996; Rezig *et al.,* 2004; Santti, *et al.,* 2000).

Enteroviruses tend to have rates of spontaneous mutation of approximately one mutation per genome per replication (Drake, and Holland, 1999). Consequently, enteroviruses exist as a dynamic mutant population termed as quasispecies corresponding to a swarm of sequence variants (Holland and Domingo, 1998). Due to the absence of proofreading activity, the misinsertion rate by the 3D polymerase is high, and mutations accumulate during replication (Drake, 1993; Holland *et al.,* 1982). It was recently demonstrated that recombination is a significant and relatively frequent mechanism in the evolution of enterovirus genomes. Bootstrap and genetic similarity analyses of viruses have revealed that genetic exchanges could occur within a given serotype (intratypic recombination) and between different serotypes (intertypic recombination) (Santti *et al.,* 1999). Mutations and recombinations are the mechanisms for enterovirus evolution. Recombination has been recognized as a frequent event in Poliovirus (Blomqvist *et al.,* 2003; Cuervo *et al.,* 2001; Karakasiliotis, *et al.,* 2004; Liu *et al.,* 2000) and non-polio Enterovirus evolution (Lukashev *et al.,* 2003; Kyriakopoulou *et al*,. 2010; Oberste *et al.,* 2004; Oprisan *et al.,* 2002; Santti *et al.,* 1999) usually located in the non-capsid coding region. Intra- and interserotypic recombination events in the VP2–VP1 coding region have been reported to occur very rarely (Blomqvist *et al.,* 2003; Kyriakopoulou *et al.,* 2006). Genetic exchanges between enteroviruses can give rise to new viral genotypes that may be extremely virulent and dangerous for public health.

1.8 Vector

Mosquitoes are vectors of parasites and viruses that cause diseases of immense importance in public health. In particular, mosquito-borne viruses are of increasing interest and concern because of recent emerging and re-

emerging arboviral diseases and global warming that may facilitate the expansion of the distribution of vector mosquitoes (Kobayashi *et al.,* 2002). Mosquitoes undergo a complete metamorphosis through four stages: egg, larvae, pupae, and adult. Under optimum temperatures and with ample food supplies, eggs hatch out in one to three days; larvae develop through all four stages in seven to ten days; and pupae develop into adult emergence in one to three days. The entire life cycle of the mosquito usually takes at least 10 to 16 days. The primary food source for both male and female mosquitoes are flower nectar and plant juices, however female mosquitoes use the proteins in blood meals to develop her eggs (MVCAC, 1996). A female, depending on her species, can lay between 50 – 500 eggs in her first brood.

Since the dispersal of *Ae.albopictus* from Asia to Europe and the Americas is largely the result of human commercial activities, the adaptation of CHIKV to *Ae. albopictus* provides a fascinating demonstration of how viruses can readily circumvent the impact of human interference on the ecosystem. Mosquitoes or ticks infected with virus, either through horizontal or vertical transmission, are infected for life. Multiple host contacts in a gonotrophic cycle increases the chance of acquiring and transmitting the pathogen (De Foliart*et.al*., 1987). Mosquitoes attempting to feed on the host by repeated penetration of mouth parts may be of epidemiologic significance even when blood is not ingested because the infected mosquitoes secrete fluids along with virus during exploratory movements (Magnarelli, 1979; Hurlbut, 1966). Multiple feeding on various hosts may favour the transmission of the vector feeds on potential hosts or reduce transmission if the vector feeds on unimportant hosts. *Ae.albopictus* can easily adapt to urban environments, exploiting all kinds of artificial containers as larval habitats. It feeds on many

vertebrates, including birds, reptiles and amphibians, but prefers mammals, and in particular humans (Hawley, 1988). Surveillance of mosquito populations for known viruses continues to provide clues about the geographical distribution of the viruses and serves to identify regions that may pose higher risk for transmission to humans (Bennett *et al.,* 2011). In nature, two types of vertebrate hosts are of particular note: those that serve as main sources of infections for vectors, and those that do not but in which overt disease can occur. The latter are important to us in relation to human disease and domestic animal loss; epidemiologically, the former are more significant because, together with the vector, they serve as virus reservoirs, disseminators, and amplifiers.

Arboviruses can persistently infect and cause limited damage to mosquito vectors. These viruses exhibit significant morbidity and mortality in the vertebrate host. However, virus persists in the mosquito vector with minimal associated pathology. The persistent nature of arbovirus infection of a vector suggests a commensal rather than parasitic relationship. The salivary glands are an important organ for successful transmission of arboviruses (Cirimotich *et al.,* 2009). Temperature also plays a vital role in determining whether mosquitoes can serve as efficient vectors of viruses (Dohm *et al.,* 2002). Higher temperature further abets transmission by shortening the incubation period of the virus in the mosquito (Watts *et al.,* 1987). On global scales, several studies have highlighted common climate characteristics of areas where transmission occurs (Hales *et al.,* 2002; Peterson, 2005; Rogers, 2006). *Ae.albopictus* breeds profusely in rainwater that collects in the hemispherical containers fitted to the trunks of rubber trees for latex collection in the region (National Informatics Centre, Government of India, 2008;

NVBDCP, 2008). Horizontally transmitted mosquito-borne viruses enter the midgut with a blood meal and then disseminate to infect the salivary glands. En route to the salivary glands, these viruses encounter the plasma (haemolymph) and blood cells (haemocytes). Mosquito-borne viruses initiate infection in the midgut of the mosquito following ingestion of a viraemic blood meal. As the infection progresses, the virus replicates and disseminates from the midgut then invades and replicates in the salivary glands (Parikh, 2009). *Ae. albopictus* has a worldwide geographical distribution, is particularly resilient, and can survive in both rural and urban environments. The mosquito's eggs are highly resistant and can remain viable throughout the dry season, giving rise to larvae and adults the following rainy season. *Ae. albopictus* is relatively long-lived (4–8 weeks) and has a flight radius of 400–600 m. It is aggressive, silent, and diurnal, meaning that bed nets are ineffective. The adult female appears to transmit the virus vertically to her eggs, although this remains to be documented in the Indian Ocean outbreak (Higgs, 2006). *Ae. albopictus* is known to be capable of adaptation in contrasting environments. For instance, it was shown that *Ae. albopictus* has the ability to adapt to colder temperatures, and these adaptive capacities are likely due to its ability to synthesize a high amount of lipids which consequently can provide eggs with substantial yolk resources to maintain them through diapause (Hawley, 1988; Kobayashi, *et al.,* 2002). *Ae. albopictus* was suspected of being a vector or secondary vector of several viruses, particularly of the viruses that cause dengue fever and dengue hemorrhagic fever (Dengue virus; DENV) (Black *et al.,* 2002). One major difference between the species is that *Ae. albopictus* has the ability to adapt to cold temperatures by becoming dormant during the winter of temperate regions. *Ae. albopictus* have the ability to breed continuously and survive at a mean temperature above 10°C in Japan and Re-union (Kobayashi *et al.,* 2002)

and at -5°C populations in USA (Mitchell, 1995). In temperate climates (e.g., Europe and the USA), the eggs are able to enter dormancy when the temperature decreases below these thresholds (Mitchell, 1995; Nawrocki, and Hawley, 1987). The ability of *Ae. albopictus* to resist cold temperatures is likely linked to its ability to synthesize a high amount of lipids and to produce larger amounts of yolk lipid in cold temperatures. The larval lipogenesis of *Ae. albopictus* was found to be far more efficient than that of *Ae. aegypti* which partially explains the ability of *Ae. albopictus* to spread further (Briegel and Timmermann, 2001). The relationship between the spread of *Ae.albopictus* and human activities have been well illustrated by the intercontinental shipment of tyres, which was responsible for the introduction of *Ae. albopictus* into the USA in 1985 (Reiter and Sprenger, 1987). Today, *Ae.Albopictus* can be found in a number of American countries (ranging from the USA to Argentina), numerous Pacific Islands (e.g., Hawaii, the Solomon Islands and Fiji) and in Australia. In Africa, *Ae. albopictus* was first detected in 1989 in South Africa (where it has been controlled) and later in Nigeria, Cameroon, Equatorial Guinea and Gabon. The first report of the species in Europe was in Albania in 1979, and since, it has been detected in other countries such as Bosnia and Herzegovina, Croatia, Greece, France, Italy, Montenegro, the Netherlands, Serbia, Slovenia, Spain and Switzerland. *Ae. albopictus* is mainly a daytime and exophagic mosquito, preferring to bite in the early morning and late afternoon; although, many exceptions have also been recorded dependent on the season, region, host availability and the nature of the human habitat. The mosquito preferentially bites mammals; however, the females can feed upon most groups of vertebrates from cold- to warm-blooded animals, including reptiles, birds and amphibians.

Once introduced into a new area, the subsequent establishment and spread of *Ae. albopictus* is mediated by abiotic (e.g., climate and the environment) and biotic factors (e.g., interactions with predators or pre-existing species that exploit the same resources across the targeted ecological niche). Such competitive interactions, shaped by environmental parameters (including resources), can lead to the exclusion of some species (including *Ae. albopictus* itself) or to a stable coexistence. In areas where Ae. *albopictus* and *Ae. aegypti* co-occur both species often share the same larval habitats (Paupy *et al.,* 2009). In addition, due to its opportunistic behaviour, *Ae. albopictus* may also help to serve as a ‘‘bridge vector’’ for the transmission of emerging viruses between animals (wildlife and domestic) and humans, particularly in newly colonized areas, such as Central Africa, a known hot spot of viral diversity. The ability of *Ae. albopictus* to serve as a vector and transmit disease has been experimentally demonstrated for a wide range of arboviruses (Moore and Mitchell, 1997; Gratz, 2004). *Ae. albopictus*has been shown to be capable of transmitting 26 viruses that belong to the Flaviviridae (genus Flavivirus), Togaviridae (genus Alphavirus), Bunyaviridae (genus Bunyavirus and Phlebovirus), Reoviridae (genus Orbivirus) and Nodaviridae (genus Picornavirus) families (Paupy *et al.,* 2009).

1.9 Tissue Culture

The scientific discipline of virology essentially began with the ability to culture cells in vitro. The ability to culture cells in vitro has enabled enormous advances in biology, particularly in virology (Cann, 1999). The judicious use of two or three cell lines, such as a monkey kidney line, a human continuous cell line and a human fibroblast line, will allow the detection of the majority of cultivable viruses of clinical importance, such as herpes simplex virus (HSV), varicella

zoster virus (VZV), cytomegalovirus (CMV), enteroviruses, respiratory syncytial virus (RSV), adenovirus, parainfluenza viruses, influenza viruses and rhinoviruses. Isolation of viruses in tissue culture still remains an important technique for the diagnosis of virus infections. The high degree of sensitivity and specificity inherent in virus isolation are the 'gold standard' against which new techniques should be measured (Cann, 1999). It is of utmost importance to support and conduct research on basic and applied aspects of host, pathogen and environmental factors that influence disease emergence and re-emergence as well as transmission (Devaux, 2012). The advantages of virus isolation include: the ability to undertake further examination of the isolate, such as drug susceptibility assays or typing; the provision of epidemiological information on viruses of public health importance; and the culture and identification of previously unrecognised viruses, e.g. human metapneumovirus (Van den Hoogen *et al.,* 2001) and SARS-associated coronaviruses. The availability of continuous insect cell cultures provides the opportunity to study how differences in the biology of vertebrate and invertebrate cells affect the biology of a particular infecting virus.

Most virus-infected cells will undergo morphological changes, for example, swelling due to alteration of membrane permeabilities, shrinkage due to cell death, and formation of giant multinucleate cells, which are visible under the light microscope, due to the presence of a fusion protein encoded by the virus. Collectively, these changes are referred to as cytopathic effects (CPE) (Cann, 1999). Examples of well-known cell types that are standard for environmental virology laboratories are buffalo green monkey (BGM) kidney cells, African green monkey kidney (Vero) cells, primary liver carcinoma (PLC/PRF/5) cells, fetal rhesus monkey kidney cells (FrhK-4), human epithelial type 2 (HEp-2) cells, human lung carcinoma cells (A549), rhabdomyosarcoma (RD) cells, human

colon carcinoma cells (CaCo-2), and simian kidney epithelial (MA104) cells (Abad *et al.,* 1998; Ali and Abdel-Dayem, 2003; Ali *et al.,* 2004; Bosch *et al.,* 2004; Chapron *et al.,* 2000; Dahling *et al.,* 1974; Dahling and Wright, 1986; Ehlers *et al.,* 2005; El-Senousy *et al.,* 2007; Fout *et al.,* 1996; Frosner *et al.,* 1979; Hashimoto *et al.,* 1991; Ko *et al.,* 2003; Morris, 1985; Pinto *et al.,* 1994; Rodriguez *et al.,* 2008; Sedmak *et al.,* 2005). Combination of more than one cell line may allow the detection of unanticipated agents, rather than focusing on the detection of only one or a few specific viruses.

1.10 Challenges

Vector-borne diseases are among the most complex of all infectious diseases to prevent and control. Not only is it difficult to predict the habits of the vectors, but most vector-borne agents can infect animals as well (Centre for Disease Control). A challenging aspect of arbovirology is identifying and disentangling important mechanisms that influence the current state of transmission (Carver, 2009). Because diseases can cross across all borders and oceans, there is a growing need for health practitioners of all sorts to understand the systems, rationale, and mechanics of public policy on national and global scales (Brown, 2010). In general, RNA viruses are much more likely to evolve by genome changes than DNA viruses, and this in part reflects the proof-reading functions present in the host cell that preserve the integrity of host cellular DNA. Such proof-reading mechanisms do not exist for RNA, and so RNA viruses are much more mutable. As with other RNA viruses, the presence of an error-prone polymerase that lacks proofreading function coupled with the large population sizes occurring in both infected avian hosts and mosquito vectors allow for the rapid selection of novel genotypes.

The major factors that contribute in emergence of viruses include- microbial adaptation and change, human vulnerability, climate and weather, changing ecosystems, economic development and land use, human demographics and behaviour, technology and industry, international travel and commerce, breakdown of public health measures, poverty and social inequality, war and famine, lack of political will and intent to harm (Smolinski *et al.,* 2003). Another important factor that has contributed to the emergence of viral diseases is the vector population density which is particularly true of mosquito-borne viruses. Once well-controlled, the mosquito population, including *Ae.aegypti* and *Ae. albopictus*, has expanded greatly since the cessation of the use of DDT, resulting in increasing cases of dengue fever worldwide. Translocation of a vector-borne virus into a new geographical region can absolutely result in the emergence of a dramatic disease outbreak. The vertebrate host spectrum varies for each virus; generally, particular species of smaller vertebrates with high population replacement rates, such as reptiles, birds or rodents, serve as hosts, but in some instances larger mammals can be involved. Reptiles, amphibians, and bats have been suspected of serving as over- wintering hosts for a few of these viruses, but there still is doubt of their significance in this role (Calisher, 1994).

Vector-borne infections are major killers, particularly of children, in developing countries. Over the past few decades, more comprehensive and transparent methods of measuring health have improved understanding of the importance of these diseases. Despite technological advances and increasing affluence in many regions, vector-borne infectious diseases remain amongst the most important causes of global ill-health (Campbell-Lendrum and Molyneux, 2005). The environmental pressures generated by human activities

may act through direct or complex mechanistic pathways, and their impacts are therefore situation-specific. The results of such processes may be trade-offs, where interventions is to increase an ecosystem service that are accompanied by increasingly frequent or severe disease outbreaks in the human populations. The long-term positive or negative effects of ecosystem changes may emerge some time later, often complicating initial assessment of the likely ecosystem impacts of development. Some environmental changes triggered by human activities impact on vector habitats, hosts, or disease transmission, in ways that are even more diffuse and difficult to predict accurately. Global, regional, and national development programs, which are designed to increase material well-being and alleviate poverty, have indirect and diffuse but nonetheless profound influences on infectious diseases. For instance, policies to increase trade can lead to increased deforestation and irrigation, with consequent impacts on vector-borne diseases. The Chikungunya outbreak highlights the importance of monitoring vector-borne and zoonotic diseases. These outbreaks have provided an opportunity to improve our previously poor knowledge of the biology, epidemiology, dynamics, and immunology of Chikungunya virus infection (Pialoux *et al.,* 2007). The relationship between infected mosquitoes and infectious mosquitoes (infected mosquitoes capable of transmission) may be affected by factors such as Extrinsic Incubation Time. Because an infected mosquito may be incapable of transmitting a virus at a particular point in time, an estimate based on the mosquito infection rates may overestimate risk of transmission to humans. By increasing our understanding of how arbovirus infection and transmission rates in mosquitoes correlate under field conditions and determining what factors influence changes in transmission rates, we may also be able to anticipate conditions when there is a greater risk of transmission to humans. Assessing the time periods that

involve increased risk to humans should increase the efficacy of education, mosquito control, and personal protection measures, reducing the overall risk of transmission to human populations (Vitek andRichards, 2008). Alarming trends in the resurgence of vector-borne diseases are anticipated to continue unless more effective action is taken to address the variety of underlying causes. Social factors, anthropogenic environmental modifications and/or ecological changes appear to be the primary drivers. The ecological dimension of vector-borne disease research and management is a pervasive element because this issue is essentially an ecological problem with biophysical, social, and economic dimensions (Ellis and Wilcox, 2009). Vector-borne diseases pose a significant public health problem today, with a number of "old" diseases resurging in recent decades alongside newly emerging infectious diseases (Smolinski *et al.,* 2003).

Some of those viruses which were effectively controlled just 50 years ago, but these previous hard-won gains are now threatened or have already been lost (Gubler, 2002). Dengue is perhaps the most striking example; from 1950-1959 less than 1,000 cases were reported world-wide (Guha-Sapir, and Schimmer, 2005), now an estimated 50-100 million cases occur annually. At least 20 other vector-borne diseases have also emerged during this time, having increased incidence and/or expanding their geographical range. Coinciding with this increase in vector-borne diseases there have been dramatic ecological changes. Marked increases in the rate and extent of environmental degradation over the last century, largely attributable to human activity, have fuelled growing concern and acceptance of the interdependence of man and the environment (WHO, 2005). Vector-borne diseases in general are especially ecologically sensitive since environmental conditions can have

dramatic effects on the vectors, pathogens, and potential hosts involved in transmission (Campbell-Lendrum and Molyneux, 2005). Monitoring the emergence and re-emergence of viral diseases with the goal of containing the spread of viral agents requires both adequate preparedness and quick response. Identifying the causative agent of a new epidemic is one of the utmost important steps for effective response to disease outbreaks. Traditionally, virus discovery required propagation of the virus in cell culture, a proven technique responsible for the identification of the vast majority of viruses known to date (Mokili *et al.,* 2012). The risk of arbovirus emergence in an area can be affected by three factors: receptivity, infectivity and vulnerability. Receptivity is expressed by the vectorial capacity index and is calculated using the formula: $ma^2pn/-ln\ p$. It refers to the expected number of new viral infections of man, per infective viremic case, per day, that the *Ae. albopictus* population would distribute to humans, assuming that all vector females biting the viremic host become infected and are subsequently able to transmit the virus. Infectivity is defined as the degree of susceptibility of the *Ae. albopictus* populations to different arboviruses (or virus strains). Vulnerability is the number of arbovirus cases or more precisely, viremic hosts, imported into the area. In other words, the risk of local transmission of an arbovirus (or another *Ae. albopictus*-borne pathogens) is the result of the simultaneous presence of the virus, competent mosquitoes in a favourable environment, susceptible human hosts and contact between these three entities (WHO, 1979).

1.11 Arbovirus

To establish and maintain an arbovirus transmission cycle three factors are essential: the arbovirus, the arthropod, and the vertebrate. As a prerequisite for continuous circulation of the virus between arthropod vector and vertebrate

host, all factors must be available in sufficient numbers, at the same time and at the same place (Carver, *et al.,* 2009; Lambrechts, *et al.,* 2009; Pfeffer and Dobler, 2009; Kuno and Chang, 2005). In theory, a single virion can initiate an infection, but in practice it is often found that a host must be inoculated with a minimum number of virions in order for that host to become infected. This minimum amount of virus required for infection of a host is known as the minimum infective dose (Carter and Saunders, 2007). Arboviruses are serious pathogens for humans but cause little damage to their arthropod vectors. Arboviruses are important causes of epidemic and endemic disease in many countries all over the world (Woolhouse and Gaunt, 2007; Kyle and Harris, 2008). In their vertebrate hosts, arbovirus generally counteracts innate defences and triggers a highly cytopathic infection that leads inevitably to cell death. However, in mosquito cells an early phase of efficient viral protein synthesis and virus production is followed by a persistent infection with low levels of viral protein expression and virus release (Newton *et al.,* 1981; Scallan and Elliott, 1992). These viruses are transmitted between susceptible vertebrate hosts by blood-feeding arthropods, and may be transmitted either by blood transfusion, tissue and organ transplantation and even breast feeding. The lifecycle of arboviruses is influenced by changes in temperature, rainfall, humidity, length of day, average daily solar radiation and or storm patterns, as well as changes in the frequency of rare events such as floods or droughts. Droughts were also known to increase the dissemination of arboviral diseases in urban areas by allowing a boost in the population of mosquitoes in foul water concentrated in catch basins where they breed. Droughts also cause a decline in mosquito predators like frogs, darning needles and dragonflies. In addition, birds congregate around shrinking water sites, enhancing circulation of viruses among birds and mosquitoes. The lifecycle of arboviruses involves

the vector (arthropod) where the virus replicates reaching levels that allow transmission to the natural host (vertebrate: birds, mammals and rodents). In the natural host, the virus replicates to levels that can be transferred infectious to a non-infected vector. Additionally, several arboviruses can be transferred by vertical or transovarial transmission between generations of the vector through the egg and larvae stage. Viruses are the most abundant and diverse genetic entities on earth; however, broad surveys of viral diversity are hindered by the lack of a universal assay for viruses and the inability to sample a sufficient number of individual hosts. Viruses present in mosquitoes can include viruses that are biologically or mechanically transmitted by these vectors, as well as other viruses that are not transmitted by mosquitoes but are drawn indiscriminately from host reservoirs. Broad surveys of natural viral diversity are technically challenging due to the inability to sample a sufficient number of individuals from different host species and the difficulty of characterizing previously undescribed viruses (Ng *et al.,* 2011). Since female mosquitoes draw blood from a wide range of vertebrate hosts including humans, non-human primates, other mammals and birds and also feed on plant nectar, they effectively sample numerous important viral reservoirs (Molaei *et al.,* 2006). It is postulated that the virus requires (i) a means to amplify after its escape from midgut epithelial cells, so that it can infect the salivary glands efficiently (Girard, 2004; Hardy *et al.,* 1983), and (ii) a vehicle to ensure dissemination from the midgut to the salivary glands (Romoser *et al.,* 2004).

To reduce the effects of defective interfering particle formation during the propagation of virus, it may be useful to infect a particular cell line with a stock virus which was previously grown in a different cell line (Pyke *et al.,* 2004). Pathogen transmission seems to progress most effectively under

warmer temperatures because vector populations increase rapidly in abundance and generation times are shortened; blood feeding and oviposition occur much more frequently, increasing the frequency of host-vector contact and rapid pathogen development within the vector shortens the duration of the extrinsic incubation period, thereby increasing the efficiency of transmission (Reisen, 2010). The extrinsic incubation period, the interval between ingestion of an infectious blood meal and the time mosquitoes are capable of transmitting the virus, is an important component of vector competency (Anderson, 2008). Viral amplification in the mosquito's salivary glands requires an average temperature of above 22°C for more than 12 days. Generally, the expansion of vector populations, as a result of climate change, into disease-free areas or areas where disease endemicity is insufficient to elicit good protective immunity, will often lead to initial high rates of disease transmission that will decrease in time as the population develops immunity (Ramasamy and Surendran, 2012). A population exposed to a mosquito-borne disease will over time generate protective immune responses to the pathogen that can result in complete or partial immunity to reinfection (Guzman and Vazquez, 2010). Global climate change can potentially increase the transmission of mosquito vector-borne diseases such as malaria, lymphatic filariasis, and dengue in many parts of the world (Ramasamy andSurendran, 2012). Arbovirus transmission by infected mosquitoes has been examined under laboratory conditions and, to a lesser degree, in the field. In the laboratory, transmission ability is often assumed after a disseminated infection (Dohm *et al.,* 2002, Girard *et al.,* 2004), although virus dissemination does not always allow successful transmission (Turell *et al.,* 2000, 2005,). Transmission rates of infected mosquitoes may vary at different times of the year or in different locations due to changes in the environment or mosquito populations.

A key step in the virus cycle is the process of entry into a new cell, during which the viral genome is translocated across the membrane of the target cell. This process requires specific interactions with a cell surface receptor, or more often, several surface receptors. This can lead to mere 'attachment', resulting in accumulation of virus particles at the cell surface, and the cellular molecules involved are termed attachment factors. A different set of interactions at the cell surface results in an active entry process, either by inducing a conformational change in the virus particle that is necessary for entry, or by inducing uptake of the virion into an endosomal compartment. In this second case, the local environment of the endosome, its acidity, or the presence of cellular proteases, termed convertases, activates the virion to interact with the membrane in such a way that the viral genome is delivered across into the cytoplasm. The cell surface molecules involved in these active processes are called entry receptors. Each virus has a different set of cellular surface molecules with which it interacts for entry, and the identification of these receptors is a major goal in contemporary virology (Backovic and Rey, 2012). Positive-strand RNA viruses have messenger RNA's as their genomes, which are directly translated into replicase polyproteins in the cytoplasm of the infected cell (Salonen *et al.,* 2005; Denison, 2008; Miller and Krijnse-Locker, 2008).

1.12 Electron Microscopy

Electron microscopy (EM) is the only technique available for directly visualising viruses, and therefore has a wide range of applications beyond purely diagnostic purposes. Preparation of specimens and the technique of negative staining are straightforward and quick, and the method is a 'catch-all' approach to detecting viruses. Nevertheless, it has a limit of sensitivity of

approximately 10^6 viral particles per millilitre of fluid. The area of most rapid development in diagnostic virology relates to molecular amplification assays to provide qualitative and quantitative results. PCR and other similar molecular assays have been applied to the diagnosis of virtually all human viruses. The preparation of specimens for EM does not require any special or expensive reagents. EM visualizes all infectious agents that are contained in the analysed specimen regardless of whether or not they are suspected by the clinician (Curry, 2003). This method can rapidly detect even very small viruses (Hazelton and Gelderblom, 2003). Since the virus families differ from each other in fine structure, an agent visualized by EM can be classified into the relevant family based on morphology. Nevertheless, the development of other techniques, *e.g.*, immunofluorescence, enzyme-linked immunosorbent assays (ELISA), and above all molecular biological methods such as polymerase chain reaction (PCR), progressively reduced the importance of EM in virus diagnosis and microbiology. However, in comparison with other diagnostic methods, EM still benefits from its rapidity and "open view", *i.e*. the capability of detecting all the pathogens present in a clinical specimen (Gentile and Gelderblom, 2005). Therefore EM should be utilized as a front line method in infectious diseases emergencies and or in suspect cases of bioterrorism (Hazelton and Gelderblom 2003; Madeley 2003). The negative staining technique developed by Horne and colleagues was a major advance in diagnostic virology (Brenner and Horne 1959; Horne and Wildy 1963, 1979). This technique uses heavy metal salts to provide contrast to viruses. In EM viruses will appear translucent, while the electron dense stain forms a dense, highly detailed halo. Phosphotungstic acid (PTA) is probably the most commonly used negative stain within diagnostic microbiology,

1.13 Future Prospects

There is an acute need for reliable predictions of within year and between-year epidemic events. The prerequisite for developing any system of early warning is a detailed understanding of the factors involved in epidemic genesis (Hay *et al*., 2000).The evident fluctuation of climatic parameters such as the annual minimum temperature in temperate regions and the increase of international trade and travel, as well as changes in vector and virus ecology associated with human intervention, altogether increase the abundance and diversity of arbovirus infections encountered in clinical practice (Grywana, 2010). Detection or accurate prediction of virus activity in vector populations and specific diagnosis of infection in the human or animal host are crucial components of effective control and treatment strategies and facilitate early warning of potential or existing outbreaks and initiation of vector management and/ or vaccination programs (Hall *et al.,* 2012). A serologically confirmed infection with a virus requires demonstration of a significant (fourfold or greater) increase or decrease in antibody titre between paired acute-phase and convalescent-phase serum samples collected days to weeks apart (Calisher, 1994).

The enhanced technologies for the specific detection of viral nucleic acid, such as multiplexed real-time PCR protocols, provide more rapid, sensitive, and specific approaches for detecting virus activity in vector populations. Furthermore, the recent application of next-generation sequencing technologies to rapidly analyse nucleic acid of unidentified viral isolates provides a revolutionary approach for the discovery and genetic characterization of new vector-borne viruses. The genomes of most DNA viruses are replicated in the nucleus, but those of some dsDNA viruses are replicated in the cytoplasm. The

genomes of most RNA viruses are replicated in the cytoplasm, but those of the minus-strand RNA viruses with segmented genomes are replicated in the nucleus (Carter and Saunders, 2007). Analysing mosquito pools for the presence of human pathogenic viruses is one of the primary sources of arbovirus surveillance data. Such information is important for determining when control measures are necessary and the form they should take for greatest effectiveness (Huang *et al.,* 2001). Traditional generic methods for identifying and characterizing novel viral diseases have included electron microscopy, virus isolation in cell culture, immunological approaches and PCR. Recently, technologies such as diagnostic microarrays and mass spectrometry have also been proposed as generic tools for identifying viruses, but all these methods require some prior knowledge of the agents to be identified (Mumford *et al.,* 2006). High throughput sequencing as a next generation sequencing technology has been developing rapidly during the last few years and has found various applications in different biological and medical research fields. Recent advances in this technology have made its application easier, cheaper, more convenient and more efficient allowing it to evolve into a powerful tool for identification of novel human pathogens (Palacios *et al.,* 2008). Virus isolation is based on inoculation of mosquito cell cultures, mosquitoes, mammalian cell cultures, or mice (Pialoux *et al.,* 2007). Currently, the accepted techniques for virus isolation, identification, and characterization include cell culture, animal inoculation, hemagglutination inhibition assay, plaque-reduction neutralization assay, enzyme-linked immunosorbent assay (ELISA), and viral serology (Berzofsky, 1999). An additional technique referred to as a real-time polymerase chain reaction (RT- PCR) could easily be adapted for field use and could be considered as a candidate for development as the next generation of nucleic acid−based field diagnostics assay (O'Guin

et al., 2004). The accurate and timely serological diagnosis of arboviral infections requires the propagation, inactivation, purification and concentration of suitable antigens that can be produced efficiently, safely and reliably. The serological diagnosis of arbovirus infections usually involves the testing of paired acute and convalescent sera in parallel, and requires stable, reliable antigens that are reproducible and provide consistent results (Pyke *et al.,* 2004). In addition, production of antigens that are suitable for multiple serological assays such as ELISA and the haemagglutination inhibition (HAI) assay improves the efficiency of the diagnostic laboratory and increases the accuracy, reproducibility and reliability of the results produced. The production of antigens requires the propagation of large quantities of high titre arboviral suspensions in cell culture. Parameters such as choice of cell line, multiplicity of infection (m.o.i.) and length of incubation period, should be considered to obtain optimal viral growth (Brinton, 1986; Monroe, 1982).

Viral diagnostics are becoming increasingly important in clinical medicine and public health. Factors in raising global concern with respect to acute viral diseases include burgeoning inter-national travel and trade, political instability and bioterrorism, climate change and its effects on vector distribution, and the emergence and re-emergence of zoonoses (Liu *et al.,* 2008). The ability of viruses rapidly to expand their geographic range and appear in unexpected locations is well illustrated by the worldwide spread of the human immunodeficiency virus, the transfer of West Nile virus to the western hemisphere and its subsequent dissemination throughout North and South America, and the recent emergence of chikungunya virus in Europe (Rezza *et al.,* 2007).

In the event of a viral outbreak, the situation warrants rapid detection and identification of the etiological agents. The molecular devices therefore become handy for the detection and characterization. Reverse transcription polymerase chain reaction (RT-PCR) using primers designed for structural and non-structural domains has been found useful in the rapid diagnosis of CHIKV (Pastorino *et al.,* 2005). Viral metagenomic analyses of environmental samples suggest that the field of virology has explored less than 1% of the extant viral diversity. Metagenomics is an alternative culture-independent and sequence-independent approach that does not rely on the presence of any particular gene in all the subject entities (Mokili *et al.,* 2012). The first application of metagenomics to the field of virology was in the analysis of the viral communities sampled at two near-shore marine locations in San Diego (Breitbart *et al.,* 2002). Since then, it has been used to survey several viruses in numerous environments including freshwater, marine sediment, soil and the human gut (Mokili *et al.,* 2012). High-through-put sequencing technologies can now be applied to all types of genomes, including single-stranded DNA and RNA. Early detection of arboviral activity provides useful information for projection of the potential course of transmission later in the season and for unravelling the environmental factors associated with the maintenance and establishment of enzootic cycles (De Foliart *et al.,* 1987; Komar, 2003; Nasci *et al.,* 2002). Early detection is also crucial since the timing of virus activity is important for understanding the intricacies of viral amplification, as well as extending the window for intervention. In temperate areas, the initiation of arboviral transmission cycles is often associated with low vector densities and low levels of transmission. Collection of sufficient mosquito samples for obtaining sensible detection power can be difficult when mosquito abundances are very low.

Vector-borne diseases occur when infectious agents (virus, protozoa, bacteria, or helminthes) are transmitted to their hosts by a carrier organism. The term 'emerging virus' is used in a number of contexts: it may refer to a virus that has recently made its presence felt by infecting a new host species, by appearing in a new area of the world or by both. Sometimes a virus is described as a 're-emerging virus' if it has started to become more common after which it was becoming rare (Carter and Saunders, +9-+2007). Diet and lifestyle changes associated with growing urbanization as well as increased substance abuse, environmental degradation, population growth, and levels of regional and local conflict are expected to lead to a surge in non-communicable diseases, including those associated with cerebrovascular events, depressive illness, conflict-related conditions, road traffic accidents, and cancers (Campbell-Lendrum and Molyneux, 2005). For instance, projections made in 1996 for the burden of disease from malaria and dengue in 2000 were subsequently found to have underestimated the true burden by 32% and 11%, respectively (WHO, 2001).

Whatever the precise nature of future trends, firm response measures to curb infectious disease will be required for the foreseeable future and these measures will inevitably affect or interact with other ecosystem services. In addition to growing human populations and increased demand for ecosystem services or natural resources means that there will be continuing, and possibly increasing, human interactions within the natural processes that influence infectious disease transmission. It should also be noted that vector-borne disease infections are influenced by a wide range of factors that are not directly related to ecosystem services. These include the provision of basic public health services to prevent, detect, and treat

disease, as well as ‘‘good governance’’ ensuring that these services are responsive to every citizens needs and that resources for disease control are not lost to corruption and inefficiency (Lendrum and Molyneux, 2005). Disease transmission is inherently an ecological process involving a network of species (Keesing *et al.* 2006). Mosquito-borne infectious diseases have been emerging and re-emerging in many areas of the world, especially in tropical and subtropical areas where agents such as West Nile virus (WNV), dengue virus (DENV), chikungunya virus (CHIKV) and yellow fever virus (YFV) are present. Surveillance of infectious agents carried by mosquitoes is important for predicting the risk of vector-borne infectious disease outbreaks. The changing global epidemiological environment is characterized by incursions of human populations into new environments, increasing overlap of the range of disease vectors with human habitation and concomitant exposure to a wider range of infectious agents (Mackenzie *et al.,* 1994). Not only are humans changing land usage patterns and entering new disease environments, but rapid transportation of disease agents is constantly increasing between continents (Sreenivasan *et al.,* 1986). During the last decades the appearance of new infectious diseases and an increasing invasion of diseases into new areas created a new category of pathogens: emerging and re-emerging pathogens. Most of the emerging viruses are zoonotic which means they can infect both animals and humans (Bengis *et al.,* 2004). Modern transportation has produced a quantum leap in the mobility of vectors and pathogens, and the consequences of this globalisation will continue to surprise us (Reiter *et al.,* 2006). The future perspectives in virology appear to be the metagenomic approach which will generate a plethora of genetic information from unknown and potentially infectious agents, some of which could be

associated with human diseases. The discovery of viruses will start to precede the characterization of the diseases they cause, well before the pathogenicity of these agents is defined (Mokili *et al.,* 2012).

1.14 Surveillance

The importance of mosquito surveillance was underscored by Reeves in his statement ‘‘ each epidemic that was evolved in recent years could have been prevented or abated early in the course of its development by means of surveillance and vector abatement’’ (Reeves, 1980). Arboviral transmission, in general, is maintained at low levels in vector mosquitoes and reservoir hosts, with transient, sporadic outbreaks among humans. When environmental variables are favourable, arboviral circulation may exhibit explosive dynamics with high prevalence of infection in vector mosquitoes and avian hosts, as manifested by WNV transmission in North America (Hayes *et al.,* 2005; Lampman *et al.,* 2006). Arthropod-transmitted flaviviruses are responsible for considerable morbidity and mortality, causing severe encephalitic, hemorrhagic, and febrile illnesses in humans (Scaramozzino *et al.,* 2001).

One of the key elements of the control of arbovirus transmission is the early detection of virus activity or increased virus activity in vector populations (Hall, 2012). The principle of vector transmission is that the vector acquires a virus when it feeds on an infected host and subsequently transmits the virus to one or more new hosts. Some viruses are transmitted after virions have become attached to the mouthparts of their vectors during feeding. Transmission in this way may occur within seconds or minutes of the vector acquiring virus. Many vector-transmitted viruses, however, cross the gut wall of the vector and enter its circulatory system. The virus ultimately reaches the salivary glands and is

secreted into the saliva, which may transport virus into new hosts when the vector feeds. This mode of transmission is said to be circulative, and transmission does not occur until hours or days after the vector has acquired the virus. Some circulative viruses replicate in one or more tissues and organs of their vectors thus there are viruses that can replicate in both invertebrates and plants, and viruses that can replicate in both invertebrates and vertebrates. Many of the invertebrates appear to suffer little or no harm when they are infected, so it has been hypothesized that many of the modern 'plant viruses' and 'vertebrate viruses' are descended from viruses of invertebrates that later extended their host ranges to plants or vertebrates (Carter and Saunders, 2007). If suitable vectors are present in adequate densities, the resurgence or recrudescence of transmission of a vector-borne disease in a given area is possible, especially if the infection was previously endemic (Gratz, 1999). Vector-borne diseases, including malaria, dengue and viral encephalitides, are among the most sensitive to climate conditions and climate change, particularly changes in temperature and humidity, factors that affect vector population dynamics and disease transmission. Arthropods do not have self-regulated body temperature, which depends on climate variation. Vector-borne diseases, including malaria, dengue and viral encephalitis are very sensitive to extreme climate changes that affect the agent itself and the susceptibility and ability of the human host to survive the infection, with consequent changes in morbidity and mortality. *Ae.albopictus* is a competent vector for a wide variety of arboviruses under experimental conditions, and has been found to be naturally infected with DEN, Japanese encephalitis, Potosi, KEY, Tensaw, CV, and EEE viruses, and can serve as an epidemic vector of DEN viruses. *Ae.albopictus* is both a nuisance as well as a potential disease vector (Moore and Mitchell, 1997). A high temperature stress of 44.5°C for 10 minutes on the larval stages was found to affect the

susceptibility of adult *Ae. aegypti* mosquitoes to Chikungunya virus. At this temperature, the mortality of the mosquito larvae was found to be approximately 95%, whereas a temperature greater than 45°C for 10 minutes was found to be lethal. Among the environmental factors, temperature has a direct influence on the extrinsic incubation period (Mourya *et al.,* 2004). Breeding sites must be removed, destroyed, frequently emptied, and cleaned or treated with insecticides (WHO Report, 2006). Large-scale prevention campaigns using DDT have been effective against *Ae. aegypti* but not *Ae. albopictus* (Reiter, 2006).

Reducing vector density by eliminating or managing preimaginal development habitats, larviciding, and space and residual spraying near infection foci are therefore central to the control of *Ae. aegypti* and *Ae. albopictus*, the principal vectors of dengue. Arbovirus vector transmission is interrupted when vector abundance decreases or disappears in temperate regions during the winter season. Importantly, larval source management and reduction strategies are presently directed exclusively toward freshwater habitats, because of the long and widely held view that the two *Aedes* species only develop naturally in fresh water (Barraud, 1934; Chan *et al.,* 1971; Kulatilaka and Jayakuru, 1998; Ooi *et al.,* 2006; World Health Organization, 2009). Unlike *Ae. aegypti*, *Ae. albopictus* has developed a diapausing egg stage that has enabled it to survive winters and spread to temperate regions, causing for example a chikungunya epidemic in northern Italy in 2007 (Rezza *et al.,* 2007) and two autochthonous cases of dengue in southern France in 2010 (La Ruche *et al.,* 2010). In tropical areas, arbovirus activity is homodynamic, since vector populations remain active year-round. On the other hand, vector transmission of arboviruses is interrupted when vectors

decrease in abundance or disappear in temperate regions during winter season (Kramer and Ebel, 2003). Measures to control the spread of infectious diseases include surveys of the dynamics of parasite transmission. Given the complexity of arboviral transmission, it should be realized that there are no ubiquitous solutions and sampling programmes must be designed with the best knowledge available regarding the ecology of vector species and arboviral transmission (Gu *et al.,* 2008).

1.15 Challenges

Water is a common vehicle for the transmission of many enteric viruses. The importance of water as a vehicle for enteric virus transmission was recognized in 1960s and since then many improvements in virus detection methods and preventive measures for drinking water treatment were implemented (Cliver, 2010). Global estimate suggests that 19% of total child deaths are attributable to diarrhoea and 73% of these deaths are concentrated in 15 developing countries. Enteroviruses are among the most common and significant causes of acute viral illness worldwide (Cherry, 1992; Modlin and Rotbart, 1997). Viruses will undoubtedly remain a major health threat, and the key scientific issues will have to be addressed to detect and characterise rapidly evolving old and new viral pathogens. One such example is the potential emergence of diverse polioviruses from C-cluster Coxsackie A viruses and its implication in a poliovirus-free world with a poliovirus-antibody naıve population (Jiang *et al.,* 2007). Genetic exchanges between enteroviruses can give rise to new viral genotypes that may be extremely virulent and dangerous for public health. The high economic impact of sporadic gastroenteritis cases and gastroenteritis outbreaks is now recognized,

thus prevention of enteric virus infection is one of the main goals in healthcare prevention programs (Ramani and Kang, 2009; Santosham *et al.,* 2010).

The fact that enteric viruses cause disease in individuals who are exposed to contaminated water has been shown directly through outbreaks associated with recreational use and inadequately treated drinking water (Baron *et al*. 1982; Gray *et al*. 1997; Levy *et al*. 1998; Kukkula *et al*. 1999), and indirectly through studies of swimming-related disease (Cabelli *et al.,* 1982). Enteric viruses survive longer in fresh and marine water than coliform bacteria, which are used to monitor water quality (Craun, 1984; Havelaar *et al.,*1993). Additionally, viruses in coastal waters have been detected far from the original source of pollution and in the absence of bacterial indicators (Metcalf and Stiles, 1967).Human enteric viruses are obligate parasites of man that infect and replicate in the gastrointestinal tract of their hosts. Patients suffering from viral gastroenteritis or viral hepatitis may excrete about 10^5 to 10^{11} virus particles per gram of stool (Bosch, 1998). Most health-significant waterborne viruses have RNA genomes and a major challenge in the development of molecular techniques for the diagnosis of RNA viruses derives from the facts that they depend on error-prone polymerases that generate high mutation rates and the occurrence of recombination events that altogether lead to complex mutant genome populations or quasispecies (Domingo and Vennema 2008; Bailly *et al.,* 2000; Grandadam *et al.,* 2004; Etherington *et al.,* 2006). The significance to human health of many of the non-human animal viruses present *in* environmental samples is less well understood although it is remarkable that zoonotic viruses infecting humans continue to be discovered or appear to re-emerge as important human pathogens (Bosch, *et al.,* 2008). Due to their stability and persistence, enteric viruses subsequently

become pollutants in environmental waters resulting in human exposure through pollution of drinking water sources and recreational waters, as well as foods. The performance of wastewater treatment systems is at present monitored largely by the use of bacterial indicator organisms. Considering that infectious viruses have been isolated from aquatic environments meeting bacterial indicator standards, in some instances in connection with virus related outbreaks (Karmakar *et al.,* 2008) the use of bacterial indicators has thus been considered an insufficient tool to monitor wastewater quality because bacterial and viral contaminations are not necessarily associated and linked with each other (He and Jiang, 2005).

Background of the present study:

The present study was initiated when several massive outbreaks of Chikungunya, Dengue and Japanese Encephalitis were frequently reported across the State of Kerala. Multiple symptoms persisted among the affected individuals and the public health officials were in search of aetiological agents responsible for the out breaks and, other than clinical samples no resources were available. In this context, a study was undertaken to focus on mosquito larvae to investigate the viruses borne by them which remain silently prevalent in the environment. The study was not a group specific investigation limited to either arbovirus or enterovirus, but had a broad spectrum approach. The study encompassed the viral pathogens that could be isolated, their impact when passaged through cell lines, growth kinetics, titer of the working stocks in specific cell line, the structure by means of transmission electron microscopy (TEM), the one step growth and molecular characterization using molecular tools.

Objectives

1) Screening for viruses from mosquito larvae: isolation and characterization in various cell lines.

2) Enumeration and electron microscopy of the isolated viruses.

3) Molecular characterization.

Chapter 2

Primary Screening for Viruses from Mosquito Larvae, Isolation and Characterization in Various Cell Lines

2.1 Introduction

An effective disease surveillance and response system helps in the early detection and control of outbreaks of epidemic-prone diseases. Epidemics are public health emergencies which disrupt routine health services and are a major drain on resources. Besides direct costs involved on the epidemic control measures and treatment of patients, the indirect costs are also inherent due to their negative impact on domestic and international tourism and trade. To cite as example, plague which was not reported from any part of India for almost a quarter of century, caused a major outbreak in Beed district in Maharashtra and Surat in Gujarat in 1994 and resulted in an estimated loss of almost US$ 1.7 billion. Zoonotic diseases, which accounts for a substantial burden of morbidity and mortality due to endemic as well as emerging diseases, are a major concern. A strong coordination is needed between human

health and animal health sectors to control the zoonotic diseases like avian influenza, plague, rabies, leptospirosis.

The communicable diseases like Tuberculosis, Leprosy, Vector borne diseases (Malaria, Kala-Azar, Dengue, Chikungunya, Filariasis, Japanese Encephalitis, etc.), Water-borne diseases (Cholera, Diarrhoeal Diseases, Viral Hepatitis A & E, Typhoid Fever etc.), Zoonotic diseases (Rabies, Plague, Leptospirosis, Anthrax, Brucellosis, etc), and Vaccine preventable diseases (Measles, Diphtheria, Tetanus, Pertussis, Poliomyelitis, Viral Hepatitis B etc) are endemic in many parts of the world and continue to be a major public health problem. Many expert committees, dating back to the Bhore Committee in 1946, reviewed the existing health infrastructure or situation in the country and made further recommendations required to control diseases including communicable, non-communicable and emerging diseases. More recently, the expert committee on Public Health System (1996) and the National Commission on Macroeconomics and Health (2005) examined these issues. National Five Year Plans, National Health Policy (1983, 2002) and many International initiatives such as Health for All by 2000, Calcutta Declaration on Public Health in South-East Asia (1999), U.N. Millennium Development Goals (2000), Global Commission on Macroeconomics and Health (2001), Revised International Health Regulations (2005) and Asia Pacific Strategy for Emerging Diseases (2005, 2010) have also provided strong policy directives for the development of health care delivery system to control/prevent diseases.

The National Vector Borne Disease Control Programme (NVBDCP) is an umbrella programme for prevention and control of six vector borne diseases namely Malaria, Dengue, Chikungunya, Japanese Encephalitis (JE) Lymphatic Filariasis and Kala-azar. The strategy employed to prevent or control these

diseases include disease management including early case detection and prompt treatment, strengthening of referral services; integrated vector management including indoor residual spraying, use of insecticide treated bed nets or Long Lasting Insecticidal Nets (LLIN), larvivorous fish and supportive interventions like human resource development, behavioral change communication, public private partnership, monitoring and evaluation, and operational research. Presently, about 1.5 million cases of malaria and less than 1000 deaths are reported every year. About 80% of malaria burden is in Northeastern (NE) states, Chhattisgarh, Jharkhand, Madhya Pradesh, Orissa, Andhra Pradesh, Maharashtra, Gujarat, Rajasthan, West Bengal and Karnataka. The integrated vector management for transmission of vector borne risk reduction necessitates the need for entomological surveillance including larval surveys in addition to which recommends anti larval measures such as source reduction, chemical larvicides or biocide, larvivorous fish and environmental management.

In a study, Crabtree *et al*. (2009) reported the isolation and characterization of a new mosquito borne flavivirus isolated from a pool of *Culex tritaeniorhyncus* mosquitoes which were collected from Vietnam in 2002 which was later identified to be genetically different from mosquito of flaviviruses reported earlier which they named as Quang Binh virus. Gerhardt *et al*. (2001) isolated La Crosse virus (LAC) encephalitis from pools of the exotic mosquito *Aedes albopictus* in United States. The lysate was cultured in Vero cells and the positive pools were identified by Polymerase Chain Reaction. Hribar *et al*. (2004) determined from isolation of virus the species of mosquitoes that could vector West Nile Virus (WNV) with which he studied with 34 species of mosquitoes in Florida and found that five species:

Culexerraticus, Cx. nigripalpus, Cx. quinquefasciatus, Ochlerotatus condolescens, and Oc. taeniorhynchus could transmit WNV and further no significant differences in the infectivity of each species was observed. In another study Hoshino et *al*. (2009) isolated a new flavivirus from *Ae. albopictus* and *Ae. flavopictus* mosquito in Japan. The virus designated as *Ae. flavivirus* (AEFV) was found to replicate only in mosquito cell line and shown to produce a mild cytopathic effect. O'Guinn *et al*. (2004) reported the field level detection of Eastern Equine Encephalitis Virus (EEEV) from *Culex melanoconionpedroi* mosquitoes from the Amazon region of Peru which were identified by way of Reverse Transcriptase-PCR. A novel flavivirus was isolated from mosquitoes in Finland which represented the first mosquito-borne flavivirus isolated from Northern Europe by Huhtamo *et al*. (2009) designated as Lammi virus (LAMV), had the biological properties of preferring replication in mosquito cells and since the genetic relatedness of the viruses is associated with vertebrate hosts, the situation warranted an immediate search for disease outbreaks. In another study Isawa *et al*. (2011) reported that two infectious agents were isolated from *Culex* species mosquitoes in Japan which were identified as distinct strains of a new RNA virus by a method for sequence-independent amplification of viral nucleic acids. The virus designated Omono River virus (OMRV) replicated in mosquito cells in which it produced a severe cytopathic effect. Phylogenetic analysis of the virus revealed that OMRV was a new member of the family of non-segmented dsRNA viruses infecting arthropod hosts excluding fungal or protozoan hosts. Jones *et al*. (2009) reported a new flavivirus from a new vector *Uranotaenia mashonaensis*, a mosquito genus which were not previously known to harborflaviviruses. The novel virus was termed Nounane¨ National Park, Co virus (NOUV) which grew only on C6/36 insect cells and

not on vertebrate cells. Phylogenetic analysis revealed that only a distant relationship to other known flaviviruses were found, indicative that NOUV is a distinct novel lineage within the family Flaviviridae. Kim *et al*. (2009) reported a flavivirus similar to the cell fusing agent viruses (CFAV) that are capable of naturally infecting a wide variety of mosquito species. This newly recognized insect-specific virus comprises a distinct CFAV complex within the genus Flavivirus which was isolated from mosquitoes collected in the East Texas and Trinidad of United States. In another study conducted by Flores *et al*. (2010) detected vertical transmission of SLEV in *Culex quinquefasciatus* mosquitoes. Under laboratory conditions, SLEV vertical transmission was detected in larvae at 1:256 and adults F1 1:406. These were the first studies to confirm experimental vertical transmission of SLEV in *Cx. quinquefasciatus* populations from Argentina, through additional overwintering mechanisms.

Arboviruses obligately cycle between vertebrates and arthropods, a process that imposes additional selective constraints on evolution and adaptation. Sequence comparisons of RNA arbovirus isolates showed that they were relatively stable (Holmes and Twiddy, 2003) and genetic studies revealed that evolution is dominated by purifying selection (Jerzak, *et al.*, 2005, 2007 and 2008). In nature, virulence is likely to be maintained by continuous alternating passages between vertebrates and insect hosts. Therefore it has been informative to compare the evolution of viruses that have been serially passaged in a single host versus viruses passaged in alternation between two different hosts. For example, Ross River Virus, an alphavirus, showed phenotypic stability during alternating passages in *Ae. aegypti* mosquitoes and mice but increased in neurovirulence after serial passage in mouse brains, suggesting that host alternation restrains the

evolution of virulence (Taylor and Marshall, 1975a and 1975b). The requirement to replicate in both vertebrate and invertebrate hosts is thought to limit the introduction of genetic changes into the genome of arboviruses. Serial passage under laboratory conditions will overcome this limitation allowing for genetic changes to be introduced and affecting the virulence of the virus for animals. Passage of Venezuelan equine encephalitis virus in tissue culture, eggs or mice resulted in 11 nucleotide or amino acid changes but no significant change in the virulence of the virus for mice whereas passage of Japanese encephalitis virus (JEV) under the identical conditions observed 22 nucleotide or amino acid changes that often resulted in improved survival probabilities . The changes in both the genotype and phenotype of the related viruses have been observed after extensive passages in cell culture (Bradt, 1990).

Emergence of pathogenic RNA viruses is particularly associated with their genomic variability and environmental changes that lead to novel host contacts (Domingo and Holland, 1997). RNA viruses have the capacity for significant genetic variation due to the error prone nature of the viral RNA-dependent RNA polymerase which is responsible for replication of the viral genome attributed to the absence of proofreading mechanisms (Drake and Holland, 1999; Duarte *et al.,* 1994; Steinhauer *et al.,* 1992). The genomic sequence of arthropod-borne RNA viruses, such as those of the alphavirus and flavivirus genera, remain stable over longer periods of time when compared to other viruses that are transmitted directly between susceptible hosts which may be likely due to the requirement for alternate replication in vertebrate and invertebrate hosts (Jenkins *et al.,* 2002; Vasilakis *et al.,* 2009). Experimental evolution studies have been performed to study fitness trade-offs and the

unique ability of arboviruses to simultaneously evolve to vertebrate and invertebrate hosts (Cooper and Scott, 2001). Another reason for the genetic stability of arboviruses could be that vertebrate and invertebrate hosts impose different selective forces and that adaptation for optimal fitness in either host type involves compromises for fitness in the other. In other words, the alternating host transmission cycle may constrain arbovirus evolution by requiring these viruses to adopt a compromise fitness genome for alternating replication in each host (Strauss and Strauss, 1994). Alternate replication in vertebrates and invertebrate vectors has been hypothesized to explain the evolutionary stability of many arboviruses including West Nile Virus (Scott *et al.,* 1994). Viruses which were forced to alternate between several cell types achieved fitness increases in both cell types comparable to that of each respective specialist which was again contradictory to the hypothesis that alternation constrains adaptation by arboviruses (Cooper and Scott, 2001). Arboviruses usually do not cause the death of mosquito cells (Karpf and Brown , 1998) but they do induce apoptosis in vertebrate cells. The theory of constrained evolution as a result of alternating hosts is supported by several studies in which alphaviruses or flaviviruses were passed sequentially within or between vertebrate and invertebrate hosts (Ciota *et al.,* 2009; Coffey *et al.,* 2008; Greene *et al.,* 2005; Jerzak *et al.,* 2007; Jerzak *et al.,* 2008; Vasilakis *et al.,* 2009; Weaver *et al.,* 1999). The slow rate of arbovirus evolution may result from very different environments provided by unrelated hosts, the mammalian host as well as the insect host (Steele and Nuttall, 1989). It was reported that differences between the replication strategy in the insect vector which are persistent or non-cytolytic and in the mammalian host which are productive and cytolytic may be more important since insect and mammalian cells can constitute similar environments for viral replication (Novella

*et al.,*1999). There are different selective pressures existing for virus replication within each period of the two-host life cycle (Cooper and Scott, 2001). In the case of flaviviruses it is suggested that host-virus interactions are different in different combinations of virus and host. Cellular factors such as signal peptidase, protease or translation factors are supposed to contribute to genetic changes, leading to evolution in the long run (Cooper and Scott, 2001; Schneider, *et al.,* 2001). The gene encoding for E protein on the surface of flaviviruses is known to be the major site of mutations (Lobigs, *et al.,* 1990).

Polymerases of RNA viruses lack proofreading to repair errors, leading to one substitution per approximately 10^{-4} nucleotides (nt) copied corresponding to one error per 10-kb genome (Domingo and Holland, 1997). This polymerase infidelity leads to diversification that produces closely related but non-identical RNAs that together form a spectrum of mutants (Aaskov *et al.,* 2006, Craig *et al.,* 2003, Jerzak *et al.,* 2005, Wang *et al.,* 2002). High mutation rates and shorter replication times allow almost all RNA viruses to evolve at rates several orders of magnitude very faster than DNA-based viruses (Henchal and Putnak, 1990; Novella *et al.,* 1999). RNA viruses have the exclusive capacity for rapid evolution due to their high mutation rates, short replication times, and large population sizes (Drake and Holland, 1999). Studies reveal that RNA viruses can tolerate increased niche breadth when they evolve in heterogeneous environments (Turner and Elena, 2000), a trait which may help in easier emergence and therefore help explain why arboviruses frequently emerge or reemerge to cause human and veterinary disease. Emergence of pathogenic RNA viruses is usually linked with genomic variability and environmental changes that lead to novel host contacts

(Domingo and Holland, 1997). It is reported that host-virus interactions are different in different combinations of virus and host. Cellular factors such as signal peptidase, protease or translation factors are supposed to contribute to these genetic changes leading to evolution of flaviviruses in near future (Cooper and Scott, 2001). Virulence or pathogenicity with respect to a virus can be considered as a consequence of virus efforts to maximize its fitness, the virus must replicate extensively in a host to ensure its transmission to the next host. As viral replication damages host tissues leading to host death, it can be considered as seriously deleterious for virus survival (Anderson and May, 1982; Ebert, 1999). This constancy of consensus sequence may derive from the need to infect disparate hosts that present conflicting demands for adaptation where sequence changes that improve fitness in one host may not be maintained in the alternate organism (Coffey and Vignuzzi, 2011).

Adaptation to alternating passage requires more replication-competent genomes at the cost of expanding genetic diversity. In contrast, serial passaging results in the accumulation of more mutations, many of which result in the formation of nonviable RNAs (Coffey and Vignuzzi,2011). During alternating passage, selection may be focused on maintaining replication competence in both hosts by favouring generalist genomes of relatively high fitness for both cell types. Understanding the adaptability and selective pressures that drive genetic and phenotypic changes in arboviruses are crucial for predicting their ability to persist as well as re-emerge (Ciota *et al.,* 2007). Compromises in replicative ability are made regularly by virus populations in both the arthropod vectors as well as vertebrate hosts due to differential selection in each. Precisely, mutations are exclusively advantageous as either hosts are purged by purifying selection if they are detrimental to replication in

the alternative host and positive selection would then generally results from the infrequent mutations that result in coadaptation (Ciota *et al.,* 2007). *In vitro* evolution studies reveals that there are three general patterns of arbovirus evolution: (i) fitness gains after serial passage in vertebrate or invertebrate cells except in certain cases (Cooper and Scott, 2001) and losses in bypassed host cell types DENV, Eastern equine encephalitis virus (EEEV), Sindbis virus (SINV), and vesicular stomatitis virus (VSV) (Greene *et al.*, 2005, Novella *et al.,* 1999, Vasilakis *et al.,* 2009 and Weaver *et al.,* 1999); (ii) reduced fitness in new cells (VSV) (Novella *et al.,* 1999), and (iii) fitness increases after alternating (invertebrate to vertebrate) passage (DENV, EEEV, SINV, VSV) (Greene *et al.,* 2005, Novella, 1999, Vasilakis, *et al.,* 2009 and Weaver, *et al.,* 1999). Studies have reported that there are differences between the replication approaches of the virus with respect to the insect vector which may be persistent or non-cytolytic, and in the mammalian host they are considered to be productive and cytolytic, which are important in this respect as insect and mammalian cells constitute similar environments for viral replication (Novella *et al.,* 1999). When a virus is passaged sequentially in a specific host type there resulted in the introduction of genetic changes and corresponding fitness gains for that host, whilst diminishing fitness in the alternate host. Alternately passing an alphavirus or a flavivirus between vertebrate and invertebrate hosts, as occurs in nature, resulted in fewer genetic substitutions and either no change in the fitness of the virus or increased fitness for both hosts.

Studies confirm that serial passage in vertebrate cells especially loosens constraints on increases in genetic distance, while alternating passage restricts increases in distance to retain RNAs that are more genetically similar to each

other (Coffey and Vignuzzi, 2011). Similarly in another study an alphavirus, Venezuelan equine encephalitis (VEEV), lineages passaged 10 times in *Ae. aegypti* exhibited an increase in mosquito infectivity relative to viruses passaged in alternation between rodents and mosquitoes, while rodent-specialized strains produced much higher viremia in rodents relative to alternately passaged virus. Moreover, both the serially-passaged VEEV lines exhibited fitness declines in the bypassed host, while lineages passaged in mosquitoes and rodents in alternation demonstrated no detectable fitness gains, nor losses in either mosquitoes or vertebrates (Coffey *et al.,* 2008). Vasilakis *et al*. (2009) reported from their studies that uncloned DENV were generally consistent with the hypothesis that releasing arboviruses from an alternating replication cycle results in the acquisition of higher fitness for the retained host cell and loss of fitness in the bypassed cells while in contrast cloned viruses showed rather fitness gains in all cell types following passage irrespective of the passage regimen. In another study when VSV was passaged alternately in sand fly and baby hamster kidney (BHK) cells or when allowed to specialize on one cell type through serial passages, fitness increases were observed in all cases, but when VSV was passaged exclusively in hamster cells fitness also increased in sand-fly cells indicating that specialization did not result in cell-specific adaptation (Novella *et al.,* 1999). Mosquito-borne alpha viruses which replicate alternately and obligately in mosquitoes and vertebrates appears to experience lower rates of evolution than do many RNA viruses that replicate solely in vertebrates. This genetic stability is hypothesized to result from the alternating host cycle, which constrains evolution by imposing compromise fitness solutions in each host (Greene *et al.,* 2005). Arbovirus adaptation to different hosts and cells was examined by Taylor and Marshall (1975) in their studies using the alphavirus Ross River

virus which revealed that serial passage in cell cultures depressed virulence, while mouse passage raised the level of virulence in a step-wise manner.

The use of cell culture passage producing virus strains which are attenuated in vitro and in vivo systems is well documented for the flaviviruses Dengue and Yellow fever (Barrett *et al.,* 1990; Butrapet *et al.,* 2000; Halstead and Marchette, 2003). Live attenuated vaccines for many different viruses, including VEEV and JEV, have been developed by repeated *in vitro* passage (Ada, 2001; Kinney *et al.,* 1993; Ni *et al.,* 1995; Nitayaphan *et al.,* 1990). A thorough knowledge about the spectrum of phenotypic changes and degree of genetic alteration in virus that were serially passaged in a single host cell type will help us to understand the evolutionary stability observed when virus cycles in nature (Ciota *et al.,* 2007). When a virus was first isolated it may replicate very poorly in cells under the laboratory conditions, but after it has gone through a number of replication cycles it may replicate much more efficiently than before. Each time the virus is 'sub-cultured' it is said to have been passaged. After a number of passages the virus may be genetically different to the original wild strain, in which case it is now a laboratory strain (Carter *et al*., 2007). Repeated passage of some viruses *in vivo* has also been used to develop live attenuated vaccines in the past (Ada, 2001).

The most basic molecular requirement for virus replication is to induce either profound or subtle changes in the host cell so that the viral genes in the genomes are replicated and viral proteins are expressed resulting in the formation of new progenies of viruses which are far greater than those used to infect the cells. While reproducing, the viruses use at least part of the cells as equipment for replication of viral nucleic acid and expression of genes and also use the protein synthetic machinery and metabolic energy resources of the

cell as well. Any infection that results in production of more infectious virus at the end than the initial is said to be a productive infection. The actual number of infectious virus produced in an infected cell defined as the burst size ranges from less than to more than 10,000 viruses which wholly depend on the type of cell infected, the nature of the virus along with other factors. Infection with many viruses completely transforms the cells into a factory for the replication of new viruses, while under certain circumstances the virus infection would lead to a state of coexistence between the cells and the infecting virus which persist together as long as the life of the host. This process can be a dynamic one in which a small amount of virus is produced consistently or a passive form in which the viral genome acts as a carrier in the cell with little or no evidence of viral gene expression (Wagner, 2006). Sequence comparisons of RNA arboviruses reveal that they are relatively stable in nature due to their alternating host cycle between the vertebrate and invertebrate hosts which in turn constrains viral evolution by a strong conservative sequence selection. This sequence stability may result from the requirements for replication in two separate hosts that present conflicting niches for replication and adaptation (Strauss and Strauss, 1994). Virulence can be considered as a consequence of virus efforts to maximize its fitness; here the virus must replicate extensively in a host to ensure its transmission to the next host though the viral replication damages host tissues, leading to host death that is seriously deleterious for virus survival (Anderson and May, 1982; Ebert, 1999).

At times several serial passages may be needed to establish evidence of isolation or to raise pathogenicity for animals or cells to levels considered necessary for laboratory experimentation (Bradish *et al.,* 1971). Any disease transferred from other vertebrates to humans is termed as zoonosis. Viral zoonosis

often requires the mediation of an arthropod vector for spreading it to humans. The role of an arthropod vector in the spread can be mechanical and passive in cases where it inoculates from a previous host into a current one without virus replication though the arthropods role can be dynamic. In the case of viruses with RNA genomes that are transmitted between the host and arthropods, the virus replication in the vector provides a secondary reservoir and a means of amplification. This events cause spread to a human host highly efficient as even a small inoculation of virus into the vector would result in a large increase in virus for transmission to the succeeding host (Wagner, 2008). Adaptation to a new environment either in the laboratory or the field implies a mechanism for variation which is usually ascribed to random mutation or selection of variants (Fennerand John, 1959). Studies were done on CHIKV population passaged alternately between invertebrate and vertebrate cells i.e invertebrate to vertebrate to simulate natural alternation and contrasted the results with those for populations that were artificially released from cycling by passage in single cell types, and then CHIKV populations were characterized by measuring genetic diversity, changes in fitness, and adaptability to novel selective pressures. In this case the greatest fitness increases were observed in alternately passaged CHIKV without any change in population diversity, however, the greatest increases in genetic diversity were observed after serial passage and were correlated with greater adaptability (Coffey and Vignuzzi, 2011).

Having this as the background a primary screening for viruses was undertaken from mosquito larvae, besides their isolation and characterization in various cell lines. The cell lines used were C6/36, VERO and HEp -2.

2.2 Materials and Methods

Most of the virus-infected cells will undergo morphological changes, which includes swelling of cells due to alteration of membrane permeability or shrinkage due to cell death and even the formation of giant multinucleate cells, which are all visible under a light microscope. Collectively, these changes are referred to as cytopathic effects (CPE) which are observations in an inoculated monolayer over a period of time; a straightforward screening method for determining whether or not a virus is present in the cell culture (Cann, 1999).

If ever there arises any doubt as to the origins of a CPE, that particular culture should be passaged into fresh cells, which are then incubated in place of the original and examined regularly to see if the effect was reproducible. If the original CPE arose because of a virus infection, then it should reappear in all the subsequent passages. As the titre of virus will have increased within the first culture, the time for appearance of CPE in the next passage should then be considerably shortened. Alternatively, if the original CPE was due to non-specific toxic factors, then these should have been diluted during passage, and the subsequent damage to the cell sheet in the passaged flask should appear more slowly and less extensively as well. There are also other reasons why it may be necessary to passage viruses. Some viruses fail to produce a visible CPE on initial invitro culture, despite viral replication. However, adaptation of the virus to the particular cell substrate may occur on repeat passaging, resulting in the production of a visible CPE. This process of blind passaging in order to encourage the appearance of a visible CPE is often essential (Cann, 1999).

2.2.1 Selection of Sampling Sites

Pathanamthitta District of Kerala, South India is of unique importance characterized by high lands and sylvatic environments that are nourished with

ample quantities of rich rubber and pineapple plantations as well. In addition, during the last epidemic of 2006 and 2007, this was one among the badly affected Districts of the State (Thenmozhi *et al.*, 2007; Kannan *et al.*, 2009). The District has tourist and pilgrimage sites where a large in flow of migrants invades the locality during various seasons of the year. Considering all of the above factors Pathanamthitta was selected as an ideal study area for the collection of mosquito larvae by which silently prevailing viruses including arboviruses could be documented. The study originated from the realization of the importance of early detection of silent viruses which prevail in the environment and also to point out the need to work out rapid prevention strategies for future viral epidemics in the long run.

2.2.2 Selection of Samples

Since it is known that enormous numbers of viruses are known to be transmitted by arthropods through both vertical and horizontal routes, samples were fixed so as to select the larvae of mosquitoes which help in documenting those viruses which remain silent in nature through vertical transmission or transo-ovarial transmission routes. The samples were collected from four main locations. From each main location an average of seven samples were subsequently collected during various seasons of the year. Each location was identified using GPS co-ordinates and the Google Earth images were used for map composing using the ArcGIS version 9.3. Although there are over 2500 different species of mosquitoes throughout the world, we have altogether only four types of disease transmitting mosquitoes of the Genus *Culex*, *Anopheles*, *Aedes* and *Mansonia* of which 80% mosquito population belongs to *Culex*.

2.2.3 Collection of Samples

The mosquitoe larvae were obtained from rain water collected in discarded tyres, utensils as well as in barks. Such types of still waters are essential for egg laying by almost all species of mosquitoes. The samples were selected from multiple sites (Farfan-Ale *et al.,* 2009; Huanyu. *et al.,* 2012) which were supported with thick vegetation of both rubber plantation and pineapple gardens during the pre-monsoon and monsoon periods during which massive explosive outbreaks of Chikungunya was reported across the state (Niyas *et al.,* 2010).

2.2.4 Transportation

Mosquito larval samples were collected in polythene bottles along with the water in which they were seen providing sufficient head space. The samples were soon transported to the laboratory with minimum disturbances.

2.2.5 Processing of Samples

Samples transported to the laboratory were sieved out of the holding water, washed three times in sterile tap water, cleared of debris and transferred to sterile mortar kept in laminar hood. Larvae were subjected to second step of washing thrice using Phosphate Buffered Saline (PBS-1X). They were then ground in 2 ml of Minimal Essential Medium (MEM) and centrifuged in 1mL aliquots at 10,000*g*for 20 minutes at 4ºC. The clarified mosquito homogenates were filter sterilized using 0.22µm polyvinylidene fluoride membrane (PVDF) filters and aliquoted in to sterile cryo vials and stored at -80°C (Crabtree *et al.,* 2009; Farfan-Ale *et al.,* 2009; Isawa *et al.,* 2011; Huanyu *et al.,* 2012; Roiz *et al.,* 2012).

2.2.6 Inoculation on to cell lines

2.2.6.1 Continuous Cell Lines

The Human Epithelial Larynx (HEp-2), African Green Monkey Kidney Cells (VERO) and the Insect Cell (C6/36) lines provided from the cell culture Repository at National Center for Cell Science (NCCS), Pune, were used in the study. VERO and C6/36 were used for isolation of viruses, while the HEp-2 was also included in the study to examine the possibility of pathogenesis in humans and in anticipation of human epidemic episodes in near future (Isawa *et al.*, 2011). All cells were grown in 25cm^2 angle neck pre sterile cell culture flasks of 50ml (Greiner Bio One -690160).

2.2.6.2 Insect cell line (C6/36)

The mosquito cell line derived from larvae of *Aedesalbopictus*, a cell line that is frequently used in arboviral isolations, was incorporated in this study. C6/36 was maintained in Minimal Essential Medium (MEM) supplemented with 5% Foetal Bovine Serum (FBS)as closed system and incubated at 28 ^{0}C.

2.2.6.3 African Green Monkey Kidney cell line (VERO)

The VERO Cell line was maintained in MEM supplemented with 10% FBS and incubated at 37°C as closed system throughout the study; however FBS concentration was brought down to 2% when virus lysates were inoculated.

2.2.6.4 Human Epithelial Larynx cell line (HEp-2)

The HEp-2 Cell line was maintained in MEM supplemented with 10% Foetal Bovine Serum (FBS) and incubated with 37°C as closed system. When the cell line was inoculated with virus lysate 2% FBS was supplemented.

2.2.7 Preservation of Cell Lines

2.2.7.1 Cryopreservation

For the cryopreservation of cells, cell culture flasks with confluent monolayer were selected, drained of the holding medium and washed with 2ml of Trypsin EDTA solution (Sigma T4174 -10X).Drained of the fluid and added another 2ml of Trypsin EDTA solution by which the cells started rounding off, drained off the fluid and tapped the bottle to effect total dislodgement. An aliquot of 1ml Foetal bovine Serum (FBS) was added to stop the reaction of trypsin, agitated well and added 2ml growth medium. An aliquot of 920µl cell suspension was transferred to cryovials and supplemented with 80 µl DMSO and mixed and kept at 4°C for one hour. After one hour the cryovials were wrapped in tissue paper and covered with aluminum foil and kept at -80°C (New Brunswik Scientific, Enfield, CT 06082-4444, USA) to achieve cooling at the rate of 1°C per min.

2.2.7.2 Revival of Cell Lines

For the revival of cells, the vials kept at -80°C were thawed rapidly by immersing in sterile distilled water kept at 37°C in a water bath. Once thawed they were dispensed into sterile centrifuge tubes (Tarsons Products Private, Ltd., Calcutta) and made up the volume to 10ml by adding MEM and mixed gently. After 30 minutes the tubes were centrifuged at 1500 rpm at 28^0 C and the supernatant discarded. To the pelleted cells 10ml MEM was again added at room temperature and after maintaining for a while, centrifuged again at the same speed at the same temperature. The process was repeated once more. The supernatant was discarded and added 5ml fresh medium at room temperature, mixed gently and dispensed in to new cell culture flasks and maintained at the required temperature. Healthy cells began to adhere within an hour or two after seeding.

All the cell lines used in the study were successfully maintained in Eagles Minimal Essential Medium (EMEM) (Himedia AT017A–20L). It was prepared by dissolving 9.5 g readymade medium per liter distilled water supplemented with 200mM L-Glutamine, 7.5% sodium bicarbonate and 0.1% sodium pyruvate, 10% (v/v) fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 U/ml penicillin streptomycin. In most cases throughout the study the cell culture medium was supplemented with 10% Foetal Bovine Serum (Himedia RM1112-500ml) except when it was used for inoculating virus during which 2% FBS was used. Phenol red (Himedia) was used as the pH indicator and pHof 7.0-7.2 was maintained.

2.2.8 Subculturing (Passaging)

Fully confluent HEp-2 and VERO cell lines were passaged using Trypsin EDTA solution (Sigma T4174 -10X) which was reconstituted to 1X with Phosphate Buffered Saline as the working stock. The medium from the cell culture flasks was drained off and added with 2ml trypsin EDTA mixture and gently swirled and drained off. Subsequently, 2ml Trypsin EDTA solution was added and waited for the beginning of rounding off of the cells and the solution drained off, tapped the bottle and added fresh medium containing FBS, dislodged the cells by gentle pipeting and transferred to fresh bottles at split ratio of 1:2. To subculture the Insect cell line (C6/36) the holding medium was drained off, tapped the bottles gently, fresh medium added, gently pipetted to dislodge the cells and transferred to fresh bottles at a split ration of 1:3. For subculturing C6/36 trypsinization was not required.

2.2.9 Primary Screening

The mosquito larval homogenates (29 Nos.) prepared and maintained at – 80 ^{0} C was used as the samples for examining the presence of viruses. An aliquot of 0.5 mL was inoculated on to a monolayer of C6/36 cell line maintained in MEM and incubated at 28ºC for 7 days. From each flask 1mL medium was inoculated to the monolayer of both VERO and HEp-2 cell lines and incubated at 37 $^{\circ}$C and observed for CPE. Those bottles which showed CPE were segregated and prepared for confirmation. They were subjected for three cycles of freezing and thawing and the content was subjected for centrifugation at 10,000 rpm. The supernatant was saved and maintained at –80°C.

2.2.9.1 Screening with Insect Cell line

C6/36 cell line bottles were inoculated with 1ml of mosquito homogenate and incubated at 28°C for a week. After incubation, anticipating the presence of virus the cell line was subjected to three freeze thaw cycles and the supernatant (0.5 ml) was passaged to VERO and HEp-2 cell lines simultaneously. All those bottles which showed CPE, were successively passaged three times.

2.2.10 Heat inactivation of the lysates

Lysates from all three cell lines in which CPE observed were subjected to heat inactivation to prove the presence of biological entity. They were incubated at 60° C in a water bath for 1hr and inoculated to the respective cell lines, incubated and observed for CPE following Issacs *et al.* (1957).

2.2.11 Serial passage of the lysates

It was required to determine whether multiple successive cell culture passages would increase the number of infectious particles (Viruses) allowing enhanced cytopathic effect determined by way of MTT assay (Mosmann, 1983). From 29 samples 22 positive lysates could be obtained which were subsequently passaged three times in the respective cell lines. C6/36, HEp -2 and VERO cell lines were grown in 24 well plates (Greiner Bio-one (Cat #662160) in quadruplicate and after 24 h of incubation the growth medium was removed and added with 500 µL lysates maintained at -80^{0}C after bringing them to room temperature (28^{0}C) along with control wells in quadruplicates without the addition of the lysates. On completion of 48hrs of incubation cytopathic effect was examined and transferred 500 µl of culture supernatant to the next batch of wells with confluent growth of cell lines. Subsequently, they were subjected for MTT assay based on which the relative percentage survival of cells was calculated based on the equation (Blank absorbance - Well absorbance/ Blank absorbance) * 100

The process was repeated three times and the progression in the mortality of cells was determined.

2.2.12 MTT Assay

The assay is a colorimetric method based on the determination of cell viability utilizing the reaction of a tetrazolium salt (3-4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, MTT) with the mitochondria of metabolicalIy active cells. The reduction of the tetrazolium salt bynicotinamide adenine dinucleotide dehydrogenase (NADH) and nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) within the cells produces insoluble purple formazan crystals, which are later solubilized yielding a purple-colored

solution (Mosmann, 1983). After replacing the medium, 50 µl of MTT (Sigma) solution (5mg per ml in PBS) was added to each well and incubated for 5 hours in dark. Control consisted of medium alone with MTT added. After incubation, the medium was removed and MTT-formazan crystals were dissolved in 100 µl dimethylsulfoxide (DMSO). Absorbance was recorded immediately at 570nm in a microplate reader (TECAN Infinite Tm, Austria).

2.2.13 Statistical analysis

Relative percentage survival of cells in each cell line (C6/36, VERO and HEp -2) in terms of MTT values between each other on inoculating with 22 lysates from HEp -2 cell line was subjected to one way ANOVA at $P< 0.05$ at each passage.

2.3 Results

2.3.1 Primary screening

The results of the positive samples with their respective co-ordinates and sampling dates are represented in Fig.1 and the sampling points and their co-ordinates given in Table.1. The results of primary screening of the homogenates of mosquito larvae for virus isolation are represented in Fig.2. Out of 29 lysates 22 were positive in terms of CPE on three successive passages in both VERO and HEp-2 cell lines accounting for 76 % mosquito homogenates harbouring viruses of unknown nature. The negative lysates were the ones which either did not show CPE at any stage in any of the cell line or the ones which showed CPE at the initial stage and disappeared during the subsequent passages.

2.3.2 Temperature Inactivation

All the 22 positive lysates could be successfully heat inactivated. None of the lysates could successfully revive or produce cytopathic effects when further re-infected into confluent monolayer of susceptible cells confirming that the biological entity got denatured on heat treatment.

2.3.3 Comparative susceptibility of cell lines to the lysates

All the 22 samples which responded positively with VERO and HEp-2 cells were subjected to three rounds of adaptability studies by passaging through three different cell lines such as VERO, HEp-2 and C6/36. The results of each set of experiments with each sample are given separately. Among the three cell lines in general C6/36 was least adapted or susceptible, VERO cells in the second position and HEp-2 most susceptible or adapted as shown in Figs 3 to 24. Substantial decrease of relative percentage survival of HEp-2 cells was observed at all stages of the study.

Virus lysates

i) MCCV1A (Fig. 3)

In the case of MCCV1A, during first passage the relative percentage survival of C6/36 was 33.44 which increased to 56.35 in the second passage and to 72.12 during the third passage. The relative percentage survival of VERO was 15.04 during the first passage, which increased to 27.27 during the second passage and to 66.18 during the third passage. In the case of Hep-2 the relative percentage survival was 5.41 during the first passage, 16.13 during the second passage which declined to 4.38 during the third passage. At every passage the variations in relative percentage survival between C6/36, VERO and HEp-2 were statistically

significant (P< 0.05). Among the three cell lines Hep–2 was the most adapted/susceptible to MCCV1A.

ii) MCCV1B (Fig. 4)

In the case of MCCV1B, during first passage the relative percentage survival of C6/36 was 42.95 which increased to 53 in the second passage and to 80.70 during the third passage. The relative percentage survival of VERO was 24.45 during the first passage, which increased to 53.55 during the second passage and to 70.74 during the third passage. In the case of HEp-2 the relative percentage survival was 22.31 during the first passage, 40.47 during the second passage which declined to 36.35 during the third passage. At every passage the variations in relative percentage survival between C6/36, VERO and HEp-2 were statistically significant (P< 0.05) except the case with C6/36 and VERO during the second passage. Among the three cell lines Hep-2 was most the adapted/susceptible to MCCV1B.

iii) MCCV1D (Fig. 5)

In the case of MCCV1D, during first passage the relative percentage survival of C6/36 was 41.69 which increased to 55.78 in the second passage and to 61.22 during the third passage. The relative percentage survival of VERO was 43.45 during the first passage, which increased to 45.85 during the second passage and to 59.44 during the third passage. In the case of HEp-2 cells the relative percentage survival was 16.19 during the first passage, 24.57 during the second passage and 25.44 during the third passage. At every passage the variations in relative percentage survival between C6/36, VERO and HEp-2 cell lines were statistically significant (P< 0.05) except the case with C6/36 and VERO

during the first and second passages. Among the three cell lines HEp-2 was the most adapted/susceptible to MCCV1D.

iv) MCCV1E (Fig. 6)

In the case of MCCV1E, during first passage the relative percentage survival of C6/36 was 32.26 which increased to 59.36 in the second passage and to 75.04 during the third passage. The relative percentage survival of VERO was 39.37 during the first passage, 46.87 during the second passage and to 47.56 during the third passage. In the case of HEp-2 cells the relative percentage survival was 21.37 during the first passage, 24.87 during the second passage and 23.56 during the third passage. At every passage the variations in relative percentage survival between C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$). Among the three cell lines HEp-2 was the most adapted/ susceptible to MCCV1E.

v) MCCV1G (Fig. 7)

In the case of MCCV1G, during first passage the relative percentage survival of C6/36 was 48.18 which increased to 74.94 in the second passage and to 64.14 during the third passage. The relative percentage survival of VERO cells was 46.86 during the first passage, which increased to 54.03 during the second passage and to 59.59 during the third passage. In the case of HEp-2 cells the relative percentage survival was 20.08 during the first passage, 25.24 during the second passage and 12.31 during the third passage. At every passage the variations in relative percentage survival between C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$) except between C6/36 and VERO in

the first passage. Among the three cell lines HEp– 2 cell line wasthe most adapted/susceptible to MCCV1G.

vi) MCCV1H (Fig. 8)

In the case of MCCV1H, during first passage the relative percentage survival of C6/36 was 29.21 which increased to 59.66 in the second passage and to 87.04 during the third passage. The relative percentage survival of VERO was 28.12 during the first passage, which increased to 56.30 during the second passage and to 84.62 during the third passage. In the case of HEp-2 the relative percentage survival was 22.30 during the first passage, 43.15 during the second passage and 16.98 during the third passage. At every passage the variations in relative percentage survival between C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$) except between C6/36 and VERO in all three passages. Among the three cell lines HEp-2 was the most adapted/susceptible to MCCV1H.

vii) MCCV1I (Fig. 9)

In the case of MCCV1I, during first passage the relative percentage survival of C6/36 was 36.08 which increased to 55.85 in the second passage and to 73.91 during the third passage. The relative percentage survival of VERO was 42.59 during the first passage, 45.86 during the second passage and to 81.42 during the third passage. In the case of Hep-2 cells the relative percentage survival was 13.33 during the first passage, 23.45 during the second passage and 34.48 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 cell lines were statistically significant (P<

0.05) except between C6/36 and VERO in first passage even though there was an increase in percentage survival of cell line from first to third passage. Among the three cell lines HEp-2 was the most adapted/susceptible to MCCV1I.

viii) MCCV1J (Fig. 10)

In the case of MCCV1J, during first passage the relative percentage survival of C6/36 was 49.26 which increased to 89.62 in the second passage and to 84.87 during the third passage. The relative percentage survival of VERO cells was 41 during the first passage, which increased to 69.02 during the second passage and to 70.30 during the third passage. In the case of HEp-2 cells the relative percentage survival was 4.28 during the first passage, 24.28 during the second passage and 11.03 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$). Among the three cell lines HEp-2 cell line was the most adapted/susceptible to MCCV1J.

ix) MCCV1K (Fig. 11)

In the case of MCCV1K, during first passage the relative percentage survival of C6/36 was 84.14 in the first passage, 76.61 in the second passage and 43.32 during the third passage. The relative percentage survival of VERO cells was 54.61 during the first passage, which slightly decreased to 51 during the second passage and to 48.25 during the third passage. In the case of HEp-2 the relative percentage survival was 29.71 during the first passage, 38.61 during the second passage and

21.94 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$) except between C6/36 and VERO in the third passage. Among the three cell lines HEp-2 cell line was the most adapted/susceptible to MCCV1K.

x) MCCV1L (Fig. 12)

In the case of MCCV1L, during first passage the relative percentage survival of C6/36 was 67.45 during first passage, 84.33 in the second passage and to 54.86 during the third passage. The relative percentage survival of VERO cells was 55.76 during the first passage, 75.08 during the second passage which declined to 49.43 during the third passage. In the case of HEp-2 cells the relative percentage survival was 25.37 during the first passage, 44.34 during the second passage and 16.58 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$) except between C6/36 and VERO in the third passage. Among the three cell lines HEp-2 was the most adapted/susceptible to MCCV1L.

xi) MCCV1M (Fig. 13)

In the case of MCCV1M, during first passage the relative percentage survival of C6/36 was 46.87 during first passage, 67.12 during the second passage and to 55.97 during the third passage. The relative percentage survival of VERO cells was 17.34 during the first passage, 55.08 during the second passage and to 61.26 during the third passage. In the case of HEp-2 cells the relative percentage survival was 13.69

during the first passage, 31.66 during the second passage and 21.22 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$). Among the three cell lines HEp-2 was the most adapted/susceptible to MCCV1M.

xii) MCCV1N (Fig. 14)

In the case of MCCV1N, during first passage the relative percentage survival of C6/36 was 31.74 during first passage, 56.07 in the second passage and to 82.69 during the third passage. The relative percentage survival of VERO was 35.12 during the first passage, 42.37 during the second passage and to 52.97 during the third passage. In the case of HEp-2 cells the relative percentage survival was 12.04 during the first passage, 31.74 during the second passage and 26.98 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$) except between C6/36 and VERO during the first passage. Among the three cell lines HEp-2 was the most adapted/susceptible to MCCV1N.

xiii) MCCV1O (Fig. 15)

In the case of MCCV1O, during first passage the relative percentage survival of C6/36 was 39.78 during first passage, 71.17 in the second passage and to 74.38 during the third passage. The relative percentage survival of VERO was 17.93 during the first passage, 56.72 during the second passage and to 63.94 during the third passage. In the case of HEp-2 cells the relative percentage survival was 12.78 during the first

passage, 40.79 during the second passage and 34.65 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 cell lines were statistically significant ($P< 0.05$). Among the three cell lines HEp–2 cell line was the most adapted/susceptible to MCCV1O.

xiv) MCCV1S (Fig. 16)

In the case of MCCV1S, during first passage the relative percentage survival of C6/36 was 58.15 during first passage, 75.12 in the second passage and to 79.67 during the third passage. The relative percentage survival of VERO cells was 15.42 during the first passage, 80.70 during the second passage and declined to 61.31 during the third passage. In the case of HEp-2 cells the relative percentage survival was 13.06 during the first passage, 27.46 during the second passage and 14.71 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 cell lines were statistically significant ($P< 0.05$) except VERO and HEp-2 in the first passage. Among the three cell lines HEp-2 was the most adapted/susceptible to MCCV1S.

xv) MCCV1T (Fig. 17)

In the case of MCCV1T, during first passage the relative percentage survival of C6/36 was 68.38 during the first passage, 74.62 in the second passage and to 85.29 during the third passage. The relative percentage survival of VERO was 35.53 during the first passage, 56.41 during the second passage and to 78.18 during the third passage. In the case of HEp-2 cells the relative percentage survival was 11.53 during the first passage, 32.24 during the second passage and 42.62 during the third

passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 were statistically significant (P< 0.05). Among the three cell lines HEp-2 was the most adapted/susceptible to MCCV1T even though there was an increase in percentage survival of cells from first to third passage.

xvi) MCCV1U (Fig. 18)

In the case of MCCV1U, during first passage the relative percentage survival of C6/36 was only 37.32 during first passage, 63.49 in the second passage and to 84.49 during the third passage. The relative percentage survival of VERO cells was 34.19 during the first passage, 44.41 during the second passage and to 62.44 during the third passage. In the case of Hep-2 cells the relative percentage survival was 9.40 during the first passage, 17.05 during the second passage and 14.14 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 were statistically significant (P< 0.05) except C6/36 and VERO in the first passage. Among the three cell lines Hep – 2 cell line was most adapted/susceptible to MCCV1U.

xvii) MCCV1V1 (Fig. 19)

In the case of MCCV1V1, during first passage the relative percentage survival of C6/36 was only 81.77 during first passage, 73.77 in the second passage and to 78.24 during the third passage. The relative percentage survival of VERO cells was 80.91 during the first passage, 68.91 during the second passage and to 61.82 during the third passage. In the case of Hep-2 cells the relative percentage survival was 36.97

during the first passage, 22.97 during the second passage and 28 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 cell lines was statistically significant ($P< 0.05$) except C6/36 and VERO in the first and third passages. Among the three cell lines Hep-2 cell line were the most adapted/susceptible to MCCV1V1.

xviii) MCCV1X (Fig. 20)

In the case of MCCV1X, during first passage the relative percentage survival of C6/36 was only 47.87 during first passage, 80.75 in the second passage and to 59.01 during the third passage. The relative percentage survival of VERO cells was 45.12 during the first passage, 81.57 during the second passage and to 52.45 during the third passage. In the case of HEp-2 cells the relative percentage survival was 21.39 during the first passage, 37 during the second passage and 4.21 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$) except in C6/36 and VERO in the first and second passages. Among the three cell lines HEp-2 was the most adapted/susceptible to MCCV1X.

xix) MCCV1Y (Fig. 21)

In the case of MCCV1Y, during first passage the relative percentage survival of C6/36 was only 57.06 during first passage, 69.01 in the second passage and to 84.90 during the third passage. The relative percentage survival of VERO cells was 53.71 during the first passage , 45.32 during the second passage and to 61.57 during the third passage.

In the case of HeP-2 cells the relative percentage survival was 14.38 during the first passage, 23.71 during the second passage and 22.21 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$) except C6/36 and VERO in the first passage. Among the three cell lines HEp–2 was the most adapted/susceptible to MCCV1Y.

xx) MCCV1Z (Fig.22)

In the case of MCCV1Z, during first passage the relative percentage survival of C6/36 was only 54.48 during first passage, 85.02 in the second passage and to 85.93 during the third passage. The relative percentage survival of VERO cells was 31.14 during the first passage, 80.42 during the second passage which declined to 40.98 during the third passage. In the case of HEp-2 cells the relative percentage survival was 15.75 during the first passage, 49.83 during the second passage and 20.21 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$). Among the three cell lines HEp-2 was the most adapted/susceptible to MCCV1Z.

xxi) MCCV2A (Fig. 23)

In the case of MCCV2A, during first passage the relative percentage survival of C6/36 was 72.86 during first passage, 89.41 in the second passage and to 65.49 during the third passage. The relative percentage survival of VERO was 56.54 during the first passage, which increased to 59.90 during the second passage and declined to 49.16 during the third

passage. In the case of HEp-2 cells the relative percentage survival was 23.86 during the first passage, 36.72 during the second passage and 28.40 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$) except between VERO and Hep-2 in the first passage. Among the three cell lines HEp–2 cell line was the most adapted/susceptible to MCCV1Z.

xxii) MCCV2B (Fig.24)

In the case of MCCV2B, during first passage the relative percentage survival of C6/36 was 43.96, 77.46 during the second passage and to 73.01 during the third passage. The relative percentage survival of VERO cells was 32.08 during the first passage, 57.53 during the second passage and to 65.09 during the third passage. In the case of HEp-2 cells the relative percentage survival was 9.77 during the first passage, 22.98 during the second passage and 22.83 during the third passage. At every passage the variations in relative percentage survival of C6/36, VERO and HEp-2 were statistically significant ($P< 0.05$) except between C6/36 and VERO in the first passage. Among the three cell lines HEp-2 was the most adapted/susceptible to MCCV2B.

2.4 Discussion

Adaptation refers to the emergence of variants that are capable of multiplying more efficiently in the host cells. Following adaptation with a cell line, infectivity of the virus to the adapted cells is found to be increased and the virulence decreased. Often such adapted variants damage the original host less severely than the wild type virus and are therefore less virulent (Ahamed

et al., 2004). Surveillance of mosquito populations for known viruses continues to provide clues to the geographical distribution of the viruses and serves to identify regions that may be at higher risk for transmission to humans (Bennett *et al.,* 2011). Screening, isolation and identification are the key steps involved for the surveillance of novel viruses isolated from the environment. We have made an attempt to isolate the viruses from the environment from an endemic site of the state providing a means of earlier information before an outbreak prevails. A total of twenty nine samples were collected from multiple sites of the district of Pathanamthitta, Kerala. The mosquito larval samples were retrieved from their natural environments and were processed separately. Each pool consisted of approximately 80-100 larvae which were separately inoculated into cell lines such as C6/36 and VERO. Since the effects on insect cell line C6/36 were all non cytolytic (Novella *et al.,* 1999), all samples were provided with an incubation period of one week. From C6/36 and VERO the lysates were inoculated to HEp -2, passaged three times and all lysates which showed CPE were saved and maintained at -80^0C. A decline of incubation time was observed following each passage as pointed out by Carter and Saunders (2007). In some studies the pathogenicity of the isolated virus were checked by alternate inoculation into mice and monkeys (Bennett *et al.,* 2011). The samples were considered as positive only when the cytopathic effects were repeated in all passages. Ciota *et al*. (2007) studied the cell specific adaptation of flavivirus using WNV and SLEV which when serially passaged into insect cells resulted in mild genetic changes which further led to increased relative fitness and replicative ability of the virus in the homologous cell line C6/36. Some lysates exhibited cell rounding, while others resulted in syncytia formation and following which cytolysis of the cells were common altogether which defined the term Cytopathic Effects. Each sample was coded

separately and stored at -80°C until used. An aliquot of each sample was then subjected to heat treatments to confirm whether the effects could be reproduced after inactivation. All samples responded in such a way that none of them could re - initiate CPE when inoculated.

All the samples were then subjected to serial passage studies in three separate hosts with three subsequent passages in each with slight modifications as done by Chen *et al*. (2003) during which they studied the changes of envelope and non-structural proteins in Dengue virus when serially passaged in mammalian and mosquito cells. Clear and consistent CPEs were observed in both VERO and HEp-2 cells. The results clearly confine to the fact that the response of each virus is unique in terms of hosts as well as to lysates of viruses. Adapting to different host *in vitro* is a result of mutation which might have occurred since isolation and also serially passaging in a single cell type would increase in fitness gains in it while losses adaptability over the other. Coffey *et al*. (2011) alternately passaged Chikungunya virus between vertebrate and invertebrate hosts, and established that host alternation was necessary for virus increasing the fitness gains in each hosts. CHIKV was alternately passaged between BHK or HeLa and C6/36 cells or serially passaged in BHK, HeLa, or C6/36 cells for a total of seven passages. The lower level of genetic variation in virus from alternating passage between VERO and C6/36 cells might have resulted due to a lower frequency of host-virus interaction as pointed out by Schneider and Roossinck (2001). The Passages were mainly done to study the virulence, attenuation (Barrett *et al.,* 1990; Butrapet *et al.,* 2000; Halstead and Marchette, 2003; Yamada *et al.,* 2012) and amino acid substitutions in the vector *Ae. albopictus*, by the acquisition of a single adaptive mutation providing selective advantage for

transmission by the vector as well as competition assays (Ciota *et al.,* 2007; Coffey *et al.,* 2011) which compared the fitness of the viruses over the parent strain or between cell lines. Since a family signature of the viruses could not be arrived at this stage, MTT assay was undertaken, the reagent of which was successively added to the infected cells following incubation. As the reagent could only percolate to the mitochondria of viable cells an absorbance read at 570nm in an Elisa reader provided the information about the % of viable cells remaining after each course of infection. From the graphs plotted, the human cells (HEp-2) showed an expanded mortality accounted with the least survival rates especially at the third passage level. The insect cell line (C6/36) responded with significant increase of viable cells and better survival percentages than rest of the other two cell lines (C6/36 and VERO). In the case of VERO cell line increased adaptability was noticed by a decrease in the survival rate which might be co related with a sylvatic transmission cycle in forests were in the primates were known to be involved. The study could provide baseline information about the isolation and the host specificity evident within each host infection during different passages. Very few correlations existed with respect to the same virus in different hosts, pointing out that the cellular events with respect to each cells and viruses were unique.

In the present study C6/36 was the least susceptible, HEp-2 the most susceptible and VERO in between in terms of MTT assay. This situation points out the fact that the virus must be more adapted to human cell line than to primate and insect cell lines. This suggests that the efforts for identification of the virus must be extended beyond arboviruses.

Table. 1 Positive sampling sites and their respective dates with latitude and longitude

Location ID	Sample Code	Date	Location(Degree,Minutes, Seconds)
1	1A	20-06-07	76°45'41.131"E 9°12'27.707"N
2	1B	20-06-07	76°46'23.97"E 9°12'44.916"N
3	1D	20-06-07	76°45'42.961"E 9°13'18.601"N
4	1E	20-06-07	76°46'29.462"E 9°13'50.456"N
5	1G	20-06-07	76°46'37.883"E 9°11'0.198"N
6	1H	20-06-07	76°46'30.56"E 9°10'3.811"N
7	1I	24-12-07	76°45'55.777"E 9°12'50.774"N
8	1J	24-12-07	76°46'20.308"E 9°12'11.23"N
9	1K	24-12-07	76°46'15.548"E 9°13'18.601"N
10	1L	24-12-07	76°46'48.868"E 9°13'40.936"N
11	1M	24-12-07	76°45'47.355"E 9°10'40.426"N
12	1N	24-12-07	76°46'13.352"E 9°10'0.882"N
13	1O	24-12-07	76°47'2.781"E 9°10'3.445"N
14	1S	18-04-08	76°45'38.568"E 9°12'10.498"N
15	1T	18-04-08	76°47'2.049"E 9°13'41.668"N
16	1U	18-04-08	76°47'16.329"E 9°11'9.718"N
17	1V	18-04-08	76°45'33.808"E 9°11'13.745"N
18	1X	18-04-08	76°46'48.135"E 9°9'44.039"N
19	1Y	22-06-08	76°46'48.502"E 9°12'5.372"N
20	1Z	22-06-08	76°47'23.286"E 9°13'5.786"N
21	2A	22-06-08	76°46'58.021"E 9°10'53.973"N
22	2B	22-06-08	76°47'9.372"E 9°9'44.772"N

Table.2 Screening of mosquito larvae homogenates for CPE indicating presence of virus

Sl.No.	Sample Code	Initial cell line used for inoculating mosquito larvae homogenate (C6/36)	First Passage VERO &HEp -2	Second Passage VERO &HEp- 2	Third Passage VERO &HEp -2	Results
1.	1A	7 days	Positive	Positive	Positive	Positive
2.	1B	7 days	Positive	Positive	Positive	Positive
3.	1C	7 days	Positive	Positive	Negative	Negative
4.	1D	7 days	Positive	Positive	Positive	Positive
5.	1E	7 days	Positive	Positive	Positive	Positive
6.	1F	7 days	Positive	Negative	Negative	Negative
7.	1G	7 days	Positive	Positive	Positive	Positive
8.	1H	7 days	Positive	Positive	Positive	Positive
9.	1I	7 days	Positive	Positive	Positive	Positive
10	1J	7 days	Positive	Positive	Positive	Positive
11.	1K	7 days	Positive	Positive	Positive	Positive
12.	1L	7 days	Positive	Positive	Positive	Positive
13.	1M	7 days	Positive	Positive	Positive	Positive
14.	1N	7 days	Positive	Positive	Positive	Positive
15.	1O	7 days	Positive	Positive	Positive	Positive
16.	*1P	7 days	Positive	Positive	Positive	Positive
17.	1Q	7 days	Positive	Negative	Negative	Negative
18.	1R	7 days	Positive	Positive	Negative	Negative
19.	1S	7 days	Positive	Positive	Positive	Positive
20.	1T	7 days	Positive	Positive	Positive	Positive
21.	1U	7 days	Positive	Positive	Positive	Positive
22.	1V1	7 days	Positive	Positive	Positive	Positive
23.	1V	7 days	Positive	Negative	Negative	Negative
24.	1W	7 days	Positive	Positive	Negative	Negative
25.	1X	7 days	Positive	Positive	Positive	Positive
26.	1Y	7 days	Positive	Positive	Positive	Positive
27.	1Z	7 days	Positive	Positive	Positive	Positive
28.	2A	7 days	Positive	Positive	Positive	Positive
29.	2B	7 days	Positive	Positive	Positive	Positive

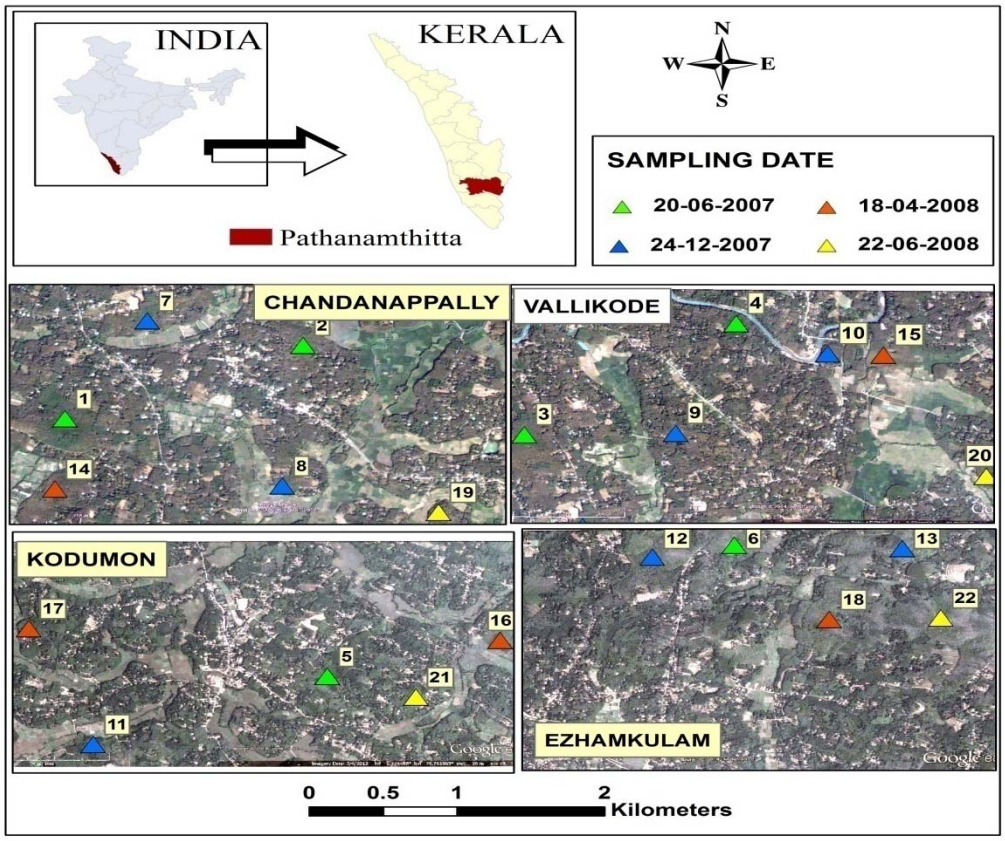

Fig.1 Sampling sites with latitude and longitude

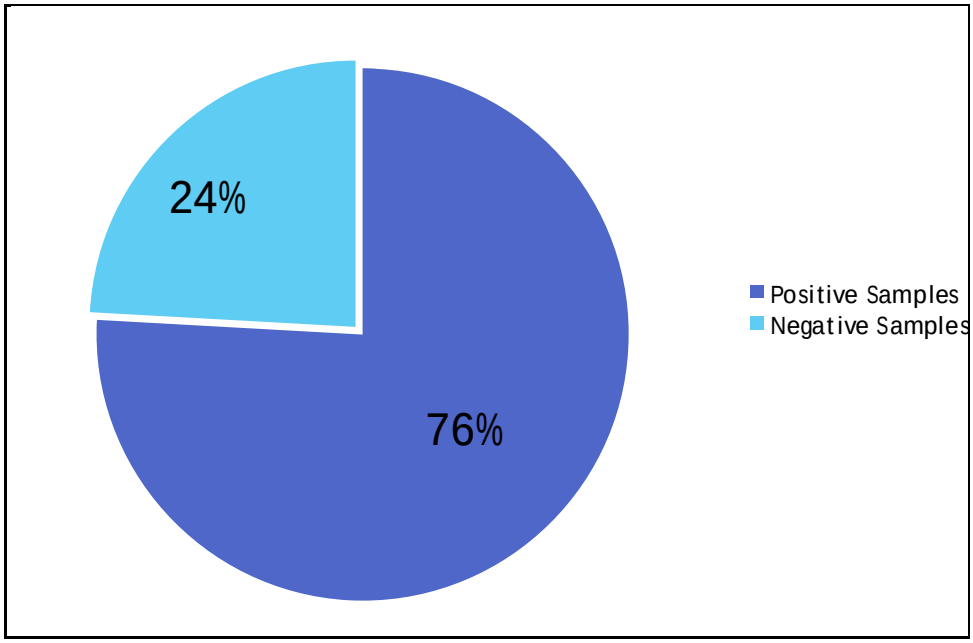

Fig.2 Primaryscreening for virus isolation indicating the percentage of positive and negative samples obtained.

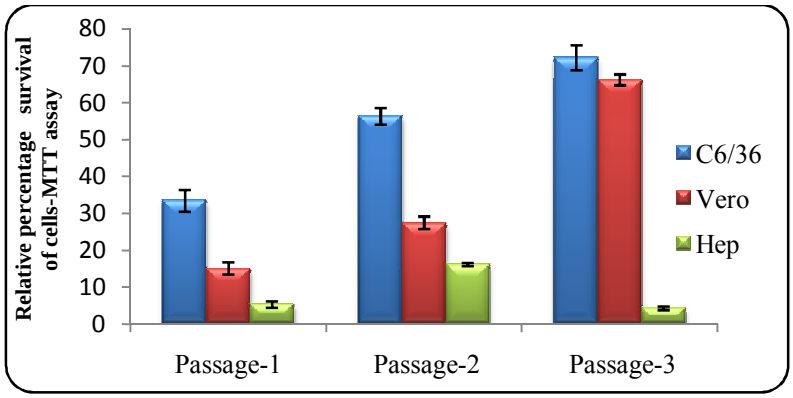

Fig. 3 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1A

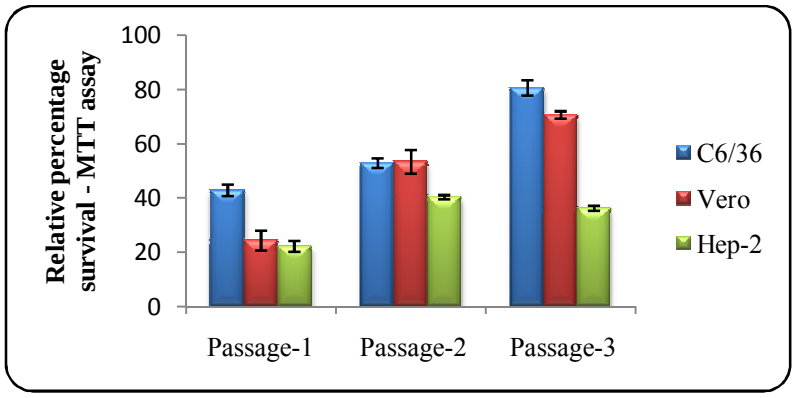

Fig. 4 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1B

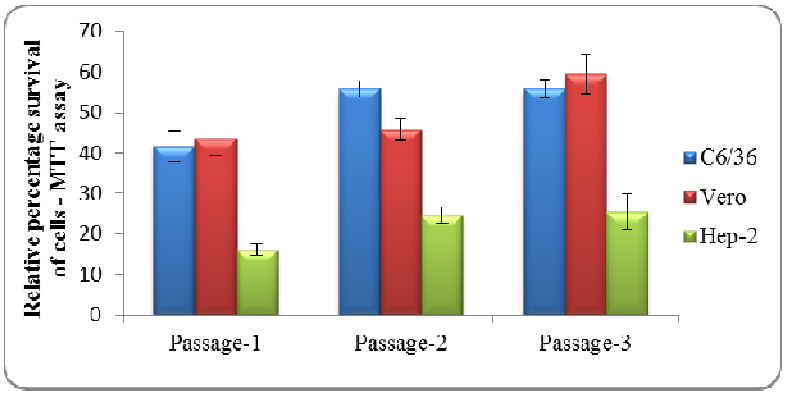

Fig. 5 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1D

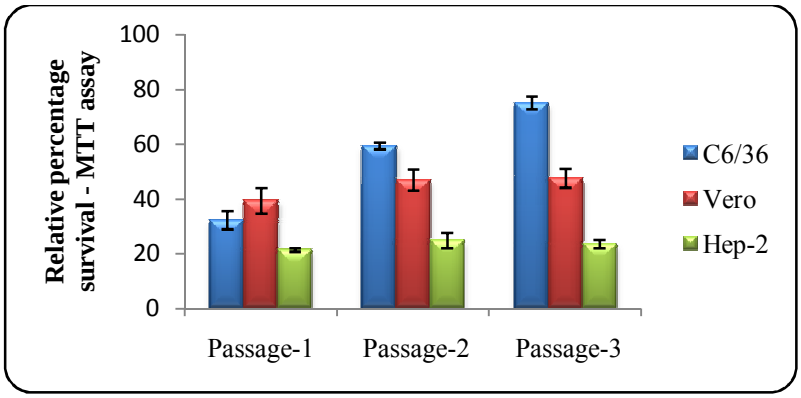

Fig. 6 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1E

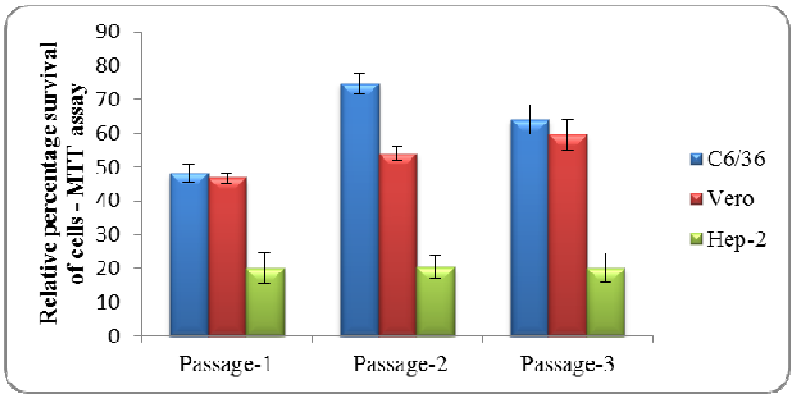

Fig. 7 Relative Percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1G

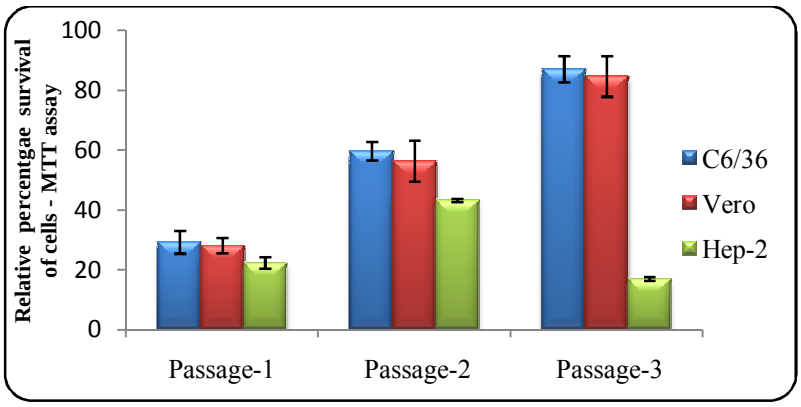

Fig. 8 Relative Percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1H

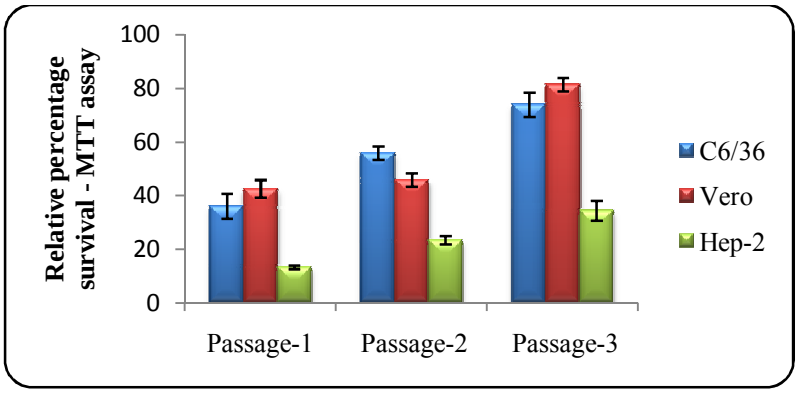

Fig. 9 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1I

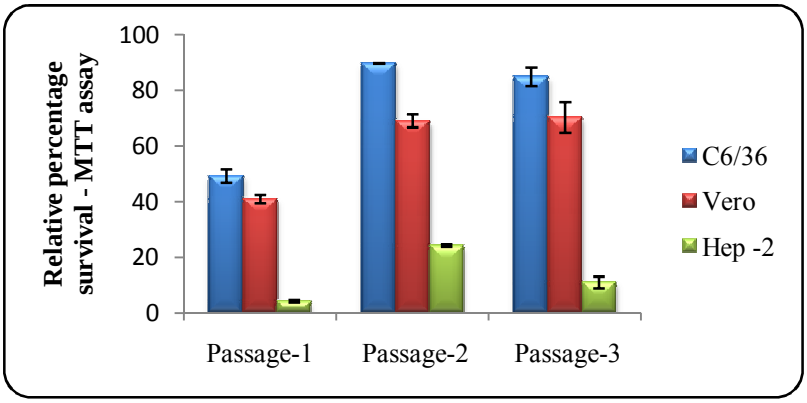

Fig. 10 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1J

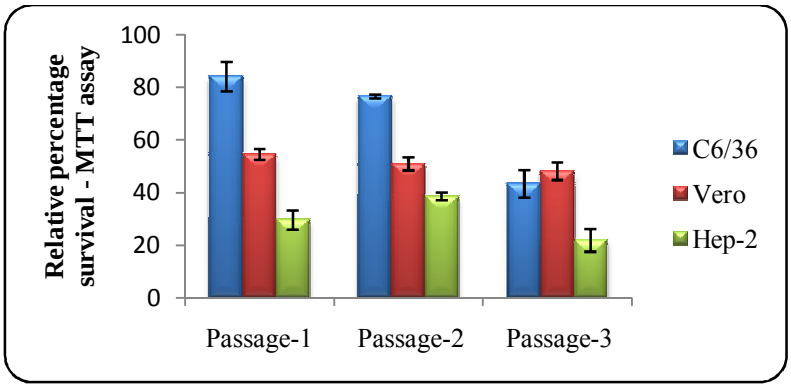

Fig.11 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1K

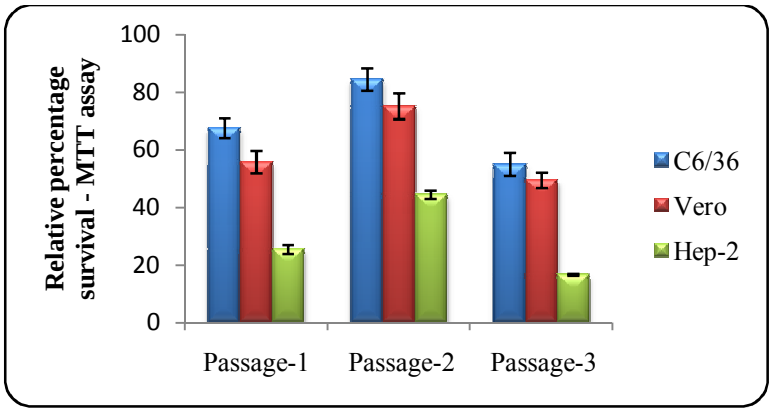

Fig. 12 Relative percentage survivals of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1L

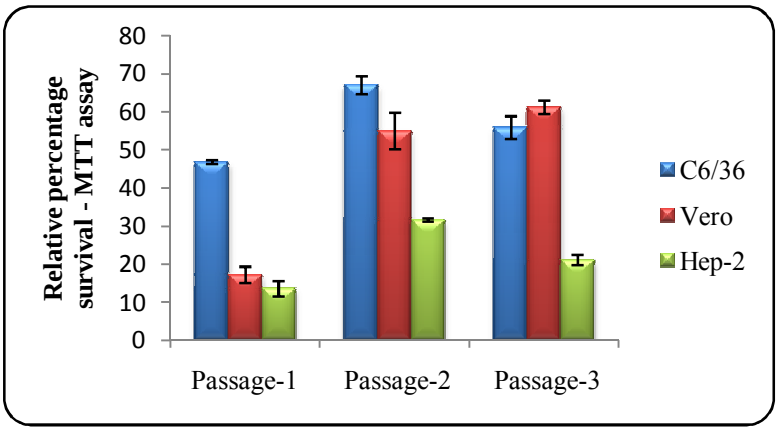

Fig. 13 Relative percentage survivals of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1M

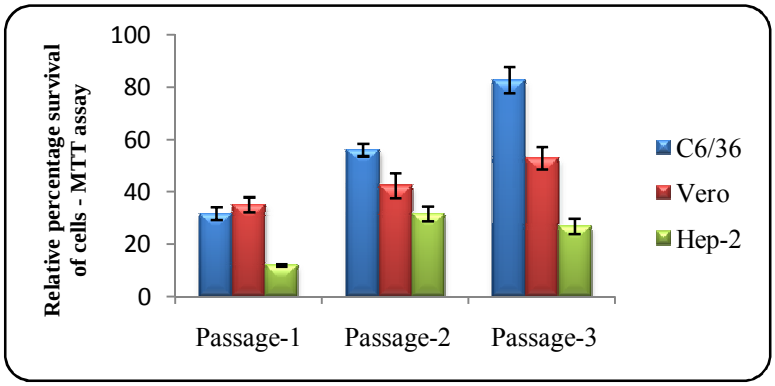

Fig. 14 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1N

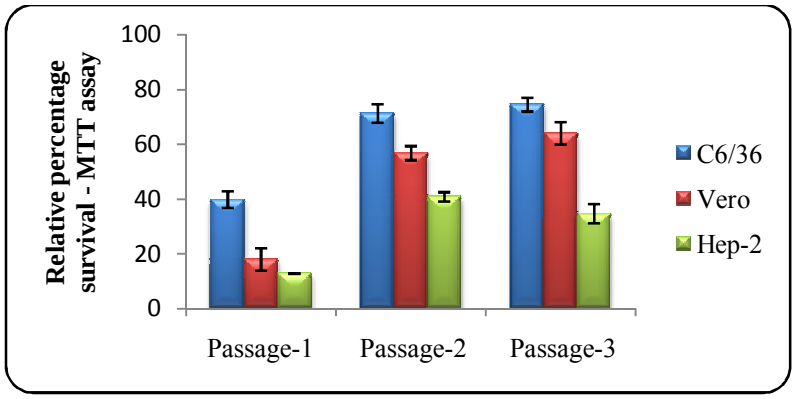

Fig. 15 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1O

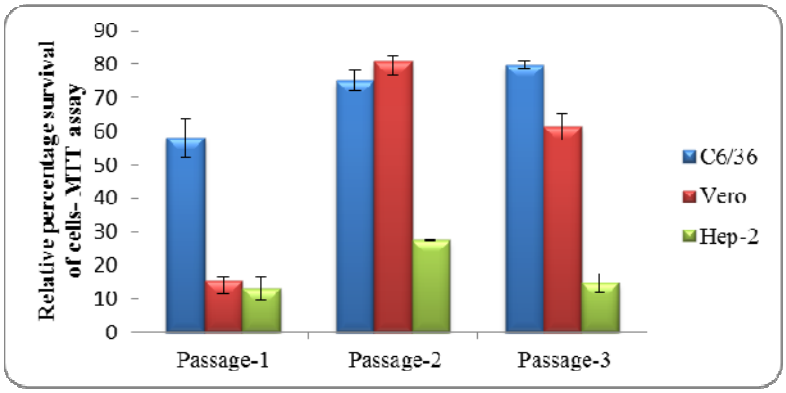

Fig. 16 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1S

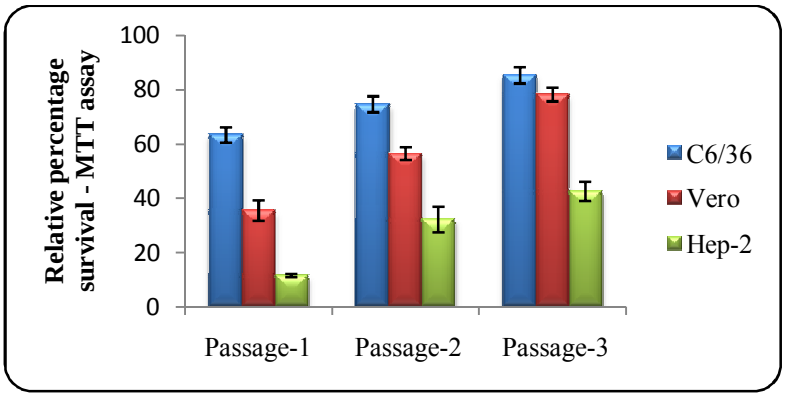

Fig. 17 Relative percentage survivals of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1T

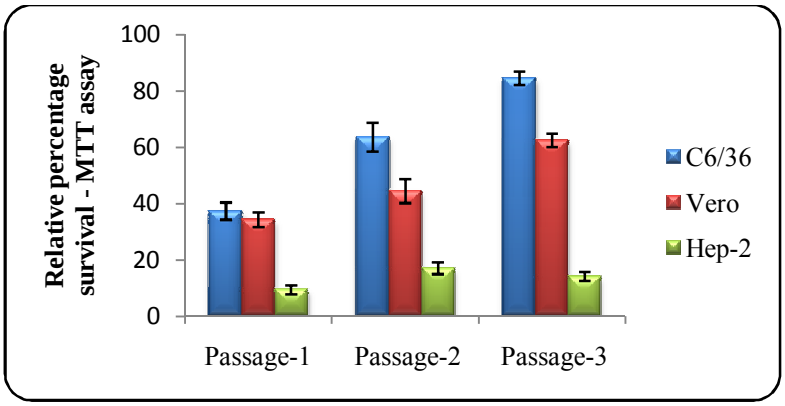

Fig.18 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1U

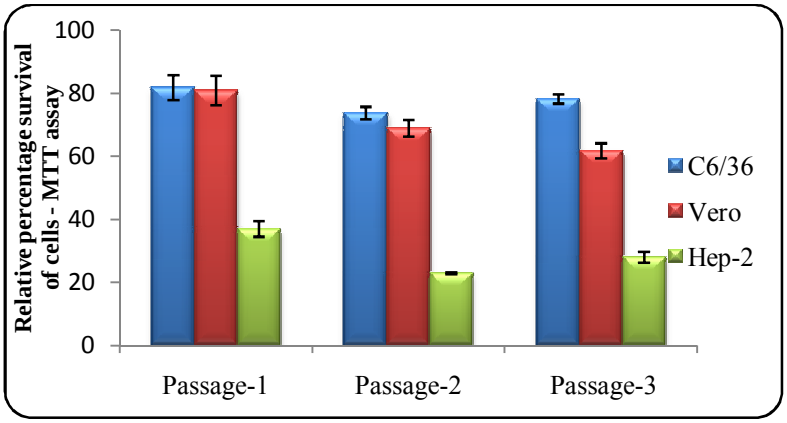

Fig. 19 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1V1

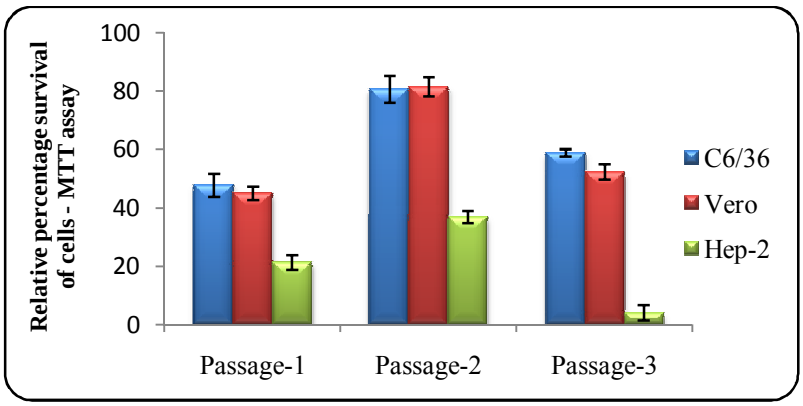

Fig. 20 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1X

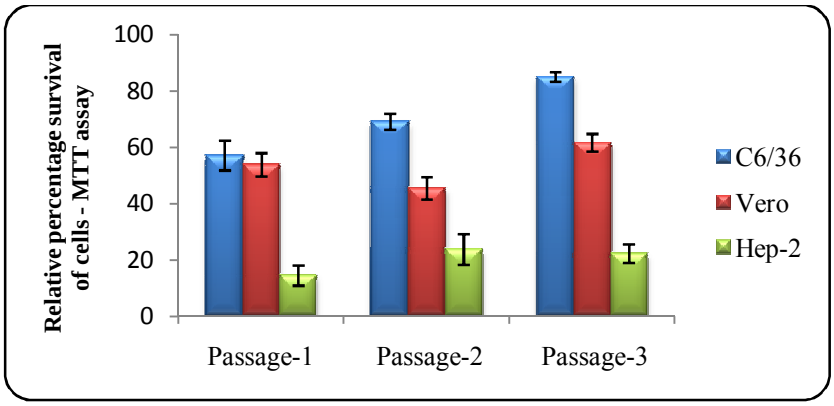

Fig. 21 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1Y

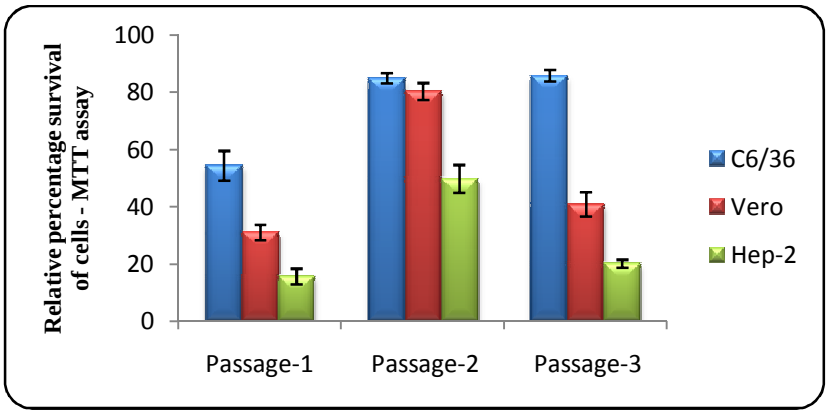

Fig. 22 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV1Z

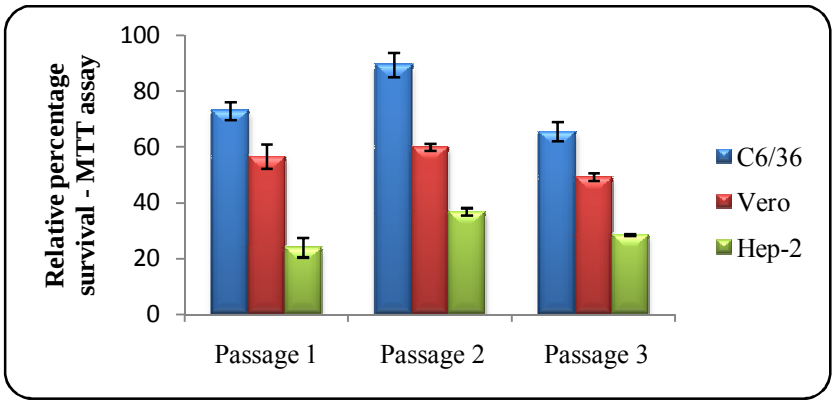

Fig. 23 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV2A

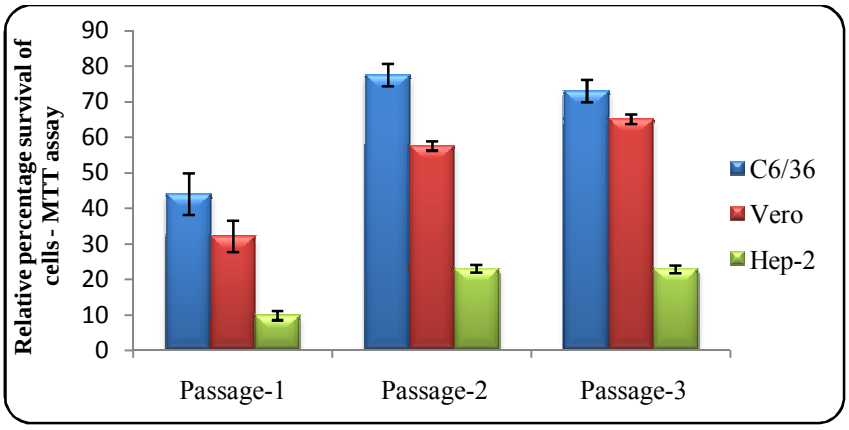

Fig. 24 Relative percentage survival of cells and successive passage in three cell lines after inoculating with virus lysate- MCCV2B

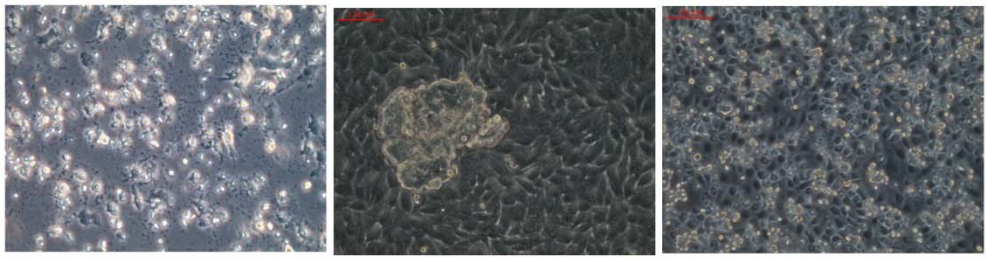

Fig.25 Representatives from first stage infection in VERO, HEp-2 & C6/36 Cells (From left to right) (Magn: 20X)

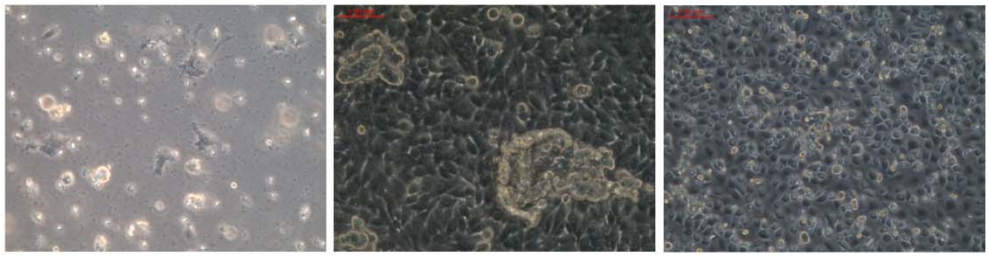

Fig.26 Representatives from second stage infection in VERO, HEp-2 & C6/36 Cells (From left to right) (Magn: 20X)

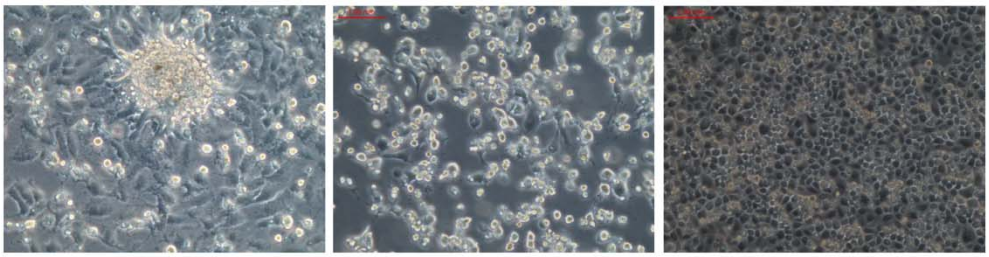

Fig.27 Representatives from third stage infection in VERO, HEp-2 & C6/36 Cells (From left to right)(Magn: 20X)

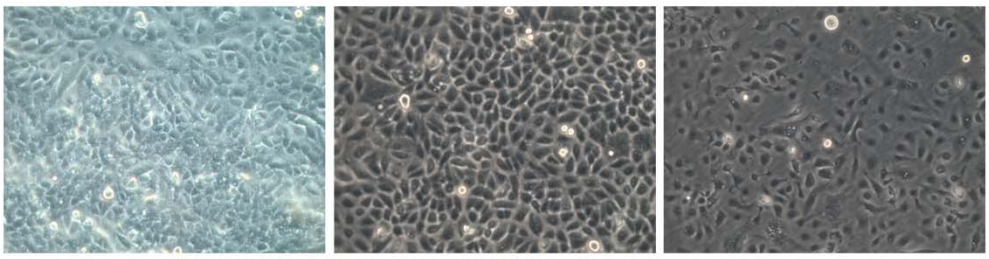

Fig.28 Controls of VERO, HEp-2 and C6/36 Cells (From left to right) (Magn: 20X)

Chapter 3

Enumeration and Morphological Characterization of the Isolated Viruses

3.1 Introduction

One of the requirements in virology is the determination of the concentration of virus or estimation of virus titer. The titer may be determined by inoculating appropriate dilutions of virus into host cell cultures, chicken embryos or laboratory animals and monitoring their progression. The responses obtained is expressed quantitatively as plaques, fluorescent foci, infectious centers or transformation or all or none in which the presence or absence is measured as the end-point dilution assay (Flint *et al.*, 2009). In almost all virological experiments it is necessary to determine the concentration of the total or infectious virus particles that are present in any given virus suspension. Such quantification experiments are very useful in the determination of one-step growth curves, examining the neutralization of virus infectivity, assessing the activity of chemotherapeutic agents, monitoring the stages of virus purification, and assessing virus pathogenicity or virulence. Animal viruses are usually quantified by way of infectivity assay (e.g.,

$TCID_{50}$, Egg Infective $Dose_{50}$ (EID_{50}), Lethal $Dose_{50}$ (LD_{50}), pock assay), other biological or chemical assays such as haemadsorption, haemagglutination, total protein, or by direct total virus particle counting using the electron microscope. Even though the plaque assay is considered to be sensitive, easiest and accurate, the plaqueing efficiency of several viruses are considered to be poor and hence alternate methods have been used (Mahy and Kangro, 1996).

The most important attribute of a virus is its ability to infect and replicate within a particular cell. The virus replicative cycle is accompanied by a sequence of biochemical and morphological changes of cells which usually results in cell death. These morphological changes are collectively termed as cytopathic effects (CPE) which may range from cell rounding to cell fusion or syncytia formation and finally lysis leading to death of the cell (Mahy and Kangro, 1996). Once the virions enter a multicellular organism they may have to travel further unless suitable host cells are encountered. During this final stage of their journey the virions will encounter hazards in the form of host defense mechanisms, which must be survived if the virions are to remain infective when they reach their destination (Carter and Saunders, 2007).

An infectivity assay measures the titer or the concentration of infective stock of a virus in a specimen or a preparation. Samples are inoculated into suitable hosts, by which a response can be observed if an infective virus is present. A suitable host includes animals, plants or cultures of bacterial, plant or animal cells. Infectivity assays fall into two classes: quantitative and quantal assays. Quantitative assays are those in which each host response can be any one of a series such as number of plaques giving an estimate of the concentration of infective virus which are expressed in plaque-forming units or PFU (Carter and Saunders, 2007). Infective assays like other forms of

assays are derived so as to calculate the actual titer of the virus which is the number of infectious units per unit volume (Mahy and Kangro, 1996). In theory, a single virion can initiate an infection, but in practice it is often found that a host must be inoculated with a minimum number of virions in order for that host to become infected. The reasons for this might be many and varied and also the stock of virus used may include some virions being defective and some being inactivated by the host's immune systems. This minimum amount of virus required for infection of a host is known as the minimum infective dose (Carter and Saunders, 2007).

The type of cell that is infected by the virus has a profound effect on virus replication. In the case of prokaryotic viruses, the replication to an extent reflects the relative simplicity of a host cell whereas in the case of eukaryotes the process is a rather complex one. Many animal viruses undergo different replicate cycles in different cell types, however, the coding capacity of virus genomes forces all viruses to choose a similar strategy of replication. In some cases they might be solely depending on the host cell in which the viral genome can be very compact requiring only the encoding of a few essential proteins (Parvoviruses). Whereas the large and complex genomes such as Pox viruses, encode most of the information necessary for replication and virus minimally depends on the host cells for want of energy and macromolecule synthesis such as ribosomes.

A virus with RNA genome has no requisite to enter the nucleus of the host cell though there are rather few exceptions which in most cases replicates in cytoplasm. Most DNA viruses replicate in the nucleus where the host DNA is replicated and where the biochemical apparatus necessary for the process is located though there are also exceptions such as Poxviruses which confers

sufficient biochemical capacity to replicate in the cytoplasm with minimum requirements from host cell functions (Cann, 2012).

The outer surface of the virus is responsible for the recognition and interaction with the host cell. Initially the virus binds with special attachment proteins through cellular receptor molecule. In some cases the capsid also plays a vital role for initiation of infection by delivering in a form which can help to associate with the host. In a few case it is just a dumping of the virus genome into the cytoplasm of the cell, whereas it is much more complex in retroviruses which carry out extensive modifications while inside the virus particle itself converting two molecules of single stranded RNA to one molecule of double stranded DNA before delivering it to the nucleus. Here the function of capsid is vital to establish an infection (Cann, 2012). In a viral infection, firstly the virus specifically interacts with the host cell surface through host protein receptors and the viral genome is introduced into the cell followed by expression of viral genes utilizing host cell process. The viral proteins then modify the host cell and allow the viral genome to replicate using the host and viral enzymes, and the viral coat proteins assemble into capsids along with the viral genome and mature virions are released (Wagner, *et al.,* 2006). In order to form mature virus particles firstly they must assemble themselves and secondly they should form regular geometric shapes from irregular proteins (Cann, 2012). Biochemical analysis of virus replication in eukaryotic cells has been used to study the levels of cellular and viral protein as well as nucleic acid synthesis and the extracellular events occurring due to synchronized infections (Cann, 2012).

Studies on the effects of viral infection on hosts can be broadly defined as the study of viral pathogenesis and sum total of the virus encoded

functions which contribute to virus propagation in infected cells and in the host organism which are collectively defined as pathogenicity of the virus. In other words, pathogenicity, precisely, is the ability of a given specific population of virus to cause disease and extent through populations that are primarily reliant on their genetic makeup or genotype. Virulence may be defined as a convoluted combination of expression of viral genes controlling pathogenicity, physiological response of the infected cells to these pathological determinants and the response of the population of cells to the virus propagating in them. Some viruses generated during the course of a disease that contain genes that are not optimally efficient in causing symptoms are due to the fact that they may have accumulated as much as mutations in pathogenic genes and so cannot induce any disease, and they are referred to as avirulent or non pathogenic and when such virus infects a host may lead to partial or complete immunity to the host (Wagner *et al.,* 2006). The major difference between the bacteriophage and that of the virus are the time required to initiate an infection which is about few minutes in the case of bacteriophages while it may require hours in terms of viruses which in part implies the slower growth of eukaryotic cells as well as the complexity of virus replication in compartmentalized cells (Cann, 2012). All viral genomes in common are packed in particles that help in transmission from host to host. The genome contains information for initiating and completing an infectious cycle within a susceptible and permissible cell. The infectious cycle of a virus can be enumerated as a sequence of events which include the attachment and entry of virus particle, decoding of the genetic information, and translation of viral mRNA by host ribosomes, genome replication and finally assembly of viral particles containing the genome (Flint *et al.,* 2009). Viruses are composed of a nucleic acid genome which is the genetic material surrounded

by a capsid made of virus encoded proteins. The viral protein encodes the structural proteins of the capsid and other proteins responsible for other functions such as initiation of virus replication. Altogether the genome, the capsid envelope, if present, constitutes the virion or virus particle (Wagner *et al.,* 2006).

Mainly two types of assay have been envisaged for the detection of viruses which includes physical and biological. Since the earlier assays focused on their infectivity biological assays such as the plaque assay and the end point titration methods eliminates the enumeration of non- infectious viral particles. In such cases employing physical assays can be done by means such as electron microscopy or the immunological methods. An information about the number of non- infectious particle in a lysate helps in the assessment of the quality of the virus preparation (Flint *et al.,* 2009). The commonly used assays for the enumeration of viruses are as follows:

3.1.1 Plaque Assay

The plaque assay was developed and modified by Dulbecco (1952) for the determination of titer stocks of bacteriophages for use in animal virology. Later on, the methodology was adopted for the reliable determination of the titers of a wide variety of viruses. Monolayers of cell cultures are initially incubated with a preparation of virus and after sufficient incubation the inoculum is removed and overlaid with a nutrient supplement such as agar. The main aim of overlaying with agar is that when the original infected cells releases a new progeny, the diffusion of the virus to the neighboring uninfected cells is restricted by the gel. This results in the formation of a circular zone of infected cells, the plaque. When the infected cell becomes

damaged the formation of plaques can be distinguished from rest of the monolayer even much more distinguishable with a naked eye over time. The main disadvantage of this method is that only those viruses which cause visible plaques can be observed or measured. For majority of animal viruses there is a linear relationship between the number of infectious virus particle and the plaque count and in such cases one infectious particle is necessary to initiate an infection and the virus said to infect the cells with one-hit kinetics. The titer of a virus is expressed as plaque forming units (PFUs) per milliliter.

3.1.2 Fluorescent-Focus Assay

The fluorescent focus assay (Flint *et al*., 2009) is a modification of the plaque assay useful in the determination of viruses that do not kill the cells. Initial procedures are the same as that for plaque assay, but after a period of sufficient adsorption and gene expression the cells are permeabilized and incubated with an antibody raised against a viral protein, later a second antibody which recognizes the first is added that is usually conjugated to a fluorescent indicator such as fluorescin. The cells are then observed under a suitable fluorescent microscope with the appropriate wavelength and the titer is expressed as fluorescent-focus-forming-units per milliliter.

3.1.3 Infectious-centers Assay

Infectious-centers assay (Flint *et al*., 2009) is yet another modification of plaque assay which determines the fraction of cells in a monolayer that is infected with a virus. The monolayers of cells are dispensed before the infected cells starts to produce new progenies. Dilutions of a known number of infected cells are then plated on to monolayers of susceptible cells covered with an overlay. The number of plaques visible on indicator cells reveals the

measure of the number of cells infected in the original population, mainly used to measure the proportion of infected cells in persistently infected cultures.

3.1.4 Transformation Assay

The transformation assay (Flint *et al*., 2009) is particularly useful for determining the titres of viruses that specifically do not form plaques. As a result of this assay the cells lose their contact inhibition, (the property by which cells when cultured together grow as a confluent monolayer) and become stacked upon one another forming small piles or foci which are easily distinguishable from rest of the monolayer. Infectivity of the virus is expressed here in terms of focus forming units per milliliter.

3.1.5 End-point Dilution Assay

The end-point dilution assay (Flint *et al*., 2009) measures the titer of the virus prior to the development of plaque assay. Serial dilutions of virus stocks are inoculated into replicate test units and the number of test units that has been infected is determined at each dilution. At higher dilution none of the cell cultures are infected because no viruses are delivered onto the cells and at lower dilutions all test units are found to be infected. End point dilution assay can be defined as the dilution of the virus that affects 50% of the test units which is calculated from the data obtained and expressed as 50% Infectious Dose $1D_{50}$ per ml. When the end-point dilution assay is used to measure the virulence of a virus or the capacity to cause a disease expressed in terms of 50% lethal dose LD_{50} per milliliter or Paralytic dose PD_{50} per milliliter is determined. If the titer of a virus can be determined by plaque assay, the 50% end point determined in an animal host can be used to relate this parameter.

3.1.6 Efficiency of Plating

The efficiency of plating is the ratio of viral titers obtained on two different host cells. The value may be more or less the one which depends on how well the virus grows in different host cells. A very different value is the absolute efficiency of plating defined as the plaque titer divided by the number of virus particles in the sample. For most animal viruses the value ranges from 1 to 10,000 which further complicate the study of animal viruses (Flint *et al.*, 2009).

In the present study virus titre is determined by MTT assay, haemagglutination assay, determination of growth kinetics which include one step growth curve of the virus lysates in C6/36 assayed using HEp -2 cell line and assayed by C6/36 itself and morphology of the virus is determined by electron microscopy.

3.2 Materials and Methods

3.2.1 Determination of titer by means of MTT Assay

The colorimetric assay based on the determination of cell viability utilizing the reaction of a tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, MTT) with the mitochondria of metabolically active cells was used as means of measuring the stock of the virus. Cells were seeded with appropriate virus dilution with a maximum of eight wells per dilution and then incubated. The holding medium from each well including the controls were withdrawn and a fresh MEM of 50µl was added into each well. Subsequent to it 50 µl MTT reagent was added onto each well and incubated in dark at 37ºC for 5 hrs (Mosmann, 1983). The medium was pipetted out and the formazan crystals solubilized in 50 µL DMSO and the absorbance read at

570nm using a micro plate reader (Tecan Infinite, Austria). The values obtained against each dilution were then averaged and probit analysis was performed using the SPSS platform for windows (SPSS: 13 Inc. USA).

3.2.2 Haemagglutination Assay

Certain viruses contain proteins that can bind to erythrocytes linking multiple cells resulting in the formation of a lattice network, the property of which is referred as haemagglutination. The influenza virus contains an envelope glycoprotein called hemagglutinin which binds to N-acetyl -9- O - acetylneuraminic acid containing glycoproteins on erythrocytes. Two fold serial dilutions of virus stock are prepared and mixed with a defined quantity of red blood cells and added on to microtitre plates. Un-agglutinated red blood cells tumble to the bottom of the well and form a sharp dot or button whereas the agglutinated red blood cells form a diffuse lattice that coats the well. Since the assay is a rapid method requiring 30 minutes of incubation, it is often used as a quick indicator of the relative quantities of virus (Flint *et al.,* 2009).

Chick erythrocytes were prepared from 10 ml of blood drawn from chicken mixed with equal volume of Alsever's solution (Sigma A3551), kept in ice and transported to the laboratory. The blood cells were then washed initially at 400 rpm for 10 minutes and the supernatant carefully drained off and added 10ml Alsever's solution and continued the process 3 times at 400 rpm for 5minutes. After the final wash, the cells were resuspended in 2ml of Alsever's solution and stored at 4°C until used. Working stocks were prepared with 1ml stock and 19ml PBS (1X). U bottom well plates were seeded with 50 µl erythrocytes to which aliquots of 50 µl diluted virus (1:2 to 1:1024) were added. Control was maintained with erythrocytes in the same proportion mixed with PBS in the same volume of

virus suspension. All preparations were incubated at room temperature for 60 m and the results recorded.

3.2.2 Growth Kinetics of the virus lysates

3.2.2.1 One Step Growth Curve of the virus lysates in C6/36 assayed using HEp-2 cell line

For the conduct of one step growth curve experiment C6/36 was considered due to the fact that in the previous chapter it was concluded that C6/36 was least adapted to the virus lysates. This finding evoked the question whether the viruses do multiply in the cell line at all. To ascertain this, one step growth curve was constructed using C6/36 as the cell line

Monolayer of 24 h grown insect cell line C6/36 in 25 cm^2 cell culture bottle was used for the study. The medium was drained off and inoculated with 1 ml virus lysate maintained at -80 ^{0} C. After 3 h of incubation for virus adsorption the inoculum was drained off and washed with 1X PBS and added 9 ml MEM and incubated at 28 $^\circ$ C for 48 h. During this period at every 6 hr intervals an aliquot of 400 µl was drawn and mixed with 400 µl MEM and inoculated at 100 µl aliquots each in 8 microwells in 96 microwell plates with fully grown HEp -2 cell line from where medium had been drained off. The plates were incubated at 37°C for 12 hours. A set of control with fresh medium added was also maintained. The medium with virus lysate was drained off and added with a mixture of 50 µl fresh MEM with 50 µl of MTT reagent at 1:1 ratio. The plates were incubated at 37°C in dark for 5hrs. Following incubation the supernatant was drained off and the formazan crystals were dissolved in 100 µl DMSO and read in a Microplate Reader (Tecan Infinite ^{TM}, Austria) at 570nm. The process was repeated once in 6 h for 8 times and the MTT %

value was plotted against time giving % cell death (Heldt *et al*., 2006). MTT % value = Blank Absorbance – Well Absorbance/ x 100

3.2.2.2 One Step Growth curve of the Virus lysates in C6/36 assayed by C6/36

In this experiment one step growth curve of virus lysate was constructed by using C6/36 as the assay system to reconfirm the multiplication of the virus lysates in the insect cell line as no CPE was noticed. The protocol remained the same except the fact that C6/36 was used for the assay. MTT % value was plotted against time giving % cell death (Heldt *et al*., 2006).

3.2.3 Electron Microscopy

3.2.4.2 Transmission Electron Microscopy

The virus lysates were grown in HEp -2 cell line for 24 hrs or till the cell line got fully lysed, subjected for three freeze thaw cycles and centrifuged at 10,000 rpm at 25°C for 3 min and supernatant passed through PVDF membranes of 0.22 µm (Millipore India (P) Ltd). The suspension was subjected for negative staining and observed under transmission electron microscope (Philips CM 10 – The Netherlands)

3.3 Results

3.3.1 Determination of titer of virus lysates in terms of $TCID_{50}$

Titer of the virus lysates was determined using MTT assay and Probit analysis with SPSS Version13 for windows.

3.3.1.1 Determination of titer by means of MTT assay and Probit Analysis

The titer of the virus lysate determination by MTT assay and probit analysis yielded wide variations in the titre among different samples studied **(Table -1)**. The sample MCCV 1A exhibited a titre of 294.69 ± 143.03 $TCID_{50}$ per ml

(Fig.1), the sample MCCV 1B had a titre of 19.89 ± 5.58 $TCID_{50}$ per ml (Fig. 2), sample MCCV 1D, 114.23 ± 25.43 $TCID_{50}$ per ml (Fig.3), sample MCCV 1E, 21.36 ± 7 $TCID_{50}$ per ml (Fig. 4), sample MCCV 1G, 72.66 ± 28.45 $TCID_{50}$ per ml (Fig. 5.), sample MCCV 1H, 71.29 ± 21.51 $TCID_{50}$ per ml (Fig. 6), sample MCCV 1I, 36.39 ± 9.93 $TCID_{50}$ per ml (Fig. 7), sample MCCV 1J, 26.94± 6.98$TCID_{50}$ per ml (Fig. 8), sample MCCV 1K, 59.75±19.66 $TCID_{50}$ per ml (Fig. 9), sample MCCV 1L, 93.74 ± 45.32 $TCID_{50}$ per ml (Fig. 10), sample MCCV 1M, 105.61 ± 41.83 $TCID_{50}$ per ml (Fig. 11); sample MCCV 1N, 40.06 ± 15.8 $TCID_{50}$ per ml (Fig. 12), sample MCCV 1O, 136.06 ±43.81 $TCID_{50}$ per ml (Fig. 13), sample MCCV1S, 49.90±18.24 $TCID_{50}$ per ml (Fig. 14), sample MCCV 1T, 64.58±15.89 $TCID_{50}$ per ml Fig. 15); sample MCCV 1U, 40.79 ± 11.16 $TCID_{50}$ per ml (Fig. 16), sample MCCV 1V 50.68 ± 12.92 $TCID_{50}$ per ml (Fig. 17), Sample MCCV 1X 52.79 ± 14.87 $TCID_{50}$ per ml (Fig. 18), sample MCCV 1Y 26.23 ± 5.47 $TCID_{50}$ per ml (Fig.19), sample MCCV 1Z, 22.42 ± 5.45$TCID_{50}$ per ml (Fig.20); sample MCCV 2A, 50.09 ± 22.9 $TCID_{50}$ per ml (Fig. 21) and the sample MCCV 2B 132.84 ± 43.26 $TCID_{50}$ per ml (Fig.22). This suggested that 22 virus lysates were different from each other in terms of multiplication rate and final output of the virus titre. Precisely, the highest cell death was observed with the lowest dilution and the lowest with the highest dilution.

3.3.1.2. Percentage death of cells (HEp-2) versus dilutions on inoculating with the virus lysate

1. MCCV1A

The highest percentage cell death of 87 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 31 with the highest dilution (1: 1024) during a period of 48hrs (Fig.1)

2. MCCV1B

The highest percentage cell death of 77 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 14 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 2)

3. MCCV1D

The highest percentage cell death of 91 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 13 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 3).

4. MCCV1E

The highest percentage cell death of 73 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 16 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 4)

5. MCCV1G

The highest percentage cell death of 86 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 21 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 5).

6. MCCV1H

The highest percentage cell death of 86 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 14 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 6)

7. MCCV1I

The highest percentage cell death of 80 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 14 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 7)

8. MCCV1J

The highest percentage cell death of 86 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 14 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 8)

9. MCCV1K

The highest percentage cell death of 91 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 19 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 9)

10. MCCV1L

The highest percentage cell death of 87 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 23 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 10).

11. MCCV1M

The highest percentage cell death of 82 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 23 with the highest dilution (1: 1024) during a period of 48hrs.(Fig. 11).

12. MCCV1N

The highest percentage cell death of 84 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 12 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 12).

13. MCCV1O

The highest percentage cell death of 88 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 24 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 13).

14. MCCV1S

The highest percentage cell death of 87 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 19 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 14).

15. MCCV1T

The highest percentage cell death of 88 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 21 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 15).

16. MCCV1U

The highest percentage cell death of 85 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 22 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 16).

17. MCCV1V1

The highest percentage cell death of 83 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 17 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 17).

18. MCCV1X

The highest percentage cell death of 83 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 24 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 18).

19. MCCV1Y

The highest percentage cell death of 87 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 31 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 19).

20. MCCV1Z

The highest percentage cell death of 80 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 10 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 20).

21. MCCV2A

The highest percentage cell death of 87 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 20 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 21).

22. MCCV2B

The highest percentage cell death of 88 was observed with the lowest dilution (1:2) of the lysate and the lowest percentage cell death of 24 with the highest dilution (1: 1024) during a period of 48hrs (Fig. 22).

3.3.2. Growth Kinetics of the Isolated Viruses

3.3.2.1. One Step Growth Curve of the virus lysates in C6/36 assayed using HEp-2 cell line.

1) Virus Lysate MCCV1A

During the initial hours(6hr) the percentage cell death was 44 which increased to 75 by the end of 30th h of post infection followed by a decline phase which went down to 27 at the end of 48th h (Fig. 23) indicating that by 30th h of incubation cell lysis was completed releasing virions.

2) Virus Lysate MCCV1B

During the initial hours(6hr) the percentage cell death was 39 which increased to 55 by the end of 24th hour post infection followed by a decline

phase which went down to 14 at the end of 48hr (Fig. 24) indicating that by 24th hour of incubation cell lysis was completed releasing virions.

3) Virus Lysate MCCV1D

During the initial hour (6hr) the percentage cell death was 35 which increased to 44 by the end of 18th hour of post infection followed by a decline phase which went down to 23 at the end of 48hr (Fig. 25) indicating that by 18th hour of incubation cell lysis was completed releasing virions.

4) Virus Lysate MCCV1E

During the initial hour (6hr) the percentage cell death was 37 which increased to 50 by the end of 24th hour of post infection followed by a decline phase which went down to 28 at the end of 48hr (Fig.26) indicating that by 24th hour of incubation cell lysis was completed releasing virions.

5) Virus Lysate MCCV1G

During the initial hour (6hr) the percentage cell death was approximately 59 which increased to 71 by the end of 24th hour of post infection followed by a decline phase which went down to 18 at the end of 48th hr (Fig. 27) indicating that by 24th hour of incubation cell lysis was completed releasing virions.

6) Virus Lysate MCCV1H

During the initial hours (6hr) the percentage cell death was 19 which increased to 49 by the end of 30th hour of post infection followed by a decline phase which went down to approximately17% at the end of 48th

hr (Fig. 28) indicating that by 30th hour of incubation cell lysis was completed releasing virions.

7) Virus Lysate MCCV1I

During the initial hour (6hr) the percentage cell death was approximately 36 which increased to 62 by the end of 24th hour post infection followed by a decline phase which went down to approximately 24 at the end of 48th hr (Fig. 29) indicating that by 30th hour of incubation cell lysis was completed releasing virions.

8) Virus Lysate MCCV1J

During the initial hour (6hr) the percentage cell death was 45 which increased to 64 by the end of 24th hour post infection followed by a decline phase which went down to approximately 37 at the end of 48th hr (Fig.30) indicating that by 30th hour of incubation cell lysis was completed releasing virions. .

9) Virus Lysate MCCV1K

During the initial hour (6hr) the percentage cell death was 27 which increased to 61 by the end of 30th hour post infection followed by a decline phase which went down to approximately 22 at the end of 48th hr (Fig. 31) indicating that by 30th hour of incubation cell lysis was completed releasing virions..

10) Virus Lysate MCCV1L

During the initial hour (6hr) the percentage cell death was 73 which increased to 80 by the end of 24th hour of post infection followed by a decline phase which went down to approximately 28 at the end of 48th hr

(Fig. 32) indicating that by 24th hour of incubation cell lysis was completed releasing virions.

11) Virus Lysate MCCV1M

During the initial hour (6hr) the percentage cell death was approximately 62 which increased to 67 by the end of 24th hour of post infection followed by a decline phase which went down to approximately 23 at the end of 48hr (Fig. 33) indicating that by 24th hour of incubation cell lysis was completed releasing virions.

12) Virus Lysate MCCV1N

During the initial hour (6hr) the percentage cell death was 14 which increased to 35 by the end of 24th hour post infection, increased to 60 at the end of 48hr (Fig.34). The multiplication extended beyond 48th hour of incubation.

13) Virus Lysate MCCV1O

During the initial hour (6hr) the percentage cell death was 13 which increased to 25 by the end of 24th hour post infection and to 49 at the end of 48th hr (Fig. 35). The multiplication extended beyond 48 hr of incubation.

14) Virus Lysate MCCV1S

During the initial hour (6hr) the percentage cell death was 38 which increased to 71 by the end of 30th hour post infection followed by a decline phase to 33% at the end of 48th hr (Fig. 36) indicating that by 30th hour of incubation cell lysis was completed releasing virions.

15) Virus Lysate MCCV1T

During the initial hour (6hr) the percentage cell death was 57 which increased to 67 by the end of 24th hour post infection followed by a decline to 24 at the end of 48th hr (Fig.37) indicating that by 24th hour of incubation cell lysis was completed releasing virions.

16) Virus Lysate MCCV1U

During the initial hour (6hr) the percentage cell death was 56 which increased to 71 by the end of 30th hour post infection followed by a decline to 37 at the end of 48th hr (Fig. 38) indicating that by 30th hour of incubation cell lysis was completed releasing virions.

17) Virus Lysate MCCV1V1

During the initial hour (6hr) the percentage cell death was 57 which increased to 70 by the end of 24th hour post infection which further increased to 81 at the end of 48th hr (Fig. 39). The multiplication extended beyond 48th hour of incubation.

18) Virus Lysate MCCV1X

During the initial hour (6hr) the percentage cell death was 23 which increased to 58 by the end of 24th hour post infection and extended up to 83 at the end of 48hr (Fig. 40). The multiplication extended beyond 48th hour of incubation.

19) Virus Lysate MCCV1Y

During the initial hour (6hr) the percentage cell death was 50 which increased to 73 by the end of 24th hour post infection extending to 88 at

the end of 48hr (Fig. 41). The multiplication extended beyond 48 hrs of incubation.

20) Virus Lysate MCCV1Z

During the initial hour (6hr) the percentage cell death was approximately 55 which increased to 61 by the end of 18th hour of post infection followed by decline to 27 at the end of 48hr (Fig. 42) indicating that by 18th hour of incubation cell lysis was completed releasing virions.

21) Virus Lysate MCCV2A

During the initial hour (6hr) the percentage cell death was approximately 58 which increased to 77 by the end of 18th hour post infection followed by decline extending to 14 at the end of 48hr (Fig. 43) indicating that by 30th hour of incubation cell lysis was completed releasing virions.

22) Virus Lysate MCCV2B

During the initial hour (6hr) the percentage cell death was 47 which increased to 64 by the end of 24th hour post infection followed by decline to 38 at the end of 48hr (Fig. 44) indicating that by 24th hour of incubation cell lysis was completed releasing virions.

3.3.3 One Step Growth Curve Analysis of Viruses with C6/36 assayed with C6/36

1). Virus Lysate MCCV1D

During the initial hour (6hr) the percentage cell death was approximately 26 which increased to 45 by the end of 42nd hour post infection followed by a decline phase which extended up to 33 at the end of 48hr (Fig.45) indicating that there was multiplication of the virus in C6/36 and release of virion.

2). Virus Lysate MCCV1I

During the initial hour (6hr) the percentage cell death was approximately 22 which increased to approximately 28 by the end of 18th hour of post infection followed by a decline phase which extended up to 6 at the end of 48hr (Fig. 46) indicating that there was multiplication of the virus in C6/36 and release of virion.

3). Virus Lysate MCCV1V1

During the initial hour (6hr) the percentage cell death was 41by the end of 30th hour of post infection followed by a decline phase which extended up to 18 at the end of 48hr (Fig. 47) indicating that there was multiplication of the virus in C6/36 and release of virion.

4). Virus Lysate MCCV2A

During the initial hour (6hr) the percentage cell death was 58 which increased to 77 by the end of 18th hour of post infection followed by a decline phase which extended up to 15 at the end of 48hr (Fig. 48) indicating that there was multiplication of the virus in C6/36 and release of virion.

To sum up multiplication extended beyond 48hours in the case of viruses MCCV IN, MCCV 1O, MCCV 1VI, MCCV 1X, MCCV 1Y, completed by 18th hour in the case of MCCV 1Z and MCCV 1D, 24th hour in the case of MCCV 2B, MCCV 1T, MCCV 1M, MCCV 1L, MCCV 1G, MCCV 1E, and MCCV 1B and 30th hour in the case of MCCV 2A, MCCV 1U, MCCV 1S, MCCV 1K, MCCV 1J, MCCV 1I, MCCV 1H and MCCV 1A.

3.3.4 Electron Microscopy

All samples under the study were fixed by gluteraldehyde and karnovsky's fixatives. Virus particles could be observed in samples fixed with gluteraldehyde only (Fig. 49 & 50). They were 15 -20 nm in diameter with icosahedral symmetry.

3.3.5 Haemagglutination Assay

All 22 virus lysates were tested for heamagglutination property with undiluted and diluted stocks of viruses (1:1 to 1:1024 with Phosphate buffered saline (PBS 1X)) and none showed haemagglutination property (Fig. 51 & 52).

3.4 Discussion

Determining the viral titers is of importance in many virological studies and the standard methods used are often laborious and time consuming. Accurate measurements of virus titers are necessary for studies involving pathogenicity, virulence, evolution and environmental adaptation. The MTT assay proved to be a reliable method of choice for replacing traditional measurements of viral titers with standard virological methods (Andersson et al. 2005). MTT assay measures cellular dehydrogenase, which is a reflection of the down regulation of the gene during the course of viral infection (Taylor *et al.,* 2000; Wen *et al.,* 2003).

Virus infection in permissive cells results in productive virus replication accompanied by death and lysis of the infected cells leading to the release of viral progeny (Melnick., 1996). The physiological and morphological changes displayed by apoptotic cells include cell shrinkage, plasma membrane blebbing, chromatin condensation, oligonucleosomal DNA degradation and

fragmentation into membrane bound bodies (Schwartzman and Cidlowski, 1993). Dramatic cell shrinkage was observed under Phase Contrast Microscope which may be a sign of apoptosis that occurred within 3hr post infection. Apoptosis has been identified as an important host mechanism for eliminating infected cells as well as interfering with virus replication (Hay and Kannourakis, 2002).

The MTT assay has been widely used to study various facets of viral multiplication such as determining titers of influenza virus in cells which do not cause cytopathic effects (Levi *et al.,* 1995), studies involving antiviral compounds including those targeting human immunodeficiency virus (de Jalon *et al.,* 2003, Ayisi *et al.,* 1991; Kimura *et al.,* 2000) herpes virus (Park *et al.,* 2003; Sudo *et al.,* 1994; Takeuchi *et al.,* 1991), studies involving pharmacological compounds (Goodwin *et al.,* 1996) and also used successfully in the titration of vertebrate viruses (Levi *et al.,* 1995; Heldt *et al.,* 2006; Andersson *et al.,* 2005). The highest Probit values such as 294 ±143.03 for MCCV1A, 136.06±43.81 for MCCV1O, 132.84±43.26 for MCCV2B, 114.23±25.43 for MCCV1D and 105.61±41.83 for MCCV1M indicated that higher dilutions were required to obtain 50% response in terms of MTT%. However, the probit values were lower such as 19.89±5.58 for MCCV1B, 21.36 ±7 in the case of MCCV1E, 22.42±5.45 for MCCV1Z, 26.23±5.47 for MCCV1Y and 26.94±6.98 for MCCV1J for attaining MTT 50 in HEp-2 cells studied. The lysates were observed with broad range of titers on multiplying in cell lines.

The information on association of hemagglutinins (HA) with viruses has increased substantially over the years since the classical work with influenza virus (Hirst. 1942). Haemagglutination assays have been widely used for the

detection of arthropod-borne viruses and the antibodies developed to these viruses (Clarke and Casals., 1958). These assays exploit the ability of the envelope glycoprotein to bind and agglutinate avian erythrocytes so that they form a visible lattice in a U-bottom microtitre plate. Preliminary studies on heamagglutinin erythrocytes with enteric viruses indicate that this property may be altered to exhibit no heamgglutination by homologus virus (Goldfield *et al.*, 1957).

Not all known viruses are capable of eliciting heamagglutination and those which are capable will only hemagglutinate under stringent conditions of pH and ionic strength, rest of the viruses may give a result with whole range of RBC's in a saline condition. Also the viruses should be present in sufficient concentration to form cross bridges between RBC's aiding heamgglutinaation. Though the assay is a common indirect means of measuring titer of viruses, it doesn't measure infectivity rather measures the virus particle present in a suspension capable of causing heamagglutination (Mahy and Kangro., 1996). In the present study, to conclude that the virus had not heamagglutinating property virus stocks without dilution were also used and confirmed that they did not possess the characteristic.

For accurate titrations of viruses based on infectivity, virus-containing wells must be clearly distinguishable from uninfected ones. The virus must be able to cause significant, quantifiable CPE in the cell line used, and the system must allow for multiple rounds of infection (DiStefano *et al.*, 1995). Synchronous infection, the key to one step growth cycle, is accomplished by infecting cells with a sufficient number of viruses so that all cells were infected rapidly. The one step growth curve assay in HEp-2 cells was studied by inoculating cells with virus and incubating for 48hrs.

The infectivity of viruses clearly improves as infection progresses and the ratio of total particles-to-infectious particles is purely time dependent (Sokoloski *et al.,* 2012). Here the productivity of virus in C6/36 was assayed through HEp-2 cells which were accomplished by means of percentage cell death. In the present study with virus lysates, the lag phase of virus productivity was observed to be extended from 6-12hrs post infection, followed by a log phase ranging between 18- 30^{th} hr. In the case of five virus lysates the log phase extended beyond 48hrs. The trend of increasing cell death versus time was observed eliciting a continued increase of titer throughout the period of study. These observations were recorded with the percentage cell death at each 6hr intervals based on the reduction of tetrazolium salt in to formazan crystals, which corresponded to the virus titer generated (Andersson *et al.,* 2005). The assay successfully detected and quantified dead cells as observed from the downward slope of the curve as reported by Heldt *et al*. (2006). The data implied that the progeny viruses were detectable even before 6hr post infection, however the burst period varied with individual lysates.

The comparative efficiency of virus productivity in C6/36 was assessed using C6/36 itself. The percentage cell death was very low and ranged to a maximum of 55 (MCCV2A) among the four representatives studied. The data unequivocally proved that the viruses do multiply in C6/36 even though in low order compared to that in HEp-2. But, the multiplication do not produce any visible cytopathic effect, however, low levels of progression was evident which was accompanied by an increase of cell death.

In the early 1960s, electron microscopy (EM) became widely used in viral diagnosis, primarily due to the introduction of the negative staining

techniques (Brenner and Horne, 1959). Later, during 1970s and 1980s, EM made it possible to detect in diagnostic cell cultures many clinically important infectious agents such as adenoviruses, enteroviruses, orthomyxoviruses, paramyxoviruses and reoviruses (Tyrrell and Almeida 1967). Since the virus families differ from each other in their fine structure, an agent visualized by EM can be classified into the relevant family based on morphology. Hence EM should be utilized as a front line method in infectious disease emergencies or in suspect cases of bioterrorism (Hazelton and Gelderblom, 2003; Madeley, 2003).

In the present study small non-enveloped viruses with an icosahedral symmetry in general were visible at a size range between 15–25 nm in diameter. The presence of virus particles could be confirmed by way of electron microscopy, the lysates producing successful generations or progenies in HEp-2 cells. The viral particles could be observed by means of Negative staining with gluteraldehyde fixation in supernatants.

There existed wide variation in the virus titre in the virus stock between 294±143.03 and 19.89 ± 5.58 in terms of probit analysis. The virus lysates were non – haemolytic. Multiplication of the viruses could be demonstrated in C6/36 cell line also. The viruses could be broadly divided in to those which complete burst within 18hrs, 24hrs and 30hrs. The virions were observed with icosahedral symmetry, non enveloped having 15-20 nm in diameter.

Table 1: $TCID_{50}$ values of individual lysates obtained in HEp-2 by Probit Analysis

Sl.No.	Virus Lysate	Probit Value ($TCID_{50}$)
1.	MCCV1A	294.69±143.03
2.	MCCV1B	19.89± 5.58
3.	MCCV1D	114.23± 25.43
4.	MCCV1E	21.36±7
5.	MCCV1G	72.66± 28.45
6.	MCCV1H	71.29± 21.51
7.	MCCV1I	36.39+/- 9.93
8.	MCCV1J	26.94± 6.98
9.	MCCV1K	59.75± 19.66
10.	MCCV1L	93.74±45.32
11.	MCCV1M	105.61±41.83
12.	MCCV1N	40.06±15.8
13.	MCCV1O	136.06±43.81
14.	MCCV1S	49.90±18.24
15.	MCCV1T	64.58±15.89
16.	MCCV1U	40.79±11.16
17.	MCCV1V1	50.68±12.92
18.	MCCV1X	52.79±14.87
19.	MCCV1Y	26.23±5.47
20.	MCCV1Z	22.42±5.45
21.	MCCV2A	50.09±22.9
22.	MCCV2B	132.84±43.26

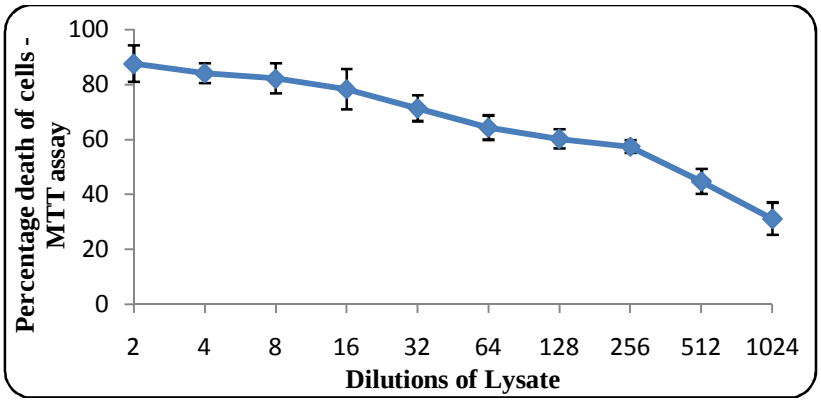

Fig.1 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1A

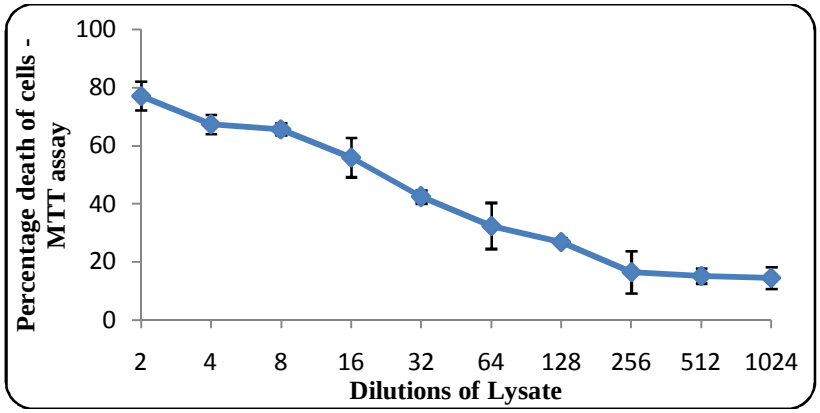

Fig.2 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1B

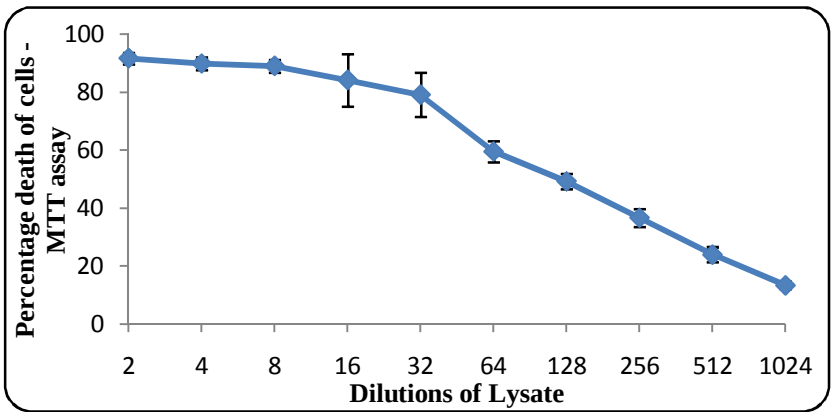

Fig.3 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1D

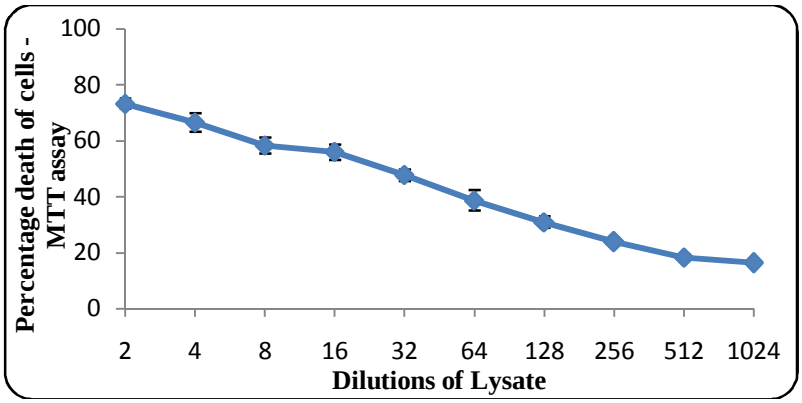

Fig.4 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1E

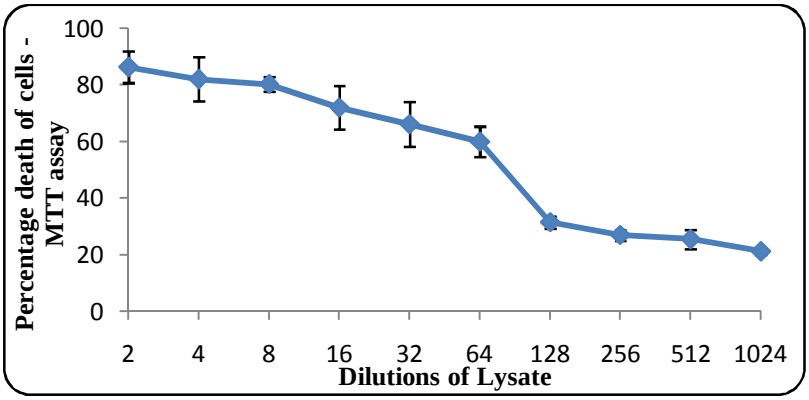

Fig.5 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1G

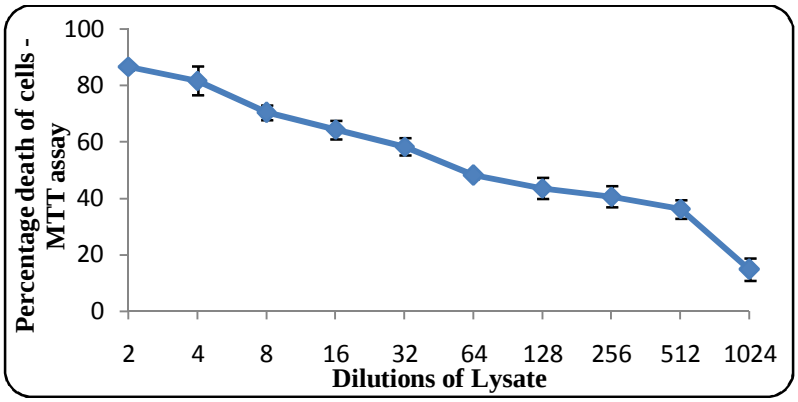

Fig.6 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1H

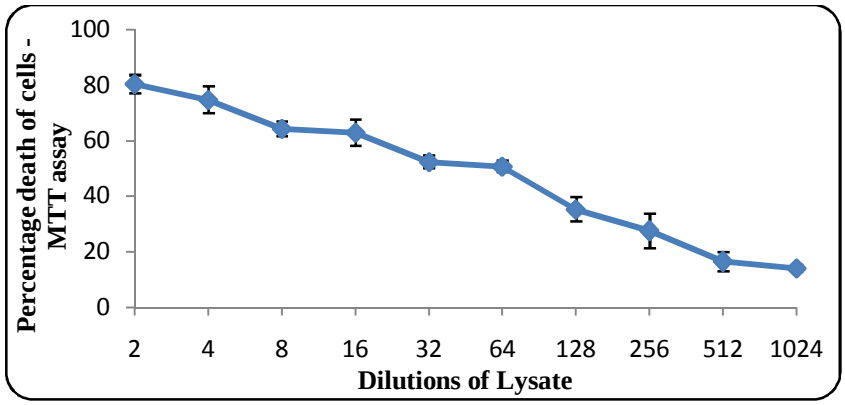

Fig.7 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1I

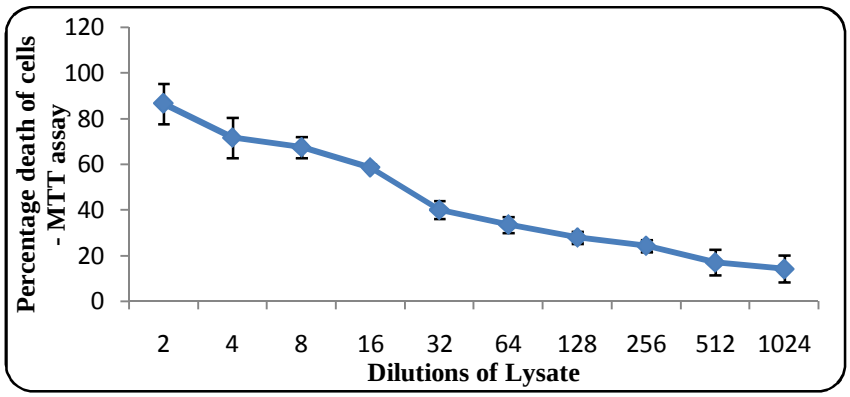

Fig.8 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1J

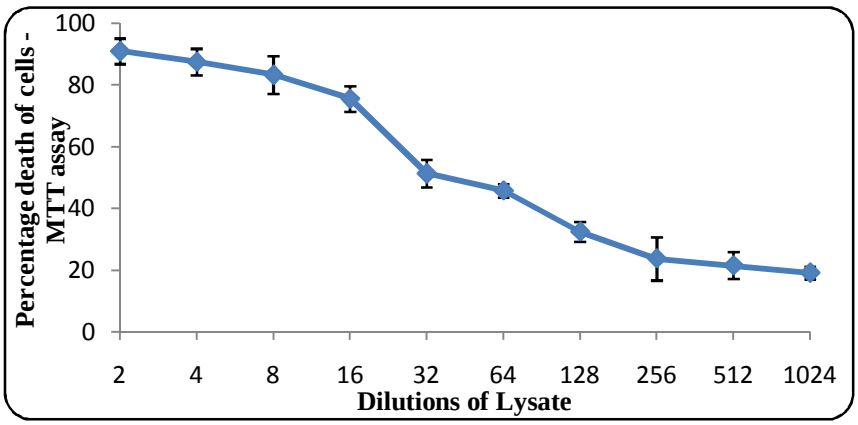

Fig.9 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1K

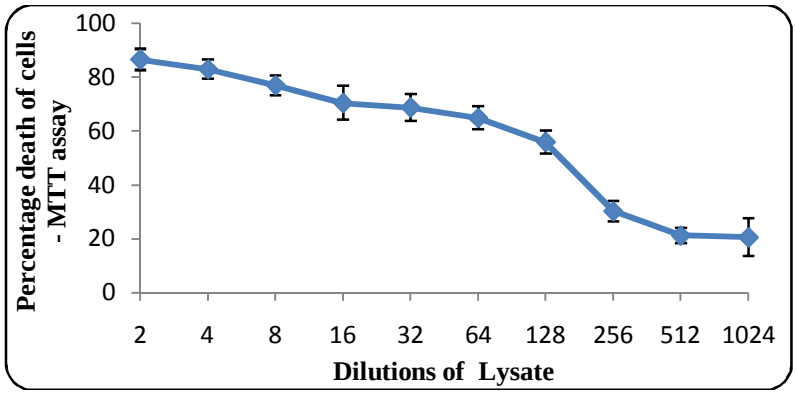

Fig.10 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1L

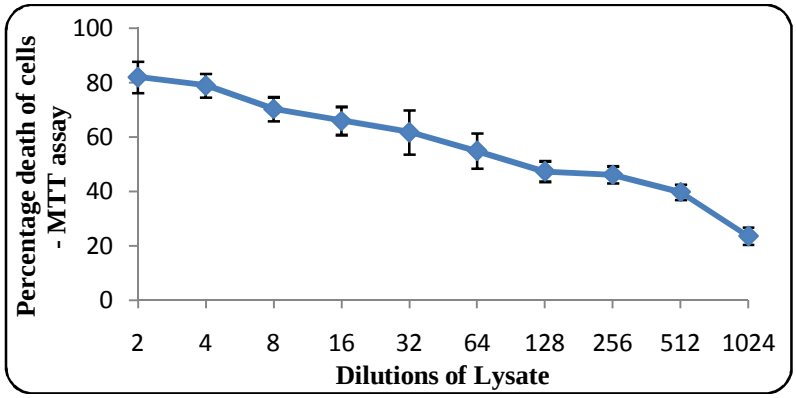

Fig.11 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1M

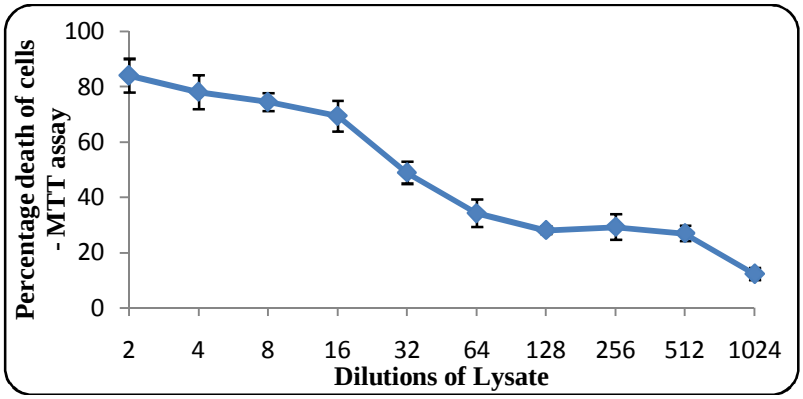

Fig.12 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1N

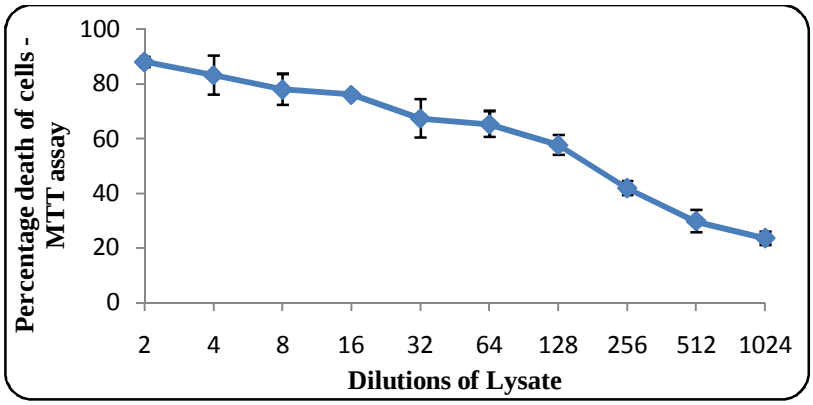

Fig.13 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1O

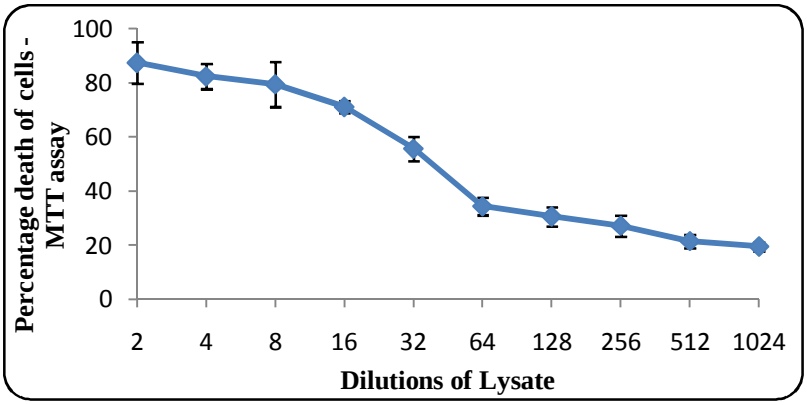

Fig.14 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1S

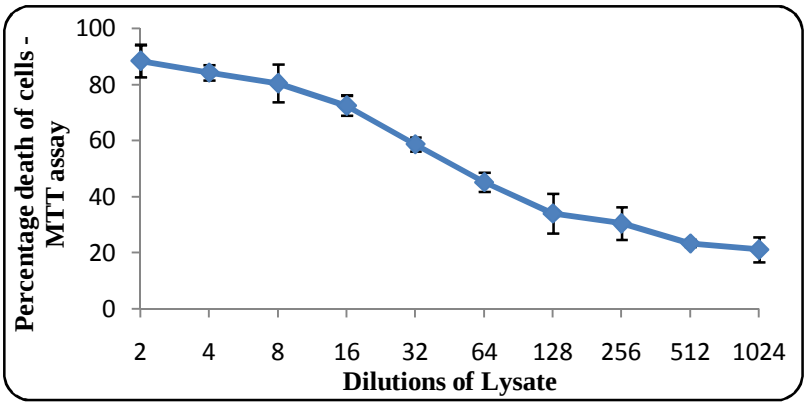

Fig.15 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1T

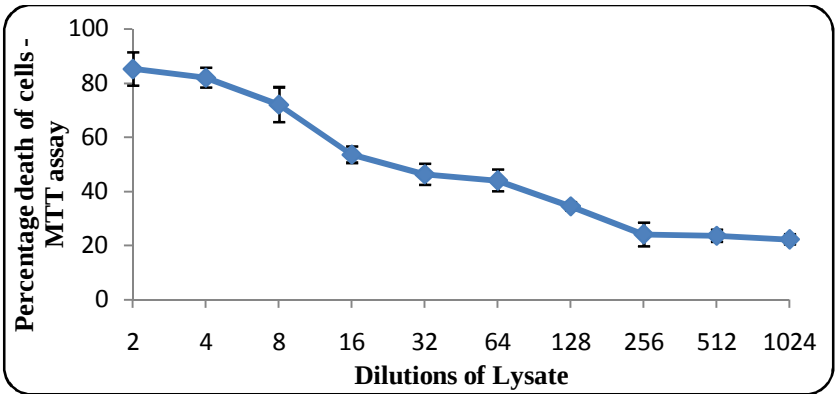

Fig.16 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1U

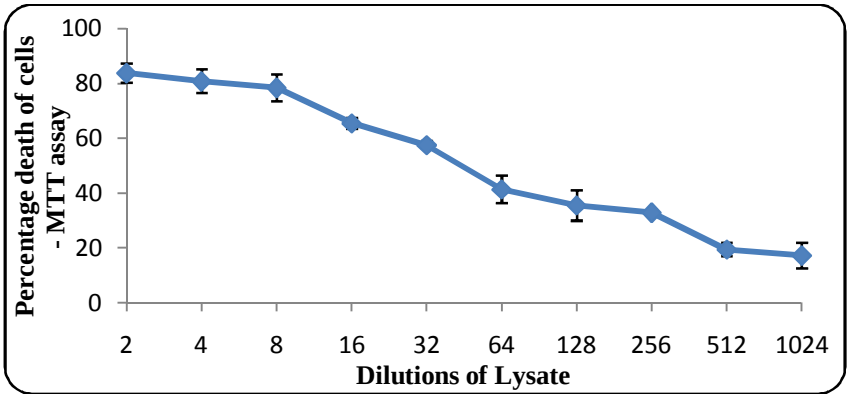

Fig.17 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1V1

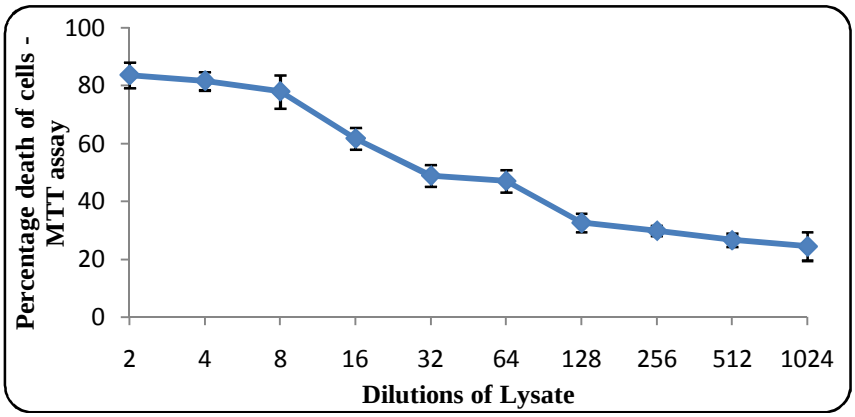

Fig.18 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1X

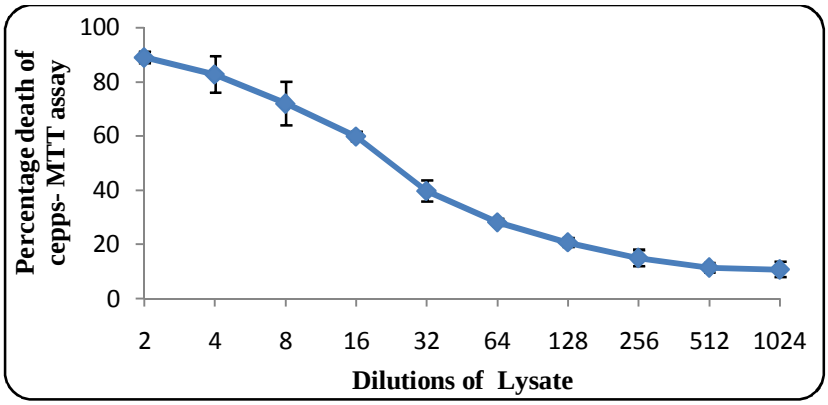

Fig.19 Percentage death of HEp-2Cells versus dilutions on inoculating with Virus lysate- MCCV1Y

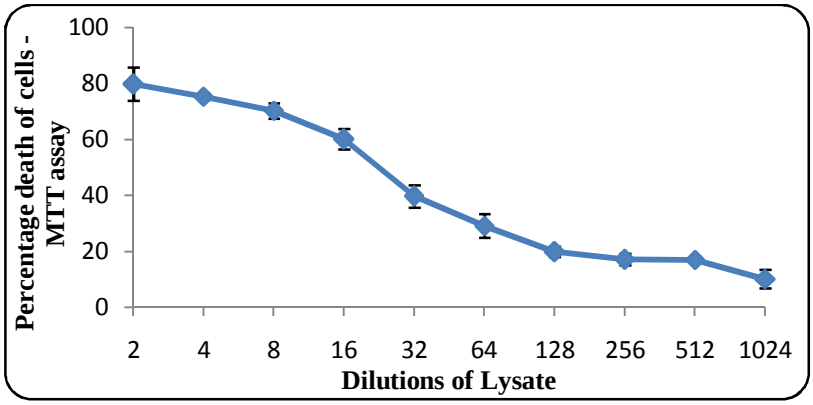

Fig.20 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV1Z

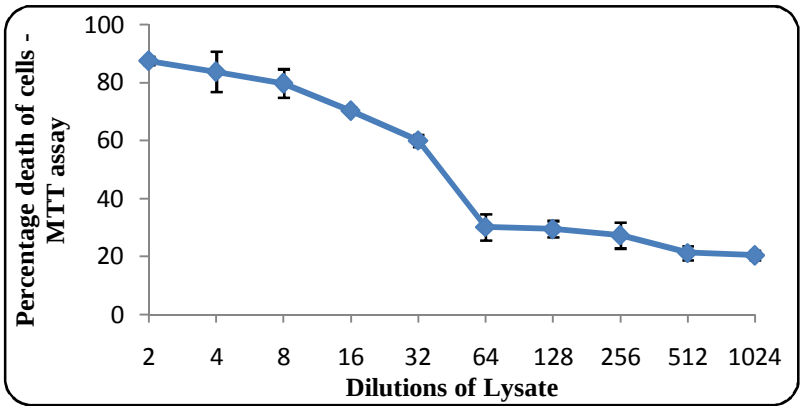

Fig.21 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV2A

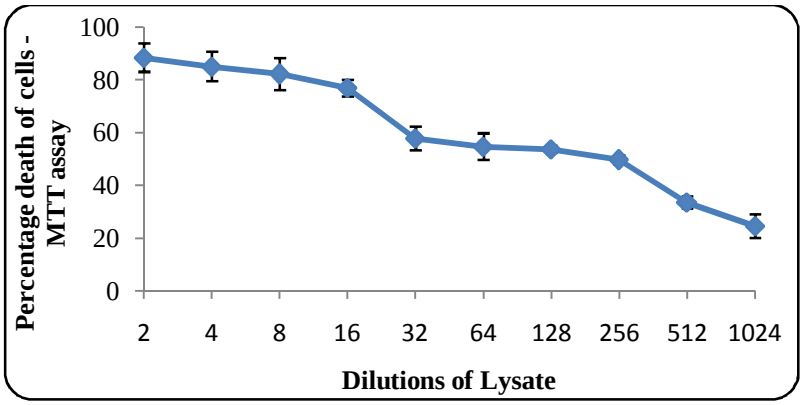

Fig.22 Percentage death of HEp-2 cells versus dilutions on inoculating with virus lysate- MCCV2B

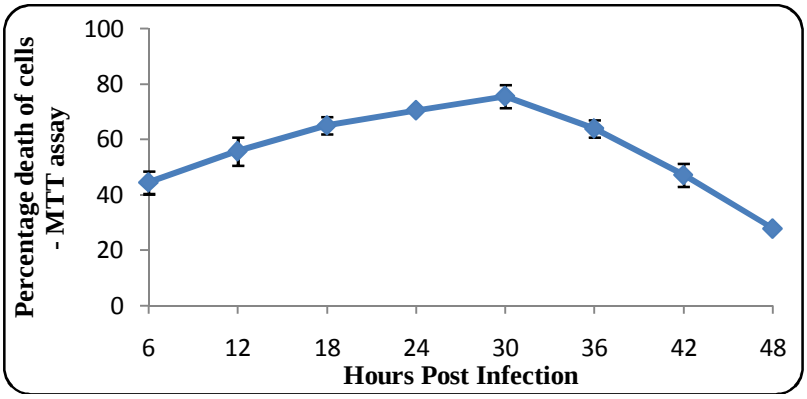

Fig .23 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1A

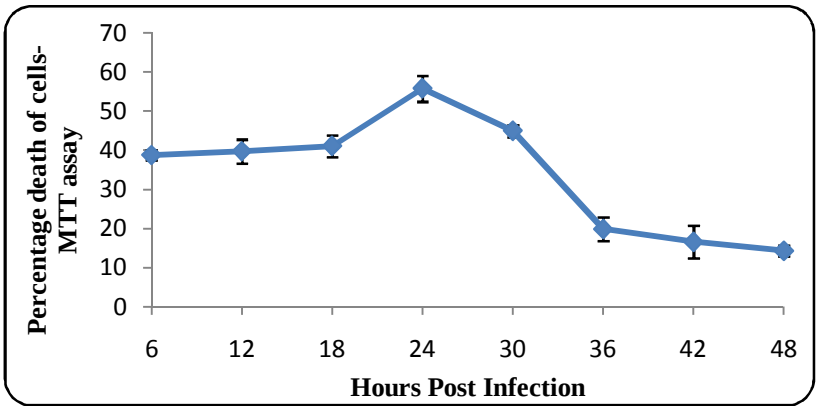

Fig .24 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1B

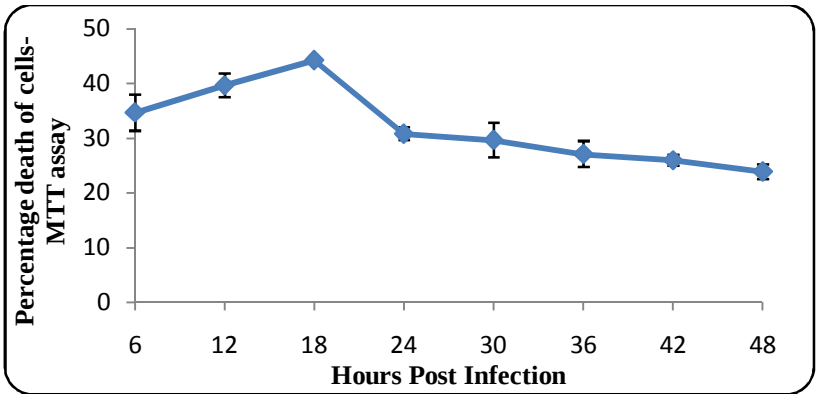

Fig .25 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1D

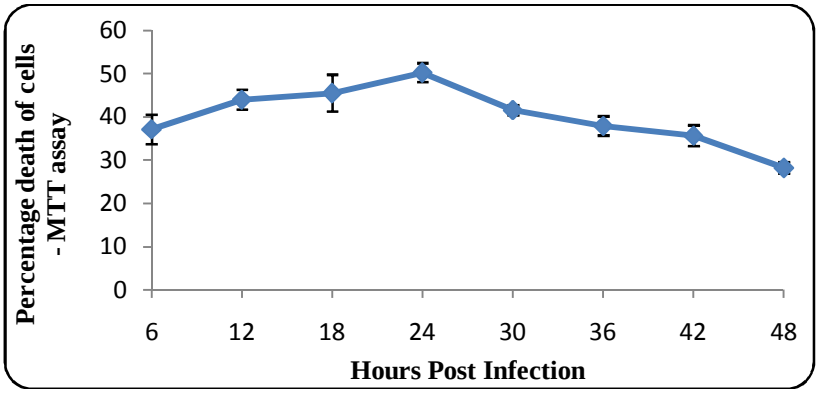

Fig .26 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1E

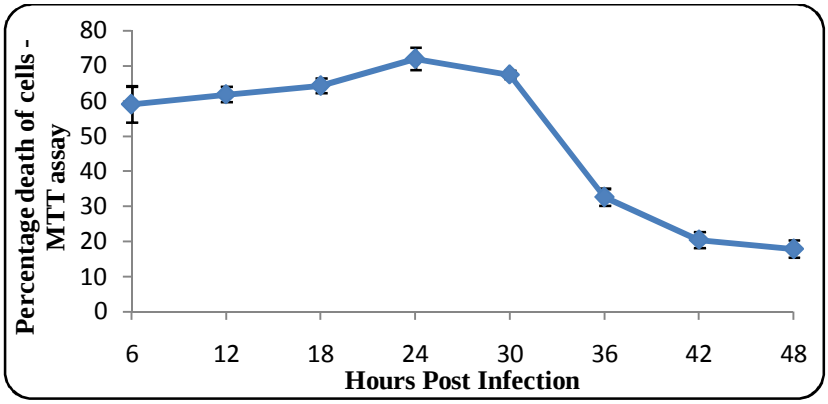

Fig .27 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1G

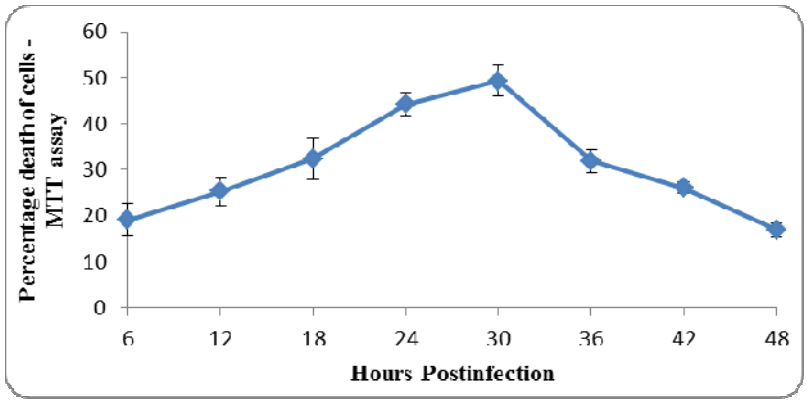

Fig .28 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1H

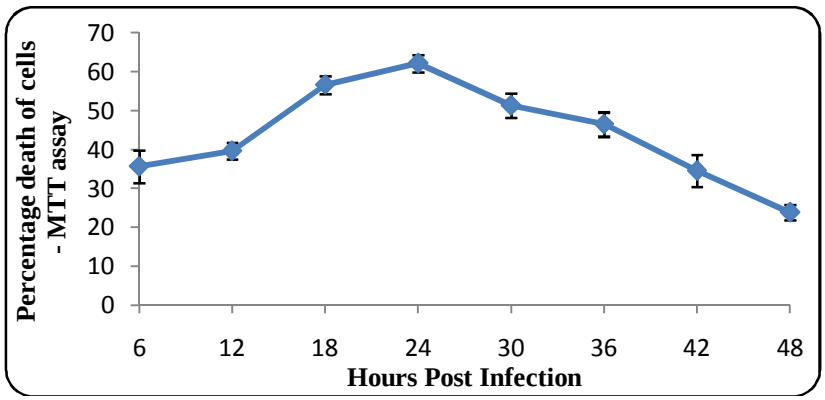

Fig .29 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1I

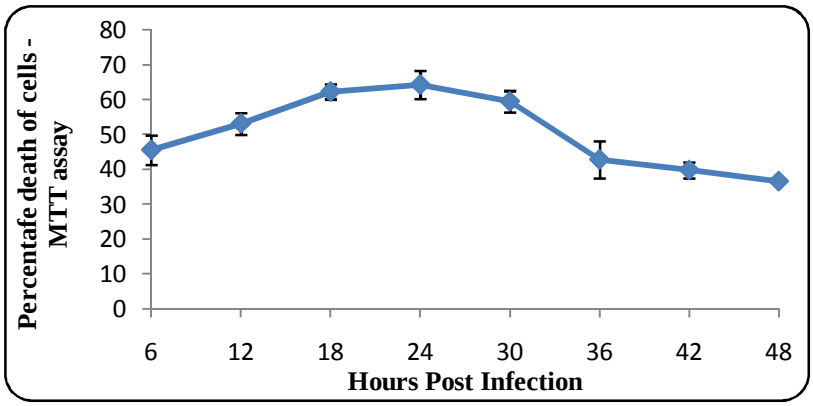

Fig .30 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1J

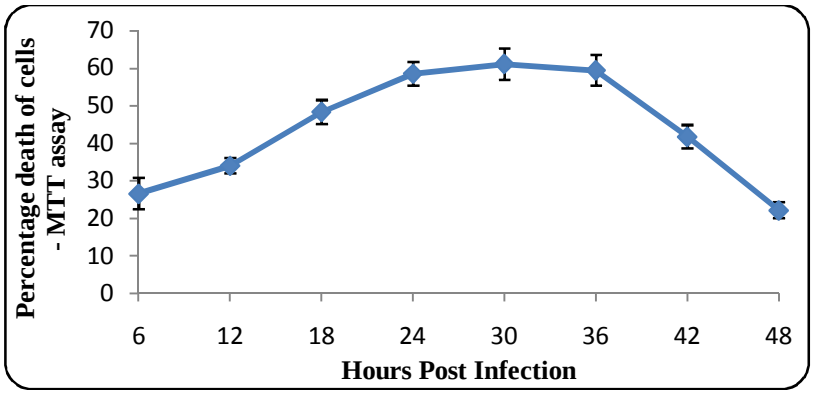

Fig .31 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1K

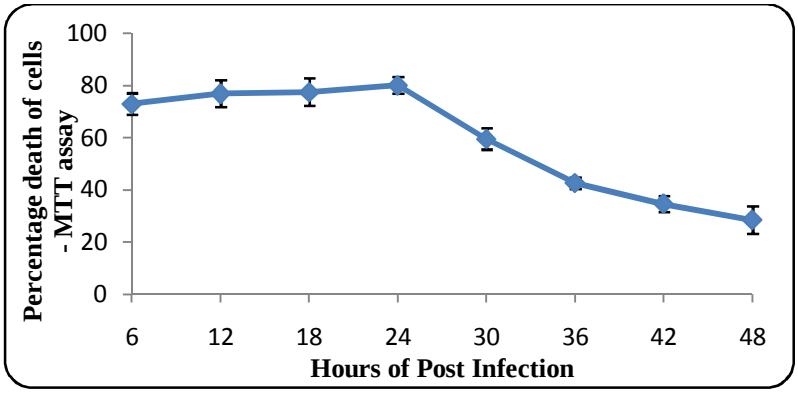

Fig .32 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1L

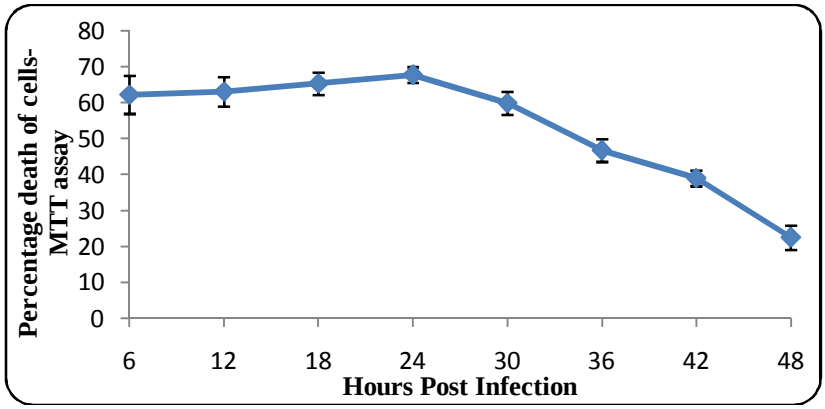

Fig .33 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1M

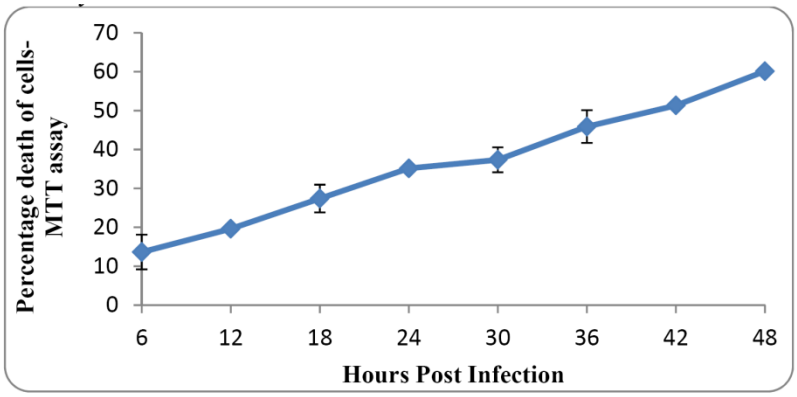

Fig .34 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1N

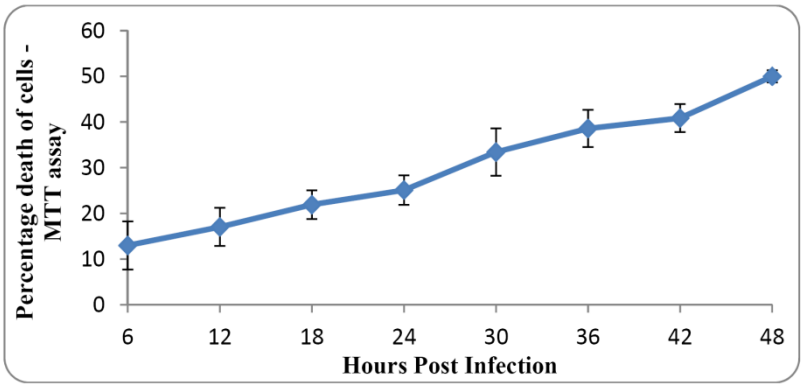

Fig .35 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1O

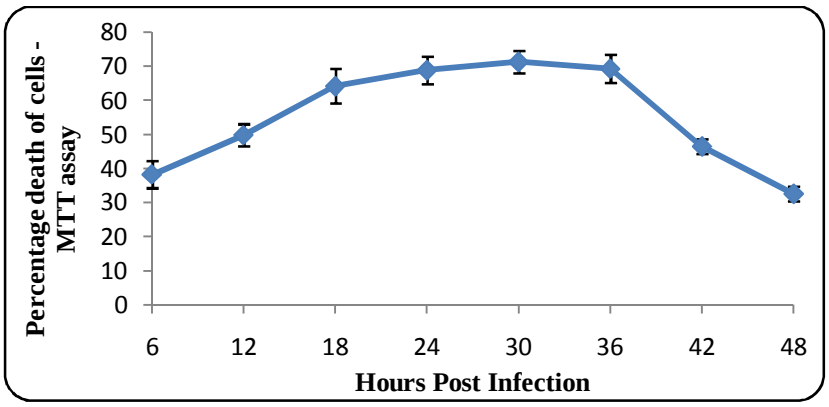

Fig .36 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1S

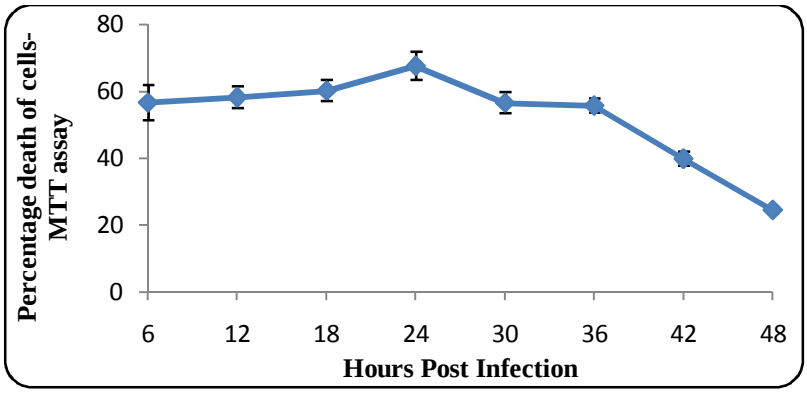

Fig .37 Percentage deaths of HEp-2cells with hours post infection on inoculating with lysate- MCCV1T

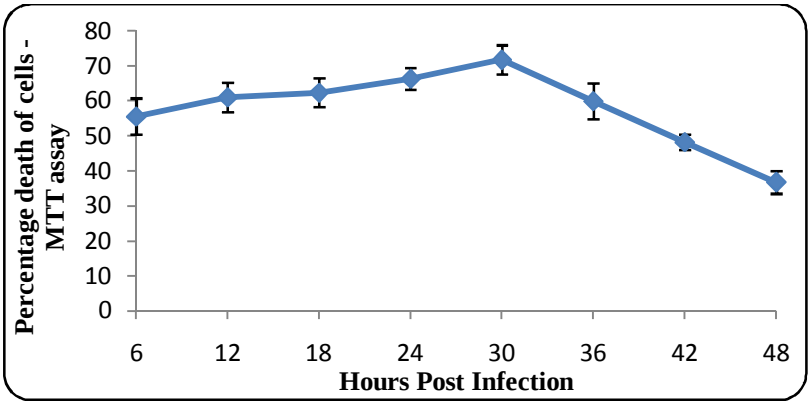

Fig .38 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1U

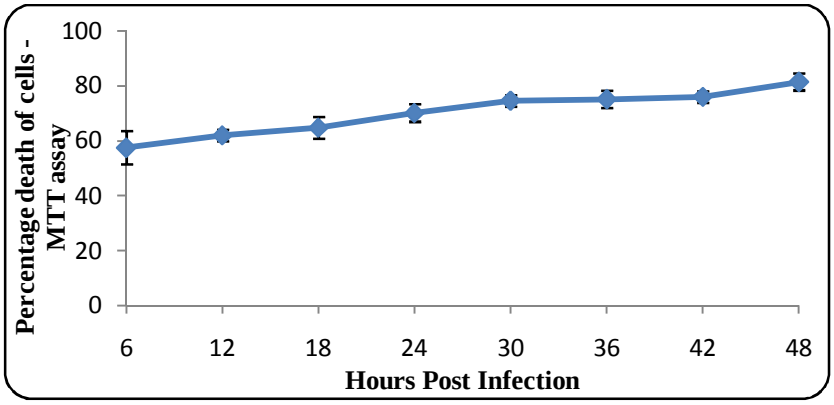

Fig .39 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1V1

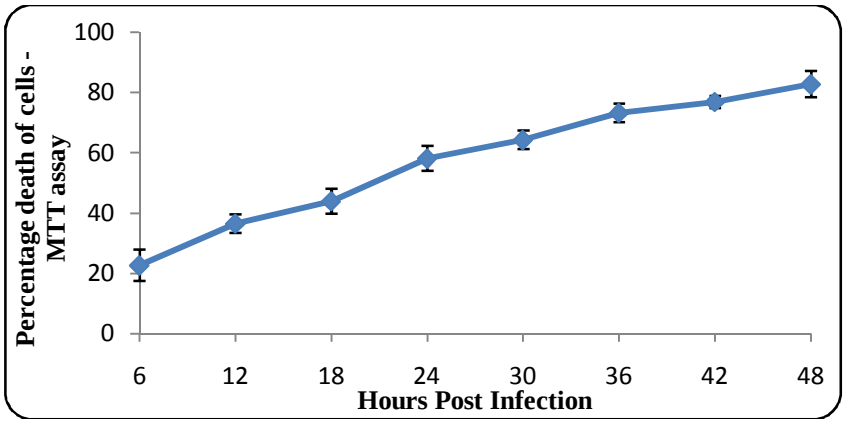

Fig .40 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1X

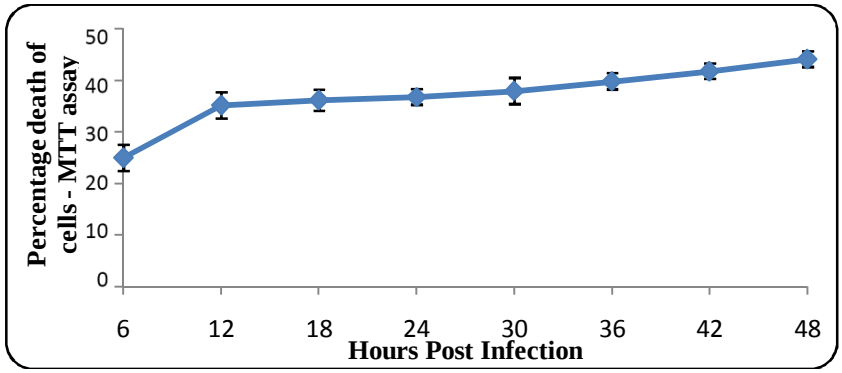

Fig .41 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1Y

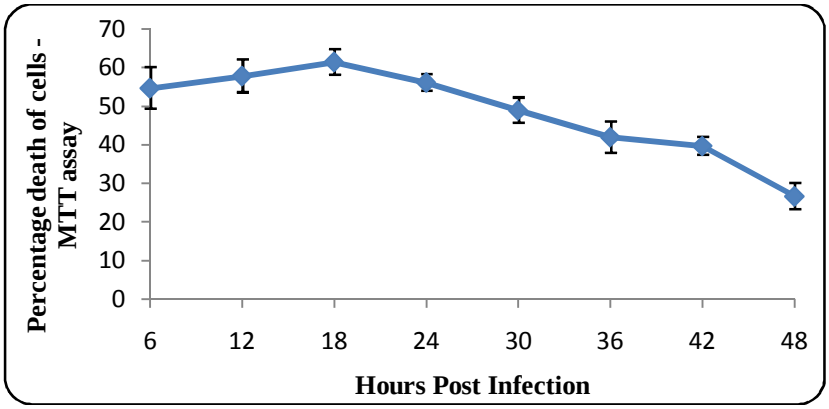

Fig .42 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV1Z

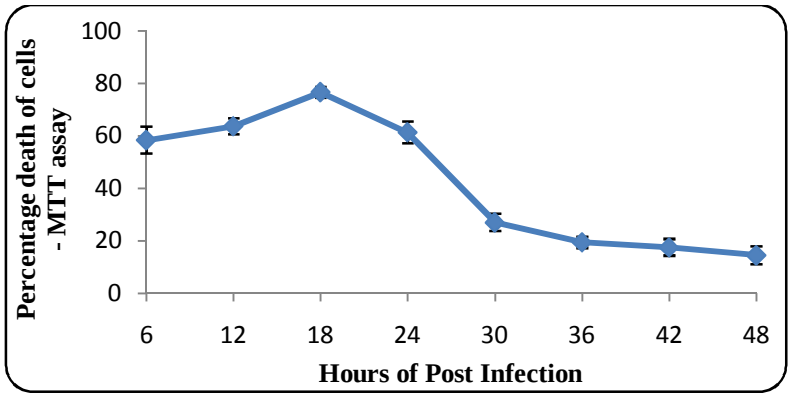

Fig .43 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV2A

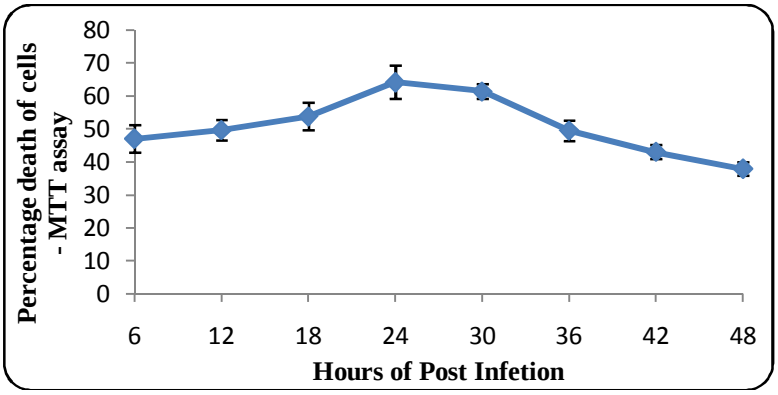

Fig .44 Percentage death of HEp-2 cells with hours post infection on inoculating with lysate- MCCV2B

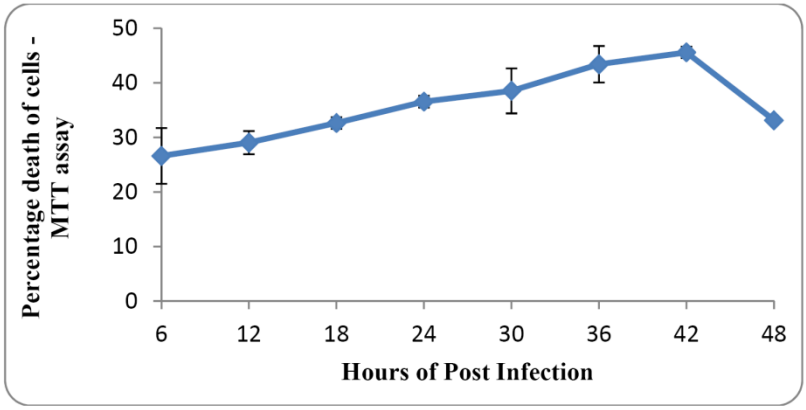

Fig .45 Percentage death of HEp-2 cells with hours post infection on inoculating C6/36 with lysate- MCCV1D

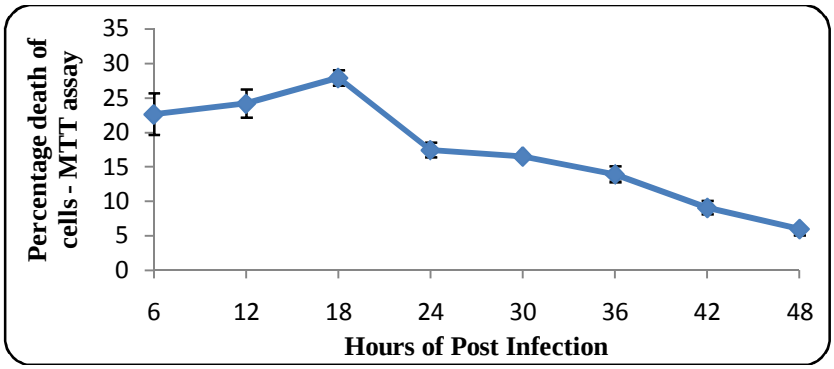

Fig .46 Percentage deaths of HEp-2 cells with hours post infection on inoculating C6/36 with lysate- MCCV1I

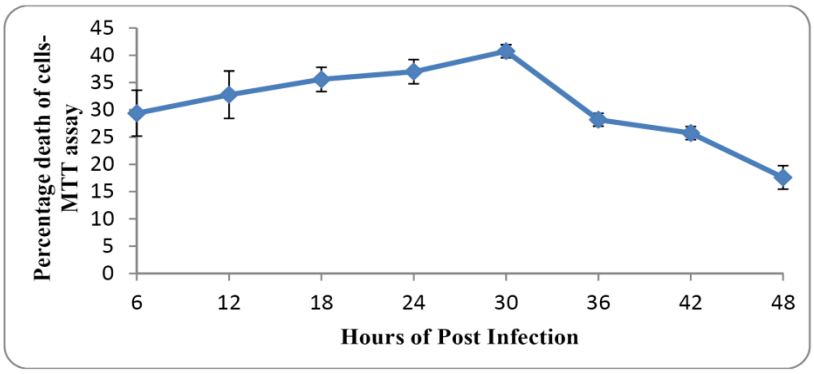

Fig .47 Percentage deaths of HEp-2 cells with hours post infection on inoculating C6/36 with lysate- MCCV1V1

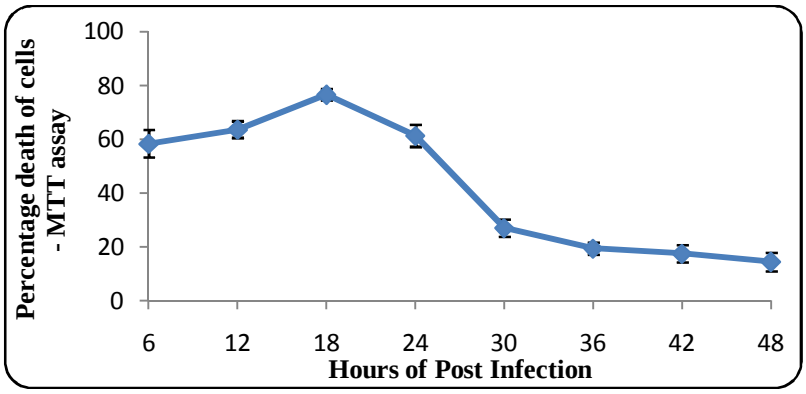

Fig .48 Percentage deaths of HEp-2 cells with hours post infection on inoculating C6/36 with lysate- MCCV2A

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Electron Microscopy

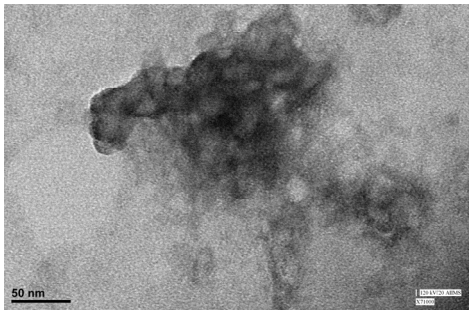

Figure 49. MCCV1V$_1$

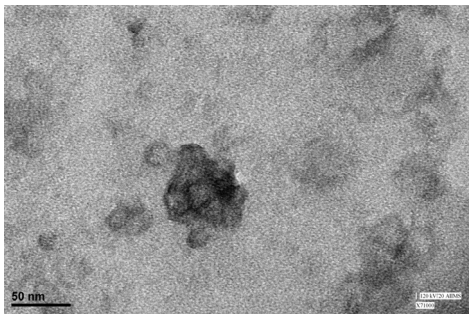

Figure 50. MCCV2B

Hemagglutination Assay

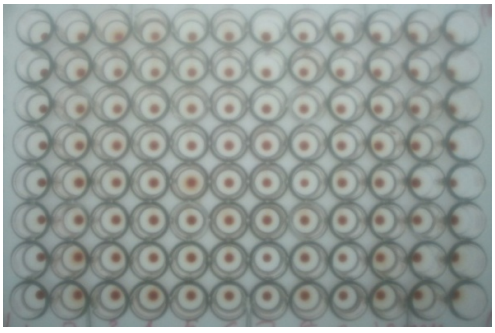

Figure 51. MCCV1D

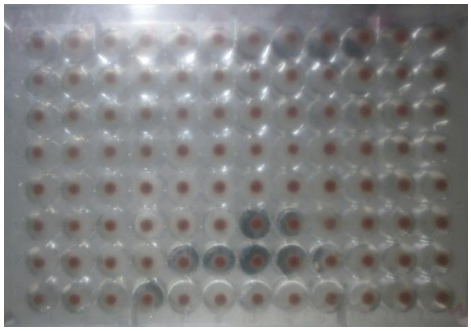

Figure 52. MCCV1N

Chapter 4

Molecular Characterization of the Isolated Viruses

4.1 General Introduction

In general, multiplication of viruses demands, cellular energy resources as well as protein synthetic machinery. In addition many viruses do make use of all or parts of the cell machinery to extract information maintained in the viral genome and convert them into mRNA through translation. Virus genomes are composed of either RNA or DNA, single or double stranded and may be linear, circular or segmented. Segmented genomes are those which are divided into two or more physically separated molecules of nucleic acid all of which are then packed into a single virus particle. Single stranded virus genomes may be either positive sense having the same polarity or the nucleotide sequence as the mRNA, negative sense or ambisense composed of a mixture of the two. The problem of strand breakage is particularly important in the case of single stranded RNA where they are less chemically stable than double stranded DNA. Physical breakage of the genome results in biological inactivation as it cannot be completely transcribed, translated or replicated.

Virus genomes range in size from approximately 2.5Kbp as in the case with Gemini virus with ssDNA to 1.2 million base pairs as with Mimivirus which is twice as big as the smallest bacterial genome (Cann, 2012). All virus genomes experience pressure in building up their size. Viruses with prokaryotic hosts must be necessarily be able to replicate quickly to keep up with their host cells which is evident in the compact nature of many bacteriophages whereas in the case of eukaryotic viruses the genomes experience pressure on genome size, in addition to which the pressure is mainly on packaging the virus particle; the amount of nucleic acid that can be packaged in a virion (Cann, 2012). Since viruses are parasitic on the molecular process of gene expression and its regulation on the host cell, an understanding of the viral genomes and viral replication could provide base line information on cellular process in general. The most basic molecular requirement for virus replication is to induce either profound or subtle changes in the cell so that the viral genome in the cell gets replicated and viral proteins are expressed. The coexistence in the cell would lead to changes in the protein composition of the cell surface, the immune signature of the cell and in turn functional changes termed lysogeny in the case of bacterial cells and transformation in the case of plant and animal cells. Isolation of the purified virions provides basic information about their nature and molecular size and is one of the first-hand information about a newly isolated virus. This information together with the general virion characteristics can be interpreted as a preliminary data to interpret the relationship between the new virus and the known virus families, finally whole determination of nucleotide sequence of viral genomes will provide information about the number and specific amino acid sequences encoded by the protein. A statistical analysis of the frequency and numbers of the base change in the genes encoding the conserved enzymes and proteins mediating essential

metabolic and other cellular processes can be used for measuring the degree of relatedness between greatly divergent common ancestors, the information of which is used to generate a phylogenetic tree which graphically represents such relationships (Wagner *et al.*, 2006).

Classical System of Classification

In 1962 Lwoff, Horne and Tournier advanced a comprehensive scheme for the classification of all viruses of bacteria, plant and animal under the classical lineal hierarchial system consisting of phylum, class, order, family, genus and species. Four characteristics are being continuously used for the classification of all viruses. These include – nature of nucleic acid in the virion (DNA or RNA), symmetry of protein shell (the capsid), presence or absence of lipid membrane (envelope) and Dimensions of the virion and capsid. Genomics, by elucidating the evolutionary relationship by analysis of nucleic acid and protein sequence similarities is being increasingly used to assign viruses to a particular family and to order members within a family (Flint *et al.*, 2009).

Baltimore System of Classification

A universal function of viral genomes is to specify proteins, since viral genomes do not encode the machinery needed to carry out protein synthesis all viral genomes must be copied to produce mRNAs that can be read by host ribosomes. A second principle is unity in diversity even with immeasurable period of time; evolution has led to the formation of seven major types of viral genome. The Baltimore system of virus classification integrates these two principles to construct an elegant molecular algorithm for virologists. The elegance of Baltimore system is that by knowing only the nature of the viral

genome one can elute out the steps required to produce mRNA. The Baltimore system excludes the universal function of viral genomes i.e to serve as template for synthesis of progeny genomes. A virus with DNA genomes replicates and mRNA synthesis presents no challenges as cell use DNA based mechanisms while animal cells possess no known mechanisms to copy viral RNA template and to produce mRNA from them. RNA viruses to survive RNA genomes must encode a nucleic acid polymerase for genome replication and mRNA synthesis (Flint *et al.*, 2009).

International Committee on Taxonomy of Viruses

In 1966, the International Committee on Taxonomy of Viruses (ICTV) was formed which were bound to provide a universal taxonomic scheme for addressing the nomenclature and classification of viruses. The system developed by ICTV is based on the hierarchial levels which include Order-(-virales), Family-(-viridae), Subfamily (-virinae), Genus (-virus) followed by commonly used names. In the latest report of ICTV published to date, the ICTV 2012 master species list consists of the 8 orders consisting of Caudovirales, Herpesvirales, Ligamenvirales, Mononegavirales, Nidovirales, Picornavirales, Tymovirales including several of which are unassigned viruses altogether consisting of 91 families including families of the unassigned Orders together with an unassigned family altogether consisting of a total of 2619 species (Shors, 2009).

4.1.1 Types of Viral Genomes

Unlike other organisms viruses contain either DNA or RNA and single stranded or double stranded alone. Knowledge about the sequence of viral genomes possesses greater advantages for their classification. Sequence analysis helps to provide relationships among diverse viral genomes providing

considerable insights into the evolution of viruses and even in the case of epidemics of viral infection, partial genome sequence can provide information about the identity of the virus to combat it (Flint *et al.*, 2009).

4.1.1.1 Smaller DNA Genomes

The genome of Bacteriophage M13 consists of 6.4kb single stranded positive sense circular DNA encoding almost ten genes. T4 and Lamdaphages are another examples of linear virus genomes. In the case of Lambda phage the substrate packaged into the phage heads during assembly consists of long repetitive strings of phage DNA produced in the later stages of genome replication. Further examples include two groups of animal viruses - the parvoviruses and the polyomaviruses. Parvovirus genomes are linear, non-segmented, single stranded DNA of 5kb, usually negative sense but in some parvoviruses package equal amounts of positive and negative strand virions. The parvovirus genome consists of only two genes: *rep* which includes proteins involved in transcription and *cap* which encodes the coat proteins. The genomes of polyoma virus consists of double stranded circular DNA molecules approximately 5kb in size. Within the particle the virus DNA assumes a supercoiled form and is associated with four cellular histones; H2A, H2B, H3 and H4. The genomic organization of these viruses have evolved to pack the maximum information about six genes into a minimal size of 5kb which are achieved by using both strands of genomic DNA and overlapping genes. VP1 is encoded here by a dedicated open reading frame (ORF) whereas the VP2 and the VP3 genes overlap so that VP3 is contained within VP2. The origin of replication is surrounded by non-coding regions that control transcription (Cann, 2012). Among the five families of ssDNA genomes Circoviridae, and Parvoviridae are only known to infect mammals (Flint *et al*., 2009).

4.1.1.2 Large DNA Genomes

A number of virus groups have DNA genomes of considerable size and complexity many of which are genetically similar to the host they infect. The genome of adenovirus consists of linear double stranded DNA of about 30-38Kbp containing 30-40 genes. The terminal sequence of each DNA strands is an inverted repeat of 100 to 140bp and the denatured single strands can form panhandle structures. The family Herpesviridae is composed of large genomes consisting of about 235Kbp of linear dsDNA and correspondingly large and complex virus particles containing about 35 virion polypeptides. The different members of the family are widely separated in terms of genomic sequence and proteins but all are similar in terms of structure and genome organization. Poxvirus genomes are linear in size ranging from 140 to 290Kbp and as with herpes virus each genome tends to express from its own promoter. The central regions of the poxvirus tend to be highly conserved and contain genes that are essential for replication in culture while the outer regions of the genome are more variable in sequence. More recently, viruses with even larger genomes have been reported which ranges to about 500Kbp in the case of Phycodnaviruses and up to 1.2Mbp in the case of Mimi virus both of which are known to infect a wide range of prokaryotic and eukaryotic hosts (Cann, 2012). Altogether there are about 24 families of viruses with dsDNA genomes among which mammalian viruses include Adenoviridae, Herpesviridae, Papillomaviridae, Polyomaviridae and Poxviridae (Flint *et al.,* 2009).

4.1.1.3 Positive Sense RNA Genomes

The ultimate size of ssRNA viruses are limited by the relatively fragile nature of RNA and the tendency of long strands to break. The RNA genomes tend to have higher mutation rates than those composed with

DNA since they are copied less accurately although the virus encoded RNA dependent RNA polymerase responsible for the replication of the genomes do possess some repair mechanisms which tends RNA viruses to have smaller genome sizes. Single stranded RNA (ssRNA) viruses vary in size, from those of coronaviruses, approximately 30 Kb long to bacteriophages such as MS2 and Qβ to about 3.5 Kb. Purified positive sense virus RNA is directly infectious when applied to susceptible host cells even in the absence of any proteins.

The picornavirus genomes consists of a single stranded positive sense RNA molecule ranging from 7.2 Kb in the case of Rhinovirus to 8.5 Kb in foot and mouth disease virus incorporating a number of common features conserved in all picornaviruses. They contain a long untranslated region (UTR) at the 5' end responsible in translation, virulence and encapsidation and a short 3' UTR end responsible for negative strand synthesis during replication. Another family Togavirus composed of a single stranded positive sense segmented RNA of 11.7 Kb in the genome resembles cellular mRNA in that it has a 5' methylated cap and a 3' poly A sequence. The genome of flavivirus is single strand positive sense of about 10.5 Kb with a methylated cap and a poly A tail as that with Togaviruses and the only difference between the two is that the genetic organization differs from the Togaviruses in that the structural proteins occupy the 5' part of the genome and nonstructural proteins in the 3' part. In the case with coronaviruses the genomes are non-segmented single stranded positive sense RNA of 27-30 Kb which are the longest of the known RNA viruses and also possess a 5' methylated cap and a 3' poly A tail and the viral RNA functions directly as mRNA. The 5' 20 Kb segment of the genome is firstly translated to produce a virus polymerase which then

produces a full negative sense strand used as a template to produce mRNA as a nested set of transcripts (Cann, 2012).

4.1.1.4 Negative Sense RNA Genomes

Viruses with negative sense RNA genomes are more diverse than positive sense. Because of the difficulties of gene expression and genome replication, they usually tend to have larger genomes encoding more genetic information and due to which several viruses among the group appear to be segmented. Since most of the eukaryotic cells do not contain enough RNA dependent RNA polymerase activity to support enough virus replication, the negative sense RNA genomes cannot be easily translated to mRNA and therefore these viruses are effectively inert. Ambisense viral genomes occur in both plant viruses –Tospovirus Genus of Bunyavirus and Tenuiviruses such as rice stripe viruses and animal viruses- phlebovirus genus of Bunyavirus and Arenavirus.

The members of the family Bunyaviridae have single stranded negative sense RNA genomes composed of three molecules - L segment with 8.5kb size, M segment with 5.7 Kb size and S segment with 0.9 Kb in size. As with all negative sense RNA's the 5' ends are not capped and the 3' ends are not polyadenylated. The family Arenaviruses consists of linear single stranded RNA with two genome segments - the L segment with 5.7 Kb and S segment with 2.8kb both segments of which have an ambisense mode of organization. Viruses of the Rhabdoviridae family have segmented negative sense RNA of 11 Kb, a leader region constituting of about 50 nt at the 3' end and a 60 nt UTR region at the 5' end of the vRNA (Cann, 2012).

4.1.2 Means of Determining Viral Genomes

Once a pure virus has been obtained the viral structural proteins can be obtained by gentle disruption of virions with mild detergents and with appropriate salt treatments. Small amounts of nucleic acid can be digested into nucleotides or proteases for protein digestion which can then be separated according to size, charge or even both.

An accurate determination of the viral genomes nature and molecular size is one of the primary requisite while isolating a virus particle. Such information helps in establishing a basic idea of virus genetic complexity. The information together with the general characteristics of virion such as enveloped or non enveloped, icosahedral, helical or complex can be used to make a preliminary data of the isolated virus with known virus families. The determination of full nucleotide sequence of the viral genome would provide information about the number and specific amino acid sequences of the proteins it encodes and its measure of degree of relatedness to other viruses.

4.1.2.1 Measuring Size of Viral Genomes

While nucleotide sequence determine molecular size, a number of physical and biochemical methods were developed in advance of sequencing. The entire size of ds DNA genomes in the range of DNA sizes can be directly visualized in an electron microscope with shadowing and spreading methods which ensures that they are very long flexible DNA and not tangled to be immeasurable. By employing proper experimental techniques viral genomes as large as 250-300kbp can be isolated without degradation. Under proper spreading and shadowing conditions the length of DNA molecule is a direct function of their size in base pairs (about 3μ/kbp).

4.1.2.2 Rate Zonal Sedimentation for Measuring Size

In contrast to DNA molecules possessed by certain viruses, several other viruses contain RNA which are highly susceptible to chemical degradation at relatively mild pH changes (<3 and >9). Ribonuclease that readily degrade are notoriously difficult to inactivate and are excreted by bacteria, fungi and even the sweat in hands are all potential sources of ribonuclease. Unfortunately ssRNA is not very easy to work using the electron microscope due to its flexibility and ability to form hairpin loops that makes it very difficult to spread for the accurate measurement of length.

The size of viral genomes are convenient for rate zonal sedimentation in sucrose gradients and conversely for size fractionation on low density acrylamide gel using electrophoresis. Since all ssRNA molecules and ssDNA molecules will have the same shape in solution, the sedimentation rate in a centrifugal speed will only be a function of their molecular size. Sedimentation rate under standard conditions is termed the sedimentation constant (s value) which is related to molecular size by logarithmic function. The size range of RNA and ssDNA molecules is easily separable by gel electrophorsesis, while most dsDNA molecules are too large for gel electrophoresis where the use of restriction endonucleases can help to resolve the problem and if highly purified virion DNA is present they can be easily digested with restriction enzymes by which fragments of a convenient size can be produced and fractionated on high porosity agarose gels (Wagner *et al.,* 2006).

4.1.2.3 Polymerase Chain Reaction

The ability to characterize and work with viruses was limited by the fact that they are present in small quantities in the given cell, tissue or host. The

use of a fluorescent stain such as ethidium bromide helps in easy detection of 100ng or less of DNA. The problem of visualizing and manipulating extremely small quantities of DNA was overcome in large part by the development of Polymerase Chain Reaction (PCR). To work with, the oligonucleotide primers must be long enough to be highly specific and short enough about 20-30bp to allow frequent priming along with the ability to do denaturation and renaturation in a single step several times accomplished by mans of heat stable DNA polymerase isolated from *Thermophilusaquaticus(Taq).*

PCR can also be employed to detect vRNA genomes or even transcripts present in very low amounts accomplished by generating a cDNA copy of the RNA by use of retrovirus reverse transcriptase followed by PCR with a known set of primer. If in case oligodeoxythymidine is used as primer it will anneal to the poly A tail of mRNA for the generation of cDNA and if correct primers are used, PCR can detect very minute traces of transcripts (Wagner *et al.,* 2006).

4.1.2.3.1 Real Time PCR

In addition to the value of detecting very small quantities of viral genomes, PCR can also be employed to measure extremely precise quantities or amounts of viral genomes or transcripts present in different tissues or under different conditions of infection. The real time PCR provides a much more reliable and precise method of quantitatively measuring products of PCR accomplished by means of measuring it continuously throughout all cycles of annealing and chain elongation. This is accomplished either by SYBR green chemistry or by TaqMan Chemistry (Wagner *et al.,* 2006).

4.1.2.2 Restriction Mapping

Restriction enzymes have a very high specificity for DNA sequence and thus could digest DNA in different locations within the amplicon. Single cleavage of circular DNA molecule would result in genome becoming linear; the same principle can be applied in mapping circular genomes. Also depending on the size of the viral genome and specificity of the restriction enzymes being mapped, a few large segments or many small fragments can be generated by digestion with restriction enzymes. Separate digestion of large fragments could provide information about the arrangement of sites of the enzymes which it could cut frequently, so that maps of any genome can be built with any of the specific enzymes. Restriction enzymes do not cleave ssDNA or ssRNA while restriction maps can still be generated by converting them enzymatically into double stranded forms with DNA polymerase while RNA can be converted into DNA using reverse transcriptase which allows RNA genomes to be manipulated as that with DNA genomes, the approach of which is called as reverse genetics (Wagner *et al.*, 2006).

4.1.2.3 Molecular screening for viruses

The previous two chapters pointed out that out that the search for viruses should be extended beyond Arboviruses (Alphavirus, Flavivirus and Bunyavirus) as the adaptability/susceptibility of cell lines to the virus lysates was in the decreasing order, HEp-2, VERO and C6/36. As there was the possibility of Enteroviruses in the lysates, PCRs were carried out for them as well.

4.1.2.4 Overview of Enteroviruses

Enteroviruses are RNA viruses of the family Picornaviridae which are small, naked, icosahedral viruses that multiply in the mucosa of the gut. These

are ubiquitous pathogens transmitted from person to person via oral feacal route. Most infections occur during childhood resulting in a lifelong immunity. Enteroviruses are able to pass through stomach and small intestine as they are resistant to pH levels as low as three and to several proteases. Enteroviruse particles are non-enveloped making it resistant to detergents, 70% alcohol, and other lipid solvents such as ether and chloroform and also to disinfectants such as 5% lysol and 1% quaternary ammonium compounds. As these viruses are present in organic matter such as faeces standard treatment methods may require prolonged contact time. Chemical inactivation can be achieved with chlorine, hydrochloric acid and aldehydes. Heating at 50°C for one hour in the absence of calcium and magnesium will also inactivate enteroviruses. They are stable for several days to weeks at 4°C. There are over 100 distinct human enteroviruses and more than 20 recognizable syndromes that have been associated with enterovirus infections (Shors, 2009).

This family constitutes non-enveloped, single-stranded, positive-sense RNA viruses, the RNA genome of these viruses exhibits a similar organization among all genera. A virus encoded protein (VPg) is covalently linked (Lee, 1977)to a long 5' non-translated region (5'NTR), followed by a single, large open reading frame (ORF) that encodes for a single polyprotein. Finally, a 3' NTR, and a genetically templated 3'poly (A) tail complete the RNA genome (Wimmer, *et al.,* 1993).Enteroviruses are responsible for a wide variety of diseases and represent a major public health hazard (Melnick, 1996) although many of their infections may often remain mild or asymptomatic (Melnick, 1996). Although most infections with human enteroviruses are asymptomatic or result in mild infection, human enteroviruses can cause diverse clinical syndromes ranging from minor febrile illness to severe and potentially fatal

diseases such as acute haemorrhagic conjunctivitis, aseptic meningo-encephalitis, and acute flaccid paralysis (Palacios and Oberste, 2005). Historically, human Enteroviruses have been classified into Echoviruses (EV), Coxsackievirus groups A and B (CV-A and CV-B), and Polioviruses (PV), according to their phenotypic and pathogenic properties.

Enteroviruses of the human Picornaviridae, such as the group B coxsackieviruses (types 1 to 5), are the most common agents known to cause viral myocarditis (Abelmann, 1973; Johnson and Palacios 1982; Melnick, 1985; Reyes and Lerner, 1985). The group of Coxsackie causes several diseases which include pericarditis, myocarditis, paralysis, macular skin rash, hepatitis, summer fever illness, upper respiratory illness and pneumonia, aseptic meningitis, pleurodynia, meningoencephalitis and myocarditis in children (Shors, 2009). The division of coxsackie viruses into A and B subgroups was mainly based on lesions observed in newborn mice. The Cosxackie A viruses affect skeletal muscle, while Coxsackie B viruses cause pathological changes in several tissues, including the central nervous system, pancreas, liver and brown fat. The more typical epidemiological manifestations of Coxsackie A and B viruses are herpangina and myocarditis respectively (Modlin and Rodbart, 1997).

4.1.2.3 Amplification of the VP1 region of Enterovirus

As a number of important neutralization epitopes as well as receptor recognition sequences lies within the VP1 capsid protein, it may be expected that the VP1 coding sequence or a partial sequence of it would very well correlate with its serotype. Because of this reason it has been suggested that nucleotide sequence analysis of this genomic region is more appropriate for

molecular typing of wild type enteroviral isolates than analysis of other genomic regions (Caro *et al.,* 2001; Norder *et al.,* 2001; Oberste *et al.,* 1999a; Oberste *et al.,* 1999b; Mulders *et al.,* 2000; Bailly *et al.,* 2000; Peng *et al.,* 2000). Genetic characterization by sequencing the VP1 capsid region can be achieved very rapidly and the results very well corelated with their serotype classification (Oberste *et al.,* 1999a). VP1 capsid protein carries the main antigenic sites, and it has been shown that there is an absolute serotype or genotype correlation in that specific genomic region (Oberste *et al.,* 1999b; Caro *et al.,* 2001). This attribute has been successfully used for molecular identification of enteroviruses and epidemiological studies (Mirand *et al.,* 2006; Chu *et al.,* 2010; Tryfonos *et al.,* 2011).Therefore, the sequencing of the VP1 region appears to be the method of choice for the precise characterization of Entericvirus isolates.

4.2 Material and Methods

4.2.1 Differentiation between RNA and DNA Genome

The Isolated viruses were to be checked for the identity of the genome. Aliquots of same virus lysates were treated with both *DNAse* and *RNAse*. For *DNAse* treatment the virus lysates (500 µl) were incubated with 2µl *DNAse* I (New England Bio labs, UK) and 1µl of *DNAse* Buffer in a 37ºC water bath for 20 min. The lysate were then inoculated to confluent monolayer of HEp-2 cells and incubated at 37ºC as closed system. Separate untreated controls were also included.

For *RNAse*treatment, aliquots of same lysates (500 µl) were incubated with 2µl *RNAse* A (New England Bio labs, UK) and incubated in a 37ºC water bath for 2 hrs. After incubation the lysates were inoculated into HEp-2 cells and incubated at 37ºC for observing cytopathic effects.

4.2.2 Sucrose Gradient

Six bottles ($25cm^{2)}$ of confluent HEp-2 cell line were infected each with 500µl virus lysates after removing the medium. After 30 min, aliquots of 2.5ml MEM were added and the bottles were incubated at 37ºC and observed for cytopathic effects under phase contrast microscope. When the cells were found to be lysed fully the bottles were subjected for three freeze thaw cycles, harvested the content and clarified by centrifugation (Eppendorf, India) at 3,000rpm for 10 mins at 4ºC. The clarified supernatant was filter sterilized using 0.22µm pore sized PVDF filters. Polyethylene Glycol (PEG 6000 Himedia) was added at 7% W/V followed by the addition of 0.5M/L NaCl. Sufficient time was given for the PEG to dissolve and subsequently centrifuged at 10,000rpm for 30 min to precipitate the virus. The supernatant was drained off and the precipitate dissolved in GTNE buffer in minimal volume (1000 µl) (Shukla *et al.,* 2009).

Discontinuous gradients of 20 and 50% sucrose in GTNE buffer was loaded into ultracentrifuge tubes (Thermo Scientific, India) in the ratio 1ml of 50% sucrose as the bottom layer followed by 3ml of 20% sucrose in the upper layer. The precipitated virus suspensions (500 µl) was layered on top of the gradients and were then run in an ultracentrifuge (Thermo Scientific, USA) at 1,00,000 x*g* for 2hrs at 4ºC. The separated whitish band was observed under visible light, the tube punctured and the band transferred using a sterile 2ml syringe and stored in sterile vials at -80ºC until used.

4.2.2.1 SDS - PAGE

Analysis of viral structural proteins on SDS-PAGE was conducted using a 4% stacking gel and 10% resolving gel (Laemmli, 1970). Viral proteins

were dissociated by mixing an equal volume of purified virus and 2X denaturing buffer (0.125M Tris pH 6.8, 4% SDS, 20% glycerol and 10% mercaptoethanol) heated in boiling water for 1.5min and then chilled in ice. Following the separation of constituent proteins by electrophoresis at 30ma/200V for 4hrs gels were stained with coomassie brilliant blue and the molecular weights of the viral proteins were determined by comparing their relative mobility with internal SDS-PAGE molecular weight standards (Bangalore Genei, India)

4.2.3 RNA Extraction

Fully confluent monolayers of HEp-2 cell lines were inoculated with 500 µl each suspension of lysates after decanting the medium from the culture bottles. After three hours 500µl fresh MEM was also introduced. The flasks were incubated at 37ºC as closed system and observed for CPE under anInverted phase contrast microscope (Leica, Switzerland). The flasks were incubated for 48 hrs and the entire contents were harvested and centrifuged at 10,000 rpm at 4 ^{0}C. for 10min. From the clarified supernatant140 µl was used for RNA extraction. The vRNA was extracted using Q1Amp Viral RNA kit(Qiagen, Venlo, Limburg, Netherlands) following the manufacturers' protocol. The final elute was adjusted to 35 µl using DEPC treated water.

4.2.3.1 DNAse Treatment

Aliquots of RNA (5 µl) were treated with 2 µl of *DNAse*I and 2 µl of *DNAse* Buffer (New England BioLabs, Inc., UK) in equal proportions at 37°C for 30mins. *DNAase* activity was arrested by holding at 75°C for 10 min. The treated RNA was used for RT-PCR for which aliquots were stored at -80°C until used.

4.2.4 Molecular screening of the virus lysates for identification

The extracted RNA was used for screening viruses using the primers as summarized in Table -1. Since the viruses were isolated from the environment, mosquito borne arboviruses and water borne enteroviruses were incorporated in the study. Arboviruses screened were Alphavirus, Flavivirus and Bunyavirus, as the mosquito larvae were reported to harbor several viruses throughout the year, while the Enteroviruses were considered as the larvae used to feed on micro organisms and organic matter present in water.

4.2.4.1 Screening for Arboviruses with different Primers

The annealing temperatures of the primers used in the study are given in Table -1. The PCR conditions include cDNA synthesis at 50 °C for 30 min in a reaction mixture containing 12.5 µL enzyme mix, 1 µL primers each of forward and reverse, 2 µL template RNA made up to 25 µL with 6.5 µL DEPC treated water (One Step Qiagen Limburg, The Netherlands), followed by PCR activation at 95°C for 50 min. followed by 35 cycles each consisting of 94°C for 1 min., 53°C for 1 min. 20 sec and 68 ° C for 2 min. with a final extension at 68°C for 6 min.

4.2.4.2 Screening for Enteroviruses with the highly conserved 5' UTR region

As the larval stages of mosquitoes are prolific feeders on detritus and micro organism in the breeding environment, enteric viruses have also been kept into consideration. The primers used and their annealing temperature are summarized in Table -1.

The Reverse transcriptase PCR (RT-PCR) was carried out in 20µl reaction mix (Orion X Onestep RT – PCR Kit, Origin Diagnostics and Research, India) containing One Step Enzyme 2µl, RT Buffer 6 µl, 10mM dNTP 1 µl, Primer 1 5µl (50 pmol), vRNA 5µl, and Milli Q 1µl, The reaction mix was incubated at 37°C for one hour. After incubation the second step PCR was done for which added with second primer set (Primer 2 - 5µl (50 pmol)) and made up the volume to 30 µl using 5 µl Milli Q. The reaction conditions consisted of 95ºC for 15mins to activate the Hot Start Taq followed by 35 cycles each consisting of denaturation at 94ºC for 1min, primer annealing at temperatures mentioned inTable 1 for 40sec followed by extension at 72 ºC for 2mins with a final extension at 72 ºC for 10 min.

From the PCR product nested PCR (n-PCR) was done with similar PCR conditions with a reaction mix composed of PCR product 5 µl, enzyme 2µl, buffer 6µl, 10Mm dNTP 1µl, primer 3 5µl (50 pmol), primer 4 - 5µl (50 pmol), and Milli Q 1µl.

4.2.5 Restriction Digestion and RFLP Analysis

Reverse transcriptase–polymerase chain reaction (RT-PCR) amplification combined with restriction fragment length polymorphism analysis (RFLP) provides alternatives to enterovirus detection and typing which over performs the specificity of conventional diagnostic serotyping (Georgopoulou *et al.,* 2001). All the lysates studied were digested with three enzymes namely: *Hha*I which classifies enterovirus into a total of 18 clusters (Kyriakopoulou *et al.,* 2013); *Hpa*II for the classification into five clusters and finally BSaJ1 for differentiating between serotypes of Coxsackie viruses.

Digestion of PCR product with the *Hha*I was done as follows: An aliquot of 5µl of 440bp PCR product amplified was digested with 2µl of Cut Smart Buffer and (0.2µl) 10 units of *Hha*I (New England Bio Labs, UK) and made up to 20µl with nuclease free double distilled water. It was then incubated at 37ºC for 2hr. After incubation the reaction was stopped by inactivation at 65°C for 10mins. The fragments were then loaded into 3% agarose gel electrophoresis (Amersham Biosciences Ltd, India) and documented using a Gel Documentation system (Bio Rad, India, Private Limited)

Aliquots of 5µl of the PCR amplicons were digested singly with (0.2µl) 20 U of restriction enzyme *Hpa*II (New England Biolabs, UK) and 2µl of Cut Smart Buffer into a final volume of 20 µl at 37°C for 3 hours as per the manufacturer's instruction. After incubation the reaction was stopped by inactivation at 65°C for 10min and the digested products were run in 3% agarose gels and documented using the Gel Documentation System (Bio-Rad, India Private Limited).

The differentiation within the Coxsackie group B viruses from all serotypes from Coxsackie B1- B6 was achieved by digestion with BsaJ1 (New England Bio Labs, UK) as follows; 5µl of PCR product was digested with buffer containing (0.2µl) 5 units of BsaJ1 (New England Bio Labs, UK) and incubated at 55ºC for 3hr followed by enzyme inactivation at 80ºC for 10 min. The digested products were then electrophoresed in 3% agarose gel and documented with a Gel Documentation system (Bio - Rad, India, Private Limited).

4.2.6 Amplification of the VP1 region of Coxsackievirus B3

The amplification of VP1 capsid gene (340bp) was done using One-Step RT-PCR kit (Orion X One step RT–PCR Kit, Origin Diagnostics and Research, India) as that with UTR amplification. The reaction mix consisted of RT buffer 6µl, One Step enzyme 2µl,10mm dNTP's 1µl, 728 F primer (292) - 2µl (20 pmol), 728 R primer (222) - 2µl (20 pmol), RNA - 5µl, RNAse free Water 12µl.

The reaction mixtures were adjusted to a final volume of 30µl and run under the following conditions in a thermal cycler (Eppendorf, India, Limited). Initial RT-PCR step at 50°C for 30min, followed by inactivation of RT-PCR step at 95°C for 15min followed by PCR step at 94°C for 3min and 35 cycles of 94°C for 30 Sec, 42°C for 30 Sec and 60°C for 30 sec, followed by a final extension at 72°C for 10min (Oberste *et al.,* 2004). The PCR amplified products were sequenced (at SciGenome labs Pvt.Ltd., Cochin, India). The sequences were subjected for BLAST search (nucleotide) in NCBI database for confirmation.

4.2.7 Phylogenetic Tree and Grouping of Isolates

Nucleotide sequence and phylogenetic analyses of all the 22 lysates were conducted using BLAST program (http://www.ncbi.nlm.nih.gov /BLAST/) (Altschul et al. 1997) and Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.2 (Tamura *et al.,* 2007). The 440bp UTR-5' nucleotide sequences were aligned using CLUSTALW. The aligned sequences were then used to generate phylogenetic trees using the software program MEGA, version 5.2. using Kimura 2-parameter models of nucleotide substitution, and substitution rates were assumed to be gamma distributed with invariant sites

at which there was a gap in any of the aligned sequences were excluded from all comparisons, and distances were corrected by using Kimura's two-parameter method. The reliability of the branching orders was estimated by bootstrapping using 1000 replicates. The final trees obtained were optimized using the heuristic nearest-neighbour-interchange (NNI) method. Separate Phylogenetic trees were generated using the UTR region, first using the highest similarity deposits from GenBank and secondly within the 22 UTR sequences studied.

In the case of VP1, amino acids were aligned using CLUSTALW as a guide to obtain the final data set. The amino acid translation of the VP1 nucleotide sequences were formulated using ExPASy Proteomics server (http://us.expasy.org/tools/pi_tool.html) and the translated amino acid contigs were aligned using CLUSTALW. The maximum-likelihood phylogenetic tree were then generated using the software program MEGA, version 5.2. Kimura 2-parameter models of amino acid substitution were used, and substitution rates were assumed to be gamma distributed with invariant sites (G+I). The final trees obtained were optimized using the heuristic nearest-neighbor-interchange (NNI) method. Separate phylogenetic trees were drawn using the translated amino acid contigs both with the NCBI BLAST deposits as well as within the representatives. The numbers on each branch indicate their respective bootstrap values.

4.2.8 DNA Barcoding ofMosquito Larvae (Vectors from Which Virus Lysates were Obtained)

4.2.8.1 Isolation of DNA

The stocks of mosquito larval samples maintained at -80°C were brought down to room temperature. Aliquots of 1ml each of the samples were dispensed

with equal volumes of DNAzol and incubated for 30min at room temperature gently inverting 2-3 times. This was followed by centrifugation at 15,000x*g* for 15mins. at 4°C. The supernatants were then transferred into fresh 1.5ml micro centrifuge tubes to which aliquots of equal volumes of absolute ethanol were added. This was then incubated at -20°C for 30mins during which the samples were gently inverted 2-3 times in between. After incubation the samples were centrifuged at 20,000x*g* for 15mins at 4°C and the supernatants were discarded. The precipitate was then washed two times with 200µl of 75% ethanol centrifuging at 20,000x*g* for 15 min at 4°C. The pellet was air dried and finally eluted in 50µl sterile Milli Q water (Millipore, India). The eluted DNA was stored at -20°C until used.

4.2.8.2 Molecular identification of Vectors

Thee DNA extracted from mosquito larvae used for barcoding employing the primers according to Tyagi *et al.,* 2009 and Daravath *et al.,* 2013 which amplified the Mitochondrial Cytochrome Oxidase Subunit I Gene Sequence (COI) giving productsize of 710bp.

LCO1490 - GGTCAACAAATCATAAAGATATTGG

HCO2198 - TAAACTTCAGGGTGACCAAAAAATCA

The reaction mix consisted of 10X PCR Buffer 2.5µl, dNTPs 2µl, Taq polymerase 1µl, primer LCOI1490 1µl, HCO2198 1µl, MilliQ 14.5µl, DNA 3µl. The reaction conditions were 95°C for 5min for initial denaturation, followed by 35 cycles of 94°C for 30sec, 51°C for 30 sec and 72° for 30 sec. This was followed by a final extension at 72° for 10 min.

4.3 Results

4.3.1 Differentiation of RNA and DNA Genomes

All samples were treated with *DNase I* and *RNAse A* and checked for infectivity in HEp-2 cells. The *DNaseI* treated samples did not show loss of infectivity nor delayed activity and were similar in response to virus control. In the case of *RNAse A* treated samples, they could neither reproduce their infectivity in cell culture nor delayed activity concluding that all lysates were possessing RNA genome.

4.3.2 Sucrose Gradient

Through discontinuous sucrose gradient a discrete band of virus could be generated at the interphase between 20 and 50% sucrose.

4.3.3 SDS – PAGE

Three discrete bands were obtained of size of 60, 66 and 80 kDa(Fig.1).

4.3.4 RNA Extraction

The vRNA was successfully extracted from 140µl of tissue culture supernatant which were confirmed with absorbance values A260/A280 greater than 1.5 using a UV - Vis spectrophotometer (Schimadzu, KYT, Japan).

4.3.4.1 DNAse Treatment

The isolated RNA was purified with *RNAse* free *DNAse* and stored in a low temperature cabinet at -80ºC (New Brunswick Scientific, USA) until used. The purity was confirmed spectrophotometrically (A_{260}/A_{280}).

4.3.5 Molecular Screening

4.3.5.1 Screening for Arboviruses

The isolated viruses were checked for Alphaviruseswith their conserved region, but no visible bands or amplicons could be observed in the 434 bp region confirming that the viruses under study did not belong to this group. Similar was the result with Flavivirus and Bunyavirus where no amplificonsat 958bp and 510bp could be observed. With the above results it could be concluded that the viruses isolated did not belong to arbovirus group reported in India.

4.3.5.2 Screening for Enteric viruses

All the viral lysates under study could successfully be amplified in the 440bp 5'UTR and nested product having 330bp. The gel images of all virus lysates are given in Fig.2 and 3 of the UTR and Fig. 4 and 5 with respect to the nested product. All the 22 virus lysates coded from MCCV1A-MCCV2B have been deposited in the GenBank. The codes of lysates and their individual accession numbers are given in Table 2. The primer sequences which gave the amplification were Primer 1: ATTGTCACCATAAGCAGCCA, Primer 2: ACCTTTGTACGCCTGTT followed by two nested primers 3: AAGCACTTCTGTTTCCC and Primer 4: ATTCAGGGGCCGGAGGA. All were identified as Coxsackie – B3.

4.3.6 Restriction Digestion and RFLP Analysis

The amplified UTR of all 22 virus lysates (440bp product) were subjected to digestion with three enzymes such as *Hha*I, BsaJ1and *Hpa*II.

4.3.6.1 Restriction digestion using *Hha*I

The 440bp UTR was digested with the enzyme *Hha*I,no specific restricted bands were observed (Fig. 6 and 7).

4.3.6.2 Restriction digestion using BSaJ1

The restriction of 440bp UTR with the enzyme BSaJ1generated four fragments of 200bp, 125bp, 85bp and 30bp (Fig. 8 and 9).

4.3.6.3 Restriction digestion using *Hpa*II

When a 440bp UTR product was digested with the enzyme *Hpa*II, three fragments such as 210bp, 150bp and 125bp were generated, which were found to be similar in all the 22 lysates (Fig. 10 and 11).

4.3.7 Amplification of the VP1 region

The VP1 gene amplified from 22 lysates gave a product of 340bp(Fig. 12 & 13). The PCR products were sequenced for confirmation and further sequence analyses. The sequences obtained were multiple aligned using CLUSTALW for phylogenetic tree construction using MEGA version 5.2.

4.3.8 Phylogenetic Tree and Grouping of Isolates

All the 22 lysates were found to evolve from a common ancestral lineage Coxsackievirus B3. The sequences obtained were compared and pairwise distance matrices showing the nucleotide sequences were calculated. For the phylogenetic tree construction all the homologous sequences derived from NCBI database of Coxsackievirus B3 with higher percentage similarity (96-99%) were used. In the case with UTR, the phylogenetic analyses revealed the percentage similarity within serotypes to range from 4 - 95% while with the reference serotypes it was

from 23% to 99%. Accordingly, the common lineage encompassing the 22 lysates consisted of six sub-clusters with the homogenous sequences from NCBI database (Fig 14). Phylogenetic tree constructed revealed two highly divergent lysates MCCV1O and MCCV1T (Fig 14).However, the UTR sequences within the representatives studied resulted in four clusters with two highly divergent out group MCCV1O and MCCV1T (Fig 15).

The Phylogenetic tree constructed using the translated amino acidsequence of VP1 with nucleotides using GenBank reference sequences (amino acid) resulted in seven clusters. Among the seven clusters the interesting factor observed was that the first three clusters (Cluster I, II and III) consisted of only the GenBank references, while the remaining four clusters formed within the serotypes (Fig 16). Two serotypes MCCV1AVP1 and MCCV2A diverged from the clusters formed. The phylogenetic analysis of VP1 (amino acid) representatives within the serotypes resulted in five clusters with three highly divergent isolates MCCV1BVP1, MCCV1DVP1 and MCCV1V1VP1 (Fig 17).

4.3.9 DNA barcoding for vectors

The extracted DNA amplified successfully a 710bp product (Fig. 18). This was then sequenced and the NCBI blast results provided with 99-100% identity. These amplified sequences were then submitted to the GenBank using the barcode submission tool. The sequences were assigned with the following accession numbers.

1) Vec2- KJ410333
2) Vec4- KJ410334
3) Vec6- KJ410335

Accordingly, the mosquito larvae were confirmed as belonging to *Aedesalbopictus* and *Armigeressubalbatus*

4.4 Discussion

In this chapter differentiation of the virus either as DNA or RNA viruses, concentration and purification the virus through sucrose gradient centrifugation, SDS – PAGE of capsid protein, identification of the mosquito larvae through bar coding, molecular screening of arboviruses and entero viruses, RFLP of the amplified UTR of enteroviruses, amplification and sequencing of VP 1 of enteroviruses, construction of phylogenetic tree and grouping of the isolates have been accomplished.

When each stocks of the virus lysate was treated separately in parallel with *DNAse I* and *RNAse* A (Allander *et al.,* 2001), the stock of aliquots of virus lysate treated with *DNase* I were unaffected by the treatment which implied that the lysates did not belong to DNA viruses. Meanwhile the RNAse A treated samples could not produce visible CPE.

The purification of representative lysates was accomplished using discontinuous sucrose gradient (Shukla *et al.,* 2009). Purified fraction was subjected for SDS - PAGE which resulted in three proteins having mass of 60, 66 and 80 KDa which however, do not match with the SDS –PAGE of the coat protein of Coxsackie – B3 (Wang *et al.,* 2002) .

Viral RNA was extracted successfully from the cell culture supernatants using a QIAmp viral RNA purification kit according to the manufacturer's protocol (Qiagen, Chatsworth, CA) (Chu *et al.,* 2010; Kokkinos *et al.,* 2010;Park *et al.,* 2010;Chiang *et al.,* 2012) which was eluted into 35µl of

RNase free water and stored at -80°C until used. A quantity of 5µl each of extracted RNA was used for PCR.

The extracted RNA was made *DNase* free by treatment with *DNase I* (Allander *et al.*, 2001) to remove the host cell contamination which was confirmed by having spectrophotometric reading of 1.5 (A_{260}/A_{280}) with all lysates.

Screening for Arboviruses with Generic level universal primers was performed following Pfeffer *et al*. (1997) for Alphavirus which were highly sensitive alternative to virus isolation for rapid screening and even post-mortem diagnosis. Even though the use of species-specific primers results in the risk of false-negative results, these were overcome by incorporating more conserved nonstructural gene regions and by the design of degenerate primers. However, this could not amplify any alphavirus gene. Similarly the PCR method reported by Fulop *et al*. (1993) was used for screening flavivirus. The study could easily ascertain the nonexistence of Flavivirus in all lysates as it could not amplify the NS5 region of Flavivirus. No amplification of Bunya virus also could be obtained.

Authenticity of the primers was verified with positive controls provided by Center for Disease Control and Prevention (CDC, DVBID, Fort Collins, Colarado). This consisted of Usutu SAAr 1776 RNA lysate which amplified NS5 gene of Flavivirus producing a product size of 958bp as described by Fulop *et. al* (1993). Similarly, NS1 gene of Alphavirus was amplified by producing a product size of 434bp as described by Pfeffer *et al.* (1997) with O'nyong-nyongUg MP 30 RNA lysate of CDC.

Meanwhile discrete bands of 440bp could be generated from all lysates, sequenced and found to belong to Coxsackievirus B3. Human infections with enteroviruses are of continued occurrence. Recombination events between different human enteroviruses are frequent which gives rise to a wide range of genotypes, of which some are reported to exhibit threatening pathogenic properties. Modern molecular techniques, based on the reverse transcription polymerase chain reaction, provide a crucial role in common diagnostics of enteroviral infections and also identification and typing of non-polio enteroviruses (Bolanaki *et al.* 2005). By employing nested PCR with specific primers derived from the 5'UTR and consensus region of the enterovirus genome, it was possible to detect enterovirus RNA from different serotypes of prototypic strains (Kuan, 1997). The 5'UTR of picornaviruses regulates not only translation, but also replication, and contributes to the control of viral pathogenesis and tissue tropism (Semler, 2004). Explicitly, the highly conserved 5'UTR is the region of choice for enterovirus detection (Bolanaki *et al.,* 2005; Georgopoulou *et al.,* 2000). Molecular analysis of the 5'UTR in combination with that of VP1 has been considered as a more reliable approach in investigations of epidemics (Zhou *et al.,* 2011; van der Sanden *et al.,* 2011). Conclusively, the analysis based on the sequencing of the VP1 genomic region in combination with the 5'UTR and RFLP assay provided with concise epidemiological information about the circulating and co-circulating enteroviruses, as well as about the epidemiological relatedness of an isolate with the dominant strains of an epidemic (Kyriakopoulou *et al.,* 2013). The UTR amplification followed by the nested PCR as done by Kuan (1997) resulted in the confirmation that all the lysates belonged to the same group enterovirus.

An RFLP-based method may provide with a fairly rapid means of virus typing, since no further amplification steps are required and the laborious process of sequencing is circumvented. A key issue for the design of a successful typing method is that it must have high differentiation power. It must be able to clearly differentiate unrelated strains, such as those that are geographically distinct from the source organism, and at the same time to demonstrate the relationship of all organisms isolated from individuals infected through the same source (Olive and Bean., 1999). The advantages of enterovirus detection and differentiation using RT-PCR, RFLP have been reported (Leparc *et al.,* 1994; Muir *et al.,* 1998; Nicholson *et al.,* 1994; Severini *et al.,* 1993; Zoll *et al.,* 1992). The genomic region of study was chosen from the highly conserved 5'-UTR in an attempt to reduce intra-serotypic variation and at the same time, allow the possible detection of any inter-serotypic differences in the studied region of the genome as with Siafakas *et al*. (2002).

The restriction enzyme *Hha*I was selected for enterovirus sub-classification which classified them into 18 groups, based on its ability to recognize a stable GC-rich restriction site sequence. This method of choice offers a better sub-classification of Enteroviruses, grouping them into 18 clusters consisting 1 to 23 serotypes (Kyriakopoulou *et al.,* 2013). On restricting the 5'UTR of the 22virus lysates in the present study with this enzyme no restrictions could be obtained which according to Kyriakopoulou *et al*. (2013) forms the most crowded group of cluster 1 consisting of 23 serotypes where no cleavage was obtained with *Hha*I. The serotypes of cluster 1 represent HEV-A (3 serotypes) and HEV-B (20serotypes) since all the virus

lysates belonged to CVB3 no cleavage was obtained confirming that the lysates belonged to Cluster 1(Kyriakopoulou *et al.*, 2013).

Restriction digestion using BSaJ1 was performed on the amplified 5' UTR. In a study, Patel *et al*. (2004), developed a simple restriction fragment length polymorphism (RFLP) assay for typing group B Coxsackieviruses into six serotypyes (CVB1-B6) and proposed it as an useful approach to differentiate Coxsackie B virus into their subtypes and is a valuable supplement to enterovirus identification for diagnostic and epidemiological studies. Digestion with the enzyme resulted in four restriction sites which were similar with that of as reported with CVB3 reference strains.

Restriction digestion using *Hpa*II was performed on the amplified 5'–UTR**.** In a previous study by Siafakas *et al*. (2003) prototype Enterovirus strains were sub-clustered into five restriction profile clusters using RFLP analysis despite intratypic genetic variability in the 5'-UTR. The results of the sequence analysis, as well as data from the RFLP analysis of 61 enterovirus reference strains from 60 different serotypes and 123 clinical isolates showed that one restriction endonuclease, *Hpa*II, contributed to a reliable sub-classification of CAVs and the rest of enteroviruses, which was reported to be particularly useful in clinical and epidemiological studies. Since the CVB3 belonged to Cluster 1, three cleavage sites could be obtained such as 213bp, 150bp and the 125bp specific to the first cluster.

Phylogentic tree and clustering of the lysates study analyzed the partial VP1 and UTR nucleotide sequences of the 22 isolates. The phylogenetic relationships of the 22 sequential isolates of Coxackievirus B3 were constructed by using the sequences from the 5'UTR regions and the VP1-

coding sequence. The VP1-coding sequence was chosen for general and specific reasons. First, VP1 is the largest capsid polypeptide in enteroviruses and comprises both stable and variable domains that correspond approximately, in the complete virion structure, to internal β-sheets and exposed loops, respectively. Accordingly, the sequences encoding these domains would be expected to give different phylogenetic information. The phylogenetic tree obtained by the maximum likelihood method showed that the 22 consecutive isolates were directly related to the prototype strain. The branching order of the viral sequences showed that all isolates diverged from a common ancestor and constituted a monophyletic group distinct from the control viruses used from the GenBank database. The phylogenetic tree (Fig.14) reconstructed from maximum likelihood distances with representatives from GenBank was similar to the tree obtained by the tree constructed using within representatives (Fig.15). The phylogenetic analysis of the entire VP1-coding sequence showed that genetically all isolates were closely related to one another and were directly related to the Coxsackievirus B3 prototype strain (Fig. 16 & 17), thereby confirming the serotypes (Bailly *et al.,* 2000). All the sequences generated within the UTR region were deposited in the GenBankwith accession numbers from KF 986388 to KF 986409 as given in Table 1.

Amplification of the VP1 region of Coxsackie virus B3 (CVB3) (Fig. 12 and 13) could be accomplished with all 22 viral lysates. The essential function of the capsid is to protect the functional integrity of the viral RNA when the virion is outside the host cell and to initiate the infectious process when a receptor on a suitable host cell is encountered (Cliver, 2009). The sequence identity of the enterovirus VP1 gene has been shown to correlate very well

with the serotype concept. Enterovirus molecular typing methods rely on sequencing of the VP1 genomic region for clustering of VP1 sequences within a homologous serotype (Thoelen *et al.,* 2004). The biosynthesis of RNA viruses occurs in the cytoplasm. The capsid proteins of RNA viruses are also distributed in the cytoplasm to assemble the progeny virions (Wychowski *et al.,*1985). CVB3 genome is a single-stranded RNA of 7.4 kb with positive polarity (Lindberg *et al.,* 1987). With only one open reading frame, CVB3 genome encodes a large polyprotein, which is cleaved into the mature viral proteins by viral proteinases 2A and 3C (Lindberg *et al.,* 1987; Sean and Semler, 2008). CVB capsid is composed of four structural proteins, VP1, VP2, VP3, and VP4. The VP1 protein, which locates on the surface of capsid is the main neutralization agent of the virus (Muckelbauer and Rossmann, 1997). VP1 protein not only is a structural unit of the capsid, but also involves in the viral pathogenesis. A single amino acid mutation in the VP1 protein can significantly change the plaque phenotype of CVB3. Mutations in the 5' untranslated region and VP1 - coding sequence can attenuate the cardiovirulence of CVB3 (Cameron-Wilson *et al.,* 1998). Single amino acid changes within the exposed region of the VP1 protein are reported to change the cytolytic and apoptotic abilities of CVB2 (Gullberg *et al.,* 2010). These findings indicate that the VP1 protein plays a critical role in the pathogenesis of CVB.

Molecular epidemiological characteristics are necessary to understand the impact of Coxsackievirus B3 (CV-B3) infection. The CV-B3 has evolved and circulated for over the past 60 years which consists of nucleotide sequence, the VP1 which is highly variable, amino acids were relatively conserved within the same genotype of CV-B3 (Chu *et al.,* 2010). All the

lysates were amplified and identified by sequencing, showing varied relations of diversity which were evident with the Bootstrap analysis done using 1000 replicates.

DNA barcoding for vectors was performed from which virus was isolated. The Genomic DNA was successfully extracted using DNA-zol-BD (Molecular Research Center, Cincinnati, OH) following the procedures of Lee *et al*. (2002). Quality of the DNA was confirmed spectrophotometrically by measuring A_{260}/A_{280}. The universal DNA primers, LCO1490 and HCO2198, amplified a 710-bp region of the mitochondrial cytochrome oxidase subunit I gene. Accordingly, the species of mosquito larvae were identified as *Aedesalbopictus* and *Armigeressubalbatus*. The study could provide with the utility of larval identification as that done with Folmer *et al*.(1994) using the primers. The mitochondrial cytochrome C oxidase subunit I (COI) gene.could serve as the uniform target gene for a bio identification system.

The study revealed that the viruses isolated from the mosquito larvae of *Aedesalbopictus* and *Armigeressubalbatus* were Coxsackie B3. It has to be pointed out that the mosquito larvae were collected from small quantity of water in used tyres, rubber - milk collection pots, discared pots etc. The question remains how enteric viruses could be isolated from mosquito larvae reared in a little volume of water. In general, for concentrating enteric viruses 100litres of water are required. In its place the enteric viruses could be isolated from mosquito larvae collected from a little volume of water in to appropriate cell cultures. It is hypothesized that the little quantity of water in the pots and tyres might have got contaminated from bird droppings or from terrestrial human sources. As mosquito larvae were filter feeders they could accumulate

the virus in the intestine where it multiplied in the epithelial lining as evidenced by their multiplication in C6/36 cell line. If it is so the mosquito larvae could be used as an indicator system for the detection and isolation of enteric viruses especially Coxsackie virus B3 from a region.

Enteroviruses are responsible for a wide spectrum of diseases posing greater public health hazards. They cause disease of varying severity ranging from minor febrile illness to severe and potentially fatal diseases, such as acute hemorrhagic conjunctivitis, aseptic meningo-encephalitis, and acute flaccid paralysis while higher implications are due to herpangina and myocarditis. Enteric viruses are considered as one of the major causes of gastrointestinal illnesses such as diarrhoea and gastroenteritis in humans. In addition, they are also known to be associated with respiratory tract infections, conjunctivitis, and hepatitis. Occasionally the infection leads to severe neurological disorders or other diseases, e.g. meningitis, encephalitis, pleurodynia, myocarditis, conjunctivitis or severe systemic infections in neonates and are also thought to have a pathogenetic relationship to dilated cardiomyopathy. Since even low infectious doses can lead to elevated public health risks, it is very much important to continue surveillance and also efforts to develop and improvise methods of detection of infectious enteric viruses The potential application of Enterovirus as a source of water quality in different potable water systems had not been fully studied and hence the potential of epidemics in the environment could not be dealt with. These viruses can survive for long periods of time in the environment through contaminated or insufficiently treated tap water, and private wells. Enteroviruses exist as a dynamic mutant population termed as quasispecies corresponding to a swarm of sequence variants due to the absence of proofreading activity, the misinsertion rate by the 3D polymerase is high,

and mutations accumulate during each course of replication. Genetic exchanges between enteroviruses can give rise to new viral genotypes that may be extremely virulent and impose greater risk for public health. The samples were collected from Pathanamthitta district of Kerala in which the sampling sites were supported by thick vegetation with least number of households. The possibility of enteric viruses circulating in the plantations might be an indication of the quality of the potable water in the region. However, the mosquito larvae as a source of natural enrichment for enteric viruses has to be seriously considered. The study could be utilized possibly in three ways such as; for determining the quality of potable water systems, the circulating viruses in the environment and as a means of utilizing the mosquito menace as a biological concentrator for viruses circulating in the environment. Considering an urban and a rural environment, the urban areas will be thickly populated with least vegetation and maximum possible pollution throughout all sources in the environment. However, the rural areas will be least populated with highly supported vegetation and hence environmental pollutants are lesser to a some extend with improved sanitation. The samples were isolated from the rural areas where environmental pollution happened to be least which demands greater scientific studies. Moreover, since the isolated viruses are RNA viruses, the mutation inherent in the translation of the genome will result in dissemination through novel or unidentified modes which were practically known to be impossible such as its entry as a zoonotic pathogen might enhance the disease spectrum implicating future public health hazards. Hence surveillance for the circulating viruses in the environment should strictly be carried out in adherence with the public health.

The present study points out three pertinent factors:

1) Mosquito larvae may be used advantageously for detecting enteric viruses in a region through Integrated Cell Culture–PCR (ICC–PCR) instead of filtering hundred litres of water for virus concentration.

2) The region from where the Coxsackievirus B3 could be isolated is contaminated with faeces which warrant attention in adopting adequate measures for sanitation.

3) It could be hypothesized that the virus might be multiplying in the intestinal epithelium of mosquito larvae suggesting the possibility of amplification of enteric virus for easy detection

Table 1 List of Primers Used in the Study

1. Arbovirus

a) Alpha virus

Sl.No.	Primer	Annealing Temp.	References	Product Size
1.	627 F TTTAAGTTTGGTGCGATGATGAAGTC	54	Grywna *et al.*, 2010	456bp
2.	627 R GCATCTATGATATTGACTTCCATGTT	54	Grywna*et al.*, 2010	456bp
3.	628 F-CTATGATATTGACTTCCATGTTCATCCA	56	Grywna*et al.*, 2010	512bp
4.	628 R CTATGATATTGACTTCCATGTTCAGCCA	56	Grywna*et al.*, 2010	512bp
5.	714 F TCTAGACATGGTGGACGGGTCGGAGAG	61	Designed	430bp
6.	714 R CCCGTCATAACTTTGTACGGCGGTCCT	61	Designed	430bp
7.	M2W- YAGAGCDTTTTCGCAYSTRGCHW	58	Pfeffer*et al.*, 1997	434bp
8.	CM3W- ACATRAANKGNGTNGTRTCRAANCCDAYYCC	58	Pfeffer*et al.*, 1997	434bp

b) Flavivirus

Sl.No.	Primer	Annealing Temp.	References	Product Size
1.	Flav100F AAY TCI ACI CAI GAR ATG TAY	58	Maher-Sturgess, 2008	620bp
2.	Flav200R CCI ARC CAC ATR WAC CA	58	Maher-Sturgess, 2008	620bp
3.	630 F- PF3S - ATH TGG TWY ATG TGG YTD GG	55	Moureau*et al.*, 2010	560bp
4.	630 R –PF2Rbis - GTG TCC CAi CCN GCN GTR TC	55	Moureau*et al.*, 2010	560bp
5.	152 F GCTGATGACACCGCCGGCTGGGACAC	57	Kuno*et al.*, 1998	420bp
6.	152 R- AGCATGTCTTCCGTGGTCATCCA	57	Kuno*et al.*, 1998	420bp
7.	715 F- CCCTAGAGGCTGACGTCACCCTC	61	Designed	500bp
8.	715 R - CCCGGTTGTAGGTCAGCATCCAC	61	Designed	500bp
9.	154 F-GCTGATGACACCGCCGGCTGGGACAC	59	Kuno*et al.*, 1998	844bp
10.	154 R- [cFD3]-AGCATGTCTTCCGTGGTCATCCA	59	Kuno*et al.*, 1998	844bp
11.	F-AJUN]-CT (CG)TACGG(CT)(TG)(GA)(AT)CCTAAT	51	Miller *et al.*, 2000	650bp
12.	R-[cCAP]-(AG)TA(CT)TG(CG)-AC(AT)GC(TG)CC(GA)TG(GA)TGCCA	51	Miller *et al.*, 2000	650bp
13.	FG1- TCAAGGAACTCCACACATGAGATGTACT	55	Fulop*et al.*, 1993	958bp
14.	FG2- GTGCCCATCCTGCTGTGTCATCAGCATACA	55	Fulop*et al.*, 1993	958bp

c) Bunyavirus

Sl.No.	Primer	Annealing Temp.	References	Product Size
1.	LCS308C-F 647F CATTTTCCTGGAAACAGGAACAA	52	Kuno*et al.*, 1996	330bp
2.	LCS824V- R 647R AATTTAGAACCTAATTTGAATG	52	Kuno*et al.*, 1996	330bp
3.	SHS305C –648F-CATTTTCCTGGAAACAGGAACAA	56	Kuno*et al.*, 1996	510bp
4.	SHS698V- 648R-TCAGGCTCTTGGCAATGGCCGTC	56	Kuno*et al.*, 1996	510bp
5.	F[BCS82C]ATGACTGAGTTGGAGTTTCATGATGTCGC	62	Kuno*et al.*, 1996	250bp
6.	Reverse-[BCS332V]-TGTTCCTGTTGCCAGGAAAAT	62	Kuno*et al.*, 1996	250bp

d) Enteric viruses

Sl.No.	Primer	Annealing Temp.(°C)	Reference	Product Size
1.	713 F- CCTACCAATGGTGACATATGTGAAGGATG	53	Designed	620bp
2.	713 R- ATGCGTCCTTTACCAACCTGAGGAAAATC	53	Designed	620bp
3.	**719- ATTGTCACCATAAGCAGCCA**	**40**	Kuan*et al.*, 1997	440bp
4.	**720: ACCTTTGTACGCCTGTT**	**40**	Kuan*et al.*, 1997	440b p
5.	**721: AAGCACTTCTGTTTCCC**	**40**	Kuan*et al.*, 1997	330bp
6.	**722: ATTCAGGGGCCGGAGGA**	**40**	Kuan*et al.*, 1997	330bp
7.	UG52 F- CAAGCACTTCTGTTTCCCCGG	56	Papaventsis*et al.*, 2005	492bp
8.	UC53 R- TTGTCACCATAACCAGCCA	56	Papaventsis*et al.*, 2005	492bp
9.	724- TCAAGCACTTCTGTT	56	Poyry*et al.*, 1996	440bp
10	725 – GTTTCGCTCAGCACTACC	56	Poyry*et al.*, 1996	440bp
11	728 F (292) MIGCIGYIGARACNGG	48	Papaventsis*et al.*, 2005	340bp
12	728 R (222) CICCIGGIGGIAYRWACAT	48	Papaventsis*et al.*, 2005	340bp
13	729 F ACCAGAGCTTGGGTGCCGCG	48	Santti*et al.*, 2000	490bp
14	729 R ACAACACCTTCNCCNCCCAT	48	Santti*et al.*, 2000	490bp

The lysates were identified using the bolded primers

Table 2. Lysates Isolated and Deposited in the GenBank

Sl. No.	Virus Lysate	GenBank accession no.	Region Amplified	Date of Sample Collection
1	MCCV1A	Coxsackie KF986388	5'UTR	20-Jun-07
2	MCCV1B	Coxsackie KF986389	5'UTR	20-Jun-07
3	MCCV1D	Coxsackie KF986390	5'UTR	20-Jun-07
4	MCCV1E	Coxsackie KF986391	5'UTR	20-Jun-07
5	MCCV1G	Coxsackie KF986392	5'UTR	20-Jun-07
6	MCCV1H	Coxsackie KF986393	5'UTR	20-Jun-07
7	MCCV1I	Coxsackie KF986394	5'UTR	24-Dec-07
8	MCCV1J	Coxsackie KF986395	5'UTR	24-Dec-07
9	MCCV1K	Coxsackie KF986396	5'UTR	24-Dec-07
10	MCCV1L	Coxsackie KF986397	5'UTR	24-Dec-07
11	MCCV1M	Coxsackie KF986398	5'UTR	24-Dec-07
12	MCCV1N	Coxsackie KF986399	5'UTR	24-Dec-07
13	MCCV10	Coxsackie KF986400	5'UTR	24-Dec-07
14	MCCV1S	Coxsackie KF986401	5'UTR	18-Apr-08
15	MCCV1T	Coxsackie KF986402	5'UTR	18-Apr-08
16	MCCV1U	Coxsackie KF986403	5'UTR	18-Apr-08
17	MCCV1V1	Coxsackie KF986404	5'UTR	18-Apr-08
18	MCCV1X	Coxsackie KF986405	5'UTR	18-Apr-08
19	MCCV1Y	Coxsackie KF986406	5'UTR	22-Jun-08
20	MCCV1Z	Coxsackie KF986407	5'UTR	22-Jun-08
21	MCCV2A	Coxsackie KF986408	5'UTR	22-Jun-08
22	MCCV2B	Coxsackie KF986409	5'UTR	22-Jun-08

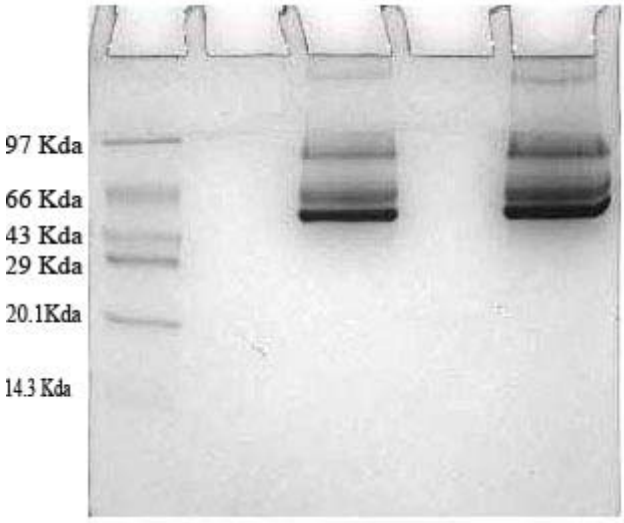

Fig.1 SDS-PAGE of the virus lysate purified by sucrose gradient

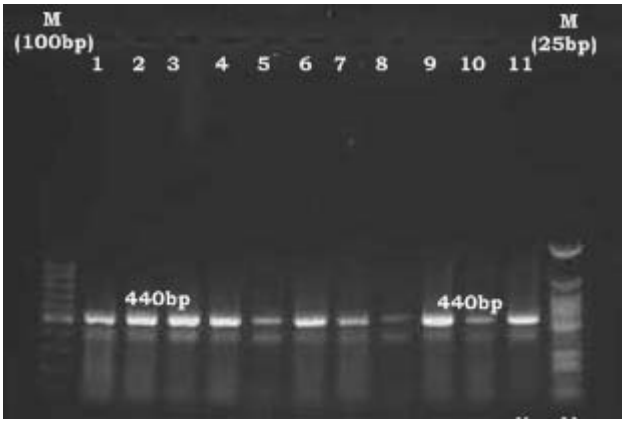

Fig.2 Amplification of UTR region of lysates from MCCV1A- MCCV1N

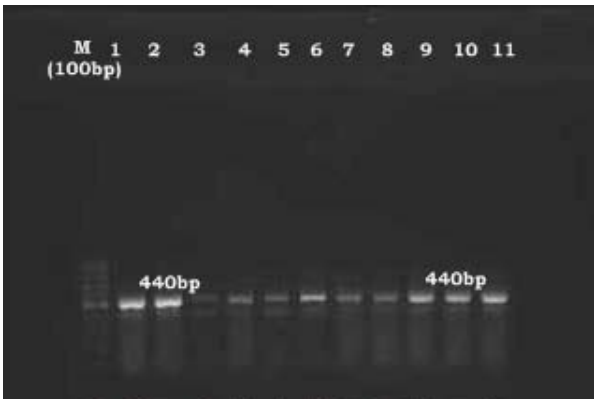

Fig.3 Amplification of UTR region of lysates from MCCV1O- MCCV2B

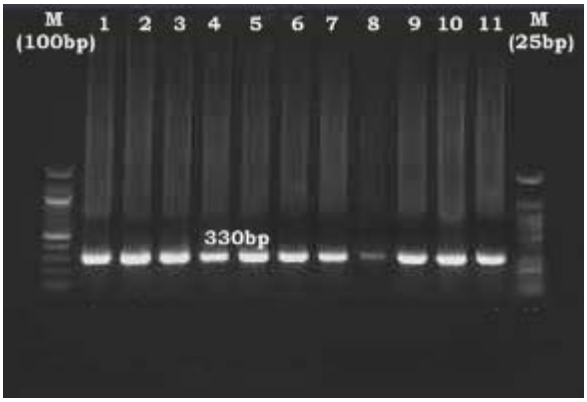

Fig.4 Amplification of nested PCR of lysates from MCCV1A- MCCV1N

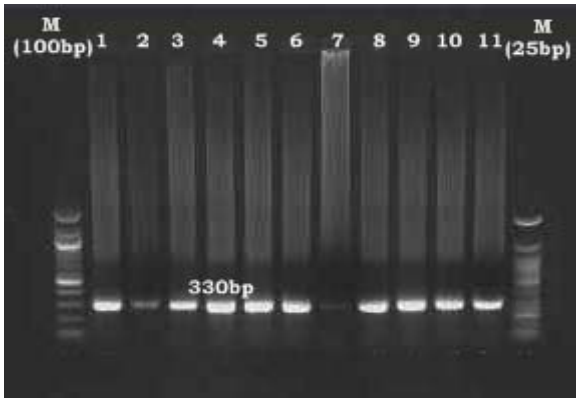

Fig.5 Amplification of nested PCR of lysates from MCCV1O- MCCV2B

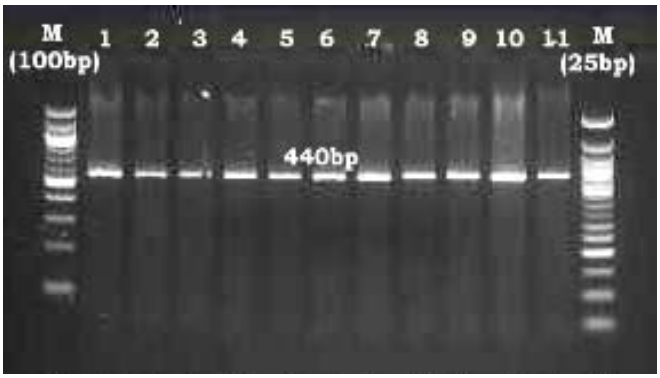

Fig.6 Restriction digestion with *Hha*I of lysates from MCCV1A – MCCV1N

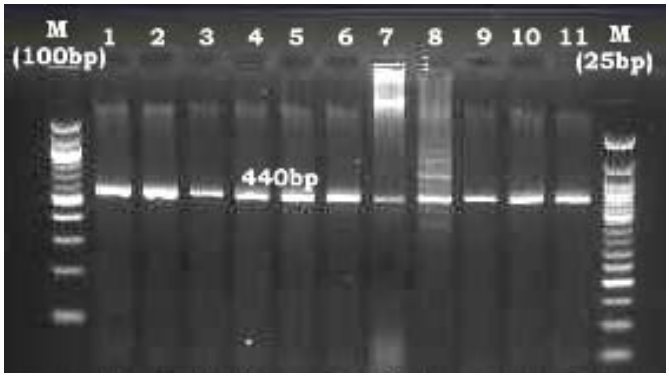

Fig.7 Restriction digestion with *Hha*I of lysates from MCCV1O – MCCV2B

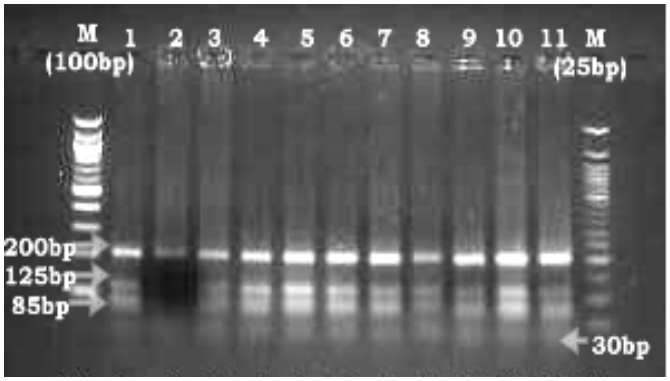

Fig.8 Restriction digestion with BSaJ1 of lysates from MCCV1A – MCCV1N

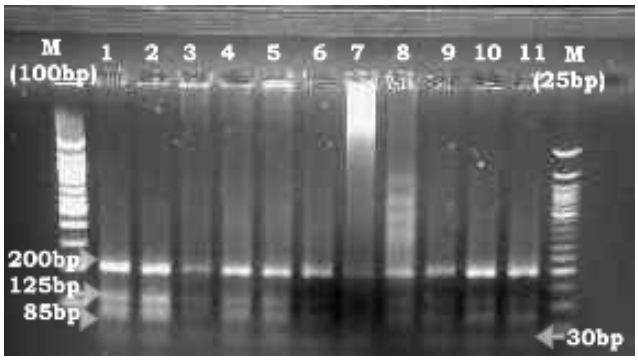

Fig.9 Restriction digestion with BSaJ1 of lysates from MCCV1O – MCCV2B

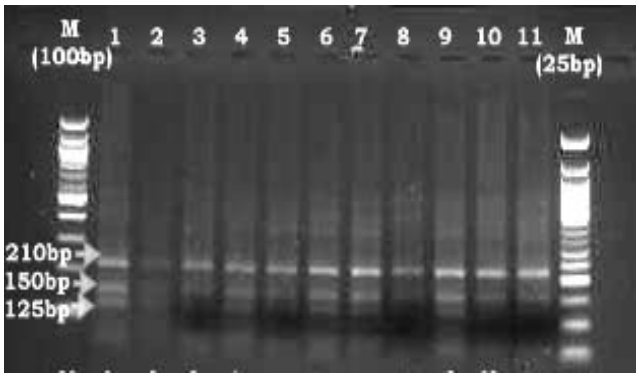

Fig.10 Restriction digestion with *Hpa*II of lysates from MCCV1A – MCCV1N

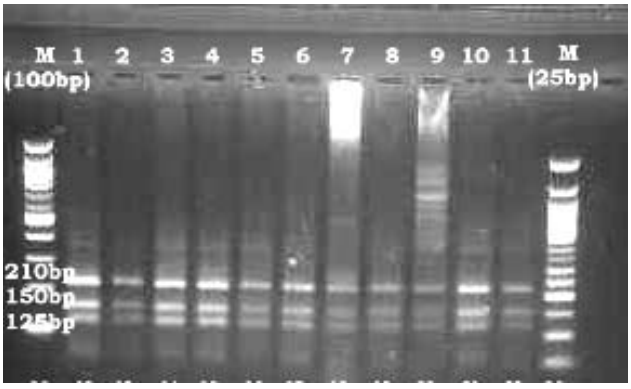

Fig.11 Restriction digestion with *Hpa*II of lysates from MCCV1O – MCCV2B

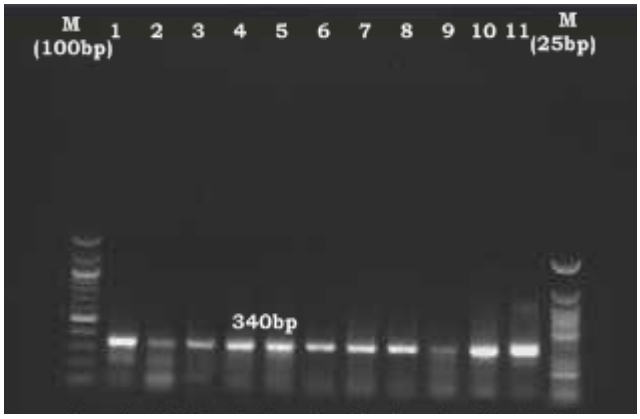

Fig.12 Amplification of VP1 region of lysates from MCCV1A- MCCV1N

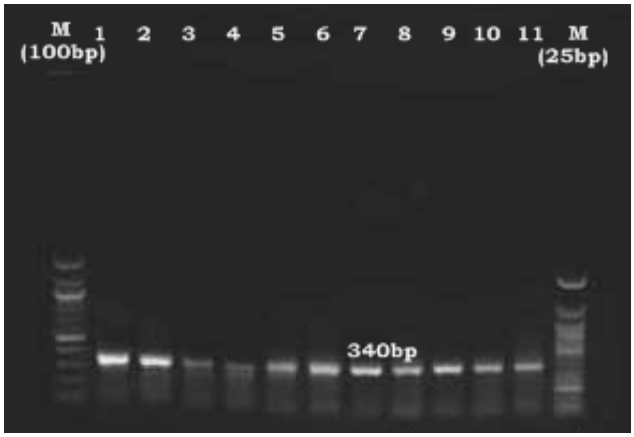

Fig.13 Amplification of VP1 region of lysates from MCCV1O - MCCV2B

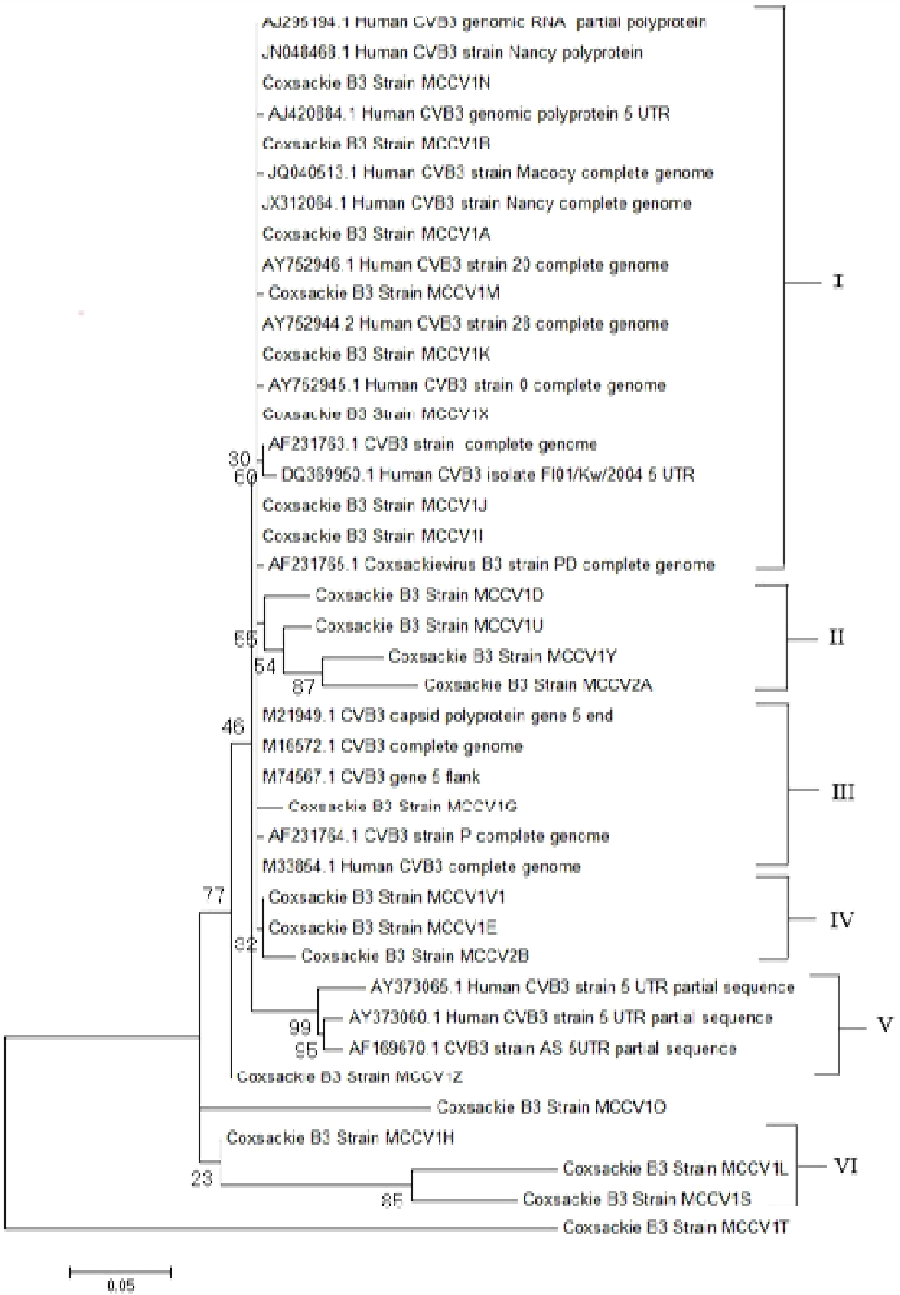

Fig.14 Phylogenetic relationship of UTR of (440bp) 22 CVB3 isolates with reference strains. Pairwise maximum likelihood distances were estimated using Kimura 2-parameter models of nucleotide substitution. The reliability of internal branch trees indicates (percent) how often the corresponding cluster is found among 1,000 intermediate trees.

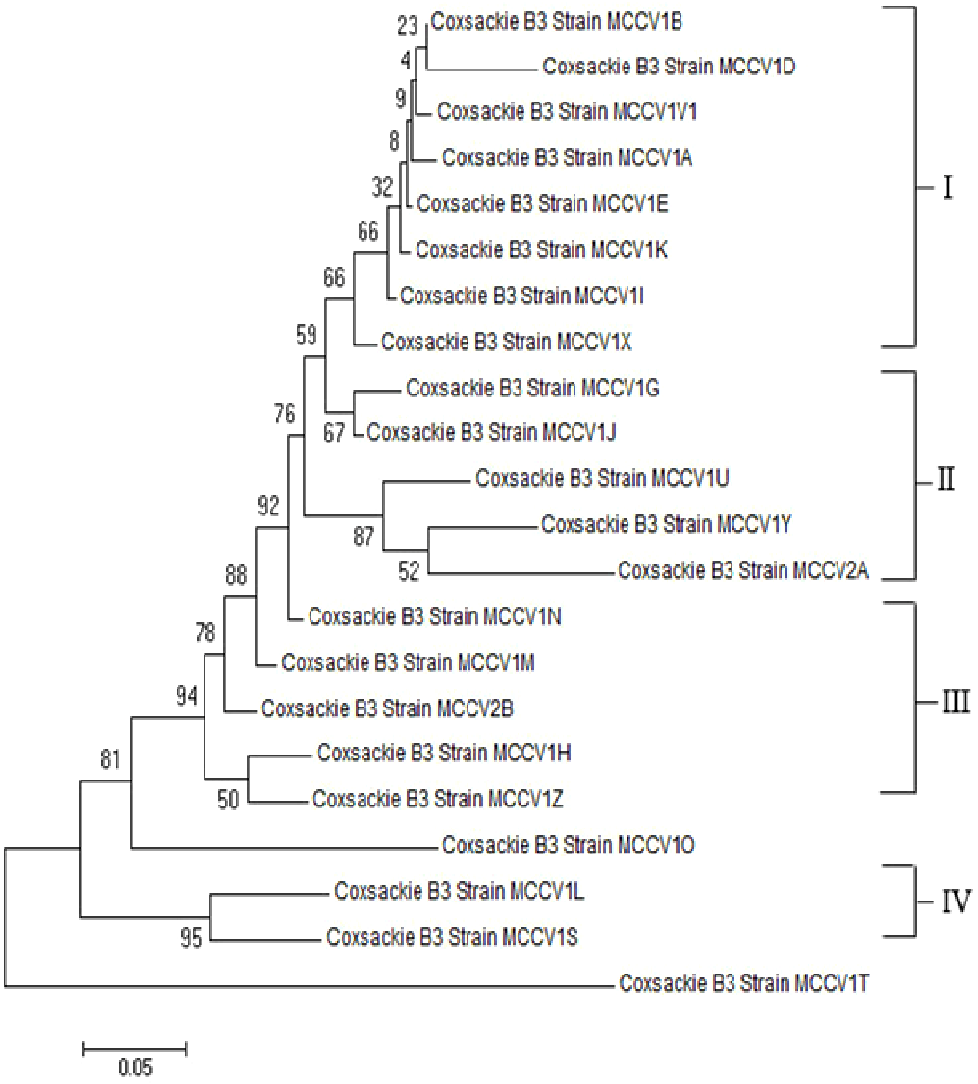

Fig.15 Phylogenetic relationship of UTR of (440bp) among 22 CVB3 isolates . Pairwise maximum likelihood distances were estimated using Kimura 2-parameter models of nucleotide substitution. The reliability of internal branch trees indicates (percent) how often the corresponding cluster is found among 1,000 intermediate trees.

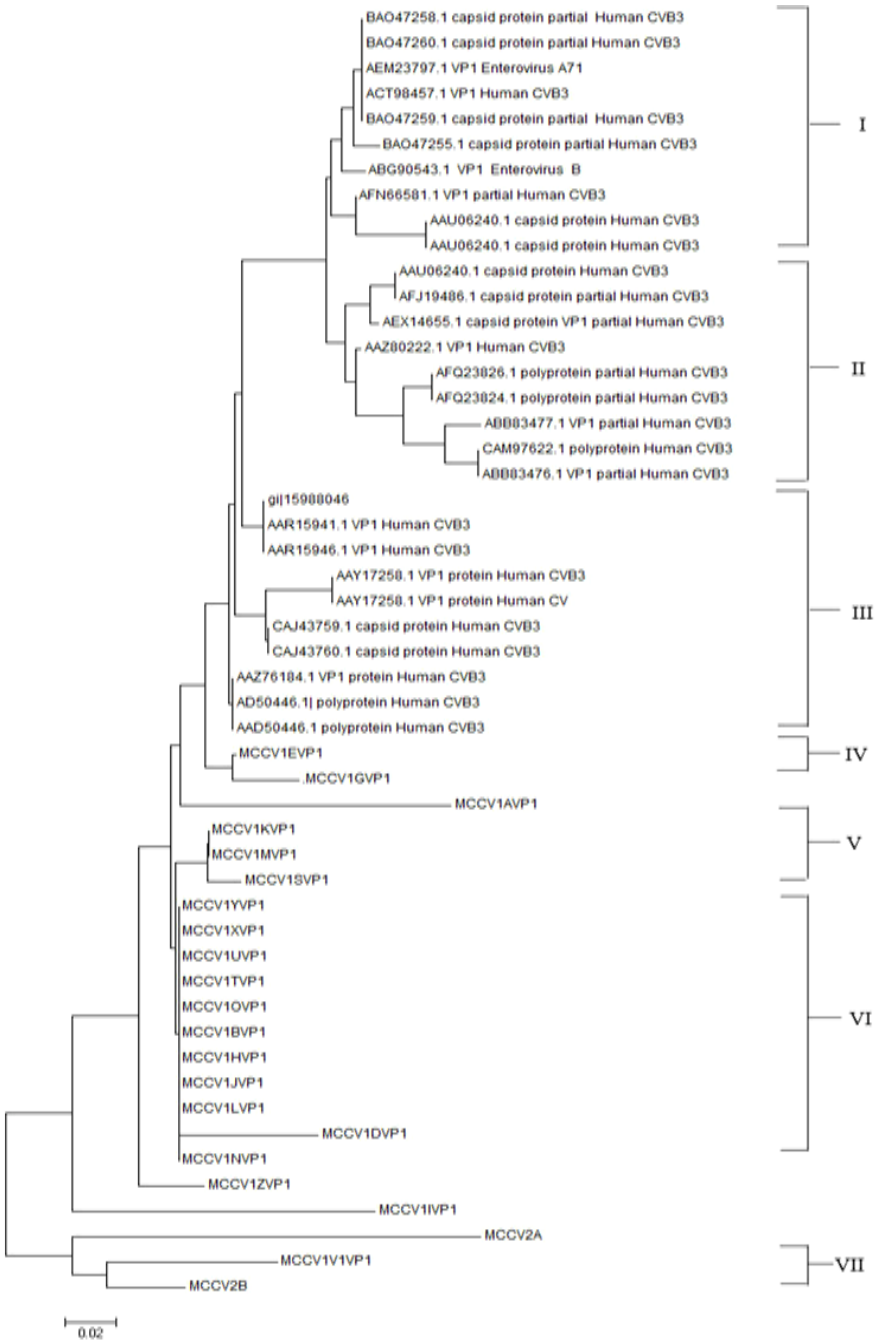

Fig.16 Phylogenetic analysis of 22 CVB3 based on 340bp aa of VP1 sequences with the reference strain using MEGA 5.2, which was used to construct the maximum likelihood tree. Bootstrap values in1000 pseudo-replicates strains designated by accession numbers and the strains generated in the study are given in codes from MCCV1A-MCCV2B.

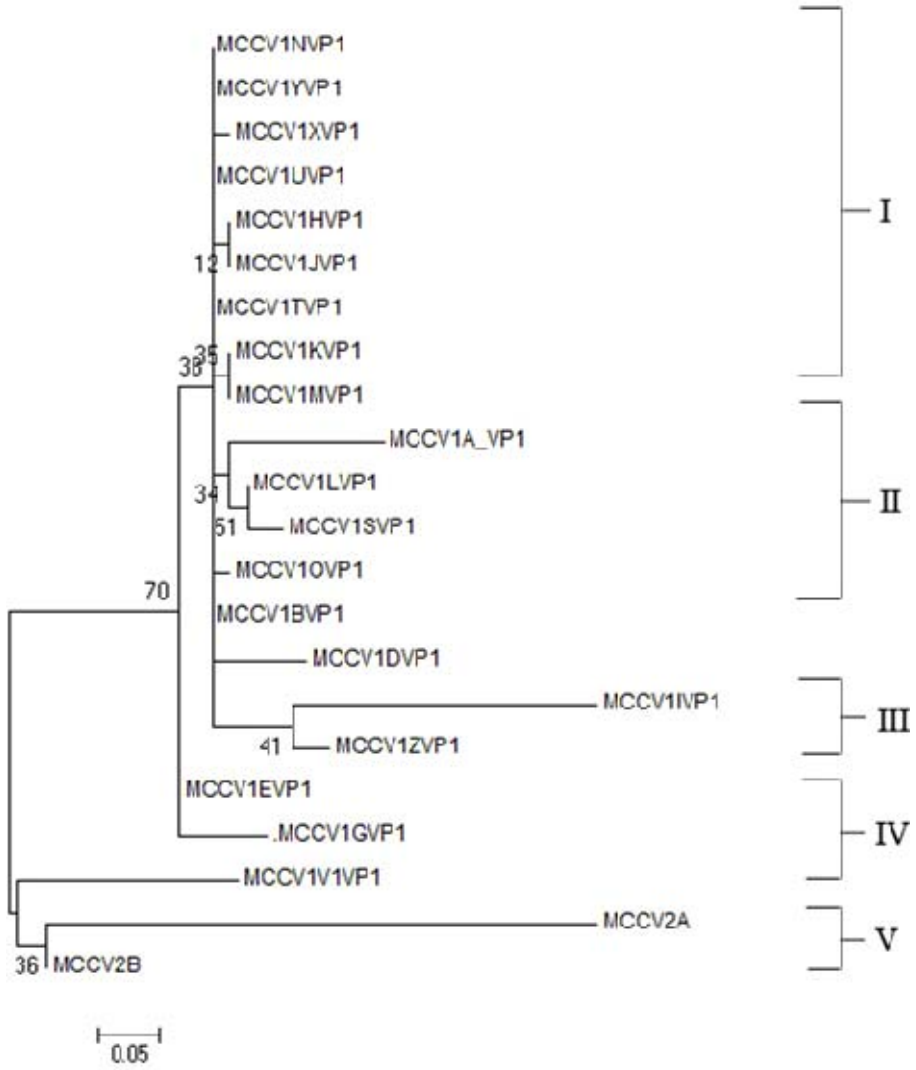

Fig.17 Phylogenetic analysis of 22 CVB3 based on 340bp nt of VP1 sequences using MEGA 5.2, which was used to construct the maximum likelihood tree. Bootstrap values in1000 pseudo-replicates designated are given in codes from MCCV1A-MCCV2B.

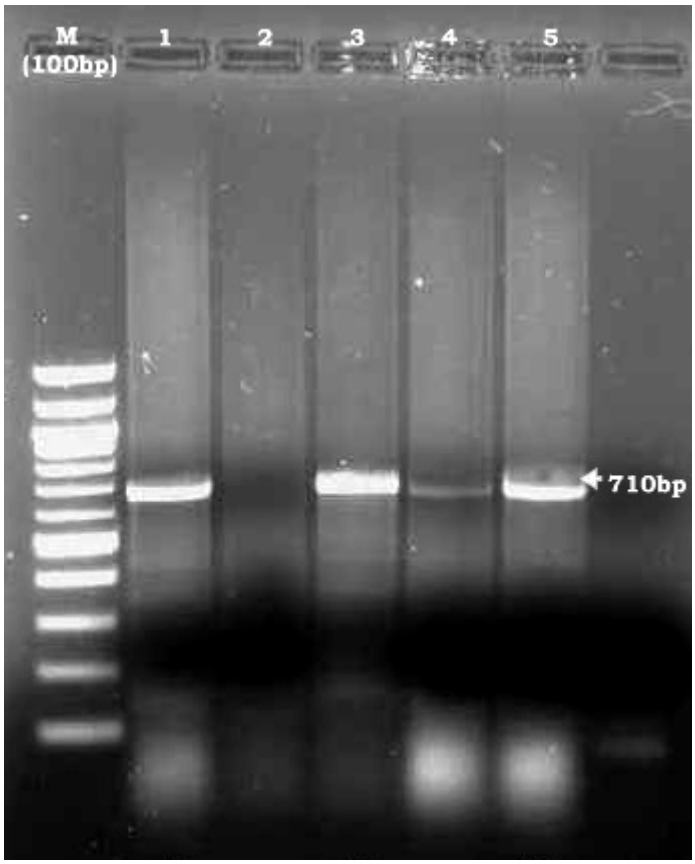

Fig.18 DNA Barcoding of vectors from which the viruses were isolated

Chapter 5

Conclusion and Scope for Further Research

The present study deals with screening of arboviruses as well as enteric viruses in mosquito larvae collected from a specific region in Kerala. Molecular tools were used for screening based on PCR employing several primer sets designed for both the above groups of viruses. In this process amplicons were obtained when screened with a primer set oriented towards enteric viruses which was sequenced and identified as the enterovirus, Coxsakieviruses B3. Among 29 mosquito lysates 22 were identified to belong to the above group with variations.

Human Enteroviruses (HEV) is an important class of virus, disseminated worldwide and transmitted by both fecal-oral and respiratory routes. All the identified human pathogenic viruses that pose significant public health risk in water environments are borne through the faecal oral route. The health impact of these viruses varies; some cause gastroenteritis, respiratory infections, conjunctivitis, hepatitis, aseptic meningitis, encephalitis and paralysis. Moreover, the infective dose can be very low, for instance it has been estimated that approximately 10-100 virions of norovirus are capable of causing human infection. Thus even the presence of a few viral particles poses a threat to public health. Their pathological significance as causal agents for

diarrhoeal diseases and their capability to cause illnesses even at low viral loads and their viability in the environment for long periods of time and tolerance to changing environmental conditions make this group a serious pathogen. Despite the rapid progress in water and wastewater treatment technology, waterborne diseases still have public health and socioeconomic implications in both developed and developing countries.

Objectives of the study were as follows:

1) Screening for viruses from mosquito larvae: isolation and characterization in various cell lines.

2) Enumeration and electron microscopy of the isolated viruses.

3) Molecular characterization.

Overall accomplishments of this work are as summarized below:

1) A pool of 29 samples of mosquito larvae were collected from the study area specifically marked using GPS co-ordinates and the location were mapped with the positive representatives with ArcGIS version 9.3 using Google images. The samples were collected in two seasons of a year for two consecutive years and all the samples were piled up from a common ecological background, the rubber plantation where the human inhabitants were found to be lower.

2) The mosquito larval samples were processed by maceration and filter sterilization and stored at -80 $^{\circ}$C until used. They were then subjected to screening with three cell lines such as C6/36, VERO and HEp-2.

3) The primary screening resulted in the isolation of 22 positive viral lysates which could be successfully propagated in all the three cell lines,

even though there were variations in the adaptability /susceptibility among them. The insect cell line (C6/36) resulted in non-cytolytic infection, while the mammalian (VERO) and human cell line (HEp-2) represented cyotolytic infection in all cases studied.

4) The serial passages conducted using the three cell lines were supported with MTT assay and plotted as relative percentage cell death. Study suggested that C6/36 was least adapted, HEp -2 most highly adapted and VERO in between.

5) HEp-2 cell line was subsequently used for further studies as the yield was higher within a short duration. Enumeration of the 22 positive lysates were documented using HEp -2 cell line using dilutions ranging from 1:2 to 1:1024. Lowest dilution resulted in higher percentage cell death which was found to decrease with higher dilution. Probit values were calculated using SPSS platform for windows and found out $TCID_{50}$ which varied widely among the lysates.

6) One step growth kinetics of all lysates were determined in C6/36 employing primarily HEp-2 as the assay system and secondarily using C6/36 itself for the assay. The percentage cell death was found maximum from the 6^{th} hour to 30^{th} hour incubation on average in most of the cases. However, percentage cell death with respect to lysates such as MCCV1N, MCCV1O, MCCV1V1 and MCCV1X was found increasing beyond 48 hours of incubation.

7) Transmission Electron microscopic studies of the viruses revealed them as icosahedral non-enveloped measuring 15-20nm diameter.

8) The virus lysates were non -haemagglutinating in chick erythrocytes.

9) Molecular screening was conducted using primers oriented towards the conserved regions of Arbovirus and Enterovirus genome. No amplification was obtained when the primers of Arboviruses were used. Meanwhile, the 5' UTR region of Enteroviruses could be amplified by way of PCR in all cases studied. On comparing with GenBank data base the virus lystaes were identified as Coxsackievirus B3, which was confirmed through nested PCR.

10) The 5' untranslated region containing cloverleaf and internal ribosome entry site secondary structures, seems to be the most conserved region among enteroviruses and is therefore targeted widely in enterovirus diagnostic procedures.

11) The amplified regions were sequenced and the 22 lysates were deposited in Gen Bank with individual accession numbers.

12) VP1 capsid is the major surface-accessible protein in enteric viruses which encodes several important serotype-specific neutralization epitopes, and it was subjected for PCR amplification resulting in the generation of 340 bp amplicons.

13) They were sequenced and compared using BLAST search algorithm and found matching 99% with GenBank database, and deposited in GenBank with individual accession numbers.

14) The results of the RFLP analysis with H*haI* on 5'UTR confirmed the absence of cleavage and hence supported to be placed the viruses in Cluster I as per the classification of Kyriakopoulou *et al.*, 2013 which included Coxsackievirus B3.

15) The restriction digestion with the enzyme B*sa*JI proved to distinguish all six serotypes of Coxsackie B viruses.

16) The restriction digestion with H*paII* resulted in three products matching with that of the reference strain of Coxackievirus (B1-B6).

17) Phylogentic tree and clustering of the lysates were studied by analysing the partial VP1 and UTR nucleotide sequences of the 22 isolates. The phylogenetic relationships of the 22 sequential isolates of Coxackievirus B3 were constructed by using the sequences from the 5'UTR regions and the VP1-coding sequence.

18) The phylogenetic tree obtained by the maximum likelihood method showed that the 22 consecutive isolates were directly related to the prototype strain. The branching order of the viral sequences showed that all isolates diverged from a common ancestor and constituted a monophyletic group distinct from the control viruses used from the GenBank database. The phylogenetic tree were reconstructed from maximum likelihood distances with representatives from GenBank was similar to the tree obtained by the tree constructed using within representatives.

19) The phylogenetic analysis of the entire VP1-coding sequence showed that genetically all isolates were closely related to one another and were directly related to the Coxsackievirus B3 prototype strain thereby confirmed their serotypes.

20) Based on barcoding using 5' region of the mitochondrial cytochrome C oxidase-I (CO-I) gene the vectors from which the virus lysates were obtained have been identified as *Aedus albopictus* and *Armigeres subalbatus*.

21) It has been concluded that mosquito larvae could be used for monitoring Enteroviruses from a region through Integrated Cell Culture - PCR (ICC-PCR) approach.

Scope for Future Research:

1) Sudden onset of infection of cells within 6hrs post inoculation helps to explore the viral progression as a suitable model for mapping viral events starting from attachment to the host to the stage of release of mature virion.

2) Comparative progression in different cell lines and their susceptibility at different intervals along with blocking of the viral sequences in the events of antiviral studies.

3) Interaction of the viruses with the mosquito larvae, either as symbionts or mutualists and their progression in the midgut of the larvae.

4) Use of mosquito larvae as source of enteric viruses for monitoring faecal pollution in a region.

5) The mutation accompanied in the RNA polymerase due to lack of proof reading mechanisms from the point of attachment to the events of release of mature virion and the amino acid substitution in the VP1 proteins.

6) Role of other genes such as VP2, VP3 and VP4 in cytopathogenesis.

7) Whole genome sequencing of the virus lysates.

8) Studies involving animal models to reveal their pathogenicity.

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