UNIVERSITY OF NAIROBI

CHARACTERIZATION OF CIRCULATING COXSACKIEVIRUSES IN KENYA IN 2007

By Johnstone Otieno Omia
Bachelor of Education Science (Honors)
Egerton University
Reg. No: I56/P/9282/05

A thesis submitted in partial fulfilment of the requirements for the award of Master of Science Degree in Health and Environmental Biotechnology at the Center for Biotechnology and Bioinformatics (CEBIB), University of Nairobi.

2020

DECLARATION

I declare that this thesis is my original work and it has not been presented elsewhere for examination, the award of a degree in any other university or publication. Where other people's work or my work has been used, this has properly been acknowledged and referenced per the University of Nairobi's requirements.

Signature: ______________ Date 30/11/2020

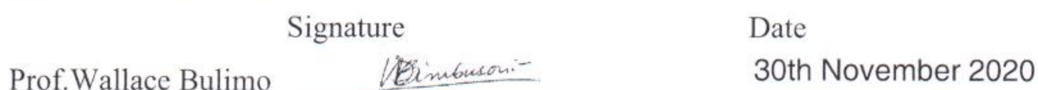
Name: Omia, Johnstone Otieno

Reg. No: I56/P/9282/05

Centre for Biotechnology and Bioinformatics (CEBIB)

University of Nairobi

This thesis is submitted with the approval of the undersigned supervisors for research leading to the award of Master of Science in Health and Environmental Biotechnology.

	Signature	Date
Prof.Wallace Bulimo		30th November 2020

Department of Biochemistry

University of Nairobi

Dr. Edward Muge		30/11/2020

Department of Biochemistry

University of Nairobi

Dr. George Obiero		

Center for Biotechnology and Bioinformatics

University of Nairobi

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LIST OF ABBREVIATIONS AND ACRONYMS

BGMK	Buffalo green monkey kidney
BLAST	Basic local alignment search tool
BSL4	Biosafety level 4 laboratory
cDNA	Complementary DNA
CPE	cytopathic effects
CsCl	Cesium Chloride
CV	Coxsackievirus
CV_A	Coxsackievirus species A
CV_A1	Coxsackievirus serotype A1
CV_B	Coxsackievirus species B
CV_B1	Coxsackievirus serotype B1
CVDNA	Coxsackievirus DNA
CVIs	Coxsackievirus infections
D1-D5	Domains1-5
DAES	Datamonkey Adaptive Evolution Server
DAF	Decay accelerating factor
DEID	Department of Emergency and Infectious Disease
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
SERU	Scientific Ethics Review Unit
ENA	European Nucleotide Archive
FAT	Fluorescein Conjugated Antibody
FBS	Fetal Bovine Serum
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
HeLa	Henrietta Lacks
HFMD	Hand, foot and Mouth Disease

ICTV	International Committee on Taxonomy of Viruses
IDDM	Insulin-Dependent Diabetes Melitus
IFA	Immunofluorescent Assays
IFAT	Indirect Fluorescein Conjugated Antibody Test
IgSF	Immunoglobulin-like Superfamily
ILIs	Influenza- like-illnesses
IRB	Internal Regulatory Board
LRT	Likelihood Ratio Tests
mg/ml	Milligrams per Milliliter
MMLV	Mouse Moloney Leukemia Virus
MRCA	Most Recent Common Ancestor
NCBI	National Center for Biotechnology and Information
NIC	National Influenza Center
NP	Nasophryngeal
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PCVIs	Prevalence of CVIs
pMol	Picomoles
RD	Rhabdomyosarcoma
RdRP	RNA-dependent RNA polymerase
SCVIs	Secondary Coxsackievirus Infections
SP	Seroprevalence
ssRNA	single stranded Ribonucleic Acid
USAMRD-K	United States Army Medical Research Directorate–Kenya
VCVS	Virulent Coxsackievirus Strains
VP1-4	Viral proteins 1, 2, 3 and 4
VPg	Viral peptide g

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ABSTRACT

Coxsackieviruses (CVs) infect humans and cause various Coxsackievirus infections (CVIs). CVs are classified in *Picornaviridae* family, genus *Enterovirus* and are of species A and B. Currently, CVIs have no specific cure but are managed by various methods. Unmanaged CVIs may be fatal. In 2007, a high prevalence of influenza-like illnesses (ILI) was detected by clinicians engaged to carry out ILI survey in selected regions of Kenya by the US Army Medical Research Directorate Kenya (USAMD-K). Twenty-two (22) out of 2925 nasopharyngeal samples collected from patients were positive for CVIs through IFA procedure. Whereas CVs caused some of the ILIs, the prevalence of CVIs, the identity of the CVs and other virologic characteristics were unknown. To begin to address this knowledge gap, this study was carried out with the main objective being to serologically and molecularly characterize CVs in the 22 samples to determine the prevalence of CVIs, species, serotypes, the phylogeny, and the evolution of the CVs to relate the data to ILIs of 2007. The CVs were multiplied in Rhabdomyosarcoma (RD) cells, their RNAs extracted and their partial VP1 genes amplified and sequenced. Twelve (12) CVs of CV_B species; serotypes CV_B2-CV_B5 were identified, 11NS were assigned accession no.s MH522779 - MH522789 and uploaded on GeneBank servers on 10th March 2019. Seroprevalence nice actual prevalence of CVIs were 0.74% and 0.41% respectively. All the CVs had mutations and only of CVs of serotypes CV_B2 and CV_B3 had evolved divergent VP1 proteins with high infectivity that partly caused the high prevalence of ILIs in 2007. Data from this study enriches the information base on CVs in Kenya and can be used to develop diagnostics and therapeutics against CVIs.

CHAPTER 1.0 INTRODUCTION

1.1.0 Background information

Coxsackievirus (CV) is a common human virus that infects children under the age of sixteen years as well as adults, especially those with weak or compromised immune systems. Coxsackievirus infections (CVIs) cause a wide range of illnesses which can be mild or severe. Mild CV illnesses include common cold, sore throat, tonsillitis and headache while severe illnesses include myocarditis, encephalitis, meningitis, pancreatitis, hepatitis, cancer of the pancreas and paralysis (Zeng et al. 2015).

Severe CV illnesses occur when CVs are carried in lymph and blood and infect vital body organs, a process referred to as secondary infection. Secondary infection of body organs by CVs cause serious, sometimes fatal complications (Steinke et al. 2013). Coxsackieviruses can also infect pregnant women and spread to their fetuses resulting in abortions, fetal myocarditis stillbirths (Ornoy and Tenenbaum 2006).

Infections with CV species are known to occur all over the world but are more prevalent in people living within tropical and sub-tropical regions of the world (Ornoy and Tenenbaum 2006). In temperate regions, CVIs are prevalent during warmer months of the year. The incubation period varies vary greatly depending on the type of infecting CV species or serotype, type of illness caused as well as the immune status of the patient. However, more than 90% of CVIs are mild and can be managed easily using commonly available medical procedures and treatments (Tao et al. 2012).

People infected with CVs may be asymptomatic or may have nonspecific febrile illnesses. Symptomatic individuals often show a variety of symptoms which range from

mild to more severe, life-threatening and occasionally fatal ones. People with mild CV illnesses show symptoms that resemble those of influenza disease including high fever that lasts for about 3 days, headache, muscle aches, sore throat and abdominal discomfort. Coxsackieviruses also cause syndromes such as non-specific febrile illnesses characterized by fever, headache, joint and muscle aches as well as general body weakness (Harvala et al. 2003).

Other illnesses caused by CVIs include hand, foot and mouth disease (HFMD) and Herpangina. Hand, foot and mouth disease is characterized by painful red blisters in the throat and on the tongue, gums, hard palate, inside of cheeks, and on the palms of hands as well as soles of feet. Herpangina, an infection of the throat which causes red-ringed blisters and ulcers on the tonsils and soft palate as well as on the fleshy back portion of the roof of the mouth (Pond and WHO 2005).

Besides, CVs cause hemorrhagic conjunctivitis, an infection of the whites of the eyes which begins as a pain in the eye. The infected eye becomes red, watery, sensitive to light and swells. This results in blurred vision and may cause blindness. Coxsackieviruses can cause more serious infections that may need hospitalization such as viral meningitis, encephalitis and myocarditis (Santti et al. 2000). Newborn children are more susceptible to serious symptoms of CVIs which often develop within 2 weeks after birth. This could be attributed to their developing immune systems (Pond and WHO 2005).

There are two main species of CVs which are designated as Coxsackievirus A (CV_A) and Coxsackievirus B (CV_B). Coxsackieviruses of CV_A species has twenty-three (23) distinct serotypes designated as CV_A1- CV_A22 and CV_A24 while CV_B spec s has six distinct serotypes designated as CV_B1- CV_B6. Both CV_A and CV_B

species enter the body through epithelial cells of the skin and mucous membranes of the alimentary canal (Yin-Murphy and Almond 1996).

Generally, CV_A species cause less severe illnesses while CV_B species cause more serious illnesses especially when they enter blood and tissue fluid where they are carried to different body organs (Tao et al. 2012). Coxsackieviruses transported in blood and lymph cause secondary Coxsackievirus infections (SCVIs) of vital body organs such as the heart, pleura, pancreas, liver, the brain and the spinal cord (Sin et al. 2015).

Secondary Coxsackievirus infections of vital body organs can result in serious complications such as pleurodynia, myocarditis, pericarditis, hepatitis, pericardial effusion, muffled heart sounds and abnormal pulses, pancreatitis and insulin-dependent diabetes (IDDM) (Jaïdane and Hober 2008). The complications associated with SCVIs can be fatal if not well managed using appropriate medical procedures and treatments.

Species of CV were first discovered faecal specimens of children who were clinically diagnosed to be suffering from polio by Dalldorf in 1948 when he was searching for a cure for poliomyelitis (Tao et al. 2012). Dalldorf discovered that CVIs in humans cause symptoms that are similar to those shown by patients suffering from non-paralytic polio (Tao et al. 2012). Coxsackieviruses are similar to other picornaviruses but can be distinguished serologically by use of monoclonal antibody tests as well as by the use of their VP1 gene encoding their immunogenic Viral Protein 1(VP1) (Yin-Murphy and Almond 1996).

Morphologically, each CV has an icosahedral shape with no viral envelope. It is about 25-30 nm in diameter and contains a linear ssRNA genome of positive-sense whose size is about 7.4 Kb. The CV RNA genome is covalently bonded to viral peptide VPg at

the 5’ end and polyadenylate (PolyA) tail at the 3’end (Lincez, Marine, and Horwitz 2010).

The genome contains an open reading frame which encodes a large polyprotein that is cleaved by proteases to produce eleven (11) CV proteins comprising of four (4) viral capsid proteins (VP1–VP4) and seven (7) functional or non-structural proteins VPg, proteases and RNA dependent RNA polymerase (Lincez 2010). Infections of the human body cells by enteroviruses as well as CVs present similar symptoms. Enteroviruses and CVs are thus classified in the family Picornaviridae and genus Enterovirus (Yin-Murphy and Almond 1996).

Some CVIs can be diagnosed differentially by use of their clinical symptoms. For example, H FMD presents with blisters or ulcers on the hand, foot and mouth of a patient (Abedi et al. 2018). However, clinical diagnoses of CVIs may not be relied upon as some of the symptoms these infections may change with the onset of new outbreaks and the fact that some symptoms are similar to those of other viral diseases such as influenza, measles and polio. Thus, confirmatory laboratory diagnoses are usually recommended (Martha 2018).

These diagnoses involve the culture of CVs in suitable cell lines such as mouse c Blue-green monkey kidney (BGMK), Henrietta Lacks (HeLa) cells or Rhabdomyosarcoma (RD) cells and observation of cytopathic effects (CPE) 2-6 days after inoculation (Martha 2018).

Coxsackieviruses can also be diagnosed using serological tests involving the use of specific monoclonal antibodies that identify their species and serotypes (Pattison 1983).

Molecular diagnosis involving CV VP1 gene nucleotide sequence comparisons with those of their prototypes at GeneBank is the confirmatory diagnosis for CVs (Martha 2018). Samples used for confirmatory laboratory diagnoses are obtained from patients' throat, faeces or body fluids like saliva, blood or cerebrospinal fluid (Martha 2018).

Currently, there is no cure or vaccine against CVIs. Mild CVIs may subside within ten days in people with strong immune systems (Lydyard et al. 2010). However, in some people, especially those with weak immune systems, serious CVIs may require management of severe symptoms to prevent severe illness that may cause complications or even death (Pond and WHO 2005). The management involves the use of anti-inflammatory drugs such as diphenhydramine, use of pain relievers such as Acetaminophen, regular administration of insulin in case of diabetes mellitus as well as correctional surgical procedures including organ transplants (Yi et al. 2017).

Characterization of circulating CVs in selected regions of Kenya in 2007 using serology and molecular methods was performed to determine the seroprevalence and the actual prevalence of CVIs, identifying the species and serotypes of CVs as well as establishing phylogenetic and evolutionary relationships through comparisons of their VP1 gene nucleotide sequences. Bioinformatics analyses of the VP1 gene NS would provide an insight into factors that led to increased pathogenicity as well as other factors that are attributed to their spread. This would enhance management and prevention of CVIs. Moreover, the nucleotide sequence data obtained would enhance understanding of CVs in Kenya for future preparedness in case of outbreaks.

In 2007, medical personnel working for the US Army Medical Research Directorate Kenya (USAMD-K) on ILI surveillance program in selected regions of Kenya, detected a high prevalence of ILIs in 2925 samples from patients in study sites. These regions are located on either side of the equator or are close to the equator and have warm and occasionally humid weather conditions which are favourable for replication, transmission and infectivity of CVs (Khetsuriani et al. 2006).

Moreover, these regions are inhabited by a significant proportion of the population of Kenya which is currently 47.21 million people. Western Kenya has a population of 4.334 million people, Kenya Highlands have more than 12 million people, Central Kenya has 4.384 million people, North Eastern Kenya has 2.311 million people and the Coast of Kenya has 3.325 million people (Kenya_National_Bureau_of_Statistics 2019).

Majority of the inhabitants of these regions have low incomes and many of them especially those in urban slums in live crowded houses with poor sanitation. They also have limited access to proper medical care and the majority of them have weak immune systems due to malnourishment. This is because many of them cannot afford regular balanced diets or they suffer from diseases that weaken their immune systems such as HIV/AIDS and cancer (Macharia, Mureithi, and Anzala 2018). These conditions are also ideal for viability, replication, high rates of transmission and infectivity of CVs (Pond and WHO 2005).

As a result of these factors, some of the patients who presented ILI symptoms were not infected with the influenza virus but were infected with CVs. It is against this background that molecular characterization of CVs that circulated in the selected regions

of Kenya in 2007 was carried to provide useful information that could be used for the formulation of policies and strategies for future outbreak preparedness and management of CVIs by the ministry of health and other players in the health sector.

1.2.0 Statement of the problem

Coxsackievirus infections are a major health concern to humans in many parts of the world as they cause mild, severe and occasionally fatal illnesses. These illnesses have no specific cures. The lack of cures is attributed to the diversity of the causative viruses, many of which have not been fully characterized. Due to this, the US military has established surveillance networks which regularly carry out studies on CVs as well as other viruses which cause ILIs in humans.

A retrospective assessment of the sample records for the year 2007 obtained from study sites located in selected regions of Kenya, showed that out of 2925 samples collected, 22 of them had tested positive for CVIs by indirect IFA procedure.

Whereas the previous findings established that CVs were some of the etiological agents that caused the ILIs in patients from the selected regions in 2007, there existed a research gap to provide information on the seroprevalence and actual prevalence of CVIs in the study regions. Also, information on the identity of the causative CV species, their serotypes, phylogeny, evolutionary relationships and their other virologic characteristic was lacking. Furthermore, it was not clear whether the CVs associated with the ILIs in the selected regions were introductions from other parts of the world or they were just those in circulation before the survey, only that they had evolved into more pathogenic CVs that were easily transmissible.

1.3.0 OBJECTIVES OF THE STUDY

1.3.1 General Objective

To characterize Coxsackieviruses that circulated in selected regions of Kenya in 2007 during influenza-like-illness surveillance.

1.3.2 Specific objectives

1. To determine the seroprevalence and actual prevalence of CVIs among patients with ILIs in selected regions of Kenya between January 1st to December 30th 2007 using serology and molecular methods of characterization of CVs.
2. To identify the species and serotypes of CVs in present in nasopharyngeal samples obtained from patients with ILI from the selected regions of Kenya in 2007 using molecular methods of characterization of CVs.
3. To establish phylogenetic relationships of the characterized CVs using phylogenetic methods.
4. To establish evolutionary relationships of the characterized CVs using evolutionary methods.

1.4.0 Justification and significance of the study

Coxsackieviruses infect young children, adults and even the unborn babies causing a wide range of mild and severe illnesses (Abedi et al. 2018). Most secondary Coxsackievirus infections of vital body organs are fatal (Cree et al. 2003). Coxsackievirus infections can be therefore considered as serious health and economic threat to humanity.

Currently, CVIs have no cure. The lack of cure is largely attributed to the fact that CVs are diverse and that most of them have not been fully characterized (Khetsuriani et al. 2006). Besides CVs are known to undergo mutations and recombinations which result in mutants that cause more disease as they become more virulent and easy to spread (Oberste, Peñaranda, and Pallansch 2004).

It is for this reason that this study was undertaken to determine the prevalence of CVIs, identify the causative species and their serotypes, their phylogenies, their evolutionary relationships as well as establish factors that may have caused easy spread and a high prevalence of CVIs between January 1st and December 30th 2007.

The information obtained from the study would be useful in guiding policy formulation and development of CVIs management strategies for surveillance purposes, future emergency preparedness in case of outbreaks and also for the development of molecular diagnostics by the stakeholders in the health sector. The nucleotide sequence data of the molecularly characterized CVs was be deposited in the GeneBank to contribute to the knowledge base on CVs in Kenya and the world.

CHAPTER 2.0 LITERATURE REVIEW

2.1.0 The Coxsackieviruses

2.1.1 Taxonomy of Coxsackieviruses

Coxsackieviruses are classified in a family of small viruses known as *Picornaviridae* and genus *Enterovirus* (Yin-Murphy and Almond 1996). They are closely related in structure and chemical characteristics to other enteroviruses such as poliovirus, echovirus and rhinoviruses (Cohen et al. 2001).

The two main species of CVs are Coxsackievirus species A (CV_A) and Coxsackievirus species B (CV_B). Serologically, there are 23 serotypes of CV_A designated as CV_A1-CV_A22 and CV_A 24 and six serotypes of CV_B designated as CV_B1-CV_B6 (Peci et al. 2014).

2.1.2 Epidemiology of Coxsackievirus Infections

Species of CVs were first discovered in Coxsackie town, New York in the USA by Dr. Dalldorf amongst children who were clinically diagnosed to be suffering from polio (Tao et al. 2012). Since their discovery, CVs have been identified in many parts of the world and are known to infect people of all races and age groups (Khetsuriani et al. 2006). However, CVs are known to mostly affect children under the age of sixteen years. People who live in warm climates especially within the tropics and subtropical areas of the world are the most affected (Peci et al. 2014). Coxsackievirus infections are also common in regions with temperate climates where the number of people infected, increases greatly during the warmer months of the year, usually June through October (Lydyard et al. 2010). However, it has been found out that an increase in altitude within

the tropics as well as a change in seasons from warmer months of the year to colder ones in temperate regions results in a decrease in the prevalence of CVIs in the affected regions (Abedi et al. 2018).

High prevalence of CVIs within the affected regions is attributed to warm temperatures found in these regions. High temperatures favour high replication and infectivity rates of CVs (Lydyard et al. 2010). In cold months within the temperate regions, the prevalence of CVIs is low because CVs lose infectivity, although they remain viable (Pond and WHO 2005).

Coxsackieviruses have caused many epidemics in many parts of the world. In 1957 CVIs were associated with HFMD in many parts of the world. In Britain epidemics of CVIs occurred in 1988 and 1994 and a total of 952 cases were reported (Tao et al. 2012). These cases mainly involved children aged 1-4 years. In the United States of America (USA), it is estimated that about 10 million symptomatic CVIs occur annually and four major epidemics caused by infection with CV_B species were reported (Abedi et al. 2018).

The epidemics in the US occurred in 1961, 1967, 1971 and 1988. Between 2002-2004, about 16.4-24.3% of all illnesses attributed to enteroviruses in the USA were caused by species of CV with CV_B1 being the most predominant serotype (Khetsuriani et al. 2006).

The Center for Disease Control (CDC) established that CVIs caused about 25% of all neonatal enterovirus infections comprising of 26,737 cases between 1983 and 2003 in the USA. During that period, CVs of serotype CV_B4 were associated with higher mortality rates than any other serotype (Khetsuriani et al. 2006).

In East and South-East Asia, large outbreaks of HFMD caused by infection with CVs have been reported and about 71 cases confirmed. The HFMD affected children mostly since 1997 (Sasidharan et al. 2005).

In China, CVs of serotypes CVA16 were responsible for nearly 50% of all the confirmed HFMD cases in mainland China, and are reported to have caused severe complications and death of many people (Tao et al. 2012). In 2007, an outbreak of CVIs occurred in Eastern China where more than 800 people were affected, 200 people mostly children were hospitalized and 22 people were killed (Tao et al. 2012). The latest CVI outbreak in China occurred between June and September 2008 in Shandong province, where a total of 887 patients comprising of 596 males and 291 females, were hospitalized. The ages of the patient ranged between 2 months to 64 years, majority of them being children under the age of 15 years (Tao et al. 2012).

Individual cases of CVIs and outbreaks occur around the world throughout the year and high rates of transmissions usually take place in group settings such as schools, childcare centres as well as other social and political meetings (Pond and WHO 2005).

In Kenya, a high prevalence of CVIs was detected in patients presenting symptoms of ILIs from study sites located in selected regions of Kenya in 2007. This was during ILI surveillance exercise carried out by medical personnel engaged by the USAMD-K. Nasopharyngeal samples were taken from the patients and upon testing 2925 samples using IFA procedure, 22 of them turned positive for CVIs.

The number of people confirmed positive for CVIs could have been higher than 22 because the processes of sampling and testing were not extensive as the selected regions have tropical and sub-tropical climatic conditions favourable for high rates of

replication and infectivity of CVs (Pond and WHO 2005). Besides, there are frequent random movements of people into and out of these regions for social, political, religious and economic reasons. Poor personal hygiene and sanitation, unavailability of adequate medical care as well as lack of regular nutritious food by most families due to low-income levels are risk factors for CVIs and are all existent in the selected regions. Poverty and low-income levels make it difficult for residents of these regions to afford medical insurance covers.

The health effects of the CVIs on the residents found in the selected regions of in Kenya in 2007 are not fully understood because of lack of adequate documented information as many residents in the selected regions were not tested for CVIs. Besides the species and serotypes of the causative, CVs were unknown. Consequently, the number of people who were infected, treated or hospitalized as well as those who succumbed to CVIs were also unknown.

Therefore, there was need to provide factual scientific data on the seroprevalence and actual of CVIs, the species, serotypes, phylogenetic and evolutionary relationships of the causative CVs through characterization of CVs that circulated in the selected regions in the year 2007. The information obtained from the study would be used for future emergency preparedness and developments of diagnostics and therapeutics.

2.1.3 Economic Importance of Coxsackievirus Infections

Unmanaged CVIs are known to cause a serious economic impact on the people living in the affected areas. The affected populations spend a lot of money on medical bills to treat severe illnesses and complications caused by secondary Coxsackievirus

infections such as myocarditis (Steinke et al. 2013). Some of the affected people are in the most productive years of their lives and waste a lot of man-hours in hospitals either when taking care of their loved ones admitted in hospitals suffering from diseases associated CVIs or when they are themselves admitted in the hospitals suffering from diseases caused by CVIs. Some of the infected people may become disabled due to paralysis caused by CVIs (Angez et al. 2017). Some of the infected people may succumb to severe illnesses caused by secondary Coxsackievirus infection of vital body organs resulting in reduction and loss in skilled labour in Kenya and the world in general. Reduction or loss of skilled labour results in an economic loss of a huge magnitude.

2.2.0 Biology of Coxsackieviruses

2.2.1 Size and morphological structure of Coxsackieviruses

Typical Coxsackie virions are small in size, have icosahedral shape and lack viral envelopes (Lincez, Marine, and Horwitz 2010). The protein coat of each of the virions is about 25-30nm in diameter and consists of 60 protomers of 4 coiled polypeptides referred to as viral proteins (VP) designated as VP1, VP2, VP3 and VP4 (Yin-Murphy and Almond 1996).

The protein coat has 12 fivefold vertices, each surrounded by a depression that is about 15A deep (Lincez, Marine, and Horwitz 2010). The charged amino acids in the component polypeptides in these depressions are responsible for binding CV to host cell surface membrane receptors (Lincez, Marine, and Horwitz 2010).

2.2.2 The Genome of Coxsackieviruses

Coxsackieviruses have ssRNA genomes of positive sense. Their RNA genomes are about 7.4 kb in size (Lincez, Marine, and Horwitz 2010). The CV RNAs have open reading frames with viral peptides (VPg) covalently attached at the 5'UTR of the RNAs while the 3' UTRs end have poly-A tails. The 5'UTR forms about 10% of the CV RNA genomes and contain internal ribosome entry site (IRES) as well as regulatory signals for CV RNA genome replications and initiation of CV protein synthesis (Liu et al. 2014).

The 3' UTR has signals for initiation of the synthesis of negative CV RNA strand and formation of poly-A tail. The genomes of many CVs species and serotypes have not been fully characterized. However, CV RNA genomes are known to have similar nucleotide sequences with UUAAAACAGC at their 5' termini (Lincez, Marine, and Horwitz 2010). The VP1 gene nucleotide sequences vary in CV species and serotypes and are used for their identification. The variations in the VP1 gene nucleotide sequences of CVs may occur naturally or may occur as a result of genetic recombinations with nucleotide sequences of other related viruses through 'copy choice' mechanism (Oberste, Peñaranda, and Pallansch 2004). This mechanism involves witching of the template RNA molecule by a viral polymerase in the course of negative-strand RNA synthesis. The process of recombination results in more virulent CVs (Oberste, Peñaranda, and Pallansch 2004).

2.2.3 Synthesis of Coxsackievirus proteins within the cytoplasm of the host cells

Both the original CV ssRNA genome strands of negative sense and their complementary ssRNA strands of positive sense serve as messenger RNAs(mRNAs) for

the synthesis of the large CV polyprotein within the ribosomes attached to RER of host cells (Liu et al. 2014).

The amino acids that are assembled to form the CV polyprotein are obtained from the cytoplasm of the host cells. The fully synthesized CV polyproteins emerge from rough endoplasmic reticula (RER) and are cleaved by viral proteases into the seven functional proteins: (2A, 2B, 2C, 3A, 3B, 3C, and 3D) and smaller structural viral proteins (VP) designated as VP4, VP3, VP2, VP and VP1. The VPs are then assembled to form the icosahedral CV capsid (Liu et al. 2014).

2.3.0 Hosts for Coxsackieviruses

Humans are the only known natural hosts for CVs. However, CVs can be propagated in laboratories using other mammalian cells such as those of mice and monkeys and also in chicken egg cells (Pond and WHO 2005).

2.4.0 Transmission of Coxsackieviruses

Coxsackieviruses are found in body fluids such as blood, urine, tears, semen, vaginal secretions, saliva, breast milk and mucus of infected people. They can also be found in faeces, sputum and air droplets from infected people (Sin et al. 2015). Coxsackieviruses are released by infected people into the air, food, water, fomites and the environment in general. People infected with CVs are infectious during the first week of infection and may continue being infectious for days or weeks after symptoms have subsided (Pond and WHO 2005). Coxsackieviruses are transmitted from infected people or contaminated surfaces, food, water, and fomites through the following modes:

i) Faecal-oral transmissions

This involves the transmission of CVs through consumption of contaminated food, drinks, water or by use of contaminated utensils when serving food and drinks. It also involves the transmission of CVs from infected food handlers. Faecal-oral transmission is the most common mode of transmission of CVs as they are usually shed in oral secretions of infected persons for several weeks and several months in their faeces (Zeng et al. 2015).

ii) Direct Contact Transmissions

Direct body contacts with the skin of an infected person as well as contact with their faeces and body fluids such urine, sweat, nose and throat secretions, saliva, sputum, or nasal mucus, blood and cerebrospinal fluid results in rapid CVs transmissions (Abedi et al. 2018).

iii) Respiratory transmissions

These occur as a result of inhalation of contaminated air droplets released by people infected with CVs or inhalation of air contaminated with CVs from the body fluids, faeces or contaminated fomites. CVs may be shed in oral secretions of infected persons for several weeks and when the infected person cough, spits or sneezes, CV are released into the environment and can spread fast in crowds and places with poor air circulation (Yi et al. 2017).

iv) Congenital transmission

Babies can be infected with CVs by their infected mothers during pregnancy, vaginal delivery and during breastfeeding. Transmission of CVs during pregnancy though

rarely occurs by placental infection mainly through Hofbauer cells, syncytiotrophoblastic and trophoblastic cells of the terminal villi (Ornoy and Tenenbaum 2006). About 30-50% of expectant women with CVIs may transmit CVs to their babies during vaginal delivery (Pond and WHO 2005).

Other less common forms of transmission of CVs include all forms of unprotected sexual intercourse with infected sex partners, kissing infected persons as well as sharing of unsterilized contaminated medical instruments such dialysis machines, needles and syringes with infected persons (Pond and WHO 2005). Like in other cases of human enterovirus infections, young children are the most susceptible primary target segment of the human population and play an important role in the transmission of CVs (Abedi et al. 2018). The susceptibility of children to CVIs and the huge role they play in the transmission of CVs is attributed to the fact that childrens' immune systems are still developing and are more exposed to contaminated environments than adults (Abedi et al. 2018). Nevertheless, older children, adolescents and adults are still susceptible and capable of transmitting CVs (Zeng et al. 2015).

2.5.0 Attachment and entry of Coxsackieviruses into host cells

After transmission, CVs attach to epithelial cells of the gut wall or mucous membranes using Coxsackievirus and adenovirus receptors (CARs) on their surfaces assisted by decay assistance factors (DAF) located on the cell surface membranes of the human epithelial cells (Cohen et al. 2001).

Coxsackieviruses in the blood can also in infection leucocytes. This affects their immunological functions leading to Immuno-compromisation (Vuorinen et al. 1994). Also, Coxsackieviruses especially CV_B species in lymph or blood can then enter cells

of the lymph nodes or other organs of the body resulting in severe, often fatal SCVIs (Legay et al. 2007). Once CVs have entered cytoplasms of the host cells, they uncoat to release their ssRNA genomes of positive-sense for the synthesis of viral proteins through transcription in the cytoplasm and translation in ribosomes of the host cells (Lincez, Marine, and Horwitz 2010).

2.6.0 Replication of Coxsackieviruses within Host cells

The CVs RNA genome undergoes further transcription in the cytoplasms of infected host cells to produce other similar RNA genomes for their replication (Lincez, Marine, and Horwitz 2010). Assembly of new daughter CVs also occurs in the cytoplasm of the infected cells (Lincez, Marine, and Horwitz 2010). The 5'UTR of their RNA genome contain regulatory signals for CV genome RNA replication and an internal ribosome entry site (RES) for regulating the initiation of translation while the 3'UTR of CV RNA has signals for initiation of negative RNA strand synthesis and formation of polyadenine (poly-A) tail (Lincez, Marine, and Horwitz 2010).

A large polyprotein composed of fused CV proteins; four (4) structural viral proteins VP1-4 and seven (7) functional proteins: 2A, 2B, 2C, 3A, 3B, 3C, and 3D emerges from translation at the rough endoplasmic reticulum (ER) of the infected host cells (Lincez, Marine, and Horwitz 2010). The polyprotein is then cleaved into its structural and functional proteins by proteases. The 3D VP is the RNA-dependent RNA polymerase (RdRP) which catalyzes the synthesis of a negative-sense CV RNA strands using the ssRNA genome as the template (Liu et al. 2014)

The resultant negative sense CV RNA strands are then used as templates for the assembly of complementary positive-sense ssRNA strands, which serve as the genome

RNA in daughter CVs (Liu et al. 2014). The daughter CVs exit the infected host cells after the rapture of their cell surface membranes through a mechanism mediated by VP 2B. The new CVs then infect other uninfected host cells or are transported in the blood, tissue fluid and lymph to infect other organs of the body (Lincez, Marine, and Horwitz 2010). Figure 2 shows the main stages of replication of CVs in host human cells.

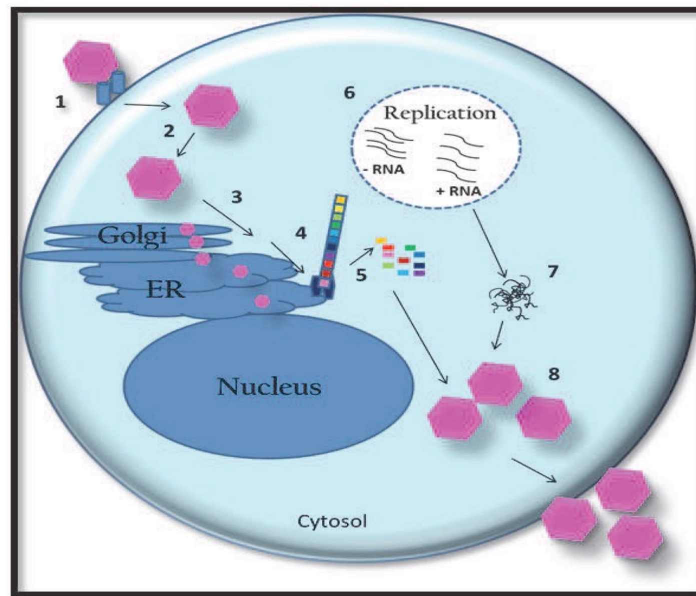

Fig.2.7: The life cycle of CVs (Lincez, Marine, and Horwitz 2010).

The key steps involve in binding and replication of CVs in host cells are:

1. Entry of CVs into host cells by binding to CAR and DAF
2. Internalization and transport of CVs to the Golgi and ER
3. CV uncoating
4. Release of the CV RNA genome
5. Translation of CV genome RNA by ribosomes on the RER into CV polyprotein
6. Autocleavage of CV polyprotein into structural and functional VP
7. Transcription positive and negative sense strands of CV ssRNA to replicate CV

RNA genome and release of replicated CV RNA genome into the cell cytoplasm to encapsidate with structural VP

8. Assembly and release of newly formed CV daughter virions

2.7.0 Clinical disease

Replication and spread of CVs in human cells cause a wide range of human illnesses. The illnesses can be mild, severe and occasionally fatal. Mild illnesses are usually caused by infection with CV_A species of CVs and include common cold, skin rashes, herpangina, sore throat and HFMD CV_B2 (Osterback et al. 2009). More severe, often fatal secondary coxsackievirus infections (SCVIs) are often associated with infection of body organs by CV_B species (Steinke et al. 2013). The illnesses include myocarditis, pericarditis, encephalitis, hepatitis, cancer of the pancreas, paralysis, type 1 diabetes among other severe illnesses (Jaïdane and Hober 2008).

People who mostly susceptible to illnesses caused by CVIs include those with developing such as children under the age of sixteen years, those with suppressed immune systems such as patients who have had organ transplants, expectant women as well as other adults whose immune systems have been weakened or compromised by other illnesses such as cancer, tuberculosis and HIV/AIDS (Pond and WHO 2005). Both CV_A and CV_B species can also cause nonspecific febrile illnesses, rashes, upper respiratory tract illnesses and aseptic meningitis (Sasidharan et al. 2005).

2.7.1 Symptoms of Coxsackievirus Infections

Coxsackievirus infections have variable incubation periods which range from 2-35 days after infection. The length of incubation periods following CVIs depends on the

immunity of the infected persons and the infecting CV species and serotypes (Pond and WHO 2005). Persons with weak immune systems show symptoms of CVIs much earlier than those with strong immune systems. Symptoms associated with infection with CV_B species take longer to manifest that than caused by infection with CV_A species. For example infection with CV_A9 serotype presents symptoms between the 2-3 days after infection while infection with CV_B5 serotypes presents symptoms between 3-12 after infection (Harvala et al. 2003). The symptoms usually last for a period of 1 to 2 weeks.

Some of the most common symptoms of CVIs include sudden fever, cough, sore throat, sores in the mouth that turn into small blisters which ulcerate, poor appetite, mild headache, muscle ache, tiredness, malaise, chest pain, stomach ache, abdominal discomfort, nausea, vomiting, diarrhoea, itchy rashes on palms of the hand or soles of the feet (Lydyard et al. 2010).

2.7.2 Illnesses, Syndromes and Complications caused by Coxsackievirus Infections

Coxsackieviruses infect can infect various parts of the human body and cause various illnesses and syndromes. These include:

i) Hand, foot and mouth disease (HFMD)

This is a syndrome caused by infection of the body with CVs of serotypes CVA6 and CV_A16. This syndrome is characterized by painful red blisters in the throat and on the tongue, gums, hard palate, inside of the cheeks, and the palms of hands and soles of the feet (Zeng et al. 2015). Other symptoms of HFMD are sore throat, fever of about 100.3^{o} F that lasts for four days, headache, stomach pains and lack of appetite (Zeng et al. 2015). HFMD caused by CVIs is specific to humans and is not related to the foot and

mouth disease (FMD) caused by foot and mouth disease virus (FMDV) that affect animals such as cattle.

ii) Herpangina

This is an infection of the throat by CVs of CVA species. The specific CVA serotypes known to cause herpangina are CV_A2, CV_A4, CV_A5, CV_A6 and CV_A10. The disease is characterized by red-ringed blisters and ulcers on the tonsils and soft palate, the fleshy back portion of the roof of the mouth (Khetsuriani et al. 2006).

iii) Hemorrhagic conjunctivitis

This is an infection of the whites of the eyes by CVs of CV_A 24 serotype. (Drescher et al. 2004). Hemorrhagic conjunctivitis usually begins as a pain in the eyes followed quickly by reddening of the eyes. The eyes become watery and swell. They then become sensitive to light resulting in blurred vision (Khetsuriani et al. 2006). If unmanaged, hemorrhagic conjunctivitis can cause blindness and other serious medical complications that may require hospitalization. People with weak immune systems especially infected newborns are at a high risk of developing serious complications after developing hemorrhagic conjunctivitis within 2 weeks after birth (Khetsuriani et al. 2006).

Serotypes of CV_B species cause serious medical complications which include:

Neonatal myocarditis and Type 1 diabetes mellitus caused by infection of the fetal pancreas by CVs of serotypes CVB2-4 (Drescher et al. 2004). Majority of the newborn babies with CVIs and that develop serious medical complications are likely to die prematurely. These complications include myocarditis and pericardial infection caused by

infection of the heart muscle by CV_B3, hepatitis due to infection of the liver by CV_B3, pancreatitis and Type-1 insulin-dependent diabetes due infection of the pancreas by CV_B3, renal failure due to complications caused by infection of the kidney by CV_B5 and arthritis caused by infection joints by serotypes of CV_B species (Drescher et al. 2004).

Neurological complications occur when CVs enter the cerebrospinal fluid and migrate to the central nervous system (CNS). Complications associated with the infection of CNS by CVs include CV meningitis and meningoencephalitis caused by infection of the meninges and the brain by CVs of serotype CV_B4 (Cree et al. 2003). Sporadic cases of paralysis in children are usually associated with CVs of serotypes CV_B2-6 (Angez et al. 2017).

2.7.3 Management, Control and prevention of Coxsackievirus Infections

Currently, there are no specific antiviral agents for the treatment or prevention of CVIs. Normally, the majority of the mild CVIs in people with strong immune systems are self-limiting therefore do not require medical treatments (Pond and WHO 2005). However, serious CVIs especially in persons with weak or compromised immune systems, management of symptoms is essential. Management of symptoms necessary to prevent the development of complications that can cause death (Pond and WHO 2005).

Management of symptoms of CVIs involves administration of supportive anti-inflammatory drugs such as diphenhydramine, an antihistamine that blocks the inflammatory effects of histamine as well as the use of pain relievers such acetaminophen to reduce pain. More severe symptoms and complications of CVIs may require inpatient

admission in a hospital for further medical examination and treatment or other forms of intervention (Lydyard et al. 2010).

Normally no surgical intervention is required unless patients develop complications such as meningitis or encephalitis where increased intracranial pressure requires ventriculostomy. In the case of heart failure, surgical valve repair or heart transplant may be carried out (Pallansch 1997, Lincez, Marine, and Horwitz 2010). Type 1 diabetes mellitus caused by CVIs of the pancreas is managed by daily intravenous insulin injections (Jaïdane and Hober 2008).

Control and prevention of CVI involve good hygiene practices, proper cooking and handling of food and drinks as well as proper sewage disposal. Infectious patients especially those with SCVIs should be isolated from other members of the society to minimize CVs transmissions (Pond and WHO 2005).

2.8.0 Diagnosis of Coxsackievirus Infections

2.8.1 Differential Diagnosis of Coxsackievirus Infections

This involves distinguishing between CVIs and other illnesses with similar clinical signs. Symptoms caused by CVIs such as fever, sore throat, diarrhoea, head and muscle aches are similar to those caused by infection of the body by other pathogens such as bacteria, protozoa and parasites (Yi et al. 2017). Differential diagnosis involves the use of specific drugs to treat illnesses with similar symptoms as CVIs and if the treatment is successful after completion of the dose, then the illnesses are not caused by CVIs but if the symptoms of the illnesses persist then the illnesses could be caused by CVIs. For instance antibiotics such penicillin could be administered to patients presenting

symptoms such as sore throat and diarrhoea but if these symptoms do not subside then bacteria as the causative agents are ruled out (Martha 2018).

Differential diagnosis of CVIs is well applied where the illnesses have distinct symptoms such as HFMD in children. However, differential diagnosis cannot be relied upon as most illnesses caused by CVIs are asymptomatic. This method is also expensive, time-consuming and cumbersome hence the need for definitive laboratory diagnoses for CVIs (Martha 2018).

2.8.2 Definitive laboratory diagnoses of Coxsackievirus Infections

Definitive laboratory diagnoses are carried out to characterize CVs using serology and molecular methods. The serological method detects antibodies against CVs in samples using IFA procedure while molecular method detects the VP1 gene of the CVs suspected to be present in samples using PCR and DNA sequencing (Martha 2018). Samples for laboratory diagnoses are usually taken from the patient's stool, swabs from the nose, pharynx or rectum as well as body fluids such as saliva, blood and CSF fluid (Martha 2018).

Laboratory diagnoses may involve propagation of CVs by culture in suitable cell lines such embryonic mice cells (EMC), Henrieta Lucks (Hela), Buffalo green monkey kidney (BGMCK) cells or RD cells followed by microscopic observation of the cytopathic effects (CPE) of infected cells, usually 2-6 days after inoculation with CVs (Martha 2018).

The supernatant and cell debris from CVs cell culture flasks can be used for further diagnoses which include serological tests involving specific Immunofluorescent assays (IFA) using monoclonal antibodies that identify different species of CVs as well

as their serotypes (Sin et al. 2015). Also, molecular diagnosis involving comparisons of nucleotide sequences of the hypervariable VP1 gene encoding the immunogenic VP1 proteins of CVs (Liu et al. 2014).

Diagnosis of CV by cell culture is estimated to have 30-35% sensitivity while serological diagnostic methods by use of antibody tests have approximately 66-90% sensitivity. Diagnosis of CVs using VP1 gene nucleotide sequence comparisons is confirmatory (Martha 2018).

2.9.0 Importance of the study vis-à-vis taxonomy of Coxsackieviruses

Characterization of CVs that circulated in selected regions in Kenya in the year 2007, is a study that provided an opportunity to obtain information on the prevalence of the identified CV species that circulated in the selected regions of Kenya in 2007 by characterizing them serologically and molecularly. The study provided information on the identity of the CVs species and their serotypes as well as other useful virologic characteristics of these viruses.

The information on mutation occurring on their VP1 gene and evolution of CVs into forms that are more virulent and spread easily is useful for explaining the high prevalence of CVIs in the selected regions in 2007. This information would be useful in guiding the development of diagnostic and therapeutic agents and future emergency preparedness to avert health and economic problems caused by CVIs.

CHAPTER 3.0 MATERIALS AND METHODS

3.1.0 Samples and Isolates used in the study

Two thousand nine hundred and twenty-five (2925) samples from patient's nasopharyngeal specimens obtained from study sites established by the US Army Medical Research Directorate Kenya (USAMD-K) in selected regions of Kenya between January 1st and December 30th 2007 were obtained. The samples were obtained from patients who had been clinically diagnosed to have ILIs.

The study sites were in Kisumu and Alupe in Western Kenya, in Kericho and Kisii in the Kenya Highlands, at Mbagathi in Central Kenya, at Isiolo in North Eastern Kenya and Port-Reitz and in Malindi in the Coast of Kenya.

The samples were collected by passing flexible, fine shafted swabs into the posterior of the pharynx and in the nostrils parallel to the palate, avoiding the tongue of patients.

The swabs were allowed to absorb secretions from the pharynx or the nostrils for 5 seconds each; they were carefully removed by gently rotating them and were placed in sterile sample collection tubes containing 3.0 ml of viral transport media. The tubes were then covered immediately with leak-proof screw-cap before they were placed on ice packs at 5°C. The samples were transported within 72 hours to the laboratory where they were stored at -80°C (Martha 2018).

Twenty-two (22) isolates out of the 2925 samples archived in freezers at -80°C of the ongoing USAMRD-K surveillance program in the Department of Emerging and Infectious Diseases (DEID) at KEMRI in Nairobi, Kenya, had been tested and found to be positive for CVIs by IFA procedure. These isolates were suspected to contain CVs, they were retrieved from the archives and were used in this study to characterize the CVs.

3.1.1 Ethical and Regulatory approval

Ethical and regulatory approvals from the Internal regulatory boards (IRBs) of Kenya Medical Research Institute (KEMRI) were sought and granted before the commencement of the study. These approvals were from the Ethics and Review Committee (ERC) and the Scientific Ethical Review Unit (SERU).

3.2.0 Methods used in the study

3.2.1 Study design

This was a retrospective cross-sectional study which involved the use of archived CVs isolates from patients who were clinically diagnosed to be with ILI in 2007 from the selected regions of Kenya under protocol whose identity is WRAIR#1267. This protocol allowed retrospective analyses of virus isolates to characterize diseases of patients whose samples had been collected earlier.

3.2.2 Laboratory Methods

3.2.3 Serological identification of Species coxsackieviruses

Immunofluorescent assays(IFA) using monoclonal antibodies had been carried out on the isolates to confirm the presence of antibodies against CV by the National Influenza Center (NIC) laboratory personnel immediately after sample collection according to manufacturer's instructions on the procedure for preparing slides described in the MBL Bion's Coxsackievirus B Antigen Substrate Slides (Bion 2012).

Briefly:

Fifty (50) µl of each sample were put in wells of a pre-labelled plate and incubated for 1hour at room temperature. The plates were then washed to discard the well solution. Into each well, 200 µl of PT were added and left for 2 min. The procedure was repeated twice to discard the well solution. The plates were then blocked with 150 µl of blocking solution, incubated for 1h at room temperature before discarding the blocking solution. Into each well, 50 µl of the hybridoma supernatants were added and the plates incubated for 30 min at room temperature before washing and 50 µl of the peroxidase-coupled anti-mouse antibody (1/5000 in blocking solution) added. The plates were then incubated for 30 min at room temperature, washed and 100 µl of developing solution added after which the plates were incubated until a colour change was evident. The reaction was stopped by adding 50 µl of 2.5 M H_2SO4 per well. The absorbance of each sample was measured at 492 nm and recorded.

3.24 Culture of Rhabdomyosarcoma (RD) cells and propagation of CVs.

Two T-25 cell culture flasks were used to culture RD cells up to 80 % confluence. The RD cells were obtained from the NIC cell culture laboratory and were of the second passage. They were cultured in the growth medium that comprised of 4.0% autoclavable Eagle's minimal essential medium, modified Earles salts (Irvine Scientific, Santa Ana, CA, USA), 8.0 mM HEPES, 0.0075% NaHCO3, 80.0 mM l- glutamine, 10.0 mM minimal essential medium non-essential amino acids (Gibco BRL, Grand Island, NY, USA), 100 U/ml penicillin and 100 g/ml streptomycin) 2.0 U/ml nystatin, and 2.0 mg/ml kanamycin) containing 8.0% fetal bovine serum (FBS) at 37 °C in presence of 5% CO_2 (HyClone™ 2012).

The 80% confluent RD cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Waltham, MA, USA) supplemented with 10% FBS (Gibco; Waltham, MA, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in presence of 5% CO_2. The 22 samples that had tested positive for the presence of antibodies against CVs using IFA were filter-sterilized and the filtrate obtained used for inoculating the 80% confluent RD cells. The infected cells were incubated for 48 hours at 37°C in 4.0% CO_2 for 6 days as observations of cytopathic effects (CPE) were made daily. After CPE had been observed, the infected cells/lysates were stored at −80°C.
The CVs in the lysates and the supernatant were filter-sterilized and CVs harvested in the filtrate. The CVs in the filtrate were then partially purified by chloroform extraction and stored at −80°C before other experimental procedures were carried out (HyClone™ 2012).

3.2.5 Extraction of Coxsackievirus genomes

Coxsackievirus RNA genomes were extracted for determination of their VP1 gene nucleotide sequences, using the Qiagen spin Protocol for purification of Viral RNA from cell-culture supernatant according to manufacturer's instructions (Qiagen 2014a).

Briefly, 560 µl of prepared Buffer AVL containing carrier RNA were pipetted into of 22 separate pre-labelled 1.5 ml microcentrifuge tubes, 140 µl of RD cell-culture supernatant from each of the 22 RD cell culture flasks were added to each of the Buffer AVL–carrier RNA mixture in the microcentrifuge tubes before mixing each by pulse-vortexing for 15 s. The mixtures were then incubated at room temperature (25°C) for 10

min and the tubes were centrifuged briefly at 6000 x g (8000 rpm) for 1 min to remove drops from the insides of their lids.

Five hundred and sixty (560) µl of 96% ethanol were added to each of the relabeled tubes, the contents of each tube were mixed by pulse-vortexing for 15 s and then the tubes were briefly centrifuged at 6000 x g (8000 rpm) for 30 sec to remove drops from the inside of their lids. Six hundred and thirty (630) µl of the resultant solution were carefully applied to the QIAamp Mini column (each in a 2 ml collection tube) without wetting their rims. Their caps were closed and the columns were centrifuged at 6000 x g (8000 rpm) for 1 min. The columns were placed in clean 2 ml collection tubes and the collection tubes containing the filtrate were discarded.

The columns were carefully opened and another 630 µl of each of the remaining the solutions were carefully applied to each column, without wetting their rims again. Their caps were closed and they were centrifuged again at 6000 x g (8000 rpm) for 1 min. After centrifugation, the columns were carefully opened and 500 µl of Buffer AW1 were separately added to each, their caps were closed, and they were centrifuged at 6000 x g (8000 rpm) for 1 min. The columns were then placed in clean 2 ml collection tubes and the tubes containing their filtrates were discarded.

The columns were carefully opened and 500 µl of Buffer AW2 were added to each. Their caps were then closed and were centrifuged at full speed (2000 x g; 14,000 rpm) for 3 min. The columns were then placed in new 2 ml collection tubes and old collection tubes with their filtrates were discarded. The columns were then centrifuged at full speed for 1 min before they were placed in clean 1.5 ml microcentrifuge tubes and the old collection tubes containing their filtrates were discarded. The QIAamp Mini

columns were carefully opened and 60 µl of Buffer AVE equilibrated to room temperature added to each. Their caps were closed and they were incubated at room temperature before centrifugation at 6000 x g (8000 rpm) for 1 min to elute CV RNAs which were then stored at –70°C to be used as templates for CV cDNA synthesis by reverse transcription (Qiagen 2014a).

3.2.6 Optimization of oligonucleotide primers for amplification of VP1 genes of CV

Oligonucleotide primers that targeted the 3' end of the VP1 gene of CV genome RNA were optimized. The forward primer (Fp) was used for reverse transcription (RT) to synthesize CV cDNAs after which the reverse primer (Rp) was used for CV DNA synthesis by PCR amplification. The nucleotide sequences of the primers and the specific positions of CV genome RNAs where they annealed are as follows:

Primer	Sequence	P position
292-Fp	5'-MIGCIGYIGARACNGG-'3	2612-2627
222-Rp	5'-CICCIG GIGGIAYRWACAT-'3	2969-2951

3.2.7 Amplification of Coxsackievirus genomes

Reverse transcription (RT) reaction to synthesize cDNAs from genome RNAs of CVs was carried out using Mouse Moloney Murine Leukemia Virus (MMLV) reverse transcriptase with a slight modification of the Protocol for First-strand cDNA synthesis by Qiagen, Inc., San Valencia, CA, USA.

Briefly:

In a sterile 1.5 ml, microcentrifuge tube a master mix for 22 reactions each comprising of 2.0 µL RNase free water, 16.0 µL of 2x reaction Mix, 2.0 µL of 20 pmol of forward primer (Fp), 2.0 µL of 20 pmol of reverse primer and 1.0 µL superscript II RT/platinum DNA polymerase Mix was prepared. The master mix was then vortexed briefly to mix the reagents and then centrifuged at 3000 rpm for 30 sec. twenty-one(21.0) µL of the master mix were then pipetted into 22 separate pre-labelled 1.2 ml PCR reaction tubes and 4.0 µL of each of the extracted RNAs added to 80 1.2 ml PCR tubes and 4.0 µL of control RNA added to the remaining 1.2 ml PCR reaction tube as control. Each PCR reaction tube was then vortexed briefly then they were all centrifuged at the same speed for 30 sec. before they were all placed in the thermocycler.

The PCR reaction tubes were preheated at 50°C for 45.0 minutes for the synthesis of cDNAs from template RNAs by reverse transcription take place. After cDNA synthesis, the tubes were then heated to 94.0 °C to denature the superscript II RT and activate platinum DNA polymerase before final amplification of the cDNA into double-stranded CV DNA at 94.0 °C for 30 sec., 42.0 °C for 1 minute and 60.0 °C for 2 minutes for 40 cycles. The final extension phase was then carried out by incubating the reaction mixtures at 60.0 °C for 10 minutes and holding the reactions at 4°C before the cDNAs of the CVs were amplified by PCR.

Amplification of CV cDNAs was carried out by long-range PCR using the procedure described in the protocol by Thermo Scientific mini handbook; Thermo Scientific, Inc., Waltham, Massachusetts, USA. The NS of the Fp and Rp and the positions on CV genome RNA where the primers annealed are as follows:

Primer	Sequence	Position
292-Fp	5’-MIGCIGYIGARACNGG-‘3	2612-2627
222-Rp	5’-CICCIG GIGGIAYRWACAT-‘3	2969-2951

Briefly:

In each of the 22 pre-labeled 1.2 ml PCR tubes the following were added; 4.0 μL nuclease-free water, 2.5μL of 10X PCR Buffer, 6.0 μL 10mM dNTPs, 2.5 μL of 10.0 mM Magnesium Chloride, 7.0 μL (50pmol/ μL) of the reverse primer, 7.0 μL CV cDNA solution and 1.0 μLTaq DNA polymerase.

The tubes were heated at 94.0°C for 5.0 minutes, followed by cycling at 94.0°C for 40.0 seconds, 55.0°C for 40.0 seconds, 68.0°C for 8.0 minutes for 30.0 cycles.

This was followed by a final strand extension phase at 72.0°C for 10.0 minutes and the reaction was be held at 4.0°C before gel electrophoresis was carried out.

3.2.8 Gel Electrophoresis of PCR products

Each of the 22 PCR products was loaded in wells made close to one end of Ethidium Bromide (EtBr)-stained agarose gels, electrophoresed in Tris Borate-EDTA Buffer solutions and viewed under Ultra Violet (UV) light and gel photographs were taken.

Briefly;

Seven (7) µL of the PCR products were loaded into wells in 1% (g/ml) agarose gel that had been stained with 5 µL of 12% (mg/ml) EtBr solution. These were then electrophoresed in 0.8X Tris Borate-EDTA Buffer solutions for 30 minutes at 100 volts and 220 mA. The agarose gel was viewed under UV light to locate the positions of the PCR amplicons on the gel relative to those of the ladder DNA used. A total of twelve (12) usable PCR amplicons were electrophoresed. Genomes of 10 CVs were neither amplified nor electrophoresed hence were not observed under UV light in the electrophoresed gel.

3.2.9 Extraction and initial purification of usable PCR Amplicons

The 12 usable PCR amplicons viewed under UV light after gel electrophoresis were extracted from the Agarose gel using the procedure described in QIAquick PCR Purification Kit by Qiagen, Inc., Valencia, USA.

Briefly;

The wash buffer was diluted according to the manufacturer's instruction and the cutting of the bands of usable PCR amplicons located in the agarose gel was done under long-wave UV rays using a scalpel. The slices of PCR amplicons were transferred to pre-

weighed tubes, and their respective weights were recorded in a table. The slices of PCR amplicons and the capture (CB) in each tube were placed in a water bath heated to 60°C and mixed by inversion for every three minutes until the agarose slices dissolved completely.

The PCR amplicon purification required one GFX column placed in a collection tube followed by brief centrifugation and the PCR amplicons–CB mixture; were collected at the bottom of each collection tube. About 600.0 µL of each of the sample-capture buffer mix collected were transferred to GFX column and incubated at room temperature for 1 minute. The tubes were centrifuged at 13,000 Rpm for 30 sec.

The flow-through was discarded by emptying each of the collection tubes. This was repeated until all the sample-capture buffer mix in each tube was loaded into each of the corresponding tubes. The flow-through was discarded by emptying the collection tubes. Each of the GFX columns was placed back in the collection tube and 500.0 µL Wash buffer (WB) were added to the column and centrifuged at 13,000 Rpm for 30 Sec. An additional wash step was performed to ensure the purity of the captured PCR amplicons. The collection tubes were discarded and the GFX columns transferred into clean sterile 1.5 microcentrifuge tubes.

Fifty (50.0) µL of elution buffer (EB) were added directly to the top of each of the glass fibre matrix in the GFX columns. The GFX columns were then incubated at room temperature for 1 minute followed by centrifugation at 13,000 Rpm for 1 minute for the recovery of the purified amplicons which were stored at -20 °C. Gel electrophoresis of 8.0 µL the purified amplicons was carried out on a 1.0% agarose gel and gel photographs were taken (Qiagen 2014b).

3.3.0 Template DNA purification for Big Dye Sequencing

To obtain template DNA strands for cycle sequencing from the purified PCR amplicons, Exonuclease I /Shrimp-Alkaline Phosphatase (EXO-SAP) enzyme was added according to manufacturer's instruction (Affymetrix, Inc., USA) to remove unused dNTPs as well any remaining forward and reverse primers used for RT and PCR amplifications.

Briefly;

Five (5.0) µL of each of the amplicons that had been visualized on the gel were mixed with 2.0µL of Exo-Sap in a 0.2µL reaction tube and the mixture was incubated in a 9700 FAST thermocycler (Applied Biosystems, USA) under the following cycling conditions; 37 °C for 15 minutes; 80 °C for 15 minutes.

3.3.1 Purification of template DNA Using Sephadex

Purification of template DNA for pre-sequencing PCR reaction was carried out using Sephadex Spin columns:

Briefly;

Dry Sephadex G-50 (Sigma Aldrich Co., USA) were loaded into a 96-well Multiscreen HV plate (Millipore Corporation, USA) using a column loader. 300µL of Milli-Q water was added into each of the wells containing Sephadex to swell the resin.

The plates were then incubated at room temperature for 3 hours. After that, the Multiscreen HV plates were placed on top of optical 96-well reaction plates and balanced with another multiscreen HV plates before they were centrifuged at 2144 rpm in a 5810R

centrifuge (Eppendorf, AG, Germany) for 5 minutes to remove excess water out of the Sephadex columns.

The Multiscreen HV plates that were used, were transferred on top of new optical 96-well reaction plates and 10µL of each of the products was carefully added to the centre of each well using a multichannel pipette (Sephadex separation matrix).

The plates were once again centrifuged to collect purified DNA fragments in the optical plates as filtrate. The used Multiscreen HV plates were discarded but the optical plates containing the obtained filtrate were retained. Then 10 µL of Hi-Di Formamide were added into the wells containing the samples and mixed.

The sample plate was carefully covered with septa and placed it into the plate base. The plate retainer (cover) was snapped onto the plate, septa and plate base and verification of the alignment of the holes of the plate retainer and the septa strip was done before loading the plate into the Genetic Analyzer.

3.3.2 Pre-Sequencing PCR reaction of the purified template DNA

Pre-Sequencing PCR of the purified template DNA was carried out using the Big Dye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, USA) which incorporates fluorescent-labelled dideoxy-chain terminators.

Briefly;

Two master mixes were prepared; one with the forward primer and the other with reverse primer in two separate prelabelled1.5ml Eppendorf tubes as shown in table 3.

The volumes of the master-mixes were replicated to make enough master mix for the 22 samples to be sequenced. 8.0µL of the prepared master mix was transferred into 0.2µL

PCR reaction tubes and then 4µL of the purified amplicons were added. The reaction tubes were transferred into a 9700 FAST ABI thermocycler (Applied Biosystems, USA) and incubated at 94°C for 5 minutes; followed by 30 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 68°C for 2 minutes 30 seconds and a final extension at 60°C for 10 minutes.

Table 3: Reagents used in CVDNA pre-sequencing PCR

Reagent	**Quantity**
Double distilled water	4.0µl
5x Big Dye buffer	2.0 µl
Forward /Reverse primer (4µM)	1.0 µl
Big Dye 3.1	1.0 µl
Total	8.0 µl per well

3.3.3 Determination of the nucleotide sequences of the pre-sequenced template DNA

Direct nucleotide sequence determination by dye termination cycle sequencing technique was used to determine the nucleotide sequences of the present in the pre-sequenced template DNA using primers that used during PCR amplification. The ABI 3500XL Genetic Analyzer was used.

Twelve (12) nucleotide sequences were obtained from the 12 pre-sequenced template DNAs. The nucleotide sequences were used as queries in the GeneBank nucleotide sequence searches using basic local alignment search tool for nucleotides (BLASTN)

algorithm to confirm if they belong to the VP1 gene of CVs and for their classification into species and their serotypes. The twelve (12) nucleotide sequences were then sent to the GeneBank for processing and assignment of GeneBank accession numbers. Table 4.1 shows samples identities and their assigned GeneBank accession numbers.

3.3.4 Nucleotide Sequence analyses

The nucleotide sequences of VP1 gene of prototype strains of CV_B2, CV_B3, CV_B4 and CV_B5 were downloaded from the GeneBank and saved as separate data files. The downloaded nucleotide sequences belong to strain "Ohio-1" (AF085363.1), the prototype strain of CV_B2 serotype isolated in Ohio in 1998, strain "Nancy" (M16572), the prototype strain of CV_B3 serotype isolated in Sweden in 1987, strain "J.V.B Benschoten" (X05690.1), the prototype strain of CV_B4 serotype isolated in the UK in 1987 and strain "Faulkner" (AF114383) the prototype strain of CV_B5 serotype isolated in Sweden and reported in 1998.

The VP1 nucleotide sequences of each of the identified CVs of a specific serotype of CV_B species were all put together, aligned with each other, and the overhang nucleotide sequences trimmed using Bio-Edit, a software used for trimming the nucleotide sequences and the data saved identified CV_B2,C V_B3, CV_B3, CV_B4 and CV_B5 alignment data files. Comparisons of nucleotide sequence amongst themselves were carried out using BioEdit software and their percentage calculated and recorded.

The VP1 gene nucleotide sequences of each of the prototype were then included in each sequence alignment and the data files saved. Comparisons nucleotide sequences to one another were then made using BioEdit software and their percentage nucleotide

sequence identities were calculated and recorded. Tables 4.2, 4.3, 4.4 and 4.5 show the results of these analyses.

3.3.6 Determination of prevalence of CVIs in the selected regions of Kenya in 2007

In this study, the prevalence of CVIs (PCVIs) was differentiated into seroprevalence that was calculated based on IFA test results from NIC and actual prevalence that was calculated based on the number of samples identified to contain CVs. The total number of samples identified to contain antibodies to CVs of CV_B species using IFA procedure was 22 while the total number of samples molecularly determined to contain CVs by sequencing of usable PCR amplicons was 12. The total number of samples collected from all the selected regions of Kenya during the 2000 ILI survey was two thousand nine hundred and twenty-five (2925).

Seroprevalence (SP) and actual AP of CVIs in the selected regions of Kenya during the 2007 ILI surveillance exercise were calculated using the following formulae:

$$\text{SP of CVIs} = \frac{\text{Total no. of samples that tested positive for CVIs using IFA} \times 100}{\text{Total no. of samples collected from selected regions of Kenya in 2007}}$$

$$\text{AP of CVIs} = \frac{\text{Total no. of samples identified to contain CVs of CV_B species} \times 100}{\text{Total no. of samples collected from selected regions of Kenya in 2007}}$$

3.3.6 Establishment of phylogenetic relationships of the identified CVs

Nucleotide sequences of VP1 gene of "Travis"; the prototype strain of Echovirus12 (GeneBank Acc. No. X79047.1) were downloaded from the GeneBank used as an outgroup in the establishment of phylogenetic relationships of the identified CVs.

Echovirus 12 is an enterovirus that belongs to the Picornaviridae family in which CVs are classified. Strain “Travis” was isolated in Germany and reported in 1994. The VP1 gene nucleotide sequences of the identified CVs of serotypes CV_B2, CV_B3, CV_B4, CV_B5, those of their prototype strains as well as those of Echovirus 12 “Travis” were put together, aligned and trimmed using Bio-Edit software.

The nucleotide sequence alignment data file was then converted into nexus file format using concatenator bioinformatics software general time-reversible model (GTR) script was added at the end of the nucleotide sequence alignments to enable the file to be read and executed by MrBayes, a software for constructing phylogenetic trees. The phylogenetic tree code generated by MrBayes was viewed using FigTree v.1.4.0, the software for viewing phylogenetic trees. Fig. 4.1 shows the phylogenetic tree obtained.

3.3.7 Establishment of evolutionary relationships amongst the identified CVs

Establishment of evolutionary relationships amongst the identified CVs was carried out by identifying natural selection events that occurred in their VP1 gene segments and at codon sites within the nucleotide sequences of this gene using evolutionary methods implemented in the on-line Datamonkey Adaptive Evolution Server (DAES) at http://www.datamonkey.org.

Nucleotide sequences of VP1 gene of the identified CVs, those of their prototypes and those belonging to other CVs of the same serotypes from Kenya and other countries were downloaded from the GeneBank and used in these analyses. The nucleotide sequences of each serotype were put together, aligned and trimmed using BioEdit

software before evolutionary analyses were carried out using evolutionary methods implemented in DAES.

The methods used were Branch-site Unrestricted Statistical Test for Episodic Diversification (BUSTED), Fixed likelihood (FEL), Fast, Unconstrained Bayesian AppRoximation (FUBAR), and Single-likelihood ancestor counting (SLAC) methods.

The BUSTED method was used to provide a gene-wide (not site-specific) test for positive selection in the VP1 gene of the CVs by determining whether the VP1 gene had experienced positive selection on at least one site on at least one branch or not. FEL, FUBAR and SLAC methods were used to infer rates of positive and negative selection events at specific codon sites basing on dN/dS metric (Murrell et al. 2015).

FEL method was used to infer rates of non-synonymous (dN) and synonymous (dS) substitution events for each cordon site in a small number of the VP1 gene nucleotide sequence alignments data sets with the assumption that the selection pressure for each codon site is constant along the entire VP1 gene phylogenies. FEL was used to establish evolutionary relationships between nucleotide sequences of the CVs based on a large number of rates of the ratios of non-synonymous substitution events, branch lengths and other parameters (Kosakovsky Pond and Frost 2005).

SLAC method was used to infer rates of substitution of non-synonymous (dN) and synonymous (dS) on a per-site basis in the VP1 gene nucleotide sequence alignments and corresponding phylogenies with the assumption that the selection pressure for each site is constant along the entire VP1 gene phylogenies for CVs (Kosakovsky Pond and Frost 2005).

SLAC uses a combination of maximum-likelihood (ML) and counting approaches establish evolutionary relationships by determining the number of changes that have occurred along the (VP1) gene using estimates of topology, branch lengths of phylogenies in addition to codon-based substitution models.

FUBAR was used to infer rates of non-synonymous (dN) and synonymous (dS) substitution events on a per-site basis in the VP1 gene nucleotide sequence alignments and corresponding phylogeny by using the Bayesian approach. This method assumes that the selection pressure for each site is constant along the entire phylogeny (Murrell et al. 2013).

FUBAR method was used to verify the results produced by FEL and SLAC to eliminate the possibility of having misleading results when sites assigned to the same size class, experience different levels of positive or purifying selection when using a small number of sites.

4.0 RESULTS

4.1.0 Results of the determination of the prevalence of CVIs in the selected regions of Kenya in 2007

Seroprevalence (SP) and AP of CVIs in the selected regions of Kenya during the 2007 ILI surveillance exercise were calculated and found to be 0.75% and 0.48% respectively as shown below.

SP of CVIs = Total no. of samples that tested positive for CVIs using IFA x 100

Total no. of samples collected from selected regions of Kenya in 2007

= 22/2925 x 100

= 0.75%

AP of CVIs = Total no. of samples identified to contain CV_B species x 100

Total no. of samples collected from selected regions of Kenya in 2007

= 12/2925 x 100

= 0.41%

The AP of each serotype of CV_B species was calculated using the formula:

AP of CVs of serotype = Total no. of samples with CV_B2 serotypes x 100

Total no. of samples collected in 2007

Therefore the:

AP of CVs of serotype CV_B2 = 8/2925x100

=0.3%

AP of CVs of serotype CV_B3 = 1/2925x100

=0.03%

AP of CVs of serotype CV_B4 = 1/2925x100

$=0.03\%$

AP of CVs of serotype CV_B5 = 2/2925x100

$=0.07\%$

4.1.1 Results of the determination of partial VP1 gene nucleotide sequences of CVs

Twelve (12) nucleotide sequences were determined from the 12 usable PCR amplicons using the ABI 3500XL Genetic Analyzer (Applied Biosystems Inc., Grand Island, New York, USA). Comparative nucleotide sequence searches of the GeneBank using the determined nucleotide sequences as queries using BLASTN algorithm identified CVs of CV_B species whose serotypes were CV_B2, CV_B3, CV_B4 and CV_B5 as shown in Table 4.1.

Eleven (11) out 12 nucleotide sequences sent to the GeneBank were processed and assigned GeneBank Acc. No. MH522779- MH522789. These nucleotide sequences were made available to the public by uploading on the servers of the GeneBank on 10th March 2019. The sample identities, the GeneBank accession numbers and nucleotide sequences of these viruses are shown in table 4.1 and appendix 3.

Table 4.1: Sample identities of the identified Coxsackieviruses of CV_B serotypes and their assigned GeneBank accession numbers.

Sample Identity	Assigned GeneBank Accession No.
Sample 15(CV_B2.sq JBSK15)	MH522779
Sample 28 (CV_B2.sq JBSK28)	MH522780
Sample 59 (CV_B2.sq JBSK59)	MH522781
Sample 61(CV_B2.sq JBSK61)	MH522782
Sample 62 (CV_B2.sq JBSK62)	MH522783
Sample 63 (CV_B2.sq JBSK63)	MH522784
Sample 64 (CV_B2.sq JBSK64)	MH522785
Sample 65(CV_B2.sq JBSK65)	MH522786
Sample 58 (CV_B3.sq JBSK58)	MH522787
Sample 55 (CV_B4.sq JBSK55)	MH522788
Sample 52 (CV_B5.sq JBSK52)	MH522789

4.1.2 Results of comparing the nucleotide sequences of the identified serotypes of CV_B species amongst themselves and with those of their prototype strains

The nucleotide sequence identities of CVs of serotype CV_B2 identified in this study ranged from 87.5% (samples 15 and 28) to 100% (samples 62 & 64; 63 & 65).
A comparison of these NS with those of the prototype CV_B2 strain "Ohio-1"(GeneBank Acc. No. AF085363.1) showed that their nucleotide sequence identities range from 82.4 % (samples 59) to 84% (sample 28). The results of these comparisons are shown in Table 4.2.

Table 4.2: Comparative identity matrix of the identified CV_B2 amongst themselves and with their prototype CV_B2 strain "Ohio-1" (GeneBank Acc. No. AF085363.1).

Sequence Source (Sample)	15	28	59	61	62	63	64	6	AF085363.1
15	ID	0.876	0.969	0.880	0.886	0.883	0.886	0.883	0.827
28	0.876	ID	0.898	0.969	0.975	0.972	0.975	0.972	0.840
59	0.969	0.898	ID	0.901	0.907	0.904	0.907	0.904	0.824
61	0.880	0.969	0.901	ID	0.993	0.996	0.993	0.996	0.830
62	0.886	0.975	0.907	0.993	ID	0.996	1.000	0.996	0.836
63	0.883	0.972	0.904	0.996	0.996	ID	0.996	1.000	0.833
64	0.886	0.975	0.907	0.993	1.000	0.996	ID	0.996	0.836
65	0.883	0.972	0.904	0.996	0.996	1.000	0.996	ID	0.833
AF085363.1	0.827	0.840	0.824	0.830	0.836	0.833	0.836	0.833	ID

4.1.3 Results of comparing the nucleotide sequences of serotype CV_B3 with those of its prototype strain

The only CV of serotype CV_B3 identified in sample 58 was found to be 24.5 % identical to the prototype CV_B3 strain "Nancy" (GeneBank Acc. No. M16572) isolated in Sweden, in 1987. The results of this analysis are shown in Table 4.3.

Table 4.3: Comparative identity matrix of CV_B3 serotype and its prototype CV_B3 strain Nancy (GeneBank Acc. No. M16572).

Sequence Source	**Sample 58**	**M16572.1**
Sample 58	ID	0.245
M16572	0.245	ID

4.1.4 Results of comparing the nucleotide sequences of serotype CV_B4 with those of its prototype strain

The only CV_B4 serotype identified in sample 55 was found to be 79.1 % identical to its prototype CV_B4 strain "J.V.B Benschoten" (GeneBank Acc. No. X05690.1) isolated in the UK in 1987. The results of this analysis are shown in Table 4.4.

Table 4.4: Comparative nucleotide sequence identity matrix of CVs of serotype CV_B4and its prototype CV_B4 strain "J.V.B Benschoten" (GeneBank Acc. No. X05690.1)

Sequence Source	**Sample 55**	**X05690.1**
Sample 55	ID	0.791
X05690.1	0.791	ID

4.1.5 Results of comparing the nucleotide sequences of serotype CV_B5 between themselves and with those of their prototype strain

The two CVs of serotype CV_B5 obtained from samples 52 and 53 were found to be 99.7 % identical to each other. The two CVs in samples 52 and 53 were 76.4 % and 76.6 % identical to the prototype CV_B strain "Faulkner" (GeneBank Acc. No. AF114383.1) isolated in Sweden and reported in 1998 respectively. Table 4.4 shows these comparisons.

Table 4.5: Comparative nucleotide sequence identity matrix the two CVs of serotype CV_B5 with the prototype CV_B5 strain "Faulkner" (GeneBank Acc. No.AF114383.1)

Sequence Source	**Sample 52**	**Sample 53**	**AF114383.1**
Sample 52	ID	0.997	0.764
Sample 53	0.997	ID	0.766
AF114383.1	0.764	0.766	ID

The VP1 gene nucleotide sequence comparisons show that CVs that circulated in the selected regions of Kenya in 2007 had all undergone mutations in their VP1 genes. Coxsackievirus of serotype CV_B3 had the highest percentage of mutations (75.5%) in its VP1 gene while the CVs of serotype CV_B2 had the least percentage of mutations (16.8%) in their VP1 gene. Coxsackieviruses of serotypes CV_B4 and CV_B5 had 20.9% and 23.5 % mutations respectively in their VP1 genes. Table 4.6 shows the percentage of mutations that occurred in the VP1 genes of the identified and characterized CVs found in selected regions of Kenya in 2007.

Table 4.6: Comparison of the percentages of VP1 gene nucleotide sequence identities of CVs in relation to their prototypes and the percentage of detected mutations

Serotype	Sample Identity	Comparison of % nucleotide sequence identity with prototype strain	Mean % of mutation in the VP1 gene
CV_B2	Sample 15	82.7	16.8
	Sample 28	84	16.8
	Sample 59	82.4	16.8
	Sample 61	83	16.8
	Sample 62	83.6	16.8
	Sample 63	83.3	16.8
	Sample 64	83.6	16.8
	Sample 65	83.3	16.8
CV_B3	Sample 58	24.5	75.5
CV_B4	Sample 55	79.1	20.9
CV_B5	Sample 52	76.4	23.5
	Sample 53	76.6	23.5

4.2.0 Results of the phylogenetic analyses on the nucleotide sequences of the identified CVs in relation to other related viruses

A dendrogram comprising of CVs of serotypes CV_B2, CV_B3, CV_B4 and CV_B5 identified in this study, their prototypes and the outgroup "Travis"; the prototype strain of Echovirus12. The dendrogram showed that CVs of serotypes CV_B2-CV_B5 were related to their prototypes and Echoviruses as they had a common root node. The dendrogram showed that CVs and Echoviruses diverged into two main branches. One main branch comprised of Echoviruses represented by the outgroup "Travis"; Echovirus12 and the other main branch comprised of CVs of CV_B species. Fig. 4.1.

The main branch with CV_B species comprised of three main clusters each with respective CVs of the same serotype and its prototype. The clusters were of serotypes CV_B2, CV_B3 and CV_B5. One of the clusters comprised of CVs of CV_B3 serotype represented by its prototype strain "Nancy" (GQ329766.1) and the isolate in sample 58. Strain "Nancy" and the isolate that were found to be closely related with as and this 100% posterior probability value at the branching point from their node as shown in Fig 4.1.

The second cluster comprised of isolates of CV_B5 serotypes that were in samples_52, 52b, and 53. The CVs of CV_B5 serotype had a common recent ancestor with their prototype strain "Faulkner" (X513586.1) with 77.78% posterior probability value between the branches. The third cluster comprised of CVs of CV_B2 serotypes were in samples 15, 59 61,62,63,64 and 65 and their prototype CV_B2 strain "Ohio-1" (AF085363.1). The posterior probability between the prototype and the isolates 100%. This showed relatedness of CVs in this cluster. Fig. 4.1.

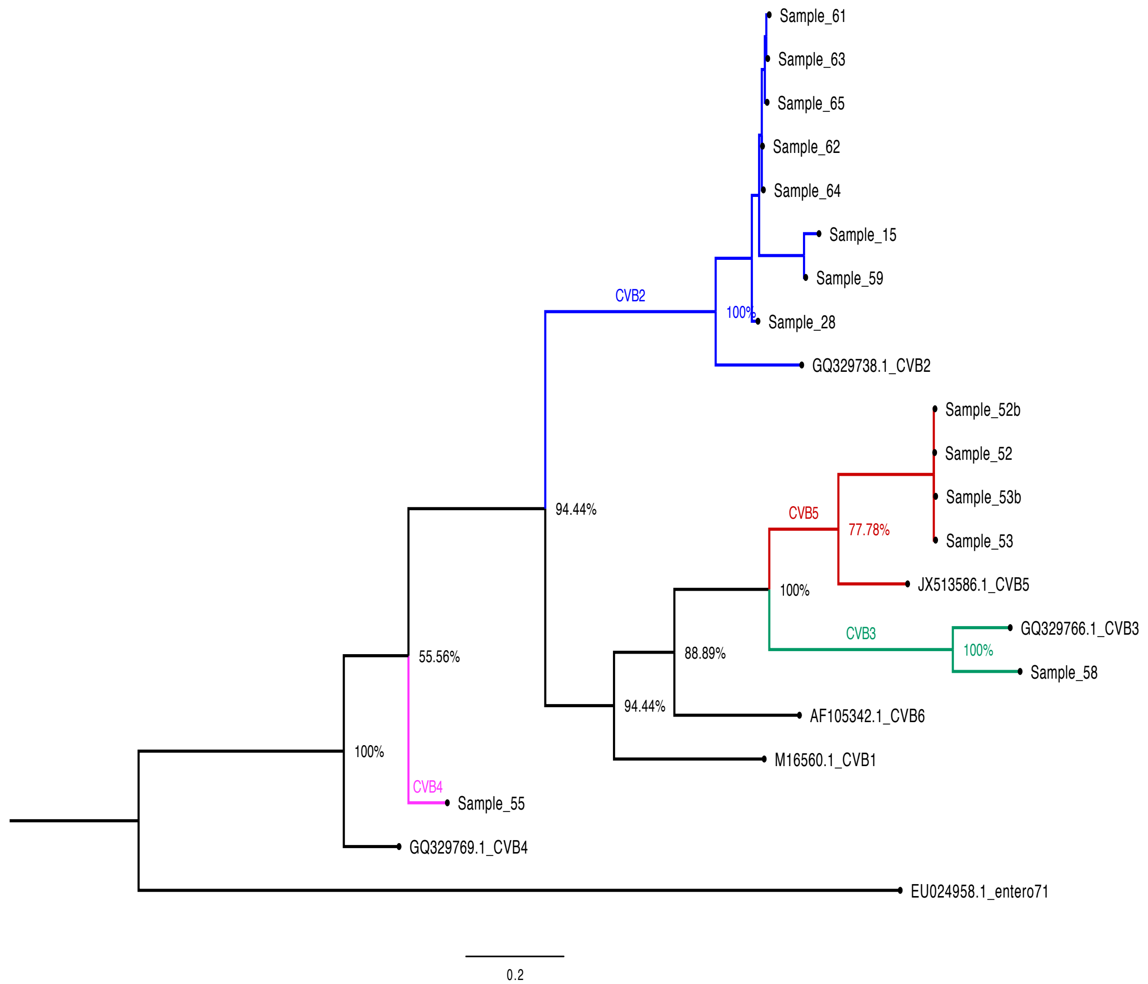

Fig. 4.1: A dendrogram of the phylogeny of the identified CVs, their prototypes and other related viruses generated using their VP1 gene phylogenies.

4.3.0 Results of the establishment of evolutionary relationships of the identified CVs amongst themselves, with their prototype strains and with other related viruses

4.3.1 Results of the evolutionary analysis on nucleotide sequences of serotype CV_B2 in relation to those of their prototype strain using the BUSTED method

BUSTED found evidence (LRT, p-value = 0.000 ≤ .05) of gene-wide episodic diversifying selection in the selected test branches of the VP1 gene phylogenies (Murrell et al. 2015). Therefore, there is evidence that at least one site on at least one test branch of the VP1 genes of CVs of serotype CV_B2 has experienced diversifying selection as shown in Fig. 4.2.

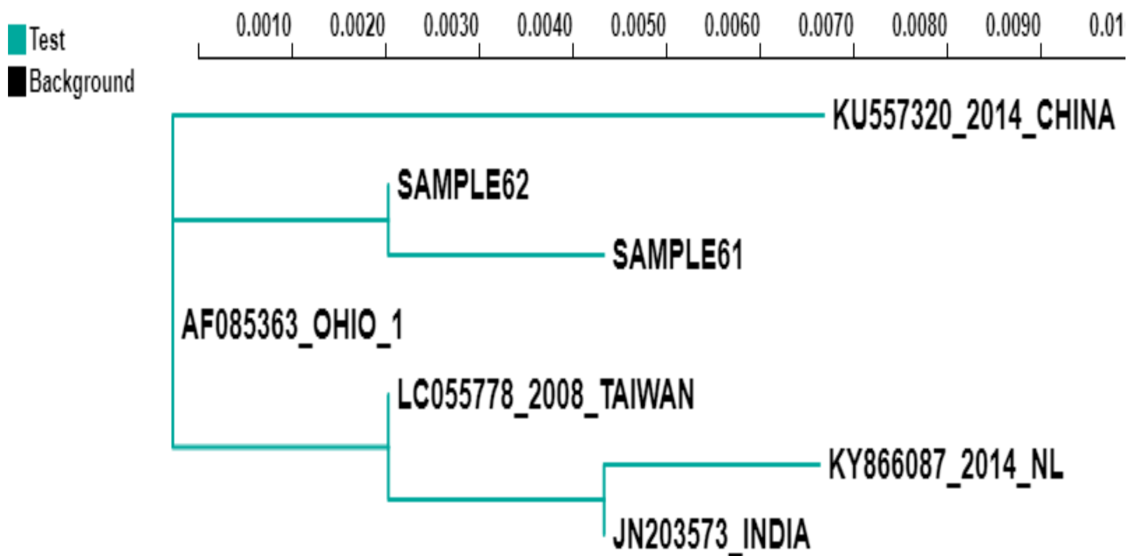

Fig. 4.2: Evolutionary tree of CVs of serotype CV_B2 generated by using BUSTED method implemented in DAES using their VP1 gene nucleotide sequences (Murrell et al. 2015).

4.3.2 Results of evolutionary analyses on nucleotide sequences of serotype CV_B2 in relation to those of their prototype strain using FEL method

FEL found evidence of pervasive positive/diversifying selection at 0 sites and pervasive negative/purifying selection at 0 sites with a p-value threshold of 0.05 (Kosakovsky Pond and Frost 2005). The results of this analysis are shown in Fig. 4.3.

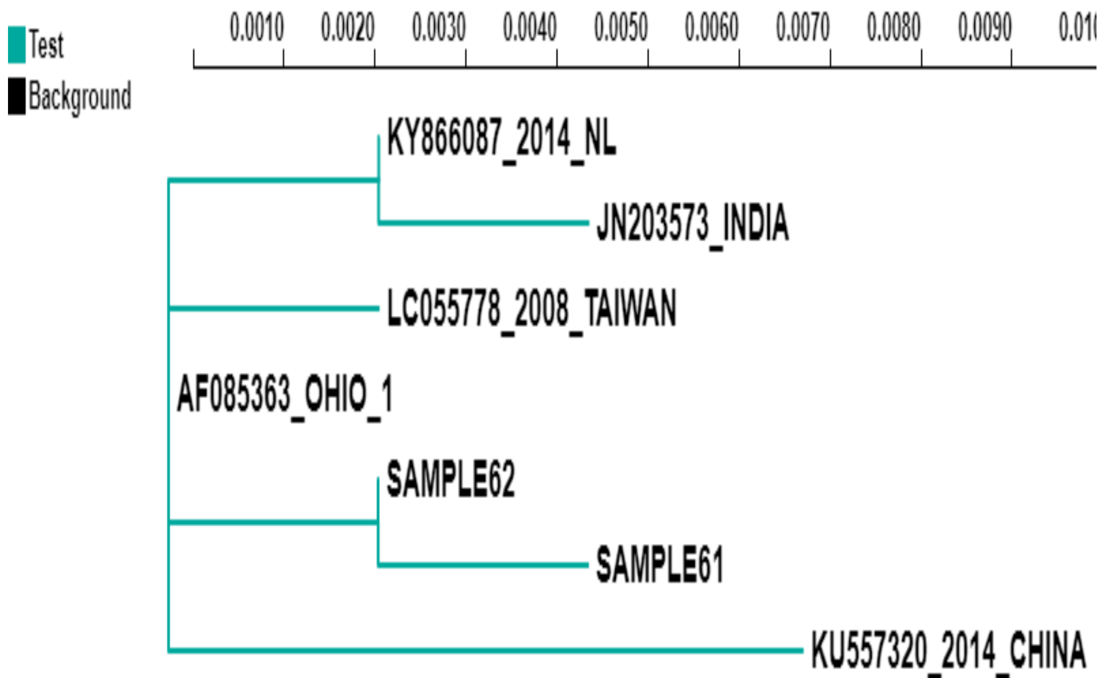

Fig. 4.3: Evolutionary tree of CVs of serotype CV_B2 generated using FEL method implemented in DAES using their VP1 gene nucleotide sequences (Kosakovsky Pond and Frost 2005).

4.3.3 Results of Evolutionary analyses on nucleotide sequences of serotype CV_B2 in relation to those of their prototype strain using SLAC method

SLAC found evidence of pervasive positive/diversifying selection at 0 sites and negative/purifying selection at 0 sites with a p-value threshold of 0.01 (Kosakovsky Pond and Frost 2005). The results of this analysis are summarized in Fig. 4.4.

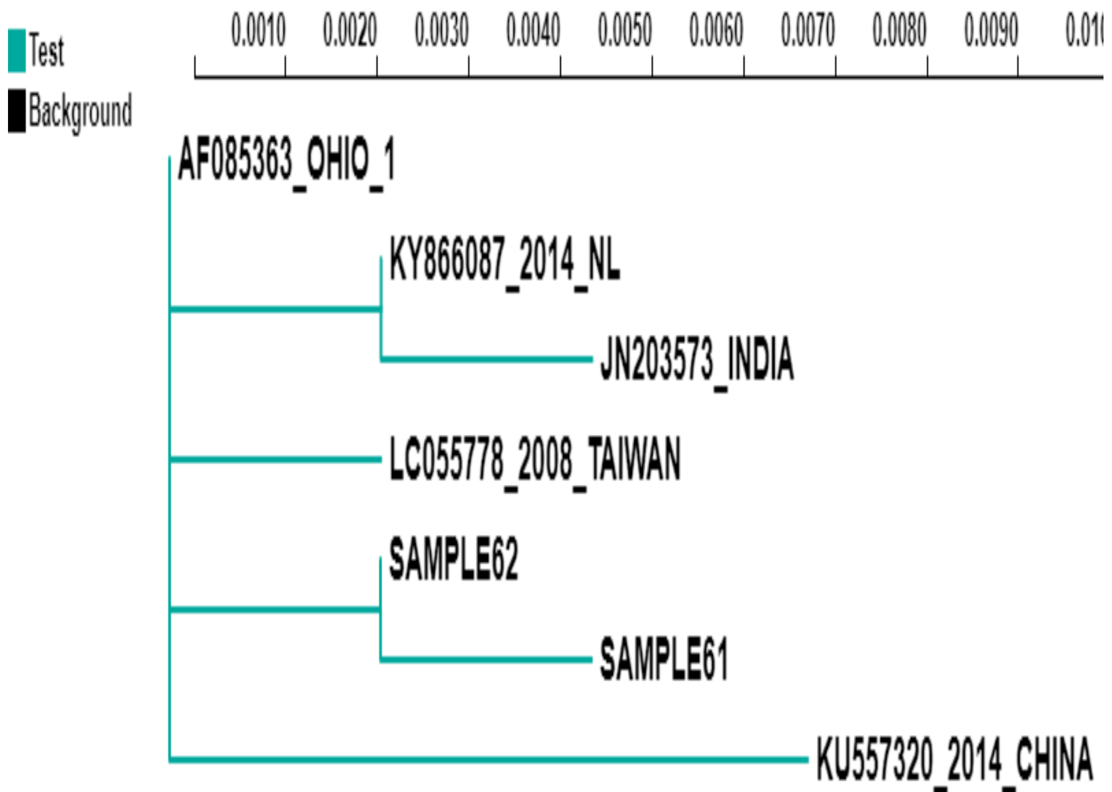

Fig. 4.4: Evolutionary tree of CVs of serotype CV_B2 generated using SLAC method implemented in DAES using their VP1 gene nucleotide sequences (Kosakovsky Pond and Frost 2005).

4.3.4 Results of Evolutionary analyses on nucleotide sequences of serotype CV_B2 in relation to those of their prototype strain using FUBAR method implemented in DAES

FUBAR found evidence of episodic positive/diversifying selection at 2 sites (17 and 50) and episodic negative/purifying selection at 0 sites with a posterior probability of 0.9 (Murrell et al. 2013). The results of this analysis are summarized in table 4.7 and Fig. 4.5.

Table 4.7: Results of evolutionary analysis of CVs of serotype CV_B2 in relation to their prototype strain using FUBAR method implemented in DAES.

Site	**A**	**B**	**β-α**	**Prob[α>β]**	**Prob[α<β]**	**BayesFactor[α<β]**
17	2.946	32.464	29.518	0.022	0.952	22.748
50	5.290	37.392	32.102	0.047	0.916	12.543

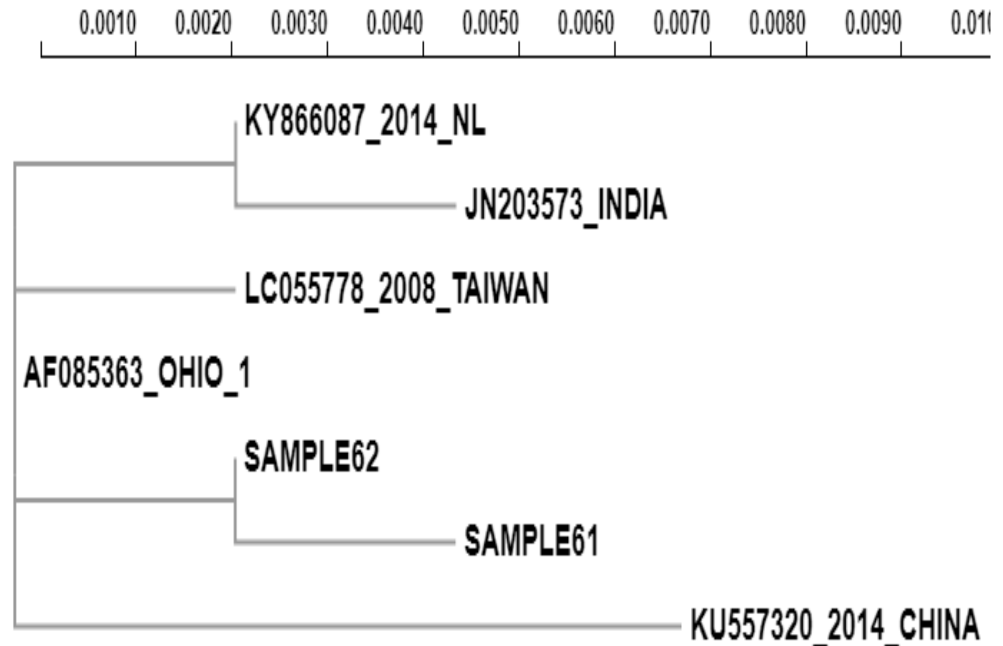

Fig. 4.5: Evolutionary tree of CVs of serotype CV_B2 generated using FUBAR method implemented in DAES using their VP1 gene nucleotide sequences (Ben Murrell and Sasha Moola 2013).

4.3.5 Results of evolutionary analyses on nucleotide sequences of CVs of serotype CV_B3 in relation to those of their prototype strain using the BUSTED method

BUSTED found evidence (LRT, p-value = 0.000 ≤ .05) of gene-wide episodic diversifying selection in the selected test branches of VP1 gene phylogenies. Therefore, there is evidence that at least one site on at least one test branch of VP1 genes of CVs of serotype CV_B3 has experienced diversifying selection (Murrell et al. 2015). The results of this analysis are shown in Fig. 4.6.

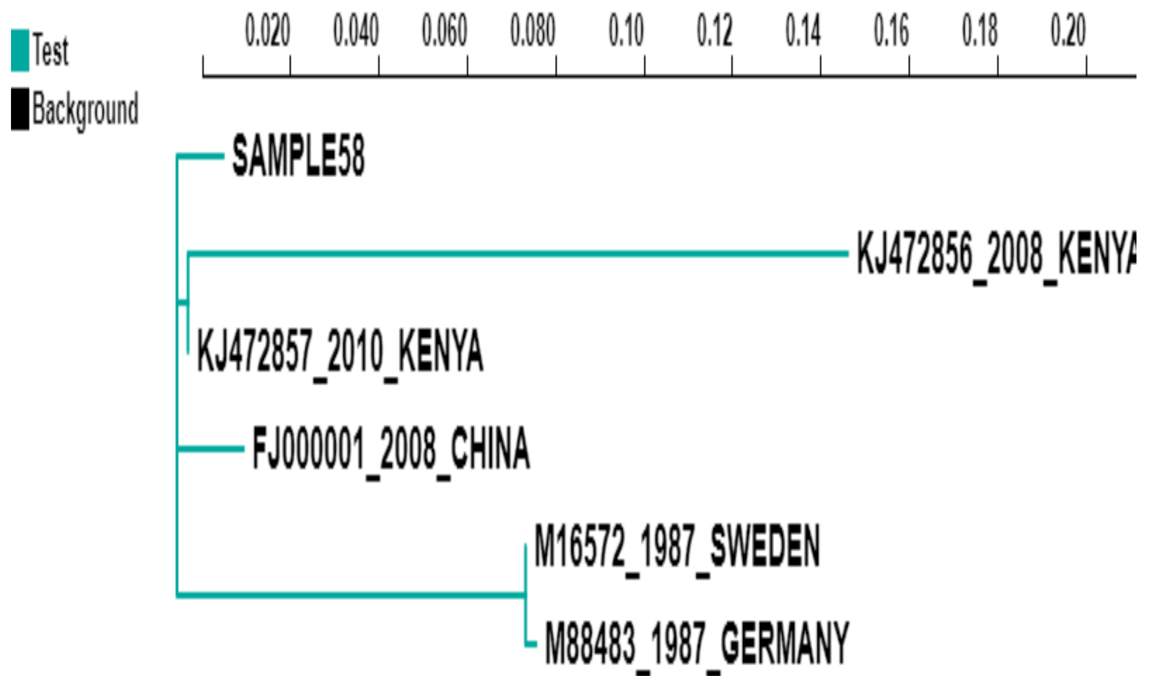

Fig. 4.6: Evolutionary tree of CVs of serotype CV_B3 generated by using BUSTED method implemented in DAES using their VP1 gene nucleotide sequences (Murrell et al. 2015).

4.3.6 Results of evolutionary analysis of the CVs of serotype CV_B3 in relation to their prototype strain using FEL method

FEL found evidence of pervasive positive/diversifying selection at 0 sites and pervasive negative/purifying selection at 0 sites with a p-value threshold of 0.05 (Sergei 2005). The results of this analysis are shown in Fig. 4.7.

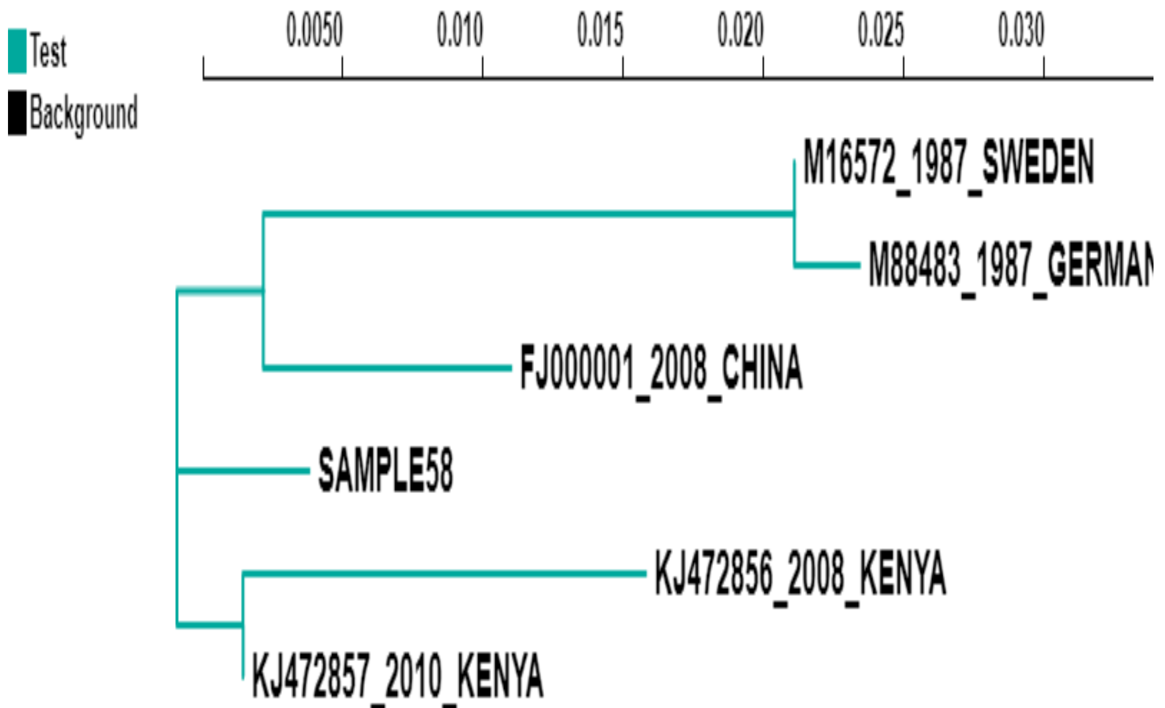

Fig. 4.7: Evolutionary tree of CVs of serotype CV_B3 generated using FEL method implemented in DAES using their VP1 gene nucleotide sequences (Sergei 2005).

4.3.7 Results of evolutionary analyses on nucleotide sequences of CVs of serotype CV_B3 in relation to those of their prototype strain using SLAC method

SLAC found evidence of pervasive positive/diversifying selection at 0 sites and negative/purifying selection at 0 sites with a p-value threshold of 0.01 (Kosakovsky Pond and Frost 2005). Fig. 4.8 shows the results of this analysis.

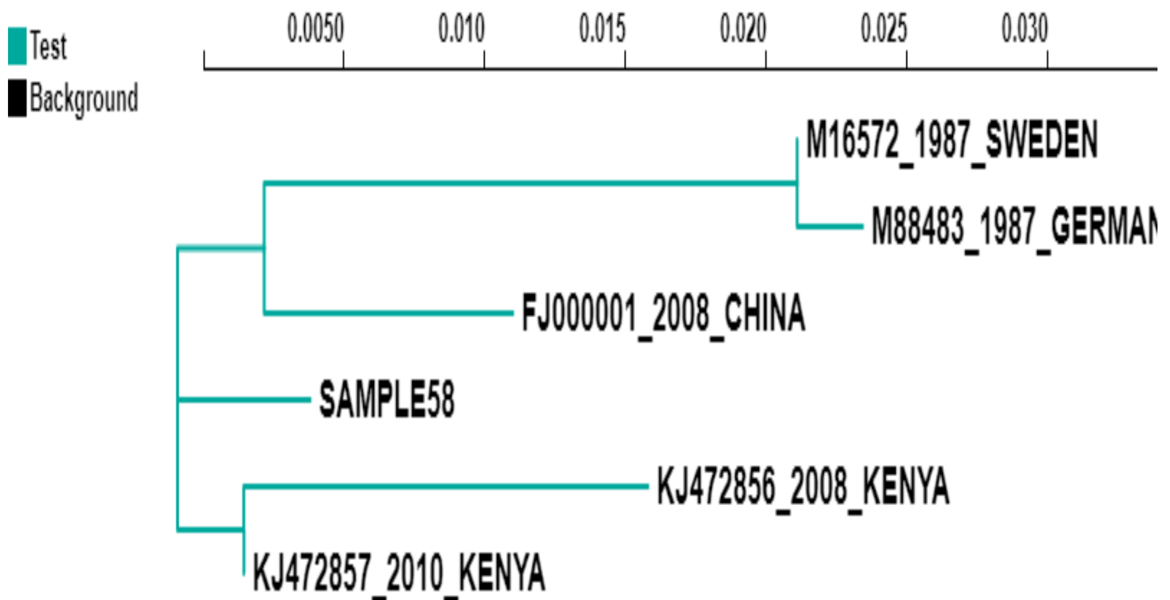

Fig. 4.8: Evolutionary tree of CVs of serotype CV_B3 generated using SLAC method implemented in DAES using their VP1 gene nucleotide sequences (Kosakovsky Pond and Frost 2005).

4.3.8 Results of evolutionary analyses on nucleotide sequences of serotype CV_B3 in relation to those of their prototype strain using FUBAR method implemented in DAES

FUBAR found evidence of episodic positive/diversifying selection at 1 site and episodic negative/purifying selection at 0 sites with a posterior probability of 0.9 (Ben Murrell and Sasha Moola 2013). Table 4.8 and Fig. 4.9 show the results of this analysis.

Table 48: Results of evolutionary analysis of CVs of serotype CV_B3 in relation to their prototype strain using FUBAR method implemented in DAES.

Site	**A**	**B**	**$\beta-\alpha$**	**Prob[$\alpha>\beta$]**	**Prob[$\alpha<\beta$]**	**BayesFactor[$\alpha<\beta$]**
43	1.808	12.311	10.503	0.065	0.900	10.679

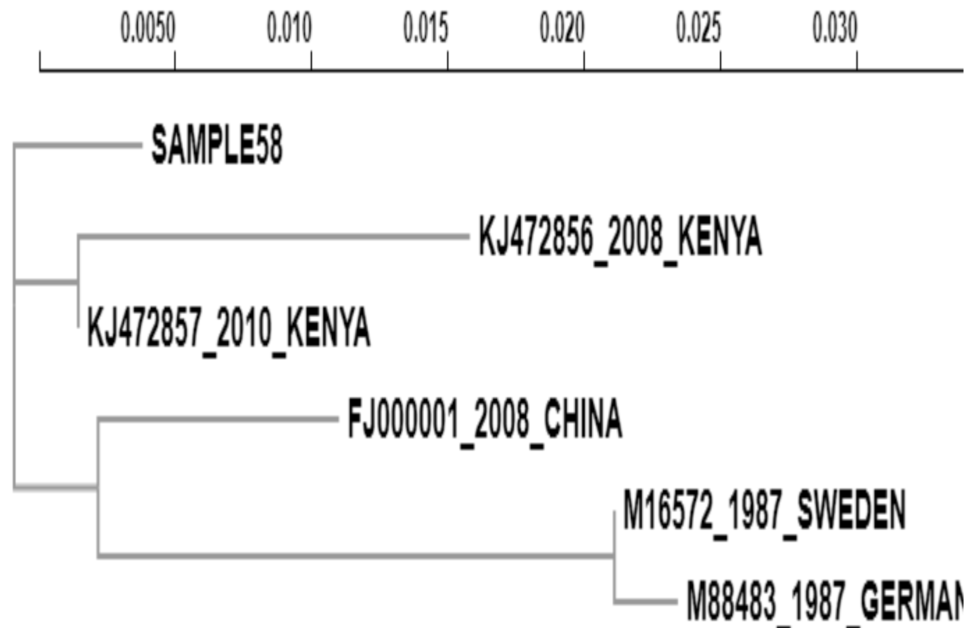

Fig.4.9: Evolutionary tree of CVs of serotype CV_B3 generated using FUBAR method implemented in DAES using their VP1 gene nucleotide sequences (Ben Murrell and Sasha Moola 2013).

4.3.9 Results evolutionary analyses on the nucleotide sequences of serotype CV_B4 in relation to those of their prototype using BUSTED, FEL, FUBAR and SLAC methods implemented in DAES

4.4.0 Results evolutionary analyses on the nucleotide of serotype CV_B4 in relation to those their prototype using the BUSTED method

BUSTED found no evidence (LRT, p-value = 0.752 ≥ .05) of gene-wide episodic diversifying selection in the selected test branches of VP1 gene phylogenies. Therefore, there is no evidence that any sites in VP gene nucleotide sequences experienced

diversifying selection along the test branches (Murrell et al. 2015). The results of this analysis are shown in Fig. 4.10.

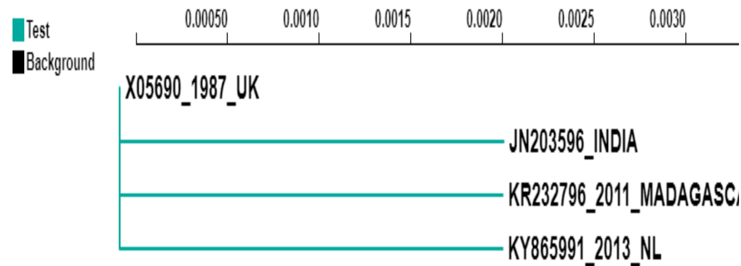

Fig. 4.10: Evolutionary tree of CVs of serotype CV_B4 generated using BUSTED method implemented in DAES using their VP1 gene nucleotide sequences (Murrell et al. 2015).

4.4.1 Results of evolutionary analyses on nucleotide sequences of serotype CV_B4 in relation to those of their prototype using FEL method

FEL found evidence of pervasive positive/diversifying selection at 0 sites and pervasive negative/purifying selection at 0 sites with a p-value threshold of 0.05 (Kosakovsky Pond and Frost 2005). Fig. 4.11 shows the results of this analysis.

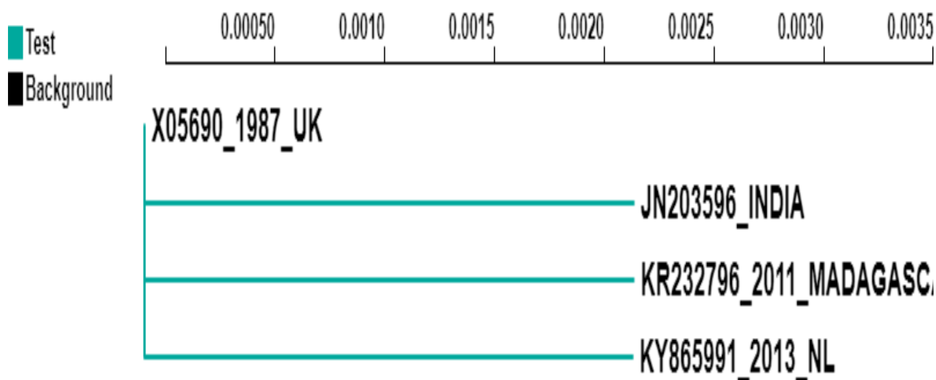

Fig. 411: Evolutionary tree of CVs of serotype CV_B4 generated using FEL method implemented in DAES using their VP1 gene nucleotide (Kosakovsky Pond and Frost 2005).

4.4.2 Results of evolutionary analyses on the nucleotide sequences of serotype CV_B4 in relation to those of their prototype strain using SLAC Method

SLAC found evidence of pervasive positive/diversifying selection at 0 sites and negative/purifying selection at 0 sites with a p-value threshold of 0.01 threshold (Sergei 2005). Fig. 4.12 shows the results of this analysis.

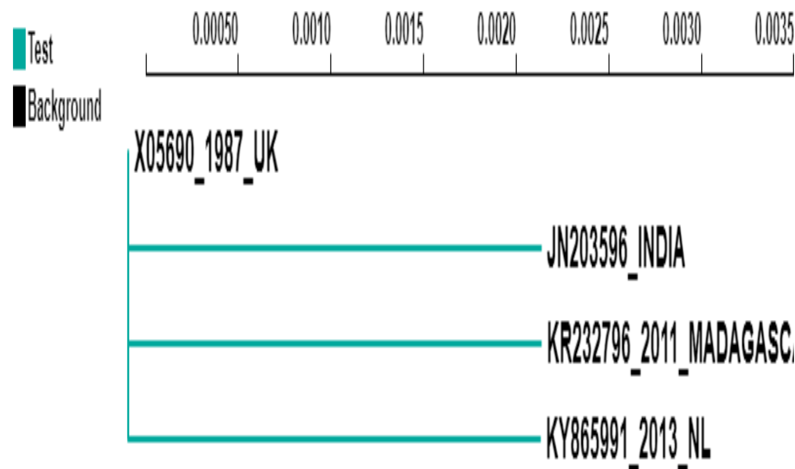

Fig. 4.12: Evolutionary tree of CVs of serotype CV_B4 generated using SLAC method implemented in DAES using their VP1 gene nucleotide sequences (Kosakovsky Pond and Frost 2005).

4.4.3 Results of evolutionary analyses on the nucleotide sequences of serotype CV_B4 in relation to those of their prototype using FUBAR method

FUBAR found evidence of episodic positive/diversifying selection at 0 sites and episodic negative/purifying selection at 0 sites with a posterior probability of 0.9 (Murrell et al. 2013). Fig. 4.13 illustrates these results.

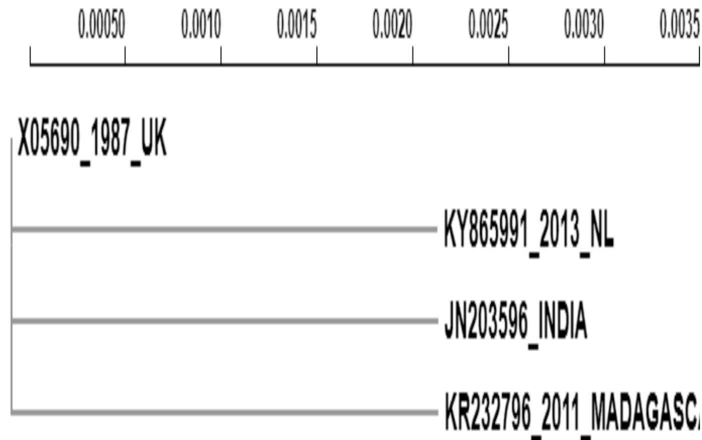

Fig. 4.13: Evolutionary tree of CVs of serotype CV_B4 generated using FUBAR method implemented in DAES using their VP1 gene nucleotide sequences (Murrell et al. 2013).

4.4.4 Results of evolutionary analyses on the nucleotide sequences of serotype CV_B5 in relation to those of their prototype using BUSTED, FEL, SLAC and FUBAR methods implemented in DAES

Evolutionary analysis of CVs of serotype CV_B5 using BUSTED, FEL, FUBAR and SLAC methods showed no natural selection events in their VP1 gene or their codon sites because nucleotide sequences were not different from one another.

Table 4.9 shows a summary of the results of revolutionary analyses carried out on the nucleotide sequences of VP1 genes CVs of serotypes CV_B2, CV_B3 and CV_B4.

Table 4.9: Summary of results of evolutionary analyses of serotypes of CV_B species

Type of selection and method used	No. of selection Events detected	Nucleotide sequences	No. of Sites	CV_B serotype
Diversifying using BUSTED	At least 1 in at least 1 test branch	7	99	CV_B2
	At least 1 in at least 1 test branch	6	100	CV_B3
	0	4	93	CV_B4
Pervasive positive/diversifying Using FEL	0	7	99	CV_B2
	0	6	100	CV_B3
	0	4	93	CV_B4
Pervasive negative/purifying Using FEL	0	7	99	CV_B2
	0	6	100	CV_B3
	0	4	93	CV_B4
Pervasive positive/diversifying using SLAC	0	7	99	CV_B2
	0	6	100	CV_B3
	0	4	93	CV_B4
Pervasive negative/purifying using SLAC	0	7	99	CV_B2
	0	6	100	CV_B3
	0	4	93	CV_B4
Episodic positive/diversifying Using FUBAR	2	7	99	CV_B2
	1	6	100	CV_B3
	0	4	93	CV_B4
Episodic negative/purifying Using FUBAR	0	7	99	CV_B2
	0	6	100	CV_B3
	0	4	93	CV_B4

4.5.0 DISCUSSION OF RESULTS

Prevalence of CVIs: Seroprevalence (SP) and actual prevalence(AP)

Determination of prevalence of CVIs was a preliminary step in the characterization of CVs that circulated in selected regions of Kenya in 2007. The overall seroprevalence of CVIs in patients caused by serotypes of CV_B species was estimated to be 0.75% while the overall actual prevalence was 0.41%. The positivity of the serotypes were: CV_B2 (0.3%), CV_B3 (0.03%), CV_B4 (0.03%) and CV_B5 (0.07%). Comprehensive data on the seroprevalence of CVIs in people living in Kenya and many parts of Africa is unavailable for comparison.

However, this seroprevalence is lower than that found in Italy where a similar study was carried out and reported on 5th November 2020. The positivity of the serotypes of CV_B species in Italy were CV_ B4 69.1%, CV_B3 (33.3%), CV_B5 (26.2%), CV_B1 (12.7%), B2 (11.0%), and B6 (1.7%) (Sciandra et al. 2020).

The low seroprevalence in this study could be attributed to the fact that samples were taken from a small number of patients. Samples from a significant number of people both healthy and ill people were not taken for characterization. Besides, the prevalence was low because some CVs that may have been in the 22 positive samples could have been destroyed while on transit to the laboratory from sampling sites, during the numerous freeze-thaw cycles and storage period inside the NIC laboratory freezers, about 5 years after they were collected from the study sites.

Genetic Identity of the Coxsackieviruses

The study identified CV_B species of serotypes CV_B2 (67%), CV_B3 (8%), CV_B4(8%) and CV_B5(17%) as the CVs that contributed to the prevalence of ILIs in the selected regions of Kenya in 2007. Eleven (11) out 12 nucleotide sequences obtained were assigned GeneBank Accession numbers ranging from MH522779 to MH522789. These nucleotide sequences were made available to the public by uploading on the GeneBank servers on 10th March 2019. The sample identity, serotypes and the GeneBank accession numbers of these viruses are shown in table 4.1 and appendix 3.

One Isolate of serotype CV_B5 identified in sample 53 was not assigned a GeneBank accession number. This was because this isolate was largely identical (99.7 %) to isolate MH522789 identified in sample 52. Table 4.5 shows the comparative sequence identity matrix of both isolates and appendix 4 shows the nucleotide sequence of the isolate that was in sample 53. The study did not identify any isolates of serotypes CV_B1 and CV_B6. Table 4.5.

Results of the genetic identities of CVs identified in this study were compared to those of a similar study carried out in Pakistan in 2013, where serotypes CV_B species isolated in stool samples of children suffering from non-polio acute flaccid paralysis were characterized (Angez et al. 2017). The study findings were: CV-B1(12.5%), CV-B2 (4.1%), CV-B3 (8.3%), CV-B4 (4.1%), CV-B5 (50%) and CV-B6 (21% (Angez et al. 2017). A comparison of the two studies show that CVs of serotypes CV_B2 and CVB3 were higher in the selected regions of Kenya than in Pakistan and contributed significantly to the high prevalence of ILIs witnessed in 2007 in Kenya because these serotypes are known to cause severe illnesses (Angez et al. 2017).

Besides serotypes of CV_B species, especially those of CV_B3, may have contributed to immuno-compromisation of the residents as they infect lymphocytes. (Vuorinen et al. 1994). Immuno-compromisation of residents may have made the infected people vulnerable to many diseases causing a high prevalence of ILIs witnessed in the selected regions in Kenya in 2007.

The absence of isolates of serotypes of CV_B1 and CV_B 6 amongst the samples used in this study could be attributed to the small number of samples collected from the study sites, loss of some viruses during transportation of samples to the laboratory and long storage period before characterization of these viruses was carried out in this study. Besides, some viruses may have been lost during the many thaw-freeze cycles the samples were subjected to when they were being used for other molecular work on in the laboratory.

Comparisons of the nucleotide sequence identities of the identified CVs showed that CVs of the same serotypes are closely related. However, the identity matrices show that all the viruses identified in the study had undergone mutations. The only isolate of serotype CV_B3 in this study had the highest percentage of mutations (75 %) while the CVs of serotype CV_B2 had the least mean percentage of mutations in their VP1 gene nucleotide sequences (16.8 %) as shown in Table 4.5.

The mutations on the VP1 genes or at codon sites of these genes may have been due to selection pressure by the immune systems (antibodies) of the patients or due to recombination of CV RNA with genome RNAs of other related viruses in infected cells of the patients. RNA recombination through “copy” choice mechanism amongst CVs of CV_B species with those of related viruses may have resulted in increased virulence and

rapid spread of CVs. Consequently, this may have led to a high prevalence of CVIs and ILIs in the selected regions of Kenya (Oberste, Peñaranda, and Pallansch 2004).

Phylogenetic analyses of the Coxsackieviruses

The dendrogram of phylogeny constructed from nucleotide sequences of the identified CVs, their prototypes and Echoviruses showed phylogenetic clustering among the CV isolates. The dendrogram also showed some genetic diversity amongst the isolates. The genetic relationships of the study isolates within and between CV_B serotypes were further analyzed and showed that CVs and Echoviruses diverged from each other to form two main branches. One main branch consisted of the outgroup "Travis" (EU024958.1), the prototype strain of Echovirus12 isolated in Germany and reported in 1994. The other main branch comprised of CVs of CV_B serotypes.

Members of CV_B serotypes diverged to form two branches. One branch consisted of "J.V.B Benschoten" (GQ329769.1), the prototype strain of CV_B4 serotype isolated in the UK, in 1987. The other branch comprised one CV of CV_B4 serotype that was present in sample 55(MH522788) as well as those of other serotypes; prototype CV_B1 "Nomoto" strain (M16560.1) reported in 1987 in Japan, prototype CV_B6 strain "Schmitt" (AF105342.1) reported in 1998. Isolates CV_B2, CV_B3 and CV_B5 clustered into three distinct CV_B clusters together with their respective prototype strains.

One of the clusters comprised of CVs of serotype CV_B3 with strain GQ329766.1 from Shandong China reported in 2008 and isolate MH522787 identified in

Sample 58 in this study. The two viruses are closely related to each other as they have 100% posterior probability value between their branches.

The second cluster comprised of CVs of CV_B5 serotype that were in samples_52 (Isolate MH522789), 52b and 53 and their prototype strain JX513586.1. The mean nucleotide divergence between the isolates and their prototype strain was 77.75% posterior probability value. This showed that the isolates were closely related but were diverging from their prototype strain. This is confirmed by their genetic identity matrix in table 4.4.

The other cluster comprised of CVs of CV_B2 serotype that were in samples 15(Isolate MH522779), 59(Isolate MH522781), 61(Isolate MH522782), 62(Isolate MH522783), 63 (Isolate MH522784), 64 (Isolate MH522785) and 65 (Isolate MH522776) and their prototype strain (GQ329738.1). The CVs in this cluster were found to be closely related to each other as they had 100% posterior probability between their branches. In general, the phylogenetic analyses show that the CVs that contributed to a high prevalence of ILIs in Kenya are related to others globally and were not native to the selected regions in Kenya but were spread to residents of these areas.

Evolutionary Analyses of the identified Coxsackieviruses

Evolutionary analyses using BUSTED, FEL, FUBAR and SLAC methods implemented in DAES found no evidence of natural selection events in the VP1 genes or at codon sites of this gene in the nucleotide sequences of serotypes CV_B4 and CV_B5 identified in this study. Figures 4.10- 4.3.

However, the BUSTED method found gene-wide episodic diversifying selection events in the selected test branches of CV_B2 and CV_B3 VP1 gene phylogenies (LRT, p-value = 0.752 ≥ .05). At least one site on at least one test branch of each of the VP1 genes of the CVs of these serotypes has experienced diversifying selection event over time (Murrell et al. 2015). Figures 4.2 and 46.

Furthermore, FUBAR method found evidence of episodic positive/diversifying selection events at codon sites on the VP1 genes of CVs of serotypes CV_B2 and CV_B3. The pieces of evidence were found at 2 sites (sites 17 and 50) in the VP1 gene of CVs of serotype CV_B2 and at 1 site (site 43) in the VP1 gene of CV of serotype CV_B3 identified in this study as shown in Tables 4.9 and Fig 4.13 (Murrell et al. 2013).

The BUSTED and FUBAR methods provided evidence that the immunogenic VP1 proteins of CVs of serotypes CV_B2 and CV_B3 identified in this study had evolved into divergent forms as they experienced gene-wide episodic diversifying selection events in the selected test branches of their VP1 gene phylogenies and at codon sites of this gene when compared to other members of their serotypes in Kenya and other parts of the world (Guéguen and Duret 2018). Table 4.9.

The resultant divergent forms of the VP1 proteins of these viruses could have enabled these viruses to evade the human immune system, become more pathogenic and spread easily. This may have led to a high prevalence of CVIs, consequently, a high prevalence of ILIs witnessed in 2007 in the selected regions of Kenya. The exact cause of mutations that resulted in diversifying selection events in the VP1 genes or their codon sites of CVs of CV_B2 and CV_B3 and is a subject for further investigation.

The information provided by this study provides useful information base on CVs in Kenya and can be used for management, control and future preparedness of CVIs in case of outbreaks. Besides the findings of this study can be used in the development of agents for diagnosis, treatment and prophylaxis of CVIs.

4.6.0 CONCLUSION AND RECOMMENDATIONS

4.6.1 Conclusion

The findings of this study show that the seroprevalence and the actual prevalence of CVIs among patients with ILI in selected regions of Kenya in 2007 were 0.75% and 0.41% respectively. These percentages, although low, indicate that CVIs contributed to the prevalence of ILIs among patients from these regions.

No Coxsackieviruses of species A were found in circulation during the study period. However, Coxsackieviruses B species were identified. These consisted of serotypes CV_B2, CV_B3, CV_B4 and CV_B5.

The findings of the study also show that the identified CVs were phylogenetically related to their prototypes, although not identical implying that they were evolved variants.

Coxsackieviruses of serotypes CV_B2 and CV_B3 evolved at a faster rate than CVs serotypes CV_B4 and CV_B5. Thus, due to their higher evolution rate Coxsackieviruses of serotypes CV_B2 and CV_B3 have the potential diversify, especially in the VP1 region making them evade the human immune system, and cause more serious outbreaks in Kenya and other probably parts of the world.

The findings also reveal that CVs of serotype CV_B2 and CV_B3 to have evolved into divergent VP1 proteins that evade the immune system, are more pathogenic and spread easily thus cause more prevalence disease. Serotypes CV_B 4 and CV_B5 had not evolved despite having undergone some mutations in their VP1 genes.

4.6.2 Recommendations of the study

The following recommendations should be considered concerning CVIs in Kenya:

1. Surveillance and characterization of CVs should continue to be routinely carried out in Kenya. This will provide comprehensive information on prevalence, identity and other virologic characteristics as the results of this study cannot be generalized, since they did not cover the whole country.
2. Furthermore, the species and serotype diversity observed by this study can guide development of policies for the control and preparedness of outbreaks of CVIs.

5.0 APPENDIXES

Appendix 1. Hand, Foot and Mouth Disease (HFMD) presentation in humans

Coxsackieviruses infect human hands, feet and mouth causing HFMD that is characterized by reddish itchy blister-like sores. Fig. 5a shows symptoms of HFMD disease in humans.

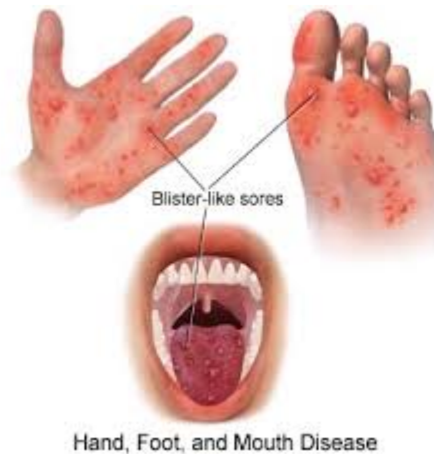

Fig.5.1: Symptoms of hand, foot and mouth disease in humans caused by CVs.
Source:https://encryptedtbn0.gstatic.com/images?q=tbn:ANd9GcSAI6mRKIY877vyUF UHZnskK7YVgJbMPgpH2lRcR8oFlfef14r5RA

Appendix 2. Secondary Coxsackievirus B infection of body organs

Coxsackievirus B (CV_B) species infect the human body and spread through blood and lymph to infect other body organs. These secondary CVIs (SCVIs) are known to cause severe life-threatening health complications such as diabetes type I, encephalitis, inflammation of body organs, autoimmune diseases, myocarditis and blood cancers. SCVIs of human body tissues are illustrated in fig. 5.3.

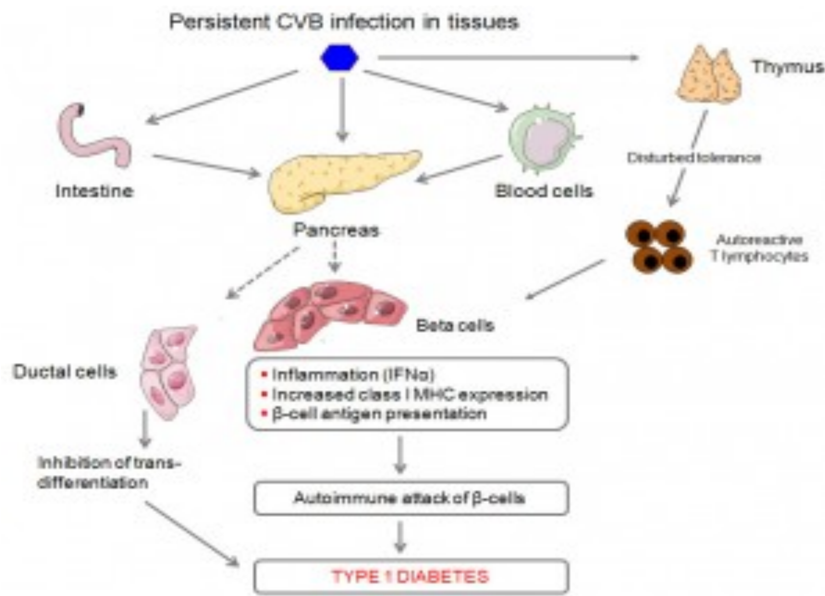

Fig. 5.3: Diagrammatic representation of secondary infection of body organs by CV_B species and health complications that occur after these infections.

Source: https://www.google.com/imgres?imgurl

Appendix 3. Nucleotide sequences of Coxsackieviruses Isolated in Kenya during the 2007 Influenza-like disease surveillance and their GeneBank accession numbers

```
>Sample 15 CV_B2.sq JBSK15 (MH522779)
      1 CATACCTCAC AAGTCACACC AAGCGACACA ATGCAAACCA GGCATGTGCA TAATTACCAT
     61 TCAAGGTCAG AGTCCAGTGT AGAGAATTTC CTAGCGCGAT CGGCATGCGT GTTTTATACA
    121 ACATATACAA ATAGCAAAAA TGCCGCCAAG GAGAAAAAGT TTGCAACATG GAAAGTAAGC
    181 GTTAGGCAGG CTGCACAATT GAGAAGAAAG TTAGAATTAT TCACATATCT ACGCTGTGAC
    241 ATTGAACTCA CGTTCGTCAT TACTAGTGCA CAGGACCCAT CAACAGCCAC CAATCTAGAT
    301 GTGCCAGTGT TGACACACCA

>Sample 28 CV_B2.sq JBSK28 (MH522780)
      1 ACCTCACAGG TCACACCAAG TGATACGATG CAAACTAGGC ATGTGCACAA TTACCATTCA
     61 AGGTCAGAAT CCAGTGTGGA GAACTTCTTA GCACGATCAG CATGTGTGTT TTACACAACA
    121 TACACAAACA GCAAAAATGC TGCTAAGGAG AAAAAGTTTG CGACATGGAA AGTGAGTGTT
    181 AGACAAGCTG CGCAATTGAG AAGGAAGTTG GAATTGTTCA CGTACTTGCG CTGTGATATT
    241 GAACTCACAT TCGTCATTAC CAGTGCGCAG GACCCGTCAA CAGCCACCAA TTTAGATGTG
    301 CCAGTGTTAA CACACCA

>Sample 52 CV_B5.sq JBSK52 (MH522789)
      1 CGGGGCACAC GTCCCAGGTG GTCCCGGCAG ACACCATGCA AACCAGGCAC GTAAAGAATT
     61 ATCACTCGCG ATCTGAATCC ACAGTAGAGA ACTTTCTGTG TAGATCCGCG TGCGTGTATT
    121 ACACAACCTA CAAGAACCAT GGAACCGATG GGGACAATTT CGCCTACTGG GTGATTAACA
    181 CAAGACAGGT TGCGCAATTA CGCCGCAAAC TAGAAATGTT CACATATGCC AGATTTGATT
    241 TAGAGCTCAC CTTTGTGATA ACAAGCACAC AAGAACAATC CACCATCCAA GGTCAAGACT
    301 CGCCTGTGCT AACTCATCAA ATAATGTACA TCCCCCCCGG CG

>Sample 55 CV_B4.sq JBSK55 (MH522788)
      1 GGAGCTGGAC ACACATCGCA GGTTGATCCA AGTGACACCA TGCAAACCAG GCACGTGCAC
     61 AACTATCACT CAAGGTCTGA GTCGTCAATA GAGAACTTTT TGTGTAGGTC TGCATGTGTA
    121 ATTTACATCA AATATTCAAG TGCCGAATCT AACAACTTAA AGCGTTATGC AGAGTGGGTC
    181 ATTAACACAA GACAGGTGGC ACAGCTGCGA CGGAAAATGG AAATGTTCAC ATACATTCGC
    241 TGTGACATGG AATTGACATT TGTCATAACC AGCCACCAGG AAATGTCCAC AGCTACCAAT
```

301 TCTGATGTCC CAGTTCAAAC ACATCAAATA ATGTACATCC CCCCCGGCGC C

>Sample 58 CV_B3.sq JBSK58 (MH522787)

1 CACACGTCGC AGGTGATACC AGGGGACACT ATGCAGACAC GCCACGTGAA AAATTACCAC

61 TCGAGATCCG AGTCAACGAT CGAGAATTTC CTGTGCAGAT CTGCCTGCGT TTACTTCACT

121 GAGTATGAGA ATTCTGGGTC TAAGCGTTAT GCTGAGTGGG TAATAACAAC ACGTCAGGCA

181 GCCCAGCTTA GGAGGAAGCT TGAATTCTTT ACGTACATAA GATTTGATTT AGAACTCACC

241 TTTGTAATCA CGAGCACGCA GCAACCCTCC ACAACTCAGA ATCAAGATGC ACAAATCCTC

301 ACCCATCAGA TTATGTGCAT CCCCCCCGGC GGG

>Sample 59 CV_B2.sq JBSK59 (MH522781)

1 CATACCTCAC AAGTCACGCC AAGCGATACA ATGCAAACCA GGCATGTGCA TAATTACCAT

61 TCAAGGTCAG AATCCAGTGT AGAGAATTTC TTAGCGCGAT CGGCATGCGT GTTTTACACA

121 ACATATACAA ATAGCAAAAA TGCTGCCAAG GAGAAAAAGT TTGCAACATG GAAAGTAAGC

181 GTTAGGCAAG CTGCACAATT GAGAAGAAAG TTAGAATTAT TCACATATTT ACGCTGTGAC

241 ATTGAACTCA CGTTCGTCAT TACCAGTGCA CAGGACCCAT CAACAGCCAC CAATCTAGAT

301 GTGCCAGTGT TGACACACCA

>Sample 61 CV_B2.sq JBSK61 (MH522782)

1 CACACCTCAC AGGTCACACC AAGTGATACG ATGCAAACCA GGCATGTGCA CAATTACCAT

61 TCAAGGTCAG AATCCAGTGT GGAGAACTTC TTGGCACGAT CTGCATGTGT GTTTTACACA

121 ACATACACAA ACAGCAAAAA TGTTGCTAAG GAGAAAAAGT TTGCGACATG GAAAGTGAGT

181 GCTAGGCAAG CTGCACAATT GAGAAGGAAG TTGGAATTGT TCACGTACTT GCGCTGTGAT

241 ATTGAACTCA CGTTCGTCAT TACCAGTGCG CAGGACCCGT CAACAGCCAC CAATTTAAAT

301 GTGCCAGTGT TGACACACCA

>Sample 62 CV_B2.sq JBSK62 (MH522783)

1 CACACCTCAC AGGTCACACC AAGTGATACG ATGCAAACCA GGCATGTGCA CAATTACCAT

61 TCAAGGTCAG AATCCAGTGT GGAGAACTTC TTGGCACGAT CTGCATGTGT GTTTTACACA

121 ACATACACAA ACAGCAAAAA TGTTGCTAAG GAGAAAAAGT TTGCGACATG GAAAGTGAGT

181 GTTAGGCAAG CTGCACAATT GAGAAGGAAG TTGGAATTGT TCACGTACTT GCGCTGTGAT

241 ATTGAACTCA CGTTCGTCAT TACCAGTGCG CAGGACCCGT CAACAGCCAC CAATTTAGAT

301 GTGCCAGTGT TGACACACCA

>Sample 63 CV_B2.sq JBSK63 (MH522784)

```
  1 CACACCTCAC AGGTCACACC AAGTGATACG ATGCAAACCA GGCATGTGCA CAATTACCAT
 61 TCAAGGTCAG AATCCAGTGT GGAGAACTTC TTGGCACGAT CTGCATGTGT GTTTTACACA
121 ACATACACAA ACAGCAAAAA TGTTGCTAAG GAGAAAAAGT TTGCGACATG GAAAGTGAGT
181 GTTAGGCAAG CTGCACAATT GAGAAGGAAG TTGGAATTGT TCACGTACTT GCGCTGTGAT
241 ATTGAACTCA CGTTCGTCAT TACCAGTGCG CAGGACCCGT CAACAGCCAC CAATTTAAAT
301 GTGCCAGTGT TGACACACCA
```

>Sample 64 CV_B2.sq JBSK64 (MH522785)

```
  1 CACACCTCAC AGGTCACACC AAGTGATACG ATGCAAACCA GGCATGTGCA CAATTACCAT
 61 TCAAGGTCAG AATCCAGTGT GGAGAACTTC TTGGCACGAT CTGCATGTGT GTTTTACACA
121 ACATACACAA ACAGCAAAAA TGTTGCTAAG GAGAAAAAGT TTGCGACATG GAAAGTGAGT
181 GTTAGGCAAG CTGCACAATT GAGAAGGAAG TTGGAATTGT TCACGTACTT GCGCTGTGAT
241 ATTGAACTCA CGTTCGTCAT TACCAGTGCG CAGGACCCGT CAACAGCCAC CAATTTAGAT
301 GTGCCAGTGT TGACACACCA
```

>Sample 65 CV_B2.sq JBSK65 (MH522786)

```
  1 CACACCTCAC AGGTCACACC AAGTGATACG ATGCAAACCA GGCATGTGCA CAATTACCAT
 61 TCAAGGTCAG AATCCAGTGT GGAGAACTTC TTGGCACGAT CTGCATGTGT GTTTTACACA
121 ACATACACAA ACAGCAAAAA TGTTGCTAAG GAGAAAAAGT TTGCGACATG GAAAGTGAGT
181 GTTAGGCAAG CTGCACAATT GAGAAGGAAG TTGGAATTGT TCACGTACTT GCGCTGTGAT
241 ATTGAACTCA CGTTCGTCAT TACCAGTGCG CAGGACCCGT CAACAGCCAC CAATTTAAAT
301 GTGCCAGTGT TGACACACCA
```

Appendix 4. Nucleotide sequences of Coxsackievirus of serotype CV_B5 identified in sample 53 and not assigned GeneBank due to its close identity with the one in sample 52

```
> Sample 53 CV_B5.sq JBSK53
      1 CGGCACACGT CCCAGGTGGT CCCGGCAGAC ACCATGCAAA CCAGGCACGT AAAGAATTAT
     61 CACTCGCGAT CTGAATCCAC AGTAGAGAAC TTTCTGTGTA GATCCGCGTG CGTGTATTAC
    121 ACAACCTACA AGAACCATGG AACCGATGGG GACAATTTCG CCTACTGGGT GATTAACACA
    181 AGACAGGTTG CGCAATTACG CCGCAAACTA GAAATGTTCA CATATGCCAG ATTTGATTTA
    241 GAGCTCACCT TTGTGATAAC AAGCACACAA GAACAATCCA CCATCCAAGG TCAAGACTCG
    301 CCTGTGCTAA CTCATCAAAT AATGTACATC CCCCCCGGCG
```

Appendix 5. Coxsackievirus genome and the encoded structural and non-structural polyprotein

Coxsackievirus genome RNA encodes a large polyprotein which is cleaved into smaller proteins by enzymes into structural and non-structural proteins. VP1 gene nucleotide sequences encode the immunogenic VP1 protein on the protein coat of CV. The VP1 gene nucleotide sequences are used for identification, classification and establishment of phylogenetic as well as evolutionary relationships of CVs. Fig. 54 shows the CV genome, encoded proteins and their functions.

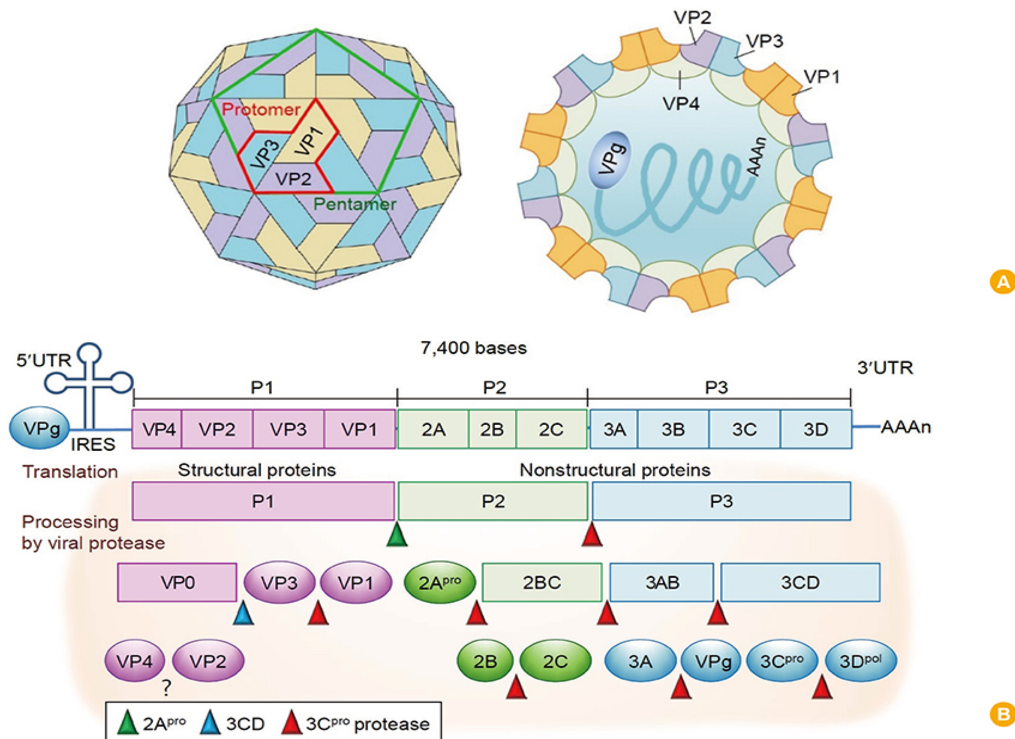

Fig. 5.4: Coxsackievirus genome that encodes a large polyprotein that is cleaved by enzymes into structural and non-structural proteins.

Source:
https://www.researchgate.net/publication/313317448_Enterovirus_71_infection_and_vaccines.

Appendix 6. KEMRI Ethical Approval

Appendix 7. WRAIR Ethics Approval

Appendix 8. Plagiarism Report

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