ISOLATION AND CHARACTERIZATION OF HUMAN ENTEROVIRUSES FROM STORED SAMPLES OF CHILDREN WITH ACUTE RESPIRATORY INFECTIONS ATTENDING KENYATTA NATIONAL HOSPITAL, NAIROBI

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Isolation and characterization of human enteroviruses from stored samples of children with acute respiratory infections attending Kenyatta National Hospital, Nairobi

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DECLARATION

I declare that this thesis is my original work and has not been presented for a degree in any other university.

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This thesis has been submitted for examination with our approval as the university supervisors:

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The Late Dr. Walter Ochieng

KEMRI, KENYA.

DEDICATION

This thesis is dedicated my parents.Mr and MrsMureithi. God bless them.

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ABBREVIATIONS AND ACRONYMS

ARI:	Acute Respiratory tract Infections
BLAST	Basic Local Alignment Search Tool
CPE:	Cytopathic effects
CSF:	Cerebrospinal fluid
CNS:	Central nervous system
DFA:	Direct Fluorescent Antigen
EDTA:	Ethylene Diamine Tetra acetic Acid
DMEM:	Dubelcoss Modified Eagles Medium
EIA:	Enzyme Immuno Assay
EVs:	Enteroviruses
ERC:	Ethical Review Committee
GI:	Gastrointestinal Infections
HEV:	Human Enterovirus
HFMD	Hand Foot Mouth Disease
IDFAT:	Indirect fluorescent antibody test
Ig:	Immunoglobulin
ILRI:	International Livestock Research Institute
ITROMID:	Institute of Tropical medicine and infectious diseases
JKUAT:	Jomo Kenyatta University of agriculture and technology
KEMRI:	Kenya Medical Research Institute
LRTI:	Lower Respiratory Tract Infection
MEGA	Molecular Evolutionary Genetic Analysis

NPA: Nasopharyngeal aspirates

NPEV: Non Polio Enteroviruses

NCR : Non CodingRegion

OPV: Oral Poliovirus Vaccine

PCR: Polymerase ChainReaction

PBS : Phosphate Buffered Saline

PV : Polio Virus

RD Cells: Rhabdomysarcoma cells

RPM : Revolutions Per Minute

RT-PCR: Real Time Polymerase Chain Reaction

RNA: Ribonucleic Acid

SSC: Scientific Steering Committee

SPSS: Statistical Package for Social Scientists

ssRNA: Single StrandedRibonucleic Acid

TBE: Tris Borate EDTA Buffer

URTI: Upper Respiratory Tract Infection

VTM: Virus Transport Medium

ABSTRACT

Enteroviruses are small non-enveloped isometric viruses that multiply in the gut mucosa and are transmitted from person to person by the faecal-oral route. Enteroviruses are a major cause of respiratory disturbances. Most infections occur during childhood, and they are usually transient but produce lifelong immunity. Clinical are generally mild, but occasionally infections may cause serious disease e.g. meningitis. In this cross sectional laboratory based study, the prevalence of enteroviruses infection was assessed in stored 287 samples from children with Acute Respiratory Infection (ARI) attending Kenyatta National Hospital in Nairobi. Throatswabsamples were inoculated in human Rhabdomysarcoma cells (RD cells) where positive samples were further characterized using indirect fluorescent antibody tests (IDFAT) to confirmenteroviruses.Ribonucleic Acid (RNA) was extracted frompositively identified enteroviruses and polymerase chain reaction (PCR) was performed to confirm them. These were sequenced, compared to otherenterovirusesdeposited at GenBank and phylogenetic trees were drawn to establish genetic relatedness. There were 24 (8.4%) CPE positive samples which were confirmed as enteroviruses. Of these positive samples, 10 (41.7%) were further identified as enterovirus, 9 (37.5%) coxsackie virus, 4 (16.7%) echovirus and 1 (4.2%) poliovirus. Eventhough no significant correlations (χ^2 (df=3) = 0.320; p = 0.956)were noted, enteroviruses were more common in patients in their 1st year (n=19; 79.2%) followed by the 2nd year (n=5; 20.8%).In the 3 months periodof sample collection, between April and June, most infections were detected in the month of April followed by May and June respectively.Male patients had slightly more (n=13; 54.2%) infections than females . PCR and sequencing results confirmed this study's isolates to a range of 90-99% to other similar enterovirusesin GenBank. Phylogenetic analysis of this study's isolates gave bootstrap values ranging from 36%-87% when compared to other enteroviruses in the GenBank. In conclusion, the detection of these virusessuggests theinvolvement of enteroviruses in paediatrics in Kenya.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Enteroviruses, a group of single-stranded sense RNA viruses, are commonly encountered infections, especially in infants and children. Enteroviruses belong to the Picornaviridae (small RNA viruses) family. The enteroviral group includes coxsackievirus, echovirus, and poliovirus. Enteroviruses are believed to have 2 distinct classes: polioviruses (types 1, 2, and 3) and nonpolioviruses (coxsackievirus, enterovirus, echoviruses, and unclassified enteroviruses). Enteroviral infections consist of 23 coxsackievirus A, 6 coxsackievirus B, 28 echovirus, and 5 unclassified enteroviruses(Kogon *et al*., 1969, Apostol *et al*., 2012).

More recently, a related genus of viruses, *Parechovirus,* has been described; two enterovirus species (echovirus types 22 and 23) were reassigned as parechovirus. To date, more than a dozen parechovirus strains have been described; however, not all sequences have been published. The clinical appearance of *Parechovirus* infection can be similar to enteroviral infections, but tests for *Parechovirus* are mostly confined to research laboratories(Craig *et al*., 2003).

Enterovirus 71 has gained notoriety in recent years for causing a rapidly fatal rhombencephalitis, in association with epidemics of HFM disease in East

Asiancountries. This appears to be a particularly aggressive neutrophicserotype of enterovirus(Moore, 1982). Each virus obtains its antigenicity from the capsid proteins that surround the RNA core. According to the Centers for Disease Control and Prevention(CDC), 65 human serotypes of enteroviruses have been identified; however, a small number cause most outbreaks.

Classification of HumanEnteroviruses:

Group: **Group IV (+)ssRNA)**
Order: *Picornavirales*
Family: *Picornaviridae*

Genus:	*Enterovirus*	*Cardiovirus*
	Aphthovirus	*Casavirus*
	Aquamavirus	*Dicipivirus*

Species:	*Bovine enterovirus*	*Human rhinovirus A*
	Human enterovirus A	*Human rhinovirus B*
	Human enterovirus B	*Human rhinovirus C*
	Human enterovirus C	*Porcine enterovirus B*
	Human enterovirus D	*Simian enterovirus A*

Within these ten species are the serotypes:

Coxsackie: Coxsackie viruses are a non-phylogenetic group. Coxsackie A viruses are mainly associated with human hand, foot and mouth disease. Coxsackie B viruses can cause mild signs and symptoms, similar to a "cold", but these viruses also can lead to more serious diseases, including myocarditis (inflammation of the heart); pericarditis(inflammation of the sac lining the heart); meningitis (inflammation of the membranes that line the brain and spinal cord); and pancreatitis :inflammation of the pancreas, (Li *et al*., 2005).

Echoviruses: Echovirusesare a cause of many of the nonspecific viral infections. They are mainly found in the intestine, and can cause nervous disorders. The usual symptoms of Coxsackie and echovirus are fever, mild rash, and mild upper respiratory tract illness (Chonmaitree *et al*., 1988).

Enterovirus 71:(EV-71) is notable as one of the major causative agents for hand, foot and mouth disease, and is sometimes associated with severe central nervous system diseases.EV71 was first isolated and characterized from cases of neurological disease in California in 1969.To date, little is known about the molecular mechanisms of host response to EV71 infection, but increases in the level of mRNAs encoding chemokines, proteins involved in protein degradation, complement proteins, and proapoptotis proteins have been implicated (Oberste *et al*., 1999).

Poliovirus: There are three serotypes of poliovirus, *PV1*, *PV2*, and *PV3*; each with a slightly different capsid protein. Capsid proteins define cellular receptor specificity and virus antigenicity. *PV1* is the most common form encountered in nature; however, all three forms are extremely infectious. Poliovirus can affect the spinal cord and cause poliomyelitis (Horstmann and McCollum, 1953, Kew *et al*., 2005, Apostol *et al*., 2012).

Rhinovirus: There are three species of Rhinoviruses: Human Rhinovirus A, Human Rhinovirus B, and Human Rhinovirus C which contain over 100 serotypes. Rhinoviruses are the most suspected causative agents of the common cold. This makes it difficult to develop a single vaccine against so many serotypes (Rueckert, 1996)**.**

Enterovirusesare associated with several human and mammalian diseases. Serological studies have distinguished 66 human enterovirusserotypes on the basis of antibody neutralization tests (Melnick, 1993). Additional antigenic variants have been defined within several of the serotypes on the basis of reduced or nonreciprocal cross-neutralization between variant strains. On the basis of their pathogenesis in humans and animals, the enteroviruses were originally classified into four groups, polioviruses, Coxsackie A viruses, Coxsackie B viruses, and echoviruses, but it was quickly realized that there were significant overlaps in the biological properties of

viruses in the different groups. Enteroviruses(EVs) isolated more recently are named with a system of consecutive numbers: EV68, EV69, EV70, and EV71(Nijhuis *et al*., 2002).

Enteroviruses affect millions of people worldwide each year, and are often found in the respiratory secretions such as saliva, sputum, or nasal mucusand stool of an infected person(Chonmaitree *et al*., 1988). Historically, poliomyelitis was the most significant disease caused by an enterovirus, Poliovirus. There are 62 non-polio enteroviruses that can cause disease in humans: 23 Coxsackie A viruses, 6 Coxsackie B viruses, 28 echoviruses, and 5 other enteroviruses. Poliovirus, as well as coxsackie and echovirus are spread through the fecal-oral route. Infection can result in a wide variety of symptoms ranging from mild respiratory illness (common cold), hand, foot and mouth disease, acute hemorrhagicconjunctivitis, aseptic meningitis, myocarditis, severe neonatalsepsis-like disease, and acute flaccid paralysis(Oberste *et al*., 1999).

Enteroviruses are members of the picornavirus family, a large and diverse group of small RNA viruses characterized by a single positive-strand genomic RNA. A picornavirus is a virus belonging to the family*Picornaviridae*. Picornaviruses are non-enveloped, positive-stranded RNA viruses with an icosahedralcapsid. The genomeRNA is unusual because it has a protein on the 5' end that is used as a primer for transcription by RNA polymerase(Manayani *et al*., 2002).

Although the majority of human enterovirus infections remain asymptomatic, these viruses are associated with diverse clinical syndromes,ranging from minor febrile illness to severe and potentiallyfatal pathologies, including aseptic meningitis, encephalitis, myopericarditis, acute flaccid paralysis, and severe neonatalsepsis-like disease(Wadia *et al*., 1983, Khetsuriani *et al*., 2006). Moreover, enteroviruses can induce nonspecificrespiratory illnesses in infants or adults, including upperrespiratory tract infections but also lower respiratory tractinfections (LRTIs), resulting in bronchitis, bronchiolitis and pneumonia (Craig *et al.*, 2003).

Enteroviruses are important etiologic agents of childhood lower respiratory tract diseases and these viral agents can be isolated from the nasopharyngeal tracts of infants with respiratory symptoms. However, like other human picornaviruses, EVs can be isolated by cell culture systems or detected by RT-PCR assays in the nasopharynges of infants without EV-related respiratory symptoms (Nijhuis *et al*., 2002).

1.2 Virology of Enteroviruses

The enteroviruses are icosahedral nonenveloped viruses that are approximately 30 nm in diameter. They have a capsid composed of 60 subunits each formed from 4 proteins. They are stable at a pH from 3-10, distinguishing them from other picornaviruses (including rhinoviruses), which are unstable below pH 6. Enteroviruses resist lipid solvents, ether, chloroform, and alcohol. They are

inactivated at temperatures above 50°C but remain infectious at refrigerator temperature. Magnesium Chloride ($MgCl_2$) reduces thermolability at higher temperatures.

The viruses are inactivated by ionizing radiation, formaldehyde, and phenol (Klein *et al* 2008). Enteroviruses cause a wide range of infections. Poliovirus, the prototypical enterovirus, can cause a subclinical or mild illness, meningitis or paralytic poliomyelitis, a disease that has been eradicated in the United States and other developed countries. The nonpolio viruses (group A and B coxsackieviruses, echoviruses, enteroviruses) continue to be responsible for a wide spectrum of diseases in persons of all ages, although infection and illness occur most commonly in infants (Horstmann and McCollum, 1953).Coxsackievirus infection is the most common cause of viral heart disease. Group Acoxsackieviruses may cause flaccid paralysis, while group B coxsackieviruses cause spastic paralysis. Other diseases associated with group A coxsackievirus infections include hand-foot-and-mouth disease (HFMD) and hemorrhagic conjunctivitis, while group B coxsackieviruses are associated with herpangina, pleurodynia, myocarditis, pericarditis, and meningoencephalitis. Aseptic meningitis and the common cold are associated with both groups. Diseases caused by echoviral infections range from the common cold and fever to aseptic meningitis and acute hemorrhagic conjunctivitis(Craig *et al*., 2003).

1.3 Pathologyof HumanEnteroviruses

Enteroviruses are spread via the fecal-oral route. The ingested viruses infect cells of the oral-pharyngeal mucosa and lymphoid tissue (tonsils) where they are replicated and shed into the alimentary tract. From here they may pass further down the gastrointestinal tract (Couch *et al.*, 1970). Due to the acid stability of these viruses; they can pass into the intestine and set up further infections in the intestinal mucosa.

The virus also infects the lymphoid tissue (Peyer's patches) underlying the intestinal mucosa. At these sites, the viruses replicate and are shed into the feces often for months after the primary infection. In the primary viremicphase, the virus also enters the bloodstream at low levels. The tissues that are then infected depend on the expression of the correct receptors. For example, CD155, the polio virus receptor, is expressed in spinal cord anterior horn cells, dorsal root ganglia, skeletal muscle, motor neurons and some cells of the lymphoid system(Gelfand *et al.*, 1963).

Expression of CD155 within embryonic structures giving rise to spinal cord anterior horn motor neurons may explain the restrictive host cell tropism of polio virus for this cellular compartment of the central nervous system. There are three polio virus serotypes and all of them bind to the CD155 receptor protein. For unknown reasons, polio virus does not spread to the cells of the central nervous system in all patients. The Coxsackie virus receptor (which also binds adenovirus) is a surface protein with two immunoglobulin-like domains(Rueckert, 1996).

At this stage symptoms may occur and the patient may experience fever and malaise. A secondary viremia may occur at this time. The spread of the virus fromthe gastro-intestinal tract and the secondary viremia that occurs about 10 days after the initial infection leads to a humoral and cell-mediated immune response (the latter being of less importance). This rapidly limits the further replication of the virus in all tissues except the GI tract because the virus must pass through extracellular space to infect another cell. In the GI tract, replication may be sustained for several weeks even though a high titer of neutralizing antibody is achieved.

The cells in which this replication occurs are not known and it is unclear why replication occurs in the presence of the neutralizing antibody. Although each group of enteroviruses share a receptor, the various serotypes of a group are usually not blocked by group-specific antibodies even though it would be expected that they would have a common receptor binding site. The reason for this appears to be that the cell receptor protein binds to a viral protein at the bottom of a canyon into which the cell protein can fit but notan antibody(Manayani *et al*., 2002).

1.4 Immunity and immune response

Immunity to enterovirus is serotype-specific. Intact humoral immunity is required for the control and eradication of enteroviral disease. T lymphocytes do not contribute to viral clearance and, in coxsackievirus B3 myocarditis, may contribute to myocardial

inflammation. Humoral immunity (antibody-mediated) mechanisms operate both in the alimentary tract (to prevent mucosal infection) and in the blood (to prevent dissemination to target organs (Rueckert, 1996).

Secretory immunoglobulin A (IgA) appears in nasal and alimentary secretions 2-4 weeks after the administration of live-attenuated oral poliovirus vaccine (OPV) and persists for at least 15 years. Upon re-exposure to infectious virus, high titers of secretory IgA antibodies prevent or substantially reduce poliovirus shedding; higher secretory IgA titers lead to better immunity (Rueckert, 1996).

Immunoglobulin M (IgM) antibodies appear as early as 1-3 days after enteroviral challenge and disappear after 2-3 months. Immunoglobulin G (IgG) antibody, which is generally detected 7-10 days after infection, is mostly of the IgG_1 and IgG_3 subtypes. Serum neutralizing IgG antibodies persist for life after natural enteroviral infections. Macrophage function is also a critical component of the immune response in enteroviral infections; ablation of macrophage function in experimental animals markedly enhances the severity of coxsackievirus B infections (Portes *et al*., 1998).

1.5 Statement of the problem

ARI is a leading cause of morbidity and mortality after malaria (WHO, 2012).In Kenya;few studies on viral etiologies of ARI have been carried out. In a study by Hazlet *etal*., (1988) at theKenyatta National Hospital, enteroviruses were detected from recruitedpatients. Most enterovirusinfections are subclinical, especially in

young children, but when they do cause clinically apparent disease, they can cause a wide range of clinical syndromes and can involve many of the body systems. Non-polio enteroviruses most commonly cause rashes, upper respiratory tract infections (URTIs) and colds. They can also cause neurological disease and are the most common cause of meningitis. In general, Coxsackievirus infections tend to cause more severe complications than echovirus infections resulting in: carditis, pleurodynia, herpangina, hand-foot-and-mouth disease and occasionally paralysis, all of which are rarely seen in echovirus infection. Detection of a broad number of respiratory viruses is not undertaken currently for the diagnosis of acute respiratory infection due to the large and always increasing list of pathogens involved. There is little information from KNH, a national reference hospital.

1.6 Justification

Enteroviruses are importantetiologic agents of childhood lower respiratory tract diseasesand these viral agents can be isolated from the nasopharyngeal tracts of infants with respiratory symptoms. Enteroviruses infecting humans are found worldwide and humans are the only known natural hosts. Young children are most susceptible to infection. These infections are among the most common reasons that small children are admitted to hospital in order to rule out a bacterial cause. The virus can spread transplacentally or from maternal fecal material and is most severe in infants born to mothers who contract the viral infection shortly before giving birth or in infants who contract the virus after birth. This is because the mother has not had

time to develop a protective immune response and pass protective antibody to the infant. Disease normally resolves but can be of consequence in the very young. Coxsackie B virus may result in severe neonatal disease including hepatitis, meningitis, myocarditis and adreno-cortical problems. Infections often spread through nurseries and are difficult to stop because of the resistance of the virus to disinfecting agents. Due to lack of current information, this study aimed to provide information on how rampant enteroviruses are in Kenya.

1.7 Research Questions

1. What is the prevalence of enteroviruses from the stored samples from children attending Kenyatta National Hospital?
2. What is the association of enteroviruses infection to the gender and age of stored samples from children attending Kenyatta National Hospital?
3. What are the genetic and phylogenetic characteristics of the enteroviruses detected from stored samples from children attending Kenyatta National Hospital?

1.8 Hypothesis

1.8.1 Null hypothesis

Samples collected from children at Kenyatta National Hospital (KNH) with acute respiratory infections do notharbour human enteroviruses.

1.9 Objectives

1.9.1 General objective

To Isolate and characterize enteroviruses from stored throat swabs of children with acute respiratory infection attending KNH.

1.9.2 Specific objectives

1. To isolateEnteroviruses from stored throat swab samples of children at KNH.
2. To determine the association between gender, age and Enterovirus infection in the children.
3. To determine thegenotypic relationship of the isolates.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology of Human Enteroviruses

Human enteroviruses are found worldwide, and humans are the only natural hosts. Enteroviruses are transmitted primarily by the fecal-oral route, mainly through the contamination of food or water. They are stable in low pH, and therefore are able to survive in the acidic environment of the stomach and gastrointestinal tract. In the gut, they are able to replicate and then be shed in the stool(Wolf *et al*., 1981). Respiratory spread is possible with some of the Coxsackieviruses, which can cause upper respiratory tract infections (URTIs). Young children are most at risk for infection, which is usually inapparent, while older children and adults are more at risk for complications.

These viruses are also able to spread from the gastrointestinal tract and enter the blood stream, causing viremia, and spread to other organs. An example of this is the spread of the poliovirus to motor-neurons of the central nervous system, causingparalytic poliomyelitis. Most infections are asymptomatic; they do, however usually result in protective immunity(Wolf *et al*., 1981).

In less developed areas of the world, most children become infected early in infancy, while in the developed world, first infections often do not occur until adolescence. Boys are more susceptible to the development of clinically apparent

diseases than girls(Melnick, 1993). The virus may be shed in the stool for many weeks. Enteroviruses have been found in water, soil, vegetable, and shellfish. Thus, they may also be transmitted by contact with contaminated food or water(Moore, 1982).

2.2 Clinical syndromes ofHuman Enteroviruses

Replication of the enterovirusesbegins in the gastrointestinal or respiratory tract and once the virus is present in the blood stream, the infection may affect various tissues and organs, causing a variety of diseases(Chang *et al*., 2004). The majority of infections are symptomless or mild in nature, the most common effect being a non-specific illness, with fever. Other manifestations include exanthems (rashes), herpangina (vesicular eruption and inflammation of the throat), acute respiratory disease, conjunctivitis, aseptic meningitis, encephalitis (inflammation of the brain), myopericarditis (inflammation of the heart tissue), and, occasionally, paralytic disease (Oprisan *et al*., 2002).

Many enteroviruses are associated with specific syndromes: for example, the viruses within the *Human enterovirus B* species more commonly cause meningitis or myopericarditis and those within the *Human enterovirus A* species more commonly cause hand-foot-mouth disease (rash especially on the palms and soles with vesicular eruption and inflammation of the mouth(Nijhuis *et al.*, 2002).

2.3 Diagnosis of HumanEnteroviruses

Clinically, it is difficult to distinguish the specific cause of most enteroviral infections. Diagnostic testing for non-polio enteroviruses requires specialized laboratory facilities. Diagnosis is made by detecting the virus in throat orfaecal samples or, more convincingly, from specimens collected from the affected part of the body, for example, cerebrospinal fluid (CSF), biopsy material, and skin lesions. A four-fold rise in the level of neutralizing antibody in specimens collected during the acute and convalescent phases of illness provides the best evidence for a recent infection(Gelfand *et al*., 1963).

2.4 Treatment ofHuman Enteroviruses

No specific antiviral agent is available for therapy of enterovirus infection. Treatment focuses on management of complications (for example, meningitis, abnormal cardiac rhythms, and heart failure). Intravenous administration of immune globulin may have some use in preventing severe disease in immunocompromised individuals or those with life-threatening disease(Manayani *et al*., 2002).

Currently there are no vaccines available for the non-polio enteroviruses. Prevention includes improved sanitation and general hygiene, in addition to quarantine and possible closing of schools in the case of recognized epidemics. Transmission of

enterovirus infections is increased by poor hygiene and overcrowded living conditions.

Improved sanitation and general hygiene are important preventative measures (Bourlet *et al.*, 2003). Measures that can be taken to avoid getting infected with enteroviruses include frequent hand washing, especially after diaper changes or going to the toilet, disinfection of contaminated surfaces with jik(sodium hypochlorite) and washing soiled articles of clothing. The viruses are resistant to many disinfectants so it is important to use chlorinated jik(sodium hypochloride) or iodized disinfectants.

During recognized epidemics, it may be advisable to close certain institutions such as schools or child care facilities in order to reduce transmission especially among young children(Nijhuis *et al*., 2002).In addition to having no vaccines, there are no specific antiviral agents currently available for clinical use. Treatment is symptomatic and focuses on complications associated with infection. Administration of immune globulin may be useful in preventing severe disease in immunocompromised individuals or in those with life-threatening disease(Bourlet *et al*., 2003).

Patients with enterovirus infections may present with symptoms as benign as an uncomplicated cold or as threatening as encephalitis, myocarditis, or neonatal sepsis. Enteroviral infections annually result in a large number of physician and emergency department visits. In 1998, Pichichero performed a prospective study and found that nonpolioenteroviral infections resulted in direct medical costs ranging from $69-771 per case.

In addition, patients with nonpolioenteroviral infections missed a minimum of 1 day of school or camp; some missed as many as 3 days of school or camp(Chang *et al*., 2004). The significant economic and medical impacts of enteroviral infections occur mostly during the peak months of summer and fall. In temperate climates, enteroviral outbreaks occur year-round.

2.5.Risk Factors

Enteroviral risk factors include poor sanitation, crowded living conditions, and lower socioeconomic class status. In addition, children younger than 5 years are more susceptible because of poor hygiene habits and lack of prior immunity(Nijhuis *et al*., 2002).

Neonatal infections are most likely acquired after birth rather than transplacentally. Exposure from an infected mother or another infant in the nursery during the first 2 weeks of life is the probable mode of transmission. The enteroviral exposure may be

perineally acquired during the delivery process (Kogon *et al.*, 1969).A B-cell response is needed for the host to properly fight off the enteroviral infection and to prevent entry to the Central nervous system (CNS). Children who lack a functioning B-cell system, such as those with X-linked agammaglobulinemia, are at risk of serious enteroviral infection, such as meningoencephalitis. Poliovirus is a consideration in all unimmunized or partially immunized children(Manzara *et al.*, 2002).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study site.

In this cross sectional laboratory based study, throat swab samples from children with acute respiratory illness attending the pediatric clinic at Kenyatta National Hospital (KNH) were collected and stored for 1 year at-80^{o}C in a previous study:"Prevalence of viral lower respiratory tract infection in children attending paediatric clinic in Kenyatta National Hospital"Ref: KNH-ERC/A/305.Currently KNH is the largest referral, teaching and research hospital in Kenya.

3.2. Study population

Study involved stored samples of children in the1 month to 10years age category.

3.3. Selection of patients

3.3.1. Inclusion Criteria

Stored samples that had been collected and used in a previous study (KNH-ERC/A/305) of children who had signs and symptoms of Acute Respiratory Infections (ARI).

3.3.2 Exclusion Criteria

Stored samples that had been collected and used in a previous study (KNH-ERC/A/305) of children without signs and symptoms of (ARI).

3.4 Sampling

The study used stored samples that were obtained from another study that had been approved by University of Nairobi and Kenyatta National Hospital review bodies for a student pursuing herMaster'sdegree. Samples had been collected and stored in the Kenya Medical Research Institute (KEMRI) freezers for 1 year at -80^{o} C.

The sample size for the study was determined using Fishers formula (Mugenda, 1999).

Where by= $(z^2xpq) \div d^2$

n= the required sample size

z^2= 1.96 [the normal deviate at 5 % level of significance

p= estimated prevalence (10%) based on a previous study of Enteroviruses byGrondahl*et al*., (1999).

q= 1-p

d = the precision of estimate which is considered to be 5%= 0.05

n= $(z^2xpq) \div d^2 = (1.96^2x0.1x0.9) \div 0.05^2 = 138.27$

≈ 139

3.4.1. Sample collection and storage

Throat swabsamples were collected from patients at the paediatric emergency clinic and the paediatric general wards of Kenyatta National Hospital presenting with ARI

and frozen at -80^{o}C.These samples were collected between April 2008 and July 2008.For every sample collected information on gender and age was recorded.

3.5 Viral isolation

Enteroviruses were isolated according to appropriate protocols developed and standardised by the National Influence Centre in KEMRI. Samples that had been frozen at -80^{o}C were partially thawed and kept on ice.The cell line human rhabdomysarcoma cells (RD Cells) had been cultured in Dubelcoss Modified Eagles Medium (DMEM, Gibco, USA) and prepared from a mother cell in a 75cm^{2} cell culture flask and inoculated in 24-well plates then incubated at37^{o}C for 3 days to obtain 70-80 % confluence. The growth mediumwas poured off and 100µl of the sample was inoculated on to the cell line and incubated for 1 hour at 33^{o}C and later overlaid with maintenance mediumand incubated at 33^{o}C for up to 10 days. The culture flasks were examined daily forcytopathic effects (CPE). After 10 days, the plates were all placed in the -80^{o}C freezer before the immunofluorescent assay.

3.6 Indirect Immunoflourescence Assay Test

Immunoflourescence assay was then performed on all samples using the manufacturer's kit instructions (Millipore, MA, and USA). First, the supernatant inall the 24 well plates was aspirated to individual cryovials and stored in the -80^{o}C. Then 1ml ofsterile phosphate buffered saline (PBS) solution was added to each of the 24 wells in the well plates which contained the cells. The wells were also slightly

scraped in order to release cells. They were dispensed later to a sterile 15ml centrifuge tube and spun at 3000Revolutions per Minute (RPM) x g for 10 min. The supernatant was poured off and PBS was again added and the procedure above repeated. After the third wash, 20-25 µl of the cell suspension was added to a multiwell slide and left in the biosafety cabinet to dry overnight. The next day, the slide was fixed in cold acetone for 10 min at 4^{o}C, 25 µl of 10% skim milk was added to the wells and incubated at 37^{o}C for30 minutes. After incubation, this was washed off usingPBS/Tween 20 (wash buffer) to eliminate non-specific staining. Then 25µl of Pan Enterovirus specific monoclonal antibody was added and incubated at 37^{o}C for30 min. After incubation, the slides were washed in PBS/Tween 20 (wash buffer) and left to dry. A drop of mounting fluid was added to each well and a cover slip put on the slide. The slides were then examined under a fluorescent microscope. Samples that were positive for Pan-enterovirus were then similarly stained as per the above procedure using specific enterovirus monoclonal antibodies for either: Enterovirus, Coxsackie, Echovirus or Poliovirus to specifically identify enteroviruses affecting the patient.

Samples that were IFA and CPE positive for any EVs were extracted and processed for PCR.

3.7 Polymerase Chain Reaction (PCR)

3.7.1. RNA extraction

RNA was extracted using Qiagen mini RNA kit (Qiagen Ltd., Crawley, UK) according to manufacturer's instructions. Briefly 140µl of sample were added to 560µl of viral lysis buffer, incubated at room temperature (15-25^{o}C) for 10 min. Then 560 µl of molecular grade 100% ethanol was added and mixed by vortexing for 15 sec. This was then centrifuged briefly to remove drops from inside the eppendorf tube lid. From the lysed RNA, 630µl of RNA was then placed on to a spin column, spun at 8000(RPM) twice so as to bind the RNA to the spin column. The RNA was washed twice, first with 500µl of AW1 (Wash 1) at 8000RPM for 1 minute, then with 500µl of AW2 (Wash 2) at 13000RPM for 3 min. The RNA was eluted from the spin column by adding 60µl elution buffer (Buffer AVE) and spinning at 8000RPM for 1 min to a 1.5 ml eppendorf tube. The eluted RNA was then stored at −80^{o}C until the day when PCR would be ran on the samples.

3.7.2 PCR Procedures

Primers targeting the non coding region of all Enteroviruses(Grondahl *et al.*, 1999) were used to confirm the IDFAT positive samples.(Forward primer: ATT GTC ACC ATA AGC AGC CA and Reverse primer, TCC TCC GGC CCC TGA ATG CG)This procedure yielded a 154bp PCR product. The reverse transcriptase PCR (RT –PCR) procedure consisted of a single-step combining reverse transcription and PCR amplification performed using the one-step RT-PCR kit from QIAGEN (Hilden, Germany). The reaction mixture contained 5µl of 5× RT-PCR buffer, 1µl of 0.4mM dNTPs, 1.25µl of each of the primers(forward and reverse primers) 13µl nuclease

free water and 1µl of enzyme mix. A 2.5µl aliquot of viral RNA was added to give a final volume of 25µl. The cycling conditions for the RT-PCRs were: an initial cycle at 50^{o}C for 30 min for the reverse transcriptase, incubationand 94^{o}Cfor 15 min to inactivate the reverse transcriptase and activate the *Taq* polymerase. This was followed by 40 cycles at denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for30 sec and strand extension at 72^{o}C for 1 min. Finally, a final incubation at 72^{o}C for 10 min was carried out.

3.7.3 Gel Electrophoresis

In gel electrophoresis, 2g of agarose were mixed with 100ml of Tris-Borate (TBE buffer). The mixture was heated in a microwave till boiling, and then cooled to 45^{o}Cwhere 5µl of 10mg/ml ethidium bromide was added. The gel was poured into an electrophoresis tank cast in order to solidify. Ten microliters of sample were added to 5µl loading dye and placed on the gel, then 100v for 30 minutes was passed. Examination was done in an alpha imager (CA, USA).

3.7.4 Sequencing

Ten positive samples (3 Enterovirus, 3 Coxsackievirus, 3 Echovirus and 1 Poliovirus) were taken to International Livestock Research Institute (ILRI) for

sequencing using the big dye terminator method.Sequencing was carried out according to the protocol described in Appendix 1.

3.7.5 Phylogenetic analysis

The sequences obtained from this study were compared to similar nucleotide sequences in the Genbank database(Benson *et al.*, 2006) using theBasic Local Alignment Search Tool(BLAST) software,(Altschul *et al.*, 1997) to determine similarities. The sequences from this study were aligned to similar sequences from the genbankusingBioEdit version 7.0.9.0(Hall, 1999) and a phylogenetic tree constructed using a neighbor-joining clustering algorithm with bootstrap resampling (n = 1000) using (Molecular Evolutionary Genetic Analysis) , MEGA version 4(Tamura*et al.*, 2007).

3.8 Data Management

Data was entered in Microsoft excel worksheet. Data was backed up in a CD, USB disc, and hard drive disks. All data collected was kept secure and confidential by use of passwords and encryption.

3.9 Statistical analysis

Data coding and analysis was done using the Statistical Package for Social Scientists(SPSS) software. Pearson's chi-square was used to determine associations between virus detection and isolation. Level of significance was fixed at 0.05

(p=0.05). Results were presented in frequency tables, pie charts and percentages. The recorded data was analyzed using the SPSS version 10 program using t-test and chi-square.

3.10.Ethical considerations

Clearance to carry out the study was sought from all relevant institutions beingKenyatta National Hospital and the Kenya Medical Research Institute, Scientific Steering Committee, and Ethical Review Committee. This study involved stored samples hence informed consent was not sought from the participants.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic Characteristics

This study took place between the months of April and July of 2008 at the Kenyatta National Hospital paediatric clinic and wards. Two hundred and eighty seven (287) samples were collected from consenting patients with ARI. Of the patients sampled, 156 representing 54.4% were male, while 131 representing 45.6% were female as shown in Figure4.1.

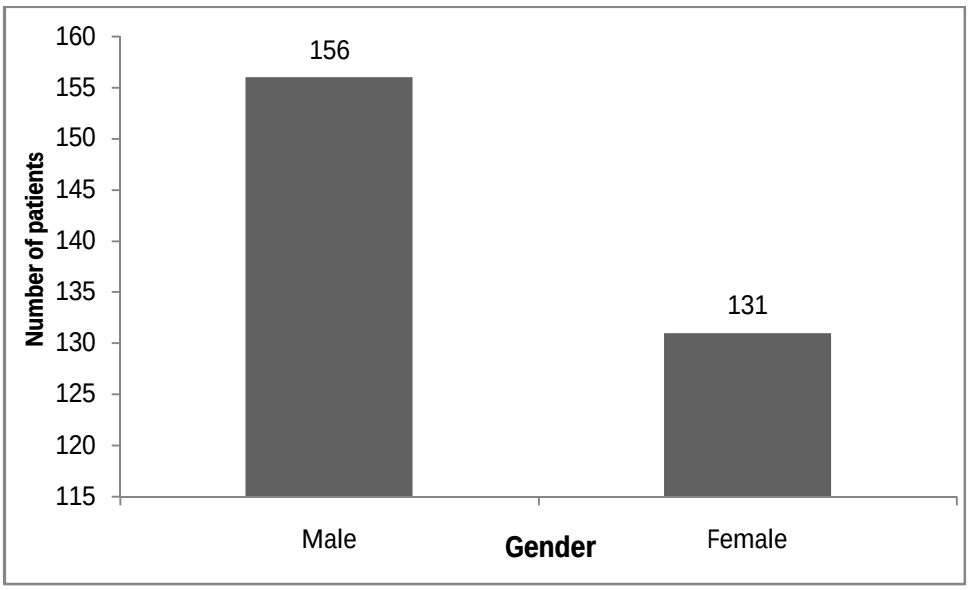

Figure 4.1: Distribution of stored samples of patients (n=287) recruited by gender.

The ages of the patients ranged from one month to ten years with majority of the patients being in the 1-12 month age cluster (n= 211, 73.5%), followed by 13-24 month range (48, 16.7%) . The mean, median and mode of the patient age were 11, 9, 7, and 2 months respectively as shown in Figure 4.2.

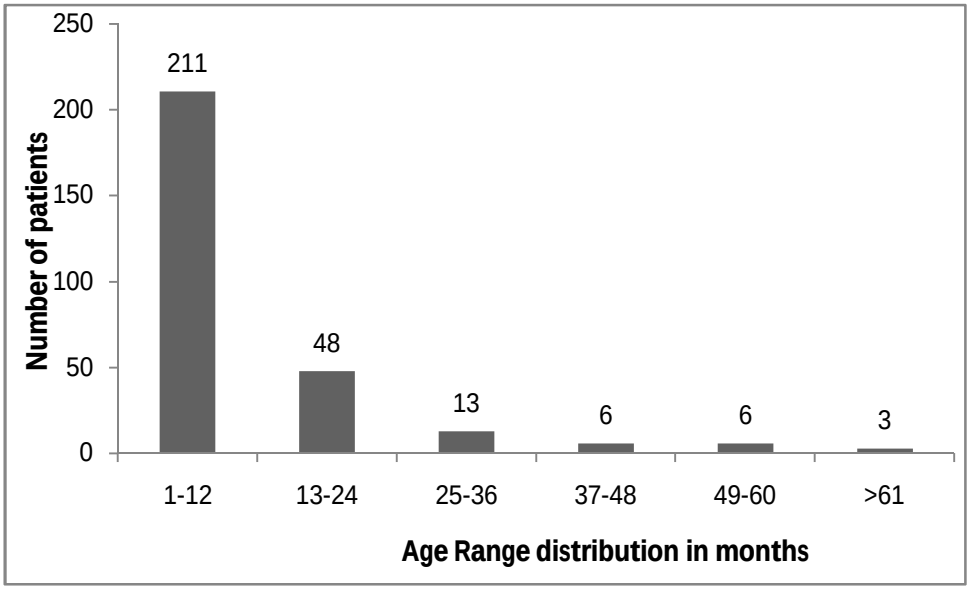

Figure 4.2: Distribution of storedsamples of patients (n=287) recruited by age.

Most of the samples were collected in the month of May (128; 44.6%) followed by April (98; 34.1%) and lastly by June (61; 21.3%) as shown by the piechart (Figure 4.3).

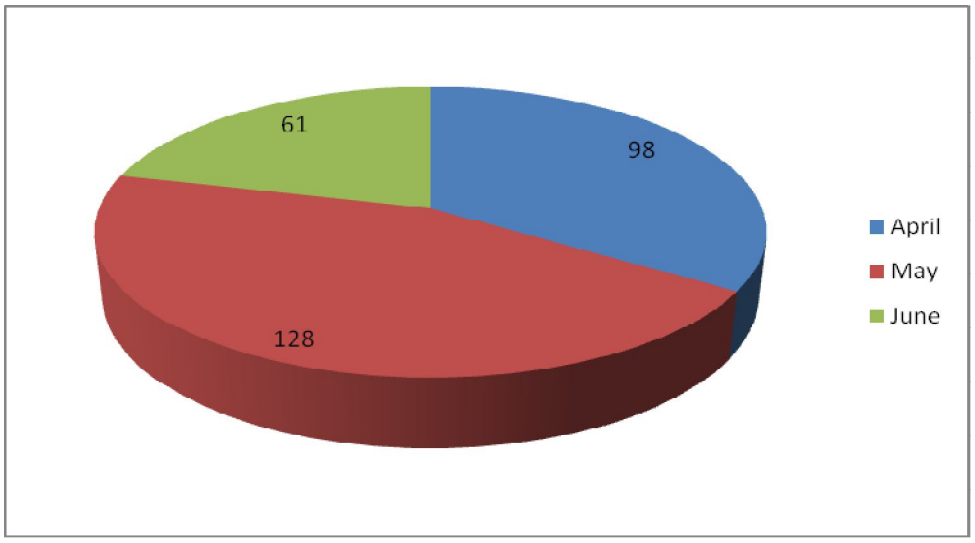

Figure4.3: Distribution in the months the samples were collected.

To determine whether there was correlation between the age range categories and gender of the patients recruited with ARI, the data was cross tabulated (Table 4.1) and the Chi-square test results showed no significant correlation (χ^2 (df=5) = 2.736; p = 0.741). Majority of the patients recruited were in 1-12month age range category (males n=113; females n=98) followed by the 13-24months age range category (males n=27; females n=21).

Correlation between the month of sample collection and gender distribution of the patients showed no significant correlation (χ^2 (df=2) = 0.132; p = 0.936).Majority of the samples were collected in the month of May (males n=71; females n=57),

followed by April (males n=52; females n=46), and lastly by June (males n=33; females n=28)as shown in Table 4.1.

Table 4.1 Age range categories, month of sample collection against gender distribution.

Age Range Categories (months)	Gender		Total
	Male (n=156)	**Female (n=131)**	
1-12 months	113 (72.4)	98 (74.8)	211
13-24	27 (17.3)	21 (16)	48
25-36	6 (3.8)	7 (5.3)	13
37-48	3 (1.9)	3 (2.3)	6
49-60	5 (3.2)	1 (0.8)	6
More than 61 months	2 (1.3)	1 (0.8)	3
Total	**156**	**131**	**287**
Month of samples collection	**Gender**		**Total**
	Male (n=156)	**Female (n=131)**	
April	52 (33.3)	46 (35.1)	98
May	71 (45.5)	57 (43.5)	128
June	33 (21.2)	28 (21.4)	61
Total	**156**	**131**	**287**

4.2 Prevalence ofEnteroviruses

4.2.1 Virus Isolation

Viruses were isolated from a total of 24 (8.4%) out of 287 samples collected throughout the study period. The isolated viruses were characterised using indirect

fluorescent antibody tests (IDFAT) where 10 (41.7%) enterovirus, 9 (37.5%) coxsackie, 4 (16.7%) echovirus and 1 (4.2%) poliovirus were identified.

Appearance of indirect flourescent antibody reaction with the virus

Infected cells (positive) appear as (apple green) while non infected cells (negative) appear as reddish (Plate 4.1).

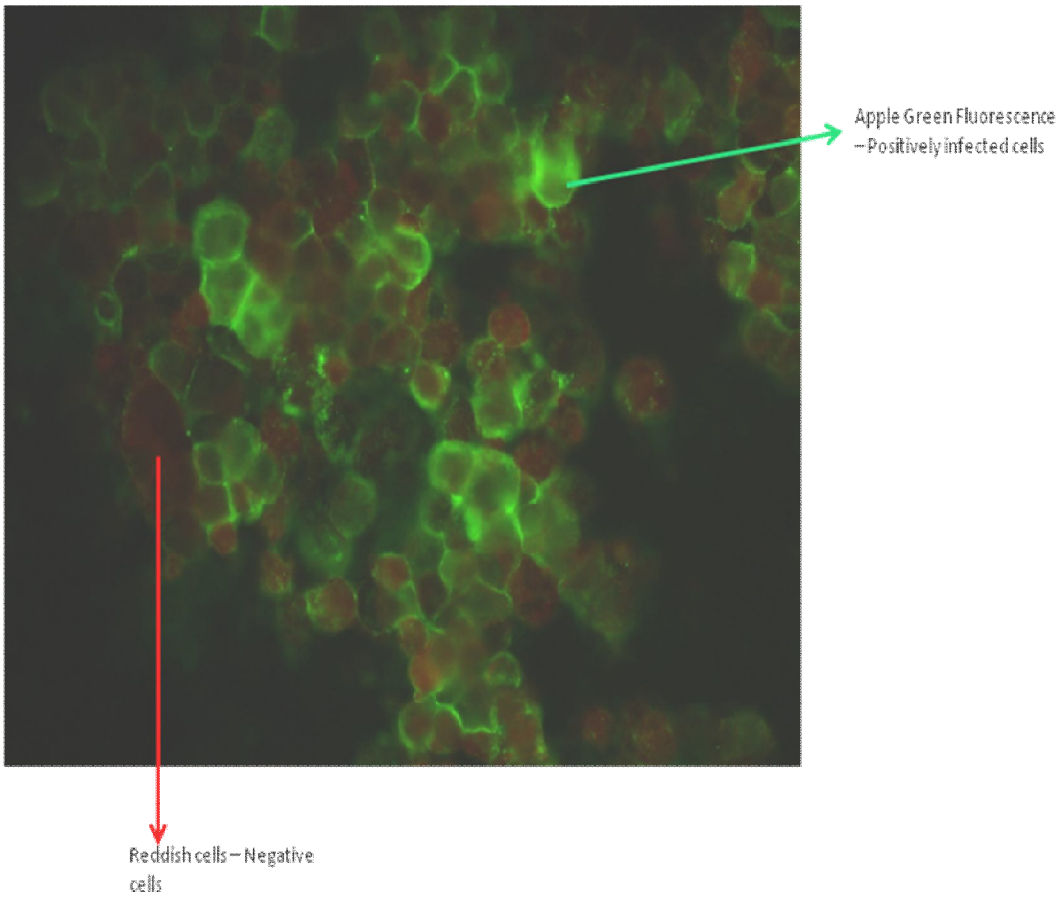

Plate 4.1: Appearance of indirect flourescent antibody reaction with the virus

4.2.2. Distribution of age range categories, month of sample collection, gender distribution against identities of the viruses.

Results of the chi-square test indicated no significant correlation (χ^2 (df=3) = 0.320; p = 0.956) between the age and viruses isolated.

Majority of the viruses were isolated from the 1-12month age range category (19; 79.2%) followed by the 13-24month age range category (5; 20.8%). The other age categories did not have any viruses detected. There was no significant correlation between the month of sample collection and the viruses detected (χ^2 (df=6) = 7.549; p = 0.273).

Majority of the viruses were detected in the month of April (14; 58.3%), followed by May (6; 25%), and lastly June (4; 16.7%). Correlation between the genders of the patients with viruses isolated from the patients indicated no significant correlation (χ^2 (df=3) = 1.958; p = 0.581) detected. Males had slightly more virus detections (13; 54.2%) when compared to females (11; 45.8%) as shown in Table 4.2.

Table 4.2Distribution of age range, month of sample collection, gender distribution against identities of the viruses.

Age range categories	IDFAT				Total
	Enterovirus	Echovirus	Coxsackie	Poliovirus	
1-12 months	8	3	7	1	19
13-24	2	1	2	0	5
25-36	0	0	0	0	0
37-48	0	0	0	0	0
49-60	0	0	0	0	0
More than 61 months	0	0	0	0	0
Total	**10**	**4**	**9**	**1**	**24**
Month of sampling	**IDFAT**				**Total**
	Enterovirus	**Echovirus**	**Coxsackie**	**Poliovirus**	
April	7	1	5	1	14
May	1	3	2	0	6
June	2	0	2	0	4
Total	**10**	**4**	**9**	**1**	**24**
Gender distribution	**IDFAT**				**Total**
	Enterovirus	**Echovirus**	**Coxsackie**	**Poliovirus**	
Male	5	3	4	1	13
Female	5	1	5	0	11
Total	**10**	**4**	**9**	**1**	**24**

4.3. PCR Results

Some of the identified isolates were subjected to PCR.The PCR of the non-coding region of the enterovirus is a 743 nucleotide 5' region having a single open reading frame that would yield a 154bp PCR product(Plate 4.2).

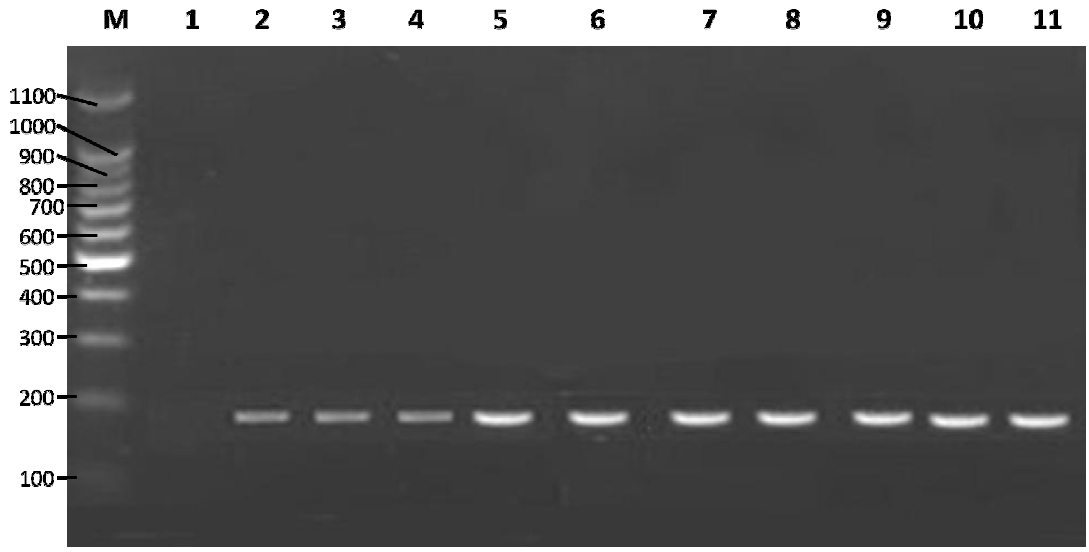

Plate4.2:PCR products of Enteroviruses.

Key: (Lane M = 100bp marker with band sizes 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, and 1100; Lane1 Negative control, Lane 2 EnterovirusPositive control; Lane 3 to Lane 11 field samples identified positive by IDFAT).

4.4 Sequencing Results

Ten of the IDFAT positive samples (3 Enterovirus, 3 Coxsackievirus, 3 Echovirus and 1 Poliovirus) were sequenced using Big Dye Terminator method at ILRI. Below listed are the nucleotide sequences that were obtained;

Sequences of Coxsackieviruses obtained

>EVsNrb/55/2008 Coxsackievirus
AGAAGGAGAAAACGTTCGTTACCCGGCTAACTACTTCGAGAAACTTAGT
AGCACCATTGAAGCTGCAGAGTGTTTCGCTCAGCACTCCCCCAGTGTAGA
TCAGGTCGATGAGTCACTGAACTCCCGACGGGCGACCGTGGCAGTGACT

GCGTTGGCGGCCTGCCTATGGGGCAACCCATAGGACGCTCTAATGCGGA
CATGGTGCGAAGAGTCTATTGAGCTAGTTAGTAGTCCTCCGGCCCCTGAA
TGCGGCTAATCCTAACTGCGGAGCGCATACCTTCAATCCAGGGGGTGGTG
CGTCGTAATGGGCAACTCTGCAGCGGAACCCACTACTTTGGATGTCCGTG
TTTCGTT

>EvsNrb/15/2008 Coxsackievirus
TGAAGGAGAAAACGTTCGTTATCCGGCTAACTACTTCGAGAAACTTAGTA
GCACCATTGAAGCTGCAGAGTGTTTCGCTCAGCACTCCCCCAGTGTAGAT
CAGGTCGATGAGTCACTGAACTCCCGACGGGCGACCGTGGCAGTGACTG
CGTTGGCGGCCTGCCTATGGGGCAACCCATAGGACGCTCTAATGCGGAC
ATGGTGCGAAGAGTCTATTGAGCTAGTTAGTAGTCCTCCGGCCCCTGAAT
GCGGCTAATCCTAACTGCGGAGCGCATACCTTCAATCCAGGGGGTGGTG
CGTCGTAATGGGCAACTCTGCAGCGGAACCCACTACTTTGGATGTCCGTG
TTTCGTT

>EvsNrb/221/2008 Coxsackievirus
TGAAGGAGAAAACGTTCGTTACCCGGCTAACTACTTCGAGAAACTTAGT
AACACCATTGAAGCTGCAGAGTGTTTCGCTCAGCACTCCCCCAGTGTAGA
TCAGGTTGATGAGTCACTGAACTCCCGACGGGCGACCGTGGCAGTGACT
GCGTTGGCGGCCTGCCTATGGGGCAACCCATAGGACGCTCTAATGCGGA
CATGGTGCGAAGAGTCTATTGAGCTAGTTAGTAGTCCTCCGGCCCCTGAA
TGCGGCTAATCCTAACTGCGGAGCGCATACCTTCAATCCAGGGGGTGGTG
CGTCGTAATGGGCAACTCTGCAGCGGAACCCACTACTTTGGATGTCCGTG
TTTCGTT

Sequences of Echoviruses obtained

>EVsNrb/77/2008
GAAACCTAGTACCACCATGGAGGTTGCGTAGTGTTTCGCTCAGCACAACC
CCGGTGTAGATCAGGCCGATGAGTCACCGCATTCCCCACGGGTGACTGTG
GCGGTGGCTGCGTTGGCGGCCTGCCTATGAGGTGACCCATAGGACGCTTC
AATACTGACATGGTGTGAAGAGTCTATTGAGCTAGTTGGTAGCCCTCCGG
CCCCTGAATGCGGCTAATCCTAACTGCGGAGCACATTCCCGCAATCCAAT
TCAAATTTCGTCGTAACGGGTAACTCCGCAGCGGAACCGACTACTTTGGG
TGTCCGTGTTTCTTTTTCATTCCTCGA

>EvsNrb/218/2008

GAAACCCAGTACCACCATGGAGGTTGCGTAGCGTTTCGCTCACCACAACC
CCGGTGTAGATCAGGCCGATGAGTCACCGCATTCCCCACGGGCGACTGT
GGCGGTGGCTGCGTTGGCGGCCTGCCTATGAGGTGACCCATAGGACGCTT
CAATACTGACATGGTGTGAAGAGTCTATTGAGCTAGTTGGTAGCCCTCCG
GCCCCTGAATGCGGCTAATCCTAACTGCGGAGCACATTCCCGCAATCCAA
TTCAAATTTCGTCGTAACGGGTAACTCCGCAGCGGAACCGACTACTTTGG
GTGTCCGTGTTTCTTTTTAATTCCTCGA

>EvsNrb/272/2008
GAAACCTAGTACCACCATGGAGGTTGCGTAGTGTTTCGCTCAGCACAACC
CCGGTGTAGATCAGGTCGATGAGTCACCGCATTCCCCACGGGTGACTGTG
GCGGTGGCTGCGTTGGCGGCCTGCCTATGAGGTGACCCATAGGACGCTTC
AATACTGACATGGTGTGAAGAGTCTATTGAGCTAGTTGGTAGCCCTCCGG
CCCCTGAATGCGGCTAATCCTAACTGCGGAGCACATTCCCGCAATCCAAT
TCAAATTTCGTCGTAACGGGTAACTCCGCAGCGGAACCGACTACTTTGGG
TGTCCGTGTTTCCTTTTCATTCCTCGA

Sequences of Enteroviruses obtained

>EvsNrb/20/2008
TAACACCATTGAAACTGCAGAGCGTTTCGTTCAGCACCTCCCCAGTGTAG
ATCAGGTCGATGAGTCACTGCGCTCCCCACGGGTGACCGTGGCAGTGGCT
GCGTTGGCGGCCTGCCTTTGGACCAATCCAAAGGACGCTTCAATGCTGAC
ATGGTGCGAAGAGCCTATTGAGCTAGTTGGTAGTCCTCCGGCCCCTGAAT
GCGGCTAATCCTAACTGCGGAGCGCATGCCTTCAAGCCAGTAGGTAATG
CGTCGTAATGGGCAACTCCGCAGCGGAACCGACTACTTTGGGTGTCCGTA
GTTCCTTTTATTCTTACAC

>EvsNrb/69/2008
TAACACCATTGAAACTGCAGAGTGTTTCGTTCAGCACCTCCCCAGTGTAG
ATCAGGTCGATGAGTCACTGCGCTCCCCACGGGTGACCGTGGCAGTGGCT
GCGTTGGCGGCCTGCCTTTGGACTAATCCAAAGGACGCTTCAATGCTGAC
ATGGTGCGAAGAGCCTATTGAGCTAGTTGGTAGTCCTCCGGCCCCTGAAT
GCGGCTAATCCTAACTGCGGAGCGCATGCCTTCAAGCCAGTAGGTAATG
CGTCGTAATGGGCAACTCCGCAGCGGAACCGACTACTTTGGGTGTCCGTA
GTTCCTTTTATTCTTACAC

>EvsNrb/207/2008
TAACACCATTGAAACTGCAGAGCGTTTCGTTCAGCACCTCCCCAGTGTAG
ATCAGGTCGATGAGTCACTGCGCTCCCCACGGGTGACCGTGGCAGTGGCT

GCGTTGGCGGCCTGCCTATGGACCAATCCAAAGGACGCTTCAATGCTGAC
ATGGTGCGAAGAGCCTATTGAGCTAGTTGGTAGTCCTCCGGCCCCTGAAT
GCGGCTAATCCTAACTGCGGAGCGCATGCCTTCAAGCCAGTAGGTAATG
CGTCGTAATGGGCAACTCCGCAGCGGAACCGACTACTTTGGGTGTCCGTA
GTTCCTTTTATTCTTACAC

Sequences of Poliovirus obtained

>EvsNrb/107/2008
CGCTCATGTACTTCGAGAAGCCTAGTATCGCTCTGGAATCTTCGACGCGT
TGCGCTCAGCACTCAACCCCGGAGTGTAGCTTGGGCCGATGAGTCTGGAC
AGTCCCCACTGGCGACAGTGGTCCAGGCTGCGCTGGCGGCCCACTTGTGG
CCCAAAGCCACGGGACGATAGTTGTGAACAGGGTGTGAAGAGCCTATTG
AGCTACATGAGAGTCCTCCGGCCCCTGAATGCGGCTAATCCTAGCCATGG
AGCAGGCAGCTGCAACCCAGCAGCCAGCCTGTCCTAACGCGCAAGTCCG
TGGCGGAACCGATTACTTTGGGTGTCCGTGTTTCCTTTTATTCTTGAACTG
CAACTA

4.5 Blast Analysis

All the study sequences obtained from ILRI were exposed to BLAST, an algorithm comparing sequences by searching for similarities between sequences in a gene database.

This study's sequences had similarities of between 90 – 99% (Coxsackievirus – 93-97%; Echovirus – 93-97%; Enterovirus – 90-91%; Poliovirus – 91-99%) with other comparable sequences in GenBank, a biorepository of genetic materials (BLAST Figures: Appendix 2).

4.6 Phylogenetic Analysis

The nucleotide sequences obtainedfrom this study and the similar sequences of enteroviruses strains obtained from BLAST analysis were aligned using BioEdit version 7.0.9.0 and a phylogenetic tree constructed using a neighbor-joining clustering algorithm with bootstrap resampling (n = 1000) using MEGA version 4 .

This study's sequences especially Coxsackievirus, Echovirus and Enterovirus clustered together indicating similarities. They also had bootstrap values of 99% indicating the confidence limits of their clustering after 1000 replicates had been analyzed and a consensus tree had been constructed (Soltis, 2003).

The phylogeny of coxsackievirus

Sequences of highly similar coxsackie virus strains that were obtained from GenBank for this analysis. The analysis was carried out using MEGA version 4 software.This study samples are supported by a bootstrap value of 99% (Figure 4.4).

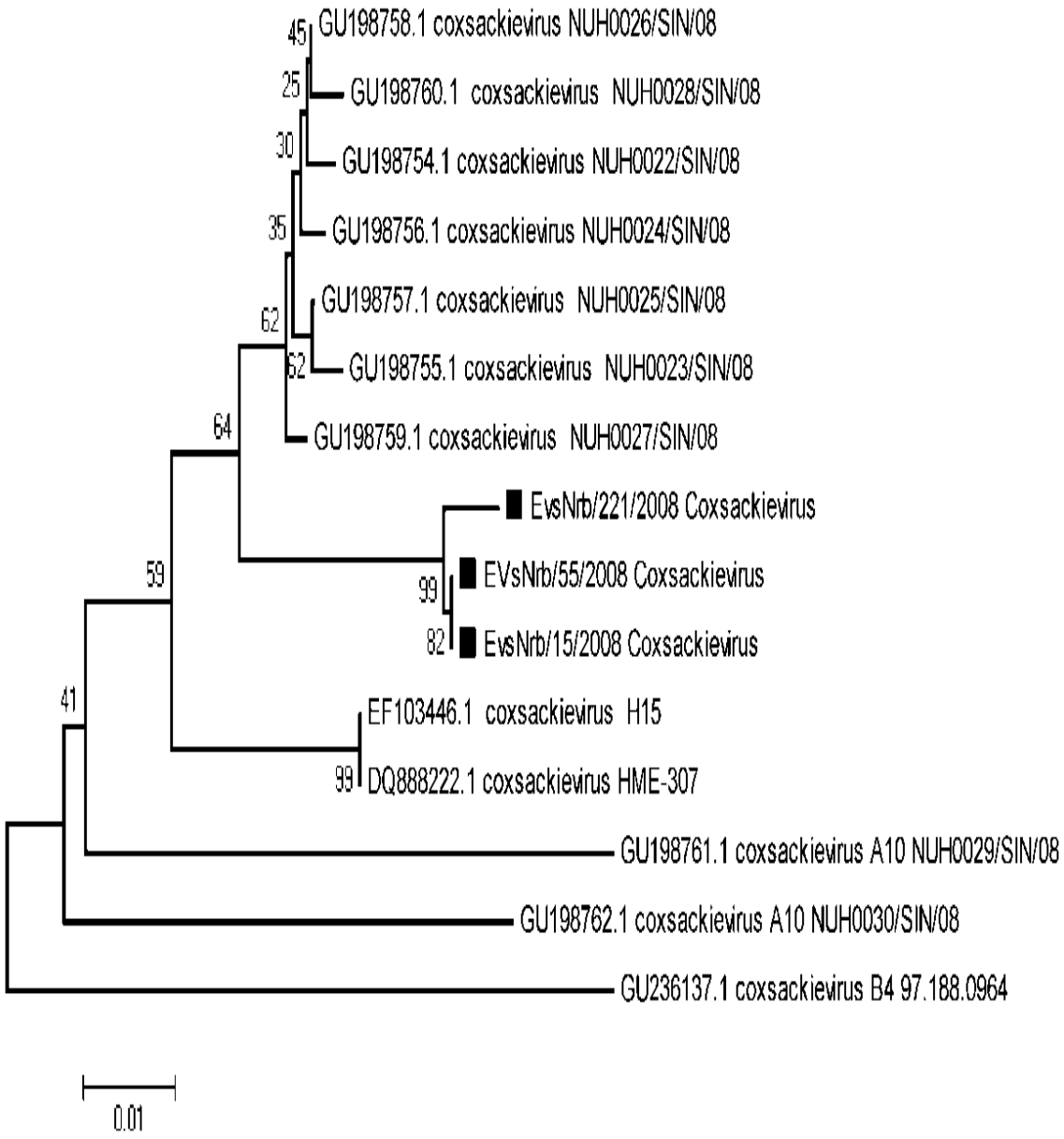

Figure 4.4: The phylogeny of coxsackievirus isolates (in dark blocks) compared to other Coxsackieviruses from GenBank using MEGA 4 software.

The phylogeny of enterovirus isolates

Sequences of highly similar enterovirus strains that were obtained from GenBank for this analysis. The analysis was carried out using MEGA version 4 software.This study samples are supported by a bootstrap value of 99% (Figure 4.5).

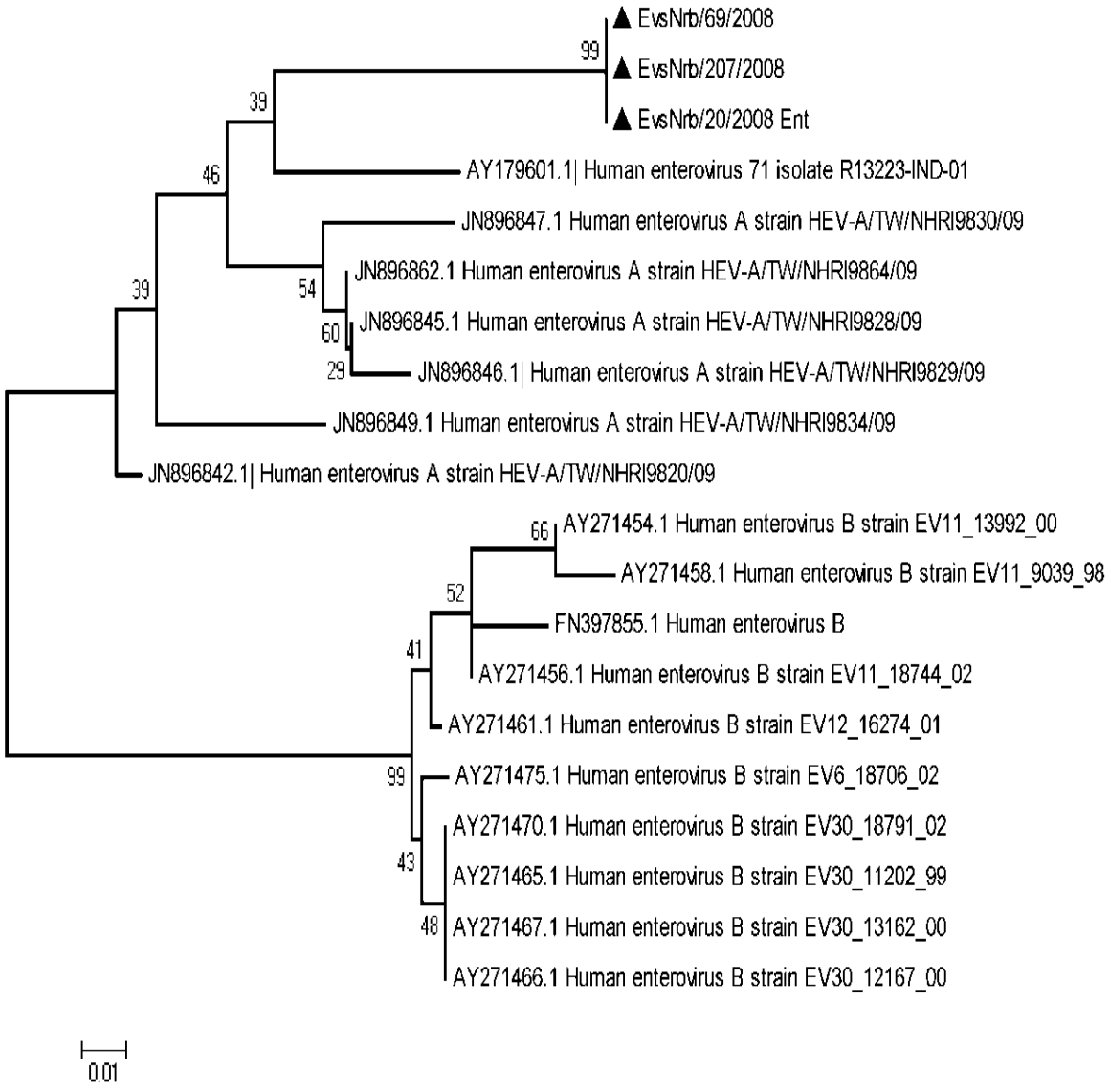

Figure 4.5: The phylogeny of enterovirus isolates (in triangles) compared to other Coxsackieviruses from GenBank using MEGA 4 software.

The phylogeny echovirus isolates.

Sequences of highly similar echovirus strains that were obtained from GenBank for this analysis. The analysis was carried out using MEGA version 4 software.This study samples are supported by a bootstrap value of 100 % (Figure 4.6).

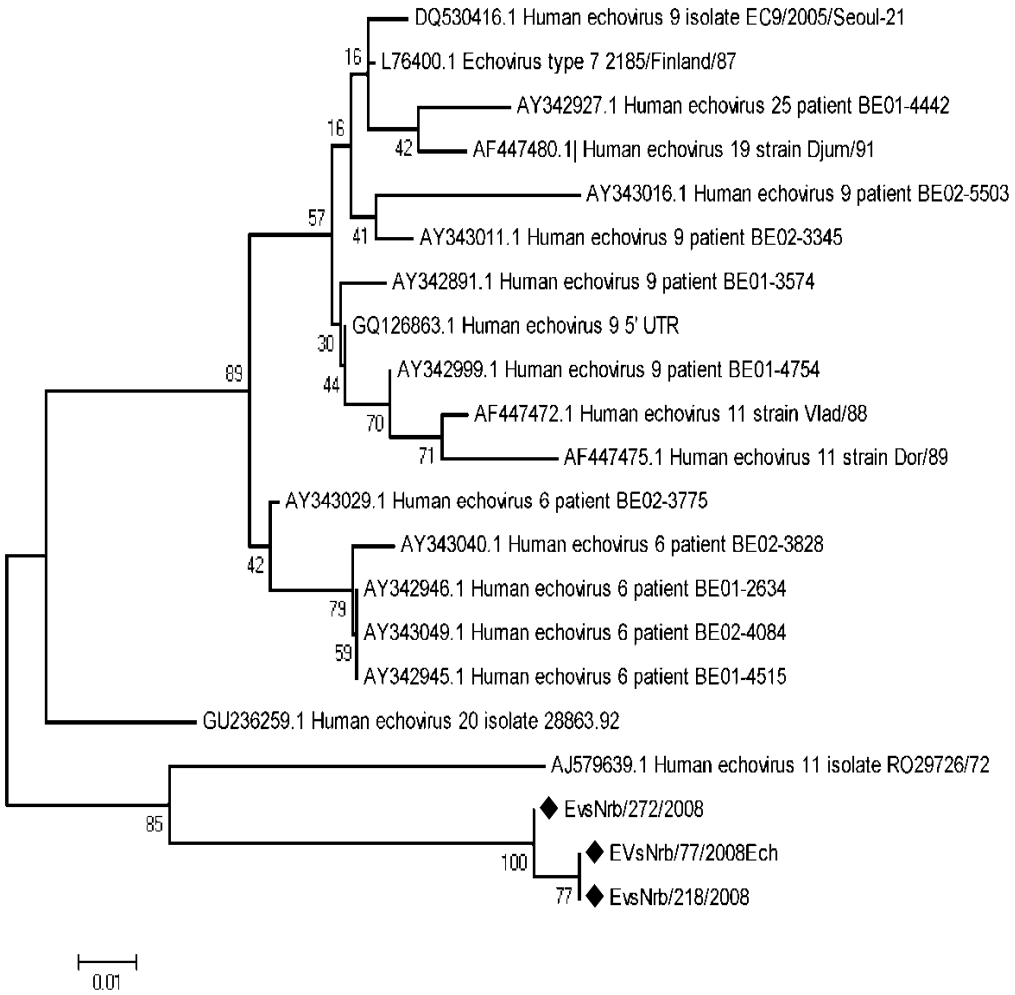

Figure 4.6: The phylogeny echovirus isolates (in diamonds) compared to other echoviruses from GenBank using MEGA 4 software.

The phylogeny of poliovirus isolates

Sequences of highly similar poliovirus strains that were obtained from GenBank for this analysis. The analysis was carried out using MEGA version 4 software.This study samples clustered on their own branch (Figure 4.7).

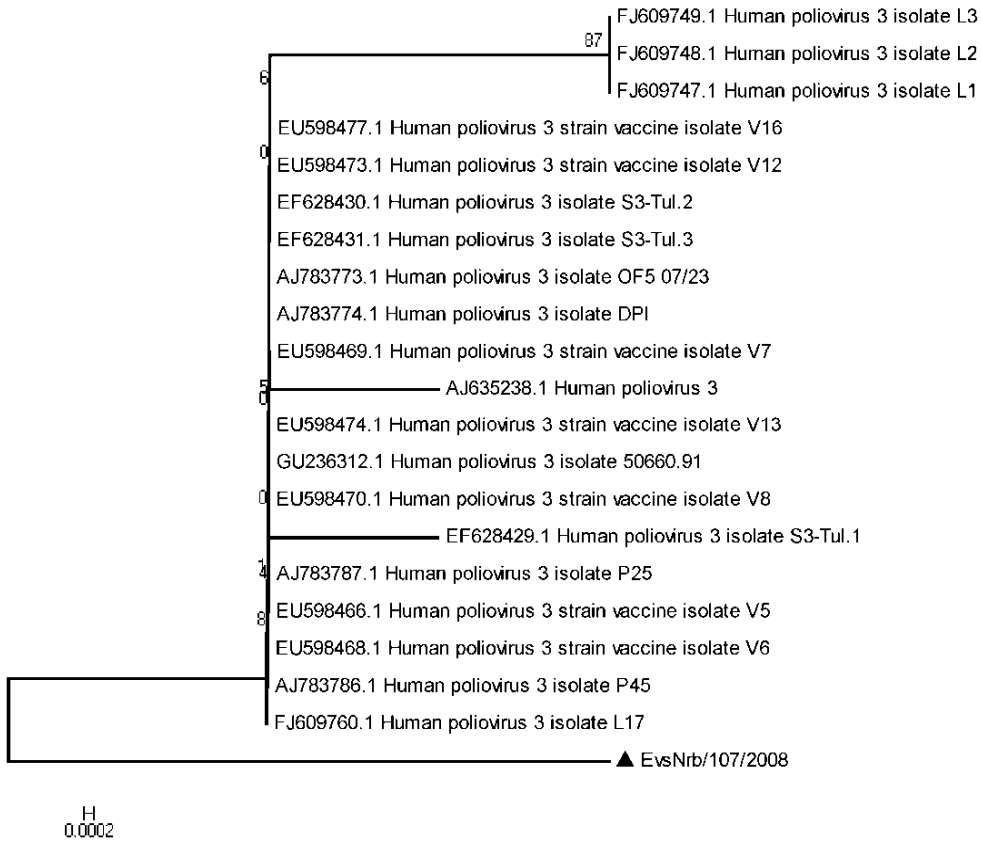

Figure 4.7: The phylogeny of poliovirus isolates (in a dark diamond) compared to other polioviruses from GenBank using MEGA 4 software

CHAPTER FIVE

5.0.DISCUSSION, CONCLUSIONS AND RECOMENDATIONS.

5.1.Discussion

In this study viruses were isolated from a total of 24(8.4%) out of 287 samples collected throughout the study period. The isolated viruses were characterised using indirect fluorescent antibody tests (IDFAT) where 10 (41.7%) enterovirus, 9 (37.5%) coxsackie, 4 (16.7%) echovirus and1 (4.2%) poliovirus were identified.Enteroviruses are known to cause minor febrile illness to severe and potentially fatal pathologies that include aseptic meningitis, encephalitis,myopericarditis, acute flaccid paralysis, and severe neonatal sepsis-like disease (Khetsuriani *et al*., 2006).

Acute respiratory infections are leading causes of childhood morbidity and mortality especially in the developing countries (WHO, 2011). Enteroviruses have been detected and associated with ARI and thus are important etiological agents of this condition (Nijhuis *et al*., 2002). Transmission and acquisition of these viruses is mainly through direct contact with infected secretions from an infected person, food, water or by contact with contaminated surfaces or objects (Moore, 1982, Nijhuis *et al*., 2002). Multiplication occurs in the gastrointestinal tract or the respiratory tract after which they spread to tissues and organs causing a variety of diseases. There is no vaccine to prevent the enteroviruses that occur. Person's hygiene is most

important in avoiding the acquisition and transmission of enterovirus infection(Chang *et al*., 2004).

The ages of the patients ranged from 1month to ten years with majority of the patients being in the 1-12 month age cluster (n= 211, 73.5%), followed by 13-24 month range (48, 16.7%).The other age categories did not have any viruses isolated from them.This could have been due to the fact that children between the ages of 1year to 5 years are more prone and susceptible to infection by agents of ARI which leads to high morbidity and sometimes mortality (Cabello *et al*., 2006, Mulholland, 2003).

Enteroviruses are common during infancy. The majority of children experience at least one enterovirus infection by one year old. Infection is also common in family members who work with young children. Adults are less likely to get infection as their immune level is higher as compared to children. Immunity can occur after infection of these viruses. However, the immunity is only to one of the enteroviruses.It does not protect against infection from the others(Oberste*et al*., 1999). From previous studies, this could be attributed mainly due to risk factors such as malnutrition, poor breastfeeding, and undeveloped immunity (Cabello *et al*., 2006, Koch *et al*., 2003, Mulholland, 2003), attending child care centers, sharing bedrooms with children of over 5years or adults (Koch *et al*., 2003), malnutrition, poor

breastfeeding practices(Cabello *et al*., 2006), and exposure to tobacco smoke (Kusel e*t al*., 2007). These factors were not assessed by this study but could be looked into in future studies.

This study had a lower prevalence when compared to a similar study carried out in the same hospital Hazlett *et al*., (1988) reported a prevalence of 19.7% n= 162 out of 822 samples. In this study, the samples had been collected, and stored in the freezer for about a year with frequent freeze thaw cycles to isolate other viruses before inoculating them to RD cell lines to isolate enteroviruses and later staining them with specific monoclonal fluorescent dyes used to identify enteroviruses. A study done by Hazlett*et al* (1988) samples received from the patients were inoculated within 2 hrsof collection. In the present study, frequent freeze thaw cycles could have led to loss of some enterovirusesthus reducing the prevalence(Nichols, 2003).

A higher prevalence was also reported in a study by Mizuta *et al*.,(2008), having a prevalence of 20.3% (n = 147 out of 723 samples). This was due to the use of a modified technique where they inoculated samples to different cell types (HEF, HEP-2, Vero, RD-18S and GMK - HHVRG). These cells were then centrifuged together with the samples and incubated at similar conditions as used by this study. In this study, only one cell line was used, and there was no centrifugation of sample together with the cells. The current study had a higher prevalence though when

compared to a seven year study carried out by Jacques *et al.,* (2008) in France. In terms of methods used to detect enteroviruses, they combined viral isolation and molecular methods and still had a prevalence of 2.4% (n=285) out of 11,509 samples(Jacques *et al*., 2008).

The subtype that was mostly detected wasenteroviruses,with 10 (41.7%) enterovirus, 9 (37.5%) coxsackie, 4 (16.7%) echovirus and 1 (4.2%) poliovirus.A Study by Mizuta *et al.*, (2008) detected more coxsackie viruses, followed by echovirus, a few enterovirus (n=2) and only one poliovirus from a six month old child who had received oral polio vaccine. A study in France by Jacques *et al.*,(2008) the most commonly detected were echoviruses, followed by coxsackie, only one enterovirus and no poliovirus. A study in Brazil Portes*et al.*, (1998), reported polioviruses (n=18) from recently vaccinated individuals, echovirus (n=12) and coxsackie (n=1). They did not isolate any enterovirus.

Demographic characteristics of patients were obtained from the patients records used during recruitment in a previous study. Majority of the detections of enteroviruses in this study were seen in children in their first year of life. This was followed by children in their second year of their life. No detections of enteroviruses were seen in other age category. This is similar to what was observed by a study carried out in the

same hospital (Hazlett*et al*., 1988), where majority of the patients (51.8%) with infections by enteroviruses were seen in patients in their first year of life.

The study by Hazlett*et al*., (1988), isolated enteroviruses throughout the year, but most were isolated and detected in February (n=35), December (n=19), May (n=17), and June (n=16). Out of the 287 samples used in this study, majority of the viruses were detected in April, followed by May and June, which are usually cold and rainy months in Nairobi. Differences were seen when this study was compared to the study by Jacques *et al*., (2008) when many of the detections were seen in June and July, which are summer months in Europe.

There was no significant difference betweenmales and females who had enteroviruses isolated from them. Even though there were slightly more enteroviruses isolated from male patients than female patients. Other studies have indicated that the male is more susceptible to respiratory illnesses due to factors such as preterm birth, malnutrition, and exposure to harsh environmental conditions (Wells, 2000). These factors were not investigated in the present study but may be looked at in future studies.

This study's sequences had similarities of between 90 – 99% (Coxsackievirus – 93-97%; Echovirus – 93-97%; Enterovirus – 90-91%; Poliovirus – 91-99%) with other

comparable sequences in GenBank, a biorepository of genetic materials. On phylogenetic analysis, an evolutionary history of the taxa analyzed, this study's sequences especially Coxsackievirus, Echovirus and Enterovirus clustered together indicating similarities. They also had bootstrap values of 99% indicating the confidence limits of their clustering after 1000 replicates had been analyzed and a consensus tree had been constructed (Soltis, 2003). This has been described as a dependable measure for the phylogenetic accuracy of the grouping (Hillis, 1993).

When these viruses (Coxsackievirus, Echovirus and Enterovirus) were compared to other similar viruses, the viruses with the highest bootstrap value (87%) were Echovirus when compared to Echovirus 11 RO29726/72 Bootstrap values over 70% could be relied on (Baldauf, 2003). This was followed by Coxsackievirus with a bootstrap value of 64% when compared to 2008 Singaporean sequences. This study's enterovirus sequences had low bootstrap values of 36% when compared to Enterovirus 71 strain A/TW/NHR19829/09. The BLAST analysis carried out on the single Poliovirus detected in this study, indicated a 91-99% similarity to other poliovirus type 3 sequences in GenBank. Poliovirus 3, virus circulates in only 5 countries including Nigeria, Niger, Pakistan, India and Sudan (Kew *et al.*, 2005). On phylogeny, this study's poliovirus isolate was on its own branch, with other poliovirus from genbank clustering in another branch. This could have been a vaccine derived polio strain.

5.2.Conclusions

- In this study viruses were isolated from a total of 24 out of 287 stored samples. The isolated viruses were characterised using indirect fluorescent antibody tests (IDFAT) where 10 (41.7%) enterovirus, 9 (37.5%) coxsackie, 4 (16.7%) echovirus and 1 (4.2%) poliovirus were identified. The detection of theseenterovirusessuggests the involvement of the viruses in paediatricenterovirus infectionsin Kenya.
- In this study, there were more samples from males than females. Slightly more enteroviruses were isolated from males than females.Of the patients stored samples, 156 representing 54.4% were male, while 131 representing 45.6% were female.
- The most common isolated enterovirus was detected in the one year olds followed by two year olds. No other age group had enteroviruses isolated from them.
- Most enteroviruses were detected in the months of April, May and June respectively whereby, most of the samples were collected in the month of May (128; 44.6%) followed by April (98; 34.1%) and lastly by June (61; 21.3%)

- This study's sequences had similarities of between 90 – 99% (Coxsackievirus – 93-97%; Echovirus – 93-97%; Enterovirus – 90-91%; Poliovirus – 91-99%) with other comparable sequences in GenBank.
- On phylogenetic analysis based on an evolutionary history of the taxa showed this study's sequences especially Coxsackievirus, Echovirus and Enterovirus clustering together indicating similarities. They also had bootstrap values of 99% indicating the confidence limits of their clustering. These findings show the role of international travel in the spread of ARI pathogens.

5.3.Recommendations

- This study had a low prevalence associated with the use of stored samples that had undergone frequent freeze thaw cycles. This could have led to loss of enteroviruses, therefore it would be recommended that samples be inoculated immediately after collection to avoid loss of enteroviruses.
- In this study only one cell line (RD Cell) was used to isolate enteroviruses. The study indicated a low prevalence and therefore as a modified technique where several cell types can be used is necessary as it will yield different results with higher prevalence.
- Future studies should be carried out to determine the prevalence, age mostly affected and subtypes of enteroviruses infecting children with ARI.

- Future studies should be carried out to determine if some factors such as preterm birth, malnutrition, exposure to harsh environment conditions are associated with more enteroviruses isolated in males than females.

5.4.Limitations of the study

- The use of stored sample could have contributed to loss of enteroviruses due samples undergoing frequent freeze thaw cycles.
- The study indicated a low prevalence that could have been associated with the use of only one cell line (RD Cell) to isolate enteroviruses.
- Studies should be carried out to determine if some risk factors such as malnutrition, poor breastfeeding and undeveloped immunity lead to infections by agents of ARI.

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APPENDICES

Appendix 1: Big Dye Terminator method of sequencing

Step 1 - Preparing the sequencing reactions for dsDNA PCR products, assuming"half-reactions".

For each reaction (template/primer combination), add the following reagents to a separate 0.2 ml centrifugetube:

Reagent	**Quantity**
Terminator Ready Reaction Mix	4.0 ml
template	2.0 ml
primer (at 3.2 M)	2.0
ddH_2O	11.5
Big dye	1.0
total volume	20 ml
mix well and spin briefly	-

Depending on size of PCR product; the DBS sequencing team at UC**Davis** suggests approximately 4 ml of a sample of dsDNA template at a concentration of 2 ng/l per

100 bp length required for each reaction. This works well for most small to moderate size PCR products and plasmids clones. For example, for a 1.0 kb template, 4 ml of a sample with a concentration of 20 ng/l (total of ca. 80 ng) would be required. We however, use about half of this amount for most PCR product reactions.

Step 2 - Sequencing on the ABI 9700 thermal cyclers.

Place the tubes in the thermal cycler and begin temperature cycling protocol. Program the thermocycleras follows: 25 cycles of [94^{o}C for 5 sec, 94^{o}C for 10 sec, 50^{o}C for 45 sec 60^{o}C, 4 min], then ramp to 4^{o}C, purify extension products as below.

Step 3 - Purifying sequencing products by ethanol precipitation.

Into a 1.5 ml microcentrifuge tubes add 2µl EDTA Ethylene diaminetetra acetic acid, 2µl 3M Sodium acetate,50µl 100 % ethanol and 20µl of the product.Mix by vortexing briefly, incubate at room temperature for 15 min to precipitate products.
The tubes are placed in a thermal cycler and temperature cycling control begins. The thermocycler is programmed as follows: 25 cycles of [94^{o}C for 5 min, 94^{o}C for 10 sec, 50^{o}C for 5 sec 60^{o}C, 4 min], then ramp to 4^{o}C, the extension products were then purified as below:

The tubes are spinned for a minimum of 20 min at maximum speed in a microcentrifuge.

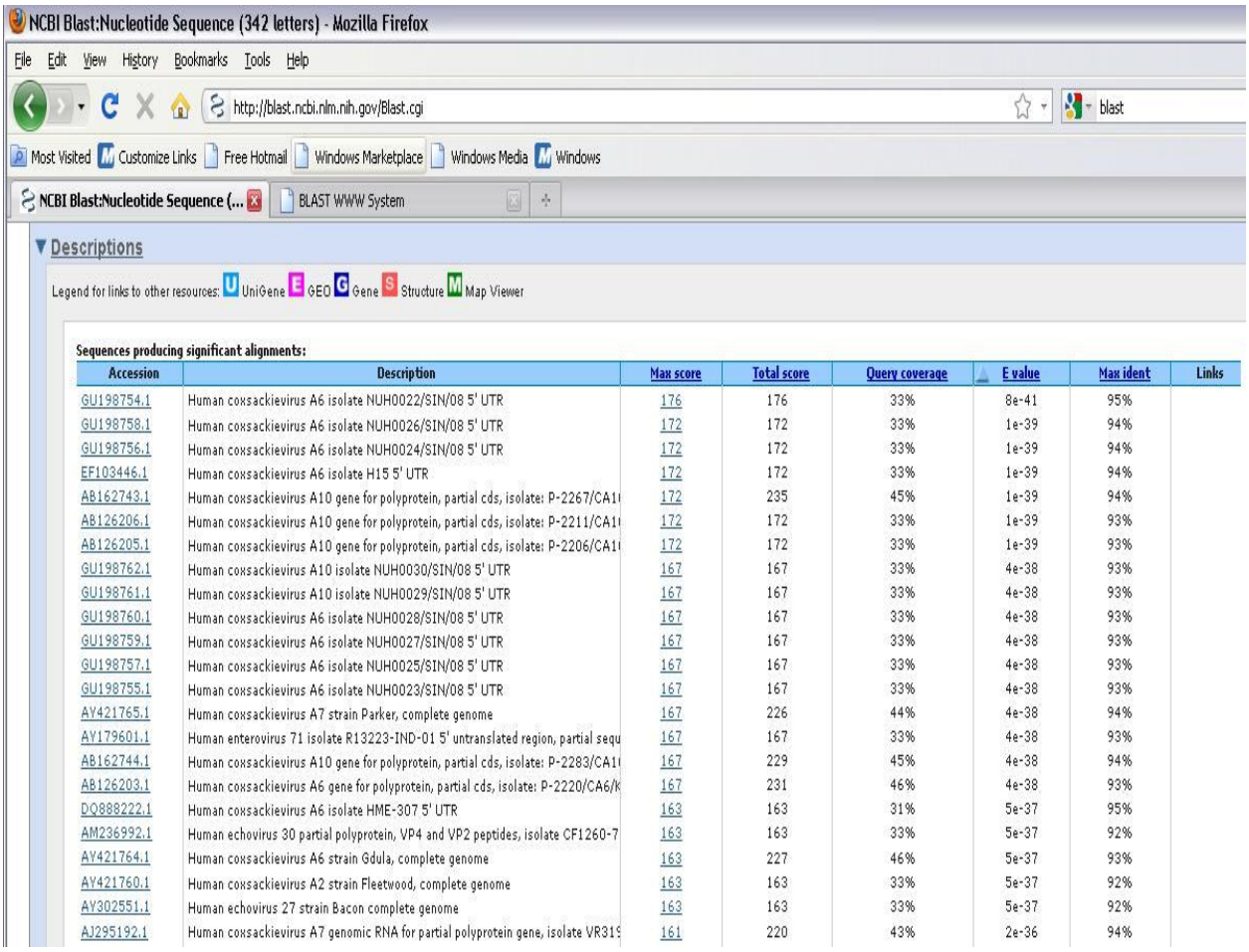
The supernatants are then aspirated completely with a separate pipet tip for each sample, being careful not to disturb the DNA pellet, and discard.

150µl of 70% ethanol was added to the tubes and vortexed briefly, and centrifuged as before for 5 min at maximum speed, and aspirate the supernatants as in step d. dry the pellets in the dark for 30 min.

Dissolve each sample pellet in 3 ml loading buffer [deionized formamide/25 mM EDTA (pH 8.0) with blue dextran (50 mg/ml), at 5:1 vol] immediately before use. Vortex and spin samples. Heat samples at 95^{o}C for 3 min, then immediately place on ice until ready to load. Load 1 - 2 ml per sample.

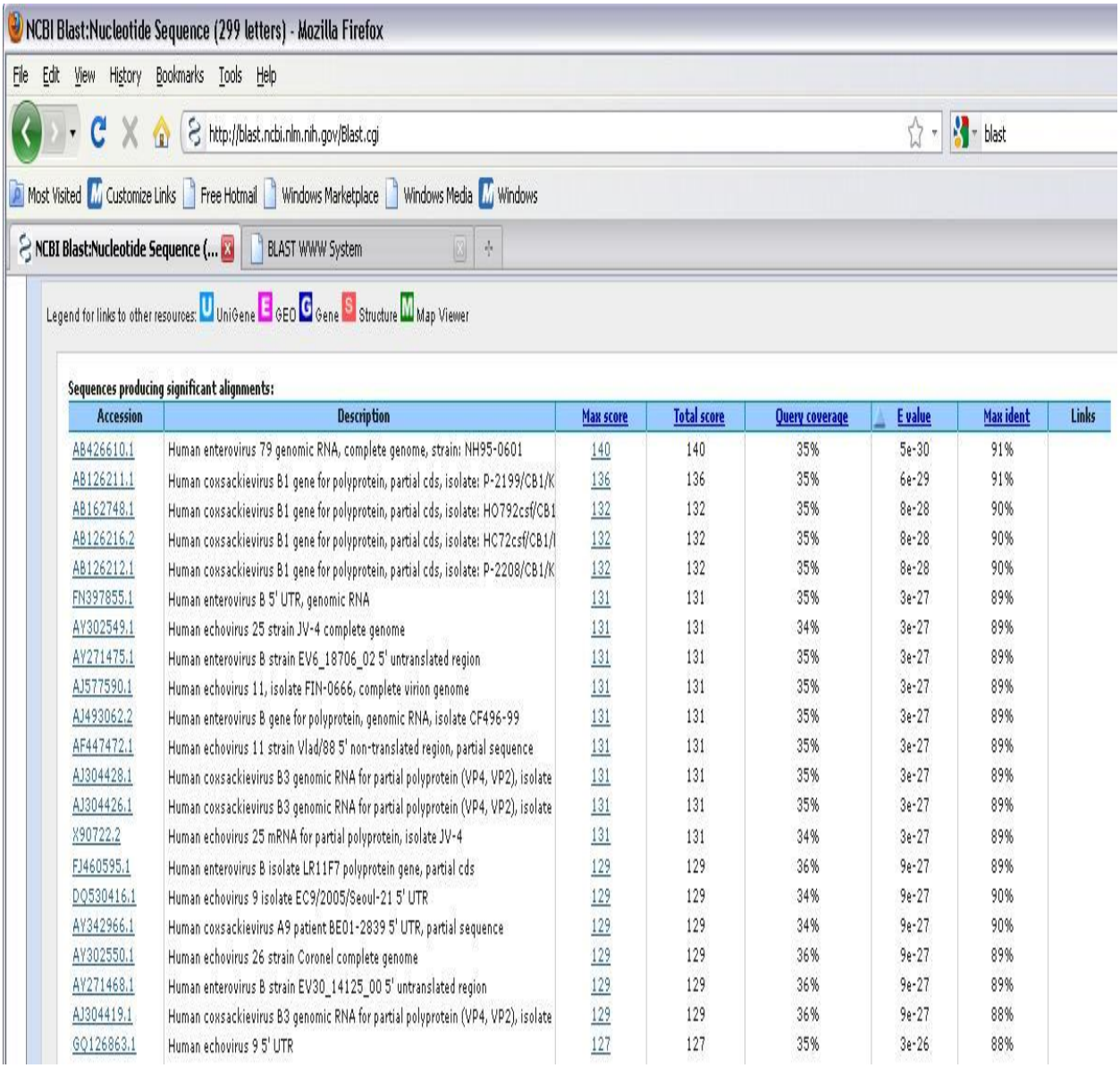
Appendix 2: Blast Analysis

The obtained sequences were compared to similar nucleotide sequences in the Genbank database using the BLAST software to determine similarities.



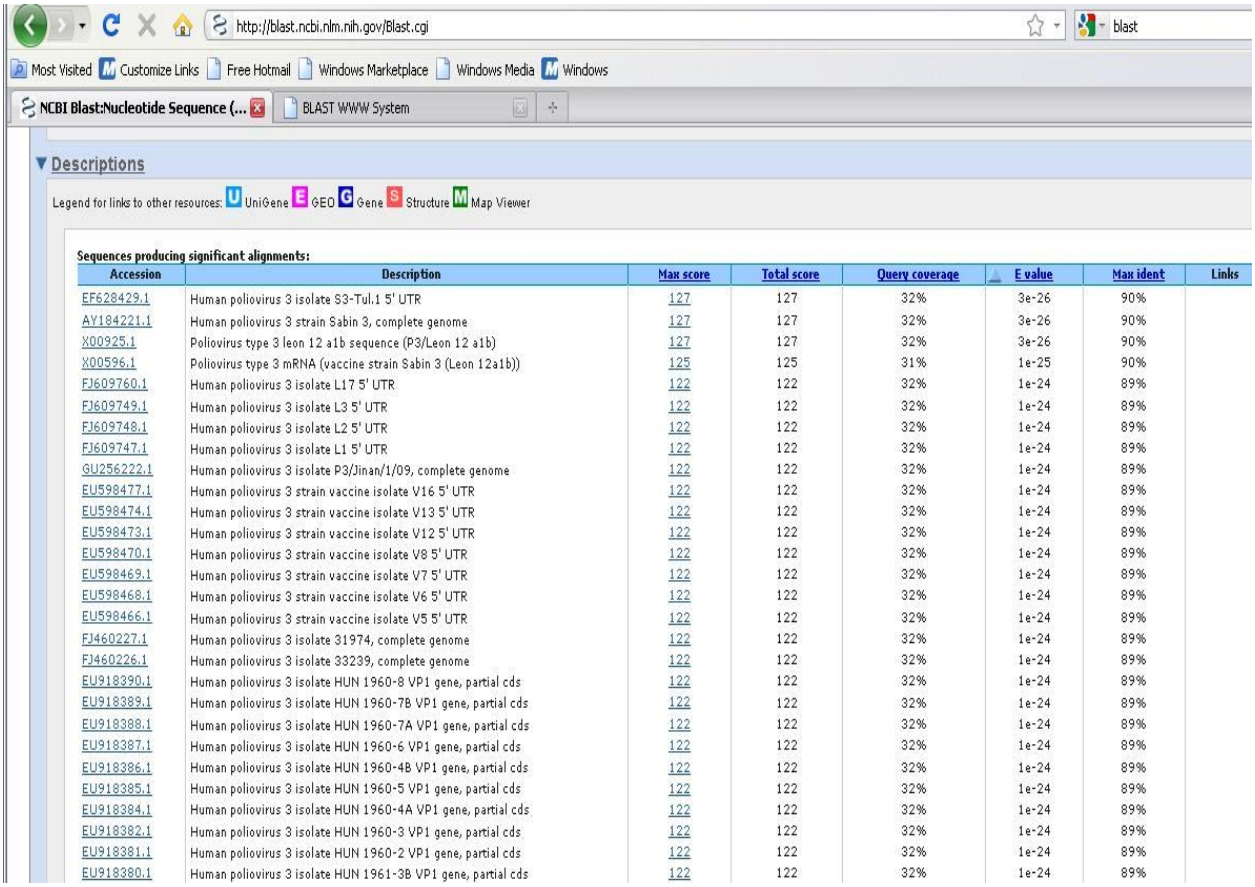
Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
GU198754.1	Human coxsackievirus A6 isolate NUH0022/SIN/08 5' UTR	176	176	33%	8e-41	95%	
GU198758.1	Human coxsackievirus A6 isolate NUH0026/SIN/08 5' UTR	172	172	33%	1e-39	94%	
GU198756.1	Human coxsackievirus A6 isolate NUH0024/SIN/08 5' UTR	172	172	33%	1e-39	94%	
EF103446.1	Human coxsackievirus A6 isolate H15 5' UTR	172	172	33%	1e-39	94%	
AB162743.1	Human coxsackievirus A10 gene for polyprotein, partial cds, isolate: P-2267/CA1	172	235	45%	1e-39	94%	
AB126206.1	Human coxsackievirus A10 gene for polyprotein, partial cds, isolate: P-2211/CA1	172	172	33%	1e-39	93%	
AB126205.1	Human coxsackievirus A10 gene for polyprotein, partial cds, isolate: P-2206/CA1	172	172	33%	1e-39	93%	
GU198762.1	Human coxsackievirus A10 isolate NUH0030/SIN/08 5' UTR	167	167	33%	4e-38	93%	
GU198761.1	Human coxsackievirus A10 isolate NUH0029/SIN/08 5' UTR	167	167	33%	4e-38	93%	
GU198760.1	Human coxsackievirus A6 isolate NUH0028/SIN/08 5' UTR	167	167	33%	4e-38	93%	
GU198759.1	Human coxsackievirus A6 isolate NUH0027/SIN/08 5' UTR	167	167	33%	4e-38	93%	
GU198757.1	Human coxsackievirus A6 isolate NUH0025/SIN/08 5' UTR	167	167	33%	4e-38	93%	
GU198755.1	Human coxsackievirus A6 isolate NUH0023/SIN/08 5' UTR	167	167	33%	4e-38	93%	
AY421765.1	Human coxsackievirus A7 strain Parker, complete genome	167	226	44%	4e-38	94%	
AY179601.1	Human enterovirus 71 isolate R13223-IND-01 5' untranslated region, partial sequ	167	167	33%	4e-38	93%	
AB162744.1	Human coxsackievirus A10 gene for polyprotein, partial cds, isolate: P-2283/CA1	167	229	45%	4e-38	94%	
AB126203.1	Human coxsackievirus A6 gene for polyprotein, partial cds, isolate: P-2220/CA6/K	167	231	46%	4e-38	93%	
DQ888222.1	Human coxsackievirus A6 isolate HME-307 5' UTR	163	163	31%	5e-37	95%	
AM236992.1	Human echovirus 30 partial polyprotein, VP4 and VP2 peptides, isolate CF1260-7	163	163	33%	5e-37	92%	
AY421764.1	Human coxsackievirus A6 strain Gdula, complete genome	163	227	46%	5e-37	93%	
AY421760.1	Human coxsackievirus A2 strain Fleetwood, complete genome	163	163	33%	5e-37	92%	
AY302551.1	Human echovirus 27 strain Bacon complete genome	163	163	33%	5e-37	92%	
AJ295192.1	Human coxsackievirus A7 genomic RNA for partial polyprotein gene, isolate VR31	161	220	43%	2e-36	94%	

*Coxsackie BLAST results



Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AB426610.1	Human enterovirus 79 genomic RNA, complete genome, strain: NH95-0601	140	140	35%	5e-30	91%	
AB126211.1	Human coxsackievirus B1 gene for polyprotein, partial cds, isolate: P-2199/CB1/K	136	136	35%	6e-29	91%	
AB162748.1	Human coxsackievirus B1 gene for polyprotein, partial cds, isolate: HO792csf/CB1	132	132	35%	8e-28	90%	
AB126216.2	Human coxsackievirus B1 gene for polyprotein, partial cds, isolate: HC72csf/CB1/I	132	132	35%	8e-28	90%	
AB126212.1	Human coxsackievirus B1 gene for polyprotein, partial cds, isolate: P-2208/CB1/K	132	132	35%	8e-28	90%	
FN397855.1	Human enterovirus B 5' UTR, genomic RNA	131	131	35%	3e-27	89%	
AY302549.1	Human echovirus 25 strain JV-4 complete genome	131	131	34%	3e-27	89%	
AY271475.1	Human enterovirus B strain EV6_18706_02 5' untranslated region	131	131	35%	3e-27	89%	
AJ577590.1	Human echovirus 11, isolate FIN-0666, complete virion genome	131	131	35%	3e-27	89%	
AJ493062.2	Human enterovirus B gene for polyprotein, genomic RNA, isolate CF496-99	131	131	35%	3e-27	89%	
AF447472.1	Human echovirus 11 strain Vlad/88 5' non-translated region, partial sequence	131	131	35%	3e-27	89%	
AJ304428.1	Human coxsackievirus B3 genomic RNA for partial polyprotein (VP4, VP2), isolate	131	131	35%	3e-27	89%	
AJ304426.1	Human coxsackievirus B3 genomic RNA for partial polyprotein (VP4, VP2), isolate	131	131	35%	3e-27	89%	
X90722.2	Human echovirus 25 mRNA for partial polyprotein, isolate JV-4	131	131	34%	3e-27	89%	
FJ460595.1	Human enterovirus B isolate LR11F7 polyprotein gene, partial cds	129	129	36%	9e-27	89%	
DQ530416.1	Human echovirus 9 isolate EC9/2005/Seoul-21 5' UTR	129	129	34%	9e-27	90%	
AY342966.1	Human coxsackievirus A9 patient BE01-2839 5' UTR, partial sequence	129	129	34%	9e-27	90%	
AY302550.1	Human echovirus 26 strain Coronel complete genome	129	129	36%	9e-27	89%	
AY271468.1	Human enterovirus B strain EV30_14125_00 5' untranslated region	129	129	36%	9e-27	89%	
AJ304419.1	Human coxsackievirus B3 genomic RNA for partial polyprotein (VP4, VP2), isolate	129	129	36%	9e-27	88%	
GQ126863.1	Human echovirus 9 5' UTR	127	127	35%	3e-26	88%	

*Enterovirus BLAST results

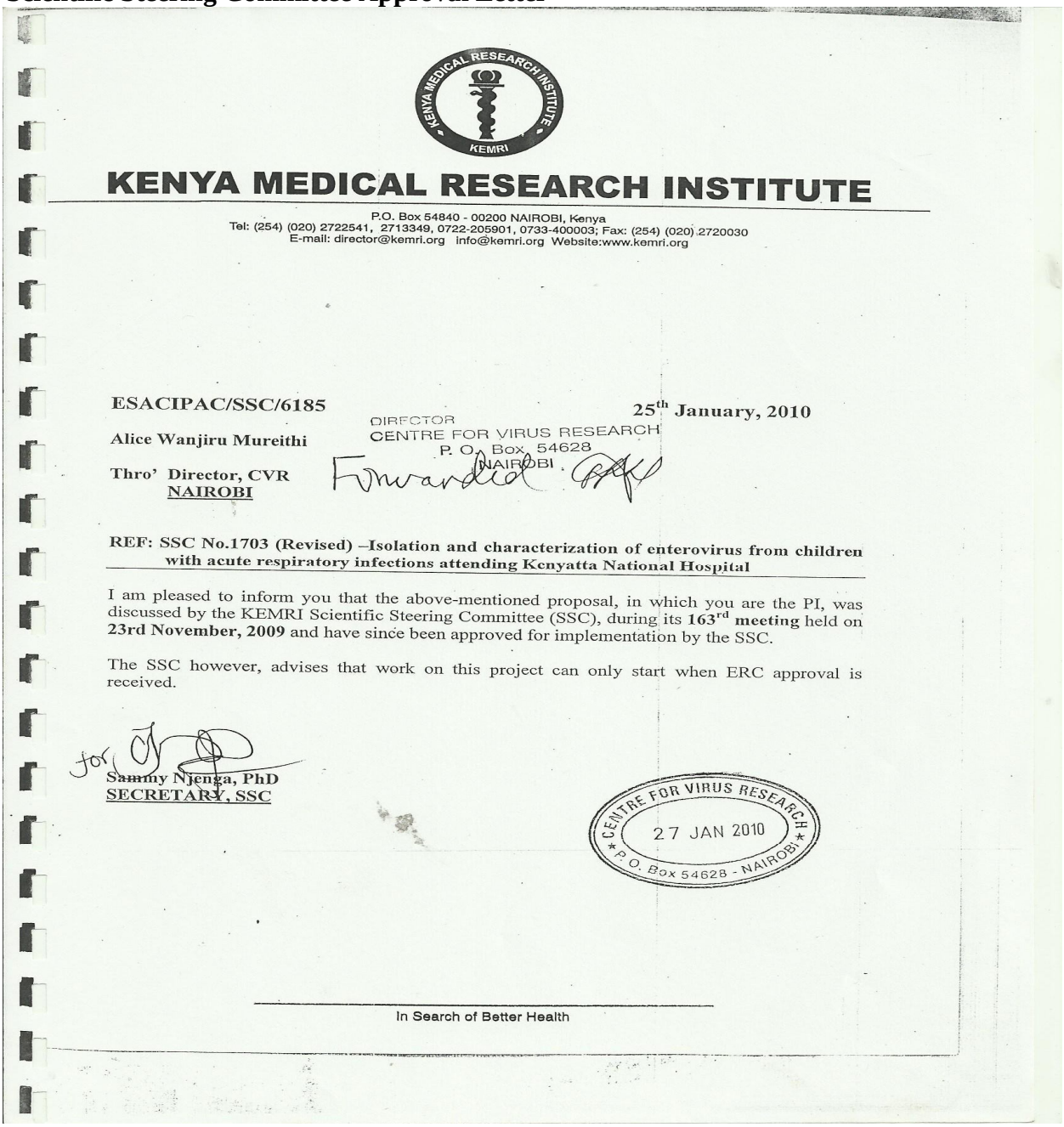


Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
EF628429.1	Human poliovirus 3 isolate S3-Tul.1 5' UTR	127	127	32%	3e-26	90%	
AY184221.1	Human poliovirus 3 strain Sabin 3, complete genome	127	127	32%	3e-26	90%	
X00925.1	Poliovirus type 3 leon 12 a1b sequence (P3/Leon 12 a1b)	127	127	32%	3e-26	90%	
X00596.1	Poliovirus type 3 mRNA (vaccine strain Sabin 3 (Leon 12a1b))	125	125	31%	1e-25	90%	
FJ609760.1	Human poliovirus 3 isolate L17 5' UTR	122	122	32%	1e-24	89%	
FJ609749.1	Human poliovirus 3 isolate L3 5' UTR	122	122	32%	1e-24	89%	
FJ609748.1	Human poliovirus 3 isolate L2 5' UTR	122	122	32%	1e-24	89%	
FJ609747.1	Human poliovirus 3 isolate L1 5' UTR	122	122	32%	1e-24	89%	
GU256222.1	Human poliovirus 3 isolate P3/Jinan/1/09, complete genome	122	122	32%	1e-24	89%	
EU598477.1	Human poliovirus 3 strain vaccine isolate V16 5' UTR	122	122	32%	1e-24	89%	
EU598474.1	Human poliovirus 3 strain vaccine isolate V13 5' UTR	122	122	32%	1e-24	89%	
EU598473.1	Human poliovirus 3 strain vaccine isolate V12 5' UTR	122	122	32%	1e-24	89%	
EU598470.1	Human poliovirus 3 strain vaccine isolate V8 5' UTR	122	122	32%	1e-24	89%	
EU598469.1	Human poliovirus 3 strain vaccine isolate V7 5' UTR	122	122	32%	1e-24	89%	
EU598468.1	Human poliovirus 3 strain vaccine isolate V6 5' UTR	122	122	32%	1e-24	89%	
EU598466.1	Human poliovirus 3 strain vaccine isolate V5 5' UTR	122	122	32%	1e-24	89%	
FJ460227.1	Human poliovirus 3 isolate 31974, complete genome	122	122	32%	1e-24	89%	
FJ460226.1	Human poliovirus 3 isolate 33239, complete genome	122	122	32%	1e-24	89%	
EU918390.1	Human poliovirus 3 isolate HUN 1960-8 VP1 gene, partial cds	122	122	32%	1e-24	89%	
EU918389.1	Human poliovirus 3 isolate HUN 1960-7B VP1 gene, partial cds	122	122	32%	1e-24	89%	
EU918388.1	Human poliovirus 3 isolate HUN 1960-7A VP1 gene, partial cds	122	122	32%	1e-24	89%	
EU918387.1	Human poliovirus 3 isolate HUN 1960-6 VP1 gene, partial cds	122	122	32%	1e-24	89%	
EU918386.1	Human poliovirus 3 isolate HUN 1960-4B VP1 gene, partial cds	122	122	32%	1e-24	89%	
EU918385.1	Human poliovirus 3 isolate HUN 1960-5 VP1 gene, partial cds	122	122	32%	1e-24	89%	
EU918384.1	Human poliovirus 3 isolate HUN 1960-4A VP1 gene, partial cds	122	122	32%	1e-24	89%	
EU918382.1	Human poliovirus 3 isolate HUN 1960-3 VP1 gene, partial cds	122	122	32%	1e-24	89%	
EU918381.1	Human poliovirus 3 isolate HUN 1960-2 VP1 gene, partial cds	122	122	32%	1e-24	89%	
EU918380.1	Human poliovirus 3 isolate HUN 1961-3B VP1 gene, partial cds	122	122	32%	1e-24	89%	

*Poliovirus BLAST results

Appendix 3

Scientific Steering Committee Approval Letter



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E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

ESACIPAC/SSC/6185

25th January, 2010

DIRECTOR
CENTRE FOR VIRUS RESEARCH
P. O. Box 54628
NAIROBI

Alice Wanjiru Mureithi

Thro' Director, CVR
NAIROBI

REF: SSC No.1703 (Revised) –Isolation and characterization of enterovirus from children with acute respiratory infections attending Kenyatta National Hospital

I am pleased to inform you that the above-mentioned proposal, in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its **163rd meeting** held on **23rd November, 2009** and have since been approved for implementation by the SSC.

The SSC however, advises that work on this project can only start when ERC approval is received.

for

Sammy Njenga, PhD
SECRETARY, SSC

CENTRE FOR VIRUS RESEARCH
27 JAN 2010
P.O. Box 54628 - NAIROBI

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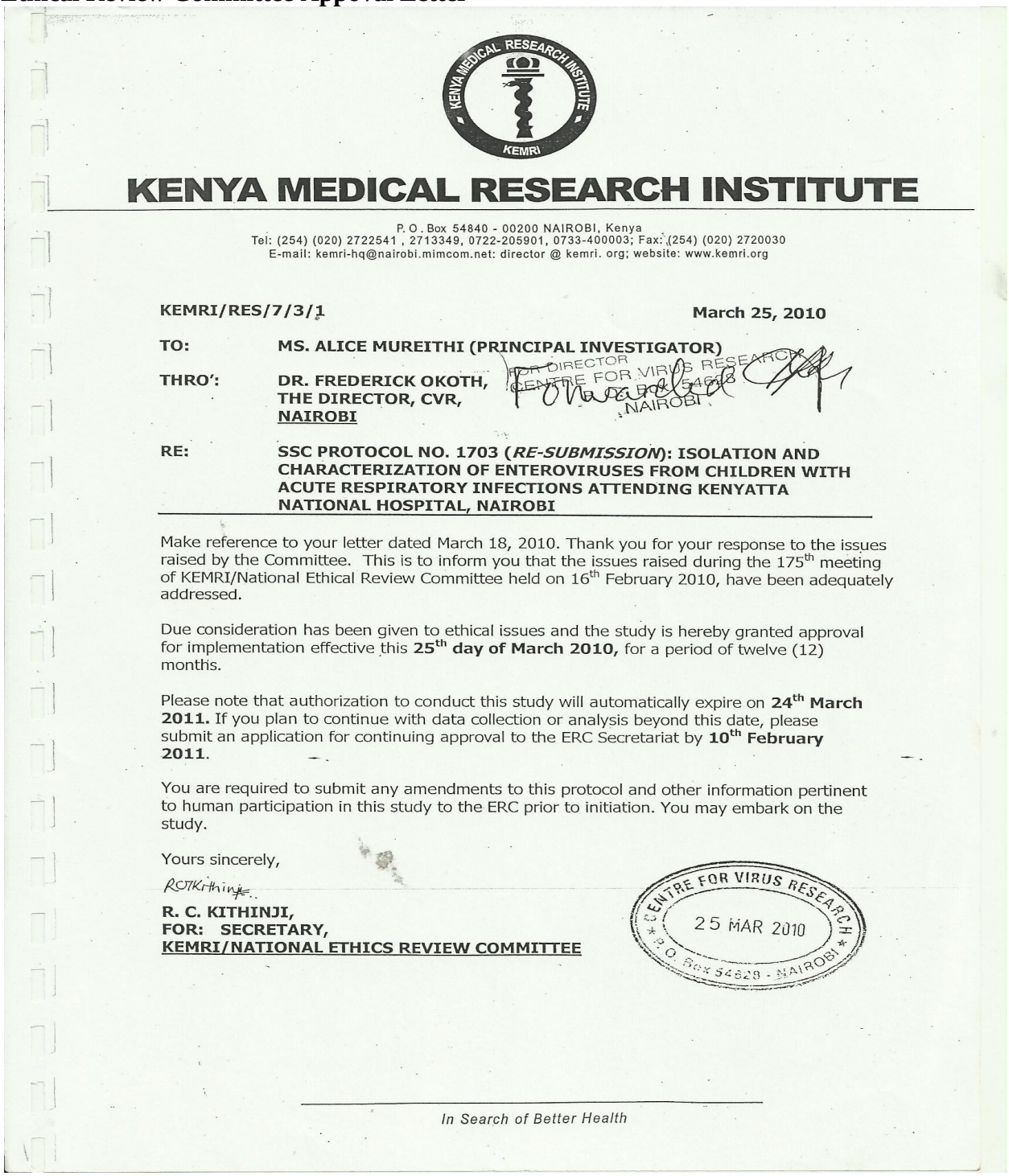
Appendix 4

Ethical Review Committee Appoval Letter



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KEMRI/RES/7/3/1 **March 25, 2010**

TO: **MS. ALICE MUREITHI (PRINCIPAL INVESTIGATOR)**

THRO': **DR. FREDERICK OKOTH, THE DIRECTOR, CVR, NAIROBI**

FOR DIRECTOR CENTRE FOR VIRUS RESEARCH P.O. Box 54840 NAIROBI

RE: **SSC PROTOCOL NO. 1703 (*RE-SUBMISSION*): ISOLATION AND CHARACTERIZATION OF ENTEROVIRUSES FROM CHILDREN WITH ACUTE RESPIRATORY INFECTIONS ATTENDING KENYATTA NATIONAL HOSPITAL, NAIROBI**

Make reference to your letter dated March 18, 2010. Thank you for your response to the issues raised by the Committee. This is to inform you that the issues raised during the 175th meeting of KEMRI/National Ethical Review Committee held on 16th February 2010, have been adequately addressed.

Due consideration has been given to ethical issues and the study is hereby granted approval for implementation effective this **25th day of March 2010,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **24th March 2011.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **10th February 2011**.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Yours sincerely,

R.C. Kithinji

R. C. KITHINJI,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

CENTRE FOR VIRUS RESEARCH 25 MAR 2010 P.O. Box 54828 - NAIROBI

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