INVESTIGATING ORIGINS AND SPREAD OF HUMAN RHINOVIRUS IN KILIFI, RURAL COASTAL KENYA BY ANALYSIS OF MOLECULAR SEQUENCE DATA

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Investigating Origins and Spread of Human Rhinovirus in Kilifi, Rural Coastal Kenya by Analysis of Molecular Sequence Data

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A thesis submitted in partial fulfillment for the degree of Master of Science in Biotechnology in the Jomo Kenyatta University of Agriculture and Technology

2019

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature: ……………………………………... Date …….…………………………….

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

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DEDICATION

This work is dedicated to my dear parents and my brothers and sisters. Thank you for your patience, prayers and support.

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First, I am grateful to God for giving me the courage and opportunity to carry to complete this work.

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LIST OF ABBREVIATIONS/ ACRONYMS

HRV	Human Rhinovirus
URTI	Upper Respiratory Tract Infection
ARI	Acute Respiratory Infection
HEV	Human Enterovirus
KHDSS	Kilifi Health and Demographic Surveillance System
KCH	Kilifi County Hospital
MOH	Ministry of Health
COPD	Chronic Obstructive Pulmonary Disease
IgA	Immunoglobin A
IgG	Immunoglobin G
VP	Viral Protein
UTR	Untranslated Region
PSA	Pairwise Sequence Alignment
KEMRI	Kenya Medical Research Institute
NPS	Nasopharyngeal Sample
ORF	Open Reading Frame
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
RdRp	RNA–dependent RNA-polymerase
TLR	Toll like Receptor
MDA-5	Melanoma Differentiation Associated Gene

RIG-I	Retinoic acid inducible protein 1
IL	Interleukin
IFN	Interferon
NK	Natural Killer Cells
WHO	World Health Organisation
KEMRI-WT	Kenya Medical Research Institute – Wellcome Trust
BLAST	Basic Local Alignment Search Tool
ML	Maximum Likelihood
MEGA	Molecular Evolutionary Genetic Analysis
SERU	Scientific and Ethical Review Unit

ABSTRACT

Human rhinovirus (HRV) is recognised as the leading cause of upper respiratory tract infections, resulting in a significant socio-economic and public health burden. Understanding of HRV transmission in communities living in low-income settings as well as their spatial-temporal patterns is currently limited. This study characterized HRV types in circulation, their origins, spread and persistence within the Kilifi Health and Demographic Surveillance System (KHDSS), Coastal Kenya. Nasopharyngeal swab (NPS) samples were collected from patients aged >7days presenting with symptoms of acute respiratory infection (ARI) at ten health facilities across a Health and Demographic Surveillance System between December 2015 and May 2016. HRV was diagnosed by real-time RT-PCR, and the VP4/VP2 genomic region (~ 400 bp) of the positive samples sequenced by Sanger method. Phylogenetic analysis was used to determine the HRV types and their genetic diversity. Of 2,150 NPS samples collected, HRV was detected in 423 of which, 306 were successfully sequenced in the VP4/VP2 genome region. HRV species A, B and C were identified in 151, 15 and 140 samples, respectively. In total, 56 HRV types were determined: 33, 4 and 19 occurred within species A, B and C, respectively. HRV types presented heterogeneous temporal patterns of occurrence (ranging from 1-5 months) and of peak occurrence most commonly in March to May. Spatially, identical HRV types occurred over a wide distance at similar times. This study records a high prevalence of HRV in outpatient presentations exhibiting remarkably high type diversity in this low-income setting. Patterns of occurrence suggest frequent and independent invasion of different HRV types into the KHDSS. Temporal differences of persistence between types may reflect variation in type-specific population immunity. Spatial patterns suggest either rapid spread of types or multiple invasion of the same HRV type.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Human rhinovirus (HRV) is the predominant cause of upper respiratory tract infections (URTIs) referred to as the common cold (Aponte et al., 2015; Iwane et al., 2011; Jan Richter et al., 2016). The virus has also been associated with lower respiratory tract illnesses including exacerbation of asthma and chronic obstructive pulmonary disease (COPD) in children under 5 years (Hershenson, 2013; Iwane et al., 2011). HRV is globally ubiquitous and infections usually occur all year-round, although peaking in the early autumn and late spring in many temperate or subtropical countries and in the rainy season in tropical countries (Briese et al., 2008; Garcia et al., 2013). Despite most HRV infections being mostly mild, they pose a considerable social and economic burden due to time lost and reduced performance of regular duties (Fendrick et al., 2003). HRV transmission, infection patterns and diversity have been rarely studied in low-income settings despite bearing the majority burden of acute respiratory illnesses (ARI) (Morobe et al., 2018; Onyango et al., 2012). HRVs fall under the genus *Enterovirus* in the family *Picornaviridae* (Palmenberg & Gern, 2015).

HRV genome occurs as a positive-sense, single-stranded RNA molecule of approximately 7.2 kb flanked by a 5' untranslated region (UTR) and 3' poly-A tail. There are three HRV species denoted as HRV-A, HRV-B and HRV-C within which ~168 genetically distinct types have been identified. Identification of HRV types presently relies on molecular typing methods based on sequence analysis of VP1 or VP4/VP2 proteins encoding regions (McIntyreet al., 2013; Palmenberg & Gern, 2015).

Previous studies investigating the transmission of HRV have shown that multiple infections occur in individuals even over short periods of time of the order of a few weeks (Howard et al., 2016). In household studies, family members experience rapid rates of reinfections up to 5 infections in adults or 12 infections in young children per year with

each infection mostly caused by a different HRV type and rarely with the same HRV type (Nguyen et al., 2016). Similar findings demonstrating reinfection among young children (<3 years) are often represent acquisition of new strains in the community, rather than same persisting HRV type have been reported (Jartti et al., 2004). These observations are consistent with evidence from immunological studies on HRV that indicate strong homologous type responses and very weak heterotypic immunity to protect individuals against multiple types (Couch et al., 1965; Fleet et al., 1968). Moreover, studies have shown that individuals develop a long-lasting (~ 1 year) type-specific neutralizing antibodies (IgG and IgA) against a specific infecting HRV-type but susceptibility to other different HRV types remains and infection will occur as long as there is exposure (Barclay et al., 1989; Message & Johnston, 2001). Hence it would be expected that at the population level the virus types would spread independent of each other.

To date, there are no approved therapeutic drugs or vaccines against HRV infections (Glanville & Johnston, 2015). Treatment of HRV common cold remains supportive with common decongestants and antihistamine (Allan & Arroll, 2014). In the case of asthma and chronic obstructive pulmonary disease (COPD) exacerbations standard corticosteroids and bronchodilators are used (Page & Cazzola, 2014). Human and animal trials on vaccine development has faced range of difficulties (Glanville et al., 2013). First, the significantly large number of HRV types (~168) with high sequence variability on the surface proteins. Second, lack of epidemiological data to identify the most important HRV types human populations should be protected against by a potential vaccine. Third, limited animal models of HRV infections to understand the pathogenesis of HRV (Glanville & Johnston, 2015; Papi & Contoli, 2011).

In Kenya, studies have shown HRV to be an important cause of hospitalized acute respiratory illness in children under the age of 5 years (Breiman et al., 2015; Onyango et al., 2012). Study in rural Kenya reported a prevalence of 22.0% (380/1759) in inpatients and 24.0% (61/254) in outpatients (Onyango et al., 2012). Moreover, HRV surveillance studies have shown circulation of wide range of HRV types in human population (Daleno et al., 2013; Onyango et al., 2012; Sansone et al., 2013). In contrast to the preceding

observations, there is a paucity of data concerning the HRV types circulating in the overall community, and their spatial-temporal patterns of prevalence. Data of this sort could improve understanding on the nature of spread of these viruses at the population level and can be of importance in developing strategies for infection control.

This study utilized sequence data to understand the origins and spread of HRV in the KHDSS. Molecular sequences data has been used to reveal the probable chains of transmission events and predicting the source of infections in epidemiological studies of livestock and humans (Cottam et al., 2008; Gire et al., 2014). In this study, time-stamped VP4/VP2 partial sequences of HRV obtained from samples collected from 9 health facilities and Kilifi County Hospital (KCH) all within the Kilifi Health and Demographic Surveillance System (KHDSS) (Scott et al., 2012) were analyzed to understand introduction, spread and persistence of HRV at the community level.

1.2 Problem statement

Despite high prevalence rates of HRV infections reported in Kenya (Breiman et al., 2015; Milanoi et al., 2016; Morobe et al., 2018; Onyango et al., 2012) including the current study area (i.e. rural coastal Kenya) there is little understanding on the mechanisms of introduction, spread, and persistence of these viruses in communities (Morobe et al., 2018). Moreover, information on the types in circulation and their spatial-temporal distribution is scarce. Molecular sequences data is important to determine the transmission dynamics and mechanism of persistence of these viruses in community.

1.3 Justification

Efforts to design effective intervention strategies against respiratory viruses are hindered by the rapid virus evolution, presence of diverse types, waning of host immunity among other factors (Glanville & Johnston, 2015; Glanville et al., 2013). Moreover, the transmission dynamics, origins, introductions, spread and mechanism of persistence of these viruses are not fully understood (Morobe et al., 2018). Genetic analyses of partial and complete viral genomes generated in real time have been used to reveal a probable chain of transmission events, predicting the source of infection and studying the evolution

of viruses (Cottam et al., 2008; Gire et al., 2014). This study used phylogenetics to analyze viral sequence obtained from samples collected from KHDSS to investigate the introduction, spread patterns and persistence of HRV. Understanding of the HRV types in circulation, their origins, introductions and patterns of spread is of significant benefit in the optimization of effective control strategies.

1.4 Research questions

1. Are HRV strains circulating in different location of KHDSS genetically different form each other?
2. Are HRV strains circulating in KHDSS genetically different to HRV strain circulating elsewhere globally?
3. Do the HRV strains circulating in the KHDSS represent multiple introduction or single introduction?
4. What is the source/origin of HRV strains circulating in the KHDSS?

1.5 Null hypothesis

HRV strains within the three known rhinovirus species circulating within different locations of KHDSS are not phylogenetically related to each other or to the contemporaneous strains circulating globally.

1.6 General objective

To understand HRV transmission patterns within Kilifi County of Coastal Kenya and the genetic relatedness of local virus strains to global circulating strains to inform spread patterns, mechanism of persistence.

1.6.1 Specific objectives

1. To investigate the phylogenetic relatedness of HRV strains circulating in different parts of KHDSS sampled over 6 months in 10 health centers.

2. To investigate the phylogenetic relatedness between rhinovirus strains circulating in Kilifi County of coastal Kenya in 2015- 2016 with contemporaneous strains circulating elsewhere globally.

3. To determine if sequence analysis of VP4/VP2 coding region of HRV is adequate to reconstruct the HRV transmission pathways in local Kilifi community.

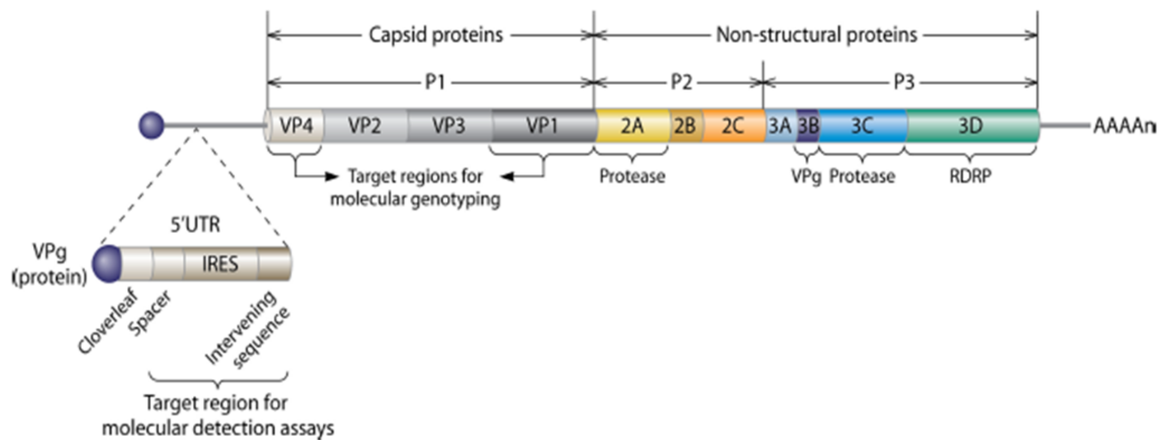
CHAPTER TWO
LITERATURE REVIEW

2.1 Genomic structure and organization of HRV

HRVs are small (30 nanometer), non-enveloped, positive-sense, single-stranded RNA viruses. HRV genome is approximately 7.2kb flanked by 5' untranslated region (UTR) and 3' poly-A tail. 5' UTR is linked to viral protein genome-linked (VPg) and acts as primer for genome replication (Figure 2.1, panel A). The 5' UTR is approximately 650 bases long; the open reading frame is approximately 6500 bases (~2100 encoded amino acids) and the 3' UTR consists of approximately 50 bases.

The viral genome open reading frame (ORF) encode for 11 gene products namely VP4, VP1, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C and 3D. The gene are divided into two; structural and non-structural genes (P1, P2 and P3). Structural gene encodes four structural proteins VP1 - VP4, while the non-structural region encodes for seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) The viral capsid VP1, VP2 and VP3 are located on the outer surface of the viral particle while VP4 is located on the inner surface of the capsid with a canyon in VP1 that serves as the site of attachment to cell surface receptors. The four viral capsid proteins form 60 promoter units forming an icosahedral structure of the mature HRV virion (Figure 2.1, panel B). Non-structural proteins (2B, 2BC and 3AB) are involved in the virus genome replication and assembly (Jacobs et al., 2013). In this study 5' UTR is a target for the diagnostics real-time PCR while VP4/VP2 regions is used in the classification of HRV to their respective HRV species and types.

A



B

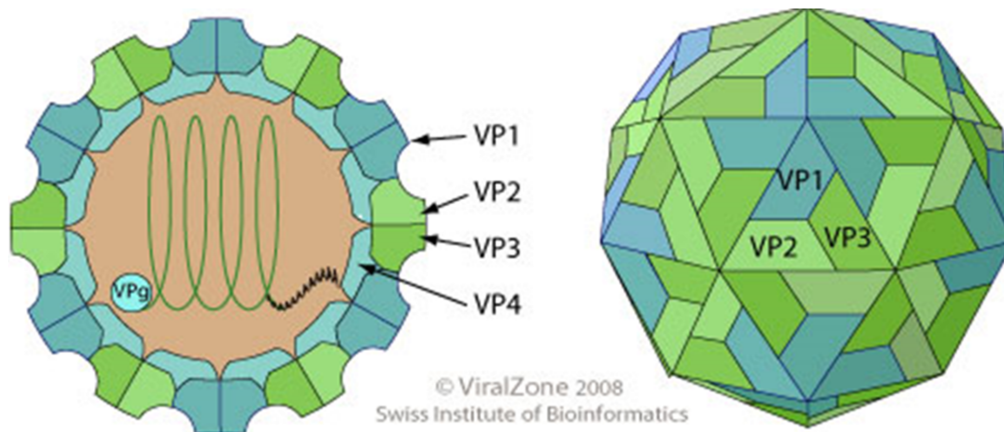


Figure 2.1: (A) HRV genomic structure (B) Structure organization of HRV

2.2 Classification of HRV

HRVs fall under the genus *Enterovirus* in the family *Picornaviridae*. The genus *Enterovirus* comprises seven species infecting humans (*Enterovirus* A-to-D and rhinovirus A to-C). Genus *Enterovirus* contains poliovirus (PV), coxsackieviruses A/B (CVA/B), enteroviruses (EV), echoviruses (E), and rhinoviruses (RV). HRV exist as three species namely, HRV-A, HRV-B and HRV-C.

Initially HRVs were classified into two species (HRV-A and HRV-B) based on antigenic cross-reactivity (Hamparian et al., 1987). HRV-C which is uncultivable in the laboratory was never detected by serological method (cross-neutralization) until 2006 after the arrival of sensitive molecular detection methods such as PCR (Lamson et al., 2006). Development of a genetic-based system has allowed classification into distinct species (A, B and C). To date, 55 different types of HRV C have been identified by using a threshold of a 13% nucleotide difference in VP1 over 821 base length region or at least a 10% nucleotide difference in the VP4/VP2 over 428 base length region (Simmonds et al., 2010). Genetic classification was extended across all the HRV species and it was established that HRV types should have at least 13% (for HRV-A), 12% (for HRV-B), or 13% (for HRV-C) nucleotide divergence from all other types in the VP1 region (McIntyre et al., 2013). Currently, there are 80 HRV-A, 33 HRV-B and 55 HRV-C classified based on VP4/VP2 nucleotide sequence divergence. The previously used term 'serotype' which reflects classification of HRVs by antigenic properties was changed to 'type' following the genetic classification (McIntyre et al., 2013; Simmonds et al., 2010) (http://www.picornaviridae.com). Classification based on VP4/VP2 nucleotide sequence correlates with the classification based on VP1 nucleotide sequence (McIntyre et al., 2013). Phylogenetic analysis of VP1 was found to be congruent to phylogenetic analysis using VP4/VP2 coding sequences (McIntyre et al., 2013).

Other classification systems have been based on the receptor they bind to in the host nasal mucosa epithelial cells (Lewis-Rogers et al., 2017). Twelve HRV-A types have been classified as "minor group" as they bind to low-density lipoprotein receptor (LDLR) while the remaining A and B types are classified as "major group" bind to intercellular adhesion molecule-1(ICAM-1). Cadherin–related family member 3 precursor (CDHR3) has been found to serve as receptor for HRV–C although the mechanism of entry and uncoating are unknown (Schuler et al., 2014).

2.3 Clinical presentation of HRV infections

HRV infections can be asymptomatic, or present as either mild upper respiratory tract infection (known as common cold syndrome including acute otitis media, rhinosinusitis)

(Cordey et al., 2010). HRV is the main cause of cases of common colds (50- 75%) (Jacobs et al., 2013). HRV have also been associated with cases of lower respiratory tract infection such as pneumonia (18 - 26%) and bronchiolitis (14%) (Jacobs et al., 2013). Solely, HRVs have been detected by RT-PCR in 15%–30% of asymptomatic individuals (van Benten et al., 2003). A study by (Peltola et al., 2008) to investigate incidence of symptomatic and asymptomatic infections within families with children, found that most rhinovirus infections in young children are symptomatic while secondary infections in older children and adults are often asymptomatic (Peltola et al., 2008). Asymptomatic infections in old age groups are probably due to acquired immunity gained after previous experience with HRV types which has allowed them to gain serum antibodies against many types. HRVs have also been linked to exacerbations of chronic obstructive pulmonary disease (COPD) and asthma development (Hershenson, 2013). Studies have shown HRV coinfection with bacteria such as *Haemophilus influenza* may increase chances of exacerbations of COPD (Wilkinson & Wedzicha, 2006).

2.4 Genetic variability of HRV

HRVs display high degree of nucleotide sequence diversity between types. Major causes of genetic diversity in RNA viruses include point mutation and recombination events (Agol, 1997; Royston & Tapparel, 2016; Zhang et al., 2015). Point mutations cause nucleotide misincorporations during replication due to the high error-rate of the viral RNA-dependent RNA-polymerase (RdRp) and lack of proof-reading activity of the viral polymerase (Domingo, Menéndez-Arias, & Holland, 1997). Recombination events cause creation of chimeric genomes, by transferring large genome regions between different viruses (Agol, 1997). RNA virus replication cycles produce many virions which possess nucleotide misincorporations in their genomes and these leads to divergent RNA sequences. Despite a large number of divergent sequences generated after replication, RNA viruses will tend to conserve essential functions in order to maintain their fitness and viability. Point mutations will occur by the random introduction of either single or multiple nucleotide changes during RNA replication which can lead to serious viral phenotypes and therefore these incremental nucleotide changes are subject to the

evolutionary forces of positive and negative selection which help to conserve the genomes. Most RNA viruses have been reported to have evolutionary rates in the range of 10^{-5} to 10^{-3} substitutions per site per year (Domingo et al., 1997).

Recombination has extensively been reported to cause genetic diversity in RNA viruses. Recombination has been explained in two models; the most widely accepted model of recombination in RNA viruses is known as the copy-choice model, whereby the RNA-dependent RNA polymerase switches template strand during synthesis of the negative strand (Agol, 1997; Kirkegaard & Baltimore, 2017). This mechanism is necessarily replication dependent and rely both on coinfection of the same host cell by two different viruses and replication of the two viruses within the same cellular compartment. The dissociation and resulting switching may be related to "pausing" of the polymerase secondary to regions of RNA secondary structure or nucleotide misincorporations (Agol, 1997). A second mechanism is proposed whereby recombination occurs by the breaking and rejoining of RNA strands and this is not replication dependent.

In HRV, studies have revealed recombination breakpoints to be found near the 5' UTR/P1, P2/P1 and P2/P3 junction regions (Kim et al., 2013; Palmenberg, Rathe, & Liggett, 2010). A study by (Kim et al., 2013) to identify recombination events in HRV-A and HRV-B found recombination breakpoints located in the region of nucleotides 472-554, which comprised of stem-loop 5 in the internal ribosomal entry site.

HRV- C has been estimated to have a substitution rate of 3.48 $x10^{-3}$ substitution /site /year in the VP1 gene (Kuroda et al., 2015). This rate is attributed to high negative selection in the capsid proteins. Negative selection is important in maintaining long-term stability of the genes by removing deleterious mutations after viral replication (Hicks & Duffy, 2011). Hypervariable regions for mutations in HRV genome include VP3, VP2 and VP1 while conserved regions include VP4, 2A and 2B (Agol, 1997; Cordey et al., 2010; Kuroda et al., 2015). Similar substitution frequency based in the VP1 gene has been observed in other members of genus *enterovirus*. EV-A71 has been shown to have a substitution rate of 4.2×10^{-3} and 3.4×10^{-3} substitutions per nucleotide per year for genotypes B and C respectively (Brown et al., 1999). Substitution rate for polioviruses is estimated at 1.19 x

10^{-2} substitutions/site/year for the VP1/capsid region and is one of the highest for RNA viruses. This high rate may be attributed to high replication rates and positive selection (Hicks & Duffy, 2011).

On comparing HRVs substitution rate with other members within the family *Picornaviridae* shows similar magnitudes in the substitution rate in the VP1 gene. Foot and mouth disease virus (FMDV) which belongs to genus *Aphthovirus* within the family *Picornaviridae* is estimated at 1.45×10^{-3} substitutions per site per year in the VP1 gene (Yoon et al., 2011). A comparison of rates for a range of enteroviruses and other RNA viruses is shown in Table 2.1. RSV is estimated to have a substitution rate of $6.47\text{x}10^{-4}$ substitutions/site/year for subgroup A, while $7.76\ \text{x}10^{-4}$ substitutions per site per year for subgroup B. In RSV B, attachment protein G is the most variable protein with a higher substitution rate ($2.78\ \text{x}\ 10^{-3}$ substitutions/site/yr.) than other RSV-B genes (Tan et al., 2012). For influenza A, the mutation rate is estimated at 2.28×10^{-3} mutations/site/infectious cycle (Xu et al., 2004). The high evolutionary rate of influenza A viruses has been attributed to positive selection by the human immune system (Nobusawa & Sato, 2006). HCoV- OC43 has been shown to have a mean substitution rate of $8.48\ \text{x}\ 10^{-4}$ per site per year in the spike gene whereas HCoV NL63 on the other hand has been estimated to have a substitution rate of around 3.10×10^{-4} in the spike gene (Ren et al., 2015; Vijgen et al., 2005).

Table 2.1: Nucleotide substitution rate in viruses.

Viral species	Gene	(No. of substitutions/site/yr.)	Family (Order)	genome structure	Length of the genome	Reference
HRV C	VP1	3.48×10^{-3}	Picornaviridae	ssRNA (+)	~7.2kb	(Kuroda et al., 2015)
FMDV	VP1	1.45×10^{-3}	Picornaviridae	ssRNA (+)	~8.5kb	(Yoon et al., 2011)
Poliovirus	VP1	1.19×10^{-2}	Picornaviridae	ssRNA (+)	~7.5kb	(Hicks & Duffy, 2011)
EV A71	VP1	4.2×10^{-3}	Picornaviridae	ssRNA (+)	~7.4kb	(Brown et al., 1999)
HRV C	VP2	1.35×10^{-3}	Picornaviridae	ssRNA (+)	~7.2kb	(Kuroda et al., 2015)
RSV-A (genomic)	-	6.47×10^{-4}	Pneumoviridae	ssRNA (-)	~15kb	(Tan et al., 2012)
RSV-B (genomic)	-	7.76×10^{-4}	Pneumoviridae	ssRNA (-)	~15kb	(Tan et al., 2012)
RSV-A	G gene	2.22×10^{-3}	Pneumoviridae	ssRNA (-)	~15kb	(Tan et al., 2012)
RSV-B	G gene	27.8×10^{-3}	Pneumoviridae	ssRNA (-)	~15kb	(Tan et al., 2012)

2.5 Mechanism of HRV infections

Nasal mucosa is the main site for rhinovirus infections (Blaas & Fuchs, 2016). Infection occurs when the virus gets access and recognition by receptors (ICAM-1 and LDLR) on the apical surface of the nasal epithelial cells. Upon binding on the receptors the virus are internalized and delivered to endosomes by receptor-mediated endocytosis (Brandenburg et al., 2007), the mildly acidic pH (6.0 -6.5) in the endosomal compartments leads to dissociation of the virus from its receptor. Low pH allows the conversion of native viruses into subviral A particles which is devoid of the capsid proteins. The viral RNA is then released into the cytoplasm and the remaining empty capsid proteins are degraded by lysosomes. Viral RNA is translated into polyprotein followed by autocatalytic cleavage into structural and non-structural proteins. The RNA is then replicated by viral polymerase. After replication, the infectious progeny is assembled into mature virions and released into the nasal cavity by pinocytosis mechanism (Blaas & Fuchs, 2016).

2.6 Host immune response to HRV

Upon HRV entry into the epithelial cells, signaling pathways are activated triggering expression and release of various cytokines including type I (IFN-α/-ß) and type III interferons (IL-28A, IL-28B, and IL-29) which are released to the site of infection and establish an antiviral state in the infected and surrounding cells. IFNs directly restrict virus replication. Cytokines IL-12 and IL-15 play important roles in cytotoxic and natural killer (NK) cell differentiation, survival, and recruitment. NK cells are also important in HRV elimination (Kennedy et al., 2012; Royston & Tapparel, 2016). Chemokine (Rantes, MCP-1, and MP-10) vasoactive peptides and growth factors are also released. Subsequently inflammatory cells including leukocytes, granulocytes and monocytes become activated resulting inflammatory process in the epithelial cell and the typical symptoms of common cold. In humoral immunity, individuals develop type-specific antibodies (IgG and IgA) against a specific type. Every different HRV - type provokes development of type-specific antibodies, subsequent infections by different type enables one to build an array of specific antibodies to all the types encountered. In a study by

(Barclay et al., 1989) demonstrated IgM antibodies are detectable at 1 to 2 weeks after inoculation and maximize after 7 weeks, antibody titers are maintained and tend to decline after 1 year.

2.7 Transmission of HRV

HRVs are easily transmitted from person to person through small particle aerosol, large particle aerosol and contact either directly or through fomites (Dicket et al, 1987; Hendley et al., 1973). Studies have shown transmission of HRV to occur in families, schools and daycare centers (Nguyen et al., 2016; Peltola et al., 2008; van der Zalm et al., 2011). Transmission rates can be influenced by number factors such as size of the household and presence of school going children in families. School going children have been linked to frequent introduction of RSV into households, leading to infection of other household members (Heikkinen et al., 2015), and similar scenario is expected in HRV. HRV can survive in the indoor environment for hours to days at favorable temperature and on undisturbed skin for 2 hours (Hendley et al., 1973) this allows HRVs to easily survive and transmit in a populated area.

2.8 Management of HRV infections

Efforts to develop therapeutic drugs and vaccines specifically for HRV prevention have been unsuccessful (Glanville & Johnston, 2015). There are no licensed drugs for treatment of HRV infection. Most drugs have not been licensed due to adverse side effects (Jacobs et al., 2013). Pleconaril, a drug tested against HRV that acts by preventing the virus from attaching to the host cell by binding to the VP4 capsid protein had its license withheld by U.S. FDA due to concerns about resistance and safety (Hayden et al., 2003). Vapendavir, a drug that prevents the release of viral RNA into the host cell by binding to VP1 capsid protein is on clinical trial (Jacobs et al., 2013). Vapendavir was found to reduce the incidence of HRV and the viral load in healthy volunteers inoculated with HRV-39 (Jacobs et al., 2013). Nevertheless, management of common cold caused by HRV infection remains supportive with common analgesics and antipyretics, decongestants and antihistamine when used as combined therapy (Allan & Arroll, 2014). Efforts to control

HRV infections have resorted to use of behavioral strategies such as social distancing, hand washing, use of alcohol-based hand disinfectant, use of gloves and respiratory mask.

2.9 Seasonality of HRV

All HRV species have been identified in temperate, tropical, subtropical, and semiarid regions (Garcia et al., 2013; Lu et al., 2014; Pretorius et al., 2014). HRV infections usually occur all year-round, although peaking in the early autumn and late spring in many temperate or subtropical countries, and in the rainy season in tropical countries. In Kilifi Kenya, HRV infections tend to rise soon after rainy seasons. High proportions of infections were detected in February 2007 at the start of dry season, June–July in 2007 end of rainy season, August– September 2008 end of rainy season, February 2009 during a dry season and April–June 2009 end of rainy season (Onyango et al., 2012). This pattern is similar to the one observed in Tanzania where HRV infections peak from June to August beginning of dry season and from October to November during short rainy season (L'Huillier et al., 2015).

2.10 Epidemiology of HRV

Epidemiological studies on HRV in sub-Saharan African countries have reported high incidence rates of HRV. A study by (Onyango et al., 2012) reported a prevalence of 22.0% (380/1759) in inpatients and 24.0% (61/254) in outpatients. In South Africa, HRV has been identified in 25.0% (1949/7641) of patients hospitalized with severe acute respiratory illnesses (Pretorius et al., 2014). In Tanzania HRV prevalence of 24.3% was reported in a study analyzing HRV and HEV circulation and their molecular epidemiology in rural and urban areas of Tanzania (L'Huillier et al., 2015). Studies have indicated enormous diversity in the HRV types in circulation in Kenya (Milanoi et al., 2016; Morobe et al., 2018). A study by (Milanoi et al., 2016) identified 20 different types in circulation in Kenya with more than one type circulating in the same region. The study included samples from 8 different geographical regions across Kenya which comprised of Malindi, Isiolo, Alupe, Port Reitz, Kisii, Kericho, Nyanza and Nairobi. Coastal region had a significant number of types in circulation that were A01, A20, A47, A49, A71, C02, and

C33. Nairobi had six types in circulation (A58, B84, C02, and C13). Five different HRV types (A58, B84, C02, and C13) were identified to be in circulation in the larger Western region of the country. Rift valley region had only one type (A01) in circulation. In terms of species, HRV-A was the found to be the predominant strain circulating in Kenya followed by HRV-C and the least common was HRV-B. However, this study had shortcomings in the sequencing phase whereby, out of 125 HRV positives detected only 26 were typed using phylogenetic analysis indicative of unreliable results on distribution of types nationwide. In another study conducted in Western Kenya, HRV was found to be the most common respiratory virus circulating in Nyanza Province, Kenya. From this study HRV was also found to co-infect with other viral respiratory viruses; 112 viral respiratory infections were detected in 103 of 197 patients (52%) and nine children had two respiratory viruses detected simultaneously, including 5 specimens with both influenza A and rhinovirus; 1 with influenza A and coronavirus; and 1 each with rhinovirus plus coronavirus, human metapneumovirus, or influenza (Waitumbi et al., 2010).

Studies conducted globally in other continents/geographic region show similar patterns experienced locally. Study from Chongqing, China over a 3-year period showed all HRVs species occurring throughout the year and displayed a characteristic biennial outbreak pattern with alternating high and low peaks during the winter and spring seasons, respectively. HRV-A (54.7%) and HRV-C (39.9%) were the predominant in HRV species in circulation (Lu et al., 2014). Study conducted in Latin America covering eight countries throughout South America and Central America over one year period to investigate the circulation of HRV and Human enteroviruses (HEV) in children and young adults also reported a similar pattern observed in other regions (Garcia et al., 2013). Temporally HRVs were present in both subtropical and tropical regions (tropical, tropical rain forest, semi-arid, tropical rainforest subtropical highland, desert highland and subtropical highland and humid subtropical) all year long. HRV-A was the predominant species followed by HRV-C and HRV-B. All the three species were detected in all countries in

the study. In terms of climatic conditions, HRV was more often detected during the end of rainy season and less in the higher temperature seasons (Garcia et al., 2013).

Epidemiological studies around the world have reported HRV types to appear in several successive seasons, with same types detected in as many as 4 consecutive seasons (Daleno et al., 2013; J Richter et al., 2014; Sansone et al., 2013). Occurrence of different types in combination with the ability of each type to remain in the population over extended period or successive epidemics is a major factor in explaining the high rate of HRV infections globally. A study in western Sweden on molecular epidemiology of rhinovirus reported 2 types (A78 and C9) to circulate in 3 successive seasons, 19 types distributed across all species circulated in 2 successive seasons (Sansone et al., 2013).

Phylogenetic and phylogeography coupled with molecular methods such as gene amplification and sequencing, have been used to explain molecular epidemiology of several respiratory viruses. Phylogenetic analysis of attachment protein gene sequences has been used to show multiple introductions of RSV genotype ON1 in Kilifi, Kenya (Otieno et al., 2016). A similar approach used to trace the introduction and spread of ON1 can be used in the case of HRV. Bioinformatics tools used in molecular epidemiology have been used to reveal the trends in respiratory virus genetics; this includes evolution, mutations and genetic relatedness between and amongst virus strains (Cordey et al., 2010; Kim et al., 2013; Palmenberg & Gern, 2015). In epidemiological studies of livestock, genetic sequence data has been used in real time to reveal the source and the probable chain of transmission of viruses in given area. A study in UK to investigate the transmission pathways of foot-and-mouth disease virus (FMDV) in beef cattle farms in the United Kingdom in 2007 utilized genetic sequence data to identify source of the FMDV outbreak in 2007 in the UK (Cottam et al., 2008).Using phylogenetics the source of the outbreak was traced to a research laboratory in Pirbright, this was by comparing the genome sequences obtained from the infected premises with the sequences from the laboratory. FMDV sequence data obtained from the first infected premise revealed a VP1 gene-identity of 99.84% to FMDV O1 British Field Sample (O1 BFS 1860) from the FMD laboratory at Pirbright. The study was also able to trace the movement/spread of the virus

from farm to farm by comparing the complete genomes acquired during the course of the epidemic. The study suggest that animal movements were not involved in the transmission of virus between premises, but a variety of local spread mechanisms such as movements of contaminated persons, objects and aerosols could account for the transmission within each geographic and temporal cluster.

2.11 HRV (VP4/VP2 coding region) dataset in the GenBank

Global search on the GenBank, a public sequence database reveals significant number of HRV nucleotide sequences (complete genome and partial sequences) which include 287 HRV B VP4/VP2 sequences, 1517 HRV A VP4/VP2 sequences and 1328 VP4/VP2 sequences belonging to HRV C. Complete genome sequences include 103 for HRV A, 34 for HRV B and 27 for HRV C (https://www.viprbrc.org). BLAST search shows that HRV sequences present in the GenBank are from different geographical regions globally (Africa, Europe, Asia, Americas and Australia). HRV sequences available in the GenBank formed a good source of contemporaneous data for this study. Reference sequences currently in the database include 76 HRV-A, 30HRV-B and 53HRV-C sequences. Reference sequences are used to assign species and types based on sequence comparisons of the VP1 protein or VP4/VP2. Sequence are assigned to a specific type if the sequence has >90 nucleotide similarity on the VP1 or VP4/VP2 with a reference sequence and if they cluster with a reference sequence in phylogenetic analysis with a bootstrap value >70. Assignment of a new strain to known types require >86–87% aligned nucleic acid identity in VP1 or VP4/VP2 (Palmenberg & Gern, 2015).

2.12 Phylogenetics and phylogeography of viruses

Phylogenetics approaches have been used to elucidate transmission patterns of pathogens by examining the clusters of transmission; phylogenetics has been used in a number of cases to identify sources and spread of pathogens in host populations (Cottam et al., 2008; Gire et al., 2014). Phylogenetics approach has been used to explain Ebola virus source and transmission during the 2014 outbreak in West Africa. Phylogenetic comparison of Ebola virus sequences obtained from the 2014 outbreak with sequences from previous

outbreak suggested that the 2014 West African virus likely spread from central Africa within the preceding decades (Gire et al., 2014). Phylogenetics also revealed that the lineages of the three most recent outbreaks (Sierra Leone, Gabon, and DRC) all diverged from a common ancestor at roughly the same time (2004). Phylogenetics can also be used to determine the subtype/ types in circulation within a host population in a geographical region (Milanoi et al., 2016; Sansone et al., 2013).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study area

This study was conducted on the coast of Kenya, within the Kilifi Health and Demographic Surveillance System (KHDSS) (Scott et al., 2012). The KHDSS area was defined and mapped for demographic surveillance, clinical and epidemiological research by the KEMRI Wellcome Trust Research Programme (KWTRP) in the year 2000. It covers an area of 891km^2 and has a population ~ 287,014 (mid-point 2016) which is heterogeneously distributed with highest population density in Kilifi township (Figure 3.1). The major economic activities in the area include fishing, tourism and subsistence farming.

The KHDSS area has 21 public health facilities, which operate under the Kenya Ministry of Health (MoH). In total, 10 of these facilities were selected for this study: Matsangoni Health Centre (39.92869° E 3.38957°), Ngerenya Dispensary (39.86235° E 3.51358° S), Mtondia Dispensary (39.86871° E 3.5788° S), Sokoke Dispensary (39.78234° E 3.52548° S), Mavueni Dispensary (39.79908° E 3.67804° S), Jaribuni Dispensary (39.74252° E 3.63265° S), Chasimba Health Centre (39.70033° E 3.73512° S), Pingilikani Dispensary(39.77805° E 3.77473° S), Junju Dispensary (39.76209° E 3.82866° S) and Kilifi County Hospital (39.85707° E 3.6317° S). The health facilities were purposively selected to provide a broad representation across the geographical regions considering major road networks and population density in KHDSS.

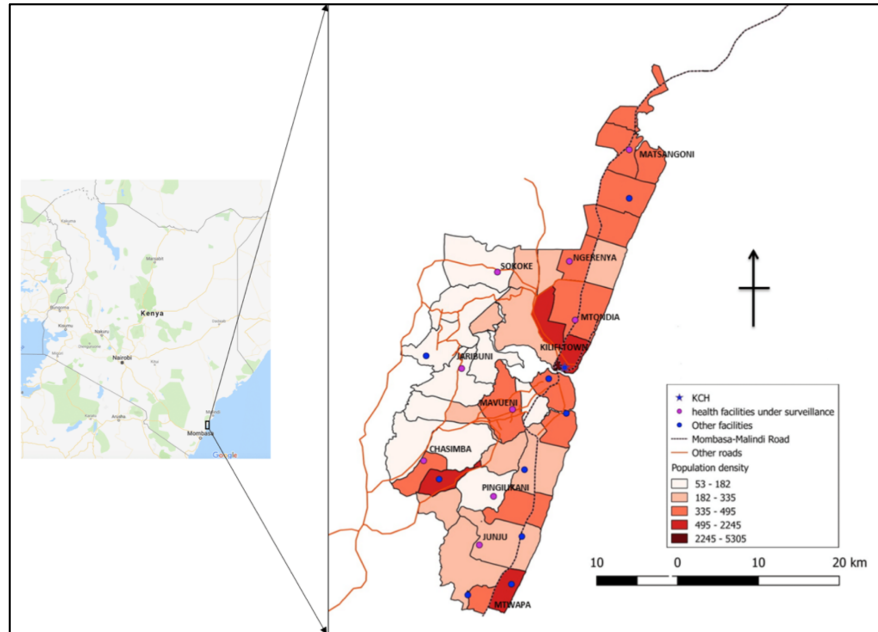


Figure 3.1: Map of the Kilifi Health and Demographic Surveillance

3.2 Study design

This study was an observational laboratory-based study on archived nasopharyngeal (NPS) samples.

3.3 Ethical approval

The study protocol was approved by KEMRI – Scientific and Ethical Review Unit (SERU# 3103) and the University of Warwick Biomedical and Scientific Research Ethics Committee (BSREC# REGO-2015-6102) (Appendix I). Individual written informed consent was obtained from all study participants aged ≥18 years (Appendix II). For those <18 years old, written consent was obtained from the parent or guardian (Appendix III)

3.4 Study Population

The study population comprised of any person presenting to the selected outpatient health facilities with symptoms of acute respiratory tract infection.

i. Criteria for inclusion of participants.

- Any person presented to the outpatient with one or more ARI symptoms i.e. cough, sneezing, nasal congestion, difficulty in breathing, increased respiratory rate for age as defined by World Health Organisation (World Health Organisation, 2013).
- Written consent for involvement in the study that includes nasal and oral specimen collection.

ii. Criteria for exclusion of participants

- Refusal to consent
- New-born brought to the health centre.
- ARI symptoms for more than 28 days.

3.5 Sample size

Fifteen (15) samples per site per week were collected between December 2015 to May 2016. The sample size estimation was based on the outpatient data on the number of respiratory infection cases seen per month per the selected health facility and also from previous reports with inpatient surveillance for respiratory viruses at KCH (unpublished). In total 2150 NPS samples were collected and tested between December 2015 -May 2016. Among the samples collected, 423/2150 (19.6%) tested positive for HRV by real-time PCR (Table 3.1).

Table 3.1: Numbers of sample collected and tested positive for HRV per month and per health facility.

Health Facility	Samples collected	Total number of samples identified as HRV positives per month						
		Dec	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Total**
Jaribuni Dispensary	202	0	4	7	3	11	10	**35**
Ngerenya Dispensary	215	1	4	7	10	6	3	**31**
Junju Dispensary	203	1	4	10	6	14	13	**48**
Pingilikani Dispensary	195	0	6	17	2	3	10	**38**
Sokoke Dispensary	199	0	5	10	8	7	6	**36**
Mtondia Dispensary	255	2	8	11	8	9	5	**43**
Mavueni Dispensary	220	2	1	5	9	8	15	**40**
Chasimba Health centre	199	1	2	6	11	11	12	**43**
Matsangoni Health Centre	250	4	7	11	14	15	9	**60**
Kilifi county Hospital	212	4	7	13	8	6	11	**49**
Total	**2150**	**15**	**48**	**97**	**79**	**90**	**94**	**423**

3.6 Patient recruitment and specimen collection

Participant recruitment and specimen collection was incorporated within the routine patient care at the ten selected outpatient facilities led by a resident clinician or nurse. Each facility had one or two sampling days per week, usually scheduled from Monday to Friday. On each sampling day, a study fieldworker stationed at the health facility, assisted by the resident clinician or nurse, would describe the study to the attending patients. Any person who presented with listed signs and symptoms of ARI would be asked to see the fieldworker for further screening and obtainment of consent as they await review by the nurse or clinician. The selection of participants each week was on a 'first-come first-served' basis as they presented to the health facility on the set sampling days. A standardized questionnaire (Appendix IV) was used to collect biodata including health facility, age, sex, presenting symptoms as well as the treatment provide. A nasopharyngeal swab (NPS) was collected from each participant by inserting a sterile nylon-flocked plastic-shafted swab (503CS01, Copan Diagnostics, Flocked Swab Technologies, Italy) into one nostril to a distance where the tip located the deep nasopharynx and was twisted 3 times before it was gently removed (in total taking about 10 seconds). The swab collected from each participant was inserted into a single 1ml vial of viral transport media containing 0.04 phosphate buffer (disodium hydrogen phosphate, potassium dihydrogen phosphate, pH 7.5), glycerol 50% (vol/vol), gentamicin sulphate solution (50mg/ml) and amphotericin B (250ug/ml). The sample was then kept at approximately 8°C in ice-packed cool box for up to 6 hours and thereafter transported to Kenya Medical Research Institute laboratories within the same day.

3.7 Primer design

Primers and probes for multiplex diagnostic real-time RT-PCR method were based on previously published work (Gunson et al., 2005). Table 3.2. show primers used in the diagnostic multiplex real-time PCR. Similarly, primers used in the amplification of VP4/VP2 region were also based on previously published work (Wisdom et al., 2009)

Table 3.2: Primers used for the amplification of 5’ UTR and VP4/VP2 coding region on the HRV genome

Target Region	F/R	Tm (°C)	GC content	Position	Sequence
5’ UTR	Forward	61.1	57.9%	201	TGGACAGGGTGTGAAGAGC
5’ UTR	Reverse	60.0	50.0%	490	CAAAGTAGTCGGTTCCCATC
Probe	-	65.6	66.7%	-	TCCTCCGGCCCCTGAATG
VP4/VP2	Forward	64.7	64.3%	458	CCGGCCCCTGAATGYGGCTAA
VP4/VP2	Reverse	63.0	53.6%	1087	TCWGGHARYTTCCAMCACC

3.8 Laboratory based Methods.

3.8.1 RNA extraction

Viral RNA was extracted from each NPS sample using QIAamp Viral RNA kit (Qiagen Inc., Valencia, California, USA) according to the manufacturer’s instructions. The samples were removed from the -80 °C freezer and left to thaw at room temperature. The samples were then pulse vortexed for 15 seconds to ensure homogenization. One hundred and forty microliters of the sample was transferred to 1.5ml tube and 560 µl of the lysis buffer (Buffer AVL-carrier RNA solution) was added and pulse vortexed for 15 second to ensure efficient lysis. The mixture was then incubated at room temperature (15-25°C) for 10 minutes. Ethanol (100%) was added to each sample and mix thoroughly by pulse vortexing for 15 seconds. Ethanol is responsible of precipitating the nucleic acid out of the aqueous solution. The samples were then centrifuged briefly to remove drops from inside the Epperndorf tube lid. 630 µl of the solution was then applied to the spin column and spun at 8000 rpm for 1 minute twice to bind RNA on the spin column. The RNA was washed twice, first with 500µl of AW1 and spun at 8000rpm for 1 minute, then with 500µl of AW2 at 13000 rpm 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

Epperndorf tube. Presence and concentration of RNA in the eluted volume was confirmed was confirmed using Nanodrop. Appendix V show RNA concentration of 423 HRV samples. RNA was then stored at -80°C ready for diagnostics real-time RT-PCR.

3.8.2 Diagnostic real-time polymerase chain reaction (qRT-PCR)

Virus detection was done using real-time PCR as described by (Gunson et al., 2005) using primers targeting 5' UTR. QuantiFast Multiplex RT-PCR Kit (Qiagen, Valencia) was used in the multiplex real-time PCR assay. Reaction mix was prepared as follows: 5µl of 2x Quantifast buffer, 0.2µl of 50x ROX, 0.5µl of 5pm/µl probe, 0.5µl of 10pm/µl Forward and 10pm/µl reverse primer, 2.2 µl of nuclease free water and 1µl RNA template. Reverse transcription was carried out for 20 min at 50°C. Taq polymerase was deactivated at 95°C for 5 min, followed by 40 cycles of PCR performed at 95°C for 15 s and 60°C for 30 s. The assays were run using the Qiagen Quantifies multiplex RT-PCR kit (Qiagen, United Kingdom) in triplex sets and analyzed on an ABI 7500 system (Applied Biosystems, United Kingdom). Samples with a cycle threshold (Ct) values of less than 35.0 were considered positive and taken through to sequencing.

3.8.3 VP4/VP2 PCR amplification

PCR (RT –PCR) procedure consisted of a single-step combining reverse transcription and PCR amplification performed using the One-step RT-PCR kit (Qiagen Valencia CA). The reaction mixture was prepared as follows; 5µl of 5x RT-PCR buffer, 1µl of 0.4mM dNTPs, 2.5µl of 10pm/µl of forward primer, 2.5µl of 10pm/µl of reverse primer, 0.5µl RNaseOUT™ 5000U, 7.8µl nuclease free water and 1µl of enzyme mix. A 2µl aliquot of viral RNA was added to give a final volume of 20µl. Thermocycling conditions were set as follows: Reverse transcription of the RNA to cDNA at 50°C for 30 minutes, in-activation of reverse transcriptase and activation of Taq polymerase at denaturation at 95°C for 15 minutes. This was followed by 40 cycles at denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and strand extension at 72°C for 1 min and final incubation/extension at 72°C for 10 minutes.

3.8.4 Gel electrophoresis

Agarose gel was prepared by mixing 1.5 mg of Agarose (AGTC Bioproducts) with 100ml of 0.5% Tris base/Boric acid/ EDTA (TBE) buffer. The mixture was heated in a microwave until boiling and allowed to cool. Red Safe dye (iNtRon Biotechnology) was added to enable visualization of DNA. Gel combs were fixed into position. Gel was then poured into an electrophoresis tank cast and allowed to solidify. 5 µl of each PCR product was mixed with 2µl loading dye (Blue/Orange Loading Dye 6X, Promega) and loaded onto a gel and run at 95 volts for 45 minutes. The gel was visualized under UV light (Bio-Rad machine) and gel images were printed for analysis. Only samples that fluoresced under UV illumination at the expected size proceeded to purification stage

3.8.5 PCR products purification

PCR products were purified using Qiagen PCR purification kit. Purification is essential to get rid of left-over primers, unincorporated nucleotides and non-specific products. PCR products were transferred to an autoclaved 1.5ml Epperndorf tube. Buffer PB (5times the volume of the PCR product) was added to the 1.5ml Epperndorf tube. To bind the DNA, the solution was applied into a QIAquick spin column and centrifuged for 1 minute at 13000 rpm, the flow through was discarded and the QIAquick column was placed in a new collection tube. The PCR product was then washed with 750 µl of Buffer PE by spinning for 1 minute at 13000 rpm. The flow through was discarded and the QIAquick column was placed in a new collection tube and centrifuged for additional 1 minute at 13000rpm to get rid of residual ethanol in buffer PE. The column was then placed in clean sterile 1.5ml tube. To elute the DNA, 30µl of sterile nuclease free water was added to the center of the QIAquick column and let to stand for 10 minutes, and then centrifugation of 13000 rpm was performed for a minute. Eluted PCR products were stored at -20°C until ready for sequencing reactions.

3.8.6 Cycle sequencing and precipitation

Purified PCR products were sequenced using Big Dye Terminator 3.1 chemistry (Applied Biosystems, Foster City, California, USA) using the PCR primers in both forward and reverse direction in an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Sequencing reaction mixture was prepared as follows: 0.5µl Big Dye Terminator, 1.75µl Big Dye 5X sequencing buffer, 1µl 5pmol/ul of forward primer and 1µl 5pmol/ul of reverse primer, 4.75µl distilled water, 2µl of approximately DNA. The plate was then loaded to a thermal cycler machine. The thermocycler conditions were set as follows: 96 °C for 30 seconds, 50 °C for 15 seconds and 60 °C for 4 minutes. This was repeated for 25 cycles.

Precipitation was done to remove any unused material after cycle sequencing. 1µl of EDTA and 1µl 3M Sodium acetate was added to each well containing DNA making sure these solutions reach the bottom of the well. 25µl ethanol (100%) was added to each well. The plate was then sealed followed by pulse vortexing for 1 minute before incubating at room temperature for 15 minutes to precipitate products. The plate was then spun for a minimum of 20 minutes at 4000 rpm at 20° C. After spinning, the supernatant was drained off by gently inverting the plate on a fresh paper towel and spun again at 400 rpm for 1 minute. 100µl of ethanol (70%) was then added to each well and spun for 5 minutes at 4000 rpm for 20° C. Supernatant was drained off by gently inverting the plate on a fresh paper towel and spun again at 400rpm for 1 minute to get rid of excess ethanol. The plate was then air dried for 60 minutes to ensure ethanol completely dried off so as not to interfere with capillary electrophoresis. 10µl of Hi-Di was added to each well before proceeding to denaturation at 95C for 3mins. After denaturation the plates were chilled on ice and stored at 4°C before transportation to sequencing facility. The sequences were read in ABI 3730 XL machine.

3.9 Sequence assembly

Raw sequence reads were assembled into contigs using Sequencher software v5 (version 5, Gene Codes Corporation, Ann Arbor, USA). Forward and reverse reads were assembled to form consensus; consensus sequence was formed when the reads overlapped.

3.10 HRV comparison dataset in the GenBank.

VP4/VP2 gene sequences deposited in the GenBank as of 31 May 2016 and whose sequenced regions overlapped the Kilifi sequences and derived from viruses collected between 1 January 2010 and May 2016 were collated and phylogenetically compared with the Kilifi VP4/VP2 sequences using MEGA 7 v.6.0. The phylogenetic analysis was aimed to determine the relatedness of the Kilifi viruses to those circulating around Kenya and the rest of the world to understand their global context and to determine whether the patterns observed in this rural population are more widely extended. This included sequences from Africa, Europe Asia and USA (list of GenBank accession number of global sequences have been listed in the Appendix VI. Sequences sampled in Kilifi, Kenya from 2010 to 2015 were also included.

3.11 Sequence analysis and phylogenetic tree reconstruction

Multiple sequence alignments (MSA) were prepared using MAFFT v7.220 (Katoh & Standley, 2013). The analyzed fragment was ~395nt in the VP4/VP2 region. Phylogenetic trees were constructed in MEGA v.6.0 (Tamura et al., 2013) with maximum likelihood methods under the general time reversible (GTR) model with gamma distribution and allowing for invariant sites. Branch support was assessed using 1000 bootstrap iterations. Types were assigned based on >90% nucleotide similarity to rhinovirus prototype sequences (also referred to as reference sequences) available in GenBank or phylogenetic clustering with reference sequences (with a bootstrap support value above 70%).

CHAPTER FOUR
RESULTS

4.1 Socio demographic characteristics of HRV cases in the KHDSS.

Among the 2150 NPS samples collected during the 6 months, 423 (19.7%) tested positive for HRV by real-time PCR. In all HRV positive cases, the age ranged from 1 month - 74 years. The mean age was 5 years and the median age was 1.0 year. 221/423 (52.4%) of the participant were females while 202/423 (47.8%) were males.

Children up to 5 years of age accounted for 312/423 (73.8%) of all HRV cases, followed by 5-9 years age group who accounted for 39/423 (9.2%), individuals above 15 years old accounted for 51/423 (12.1%) of all HRV cases. HRV-A species was identified in 151 (49.3%) samples, HRV-B was identified in 15 (4.9%) samples while HRV-C was identified 141 (45.7%) samples. There was no significant difference in the distribution of HRV cases in relation to age (P-value = 0.728) or sex (P value = 0.961) (Table 4.1)

Table 4.1: Baseline characteristics of HRV cases in the KHDSS.

Demographic Characteristics		HRV Positives n=423	HRV typed n=306	HRV-A n=151	HRV-B n=15	HRV-C n=141	P-value
Age (Years)	Median	1	1	1	1	1	
Sex	Female	221	156	76	7	73	0.961
	Male	202	150	75	8	68	
Age Bands	<1	149	108	53	5	49	0.728
	1-4yrs	163	120	56	5	58	
	5-9yrs	39	31	13	1	17	
	10-15yrs	21	13	7	0	5	
	>15yrs	51	35	22	3	12	

4.2 Quantitative RT-PCR.

423/2150 samples tested positive for HRV and displayed low cycle threshold (ct)value ranging from 22 – 35 (Figure 4.1). A sample was considered HRV positive if the ct value was below 35.

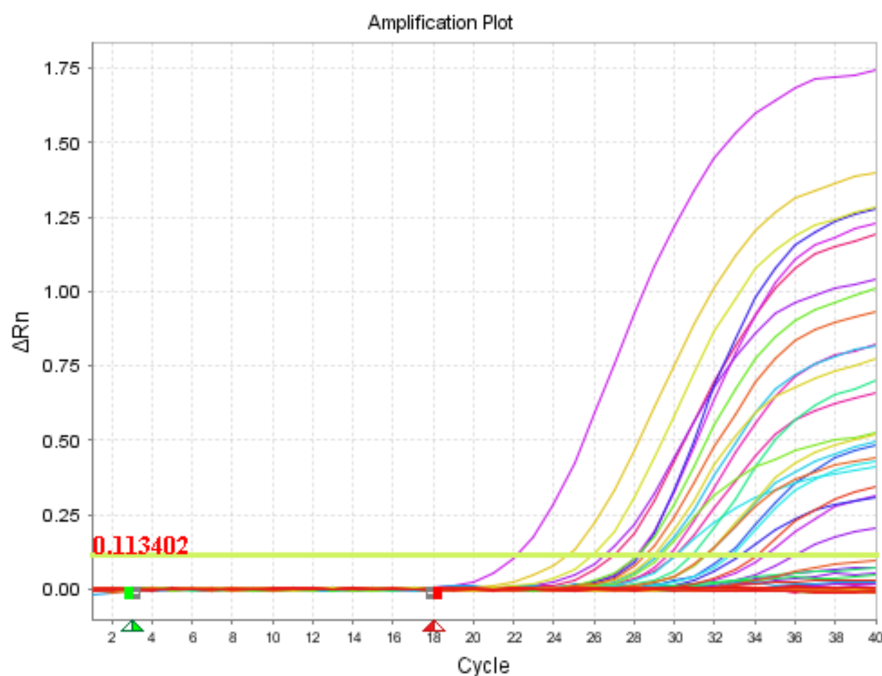


Figure 4.1: Real-time PCR amplification curves of NPS samples.

4.3 Conventional PCR

HRV VP4/VP2 coding region with an expected size of 678bp was amplified using PCR and the product visualized by a UV trans-illuminator (Bio-Rad machine) (Figure 4.2). A total of 306/423 samples showed bands which was evidence for amplification of VP4/VP2 in these samples. No amplification was observed with the negative control (Figure 4.2). Amplicons obtained were purified and sequenced. Forward and reverse reads then were assembled to form consensus using Sequencher software v5.

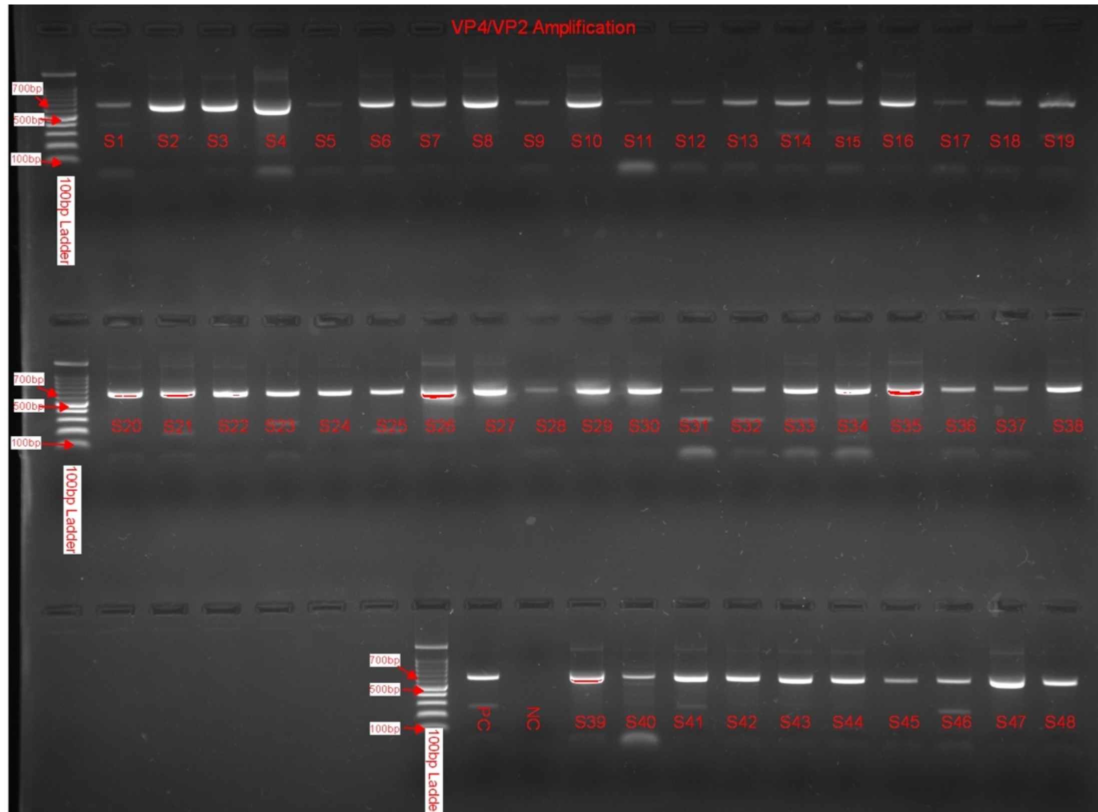


Figure 4.2: Gel electrophoresis photo showing amplification of VP4/VP2 coding region in the HRV positives samples (48 samples). PC represents positive control while NC represent negative control.

4.4 Phylogenetic analysis of HRV types circulating in KHDSS.

The first objective of this study was to investigate the genetic relatedness of HRV strain circulating in different regions of the KHDSS. First, phylogenetic analysis was used to classify the viruses into their respective HRV species and types based on the VP4/VP2 sequences generated. Subsequently, phylogenetic analysis and genetic identity analysis were used to investigate the relatedness of the different HRV strains identified in the KHDSS.

4.4.1 HRV species and types classification

Classification of the viruses into their respective HRV species and types was based on criteria outlined in Section 3.11.As ascribed above VP4/VP2 (~420 bp fragment size) was successfully amplified in 306/423 sample and sequenced for 306/423 (72.3%) samples;117 (27.7%) samples either totally failed amplification or had short consensus sequences recovered (<300 nucleotides) and were not included in subsequent phylogenetic analysis. Failure to sequence the VP4/VP2 region of 117 samples was largely influenced by the low viral load (inferred from cycle threshold (Ct) values determined during the diagnostic real-time RT-PCR). Mean Ct value of the samples that failed sequencing was higher (Ct value of 32) compared to mean Ct value of 30 for samples that were successfully sequenced.

Phylogenetic analysis of 306 VP4/VP2 sequences showed the three HRV species comprising of 151 (49%) HRV-A, 141 (46%) HRV-C and 15 (4.9%) HRV-B (Figure 4.3) Five sequences were not assigned to any HRV species and these were identified as Enterovirus D68 (n=3) and Coxsackievirus B5 (n=2).

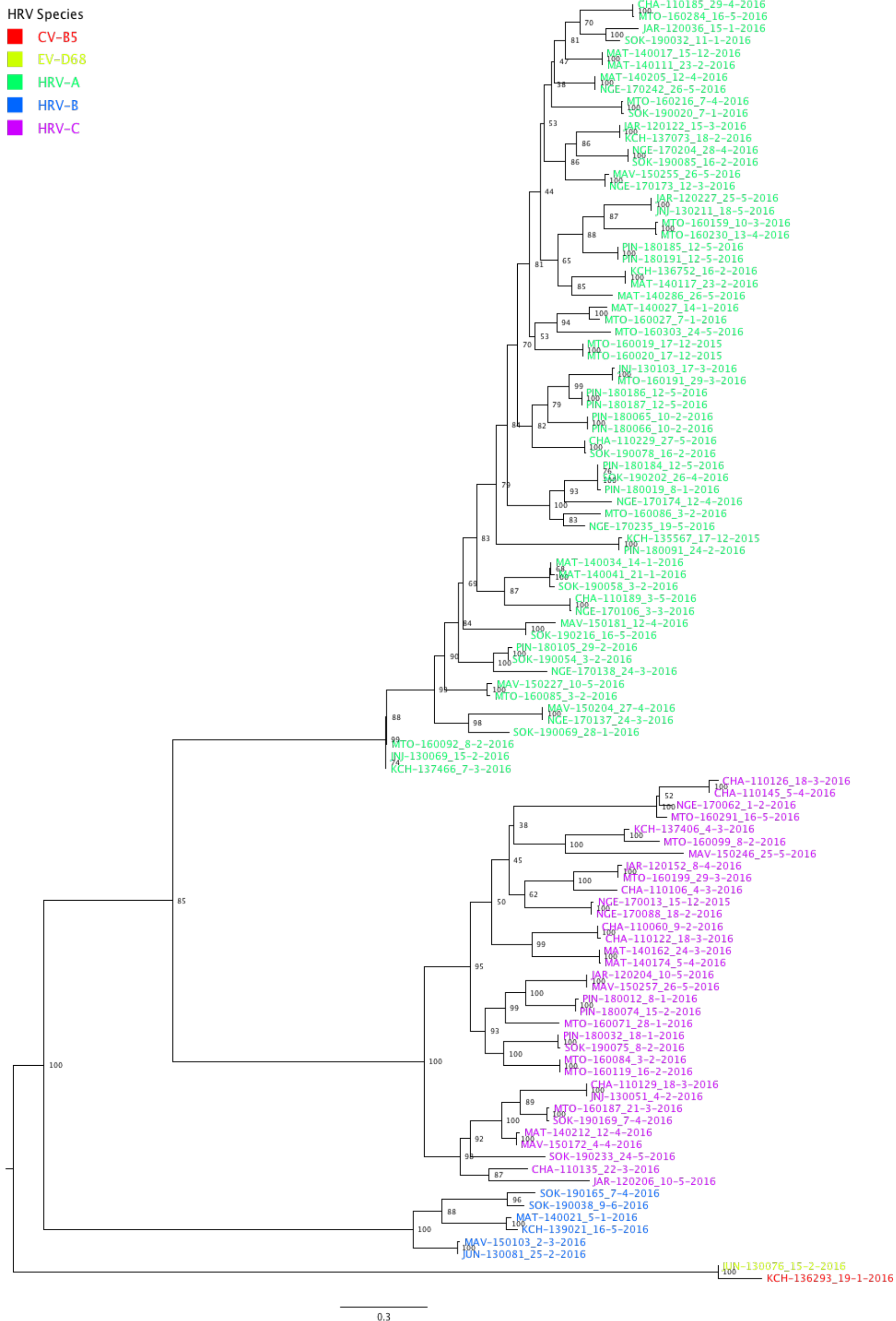


Figure 4.3: Maximum likelihood phylogenetic analysis of HRV VP4/VP2 sequences from the KHDSS. Phylogenetic tree included <2 sequences for every HRV type.

In total, 56 different HRV types were identified: 33 within species HRV-A, 4 within HRV-B and 19 within HRV- C. Among the 33 different HRV-A types identified to circulate during the six months period (Figure 4.4), the most common types were A8 (n=12), A101 (n=12), A41 (n=11), A12 (n=10) Other HRV-A types occurred at lower frequencies, ranging from 1 to 8 cases.

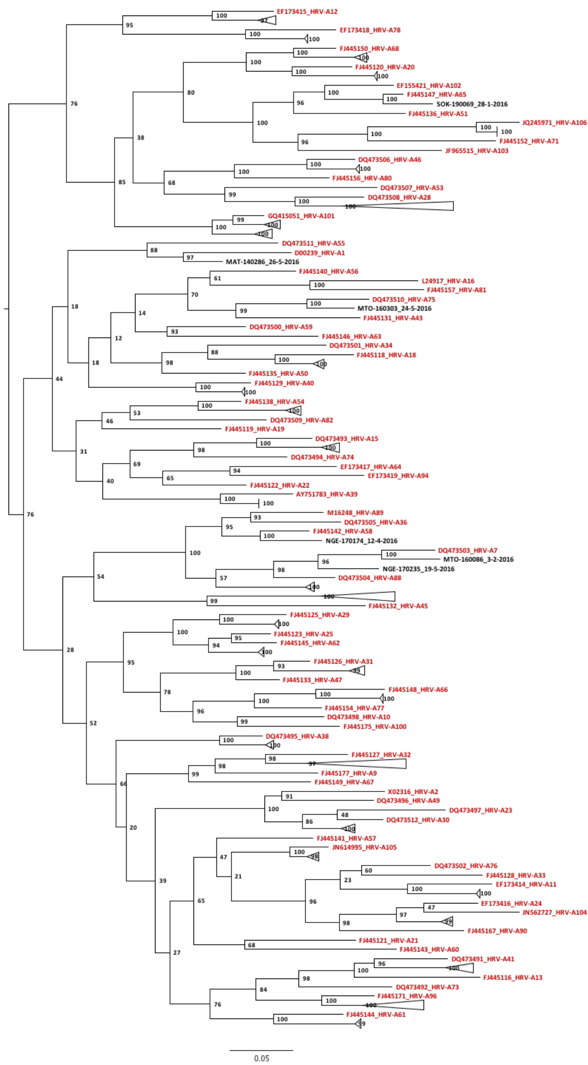


Figure 4.4: Maximum likelihood phylogenetic analysis of 151 HRV-A VP4/VP2 sequences from the KHDSS. Black tip labels represent KHDSS sequences while red tip labels represent reference sequences. Collapsed clades represent Kilifi VP4/VP2 HRV strains with more than two sequences.

A total of nineteen (19) different HRV-C types were identified to circulate during the six months period (Figure 4.5), the commonly detected types were C11 (n=35) C36 (n=18), C14 (n=15), C1 (n=10) and C6 (n=10).

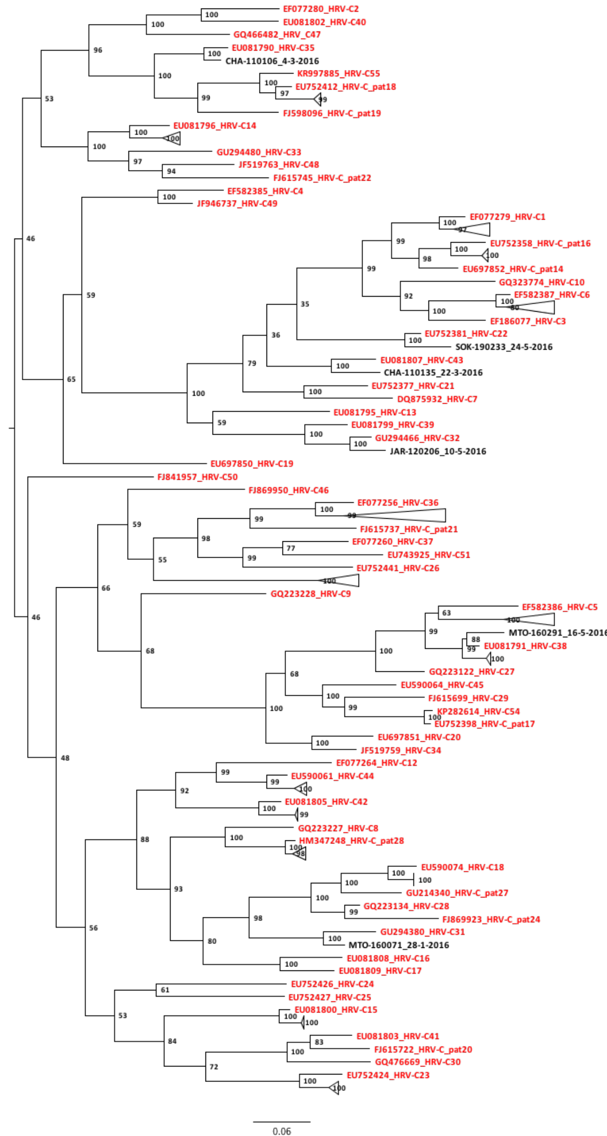


Figure 4.5: Maximum likelihood phylogenetic analysis of 141 HRV-C VP4/VP2 sequences from the KHDSS. Black tip labels represent KHDSS sequences while red tip labels represent reference sequences. Collapsed clades represent Kilifi VP4/VP2 HRV strains with more than two sequences

HRV-B strains were the least detected with only 4 different types detected (B27, B35, B91, B70) (Figure 4.6)

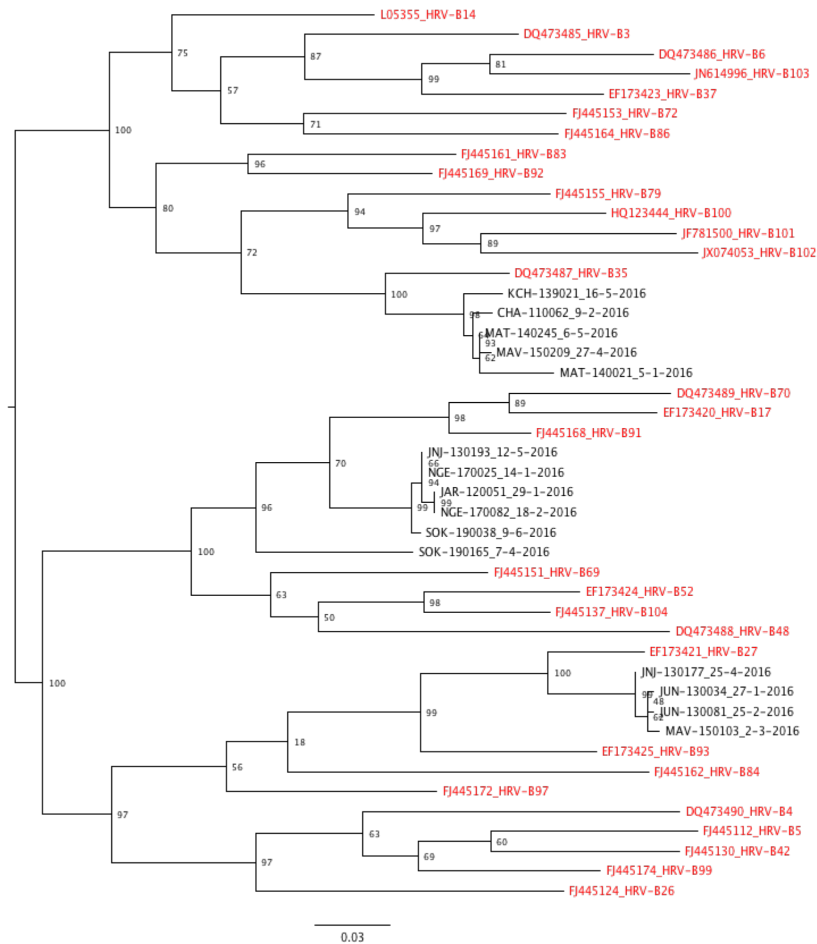


Figure 4.6: Maximum likelihood phylogenetic analysis of 15 HRV-B VP4/VP2 sequences from the KHDSS. Black tip labels represent KHDSS sequences while red tip labels represent reference sequences. Collapsed clades represent Kilifi VP4/VP2 HRV strains with more than two sequences.

4.4.2 Phylogenetic analysis of HRV types circulating in KHDSS.

Based on maximum likelihood phylogenies of VP4/VP2 genes, sequences of most HRV types largely clustered together irrespective of health centre of sampling (geographical region) indicating close genetic relatedness between these viruses. This clustering suggests that there was some degree of transmission within the KHDSS and perhaps the infections reported were caused by a genetically similar strain of an HRV type. For example, VP4/VP2 sequences of HRV- A8 detected in 5 different health facilities (Matsangoni, Pingilikani, Jaribuni, Kilifi County Hospital and Chasimba) and in recorded to be in circulation for 5 months (December 2015, February – April 2016) clustered together on the phylogenetic tree (bootstrap value of 92%), such clustering could suggest circulation of a genetically identical HRV- A8 lineage in the KHDSS (Figure 4.7)

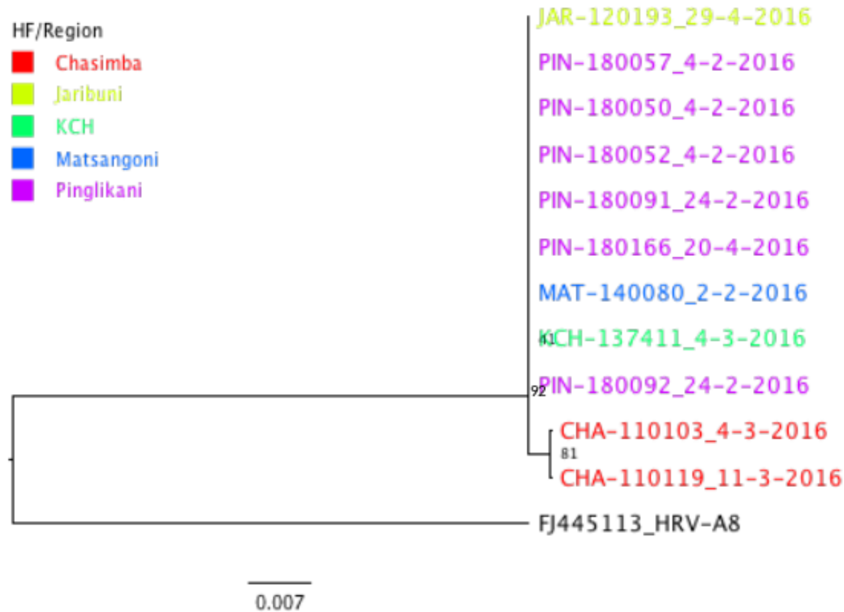


Figure 4.7: Maximum likelihood phylogenetic analysis of HRV-A8 VP4/VP2 sequences from the KHDSS.

Similarly, HRV-A12 which was detected in 5 health facilities (Matsangoni, Sokoke, Mtondia, Pingilikani and Kilifi County Hospital) and found to circulate for 5 months (January 2016 - May 2016) also formed single cluster with 90% bootstrap support on the phylogenetic tree suggesting circulation of genetically similar HRV-A12 lineage in the community (Figure 4.8).

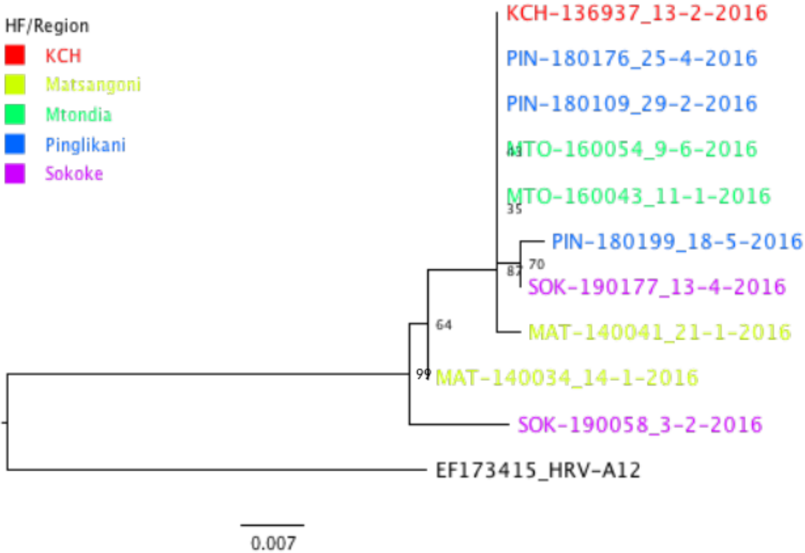


Figure 4.8: Maximum likelihood phylogenetic analysis of HRV-A12VP4/VP2 sequences from the KHDSS.

HRV-A41 detected in 5 different health facilities (Junju, Sokoke, Chasimba, Mtondia and Pingilikani) and found to circulate in 2 out of the 6 months of study (January 2016 - May 2016) formed a single cluster with 95% bootstrap support on the phylogenetic tree suggesting circulation of genetic similar HRV-A41 lineage between the 5 region listed above (Figure 4.9).

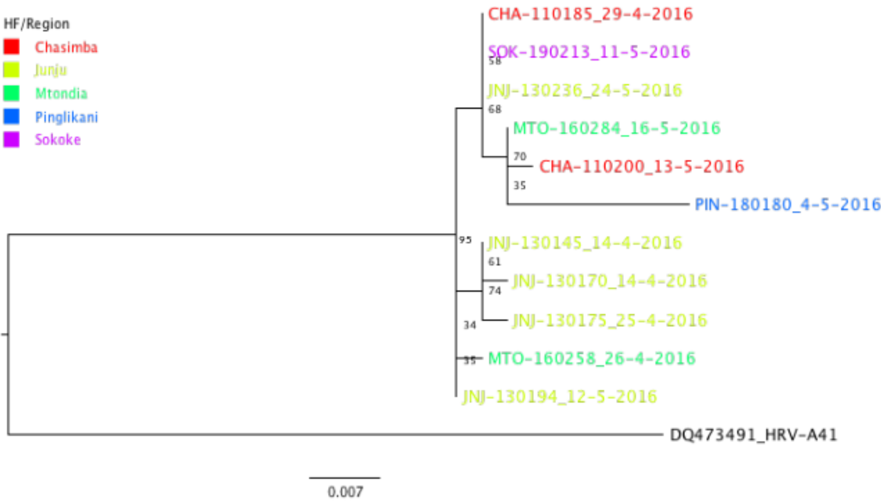


Figure 4.9: Maximum likelihood phylogenetic analysis of HRV-A41 VP4/VP2 sequences from the KHDSS.

Similar observations were recorded in HRV-A106 strain which circulated in the study population for 6 months. HRV-A7, A30, A40, A66, A68, and A105 circulated for 4 months, HRV-A15, A18, A20, A28, A29, A31, A38, A54 and A61 for 3 months, while HRV-A11, A24, A25, A41, A46, A58, A78, A106 for 2 months. In some cases, sequences formed multiple clusters suggesting circulation of divergent lineages belonging to the same HRV strain. A101 formed 2 clusters on maximum likelihood phylogenetic trees an indication of two divergent HRV-A101 lineages co-circulating in the KHDSS. Each cluster had sequences from different health centers. Variant 1 (Mavueni, Matsangoni, Chasimba and Junju) and variant 2 (Junju, Matsangoni, Mtondia and Mavueni) (Figure

4.10). Similar observations were made for A31, A7, A18, A38, A54, A11, A24, A32, A68, A20, A46 and A28 formed 2 clusters while A15 and A105 formed three clusters. Some types were only detected in a single month, these includes A1, A32, A39, A58, A65, A96.

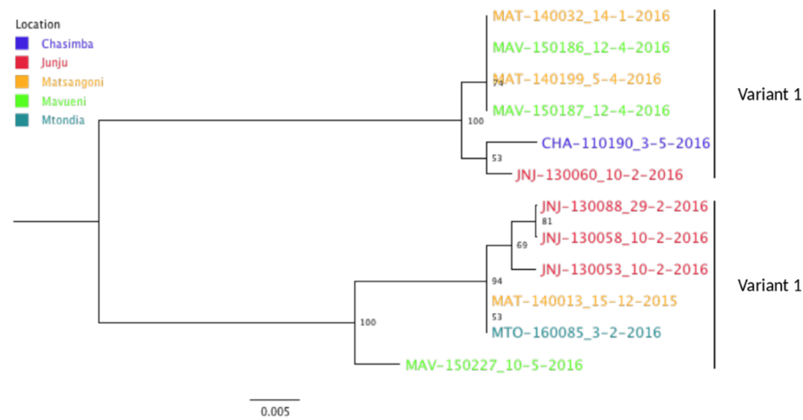


Figure 4.10: Maximum likelihood phylogenetic analysis of HRV-A101 VP4/VP2 sequences from the KHDSS

Total of nineteen (19) different HRV-C types were identified to circulate during the six months period, the commonly detected types were C11 (n=35) C36 (n=18), C14 (n=15), C1 (n=10) and C6 (n=10). VP4/VP2 sequences of HRV- C11 detected in all the 10 health facilities and found to be in to be in circulation for 3 consecutive months (from March 2016 to May 2016) formed a two cluster on the phylogenetic tree an indication of two divergent HRV- C11 variants co-circulating in the KHDSS (Figure 4.11). Variant 1 had sequences from all the health facilities while cluster representing variant 2 sequences sampled from 2 health facilities (Mavueni and Matsangoni).

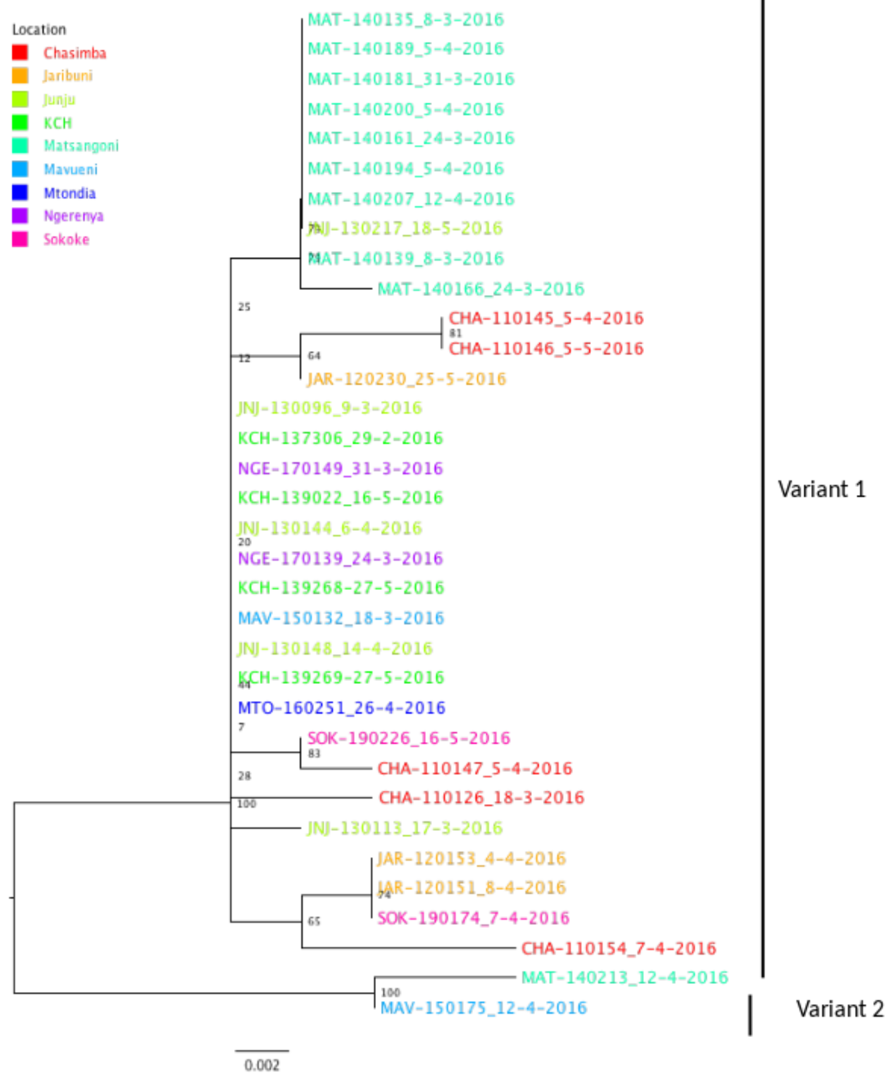


Figure 4.11: Maximum likelihood phylogenetic analysis of HRV-C11 VP4/VP2 sequences from the KHDSS.

HRV-C6 was detected to be in circulation for 5 months (December 2015 -May 2016) and detected in all 5 health facilities (Mtondia, Pingilikani, Junju, Kilifi County Hospital and Chasimba) also clustered into separate cluster suggesting co-circulation of different lineage of C6 in the KHDSS. Variant 1 had sequences from (Mtondia, Pingilikani and Kilifi County Hospital) while variant 2 had sequences from (Pingilikani, Junju and Chasimba) (Mtondia, Pingilikani and Kilifi County Hospital) (Figure 4.12), others include C14, C36, and Cpat28s which were found to circulate for 4 months in various health

facilities. HRV- C38 and Cpat16 circulated for consecutive 3 months while C15, C23 and C44 circulated in 2 months. Some types were only detected in a single month, these includes C22, C31, C32, C35 and C43.

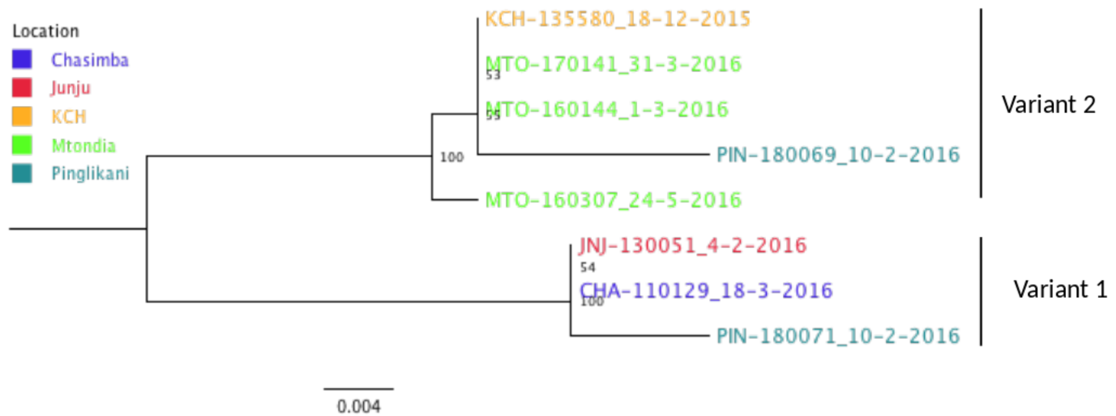


Figure 4.12: Maximum likelihood phylogenetic analysis of HRV-C6 VP4/VP2 sequences from the KHDSS.

Similar observation was observed in C1, which circulated in the study area for 4 months and occurred in 8 health facilities (Matsangoni, Mavueni Pingilikani, Junju, Sokoke, Mtondia, Kilifi County Hospital and Chasimba) (Figure 4.13). others include C14, C36, and Cpat28s which were found to circulate for 4 months in various health facilities. HRV-C38 and Cpat16 circulated for consecutive 3 months while C15, C23 and C44 circulated in 2 months. Some types were only detected in a single month, these includes C22, C31, C32, C35 and C43.

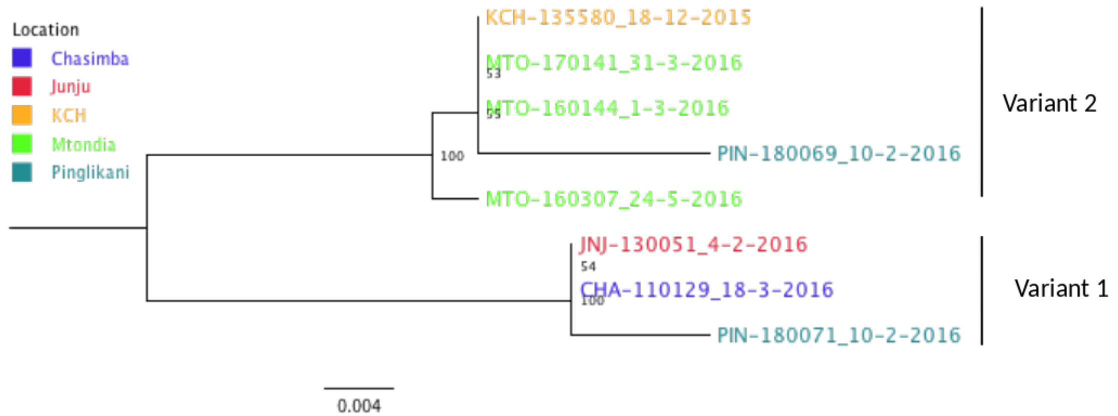


Figure 4.13: Maximum likelihood phylogenetic analysis of HRV-C1 VP4/VP2 sequences from the KHDSS.

In addition to phylogenetic analysis aimed to understand the genetic relatedness of HRV strains circulating in the KHDSS, detailed sequence comparison of VP4/VP2 fragments revealed minimum nucleotide variations within most of the HRV types in circulation. Within HRV-A8 strain, VP4/VP2 sequences from 5 health facilities displayed a single nucleotide difference when comparing the HRV-A8 sequences. (Figure 4.14). VP4/VP2 sequences from Matsangoni (MAT-140080_2-2-2016), Pingilikani (PIN-180052_4-2-2016, PIN-180057_4-2-2016, PIN-180050_4-2-2016, PIN-180091_4-2-2016 and PIN-180092_4-2-2016), Jaribuni (JAR-120193_29-4-2016) and Kilifi County Hospital (KCH-137411_4-3-2016) did not have any nucleotide difference between them. Two sequences from Chasimba (CHA-110103_4-3-2016 and CHA-110119_11-3-2016) that clustered separately in the phylogenetic tree had 1 nucleotide difference in the VP4/VP2 sequence when compared with sequences from the other health facilities.

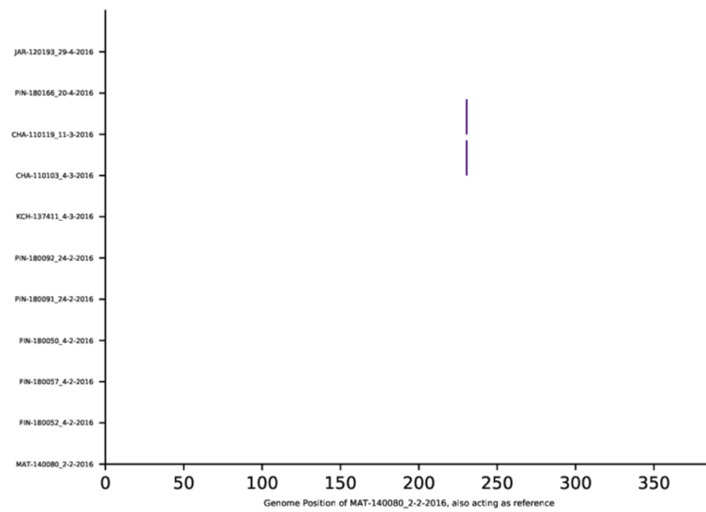


Figure 4.14: Nucleotide differences between HRV-A8 viruses detected from 5 health facilities. Vertical colored bars show the nucleotide differences

HRV-A12 VP4/VP2 sequences sampled from 5 health facilities displayed 11 nucleotide difference at various positions on the VP4/VP2 sequences. To note, 5 sequences from sampled from Mtondia (MTO-160043_11-1-2016 and MTO-160054_9-6-2016), Kilifi County Hospital (KCH-136937) and Pingilikani (PIN-180109_29-2-2016 and PIN-180176_25-4-2016) did not have any nucleotide difference in the VP4/VP2 sequences when compared to sequences from other or same health facilities (Figure 4.15).

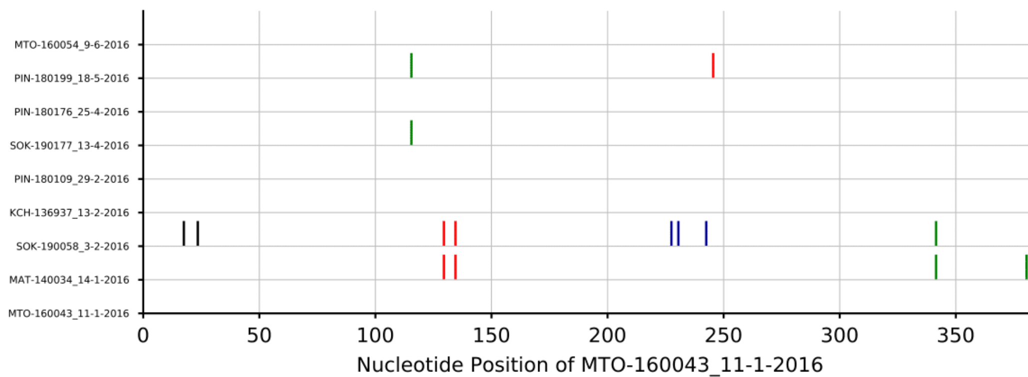


Figure 4.15: Nucleotide differences between HRV-A12 viruses detected from 6 health facilities. Vertical colored bars show the nucleotide differences

In HRV -A101, sequence analysis was done separately for the two variants. Variant 1 had 9 nucleotide difference between sequences from different health facilities including Junju (JNJ-130088_10-2-2016, JNJ-130058_10-2-2016 and JNJ-130053_10-2-2016), Matsangoni (MAT-140013_15-12-2015), Mtondia (MTO-160085_2-2-2016) and Mavueni (MAV-150227_10-5-2016) (Figure 4.16a).Variant 2 had 6 nt difference between sequences from different health facilities including Matsangoni (MAT-140032_14-1-2016 and MAT-140199_5-4-2016), Mavueni (MAV-150186_12-4-2016 and MAV-150186_12-4-2016), Chasimba (CHA-110190_3-5-2016) and Junju (JNJ-130060_10-2-2016) (Figure 4.16b). To note, there was 28 nucleotide difference between variant 1 and variant 2 which were observed at same genomic positions in the VP4/VP2 sequence between the two variants

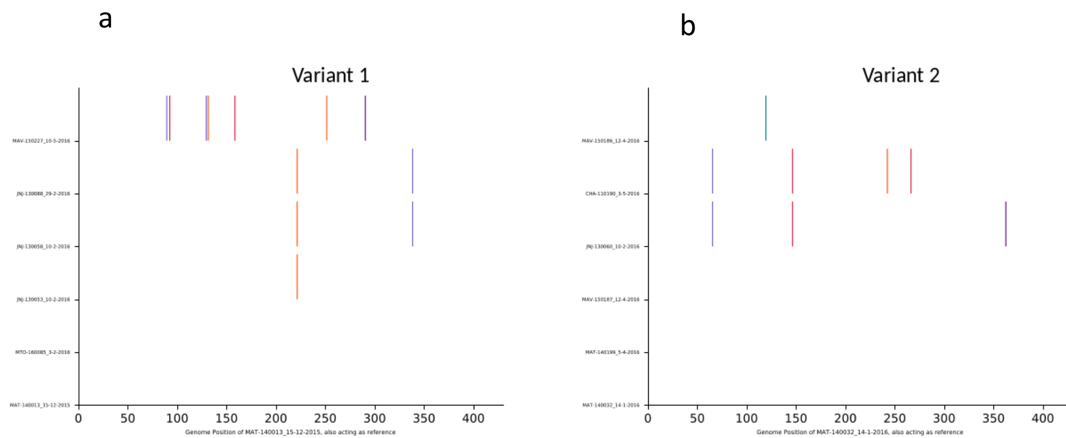


Figure 4.16: Nucleotide differences in 2 variants of HRV-A101 viruses detected from 6 health facilities. Vertical colored bars show the nucleotide differences

HRV-C11 VP4/VP2 sequences from all the health facilities displayed 19 nucleotide difference in various positions on the VP4/VP2 sequences. To note, sequences from KCH (sample id KCH-137306_29-2-2016), Junju (samples id JNJ-130096-9-3-2016 and JNJ-130148-14-4-2016), Mavueni (sample id MAV-150132_18-3-2016), Ngerenya (samples id NGE-170139-24-3-2016 and NGE-170149-24-3-2016) did not have any nucleotide differences (Figure 4.17).

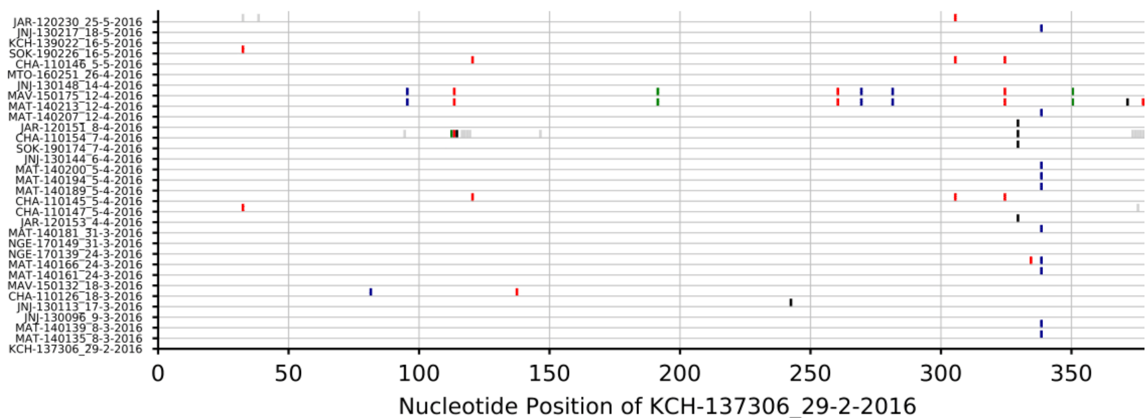


Figure 4.17: Nucleotide differences between HRV- C11 viruses detected from 8 health facilities. Vertical colored bars show the nucleotide differences

HRV-C6 sequence analysis was also done separately for the two variants. Within variant 1, one sequence from Pingilikani (180069_10-2-2016) had 5 nucleotide differences when compared to the rest of variant 2 sequences while another sequence from Mtondia (MTO-160307_24-5-2016) had 2 nucleotide difference from the rest of variant 1 sequences) (Figure 4.18) within variant 2, sequence from Pingilikani (180071_10-2-2016) had 3 nt difference when compared the rest of variant 2 sequences from Chasimba (CHA-110129_18-3-2016) and Junju (JNJ-130051_4-2-2016). To note, there was 16 nucleotide difference between variant 1 and variant 2 which were observed at same genomic positions in the VP4/VP2 sequence between the two variants

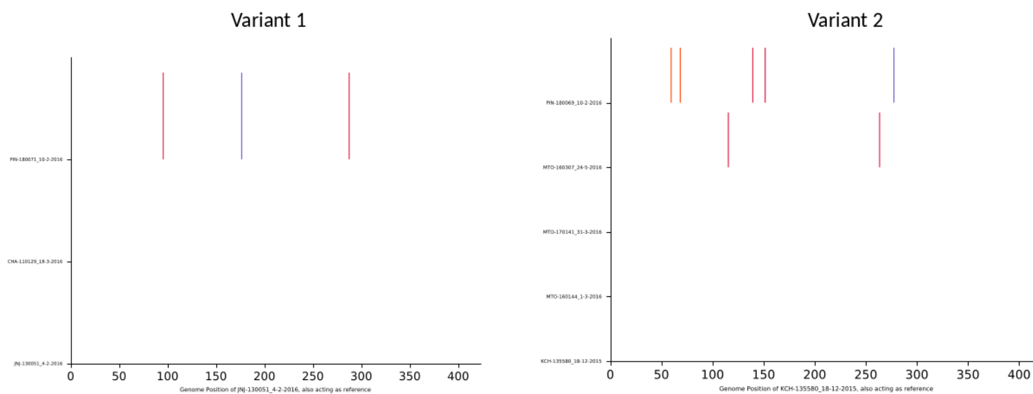


Figure 4.18: Nucleotide differences between HRV- C6 viruses detected from 6 health facilities. Vertical colored bars show the nucleotide differences

Similar to HRV-C6, sequence analysis in HRV-C1 was also done separately for the two variants. Variant 1 had 5 nucleotide difference between sequences from different health facilities including Matsangoni (MAT-140212_12-4-2016), Mtondia (MTO-160103_8-2-2016), Sokoke (SOK-190124_10-3-2016) KCH (KCH-137091_19-2-2016) and Mavueni (MAV-150172_4-4-2016). Variant 2 sequences did not have any nucleotide difference (Figure 4.19).

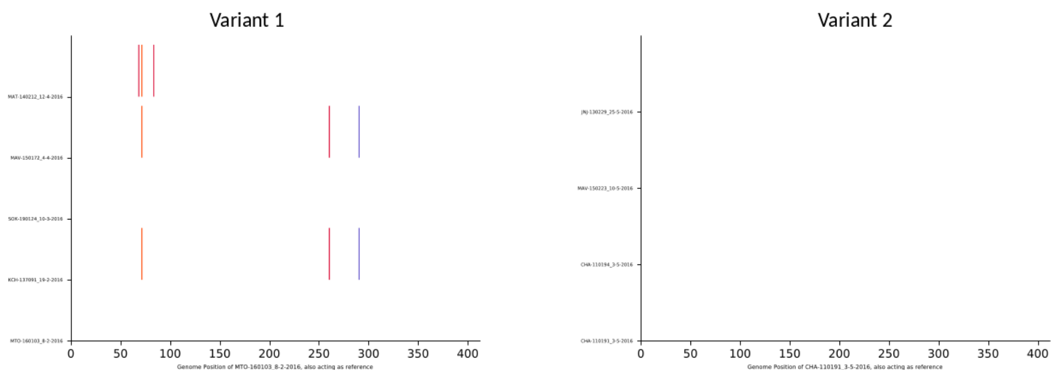


Figure 4.19: Nucleotide differences between HRV- C1 viruses detected from 4 health facilities. Vertical colored bars show the nucleotide differences

4.4.3 Genetic identity analysis within Kilifi HRV strains.

Analyses of genetic identities revealed that intratype identities within Kilifi HRV strains ranged from 91 to 100% at the nucleotide level and 99% to 100% at the amino acid level. Within species the HRV-A species, nucleotide identity within the Kilifi HRV-A strains strain ranged from 91% to 100% and the corresponding amino acid identities were between 95% and 100%. HRV-B strains displayed 96% –100% and 99 –100% sequence identity within the various HRV-B strains. Within species the HRV-C species, nucleotide identity within the Kilifi HRV-C strains strain ranged from 93% to 100% and the corresponding amino acid identities were between 97% and 100% (Table 4.2).

Table 4.2: Type-specific nucleotide and amino acid identities comparison within Kilifi HRV strains

Species and Type	% Nucleotide identity within Kilifi sequences	% Amino acid identity within Kilifi sequences
A8	99-100	100
A11	99-100	100
A12	98-100	99-100
A15	99- 100	100
A18	99 -100	100
A20	100	100
A24	99 – 100	100
A25	98	100
A28	99-100	100
A29	100	100
A30	98-99	100
A31	98-100	100
A32	87-93	92-96

Species and Type	% Nucleotide identity within Kilifi sequences	% Amino acid identity within Kilifi sequences
A38	99-100	100
A39	100	100
A40	100	100
A41	97-100	96-100
A46	100	100
A54	98-100	100
A61	100	100
A66	100	100
A68	99-100	100
A78	99	99
A88	99-100	100
A96	91- 99	95-98
A101	92-100	100
A105	99 -100	100
A106	100	100
B27	98-99	99-100
B35	96-100	100
C1	96-100	100
C6	93-100	97-100
C11	96-100	98-100
C14	97-99	99-100
C18	99	100
C15	99	100
C23	99	99-100
C36	90-100	99-100
C38	93-100	99-100

Species and Type	% Nucleotide identity within Kilifi sequences	% Amino acid identity within Kilifi sequences
C42	99-100	100
C44	98-100	100
C_pat16	99-100	100
C_pat18	99-100	100
C_pat28	98-100	100

4.5 Temporal patterns of HRV cases over the 6 months period

During the 6-months period, HRV was in circulating throughout the study period (Figure 4.20). HRV incidence peaks were observed in March up to the end of the surveillance in May, while troughs were observed in December 2015 and January 2016, which was likely due to fewer samples collected in the first two months of the study. The study period was however not enough to enable an absolute description of seasonality of HRV.

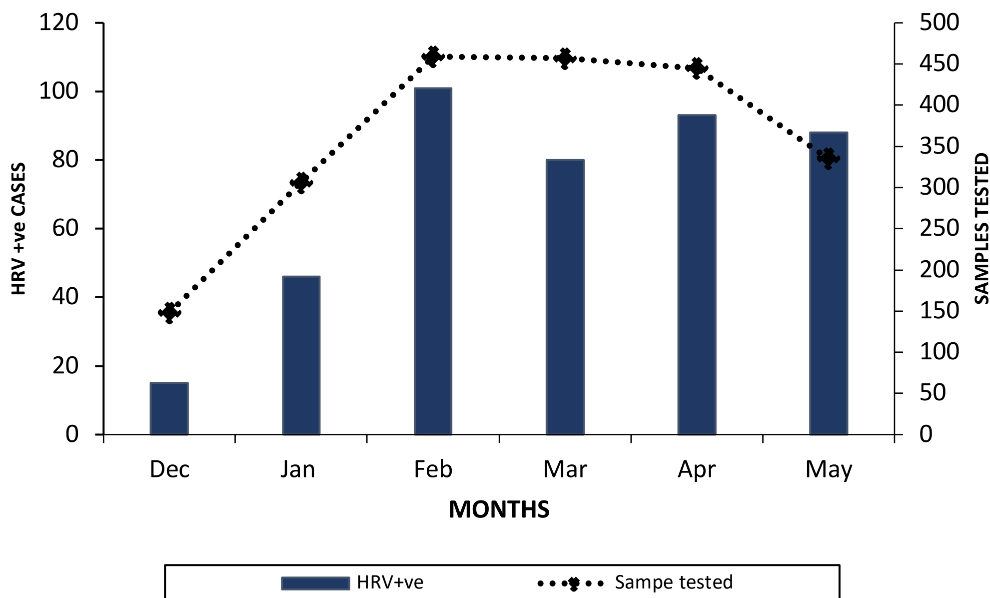


Figure 4.20: Monthly distribution of NPS samples tested and HRV positive cases.

All HRV species were detected in all the months except in December 2015 when only A and C were detected. There was a shift in dominance between HRV-C and HRV-A with time (Figure 4.21). HRV- A appeared dominant in December, January, April and May while HRV-C dominated in the months of February and March (Figure 4.21).

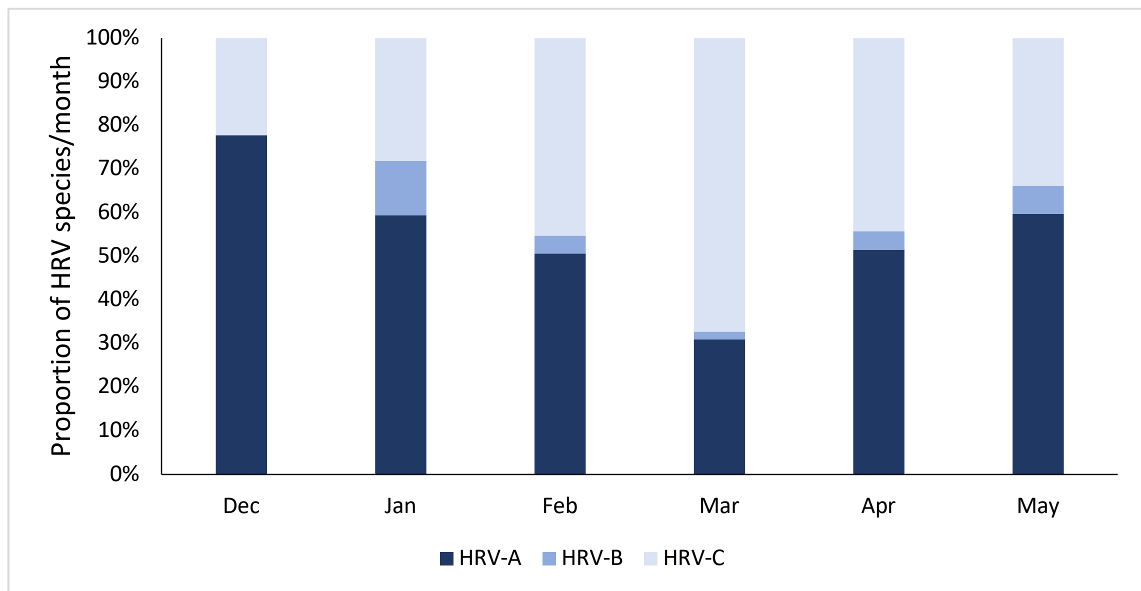


Figure 4.21: Proportion of HRV species per month.

Further analysis was done to evaluate the temporal patterns for each HRV type detected (Figure 4.22). To note, several HRV types circulating simultaneously presented different occurrence period in population that lasted between 1 - 5 months. Each HRV type showed a considerable different occurrence pattern, with some type having their peak detection around February-March 2016 while other had their peaks around April-May 2016 (Figure 4.22). Some HRV types were found across several months, such as HRV-A101, A68, C14 and C_pat28 which appeared in five months of the study. HRV-A105, A12, A8, B35, C1, C11, C36 and B27 appeared for 4 months. Other HRV types had detections ranging from 1 – 3 months.

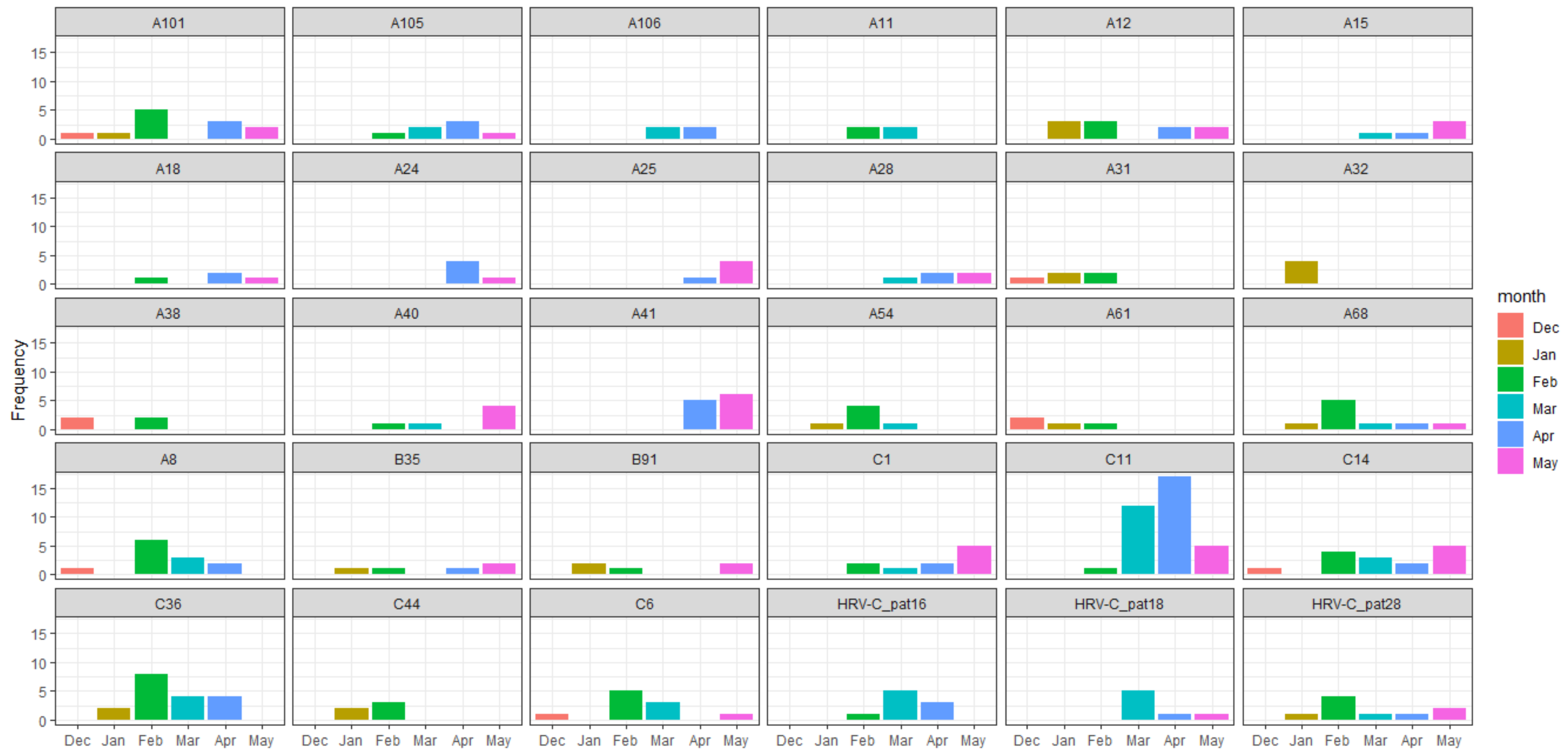


Figure 4.22: Temporal patterns by month of HRV types in the KHDSS, December 2015 to May 2016.

continuation

4.6 Geographical distribution of HRV types in the KHDSS.

Spatial analysis revealed wide geographical distribution of identical HRV-types across the KHDSS. HRV-C11 was detected in all health facilities, C1 and C14 were detected 8 health facilities while HRV-A101, A41, A8 and A12 were detected in 5 different health facilities spread across the KHDSS (Figure 4.23)

HRV- C11 was identified in all health facilities. First, it was reported in February in KCH, and later in other health facilities across the KHDSS for 4 months of the study period. To note, HRV-C11 sequences from the different health facilities had minimal nucleotide variation on the VP4/VP2 genomic region in viruses circulating simultaneously at different locations in the KHDSS suggesting spread of a genetically identical HRV- C11 type in the KHDSS. HRV-C1 was detected in KCH, Junju, Chasimba, Matsangoni, Mavueni, Mtondia, Ngerenya, Sokoke and Pingilikani.

HRV-C14 was detected in KCH, Jaribuni, Junju, Matsangoni, Mtondia, Ngerenya, Sokoke and Pingilikani. HRV-A12 was detected in KCH, Junju, Matsangoni, Pingilikani, Sokoke and Mtondia, similar observation was made on HRV-A8 which was detected in Matsangoni, Pingilikani, Chasimba, KCH, and Jaribuni. HRV-A41 was detected in Junju, Mtondia, Chasimba, Sokoke and Pingilikani. HRV- A101 was also detected 5 health facilities namely Junju, Chasimba, Matsangoni, Mavueni and Mtondia (Figure 4.23). Other HRV types had detections ranging from 1 – 4 health facilities.

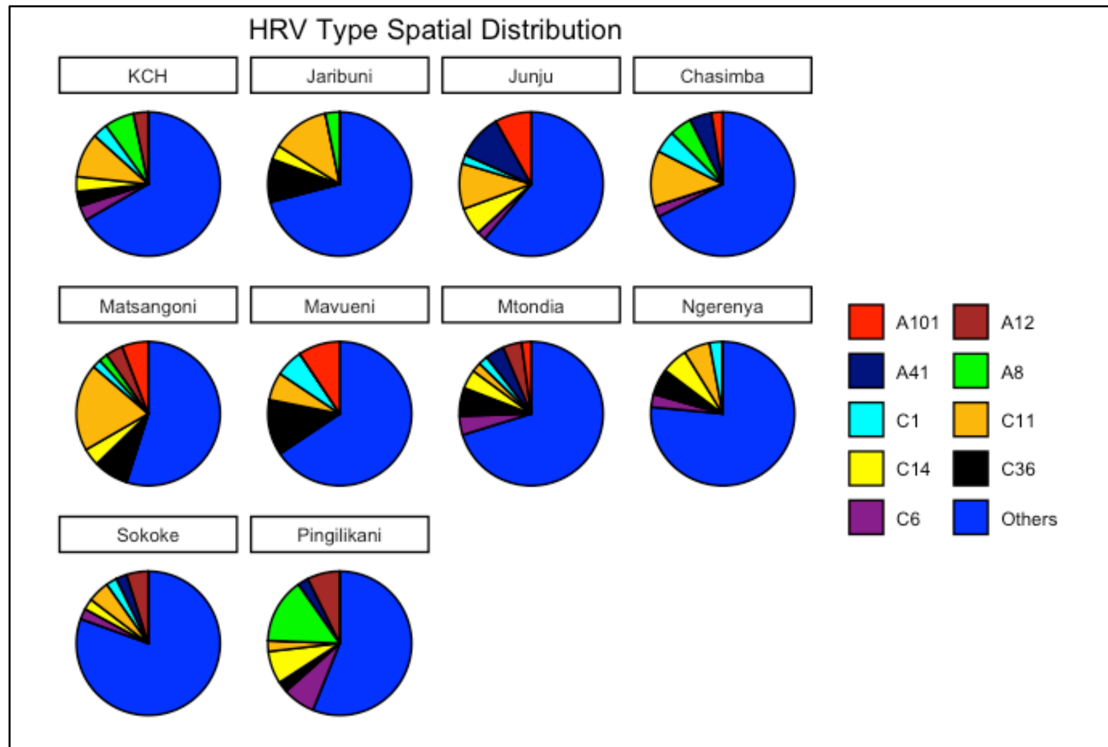


Figure 4.23: Distribution of HRV types by site

4.7 Genetic relatedness of Kilifi to global HRV viruses

4.7.1 Phylogenetic relatedness of Kilifi and global HRV strains

The second objective of this study was to investigate the genetic relatedness of HRV strains circulating in Kilifi to HRV strains circulating elsewhere globally. VP4/VP2 gene sequences deposited in the GenBank as of 31 May 2016 and derived from viruses collected between 1 January 2010 and May 2016 were collated and phylogenetically compared with the Kilifi VP4/VP2 sequences. The data set comprised 345 VP4/VP2 sequences from 14 countries including USA, Spain, Mongolia, Poland, China, Italy, Kenya, Viet-Nam, Thailand, France, Paraguay, Panama, Egypt, Indonesia, Cyprus.
Genetic relatedness was assessed at HRV type level using phylogenetic. In most HRV strains, sequences clustered together with respect to the country of origin (country of collection). HRV strains of types A68, A105, A25 and A106 had higher sequences

similarity (87-100%) to sequences from elsewhere globally and clustered together on the global phylogenetic tree.

Kilifi HRV-A68 sequences obtained from viruses collected between January - May 2016 clustered together with a sequence from USA dated 2016 (bootstrap support value of 86%), perhaps depicting likely origin of this HRV strain or subsequent export to this area considering they were circulating at the same period (2016) (Figure 4.24). HRV-A68 sequences from Kilifi also clustered together with sequences from France and Thailand all dated 2014 (Figure 4.24).

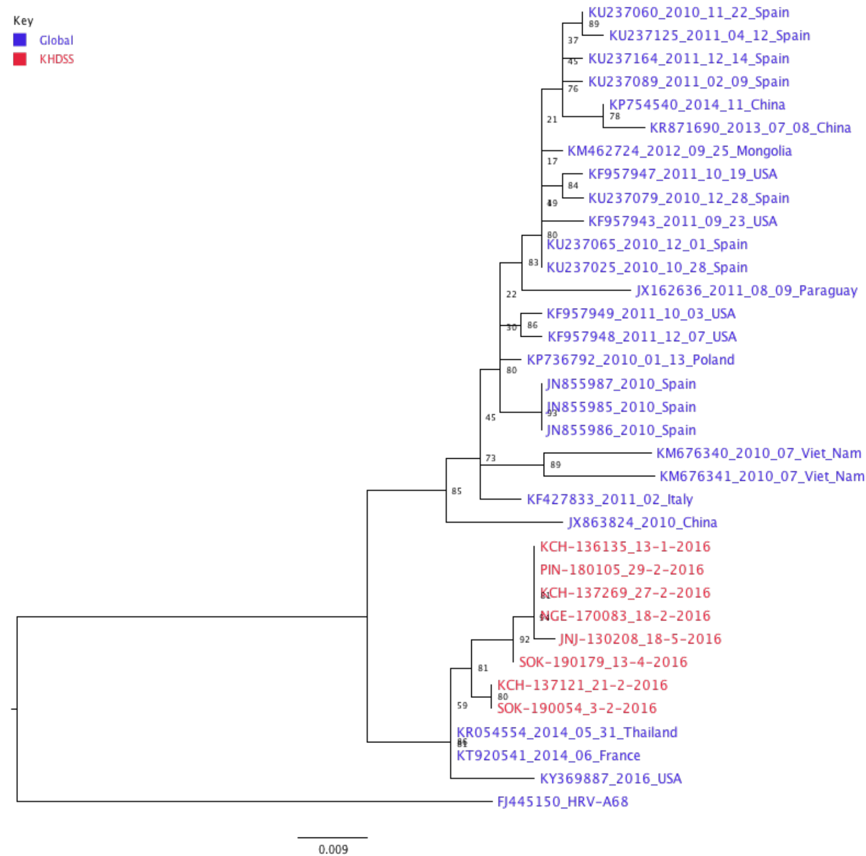


Figure 4.24: Maximum likelihood phylogenetic tree of A68 sequences from KHDSS and global sequences from the GenBank (2010 – 2016).

In HRV-A105, Kilifi sequences clustered closely with sequences from USA dated 2016 and from Thailand dated 2013 (bootstrap support value of 100 %) (Figure 4.25).

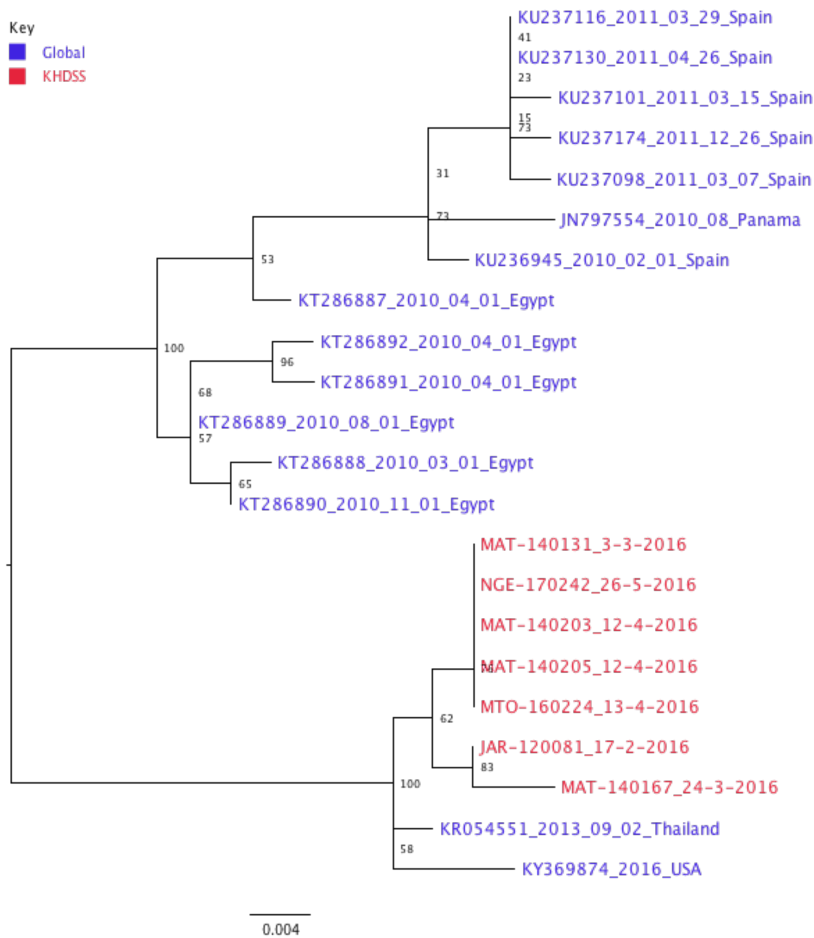


Figure 4.25: Maximum likelihood phylogenetic tree of A105 sequences from KHDSS and global sequences from the GenBank (2010 – 2016).

HRV-A106 sequences from Kilifi clustered together with four sequences from China dated 2014, one sequence from France dated 2013 and one sequence from Cyprus dated 2012 (bootstrap support value of 95%) (Figure 4.26).

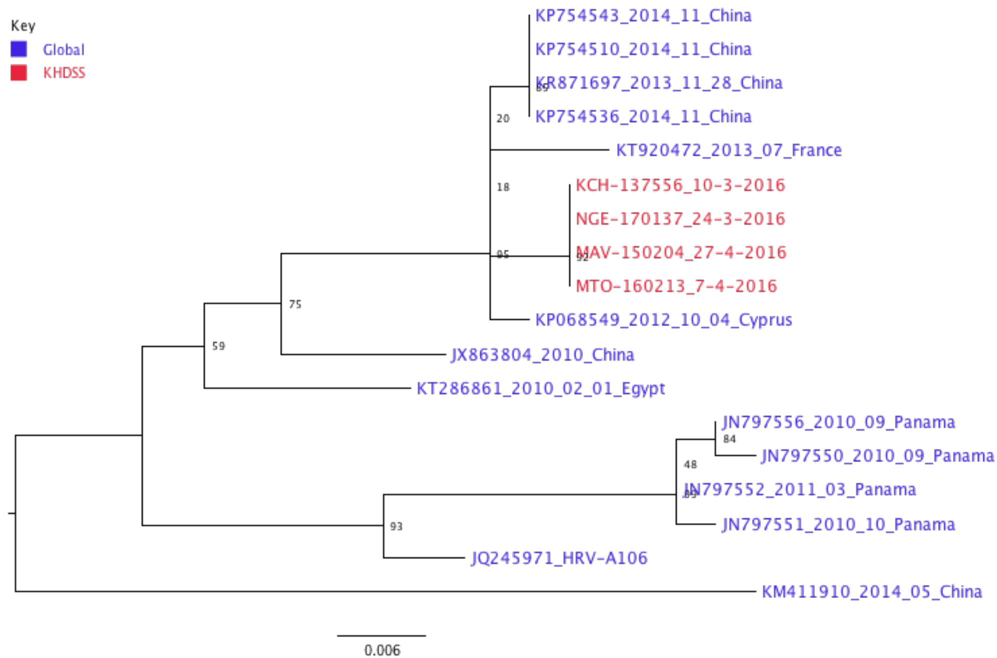


Figure 4.26: Maximum likelihood phylogenetic tree of A106 sequences from KHDSS and global sequences from the GenBank (2010 – 2016).

HRV-A 25 sequences form Kilifi clustered closely with one sequence from China dated 2014 (bootstrap support value of 97 %) (Figure 4.27). To note, Kenya sequences dated 2010 clustered separately from the Kilifi 2016 sequences and closely with sequences from Poland and Slovakia all sample all dated 2010.

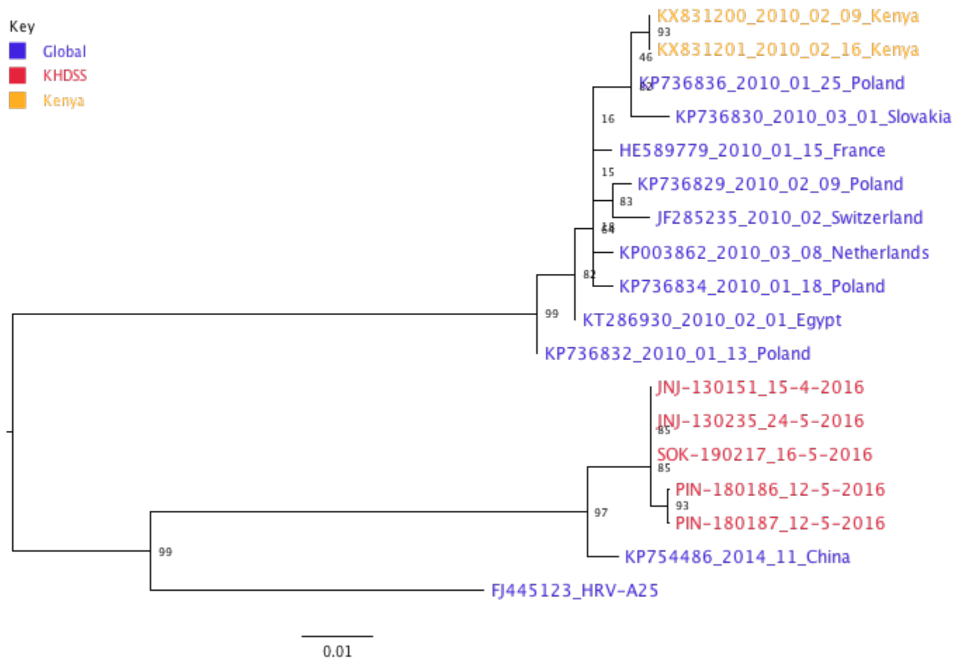


Figure 4.27: Maximum likelihood phylogenetic tree of A25 sequences from KHDSS and global sequences from the GenBank (2010 – 2016).

Similar to HRV-A strains, HRV strains of types C6, C14 and Cpat_16 had close sequences similarity (>90%) to sequences from elsewhere globally and clustered together in the global phylogenetic trees. 2 divergent strain of HRV-C6 from Kilifi clustered formed separate cluster on the global phylogenetic tree. 1 variant clustered with sequences from Tunisia, Egypt and China all dated 2010 (bootstrap support value of 98%). Second variant of HRV-C6 clustered with sequences from China dated 2014, and also sequences from Malaysia and Vietnam dated 2010 (bootstrap support value of 85%) (Figure 4.28).

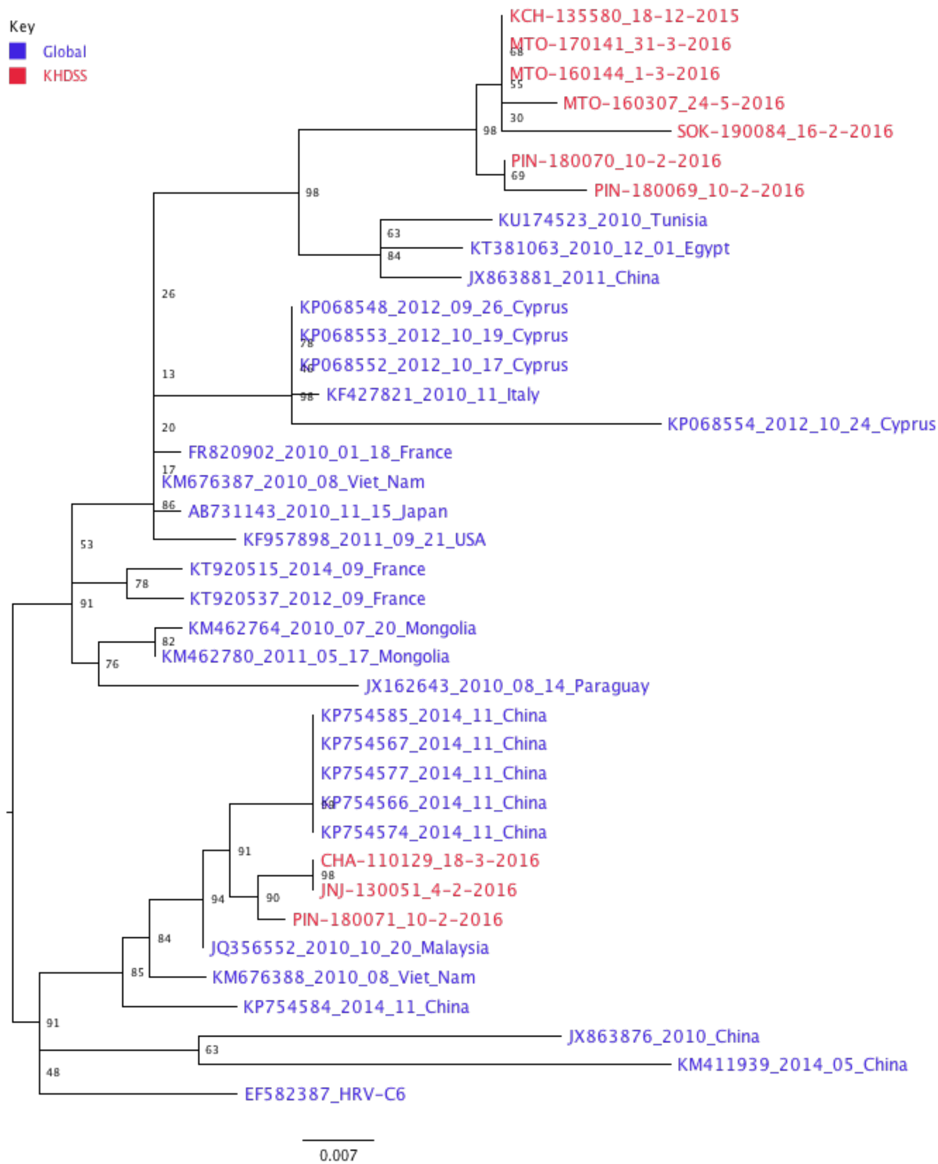


Figure 4.28: Maximum likelihood phylogenetic tree of C6 sequences from KHDSS and global sequences from the GenBank (2010 – 2016).

HRV-C14 sequences from Kilifi clustered together with sequences from India dated 2015 (bootstrap support value of 89 %) (Figure 4.29).

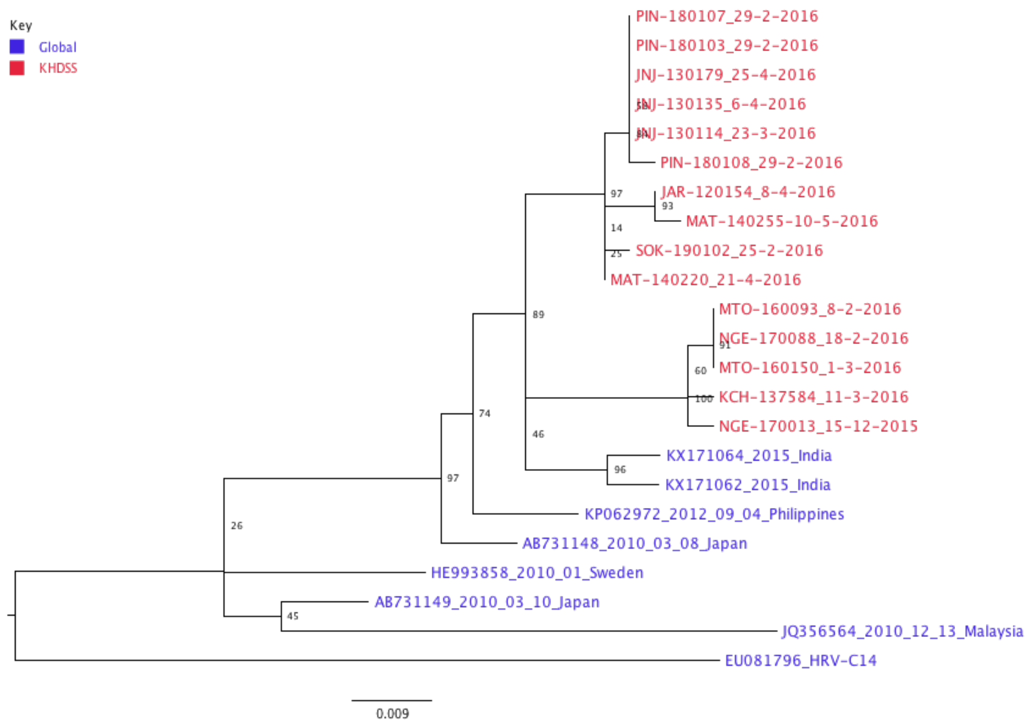


Figure 4.29: Maximum likelihood phylogenetic tree of C14 sequences from KHDSS and global sequences from the GenBank (2010 – 2016).

HRV-Cpat 16 sequences from Kilifi clustered together with one sequence from the Netherlands dated 2012 (bootstrap support value of 95%). To note, 2 Kenyan sequences dated 2010 clustered separately from the Kilifi 2016 sequences (bootstrap support value of 38%) (Figure 4.30).

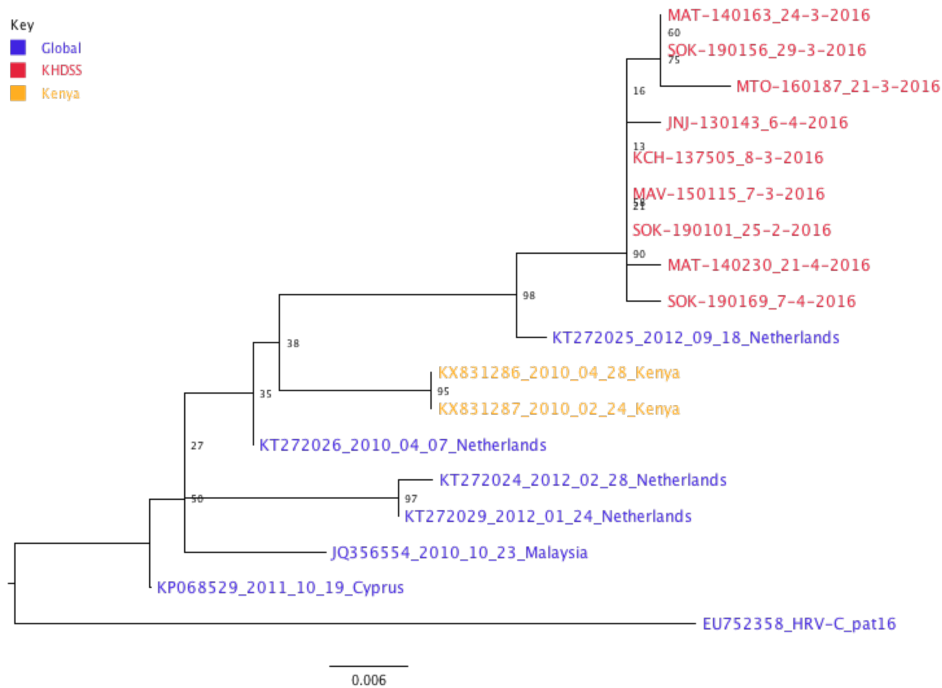


Figure 4.30: Maximum likelihood phylogenetic tree of Cpat 16 sequences from KHDSS and global sequences from the GenBank (2010 – 2016).

Despite close genetic relationship in some Kilifi HRV strains to HRV strains from elsewhere globally, some HRV-A strains from Kilifi were distantly related strain circulating elsewhere. This was observed in strains of type A41, C_pat28, A8 and A54.

HRV-A41 from Kilifi appeared to be genetically different from the HRV-A41 strain circulating elsewhere globally (France, China, Switzerland and USA) suggesting evolutionary divergence between HRV-A41 viruses circulating in Kilifi and those circulating elsewhere globally (Figure 4.31).

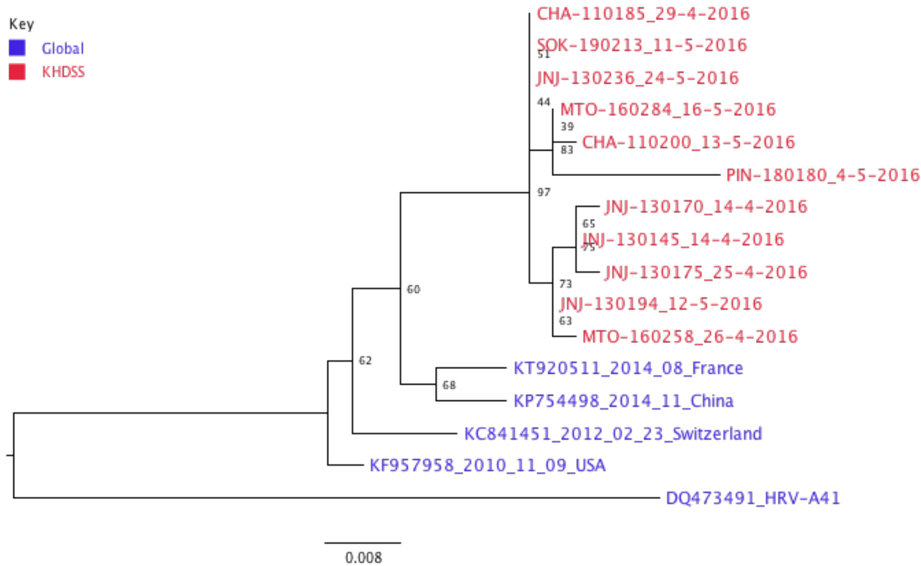


Figure 4.31: Maximum likelihood phylogenetic tree of A41 sequences from KHDSS and global sequences from the GenBank (2010 – 2016)

Similar observations were seen in HRV-A54. HRV-A54 strain circulating in Kilifi sequences clustered separately to the HRV-A54 strains sequences circulating elsewhere globally (Figure 4.32). The genetically divergent sequences originated from India, Thailand, Sweden, China, Tunisia and Spain.

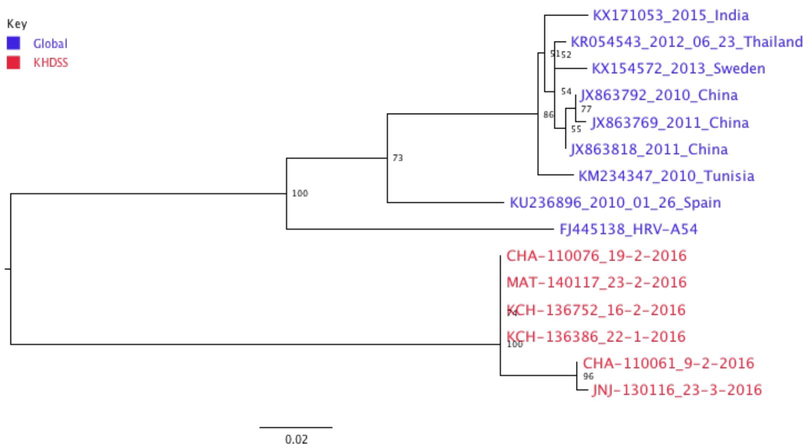


Figure 4.32: Maximum likelihood phylogenetic tree of A54 sequences from KHDSS and global sequences from the GenBank (2010 – 2016).

Consistent observation was seen in HRV-Cpat28, where strain circulating in Kilifi sequences formed monophyletic cluster separate to the HRV-A54 strains sequences circulating elsewhere globally (Figure 4.33).

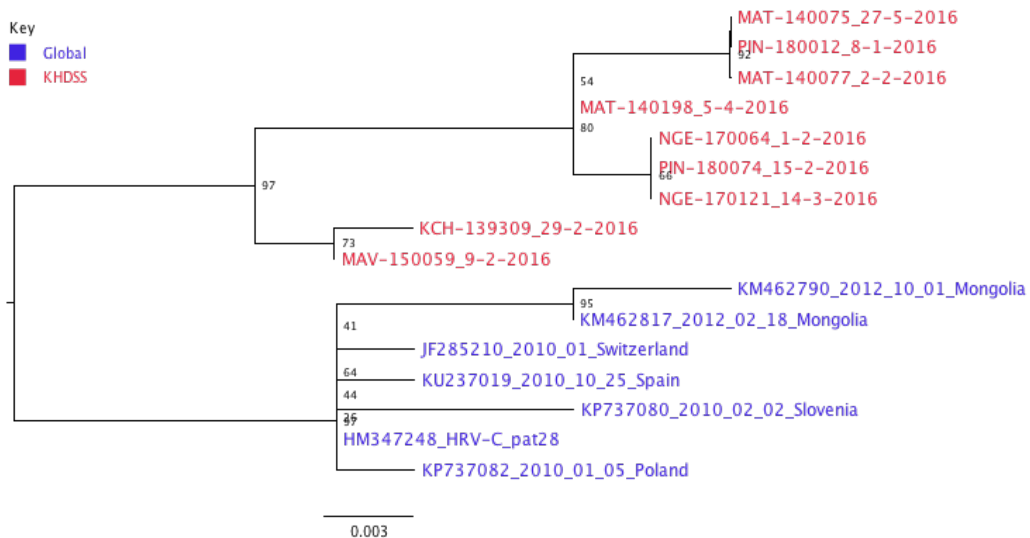


Figure 4.33: Maximum likelihood phylogenetic tree of Cpat 28 sequences from KHDSS and global sequences from the GenBank (2010 – 2016).

4.7.2 Genetic identity analysis between Kilifi and global HRV strains.

Nucleotide identity of the Kilifi HRV -A strains to other contemporaneous strains ranged from 86% to 100% and the corresponding amino acid identities were between 91% and 100%. HRV-B strains displayed 92–99% nucleotide identities and 99 – 100% amino acid identities with other HRV-B contemporaneous strains. Nucleotide identity of the Kilifi HRV-A strains to other contemporaneous strains ranged from 86% to 100% and the corresponding amino acid identities were between 91% and 100%. Table 4.4 shows the nucleotide identity and amino acid identity between sequenced from KHDSS and contemporaneous sequences

Table 4.3: Type-specific nucleotide and amino acid identities comparison of Kilifi HRV strain to contemporaneous HRV strain.

Species and Type	% Nucleotide identity between Kilifi and contemporaneous sequences	% Amino acid identity between Kilifi and contemporaneous sequences
A1	88%	99-100
A7	92-94	99-100
A8	95-97	97-100
A11	93-98	100
A12	92-99	97-100
A15	94 -98	100
A18	94 – 98	99-100
A20	93-98	99-100
A24	97-98	100
A25	91-99	99-100
A28	91-98	99-100
A29	89-99	98-100

Species and Type	% Nucleotide identity between Kilifi and contemporaneous sequences	% Amino acid identity between Kilifi and contemporaneous sequences
A30	94-98	100
A31	93-98	98-100
A32	90-99	94-99
A38	97-99	99-100
A39	100	100
A40	88-99	100
A41	95-98	97-100
A46	97-98	99-100
A54	86-89	100
A58	93-99	99-100
A61	89-98	99-100
A65	95-97	99-100
A66	93-99	99-100
A68	91-100	96-100
A75	93-98	99-100
A78	86-99	97 -100
A88	86 -88	97
A96	90-94	97-98
A101	91 -99	91- 99
A105	95-99	100
A106	94-99	99
B27	92-94	99-100
B35	96-99	99-100
C1	92-95	98-100
C6	92-99	91-100

Species and Type	% Nucleotide identity between Kilifi and contemporaneous sequences	% Amino acid identity between Kilifi and contemporaneous sequences
C14	93-98	99-100
C18	96-97	99-100
C15	93-99	97-100
C22	94-97	99-100
C23	92-93	98-100
C31	93-99	99-100
C32	93-97	99-100
C35	91-96	99-100
C36	89-96	98-100
C38	94-98	98-99
C42	92-98	98-100
C43	97-99	99-100
C44	97-99	99-100
C_pat16	96-99	99-100
C_pat18	97-98	100
C_pat28	95-97	99-100

4.8 Introductions and persistence of HRV strains into the KHDSS

To investigate possible introductions and persistence of HRV strains in the KHDSS from elsewhere i.e. other locations within Kenya or global, VP4/VP2 sequences from viruses sampled at KCH between 1 January 2010 and 31 May 2016, and related sequences from GenBank retrieved by BLAST were collated and phylogenetically compared to Kilifi HRV strains sampled from the KHDSS between December 2015 - May 2016.

Phylogenetic analysis revealed that some HRV types persisted for longer periods with detections over many years/seasons. HRV strain was characterized as persistent if the viruses sampled in the immediate years clustered together with viruses circulating

between December 2015 -May 2016 in phylogenetic tree. Alternatively, if there was no persistence, strains detected over the immediate years (2010 to 2015) would be more similar to variants from elsewhere around the world than strain circulating in KHDSS. Moreover, separate clustering of Kilifi HRV strain collected between 2010 – 2016 is an indication of separate introductions in the KHDSS.

In HRV-C6, sequences identified in 2015/2016 Kilifi together with sequenced from earlier years (2013 and 2014) suggesting close genetic relatedness and persistence of this HRV-C6 strains for since 16-9-2013 when it was first detected at KCH. A separate variant of HRV-C6 also formed single cluster with sequence from 2010 and 2013. Generally, these observations indicate there were two introductions of HRV-C6 between 2010 and 2016. HRV-C6 variant 1 was first introduced into the KHDSS around 2010 and persisted through to 2016 with detections in 2013 while variant 2 was introduced in the KHDSS in 2013 and persisted through to 2016 (Figure 4.34).

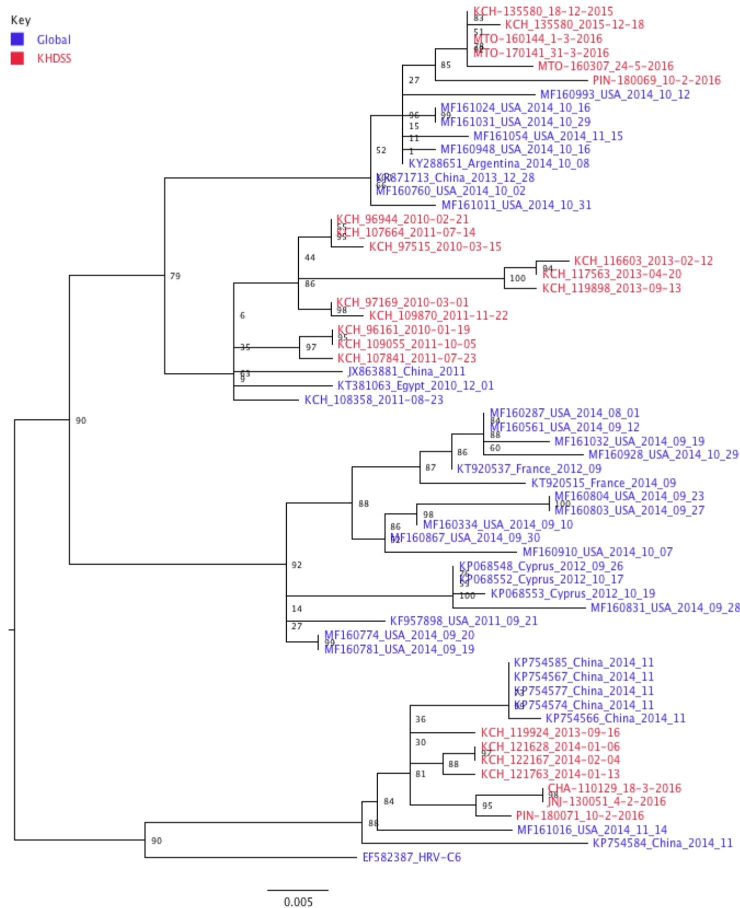


Figure 4.34: Maximum likelihood phylogenetic tree of HRV-C6 sequences from KHDSS and global sequences from the GenBank (2010 – 2016).

In HRV-A101, Kilifi sequences separated into four separate clusters suggesting separate introductions into the KHDSS since 2010. Phylogenetic analysis revealed circulation of 2 genetically divergent variants in 2016 possibly coming from 2 introductions this is indicated by the separate/distant clustering of the 2015/2016 sequences. Moreover, the tree reveals there was an introduction virus of HRV-A101 circa 2010 which persisted in the KHDSS for 30 months (07 March 2010 to 30 April 2013) (Figure 4.35).

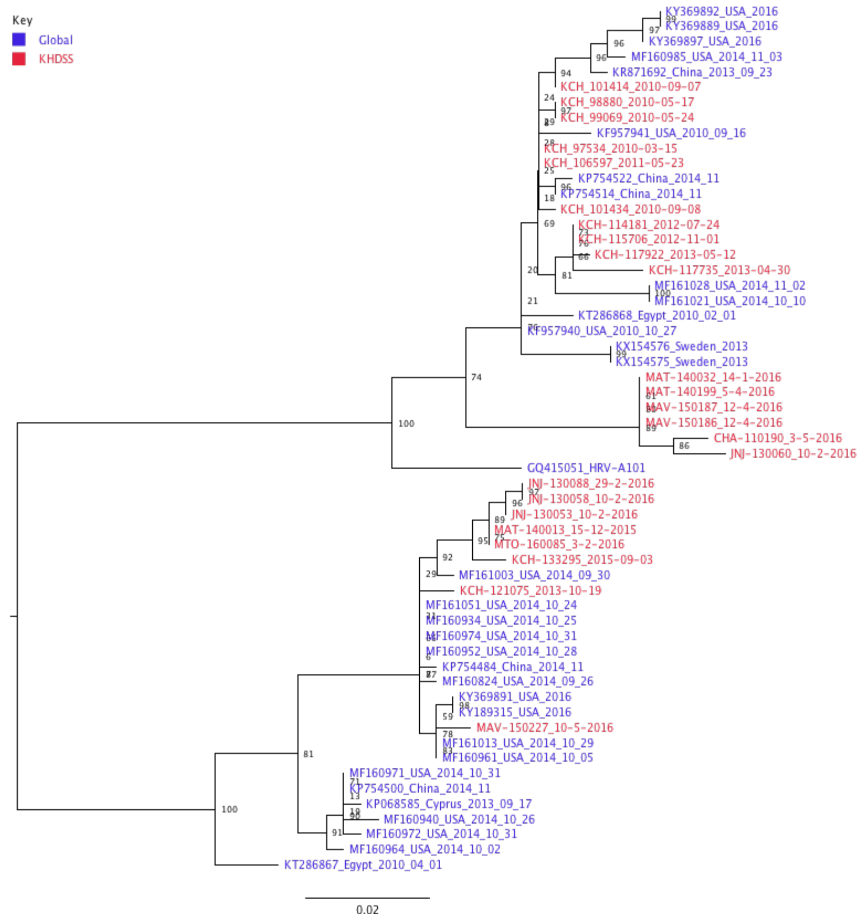


Figure 4.35: Maximum likelihood phylogenetic tree of HRV-A101 sequences from KHDSS and global sequences from the GenBank (2010 – 2016).

In another similar case, HRV- A12 sequences circulating in 2016 clustered together with sequences from 2014 in a phylogenetic tree indicating close genetic relatedness (bootstrap value > 70%). This variant of HRV-A12 circulated and persistence in the KHDSS for 23 months (9 June 2014 – 18 May 2016). The phylogenetic analysis also revealed circulation of divergent strain that circulated and persisted for 9 months (28 November 2012 – 8 July 2013) and another variant that circulated in for 3 months in 2010 (Figure 4.36).

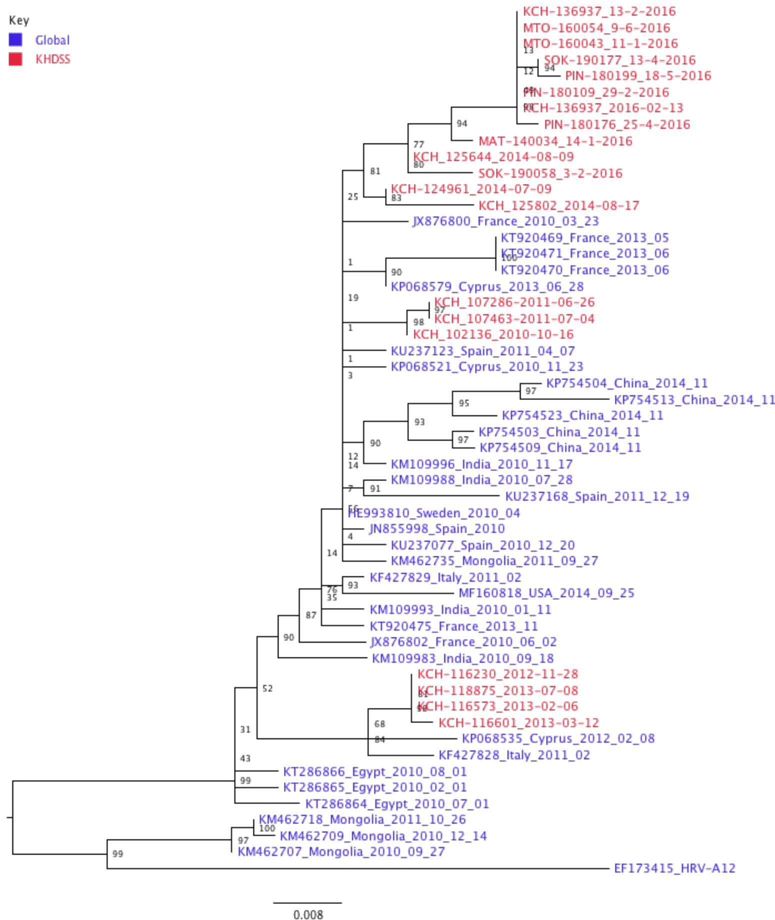


Figure 4.36: Maximum likelihood phylogenetic tree of HRV-A12 sequences from KHDSS and global sequences from the GenBank (2010 – 2016).

4.9 Reconstructing of transmission pathways of HRV strains in the KHDSS

Although this study shows evidence of introductions and spread of HRV strains into the KHDSS, it was not possible to reconstruct the transmission pathways of HRV from one individual to another in KHDSS population. General limitation of phylogenetic approach to elucidate transmission patterns is the interpretation of a definitive linkage between people infected with the same HRV or to a common infecting source. In addition to having molecular sequence data such studies will also need to collect social contact patterns of individuals who report infections with the same HRV type to allow mapping of who infected whom and also spread of the virus in the population, this data was not available for this study. Moreover, such study will require analysis of full genome sequence which

will provide better phylogenetic resolution in tracking spread of viruses compared to analysis of VP4/VP2 sequences.

CHAPTER FIVE

DISCUSSION

5.1 Socio-demographic characteristics of HRV cases in the KHDSS.

In this study, HRV was detected in 423/2150 (19.6%) NPS samples collected during the six months period (December 2015 -May 2016). There was a decrease in the prevalence of HRV cases with increase in the age of the patients, where majority of the HRV positive patients were children under 5 years of age 312/423(73.8%). The detection rates within different age groups are in line with previous studies that reported ratios of 50 – 80 % in children under the age 5 years (Mackay et al., 2013; Peltola et al., 2008). Findings of high proportions of HRV infections in children under the age of 5 years reiterates the need to focus control strategies to this age group since they are the most vulnerable, and presumably makes the greatest contribution to community transmission. Low incidence rates of HRVs reported in adults may reflect the gradual development of type-specific immunity due to increased exposure to HRV types throughout life (Peltola et al., 2008) or it could be that the most adult do not seek medical attention in health facilities when they get mild respiratory infections.

In this study, distribution of HRV species among the age groups did not differ by age and gender. Contrary to our findings, studies carried out in different continents including Africa have found that, although all of the HRV species may be associated with any type of respiratory disease, RV-C seems to be more frequent in children under 5 years (Baillie et al., 2019).

5.2 HRV species and types classification

Classification of HRV viruses to their respective HRV species and types was based on the phylogenetic analysis of VP4/VP2 coding sequences (McIntyre et al., 2013; Simmonds et al., 2010). A wide range of HRV types (57 HRV types) belonging to the three species was reported, demonstrating an extensive representation of the different HRV types in the KHDSS. HRV-A and HRV-C were the predominant species, while HRV-B detections were low. The low detection of HRV-B in comparison with the other HRVs is similar to

other HRV epidemiology studies in children with respiratory infections, regardless of geographical location (Milanoi et al., 2016; Morobe et al., 2018; J Richter et al., 2014; Sansone et al., 2013).To note, the proportions of HRV observed in our findings is in agreement with a previous study conducted in rural coastal Kenya (Onyango et al., 2012) and also studies conducted in other parts of the world (Garcia et al., 2013; Pretorius et al., 2014; J Richter et al., 2014), where HRV-A and HRV-C predominated in the hospital detections.

The most prevalent HRVs types were A101, A8, A12 and A41 for HRV-A; B27 and B35 for HRV-B; and C11, C36 and C6 for HRV-C. Five samples diagnosed as HRV positives by real-time RT-PCR were found to belong to a different species within the genus *Enterovirus.* They were correctly identified as Enterovirus D68s (n=3) and one Coxsackievirus B5 (n=2) after online BLAST search. This is not unusual as the PCR target region (5' UTR) used for the detection of HRV is genetically close between HRV and other members of the genus *Enterovirus* (Zell, 2018). This problem can be overcomed by using primers that anneal only to the highly conserved region on the 5' UTR of all the HRV species to provide the most sensitive assay for the detection of HRV strains (Bochkov, Grindle, & Vang, 2014). To note, failure to sequence and type 117/423 (27.7%) of HRV positive samples was largely attributed to PCR failure due to low virus titers in the nasopharyngeal samples inferred from high Ct value (Ct value>33) in these samples compared to a Ct value of 32 in the successful sequenced samples. Furthermore, some samples failed at the PCR stage, which could have resulted from mismatches in the primer binding sites or assay sensitivity given that we were using a single step rather than nested PCR which more sensitive (Bochkov et al., 2014).

5.3 Phylogenetic analysis of HRV types circulating in the KHDSS.

Phylogenetic analysis revealed close genetic relationship between similar HRV strain from different health facilities. High bootstrap support value (> 92%) in these clusters justified the reliability in these analyses. Moreover, high intra-type genetic identities between the sequences from different health facilities (92-100%) reaffirms the close association between the HRV strains in circulation and could suggest a probable spread

of the same virus strains in KHDSS and probably as a result of a single introduction. In some cases, the identical HRV-types were circulating simultaneously in different geographical regions separated by a distance as far as 30 km apart. Low genetic variation in the VP4/VP2 coding sequences and the concurrent distribution between these identical HRV-types it is probable that there is a rapid spread of HRV-types within the KHDSS once introduced and or there could be multiple introductions of the same type into different areas of the KHDSS. Despite phylogenetic evidence for rapid spread across the KHDSS, there was variation in the distribution of HRV-types between health facilities. An obvious mechanism that accounts for the variation in HRV-type distribution between health facilities was that transmission occurs more frequently between people attending the same health facility compared to those who attend other health facilities. In this case, sharing a health facility is a proxy for being more likely to live nearby and share the same social amenities and gatherings which are hotspot for transmission of respiratory viruses (Morobe et al., 2018).

5.4 Temporal patterns of HRV cases over the 6 months period.

HRV circulated throughout the study period without clear seasonal pattern, although it will be false to state if at all there was no seasonal pattern due to the short study period. HRV-A was the predominant species followed by HRV-C; HRV-B was the least commonly detected. Occurrence of HRV throughout the year appeared to be sustained by the existence of simultaneous and successive mini epidemics each caused by a different HRV type introduced into the community independently (Morobe et al., 2018). Occurrence of the multiple typical mini-epidemics caused by specific type in the community, is possibly due to homotypic immunity caused by HRV types, if the community population had stronger herd heterotypic immunity against various types we would expect minimum number of HRV cases, moreover we would expect the virus to remain in the community shorter time periods (few months). Therefore, due to the homotypic immunity in HRV, the population will continue to report HRV incidence as other new HRV types are introduced into the community. Also, the differences in circulation periods observed among HRV-types could be as a result of stochastic

differences in frequency of introduction and onward transmission of the HRV types, alternatively, it could be due to differences in the community level immunity to the different types given their differences in incidence in the recent past (Mackay et al., 2013; Peltola et al., 2008). Similar to our findings, A study in Sweden also presented similar observations where simultaneous and successive epidemics caused by different HRV types contributed to HRV high incidence and enabled HRV to remain in the local population for extended periods (Sansone et al., 2013). In some cases, type-specific epidemics were served with different variants of the same HRV type. This represent a classic case of multiple introductions of a single type into the area. Such variants were observed to be substantially diverse displaying two clusters on the type-specific phylogenetic trees.

5.5 Geographical distribution of HRV types in the KHDSS.

There appeared to be no organised spatial pattern of the types detected; most HRV types were circulating simultaneously in different geographical regions separated by a distance as far of 30 km apart. There are several potential explanations for these patterns. The HRV strain may be widely distributed across the region and infections are being acquired locally, or it may be that people are traveling and becoming infected from a common point source. Unfortunately, in this study social contact data and travel histories were not available to explain the point-source transmission scenario. However, the data available still suggests that HRV strains widely spread across the region and then the lack of genetic variation among isolates suggests that the spread of HRV strain throughout the region occurred relatively rapidly, with insufficient time elapsed for mutations to accumulate.

5.6 Genetic relatedness of Kilifi to global HRV strains.

A phylogenetic comparison of the Kilifi VP4/VP2 sequences obtained from viruses sampled between January 2015 and May 2016 and others from global locations found that most of the contemporaneous sequences clustered by country of collection. In most HRV types, Kilifi viruses clustered separately from the rest of the viruses circulating elsewhere globally. Such clustering indicates circulation of different variants of HRV-type per

country or rather the circulation of the types is localized within countries. Furthermore, if the distinct clusters represent different variants in circulation globally, we conclude that the variants circulating in Kilifi are phylogenetically distant from the variants in circulation globally. Monophyletic clustering of Kilifi VP4/VP2 sequences on the global phylogeny could be an indication of single introduction/single variant in circulating in the population. Notwithstanding, some contemporaneous sequences were dispersed in Kilifi dominated clusters suggesting globalized circulation of these HRV-types and these countries could be possible sources of these viruses detected in Kilifi, having in mind Kilifi which lies in the coastal Kenya is a popular international tourist destination where individual from all over the world visit. However, like many phylogenetic studies, directionality of transmission could not be ascertained but phylogenetics can identify viruses that shared a transmission chain.

5.7 Introductions and persistence of HRV strains into the KHDSS.

Separate phylogenetic clustering of sequences from in Kilifi between 2010 and 2016 indicated cases of separate introductions into the KHDSS within this period, or there could have been replacement of variants. Alternatively, it could be the same variants that circulating in the preceding year had evolved to the genetic shape seen lately. If HRV strain persisted for longer periods in the community, we would expect the variants to cluster together in a phylogenetic tree. Alternatively, if there was no persistence, variants detected over the years would be more similar to variants from elsewhere around the world. These claims could have been clear if there was intensive community surveillance in the years preceding this study. Moreover, detection of the some HRV-type across several months (up to 6 months) suggests prolonged /extended circulation providing additional explanation of HRV persistence. Moreover, persistence of HRV strain in the population can be explained by 3 causes 1.) re-introduction of new variants of the same HRV type into the area 2.) Fading of type specific (homotypic) immunity which last for approximately 1 year (Barclay et al) which allows/facilitate introduction and spreading. also, limited/lack of cross-neutralization among HRV types facilitate introduction, spreading and continuous circulation of HRV in the community.

5.8 Conclusion.

This study recorded co-circulation of numerous HRV types over the six months. HRV types from different health facilities (representing different locations of the KHDSS) were phylogenetically related to each other suggesting circulation and spread of the same HRV strains in the KHDSS.

There was no discernable spatial distribution pattern in the HRV types, Most of the HRV types were detected at the same times across different health facilities which were widely distributed in the KHDSS.

Different HRV types presented different temporal patterns of occurrence with peaks mainly in February-March 2016 and April-May 2016. Nevertheless, there was considerable variation in the spread of the seasonal occurrence and modal month.

HRV strains circulating in the KHDSS were phylogenetically distant to contemporaneous HRV strains circulating elsewhere globally an indication that the viruses circulating in the KHDSS might have been seeded or originated from a local source (within Kilifi). However, some strains were phylogenetically close to strain circulating in USA, Cyprus, Egypt, Tunisia, Thailand and India suggesting possible origins of these HRV strains.

There were frequent introductions of HRV types into the KHDSS, in some cases multiple strains of the same HRV type were introduced into this area at different times.

Persistence of HRV in the KHDSS population was characterized by multiple introductions of HRV types, in some cases introduction were primarily new-lineage introductions within the same HRV type.

This study fell short to fully reconstruct the transmission patterns of HRV infection due to lack of social contact patterns of individuals or epidemiological link between people who reported infection with same HRV-type which could have allowed for the mapping of who infected whom and also spread of the virus in the population. In future, such studies should consider collecting such data which is crucial in reconstructing transmission pathways. Moreover, such studies should consider use of whole genome sequences compared to partial sequences to have a better resolution of transmission networks instead of partial sequences. Whole genome sequences provide better

phylogenetic resolution in tracking RSV spread compared to analysis of small partial sequences. Overall, the results of this led me to reject the hypothesis that HRV strains within the three known rhinovirus species circulating within different locations of KHDSS are not phylogenetically related to each other or to the contemporaneous strains circulating globally.

5.9 Study limitations

This study had three major limitations. First, failure to sequences VP4/VP2 coding region of 117/423 HRV positives samples might have led to missing out on some HRV types circulating in the KHDSS. Secondly, there was low patient recruitment in the initial study months (December 2015 and January 2016) due to clinic closure on public holidays or people migrating to other areas of the country during festive season. This may have contributed to the lower observed HRV prevalence compared to the other months of study. Finally, asymptomatic infections were not included in the study, and since HRV infections can present asymptomatically in adults there could be bias in the prevalence and the actual HRV types in circulation in the KHDSS.

5.10 Recommendations

This study recommends inclusion of asymptomatic patients in similar studies. Since asymptomatic individuals can carry HRV without observable clinical symptoms yet there can still transmit the virus, it will be important to include this group in similar future study. This study recommends further studies to characterize the introduction and mechanism of persistence of HRV types in the community over a long period (~10 years). Studies covering long periods will be able to elucidate the issue of successive epidemics caused by the introductions of divergent variants of the same HRV types in the community over time. Moreover, such kind of data will be important to understand the mechanism by which strains of the same re-invades the KHDSS over time. Future studies should consider including whole genome analysis with other epidemiological information (e.g. symptoms onset, date of sample collection, social contact patterns, etc.) to allow for the reconstruction of transmission pathways of HRV within human population.

In summary, insights from this study contribute to the ongoing research to understand the molecular epidemiology if HRV in terms of HRV types in circulation, their spread patterns and mechanism of persistence.

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APPENDICES

Appendix I: Ethical approval letter

The protocol for this study was approved by the Scientific Steering Committee (SSC) and the Ethical Review Boards of the Kenya Medical Research Institute (KEMR. Below is the approval letter from the National Ethics Review Committee.

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **September 11, 2015**

TO: **JOYCE UCHI NYIRO**
PRINCIPAL INVESTIGATOR

THRO': **DR. BENJAMIN TSOFA,**
THE DIRECTOR, CGMR-C,
KILIFI

Dear Madam,

RE: PROTOCOL NO. KEMRI/SERU/CGMR-C/015/3103 (*RESUBMISSION*): CHARACTERIZATION OF TRANSMISSION PATHWAYS OF VIRAL RESPIRATORY INFECTIONS IN KILIFI *(VERSION 1.1 DATED 6TH SEPTEMBER 2015).*

Reference is made to your letter dated 25th August, 2015 of which the KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt on the 31st August 2015.

This is to inform you that the Committee determined that the issues raised at the 241st meeting of the KEMRI/Ethics Review Committee held on July 21, 2015, have been adequately addressed. Consequently, the study is granted approval for implementation effective this day, **11th September, 2015.** Please note that authorization to conduct this study will automatically expire on **10th September, 2016**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to SERU by **July 30, 2016**.

You are required to submit any proposed changes to this study to the SERU for review and the changes should not be initiated until written approval from the SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the SERU and you should advise them when the study is completed or discontinued.
You may embark on the study.

Yours faithfully,

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

RECEIVED
15 SEP 2015
DIRECTOR'S OFFICE

In Search of Better Health

Appendix II: Information and consent form

Health Facility – based sampling study ICF

KEMRI Wellcome Trust Research Programme: Information Sheet and Consent Form

Investigating patterns of spread of viral respiratory infections in Kilifi County

Introduction: Your child's illness/symptoms

You (your child) have come to this hospital because of a severe cold. Severe cold is caused by germs which spread through close contacts via large nasal droplets among people from infected persons. Commonly, people (especially older children and adults) have this germ in their noses and throats without any symptoms at all. However, when they pass it on to someone else, that person may develop symptoms. Some germs have treatment while others do not. That is why KEMRI is conducting a study to get knowledge of these germs that will be used to find ways to stop their spread. In this hospital, the doctor will give you standard care of your illness and where possible some tests will be done to confirm the exact cause of your illness. As you continue with the standard procedures for your care, we would like to involve you in our study procedures too.

Who is carrying out this study?

This study is being conducted by KEMRI. KEMRI is a government organization that carries out medical research to find better ways of preventing and treating illness in the future for everybody's benefit.

What is this study about?

- One of the research problems that KEMRI currently seeks to understand is how germs that cause infection of the airways spread in the community, their source and how they change with time. In this study, we aim to find out the spread of airways germs

including, their routes of introduction, pattern of spread, persistence and disappearance at different times and locations using genetic fingerprinting methods. This is because some of these germs cause respiratory infections like pneumonia or severe cold which leads to hospitalization among children under 5 years. Vaccination is the ideal method of preventing diseases caused by these germs, but there is no licensed vaccine to most of them yet. To control infections and spread of these germs requires knowledge on the networks of their spread which this study seeks to answer.

- This study will involve all people presenting to this health facility with symptoms of a severe cold or pneumonia without any preference to either age or sex. We are conducting this study within 9 selected health facilities (Matsangoni, Ngerenya, Sokoke, Mtondia, Mavueni, Jaribuni, Chasimba, Pingilikani and Junju). We plan to do this study for 2 years from September 2015 to August 2017 and we expect to recruit about 14000 people. We are asking all people coming to this hospital with a severe cold or pneumonia to participate in this study by providing a nasal and an oral sample.
- We are asking if you/your child can participate in this study to help us learn more about the networks in which these germs spread since you have been found to have signs of the illness we are studying.

What will it involve for me/my child if I agree?

If you agree / allow your child to participate in this research, the following observations will be done on you / your child **in addition** to those needs for normal care:

- We will take a sample of mucus from the nose during this hospital visit. The nasal specimen will be collected by inserting a sterile cotton swab in one nostril and twisting 3 times before gently removing it (takes about 10 seconds) [*show devices and methods*].
- We will also take another sample from the mouth.
- We will also like to ask you some simple questions about you/your household related to history of respiratory illness. (I will ask you those questions that the clinician did

ask you, while tests and measurements done for this illness, I will obtain that from the clinician's report).

- Everything else that is done over the course of this visit will be part of the normal tests and treatment requested by the doctor.

Are there any risks or disadvantages to me or my child in taking part?

- Our priority for every patient is his/her care.
- When we collect a nasal mucus sample you/your child may experience some mild discomfort but there are no known risks associated with taking this sample. All the procedures will be undertaken by trained and experienced staff.
- The study procedures will take about 30 minutes of your time within this health facility.

Are there any advantages to me/my child of taking part?

There will be no direct benefits to you/child for participating in this study. However, by participating in this study, you will have helped us to understand more on the networks in which the respiratory germs spread and infect individuals within Kilifi, which will help to inform on the most effective prevention strategies. The results of this study may have the potential to inform on the best design of prevention strategy against common respiratory germs and may improve the care of other people with this illness in the future.

What happens if I refuse to participate?

All participation in this research is voluntary. You are free to decide if you want / your child to take part. You/your child will still receive the recommended standard of care even if you/they do not take part. If you do agree you can change your mind at any time and withdraw [your child] from the research. This will not affect you/your child's care now or in the future.

What happens to the samples?

- Individual names are removed from all samples and replaced by codes, to ensure that samples can only be linked to the participants by people closely concerned with the research.
- Most of the research tests that will be done on the sample will be done here in Kilifi. However, for some test especially the genetic fingerprinting tests that cannot be done in Kenya will require part of the samples to be sent to our collaborators laboratories overseas in the UK.
- After the research, a small portion of the nasal samples will be stored in the freezer at KEMRI Kilifi. In the future, new research about illness caused by infection of airways, or pneumonia may be done on these samples. Future research must first be approved by a national independent expert committee to ensure participants' safety and rights are respected.

Who will have access to information about me/my child in this research?

- All our research records are stored securely in locked cabinets and password protected computers. In future, information collected or generated during this study may be used to support new research by other researchers in Kenya and other countries on other health problems. In all cases, we will only share information with other researchers in ways that do not reveal individual participant identities. For example, we will remove information that could identify people, such as their names and where they live, and replace this information with number codes. Any future research using information from this study must first be approved by a local or national expert committee to make sure that the interests of participants and their communities are protected.
- Sharing of information on genomic data of the respiratory germs arising from this study will not include any participant identifiers.
- We will share anonymized individual and summary information we collect or generate with our collaborators in the UK in ways that do not reveal individual participants' identities.

Who has allowed this research to take place?

All research at KEMRI has to be approved before it begins by several national and international committees who look carefully at planned work. They must agree that the research is important, relevant to Kenya and follows nationally and internationally agreed research guidelines. This includes ensuring that all participants' safety and rights are respected.

What if I have any questions?

You may ask any of our staff questions at any time. You can also contact the research team using the contacts below:

Ms Joyce Nyiro, Dr. Patrick Munywoki or Prof. James Nokes, KEMRI Wellcome Trust Research Programme, P. O. Box 230, Kilifi. Telephone: 0722558143, 0725514400 or 0722 203417, 0733 522063, 041 7522063

If you want to ask someone independent anything about this research, please contact:

Community Liaison Manager, KEMRI Wellcome Trust Research Programme, P.O. Box 230, Kilifi. Telephone: 0723 342 780/0738 472 281 or 041 7522 063

And

The Secretary - KEMRI/Scientific and Ethics Review Unit SERU, P. O. BOX 54840-00200, Nairobi, Tel number: 020 272 2541 Mobile: 0722 205 901 or 0733 400 003

KEMRI-Wellcome Trust Research Programme Consent form for investigating patterns of spread of viral respiratory infections in Kilifi County

I, [being a parent/guardian of ________________________________ (Name/name of child)], have had the research explained to me. I have understood all that has been read/explained and had my questions answered satisfactorily.

Please tick the boxes below where relevant:

I agree to take part/allow my child to take part in this research

I agree to samples being stored and used for future research

I agree to samples being exported

I understand that I can change my mind at any stage and it will not affect me/my child in any way.

Subject/Parent/guardian's signature: ___________________________**Date**_______

Subject/Parent/guardian's name: ______________________________**Time** _______

(Please print name)

Where parent/guardian cannot read, ensure a witness* observes consent process and signs below:

I attest that the information concerning this research was accurately explained to and apparently understood by the subject/parent/guardian and that informed consent was freely given by the participant/parent/guardian.

Witness' signature: ___________________________________ **Date** _____________

Witness' name: ______________________________________ **Time** ______________

**A witness is a person who is independent from the study or a member of staff who was not involved in gaining the consent.*

Thumbprint of the parent as named above if they cannot write: -------------------------------

[Following section is recommended, and in some cases, must be signed by person undertaking informed consent]

I have followed the study SOP to obtain consent from [participant/guardian]. S/he apparently understood the nature and the purpose of the study and consents to the participation [of the child] in the study. S/he has been given opportunity to ask questions which have been answered satisfactorily.

Designee/investigator's signature: ______________________________ **Date** _____________

Designee/investigator's name: ______________________________**Time** _____________

(Please print name)

THE SUBJECT/PARENT/GUARDIAN SHOULD NOW BE GIVEN A SIGNED COPY TO KEEP

Appendix III: Information and assent form for children 13-17 years

Title: Characterization of transmission pathways of viral respiratory infections in Kilifi County

Lay Title: Investigating patterns of spread of viral respiratory infections in Kilifi County

Who is carrying out this study?

This study is being carried out by KEMRI. KEMRI is a government organization that carries out medical research to find better ways of preventing and treating illness in the future for everybody's benefit.

What is this research study trying to find out and why are you being asked to participate?

KEMRI is currently doing research to understand how germs that cause infection of the airways spread in the community, their source and how they change with time. In this study, we aim to find out the spread of airways germs including, their routes of introduction, pattern of spread, persistence and disappearance at different times and locations using genetic fingerprinting methods. This is because some of these germs cause airway infections like pneumonia or severe cold which leads to hospitalization among children under 5 years. Vaccination is the ideal method of preventing diseases caused by these germs, but there is no licensed vaccine to most of them yet. To control infections and spread of these germs requires knowledge on the networks of their spread which this study seeks to answer.

We are asking all children between 13 to 17 years to participate in the study by providing a nasal and oral sample. This study will involve all children attending this health facility with signs of respiratory infection. We are asking if you would participate in this study.

You can choose if you want to be in this study:

- We have spoken to your parent about this research and they are aware that we are talking to you. All participation in research is voluntary, and you are free to decide whether you want to take part or not. If you agree now, you can change your mind and

stop participating in the future and no one will be upset with you. This will not affect you/your health care now or in the future.

- If you agree to take part in this research, the following things will happen;
- We will take a sample of mucus from the nose during this hospital visit. The nasal specimen will be collected by inserting a sterile cotton swab in one nostril and twisting 3 times before gently removing it (takes about 10 seconds) [*show devices and methods*].
- We will also take another sample from the mouth.
- We will also like to ask you some simple questions about you/your household related to history of respiratory illness. (I will ask you some questions about your family and this illness, while questions about the tests and measurements done for this illness, will be obtained that from the clinician's report).
- Everything else that is done over the course of this visit will be part of the normal tests and treatment requested by the doctor.

Are there any disadvantages of taking part?

- Taking care of your health and wellbeing is important to us.
- When we collect a nasal mucus sample you child may experience some mild discomfort but there are no known risks associated with taking this sample. All the procedures will be undertaken by trained and experienced staff.

What will I gain from participating in this research study?

There will be no direct benefits to you for participating in this study. However, by participating in this research, you will have helped us to understand more on the networks in which the respiratory germs spread and infect individuals within Kilifi, which will help to inform on the most effective prevention strategies. The results of this study may have the potential to inform on the best design of prevention strategy against common respiratory germs and may improve the care of other people with this illness in the future.

What happens to the samples?

- Individual names are removed from all samples and replaced by codes, to ensure that samples can only be linked to the participants by people closely concerned with the research.
- Most of the research tests that will be done on the sample will be done here in Kilifi. However, for some test especially the genetic fingerprinting tests that cannot be done in Kenya will require part of the samples to be sent to our collaborators laboratories overseas in the UK.
- After the research, a small portion of the nasal samples will be stored in the freezer at KEMRI Kilifi. In the future, new research about respiratory illness or pneumonia may be done on these samples. Future research must first be approved by a national independent expert committee to ensure participants' safety and rights are respected.

Who will have access to the information I give?

The information collected from this study will be stored in securely locked cabinets and password protected computers. This information will only be shared with people who are concerned with the research. The information will be summarized, and all the names of the participants will be removed from the documents. This study information may be used for future work; the information will only be provided after a national independent committee checks and agrees that you will not be affected in any way.

What if I have any questions?

You are free to discuss your decision about taking part in this study with your parent/guardian or other people and you can ask to be given time to go and discuss this with them.

You are free to ask questions to any of the staff at any time. You can also contact the research team using these contacts:

Ms Joyce Nyiro, Dr. Patrick Munywoki or Prof. James Nokes, KEMRI Wellcome Trust Research Programme, P.O. Box 230, Kilifi. Telephone: 0722558143 or 0722 203417, 0733 522063, 041 7522063

If you want to ask someone who is not related to this research about this work please contact:

Community Liaison Manager, KEMRI Wellcome Trust Research Programme, P.O. Box 230, Kilifi. Telephone: 0723 342 780 or 041 7522 063

And

The Secretary, KEMRI/Scientific and Ethics Review Unit, P. O. BOX 54840-00200, Nairobi, Tel number: 020 272 2541 Mobile: 0722 205 901 or 0733 400 003

[Optional: If child is able to sign]

KEMRI-Wellcome Trust Research Programme Assent form for investigating patterns of spread of viral respiratory infections in Kilifi County

Appendix IV: Study questionnaire

Characterization of transmission pathways of viral respiratory infections in Kilifi County

Date today (DD/MM/YYYY) __ __ /__ __/ 20__ __ Time: ___: ___hrs.

Section I: Person details

Patient Names: 1____________________ 2____________________ 3____________________

Study Person id: __ __ __ __ ___ Date of birth: __ __ __ __ ___ __ Sex: ____ (M/F)

DSS PID: __ __ __ __ __ __ __ Residence (Village): _____________

For Non-DSS Residents;

Specify County: ___________________ Residence (Location): ___________________

Recent travel outside KHDSS in last 2 weeks: [ ] y/n

Where from: ___________________

Education level: 1. Primary [ ] 2. Secondary [ ] 3. Tertiary [ ] [ ]

Ethnicity (specify): ___________________

Occupation (Specify): ___________________

Brought to health facility by (caretaker names): _______________________________

Relation to the patient: ________________

Referred by: ____________FW/Self/Study clinician

Section II: Anthropometrics (*Fieldworker to fill**)*

i) Anthropometrics: (For children <12 years)

Weight [ ] [ ] [ ]. [ ] [ ] *kg*

MUAC [ ] [ ]. [ ] *cm*

Height/Length [ ] [ ] [ ]. [ ] *cm*

ii) Oxygen saturation [ ] [ ] [ ] %

iii) Heart rate [ ] [ ] [ ]/min

iv) Axillary temperature [ ] [ ].[ ] 0C

v) Respiratory rate [ ] [ ]/min

Section III: Clinical assessment (*Clinician to fill****)***

vi) How long has the patient been sick? [ ] [ ] days

vii) What are the main complaints? (*Please indicate Yes or No*)

Fever [ ] y/n

Cough [ ] y/n

Nasal discharge/congestion [ ] y/n

Difficulty in breathing [ ] y/n

Nasal flaring [ ] y/n

Sore throat [ ] y/n

Chest wall indrawing [ ] y/n

Crackles [ ] y/n

Wheezes [ ] y/n

Unable to feed [ ] y/n

Head nodding [ ] y/n

Cyanosis [ ] y/n

viii) Conscious level (*please tick one)*

Alert [ ] Lethargic [ ] Prostrate [ ] Unconscious [ ]

ix) Other complaints *(specify in the box below)*

x) Laboratory tests:

Malaria test: *Indicate results as either positive, negative, equivocal or not done*

Rapid diagnostic test: [ ]

Peripheral blood slide: [ ]

xi) Primary diagnosis (s*pecify*): ____

xii) Other diagnoses *(specify in the box below)*

xiii) Treatment given *(List all the drugs prescribed in the box below)*

xiv) Does the person require referral for hospital (KDH) admission [ ] (y/n)

xv) Initials of the clinician reviewing the patient [ ]

Section III: Specimen Collection *(Fieldworker to fill)*

i) Is the primary diagnosis ARI? [ ] (y/n)

ii) If yes, has the swab been collected: [ ] (y/n)

iii) If nasal sample is not collected specify reason: ________________________________

iv) Nasal specimens collected by *(indicate your initials)* [ ]

<u>Notes for the completion of the Health facility study Clinic visit proforma</u>

General instructions

- This clinical assessment form is to be filled for all clinic visits made by the HF study participants presenting at the following health facilities, Matsangoni, Ngerenya, Sokoke, Mtondia, Mavueni, Jaribuni, Chasimba and Junju with symptoms of acute respiratory infection.
- The form will be filled by the study field worker assisted by the clinician.
- Nasal or oral specimen should be taken to all eligible participants with ARI symptoms.

Section I: Patient details

Indicate the patient ***full names***, the ***person id*** (unique study number) and the ***DSS PID***.

Sex: Indicate whether male or female

Date of birth: Indicate the day month and year of birth.

Residence: This should be the village of residence

Caretaker: Refers to the person who brought or accompanied the patient to the clinic. Note the ***relation*** of the caretaker with the patient. He/she could be from the same household as the patient or not.

Referral: Establish who referred the patient to the clinic. Whether it's the study FWs, clinician or it's a self-referral

Section II: Clinical assessment

Weight: measured in Kgs into 2 decimal places. Ensure the weighing scales are calibrated every week.

MUAC: mid-upper arm circumference; reported in cm. This will only be collected in children who are below 13 years old.

Height/Length: measure in cms

Oxygen saturation: Use pulse oximeter to measure oxygen saturation. Recheck if the oxygen saturation is below 95% and if it is less than 50% there is likely to be a problem with the Oximeter – repeat measurement. If less than 90% repeat the procedure and compare the two readings. Ensure person is at rest and at ease.

Heart rate*: or pulse rate:* Use the readings from the pulse oximeter or count peripheral pulse rate for one complete minute!

Axillary temperature: Take axillary temperature using a digital thermometer e.g. accuracy 37.3

Respiratory rate: Also referred to as breaths per minute. Count respirations for one complete minute. Ensure person is in calm stated and rested before starting. If a child it should not be agitated or crying for example. Record the breathing rate as accurately as possible. Ensure the participant has settled before recording. Assess if child has fast breathing (i.e. >50 for all ages, and >40 if 12 months or over).

Duration sick: Enquire the duration (number in days) the patient has been ill. If the person has been ill for more than a month exclude from the study.

Main complaints: during the clinical assessment of the illness establish whether any of the listed respiratory-related signs and symptoms are reported.

Other complaints: Note the presence of any other complaints which might be accompany respiratory illness e.g. diarrhoea, vomiting

Laboratory tests: Please indicate any malaria parasite test results for ***Rapid diagnosis*** and or ***blood slide*** as positive, negative, equivocal or not done.

Primary diagnosis: Indicate the final/main diagnosis based on patient complains and any laboratory tests done.

Other diagnoses: Note any other diagnosis where applicable.

Referral: Indicate if the person requires further clinical care (hospital admission) in the Kilifi District Hospital.

Section III: Specimen Collection

Collect a nasal and oral specimen if the person has symptoms of ARI. If nasal sample is not collected specify ***reasons***. Please indicate the **initials** of the FW/clinician collecting the sample.

Appendix V: RNA concentration of HRV positive samples

	Sample_ID	RNA Conc.(ng/uL)		Sample_ID	RNA Conc.(ng/uL)
1	CHA-110005_2015-12-14	41.71	35	CHA-110194_2016-05-03	86.34
2	CHA-110026_2016-01-12	77.61	36	CHA-110191_2016-05-03	159.42
3	CHA-110036_2016-01-22	78.09	37	CHA-110189_2016-05-03	32.51
4	CHA-110061_2016-02-09	55.03	38	CHA-110200_2016-05-13	86.55
5	CHA-110060_2016-02-09	32.86	39	CHA-110219_2016-05-17	117.52
6	CHA-110062_2016-02-09	148.51	40	CHA-110213_2016-05-17	15.30
7	CHA-110075_2016-02-19	84.66	41	CHA-110229_2016-05-27	48.53
8	CHA-110068_2016-02-19	47.27	42	CHA-110221_2016-05-27	120.17
9	CHA-110076_2016-02-19	15.71	43	CHA-110234_2016-05-27	146.02
10	CHA-110106_2016-03-04	171.11	44	JAR-120036_2016-01-15	167.03
11	CHA-110103_2016-03-04	103.70	45	JAR-120039_2016-01-20	150.78
12	CHA-110107_2016-03-04	19.96	46	JAR-120051_2016-01-29	180.99
13	CHA-110119_2016-03-11	46.52	47	JAR-120055_2016-01-29	42.93
14	CHA-110126_2016-03-18	119.68	48	JAR-120075_2016-02-12	80.50
15	CHA-110122_2016-03-18	138.14	49	JAR-120072_2016-02-12	19.79
16	CHA-110129_2016-03-18	39.21	50	JAR-120083_2016-02-17	172.29
17	CHA-110135_2016-03-22	51.62	51	JAR-120081_2016-02-17	167.13
18	CHA-110140_2016-03-30	45.09	52	JAR-120079_2016-02-17	113.24
19	CHA-110142_2016-03-30	19.13	53	JAR-120087_2016-02-26	53.04
20	CHA-110141_2016-03-30	13.46	54	JAR-120098_2016-02-26	136.11
21	CHA-110147_2016-04-05	32.58	55	JAR-120101_2016-03-02	30.82
22	CHA-110146_2016-04-05	68.34	56	JAR-120122_2016-03-15	134.22
23	CHA-110145_2016-04-05	70.09	57	JAR-120135_2016-03-23	161.07
24	CHA-110154_2016-04-07	79.74	58	JAR-120154_2016-04-08	21.88
25	CHA-110170_2016-04-19	114.43	59	JAR-120152_2016-04-08	85.71
26	CHA-110175_2016-04-19	35.46	60	JAR-120151_2016-04-08	28.86
27	CHA-110176_2016-04-19	27.73	61	JAR-120153_2016-04-08	158.04
28	CHA-110169_2016-04-19	26.66	62	JAR-120163_2016-04-12	59.15
29	CHA-110174_2016-04-19	117.85	63	JAR-120164_2016-04-12	70.15
30	CHA-110185_2016-04-29	105.33	64	JAR-120166_2016-04-12	177.14
31	CHA-110181_2016-04-29	81.33	65	JAR-120182_2016-04-27	70.67
32	CHA-110195_2016-05-03	61.20	66	JAR-120190_2016-04-29	185.37
33	CHA-110198_2016-05-03	127.07	67	JAR-120193_2016-04-29	35.55
34	CHA-110190_2016-05-03	173.35	68	JAR-120188_2016-04-29	153.83

	Sample_ID	RNA Conc.(ng/uL)		Sample_ID	RNA Conc.(ng/uL)
69	JAR-120204_2016-05-10	7.39	104	JNJ-130142_2016-04-06	104.32
70	JAR-120196_2016-05-10	107.99	105	JNJ-130143_2016-04-06	90.84
71	JAR-120205_2016-05-10	42.88	106	JNJ-130144_2016-04-06	37.78
72	JAR-120206_2016-05-10	91.73	107	JNJ-130145_2016-04-14	56.36
73	JAR-120212_2016-05-20	81.67	108	JNJ-130148_2016-04-14	140.11
74	JAR-120210_2016-05-20	162.68	109	JNJ-130151_2016-04-15	109.65
75	JAR-120232_2016-05-25	60.76	110	JNJ-130153_2016-04-15	104.72
76	JAR-120227_2016-05-25	122.97	111	JNJ-130170_2016-04-20	133.92
77	JAR-120229_2016-05-25	62.62	112	JNJ-130175_2016-04-25	148.53
78	JAR-120230_2016-05-25	141.36	113	JNJ-130176_2016-04-25	48.99
79	JNJ-130007_2015-12-16	177.59	114	JNJ-130177_2016-04-25	185.13
80	JNJ-130025_2016-01-13	120.73	115	JNJ-130179_2016-04-25	169.86
81	JNJ-130038_2016-01-27	83.67	116	JNJ-130188_2016-05-04	95.00
82	JNJ-130034_2016-01-27	176.11	117	JNJ-130185_2016-05-04	92.41
83	JNJ-130037_2016-01-27	60.89	118	JNJ-130194_2016-05-12	176.90
84	JNJ-130051_2016-02-04	143.85	119	JNJ-130193_2016-05-12	42.57
85	JNJ-130042_2016-02-04	161.49	120	JNJ-130195_2016-05-12	40.01
86	JNJ-130060_2016-02-10	153.36	121	JNJ-130211_2016-05-18	48.95
87	JNJ-130058_2016-02-10	136.43	122	JNJ-130208_2016-05-18	174.48
88	JNJ-130053_2016-02-10	185.68	123	JNJ-130209_2016-05-18	83.31
89	JNJ-130076_2016-02-15	142.29	124	JNJ-130217_2016-05-18	143.26
90	JNJ-130069_2016-02-15	184.45	125	JNJ-130236_2016-05-23	115.12
91	JNJ-130078_2016-02-24	142.97	126	JNJ-130232_2016-05-23	115.75
92	JNJ-130081_2016-02-25	109.61	127	JNJ-130229_2016-05-23	139.52
93	JNJ-130088_2016-02-29	47.59	128	JNJ-130225_2016-05-23	182.07
94	JNJ-130092_2016-02-29	65.10	129	JNJ-130235_2016-05-23	171.17
95	JNJ-130096_2016-03-09	26.52	130	MAT-140017_2015-12-15	104.93
96	JNJ-130100_2016-03-09	76.60	131	MAT-140012_2015-12-15	87.97
97	JNJ-130103_2016-03-17	27.70	132	MAT-140016_2015-12-15	147.00
98	JNJ-130113_2016-03-17	149.51	133	MAT-140013_2015-12-15	140.68
99	JNJ-130116_2016-03-23	16.57	134	MAT-140021_2016-01-05	80.43
100	JNJ-130114_2016-03-23	58.45	135	MAT-140020_2016-01-05	185.83
101	JNJ-130125_2016-04-01	162.96	136	MAT-140027_2016-01-14	62.48
102	JNJ-130119_2016-04-01	33.30	137	MAT-140032_2016-01-14	154.19
103	JNJ-130135_2016-04-06	179.20	138	MAT-140034_2016-01-14	83.83

	Sample_ID	RNA Conc.(ng/uL)		Sample_ID	RNA Conc.(ng/uL)
139	MAT-140041_2016-01-21	163.82	174	MAT-140207_2016-04-12	145.94
140	MAT-140061_2016-01-26	187.61	175	MAT-140203_2016-04-12	36.80
141	MAT-140075_2016-02-01	136.24	176	MAT-140205_2016-04-12	63.20
142	MAT-140080_2016-02-02	117.15	177	MAT-140213_2016-04-12	41.33
143	MAT-140077_2016-02-02	147.59	178	MAT-140212_2016-04-12	158.53
144	MAT-140089_2016-02-11	131.73	179	MAT-140223_2016-04-21	70.37
145	MAT-140085_2016-02-11	16.92	180	MAT-140220_2016-04-21	57.50
146	MAT-140102_2016-02-18	171.54	181	MAT-140226_2016-04-21	124.50
147	MAT-140107_2016-02-18	84.31	182	MAT-140230_2016-04-28	172.98
148	MAT-140111_2016-02-23	171.18	183	MAT-140245_2016-05-06	107.16
149	MAT-140116_2016-02-23	182.41	184	MAT-140255_2016-05-09	179.77
150	MAT-140117_2016-02-23	57.86	185	MAT-140268_2016-05-19	126.01
151	MAT-140115_2016-02-23	85.05	186	MAT-140286_2016-05-26	39.72
152	MAT-140133_2016-03-03	14.40	187	MAT-140292_2016-05-31	57.98
153	MAT-140132_2016-03-03	68.23	188	MAT-140296_2016-05-31	86.62
154	MAT-140131_2016-03-03	112.66	189	MAT-140291_2016-05-31	70.89
155	MAT-140135_2016-03-08	63.85	190	MAT-140294_2016-05-31	13.07
156	MAT-140139_2016-03-08	181.84	191	MAT-140299_2016-05-31	88.56
157	MAT-140140_2016-03-08	145.08	192	MAV-150014_2015-12-14	34.21
158	MAT-140154_2016-03-14	101.65	193	MAV-150005_2015-12-14	97.32
159	MAT-140161_2016-03-24	114.11	194	MAV-150028_2016-01-20	137.78
160	MAT-140167_2016-03-24	79.84	195	MAV-150042_2016-02-02	20.07
161	MAT-140166_2016-03-24	187.56	196	MAV-150059_2016-02-09	79.42
162	MAT-140162_2016-03-24	12.23	197	MAV-150075_2016-02-17	159.76
163	MAT-140163_2016-03-24	121.84	198	MAV-150066_2016-02-17	89.00
164	MAT-140181_2016-03-31	159.04	199	MAV-150093_2016-02-22	92.14
165	MAT-140184_2016-03-31	187.96	200	MAV-150100_2016-03-02	187.76
166	MAT-140175_2016-03-31	150.13	201	MAV-150103_2016-03-02	144.73
167	MAT-140199_2016-04-05	27.24	202	MAV-150116_2016-03-07	43.28
168	MAT-140189_2016-04-05	144.32	203	MAV-150115_2016-03-07	99.88
169	MAT-140174_2016-04-05	148.81	204	MAV-150117_2016-03-07	119.89
170	MAT-140197_2016-04-05	34.82	205	MAV-150132_2016-03-18	180.52
171	MAT-140200_2016-04-05	152.05	206	MAV-150155_2016-03-30	17.38
172	MAT-140198_2016-04-05	92.48	207	MAV-150149_2016-03-30	67.74
173	MAT-140194_2016-04-05	164.13	208	MAV-150151_2016-03-30	11.05

	Sample_ID	RNA Conc.(ng/uL)		Sample_ID	RNA Conc.(ng/uL)
209	MAV-150172_2016-04-04	160.53	244	MTO-160085_2016-02-03	147.69
210	MAV-150167_2016-04-04	62.95	245	MTO-160086_2016-02-03	75.82
211	MAV-150181_2016-04-12	168.27	246	MTO-160097_2016-02-08	134.34
212	MAV-150186_2016-04-12	14.20	247	MTO-160100_2016-02-08	133.40
213	MAV-150175_2016-04-12	107.89	248	MTO-160093_2016-02-08	81.30
214	MAV-150187_2016-04-12	110.94	249	MTO-160098_2016-02-08	65.26
215	MAV-150204_2016-04-27	172.26	250	MTO-160092_2016-02-08	50.97
216	MAV-150209_2016-04-27	80.37	251	MTO-160103_2016-02-08	157.41
217	MAV-150216_2016-05-03	103.91	252	MTO-160099_2016-02-08	79.50
218	MAV-150213_2016-05-03	12.03	253	MTO-160119_2016-02-16	14.50
219	MAV-150225_2016-05-10	177.96	254	MTO-160124_2016-02-25	73.23
220	MAV-150224_2016-05-10	75.69	255	MTO-160136_2016-03-01	93.46
221	MAV-150227_2016-05-10	186.57	256	MTO-160144_2016-03-01	128.41
222	MAV-150223_2016-05-10	16.73	257	MTO-160150_2016-03-01	43.84
223	MAV-150229_2016-05-17	51.37	258	MTO-160159_2016-03-10	46.43
224	MAV-150234_2016-05-17	10.22	259	MTO-160174_2016-03-16	52.48
225	MAV-150246_2016-05-25	172.60	260	MTO-160187_2016-03-21	163.36
226	MAV-150245_2016-05-25	34.18	261	MTO-160199_2016-03-29	97.09
227	MAV-150257_2016-05-26	26.67	262	MTO-160191_2016-03-29	17.04
228	MAV-150255_2016-05-26	144.18	263	MTO-160216_2016-04-07	111.94
229	MAV-150267_2016-05-30	171.85	264	MTO-160212_2016-04-07	186.18
230	MAV-150266_2016-05-30	157.87	265	MTO-160213_2016-04-07	153.55
231	MAV-150258_2016-05-30	56.97	266	MTO-160224_2016-04-13	36.54
232	MAV-150263_2016-05-30	33.63	267	MTO-160230_2016-04-13	102.90
233	MTO-160020_2015-12-17	80.26	268	MTO-160233_2016-04-18	33.45
234	MTO-160019_2015-12-17	160.06	269	MTO-160240_2016-04-18	14.81
235	MTO-160027_2016-01-07	17.53	270	MTO-160258_2016-04-26	114.73
236	MTO-160037_2016-01-11	34.91	271	MTO-160253_2016-04-26	50.42
237	MTO-160044_2016-01-11	82.57	272	MTO-160251_2016-04-26	87.89
238	MTO-160043_2016-01-11	60.51	273	MTO-160291_2016-05-16	98.53
239	MTO-160054_2016-01-19	131.18	274	MTO-160284_2016-05-16	128.15
240	MTO-160074_2016-01-28	148.08	275	MTO-160307_2016-05-24	33.99
241	MTO-160069_2016-01-28	15.38	276	MTO-160305_2016-05-24	150.72
242	MTO-160071_2016-01-28	54.39	277	MTO-160303_2016-05-24	116.16
243	MTO-160084_2016-02-03	101.04	278	NGE-170013_2015-12-15	72.08

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280	NGE-170037_2016-01-21	74.15	315	PIN-180040_2016-01-27	16.72
281	NGE-170039_2016-01-21	134.27	316	PIN-180052_2016-02-04	20.60
282	NGE-170040_2016-01-21	187.41	317	PIN-180050_2016-02-04	82.54
283	NGE-170054_2016-02-01	158.09	318	PIN-180057_2016-02-04	14.00
284	NGE-170064_2016-02-01	115.76	319	PIN-180065_2016-02-10	148.66
285	NGE-170062_2016-02-01	89.51	320	PIN-180069_2016-02-10	160.10
286	NGE-170083_2016-02-18	169.06	321	PIN-180071_2016-02-10	137.75
287	NGE-170082_2016-02-18	41.29	322	PIN-180070_2016-02-10	108.71
288	NGE-170088_2016-02-18	161.22	323	PIN-180066_2016-02-10	126.33
289	NGE-170101_2016-02-24	152.67	324	PIN-180074_2016-02-15	187.76
290	NGE-170106_2016-03-03	90.72	325	PIN-180076_2016-02-15	92.40
291	NGE-170121_2016-03-14	80.38	326	PIN-180098_2016-02-24	86.70
292	NGE-170130_2016-03-14	55.12	327	PIN-180092_2016-02-24	23.63
293	NGE-170138_2016-03-24	150.18	328	PIN-180091_2016-02-24	10.74
294	NGE-170139_2016-03-24	67.38	329	PIN-180108_2016-02-29	100.51
295	NGE-170132_2016-03-24	163.04	330	PIN-180105_2016-02-29	102.14
296	NGE-170137_2016-03-24	29.49	331	PIN-180102_2016-02-29	37.71
297	NGE-170144_2016-03-31	116.74	332	PIN-180107_2016-02-29	42.79
298	NGE-170141_2016-03-31	43.44	333	PIN-180103_2016-02-29	80.65
299	NGE-170149_2016-03-31	99.05	334	PIN-180109_2016-02-29	53.94
300	NGE-170157_2016-04-05	27.90	335	PIN-180122_2016-03-17	15.34
301	NGE-170174_2016-04-12	46.81	336	PIN-180136_2016-03-23	147.02
302	NGE-170173_2016-04-12	45.36	337	PIN-180146_2016-04-01	20.34
303	NGE-170170_2016-04-12	134.40	338	PIN-180166_2016-04-20	185.62
304	NGE-170182_2016-04-12	94.73	339	PIN-180176_2016-04-25	154.47
305	NGE-170204_2016-04-28	141.43	340	PIN-180180_2016-05-04	122.11
306	NGE-170235_2016-05-19	87.53	341	PIN-180187_2016-05-12	104.25
307	NGE-170242_2016-05-26	170.47	342	PIN-180186_2016-05-12	14.51
308	NGE-170256_2016-05-27	135.25	343	PIN-180184_2016-05-12	66.24
309	NGE-170252_2016-05-27	120.10	344	PIN-180191_2016-05-12	186.42
310	PIN-180019_2016-01-08	63.90	345	PIN-180185_2016-05-12	167.30
311	PIN-180012_2016-01-08	44.09	346	PIN-180182_2016-05-12	57.23
312	PIN-180032_2016-01-18	185.55	347	PIN-180192_2016-05-12	77.78
313	PIN-180028_2016-01-18	111.86	348	PIN-180199_2016-05-18	104.54

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351	SOK-190019_2016-01-07	39.97	386	SOK-190233_2016-05-24	168.71
352	SOK-190032_2016-01-11	149.47	387	KCH-135344_2015-12-08	47.24
353	SOK-190002_2016-01-13	32.76	388	KCH-135355_2015-12-08	45.69
354	SOK-190038_2016-01-19	50.28	389	KCH-135567_2015-12-17	161.98
355	SOK-190054_2016-02-03	86.91	390	KCH-135580_2015-12-18	15.82
356	SOK-190058_2016-02-03	138.53	391	KCH-136128_2016-01-13	25.90
357	SOK-190062_2016-02-08	110.30	392	KCH-136135_2016-01-13	12.62
358	SOK-190075_2016-02-08	86.85	393	KCH-136293_2016-01-19	79.83
359	SOK-190069_2016-02-08	136.41	394	KCH-136386_2016-01-22	80.75
360	SOK-190073_2016-02-08	89.67	395	KCH-136418_2016-01-24	127.35
361	SOK-190084_2016-02-16	67.27	396	KCH-136481_2016-01-26	16.86
362	SOK-190085_2016-02-16	149.74	397	KCH-136523_2016-01-28	44.17
363	SOK-190078_2016-02-16	60.76	398	KCH-136657_2016-02-03	17.14
364	SOK-190101_2016-02-25	14.36	399	KCH-136752_2016-02-06	98.57
365	SOK-190102_2016-02-25	176.78	400	KCH-136787_2016-02-07	183.75
366	SOK-190108_2016-03-01	156.31	401	KCH-136937_2016-02-13	172.69
367	SOK-190106_2016-03-01	63.34	402	KCH-136977_2016-02-15	26.22
368	SOK-190124_2016-03-10	63.90	403	KCH-137057_2016-02-18	150.11
369	SOK-190134_2016-03-16	88.21	404	KCH-137073_2016-02-18	162.76
370	SOK-190151_2016-03-21	169.14	405	KCH-137091_2016-02-19	13.84
371	SOK-190145_2016-03-21	130.81	406	KCH-137121_2016-02-21	156.59
372	SOK-190156_2016-03-29	150.40	407	KCH-137269_2016-02-27	185.02
373	SOK-190160_2016-03-29	149.70	408	KCH-137285_2016-02-28	61.88
374	SOK-190174_2016-04-07	103.75	409	KCH-137297_2016-02-29	90.04
375	SOK-190169_2016-04-07	75.95	410	KCH-137306_2016-02-29	186.95
376	SOK-190165_2016-04-07	21.84	411	KCH-137406_2016-03-04	146.24
377	SOK-190177_2016-04-13	170.44	412	KCH-137411_2016-03-04	70.01
378	SOK-190179_2016-04-13	166.29	413	KCH-137466_2016-03-07	140.23
379	SOK-190197_2016-04-18	140.50	414	KCH-137505_2016-03-08	176.71
380	SOK-190202_2016-04-26	24.94	415	KCH-137556_2016-03-10	169.55
381	SOK-190213_2016-05-11	48.68	416	KCH-137584_2016-03-11	23.03
382	SOK-190226_2016-05-16	186.68	417	KCH-137936_2016-03-25	170.43
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421	KCH-138275_2016-04-11	110.10		
422	KCH-138325_2016-04-13	88.87		
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Appendix VI: GenBank accession number of contemporaneous sequences

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9	AB731148	41	JN855844
10	AB731149	42	JN855848
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21	HM044171	53	JN855933
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23	HM044178	55	JN855936
24	HM044179	56	JN855938
25	HM044196	57	JN855939
26	HQ190928	58	JN855941
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73	JQ356554	108	KF427798
74	JQ356564	109	KF427821
75	JQ356569	110	KF427828
76	JX162636	111	KF427829
77	JX162643	112	KF427833
78	JX863777	113	KF957898
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80	JX863798	115	KF957936
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83	JX863806	118	KF957941
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88	JX863816	123	KF957958
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183	KP736830	218	KR871697
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185	KP736834	220	KT272025
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