MOLECULAR CHARACTERIZATION OF HUMAN RESPIROVIRUS TYPE 3 ISOLATED IN KENYA BETWEEN 2010 AND 2013

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Molecular Characterization of Human Respirovirus Type 3 Isolated in Kenya between 2010 and 2013

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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 university supervisors

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DEDICATION

I dedicate this work to my parents for their financial support, prayers, and encouragement throughout my education.

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LIST OF ABBREVIATIONS AND ACRONYMS

ARI	Acute Respiratory Infections
CDC	Centre for Disease Control and Prevention
CPE	Cytopathic effect
CVR	Centre for Virus Research
DFAT	Direct fluorescent antibody test
FITC	Fluorescein isothiocyanate
HPIV's	Human parainfluenza Viruses
HRV1	Human respirovirus 1
HPIV2	Human parainfluenza 2
HRV3	Human respirovirus 3
HPIV4	Human parainfluenza 4
ILI	Influenza-like illness
IFAT	Immunofluorescence antibody test
KEMRI	Kenya Medical Research Institute
LLCMK2	Rhesus Monkey Kidney cell line
LRTI	Lower Respiratory Tract Infection
NIC	National Influenza Centre
OM	Otitis Media
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RSV	Respiratory Syncytial Virus
RT-PCR	Reverse transcriptase-polymerase chain reaction
SARI	Severe acute respiratory infection
URTI	Upper Respiratory Tract Infection
USAMRD-A	United States Army Medical Research Directorate – Africa
VTM	Viral Transport Media

ABSTRACT

Human respirovirus type 3 (HRV3) is among the leading causative agents of lower respiratory tract infections such as pneumonia and bronchiolitis in young children, ranking only second to the human respiratory syncytial virus (RSV) in this respect. Despite their public health significance, there is scanty information on the molecular characteristics and diversity of HRV3 strains circulating in Kenya. The general objective of this study was therefore to determine the molecular characteristics of the HRV3 virus isolated in Kenya between 2010 and 2013, during a time when there was an increase of respiratory diseases in the country worsened by the influenza pandemic. To address this gap, 35 complete hemagglutinin-neuraminidase (HN) sequences of HRV3 strains isolated in Kenya between 2010 and 2013 were analysed. Viral RNA was extracted from the isolates and the entire HN gene amplified by RT-PCR followed by nucleotide Sanger sequencing. Phylogenetic analyses of the sequences revealed that all the Kenyan isolates classified into genetic Cluster C; sub-clusters C1a, C2, and C3a. The majority (54%) of strains grouped into sub-cluster C3a, followed by C2 (43%) and C1a (2.9%). Sequence homology revealed a high similarity between the Kenyan isolates and the HRV3 prototype strain both at the nucleotide (94.3-95.6%) and amino acid (96.5 -97.9%) levels. No amino acid variations affecting the catalytic/active sites of the HN glycoprotein were observed among the Kenyan isolates. Selection analysis showed that the HN glycoprotein was evolving under positive selection. Evolutionary analyses showed that the mean TMRCA for the HN sequence dataset was estimated at 1942 (95% HPD: 1928 - 1957), while the mean evolutionary rate was $4.65x10^{-4}$ nucleotide substitutions/site/year (95% HPD: $2.99x10^{-4}$ to $6.35x10^{-4}$). Overall, the study demonstrates the introduction and co-circulation of multiple lineages of Cluster C HRV3 variants in Kenya during the study period. Furthermore, results obtained from this study provide more evidence of the conserved nature of the HRV3 HN glycoprotein. In conclusion, this study is the first to describe the genetic and molecular evolutionary aspects of HRV3 in Kenya using the complete HN gene. Accordingly, there is a need to establish constant surveillance of these important pathogens in the country. This will contribute to a better understanding of circulation trends, disease associations, and outbreaks of HRV3 strains circulating in Kenya. It will also contribute to the future development of antivirals/therapeutics, leading to better patient management. Furthermore, whole genome sequencing of Kenyan HRV3 should be utilized in future to provide deeper insights into the genetic and evolutionary dynamics of these viruses.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Human parainfluenza viruses (HPIVs) are significant aetiological agents of upper and lower respiratory tract infections (RTIs). The most susceptible groups are children, the elderly, and immunocompromised persons (Almajhdi *et al*., 2012, Schomacker *et al*., 2012, Organization, 2009). Globally, these viruses are responsible for about 40% of hospital consultations in children, with a mortality of approximately 1.9 million yearly (Nascimento-Carvalho *et al*., 2018). Viral upper respiratory tract infections (URTI) are mainly self-limiting; however, their complications are more important. These complications include the predisposition of young children to bacterial infections of the sinuses and middle ear that could lead to rheumatic fever and deafness (Mizuta *et al*., 2014). Furthermore, aspirations of the infected secretions due to URTIs can lead to lower respiratory tract infections (LRTI's), such as bronchiolitis and pneumonia. The lower respiratory tract consists of the areas covering the airways of the trachea, bronchi to the bronchioles, and alveoli. The common LRTIs are pneumonia and bronchiolitis. Bacterial and viral agents are the main aetiologies of LRTIs. These infections are responsible for about 4.3 million deaths worldwide, with the majority of these deaths occurring in developing countries (Linster *et al*., 2018). Studies show that infections with viruses lead to increased binding of bacterial pathogens, especially *Haemophilus influenzae* and *Streptococcus pneumoniae* to the cells lining the respiratory tract (Simoes *et al*., 2006, Symekhier *et al*., 2009). Nearly all children encounter HPIVs within the first few years after birth since their immunity is incomplete (Hall, 2002); hence re-infections occur throughout their life.

HPIV viruses belong to the family *Paramyxoviridae* and are grouped into four types, HPIV 1 – 4, based on their genetic and antigenic differences (Linster *et al*., 2018,

Smielewska *et al*., 2018). HPIV types 2 and 4 are members of the genus *Rubulavirus* whereas HPIV types 1 and 3 are classified as members of the genus *Respirovirus.* Thus, these two viruses are currently known as Human Respirovirus types 1 and 3 abbreviated as HRV1 and HRV3, respectively (Henrickson, 2003, Kuhn *et al*., 2010, Lee, 2012). Each of these virus types is associated with distinct respiratory disease manifestations. Amongst the respiroviruses, HRV3 is the most frequent aetiology of acute lower respiratory infections such as pneumonia and bronchiolitis in infants and young children, ranking second only to the human respiratory syncytial virus (RSV) in this regard (Almajhdi *et al*., 2012, Mao *et al*., 2012b, Linster *et al*., 2018, Bose *et al*., 2019).

HRV3 infections occur yearly, and approximately two-thirds of children are infected by this virus in the first year of life, mainly causing bronchiolitis and pneumonia (Mao *et al*., 2012b). According to the WHO (2009), HRV3 accounts for about 40% of hospital consultations and average mortality of four million globally. Although developing countries are most affected by these viruses, there exists little published literature on their epidemiology. For instance, HRV3 affects 64% and 26% of children in South Africa and Uganda respectively (Balinandi, 2012, Cohen *et al*., 2015). In Kenya, acute respiratory infections (ARI's) are a leading cause for hospital consultations and is the most critical childhood illness apart from fever and diarrhoea (Demographic, 2014).

The HRV3 genome consists of an enveloped, negative-sense, single-stranded RNA of approximately 15462 nucleotides. This genome encodes six viral proteins namely; the nucleocapsid protein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the large protein (L) (Kosutic-Gulija *et al*., 2017). The hemagglutinin-neuraminidase (HN) glycoprotein has neuraminidase and hemagglutinin activities (Lawrence *et al*., 2004). It is also involved in virus entry/budding, and it is the major virus surface antigen (Mizuta *et al*., 2014). HN protein recognizes and adsorbs to the host's cell-surface α2,6 or α2,3 sialic acid receptors, initiating virus infection (El Najjar *et al*., 2014, Schmidt *et al*., 2011, Kosutic-Gulija *et al*., 2017).

Also, the HN glycoprotein plays an essential role in promoting fusion of the F protein (Schmidt *et al*., 2011). Notably, the HN gene is antigenically and genetically highly variable; therefore, it is a favourite target in HRV3 molecular epidemiology studies (Chang and Dutch, 2012, Mao *et al*., 2012b, Kosutic-Gulija *et al*., 2017).

1.2 Statement of the Problem

HRV3 causes a broad spectrum of respiratory diseases leading to a 40% morbidity and 4.3 million mortality worldwide (Linster *et al*., 2018). These infections range from an influenza-like illness where 30-50% are complicated by ear infections (otitis media), to severe acute respiratory illness (SARI), where about 0.3% of the infected patients are hospitalised (Henrickson, 2003). Estimates from the World Health Organization (WHO, 2009), indicate that HRV3 will infect the majority of children by the age of two to five years. HRV3 is the most dominant serotype among parainfluenza viruses and has been associated with severe respiratory infections in children, the elderly, and immunocompromised individuals . These viruses circulate globally and have been studied extensively in most parts of the world, including Asia, the USA and Europe. In Africa, information is scarce concerning circulating strains of HRV3. For instance, in Kenya, a laboratory detection study on human parainfluenza viruses at the National influenza centre laboratory showed that HRV3 accounted for 80% of the human parainfluenza virus infections (Mitei *et al*., 2012). The epidemiology of HRV3 in Kenya is poorly understood due to difficulties in diagnosis in most clinical virology laboratories (Symekher *et al*., 2012, Mitei *et al*., 2012). Despite HRV3 being a pathogen of public health importance responsible for severe respiratory illnesses, especially among infants and children globally, data on genetic diversity and phylogenetic evolutionary aspects of HRV3 viruses circulating in Kenya remain scanty.

1.3 Justification

Previous studies have established that the HRV3 strains circulating globally are categorised into three main clusters; A, B, and C, with cluster C being the most dynamic (Almajhdi, 2015a).

Whereas molecular characteristics of these viruses have been studied extensively in other parts of the world, in Kenya, no information is available. This precedence calls for urgent surveillance of these viruses to provide in-depth knowledge of all the circulating strains in Kenya. Molecular characterization of HRV3 viruses is crucial in establishing the spatial and temporal circulating patterns, and the major strains of these viruses in a given season. This information is vital for proper health planning, HRV3 outbreak studies, and disease associations. Moreover, this information will contribute to future vaccine/therapeutics development efforts leading to better ARI patient management.

1.4 Research Questions

1. Were there any HRV3 strains circulating in Kenya between 2010 and 2013?
2. Were there any genetic mutations on the HN gene of HVR3 from the isolates?
3. What was the evolutionary relationship between the Kenyan HVR3 strains and the global strains?

1.5 Objectives

1.5.1 Broad Objective

To determine the molecular characteristics of HRV3 virus isolated in Kenya between 2010 and 2013

1.5.2 Specific Objectives

1. To determine the presence of HRV3 in nasopharyngeal samples from patients using combined cell culture and direct immunofluorescent assay (DFA)
2. To determine the HN gene nucleotide sequence and analyse for genetic mutations using bioinformatics tools
3. To determine evolutionary relationships of HRV3 with reference to global strains using bioinformatics tools

CHAPTER TWO

LITERATURE REVIEW

2.1 History of HRV3

During the 1955 winter, a novel virus then called the *Croup-associated virus* (CA virus) was first detected in 2 out of 12 children who had croup (Huebner *et al*., 1958b). These viruses were isolated from monkey kidney cells producing a 'sponge-like syncytial' cytopathic effect (CPE) on the cells. Type 1 haemadsorption virus (HPIV1) and type 2 haemadsorption virus (HPIV2) were detected using haemadsorption technique, while (HPIV4) was isolated from a male student who had a mild respiratory tract infection. HRV3 was also recovered from children with croup (Huebner *et al*., 1958a). The detected viruses resembled myxoviruses, but they had low growth characteristics in embryonated eggs and different antigenic sites (Chanock, 1956). As a result, these varying characteristics placed them in a new family of viruses named *Paramyxoviridae.* The Paramyxoviridae family is divided into various subfamilies. They include; Avulavirus, where the New castle disease virus belongs, the sub-family Henipavirus where the Hendravirus and Nipahvirus are found, the sub-family Morbilivirus (Measles), Respirovirus (HRV1 and HRV3), and lastly the sub-family Rubulavirus (HPIV2 and 4, Mumps), that is associated with human beings (Lee, 2012).

2.2 Classification of HRV3

The term "paramyxovirus" is a Greek word meaning (para) beyond, and (myxo) mucus or slime (Samal, 2011). HRV3 viruses belong to the order *Mononegavirale.* The term was derived from a Greek adjective *μόνος* [*monos*] that alludes to the monopartite and single-stranded genomes of most mononegaviruses. Furthermore, the Latin verb *negare* alludes to the negative polarity of these genomes, and the taxonomic suffix *-virales* (denoting a viral order) (Vainionpaa and Hyypia, 1994). This virus is further classified into the family *paramyxoviridae* and sub-family *respirovirus* (Kuhn *et al*., 2010). Phylogenetic classification of HRV3 is based on the HN and F genes. Clasification based on the highly variable HN gene categorises HRV3 strains into three main genetic clusters; A, B, and

C. (Almajhdi, 2015a, Tsutsui *et al*., 2017a). These major clusters are then divided into sub-clusters C1-C5 and lineages a, b, c, and so forth. In contrast, phylogenetic classification of HRV3 based on whole genome data established that there are two major groups of these viruses circulating globally (Bose *et al*., 2019).

2.3 Genetic Structure and Virion morphology of HRV3

The genome of HRV3 consists of a single strand of negative-sense and non-segmented RNA containing approximately 15,000 nucleotides (El Najjar *et al*., 2014). The morphological and genomic structures of HRV3 are akin to other human parainfluenza viruses, and are organized in the following format that is based on nucleotide sequence analysis; the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion glycoprotein (F), hemagglutinin-neuraminidase glycoprotein (HN), and the large protein (L) (Figures 1 and 2). These proteins are usually denoted as 3'-NP-P-M-F-HN-L-5' (Almajhdi *et al*., 2012).

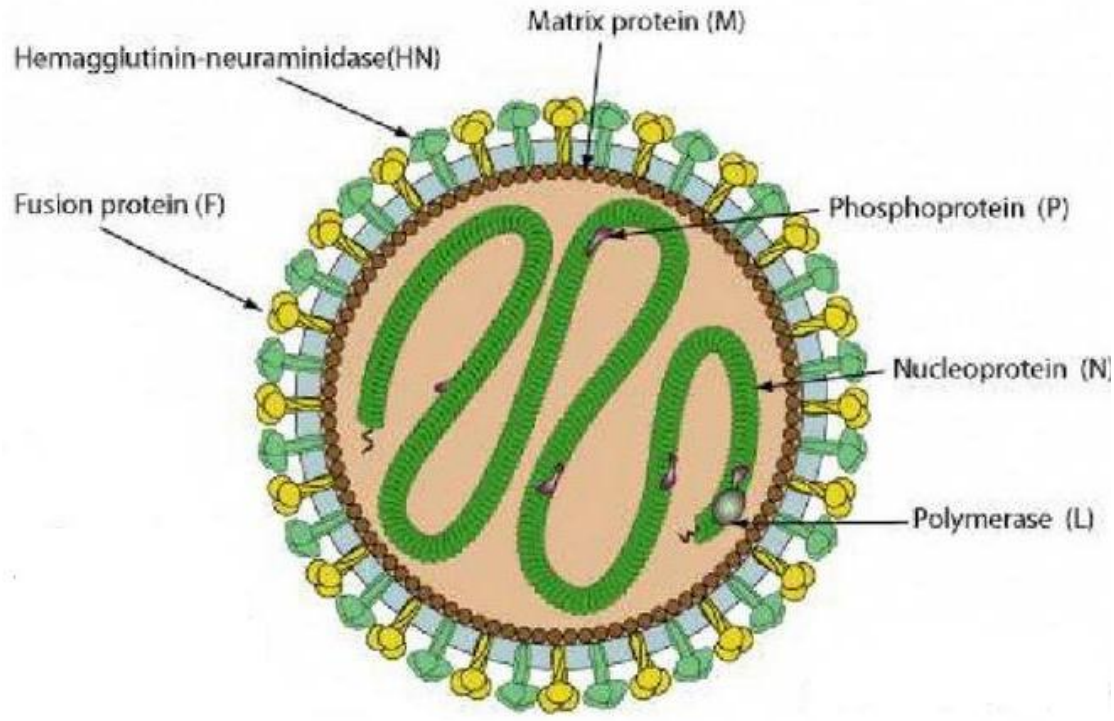

Figure 2.1 General morphological structure of HRV3 showing proteins encoded for by the viral genome

Source: (Schmidt *et al*., 2011)

The function of the nucleocapsid protein (NP) is to form a complex with the RNA genome. The phosphoprotein (P) forms part of the RNA polymerase complex. This region is multicistronic, having continuous, discontinuous, and overlapping cistrons. The matrix protein (M) is used in viral assembly and is the most conserved region in HRV3 (El Najjar *et al*., 2014). The fusion glycoprotein (F) fuses with the host cell receptors and aids in viral entry (Chang and Dutch, 2012). The entrance of the virus is achieved by the fusion of the viral envelope and the host cell plasma membrane, thus releasing the viral nucleocapsid. The large protein (L) is the largest HPIV gene and forms part of the RNA polymerase complex (Moscona, 2005). The hemagglutinin-neuraminidase glycoprotein (HN) is associated with viral attachment to host cells sialic acid receptors found on the host cell membrane and cell entry (Lawrence *et al*., 2004).

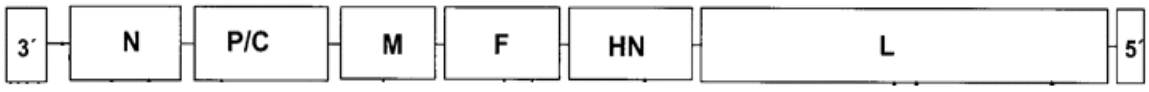

Figure 2.2 General genomic structure of HPIV3

Source:(Schmidt *et al*., 2011)

2.4 Epidemiology of HRV3

Human respirovirus 3 viruses cause a wide spectrum of respiratory tract infections that include URTIs, which can be complicated by otitis media in children and LRTIs that lead to pneumonia and bronchitis (Shi *et al*., 2015a). These viruses are the most predominant serotypes of human parainfluenza viruses. Unlike other human parainfluenza viruses that cause mild respiratory infections, HRV3 is associated with severe lower respiratory tract infections like pneumonia and bronchitis. As a result, these viruses are responsible for the morbidity of up to 40% and a mortality of around 4 million per year globally (Shi *et al*., 2015b). In the United States of America, ARIs result in a 3-18% paediatric admissions as a consequence of HRV3, detected at the rates of between 9%-30%, depending on the season (Chambers, 2009, Bellos *et al*., 2010). In Africa, there is a scarcity of information on HRV3, even though most acute respiratory infections reported as causes of hospital consultations present with pneumonia and bronchitis. These symptoms are mostly

associated with HRV3 viruses (CDC, 2012). For instance, in South Africa HPIV3 accounted for 64% of paediatric patients with SARI (Cohen *et al*., 2015). Furthermore, in Uganda, HRV3 viruses were shown to cause 26% of ARI in hospitalized children (Balinandi, 2012). In Kenya, ARI's constitute approximately 45% of paediatric hospital consultations (Mitei *et al*., 2012). Moreover, HRV3 cause LRTIs in the immunocompromised, those with chronic disease and the elderly (Henrickson, 2003). The majority of HRV3 infections in children mainly occur between the 7^{th} and 36^{th} months of life, with peaks in the second and third years of life. In adults and the elderly, yearly hospitalisations occur. These viruses circulate all year round and in all hemispheres but biennial peaks have been noted (Bose *et al*., 2019). For instance, HRV3 occurs during spring and summer outbreaks.

2.5 Clinical Features of HRV3 Infection

After infection with HRV 3, it takes between 2-7 days for symptoms to appear (Rudan *et al*., 2010, Godoy *et al*., 2016a). Symptoms for URTI include a fever of over 38˚C, running nose, and a dry cough. For LRTI, symptoms associated with HRV3 which are similar to RSV infection include laryngotracheobronchitis (swelling around vocal cords and windpipe), bronchiolitis, bronchitis, and pneumonia. HRV3 symptoms resemble those produced by other viral respiratory aetiologies; therefore, it is difficult to distinguish them without molecular and serology tests (Organization, 2014). For younger children and people with weak immunity, severe illness does occur. Other symptoms, especially for young children that are associated with HRV3 infection, include ear infection (otitis media), irritability, and decreased appetite (Song *et al*., 2016). Croup, a common feature of HRV3 disease, is a respiratory condition triggered by an acute viral infection of the upper respiratory tract. This infection leads to swelling inside the throat, which interferes with normal breathing (Johnson, 2009, Teo *et al*., 2010, Bjornson and Johnson, 2013), resulting in the typical symptoms of a "barking" cough, stridor, and hoarseness (Henrickson, 2003).

2.6 Viral Entry and Multiplication of HRV3

Viral replication of HRV 3 starts only after the successful entry of the virus into a cell. First, the HN protein recognises and attaches to the sialic acid receptors of the host cell, followed by fusion between the virus envelope and host cell lipid membrane, initiated by the F protein (Palmer *et al*., 2014). The F protein is initially in an inactive form (F_0), but proteolysis can cleave it to its active form, F_1 and F_2, which are then linked by disulphide bonds (Chambers, 2009). Once the linking has occurred, the HRV3 nucleocapsid enters the cytoplasm of the cell. Afterwards, genomic transcription occurs using the viruses' viral RNA-dependant RNA polymerase' (L protein) (Chang and Dutch, 2012). The ribosomes of the infected cells- translate and form viral proteins from the viral mRNA. Initially, the formation of a positive-sense RNA virus occurs but is an intermediate step necessary for producing viral progeny. Finally, a negative-sense RNA is formed and associated with the nucleoprotein (El Najjar *et al*., 2014, Chang and Dutch, 2012). The negative-sense RNA may then be packaged and released from the cell by budding or used for subsequent rounds of transcription and replication (Henrickson, 2003). Morphological changes can be seen in infected cells including the enlargement of the cytoplasm, decreased mitotic activity, and 'focal rounding,' with the potential formation of multi-nucleate cells (Chambers and Takimoto, 2001). These changes are termed cytopathic effects.

2.7 Diagnosis of HRV3

The HRV3 viruses can be detected by isolation and identification of the virus in cell culture, direct detection of viral antigens, and detection of the viral genome using polymerase chain reaction. Serological diagnosis of HRV3, similar to other human parainfluenza viruses, is based on the specific antibody-antigen binding. Each virus/antigen elicits the production of unique antibodies that can bind specifically. The serological technique utilizes fluorescence to capture these particular reactions. Molecular detection of Human parainfluenza viruses is based on the surface HN gene. This gene is highly variable and has been primarily used to type various serotypes of HPIV's using their specific primer sets. (Linster *et al*., 2018).

2.7.1 Isolation and Detection of HRV3

HRV3 viruses grow very poorly in fertilised or embryonated eggs but are best isolated from monkey cells (Henrickson, 2003). Primary monkey kidney (PMK) cells, including the Rhesus monkey, African green monkey, and Cynomologous monkey kidney cells have been used to isolate these viruses. The most popular cells are the rhesus monkey kidney cells. Later a secondary cell line, LLCMK2, was developed in the 1950s (Frank *et al*., 1979, Chanock, 1956). LLCKM2 pooled from six rhesus monkeys has been used for HRV3 isolation. The isolation of HRV3 in PMK and LLCMK2 is enhanced when trypsin (tpck) (2 - 3µg/ml) is added to the maintenance medium (Chanock *et al*., 2010). During the incubation of these viruses in the cells, detection is carried out using hemagglutination adsorption technique (HAd). Guinea pig red blood cells adsorb (attach) to the cells from day 3 - 21. Fluorescent antibody testing (FAT) has been described as the most rapid and accurate method for testing, detecting, and typing HRV3 isolates from inoculated cells (Henrickson, 2003). HRV3 will be identified in PMK as early as 2-10 days (Coiras *et al*., 2004). In LLCMK2 cells, it is detectable between 10-21 days.

2.7.2 Serological Detection

The enzyme-linked immunosorbent assay (ELISA) is by far the most sensitive assay where it detects antibody titres in patients with HRV3 (Yano *et al*., 2014). However, it is the least specific and detects many dual HPIV infections. This challenge is attributed to a heterologous HPIV antibody formed by the infected patient making it difficult to separate infections of HPIVs of similar serogroups as well as other viruses, for example, mumps virus (Jalal *et al*., 2007). Paired serum should be collected from a patient to detect a fourfold increase and decrease of HRV3 specific antibody in the affected patient (Henrickson, 2003). Additionally, virus isolation, DFAT, and molecular techniques can accurately determine the specific HRV3 variant affecting a patient (Almajhdi *et al*., 2012, Linster *et al*., 2018).

2.7.3 Molecular Methods

Detection of human parainfluenza viruses is based on the highly variable HN gene. Specific primer sets that particular bind regions of the HN gene have been designed to detect various HRV3 serotypes. The HRV3 RNA has to be converted to cDNA through reverse transcription (RT) before the amplification process by polymerase chain reaction (PCR) (Mao *et al*., 2012b, Almajhdi *et al*., 2012). Several studies have elucidated the sensitivity and specificity of HRV3 detection (Linster *et al*., 2018, Bose *et al*., 2019). A multiplex reverse transcriptase PCR (RT-PCR) for the detection of HPIV 1- 3 was developed in 1985 (Fan *et al*., 1998). Later a multiplex RT-PCR for the detection of all HPIVs (1-4) was also established in 2005 (Bellau-Pujol *et al*., 2005). Real-time PCR methods for either single as well as multiplex detection of all HPIVs have also been developed.

2.8 Stability and Viability of HRV3

2.8.1 Drug Susceptibility

Currently, there are no antiviral drugs against human parainfluenza viruses. HRV3 viruses just like other human parainfluenza viruses may be susceptible to ribavirin (1-[(2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1*H*-1,2,4-triazole-3-carboxamide), some interferons, and some protein inhibitors. Nonetheless, further testing is required to evaluate their efficacy (Gomez *et al*., 2009). Ribavirin is guanosine (ribonucleic) analogue used to stop viral RNA synthesis and viral mRNA capping; thus, it is a nucleoside inhibitor (Schmidt *et al*., 2011). It resembles the purine RNA nucleotides guanosine or adenosine, pairing with uracil or cytosine, thus inducing mutations in RNA-dependent replication in RNA viruses. This phenomenon leads to mutations that interfere with RNA metabolism required for viral replication (Mulholland, 2003, Pawełczyk and Kowalski, 2017).

2.8.2 Susceptibility to Disinfectants

Human respirovirus three viruses may be susceptible to iodophores (1% iodine), 2% glutaraldehyde, formaldehyde (18.5 g/L; 5% formalin in water), and hypochlorites (1% sodium hypochlorite) (Chang and Dutch, 2012). Common detergents such as soap, disinfectants, or antiseptic agents are usually efficient enough to decontaminate HRV3 from surfaces (Chambers and Takimoto, 2001).

2.8.3 Physical Inactivation

HRV3 viruses are sensitive to temperatures that are above 37°C (Chambers and Takimoto, 2001). At this temperature, a significant decrease in viral survival is observed and the virus is inactivated at 50°C for about 15 minutes. They are most stable under freezing conditions or at 4°C. Also, viral infectivity is quickly lost at a pH range of 3.0 - 3.4, under low humidity, and upon virus desiccation (Henrickson, 2003).

2.8.4 Survival Outside a Host

Existing evidence shows that HRV3 can survive for up to 10 hours on nonporous surfaces and 4 hours on porous surfaces (Teo *et al*., 2010). Furthermore, the viral survival rate on human skin is low, as these viruses lose more than 90% infectivity rates within the first 10 minutes when placed on human skin. Viral infectivity can be maintained for an extended period if frozen with reagents such as 0.5% bovine serum albumin, skimmed milk, 5% dimethyl sulfoxide (DMSO), or 2% chicken serum (Yano *et al*., 2014).

2.9 Vaccination of HRV3

Currently, there are no licensed vaccines available against human parainfluenza viruses. The first attempts at developing HRV3 vaccines began in the early 1960s; these vaccines produced either serum or mucosal neutralising antibodies in an individual and never both (Schmidt *et al*., 2011). HRV3 vaccines were developed by growing them in low temperatures for 45 passages to acquire mutations (Belshe and Hissom, 1982), which were

tolerated, safe, and immunogenic. This vaccine was however obscured by maternal antibodies (Gomez *et al*., 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

All the laboratory work was carried out at the National influenza Centre (NIC) laboratory within the Kenya Medical Research Institute (KEMRI).

3.2 Study Design

This was a retrospective laboratory-based explorative study, that described the molecular characteristics of HRV3.

3.3 Study Population

The population whose archived samples were used in this study comprised consenting outpatients above two months who presented with influenza-like illness. For children below 18 years, consent was sought from their parents/guardians. These patients attended level four and five hospitals under the Kenya respiratory virus surveillance program between 2010 and 2013. These hospitals comprised; Mbagathi sub-county hospital in Nairobi county, Isiolo sub-county hospital in Isiolo county, Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) in Kisumu County, Kisii referral hospital in Kisii county, Alupe sub-county hospital in Busia county, Port Reitz sub-county hospital in Mombasa County, Malindi sub-county hospital in Kilifi county, and Kericho sub-county hospital in Kericho county.

3.5 Ethical Consideration

The protocol used in this study was approved by the Scientific and ethical review unit (SERU) in KEMRI, under SERU#3298 (Appendix 1). This study was part of an ongoing protocol; Influenza Surveillance in Kenya; SSC#981, approved by KEMRI. The archived nasopharyngeal swab specimen had unique identities delinked from actual patient identity. Clinicians who collected the data were the only people who had access to the real patient identities. There was no link between the codes and patient identity.

3.6 Samples

This study used archived samples collected from nasopharyngeal swab specimen of consenting patients attending hospitals under the Kenya respiratory surveillance network between 2010 and 2013. All the samples (n=115) collected during this period were used in this study.

3.7 Virus Isolation

3.7.1 Cells

All the samples used in this study were propagated in Rhesus monkey kidney cell line ($LLCMK_2$) (American Type Culture Collection, ATCC® CCL-7.1™). Briefly, the cells (passage 1) were prepared in a $75cm^2$ cell culture flask (Corning, USA) and inoculated into cell culture tubes (Nunc, USA). These cells were grown in Dubelcos Modified Eagle's Medium (DMEM) [Gibco, USA], supplemented with 10% Foetal Bovine Serum (ATCC, USA), penicillin/streptomycin solution (Sigma, USA), and L-glutamine (Gibco, USA).

3.7.2 Cell culture conditions and maintenance

The cells were incubated at 37˚C in a CO_2 incubator (Napco, Japan) until the monolayer grew to 75 – 80% confluence. Before inoculation, the cells in the tubes were washed thrice using Hans balanced salt (Sigma, USA) containing trypsin (TPCK) (1µg/ml).

3.7.3 Inoculation and isolation

One hundred microliters (100µl) of the nasopharyngeal samples were inoculated into the cells, followed by an incubation step for one hour at 33˚C to adsorb the viruses onto the cells. The monolayers were then overlaid with maintenance medium containing bovine serum albumin (Sigma, USA), penicillin/streptomycin (Sigma, USA), fungizone (Sigma, USA), and 2.4µg/ml of trypsin (Sigma, USA). The infected cells were then incubated at 33˚C for up to 12 – 14 days. They were examined frequently using an inverted Olympus® microscope (Olympus, Japan), to detect any contamination or cytopathic effects (CPEs).

Any tube indicating contamination was discarded, and a fresh aliquot of the isolate was filter-sterilised through a 0.22µm pore size syringe filter (Millipore, UK) and re-inoculated into the cell line. Once the cytopathic effect (CPE) was observed on the cell line, the supernatant of the monolayer was frozen in preparation for direct fluorescent tests.

3.8 Direct Immunofluorescent Assays

To confirm the isolation of HRV3, DFAT was carried out. First, the LLCMK2 cells that showed the cytopathic effects of about 60 – 70% were frozen at -80°C to detach the cells from the tubes. The supernatant fluid from the tubes was aliquoted to labelled cryovials and stored at -80°C. The cells left adhering on the tube surface were washed three times using sterile phosphate-buffered saline (PBS) followed by centrifugation at 3500 revolutions per minute (rpm) for 10 minutes using an Eppendorf Centrifuge (USA). Furthermore, 25µl of the PBS containing the detached cells was spotted onto a multiwell slide (ThermoScientific, USA) and left overnight to air dry in a biosafety cabinet. The resulting slides were immersed in cold acetone (Sigma, USA) at 4°C for 10 minutes to inactivate the virus and fix the cells on the slide. The fixed slides were stained with 25µl of HRV3 virus-specific monoclonal antibody conjugated with a fluorescein isothiocyanate (FITC) (Millipore, USA). The stained slides were then incubated at 37°C for 30 minutes in a humid chamber. Finally, the slides were washed with tween PBS to remove excess antibodies, stained with 25µl of mounting fluid, and covered with a coverslip for examination under an Olympus BX51 fluorescent microscope (Olympus Japan) using the times 4, 10, and 40 magnifications.

3.9 Molecular Assays

3.9.1 RNA extraction

Ribonucleic acid (RNA) from the HRV3 isolates was extracted using the Qiagen RNA mini kit (Qiagen CA, USA) according to manufacturers' instructions. Briefly, 140µl of the sample was added to 560µl of viral lysis buffer and incubated at room temperature (15-25°C) for 10 minutes. 560µl of molecular grade 100% ethanol (Sigma, USA) was then

added and mixed by vortexing for 15 seconds; the resulting lysate was then centrifuged using a microcentrifuge (Applied Biosystems, USA) to remove any droplets walls of the tube. The lysate was transferred to a spin column, centrifuged at 6000rpm, that allowed binding of the RNA to the spin column. Furthermore, the column was washed twice, first with 500µl of wash buffer 1 (AW1) at 6000 x g for 1 minute, then with 500µl of wash buffer 2 (AW2) at 20,000 x g for 3 minutes. Finally, RNA was eluted from the spin column using 60µl elution buffer (AVE) at 6000 x g for 1 minute into a 1.5 ml Eppendorf tube. The eluted RNA was then stored at −80° C.

3.9.2 Polymerase chain reaction (PCR)

Three primer pairs previously described (Almajhdi *et al*., 2012), were utilised in the RT-PCR assay. These primers were chosen from highly conserved sequences of the HN gene designed to achieve optimal performance in different reaction sets. The RT-PCR was performed in three separate runs using specific primers (F1R1, F2R2, and F3R3). The RT-PCR master mix was prepared using One-step RT-PCR kit (Invitrogen Corporation, USA) following the manufacturer's instructions. 16µL of 2X reaction mix,1µL of both forward and reverse primers (10µM), 1µL of superscript/ platinum Taq enzyme mix, and 1ul of nuclease-free water (Promega Corporation, USA) were used to make the master mix, in which 5µL of RNA was added. For negative and positive control, nuclease-free water and HRV3 strain (ATCC® VR-93™) were added to the tube, respectively. The reaction tubes were incubated in the thermal cycler Gene-Amp 9700 (Applied Biosystems, Foster City, CA).

Table 3.1:Primers used in this study

PRIMER	SEQUENCE (5' - 3')	POSITION IN HN GENE[a]
HRV3-HN-F1 (sense)	CGA GAT GGA ATA CTG GAA GC	5^{up}–16
HRV3-HN-R1 (antisense)	ATC AAG TAC AAT ATC TTC TAT GCC	849-872
HRV3-HN-F2 (sense)	CTG TAA ACT CAG ACT TGT TAC CTG	688-711
HRV3-HN-R2 (antisense)	GCT GTT GAG TAA GTT ATG ACT GG	1512-1534
HRV3-HN-F3 (sense)	CAT AAT GTG CTA TCA AGA CCA GG	1353-1375
HRV3-HN-R3 (antisense)	CTG ATT GCT GAT TAC TTA TCA TAT ACT TG	50^{down}–78^{down}

The ''up'' indicates the upstream of HN gene start codon. The ''down'' indicates the downstream of HN gene stop codon. The "a" indicates Nucleotide positions relative to HRV3 HN gene sequence. The expected size of the HN gene is 1.8kb

Table 3.2: **Amplification conditions for HN gene**

Procedure		Temperature	Time
Reverse Transcription		50°C	30 minutes
Initial Denaturation		95°C	Three minutes
Cycle denaturation	35 cycles	94°C	One minutes
Annealing		50°C	One minute 30 sec
Cycle Extension		68°C	Two minutes
Final Extension		68°C	10 minutes
Hold		4°C	∞

3.9.3 Gel electrophoresis

The RT-PCR products were analysed with 100 bp DNA size marker (Qiagen), in 2% agarose gel (Sigma-Aldrich Co., USA) prepared by dissolving two grams of agarose into 100mL of tris borate EDTA buffer. This solution was warmed in a microwave and swirled gently for the complete dissolution of agarose. The warm agarose gel was then stained with 2µL ethidium bromide **(**Sigma-Aldrich Co., USA), cast on a gel tray fitted with gel combs, and left to set. The combs were then removed, and the gel was transferred into a gel tank (Bio-Rad, USA) flooded with tris borate EDTA as the running buffer. 100bp ladder (Qiagen) was loaded into the first lane as a standard. After that, the amplicons mixed with 3x blue orange dye (Promega, USA) were loaded in the rest of the wells. The gel tank was connected to the power pac^{Tm} and ran on 50 volts for an hour. The bands were then visualised under UV light using the E-box gel documentation system (Vilber Lourmat, France) following the manufacturer's instructions.

3.9.4 Purification of RT-PCR Products using ExoSAP-IT

RT-PCR products were purified using Exonuclease I/Shrimp Alkaline Phosphatase (ExoSap-IT) enzyme (Affymetrix, Inc., USA), according to the manufacturer's instructions. Briefly, 10µL of each amplicon visualised on the gel, and 3µl of ExoSAP was placed into a 0.2mL PCR reaction tube and vortexed. The mixture was then incubated in a 9700 FAST thermocycler (Applied Biosystems, USA) under cycling parameters of 37˚C for 15 minutes and 80˚C for 15 minutes.

3.9.5 Cycle sequencing PCR

Labelling of the RT-PCR amplicons was carried out using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) which incorporates fluorescent-labelled dideoxy-chain terminators, following the manufacturer's instructions. Briefly, two master mixes; one for forward primer and one for reverse primer, were prepared in a 1.5 Eppendorf tube, by adding 4µL nuclease-free water, 2µL of Big Dye buffer, 1µL of

primer (10µM forward and reverse primers), Big Dye 1µL, and lastly 3µL of purified RT-PCR product. This step was followed by incubation in a 9700 ABI thermocycler (Applied Biosystems, USA).

Table 3.3:**Amplification conditions for cycle sequencing**

Procedure		Temperature	Time
Initial Denaturation		94°C	Five minutes
Cycle denaturation	35 cycles	94°C	15 seconds
Annealing		55°C	30 seconds
Cycle Extension		68°C	Two minutes 30 seconds
Final Extension		60°C	Three minutes
Hold		4°C	∞

3.9.6 Purification of cycle sequencing products using Sephadex spin columns

The labelled cycle sequencing products were purified using size exclusion chromatography, which removed excess primers, salts, unincorporated nucleotides, and dye terminators that may interfere with the electrophoretic dynamics in the sequencing procedure (Hutchison *et al*., 2005). Dry Sephadex G-50 (Sigma-Aldrich Co., USA) resins were loaded into 96-well Multiscreen HV plates (Millipore Corporation, USA) using a column loader, according to the manufacturer's instructions. Three hundred microliters (300µl) of Milli-Q water were used to hydrate and swell the Sephadex resins. The Sephadex plate was incubated at room temperature for up to three hours and centrifuged at 910 x g in a 5810 R centrifuge (Eppendorf AG, Germany) for five minutes to remove excess water from the Sephadex columns. The Multiscreen plate was then placed on top of an optical 96 well plate and 10µl of sequencing reactions were carefully added to the centre of each well before centrifugation at 910 x g for 5 minutes.

3.9.7 Resolution of cycle sequencing products

Before the resolution process, ten microliters (10µl) of Hi-Di formamide was added to single-stranded labelled purified cycle sequencing products and resolved using a 24 capillary genetic analyser (Applied Biosystems 3500xL Dx).

3.10 Data Analysis and Management

3.10.1 Contig assembly and homology analysis

The reads obtained from the genetic analyser were edited and assembled into contigs using DNA baser v.3.5 (http://www.dnabaser.com). HRV3 HN sequences obtained were compared to the prototype (Accession number: JN089924) and other similar sequences in the GenBank database using the Basic Local Alignment Search Tool -BLAST (Ngoc Ha *et al*., 2018). The results were evaluated based on bit scores and E-values to assign serotypes to respective query sequences. HRV3 HN nucleotide and protein sequences were considered to be homotypic if they shared a similarity of over 75% and 85% respectively. Protein translations of the nucleotide sequences obtained were carried out using discovery studio gene (DS Gene) version 1.5 (Accelrys Inc.).

3.10.2 Phylogenetic analysis

Multiple sequence alignment of Kenyan HRV3 HN nucleotide sequences together with those of other reference strains retrieved from GenBank was performed using muscle v3.8 software (Ngoc Ha *et al*., 2018). Phylogenetic reconstruction was carried out using MrBayes v3.2 software (Ronquist *et al*., 2012) using the best fit HKY+G substitution model as predicted by the jModelTest software (Posada, 2008).

3.10.3 Timescale evolutionary analysis

A Maximum Clade Credibility (MCC) tree using the Markov Chain Monte Carlo (MCMC) method implemented in the BEAST package v1.8.4 was generated (Drummond *et al*., 2012). The BEAST ran for 100 million generations, sampling every 10,000 steps

with a 10% burn-in employing an uncorrelated relaxed molecular clock and Coalescent GMRF Bayesian Skyride demographic model combination. The convergence of BEAST runs was confirmed by effective sample size (ESS) values >200 for all the parameters using the Tracer program v1.6 (Rambaut *et al*., 2014). The MCC tree was summarised using Tree Annotator (Rambaut and Drummond, 2013). Both trees were visualised and annotated using Fig Tree™ version 1.4.3 (Rambaut, 2016). Estimates of evolutionary divergence between and within clusters were conducted in MEGA6 using the Kimura 2 parameter (Takahashi *et al*., 2018).

3.10.4 Natural selection and Glycosylation analyses

Natural selection was measured using the ratios of nonsynonymous (d_N) to synonymous (d_S) changes implemented in the Datamonkey web server. Positively selected sites on the HN genes of Kenyan HRV3 strains were estimated using single likelihood ancestor counting (SLAC), fast and unconstrained Bayesian approximation (FUBAR). Additionally, it employed random-effects likelihood (REL), the mixed-effects model of evolution (MEME), and fixed effects likelihood (FEL) algorithms implemented in the Datamonkey webserver (http://www.datamonkey.org) (Kosakovsky Pond and Frost, 2005). Codon sites were considered to be under positive selection if identified by two or more methods, with a p-value less than 0.05 for SLAC, FEL, & MEME or Bayes factor/posterior probability of more than 0.95 for REL and FUBAR (Bose *et al*., 2019, Mizuta *et al*., 2014). Analysis of potential N-glycosylation across the HN protein sites was performed using the online n- netnglyc.1 server (www.cbs.dtu.dk/services/netnglyc.) (Steentoft *et al*., 2013). A threshold score value of > 0.5 was regarded as suggestive of glycosylation.

3.9.2 Data management

The data generated from this study were entered into a Microsoft Office Excel spreadsheet in duplicate using codes; storing back up in a flash drive and compact disk as well as print out sheets that were kept locked. The HRV3 HN sequences were deposited in GenBank for reference purpose.

CHAPTER FOUR

RESULTS

4.1 Isolation in Culture

A total of 115 samples (n=115) were pr opagated in rhesus monkey kidney cell line ($LLCML_2$). 100 (87%) out of 115 samples displayed cytopathic effects (CPE). Direct immunofluorescence antibody test confirmed that 60 out of 100 cultures that showed CPE were positive for Human respirovirus type 3 . Confirmation of HRV3 isolation was indicated by apple-green fluorescence emanating from the cells when exposed to U.V. light.

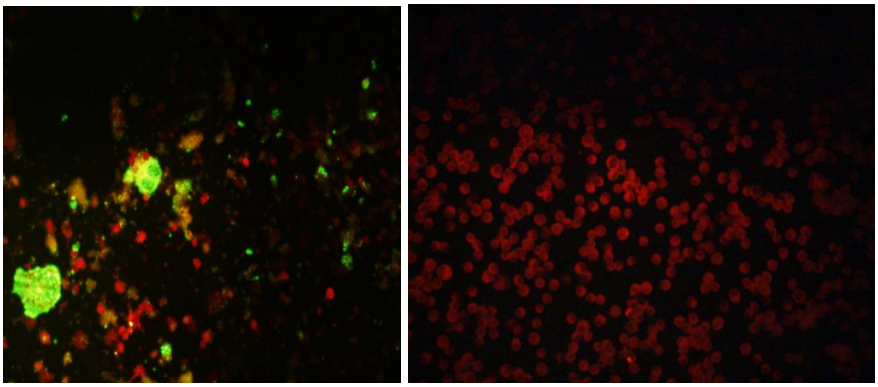

Figure 4.1:**Direct immunofluorescent antibody tests images confirming isolation of HRV3**

The image on the left shows confirmation of HRV3 isolation indicated by apple-green fluorescence emanating from the cells (red background). The image on the right shows negative results hence only red background with no sign of infection.

4.2 RT-PCR and Sequencing of the Hemagglutinin Neuraminidase gene (HN)

Only 50 (83%) out of the 60 HRV3 isolates confirmed by IFA successfully amplified using specific primers. Gel images were visualised using the E box gel documentation.

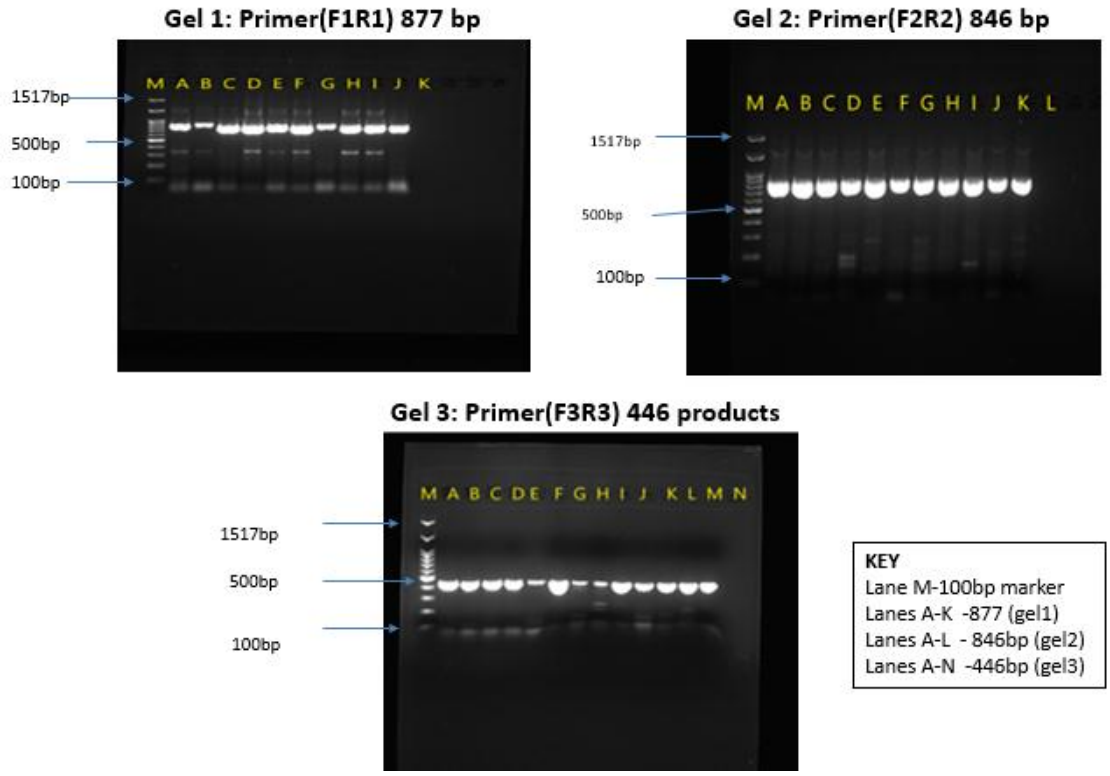

Figure 4.2:**Gel images confirming amplification of the complete HN gene using three pairs of primers**

Gel 1 indicates amplification of 877 bp HN region using (F1R1) primer pair. Gel 2 shows gel image of 846bp HN region using (F2R2) primer pair and gel 3 shows a gel image indicating amplification of 446bp HN region using primer pair (F3R3). These three regions form a contig of about 1.7kb complete HN gene. N/B Lanes A were positive controls while lines K, L, and N are negative controls.

The HN gene of all the 50 isolates was successfully amplified using RT-PCR. 35 (70%) out of 50 amplicons yielded quality complete HN sequences. Analysis by BLAST confirmed that all the 35 isolates were indeed human respirovirus type 3 viruses. The 35 complete HN sequences were deposited in GenBank and assigned accession numbers MN116649 - MN116682 and MN116749.

4.3 Sequence Homology Analyses

Relative to the prototype strain (Accession number: JN089924) from the USA, the Kenyan isolates shared a 94.3-95.6% (nucleotide) and a 96.5 to 97.9% (amino acid) identity determined by a sequence identity matrix

Table 4.1:**Nucleotide and protein identities of the Kenyan HRV3 strains relative to the prototype**

Isolate code	% Protein Identity	% Nucleotide identity	Isolate code	% Protein identity	%Nucleotide identity
KEN/NR015/2010	97.7	95.1	**KEN/KR010/2011**	97.7	95.2
KEN/NY011/2011	97.7	95.5	**KEN/PR010/2010**	97.7	95.2
KEN/KS025/2012	97.9	95.6	**KEN/KS011/2011**	97.5	95.2
KEN/KS007/2010	97.7	95.1	**KEN/IS012/2012**	97.2	95.2
KEN/IS002/2010	97.5	95.6	**KEN/KS012/2011**	97.5	95.1
KEN/ML002/2010	97.7	95.5	**KEN/NY012/2011**	97.9	95.5
KEN/NY002/2010	97.9	95.2	**KEN/KS013/2011**	97.7	95.2
KEN/KR003/2010	97.7	95.1	**KEN/PR013/2010**	97.7	95.2
KEN/NY003/2011	97.7	95.4	**KEN/KS017/2010**	97.5	95.1
KEN/PR003/2010	97.9	95.4	**KEN/PR017/2012**	97.7	95.3
KEN/IS004/2011	97.5	95.2	**KEN/KS018/2011**	97.7	95.4
KEN/KR004/2010	97.7	95.1	**KEN/AP005/2011**	96.5	94.3
KEN/NY004/2010	97.9	95.5	**KEN/ML008/2012**	97.7	95.2
KEN/KR005/2010	97.7	95.2	**KEN/KS010/2010**	97.5	95.0
KEN/NY005/2011	97.9	95.6			
KEN/IS006/2011	97.7	95.1			
KEN/PR006/2010	97.5	95.1			
KEN/IS007/2011	97.7	95.1			
KEN/KR007/2011	97.5	95.2			
KEN/NY007/2010	97.7	95.4			
KEN/KS008/2010	97.7	95.3			

Table 4.2 :Amino acid substitutions in the HN gene of the Kenyan HRV3 strains relative to the prototype strain

Strains	7	8	9	12	15	16	17	19	20	21	24	25	26	27	28	33	39	40	45	47	53	58	60	62	66	69	82	87	118	168	191	295	387	391	413	555	556
Prototype strain	T	N	H	D	N	E	L	T	S	M	H	G	N	K	I	T	I	I	S	V	I	S	K	H	L	V	M	I	M	K	V	H	G	I	L	L	D
KEN/NR01 5/2010	P	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/NY01 1/2011	.	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	I	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/KS025 /2012	.	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/KS007 /2010	.	.	.	.	.	.	.	.	.	T	N	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/IS002/ 2010	.	S	.	V	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/ML00 2/2010	.	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	I	Y	.	V	.	.	N
KEN/NY00 2/2010	.	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/KR00 3/2010	.	.	.	.	.	.	.	.	.	T	N	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/NY00 3/2011	.	.	R	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/PR003 /2010	.	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/IS004/ 2011	.	.	R	.	.	.	.	.	.	T	.	.	.	.	L	.	V	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/KR00 4/2010	.	.	.	.	.	.	.	.	.	P	N	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/NY00 4/2010	.	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/KR00 5/2010	.	.	.	.	.	.	.	.	.	T	N	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/NY00 5/2011	.	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/IS006/ 2011	.	.	.	.	.	.	.	.	.	T	.	.	.	.	L	I	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/PR006 /2010	.	.	.	.	.	.	V	.	.	T	N	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	.	N

KEN/IS007/2011	.	.	.	.	.	.	.	.	.	T	N	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/KR007/2011	.	.	.	.	.	.	.	.	.	T	N	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	F	.	N
KEN/NY007/2010	.	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	I	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/KS008/2010	.	.	Q	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/ML008/2012	.	.	.	.	.	.	.	.	.	T	.	.	S	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/KS010/2010	.	.	.	.	I	.	.	.	.	T	N	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/KR010/2011	.	.	.	.	.	.	.	.	.	T	N	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/PR010/2010	.	.	.	.	.	.	.	.	.	T	.	.	.	W	L	.	T	T	.	I	.	.	.	.	.	I	V	.	I	R	.	Y	.	V	.	.	N
KEN/KS011/2011	.	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	L	.	T	N	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/IS012/2012	.	.	.	.	.	.	.	S	T	T	.	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	I	Y	D	V	.	.	N
KEN/KS012/2011	.	R	.	.	.	.	.	.	.	T	N	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/NY012/2011	.	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/KS013/2011	.	.	.	.	.	.	.	.	.	T	N	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/PR013/2010	.	.	.	.	.	.	.	.	.	T	N	.	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/KS017/2010	.	.	.	.	.	.	.	.	.	T	N	S	.	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/PR017/2012	.	.	.	.	.	.	.	.	.	T	.	.	S	.	L	.	.	T	.	.	T	.	.	R	.	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/KS018/2011	.	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	.	T	.	.	T	.	.	R	I	I	V	T	I	.	.	Y	.	V	.	.	N
KEN/AP005/2011	.	.	R	E	.	V	V	K	.	T	N	.	Y	.	L	.	.	T	.	.	.	.	Q	R	.	I	V	T	I	.	.	Y	.	V	.	S	N

Comparatively, the prototype and all the Kenyan HRV3 isolates were conserved in the antigenic and catalytic regions of the HN gene. All the Kenyan HRV3 isolates shared eight unique amino acid substitutions from the prototype strain. These were; I28L, I40T, V69I, M82V, M118I,

4.4 Selection Analyses

Analysis of natural selection across the HN protein indicated that the Kenyan strains were generally evolving under positive selection. Overall, five codon sites on the HN gene of the Kenyan HRV3 viruses were shown to be under positive selection pressure by MEME and REL algorithms. These positively selected sites were positions; 8, 45, 66, 168, 191, and 555. The five positively selected codon sites detected by both MEME and REL resulted in changes in amino acid residues at these sites. The changes comprised; N8R, S45L, K168R, V191I, and L555S. No positively selected sites were identified by either SLAC, FUBAR, or FEL algorithms.

Table 4.3:**Positively selected sites by at least two algorithms (in bold) on the Kenyan HRV3 viruses**

Codon	SLAC (p-value)	FUBAR (Post Pr.)	MEME (p-value)	FEL (p-value)	REL (Post Pr.)
8	0.879	0.38	**0.0426**	0.273	**0.9998**
21	0.889	0.221	0.0116	0.407	N/A
33	0.89	0.225	0.0184	0.412	N/A
45	0.813	0.674	**0.029**	0.351	**0.9989**
66	0.517	0.784	0.0711	1	**0.9653**
168	0.708	0.751	**0.0263**	1	**0.9993**
191	0.708	0.742	**0.0408**	0.757	**0.9999**
555	0.43	0.816	**0.0108**	0.972	**0.9995**

4.5 Glycosylation

Analysis of N glycosylation motifs across the HN protein using the NetNglyc tool suggested that amino acid codon sites 308, 485, and 523 were likely to be glycosylated.

These sites were conserved in the Kenyan strains likened to the prototype.

```
    Proteins without signal peptides are unlikely to be exposed to
    the N-glycosylation machinery and thus may not be glycosylated
    (in vivo) even though they contain potential motifs.

    SignalP-NN euk predictions are as follows:

  # name                Cmax  pos ?  Ymax  pos ?  Smax  pos ?  Smean ?  D     ?
  washington_1957_C243_JN089924.1  0.156  59  0.112  70  0.176  58  0.079   0.099 N  0.500      SignalP-TM

    SignalP output is explained at http://www.cbs.dtu.dk/services/SignalP/output.html

##############################################################################################

Name:  washington_1957_C243_JN089924.1  Length:  572
MEYWKHTNHGKDAGNELETSMATHGNKITNKITYILWTIILVLLSIVFIIVLINSIKSEKAHESLLQDVNNEFMEVTEKI      80
QMASDNINDLIQSGVNTRLLTIQSHVQNYIPISLTQQMSDLRKFISEITIRNDNQEVPPQRITHDVGIKPLNPDDFWRCT     160
SGLPSLMKTPKIRLMPGPGLLAMPTTVDGCVRTPSLVINDLIYAYTSNLITRGCQDIGKSYQVLQIGIITVNSDLVPDLN     240
PRISHTFNINDNRKSCSLALLNTDVYQLCSTPKVDERSDYASSGIEDIVLDIVNHDGSISTTRFKNNNISFDQPYAALYP     320
SVGPGIYYKGKIIFLGYGGLEHPINENAICNTTGCPGKTQRDCNQASHSPWFSDRRMVNSIIVVDKGLNSIPKLKVWTIS     400
MRQNYWGSEGRLLLLGNKIYIYTRSTSWHSKLQLGIIDITDYSDIRIKWTWHNVLSRPGNNECPWGHSCPDGCITGVYTD     480
AYPLNPTGSIVSSVILDSQKSRVNPVITYSTATERVNELAIRNKTLSAGYTTTSCITHYNKGYCFHIVEINHKSLDTFQP     560
MLFKTEIPKSCS
..............................................................................          80
..............................................................................         160
..............................................................................         240
.....................................................................N....................  320
..............................................................................         400
..............................................................................         480
....N.....................................N.....................................         560
............                                                                           640

(Threshold=0.5)
------------------------------------------------------------------------
SeqName      Position  Potential   Jury    N-Glyc
                                 agreement result
------------------------------------------------------------------------
washington_1957_C243_JN089924.1  308 NISF   0.6200     (8/9)   +
washington_1957_C243_JN089924.1  351 NTTG   0.4651     (6/9)   -
washington_1957_C243_JN089924.1  485 NPTG   0.5513     (7/9)   +    WARNING: PRO-X1.
washington_1957_C243_JN089924.1  523 NKTL   0.5090     (4/9)   +
------------------------------------------------------------------------
```

Figure 4.3:NETGlyc tool indicating potential glycosylation sites across the HN gene of the Kenyan viruses relative to the prototype

The coloured amino acids (Asn-Xaa-Ser/Thr) indicate potential glycosylation sites.

4.6 Phylogenetic and Evolutionary Analyses

Phylogenetic analysis of the HRV3 HN gene sequences exhibited segregation of the viruses into three main clusters A, B, and C. The sequences included in the analysis comprised all the Kenyan HRV3 isolates from this study with those of global strains retrieved from GenBank. Cluster A contained the prototype HRV3 strain from North America and a single strain from Australia, while Cluster B had variants from North America, Australia, and a South African strain. All the Kenyan isolates grouped significantly (posterior probability value 100%) into cluster C among other global strains.

The majority of the Kenyan isolates were further grouped into Sub-clusters C3a (54%) and C2 (43%) among other global strains. In contrast, a small percentage of the isolates (2.9%) were grouped into Sub-cluster C1a. Notably, the Kenyan isolates belonging to Sub-clusters C1a and C2 were closely related to viruses isolated in East and Western Asia. Those belonging to Sub-cluster C3a were significantly akin to HRV3 viruses that circulated in North America and Australia.

Molecular evolutionary analyses of the HN sequences confirmed that all Kenyan HRV3 isolates belonged to Cluster C (high posterior probability values), Sub-clusters C3a (54%), C2 (43%), and C1a (2.9%). The mean TMRCA of HRV3 for the whole sequence dataset was estimated at 1942 (95% HPD: 1928 - 1957) (while the mean evolutionary rate was $4.65x10^{-4}$ nucleotide substitutions/site/year (95% HPD: $2.99x10^{-4}$ to $6.35x10^{-4}$). Further analyses suggested that the HRV3 virus infections in Kenya between 2010 - 2013 were a result of multiple introductions from various countries including India (Kenyan Sub-cluster C1a virus strains), Saudi Arabia (Kenya Sub-cluster C2 virus strains), Beijing North, and South America (Kenyan Sub-cluster C3a virus strains).

Genetic distances (P) over sequence pairs between the phylogenetic clusters A:B, A:C, and B:C were calculated as 0.050, 0.137, and 0.142, respectively. The genetic distances between sequence pairs of Kenyan HRV3 strains and those of global reference strains in clusters A, B, and C were estimated at 0.056, 0.063, and 0.025, respectively. The lower genetic distance of the Kenyan viruses with the global strains in the C cluster confirmed that the HRV3 strains detected in the study indeed belonged to cluster C.

The genetic distances between the various sub-clusters within cluster C ranged from 0.013 to 0.039.

Table 4.4: **Genetic distances within cluster C**

	C1a	C1b	C2	C3a	C3b	C3c	C4	C5
C1a								
C1b	0.016							
C2	0.035	0.039						
C3a	0.034	0.036	0.026					
C3b	0.035	0.037	0.028	0.013				
C3c	0.038	0.039	0.029	0.02	0.021			
C4	0.029	0.031	0.034	0.030	0.032	0.035		
C5	0.037	0.037	0.030	0.023	0.024	0.026	0.034	

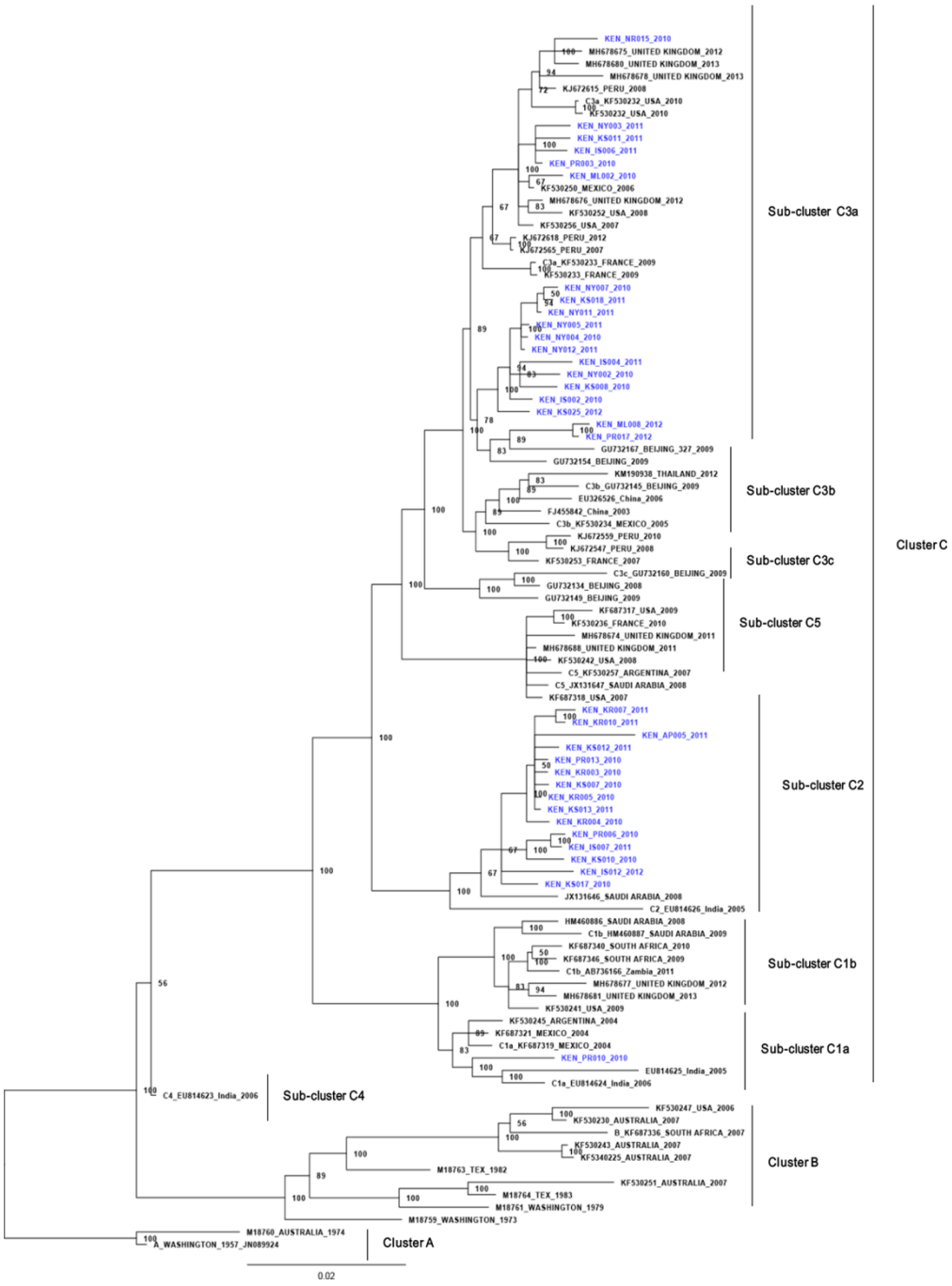

Figure 4.4:Bayesian phylogenetic tree showing the classification of HRV3 viruses based on the complete HN gene.

The tree was constructed using MrBayes v3.2. The numbers at the nodes represent posterior probability values. The scale bar indicates the number of nucleotide substitutions per site. Kenyan isolates are shown in blue.

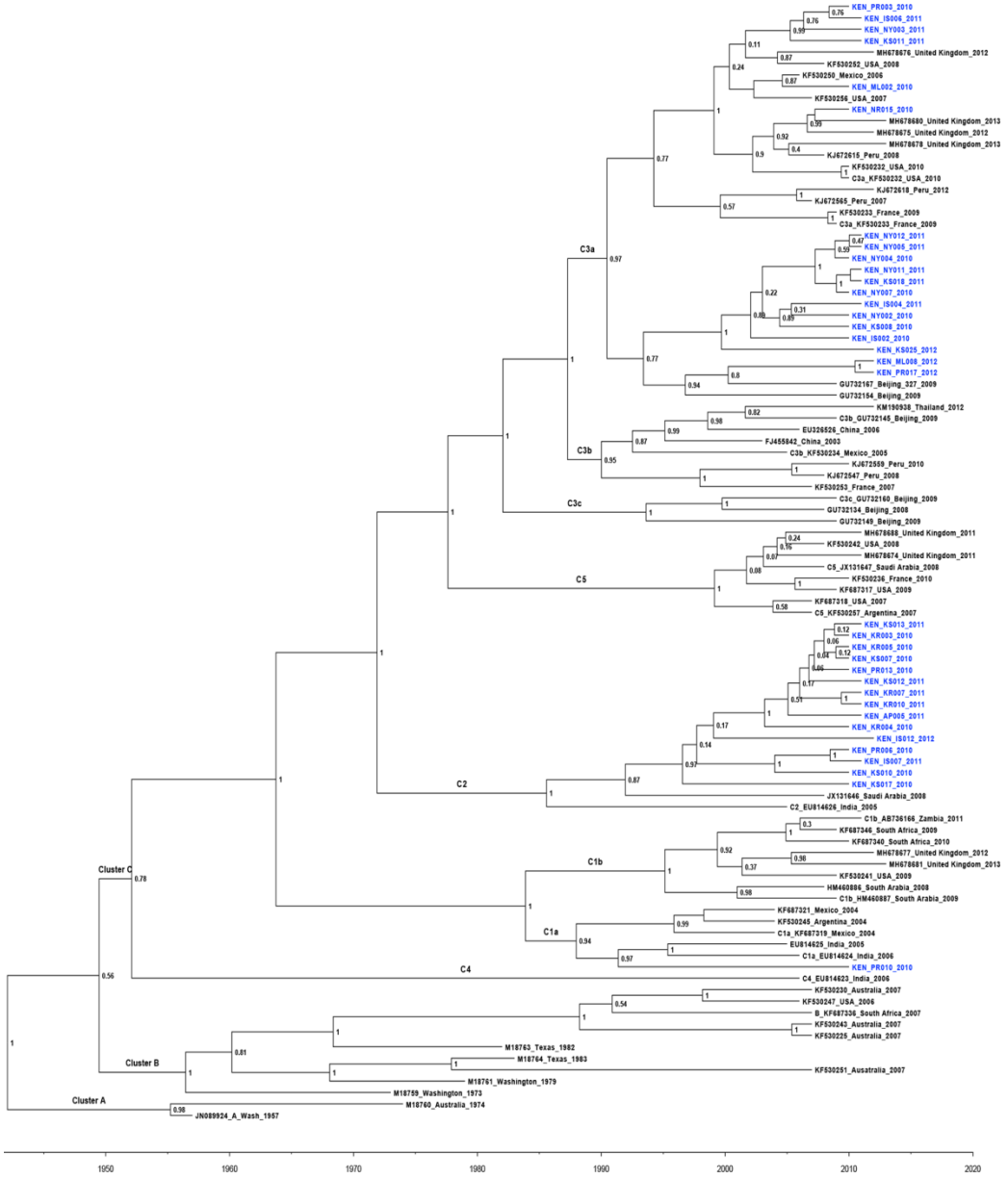

Figure 4.5:**Maximum credibility tree of the HRV3 viruses based on the complete HN coding sequences**

The Kenyan strains are shown in blue. The numbers at the nodes represent posterior probability values. The tree was constructed using BEAST software.

CHAPTER FIVE

DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

5.1 DISCUSSION

Molecular characterization of HRV3 viruses is crucial for various reasons. These reasons include; detection of newly emerging HRV3 strains, establishing their spatial and temporal circulating patterns in a given season, interpreting disease associations, and lastly, investigating outbreaks by linking disease clusters to specific HRV3 strains. Consequently, characterizing these viruses is useful for proper health planning and guidance on future vaccine/therapeutics development efforts leading to better ARI patient management. Despite the importance of characterizing these viruses and their contribution to the burden of viral infections, there exists no literature on HRV3 strains that are circulating in Kenya. To address this gap, this study utilized the HRV3 HN gene isolated from archived nasopharyngeal swab samples collected between 2010 and 2013, to establish their molecular characteristics. HRV3 HN sequence homology and phylogenetic clustering analysis relative to the HRV3 prototype strain has been used as a standard way of typing these viruses(Almajhdi, 2015b).

Only 87% of HRV3 displayed cytopathic effects. Unlike other viruses like influenza, which display distinctive CPE like cell rounding, human parainfluenza viruses rarely show CPE in cells. HRV1 and HPIV2 may display focal rounding and cell distraction in initial isolation. Similarly, HRV3 has been shown to destroy 50% of the cells due to its aggressive nature, and it may display more CPE in well-adapted cell lines (Schomacker *et al*., 2012). Some of the isolates (40%) failed to grow in culture; this failure could be a result of the loss of viability of the virus due to extended storage of more than a year. This challenge was also experienced by Opanda and co-authors (2014). Direct immunofluorescent assay was employed to confirm the isolation of HRV3 viruses. Although immunofluorescent tests have been used as a standard technique in virology for antibody detection, they have been reported to show low specificity (Yano *et al*., 2014). This may explain why some isolates failed to amplify despite being confirmed to be

positive for HRV3 by DFAT. A study on molecular classification of HRV1 used FAT and PCR to confirm HRV1 isolation (Kiptinness *et al*., 2013). Identification of these viruses plays a significant role in their treatment, prevention and control.

83% of RNA extracted from the culture supernatants amplified correctly indicated by the bright and distinct bands. The HN gene was amplified by three sets of primers previously described by Almajhdi (2012). However, a minority (17%) of the amplicons failed to amplify. This failure could be attributed to the low specificity of the primers used in this study. The challenge was also experienced by a study on HRV3 by Linster and colleagues (2018). According to their findings, changes in the primer binding regions of the HN gene of the different variants of HRV3 may have failed these isolates to amplify. BLAST analyses confirmed that all the 35 (70%) sequences obtained in this study were of human respirovirus type 3 origin. Whereas further optimization was required to increase the yield of good usable sequences, this was not possible because of financial implications.

Relative to the prototype strain, the Kenyan isolates shared a 94.3-95.6% (nucleotide) and a 96.5 to 97.9% (amino acid) identity determined by a sequence identity matrix. The majority of the nucleotide changes observed in this study did not alter the intended amino acid; which explains why the amino acid sequences of the Kenyan strains were highly identical to the prototype as opposed to the nucleotide sequences. These results mirror those of other authors across the globe (Mizuta *et al*., 2014, Linster *et al*., 2018). Interestingly, all the Kenyan variants also shared eight inimitable amino acid changes with the prototype. These findings are consistent with those from a study of HRV3 in China (Mao *et al*., 2012a), thus pointing to the diverse HRV3 strains observed currently. Further. the N terminus region displayed the most nucleotide variability, suggesting the reliance of this region for typing HRV3.

The conserved nature of the Kenyan HRV3 glycoprotein observed in this study corroborates findings from studies by other scientists (Almajhdi *et al*., 2012, Mizuta *et al*., 2014). Mutations in the catalytic region of the HRV3 HN glycoprotein may interfere with its function, inactivating it, and thus rendering it non-viable (Lawrence *et al*., 2004).

These findings may provide insights into future therapeutics against these viruses leading to better patient management. The N-linked glycosylation motifs in the Kenyan strains continue to be conserved relative to the prototype. Alterations at the N-linked glycosylation sites have been shown to hinder receptor recognition and F fusion protein cleavage. Furthermore, these alterations could also mask or introduce new epitopes on the HN protein that could either be advantageous or catastrophic to these viruses (Chu *et al*., 2013, Tsutsui *et al*., 2017b). These findings imply that these viruses remain fit in the population and are still a risk; therefore, urgent measures need to be taken to prevent infection.

Five codons involving the amino acid changes N8R, S45L, K168R, V191I, and L555S were shown to be under positive selection within the HN glycoprotein of the Kenyan viruses. These were identified by both MEME and REL methods, with strong statistical support (MEME p-value <0.05 and REL posterior probability > 0.95. The detection of amino acid residue L555S on the HN gene of the HRV3 as being positively selected by both MEME and REL algorithms has been reported elsewhere (Takahashi *et al*., 2018). The inferred positively selected sites in this study did not fall into any of the antigenic/ active sites; therefore, they may not affect the function of the HN protein. This finding may further indicate the conserved nature of the HN glycoprotein. The failure by other algorithms used in this study to detect positively selected sites with strong evidence of statistical support may be attributed to the small analysed sequence dataset. This result further shows the conserved state of the circulating Kenyan HRV3 strains. REL has been proven as the preferable model to detect positive selection (Suzuki, 2004). The positive selection gives an advantage to the virus enabling it to propagate hence survive in the population; Contrarily, negative selection infers a disadvantage to the virus making it incapable of having descendants, therefore, wiped out from the population (Biswas and Akey, 2006). The stable catalytic and antigenic HN regions suggest that the amino acid changes caused by positive selection in the HN region of circulating HRV3 were of no significance to the fitness of these viruses.

Many of HRV3 classification is based on a system laid out by Almajhdi and colleagues (Almajhdi, 2015a). This system classifies HRV3 into three main clusters; A, B, and C; with Cluster, C further divided into sub-clusters(C1-5) and lineages (a, b, c, and so forth). The phylogenetic reconstruction in this study included sub-clusters C1-C5 with lineages C1a, C1b, C3a, C3b, and C3c. A recent study on HRV3 introduced a new lineage C1c in their analysis (Bose *et al*., 2019). Phylogenetic analysis using Kenyan variants and other global strains elucidated that Cluster A encompassed an isolate from Australia and a rarely detected prototype strain. Cluster B possessed many current isolates from South Africa, USA, and Australia. However, none of the Kenyan variants clustered into A and B. Cluster C was the most widespread cluster comprising more recent isolates and all the Kenyan variants. These results are consistent with research performed in China, Saudi Arabia, Argentina, and Spain that elucidated the diversity of cluster C and the dominance of sub-cluster C3a (Godoy *et al*., 2016b, Goya *et al*., 2016, Takahashi *et al*., 2018).

The dominance of cluster C3a may indicate the efficiency of replication of this strain in cell culture compared to strain C2 and C1a. Indeed, analyses of genetic distances confirmed that the Kenyan HRV3 was closely related to the viruses in cluster C compared to those in Cluster A. This finding is consistent with that from a previous study (Takahashi *et al*., 2018) which could explain the clustering pattern observed in this study. HRV3 classification is also supported by (Mizuta *et al*., 2014), where HRV3 was classified into three lineages 1, 2, and 3. Subsequently, lineage 1 and 2 evolved into three main clusters, as observed presently. Contrary to previous findings, a study on HRV3 in the UK identified two significant groups of HRV3 strains using whole-genome data (Smielewska *et al*., 2018). The study also established a hypervariable short non-coding region between the fusion and the matrix gene. Classification of HRV3 based on this region and whole-genome data reflected a comprehensive phylogenetic profile of HRV3 compared to analysis using only the HN gene. The establishment of the three strains of HRV3 circulating in Kenya is important for studying seasonal trends of these viruses, which will enable enabling proper health planning. Furthermore, these patterns will also aid in understanding disease associations and outbreaks of these viruses.

Evolutionary analysis using BEAST algorithm showed multiple introductions of HRV3 variants from different sources into Kenya. For instance, strain C1a was seeded from India, strain C2 from Saudi Arabia, and finally strain C3b from China, North, and South America. This finding is similar to those observed in Vietnam (Linster *et al*., 2018). There is a possibility that multiple introductions maintained the epidemiology of HRV3 in Kenya. The three Kenyan HRV3 strains seemed to have been co-circulating at the same time (2010 – 2013), which is very common with viruses (Linster *et al*., 2018).

The evolution rate of HRV3 in this study was 4.56 $X10^{-4}$ (95% HPD 2.88 X 10^{-4} -6.42 X 10^{-4}) substitutions per site per year, while the mean TMRCA was1942 (95% HPD: 1928 - 1957). The 1942 TMRCA implies that HRV3 was already an established pathogen before its discovery in the 1950s (Henrickson, 2003). The moderate rate of evolution of HRV3 in this study mirrors those in other studies (Duffy *et al*., 2008, Bose *et al*., 2019), suggesting that were a vaccine formulation to be based on the prototype strain's HN protein, it may be efficient against the Kenyan viruses and hence appropriate for use in Kenya.

This study had some limitations. Since it relied on archived isolates, recovery of some HRV3 Strains in culture may have failed, introducing bias in the HRV3 variants reported in the study. Secondly, since this study used ILI instead of the SARI case definition, it missed cases with severe presentations, including bronchitis and pneumonia. The ILI case definition resulted in low detections of HRV3. Lastly, the utilisation of only the complete HN genes instead of whole genomes may fail to provide comprehensive insights into the genetic diversity and other evolutionary dynamics of HRV3 strains circulating in Kenya.

5.2 CONCLUSION

1. This study isolated 35 HRV3 viruses from Kenya patient samples between 2010 and 2013 confirming that HRV3 strains circulated in Kenya during the study period. Furthermore, this study has for the first time presented molecular evidence for the molecular characteristics of those HRV3 strains that circulated in Kenya between 2010 and 2013 using the complete HN gene
2. The study reported genetic mutations in the HN gene of HVR3 in Kenya. However, those mutations were immunologically inconsequential because they did not affect any antigenic site of this majorly immunogenic protein. The accompanying sporadic amino acid changes across the Kenyan HRV3 HN protein were point mutations, indicating that the HRV3 glycoproteins were antigenically stable. Indeed, all the observed mutations did not introduce nor abrogate the existing glycosylation patterns on this HN protein.
3. From an evolution perspective, the Kenyan HRV3 viruses were quite similar to others circulating elsewhere globally. It was established that all these viruses belonged to cluster C, with sub-cluster C3a being the dominant one in Kenya followed by sub-cluster C2 then C1. In addition, a moderate rate of evolution was observed amongst the Kenyan viruses. The conserved evolutionary stasis of the Kenyan HRV3 HN provides confidence that this protein is a possible target for vaccine development.

5.3 RECOMMENDATIONS

1. One major limitation of this study is that only the HN gene, which accounts for only 12% of the total genome, was used to examine the mutations occurring in the HRV3 genome as well as the virus's evolution. However, this is insufficient because deeper insights and comprehensive conclusions about virus mutation rates and evolutionary changes can only be inferred using whole-genome data. As a result, whole-genome sequencing of Kenyan HRV3 should be performed in the

future to gain a better understanding of the genetic and evolutionary dynamics of HRV3.

2. Due to the severe respiratory illnesses caused by these important pathogens, the country must continue to monitor HRV3 viruses. This decision will allow for the study of HRV3 outbreaks, disease associations, and the circulation patterns of these viruses, ensuring proper health planning.
3. This study established the conserved nature of the HN glycoprotein, which is supported by other research from around the world. In the future, the HN gene should be considered as a viable target for the development of HRV3 vaccines and therapeutics.

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APPENDICES

Appendix I:Scientific and ethical review unit approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 (020) 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2720030
Email: director@kemri.org info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1 August 19, 2016

TO: JULIET ELUSAH MWATA
PRINCIPAL INVESTIGATOR

THROUGH: DR. ROSEMARY SANG,
ACTING DIRECTOR, CVR,
NAIROBI

Dear Madam,

RE: PROTOCOL NO. KEMRI/SERU/CVR/004/3398 (*RESUBMISSION OF INITIAL SUBMISSION*) MOLECULAR CHARACTERIZATION OF HUMAN PARA INFLUENZA TYPE 3 VIRUS ISOLATED AMONG PATIENTS ATTENDING PUMWANI HEALTH CENTRE NAIROBI, 2013-2015 *(VERSION 2; 3RD AUGUST 2016)*

Reference is made to your letter 3rd August, 2016. The KEMRI/Scientific and Ethics review Unit (SERU) acknowledges receipt of the revised study documents on 11th August, 2016.

The Committee notes that the issues raised at the 253rd Committee A meeting of the KEMRI Scientific Ethics Review Unit (SERU) held on **13th July 2016** have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **August 19, 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **August 18, 2017.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **July 07, 2017**.

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

You may now embark on your study.

Yours faithfully,

For

DR. EVANS AMUKOYE,
ACTING HEAD,
KEMRI SCIENCE AND ETHICS REVIEW UNIT

Appendix II: Scientific and ethical review unit approval amendment

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) 2722541, 2713349, 0722-205901,0733-400003, Fax: (254) (020) 2720030
Email: director@kemri.org; info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 **November 05, 2019**

TO: **JULIET ELUSA MMATA**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CVR**
NAIROBI

Dear Madam,

RE: **SERU PROTOCOL NO. 3298 *(RESUBMISSION REQUEST FOR AMENDMENT 1)*: MOLECULAR CHARACTERIZATION OF HUMAN PARA INFLUENZA TYPE 3 VIRUS ISOLATED AMONG PATIENTS ATTENDING PUMWANI HEALTH CENTRE 2013-2015. *(VERSION 4 DATED 1 OCTOBER 2019)***

Reference is made to your letter dated October 02, 2019. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledge receipt of the revised documents on October 04, 2019

This is to inform you that the Committee determines that the issues raised during the 290th Committee C meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **August 29, 2019** are adequately addressed.

You are therefore **authorized** to implement the following amendments accordingly:

1. Change of study site from Pumwani Health Centre to Kenya
2. Change of study period from between 2013 to 2015 to between 2010 to 2013

Please note that you are responsible for submitting any further changes to the approved version of the study protocol to SERU for review and the changes should not be initiated until written approval from the SERU is received.

Yours faithfully,

ENOCK KEBENEI
THE ACTING HEAD
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix III:Media preparation

a). Growth media

To prepare 50mL of growth media add;

i. 1% L. glutamine (0.5mL)
ii. 1% Penicillin/Streptomycin (0.5mL)
iii. 1% Gentamycin (50µL)
iv. 1% Fungizone (0.5mL)
v. 10% Foetal Bovine Serum (10mL)
vi. Top up to the 50mL mark
vii. Swirl gently to mix
viii. Label the growth media with your name, preparation date, and expiry date of four weeks from the preparation date.
ix. Store at 4℃

b) Maintenance media

To prepare 50mL of maintenance media add:

i. 0.5mL L glutamine (1%)
ii. 0.5Ml Penicillin/Streptomycin (1%)
iii. 0.5Ml Fungizone (1%)
iv. 50µLTrypsin(tpck)
v. 1.35mL Bovine Serum Albumin
vi. Top up with DEMEM up to the 50Ml Mark
vii. Swirl gently to mix
viii. Label the maintenance media with your name, preparation date, and expiry date of four weeks from the preparation date
ix. Store at 4℃

c) Preparation of PBS

To prepare 1000mL of PBS, in a one-litre measuring cylinder add:

i. 100mL of PBS (10x concentration)
ii. Top up with distilled water up to the 1000mL mark and adjust the PH to 7.1-7.3
iii. Autoclave
iv. Label sterile PBS with your name, date of preparation, and expiration date of six months from the preparation date
v. Store at room temperature

d) Preparation of tween PBS

To make 1000mL of Tween PBS, in a measuring cylinder:

i. Add 800mL of distilled water and add:

- 8g of NaCl
- 0.2g of KCl
- 1.44g of Na2HPO4
- 0.24g of KH2PO4
- 2ml of tween-20

ii. Swirl gently to mix and adjust the pH to 7.2

iii. Top up with distilled water unto the one-litre mark

iv. Sterilize by autoclaving

v. Label as "Tween PBS" with, your name, preparation date, and an expiration date of 6 month

from the preparation date

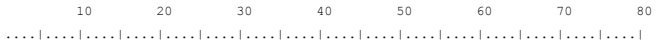
vi. Store at 4℃.

e) Preparation of Tris Borate EDTA

To make 100mL of Tris Borate EDTA buffer, in a measuring cylinder:

i. Add 900m of distilled water to 100mL of 10X TBE electrophoresis buffer.
ii. Swirl gently to mix
iii. Store at room temperature

Appendix IV: Amino acid substitutions of the Kenyan and global



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INSIKSEKAHESLLQDVNNEFMEVTEKI

MH678676_UK_2012

....XX.X.....T.X.X.XL.....T.....X.X.....X.....I..X.....

MH678674_UK_2011 **.....T.....L.....T.....I..L.....**

KF687317_USA_2009 **.....T.....L.....T.....R.....I..L.....**

KF530236_FRA_2010 **.....T.....L.....T.....R.....I..L.....**

MH678688_UK_2011 **.....T.....L.....T.....R.....I..L.....**

KF530242_USA_2008 **.....T.....L.....T.....R.....I..L.....**

C5_KF530257_ARG_2007

.....T.....L.....T.....R.....I..L.....

C5_JX131647_S.Arabia_2008

.....T.....L.....T.....N..R.....I..L.....

KF687318_USA_2007 **.....T.....L.....T.....R.....I..L.....**

KF530253_FRA_2007 **.....V.....T.....L.....T.....T.....R.....I.....**

KJ672559_PER_2010 **.....V.....T.....L.....T.....I.....T.....R.....I.....**

KJ672547_PER_2008 **.....V.....T.....L.....T.....T.....R.....I.....**

KM190938_Thai 2012 **.....V.....T..S..L.....T.....T.....R.....I.....**

GU732167_BJ_327_09 **.....T.....L.....T.....M.....T.....R.....I.....**

C3b_KF530234_MEX_2005

.....V.....T.....L.....T.....T.....R.....I.....

FJ455842_China 2003 **.....V...G..T.....L.....T.....T.....R.....I.....**

C3b_GU732145_BJ_2009

.....V.....T...S..L.....T.....T.....R....R.I.T.....

EU326526_China 2006 ..........G..T.....L.....T.....T.....R.....I.....

KEN/ML008/2012 ..........T....S.L.....T.....T.....R.....I.....

KEN/PR017/2012 ..........T....S.L.....T.....T.....R.....I.....

GU732154_BJ_2009 ..........T.....L.....T.....T.....R.....L.....

KEN/IS004/2011 .....R.....T.....L.....VT.....T.....R.....I.....

KEN/NY002/2010 ..........T.....L.....T.....T.....R.....I.....

KEN/KS008/2010 .....Q.....T.....L.....T.....T.....R.....I.....

KEN/KS025/2012 ..........T.....L.....T.....T.....R.....I.....

KEN/IS002/2010 .....S..V.....T.....L.....T.....T.....R.....I.....

KEN/NY007/2010 ..........T.....L.....T.....T.....R..I.I.....

KEN/KS018/2011 ..........T.....L.....T.....T.....R..I.I.....

KEN/NY011/2011 ..........T.....L.....T.....T.....R..I.I.....

KEN/NY005/2011 ..........T.....L.....T.....T.....R.....I.....

KEN/NY004/2010 ..........T.....L.....T.....T.....R.....I.....

KEN/NY012/2011 ..........T.....L.....T.....T.....R.....I.....

MH678678_UK_2013 ..........T.....L.....T.....T.....R.....I..L.....

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..........A.T.....L.....T.....T.....R.....I.....

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KEN/NR015/2010 .....P.....T.....L.....T.....T.....R.....I.....

KF530252_USA_2008 ..........T.....L.....T.....I....T.....R.....I.....

MH678675_UK_2012 ..........T.....L.....T.....T.....R.....I.....

MH678680_UK_2013 ..........T.....L.....T.....T.....R.....I.....

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GU732149_BJ_2009 .....T.....L.....T.....R.....I.....

C2_EU814626_India_2005

.....GKK.....T..N..L.....T.....Q.R.....I.....

JX131646_S.Arabia_2008 .....T..N..L.....T.....Q.R.....I.....

KEN/AP005/2011

.....R..E...VV.K.T..N.Y.L.....T.....Q.R.....I.....

KEN/IS012/2012 .....STT.....L.....T.....Q.R.....I.....

KEN/KS010/2010 .....I.....T..N..L.....T.....Q.R.....I.....

KEN/PR006/2010 .....V...T..N..L.....T.....Q.R.....I.....

KEN/IS007/2011 .....T..N..L.....T.....Q.R.....I.....

KEN/KS017/2010 .....T..NS..L.....T.....Q.R.....I.....

KEN/KR007/2011 .....T..N..L.....T.....Q.R.....I.....

KEN/KR010/2011 .....T..N..L.....T.....Q.R.....I.....

KEN/KS012/2011 .....R.....T..N..L.....T.....Q.R.....I.....

KEN/KR004/2010 .....P..N..L.....T.....Q.R.....I.....

KEN/PR013/2010 .....T..N..L.....T.....Q.R.....I.....

KEN/KR003/2010 .....T..N..L.....T.....Q.R.....I.....

KEN/KS007/2010 .....T..N..L.....T.....Q.R.....I.....

KEN/KR005/2010 .....T..N..L.....T.....Q.R.....I.....

KEN/KS013/2011 .....T..N..L.....T.....Q.R.....I.....

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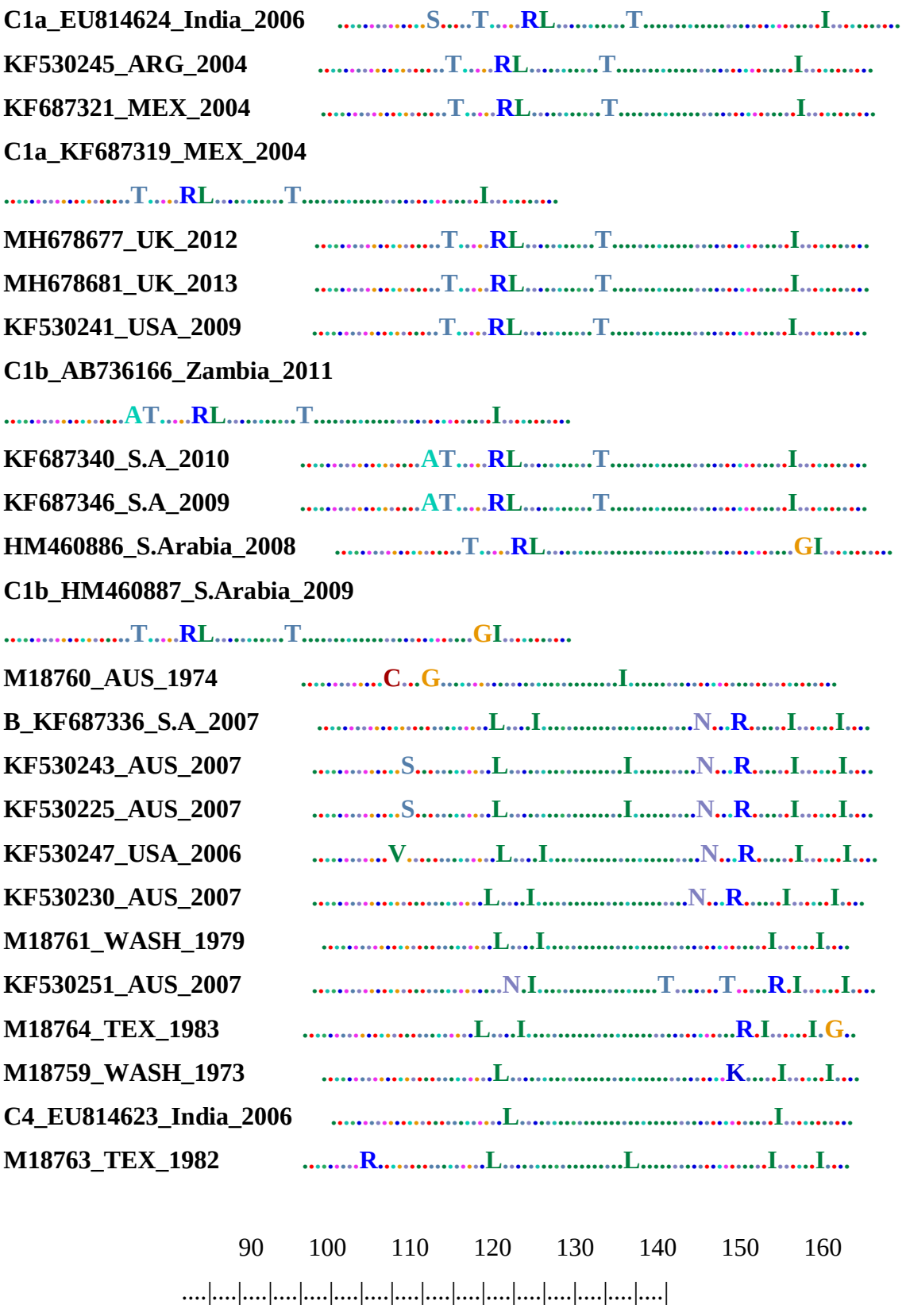
KEN/PR010/2010 .....T.....WL.....TT.....I.....I.....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KF530241_USA_2009
C1b_AB736166_Zambia_2011
KF687340_S.A_2010
KF687346_S.A_2009
HM460886_S.Arabia_2008
C1b_HM460887_S.Arabia_2009
M18760_AUS_1974
B_KF687336_S.A_2007
KF530243_AUS_2007
KF530225_AUS_2007
KF530247_USA_2006
KF530230_AUS_2007
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KF530251_AUS_2007
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90
100
110
120
130
140
150
160

A_Wash_1957_JN089924

QMASDNINDLIQSGVNTRLLTIQSHVQNYIPISLTQQMSDLRKFISEITIRNDN

QEVPPQRITHDVGIKPLNPDDFWRCT

MH678676_UK_2012

.V...T.....X.....X...I.....X.....X...X...X...

MH678674_UK_2011 .V...T.....I.....L.....

KF687317_USA_2009 .V...T.....I.....L.....

KF530236_FRA_2010 .V...T.....I.....L.....

MH678688_UK_2011 .V...T.....I.....

KF530242_USA_2008 .V...T.....I.....

C5_KF530257_ARG_2007 .V...T.....I.....S.....

C5_JX131647_S.Arabia_2008 .V...T.....I.....

KF687318_USA_2007 .V...T.....I.....

KF530253_FRA_2007 .V...T.....I.....L.....

KJ672559_PER_2010 .V...T.....I.....

KJ672547_PER_2008 .V...T.....I.....

KM190938_Thai 2012 .V...T.....I.....

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FJ455842_China 2003 .V...T.....I.....I.....

C3b_GU732145_BJ_2009 .V...T.....I.....

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KEN/ML008/2012 .V...T.....I.....

KEN/PR017/2012 .V...T.....I.....

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KEN/IS004/2011 .V...T.....I.....

KEN/NY002/2010 .V...T.....I.....

KEN/KS008/2010 .V...T.....I.....

KEN/KS025/2012 .V...T.....I.....

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KEN/NY007/2010 .V...T.....I.....

KEN/KS018/2011 .V...T.....I.....

KEN/NY011/2011 .V...T.....I.....

KEN/NY005/2011 .V...T.....I.....

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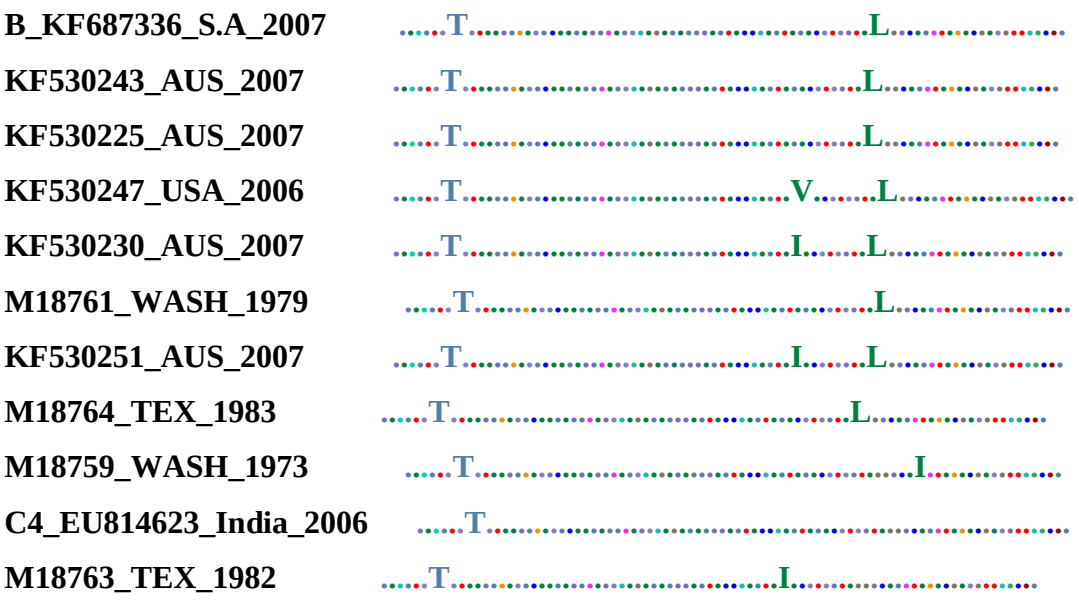
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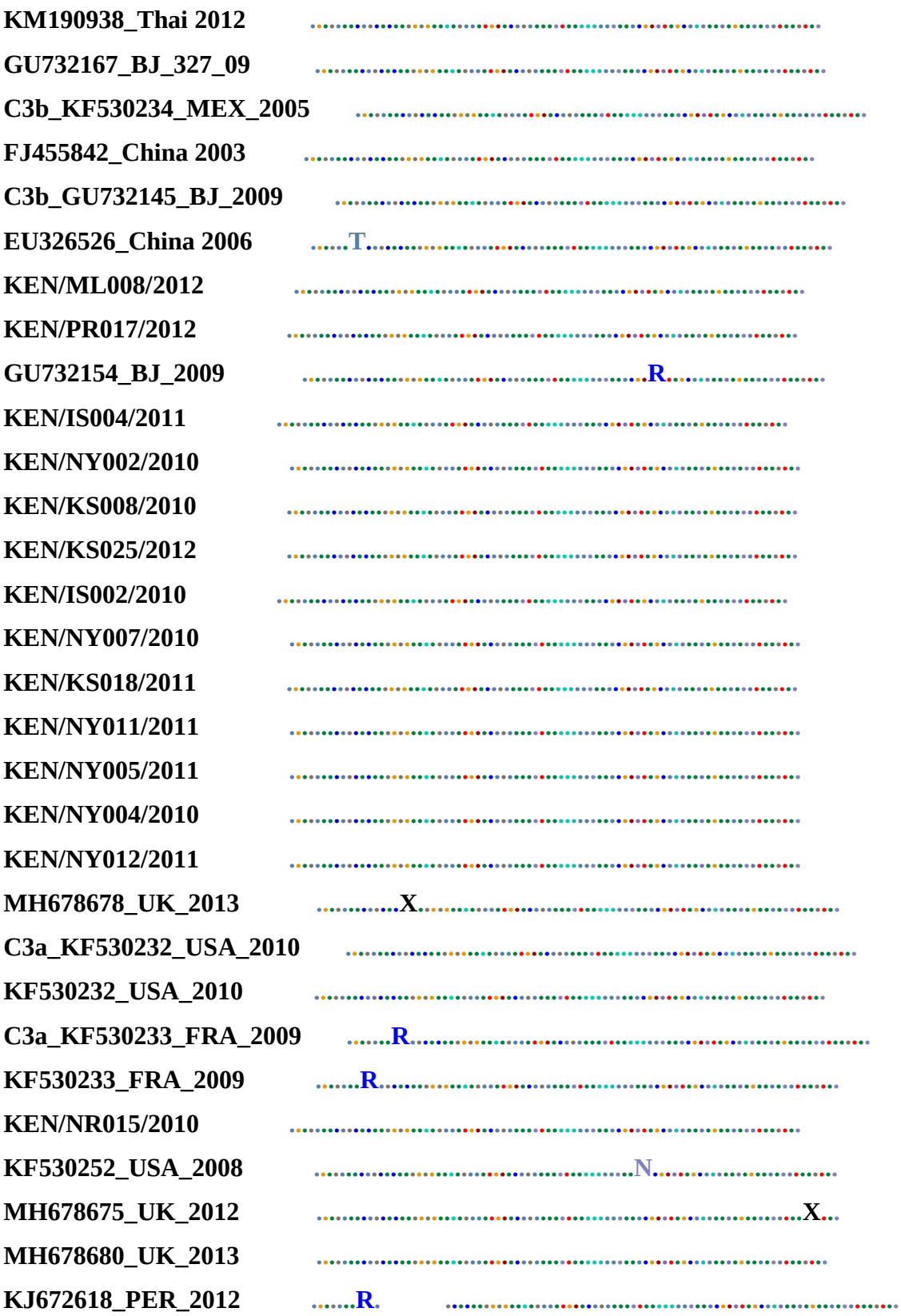
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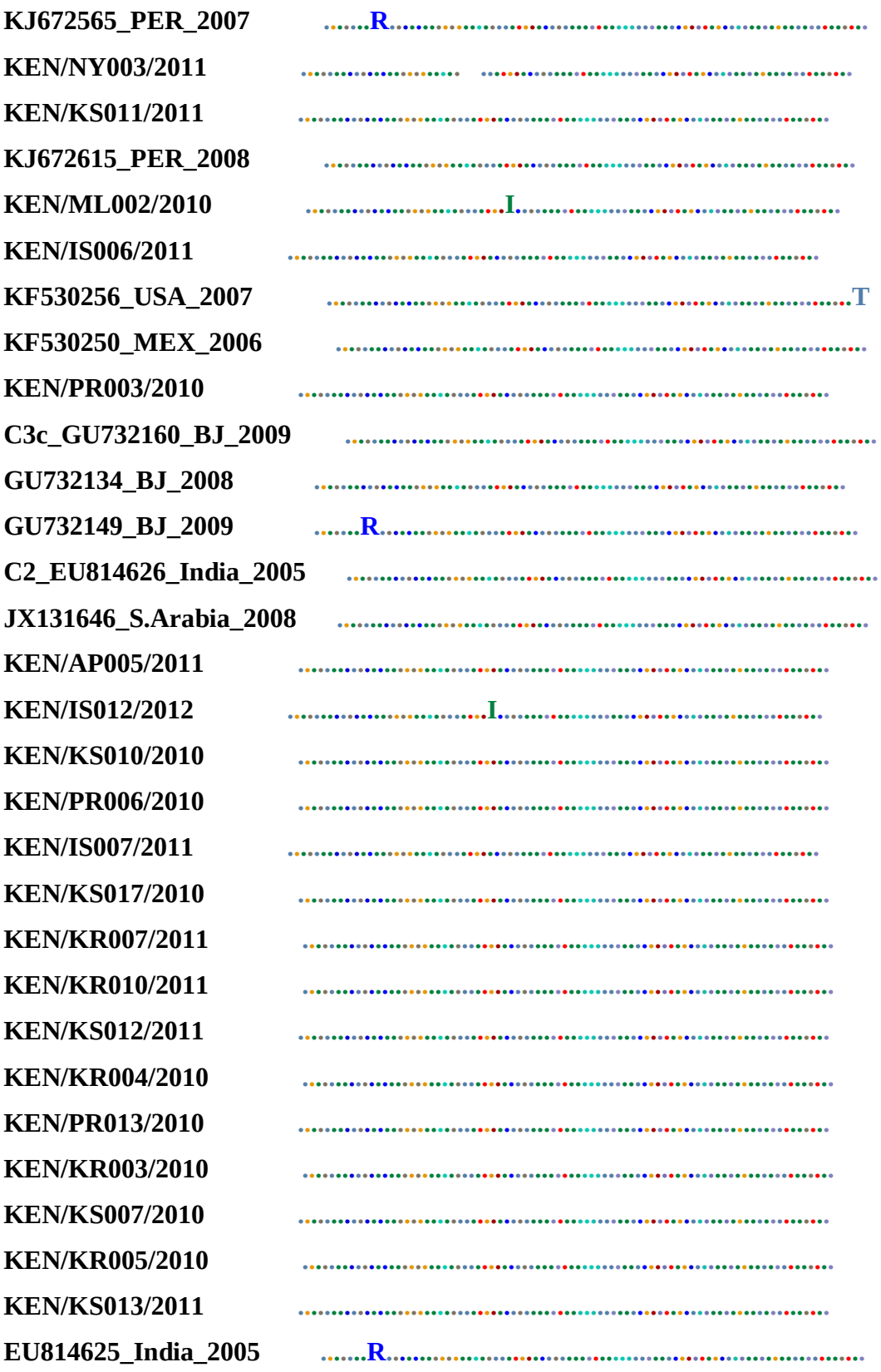
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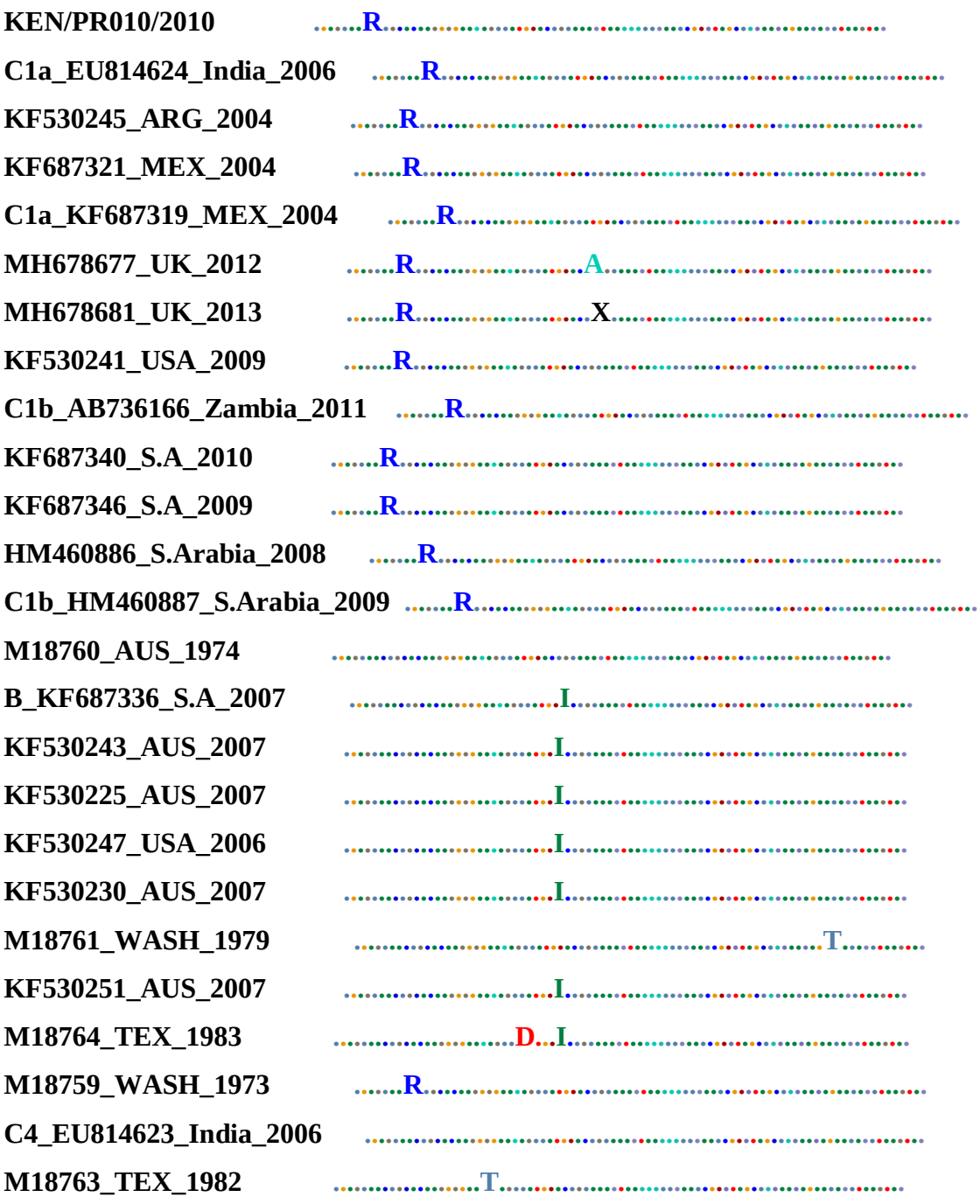
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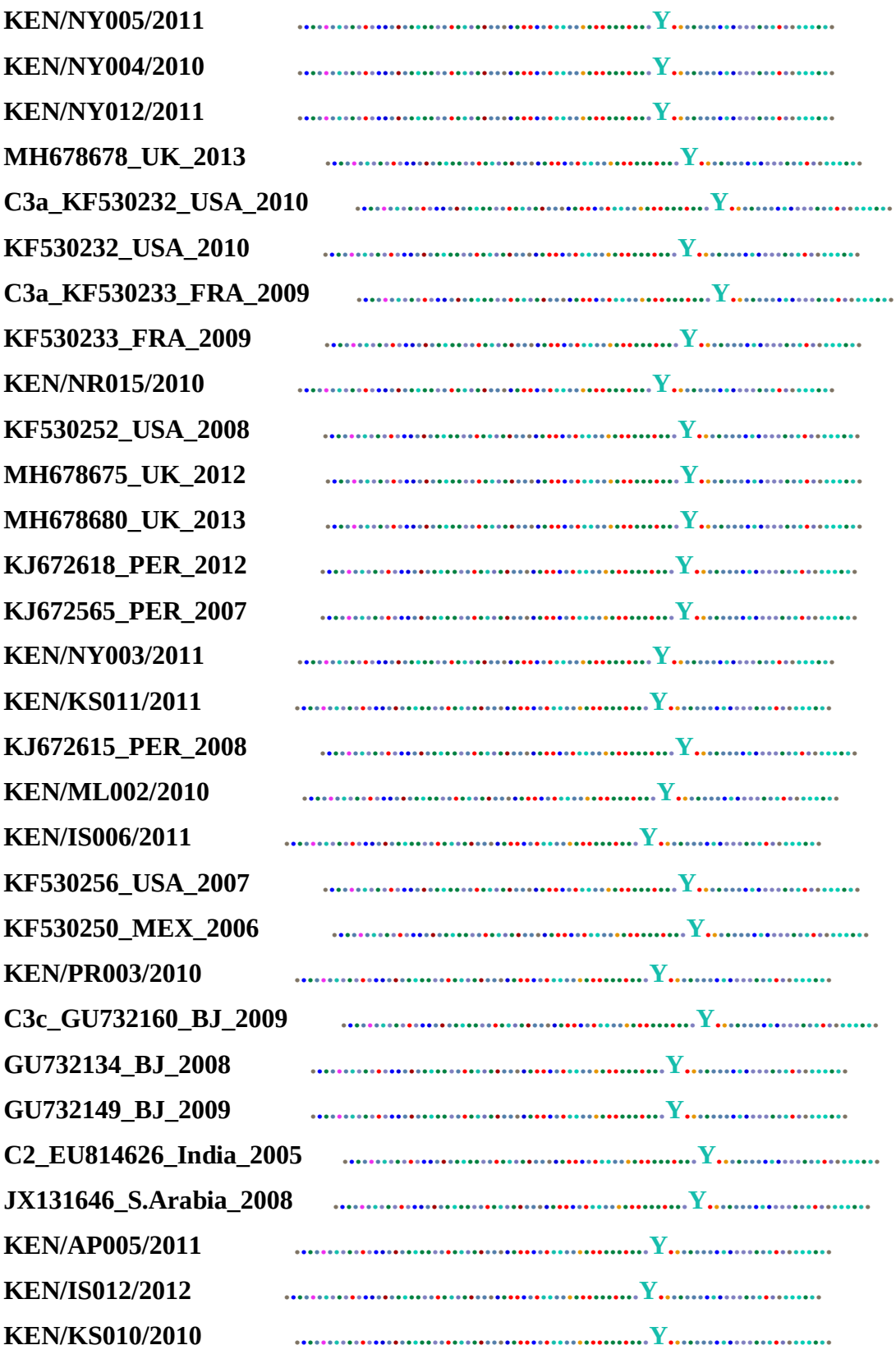
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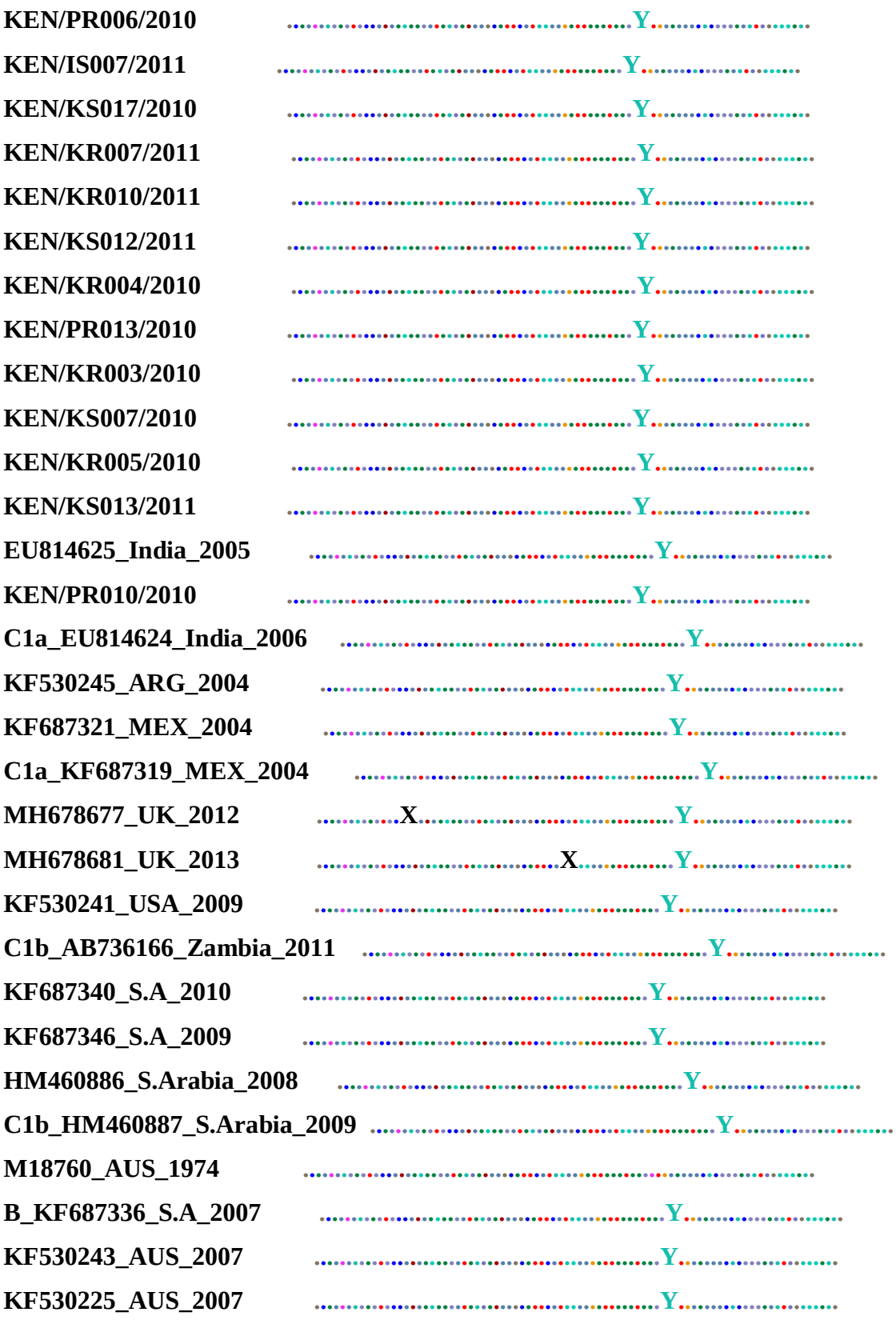
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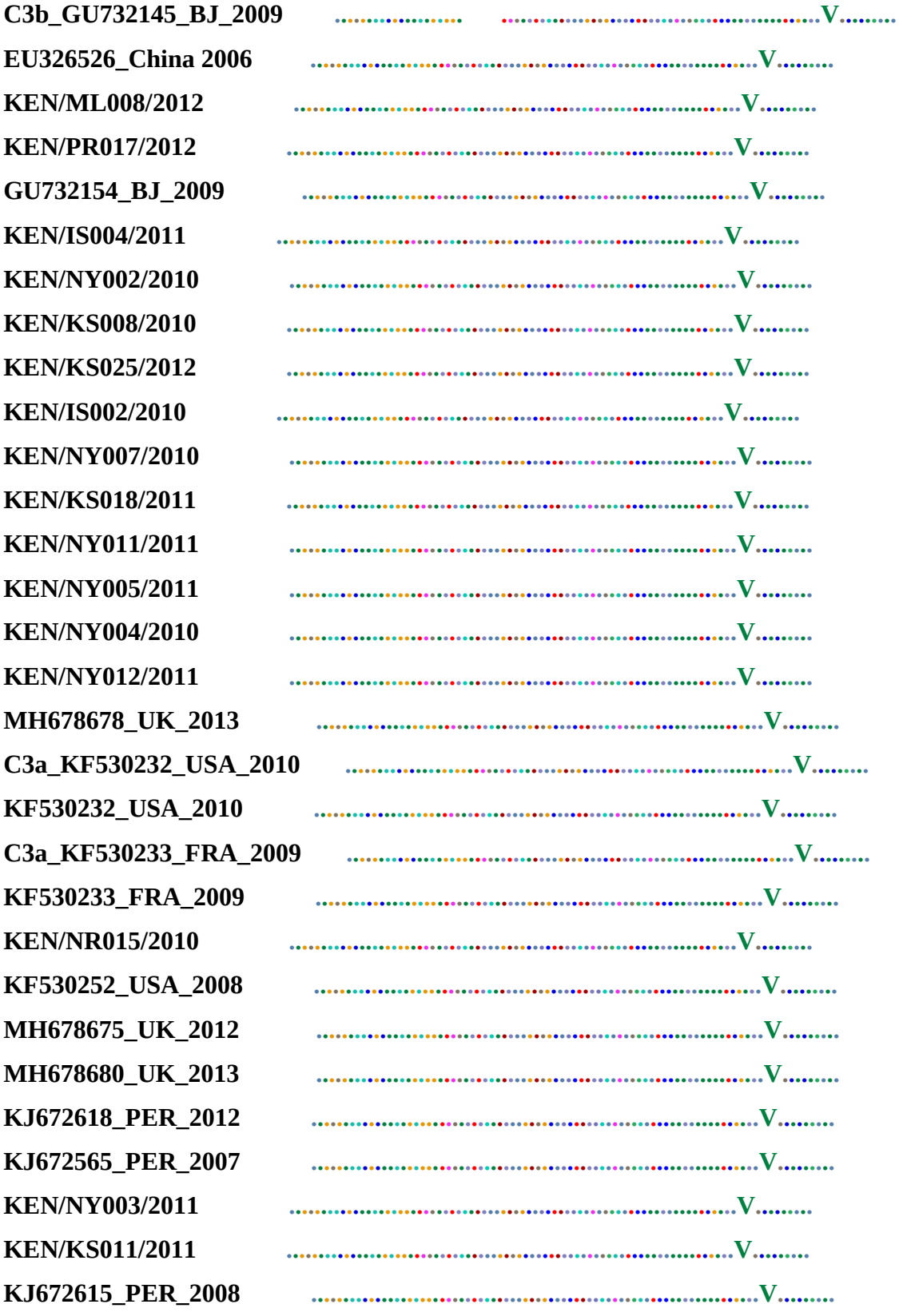
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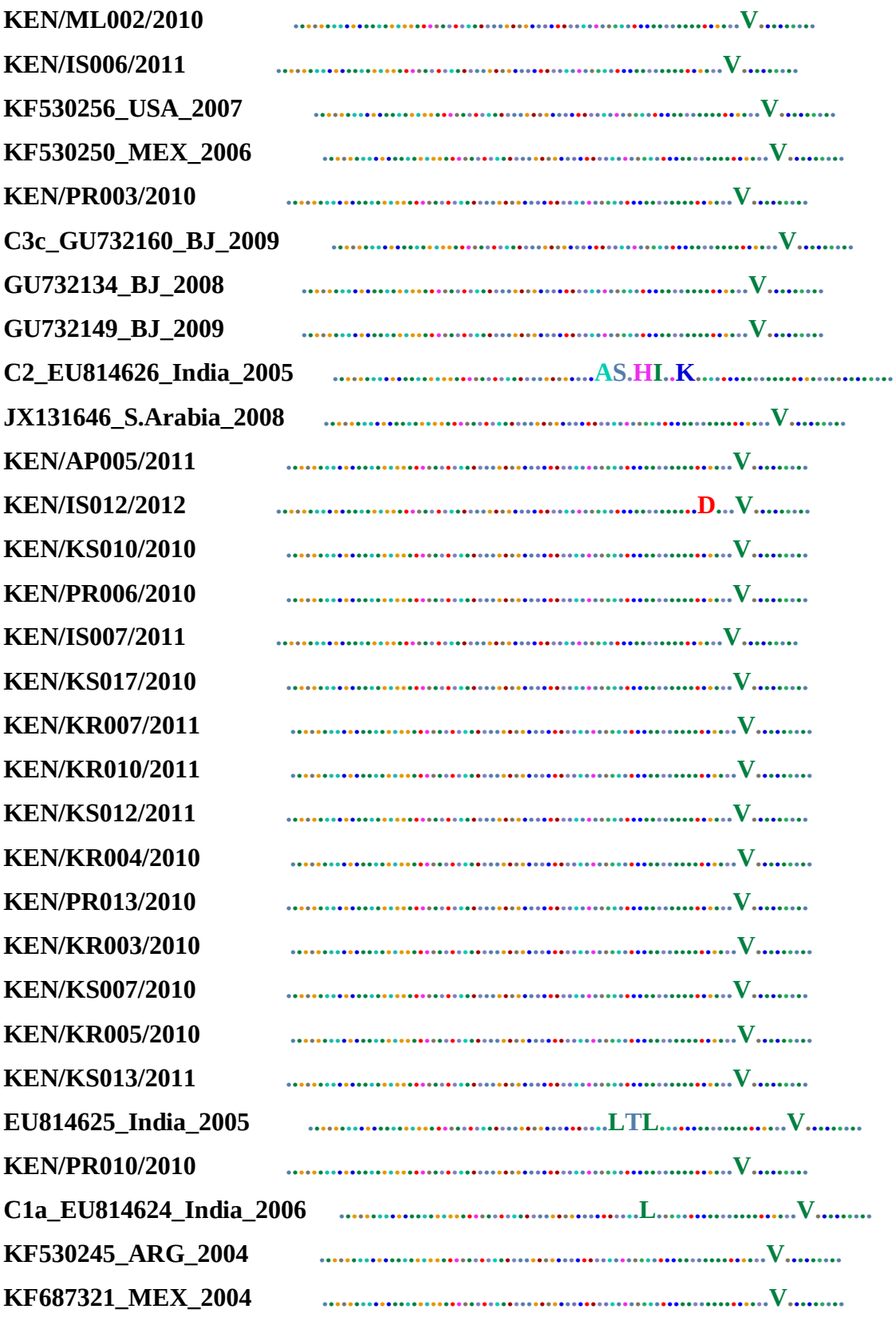
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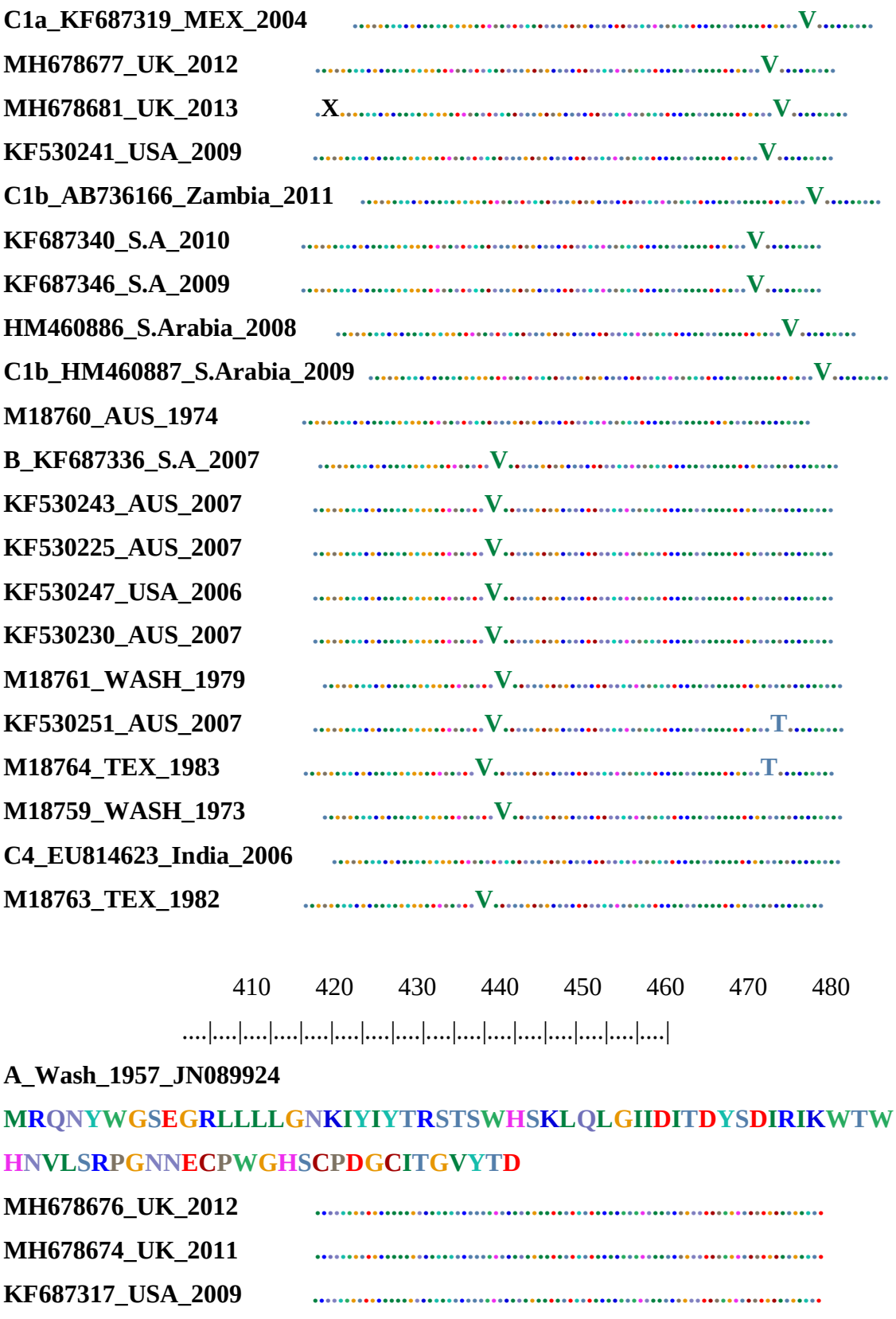
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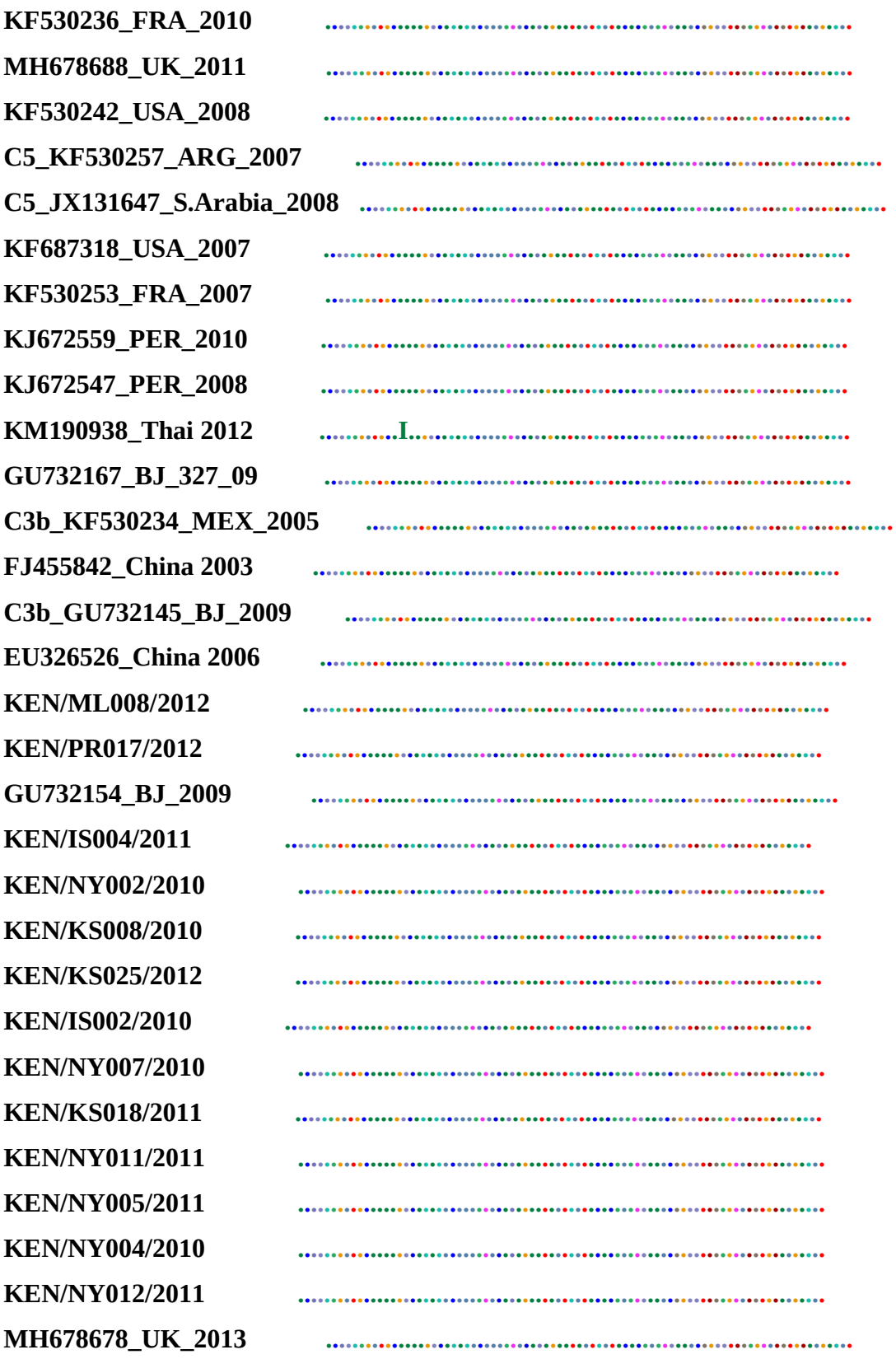
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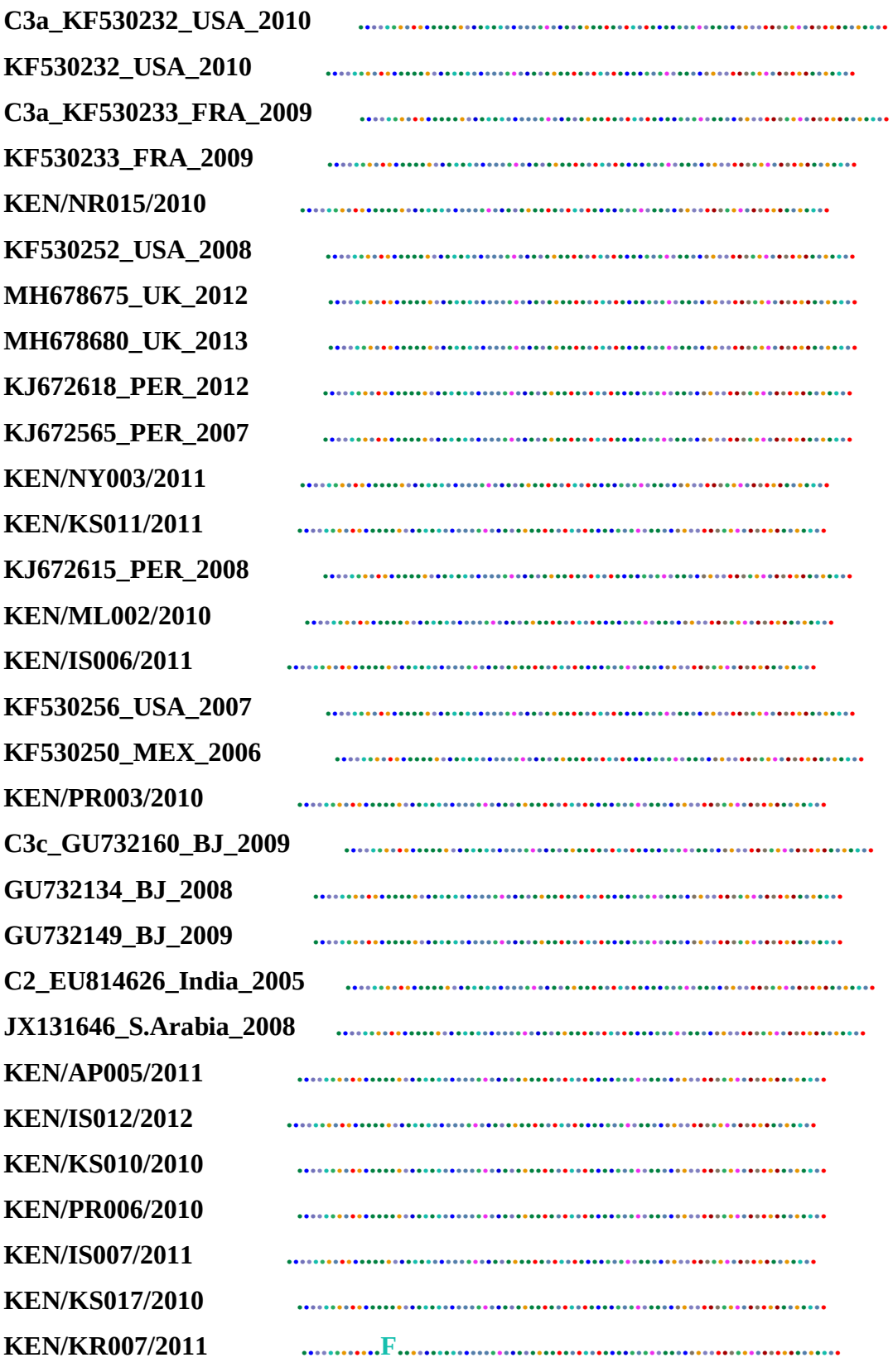
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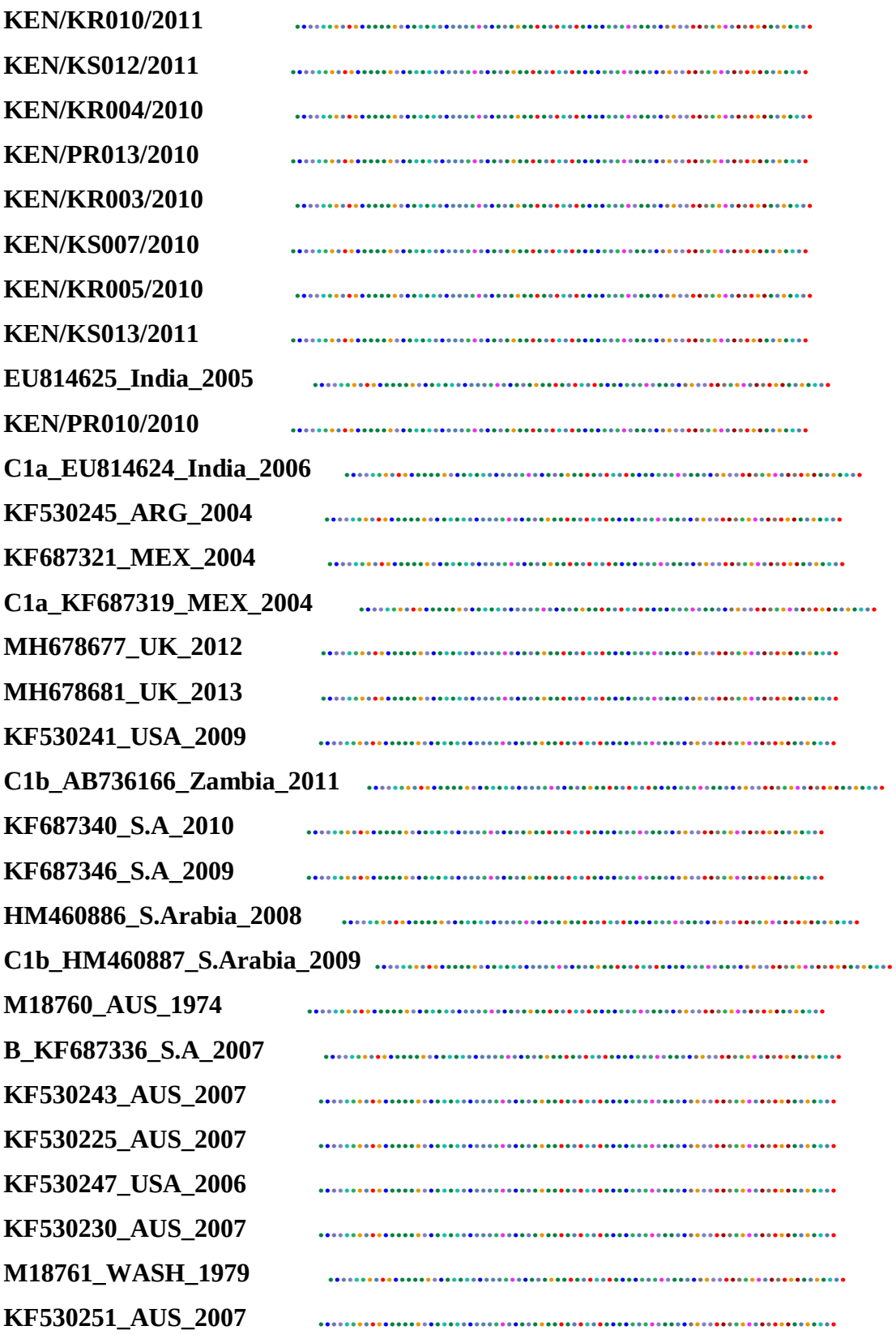
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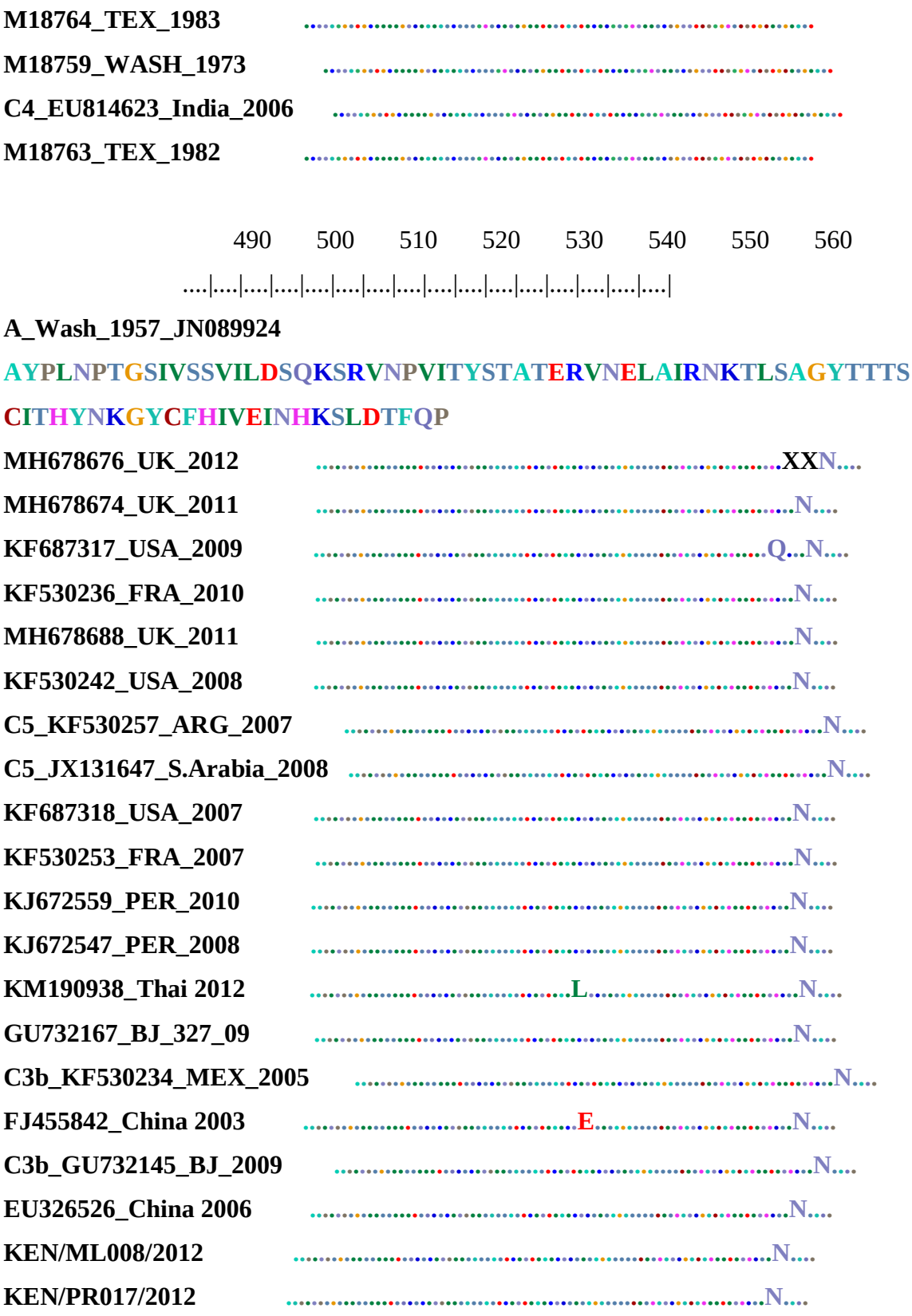
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KEN/KS017/2010 .....N....

KEN/KR007/2011 .....N....

KEN/KR010/2011 .....N....

KEN/KS012/2011 .....N....

KEN/KR004/2010 .....N....

KEN/PR013/2010 .....N....

KEN/KR003/2010 .....N....

KEN/KS007/2010 .....N....

KEN/KR005/2010 .....N....

KEN/KS013/2011 .....N....

EU814625_India_2005 .....N..NS

KEN/PR010/2010 .....N....

C1a_EU814624_India_2006 .....N....

KF530245_ARG_2004 .....N....

KF687321_MEX_2004 .....N....

C1a_KF687319_MEX_2004 .....N....

MH678677_UK_2012 .....N....

MH678681_UK_2013 .....N....

KF530241_USA_2009 .....T....

C1b_AB736166_Zambia_2011 .....N....

KF687340_S.A_2010 .....N....

KF687346_S.A_2009 .....N....

HM460886_S.Arabia_2008 .....N....

C1b_HM460887_S.Arabia_2009 .....N....

M18760_AUS_1974 .....T.....

B_KF687336_S.A_2007 .....I.R.....N....

KF530243_AUS_2007 .....R.....N....

KF530225_AUS_2007 .....R.....N....

KF530247_USA_2006 .....R.....G.N....

KF530230_AUS_2007 .....R.....N....

M18761_WASH_1979 .....R.....Q..N....

KF530251_AUS_2007 .....R.....N....

M18764_TEX_1983 .....R.....N....

M18759_WASH_1973 .....R.....SN....

C4_EU814623_India_2006 .....N....

M18763_TEX_1982 .....

570

....|....|..

A_Wash_1957_JN089924 MLFKTEIPKSCS

MH678676_UK_2012 .....

MH678674_UK_2011 .....

KF687317_USA_2009 .....

KF530236_FRA_2010 .....

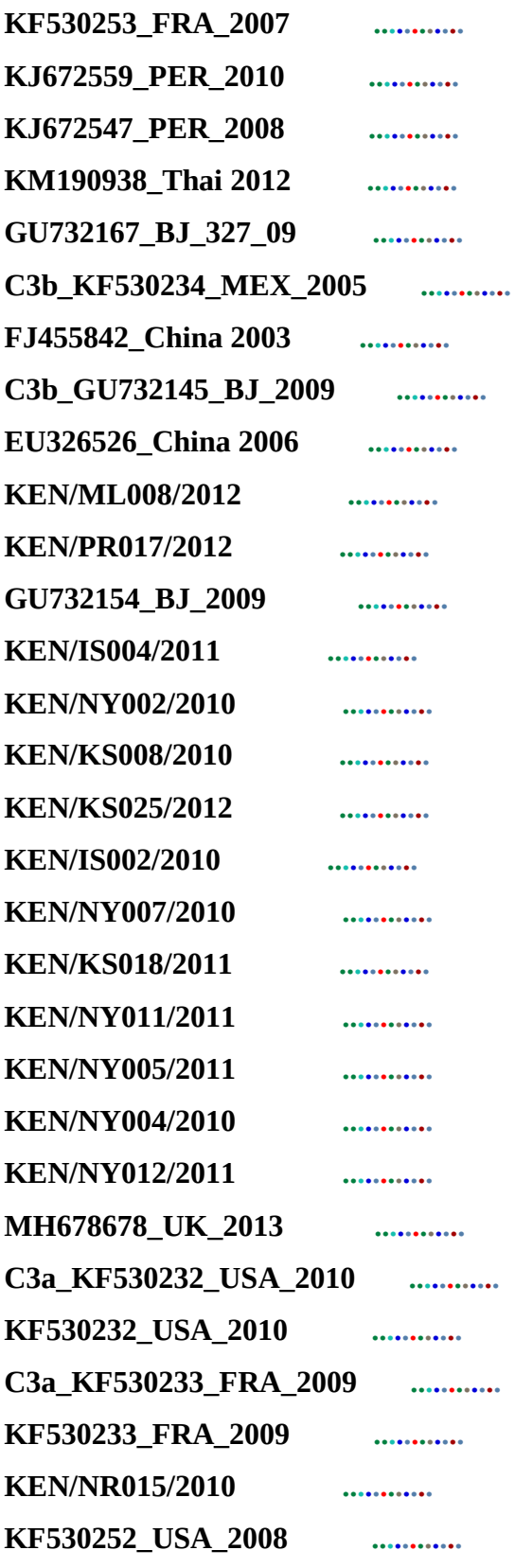
MH678688_UK_2011 .....

KF530242_USA_2008 .....

C5_KF530257_ARG_2007 .....

C5_JX131647_S.Arabia_2008 .....

KF687318_USA_2007 .....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KJ672559_PER_2010
KJ672547_PER_2008
KM190938_Thai 2012
GU732167_BJ_327_09
C3b_KF530234_MEX_2005
FJ455842_China 2003
C3b_GU732145_BJ_2009
EU326526_China 2006
KEN/ML008/2012
KEN/PR017/2012
GU732154_BJ_2009
KEN/IS004/2011
KEN/NY002/2010
KEN/KS008/2010
KEN/KS025/2012
KEN/IS002/2010
KEN/NY007/2010
KEN/KS018/2011
KEN/NY011/2011
KEN/NY005/2011
KEN/NY004/2010
KEN/NY012/2011
MH678678_UK_2013
C3a_KF530232_USA_2010
KF530232_USA_2010
C3a_KF530233_FRA_2009
KF530233_FRA_2009
KEN/NR015/2010
KF530252_USA_2008

MH678675_UK_2012

MH678680_UK_2013

KJ672618_PER_2012

KJ672565_PER_2007

KEN/NY003/2011

KEN/KS011/2011

KJ672615_PER_2008

KEN/ML002/2010

KEN/IS006/2011

KF530256_USA_2007

KF530250_MEX_2006

KEN/PR003/2010

C3c_GU732160_BJ_2009

GU732134_BJ_2008

GU732149_BJ_2009

C2_EU814626_India_2005 ....P....RR

JX131646_S.Arabia_2008

KEN/AP005/2011

KEN/IS012/2012

KEN/KS010/2010

KEN/PR006/2010

KEN/IS007/2011

KEN/KS017/2010

KEN/KR007/2011

KEN/KR010/2011

KEN/KS012/2011

KEN/KR004/2010

KEN/PR013/2010

KEN/KR003/2010

KEN/KS007/2010

KEN/KR005/2010 ....

KEN/KS013/2011 ....

EU814625_India_2005 .FLTA.....

KEN/PR010/2010 ....

C1a_EU814624_India_2006 ....

KF530245_ARG_2004 ....

KF687321_MEX_2004 ....

C1a_KF687319_MEX_2004 ....

MH678677_UK_2012 ....

MH678681_UK_2013 ....

KF530241_USA_2009 ....

C1b_AB736166_Zambia_2011 ....

KF687340_S.A_2010 ....

KF687346_S.A_2009 ....

HM460886_S.Arabia_2008 ....

C1b_HM460887_S.Arabia_2009 ....

M18760_AUS_1974 ....

B_KF687336_S.A_2007 .....V.....

KF530243_AUS_2007 .....V.....

KF530225_AUS_2007 .....V.....

KF530247_USA_2006 .....V.....

KF530230_AUS_2007 .....V.....

M18761_WASH_1979 ....

KF530251_AUS_2007 ....

M18764_TEX_1983 ....

M18759_WASH_1973 ....

C4_EU814623_India_2006 ....

M18763_TEX_1982 .....V.....

Appendix V:Reference strains used in this study

Accession Number	Country	Year
JN089924	USA	1957
M18760	AUSTRALIA	1974
M18759	USA	1973
M18761	USA	1979
M18764	TEXAS	1983
KF530251_	AUSTRALIA	2007
M18763	TEXUS	1982
KF530225	AUSTRALIA	2007
KF530243	AUSTRALIA	2007
KF687336	SOUTH AFRICA	2007
KF530230	AUSTRALIA	2007
KF530247	USA	2006
EU814624	INDIA	2006
EU814625	INDIA	2005
KF687319_	MEXICO	2004
KF687321	MEXICO	2004

KF530245	ARGENTINA	2004
KF530241	USA	2009
MH678681_	UK	2019
MH678677	UK	2012
AB736166	ZAMBIA	2011
KF687346	SOUTH AFRICA	2009
KF687340	SOUTH AFRICA	2010
HM460887_	SAUDI ARABIA	2009
HM460886	SAUDI ARABIA	2008
EU814626	INDIA	2005
JX131646	SAUDI ARABIA	2008
JX131647	SAUDI ARABIA	2008
KF687318	USA	2007
KF530257	ARGENTINA	2007
KF530242	USA	2008
MH678688	UK	2011
MH678674	UK	2011
KF530236	FRANCE	2010
KF687317	USA	2009
GU732149	BEIJING	2009

GU732134	BEIJING	2008
GU732160	BEIJING	2009
KF530253	FRANCE	2007
KJ672547	PERU	2008
KJ672559	PERU	2010
KF530234	MEXICO	2005
FJ455842	CHINA	2003
EU326526	CHINA	2006
GU732145	BEIJING	2009
KM190938	THAILAND	2012
GU732154	BEIJING	2009
GU732167	BEIJING	2009
KF530233	FRANCE	2009
KJ672565	PERU	2007
KJ672618	PERU	2012
KF530256	USA	2007
KF530252	USA	2008
MH678676	UK	2012
KF530250	MEXICO	2006
KF530232	USA	2010

KF530232	USA	2010
KJ672615	PERU	2008
MH678678	UK	2013
MH678680	UK	2013
MH678675	UK	2012
EU814623	INDIA	2006

-Appendix VI: Article published from this work

PLOS ONE

accession numbers: MN116649 MN116682 and MN116749.

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Genetic diversity and evolutionary analysis of human respirovirus type 3 strains isolated in Kenya using complete hemagglutininneuraminidase (HN) gene

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DataAvailabilityStatement: The HN sequences of HPIV3 isolates reported in this study are available in GenBank (www.ncbi.nlm.nih.gov/genbank) under

Abstract

Human respirovirus type 3 (HRV3) is a leading etiology of lower respiratory tract infections in young children and ranks only second to the human respiratory syncytial virus (HRSV). Despite the public health importance of HRV3, there is limited information about the genetic characteristics and diversity of these viruses in Kenya. To begin to address this gap, we analyzed 35 complete hemagglutinin-neuraminidase (HN) sequences of HRV3 strains isolated in Kenya between 2010 and 2013. Viral RNA was extracted from the isolates, and the entire HN gene amplified by RT-PCR followed by nucleotide sequencing. Phylogenetic analyses of the sequences revealed that all the Kenyan isolates grouped into genetic Cluster C; sub-clusters C1a, C2, and C3a. The majority (54%) of isolates belonged to sub-cluster C3a, followed by C2 (43%) and C1a (2.9%). Sequence analysis revealed high identities between the Kenyan isolates and the HRV3 prototype strain both at the amino acid (96.5–97.9%) and nucleotide (94.3–95.6%) levels. No amino acid variations affecting the catalytic/active sites of the HN glycoprotein were observed among the Kenyan isolates. Selection pressure analyses showed that the HN glycoprotein was evolving under positive selection. Evolutionary analyses revealed that the mean TMRCA for the HN sequence dataset was 1942 (95% HPD: 1928–1957), while the mean evolutionary rate was $4.65x10^{-4}$ nucleotide substitutions/ site/year (95% HPD: $2.99x10^{-4}$ to $6.35x10^{-4}$). Overall, our results demonstrate the co-circulation of strains of cluster C HRV3 variants in Kenya during the