

ASSESSMENT OF DIVERSITY AND RELATEDNESS OF NDUMU VIRUS STRAINS ISOLATED FROM MOSQUITOES FROM FOUR DISTRICTS DURING ROUTINE ARBOVIRUS SURVEILLANCE IN KENYA

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Abstract

Members of the genus Alphavirus, family Togaviridae are a diverse group of principally mosquito-borne RNA viruses that produce a variety of diseases worldwide. Being RNA viruses, they are prone to genetic transformation like other RNA viruses. The aim of this study was to investigate the genetic diversity and relatedness of ndumu virus isolates from Garissa, Ijara, Busia and Baringo districts in Kenya. It also aimed at determining the phylogenetic relatedness of the ndumu virus isolates to other known alphaviruses. This was achieved by culturing eight virus isolates, two from each district in Vero cells. The isolates induced cytopathic effects (CPE) on cell culture on day 1 post inoculation. The infected cells were harvested and the cell culture supernatant used to isolate total RNA. Some of the RNA was amplified by Reverse transcriptase polymerase chain reaction using primers specific for the ndumu envelope gene (E1) to confirm the identity of the virus. Complete genome sequencing of the isolates using 454 pyro-sequencer was done to get the sequences. A mapping assembly of the sequence reads from the 454 sequencer was done using GS runmapper with reference to the longest ndumu virus sequence available in the genbank. Nucleotide and amino acid sequence alignment was done using muscle v 3.7 and clustalX softwares respectively. Phylogenetic analysis was done using MEGA v4.0. The ndumu virus isolates from Baringo, Busia and Garissa districts exhibited minimal genetic diversity despite the geographic distance separating them. One isolate from Ijara District was distinct. This study has facilitated the identification of single nucleotide polymorphisms (SNPs) within ndumu virus genome. Such SNPs may change the protein coding sequence and affect the virus' virulence and/or host susceptibility over time. When compared with other known alphaviruses, the ndumu virus isolates were not closely related to any other alphavirus.

Key words: Ndumu, SNPS, diversity, assessment

1.0 Introduction

The family *Togaviridae* comprises two genera, *Alphavirus* and *Rubivirus* (Weaver *et al.*, 2000). As a genus, the alphaviruses are widely distributed throughout the world, inhabiting all of the continents except Antarctica. The geographic distributions of individual species are restricted because of specific ecological conditions for reservoir hosts and vectors (Johnson, 1988; Weaver *et al.*, 2000). Members of the genus *Alphavirus* are typically maintained in natural cycles involving transmission by arthropod vectors among susceptible vertebrate hosts (Strauss and Strauss, 1994). Virus-host interactions may be highly specific, and sometimes only a single mosquito species is utilized as the principal vector of a virus, as has been reported for many Venezuelan equine encephalitis complex viruses (Weaver, 1998). These specific virus-vector interactions may limit the distribution of many alphaviruses.

Originally isolated in 1959 from *Mn. uniformis* in South Africa, Ndumu virus has been found throughout Africa, and although antibodies to the virus have been identified in humans from several African countries, no human illnesses have been attributed to Ndumu Virus infection (Karabatsos 1985). Due to the fact that it belongs to the same genus as other viruses that cause human illnesses like Chikungunya virus, O'nyong-nyong virus, Semliki forest virus and Getah virus and the antibodies detected in human population, it has the potential to raise public health concerns in humans with time. The 2005/2006 outbreak of Chikungunya virus in the western Indian Ocean region, including the Comoros Islands, Mauritius, Réunion Island, Madagascar, and the Seychelles (Enserink M., 2006; Charrel *et al.*, 2007) is an example. Chikungunya virus infection which had been known to be self-limiting fever had changed and led to a serious health problem around the world. In India, Eastern Africa, Western Africa and Central Africa, this viral disease is spread by mosquito bites from *Aedes* species mosquitoes, (Reinert *et al.*, 2004; Womack M., 1993; Ann M. P and Christopher H. L., 2007). Recent research by the Pasteur Institute in Paris claims the virus has acquired a mutation that enables it to also be transmitted by *Aedes albopictus* (Tiger mosquito). A genetic change in position 226 of E1 in the Chikungunya virus made it possible for the virus to no longer need cholesterol as viruses normally need cholesterol to infect the cells of their human and mosquito hosts (Afjal *et al.*, 2002).

By October 2006 on Réunion Island alone, which has a population of 760,000, at least 266,000 cases had been reported (Flauhaut, 2007). The epidemic swept eastward into the Indian subcontinent, where by the end of the year it had caused >1.3 million cases; attack rates were 45% in some regions (WHO, 2006; Mudur, 2006). By the beginning of 2007, the epidemic was on the decline on La Réunion and the Seychelles (Barret, 2007), but it seemed to be continuing in areas of India. New outbreaks had been reported from early 2007 in Malaysia and mid 2007 in Indonesia (Barret; 2007; Banks, 2007). These events demonstrated the public health impact alphavirus infections can have. The impact of genetic change or single mutation therefore, emphasizes the importance of monitoring and detecting genetic diversity of Ndumu virus which belongs to the alphavirus genus.

2.0 Materials and Methods

2.1 Cell Culture

2.1.1 Cell Lines

Vero cells that were used to culture all the virus strains were obtained from the Viral Hemorrhagic Fever laboratory at Centre for Virus Research –KEMRI. They were cultured until they attained 80% confluency in 25cm² cell culture flasks.

2.1.2 Virus Isolates

The isolates that were used in this study were archived samples in the VHF laboratory. They were isolated during routine arbovirus surveillance work in this laboratory. These virus isolates had been grown in vero cells and preserved as virus stocks in arbovirus/Virus hemorrhagic fever (VHF) laboratory in KEMRI in the liquid nitrogen (-196°C) tank by the laboratory staff. They were identified as Ndumu virus by RT-PCR and sequencing of the diagnostic fragment (E1 protein). A total of eight isolates, two mosquito pools from each region were used. They included: Garissa- GSA/S1/936 and GSA/S5/4278; Busia- BSA/S4/2265 and BSA/S4/2268; Baringo- BAR/S2/3527 and BAR/S2/3526 and Ijara- AMH 001346 and AMH 001056.

2.2 Growth of Cells

Vero cells were grown in 25cm² flasks in Minimum Essential Medium Eagle (MEM), containing 10% FBS, 2% L-glutamine and 2% antibiotic/antimycotic solution and maintained in medium containing 2% concentration of FBS,

2% L-glutamine and 2% antibiotic/antimycotic solution. They were incubated at 37°C in a humidified incubator with 5.0 % CO₂ for one day when they became 80% confluent.

2.3 Cell culture Inoculation

The virus isolates were removed from the liquid nitrogen tank and thawed at room temperature. The cell cultures which had been grown in 25cm² tissue culture flasks to 80% confluency were washed with sterile PBS pre-warmed in the water bath at 37°C. Two hundred microlitre of thawed sample was added to the monolayer followed by incubation in 5% CO₂ incubator at 37°C for 1hr with frequent rocking at an interval of 15 minutes in order to allow for virus adsorption. After incubation the monolayer was rinsed with sterile saline and maintenance media containing: 2% FBS, 2% L-glutamine, 2% antibiotic/antimycotic was added. The cells were then incubated at 37°C incubator, the cytopathogenic changes were monitored twice a day for each virus isolate. The infected cells were then harvested and the supernatant used for molecular assay.

2.4 Harvesting of the Virus

When about 50% CPE was observed, the virus was harvested by freezing down the infected cells in the -80 °C freezer overnight to burst-open the cells thus releasing the viral particles. Then the frozen cells were thawed on ice and the suspension was transferred into a 15 ml centrifuge tube and centrifuged (KUBOTA KS – 5000 centrifuge) at 277xg for 15min to sediment the cells. The supernatant containing the virus was placed in 1ml cryovials tubes in 0.5ml aliquots and stored at -80°C freezer.

2.5 Genetic Analysis

2.5.1 RNA Extraction and RT-PCR Assays

Viral RNA was extracted from 250µl each of cell culture suspension that was harvested after 24 hours post inoculation by using the TRIZOL[®] LS reagent- Chloroform method and the RNA pellet re-suspended in 11 µl of nuclease free water. This was followed by a two-step RT-PCR where a 20-µl reaction of first strand cDNA was carried out to convert RNA to cDNA. Then PCR was carried out using primers: ND 124F 5'-CAC CCT AAA AGT GAC GTT-3' and ND 615R 5'- ATT GCA GAT GGG ATA CCG-3' (Kinney and Pfeffer, 2001; Bryant *et al.* 2005) that targeted the envelope (E1) gene to confirm the identity of the virus.

2.6 Nucleotide Sequencing

Sequence-independent single primer amplification (SISPA) method was used to convert viral RNA to cDNA using random-tagged and poly-T tagged primers (FR26RV-N and FR40RV-T). cDNA synthesis was done by combining 40pmoles of primer FR26RV-N (5'GCCGGAGCTCTGCAGATATCNNNNNN3') and 10 µl of the RNA template and incubated at 65°C for 5 minutes then immediately placed on ice. Then the following components were added to each tube: 4µl of First Strand Buffer (5X; Invitrogen), 0.2 µmoles of DTT (Invitrogen), 0.01 µmoles of dNTPs, 8U of RNase Out inhibitor and 100U of SuperScriptIII reverse transcriptase and incubated at the following conditions: 25°C for 10 min, 50°C for 50 min, 85°C for 10 min and 4°C hold temperature.

A Klenow reaction was then carried out to convert the cDNA into dsDNA with interspersed sequences of the 20 nt on one DNA strand. Second strand DNA was synthesized using Klenow exo-DNA polymerase, in the presence of random tagged and virus specific 5' end oligo primers. To the cDNA reaction (19.7µl) the following components were added: 0.5ul (2.5 units) of the 3'-5'exo- Klenow DNA Polymerase and incubated at the following conditions: 37°C for 60 minutes, 75°C for 10 minutes and 4°C hold temperature.

This was followed by Shrimp Alkaline Phosphatase/Exonuclease Treatment (SAP-EXO treatment) to degrade nucleotides and any primers left over. To the Klenow reaction the following components were added: 1 µl SAP buffer (10X), 1 µl of SAP, 0.2µl of Exo and water to top up to a total volume of 40 µl. This was followed by an incubation of 37°C for 60 minutes and at 72°C for 15 minutes.

PCR was then carried out using the Invitrogen Accuprime Kit for PCR amplification of Klenow products. The following components were combined together: 5 microlitres of Buffer I (10x), 1unit of taq polymerase, 4 microlitres Klenow Product, 40 picomoles of primer FR26RV (5'GCCGGAGCTCTGCAGATATC 3') and 38.8 µl of Water. They were then placed on a thermocycler (Applied Biosystems GeneAmp[®]PCR System 9700) set at the following conditions: 94

°C for 2 min followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec , 68 °C for 15 sec and a final extension of 68 °C for 10 min and 4 °C hold temperature.

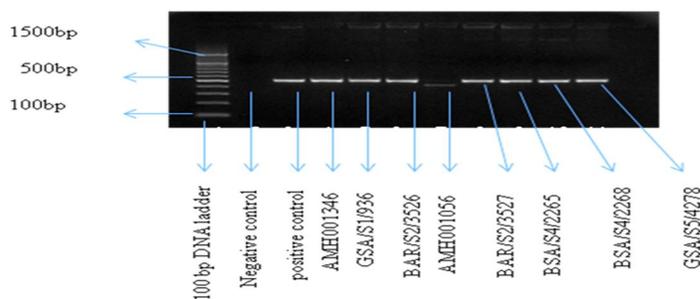
Finally, the SISPA products were sequenced using the GS FLX Titanium Sequencing Kit XLR70 (Roche) in combination with the matching GS FLX Titanium PicoTiterPlate Kit 70 × 75 (Roche) in a 454 sequencer according to the sequencing method manual. This sequencer was able to sequence the genomes for the virus isolates in short fragments (contigs) ranging from 300bp to 800bp.

The PCR products for confirmation of identity were analyzed on a 2% agarose gel (AGTC Bioproducts LTD). A mapping assembly of the nucleotide sequence data from the 454 sequencer was done using GS Runmapper (Roche 454). The sequence reads were mapped against the longest available Ndumu virus sequence (Accession No. AF339487.1). Nucleotide and translated amino acid sequence alignments were done using Muscle v 3.7 software (Dereeper *et al.*, 2008; Edgar, 2004). MEGA software version 4.0 was used for phylogenetic analysis (Tamura *et al.*, 2007) on both the nucleotide and translated amino acid sequences by use of neighbour joining tree method based on the number of nucleotide or amino acid differences.

3.0 Results

3.1 Rt-PCR Results

Figure 1. shows the gel photograph for RT-PCR results for the eight virus isolates. The E1 protein of each isolate was amplified by RT-PCR as described in the methods. Seven isolates produced bands of the expected size (508bp) which were similar to the positive control while one isolate (AMH001056) showed artifacts. This was the isolate that was identified as Semliki Forest Virus after sequencing. Initially, this sample was detected and identified as Ndumu virus using alphavirus genus primers and Ndumu virus primers.



Legend

AMH- avid mosquito homogenate

GSA- Garissa

BAR- Baringo

BSA- Busia

Figure 1: Gel photograph of 8 isolates of Ndumu virus

3.2 Sequencing Results

The SISPA products were sequenced using 454 pyrosequencer in order to obtain complete genomes of the viruses. A partial genome sequence of Ndumu virus (genbank Accession No. AF339487.1) was used as a reference. This was the longest nucleotide sequence for Ndumu virus that was available in the database and it was the one against which each isolate sequences were aligned in order to come up with a continuous sequence. Since the complete genome of Ndumu virus had not been sequenced by the time this work was being done, it was difficult to come up with a continuous sequence of Ndumu virus complete genomes for the samples being investigated. This work is still underway and the complete genome shall be made available in the genbank database through this project. The isolate AMH001056 was not included at this point because it was a strain of Semliki forest virus. Table 1 shows the number of SNPs of Ndumu virus isolates under investigation while table 2 shows amino acid differences of the isolates. A total of 118 Single nucleotide polymorphisms (SNPs) were seen in the isolates when compared to the reference sequence. There were eighty two (69.5%) variable positions in isolates from Baringo, Garissa and Busia while the Ijara isolate (AMH001346) had 93 SNPs (78%). By comparing the seven isolates under study, six out of seven isolates were almost

identical. One sample from Baringo (BAR/S2/3526) had SNPs at two positions and the two samples from Busia had SNP at one position each, BSA/S4/2268 and BSA/S4/2265 (Table 1). The two Garissa isolates and one Baringo isolates had no SNPs. The isolate AMH001346 was the most genetically different from the rest having SNPs at 49 positions. At the amino acid level, all the six isolates were very identical while the Ijara isolate (AMH001346) was different at four positions.

Table 1: Number of SNPs of Ndumu virus isolates under investigation

Sample ID	Number of SNPs and the change that has occurred								Total SNPs
	T-C	C-T	G-A	A-G	G-C	A-T	A-C	G-T	
AMH001346	12	14	8	8	1	4	1	1	49
GSA/S1/936	0	0	0	0	0	0	0	0	0
GSA/S5/4278	0	0	0	0	0	0	0	0	0
BAR/S2/3526	0	0	0	0	1	0	1	0	2
BAR/S2/3527	0	0	0	0	0	0	0	0	0
BSA/S4/2268	0	0	0	1	0	0	0	0	1
BSA/S4/2265	0	1	0	0	0	0	0	0	1

Table 2: Amino acid differences

Sample ID	Amino acid change that occurred and the frequency					
	E-V	F-L	N-H	V-L	M-T	Total
AMH001346	1	1	1	1	0	4
GSA/S1/936	0	0	0	0	1	1
GSA/S5/4278	0	0	0	0	0	0
BAR/S2/3526	0	0	0	0	0	0
BAR/S2/3527	0	0	0	0	0	0
BSA/S4/2268	0	0	0	0	0	0
BSA/S4/2265	0	0	0	0	0	0

3.3 Phylogenetic Tree Analysis

This analysis was done using Neighbor Joining method of tree construction in order to study how the isolates under study and the reference virus cluster in the tree. This provides better understanding of the virus origin or ancestry in relation to other strains of the same or similar viruses.



Figure 2: Neighbour joining tree using 1000 replicates (bootstraps) based on number of nucleotide sequence differences of Ndumu virus isolates and the reference strain from genbank

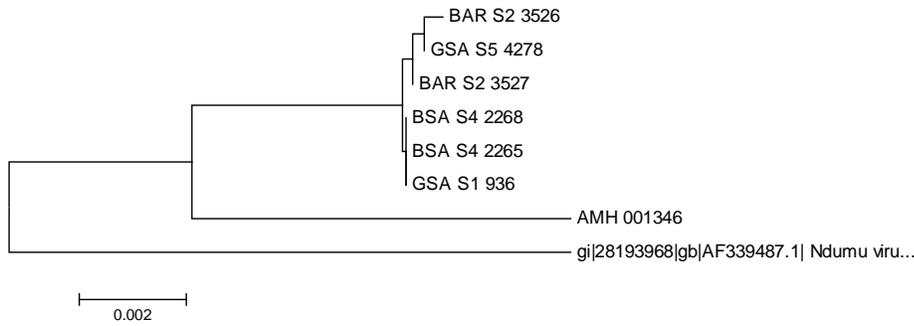


Figure 3: Neighbour joining tree using genetic distances for pairwise sequences

Figures 2 and 3 show that the six isolates are nearly identical and cluster together whereas one isolate is distinct. The Ndumu viruses have separated into 3 nodes: the reference Ndumu virus node, the sample AMH001346 node and the final node is composed of the other six isolates.

Figure 4 shows the relationship of Ndumu virus with other alphaviruses based on amino acid sequences. All the Ndumu viruses clustered together forming a taxa, a pattern which is common to most alphaviruses.

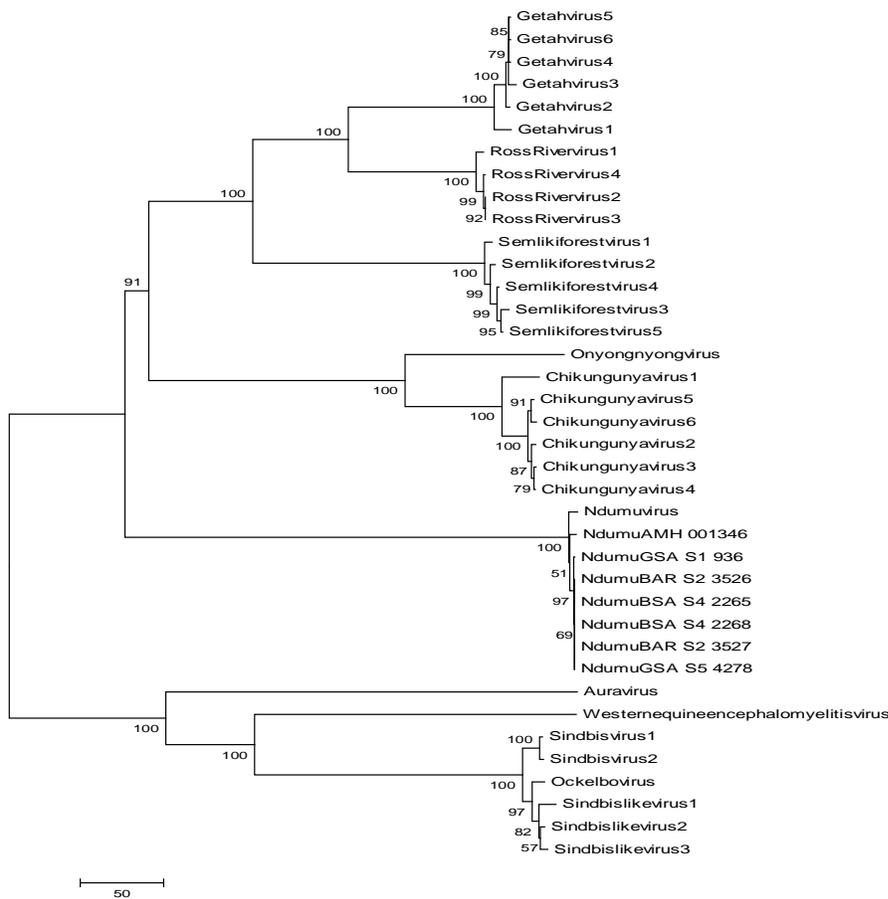


Figure 4: NJ-Tree with 1000 Bootstrap based on the number of protein sequence differences for alphaviruses

4.0 Discussion

Alphaviruses are genetically highly diverse (Weston *et al.*, 2005) creating challenges for their detection and monitoring in environmental samples such as RNA extracts from mosquitoes. Due to the diversity of alphavirus, separate assays must be performed in order to detect them. The possibility of more than one alphavirus type/species being present in a pool of mosquitoes presents additional challenge.

4.1 Molecular Analysis of Viral Isolates

Molecular analysis of the eight isolates using Reverse Transcriptase PCR yielded results that were similar to the cell culture assays. It also revealed that isolate AMH001056 from Ijara was different from the other seven isolates. The seven isolates yielded 508bp fragments as was expected. This confirmed the identity of the isolates as Ndumu viruses. However, the Ijara isolate AMH001056 yielded non-specific products. Sequencing results revealed that this was Semliki Forest Virus not Ndumu virus.

Sequence-Independent Single Primer Amplification (SISPA) method was used to generate cDNA libraries using random primers to amplify any RNA in the sample. This method does not depend on pre-existing sequences. Since complete genome of Ndumu virus had not been sequenced before, this method was suitable for this work. The challenge was to assemble all the reads to get a complete genome of the Ndumu virus due to the high degree of genetic divergence of Alphaviruses. From the phylogenetic tree (figure 4), Ndumu virus seems to be an out-group and is very distant from other alphaviruses. This study focused on the available 4700bp of the Ndumu virus genome that had already been sequenced. However, work on getting the full genome of Ndumu virus is in progress.

The sequencing results showed that one of the isolates from Ijara was not Ndumu virus but a Semliki forest virus. This may be a variant of Semliki forest virus that is more related to Ndumu virus. More studies need to be done on this sample. The other seven isolates were Ndumu virus confirming the RT-PCR results. They were analyzed based on the longest available Ndumu virus sequence from the genbank (Accession No. AF339487.1). The isolates from Garissa, Busia and Baringo districts were very similar at both nucleotide and protein levels despite the geographical distance. One of the isolates from Ijara district (AMH001346) was genetically different from the other isolates and at amino acid level also. Ijara is closer to Garissa district yet the isolates from the two areas were very different. The genetic divergence of this Ndumu virus suggests the possibility of genetic change in the virus which in turn may affect the protein which influences the virulence of the virus as was in the case of Chikungunya virus. Chikungunya virus which was generally not fatal turned into a fatal strain due to just a single mutation. In 2006 many deaths were associated with Chikungunya on Reunion Island and a widespread outbreak in India. An analysis of the virus's genetic code (Prescott L., 1993) suggested that the increased severity of the 2006 outbreak might have been due to a change in the genetic sequence, altering the virus's coat protein, which potentially allows it to multiply more easily in mosquito cells.

4.2 Phylogenetic Tree Analysis

All the seven isolates originated from a common ancestor as seen in figures 2 and 3. However, they formed two branches, one comprising of the six isolates forming the upper branch and the second lower branch is represented by the Ijara isolate AMH001346 showing how diverse it was from the other six. The two isolates from Busia and Baringo came from the same species of mosquito pools, *Coquellitidia fraseri* and *Culex rubinotus* respectively. This could be revealing that viruses isolated from the same species of mosquitoes collected from the same region might be having very little or no diversity in their genetic makeup. The Ndumu virus reference isolate was also different from the rest of the isolates. This was a South African strain while the rest were Kenyan and therefore the diversity was expected. Previous studies of Alphavirus diversification have emphasized host switching events and geographic introductions in the evolution of the alphavirus genus (Brault *et al.*, 1999; Powers *et al.*, 1997; Powers *et al.*, 2000; Weaver, 1995; Weaver *et al.*, 1997).

The analysis of translated sequences of Ndumu virus isolates in relation to a number of Alphaviruses showed that alphavirus species in general make monophyletic clusters in the phylogenetic tree. This confirms the previous report by Powers and the team that viruses within a given antigenic serocomplex were usually genetically more closely related than viruses in different complexes (Powers *et al.*, 2001). From figure 4, all viruses of the same

species cluster together in the tree. This study has shown that the Ndumu virus isolates from Busia, Baringo and Garissa exhibited minimal genetic diversity despite the geographic distance separating them. The Ndumu virus isolate from Ijara district was the most different from all the rest.

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