To Profile Nucleoside Reverse Transcriptase Inhibitor Drug-Resistance and Susceptibility Patterns of Naive HIV Positive Patients from Machakos Level 5 Hospital

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Abstract

This study focused on Nucleoside Reverse Transcriptase drug-resistance profiling and the susceptibility patterns for the plasma samples obtained from HIV-positive naïve patients enrolled at Machakos Level 5 Hospital. The research's specific objectives were to profile resistance to Nucleoside Reverse Transcriptase Inhibitor drugs and then identify the markers for resistance to Nucleoside Reverse Transcriptase Inhibitor. This study used an experimental research design; DNA was extracted from the plasma samples, and PCR was amplified using polymerase-gene specific primers and later Gel electrophoresis. Then finally, cycle sequencing of the polymerase (pol) gen. The amplified products were sequenced, and drug-resistant mutations were determined using Los Alamos HIV DR database. All amplified samples from the PCR had the gel cut/excised and cleaned using the QIA quick gel extraction kit protocol. Sequences with high relatedness were fetched in a FASTA format and aligned using the Mega Evolutionary Genetic Analysis (MEGA) software version 10 using the Neighbor Joining (NJ) algorithm and the 1000 Bootstrap resampling algorithm. The main HIV strain detected in this study was the HIV A1 subtype, the major sub-subtype in Kenya. No other subtypes were noted in the study.
Regarding NRTIs, the major mutation noted was D67E which indicated inadequate level, zidovudine resistance, and drug susceptibility to abacavir, emtricitabine, lamivudine, and tenofovir noted with no resistance to NNRTIs. However, there were minor mutations noted. Drug resistance mutations were found in high numbers associated with viral load and treatment time. Importantly, patients with triple and dual-class drug resistance should immediately alter ART regimens to alter the possibility of transmitting multi-drug-resistant HIV-1 strains.

**Keywords:** Anti retroviral therapy; Nucleoside Reverse Transcriptase Inhibitor.

1. Introduction

Globally, antiretroviral therapy coverage has grown to more than 21 million individuals. The antiretroviral therapy coverage in Sub-Saharan Africa has improved greatly [31]. According to World Health Organization, many nations, irrespective of CD4 T-cell count, have taken the advice of the WHO for the initiation of ART in all persons infected with HIV. While HIV mortality and morbidity have been reduced dramatically by ART, a sustained global expansion of HIV-resistant strains can lead to emergence and dissemination [5]. A rise in pretreatment drug resistance (PDR) in low-resource environments has successfully increased ART [7]. The latest World Health Organization study (WHO) found that the prevalence of PDR to NNRTIs in 6 of the 11 countries surveyed was more than 10%. The WHO 10 percent drug resistance level could entail improvements in the country's first line of ART regimes. In 63 countries, a new meta-analytic study showed a global rise in PDR to NNRTI (up to 23% in Southern Africa). HIV-resistant medication strains restrict treatment opportunities and risk the successful escalation of ART to monitor HIV infection by 2030 [32]. Consequently, controlling the population level of HIV drug resistance (HIVDR) is important because it helps to keep the viral load low and the CD4 cell count high. HIV medicine can make the viral load very low by preventing HIV multiplication.

According to National Guidelines on Use of Antiretroviral Drugs for Treating and Preventing HIV Infection in Kenya, the recommended first-line ART regimen for treatment-naive adults consists of two nucleoside reverse-transcriptase inhibitors (NRTIs) and an integrase inhibitor [21]. As an alternative, the use of a non-nucleoside reverse-transcriptase inhibitor (NNRTI) or boosted protease inhibitor (PI) is recommended. Despite the effectiveness of ART and considerable efforts to help control the HIV/AIDS epidemic by 2030, ART failure due to drug resistance mutations is proving a challenge for ART provision and HIV care. In 2017, the World Health Organization (WHO) published a report on HIV drug resistance addressing the alarming increase in the prevalence of DRMs in individuals initiating their first-line ART regimen, linking DRMs to treatment failure. According to the National AIDS and STIs Control Program (NASCOP Recommendations of use of ART drugs, HIV resistance testing is recommended for all individuals with HIV infection who are newly diagnosed, before they initiate ART and in People Living With HIV (PLWH) with ART failure. Genotyping DNA-based assays are the most widely used for HIV DRMs detection. In Machakos Level 5 hospital, HIV genotyping is not performed in PLWH failing their first-line regimens; it is not routinely performed for all PLWH who are treatment-naive and starting their first-line. Several studies have reported the prevalence of HIV DRMs in treatment-naive Kenyan PLWH. According to a nationally representative survey, in Kenya the prevalence of any antiretroviral (ARV) resistance drug in treatment-naive PLWH is greater than 10%. Also, this report
concluded that PWLH who initiated with NNRTI-based regimens achieved significantly lower levels of viral suppression compared to those who initiated with Protease inhibitor-based regimens. Also, Drug resistance mutations can directly confer resistance to PI, in the absence of detectable DRMs in the PR. Therefore, after reports on the general HIV-1 drug resistance is missing especially in resource limited rural settings with a longer history of ARV drug use, this research focused on drug-resistant profiling and sensitivity trends for ingenuous HIV-positive patients at Machakos levels 5 Hospital of Nucleoside Reverse Transcriptase.

2. Materials and Methods

a) Ribonucleic Acid Extraction:

Ribonucleic acid (RNA) from the plasma samples was extracted using Qiagen RNeasy kit according to manufacturers’ instructions. Briefly 140µl of sample was added to 560µl of viral lysis buffer, incubated at room temperature (15-25°C) for 10 minutes, then 560µl of molecular grade 100% ethanol [22] was added and mixed by vortexing for 15 seconds. This was then centrifuged using a micro centrifuge briefly to remove drops from inside the Eppendorf tube lid. From the lysed RNA, 630µl of RNA was then placed on to a spin column, spun at 6000 x g, twice binding the RNA to the spin column. The RNA was then 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. The RNA was eluted from the spin column by adding 60µl elution buffer (AVE) and spinning at 6000 x g for 1 minute to a 1.5 ml Eppendorf tube. The eluted RNA was then stored at −80°C until the day when the polymerase chain reaction (PCR) was carried out on the samples.

b) Polymerase Chain Reaction (PCR):

The reverse transcriptase PCR (RT-PCR) procedure consisted of one-step reverse transcription and PCR amplification, using the one-step RT-PCR kit from QIAGEN [22]. The reaction mixture contained 5µl of 5× RT-PCR buffer, 1µl of 0.4mM dNTPs, 0.75µl of each of the primers (1st round forward and reverse primers final concentration 0.6µM), 9.5µl of nuclease free water and 1µl of enzyme mix. A 2.5µl aliquot of viral RNA was added to give a final volume of 25µl. The cycling conditions for the RT-PCRs were an initial cycle at 50°C for 30 minutes for the reverse transcriptase. This was followed by incubating at 94°C for 10 minutes to inactivate the reverse transcriptase and activate the Taq polymerase. This was followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final extension of 72°C for 10 minutes. In the 2nd round PCR, the amplification was carried out using the 2mM MgSO4 (Invitrogen), 0.8mM dNTPs (Invitrogen), 0.5 units Taq polymerase (Invitrogen), 10x PCR Buffer (Invitrogen), 2ng of each 2nd round primer and the 2µl of the 1st round DNA template. The PCR cycle conditions consisted of 1 cycle of 95°C for 10 min and 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, with a final extension of 72°C for 10 min. The forward primer used for the 1st round PCR was RT18 F1 and its sequence 5’-GGAACAAAAATGTAGGGAATTGGAGG-3’. For the reverse primer, KS104 R1 was utilized where its sequence was 5’-TGACTTGCCAATTAGTTTCCACTAA-3’ [22]. Finally, for the 2nd Round PCR, KS101 F2 was used as a forward primer where its sequence was 5’-GTAGGACCTACACCTGTCCAACATAATTGGAGG-3’ and KS102 R2 as a reverse primer where its
sequence was 5'-CCCATCCAAAGAAATGGAGGAGGTTCTTTCTGATG-3' [30].

c) Gel electrophoresis:

After the 2nd round PCR, PCR products were electrophorized in 1.5% agarose gels along with a 100-bp ladder [20] and visualized under UV light by ethidium bromide staining. Briefly, 1.5g of agarose was added to 100ml of tris-borate EDTA buffer (TBE). This was then heated in a microwave until clear, then later placed on a water bath that was at 48°C. When cool, 0.5-1µl of ethidium bromide was added to the agarose, then later poured on to a gel tank that had gel combs. This was left to solidify. Once solid the comb was removed, and the gel tank filled TBE. The product 10µl was mixed with gel loading dye (gld) and electrophorized at 100v for 30 minutes. The PCR products were visualized under UV light using an HP AlphaImager®

d) QIAquick Gel Extraction Procedure:

The QIAquick®gel extraction kit was used to clean up the PCR products following the procedure described in the manufacturer's manual. Briefly, the PCR products were excised from the gel, weighed and 3 volumes of buffer QG added for every 1 volume of the gel. These was incubated for 10 minutes at 50°C to dissolve the gel. Once dissolved, 850µl of the solution was dispensed to a QIAquick spin column and centrifuged at 17,900 x g for 1 minute to bind the DNA to the matrix of the column. This procedure was repeated once more. After the last spin, 500µl of buffer QG was added to the spin column to remove traces of agarose and centrifuged at 17,900 x g for 1 minute. This was then washed by adding 750µl of buffer PE and centrifuged at 17,900 x g for 1 minute. The spin column was placed on a 1.5ml Eppendorf tube, 50µl of buffer EB was then be added to the column and centrifuged for 1 minute. The eluted DNA was stored at -20°C until nucleotide sequencing was carried out.

e) Cycle Sequencing:

The amplified fragments acquired for RT were in several base pairs. These fragments were sequenced by the Sanger sequencing method at KEMRI following the manufacturer's instructions, along with published primers.

f) Statistical Data Analysis:

This was an experimental study profiling the NRTI drug resistance and susceptibility patterns of treatment naïve HIV patients where their samples were obtained randomly, without any knowledge of drug-resistance and susceptibility patterns, from both the sample collection facility, Machakos Level IV hospital, and the diagnostic section at Kenya Medical Research Institute (KEMRI).

3. Results

Figure 1.1 below shows PCR products obtained after second-round amplification with HIV pol-RT specific primers. The expected sizes of amplified gene fragments, if positive, were approximately 697bp, whereas the gel picture below shows that, indeed, the amplified fragments were approximately 697bp as expected. All amplified
samples from the PCR had the gel removed and cleaned using the QIAquick Gel Extraction equipment protocol as described in the methods section.

![Gel electrophoresis](image)

**Figure 1.1:** Gel electrophoresis

Table 1 below shows the results from uploading of study’s sequence and uploading them to the Stanford University HIV database ([https://hivdb.stanford.edu/hivdb/by-sequences/](https://hivdb.stanford.edu/hivdb/by-sequences/)) to govern the nucleoside reverse transcriptase inhibitors (NRTIs) sequences using the default algorithms.

**Table 1: NRTI mutations and NRTI drug susceptibility**

<table>
<thead>
<tr>
<th>Sample Id</th>
<th>NRTI Mutation</th>
<th>Drug Susceptibility</th>
<th>Potential Low-level Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKseq1</td>
<td>D67E</td>
<td>ABC, FTC, 3TC, TDF</td>
<td>AZT</td>
</tr>
<tr>
<td>MKSeq2</td>
<td>D67E</td>
<td>ABC, FTC, 3TC, TDF</td>
<td>AZT</td>
</tr>
<tr>
<td>MKSeq3</td>
<td>D67E</td>
<td>ABC, FTC, 3TC, TDF</td>
<td>AZT</td>
</tr>
<tr>
<td>MKSeq9</td>
<td>D67E</td>
<td>ABC, FTC, 3TC, TDF</td>
<td>AZT</td>
</tr>
<tr>
<td>MKSeq10</td>
<td>D67E</td>
<td>ABC, FTC, 3TC, TDF</td>
<td>AZT</td>
</tr>
</tbody>
</table>

*Key:* ABC – abacavir, AZT – zidovudine, FTC – emtricitabine, 3TC – lamivudine, TDF – tenofovir
The sequences obtained from this study were then uploaded to the Stanford University HIV database (https://hivdb.stanford.edu/hivdb/by-sequences/) to determine the non-nucleoside reverse transcriptase inhibitors (NNRTIs) sequences using the default algorithms. The results obtained are shown in table 2 below.

Table 2: Non-Nucleoside Reverse Transcriptase Inhibitor Mutations

<table>
<thead>
<tr>
<th>Sample Id</th>
<th>NNRTI Mutation</th>
<th>Other Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKseq1</td>
<td>None</td>
<td>V35T, E40D, V60I, D113E, D121H, K122E, D123S, K173S, Q174K, D177E, I178L, V179I, Q207A, R211S</td>
</tr>
<tr>
<td>MKseq2</td>
<td>None</td>
<td>V35T, E40D, V60I, D113E, D121H, K122E, D123S, K173S, Q174K, D177E, I178L, V179I, Q207A, R211S</td>
</tr>
<tr>
<td>MKseq3</td>
<td>None</td>
<td>V35T, E40D, V60I, D113E, D121H, K122E, D123S, K173S, Q174K, D177E, I178L, V179I, Q207A, R211S</td>
</tr>
<tr>
<td>MKseq9</td>
<td>None</td>
<td>V35T, E40D, V60I, D113E, D121H, K122E, D123S, K173S, Q174K, D177E, I178L, V179I, Q207A, R211S</td>
</tr>
<tr>
<td>MKseq10</td>
<td>None</td>
<td>V35T, E40D, V60I, D113E, D121H, K122E, D123S, K173S, Q174K, D177E, I178L, V179I, Q207A, R211S</td>
</tr>
</tbody>
</table>

Although specific primers were used to amplify the polymerase reverse transcriptase gene (pol-RT) gene fragments from the patient samples, confirming the nucleotide sequences obtained from the amplified fragments was always necessary. The sequences obtained from the amplified products from this study were uploaded and compared to related sequences found in the GenBank database using the Basic Local Alignment Search Tool (BLAST) using the default algorithm analysis result from this study using the GENBANK database.
Figure 1.2: A representative nucleotide sequence BLAST analysis result from this study using GENBANK database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The sequences were later uploaded and compared to similar sequences found in the Los Alamos HIV database tools, Recombinant Identification Program (RIP) default algorithm. A representation of the analysis results is shown in figure 1.3 below.
Figure 1.3: A representative sequence analysis result from this study using the Los Alamos HIV database tool, Recombinant Identification Program (RIP). Ref: https://www.hiv.lanl.gov/cgi-bin/RIP3/RIP.cgi.

This study's sequences were later uploaded to the online REGA HIV, which is an HIV Drug Resistance Database subtyping database using the default algorithm. A representation of the genotyping analysis results is shown in figure 1.4 below. Figure 1.4 below confirms that the predominant HIV-1 subtype is, subtype A with 100 percent support.

Figure 1.4: Sequence Analysis from REGA HIV Subtyping
To determine the phylogenetic relatedness to similar subtype sequences, sequences with high relatedness were fetched in a FASTA format which is a text-based format for representing either nucleotide sequences or amino acid (protein) sequences, and aligned using the Mega Evolutionary Genetic Analysis (MEGA) software version 10 using the Neighbour Joining (NJ) algorithm and the 1000 Bootstrap resampling algorithm. The NJ algorithm substitution model used was the Kimura 2-parameter method. The generated tree to infer and assign HIV-1 subtypes is shown below in figure 1.5.

**Figure 1.5:** Phylogenetic Relatedness from BLAST and HIV BLAST Databases using MEGA version 10 Neighbor-Joining (NJ) algorithm at 2000 replicates Rooted with SIV (cpz)-X52.
4. Discussion

Research shows that in East Africa, most infections are caused by subtype A, plus also subtypes C and D, plus some URFs. Subtype A is also distributed in Russia and the former Soviet Union, Central Asia [3,4]. In Kenya, research investigations conducted across the nation in diverse locations ranging from the coast to western and central Kenya show the detection and, in some cases, preponderance of subtype A, as well as the existence of subtypes C and D. This study's sequences on analysis had shown genetic similarities of between 85 – 97% alignment with HIV subtype A as shown by the representative sequence BLAST and HIV BLAST results. This is similar to what other researchers have shown from previous studies carried out here in Kenya, which show the predominance of subtype A, where they range from 44% to 74% detection [1,2,6,8,10,11,13,14,15,18,24,33]. At the time of the study, the amplified and sequenced samples data indicated the presence of only subtype A and no other subtype was detected from the samples, which is a difference from other studies carried out in Kenya.

When the Recombinant Identification Program (RIP) hosted by the Los Alamos Database was used to analyze for and determine recombination in this study's sequences, from the results, the most dominant curve was that of the A1 sub-subtype. This was also confirmed by the REGA HIV subtyping tool, which also confirmed the genotyping as sub-subtype A1 with 100% support. When the phylogenetic tree was drawn using similar sequences obtained from the Genbank Database, this study's sequences aligned with other Kenyan sub-subtype A1 sequences. A unique observation is the clustering of this study's samples on the inferred phylogenetic tree after 2000 replicates. Previous Kenyan studies carried out on various groups, including expectant mothers, intravenous drug users, and HIV infected individuals, have majorly the sub-subtype A1, which is the most predominant strain detected with detection rates ranging from 44% to 86% [1]. The neighbor-joining method for constructing phylogenetic trees uses pairs of operational taxonomic units (OTU) or neighbors to minimize branch lengths, starting with a starlike tree that is repeatedly run at each replication stage to ensure the integrity of the tree structure [28]. The Kimura 2 parameter model used in this analysis has the role of estimating genetic distances between different nucleotide sequences during an evolutionary process [12,23].

a. Profile of Resistance to Nucleoside Reverse Transcriptase Inhibitor (NRTI) Drugs

Nucleoside reverse transcriptase inhibitors (NRTIs) are medicines that prevent viral DNA from being reversed within cells. They contain nucleotide base analogs that will cause the chain termination or non-extension of the DNA during reverse transcription of the HIV viral DNA while inside an infected cell using the HIV reverse transcriptase [9]. Their method of action is either discriminating, resulting in a reduction in the binding affinity of NRTI-triphosphate over the natural nucleotide at the reverse transcriptase binding site, or non-discriminatory. Again, this may decrease the NRTI-phosphate over the natural nucleotide rate of incorporation of the reverse binding site. The second mechanism is an excision process that relies on adenosine triphosphate (ATP) or pyrophosphate; hence, mutations that improve reverse transcriptase affinity for ATP or increase the rate of analog complex removal are preferred. Additionally, changes in the capacity of the residues to translocate from the active site (N-site) to the post-translocation site (P-site), and the rate of separation of the template/primer from the enzyme, may also help to improve the excision route [9]. Generally, NRTIs are drugs that comprise abacavir (ABC), zidovudine (AZT), emtricitabine (FTC), lamivudine (3TC), tenofovir (TDF), stavudine (d4T),...
didanosine (ddl), and zalcitabine (ddC) [9,21]. The findings of this study showed that all the sequenced samples had a D67E mutation, which is a change of amino acid aspartic acid (D) to glutamic acid (E). Aspartic acid (D) is an important building block used in making proteins in the body, while glutamic acid (E) is used to form proteins in the body [9]. The findings of this study is same as the findings of other HIV research, which determined that this is a non-polymorphic NRTI selected mutation at position 67 that is a change from aspartic acid (D) to either glutamic acid (E), serine (S), threonine (T), or histidine (H), that is D67G/E/S/T/H which has been associated with low-level resistance to AZT and d4T, plus also reduced susceptibility to ABC, DDL and TDF [9,17,25,26,29]. This is also similar to previous research studies from Kenya done on drug resistance and have shown resistance to the resistance mutation at position 67, where there was a change from glutamic acid (D) to asparagine (N), thus the acronym D67N [13,16]. These previous studies also noted resistance to AZT and other NRTIs.

b. Markers for Resistance to non-Nucleoside Reverse Transcriptase Inhibitor Drugs

Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs) inhibit reverse transcription by directly attaching to the enzyme reverse transcriptase (RT) and preventing it from working. Typically, they are tiny chemical compounds with a lengthy half-life [9].

Generally, NNRTIs drugs include nevirapine (NVP), efavirenz (EFV), doravirine (DOR), and delavirdine (DLV) [33,21]. No major NNRTIs resistance mutations were noted in the amplified samples sequenced in this study. Results from table 2 shows that. However, the results of this study in table 2 indicate that there was only one mutation at position 179 where valine (V) changed to isoleucine (I), thus the acronym V179I. Valine is a plant synthesized essential acid used for muscle growth stimulation, regeneration, and energy production, while isoleucine, another essential amino acid, is involved in muscle metabolism and important in immune functions and collagen production. This polymorphic mutation is frequently seen in patients receiving etravirine (ETR) and rilpivirine (RPV) but has a little direct effect on NNRTI susceptibility. The findings of this study are similar to the findings of a study carried out in Kisii County. The main aim of the study was to determine the HIV-1 genetic diversity, viral tropism, acquired and transmitted drug resistance among treatment naive and experienced HIV infected patients attending Kisii Level Five Hospital, Kenya. From this study, V179I mutation in 10 sampled patients was also detected [19]. The most commonly identified resistance mutation noted in other studies was at position 103, where lysine (K) changed to asparagine (N), thus the acronym K103N [8,13,15,16,18,27]. However, this mutation was not noted in this study's amplified sample sequences.

5. Conclusion and Recommendation

To summarize, a frighteningly high prevalence of drug resistance mutations was found. Importantly, patients with triple and dual class drug resistance should alter ART-regimens immediately to avoid the possibility of transmitting multidrug-resistant HIV-1 strains, which would have fewer treatment options. The most significant predictors of HIVDRM were viral load and treatment duration. The most striking finding was that a subject’s sex and treatment-duration independently influenced HIV DR counts, emphasizing the importance of targeted resistance monitoring and switching ART regimens while taking into account the risk of exhausting future
treatment options. More research is needed to determine the variables that contributed to the finding that a subject’s sex and treatment time independently influenced HIV-1 drug resistance mutations.

The study recommends the following

1. The provision of fresh samples could help get better amplicons and sequences data.
2. Providing patient details that include age, gender, and treatment regimens would help address research questions regarding which age or gender is mostly affected. Regarding knowing treatment regimens, resistance mutations determined would help improve patient management.

6. Study Limitations

Despite the findings of this study, it had a number of limitations. First, the samples sequenced were few. However, amplifications for most of these samples occurred and good and reliable sequence results were obtained from these samples. Secondly, no patient information regarding gender, age, and treatment regimen was provided. This was because of ethical concerns at the health facility. Lastly, some of the assays were not conducted such as phenotypic assay. This was because of the restricted availability of a facility for biosafety.

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References


Infected Patients Attending Comprehensive Care Clinic in Kisii Teaching and Referral Hospital, Kenya (Doctoral dissertation, JKUAT-COHES).


