Single nucleotide polymorphisms in the V3 region of glycoprotein 120 among Human immunodeficiency Virus positive subjects in Nigeria.

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ABSTRACT

Background: The Cysteine-Cysteine Receptor 5 (CCR5) molecule is the most important co-receptor for macrophage-tropic HIV-1. A 32-bp deletion in the gene encoding CCR5 (CCR5-del32) confers nearly complete resistance to HIV-1 infection in homozygotes, and slows the rate of progression to AIDS in heterozygous adults. This study aimed to determine the frequencies of chemokine receptor 5 (CCR5) genes alleles associated with V3 loop of glycoprotein (gp) 120 among HIV patients in Yenagoa, Nigeria.

Methods: Genomic DNA was extracted from whole 100 blood samples using Quick gDNA Mini Prep kit. A portion of the CCR5 gene and gp 120 regions of HIV-1 DNA were amplified from the gDNA by PCR with sequence specific primers and universal primers respetively followed by agarose gel electrophoresis. DNA sequencing of the resultant gp 120 amplicon was also carried out.

Results: The CCR5 gene was positive at 189bp. None was positive to the CCR5/Δ32 heterozygous and none was also positive to the Δ32 homozygous deletion. The V3 region of the HIV DNA was seen to be positive at 250bp.

Conclusion: This study shows that there is Single nucleotide polymorphism (SNP) of the protein sequence in the V3 region of gp120 among positive individuals in the study population.

Keywords: Single nucleotide polymorphism, glycoprotein 120, Humman Immunodeficiency virus, Nigeria

INTRODUCTION

Chemokines are groups of small cytokines or proteins that are secreted by cells that send immune signals. The name is as a result of their capacity to induce immune responses. All vertebrates have chemokines in them even some bacteria. There are four main subclasses of Chemokines. These are; CXC, CC, CX3C and XC. They are receptors found on the surface of target cells (1).

Chemokines and their receptors are expressed by a wide variety of non-haematopoietic cells, and that chemokine function extends far beyond leukocyte physiology. Even within the world of

leukocytes, the connections among chemokines, their receptors, and human immunodeficiency virus (HIV) infection broadens the previously narrow focus on chemokines as mere chemoattractants. Furthermore, a proliferation of animal models has more precisely defined the functions of chemokines in vivo (2).

The attraction of leukocytes to tissues is essential for inflammation and the host response to infection. The process is controlled by chemokines, which are chemotactic cytokines. Chemokines majorly act as guide for cell migration during immune response. Other chemokines help in the development and growth of new blood vessels. During inflammation, chemokines are released from variety of cells against pathogens. Chemokines can also be activated to promote wound healing during inflammation, maintenance of homeostasis, and are involved in driving the leukocytes to the points of inflammation. This process is known as homing (3).

Basal Chemokines: The site of production of basal chemokines for homeostasis is the thymus and also the lymphoid tissues. Few examples are CXCR5, CCR9 and CCR10. They are produced in the bone marrow (4). Inflammatory Chemokine: When injury or infection occurs, there is increased production of inflammatory chemokines which drive leukocytes to the points of infection or injury. Few examples are: CCL2 and CXCL1 (5).

Statement of the Problem

Sexual active age of an individual shifts significantly between various societies and varies seasonally. The act of sexual activity is part of human natural physiology though has its associated risks. The major risk factors include HIV transmission among many others. Human immunodeficiency virus (HIV) transmission in sub-Saharan Africa is mostly by heterosexual contact. Unprotected sexual intercourse places a substantial number of sexually active individuals at risk for sexually transmitted disease (STD) and HIV infection(6).

HIV-1 infection has spread to all population groups in Africa and has reached epidemic proportions. The rate of progression of HIV-1 disease exhibits a remarkable variation among different individuals. Many host genetic factors are now known to affect the disease progression rates, especially polymorphisms in genes encoding chemokine receptors (7).

Justification of the Study

The adverse effect of HIV is global; However, sub-Saharan Africa bears most burden of HIV infection. The discovery of the delta 32 (CCR5Δ32) allele of CCR5 brought to the fore the importance of CCR5 in HIV infection and disease progression (8). It has been reported that persons who lack functional CCR5 (i.e. CCR5Δ32) in the homozygous state, may be completely protected from HIV infection. On the other hand, persons with both alleles of CCR5 are slow progressors if infected with HIV (9). In Nigeria, there is no report about the heterogeneous or homogeneous allele of the mutant CCR5.

However, it has been reported that this genetic mutation is found in Caucasian rather than non-Caucasian population (10). Genetic relationships among ethnic groups are not uniform across the geographical region. Although no studies on chemokine receptor gene alleles have been reported in Yenagoa Bayelsa State, Nigeria. On this assumption, the need to determine the pattern of distribution of CCR5 in our geographical region becomes imperative. This study was therefore aimed at determining the frequencies of cysteine-cysteine receptor 5 (CCR5) genes V3 loop region of glycoprotein 120 in HIV subjects in Yenagoa.

MATERIALS AND METHODS

Study area

The study was conducted in Yenagoa and analyzed at the medical laboratory science department (Molecular laboratory) of the Niger Delta University, Amasoma both in Bayelsa.

Yenagoa is the headquarters of Yenagoa Local Government Area and also the capital of Bayelsa State. It is a Wetland located in Delta, South-south

region of Nigeria. It is inhabited mostly by people of the Ijaw tribes. The official language is English but several other Ijaw dialects are also spoken in Yenagoa, some of the Ijaw dialects are: Epie-Atissa, Ekpetiama, Gbarian, Zarama and Biseni. It has an area of 706 km² and a population of 352,285 according to the 2006 census with geographical location of 4°55'29"N 6°15'51"E.

They are mainly farmers and also engage in fishing. Some of their staple foods include cassava, plantain and rice.

HIV positive patients from the state owned hospitals with heart to heart centres were recruited for the study. Both male and female ages between 18 and 60 years.

Determination of sample size

Test size was resolved utilizing G-power measurable programming, with a power of 80%. A specimen size of 100 has 80% power to identify an expansion of 9% with level (alpha) of 0.05 (two-followed). In this way a specimen size of 100 was utilized for the study.

Experimental Design

A cross-sectional observational study design was employed in this study. It was descriptive/diagnostic study. Subjects were randomly selected without bias.

Ethical Consideration

Ethical approval for this study was received from the Bayelsa State Ethical Committee of the Ministry of Health. Subjects were educated on the objects, advantages and procedures of the study and were guaranteed the confidentiality of their data. All methods were performed in accordance with relevant guidelines and regulations.

Informed Consent

Informed consent was obtained from all subjects and experiments were performed in accordance with relevant guidelines and regulations as enshrined in the Helsinki declaration, 2014. The study had no financial involvement on the part of the enlisted subjects.

Inclusion criteria: All HIV positive patients between age 18 and 60 who consented to the study were included in the research.

Exclusion criteria: All HIV negative patient of any age where excluded from the study.

Collection of sample

Blood samples (4mls) were collected from each subject with normal stasis by venipuncture from either the antecubital vein or the dorsal vein under sterile condition and dispensed into tubes containing the anticoagulant ethylene diamine tetra acetic acid (EDTA). The specimens were labeled with the subject's age, sex and identification number. The EDTA samples were kept in the refrigerator at a temperature of 2-8o^{C} until processing.

MOLECULAR STUDIES

DNA extraction

Genomic DNA was isolated from the EDTA anti-coagulated whole blood samples using Quick gDNA Mini Prep kits supplied by Zymos Research Inqaba, South Africa. Procedures for extraction was done according to the manufacturer's instructions as shown below:

To a 1.5 ml micro-centrifuge tube, 50 µl of anti-coagulated whole blood was added, then 200 ul of Genomic Lysis Buffer was added. This was mixed completely by vortexing for 4-6 seconds and incubated at room temperature for 10 minutes. The mixture was transferred in a collection tube into a Symo-Spin and was centrifuged at 10,000 or 13,000 rpm for one minute and the collection tube was discarded with the flow-through. The micro-centrifuge tube was transferred into a new collection tube and 200µl of DNA Pre-Wash Buffer was added to the tube and was centrifuged for one minute at 10,000 or 13,000 rpm, 500 µl of Genomic DNA Wash Buffer was added to spin column and was centrifuged for 10,000rpm for one minute. The spin column was transferred into a clean micro-centrifuge to which was added 10 µl of DNA Elution

Buffer. It was incubated at room temperature for 5 minutes and then centrifuged at high speed for 30 seconds to elute the DNA. The DNA was stored at –20°C for later use.

DNA quantification

This was done using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer. One(1)µl of samples was placed on the pinhole of the nanodrop and the pedestral lowered to establish contact with the sample. The concentration of the DNA was read through the nanodrop software installed on a desktop computer.

Amplification of CCR5 gene

The amplification of the CCR5 gene was done on 2700 ABI thermal cycler in 20µl volume. The components were 2X master mix containing taq polymerase 1.5µl, dNTPs 200, 0.5µm of forward primers (P1(2975), 5´CAAAAAGAAGGTCTTCATTACACC-3 and reverse primers P2(2976), 5´-CCTGTGCCTCTTCTTCTCATTTCG – 3´, and 3µl of DNA extract. The PCR condition were initially denatured at 95°C for 5min, 30 cycles of denaturation at 95°C for 30sec. Annealing at 55°C for 30sec and extension at 72°C for 50 sec and final extension at 72°C for 30min.

Amplification of the V3 region of Glycoprotein 120

The PCR reaction was done using a nested approach with the outer and inner primers 5'C2V3: TGTACACATGGAATTAGGCCA and 3'V3:

ATGAATTCATTACAGTAGAAAAATTCCC respectively. The second round of PCR was done using the primers J5'-2KSI: ATAAGCTTGCAGTGTAGCAGAAGAAGA and 3'C2V3: ATTTCTGGGTCCCCTCCTGAGG as outer and inner primer. All the reaction were on a final volume of 20ul with the following component , 2x master mix composing of taq polymerase dNTPs, mgcl, 0.2um of each primers. The conditions for the first rounds were as follows 95°C for 5min, 30 cycles of denaturation 95°C for 30sec. annealing 56°C for 30sec and extension 72°C for 1min with final extension at 72°C for 5min. The condition for the second round were similar for that of the first round except the annealing which was 52°C for 30sec .

The amplified products were resolved on a 1.5% agarose gel alongside a 100bp ladder and visualized on a UV trans-illuminator.

Sequencing of V3 region and CCR5 amplicons

Sequencing was done using the Big Dye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria, South Africa. Sequences were subjected to bioinformatics analysis using MEGA 6.0 software.

Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace Edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using Clustal X. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (11). The bootstrap consensus tree inferred from 500 replicates (12) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (13).

Results

Of the 100 subjects enrolled for the study, 27(27%) were males and 73(73%) were females. The age distribution of subjects showed that of the 27 males, 8(30%) and 19(70%) belonged to age bracket 40 -50 and 51-60 years respectively. Out of the 73 female subjects recruited, 4(5.5%), 16(21.9%), 41(56.2%) and 12(16.4%) belonged to age bracket 18-28, 29-39, 40-50, 51-60 respectively. This is represented in Table 1 below.

Table 2 shows that all the subjects were homozygote for the CCR5 Gene. None were heterozygote or homozygote for the CCR5/Δ32 or Δ32/Δ32 mutant respectively.

The translation of protein sequence of various V3 region of the HIV isolates aligned using clustal X and translated to protein sequences on mega 6.0 showed few conserved amino acids among the HIV isolates as shown in table 4.4 . This indicates polymorphism and strain variation among the HIV viruses in Yenagoa.

Agarose gel electrophoresis of the V3 region of GP 140 of the HIV Virus showed bands of 250bp when read against the 1kp molecular ladder. S1-S10 represent the V3 bands, L1 represents a 100bp ladder while L2 represents a 1kb molecular marker. The V3 region of the HIV also showed bands of 300bp when run alongside a 100bp molecular ladder. This is shown in Figure 1

Figure 2 represents the agarose gel electrophoresis showing the CCR5 bands lane 1-10 represents the CCR5 gene bands. M and L represent the 100bp and 1kb ladder respectively.

The agarose gel of the CCR5 also showed single band of 189bp when run alongside a 100bp molecular ladder electrophoresis as shown in figure 2.

The phylogeny of the HIV isolates using Neighbour Joining revealed that of the 17 HIV V3 regions successfully sequenced, 2(%) showed a highly relatedness to the indigenous HIV-1 isolate 01NGPL0567 from Nigeria, 1 (%) was related to the HIV isolate 05CVHAN14 from Cape Verde, 1(%), 1(%), 8(%), 3(%) and 1(%) related to strain J11243 from Saudi Arabia, strain NGIB.05_026 from Nigeria, strain TWG3.2 from Taiwan, strain IU6 clone G from Kenya and strain 280_10 from Cameroon respectively. (Fig 3)

Discussion

Studies on the effects of CCR5 alleles in HIV disease progression have been made in different parts of the world (14, 17),. However, less data are available for this part of the world. There are reports of high frequencies of □32 alleles across different geographic regions in the world for which Caucasians ranked highest and Africans least (15, 16). The highest recorded allele frequency is among the highly endogamous Ashkenazi Jews at above 22% (10). This study reported a 100% non-mutant CCR5 genotype for the study subjects. None was positive to the CCR5 heterozygous allele CCR5/□32 and none was also positive to the □32 homozygous deletion. Silva et al., (18) reported a 6.5% incidence of the heterozygous genotype among blood donors from São Paulo city, Brazil. Many studies have been performed to evaluate whether heterozygosity of the CCR5-del32 allele genotype affects the vertical transmission of HIV-1, or whether it affects disease progression (14, 19).

One meta-analysis has revealed that perinatal infection is not significantly altered by heterozygosity for CCR5□32 in children (20). Another meta-analysis has been performed among 10 studies including 1317 HIV-1 infected children, addressing the effects of CCR5□32. For progression to clinical AIDS, CCR5□32 showed an overall non-significant trend for protection (hazard ratio 0.84, 95% confidence interval 0.58-1.23). However, survival analyses showed a statistically significant time-dependence. The CCR5□32 genotype was associated with a decreased risk of death among perinatal infected children, although only during the first years of life (20). Multiple factors may affect HIV-1 disease progression in perinatal-infected children. Such factors include in utero versus intrapartum infection, maternal disease status at the time of delivery (21), Therapeutic and prophylactic treatment of the mother and infant, and host human leucocyte antigen (HLA) genotype. In this study population, the CCR5 genotype was unable to account for the difference in pattern of disease progression among the three groups (rapid, moderate and slow progressors). However, we cannot exclude a potential role for these genetic characteristics, since sample size in our study was limited, and the allele frequency of CCR5- del32 was too low to allow statistical comparisons with adequate resolving power. Studies with larger populations may further elucidate the role of this

allele and other host factor in the regulation of HIV-1 pathogenesis. This study also indicates polymorphism and strain variation among the HIV viruses in Yenagoa.

Table 1: Demographic characteristics of the study participants

Age (years)	Male n=27	Female n =73	Total n=100
18-28	-	4(5.5%)	4(4.0)
29-39	-	16(21.9%)	16(16.0)
40-50	8 (30%)	41(56.2%)	49(49.0)
51-60	19 (70%)	12(16.4%)	31(31.0)
TOTAL	27(100%)	73(100%)	100(100)

Table 2: Performance charactristics of the Rapid Diagnostic Test used for HIV screening

	Positive gold standard (100) (V3 PCR)	Negative gold standard (V3 PCR)
Positive by RDT	95 (True Positive)	6 (False Positive)
Negative by RDT	5 (False Negative)	94 (True Negative)
Total	100	100

Sensitivity = 95%
Specificity = 94%
PPV = 94%
NPV = 94%
Prevalence = 0.9

Table 3: Distribution of CCR5 Genes among the Subjects

Sex	CCR5	CCR5/Δ32	Δ32
Male	26 (26%)	0	0
Female	74(74%)	0	0

Table 4: Protein Sequence of the V3 Region of the Various HIV Strains

P1	YESWTWGILC NR*YNRYTIC KNRMDGDAKK GKSTAEQKL* PTLR
P13	YKSWTWGILC NR*YNRYTIC KNRMDGDAKK GKSTAEQKL* PTLR
P16	HKNWSWSVPC NRWSSRYTTC KKGLVWDVTE CNETTKQNLC LTLR
P17	HKNRTWSILC SR*NSKYTTC KEQMECDNRK GKNTAKENL* LSLR
P2	YKDWTWDILC NR*HNRYTTC KKQMDRDGKE SKSTAREKV* FILR
P8	YKDWTWDILC NR*HNRYTTC KKQMDRDGKE SKSTAREKV* FILR
P9	YKDWTWDILC NR*HNRYTTC KKQMDRDGKE SKSTAREKV* FILR
P6	YKVWTWDALC NRSHNRHATC KDKMERGNRG SKRTA*DNI* ISYR
P11	YKVWTWDALC NRSHNRHATC KDKMERGNRG SKRTA*DNI* ISCR
P4	CKNRTWGVLC KWRHNRYATC KRKMESDVGG SKKKTTRNL* PILR
P10	YKNRTWNILY NR*NNRYTIC KNKVEYNDAE GKRTAKENV* LIRG
P7	YKIRTWGVLC NG*HNRYTSC KNKME*DGTE GKSTAKENI* LTLR
P12	YKLWTWGVLC NR*YNRYTTC KSKLD*GGTA DKSTAT*KL* LILR
P15	YKLWTWGVLC NR*YNRYTTC KSKLD*GGTA DKSTAT*KL* LILR
P3	YNLWTWGVLC NR*FNRYTTC KSKLD*GGTA DKSTAT*KL* LILM
P18	YKNWTWNVLC NK*NNRHTTC KHRLE*DNT* CKSRATKNL* LILR
P5	YKNWTWGILY NR*CNRYKTC KDSMD*HAKE GTCKATKIF* LILR
P14	CTHRTRSLLC KGCSNEYTIG QNRME*NFTS GSISI*NNLQ QLLR

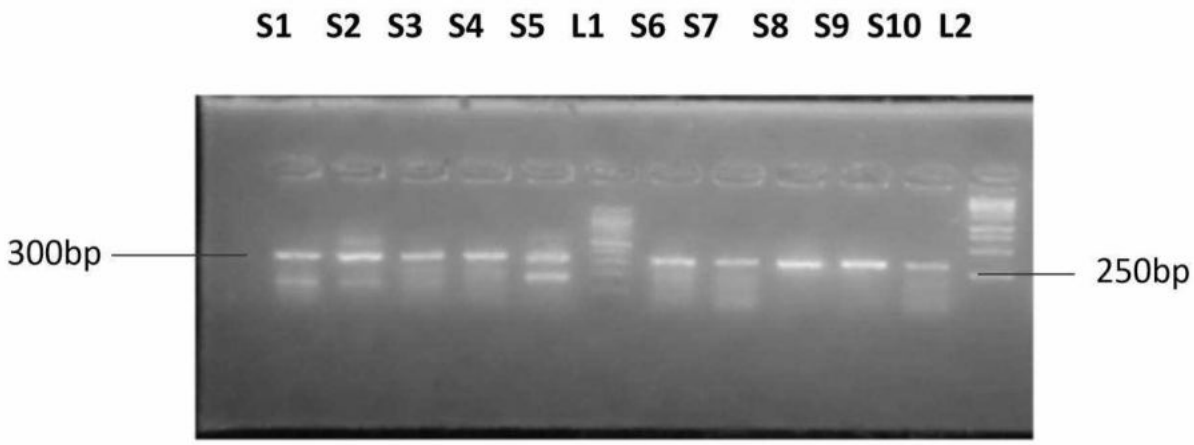


Fig 1 Agarose Gel Electrophoresis of the V3 Region

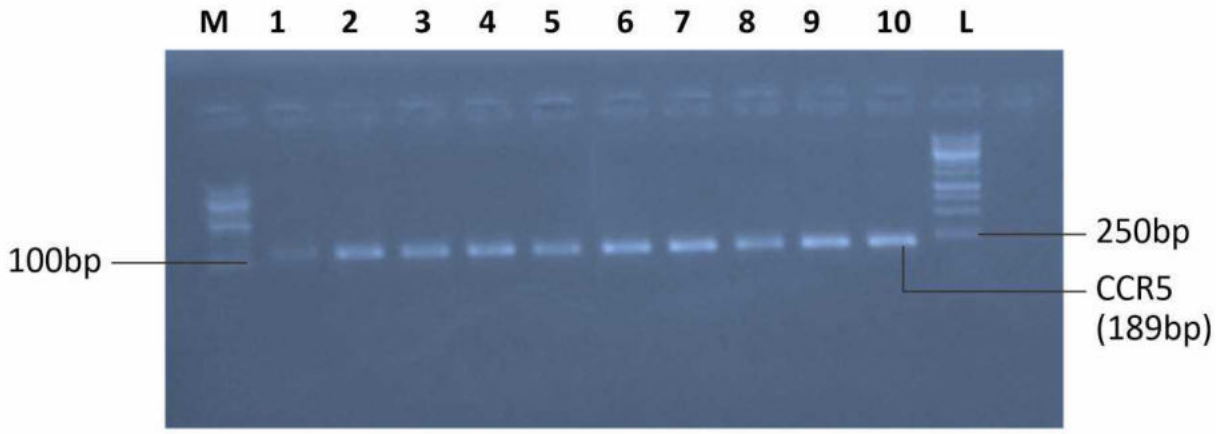
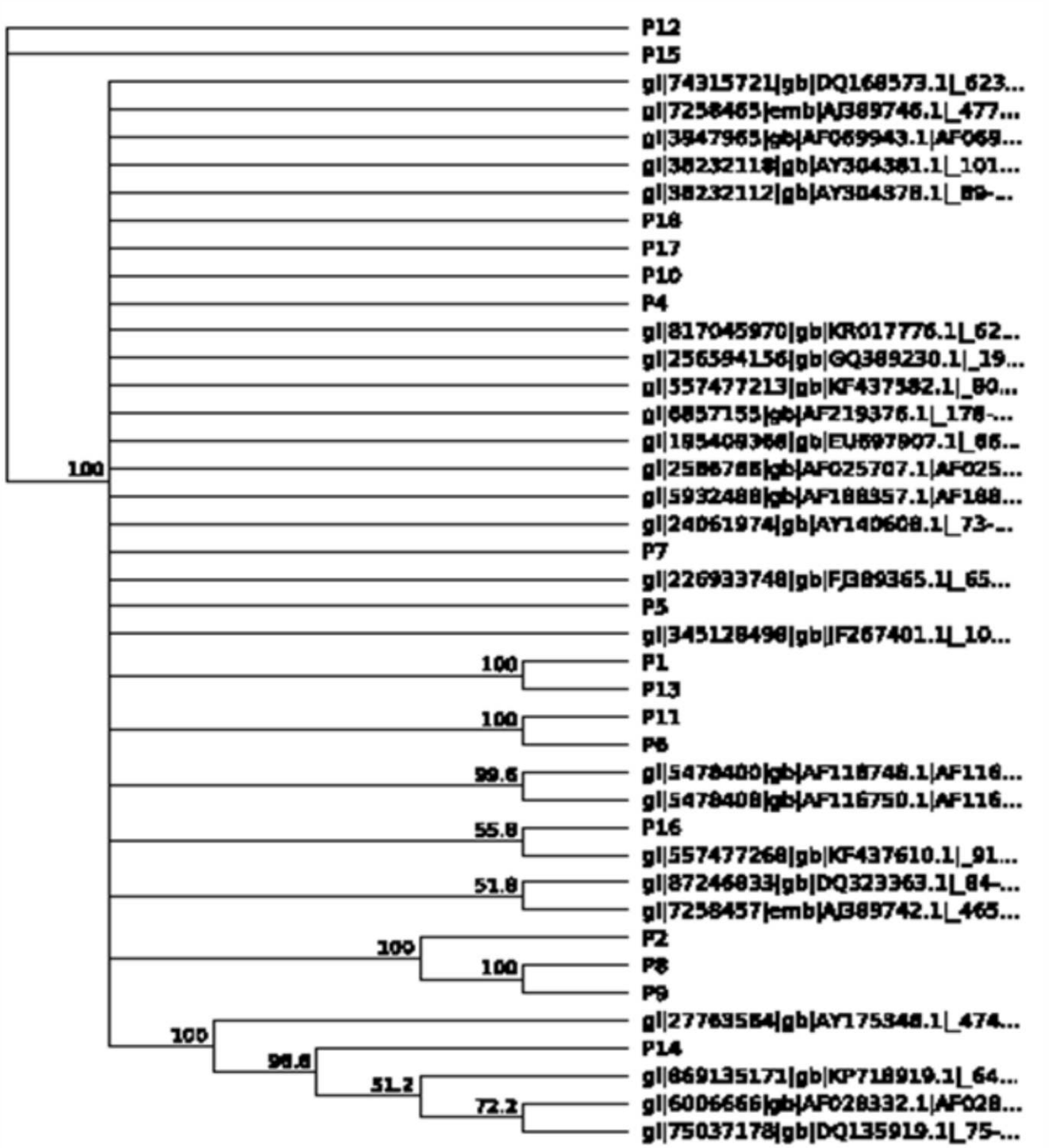


Fig 2 The Agarose Gel Electrophoresis of the CCR5

Fig. 3: Phylogenetic tree showing relationship between the HIV isolates



Conclusion

The CCR5 gene among HIV- positive individuals studied in Yenagoa is homozygous and there is no mutation. On the other hand, there is Single Nucleotide Polymorphism (SNP) of protein sequence in v3 region of p160 gene among HIV positive individuals in the study population. The study showed that the various strains of HIV virus among the studied population are CCR5 tropic.

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Additional Information

Competing Interests: The authors declare no competing financial or non-financial interests related to this work