Molecular Detection of CCR5 D32 Allele Gene Among Discordant HIV- Infected Couples in Kaduna State, Nigeria.

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Research Article

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Abstract

Background

Discordance is a situation where one partner is HIV positive while the other remains negative within couples and the CCR5 D32 gene is the mutant variant of the CCR5 wt. fusion protein which confers resistance to HIV infection. The research was conducted for molecular detection of CCR5 D32 allele gene in discordant HIV- infected couples in Kaduna State Nigeria. The objective of the research was to establish the incidence of discordance among HIV infected couples in Kaduna state and to determine the frequency of CCR5 D32 mutant allele gene among discordant HIV- infected couples. Existing literature has it that HIV discordance is low in several communities and CCR5 mutation is very rare in Africa; this underscores the need to establish the real situation in Nigeria.

Methods

A total of 158 couples and 317 individuals were recruited for the survey using a Stratified sampling technique. The 23 Local Government Areas of Kaduna State were divided into 3 strata (senatorial Zones). These respondents accessed treatment at 9 HIV treatment centers in 9 Local Government Areas of the 23 Local Government Areas of Kaduna State. Blood test was conducted to confirm their HIV status and ensure they were discordant couples. Blood samples of negative partners were analyzed using Primers of CCR5 gene in the molecular laboratory.

Results

The results showed that more women 105(33.12%) were HIV positive, and the HIV discordance prevalence for Kaduna State determined at 0.2%. The results also showed from gel electrophoresis, that the frequency of CCR5 D32 allele gene in the population was 1.3%, and there was no correlation between HIV discordance and CCR5 D32 allele gene- they both occur independently.

Conclusions

Low incidence of CCR5 D32 gene (1.3%) was discovered within the population studied. However, high presence of this gene variant in the population would have been a starting point in the research for the ultimate goal of finding a final solution in the treatment of HIV- AIDS. It is hereby recommended that more research be conducted in other states of Nigeria, and other African countries to ascertain the present status of the frequency of CCR5 D32 allele gene.

Introduction

HIV(Human Immunodeficiency Virus) Discordance among infected couples is a common phenomenon in sub-Saharan Africa and this has always been a challenge in the struggle for HIV prevention and cure in the population. (1, 2) Discordant couples are those where one partner is HIV- infected while the other is not, whereas a couple is defined as two persons in an on- going sexual relationship and each of these persons is referred to as a “partner” in the relationship. (3) HIV Discordance usually occurs as a result of several factors: it may be from initial non disclosure before marriage or relationship, traditional marriages without pre- marital counseling and testing, latency in the event of infection before marriage, and accidental infection of a partner during course of marriage or sexual relationship. (4) The phenomenon of discordance among HIV infected couples has remained a mirage and a concept that needs to be unraveled. It has remained so for as long as the discovery of the mutant alleles of the CCR5 gene came to fore. The mutant allele concept is just one possibility for discordance; there could be other reasons such as reduced viraemia or innate resistance. Knowing that majority of HIV-1 infection that occurs in Africa is among individuals in stable sexual partnership; improving our understanding on the correlates of discordance will provide information on the dynamics and risk factors of couple transmission that can assist in the development of interventions to reduce transmission within couple relationships. Chemokine receptor type 5 otherwise called CCR5 or CD195 is a protein on the surface of white blood cells that participates in the immune system as it acts as a receptor for chemokines. (5) For human beings, the CCR5 gene that encodes the CCR5 protein is located on the short (p) arm at position 21 on chromosome 3. Extensive research has revealed to us that some particular populations have inherited the Delta 32 mutation, resulting in the genetic deletion of a portion of the CCR5 gene. Homozygous carriers of this mutation are resistant to M- tropic strains of HIV1-infection. (6, 7, 8, 9, 10, 11) Research has shown that CCR5 surface protein belongs to the beta chemokine receptor family of integral membrane proteins. (12) It is a G protein- coupled receptor which functions as a chemokine receptor in the Cystein-Cystein chemokine group. Reports have it that the CCR5 is mostly expressed on T cells, macrophages, dendritic cells, eosinophils, microglia and a subpopulation of either breast or prostate cancer cells. (13, 14) The mutant CCR5-Δ32 (or CCR5-D32 or CCR5 delta 32) is an allele of CCR5 (15, 16). CCR5 Δ32 is a 32-base-pair deletion that introduces a premature stop codon into the CCR5 receptor locus, resulting in a nonfunctional receptor. (17, 12) CCR5 is required for M-tropic HIV-1 virus entry. Some individuals that are homozygous (denoted Δ32/Δ32) for CCR5 Δ32 do not express functional CCR5 receptors on their cell surfaces and are therefore resistant to HIV-1 infection, despite multiple high-risk exposures. (18) Individuals heterozygous (+/Δ32) for the mutant allele have a greater than 50% reduction in functional CCR5 receptors on their cell surfaces due to dimerization between mutant and wild-type receptors that interfere with transport of CCR5 to the cell surface. (19) Heterozygote carriers have been found to be resistant to HIV-1 infection relative to wild types and when infected, heterozygotes exhibit reduced viral loads and a 2-3-year-slower progression to AIDS relative to wild types. (17, 18, 20) Heterozygosity for this mutant allele also has shown to improve one's virological response to anti-retroviral treatment. (21) The CCR5 Δ32 has an (heterozygote) allele frequency of 10% in Europe, and a homozygote frequency of 1%. A few numbers of human beings show partial or apparently complete inborn resistance to HIV, the virus that causes AIDS. (22) The main mechanism for discordance is a mutation of the gene encoding CCR5, which acts as a co-receptor for HIV. Research reports an estimated proportion of people with some form

of resistance to HIV to be fewer than 10%. (23, 24, 25) Some particular populations have inherited the Delta 32 mutation resulting in the genetic deletion of a portion of the CCR5 gene (6, 7, 8, 9, 10, 11). In a survey conducted by Solloch et al. (26), they reported a CCR5 D32 allele frequency 0% from blood donors in Africa. Hence, there is need to investigate, and see if this gene deletion is responsible for HIV discordance among the population in Kaduna State Nigeria.

Materials And Methods

Study Area

This study was conducted in HIV treatment centers situated in all general hospitals in Kaduna State. Kaduna State Nigeria is one of the 19 northern states of Nigeria and has long been seen as the Major capital of the northern region, and now the capital of the North West. (27) It is ranked 4th by land area and 3rd by population in Nigeria. The state capital was the former capital city of the British protectorate of Northern Nigeria (1923-1966) after Zungeru (1903–1923) and Lokoja (1897–1903). It is made up of some major towns which include: Zaria, Kagoro, Kafanchan, Kachia, Zonkwa, Makarfi and Birnin Gwari. (27) Kaduna State has coordinates of 10° 20'N 7° 45' E of Nigeria. It has a total land area of 46,053 km² and a total population of 6,113,503 according to the 2006 population census of Nigeria. (28) Kaduna State has over 1,000 primary healthcare facilities to cater for every resident - even in the most remote villages or political wards of the state. In addition to these, the government has made efforts to establish General hospitals in each of the 23 LGA headquarters to enhance effective and accessible healthcare for its citizens. These General hospitals have since become treatment centers for HIV anti retroviral therapy all around the state.

The survey recruited 158 discordant couples which amounted to 317 individuals. Kaduna State of Nigeria has 23LGAs and these LGAs were further grouped into three senatorial zones/ strata, out of which three LGAs were finally selected by simple random sampling from each senatorial zone. This stratified sampling technique resulted into 9LGAs from which we got the 317 individuals. These LGAs are: Kaduna north senatorial zone (Kudan, Lere and Soba), Kaduna central senatorial zone (Kajuru, K/north and Giwa), and Kaduna south senatorial zone (Kachia, Jema'a and Kagarko). Discordant couples who declined participation were not included. See Kaduna State Map here

https://drive.google.com/file/d/1eyNtIG3tVZLDOazH8nA88lpbhZWwi7_L/view?usp=sharing

Sample collection

Ethical Consideration and Informed Consent

The study was conducted according to ethical standards for human studies as approved by the research and ethics review committee of the Kaduna State Ministry of Health and Human Services.

MOH/ADM/744/VOL.1/913. Informed consent was obtained from all participants to take part in the

study, after adequate explanations on the purpose, risk, method and benefit of the research. See link below for ethical clearance

https://drive.google.com/file/d/1_JnIr30XLQWQVNwVoAZLWY39x2cbg-mB/view?usp=sharing

A structured questionnaire containing questions on demography and other required information was issued to all the respondents who were HIV positive, with adequate guidance on how to fill- in their responses correctly, and to invite their HIV negative partners for blood sampling. This took place during group information session (GIS). See link for questionnaire here

https://drive.google.com/file/d/1_uWS7jP01VAPzbf6pmtGcm61afmWKUc6/view?usp=sharing

For each of the couples determined above, blood samples (whole blood) of negative partners were collected by Laboratory staff of haematology department of the hospitals. HIV absence was confirmed using test kits (Rapid BTS, Rapid HIV 1 and 2 Home Test Kit/ DETERMINE). Blood samples were stored in the blood bank of the hospitals at 7°C, and were later transported to DNA laboratory Kaduna in good conditioned ice packs for safe delivery. Further analysis for the presence of CCR5 D32 was conducted for the negative bloods in retrospect of CCR5 primer. Chi Square statistics and SPSS 21 was used for relevant statistical analysis.

Optimization/pooling of blood samples

This involved mixing some of the blood samples together (pooled) in one tube, and running the DNA extraction, PCR, and gel electrophoresis as 1 sample. The gel bands were expected to give a picture of a positive result, should it appear at the end of the main analysis. The optimization was done successfully and kept for future reference.

DNA Extraction

DNA was extracted using Accu prep Genomic DNA extraction kit from Bioneer. 20µl of proteinase K was added to each of 20 clean 1.5ml tubes. 200µl of whole blood was added to each of the 20 tubes containing proteinase K. 200µl of binding buffer (GC) was added to the samples and mixed immediately by vortex mixer. All of the mixture was then incubated at 60°C for 10min. 100µl of Isopropanol was added and mixed well by pipetting. After this step, the whole solution was spinned down to get the drops clinging under the lid. The lysate was carefully transferred into the upper reservoir of the binding column tube (fit in a 2ml tube) without wetting the rim. The tubes were closed and centrifuged at 8000rpm for 1min. The tubes were opened and the binding column tubes transferred to new 2ml tubes for filtration (supplied). 500µl of washing buffer1(W1) was added without wetting the rim, and all the tubes closed and centrifuged at 8000rpm for 1min. They were centrifuged again, at 12000rpm for 1min to completely remove ethanol, and check that there is no droplet clinging to the bottom of the binding column tube. The binding column was transferred to a new 1.5ml tube for elution (supplied), 200µl elution buffer added onto binding column tubes, and waited for 1min at RT (15- 25°C) until elution buffer was completely absorbed into the glass fibre of binding column tubes. To increase DNA yield, there was a waiting for

5min after adding elution buffer (EL), then the tubes were centrifuged at 8000rpm for 1min. to elute. About 180µl- 200µl was obtained using 200µl Elution buffer (for improved yield, the elution was done twice). The eluted genomic DNA which was visible at the bottom of the tube was then used for PCR.

Polymerase Chain Reaction (PCR)

The PCR was conducted using Accupower Hotstart PCR premix, Bioneer. PCR primers (forward (5'→3'):AGGTACCTGGCTGTCGTCCA;reverse(5'→3'):CTCACAGCCCTGTGCCTCTTC) designed for an amplicon covering the 32 b.p. deletion of the CCR5 D32 with 329 or 297 b.p. in length, respectively was used. PCR- was conducted using the Thermal Cycler Model PTC 100. MJ Research. 2µl DNA extract was added to each of 40 1.5ml tubes. 16µl dH₂O was also added to each of the tubes together with 1µl primer forward and 1µl primer backward primers(forward(5'→3'):AGGTACCTGGCTGTCGTCCA;reverse(5'→3'):CTCACAGCCCTGTGCCTCTTC) designed for an amplicon covering the 32 b.p. deletion of

CCR5-Δ32 with 329 or 297 b.p. in length, respectively (Bioneer inc.). The thermal cycler condition was set as follows: Pre- Denaturation- 5min at 95⁰C, Denaturation- 30sec at 94⁰C, Annealing- 30sec at 55⁰C, Extension- 30sec at 72⁰C 35 cycles, Final extension- 5min at 72⁰C. The procedure was repeated for all 159 DNA extracts.

Gel Electrophoresis

The amplified gene was run on a 1.5% Agarose gel. The gel was prepared by weighing 3g Agarose powder and dissolved in 200ml TAE (Triple Acetate Electrolyte). The mixture was heat in a microwave oven until all Agarose was completely dissolved. The solution was allowed to cool in a water bath set at 50⁰C- 55⁰C. A gel casting tray was prepared by sealing ends of gel chamber with transparent selo tape. A 26 number comb was placed in the gel tray. While the gel was cooling, 5µl Ethidium bromide was added to the gel, mixed by shaking round and poured into the gel tray. It was allowed to cool for 30min at room temperature, combs were removed and the gel placed in electrophoresis chamber and covered with buffer (TAE as used previously). 26 DNA extracts and standard Ladder (Bioneer inc.) were loaded onto the gel electrophoresis at 120V for 1hr. The DNA bands were viewed using UV light box (Biorad). The procedure was repeated for all the 159 samples.

Sequencing

The sequencing of CCR5 genes was done using sequencer model ABI 3100. Preparation of sequencing reaction was done in a 2ml tube in groups of 40 tubes each. All reagents were kept on ice while preparing the sequencing reactions. 9µl of dH₂O was added into the tube, 9µl DNA extract was also added, 2µl of primers was equally added and 8µl of Bigdye master mix added. All the mix was then put into the sequencing machine. The thermal cycling program was then set at: 96⁰C for 20sec, 50⁰C for 20sec X 30 cycles, 60⁰C for 4min.

Ethanol precipitation: A labeled sterile 0.5ml tube was prepared for each sample into which 5µl stop solution was added. Every one of the sequencing reaction was transferred into the appropriately labeled tube and mixed thoroughly. The stop solution contains 2µl of 3M Sodium acetate, 2µl of 100mM Na₂ – EDTA and 1µl of 20mg/ml of glycogen (provided in the kit). 60µl cold 95% (v/v) ethanol from -20⁰C freezer was added and mixed thoroughly. The mixture was immediately centrifuged at 14,000rpm at 4⁰C for 15min. the supernatant was carefully removed with a micropipette leaving a visible pellet. The pellet was rinsed with 200µl 70% (v/v) ethanol from -20⁰C freezer and centrifuged at 14,000rpm at 4⁰C for 2min. The supernatant was carefully removed again with a micropipette. It was then vacuum- dried for 10min. The sample was re- suspended in 40µl of the sample loading solution provided in the kit.

Sample preparation for Loading in to the Sequencer: The re- suspended samples were transferred to the appropriate wells of the sample plate. Each one of them was overlaid with one drop of mineral oil supplied in the kit. The sample plate was then finally loaded into the instrument and the sequencing started. The whole procedure was repeated for different sets until all the 159 samples were sequenced.

All chromatographs/sequence plotting as read on the Flinch TV software after sequencing showed base pair readings as homozygous for CCR5 gene.

Phylogenetic analysis

The sequence obtained above was edited using the NCBI bioinformatics data software. Similar sequences were downloaded in FASTA format using BLAST N from the NCBI bioinformatics software. Downloaded sequences were aligned using MEGA 11 alignment software and evolutionary relationship eliminated at 1000 bootstrap.

Results

The results in Figure 1 shows that majority of women (66.46%) were HIV positive than the men (33.54%) hence the type of discordance in Kaduna State is “soft discordance”. Even though the distribution of men to women in this research was 50: 50 (because they were husband and wife), the women were more positive for HIV than the men.

Table 1: Incidence of HIV Discordance in Kaduna State (N= 317)

Item	Number of People	Percentage (%)
Total Population	6, 115, 503	100
HIV Prevalence	67, 271	1.1
HIV Sero- discordance	159	0.2

Table 1 shows 0.2% incidence for HIV discordance in Kaduna State.

The results in Figures 2 shows that all the blood samples had the CCR5 gene except number 57 which means it had the CCR5 D32 allele. The 32 b.p. deletion has an approximate molecular weight 300bp which is visible on the bands when compared to the DNA ladder (Bioneer inc.). The gel picture shows that the DNA bands aligned at 300bp as expected.

Table: 2 Frequency of CCR5 D32 gene among the studied Population

Gene Variant	Frequency	Percentage (%)
Wild Type (CCR5- wt)	157	98.7
Mutant Type (CCR5 D32)	2	1.3

Table 2 shows the frequency of CCR5 D32 gene in the population. Majority of the subjects (98.7%) were homozygous for the wild type CCR5 gene. Only 1.3% of the studied population had the mutant CCR5 D32 gene.

The obtained CCR5 gene sequences from the respondents showed an exact match to BLAST sequences from data base of CCR5 genes as shown in Figure 3. The percentage similarity was between 90 – 100% in the BLAST mega search to existing sequences. The evolutionary distances shown on the tree reveals an evolutionary close relationship with trees of existing CCR5 genes, using the MEGA 11 software.

Figure 4 shows sequence alignment of CCR5 genes showing the few nucleotide polymorphisms.

Table 3: Correlation Between CCR5 D32, Use of ART, Sex dependence and Discordance (N= 317)

Item	Yes (%)	No (%)	P- Value
CCR5 D32	2 (1.3)	157 (98.7)	0.16
Use of ART	157 (49.5)	160 (50.5)	0.00*
Sex Dependence	Male (%) 158 (49.8)	Female (%) 159 (50.2)	1.00

*Statistically Significant Item

Table 3 shows that there is no significant relationship between CCR5 D32 and HIV discordance.

Discussion

Results of Figure 1 showed that in Kaduna State, more women (66.46%) were HIV positive than their male counterparts (33.54%). This means that in Kaduna State, women are two times more likely to be infected with HIV than men. This agrees with Magaji et al. (29) who reported similar incidence in Plateau State Nigeria. Biologically, women are two to eight times more likely than men to contract HIV during vaginal intercourse (30) and this is likely going to be the reason why our results somewhat agree with their report. We can also conclude that a large proportion of HIV discordance in Kaduna State is “soft discordance” according to Nnebue et al. (31) In other states of Nigeria, the case may be different as also reported by Nnebue et al. (31) A United Nations national report 2019 places Kaduna State on 1.1% HIV prevalence, and Table 1 gives us a total of 159 respondents who were HIV negative out of the total 317 respondents. Using the figures above, the incidence of HIV discordance was calculated to be 0.2% for Kaduna State as shown in Table 1. This again agrees with Magaji et al. (29) who reported low incidence of sero-discordance in Plateau State, Nigeria. In Figure 2, the molecular analysis of blood samples showed that all of the blood samples, except two (1.3%) were positive for CCR5 gene profiling- this shows clearly that 98.7% of discordant HIV subjects that participated in the study were positive for the normal allele/ wild type (CCR5- wt). The 1.3% mutant gene (CCR5 D32) observed in the gel pictures, represents a few individuals that have the capacity to resist HIV infection in the population. (22) In order to confirm the authenticity of the gene amplification process carried out, the DNA extracts were sequenced, and the sequence results was entered into the NCBI real time software for bioinformatics and the confirmation showed that the gene of the respondents was a CCR5 complete sequence. The frequency of the CCR5 D32 gene in the population (1.3%) shown in Table 2, disagrees with Solloch et al.(26) who reported a 0% frequency in Africans; and agrees with other researchers (24, 25, 23) who also stated that an estimated proportion of people with some form of resistance to HIV are fewer than 10%. This goes to show that

contrary to Solloch et al. (26) some few Africans do possess the CCR5 D32 allele gene. The 1.3% frequency for CCR5 D32 mutant gene in Kaduna State also disagrees with Ekere et al. (32) who also reported a 0% frequency in Calabar, Cross river state where all the respondents had the CCR5 wt (wild type). The evolutionary relatedness of CCR5 gene seen in the samples studied; with those of already existing CCR5 genes confirm the predominance of HIV in the population as a result of persistent transmission of the virus from person to person. (32) Statistical analysis using SPSS 21 showed no correlation between CCR5 D32 and discordance, using available data as shown in Table 3. The high p-value of 0.157 means there was no association between CCR5 D32 allele gene and the occurrence of HIV discordance, contrary to our initial expectations. This means that other factors may have been responsible for HIV discordance in the population as mentioned by Onovo et al. (4)

Conclusion

This research reaffirmed the existence of discordance among HIV infected couples in Kaduna State Nigeria; and revealed that more women (66.46%) were positive than men (33.54%). The discordance type was “soft discordance”, and the incidence was found to be 0.2% of the infected population. The CCR5 D32 allele gene frequency in Kaduna State was also found to be 1.3% contrary to initial expectations of a 0% frequency. But because with or without CCR5 D32 allele gene the population still had discordance, we can conclude that there was no correlation between CCR5 D32 and HIV discordance in Kaduna State Nigeria, using available data. It will therefore be recommended that more of this survey be conducted in other states of Nigeria, and even extend the work to other African countries. Probably, a high frequency of the CCR5 D32 allele gene in the population will open a new vista of hope in the eventual discovery of a final solution to the problem of HIV and AIDS.

Declarations

Ethics approval and consent to participate- The study was conducted according to ethical standards for human studies as approved by the research and ethics review committee of the Kaduna State ministry of Health and Human Services. MOH/ADM/744/VOL.1/913. See document here https://drive.google.com/file/d/1_JnIr30XLQWQVNwVoAZLWY39x2cbg-mB/view?usp=sharing

Informed consent was obtained from all participants to take part in the study, after adequate explanations on the purpose, risk, method and benefit of the research. LINK https://drive.google.com/file/d/1_uWS7jP01VAPzbf6pmtGcm61afmWKUc6/view?usp=sharing

Consent for Publication- Consent was received from participants. See LINK <https://drive.google.com/file/d/1eVGyJ7F-QOuDrfrIN7o9B10PjPHvr0Bs/view?usp=sharing>

Availability of data and materials- The dataset used and /or analysed in the current study are available from the corresponding author on reasonable request.

Competing interests- The authors declare that they have no competing interests.

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Authors' contributions

Danboyi D.A. conceived the study and participated in its design and coordination.

Sani M.N and Danboyi D.A. conducted the statistical analysis.

Danjuma L and Abdullahi M handled the study review.

All authors read and approved the final manuscript

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Figures

Figure 1

HIV Status of Respondents

Figure 2

Visible Bands of CCR5 Gene, showing one blank CCR5 D32 mutant allele.

Figure 3

Phylogenetic tree showing genetic relationship between CCR5 genes

Figure 4

Sequence Alignment of CCR5 genes showing nucleotide polymorphism