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Rabies Virus in Slaughtered Dogs: A Molecular Study in Agatu Local Government Area, Benue State, Nigeria

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Abstract
Rabies is a viral zoonosis that causes encephalomyelitis which results in death of almost all victims. Dogs are the main source of human rabies deaths, contributing up to 99% of all rabies transmissions to humans. This research is targeted at detection and phylogenetic analysis of a segment of N gene of rabies virus among dogs slaughtered for consumption in Benue State. Brain tissues were collected from 50 dogs slaughtered in Benue State, Nigeria. Direct fluorescent antibody test (DFAT) was used to screen for the presence of rabies virus antigen. Viral RNA isolated from DFAT positive brain tissues were subjected to the reverse transcription polymerase chain reaction (RT-PCR) followed by sequencing of the amplicons. Phylogenetic analysis was done in relation to other isolates from the Gen bank. The presence of nucleoprotein antigen using DFAT was revealed in 2% of the samples collected for the study. The DFAT positive sample J50 had fluorescence score of (++++) and also positive by RTPCR. The amplified N gene segment of the isolate sequenced, deposited into GenBank with accession number MT394532 and compared with 23 other rabies virus N gene sequences from the Gene bank. The phylogenic analysis gave an evolutionary pattern, showing that the Africa 2 cluster and MT394532 are paralogous having similar structure and indicating divergence from a common ancestral nucleoprotein gene as this might have been conserved overtime. This finding has shown that there is an in-country circulation of rabies virus strains which extends to the continent at large and possibly a cross movement within hosts of different species ranging from dogs to bats. Also, this revealed the possible exposure of the virus to both dog handlers and consumers. Rabies epidemiology dynamics in Benue State can be ascertained from this study which can serve as a direction to further rabies control programs.

Key words*: Rabies virus, slaughtered dog, direct fluorescent antibody test, Reverse transcription polymerase chain reaction and Phylogenetic analysis.*

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I. Background of Study

Rabies is a viral zoonosis that causes encephalomyelitis (Dacheux et al., 2011). It affects all warm-blooded animals (Warrell and Warrell, 2004; Adedeji et al., 2010), and having almost 100% case-fatality rate (WHO, 2016). Human mortality due to rabies is estimated to be 50,000 deaths per year worldwide, mostly reported from Asia and Africa (Dacheux et al., 2011; Yousaf et al., 2012). The disease is of worldwide public health importance (Streicker et al., 2012), and have been found to be endemic in Nigeria (Ehimiyein et al., 2010; Okpe et al., 2011). Dogs have been confirmed as the reservoirs and vectors of human rabies, accounting for 94% of the cases (Nel and Rupprecht, 2007). At least, 10,000 persons have been estimated to be exposed to rabies annually in Nigeria, which is apparently underestimated because there is no efficient monitoring and reporting system for the disease (Ehizibolo et al., 2011). In Nigeria and many other developing countries in the tropics, at least 10% of brain samples received at laboratories for rabies diagnosis are decomposed because of inadequacy of transportation and storage facilities (Dürr et al., 2008), leading to the misdiagnosis of most of the cases. For efficient transportation, various methods of preservation and transportation have been experimented (Dürr et al., 2008). The use of 50% glycerol/phosphate-buffered sulphate for preservation is commonly practiced in Nigeria, and it is assumed that the virus remains viable in the medium for over six months (Elsa and Ogunkoya, 1996).

The Nucleoprotein gene which is the first gene after the leader sequence (LDR) of approximately 50bp, has a size of 1424bp and codes for the N protein, which is the most abundant protein in the Ribonucleo Protein complex and is involved in the encapsulation of viral genome (Wunner, 2007). Besides, the N gene is also the most conserved gene in the viral genome, making it an ideal candidate for phylogenetic analysis. It was found that the N protein was phosphorylated in rabies virus possibly by a cellular casein kinase II and different phosphorylation states of N protein played an important in viral transcription and replication (Wu et al., 2009). Furthermore, previous studies have mapped several functional regions to the N protein. It has been shown that in addition to the G protein, the N protein also possessed antigenic and immunogenic properties. A number of antigenic sites have been identified in the N protein, including antigenic site I (aa 358-367), antigenic site II (aa 373-383) and antigenic site IV (aa 359=366 and aa 375-383) (Goto et al., 2000) and a phosphorylation site, Ser389, was also involved in the N antigenicity (Kawai et al., 1999). In addition, several RABV-specific T helper cell epitopes were identified in the N protein, comparing aa 21-35 and the so-called 31D (aa 404-418), the latter of which was shown to be an immunodominant epitope to stimulate RABV-specific T cell production *in vitro*. Finally, the RNA-binding site on N gene for interaction with viral RNA has been localized to region aa 298-352 of the N protein (Kouznetzoff et al., 1998)

Rabies virus is very sensitive to environmental factors. It is highly sensitive to sunlight, ultraviolet irradiation and heat at 60^0C (for five minutes), lipid solvents (70% alcohol and ether), sodium deoxycholate, trypsin, and common detergents. However, it is preserved at sub-zero temperature and in glycerol (Awoyomi et al., 2007; Adedeji et al., 2010).

Dogs have been the major reservoirs of rabies to humans in Africa. Approximately 24,000 deaths occur per year in Africa, although most of the incidents are under-reported. The sites of exposures are usually in the rural areas where prompt diagnosis and post exposure prophylaxis are not readily available, thereby leading to increase in human deaths. Some factors militate against the control and elimination of rabies in Africa; socio-cultural beliefs, lack of prompt field-based diagnostic tests, funding and illiteracy, etc. (Yousaf et al., 2012).

II. Materials and Methods

This research was aimed to carry out molecular studies on a segment of the N gene of rabies virus isolated in dogs from Benue State**,** whose objectives were; to detect the presence of rabies virus nucleocapasid antigen in brain tissues of dogs using Direct Fluorescent antibody technique (DFAT), to amplify fragment of the N gene of the rabies virus genome by PCR amplification, to study the genetic relatedness of the amplified fragment of the N gene with other rabies virus isolates and to study the evolutionary relationship of the amplified fragment of the N gene with other rabies virus isolates.

Sample Area

The study was carried out at dog slaughter points in Benue state which is in the mid-belt region of Nigeria (Figure 1). The study involved a purposive cross-sectional study of trade dogs at two Local Government Areas of Benue State (Agatu and Makurdi Local Government Areas) and experimental research involving the detection of rabies antigens in brain tissues of slaughtered dogs and PCR identification of the virus and phylogenic analysis of sequenced PCR amplicons of the N gene.

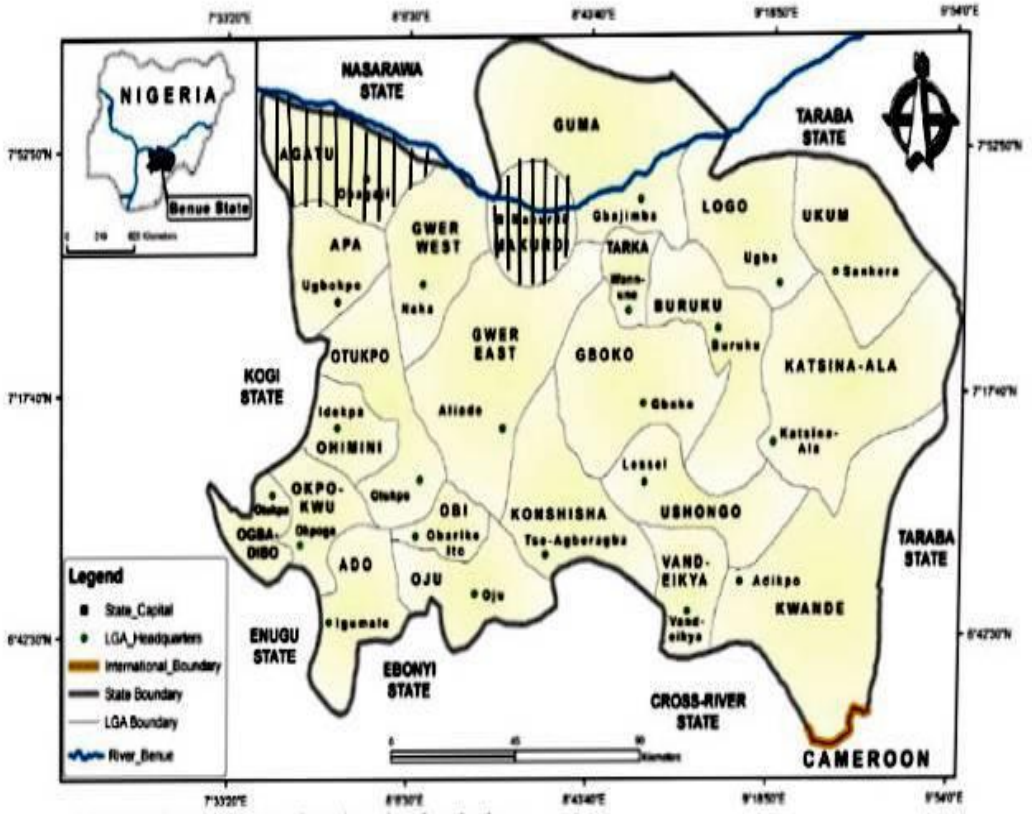

Figure 1: Map of Benue State showing the Study Area

Ethical Clearance

Ethical approval for this research was acquired from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC).

Direct Fluorescent Antibody Test

Direct Fluorescent Antibody test (DFA) was performed on the brain samples collected from slaughtered dogs as described by (Dean et al., 1996). Rabies direct fluorescent antibody assay (DFA) monoclonal antibody reagent (Fujirebio Diagnostic Inc. Malvern, P.A 19355, USA) was used, according to manufacturer's recommendations. An impression smear of part of the brain sample was made on a clean glass slide, air dried and fixed in cold acetone for 30 minutes at -20^0C. The slide was then air dried for 5 minutes and a drop of the rabies conjugate was applied on the smear using a micro pipette and this was incubated for 30 minutes at 37^0C in a humid chamber. After the incubation step, excess conjugate was removed by washing with PBS (pH 7.4) for about 5 minutes, repeated three times, and allowed to air dry. The slides were examined using the fluorescent microscope at X 40 magnification objective lens. The presence of brilliant apple green fluorescence or greenish-yellow objects against a dark background was regarded as positive and the intensity was noted and scored as +, ++, +++ or ++++ based on the number of fluorescents per unit area of the field, while the absence of specific apple green fluorescence was regarded as negative for rabies (Woldehiwet Z, 2005).

Molecular Analysis

RNA Extraction

Total RNA was extracted from all the DFAT positive samples using Trizol reagents according to the manufacturer's instruction (Life Technologies, Carlsbad, California, USA). Briefly about 0.1 g brain tissue was homogenized with 1 ml Trizol and then 200 ml chloroform (Sigma Chemical Co., St Louis, MO, USA) was added and mixed. After centrifugation of the sample at 10 000 g for 15 min, the top aqueous layer was recovered and RNA was precipitated by adding 0.5 ml isopropanol. The sample was spun at 10 000 g for 10 min, the liquid was removed and the pellet washed with 1 ml of 75% ethanol. The RNA was dissolved in RNase

free buffer provided with the kit. RNA was quantified with a spectrophotometer (Gene Quant II Pharmacia, Piscataway, NJ, USA) and stored at -70^{o}C, prior to use.

Polymerase Chain Reaction (PCR)

PCR was performed using one-step RT-PCR kit following manufacturer's protocol. For the amplification, the N gene specific primers targeting the 355 bp conserved region based upon the previous submissions in the Genbank were used (Singh et al., 2010).

Briefly, 25µl reaction mix was prepared consisting of 5µl purified RNA template, 5µl 5x QIAGEN one step RT-PCR buffer, 3µl 5x QIAGEN Q solution, 1.5µl dNTP mix (10mM), 3µl (30pmol) each of N specific forward primer; RabN-533F; 5'-CATTGCAGATAGGATAGAGC-3' and reverse primer; RabN-888R; 5'-GAGGAACGGCGGTCTCCTG-3', 1µl of QIAGEN RT-PCR enzyme mix, 0.5µl of RNase inhibitor (20U/µl), and 3µl of dH_2O. The reaction was incubated at 60^{o}C for 1minute, 42^{o}C for 10minutes, 50^{o}C for 30minutes for the cDNA synthesis (step 1) followed by denaturation at 94^{o}C for 5minutes (step 2). After the initial denaturation, the amplification was carried out for 30 cycles in three steps, 94^{o}C for 30seconds, 55^{o}C 30seconds and 72^{o}C for 30seconds (step 3), with a final extension at 72^{o}C for 5minutes.

PCR aliquots were electrophoresed through a 1.5% agarose gel, stained with ethidium bromide (5mg/ml) (Gibco-BRL, Grand Island, NY, USA), evaluated under UV light and photographed.

DNA Preparation

The DNA fragment matching the size of expected amplicon was excised from the agarose gel and weighed in a 1.5ml micro tube (A gel of 100mg is approximately equal to 100ul.), 3 gel volumes of buffer GB was added and the mixture was incubated at 50^{o}-60^{o} for 8 minutes with mixing of the tube by tapping the tube bottom every 2-3 minutes till gel was completely melted. Two gel volumes of isopropanol was added and mixed, 750ul was transferred to a DNA mini column with a collection tube and was centrifuged at 11000 rpm for 30 seconds before the flow through was discarded and the column placed back in the collection tube. This was repeated until there wass no remaining mixture, 750ul of wash buffer was added to the column and centrifuged at 11000 rpm for 1 minute at room temperature, and the flow through was discarded. The empty column was further centrifuged at 11000 rpm for 1 minute to remove the residual ethanol.

Sequencing

The PCR fragments was sequenced using the ABI PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer, USA) with primers RabN-533F and RabN-888R on an ABI 310 genetic analyzer. A labeled sterile 0.5ml tube was prepared for the sample. Fresh stop solution/glycogen mixture was prepared as follows per sequencing reaction: 2ul of 3M Sodium acetate, 2ul of 100mM Na2-EDTA and 1ul of 20mg/ml of glycogen (provided in the kit). To the labeled tube 5ul of the stop solution/glycogen mixture was added. Sequencing reaction was transferred to each of the appropriately labeled tube and mixed thoroughly. Sixty microlitres (60ul) cold 95% (v/v) ethanol from -20 freezer was added and mixed thoroughly. This was immediately centrifuged at 14,000rpm at 4^{o}C for 15min and carefully the supernatant was removed with a micropipette (the pellet being visible). The pellet was rinsed with 200ul 70% (v/v) ethanol from -20 freezer, centrifuged at 14,000rpm at 4C for a minimum of 2minutes and carefully all the supernatant was removed with a micropipette. The constituent was vacuum dried for 10min or until dry and resuspended in 40ul of the sample loading solution. The resuspended sample was transferred to the appropriate wells of the sample plate (PN 609801) and overlaid with one drop of mineral oil from the kit. The sample plate was loaded into the instrument and the desired method started.

Sequence Analyses and Phylogenetic Reconstructions

Sequence analyses and phylogenetic reconstructions was performed using Bio Edit (Hall, 1999) and MEGA 6 software platforms (Tamura et al., 2013). Phylogenetic analysis was conducted by comparing the partial rabies sequences with other rabies isolates selected from the Genbank representing rabies virus isolates that circulate in Africa.

Statistical Analyses

Data obtained were subjected to descriptive statistics and categorical evaluation of the test outcome. Results were presented in Tables and charts.

III. Results

Detection of Rabies Virus Nucleocapasid Antigen in Brain Tissues of the Dogs

Out of 50 samples tested for nucleocapasid antigen in brain tissues of the dogs using Direct Fluorescent antibody technique (DFAT), only 1 sample from Osugudu tested positive, that is, sample No. J50. The dog from which this sample was collected was showing the signs of full-blown rabies, such as hydrophobia, ferociousness and seclucsion. The DFAT positive rate is 7.1% for Oshugudu and 0.0% for the rest locations. The DFAT

positive rate among all the samples was 2.0%. out of six sampling locations only one sample from 14 in Oshugudu was positive, in general the positive rate is 2%.

PCR Amplification of DFAT Positive Sample

Out of the 50 samples tested only one was positive by DFAT, which was from a male dog in Oshugudu. The DFAT Fluorescent score of the sample was estimated to be ++++ and the sample was also positive for N gene fragment amplification by PCR. The electrophoregram shows the molecular weight marker on Lane M, the positive sample J50 on Lane 1 and the Negative control on Lane 2. The amplicon size as indicated by the arrow shows that the band as within the 355bp as to be amplified by the primer (Figure 2).

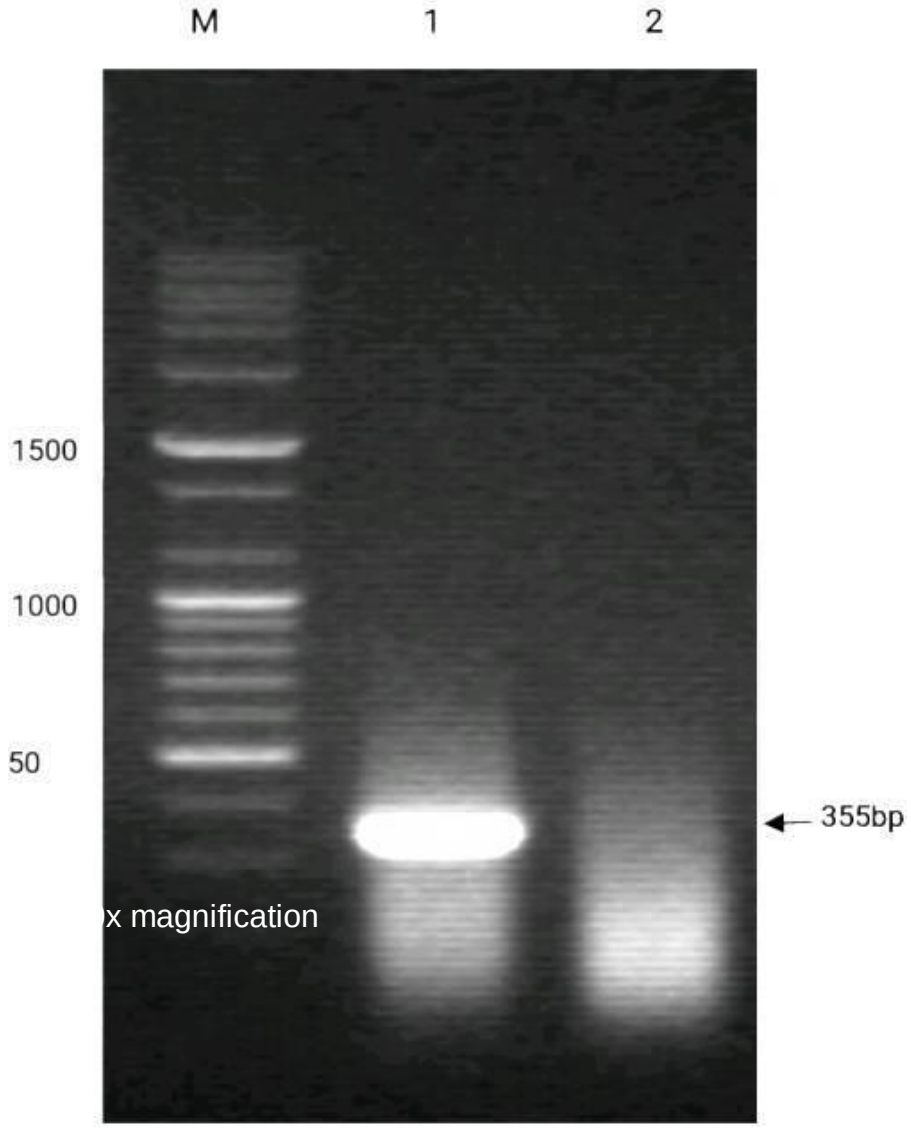

Figure 2: An Agarose Gel showing amplification of N gene

KEY: Lane M: 100bp Molecular Weight Marker, Lane 1 is Sample J50, Lane 2: Negative Control

Sequence Reads of the N Gene Segment amplified from Sample J50

The amplified N gene segment was sequenced and submitted to GenBank. The sequence was assigned accession number MT394532.

Phylogenic Analysis of the Aligned Rabies Virus Sequences

The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA 6 software program. The evolutionary history was inferred using the Maximum Likelihood method.

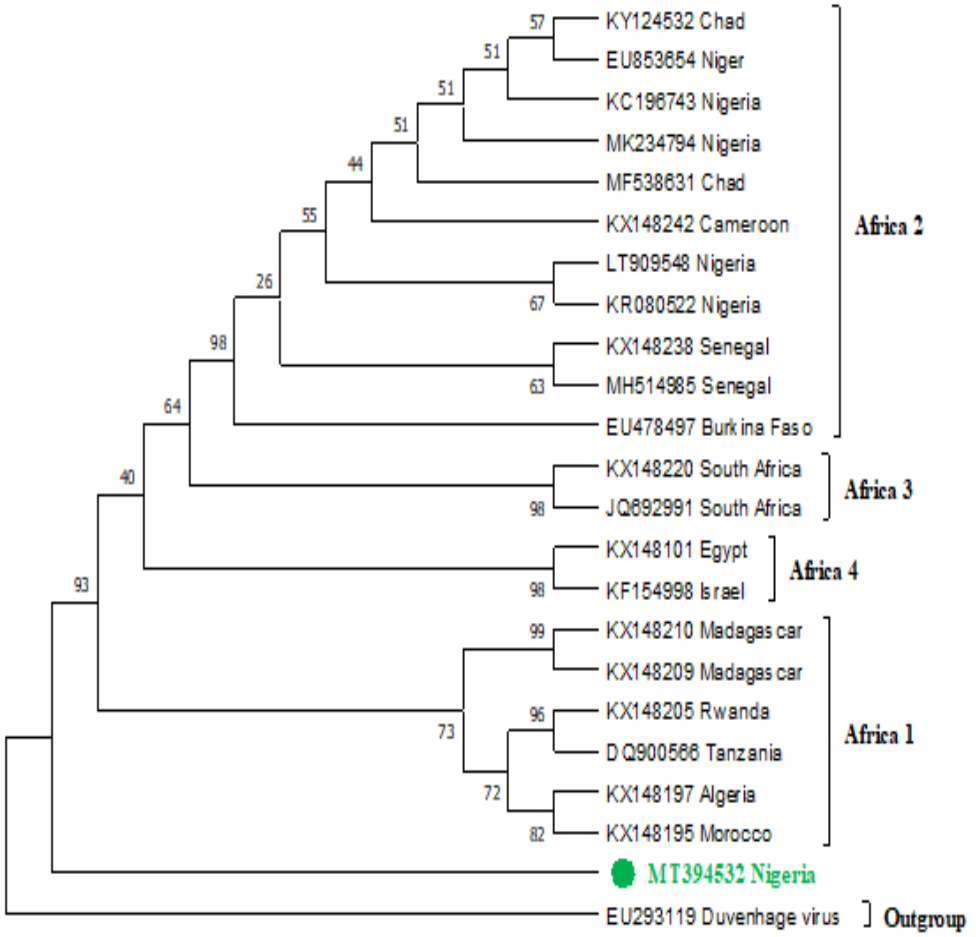

Fig. 3 Evolutionary analysis by Maximum Likelihood method of MT394532

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-1276.17) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 1.2384)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 15.98% sites). This analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

IV. Discussion

Rabies antigen was detected in 1 (2%) of the 50 dog brain samples tested. This indicates that dogs slaughtered for human consumption may be a source of infection to dog handlers and butchers. This rate is lower than those reported previously by other researchers (Akumbo, 2009; Ameh et al., 2014; Otolorin et al., 2014) Most of the individuals involved in dog meat processing have been found not to utilize personal protective equipment and also the method of slaughter predisposes them to bites from dogs during restraint. About 68.0% of the brain tissue samples tested was obtained from male dogs as more male dogs were slaughtered in the study area during the study period.

The intensity of fluorescence of the DFAT positive sample was estimated to be ++++ and the sample was also positive for N gene fragment amplification by PCR (Figure 2). The intensity of fluorescence of a sample may be a factor affecting the PCR amplification of the sample, which shows that the intensity may represent the concentration of the rabies virus in the sample. Nevertheless, PCR amplification can be affected by other factors that may cause RNA degradation. RNA degradation can affect the RNA content of a sample which can lead to total loss of RNA and unavailability of Nucleic acid for amplification. The intensity of fluorescein entities, shows that viral load was high. This was also shown by the display of signs of rabies by the dog. Often,

people mistake the presence of artifacts as being fluorescein entities by Rabies Nucleoprotein antigen, but with PCR better inference can be made (Veasna et al., 2016).

The sequenced segment of the N gene of the Rabies virus was compared with 23 other sequences from the Gen bank. Among these aligned sequences MT394532 had a maximum similarity of 97% with isolate KC196743 and a minimum similarity of 93% with isolate EU293119. The amino acid variation occurred in various positions, showing that the nucleic variations were significant, resulting in different codons for amino acid synthesis. The variation in Nucleic acid composition affects the amino acid sequence, because amino acid changes can potentially affect the folding of proteins which can have an influence on the conformational antigenic sites (epitopes) (Moore et al., 2005). The differences observed can theoretically influence the immunological characteristics of the Rabies virus. Further studies may be needed to support that, because immunogenicity and pathogenicity of rabies virus have complex nature and is not yet fully understood (Moore et al., 2005).

The phylogenic analysis gave an evolutionary pattern, showing that the MT394532 rabies virus lies around the African 1 group and the outgroup EU293119 Duvenhage virus, inferring a distance relationship lying in between the African rabies virus from dog origin and bat originated Duvenhage virus. From the inferred phylogeny, the Africa 2 cluster shows KY124532 (Chad) is the sister terminal to EU853654 (Niger) and together they form a monophyletic group. MK234794 (Nigeria) and KX148242 (Cameroon) are paraphyletic with regard to the monophyletic sister group containing the two clades from Nigeria and Senegal (fig.2). These phylogenetic terminologies are redrawn from Harrison et al. (2005) and the tree is rooted to the nucleoprotein gene of Duvenhage virus. Similarly, MT394532 (Nigeria) and EU293119 (Duvenhage virus) both form the terminals of the tree. However, MT394532 should have clustered in Africa 2 since the isolate was from a state in Nigeria (West Africa region), perhaps this is due to the fact that the other isolates in Africa 2 cluster and MT394532 are paralogous having similar structure and indicating divergence from a common ancestral nucleoprotein gene as this might have been conserved overtime. Consequently, Africa 1, Africa 2, Africa 3 and Africa 4 cluster suggest a monophyletic (orthologous) nucleoprotein gene group in the phylogeny (Harrison and Langdale, 2006).

Practices of good vaccination of dog handlers, advising dog handlers to wear protective equipment and receiving pre-exposure human anti-rabies vaccine and washing of dog bite wounds with soap and water are also indicators that the community is involved in the control of Rabies (Otolorin et al., 2014). Poor practices of seeking traditional medicine and non-washing of dog bite wounds go against the WHO recommendation of instituting medical treatment on victims of dog bites. These negative practices may be as a result of inadequate awareness among the population of the dangers of rabies. Individuals involved in the handling and processing of dog meat are constantly being exposed to rabies virus through bites from dogs, cuts or wounds not protected from infective tissue or saliva of these dogs. Presence of antigens to rabies in brain of dogs slaughtered for human consumption is very significant in the epidemiology of the disease. This means that dog meat processors and handlers are at risk of being exposed to rabies either from bites from dogs before slaughter or by coming in contact with infective tissue or saliva (Sabo et al., 2008; Akumbo, 2009; Aliyu et al., 2010; Isek et al., 2013). Sequencing data of genomes have increased exponentially now and the sequenced data have become hotspots for genomic and proteomic investigations (Bansal, 2005). The present study carried out the sequencing of a segment of the N gene of the amplicon of the PCR positive sample and deduced the nucleotide sequence of the N gene, its variation from other sequences from the Gen bank which may provide the molecular basis for further investigations on genomic and proteomic structures, biological characteristics, pathogenic mechanisms and other data for dog rabies.

This study revealed the presence of Nucleo protein antigen using the Direct Flourescent Antibody Test (DFAT) in 2% of the 50 dog brain samples tested. The DFAT positive sample was from a male dog. The positive sample was positive by Polymerase Chain Reaction (PCR) for Rabies virus N-gene segment. The phylogenic analysis gave an evolutionary pattern, showing the Rabies virus detected MT394532 and Africa 2 cluster are paralogous having similar structure and indicating divergence from a common ancestral nucleoprotein gene as this might have been conserved overtime. This finding has given additional insight into the molecular epidemiology of rabies virus in Nigeria, therefore providing more baseline information for future design of rabies control programs in the country.

V. Conclusions

This study reveals an in-country circulation of rabies virus strains which extends to the continent at large and possibly a cross movement within hosts of different species ranging from dogs to bats within Africa. Also, it reveals the possible exposure of the virus to both dog handlers and consumers. Rabies epidemiology dynamics in Benue State can be ascertained from this study which can serve as a direction to further rabies control programs.

List of abbreviations

DFAT: Direct fluorescent antibody test, RTPCR: reverse transcription polymerase chain reaction, RBV: Rabies Virus, N: Nucleoprotein, RNA: Ribonucleic Acid, DNA: Deoxyribonucleic Acid,

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