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Phylogenetic Analysis of Rabies Virus Isolates from Dogs in Plateau State, Nigeria

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Abstract: The entire Nucleoprotein gene of 28 rabies virus isolates from Plateau state, Nigeria were subjected to genetic typing using RT-PCR and nucleotide sequencing respectively. A phylogenetic tree of the isolates was constructed to determine their ancestry and study their relationships with other strains of the rabies virus from previous studies in Nigeria, other regions of the world and the Flurry LEP vaccine strains widely used within the country for vaccine production. The RABVs found in this study differed from the LEP fixed strains indicating that the disease is caused by stray dog related RABVs. These viruses also clustered away from another Nigerian isolate reported in a previous study in which it belonged to Africa 1b lineage. All Nigerian isolates from this study belong to Africa 2 lineage as do other isolates from the West African region, suggesting a common ancestry for all these isolates which in turn implies they may have been part of the same long term evolving rabies enzootics.

Keywords: Phylogenetic; Tree; Rabies; Virus; Plateau State; Nigeria.

1. Introduction

Rabies is an acute encephalomyelitis caused by all members of the *Lyssavirus* genus, classified within the *Rhabdoviridae* family [1, 2]. Rabies virus or genotype 1 is maintained in several independent enzootics by mammals within the Carnivore and Chiroptera orders. Epidemiologically, rabies is the most important member of the genus because it is responsible for more than 55, 000 human deaths, and millions of costly post-exposure prophylaxis annually. In Africa alone, rabies virus is estimated to cause more than 20, 000 deaths yearly [3, 4] Sporadically, other lyssaviruses, such as Lagos bat virus (LBV) and Duvenhage virus (DUVV) may be involved in causing human rabies in Africa. Mokola virus (MOKV) and a recently described Shimoni bat virus (SHIBV) have not yet been found to be involved in human disease in Africa [5].

Nucleoprotein sequencing coupled to phylogenetic re-constructions is frequently used to identify major rabies virus reservoirs, type the rabies virus variants associated with them, establish spatio-temporal relationship among rabies virus variants or lineages, trace dissemination histories and compare with other lyssaviruses to get them classified [1, 2, 5]. When this information is combined with descriptive epidemiological data, a more powerful tool is gained to institute more appropriate control measures [6].

In Nigeria, the domestic dog (*Canis familiaris*) is the major reservoir host for rabies and remains enzootic for this species. Thus, cross species transmission of rabies may occur rather frequently from dogs into other domestic animals (cats, livestock) and humans [7, 8]. There has been a reported increase in the prevalence of rabies in Nigeria [9]. Vaccine coverage is poor [10], and the disease is now an important public health problem [10]. A total of 1636 human cases were reported between 1987 and 1995 [11]. However, these numbers under-represents the actual figure because the majority of cases are not documented.

In Nigeria, dogs are traded alive across the country's geopolitical boundaries for the purpose of getting their meat for human consumption. In Plateau State, various dog markets exist where live dogs are purchased and transported to other regions of the country. Under these circumstances, rabies control in Nigeria has become more challenging, given that mass vaccination campaigns have no longer occurred since the 1980s and the basic infrastructure that made this program operative has broken down over the years [7]. In addition, there are speculations on the occurrence of vaccine-induced rabies cases in dogs, given the use of both HEP and LEP live-attenuated Flury strains in the production of vaccines.

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Limited data on the disease is available especially on the molecular epidemiology of rabies in Nigeria. The spatio-temporal epidemiology of lyssaviruses needs to be addressed through enhanced surveillance to provide information needed for development of policies that will govern control strategies of this disease. Thus, this study's main objective therefore was to determine the variants of the rabies virus circulating in this region and study their possible origin.

2. Materials and Methods

2.1. Specimens

Rabies infected brain materials collected from 2005-2006 were obtained from the archives of the National Veterinary Research Institute, Vom, Plateau state Nigeria. These were from dogs that have exposed humans or other animals to rabies as a result of dog bite. Hippocampi were tested by Sellers' staining technique [12], and mouse inoculation test [13], and stored in clean bottles at -20^{0}C until shipped on dry ice to the rabies laboratory of the Centers for Disease Control and Prevention (CDC).

2.2. RNA Extraction and cDNA Synthesis

Total RNA was extracted from infected brain tissue using TRIzol® (Invitrogen, life technologies, Faraday Avenue, USA) and chloroform. Isopropyl alcohol and 75% ethanol were used to precipitate and clean up the RNA and the product was resuspended in diethyl procarbonate (DEPC) water. The extracted RNA was solubilised by heating in a water bath at 56 ^{0}C for 10 minutes, and chilled immediately on ice. cDNA was generated following addition of either of the sense primers 001 or 550 to 10µl of the RNA followed by a brief period of denaturing at 94 ^{0}C for 1minute. The reverse transcriptase (RT) mix of final volume of 14µl made of 0.4µl each of avian myeloblastosis virus (AMV) and RNase inhibitor, 5.24µl of dNTPs, 10.71µl of RT buffer and 17.38µl DEPC water was added. The cocktail was cycled in a Peltier Thermal Cycler, (MJ Research) at 42 ^{0}C for 1 hour 30 minutes to generate the cDNA.

2.3. Polymerase Chain Reaction (PCR)

The cDNAs were subjected to PCR using the sense and anti-sense primers sets 001/1066B, 550F/304, 001/550B, 1066F/304, 001/1066B at 0.54µl and 0.68µl respectively. PCR mix of 37.3µl DNAase free water, 4.32µl Tris pH 8.3 and AmpliTaq at a final volume of 80µl was added into each tube containing the synthesized cDNA. 40 cycles of 94^{o}C for 1 minute, 94^{o}C for 30 seconds, 37^{o}C for 30 seconds and 72^{o}C for 1 minute and 30 seconds and an additional final elongation step at 72^{o}C for 7 minutes in Eppendorf ® Master cycler gradient 5331H was employed.

The resulting products were resolved in 1-1.5% agarose gel. Electrophoresis was achieved using TBE X1 buffer alongside 100 base pair DNA molecular weight marker (Roche Diagnostics GmbH, Germany), and stained with Ethidium bromide. Gels were photographed under UV light using a Polaroid camera (Fotodyne, New Berlin, Wisconsin).

Where products could not be visualized because of the low threshold of the amplicon, hemi-nested PCR was employed using 5µl of the primary PCR product, 7µl RT mix excluding AMV and RNase inhibitor. Approximately 38µl PCR mix was used with 0.7µl of primers 001 550B and 1066 304 where 001/1066B and 550F 304 where used in the primary PCR. The resulting products were purified using ExoSAP-IT® (USB Corporation, Cleveland, Ohio, USA) for post amplification purification and put through another round of sequencing PCR using the hemi nested PCR primers forward and reverse, BigDye® Terminator RR-100 (Foster City, Ca 94404, USA) for a total of 25 cycles, for each visible amplicon in Agorose gels. Approximately 20µl completed DyeDeoxy TM terminator reaction mixture was purified using CentriSep columns (Princeton Separations Inc. Adelphia, NJ) according to the manufacturers' instructions to remove excess primers, dye terminators, enzymes and salts.

2.4. Sequence Analysis

The samples were pelleted in an Eppendorf vacuum centrifuge (Vacufuge TM, Brinkman Instruments Inc., Cantiague, and Westbury, NY) .The purified products were sequenced by automated fluorescence sequencing in an Applied Biosystems 377 DNA sequencer (Foster City, USA) as described by de Mattos, *et al.* [14].

2.5. Phylogenetic Analysis

The data generated following sequencing were analyzed using the Bioedit sequence alignment editor version 7.0.1 [15] and Clustal X version 1.83 [16] softwares. Phylogenetic and molecular evolutionary analyses were conducted using the MEGA 2.1 software [17]. A hundred and eleven other sequences from the gene bank were included for comparisons with the 28 strains from this study. A phylogenetic tree was generated using the neighbour-joining method, using substitution model Kimura 2 parameter. A bootstrap value of 1000 replicates was used to evaluate reliability of the groupings and values <70 were considered insignificant.

3. Results

3.1. Phylogenetic Analysis

All the Nigerian isolates clustered within the Africa 2 group (Figure 1). The overall distance between the isolates is 0.016, equivalent to 1.6% difference or 98.4% average identity. Nigerian samples grouped in 4 clusters identified as 4 independent rabies foci designated as Nig 1, Nig 2, Nig 3 and Nig 4 (Figure 2.) the highest average difference between any groups is Nig 4 and Nig.3, with a value of 4.7% and the most closely related are Nig.1 and Nig.2, which are 99% identical. The most divergent or diverse group from this Nigerian study is the Nig.3, members of which are only 97.7% similar in average, while members of Nig.2 are the most homogeneous group with 99.6% of average identity. In the whole continent context RABV designated as Africa 1A differed more from all of those Nigerian isolates reported in this study with only 95.4% average identity. All the Nigerian isolates from this study were most closely related to Chadian enzootic dog RABV, presenting a 97.8% of similarity and most divergent from Africa 3 lineage which is associated with the yellow mongoose when compared to other African lineages. The Nigerian isolates studied here were not found to be closely related to any vaccine fixed strains .

4. Discussion

The occurrence of Nigerian isolates among the Africa 2 lineage agrees with the West African origin of isolates within this lineage. It can be easily explained that isolates from countries located in close geographical proximity such as the West African block with Nigeria, (pre-fix RD), Niger (NIG), Cameroon (CAM), Chad (CHAD), Benin Republic (BEN), Guinea (GUI), Cote d'Voire (CI), Central African Republic (CAF), Burkina Fasso (HAVA) and Mauritania (MAU) showed close relatedness. This suggests that West African isolates may have originated from a common ancestor, implying they have been part of the same expanding dog rabies enzootics which has evolved over the West African topography and human history.

Nigeria is located on the geographical coordinates $10^{0}00'$N, 8^{0}□□00′E, and shares land boundaries with Benin Republic, Niger Republic, Chad, and Cameroon. These borders are porous and informal animal trades are common between these countries facilitating introduction of many transboundary diseases including rabies [18]. There was a cluster of isolates from Chad that were sandwiched by Nigerian isolates in the Africa 2 lineage. This may imply that isolates were translocated across the borders evidenced by the indiscriminate movement of dogs within the region [19]. The 97.8% homology demonstrated by the Nigerian isolates and Chadian isolates and also the apparent monophyletic character of these two clusters suggests possible translocation of isolates between the two countries which is very likely based on the interactions, especially movement of livestock and possibly dogs between the 2 countries .

Nig. 1 rabies focus was present in both 2005 and 2006 and most of the strains in this study belong to this cluster. Nig. 4 was present only in 2005 and might have been imported from Cameroon since it clustered among Cameroon strains. In 2006, there were 3 active foci of infection namely Nig. 1, 2 and 3. All the isolates from this study however remain in the Africa 2 lineage with other West African strains.

A Nigerian isolate (U22488 8670 NGA) from previous studies [20] clustered with the Africa 1b lineage. Members of the Africa 1b lineage are usually circulating in Eastern Africa and the presence of this Nigerian isolate among the Aftrica 1b group was not expected. The marked divergence may be accounted for either because Nigerian isolates belong to different antigenic groups based on monoclonal antibody typing [21] and this might have come from a different region from which these new strains came. It may also be that this was introduced into Nigeria following human exposure from the East African region since the isolate previously reported came from a human. These however are just speculations and need to be verified.

The Flury strains of the RABV are used for production of live attenuated vaccine for domestic use within Nigeria. In the past there have been speculations of the vaccine reverting to virulence and causing disease following its administration in dogs [22, 23]. These reports were based on assumptions as the strains were never sequenced and compared with the vaccine strain. From this study however, the isolates clustered far away from the vaccine strains LEP and HEP on the phylogenetic tree. This confirms findings of other authors in studies using monoclonal antibodies which demonstrated difference between the Flury strains and circulating RABV in Nigeria [24, 25] It then implies that the cases occurred due to natural infection by street lineages of the virus maintained enzootic in the canine reservoir host.

Countries that succeed in controlling rabies can have the disease re-introduced from surrounding countries where it is endemic. It buttresses the growing need for countries to enhance their surveillance systems in order to have a good understanding of the epidemiology of diseases within their jurisdictions, and to work with their neighbors towards control of trans-boundary diseases. This study shows a possible exchange of rabies virus strains within close countries in Africa across land borders.

This study was limited to a certain geographical location of the country. There exists a high possibility that strains reported in this study may also be found in other parts of the country to which dogs are transported since dog trade is common and unregulated within Nigeria [19] and traders come from other regions to Plateau state to purchase dogs. It will be interesting and informative to study the genetics of rabies viruses from other regions in Nigeria to have a better picture of the molecular epidemiology of terrestial rabies in Nigeria and to plan effective strategies for the control of the disease.

5. Conclusion

All the isolates in this study belonged to Africa 2 lineage as do other isolates from West and Central Africa. The Nigerian isolates were closely related to the Chadian isolates, suggesting a common ancestry for RABVs circulating in Nigeria and Chad. The infecting strains differed from the LEP used for canine anti-rabies vaccine production in Nigeria.

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Fig-1. Phylogenetic tree constructed by neighbor joining method. Bootstrap values are indicated to the left of the branches

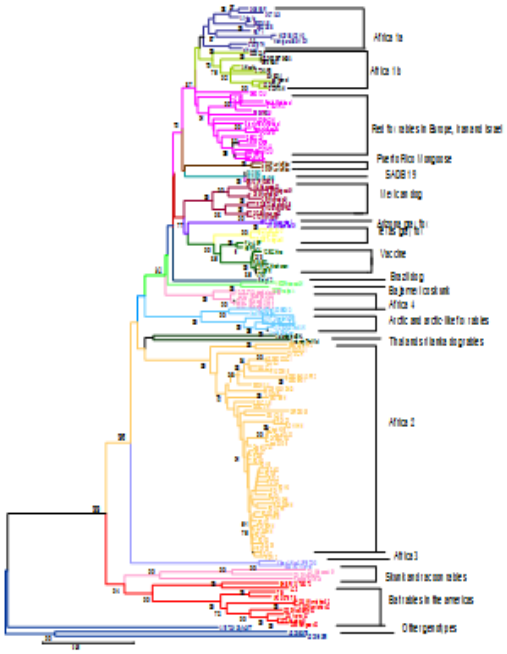

Fig-2. Phylogenetic relationships among the Africa 2 isolates and their countries of origin