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ARTICLE

Phylogenetic Evidence of the Public and Veterinary Health Threat of Dog Rabies in Nigeria

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SUMMARY

Molecular phylogenetics has been utilised to trace spillovers of rabies infection from reservoir host species to dead-end non-reservoirs including humans and other susceptible mammalian species. In this study we demonstrate phylogenetic evidence of the transmission of rabies virus infection from a reservoir host to humans and other animals. Here, we analysed phylogenetically a partial region of the nucleoprotein gene of 33 representative Nigerian rabies viruses predominantly recovered from dogs. The analyses revealed monophyletic group with 2 subclusters according to geographical locality of origin of the viruses. Furthermore, a correlation between human-dependent activities such as movement was observed. The data indicated that the virus isolates analysed here together with sequence obtained from Genbank of a human (Nigerian) formed one dominant group. This finding could be attributable to migration and translocation of animals infected with these rabies strains across the country. These molecular epidemiological data further contribute to depicting the public and veterinary health threat that rabies still poses in Nigeria and its implication to food security in this developing economy.

KEY WORDS: Nucleoprotein; Phylogenetics; Rabies virus; Nigeria

INTRODUCTION

The causative agent of rabies is a highly neurotropic virus that is generally transmitted through bites from infected animals to susceptible host species (Knobel *et al.,* 2005). Rabies belongs to the *Lyssavirus* genus of the family *Rhabdoviridae*. Seven genotypes are currently recognised within this genus based on antigenic characterization of panels of lyssaviruses (Tordo *et al*., 2006). These include classical rabies (RABV) GT1, Lagos bat virus (LBV) GT2, Mokola virus (MOKV), GT3, Duvenhage (DUVV) GT4, European bat lyssavirus types-1 and 2 (EBLV-1, GT5 & EBLV-2, GT6) and Australian bat lyssavirus (ABLV) GT7. The RABV consists of a single-stranded RNA genome of approximately 12 kb in length (Tordo, 1996a). It codes for 5 viral proteins which include the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the RNA dependent polymerase (L) (Tordo *et al.*, 1986a). The N gene of lyssaviruses is prime target for many applications including diagnosis (Dean *et al*., 1996) and the elucidation of the epidemiological and evolutionary relationships of lyssaviruses (Kissi *et al*., 1995; Sabeta *et al*., 2007).

Canine rabies in Nigeria still constitutes a significant public health threat as in most developing countries. In these countries, domestic dogs are the principal reservoir and maintenance host species responsible for the transmission of the disease to humans and other domestic animals (Fagbami *et al.,* 1981; Ogunkoya *et al*., 1984 and Obegbulum, 1994). Wildlife host species are pivotal in the epidemiology of rabies in many countries, but in this country their exact role is still poorly understood and information on wildlife rabies is scanty (Umoh and Belino, 1978).

Globally, at least 55,000 human deaths are believed to occur every year (WHO, 2005) with 99% of the deaths occurring in the developing countries of Africa and Asia. In Nigeria, an estimated 10, 000 human rabies cases occur annually (Nawathe, 1980). The first documented official report of rabies in human was in 1912 (Boulger and Hardy, 1960). Accurate statistics of both human and animal

rabies are limited primarily due to misdiagnosis, underreporting and a lack of national rabies surveillance system. All these result in underestimation of the true incidence of the disease. This study presents evidence of the domestic dog to being a perpetual threat to public and veterinary health.

MATERIALS AND METHODS

Viral isolates

Original brain tissue specimen recovered from infected animals including dogs, a cat and a goat submitted for rabies diagnosis in the Rabies Diagnostic Unit of the National Veterinary Research Institute, Vom, Nigeria and collected between 1989 and 2008 were included in the investigation. The brain tissues were kept frozen and transported as original brain samples to the Rabies Unit of the Ondersterpoort Veterinary Institute, Republic of South Africa. These samples on arrival were confirmed as rabies infected by fluorescent antibody test (FAT) ((Dean *et al*., 1996) and stored at -20C prior to molecular characterization. All virus isolates and their epidemiological information are shown in Table I.

RNA extraction, RT-PCR and viral sequencing

Total viral RNA was extracted from approximately 50-100 ng infected brain tissue using Tri reagent according to the supplier's instructions (Sigma, USA). The RNA pellets were solubilised in RNA ribonuclease-free sterile water (RNAsecure suspension solution, Ambion). Reverse transcription was performed using protocols as described previously (Markotter *et al*., 2006; Sabeta *et al*., 2007)using primer Lys001 (+) 5ˆACGCTTAACGAMAAA 3 corresponding to nucleotides 1-15 of the reference Pasteur virus strain (PV) (Tordo *et al*., 1986a) for the priming in the cDNA synthesis. A combination of these primers was used for amplification of the partial N gene Lys001 (+), 550B (-) (647-666), 304 (-) (1514-1533) (Markotter *et al*., 2006). The PCR reactions were undertaken with an ABI 9700 thermocycler with the following thermocycling conditions, initial denaturation at 94°C for 1 min, followed by 40 cycles of 94°C for 30 s, 37°C for 30 s, 72°C for 90 s and a final extension at 72°C for 7 minutes. The amplicons were visualized and evaluated under UV light after electrophoresis in 1% ethidium bromide stained agarose gels. The amplicons were gel purified using Wizard PCR purification system (Promega, Madison, WI, USA) according to supplier's protocol prior to sequencing.

Nucleotide sequencing and Phylogenetic analysis

The purified amplicons were cycle sequenced using ABI PRISM Big Dye Terminator v3.1 sequencing kit, (Applied Biosystem, USA) according to supplier's protocol. This was performed in a thermocycler (Gene Amp PCR System 9700, Applied Biosystem); with the same primers as in the amplification step with the following cycling conditions 96°C for 1 min, 50°C for 50s, 60°C for 4 min for 25 cycles. The sequencing products were further analysed using ABI 3100 DNA analyzer.

Nucleotide sequences generated were manually edited and a consensus sequence obtained after the forward and reverse sequences were aligned using the Bio-Edit program (Hall, 1999) and multiple alignments undertaken with the Clustal X version 1.82 package (Thompson *et al*., 1997). Phylogenetic analysis was performed with MEGA 4.1 version (Tamura *et al*., 2007) and a neighbor-joining tree was constructed as described previously (Saitou and Nei, 1987). Bootstrap values were estimated based on 1000 replicates and values above 70% were considered significant (Hillis and Bull, 1993). The TREEVIEW Program (Page, 1996) was used to illustrate the graphical output. Nucleotide sequences obtained in this investigation were submitted to the GenBank and the accession numbers are shown in Table I

Table I: Rabies viruses used in the phylogenetic study

Virus #	Lab #	Locality of origin	Species of origin	Year of isolation	Reference	Lat-long	Genbank #
1.	RD172/06	Kastina	Dog	2006	This study	12°59' - 7°36	EU888716
2.	RD94/07	Foron	Dog	2007	This study	9°41' - 8°56'	EU888687
3.	RD138/06	Stray	Dog	2006	This study	Unknown	EU888703
4.	RD33/06	Bukuru	Dog	2006	This study	9°47' - 8°51	EU888657
5.	RD188/06	Quaanpan	Dog	2006	This study	9°26' - 8°54'	EU888726
6.	RD159/06	Jos	Dog	2006	This study	9°54' - 8°53'	EU888707
7.	RD16/07	Fadakarshe	Dog	2007	This study	9°47' 8°17'	EU888650
8.	RD47/07	Jos	Cat	2007	This study	9°54' 8°53'	EU888662
9.	RD17/07	Jos	Dog	2007	This study	9°54' - 8°53'	EU888651
10.	RD58/06	Lagos	Dog	2006	This study	6°29' - 3°21'	EU888665
11.	RD14/07	BarkiLadi	Dog	2007	This study	9°32' - 8°53'	EU888649
12.	RD38/05	Riyom	Dog	2005	This study	9°37' - 8°45'	EU888658
13.	RD158/06	Jos	Dog	2006	This study	9°54' - 8°53'	FJ435701
14.	RD166/06	Mangu	Dog	2006	This study	9°18'09' 9°11'34'	EU888713
15.	RD79/07	Mangu	Dog	2007	This study	9°18'09' 9°11'34'	EU888681
16.	RD181/06	Bokkos	Dog	2006	This study	9°18' 9°00'	EU888721
17.	RD124/07	Mangu	Dog	2007	This study	9°18'09' - 9°11'34'	EU888697
18.	RD122/07	Mangu	Dog	2007	This study	9°18'09' - 9°11'34'	EU888695
19.	RD2/08	Mangu	Dog	2008	This study	9°18'09' - 9°11'34'	EU888643
20.	RD177/06	Bauchi	Dog	2006	This study	10°30' - 9°50'	FJ435700
21.	RD60/05	Mangu	Dog	2005	This study	9°18'09' - 9°11'34'	EU888666
22.	13136/89	Zaria	Dog	1989	This study	11°04' - 7°42'	EU888729
23.	RD173/06	Jos	Dog	2006	This study	9°54' 8°53'	EU888717
24.	RD123/07	Markurdi	Dog	2007	This study	7°43' 8°32'	EU888696
25.	RD48/07	Lafia	Dog	2007	This study	9°28' 8°53'	EU888663
26.	RD80/07	Jos	Dog	2007	This study	9°54' - 8°53'	EU888682
27.	RD168/06	Bauchi	Dog	2006	This study	10°30' - 9°50'	EU888714
28.	RD71/07	Kagoro	Dog	2007	This study	9°34'60' 8°30'	EU888675
29.	RD30/07	Jos	Dog	2007	This study	9°54' - 8°53'	EU888656
30.	RD10/07	Ibadan	Dog	2007	This study	7°22' 3°53'	EU888647
31	RD11/07	Ibadan	Dog	2007	This study	7°22' - 3°53'	EU888648
32.	RD44/07	Stray	Dog	2007	This study	Unknown	EU888660
33.	RD99/06	Abeokuta	Goat	2006	This study	7°09' - 3°20'	EU888689
34*	RV629	Nigeria	Human	1996	Johnson *et al.,* 2002	Unknown	AY103008

Key Sequence obtained from the Genbank*

RESULTS

The phylogenetic analyses were performed after nucleotide sequence determination of amplified products with the expected band size of 606 bp using thirty-three representative viruses from a panel of 100 viruses. In this analysis was included a single human sequence from the Genbank. These data revealed that the viruses included in the analyses were very closely related with at least 99% sequence identity based on Kimura-2 parameter model using the MEGA programme] (Tamura *et al*., 2007).

All the viruses were found to belong to the African 2 dog lineage (Kissi *et al.,* 1995) the main variant found to be circulating in dogs in the west African sub-region. Two major subgroups supported by bootstrap values of (100 % and 75%, cluster 1 and 2) were identified despite the close relatedness of the viruses. The sequence of a virus retrieved from the Genbank (Johnson *et al.*,2002) and recovered from woman originating from Nigeria who succumbed to rabies infection in Britain clustered with viruses from Ibadan (cluster 2). On average, the rabies viruses showed 13.2 % sequence divergence from the Pasteur virus (PV).

Phylogenetic data from the NJ tree (fig. 1) was validated with other algorithms including maximum parsimony (MP) and maximum likelihood (ML) and produced trees with similar topologies.

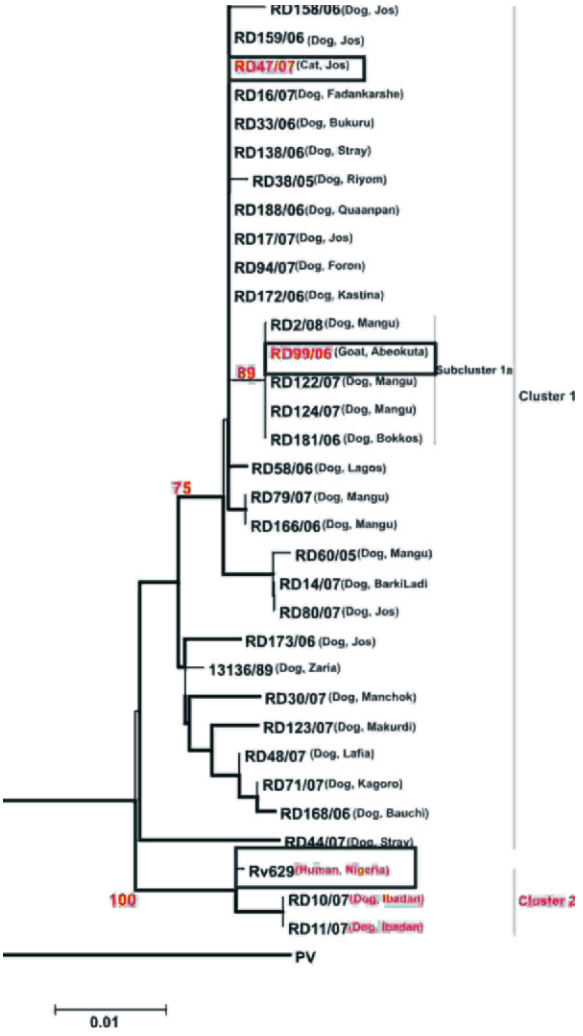

Figure 1: Neighbour-joining phylogenetic tree of 33 rabies viruses from Nigeria. Bootstrap support values were obtained from 1000 replicates and the scale bar represents nucleotide substitution per site. PV was used to root the tree and the vertical branches are set for clarity

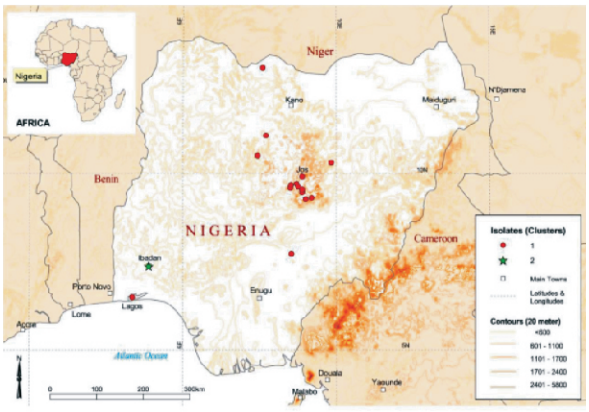

Figure 2: A map of Nigeria illustrating the geographic distribution of the representatives of the virus isolates in the clusters obtained in the study (Geographic_information_system)

DISCUSSION

During the early 1900s human rabies was first officially documented (Boulger and Hardy, 1960) in Bonny and Eket, the southern coastal parts of Nigeria but now has spread virtually to all geographical zones of the country (Ogunkoya *et al*., 1984; Ezekoli and Umoh, 1987). This study has confirmed that the use of molecular technique such as nucleotide sequencing and phylogenetics can be useful in tracing the origin of infectious pathogens such as rabies.

The results presented here indicate that the rabies viruses in the study panel were very closely related with over 99% nucleotide similarity. These viruses grouped into one dominant genetic variant (cluster 1) which further support the notion of a single progenitor for the West African rabies viruses (Kissi *et al*., 1995). The sequence obtained from the Genbank recovered from a Nigerian woman who died of rabies in the UK was very similar to those obtained from infected dogs from Ibadan in the south west of the country suggesting that this woman was probably exposed in this locality before her travel to Europe.

This finding further emphasise rabies as a travel disease and secondly the utility of phylogenetics in tracing the transmission patterns of infectious diseases. The domestic dog evidently remains the prime maintenance host species and largely responsible for transmitting rabies to humans and other domestic animal as described in some earlier studies (Fagbami *et al*., 1981). In the process, spill-over events take place to include animals such goat and cat. Isolation of a rabies virus strain from a goat from the south west of Nigeria which clustered with the subcluster 1a group from the north could likely be linked to human activities such as seasonal migration of pastoralists from north to south as well as movement of animals for commercial purposes. The identification of subcluster 1a with high bootstrap value of 89% could suggest to a local rabies outbreak in this locality where there is high concentration of dogs and activities causing local translocation of these dogs due to cultural beliefs and practices of the indigenous people (Oboegbulem, 1994).

In conclusion, findings from this study have demonstrated why we must control rabies in dogs in this country. This is to protect humans and other susceptible animals especially those

which form part of food security and a source of income to many rural households. There is also the need to increase vaccination coverage of dogs so as to break the cycle of infection in these species. It is further and urgently recommended that steps have to be taken towards controlling strays and more importantly raise awareness regarding the need to seek prompt medical attention when exposed.

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