www.ajbrui.net

Afr. J. Biomed. Res. Vol.17 (May, 2014); 101- 107

Full Length Research Paper

Efficacy of a Direct Rapid Immunohistochemical Test (DRIT) For Rabies Detection in Nigeria

Ehimiyein A.M[1]., Niezgoda M[2]., Orciari L[2]., Osinubi M.O.V[2]., Ehimiyein I.O[3]., Adawa D.A.Y[4]., Abdullahi S.U[1]., Ogunkoya A.B[1]., Rupprecht C.E[5,6].

[1]*Department of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria*
[2]*Centers for Disease Control and Prevention, Rabies, Atlanta, Georgia, USA*
[3]*Gimaf Veterinary Consult, Kaduna, Nigeria*
[4]*Ahmadu Bello University, Veterinary Teaching Hospital, Zaria*
[5]*The Global Alliance for Rabies Control, Manhattan KS, USA,*
[6]*Ross University School of Veterinary Medicine, Basseterre, St. Kitts, West Indies*

ABSTRACT

Rabies is an acute, infectious disease mostly transmitted through bites from an infected animal. Dogs majorly transmit rabies to humans. Human rabies is not curable once clinical signs commence, but can be prevented. The aim of this study was to find an appropriate diagnostic test suitable for use in Nigeria and other developing countries with infrastructural challenges. Thirty dog brain samples collected from dog markets in Kaduna State were analysed using four tests direct fluorescent antibody test (DFA), mouse inoculation test (MIT), Seller's staining test, the reverse transcriptase-polymerase chain reaction test (RT-PCR) and the direct rapid immunohistochemistry test (dRIT). A total of 15 (50%) of the samples tested positive using DFA, dRIT, RT-PCR and MIT, while the remaining 15 (50%) were negative. The results obtained using these four different tests showed concordance between those that were positive and negative. There was no statistical difference ($P > 0.05$) among the four tests. An appropriate diagnostic test must be prompt, cheap, sensitive, field-based and reliable. The direct rapid immunohistochemistry test is a new diagnostic test established by the Centers for Disease Control and prevention, Atlanta, Georgia, USA. It does not require sophisticated equipment so it can be used on the field especially in the rural areas where most of the rabies exposures occur. It is also as sensitive as the DFA and the result can be obtained within an hour. In Nigeria and most developing countries, prompt diagnosis play a major role in the prevention and control of rabies.

Key words: rabies diagnosis, direct rapid immunohistochemistry test, mouse inoculation test, RT-PCR

INTRODUCTION

Rabies is endemic in Nigeria (Fagbami *et al.*, 1981; Ogunkoya *et al.*, 2007). It is a disease of public health importance and is widespread (Nawathe, 1980; Ezebuiro *et al.*, 1980; Ogunkoya, 2007). Dogs were confirmed as the reservoirs and vectors of human rabies, accounting for 94% of the cases (Ezebuiro *et al.*, 1980; Nel and Rupprecht, 2007). At least, 10,000 persons have been

*Corresponding author:
E-mail: ajokeeo@gmail.com

Date Received: February, 2014
Date Accepted:, April, 2014

Abstracted by:
Bioline International, African Journals online (AJOL), Index Copernicus, African Index Medicus (WHO), Excerpta medica (EMBASE), CAB Abstracts, SCOPUS, Global Health Abstracts, Asian Science Index, Index Veterinarius

estimated to be exposed to rabies annually in Nigeria, though underestimated (Nawathe, 1980), because there is no surveillance and reporting system of the disease. In Nigeria and many other developing countries in the tropics, at least 10% of brain samples received at the laboratories for rabies diagnosis are decomposed because of lack of storage facilities, proximity of the exposure sites to the laboratories, inadequate means of transportation, prompt diagnostic tests that are field based (Barat, 1996; Elsa and Ogunkoya, 1996; Durr *et al.*, 2008), all these factors lead to the misdiagnosis of most cases. Diagnosis is fundamental in the surveillance and control of every major infectious disease. The aim of this study is to identify a diagnostic test that is suitable for use in Nigeria and most developing countries where there are lots of constraints. The absence of a rapid confirmatory test can result in the inappropriate management of animal bite, unnecessary administration of post exposure prophylaxis (PEP) and delays in rabies post exposure prophylaxis (PEP) leading to human deaths.

The procedure for the DFA was first described by Goldwasser and Kissling (1958). When reacted with fluorescein conjugated rabies immune serum and illuminated with ultra-violet light, rabies antigen in infected tissues appear as brightly-coloured apple green or greenish yellow, round to oval intracellular accumulations. The antibody primarily responsible for staining in the DFA test is that directed against the nucleo-capsid antigen of the virus. This antigen is believed to be the same as the classical negri body, observed on histological examination of the CNS tissue (Matsumoto, 1962). In addition to the larger stained bodies, infected tissues may contain smaller collections of antigen, which appear as granular or dust-like fluorescent particles or thread-like material (Fekadu and Smith, 1985; Chhabra *et al.*, 2007). Immunofluorescent rabies antigen may be detected in all parts of the CNS of infected animals, but because of the frequently uneven distribution of antigen, the most reliable diagnosis is made from tests which include examination of the medulla (brain stem), cerebellum and the hippocampus (Koprowski, 1973). Lately, antigen could be detected in the skin, the salivary gland and other tissues of an infected animal (Beran, 1984). The DFA which is the gold standard in rabies diagnosis (Goldwasser and Kissling, 1958; Dean *et al.*, 1996), has shown 100% sensitivity (Chhabra *et al.* 2007) but it is limited by the cost of acquiring and maintaining a fluorescent microscope.

As far back as 1940, Casals showed that the susceptibility of mice to the infection of rabies virus was related to their age; the younger, the more susceptible they are. The major problem with the MIT is the length of incubation period which mostly is unsuitable, when an exposure has occurred that needs rapid diagnosis and confirmation. This test is expensive. Mouse Innoculation Test has the ability to detect small quantities of virus in a sample. Apart from that, it has its applicability to partially decomposed specimens (Chahhbra *et al.*, 2007). It is practical, sensitive, reliable and technically non-demanding. Its main drawback, besides the inherent environmental and ethical issues with the use of live animals in the laboratory, is the typical 7-21 days waiting before the result is obtained. MIT also requires a well-maintained animal house for continuous supply of mice, a large number of mice per sample and is labour intensive (Chahhbra *et al.*, 2007).

Seller's Staining test has been found to be simple and economical. The procedure was described by Seller (1927). The negri body development is related to the length of time an animal lives after it had developed the clinical signs of rabies. Although the observation of negri bodies confirms the diagnosis of rabies, the inclusions are found in only 75-80% of specimens found positive by other more sensitive diagnostic methods. In some animal species, the sensitivity of this diagnostic method may even drop to 50% (Baer *et al*., 1968). The observation of non-specific inclusions in some animals, particularly cats and rodents, may also result in a number of false-positive rabies diagnoses in these species (Fekadu and Smith, 1985). In view of the low sensitivity and possibility of false-positive with Seller's test, its use cannot be relied upon singularly. It should be confirmed by the MIT (Goldwasser *et al*., 1958; Fekadu and Smith, 1985) or any other more sensitive method.

RT-PCR, which is a cumbersome assay, has been used for detection of rabies virus and rabies–related viruses (Heaton *et al*., 1997). Studies have shown that the nested and semi-nested RT-PCR assays were more sensitive than the fluorescent antibody (FA) test. The rabies virus N-protein gene, which is highly conserved, has been used as the target for RT-PCR analysis in several molecular epidemiological studies (De Mattos *et al*., 1999, 2000; Ito *et al*., 1999; David *et al*., 2002). The RT-PCR has been found to be effective in both ante-mortem and post-mortem cases as well as in archival samples of over 12 years but its main setback is the availability of the reagents, equipment and the expertise in most developing countries.

A direct rapid immunohistochemical test (dRIT) has been developed at the Centers for Disease Control and Prevention (CDC), USA, to detect rabies virus using an immunoperoxidase technique (Niezgoda and Rupprecht, 2006). It requires only light microscopy, which is 10 times less expensive than a fluorescence microscope.

The reduced cost can make rabies diagnosis available in cities and rural areas which will eventually contribute to rabies control in Nigeria. The result can be read in less than one hour and it detects all the Lyssa viruses. The use of dRIT in diagnosis will reduce the number of human deaths due to rabies because of its promptness. The test is simple, field-based, requires no specialized equipment or infrastructure, and can be successfully performed on samples preserved under variable conditions (Lembo *et al.,* 2006).

MATERIALS AND METHODS

Thirty dog brain samples were collected from dog markets in Kaduna State and kept in individual labeled bottles. Five visits were made to the dog markets on different days. All the samples were subjected to DFA, dRIT, MIT and RT-PCR.

Direct fluorescence antibody test: A smear or touch impression was made on a three welled slides, which were prepared from the cut surface of the cross-section that exposed multiple ascending and descending nerve tracts. Excess sample materials were blotted from the slides using paper towels. The control slides (positive and negative) were prepared in the same manner as the test sample slides. The wells were stained using 5100 Millipore DFA reagent (consisting of 3 monoclonal rabies antibodies), 5102 Millipore Light Diagnostic Negative Control and Fuji Rebio Diagnostic (consisting of 2 monoclonal rabies antibodies). Each sample brain area was of a size sufficient for examination of 40 separate views or microscopic fields at a magnification of approximately 200 x (i.e., making an impression in a 15 mm diameter well or a smear of 10 mm^2).

All the impression smears made on glass slides were air-dried completely at room temperature in a safety biocontainment hood for about 15-30 minutes. When the tissue no longer appeared wet and glistening, slides from an individual test animal were combined in one container for acetone fixation. Positive and negative control slides were put in different separate containers of acetone and fixed at the same time as test slides to control the effect of acetone fixation on test performance. The prepared slides were fixed for a minimum of 1h at -20^{o}C in an explosion proof freezer. Following the fixation of the control and test impression smear slides using acetone, the slides were air-dried at room temperature, and each anti-rabies conjugate was added by dispensing through a syringe fitted with a 0.45 µm low-protein binding filter while ensuring that the first 3 drops of the conjugate were discarded. The slides were arranged so that the positive control slide was the first to receive the conjugate (to control for any unexpected removal of antibody by filtration) and the negative control slide was the last to receive the conjugate (to control for adequate removal of excess fluorescein by the filter throughout conjugate application). The slides were then incubated for 30 minutes at 37^{o}C in a high humidity chamber. After staining, excess conjugate was drained from the slides onto adsorbent paper and the slides were given a brief rinse under a stream of PBS, followed by immersing and soaking in PBS for 3 to 5 minutes (control slides and slides from each test animal were in a separate rinse container–coplin jars). The PBS was discarded and replaced by fresh PBS in which the slides were soaked for another 3 to 5 minutes.

The slides were carefully blotted to remove excess liquid, and then briefly air dried before mounting. The mounting was done by dropping a small amount of 20% glycerol-tris buffered saline pH 9.0 onto the cover slips arranged on absorbent paper. The stained slides were inverted over the cover slips. Excess mountant was wicked into the absorbent paper by applying light pressure to the back of the slides. A small volume syringe fitted with a 0.45 µm filter and small bore rubber tubing was used to dispense the mountant in small droplets onto the cover slips. The slides were read within 2 hours following mounting. Antigen distribution and staining intensities of +4 to +1 were considered positive. The positively stained slides were then preserved for reference in a refrigerator.

Direct rapid immunohistochemistry test: Routine touch impressions of the suspected CNS tissues from the suckling mice that died after challenge were made on labeled glass microscope slides. Similarly, touch impressions of standard positive and negative controls were made. All the prepared impression slides were air-dried for 5 minutes at room temperature. The slides were immersed in 10% buffered formalin at room temperature for 10 minutes after which they were removed and dip-rinsed several times to wash off any excess fixative in Tween Phosphate Buffered Saline (TPBS). The slides were thereafter immersed in 3% hydrogen peroxide for 10 minutes. Excess hydrogen peroxide was removed by dip-rinsing the slides in TPBS. The slides were then transferred for another rinse after which, excess buffer was shaken off, and blotted from the slide edges surrounding the impression. The process was carried out one at a time leaving the remaining slides under humidity chamber (using the plastic top of a 96-well plate, on a moistened paper towel on the laboratory bench top) at room temperature with the primary antibody-biotinylated anti-rabies MAb for 10 minutes.

Primary antibody was added by drop to cover the impression.

After incubation, excess conjugate was shaken off. The slides were dip-rinsed in TPBS, after which excess buffer was shaken off and blotted from slide edges surrounding the impression. The slides were then incubated with streptavidin-peroxidase complex, in a humidity chamber at room temperature for 10 minutes. After incubation, excess is shaken off. The slides were then dip-rinsed with TPBS, and excess buffer shaken off and blotted from slide edges surrounding the impression. The slides were subsequently incubated with peroxidase substrate (i.e. AEC drop to cover the impression in a humidity chamber at room temperature for 10 minutes). After incubation, excess substrate was shaken off, and the slides were dip-rinsed in deionized distilled water to ensure removal of excess stain. The slides were then transferred to fresh distilled water and rinsed. The slides were mounted with water-solution mounting medium and covered with cover-slip. When multiple slides were stained, they were left in the deionized distilled water rinse before applying the cover-slips. The slides were viewed by light microscope, using a 20 x objective to scan the field, and a 40 x objective for higher power inspection and the results were recorded.

Mouse inoculation test: Mouse inoculation test was carried out to isolate rabies viruses. All test tubes used for each specimen were labeled with the specimen number corresponding to each brain sample number. Proper protective clothing (surgical gloves-doubled: laboratory coats) were used and all the procedures for virus preparation were carried out under a negative-pressure safety biocontainment hood. A total of 30 samples were prepared according to the mouse inoculation test (MIT) techniques.

At the animal facility where the mice were housed, the work bench surface was covered with paper towels to avoid contamination, and proper protective clothing including laboratory coat, double gloves, caps, and shoe cover were worn with additional nose mask and face shield. The cages were labeled with the appropriate sample numbers. experiment and protocol numbers, type/strain of tissue inoculated, volume of inoculum, route of inoculation, age of mice, and the date of inoculation were meticulously recorded. The syringe was held perpendicularly, with needle up and the tube and gauze still in place, and syringe was tapped to remove any air bubbles by rising to the level of the base of needle which was then expelled from the syringe into the gauze placed in the tube. Care was taken not to expel the air bubbles into the air thereby avoiding any aerosol accidental infection. Using animal forceps, each suckling mouse of about 3-4 days old was removed from the cage and held stomach down, and inoculated intra-cerebrally by inserting a needle through the cranium at a point halfway between the ear and eye, just off the midline of the skull. Twenty microliter of the specimen was injected into each mouse. This was repeated for all the mice using each sample. An average of 5 suckling mice per mother was used for each sample. Positive and negative controls were also included. The mice were checked to ensure that none died of trauma from handling. The nursing mice were well supplied with food and water and the inoculated suckling mice were observed daily for 30 days for specific signs of rabies which included ruffling of the fur, humping, trembling, lack of coordination of the hind legs, ascending paralysis, prostration, slow movements, lack of responsiveness to stimuli and lack of milk in the stomach.

Reverse transcriptase-polymerase chain reaction (RT-PCR): The reverse-transcriptase polymerase chain reaction (RT-PCR) was performed on all the thirty samples shipped to the Rabies section of the CDC, Atlanta, Georgia, USA. Total RNA was extracted from the infected dog brain samples using Trizol ™ (Invitrogen, San Diego, CA) according to the recommendations of the manufacturer. The RT-PCR was performed as described by Kuzmin *et al.* (2003), using primers for amplifying the whole N-gene. All positive PCR products were purified and subjected to direct sequencing using ABI PRISM 377 DNA sequencer (Applied Biosystems Foster city, CA, USA). Primary assembly, alignment, consensus generation and DNA translation were performed using BioEdit (Hall, 1999). Neighbour joining (NJ) phylogenetic analysis was performed using Kimura-2-parameter using MEGA software version 4.0.2 (Tamura *et al.*, 2007). Bootstrap support was estimated for 1000 replicates. Bootstrap values 70 and more were considered significant.

RESULTS

A total of 15 (50%) of the samples originating from dog markets in Kaduna State tested positive using dRIT, DFA, MIT and, RT-PCR while the remaining 15 (50%) were negative to the same tests carried out. The results obtained using these four different tests showed concordance between those that were positive and that were negative. There was no statistical difference ($P > 0.05$) among the four tests. From this study, analysis of the phylogenetic relationships performed on the complete nucleoprotein (N) coding gene, established them to belong to the genotype 1 comprising typical dog

rabies virus with their geographical location pointing to West Africa and Africa 2 lineage (Kissi *et al.*, 1995; Johnson *et al.*, 2004; Johnson *et al.*, 2004; Lembo *et al.*, 2006 and Talbi *et al.*, 2009).

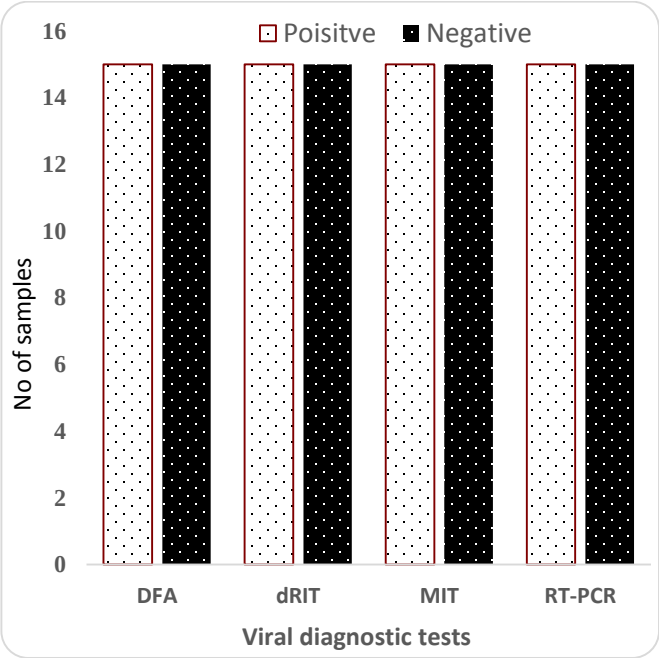

Fig. 1:
Results of the four rabies diagnostic tests (dRIT, DFA, RT-PCR and MIT) performed on dog brain samples obtained from dog markets in Kaduna State .

DISCUSSION

Rabies is an endemic disease of public health importance in Nigeria. There is a rudimentary reporting system of rabies exposures in Nigeria which may be as a result of poor surveillance system, lack of a rapid, cheap, accurate and field-based diagnostic rabies tests. This study agrees with the findings of Garba *et al.*, 2010 that rabies is endemic in some dog markets in Nigeria. Rabies was confirmed from fresh dog brain samples collected from apparently healthy and sick dogs from dog markets in Kaduna State using dRIT, DFA and MIT. All the positive samples were subjected to RT-PCR. Fifty percent of the samples obtained from some dog markets in the State were positive, the high risk of human exposure especially from unrecognized apparently healthy rabies carrier dogs.

In this study, there was equal number of positives and negatives for both DFA and dRIT. Mouse inoculation test also proved to be an accurate test for rabies, but it is capital intensive, involving the use of an animal facility and human labour. The Reverse-transcriptase polymerase chain reaction is a complicated procedure that required expertise and technical knowledge. The cost and unavailability of the reagents was a limiting factor especially in developing countries including Nigeria.

The dRIT is a new and simple diagnostic test which requires only the use of a light microscope following its application and the result can be obtained within 1h (Lembo *et al.*, 2006). It has been used under field conditions (Abraham, 2014) and has been found to yield the same result when carried in the laboratory (Durr *et al.*, 2008). The test only requires that the reagents to be kept in a refrigerator. Although the DFA test is still the gold standard test frequently used for rabies diagnosis, the dRIT, has proven to be equally efficacious and reliable (Damayanti *et al.*, 2014) with the added advantage of being cheap and prompt (Abraham *et al.*, 2013) which will serve as an effective tool for rabies diagnosis in Africa where there is limited surveillance on rabies (Nel *et al.*, 2005; Markotter *et al.*, 2006). The light microscope used in dRIT is 10 times cheaper than the fluorescent microscope used in the DFA test. Thus, the dRIT requires the use of less expensive and sophisticated equipment. This technique will be of great advantage to our environment. It has a significant potential for diagnosing rabies in low-income countries, and under field conditions where rabies diagnosis is unavailable at the moment. This new test also opens up a great potential to train technical staff and to establish rabies diagnosis without delay in low-income developing countries. The dRIT might be considered as an appropriate test for rabies diagnosis in developing countries.

REFERENCES

Abraham, A.M. (2014). Comparison of direct, Rapid Immunohistochemical Test Performance with Direct Fluorescent Antibody Test for rabies diagnosis in Ethiopia. Open Journal of Biochemistry 1 (1): 49-55.

Abraham, S.S., Anthony, M.U., Viswanathan, H. (2013). Evaluation of a direct rapid immunohistochemical test (dRIT) as a diagnostic tool for rabies in animals. Indian Journal of Vet. Pathol. 37 (1): 68-69.

Baer, G.M., Shantha, T.R., Bourne, G.H. (1968). The pathogenesis of street rabies virus in rats. Bulletin of the World Health Organization, 38: 119-121.

Barrat, J. (1996). Simple technique for the collection and shipment of brain specimens for rabies diagnosis. *In:* Meslin, F.X., Kaplan, M.M., Koprowski, H. (Eds.). Laboratory Techniques in Rabies, Geneva. World Health Organisation, 425- 432.

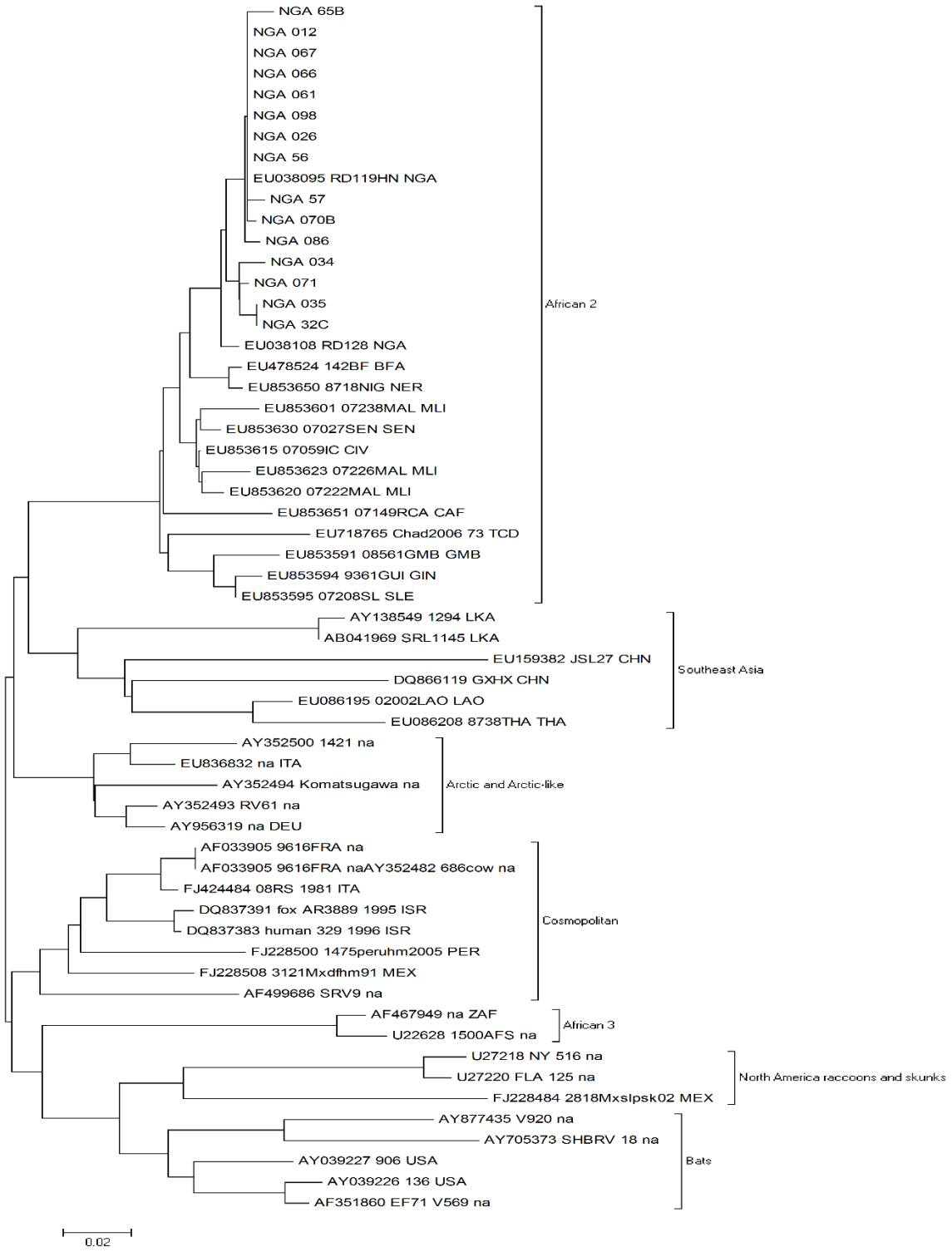

Plate 1:
Phylogeny of Nigerian rabies virus strains isolated from dog markets in Kaduna State

Beran, G.W. (1984). Rabies and infections by rabies related viruses. *In*: Beran, G.W. (Ed.). *Hand book of Zoonoses*, Viral, CRC Press. Boca Raton Florida, 307- 357.

Chhabra, M., Mittal, V., Jaiswal, R., Malik, S., Gupta, M., Lal. S. (2007). Development and Evaluation of an In vitro isolation of street rabies virus in mouse neuroblastoma cells as Compared to conventional tests used for diagnosis of rabies. Indian J. of Med. Microbiol. 25: 263-266.

Damayanti, R., Rahmadani, I., Fitria, Y. (2014). Antigen detection of rabies virus in brain smear using direct Rapid Immunohistochemistry Test. Indonesia J. of Ani. and Vet. Sci. 19(1).

David, D., Yakobson, B., Rotenberg, D. (2002). Rabies virus detection by RT-PCR in decomposed naturally infected brains. Vet. Microbiol. 87, 111-118.

Dean, D.J., Abelseth, M.K., Atanasiu, P. (1996). Routine Laboratory procedures: The fluorescent Antibody Test. *In:* Meslin, F.X., Kaplan, M.M., Koprowski. H. (Ed.). Laboratory Techniques in Rabies, 4th edition, Geneva, World Health Organization. 88-95.

De Mattos, C.C., De Mattos, C.A., Loza-Rubio, E., Aguilar-Setien, A., Orciari, L.A., Smith J.S. (1999). Molecular characterization of rabies virus isolates from Mexico; implications for transmission dynamics and human risk. American J. Trop Med and Hyg. 61, 587-597.

De Mattos, C.A., Favi, M., Yung, V., Pavietic, C., De Mattos, C.C. (2000). Bat rabies *In* urban centres in Chile. J. Wildlife Dis. 36, 231 – 240.

Dürr, S., Naïssengar, S., Mindekem, R., Diguimbye, C., Niezgoda, M., Kuzmin, I., Rupprecht, C.E., Zinsstag, J. (2008). Rabies diagnosis for developing countries. PLoS Negl. Trop. Dis. 26, e206. doi: 10. 1371/journal.pntd.0000206.

Elsa, T.A., Ogunkoya, A.B. (1996). Survival of rabies virus in 50% buffered glycerol under different temperatures. Nigeria Journal of Basic and Applied Sciences, 5(2), 15-18.

Ezebuiro, E.O., Fagbami, A.H., Anosa, V.O. (1980). A survey of rabies in man and animals in Nigeria. Report of a Commissioned Study of the Federal Livestock Department of Nigeria, 142.

Fagbami, A.H., Anosa, V.O., Ezebuiro, E.O. (1981). Hospital records of human rabies and antirabies prophylaxis in Nigeria 1969-1978. Trans. of the Roy. Soc. of Trop. Med. and Hyg. 95, 872-876.

Fekadu, M., Smith J.S. (1985). Laboratory diagnosis of rabies. *In:* Winkler, W.G. (Ed.). Rabies Concepts for Medical Professionals. 37.

Garba, A., Oboegbulem, S.I., Junaidu, A.U., Magaji, A.A., Umoh, J.U., Ahmed, A., Danbirni, S., Chiko, K.L., Habu, A.K. Masdooq, A.A. (2010). Rabies virus antigen in the brains of apparently healthy slaughtered dogs in Sokoto and Katsina States, Nigeria. Nig. J. of Parasitol. 31:123-125.

Goldwasser, R.A., Kissling, R.E. (1958). Fluorescent and staining of street and fixed rabies virus antigens. Proc. of the Soc. for Exper. Med. 98, 219-223.

Hall, T.A. (1999.) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, version Nucl. Acids. Symp. Ser. 41: 95-98.

Heaton, P.R., Johnstone, P., McElhinney, I.M., Cowley, R.O., Sullivan, E., Whitby, J.E. (1997). Heminested PCR assay for detection of six genotypes of rabies and rabies-related virus. J. Clinical Microbiol. 33(11), 2762-2766.

Ito, N., Sugiyama, M., Oraveerakul, K., Piyaviriyakul, P., Lumlertdacha, B., Aria, Y.T., Tamura, Y., Mori, Y., Minamoto, N. (1999). Molecular epidemiology of rabies in Thailand. Microbiol. and Immunol. 43, 551-559.

Johnson, N., McElhinney, L.M., Ali, Y.H., Saeed, I.K., Fooks, A.R. (2004). Molecular epidemiology of canid rabies in Sudan: Evidence for a common origin of rabies with Ethiopia. Vir. Res. 104(2), 201-205.

Kissi, B., Tordo, N., Bourhy, H. (1995). Genetic polymorphism in the rabies virus nucleoprotein gene. Virus. 209, 526-537.

Kuzmin, I.V., Orciari, L.A., Arai, Y.T., Smith, J.S., Hanlon, C.A., Kameoka, Y., Rupprecht, C.E. (2003). Bat lyssaviruses (Aravan and Khujand) from Central Asia: phylogenetic relationships according to N, P and G gene sequences. Virus. Res. 97: 65-79.

Koprowski, H. (1973). The mouse inoculation test. In Laboratory techniques in rabies, 3rd (Ed.). *In:* Kaplan, M.M., Koprowski, H. (Eds.). World Health Organization (WHO) Monograph series No.23, WHO, Geneva. 85-93.

Lembo, T., Niezgoda, M., Velasco-Villa, A., Cleavland, S., Ernest, E., Rupprecht, C.E. (2006). Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. Emerg. Infect. Dis. 12, 310-313.

Markotter, W., Randles, J., Rupprecht, C.E., Sabeta, C.T., Taylor, P.J., Wandeler, A., Nel, L. (2006). Lagos Bat Virus, South Africa. Emerg. Infect. Dis. 12, 504-506.

Matsumoto, S. (1962). Election microscopy of nerve cells infected with street rabies. Vir. 17, 198-201.

Nawathe, D.R. (1980). Rabies control in Nigeria. Bulletin d'Office International des Epizooties. 92, 129-139.

Nel, L. (2005). Vaccines for lyssaviruses other than rabies. Expert Rev. Vaccines. 4, 533-40.

Nel, L.H., Rupprecht, C. E. (2007). Emergence of lyssaviruses in old world: The case of Africa, 315, 161-193.

Niezgoda, M., Rupprecht, C.E. (2006). Standard operating procedure for the direct rapid immunohistochemistry test for the detection of rabies virus antigen. Manual: National Laboratory Training Network Course. Atlanta: US Department of Health and Human Services,Centres for Disease Control and Prevention. 1-16.

Ogunkoya, A.B. (2007). Clinical rabies in animal and man. In: Rabies-Basic Concepts,Problems and Prospects of its Control in Nigeria. Oreofe Publishers Nigeria Limited. 24-38.

Seller, T.F. (1927). A new method for staining Negri bodies of rabies. *J. of Pub. Health*, 17, 1080-1085.

Talbi, C., Holmes, E.C., Benedictis, P., Faye, O., Nakoune, E., Gamatie, D., Diarra, A., Elmamy, B. O., Sow, A., Adjogoua, E. V., Sangare, O., Dundon, W.G., Capua, I., Sall, A., Bourhy, H. (2009). Evolutionary history and dynamics of dog rabies virus in western and central Africa. J. of Gen. Virol. 90: 783-791.

Tamura, K., Dudley, J., Nei, M., Kumar, S. (2007). MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol. Biol. Evol. 24(8): 1596–159