AFRICAN JOURNAL OF CLINICAL & EXPERIMENTAL MICROBIOLOGY JUNE. 2002 ISSN1595-689 Vol 3 No2
AJCEM/2002027/2214

MOLECULAR STUDY OF NUCLEPROTEIN GENE OF RABIES VIRUS ISOLATES FROM DOG BRAINS.

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Rabies is an endemic disease in Nigeria and it produces disease in warm-blooded animal species. In this report, molecular techniques have been used to study seven rabies isolates from the brain of Dogs in Lagos. Analysis of the 1400-b.p RT-PCR products of the N gene and the views by sequencing and restriction endonuclease analysis enabled division of isolates into 3 types. The conclusion from the study is that RT-PCR and restriction endonuclease analysis of the amplified products of the N gene would allow identification and differentiation of rabies virus strains in a location and in different parts of Nigeria.

INTRODUCTION.

Rabies is an endemic disease in Nigeria (1). There had been an under reporting of the disease in the country despite the awareness of its danger and its toll on human lives (2). In the absence of protective measures, infection with rabies virus causes severe neurological disease and death (3). The fluorescent antibody test (FAT) forms the basis for routine rabies diagnosis and together with monoclonal antibody techniques they are used for the characterization of rabies viruses. In addition, viral isolation either by newborn mouse intra cerebral inoculation test or by the rabies tissue culture infection test is also performed to allow further analysis of the etiological agent.

The recent application of molecular techniques to explore the rabies genome has permitted substantial progress in the understanding of the molecular structure and mechanism of virus (4,5). The rabies virus is known to consist of a negative strand RNA genome contained in a tight neucleocapsid composed of the nucleoprotein (N), which is enclosed by a bullet shaped outer capsid made up of a glycoprotein (G) and a membrane protein (M2) (6). The virus genome encodes a further two proteins L (the polymerase) and M1 (the phosphoprotein), both of which occur in small quantities in the virus particle where they are associated with the N protein (7,8). Detailed study of the N protein, G, L, and M proteins of rabies virus genome have been used to elucidate the nature, characteristic, phylogenetic, analysis and the epidemiology of rabies (9,10,11,12,13,).

The N gene, the major component of the internal nucleocapsid, is involved In the regulation and replication of the virus (14). It has been used to study antigenic diversity amongst rabies viruses (4) and to study the molecular basis of the antigenicity and cross protection among serotypes (15,16,17,18).

In this paper, molecular techniques such as RT-PCR, direct sequencing and restriction endonuclease analysis have been used to study the N gene of rabies Virus in isolates from brain sample of nine dogs in Lagos Nigeria. These studies also made N gene a potentially useful gene for diagnosis and epidemiological studies (12).

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MATERIALS AND METHODS

Sources of viral brain sample:

Seven brain samples collected from dogs diagnosed to be positive for rabies at Nigerian institute of medical research Lagos, Nigeria were stored at 80 C until used. The brain of two dogs diagnosed as positive for rabies were also collected from government veterinary centre in Lagos. The sample has been stored for two years before amplification of the virus.

Oligonucleotide primers:

The oligonucleotide primer rabN1 and rabN5 (10) (Table2) were used successfully to amplify the nucleoprotein (N) gene for all the nine specimens. The primers used for nucleotide sequencing were shown I table 2.

RNA extraction:

Total RNA was extracted directly from rabid brain tissue using TRIzol method which is an acid phenol / guanidium method described by Kamollvarin and his group (19) thus: in a microfuge tube, o.1gm of brain tissue dissolved in 1ml of TRIzol reagent by vigorous vortexing after which 0.2ml chloroform was added. After further vortexing for 2 to 3 minutes, tubes were spun in a microfuge centrifuge (1 x 12000g) at 4 C for 15 minutes. The upper aqueous phase was removed to a fresh tube and RNA was precipitated by the addition of 0.6ml isopropyl alcohol at room temperature for 10 minutes. RNA was pelleted by centrifugation as above for 15 minutes. The pellet was washed in 75% ethanol and dried.

Subsequently the dried RNA pellet was dissolved in diethylpyrocarionate (DEPC) treated sterile water.

RT-PCR:

FOR N gene PCR, primers RabN1 and RabN5 were used. Synthesis of cDNA was primed from 2ug of RNA using 25pmol of the positive sense primer RabN1 in a 20ul reaction, containing 50mM Tris HCL pH 8.3,75mM KCL, 3mM MgCL2 10mM DTT, dNTPs at 1mM each and 200 units Moloney murine leukaemia virus reverse transcriptases (life technologies). After incubation at 37 C for 2 hours, the reaction was inactivated by heating to 90 C for 5mins, cooled on ice and then centrifuged to collect the condensate. This cDNA product was later used as the template for PCR.

In a 200ul microfuge tube containing 14.25ul of water, the following were added: 5ul of 10 x PCR buffer (22.5mM MgCL2, 500mM Tris- HCL pH 9.2, 160mM (NH4) SO4), 5ul of cDNA sample and 0.75ul of expand enzyme (Bohringer Manheim). Also added to the above mixture were the following: 1.25ul of 10mM dNTPs, 1.25ul of 10um 5' end primer (negative sense) and 22.5ul of water. The DNA amplification was carried out in led lid Perkin Elmer thermocycler. For the N gene PCR, the temperature profile was 93 C for 10 secs, 48°C for 30 secs and 68°C for mins, then 68°C for 5 mins. PCR products were electrophoresed through 1% agarose gels ethidium bromide and visualised with UV illumination.

Direct DNA sequencing:

Direct DNA sequencing of PCR products was performed by the dideoxynucleotide chain termination method using an fmol DNA sequencing kit and 32P-labelled N gene directed internal primers according to the manufacturer's specifications (Promega). DNA sequencing comparison was performed using IBI-Pustell software.

Restriction Endonucleases of PCR Products.

PCR products were purified using the Wizard PCR clean up method (Promega) prior to analysis by restriction endonuclease digestion, performed by standard procedures (20).

Aliquots of 3ul of each amplification mixture were analysed after digestion by suitable restriction enzymes on 1% agarose gel containing ethidium bromide. The following enzymes were used for the experiment: BstbN1, Hind 3, Accl, Sca1, Eco1091, EcoR1, Dde1.

RESULTS

RNA Extraction

In the RNA extraction the concentration obtained ranged from 0.4ug/ml to 2.4ug/ml. The isolates obtained for the RT-PCR amplification were found to vary in the level purity. This was from 90% of sample 3 to 98% of sample 9 (Table 1). The RNA obtained from the rabies isolates was subjected to RT-PCR process and the DNA generated was amplified.

Analysis of Rabies Isolates PCR Products:

Of the 9 dog brain samples that were analysed, seven samples 3,4,5,6,7,8,9, that were positive for rabies by fluorescent antibody test (FAT) were positive by PCR technique. They all showed a single band at the expected site of 1,400 base pairs, when analysed in agarose gel electrophoresis. (Fig 1).

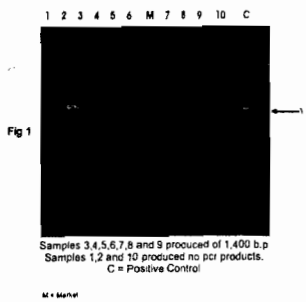

Fig 1

Samples 3,4,5,6,7,8 and 9 procuced of 1,400 b.p
Samples 1,2 and 10 produced no pcr products.
C = Positive Control

M = Marker

Genome Sequencing Determination and Analysis of the PCR Products:

Initially, partial sequencing of the PCR products of the brain samples 3,4,5,7,8 and 9 was performed using sequencing primers Nseq. 0 to Nseq. 5 and using Nseq.1 reverse primer. Both ends of the DNA were sequenced and the Nseq. 1 reverse primer was found common to the PCR products sequenced. The nucleotide sequence obtained showed that there were three distinct types of rabies virus among the samples. They were classified according to their similarities in nucleotide sequences as follows: samples 3,6,7 and 9 formed type 1; samples 4 and 5 formed type 2 and sample 8 formed type 3, though it appeared to be related to sample 4 and 5, but it was sufficiently distinct to be classified separately. One sample from each group was selected for N gene open reading frame sequencing using the additional primers detailed in Table 2. The samples selected were sample 3 for

type 1, sample 5 for type 2 and sample 8 for type 3.

In comparative analysis of sample 3 using IBI-Pustell software, samples 5 and 8 exhibited 86.3% and 85% sequence similarly respectively. However, sequence of samples 5 and 8 were 94.1% similar to each other.

The nucleotide sequences for these isolates are compared in Fig 2; positions of useful restriction endonuclease sites are also indicated.

Restricted Endonuclease Analysis:

From the nucleotide sequence analysis of each isolate using the IBI-Pustell software, a detailed restriction endonuclease site maps were determined for each sequence (Fig 2). The PCR products of the isolates were digested and the result of the enzyme digest is shown in Table 3. Samples 3,6,7 and 9 were sensitive to the following enzymes Hind 3, and Bstb1 by which they were cleaved into 2 bands of the same size. This indicated the samples to be of the same type. Samples 3,7, and 9 were grouped as type 1 by nucleotide sequencing.

Sample 4,5, and 8 were sensitive to the following enzymes Acc1, Sal 1, Sca1, and Eco01091 by which they were cleaved into 2 fragments of the same size, indicating this group of samples to be of type 2. However, samples 4 and 5 were sensitive to EcoR1 also, thereby distinguishing the two samples from sample 8 and this made sample 8 a unique type. Sample 8 is therefore designated as type 3.

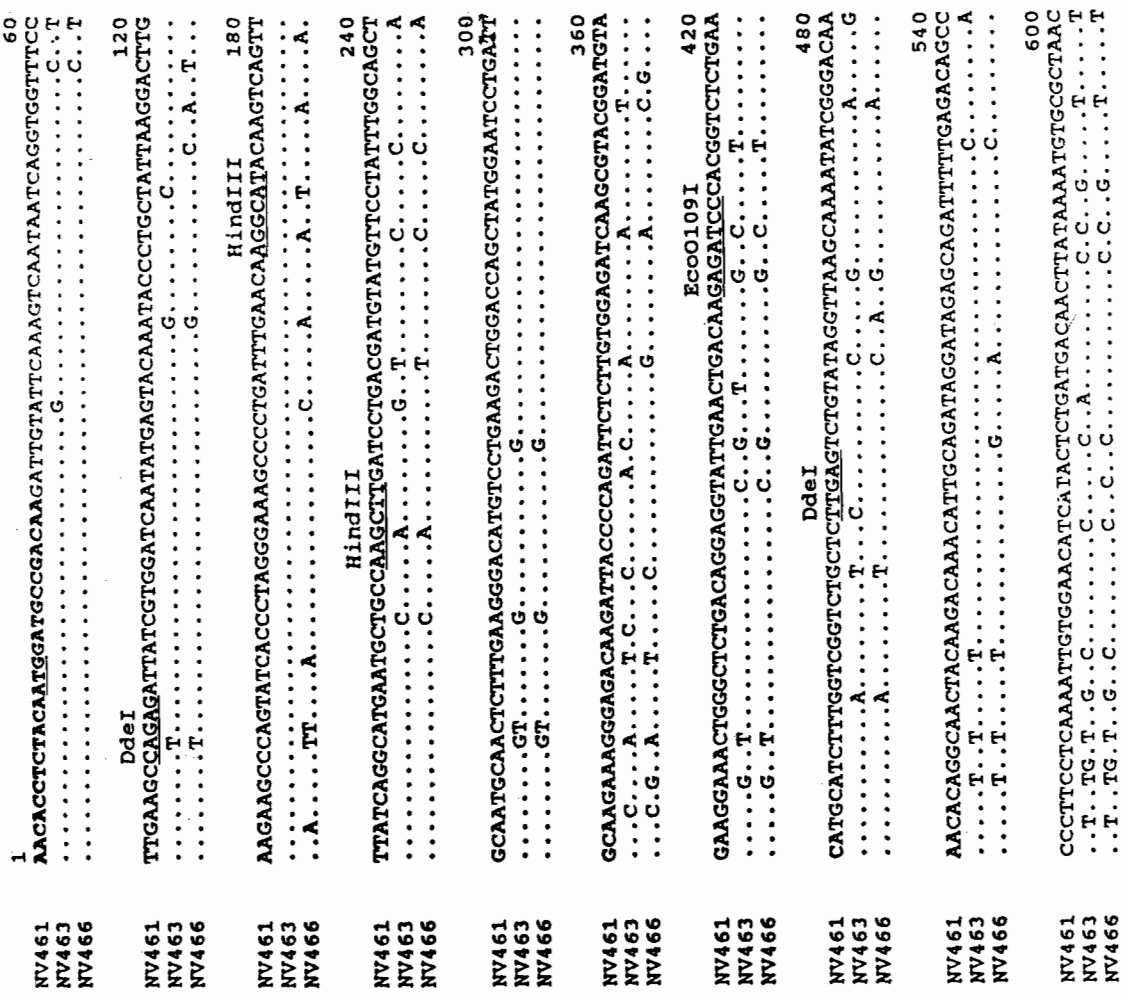

Figure 2. Comparison of the N gene nucleotide sequence of three representative rabies strains from Nigeria.

SAMPLE	OD 260	OD2 60/ 280	CONC. Ug/ml 1:500 dil.	STOCK CONC. Ug/ul	% PURITY	VOLUME OF RNA USED.u
1	0.024	1.9	1.1	0.55	94	4
2	0.011	1.89	0.5	0.25	94	6
3	0.019	1.82	0.8	0.4	90	5
4	0.059	1.83	2.4	1.2	91	1.8
5	0.038	1.92	1.6	0.8	95	2.5
6	0.036	1.95	1.5	0.75	97	2.7
7	0.044	1.95	2.0	1.0	97	2.0
8	0.042	1.88	1.8	0.9	93	2.3
9	0.019	1.96	0.8	0.4	98	5.0
10	0.010	1.90	0.4	0.2	94	6.0

Table 1
CONCENTRATION OF THE RNA OBTAINED FROM BRAIN TISSUES OF NIGERIAN ISOLATES AND THE VOLUME USED FOR PCR AMPLIFICATION

TABLE 2

PRIMERS USED FOR RT-PCR AND NUCLEOTIDE SEQUENCING

PRIMERS	NUCLEOTIDE SEQUENCE	POSITION/SENSE	
RabN 1 RT Primer	[illegible]TTACAATGGATGCCGA[illegible] 3'	1-36	-
RabN 5 cDNA PRIMER	[illegible]GATTGA[illegible]AAGATCTTGCTCAT 3'	514-536	-
SEQUENCING PRIMERS			
Nseq	[illegible]TACAA[illegible]TACCT 3'	1-24	+
Nseq 1 REVERSE.	**5'GACTTGTATGCCTTGTTCAAATTCGG 3'**	**152-176**	-
Nseq 1	5'[illegible]TTGAACAAGGCATACAAGTC 3'	151-176	+
Nseq 2	[illegible]AAAGGAGATAA[illegible]ATCACCC 3'	301-325	+
Nseq 3	5'G[illegible]ATATCTGGGCAAAACACCGG 3'	464-488	+
Nseq 4	[illegible]AACT[illegible]ATACC 3'	590-614	+
Nseq 5	5'[illegible]AAATCTCACTGCAAGGGAAGC 3'	754-779	+
Nseq 6	5'[illegible]TTGAGTGGGAAGT[illegible]CTT 3'	886-909	+
Nseq 7	5'[illegible]AGTTTTTT[illegible]GAAAGGGAA 3'	1048-1072	+
Nseq 8	5'[illegible]TACTTCTCCGGGGAAACCAGAAG 3'	1111-1214	+
Nseq 9	5'AT[illegible]AAGAGTTATTG[illegible]TACATGTG 3'	989-1012	+
Nseq 10	5'(AG)ATAGAGCAGATTTT(T[illegible])GAGA[illegible]	[illegible]	+
Nseq 11	5'AAG(A[illegible])GA[illegible]GTGGCA[illegible]GCAG[illegible]	[illegible]	+

The base position of each Primer refers to equivalent sequence of the PV strain rabies genome (Tordo et. al. 1986). Primers RabN 1 and RabN 5 were used for reverse transcription and amplification respectively, while Primers Nseq 0 to Nseq 11 were used for sequencing.

TYPE	EB	Hd	Ac	Sl	Sc	Ec	ER	Dd*
#3	-	-	-	-	-	-	-	+ * P4 & P6
#4	-	-	-	-	-	-	-	+ * P1, P2, P3 & P5
#5	-	-	-	-	-	-	-	+ * P1, P2, P3 & P5
#6	-	-	-	-	-	-	-	+ * P4 & P6
#7	-	-	-	-	-	-	-	+ * P4 & P6
#8	-	-	+	-	-	-	-	+ * P1, P3 & P5
#9	-	-	-	-	-	-	-	+ * P4 & P6

The restriction endonucleases used in this study are represented as follows: EB,EstbI; Hd,Hind3; Ac,AccI; Sl,SalI; Sc,ScaI; Ec,Eco0109I; ER,EcoRI; Dd,DdeI.
The positive sign - indicates the cleavage of the sample.
*DdeI enzyme has 6 cutting sites in the PCR products of these isolates with the positions designated as P1, P2 ,P3, P4, P5 & P6. For each isolate the position of cutting is shown in brackets.

TABLE 3.

SUMMARY OF RESTRICTION ENZYME ANALYSIS PROFILES OBSERVED FOR NIGERIAN RABIES ISOLATES IN THIS STUDY.

```
                 ScaI                                                  660
NV461    TGGAGCACCATACCGAACTTCCGATTCTTGGCCGGAACATACGACATGTATTTCTCCGG
NV463    .....T..T...........A....T..........C.........T......A...
NV466    .....T..T...........A....T..........C..T.......T......A...

                                                                       720
NV461    ATTGAACATCTATATTCAGCCATCAGAGTGGGCACAGTAGTCACTGCTTATGAAGATTGC
NV463    .....G.....G.....G..A..............G......GTCA.....T.T..C...
NV466    ..C..G.....G.....G..A..................T.....C...........C...

         DdeI                                                          780
NV461    TCTGGGCTGGTGTCGTTCACAGGGTTCATAAAACAGCAAAATCTCACTGCAAGGGAAGCA
NV463    ..A..A.....A.....T.............G...ATC........C......A.....
NV466    ..A..A.....A.....T.............G...ATC........C.............

                                                                       840
NV461    ATACTATATTTCTTCCAGAACAACTTCGAGGAAGAGATAAGAAGAATGTCCGAGCCAGGG
NV463    ................C..G.....G.....................T..........
NV466    ................C..AG.G..T.....................TT.........

                                                              DdeI  900
NV461    CAGGAGACTGCGGTTCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGG
NV463    ..A.....A..T..........T........C........T..AC.A..CT....T...
NV466    ..A.....A..T..........T..................T..AC.A..CT....T...

                                                                       960
NV461    AAATCCCCGTATTCCTCAAATGCAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGA
NV463    ..G..T..T.....A..G.....T..C........G.........................
NV466    ..G..T..T.....A........C..C........G.........................

                                                                      1020
NV461    TGTTATATGGGTCAGGTCAGGTCTTTAAATGCGACGGTCATTGCTGCATGTGCTCCTCAT
NV463    ..C..C........A.....A...C.............T..............C......
NV466    ..C...........A.....A...C.......A.....T..............C......

                                                  EcoRI          BstBI 1080
NV461    GAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAGAGTTTTTTGGAAAAGGCACATTCGAA
NV463    ..........T........C...T.G..A..G..A..C..C........G.....T...
NV466    ..........T........C...T.G..A..G.....C..C........G.....T.G.

                                                                      1140
NV461    AGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAATATGAGGCAGCTGAATTGACGAAG
NV463    .....G.....C........G........T..............G......C.A..A...
NV466    .AG.AG.....C........G........T...........A..G......C....A...

                                                  DdeI                 1200
NV461    ACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTACTTTTCC
NV463    T.C..C.................................A.....C.....C..T..C..T
NV466    ..C..C.....................T........A.....C.....C.....C...

                                                          SalI/AccI 1260
NV461    GGTGAAACCAGGAGTCCTGAAGCCGTTTATACTCGGATCATGATGAACGGAGGCCGACTA
NV463    .TG........A...T.A.....T..C........A...........T.....T.....G
NV466    ..........A...........T..C........A...........T.....T.....G

                                                                      1320
NV461    AAGAGATCTCACATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCCCGCCCAAAC
NV463    ..........T...C.....................................T......
NV466    ........A..T...C..........G.........................T......

                 DdeI                                                 1380
NV461    TCATTTGCTGAGTTCCTAAACAAAACATATTCTAGTGATTCATAGAGAGTTGAATCACAG
NV463    .....C..C..A..TT.......G..G.....A.T...C.....AG.......T.G....
NV466    ......T.G..A..TT.......CGGG.....G.....C.....AG.........G....

                                                                      1440
NV461    GATTCTAGGAAACGATAACTTGTGTACATCCTTCATGAAAAAAACTAACACCTCTCCTTG
NV463    .G.G.C..A..T.T...GA....A..T....A...................TC......T
NV466    .G.G.C..A..T.C...GA.......T....A...................TC......T

                                                          1478
NV461    CGAACCATCCTAAATATGAGCAAGATCTTTGTCAATCC
NV463    .A........C.....................T.....
NV466    ..........C..G..................T.....
```

The nucleotide sequence of sample V461 (#3) is presented; bases are numbered from the start of the N gene PCR product. Only differences from this sequence are indicated for samples V463 (#5) and V466 (#8). Positions of ATG open reading frame start codons and TAG/TAA stop codons are underlined as are the recognition sequences of the discriminatory restriction endonucleases described in the text. The sequences of the amplification primers RabN1 and RabN5 are bolded.

DISCUSSION

Molecular techniques including DNA amplification have been used as invaluable tools for virus diagnosis and typing (21). These techniques have been applied to many laboratories and street isolates of rabies virus to understand the nature and character of viral genome (22). The technology has helped to improve the knowledge of relationship between rabies strains within the same geographical areas and different geographical areas.

In this study, we have applied the use of molecular techniques for the diagnosis and typing of rabies isolates collected from the metropolitan city of Lagos in Nigeria. With the RT-PCR technique, the size of the DNA of the isolates was determined to be 1,400p. The technique is also found to be useful as a diagnostic tool for confirming the presence of the virus in a specimen, by the amplification of the converted DNA.

The agreement of the results with that obtained by conventional diagnostic procedure such as FAT showed that the detection of rabies genome by PCR amplification is both specific and sensitive, more so with the highly conserved area of N gene which is invariant (9). Therefore PCR appears as a possible future technique for routine post mortem diagnosis.

The direct nucleotide sequencing of the N gene of the rabies isolate showed that the isolates could be grouped into 3 types with isolates 3,6,7, and 9 grouped into type 1, isolates 5 and 5 grouped into type 2 and isolate 8 into type 3.

Restriction enzyme analysis of amplified DNA of the N gene using a panel of eight enzymes (Bstb1, Hind 3, Acc1, Sal 1, Sca1, Eco01091, EcoR1, Dde1), divided the isolates into the same 3 types identified by the sequencing method. Though there is no record of typing of wild rabies isolates in Nigeria using molecular techniques, the study is similar to the work of Nadin-Davies et al (11), where typing of detected strains of rabies virus was carried out in an outbreak of rabies in Central Ontario. Five strains of rabies virus were identified with respect to geographical locations studied. In Lagos where Nigerian isolates were collected, three types of strains were identified and they were from neighbouring states and country (Republic of Benin). The different strains identified showed some variations in their nucleotide differences and this could be attributed to differences in wild life population densities and movement.

The molecular technique used for this study is realised to be a very simple typing method that produces result within 24 hours. It is a useful method for comparing known viral strains in a geographical area with any emerging strain in the same or a different geographical area.

CONCLUSION

Molecular biology techniques have been used to amplify and type the rabies virus isolates and 3 types were identified by both direct sequencing and restriction enzyme techniques. These showed that the techniques are very useful especially the PCR, which apart from its usage for routine diagnosis, forms powerful tool for typing and molecular epidemiological studies. Using these techniques, it would be possible to identify and differentiate similar or other different rabies virus strains that exist in the same environment and other parts of the country Nigeria and else where.

Acknowledgement

I wish to thank W.H.O. for sponsoring the training programme and this study. I am also grateful to Dr. Nadin-Davis for the supervision and assistance she gave me when undertaking this study.

REFERENCES.

1. Fagbami. A.H.; Anosa V.O. and Ezeburo E.O.I. Hospital records of human rabies and anti-rabies prophylaxis in Nigeria. Trans. R. Soc. Trop. Med. And Hyg. 1981; 75:82-86
2. W.H.O. Conference report: Control of rabies in English Speaking West African Countries. WHO Rabies Research 1992; 93:39.
3. Charleston K.M. The pathogenesis of rabies. In Rabies 1988; p.101-150. Edited by J.B. Campbell and K.M. Charlton. Boston: Kluver Academy Publishers.
4. Dietzscold B.B.Q. Rupprecht, C.E.; Tollis M.; Lafon M.; Mattei J.; Wikto T.J. and Koprowski H. Antigenic diversity of the glycoprotein and the nucleocapsid proteins of rabies and rabies related viruses: Implications for epidemiology and control of rabies. Reviews of Infection Disease 1988; 10:5785-98.
5. Wunner W.H.; Larson J.K.; Dietzschold B. and Smith C.L. The Molecular biology of rabies virus. Review of Infections Diseases. 1988; 10:771-784.
6. Von Teichman F.; Thomson G.R.; Meredith

C.D. and Nel L.H. Molecular Epidemiology of rabies in South Africa: Evidence for two distinct groups. J. General Virology. 1995; 76:73-82.
7. Morimoto K.; Ohkubo A. and Kawal A. Structure and transcription of the glycoprotein gene of attenuated HEP-Flury strain of Rabies virus. Virology. 1989; 173:465-477.
8. Tordo N.; Poch O.; Ermine A.; Keith G and Rougeon F. Completion of the rabies virus genome sequence determination highly conserved domains among the L (polymerase) proteins of unsegmented negative-strand RNA viruses. Virology 1988; 165:565-576.
9. Sacramento D.; Bourhy H.; and Tordo N. PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. Molecular and cellular probes. 1991; 6:229-240.
10. Nadin-Davis S.A.; Casey G.A. and Wandeler A.I. Identification of regional variants of the rabies virus within the Canadian province of Ontario. J. Gen. Virol. 1993; 74:829-837.
11. Nadin-Davies S.A.; Casey G.A. and Wandeler A.I. A Molecular epidemiological study of rabies virus in Central Ontario and Western Quebec. J. Gen. Virol. 1994; 75:2575-2583.
12. Kissi B.; Tordo N. and Bourhy A. Genetic polymorphism in the rabies virus nucleoprotein gene. Virology 1995; 209:526-537.
13. Tordo. N. Contribution of molecular biology to vaccine development molecular epidemiology of rabies disease. Memoirs Institute Butantan 1991; 53 (Suppl. 1) 31-51.
14. Patton J.T.; Davis N.L. and Wertz G.W. N protein alone satisfies the requirement for protein synthesis during RNA replication of vesicular stomatitis virus. J. Virol. 1984; 49:303-309.
15. Celis E.; Rupprecht C.E.; Plotkin S.A. New and improved vaccines against rabies. In: Woodrow G.C., Levine M.M. (Ed). New generation vaccines. Decker New York 1990; pp 419-439.
16. Tollis M.; Dietzschold; Buona. Volia C. and Koprowski H. Immunization of monkeys with rabies ribonucleoprotein (RNP) confers protective immunity against rabies. Vaccine 1991; 9:134-136.
17. Perrin P. Joffret M.L.; Zenetti C.; Bourhy H.; Gontier C.; Fritzell C.; Leclerc C. and Sureau P. Rabies specific production of interleukin-2 by peripheral blood lymphocytes from human rabies vaccines. Vaccine. 1991; 549-558.
18. Ertl. H.C.J.; Dietzschold B. and Otvos L. T Helper cell epitope of rabies virus nucleoprotein defined by tri and tetrapeptides. Euro. J. Immunol. 1991; 21:1-10.
19. Kamolivarin N.; Tirawatnpong T.; Rattaanastwamoke R.; Torawatnopong S.; Panpanich T. and Hemachuda T. Diagnosis of rabies by polymerase chain reaction with nested primers. Journal of Infectious Disease 1993; 167:207-210.
20. Sambrook J.S.; Fritsch E.F. and Maniatis T. Molecular Cloning: A laboratory manual 1989; 2nd Edn. Cold Spring Harbor NY: Cold Spring Harbor Laboratory.
21. Kwok S. and Sninsky J.J. Application of PCR to the detection of human infectious diseases. In PCR Technology, principles and applications for DNA amplification. 1989; pp 235-244. Edited by H.A. Erlich. New York Stockton Press.
22. Smith J.S.; Fishbein D.B.; Rupprecht C.E. and Clark K. Unexplained rabies in three immigrants in the United States. New England Journal of Medicine 1991l 324:205-211.