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Research Article

REAGENTS, PRIMERS/PROBES FOR MOLECULAR DIAGNOSIS OF RABIES

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ABSTRACT
PCR is the most powerful technique for detection of small amounts of nucleic acids. Advanced molecular approaches for ante mortem and postmortem diagnosis of rabies through polymerase chain reaction is a rapidly growing field, impeded by lack of trained human resource and dearth of well equipped laboratories. Here, we review the techniques used, to date, various reagents kits, primers for amplification and provide information of the approaches already undertaken for those new to this field. Optimization attempted for RNA extraction, and cDNA synthesis techniques in various studies has been discussed. The choice of genes and advice on primer design to maximize specificity and detection has also been highlighted. We look ahead to new technologies that will replace the conventional tests while surpassing the obstacles of cost, complexity and infra-structure.

Key Words: *Nested RT-PCR, Taq Man Real Time PCR, Saliva, Brain, Rabies*

INTRODUCTION
Rabies is a viralzoonotic disease of mammals that infects the central nervous system, causing encephalopathy and ultimately death (Baer *et al.,* 1996; Timoney *et al.,* 1988). It is caused by single stranded RNA virus belonging to genus *Lyssavirus* of the family *Rhabdoviridae* (Wunner *et al.,* 1988). The virions of the rabies virus have a bullet shaped structure with an approximate length of 180 nm and diameter of 75nm (Tordo and Poch 1988). Rabies virus genome consists of a single stranded, negative sense, non segmented RNA, 12 kb in length. Five genes (3'-N-P-M-G-L-5') encodes for five proteins: nucleoprotein (N), phosphoprotein (P), matrixprotein (M), glycoprotein (G) and the polymerase (L) (Fauquet *et al.,* 2005).
Rabies has affected several thousand people and large number of unaccounted domestic animals worldwide, causing a significant, though often neglected health impact (Rupprecht *et al.,* 1995). Each year at least 10 million people receive treatment after being exposed to animals suspected to be rabid; however 55,000 people still die in Asia and Africa, based on the estimation by the WHO (Knobel *et al.,* 2005).
The clinical diagnosis of rabies is sometimes suggested by epidemiological (history of exposure) and clinical (e.g. paraesthesia, hydrophobia) findings (Hemachudha, 1994). However; the disease is often mistaken for other disorders (Emmons, 1979). Differentiation from other neurological diseases may require extensive investigations. Therefore, diagnosis is often confirmed late in the course of the disease or postmortem (Fishbein, 1991).Delay in diagnosis greatly increases the number of contacts that require post exposure prophylaxis. The early detection of this dreaded disease is also essential to eliminate the expenses and discomfort of unnecessary diagnostic tests and inappropriate therapy. Lately, detection of cytoplasmic inclusion or Negri bodies (Negri, 1903) in nerve tissue impression smear is rarely used to diagnose rabies. This method was successfully replaced by rabies viral antigen detection with Fluorescent Antibody Test (FAT) (Goldwasser *et al.,* 1958). WHO and OIE have recommended FAT as the primary laboratory method for diagnosis of rabies. But application of this approach is often limited to post mortem samples however, with the advent of molecular approaches; it is now possible to detect rabies ante-mortem.
Since rabies virus appears in the saliva of dogs before and during the appearance of clinical signs (Schneider, 1975), thus molecular approaches can be employed for reliable ante-mortemdiagnosis. Ante-mortem diagnosis of rabies by molecular techniques based on detecting virus or viral RNA has been attempted in body fluids of live animals such as saliva (Crepin *et al.,* 1998) and CSF (Saengseesom *et al.,* 2007).
The rabies virus is also present in nerve cells surrounding the base of hair follicles (Madhusudana and Sukumaran 2008). The ante-mortem diagnosis of rabies has been reported from skin samples taken before death.Nested (Strauss *et al.,* 2005) and Heminested PCR (Dacheux *et al.,* 2008) have been employed for ante-mortem diagnosis of rabies from skin biopsy however, there is no study which correlates the use of sensitive TaqMan real time PCR technique on skin biopsy samples for ante-mortem diagnosis of rabies.
TaqMan based real-time RT-PCR is consistently sensitive, rapid and specific for the detection of rabies virus RNA in brain samples (Hughes *et al.,* 2004). TaqMan probes are incorporated in reverse transcriptase polymerase chain reaction (RT-PCR) by (Black *et al.,* 2002) that could distinguish among the six established rabies and rabies-related virus genotypes. Primer set that targets distinct conserved region of rabies virus N gene were designed to develop TaqMan based quantitative reverse transcriptase polymerase chain reactions (qRT-PCR) for the diagnosis of rabies viral RNA (Nadin Davis *et al.,* 2009). The comparison of real time PCR with the heminested RT-PCR method revealed that the TaqMan PCR was 10-fold more sensitive than the heminested RT-PCR (Orlowska *et al.,* 2008). Real time PCR methods are more favored than conventional reverse transcription PCR methods by several laboratories (Wacharapluesade and Hemachudha 2010).

Extraction of RNA

Saliva Samples:

Various reagents have been used for isolation of rabies viral RNA from saliva samples. These include TRIzol® (Gibco BRL, USA) reagent (Elmgren *et al.,* 2002). Saengseesom *et al.,* (2007) has reported precipitation of RNA and dissolution in 20 to 40 µl of diethyl polycarbonate water and stored at -80ºC for further use.

TRIzol® (Invitrogen, Canada) reagent protocol has been reported by Shankar *et al.,* (2004) and Brito *et al.,* (2011). Former also supported addition of 100 µl lysis buffer (10 mMTris-HCL, pH 7.5, 150 mMNacl, 1.5mM $MgCl_2$ and 0.65% NP-40) with ~100 µl of the saliva sample prior to addition of Trizol®. TRIzol LS reagent (Invitrogen, Canada) has also been used for recovery of RNA from human and bat saliva samples by Nadin Davis *et al.,* (2009) and Reynes *et al.,* (2011) respectively. TRIzol LS reagent (Invitrogen, Canada) was used for RNA extraction with a Roche MagNA Pure LC (model JE 379, Roche Diagnostics, Indianapolis, Indiana, USA) system (Jackson *et al.,* 2008). Glycoblue (Ambion) and Glycogen (Ambion) have been employed as co-precipitant along with Trizol LS reagent for extraction of RNA by Hughes *et al.,* (2004) and Dacheux *et al.,* (2008) respectively.

QIAamp viral RNA mini kit (Qiagen, Germany) was used for extraction of RNA from 200 µl saliva samples byJohnson *et al.,* (2008), Coertse *et al.,* (2010) and Panning *et al.,* (2010).

Proteinase K method [200 µl of fluid sample was incubated for 2 hrs at 37ºC with 400 µl of Proteinase K buffer containing 40 µg of Proteinase K (Gibco BRL)] prior to purification by phenol-chloroform extraction was reported by Crepin *et al.,* (1998). Use of Guanidinium thiocyanate together with silica particles for purification of RNA was reported by Crepin *et al.,* (1998) and Echevarria *et al.,* (2001).Cationic surfactants (Catrimox-14; Iowa Biotechnology Corporation) and Chelating resin (Chelex 100; Bio-Rad) techniques explored by Crepin *et al.,* (1998) for production of RNA yielded negative results.

Skin Samples:

The reagents used for isolation of rabies viral RNS from skin include TRIzol® reagent (Invitrogen, Canada) that was reported by Macedo *et al.,* (2006), Nadin Davis *et al.,* (2009) and Brito *et al.,* (2011) whereas TRIzol LS reagent (Gibco BRL, USA) was used to precipitate RNA from finely chopped skin biopsy material by Elmgren *et al.,* (2002). Precipitated RNA was dissolved in 20 to 40 µl of diethyl polycarbonate treated water and stored at -80ºC.

RNeasy kit (Qiagen) was employed for extraction of RNA from skin biopsy samples by Panning *et al.,* (2010).

ALT (180 µl) tissue lysis buffer and Proteinase K (20 µl) (Qiagen)were incubated with skin biopsy samples at 37ºC for 3 hr. prior to addition of TRI Reagent LS (Molecular Research Centre) by Dacheux *et al.,* (2008) and Reynes *et al.,* (2011).

Brain Samples:

In addition to isolation of rabies viral RNA from clinical samples, reagents have also been employed for isolation of rabies viral RNA from nervous tissue. TRIzol LS reagent (Invitrogen, Canada) was used for extraction of RNA from 10% homogenate of mouse brain passaged human isolateby Madhusudana and Sukumaran (2008). Chloroform and glycogen were added as RNA carrier. RNA was precipitated using isopropyl alcohol and washed in 70% alcohol and dissolved in DEPC treated water.

Trizol® (Invitrogen, Canada) reagent protocol for extraction of total RNAs from was reported by Nadin Davis *et al.,* (1998), Wakely *et al.,* (2005), Barbosa *et al.,* (2007), Araujo *et al.,* (2008), Nagarajan *et al.,* (2009), Coertse *et al.,* (2010) and Zhang *et al.,* (2011).

Trizol® (Gibco BRL, USA) reagent has been reported for extraction of RNA from rabies virus infected BHK-21 monolayer or infected mouse brain tissue by Heaton *et al.,* (1997). 1µl of Glycoblue (Ambion) was used as coprecipitant along with 750µl of TRIzol reagent for extraction of RNA from frozen brain tissue samples by Hughes *et al.,* (2004). Dried RNA pellets were resuspended in 100 µl of nuclease free water (Promega) and stored at -80ºC until used.

Acid guanidium thiocyanate-phenol-chloroform mixture method was used by Biswal *et al.,* (2007) for extraction of RNA from archived brain samples stored at -20ºC for 5-6 yrs in 50% glycerol saline whereas, Kamolvarin *et al.,* (1993) extracted RNA from brain samples kept at room temperature (28-32ºC) by similar method.

RNeasy lipid tissue mini kit (Qiagen, USA) has also been reported by Wacharapluesadee *et al.,* (2008) for recovery of RNA from brain tissue specimens. QIAamp viral RNA kit or RNeasy kit (Qiagen, Germany) was used by Orlowska *et al.,* (2008) and Hoffmann *et al.,* (2010) for producing rabies viral RNA from brain suspension.

Synthesis of cDNA

Likewise the kits reported for synthesis of cDNA include AccessQuick® kit (Promega, USA) by Echevarria *et al.,* (2004), Hughes *et al.,* (2004) and Saengseesom *et al.,* (2007) in a one step process. Likewise, Qiagen one step RT-PCR kit (Qiagen, Germany) components were used for synthesis of cDNA by Nagarajan *et al.,* (2006), Orlowska *et al.,* (2008), Nadin Davis *et al.,* (2009) and Panning *et al.,* (2010).

Super script reverse transcriptase (100 U; Gibco BRL) and rabies specific primers were used by Crepin *et al.,* (1998) for making of cDNA from RNA. Super script II reverse transcriptase (10U and 200U, Invitrogen) was used by Barbosa *et al.,* (2007) and Dacheux *et al.,* (2008) respectively for synthesis of cDNA.

Avian myeloblastosis virus reverse transcriptase (4-12U; Promega, WI) have also been used for synthesis of cDNA by Kamolvarin *et al.,* (1993). Avian myeloblastosis virus reverse transcriptase (8U; Roche Diagnostics, USA) has been reported by Shankar *et al.,* (2004) and Jackson *et al.,* (2008).
Avian myeloblastosis virus reverse transcriptase (20U; Roche Diagnostics, Germany) has reported by Coertse *et al.,* (2010) for synthesis of cDNA. M-MLV reverse transcriptase (200 U, Gibco BRL) was used by Heaton *et al.,* (1997) for synthesis of cDNA. Moloney murine leukemia reverse transcriptase (200 U, Promega, USA) was used by Wakely *et al.,* (2005) for reverse transcription of RNA. Murine leukemia reverse transcriptase (MBI, Fermentas, USA) was used by Biswal *et al.,* (2007) for reverse transcription of rabies RNA. M-MLV reverse transcriptase (200 U, Invitrogen^{TM}) was used by Macedo *et al.,* (2006), Araujo *et al.,* (2008) and Brito *et al.,* (2011) for synthesis of cDNA.
Saliva

Molecular Approaches for Ante-Mortem Diagnosis of Rabies

Molecular approaches reported by various workers for diagnosis of rabies virus are compared. Forward and reverse primer sequences, their relative position, product size, thermocycling conditions was also discussed for standard RT-PCR (Table 1), Nested RT-PCR (Table 2), Heminested RT-PCR (Table 3). TaqMan real time PCR assay used by different workers for rabies viral diagnosis was also compared for forward primer, reverse primer and probe sequences, their relative positions, thermocycling conditions (Table 4).
Crepin *et al.,* (1998) tested an optimized reverse transcription (RT)-PCR protocol for the intravitam detection of rabies virus genomic RNA in clinical samples obtained from 28 patients suspected of having rabies, 9 of whom were confirmed to have had rabies by postmortem examination. RT-PCR using saliva combined with an immunofluorescence assay performed with skin biopsy samples allowed detection of rabies in nine patients.
Noah *et al.,* (1998) observed a higher sensitivity of >98% was obtained by using RT-PCR for ante-mortem diagnosis of rabies in human saliva samples. Isolation of rabies from saliva was attempted in 15 of the 20 cases of rabies diagnosed before death, and in 9 cases virus was found in 1 or more samples.
Nagaraj *et al.,* (2006) evaluated the utility of conventional RT-PCR and SYBR Green I Real time PCR in the ante mortem diagnosis of rabies using saliva samples. Saliva samples collected from twenty-four patients presenting with typical clinical manifestations of rabies were tested in the two assays. Real time PCR assay was more sensitive than conventional RT-PCR assay (sensitivity 75% versus 37%, p = 0.0189).
Saengseesom *et al.,* (2007) conducted a study in order to look for evidence of rabies virus in saliva and cerebrospinal fluid (CSF) of suspected live rabid dogs at the time of quarantine by using a SYBR Green real-time RT-PCR based assay for the detection of rabies virus RNA.
Dacheux *et al.,* (2008) standardized a new reverse-transcription; Heminested polymerase chain reaction (hnRT-PCR) protocol at 3 participating centre's in Cambodia, Madagascar, and France. In this study, saliva samples provided the second-best results for sensitivity testing (63.2% [57 samples in group 1] and 70.2% [84 samples in group2]). A sensitivity of 100% was obtained with the saliva sample when sanalyzed at least 3 successive samples per patient. RT-PCR on saliva for viral nucleic acid detection yielded a sensitivity of 50-70% and a specificity of 100% (Madhusudana and Sukumaran 2008).
Wacharapluesadee and Hemachudha (2010) obtained a sensitivity of 75.8% (47/62 samples) by applying nucleic acid-amplification test methods with saliva samples for ante-mortem detection in human patients.
Rabies RNA may be found in saliva, CSF, skin biopsy tissue and urine. Nested PCR techniques enhance the sensitivity. Real time PCR methods are being evaluated (Principles and Practices of Clinical Virology 2009).
Molecular techniques can improve clinical diagnosis. Although molecular diagnosis facilities of rabies are limited in developing countries, these do exist in parts of India, the Philippines, Latin America, Sri Lanka and Thailand. The best specimens include saliva, tear secretions, nuchal skin biopsy specimens, CSF and urine. Secretions of virus are intermittent in saliva, urine and even CSF (Principles of Neurologic Infectious Diseases 2005).

Skin Samples

Noah *et al.,* (1998) tested nuchal skin biopsy specimen by RT-PCR in 15 cases out of the 20 cases before death and rabies viral antigen was detected in 10 (66.6%) human patients.
Strauss *et al.,* (2005) used RT-PCR for ante-mortem diagnosis of rabies from neck skin biopsy samples and out of all the samples collected first positive result were obtained by RT-PCR on punch biopsy of neck skin sample.
Macedo *et al.,* (2006) used a RT-PCR, with primers targeted to the 3' terminal portion of the nucleoprotein gene (N), to test neck-skin samples of nine patients who had rabies in order to validate a diagnostic method that could serve as an additional tool for rabies diagnosis, particularly in ante-mortem samples and obtained a sensitivity of 70% per sample and 77.7% per patient.
Dacheux *et al.,* (2008) standardized a new reverse-transcription; Heminested polymerase chain reaction (hnRT-PCR) protocol at 3 participating centres in Cambodia, Madagascar, and France. Accuracy of the diagnosis by comparing the results obtained with use of biological fluid specimens (saliva and urine) and skin biopsy specimens with the results obtained with use of the standard rabies diagnostic procedure performed with a postmortem brain biopsy specimen were studied.

Table 1: Different primers and ThermoCycling conditions of RT-PCR for diagnosis of rabies virus.

Author	Primer Sequence		Position*		Product Size (bp)	Thermo Cycling Conditions**		
	Forward Primer	Reverse Primer	Forward Primer	Reverse Primer		Initial Den.	Den. Ann. Ext. } No. of Cycles	Final Ext.
Sacramento *et al.*, 1991)	5'TTTGAGACTGCTCCTTTT 3'	5'CCCATATAGCATCCTAC 3'	587-605	1013-1029	443bp	NR	NR***	NR
Arai *et al.*, (1997)	5'CTACAATGGATGCCGAC 3'	5'TGGGGTGATCTT(A/G)TC TCCTTT3'	66-82	365-385	320bp	NR	94°-60s 45°-60s 72°-60s } NR	NR
Crepin *et al.*, (1998)	5'GTAACACCTCTACAATG G3	5'GCTTGATGATTGGAACT G3'	57-74	1349-1368	1311bp	94°- 60s	94°-50s 50°-90s 72°-90s } **130**	72°-5min
East *et al.*, (2001)	5'AAGAACTTCAAGAAT ACG AGGC3'	5'TTCAGCCATCTCAAGAT CGG3'	1161-1182	1560-1579	418bp	94°- 1min	94°-30s 37°-30s °-90s } **40**	72°-7min
Gupta *et al.*, (2001)	5'ACTGATGTAGAAGGGA AT TG3'	5'GAACGGAAGTGGATGA AATA3'	NR	NR	533bp	4°- s	94°-60s 50°-60s 72°-60s } **35**	72°-5min
Adeiga *et al.*, (2002)	5'GCTCTAGAACACCTCTA CAATG GATGCCGACAA3'	5'GGATTGAC(AG)AAGATC TT GCTCAT3'	59-84	1514-1536	1400bp	NR	93°-10s 48°-30s 68°-60s } **NR**	72°-5min
David *et al.*, (2002)	5'GAGAAAGAACTTCAAG A3'	5'GAGTCACTCGAATATGT C3'	1156-1172	1513-1533	377bp	NR	94°-45s 37°-45s 2°-90s } **40**	NR
Junior *et al.*,(2004)	5'ATGTAACACCC/TCTACA ATTG3'	5'CAATTCGCACACATTTT GTG3'	55-73	641-660	606bp	4°- min	94°-30s 45°-30s 72°-60s } **40**	72°-7min
Langoni *et al.*, (2005)	5'ATAGAGCAGATTTTCGA GACAGC3'	5'CCTCAAAGTTCTTGTGG AAGA3'	505-527	916-937	432bp	4°- min	94°-45s 45°1min 72°-10 min } **35**	72°- 10min
Macedo *et al.*, (2006)	5'ATGTAACACCTCTACAA TTG3'	5'TTGACGAAGATCTTGCT CAT3'	55-73	1514-1533	1478bp	94°- 1min	94°-30s 37°-30s 72°-90s } **40**	72°-7min

Rojas *et al.*, (2006)	5'CGTRGAYCAATATGAGT ACA3'	5'CAGGCTCRAACATTCTT CTTA3'	66-85	806-826	760bp	NR	95°-45s 50°-30s 72°-30s } **30**	72°-10min
Barbosa *et al.*, (2007)	5'GGAAGAGATAAGAAGA T GTTTG3'	5'TTGGAGCTGACTGAGAC ATA3'	868-890	1359-1378	491bp	NR	94°-1min 55°-2min 72°-2.5min } **35**	NR
Wacharapluesadee *et al.*, (2008)	5'TAGGGAGAAGGATCGT GGAGCACCATACTCTCA3'	5'GATGCAAGGTCGCATAT GAGTACCAGCCCTGAACA GTCTTA3	611-632	769-790	179bp	NR	NR	NR
Lopes *et al.*, (2009)	5'CTACAATGGATGCCGAC 3'	5'TTGACGAAGATCTTGCT CAT3	33-49	1514-1533	1500bp	94°- 5 min	94°-45s 55°-45s 72°-90s } **35**	72°- 5min.
Brito *et al.*, (2011)	5'TATACTCGAATCATGAT GAATGGAGGTCGACT-3'	5'TTGACGAAGATCTTGCT CAT-3'	1287-1318	1514-1533	249bp	94°- 1 min	94°-30s 37°-30s 72°-90s } **40**	72°- 7min.
Qureshi *et al.*, (2011)	5'TTTGAGACTGCTCCTTTT G3'	5'CCC ATATAGCATCCTAC3	587-605	1013-1029	443bp	94°- 60s	94°-30s **30** 45°-90s 50°-20s **5** 72°-90s 72°-60s **30**	72°- 10min

All nucleotides positions are based on genomic sequence of the reference PV (Pasteur Virus) strain sequence of Nucleoprotein gene (N) (GeneBank accession number M13215)
****Den- Denaturation; Ann- Annealing; Ext- Extension***
*****NR- Not Reported***

Table 2: Comparison of different studies using Nested RT-PCR for diagnosis of rabies virus.

Author	Primer sequence				Position*				Product size (bp)		Thermo cycling Conditions**							
	1st Round		2nd Round		1st Round		2nd Round		1st Round	2nd Round	1st Round				2nd Round			
	Forward Primer	Reverse Primer	Forward Primer	Reverse Primer	Forward Primer	Reverse Primer	Forward Primer	Reverse Primer			Initial Den	Den Ann Ext } No Of cycle		Final Ext	Initial Den	Den Ann Ext } No Of cycle		Final Ext
Whitby *et al.*, (1997)	5'TTTGAGACTGCT CCTTTT3'	5'GCTTGATGAT TGGAACT3'	5'AGAATGTT TGAGCCACG GCA3'	5'TCAGGTGA AACCAGAAG TCC3'	NR	NR	NR	NR	782	396	NR	95°-90s 45°-90s 72°-90s } 5	30s 60s 60s } 30	30s 90s 10m } 1	NR	95°-120s	95°-60s 52°-60s 72°-60s } 25		72° 10min
Kulonen *et al.*, (1998)	5'GAAGCCTGAG ATTATCGTGG3'	5'CCCTTCTACA TCAGTACG3'	5'TGAGTAC AAGTACCCT GC3'	5'GGAACAT ACATCGTCA GG3'	63-82	349-367	90-107	211-229	304	139	NR	NR**			NR	NR	NR		NR
Echevaria *et al.*, (2001)	5'AAGATGTGTG CCAACTGGAG3'	5'ATGTTTGAGC CAGGGCAAGA3'	5'TACTGCTT ATGAGGATT GTTC3'	5'AAGAACT TCGAGGAA GAGATC3'	NR	NR	NR	NR	NR	NR	94°-2min	93°-60s 60°-60s 72°-60s } 30			72°-5min.	94°-2min	94°-60s 50°-60s 72°-60s } 30		72° 5 Min
Franka *et al.*, (2004)	5'GTAACACCTCT ACAATGGA3'	5'AGTTTCTTCA GCCATCTC3'	5'GGATGCC GACAAGATT GTAT3'	5'CACATTTT GTGAGTTGT CA3'	57-75	1568-1585	73-92	633-651	1529	579	94°-5min	94°-40s 56°-40s 72°-60s } 35			72°-7min.	94°-3min	94°-30s 60°-30s 72°-40s } 35		72° 7 Min
Foord *et al.*, (2006)	5'ATGTAACACC YCTACAATG3'	5'CAGTTGGCAC ACATCTTGTG3'	5'AGATCAAT ATGAGTAYA ARTAYCC3'	5'GTCATCA AAGTGTGRT GCTC3'	55-73	641-660	139-163	617-636	605	497	95°-10min	95°-90s 45°-90s 50°-20s 72°-90s } 5	30s 60s 20s 60s } 40	30s 90s 20s } 1	72°-10m	95°-10 min	95°-90s 45°-90s 50°-20s 72°-90s } 5	30s 60s 20s 60s } 40	30s 90s 20s } 1	72°-10m
Kasempi-molpom *et al.*, (2006)	5'GTAACACCCCT ACAATGGATGC3'	5'CAAAGATCTT GCTCATGTTTG G3'	5'GACATGT CCGGAAGA CTGG-3'	5'GTATTGCC TCTCTAGCG GTG3'	57-78	1508-1529	319-337	823-842	147 3	524	94	94° 60° 72° } NR			72	94	94° 60° 72° } NR		72
Vazquez Moron *et al.*, (2006)	5'AARATNGTRG ARCAYCACAC3'	5'GCRTTSGANG ARTAAGGAGA3'	5'AARATGT GYGCIAAYT GGAG3'	5'TCYTGHCC IGGCTCRAA CAT3'	538-557	892-911	574-593	814-833	374	260	94°-2min	93°-60s 53°-60s 72°-60s } 30			72°-5min.	94°-2min	93°-60s 53°-60s 72°-60s } 30		72 - 5min
Nadin Davis *et al.*, (2007)	5'AACACCTCTAC AATGGATGCCG ACAA3'	5'TTGTA/GGAT/C CAATATGAGTAC AA3'	5'GGATTGAC(AG)AAGATCT TGCTCAT3'	5'CCGGCTCA AACATTCTTC TTA3'	59-84	1514-1586	135-56	876-896	1461	762	NR	94°-60s 55°-60s 72°-120s } 30			+5s/ cycle	NR	94°-45s 55°-30s 72°-30s } 30		NR
Zienius *et al.*, (2008)	5'GTAACACCTCT ACAATGG3'	5'AGTTTCTTCA GCCATCTC3'	5'GGATGCC GACAAGATT GTAT3'	5'CTAAAGA CGCATGTTC AGAG3'	57-74	1568-1585	73-92	472-491	NR	400	95°-5min	94°-40s 56°-40s 72°-60s } 35			NR	95°-3min	94°-30s 60°-30s 72°-40s } 35		72 - 10min
Mulevaa *et al.*, (2012)	5'CTACAATGGAT GCCGAC3'	5'GAGTCACTCG AATATTGC3'	5'GACATGT CCGGAAGA CTGG3'	5'GTATTGCC TCTCTAGCG GTG3'	66-82	1402-1419	319-337	823-842	135 3	523	95°-5min	95°-60s 50°-60s 72°-60s } 35			72°-5min.	95°-5min	95°-60s 50°-60s 72°-60s } 35		72°- 5min.

Table 3: Comparison of different studies using Heminested RT-PCR for diagnosis of rabies virus.

Author	Primer sequence				Position*				Product size (bp)		Thermo cycling Conditions**							
	1st Round		2nd Round		1st Round		2nd Round		1st Round	2nd Round	1st Round				2nd Round			
	Forward Primer	Reverse Primer	Forward Primer	Reverse Primer	Forward Primer	Reverse Primer	Forward Primer	Reverse Primer			Initial Den	Den Ann Ext } No Of Cycle		Final Ext	Initial Den	Den Ann Ext } No Of cycle		Final Ext
Fraser *et al.*, (1996)	5’GAGAAAGAGA /CTG/TCAAGAA/ C/TA3’	5’CAGAGACAT ATCTG/CCG/TG /TATGTG3’	5’GAGAAAG AGA/CTG/TC AAGAA/C/T A3’	5’CTTCAC/T CG/TACCA/T C/TC/TGTTC ATCAT3’	1087	1279	1087	1227	NR	NR***	NR	94º-1m 37º-1m 72º-2m } **35**		NR	NR	94º-1m 37º-1m 72º-2m } **15**		NR
Heaton *et al.*, (1997)	5’ATGTAACACC (C/T)CTACAATT G3’	5’CAATTCGCA CACATTTTGTG 3’ + 5’CAGTTGGCA CACATCTTGTG 3’+ 5’CAGTTAGCG CACATCTTATG 3’	5’ATGTAAC ACC(C/T)CT ACAATTG3’	5’GTCATCA AAGTGTG(A /G)TGCTC3’ + 5’GTCATCAA TGTGTG(A/G) TGTTC3’ + 5’GTCATTAG AGTATGGTG TTC3’	55-73	641-660	55-73	617-636	606 bp	586 bp	95º-10min	95º-90s 45º-90s 50º-20s 72º-90s } 30s 60s 20s 60s } 30s 90s 20s **5 40 1**		72º-10m	95º-10 min	95º-90s 45º-90s 50º-20s 72º-90s } 30s 60s 20s 60s } 30s 90s 20s **5 25 1**		72º-10m
Soares *et al.*, (2002)	5’ATAGAGCAG ATTTTCGAGAC AGC3’	5’CCCATATAA CATCCAACA AAGTG3’	5’ATAGAGC AGATTTTCG AGACAGC3’	5’CCTCAAA GTTCTTGTG GAAGA3’	510	942	510	784	455 bp	295 bp	94º-3min	94º-45s 55º-60s 72º-90s } **35**		72º-10min.	94º-3min	94º-45s 55º-60s 72º-90s } **25**		72º-10min.
Cliquet *et al.*, (2003)	5’ATGTAACAC CYCTACAATG3’	5’CARTTVGCR CACATYTTRTG 3’	5’ATGTAAC ACCYCTAC AATG3’	5’GTCCCG AGTGAG ATCTTGA3’	55-74	641-660	55-74	447-465	606 bp	410 bp	95º-10min	94º-30s 55º-30s 72º-1m } **35**		72º-10min.	95º-10min	94º-30s 55º-30s 72º-1m } **25**		72º-10min.
Shankar *et al.*, (2004)	5’ATGTAACACC CCTACAATG3’	5’AGCTTGGCT GCATTCATGCC -3’	5’CAATATGA GTACAAGTA CCCGGC3’	5’AGCTTGG CTGCATTCA TGCC-3’	NR	NR	NR	NR	210 bp	122 bp	94º-1min	94º-30s 37º-30s 72º-90s } **40**		72º-7min.	94º-1min	94º-30s 37º-30s 72º-90s } **40**		72-7min.

Langoni *et al.*, (2005)	5’ATAGAGCAGATTTTCGAGACAGC3’	5’CCTCAAAGTTCTTGTGGAAGA3’	5’ATAGAGCAGATTTTCGAGACAGC3’	5’CCCATATAACATCCAACAAAGTG3’	505	937	505	779	432 bp	274 bp	94º-3min	94º-45s 55º-60s 72º-10m } **35**	72º-10min.	94º-3min	94º-45s 55º-60s 72º-10m } **35**	72º-10min.
Araujo *et al.*, (2008)	5’ATAGAGCAGATTTTCGAGACAGC3’	5’CCCATATAACATCCAACAAAGTG3’	5’ATAGAGCAGATTTTCGAGACAGC3’	5’CCTCAAAGTTCTTGTGGAAGA3’	510	942	510	784	455 bp	299 bp	95º-3min	94º-30s 55º-30s 72º-30s } **35**	72º-5min.	95º-3min	94º-30s 55º-30s 72º-30s } **35**	72º-5min.
Dacheux *et al.*, (2008)	5’ATGACAGACAAYYTGAACAA3’	5’TGACCATTCCARCARGTNG3’	5’ATGACAGACAAYYTGAACAA3’	5’GGTCTGATCTRTCWGARYAATA3’	7170	7489	7170	7419	319 bp	249 bp	94º-3min	94º-30s 56º-45s 72º-40s } **35**	72º-3min.	94º-3min	94º-30s 56º-45s 72º-40s } **35**	72º-3min.
Franka *et al.*, (2008)	5’CAGAGTTGTGCACCCCATGAA3’	5’TTGACAAAGATCTTGCTCAT3’	5’GAGAGAAGATTCTTCAGGGA3’	5’TTGACAAAGATCTTGCTCAT3’	1061-1081	1517-1536	1136-1155	1517-1536	475 bp	400 bp	94º-5min	94º-40s 56º-40s 72º-60s } **35**	72º-7min.	94º-3min	94º-30s 60º-30s 72º-40s } **35**	72º-7min.
Jackson *et al.*, (2008)	5’GARAGAAGATTCTTCAGRGA3’	5’TTGACGAAGATCTTGCTCAT3’	5’GAGAARGAACTTCARGAITA3’	5’TTGACGAAGATCTTGCTCAT3’	1136-1155	1514-1533	1157-1176	1514-1533	398 bp	377 bp	94º-1min	94º-30s 37º-30s 72º-90s } **40**	72º-7min.	94º-1min	94º-30s 37º-30s 72º-90s } **40**	72º-7min.
Orlowska *et al.*, (2008)	5’ATGTAACACCYCTACAATG3’	5’CAATTCGCACACATTTTGTG3’	5’ATGTAACACCYCTACAATG3’	5’ GTCATTAGAGTATGGTGTTC3’ or 5’GTCCCGAGTGAGATCTTGA3’	55-73	617-636	55-73	447-465	586 bp	410 bp	95º-15min	94º-30s 49º-30s 72º-1m } **35**	72º-10min.	95º-5min	94º-30s 55º-30s 72º-1m } **30**	72º-10 min.
Coertse *et al.*, (2010)	5’ACGCTTAACGAMAAA3’	5’GTRCTCCARTTAGCRCACAT3’	5’CACMGSNAAYTAYAARACNAA3’	5’GTRCTCCARTTAGCRCACAT3’	1-15	647-666	541-561	647-666	NR	126 bp	94º-1min	94º-30s 45º-30s 72º-1m } **40**	72º-7min.	94º-1min	94º-30s 45º-30s 72º-1m } **40**	72º-7min.
Panning *et al.*, (2010)	5’ATGTAACACCYCTACAATG3’	5’CAATTCGCACACATTTTGTG3’ + 5’CAGTTAGCGCACATCTTATG3’	5’ATGTAACACCYCTACAATG3’	5’GTCATCAATGTGTGATGTTC3’ + 5’GTCATTAGAGTATGGTGTTC3’	NR	NR	NR	NR	NR	NR	95º-15min	95º-20s 60º-30s 72º30s } 10 Annealing -1º/cycle In first 10 cycles 95º20s 52º30s 72º30s } 35	NR	94º-5 min	94º-20s 52º-20s 72º-30s } **35**	NR

Table 4: Comparison of different studies using TaqMan real time PCR for diagnosis of rabies virus.

Author	Name of primer/ probe/ assay			Sequence			Position*			Length (No. of Nucleotides)			Thermo cycling Conditions**			
	Forward Primer	Reverse Primer	Probe	Forward Primer	Reverse Primer	Probe	Forward Primer	Reverse Primer	Probe	Forward Primer	Reverse Primer	Probe	Reverse transcription	Initial Den	Den / Ann	No Of cycles
Hughes *et al.*, (2004)		AZ-EF		5'GAATCCTGATAGCACGGAGGG3'	5'CTTCCACATCGGTGCGTTTT3'	5'CAAGATCACCCCAAATTCTCTTGTGGACA3'	278-298	333-352	303-331	21	20	29				
		AZ-SK		5'GTCGGCTGCTATATGGGTCAG3'	5'ATCTCATGCGGAGCACAGG3'	5'TGAGGTCCTTGAATGCAACGGTAATAGCC3'	943–963	995–1013	965–993	20	19	29				
		CASK		5'TCATGATGAATGGAGGTCGACTC3'	5'TTGATGATTGGAACTGACTGAGACA3'	5'AGAGATCGCATATACGGAGAT3'	1226–1247	1296-1272	1249–1270	23	25	21		95ºC-10min	95ºC-15s / 60ºC-60s	40
		NCSK		5'GGTGAAACCAGAAGTCCGGAA3'	5'CCGTATATGCGATCTCTTTAGTCGA3'	5'CTGTCTATACTCGAATCATGA3'	1189-1209	1266–1242	1211–1227	21	25	21				
		RAC		5'TGGTGAAACCAGGAGTCCAGA3'	5'ATCTTTTGAGTCGGCCCCC3'	5'CGGTCTATACTCGGATCAT3'	1188–1208	1255–1235	1211-1227	21	19	19				
		SCSK		5'ATGATGAAGACTATTTCTCCGGTGAG3'	5'GTCGGCCTCCATTCATCATG3'	5'CGGAGGCAGTCTATAC3'	1169–1191	1246–1226	1202–1219	26	20	16				
Shankar *et al.*, (2004)	23F	20R	Probe	5'CAATATGAGTACAAGTACCCGGC3'	5'AGCTTGGCTGCATTCATGCC3'	5'AAGCCCAGTATAACCTTAGGAAA3'	NR	NR	112-134	23	20	23	50ºC- 2 min	95º-10min	95ºC-15s / 50ºC-60s	40
Wakeley *et al.*, (2005)	JW12	N 165–146	LysGT1	5'ATGTAACACCYCTACAATG3'	5'GCAGGGTAYTTRTACTCATA3'	5'ACAAGATTGTATTCAAAGTCAATAATCAG3'	55-73	165-146	81-109	19	20	29	32ºC – 30 min	94º-2min	94ºC-30s / 55ºC-30s	40

Foord *et al.*, (2006)	LYSF-YB	LYSR-YB	LYSF-YB-FAM	5’GAACGCCGCGAAGTTGG3’	5’AGATCCCCTCAAATAACTCCATAGC3’	5’CGGACGATGTTTGCTCCTACCTAGCTGC3’	191-207	240–264	211–238	17	25	28	50ºC-30min	95ºC -15min	94ºC-30s 55ºC-30s } 42
	LYSF-FF	LYSR-FF	LYSF-FF-FAM	5’TCGGGAATGAATGCTGCAA3’	5’GGCAGAYCCCCTCAAATAACTC3’	5’ACCCCGATGATGTATGTTCTTACTTAGCTGCAG3	183-201	267–247	208-239	19	21	32			
Orlowska *et al.*, (2008)	gt1L	gt1P	AWgt1	5’TACAATGGATGCCGACAAGA3’	5’CAAATCTTTGATGGCAGGGTA3’	5’TCAGGTGGTCTCTTTGAAGCCTGAGA3’	NR	NR	NR	20	21	26	NR***	NR	95ºC-15s 60ºC-60s } 40
Wacharaplue-sadee *et al.*, (2008)	1129F	1218R	RB probe	5’CTGGCAGACGACGGAACC3’	5’CATGATTCGAGTATAGACAGCC3’	5’TCAATTCTGATGACGAGGATTACTTCTCCGG3’	1129	1218	NR	18	22	31	NR	95ºC -15min	95ºC-0s 60ºC-60s } 45
	RABV D1 For	RABVD 1 Rev	RABV D1 Probe	5’ATGTAACACCYCTACAATG3’	5’GCMGGRTAYTTRTAYTCATA3’	5’56FAM/CCGAYAAGATTGTATTYAARGTCAAKAATCAGGT/3BHQ_1-3’	55 – 73	165-146	78 - 111	19	20	34	NR		
Nadin davis *et al.*, (2009)	RABV D2 For	RABVD 2 Rev	RAB D2 Probe	5’TRATGACAACYCACAARATGT3’	5’TGARCAGTCYTCRTARGC3’	5’56FAM/TAYGACATGTTTTTCTCYCGGATTGARCATC/3BHQ_1 3’	630 - 650	764-781	698-728	21	18	31	NR	95ºC -2min	95ºC-15s 50ºC-60s } 45
	RABV D3 For	RABVD 3 Rev	RABV D3 probe	5’AYTTCTTCCAYAARAACTTYGA3’	5’CATCCRACAAAGTGRATGAG3’	5’56FAM/TGYCCYGGCTCRAACATYCTYCTTAT/3BHQ_1 3’	846 - 867	1001-1020	900-875	22	20	26	NR		}

Coertse *et al.*, (2010)	550B	541lys	620lys	5’GTRCTCCARTTAGCRCACAT3’	5’CACMGSNAAYTAYAARACNAA3’	5’FAMCATCACACCTTGATGACAACTCACAA-BHQ-1 3’	647-666	541-561	620-645	20	21	26	NR	95ºC -15min	95ºC-5s 42ºC-15s **45**
Panning *et al.*, (2010)	RSS1	RSAs1	RSP	5’AGAAGGGAATTGGGCTTTGAC3’	5’AGATGCATGCTCGGGAACA3’	5’AATGGAACTGACGAGGGACCCCAT3’	NR	NR	NR	21	19	24	50º-30min	95º -15min	95ºC-10s 61ºC-30s **40**
Hoffmann *et al.*, (2010)	Jw12	N146-165	LysGT1B-FAM & LacZCy5FAM	5’ATGTAACACCYCTACAATG3’	5’GCAGGGTAYTTRTACTCATA3’	6-FAM-ACAAGATTGTATTCAAAGTCAATAATCAG-TAMRA & Cy5-TCCAGTCGG GAAA CCTGTCGTGCCA-BHQ3	55-73	146-165	81-109 & 56-80	19	20	29 25	50º-30min	95º -15min	94ºC-30s **42** 55ºC-30s
	Jw12	N146-165	RabGT1-B-FAM & LacZCy5FAM	5’ATGTAACACCYCTACAATG3’	5’GCAGGGTAYTTRTACTCATA3’	6-FAM-CAGCAATGCAGTTYTTTGAGGGGAC-TAMRA & Cy5-TCCAGTCGG GAAA CCTGTCGTGCCA-BHQ3	266-288	335-353	297-321 & 56-80	23	19	29 25			

Rabies RNA may be found in saliva, CSF, skin biopsy tissue and urine. Nested PCR techniques enhance the sensitivity. Real time PCR methods are being evaluated (Principles and Practices of Clinical Virology 2009).

Molecular techniques can improve clinical diagnosis. The best specimens include saliva, tear secretions, nuchal skin biopsy specimens, CSF and urine. Secretions of virus are intermittent in saliva, urine and even CSF (Principles of Neurologic Infectious Diseases 2005).

Postmortem Confirmation of Rabies

Molecular Approaches for Post-Mortem Confirmation:

Ermine *et al.,* (1989) attempted to improve the sensitivity of the rabies genome hybridization test, so PCR amplification was used following reverse transcription of rabies RNA extracted from infected brain.

Sacramento *et al.,* (1991) investigated the PCR amplification technique of viral nucleic acids as an alternative protocol for diagnosis and epidemiological studies of rabies virus.

Kamolvarin *et al.,* (1993) described a simple, sensitive, and specific polymerase chain reaction (PCR) protocol for detection of rabies virus. Rabies nucleocapsid sequence was amplified from all brain samples from 95 dogs and 3 humans with rabies confirmed by fluorescent antibody (FAT) and mouse inoculation tests (MIT).

McColl *et al.,* (1993) examined blood and post-mortem tissues from a 10-year-old girl and reported that results of fluorescent antibody test on brain smears, and immunoperoxidase test on formalin-fixed sections of brain were consistent with diagnosis of rabies. Polymerase chain reactions (PCRs) were conducted on a 10% suspension of a post-mortem sample from the patient's brain, and comparison with equivalent regions of known rabies viruses, confirmed that the fragments originated from a virus belonging to the rabies virus serotype. This case demonstrated the advantage of using a range of laboratory techniques to obtain a definitive diagnosis.

Nadin-Davis *et al.,* (1994) reported a protocol applying reverse transcription polymerasechain reaction (RT-PCR) and restriction endonuclease analysis (REA) to therabies virus nucleoprotein gene that was useful for discrimination of rabies virus variants in Ontario. Four main types, which showed no host species specificity but which did exhibit different geographical distributions, were identified.

Heaton *et al.,* (1997) described a heminested reverse transcriptase PCR (hnRTPCR) protocol which is rapid and sensitive for the detection of rabies virus and rabiesrelated viruses. Sixty isolates from six of the seven genotypes of rabies and rabies-related viruses were screened successfully by hnRT-PCR and Southern blot hybridization of the 60 isolates, 93% (56 of 60) were positive by external PCR, while all isolates were detected by heminested PCR and Southern blot hybridization.

Whitby *et al.,* (1997) concluded that reverse transcriptase-polymerase chain reaction (RT-PCR) was a useful additional tool for the detection of rabies and rabiesrelatedviruses, which was easy to perform and was rapid and highly sensitive.

Heaton *et al.,* (1999) reported a comparison of the sensitivity of the standard fluorescent antibody test (FAT) for rabies antigen and that of hnRT-PCR for rabies viral RNA with degraded tissue infected with a genotype 1 virus. Results indicated that FAT failed to detect viral antigen in brain tissue that was incubated at 37°C for greater than 72 h, while hnRT-PCR detected viral RNA in brain tissue that was incubated at 37°C for 360 h.

Kulonen *et al.,* (1998) compared direct immunofluorescence and PCR detection methods for sensitivity in evaluating the rabies status of archival specimens of Carnoy fixed, paraffin-embedded brain tissue. The immunofluorescence assay detected 100% (12/12) of the rabies-positive archival cases. A PCR assay designed to detect a 304-bp target spanning the 139-bp target of the first assay detected only 67% (8/12) of the original cases. No false positives were recorded. Both immunofluorescence detection of antigen and PCR detection of a short region of the nucleoprotein gene are useful in determining the rabies status of fixed, paraffin-embedded (archival) material.

Nadin-Davis (1998) reported that the relative temporally conserved nature of certain regions of the RV genome, particularly the N gene, permitted development of rapid molecular methods for RV typing wherein restriction fragment length polymorphism (RFLP) of PCR products and strain-specific PCR (SS–PCR), in which sequences of specific viral strains were amplified differentially using strain-specific primers.

Luo-Ting Rong *et al.,* (2000) developed a nested polymerase chain reaction (PCR) for detecting rabies virus and revealed that PCR could detect 3 TCID50 of rabies virus and gave a positive result with 0.8 pg of RNA. Nested PCR could identify RNA of rabies virus in the liver, heart, lung, and spleen of mice 5 days after inoculation.

David *et al.,* (2002) used the reverse transcriptase polymerase chain reaction (RTPCR), 10 decomposed brain samples collected between 1998 and 2000 that were diagnosed as negative by direct fluorescent antibody test (FAT), were found positive.

Three of the ten decomposed brains were confirmed as positive by isolation of rabies virus in tissue culture and by mouse inoculation (MIT), whereas the other seven decomposed samples were found positive only by RT-PCR.

Soares *et al.,* (2002) evaluated heminested-PCR (hnRT-PCR) using primers to the nucleoprotein-coding gene in a nested set in the detection of Brazilian strains of rabies virus (RV). A representative number of RV nucleoprotein sequences belonging to genotype 1 were aligned. Based on such alignment, primers were directed to highly conserved regions. All 42 clinical samples positive by both fluorescent antibody and mouse inoculation tests were also positive by the hnRT-PCR.

Romijn *et al.,* (2003) carried out an epidemic-geographic rabies study in which 72 animal and human brain samples were analyzed for Lyssaviruses by a direct immunofluorescent technique (DIFT) and a reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Fifty-two samples were also tested by a mouse inoculation test. Lyssavirus RNA was detected in 60 of 72 samples. All samples studied were of genotype1. With exception of the human sample, all were distinct from the reference sample.

Paez *et al.,* (2003) derived phylogenetic relationships between rabies viruses isolated in three regions in Colombia (Caribbean Region (northern Colombia), Arauca (eastern Colombia) and in the Central Region), 902ntcDNA fragments encoding the cytoplasmic domain of protein G and a fragment of protein L were obtained by RT-PCR.

This finding is the first that associates bats to rabies in Colombian dogs and humans, showing an unsuspected vector threatening animal and public health.

Hughes *et al.,* (2004) described a TaqMan PCR-based method for the detection of rabies virus (RV) RNA in tissue samples and showed the method has an acceptable linear range, is both sensitive and specific, and, importantly, correlate with the concentration of infectious virus. This study demonstrates that the genetic heterogeneity of RVs may prove a serious obstacle in the development of a diagnostic assay based on TaqMan PCR; however, the quantification of RV levels may prove to be a valuable application of this assay.

Picard Meyer *et al.,* (2004) developed a simplified hemi-nested reverse transcriptase polymerase chain reaction (hnRT-PCR) to determine specifically the European Bat Lyssa virus nucleoprotein gene (EBLV-1) and observed that compared to the rabies diagnostic methods, the hnRT-PCR showed a higher sensitivity for the detection of small amount of EBLV-1 virus.

Gupta *et al.,* (2005) used a method based on RT-PCR and restriction end onuclease digestion of amplified PCR product to differentiate rabies laboratory fixed and street viruses.

Lima *et al.,* (2005) evaluated the heminested RT-PCR for the study of rabies virus distribution in mice inoculated experimentally. Inoculation was by the intramuscular route in 150 mice, using the dog street rabies virus. HnRT-PCR was shown to be more efficient for the study of rabies virus distribution in different tissues and organs viz. brain, spinal cord, salivary gland, limbs, lungs, liver, spleen, urinary bladder, tongue and right kidney.

Wakeley *et al.,* (2005) described a single, closed-tube; none nested RT-PCR with TaqMan technology that distinguishes between classical rabies virus (genotype 1) and European bat lyssaviruses 1 and 2 (genotypes 5 and 6) in real time. The TaqMan assay is rapid, sensitive, and specific and allows for the genotyping of unknown isolates concomitant with the RT-PCR.

Nagarajan *et al.,* (2006) studied the molecular epidemiology of RV isolates in India based on nucleotide sequence analysis of 29 RV isolates originating from different species of animals in four states. Phylogenetic analysis using RT-PCR (one step RTPCR) revealed that the RV isolates belong to genotype 1 and that they were related geographically but were not related according to host species. Analysis of the data indicated that the dog rabies virus variants are the major circulating viruses in India that transmit the disease to other domestic animals and humans as well.

Junior *et al.,* (2006) produced a digoxigeninlabelled probe from the Pasteur virus strain for the detection of the rabies virus N gene. The probe hybridization was performed from amplified N gene obtained by reverse transcription polymerase chain reaction and the results by RT-PCR and hybridization showed 100% agreement.

Rojas *et al.,* (2006) used reverse transcription-polymerase chain reaction (RT-PCR) to determine the stability of rabies virus genomic RNA in brain samples. Reverse transcriptase PCR experiments were performed in 3 different inoculated brains, in which the direct fluorescent antibody (DFA) test was previously conducted to detect rabies viralantigen in the brains kept at room temperature and in the frozen brains. These results indicate that brain samples kept at ambient temperature (up to 27ºC) may reach a reference laboratory in an adequate state for rabies diagnosis by RT-PCR.

Biswal *et al.,* (2007) found examination of archival samples by molecular techniques as a valuable tool in providing retrospective and epidemiological data. Study was carried to evaluate the usefulness of RT-PCR in unfixed archival samples to assess whether a retrospective diagnosis of human rabies could be made from archival brain samples from patients suspected to have died of rabies. These results demonstrate the importance of RT-PCR in the detection of rabies virus RNA in 5-6 year old preserved samples without substantial loss.

Nadin-Davis *et al.,* (2007) compared selected Indian viruses with representative rabies viruses recovered worldwide by using nested PCR to amplify a portion of the viral N gene and showed a close association of all Indian isolates with the circumpolar Arctic rabies lineage distributed throughout northern latitudes of North America and Europe and other viruses recovered from several Asian countries.

Picard-Meyer *et al.,* (2007) evaluated the feasibility of the use of the FTA Gene Guard System (a commercial product consisting of filter paper impregnated with patented chemicals supplied by the Whatman Company) for the shipment, storage and detection of RNA rabies viruses by a simplified hemi-nested reverse transcriptase polymerase chain reaction (hnRT-PCR).

Wacharapluesadee *et al.,* (2008) developed a TaqMan real-time RT-PCR assay as an adjunct to FAT. Results were concordant with FAT. Thirteen rabies proven samples from Myanmar, Cambodia, Indonesia and India; 3 of which had up to 7 mismatches at primer/probe binding sites, could be detectable. This assay could be used as an adjunct to FAT and may serve as a rabies surveillance tool.

Araujo *et al.,* (2008) evaluated the RT-PCR and hnRT-PCR for rabies virus detection in original tissues stored at -20°C for different periods considering their use for rabies virus detection in stored and

decomposed samples. The RT-PCR and hnRT-PCR results were compared with previous results from Direct Fluorescent Antibody Test and Mouse Inoculation Test. From the 50 positive fresh samples, 26 (52%) were positive for RT-PCR and 45 (90%) for hnRT-PCR. From the 48 positive decomposed samples, 17 (34.3%) were positive for RT-PCR and 36 (75%) for hnRT-PCR.

Orlowska *et al.,* (2008) aimed at the comparison of the real-time PCR with the heminested RT-PCR method, both applied for the detection of nucleoprotein gene of rabies viruses in bats and terrestrial animals. The comparison of the methods revealed that the TaqMan PCR was 10-fold more sensitive than the heminested RT-PCR and the detection of rabies virus by this method was possible from 0.1 TCID50/mL on up.

Nadin Davis *et al.,* (2009) developed methods to detect viral RNA by TaqMan based quantitative reverse transcriptase polymerase chain reactions (qRT-PCRs) to improve timely ante-mortem human rabies diagnosis. Three sets of two primers and one internal dual-labelled probe for each primer set that target distinct conserved regions of the rabies virus N gene were designed and evaluated. These qRT-PCR assays were shown to be quantitative over a wide range of viral titre and were 100-1000 times more sensitive than nested RT-PCR.

Panning *et al.,* (2010) showed a direct comparison of virus isolation with quantitative real-time RT-PCR on human rabies samples. In this study, RT-PCR rendered to be more sensitive than virus isolation.

CONCLUSION

Day to day various reagents, readymade kits are being developed for obtaining high-quality total RNA in high yield, synthesis of cDNA from the RNA isolated, for common nucleic acid amplification applications such as Nested RT-PCR, heminested PCR, real time PCR. A great deal of valuable background work is going on developing primers, probes, reagent kits characterizing their use in the laboratory to obtain highly significant results for diagnosis of rabies. Here it was concluded that the various commercial preparations available in market for fast, reliable, and simplified processing of samples however there are many factors which limits the efficiency of them and needs to be evaluated for their proper optimization.

REFERENCES

Adeiga AA (2002). Molecular study of nucleoprotein gene of rabies virus artcle from dog brains. *African Journal of Clinical and Experimental Microbiology* **3**(2) 48-54.

Arai YT, Yamada K, Kameoka Y, Horimoto T, Yamomoto K, Yabe S, Nakayama M and Tashiro M (1997). Nucleoprotein gene analysis of fixed and street rabies virusvariants using RT-PCR. *Archives of Virology* **142** 1787–1796.

Araujo DB, Langoni H, Almeida MF and Megid J (2008). Heminestedreversetranscriptase polymerase chain reaction (hnRT-PCR) as a tool for rabies virus detection in stored and decomposed samples. *BMC Research Notes* 1-17.

Baer GM, Smith JS and Yager PA (1996). A rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus-neutralising antibody. In: Meslin FX, Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies*. 4th edn. Geneva, Switzerland WHO Baltazar 181-192.

Barbosa TFS, Medeiros DBA, Rosa EST, Casseb LMN, Medeiros R, Pereira AS,Vallinoto ACR, Vallinoto M, Begot AL, Lima RJS, Vasconcelos PFC and Nunes MR T(2007). Molecular epidemiology of rabies virus isolated fromdifferent sources during a bat-transmitted human outbreak occurring in AugustoCorrea municipality, Brazilian Amazon. *Virology* **370** 228–236.

Biswal M, Ratho R and Mishra B (2007). Usefulness of reverse transcriptase-polymerase chain reaction for detection of rabies RNA in archival samples. *Japanese Journal of Infectious Diseases* **60** 298-299.

Black EM, Lowings JP, Smith J, Heaton PR and McElhinney LM (2002). A rapid RT-PCR method to differentiate six established genotypes of rabies and rabies-related viruses using TaqMan technology. *Journal of Virological Methods* **105** 25–35.

Brito MG, Chamone TL, Silva FJ, Wada MY, Miranda AB, Castilho JG, Carrieri ML, Kotait I and Lemos FL (2011). Antemortem diagnosis of human rabies in a veterinarian infected when handling an herbivore in Minas Gerais, Brazil. *Revista Do Instituto De Medicina Tropical De* São Paulo **53**(1) 39-44.

Cliquet F, McElhinney LM, Servat A, Boucher JM, Lowings T and Goddard T (2003). Development of a qualitative indirect ELISA for the measurement of rabies virus specific antibodies from vaccinated dogs and cats. *Journal of Virological Methods* **117** 1– 8.

Crepin P, Audry L, Rotivel Y, Gacoin A, Caroff C and Bourhy H (1998). Intravitamdiagnosis of human rabies by PCR using saliva and cerebrospinal fluid. *Journal ofClinical Microbiology* **36** 1117–1121.

Coertse J, Weyer J, Nel LH and Markotter W (2010). Improved PCR Methods forDetection of African Rabies and Rabies-Related Lyssaviruses. *Journal of Clinical Microbiology* **48**(11) 3949–3955.

Dacheux L, Reynes JM, Buchy P, Sivuth O, Diop BM, Rousset D, Rathat C, Jolly N Dufourcq JB, Nareth C, Diop S, Rajerison ICR, Sadorge C and Bourhy H (2008). A Reliable Diagnosis of Human Rabies Based on Analysis of Skin BiopsySpecimens. *Clinical Infectious Diseases* **47** 1410–1417.

David D, Yakobson B, Rotenberg D, Dveres N, Davidson I and Stram Y (2002). Rabies virus detection by RT-PCR in decomposed naturally infected brains. *Veterinary Microbiology* **87**(2) 111-118.

East ML, Hofer H, Cox JH, Wulle U, Wiik H and Pitra C (2001). Regular exposure torabies virus and lack of symptomatic disease in Serengeti spotted hyenas. *Proceedings of National Academy of Sciences USA* **98** 15026–15031.

Elmgren LD, Nadin-Davis SA, Muldoon FT and Wandeler A I (2002). Diagnosisandanalysis of a recent case of human rabies in Canada. *Canadian Journal of Infectious Diseases* **13**(2) 129-133.
Echevarría JE, Avellón A, Juste J, Vera M and Ibanez C (2001). Screening of activelyssavirus infection in wild bat populations by viral RNA detection onoropharyngeal swabs. *Journal of Clinical Microbiology* **39**(10) 3678-3683.
Emmons RW (1979). Rabies diagnosis and rabies vaccine. *New England Journal of Medicine* **301** 331–332.
Fauquet CM, Mayo MA, Maniloff J, Desselberger U and Ball LA (2005). Virustaxonomy: classification and nomenclature of viruses. Eighth report of theInternational Committee on the Taxonomy of Viruses. *Family Rhabdoviridae*. Academic Press, San Diego 623–633.
Fishbein DB, Smith JS, Rupprecht C and Clark K (1991). Unexplained rabies in threeimmigrants in the United States. A virologic investigation. *New England Journal of Medicine* **324** 205-11.
Foord AJ, Heine HG, Pritchard LI, Lunt RA, Newberry KM, Rootes CL and Boyle DB (2006). Molecular diagnosis of lyssa viruses and sequence comparison of Australian bat lyssa virus samples. *Australian Veterinary Journal* **84** 225–230.
Franka R, Svrcek S, Madar M, Kolesarova M, Ondrejkova A, Ondrejka R, Benisek Z,Suli J and Vilcek S (2004). Quantification of the effectiveness of laboratorydiagnostics of rabies using classical and molecular-genetic methods. *Veterinarni Medicina 49* 259–267.
Fraser GC, Hooper PT, Lunt RA, Gould AR, Gleeson LJ, Hyatt AD, Russell GM and Kattenbelt JA (1996). Encephalitis caused by a lyssa virus in fruit bats in Australia. *Emerging Infectious Diseases* **2** 327–331.
Goldwasser RA and Kissling RE (1958). Fluorescent antibody staining of street and fixed rabies virus antigen. *Proceedings of Society for Experimental Biology and Medicine 98* 291-223.
Gupta PK, Singh RK, Sharma RN, Rao YUB and Butchaiah G (2010). Preliminary report on a single-tube, non-interrupted reverses transcription polymerase chain reaction for the detection of rabies virus in brain tissue. *Veterinary Research Communications* **25**(3) 239-247.
Hemachudha T (1994). Human rabies: clinical aspects, pathogenesis, and potential therapy, In: Lyssaviruses, edited by C. E. Rupprecht, B. Dietzschold, and H. Koprowski, Springer-Verlag, Berlin, Germany. Hoffmann B, Freuling C M, Wakeley P R, Rasmussen T B, Leech S, Fooks A R, Beer M and Muller T. 2010. Improved Safety for Molecular Diagnosis of Classical Rabies Viruses by Use of a TaqMan Real-Time Reverse Transcription-PCR "Double Check" Strategy. *Journal of Clinical Microbiology* **48**(11) 3970–3978.
Heaton PR, Johnstone P, Mcelhinney LM, Cowley R, o'sullivan E and Whitby JE (1997). Heminested PCR assay for detection of six genotypes of rabies and rabies related viruses. *Journal of Clinical Microbiology* **35** 2762–2766.
Hoffmann B, Freuling CM, Wakeley PR, Rasmussen TB, Leech S, Fooks AR, Beer M and Muller T (2010). Improved Safety for Molecular Diagnosis of Classical RabiesViruses by Use of a TaqMan Real-Time Reverse Transcription-PCR "DoubleCheck" Strategy. *Journal of Clinical Microbiology* **48**(11) 3970–3978.
Hughes GJ, Smith JS, Hanlon CA and Rupprecht CE (2004). Evaluation of a TaqMan PCR Assay to Detect Rabies Virus RNA: Influence of Sequence Variation and Application to Quantification of Viral Loads. *Journal of Clinical Microbiology* **42**(1) 299–306.
Jackson FR, Turmelle AS, Farino DM, Franka R, McCracken GF and Rupprecht CE (2008). Experimental rabies virus infection of big brown bats (*EptesicusFuscus*). *Journal of Wildlife Diseases* **44**(3) 612–621.
Junior PC, Ventura AM and Durigon EL (2006). Digoxigenin-labeled probe for rabiesvirus nucleoprotein gene detection. *Revista da Sociedade Brasileira de Medicina Tropical* **39**(2) 159-162.
Kamolvarin N, Tirawatnpong T, Rattanasiwamoke R, Tirawatnpong S,PanpanichTand Hemachudha T (1993). Diagnosis of Rabies by Polymerase Chain Reaction withNested Primers. *Journal of Infectious Diseases* **167** 207–210.
Kasempimolporn S, Sichanasai B, Saengseesom W, Puempumpanich S, ChatrapornS,Sitprija V (2006). Prevalence of rabies virus infection and rabies antibody in stray dogs: A survey in Bangkok, Thailand. *Preventive Veterinary Medicine.*
Knobel DL, Cleaveland S, Coleman PG, Fevre EM, Meltzer MI, Miranda MEG, Shaw A, Zinsstag J and Meslin FX (2005). Re-evaluating the burden of rabies in Africa and Asia. *Bulletin World Health Organization 83* 360-368.
Kulonen K, FekaduM , Whitfield S and Warner CK (1998). An Evaluation ofImmunofluorescence and PCR.Methods for Detection of Rabies in Archival Carnoy-Fixed, Paraffin-Embedded Brain Tissue. *Journal of Veterinary Medicine Series B* **46**(3) 151-156.
Langoni H, Lima K, Menozzi B D and Silva R C (2005). Rabies in the big fruit eating bat *Artibeuslituratus*from botucatu, Southeastern Brazil. *Journal Venom. Anim. Toxinsincl. Trop. Dis.* **11**(1) 84-87.
Lopes MC, Venditti LLR, Queiroz LH (2010). Comparison between RT-PCR and the mouse inoculation test for detection of rabies virus in samples kept for long periods under different conditions. *Journal of Virological Methods* **164** 19–23.
Macedo CI, CarnieliJr P, Brandao PE, Travassos da Rosa ES, Oliveira Rde N, Castilho JG, Medeiros R, Machado RR, Oliveira RC, Carrieri ML and Kotait I (2006). Diagnosis of human rabies

cases by polymerase chain reaction of neck-skin samples. *Brazilian Journal of Infectious Diseases* **10**(5) 341-345.

Madhusudana SN and Sukumaran SM (2008). Antemortem diagnosis and prevention of human rabies. *Annals of Indian Academy Neurology* **11**(1) 3–12.

Muleyaa W, Namangalac B, Mweened A, Zulud L, FandamueP,Bandad D, KimuraaT,Sawaa H and Ishii A (2012). Molecular epidemiology and a loop-mediatedisothermal amplification method for diagnosis of infection with rabies virus in Zambia *Virus Research* **163** 160–168.

Panning M, Baumgarte S,Maier SPT, Martens A and DrostenC (2010). Comparative Analysis of Rabies Virus Reverse Transcription-PC Rand Virus Isolation Using Samples from a Patient Infected with Rabies Virus. *Journal Of Clinical Microbiology* **48** (8) 2960–2962

Nadin-Davis SA (1998). Polymerase chain reaction protocols for rabies virus discrimination. *Journal of Virological Methods* **75** 1-8.

Nadin-Davis SA, Turner G, Paul JPV, Madhusudana SN, and Wandeler AI (2007). Emergence of Arctic-like Rabies Lineage in India. *Emerging Infectious Diseases* **13**(1) 111-116.

Nadin Davis SA, Sheen M and Wandeler AI (2009). Development of Real-Time Reverse Transcriptase Polymerase Chain Reaction Methods for Human Rabies Diagnosis. *Journal of Medical Virology* **81** 1484-1497.

Nagarajan T, Mohanasubramanian B, Seshagiri EV, Nagendrakumar SB, Saseendranath MR, Satyanarayana ML, Thiagarajan D, Rangarajan PN and Srinivasan VA(2006). Molecular Epidemiology of Rabies Virus Isolates in India. *Journal of Clinical Microbiology* **44**(9) 3218–3224.

Nagarajan, T, Nagendrakumar SB, Mohannasubramanian B, Rajalakshmi S, Hanumantha NR, Ramya R, Thiagarajan D and Srinivasan VA (2009). Phylogenetic analysis of nucleoprotein gene of dog rabies virus isolates from Southern India. *Infection, Genetics and Evolution* **9** 976–982.

Negri A (1903). Beitragzumstudium der Actiologie der Tollwuth. *Z Hygiene Infektions Kr 43* 507.

Orłowska A, Smreczak M, Trębas P and Żmudziński JF (2008). Comparison Of Real-Time Pcr And HeminestedRt-Pcr Methods In The Detection Of Rabies VirusInfection In Bats And Terrestrial Animals *Bulletin of Veterinary Institute of Pulawy* **52** 313-318.

QureshiZafarul A, Numan M, Manzoor AW, Afzaal S, Hussain Z (2011). Coincidence of Haemorrhagic Septicemia and Rabies. *Materia Socio-Medica.* **23**(4) 192-194.

Reynes JM, Andriamandimby SF, Razafitrimo GM, Razainirina J, Jeanmaire EM,Bourhy H and Heraud JM (2011). Laboratory Surveillance of Rabies in Humans, Domestic Animals and Bats in Madagascar from 2005 to 2010. *Advances in Preventive Medicine* 1-6.

Rojas AE, Loza-Rubio E, Manuel Banda RV and Herna´ndez BE (2006). Use of reverse transcription-polymerase chain reaction to determine the stability of rabies virus genome in brains kept at room temperature. *Journal of Veterinary Diagnostic Investigation* **18** 98–101.

Rupprecht E, Smith JS, Fekadu M and Childs JE (1995). The ascension of wildlife rabies: a case for public health concern or intervention? *Emerging Infectious Diseases* **11** 07–14.

Sacramento D, Bourhy H and Tordo N (1991). PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. *Molecular and Cellular Probes* **5**(3) 229-240.

Schneider LG (1975). Spread of virus from the central nervous system. In: Baer, G.M. (Ed.), *the Natural History of Rabies*. Academic Press, New York 273–301.

Shankar V, Bowen RA, Davis AD, Rupprecht CE and O'Shea TJ (2004). Rabies in acaptive colony of big brown bats (*Eptesicus Fuscus*). *Journal of Wildlife Diseases* **40**(3) 403–413.

Saengseesom W, Mitmoonpitak C, Kasempimolporn S and Sitprija V (2007). Real-time PCR analysis of dog cerebrospinal fluid and saliva samples for ante-mortem diagnosis of rabies. *Southeast Asian Journal of Tropical Medicine and Public Health* **38**(1) 53-57.

Soares RM, Bernardi F, Sakamoto SM, Heinemann MB, Cortez A, Alves LM, Meyer AD, Ito FH and Richtzenhain LJ (2002). A Heminested Polymerase Chain Reaction for the Detection of Brazilian Rabies Isolates from Vampire Bats and Herbivores. *Memorias do Instituto Oswaldo Cruz* **97**(1) 109-111.

Strauss R, Granz A, Wassermann-Neuhold M, Krause R, Bago Z, Revilla-Fernandez S, Simon-Soria F, Echevarria JE, Popow-Kraupp T, Allerberger F, Schonbauer Mand Hrabcik H(2005). A human case of travel-related rabies in Austria, September 2004. *European Surveillance* **10**(11).

Timoney JF, Gillespie JHand Scott FW (1988). *Hagan and Bruner's Microbiology and Infectious Disease of Domestic Animals*, 8 ed. Cornell University Press, Ithaca and London 832–846.

Tordo N, Poch O, Ermine A, Keith G and Rougeon F (1988). Completion of the rabiesvirus genome sequence determination: highly conserved domains among the L (polymerase) proteins of unsegmented negative-strand RNA viruses. *Virology* **165** 565–576.

Vazquez-Moron S, Avellon A and Echevarria JE (2006). RT-PCR for detection of all seven genotypes of Lyssavirus genus. *Journal of Virological Methods* **135** 281–287.

Wacharapluesadee S and Hemachudha T (2010). Ante-mortem and post-mortem diagnosis of rabies using nucleic acid-amplification tests. *Expert Reviews on Molecular Diagnosis* **10**(2) 1-12.

Wacharapluesadee S, Sutipanyaa J, Damrongwatanapokinb S, Phumesina P, Chamnanpoodb P, Leowijukb C and Hemachudhaa T (2008). Development of aTaqMan real-time RT-PCR assay for the detection of rabies virus. *Journal of Virological Methods* **151** 317-320.

Wakeley PR, Johnson N, McElhinneyLM., Marston D, Sawyer J and Fooks AR (2005). Development of a Real-Time, TaqMan Reverse Transcription-PCR Assay for Detection and Differentiation of Lyssa virus Genotypes 1, 5, and 6. *Journal of Clinical Microbiology* **43** 2786–2792.

Whitby JE, Heaton PR, Sullivan E and Johnstone P (1997). Rapid detection of rabies and rabies related viruses by RT-PCR and ELISA. *Journal of Virological Methods* **69** 63-72.
Wunner WH, Larson JK, Dietzschold B and Smith CL (1988). The molecular biology of rabies viruses. *Reviews of Infectious Diseases* **10** 771-784.
Zhang K, Guo J, Xu Z, Xiang M, Wu B and Chen H (2011). Diagnosis and molecular characterization of rabies virus from a buffalo in China: a case report. *Virology Journal* **8** 101.
Zienius D, Sajute K, Zilinskas H and Stankevicius A (2008). Phylogenetic Analysis of the Rabies Virus N-coding Region in Lithuanian Rabies Isolates. *Acta Veterinaria Brno* **78** 273–280.