ANTE-MORTEM DIAGNOSIS OF RABIES IN ANIMALS BY REAL TIME PCR

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE
in
VETERINARY PATHOLOGY
(Minor Subject: Veterinary Microbiology)

By

Karan Bansal
(L-2009-V-26-M)

Department of Veterinary Pathology
College of Veterinary Science
Guru Angad Dev Veterinary and Animal Sciences University,
Ludhiana 141 004

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CERTIFICATE – I

This is to certify that the thesis entitled, "**ANTE-MORTEM DIAGNOSIS OF RABIES IN ANIMALS BY REAL TIME PCR"** submitted for the degree of M. V. Sc., in the subject of **Veterinary Pathology** (Minor Subject: **Veterinary Microbiology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Karan Bansal (L-2009-V-26-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

(Dr. C.K. Singh)
Major Advisor
Senior Veterinary Pathologist
Department of Veterinary Pathology
College of Veterinary Sciences
Guru Angad Dev Veterinary and Animal Sciences University,
Ludhiana-141004, India

CERTIFICATE – II

This is to certify that the thesis entitled, **"ANTE-MORTEM DIAGNOSIS OF RABIES IN ANIMALS BY REAL TIME PCR"** submitted by **Karan Bansal (L-2009-V-26-M)**, to Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in partial fulfilment of the requirements for the degree of M. V. Sc., in the subject of **Veterinary Pathology** (Minor Subject: **Veterinary Microbiology**) has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

Head of the Department
(Dr. N.K. Sood)

Major Advisor
(Dr. C.K. Singh)

Dean, Postgraduate Studies
(Dr. S. N. S. Randhawa)

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ABSTRACT

Rabies is a progressive, fatal, zoonotic disease of mammals caused by single stranded RNA virus which belongs to genus *Lyssavirus* of family *Rhabdoviridae.* The present study was conducted on 20 animals suspected for rabies. Diagnosis was confirmed by molecular approaches along with conventional techniques. Nested RT-PCR and TaqMan real time PCR were applied on 20 skin biopsy and 11 saliva samples. Amplification with nested set of primers (Rab Nfor and Rab Nrev) yielded a 762 bp product. By nested RT-PCR, viral RNA could be detected in 36.36% (4/11) saliva samples and 45.0% (9/20) skin biopsy samples. By TaqMan real time PCR the cycle threshold (*C*t) values ranging from 26 to 35 cycles were obtained. By TaqMan real time PCR, viral RNA could be detected in 54.54% (6/11) saliva samples and 55.0% (11/20) skin biopsy samples. Sensitivity of 86.67% & 76.47% was obtained with application of TaqMan real time PCR and Nested RT-PCR, respectively on skin biopsy samples while, sensitivity of TaqMan real time PCR and nested RT-PCR on saliva samples was found to be 80% & 66.67%, respectively. Molecular and conventional approaches were applied on brain samples for post-mortem confirmation of rabies. Out of 20 brain samples, 12 were positive by nested RT-PCR, with a sensitivity of 92.85% while, TaqMan Real time analysis could detect rabies in 13/20 brain samples with a sensitivity of 100%. The results obtained were in concordance with conventional immunofluorescence that confirmed 13/20 (65%) rabies positive cases. It was concluded that skin offers greater sensitivity for ante-mortem diagnosis of rabies as compared to saliva. Among the molecular approaches, TaqMan real time PCR technique was found to be more sensitive than nested PCR for ante-mortem diagnosis of rabies from skin as well as saliva.

Key words: Ante-mortem diagnosis, Nested RT- PCR, Rabies, Skin, Saliva, TaqMan real time PCR

Signature of Major Advisor Signature of the student

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LIST OF ABBREVIATIONS

%	: percent
@	: at the rate of
+	: plus
-	: minus
µg	: microgram
µl	: microlitre
ºC	: degree celcius
bp	: base pair
cm (s)	: centimetre (s)
CSF	: cerebrospinal fluid
Ct	: threshold cycle
cDNA	: complementary deoxyribonucleic acid
DNA	: deoxyribonucleic acid
dNTP (s)	: deoxy ribonucleotide tri phosphate (s)
DPI	: days post inoculation
EDTA	: ethylene diamine tetra acetic acid
ELISA	: enzyme linked immunosorbent assay
F	: female
FAM	: fluorescent reporter dye
FAT	: fluoroscent antibody test
g	: gram
H & E	: haemotoxylin and eosin
hr/hrs	: hour (s)
hnRT-PCR	: heminested reverse transcriptase PCR
kb	: kilobases
M	: male
mA	: milli ampere
mg	: milligram
min (s)	: minute (s)

MIT	: mouse inoculation test
ml	: millilitre
mm	: milli metre (s)
mM	: milli molar
mth (s)	: month (s)
NA	: not applicable
ng	: nanogram
nm	: nanometre
NR	: not reported
nt (s)	: nucleotide (s)
OD	: optical density
PBS	: phosphate buffered saline
PCR	: polymerase chain reaction
pg	: pico gram
pH	: hydrogen ion concentration
pmol	: pico mole
RNA	: ribonucleic acid
rpm	: revolutions per minute
RT-PCR	: reverse transcriptase PCR
RV	: rabies virus
s	: second
TAMRA	: quencher dye
TBE	: tris borate EDTA
TE	: tris EDTA
U	: unit
UV	: ultraviolet ray
V	: volt
viz.	: namely
Wks	: weeks
w/v	: weight/volume
yr (s)	: year (s)

CHAPTER I

INTRODUCTION

Rabies is a viral zoonotic 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 75 nm (Tordo *et al* 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).

In India rabies is enzootic and is a serious public health and economic problem (Nagaranjan *et al* 2006). A National Rabies Survey in India was conducted by the Association for Prevention and Control of Rabies in India in collaboration with WHO found that 20,565 persons die of rabies each year (Sudarshan *et al* 2007).

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 *et al* 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 and Kissling 1958). WHO and OIE have recommended the FAT as the primary laboratory method for diagnosis of rabies. But application of this approach is often possible only post mortem; however, with the advent of molecular approaches, it is now possible to detect rabies ante-mortem with the advent of molecular approaches.

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-mortem diagnosis. 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 (Wacharapluesadee and Hemachudha 2010).

So far, systematic study has not been conducted to elucidate the significance of TaqMan real time PCR for ante-mortem detection of rabies in natural cases in animals in India. Thus, the present investigation is envisaged with the following objectives;

1. To standardize TaqMan real time PCR for ante-mortem detection of rabies virus from skin and saliva.
2. To compare the relative efficacy of molecular detection of rabies in skin samples with that in saliva in natural cases of rabies in animals.

CHAPTER II

REVIEW OF LITERATURE

2.1 Clinical symptoms

The appearance of specific rabies disease symptoms is preceded by prodromal period in which there are a number of non-specific symptoms of malaise (Bishop 1979). Sinchaisri *et al* (1992) suggested that clinical signs such as ataxia or depression leading to death may be due to the direct effect of the virus on the functions of neuronal cells, but not to inflammatory reactions. Hemachudha (1994) suggested clinical diagnosis of rabies by epidemiological (history of exposure) and clinical (e.g., paraesthesia, hydrophobia) findings. Woldehiwet (2005) described that the clinical disease can be divided into three phases: (1) Prodromal phase; characterized by marked change in behaviour, (2) Excitation phase, furious rabies or acute neurologic phase, and (3) Paralytic phase, dumb rabies or coma preceding death. However, these phases may not be distinct, as some of the early phases are not always apparent.

Martinez-Burnes *et al* (1997) observed that the animals with rabies exhibit signs of fever, photophobia, ptyalism, in coordination followed by prostration, paralysis, and loss of cutaneous sensitivity.

In rabid cattle and buffalo symptoms like anorexia, hyper salivation, constipation, conjunctivitis, corneal opacity and nervous signs were observed by (Srinunthapanth *et al* 1985) while ataxia, sluggish movements, recumbence and pharyngeal paralysis were observed by (Tanyi *et al* 1988). Salem *et al* (1995) observed clinical signs in buffaloes

that included anorexia, hydrophobia, dilatation of pupils, frequent bellowing, aggression, excitability, hypersensitivity, in coordination, recumbency and death within 5-10 days.

Hudson *et al* (1996a) observed signs of excessive salivation, behavioural changes, muzzle tremors, hyper aesthesia, aggression and pharyngeal paralysis in experimentally induced rabies. Singh and Grewal (1998b) reported the symptoms of experimental rabid buffalo calves which included bellowing, conjunctivitis, ocular and nasal discharge, arched back and sluggish movements. In experimentally inoculated buffalo calves with SRV ocular discharge, nasal discharge and sluggish movements in animals sacrificed at 30 and 60 days post inoculation (DPI). The animals revealed disinclination to move w.e.f. 50 DPI (Asha Rani 2001). Aytekin and Mamak (2009) reported the symptoms of anorexia, apathy, pain, salivation, tenesmus, bellowing with tongue hanging out, leaning against objects, attempting to bite of objects, occasional postural appearance of kyphosis, hydrophobia and dysphagia in cattle suffering from rabies. Pedroso *et al* (2009) reported that paralytic form was the most common clinical picture in cattle and included in coordination, paresis, and paralysis of the pelvic members, besides recumbence, paddling and death.

In dog's recumbency, dropped jaw, dyspnoea, ataxia, salivation, conjunctiva congestion and glazed eyes were observed by (Okolo 1986). Fekadu *et al* (1988) studied the development of symptoms and excretion of rabies virus in dogs experimentally infected by street virus. Silva *et al* (2004) reported that aggressive behaviour was observed in 77% of rabid dogs, followed by lack of coordination and paralysis 42% and 48% of these dogs were responsible for biting people or other animals.

Eng and Fishbein (1990) observed that clinical signs of rabies varied. Rabid cats were more likely than dogs to have aggressive behaviour (55% in cats and 31% in dogs). Fogelman *et al* (1993) reported major signs of rabies in cats that included behaviour change, gait abnormality, strange or unusual look in the eyes and a wound within the preceding 6 months. Frymus *et al* (2009) reported exaggerated emotional responses viz. irritability, rage, photophobia, attacking inanimate objects, seizures, muscular twitching or tremors and aimless pacing in cats.

In equine species muzzle tremors were the most common and initial sign besides pharyngeal spasm, ataxia, lethargy and furious form in experimentally induced rabies in horses (Hudson *et al* 1996b). Url *et al* (2004) described clinical and diagnostic peculiarities concerning equine rabies on the basis of a rabid horse originating from the area of Austria and reported apathy, colic and unspecific CNS symptoms in an 18 year old warm blooded gelding. Luciano *et al* (2009) reported decrease or absence of sensitivity testing of the anus, tail and varying degrees of paresis and paralysis in horses.

In sheep, Baltazar *et al* (1986) observed symptoms of experimental rabies in 18 sheep, produced by street rabies virus of fox origin by intra-muscular route and mainly included refusal to eat, drink, paresis, paralysis, muscular tremors and death ensuing in 2-4 days. Soria Baltazar *et al* (1988) studied the effect of the inoculation of a canine strain of rabies virus in sheep and clinical signs observed were anorexia, emaciation, nervous reactions and prostration before death. Rissi *et al* (2008) reported the signs of abnormal gait, trembling, lateral recumbency, convulsion, opisthotonus, and fever in sheep suffering from rabies.

In camels, Afzal *et al* (1993) reported hyper excitability, attacking inanimate objects, self-biting of forelimbs, colic, drooling of saliva, internal recumbency and paralysis of hind limbs.

In wild animals, **Delpietro *et al* (2009) reported** signs of paralysis, difficulty in walking, opisthotonus, pedalling of posterior limbs and aggressiveness**.** Oertli *et al* (2009) reported typical behaviours of rabid skunks were entering a dog pen, appearing outside during daytime, and attacking pets. Delpietro *et al* (1990) conducted seven trials, in which infected Calomys Musculinus had evident nervous symptomatology consisting of excitability aggressiveness and paralysis.

2.2 Collection of saliva samples

Cotton swabs were used for collection of saliva samples directly from oral cavity of rabies suspected animal by Larghi *et al* (1975), Crepin *et al* (1998), Shankar *et al* (2004) and Johnson *et al* (2008).

Oropharyngeal swabs were collected (from bats) and stored in tubes containing 1.5 ml lysis buffer at -80ºC by Echevarria *et al* (2001), Jackson *et al* (2008) and Reynes *et al* (2011).

Sponge tipped applicators were immersed into 2ml of phosphate buffered saline prior to swabbing the anterior surface of tongue and cheek mucosa (of the dog) for 15-20 sec. by Kasempimolporn *et al* (2000) and Saengseesom *et al* (2007).

Aspiration technique was reported by Crepin *et al* (1998) for daily collection of saliva samples (from human patients) for intravitam diagnosis of rabies.

Liquid saliva sample (200 µl) was preferred than saliva swab by Nagaraj *et al* (2006), Dacheux *et al* (2008), Hemachudha and Wilde (2009), Wacharapluesadee and Hemachudha (2010) and Brito *et al* (2011).

Use of sterile eye dropper pipette and syringe for collection of saliva sample directly from the mouth of animal was suggested by centres for disease control and prevention (CDC) and Kaw *et al* (2011).

Due to intermittent secretion of rabies virus in saliva, serial successive sampling without addition of any preservative was suggested by most of researchers.

2.3 Collection of skin biopsy samples

Skin biopsy punch (Stiefel), was used to collect skin biopsy samples (diameter, ~ 4 mm; total volume, 20 mm^3) from the nape of neck by Dacheux *et al* (2008) and Reynes *et al* (2011). According to centre for disease control and prevention (CDC), section of skin 5 to 6 mm in diameter should be taken from the posterior region of the neck at the hairline. The biopsy specimen should contain a minimum of 10 hair follicles and be of sufficient depth to include the cutaneous nerves at the base of the follicle.

Approximately $1cm^2$ samples of neck-skin biopsy were collected by Macedo *et al* (2006) and Brito *et al* (2011) for diagnosis of rabies.

Rabies virus detection from nuchal skin biopsy was also reported by Plotkin (2000), Elmgren *et al* (2002), Nadin Davis *et al* (2009) and Panning *et al* (2010).

2.4 Extraction of RNA

2.4.1 Saliva samples

TRIzol® (Gibco BRL, USA) reagent was used for extraction of RNA from saliva specimens by Elmgren *et al* (2002) and Saengseesom *et al* (2007). Precipitated RNA was dissolved in 20 to 40 µl of diethyl polycarbonate water and stored at -80ºC for further use.

TRIzol® (Invitrogen, Canada) reagent protocol was used for extraction of RNA by Shankar *et al* (2004) and Brito *et al* (2011). Former also supported addition of 100 µl lysis buffer (10 mM Tris-HCL, pH 7.5, 150 mM Nacl, 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) was 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 by Jackson *et al* (2008).

Glycoblue (Ambion) and Glycogen (Ambion) were used as coprecipitant along with Trizol LS reagent for extraction of RNA by Hughes *et al* (2004) and Dacheux *et al* (2008), respectively.

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.

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

2.4.2 Skin samples

TRIzol® reagent (Invitrogen, Canada) was used for extraction of RNA from skin biopsy samples by Macedo *et al* (2006), Nadin Davis *et al* (2009) and Brito *et al* (2011).

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.

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).

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

2.4.3 Brain samples

TRIzol® (Gibco BRL, USA) reagent was used for extraction of RNA from rabies virus infected BHK-21 monolayer or infected mouse brain tissue by Heaton *et al* (1997).

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

1µl of Glycoblue (Ambion) was used as coprecipitant along with 750µl of TRIzol reagent (Invitrogen Life Technologies, Carlsbad, Calif.) 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.

TRIzol LS reagent (Invitrogen, Canada) was used for extraction of RNA from 10% homogenate of mouse brain passaged human isolate by 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.

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.

2.5 Synthesis of cDNA

AccessQuick® kit (Promega, USA) was used by Echevarria *et al* (2001), Hughes *et al* (2004) and Saengseesom *et al* (2007) for synthesis of cDNA in a one step process.

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 (10 U and 200 U, Invitrogen) was used by Barbosa *et al* (2007) and Dacheux *et al* (2008), respectively for synthesis of cDNA.

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).

Avian myeloblastosis virus reverse transcriptase (4-12U; Promega, WI) were used for synthesis of cDNA by Kamolvarin *et al* (1993).

Avian myeloblastosis virus reverse transcriptase (8U; Roche Diagnostics, USA) has also been reported by Shankar *et al* (2004) and Jackson *et al* (2008) for converting RNA into cDNA.

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 Wakeley *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.

2.6 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).

2.6.1 Saliva

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.

Table 1: Comparison of different studies using Standard 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'TTTGAGACTGCTCCTTTT3'	5'CCCATATAGCATCCTAC3'	587-605	1013-1029	443bp	NR	NR***	NR
Arai *et al* (1997)	5'CTACAATGGATGCCGAC3'	5'TGGGGTGATCTT(A/G)TC TCCTTT3'	66-82	365-385	320bp	**NR**	94º-60s 45º-60s 72º-60s } **NR**	**NR**
Crepin *et al* (1998)	5'GTAACACCTCTACAATGG3	5'GCTTGATGATTGGAACTG3'	57-74	1349-1368	1311bp	94º-60s	94º-50s 50º-90s 72º-90s } **30** **1**	72º-5min
East *et al* (2001)	5'AAGAACTTCAAGAAT ACG AGGC3'	5'TTCAGCCATCTCAAGATCGG3'	1161-1182	1560-1579	418bp	94º-1 min	94º-30s 37º-30s 72º-90s } **40**	72º-7min.
Gupta *et al* (2001)	5'ACTGATGTAGAAGGGAATTG3'	5'GAACGGAAGTGGATGAAATA3'	NR	NR	533bp	94º-60s	94º-60s 50º-60s 72º-60s } **35**	72º-5min
Adeiga *et al* (2002)	5'GCTCTAGAACACCTCTACAATG GATGCCGACAA3'	5'GGATTGAC(AG)AAGATCTTGCTCAT3'	59-84	1514-1536	1400bp	NR	93º-10s 48º-30s 68º-60s } -	72º-5min
David *et al*	5'GAGAAAGAACTTCAAGA3'	5'GAGTCACTCGAATATGTC3'	1156-1172	1513-1533	377bp	NR	94º-45s }	NR
Ju								72º-7min
Langoni *et al* (2005)	5'ATAGAGCAGATTTTCGAGACAGC3'	5'CCTCAAAGTTCTTGTGGAAGA3'	505-527	916-937	432bp	94 - 3 min	94 -45s 45º-1 min 72º-10 min } **35**	72º-10min
Macedo *et al*	5'ATGTAACACCTCTACAATTG3'	5'TTGACGAAGATCTTGCTCAT3'	55-73	1514-1533	1478bp	94º-1 min	94º-30s 37º-30s } **40**	72º-7min

(2006)							72º-90s }	
Rojas *et al* (2006)	5'CGTRGAYCAATATGAGTACA3'	5'CAGGCTCRAACATTCTTCTTA3'	66-85	806-826	760bp	NR	95º-45s 50º-30s } **30** 72º-30s	72º-10min
Barbosa *et al* (2007)	5'GGAAGAGATAAGAAGAATGTTTG3'	5'TTGGAGCTGACTGAGACATA3'	868-890	1359-1378	491bp	NR	94º-1min 55º-2 min } **35** 72º-2.5min	NR
Wacharapluesadee *et al* (2008)	5'TAGGGAGAAGGATCGTGGAGCACCATACTCTCA3'	5'GATGCAAGGTCGCATATGAGTACCAGCCCTGAACAGTCTTCA3'	611-632	769-790	179bp	NR	NR	NR
Lopes *et al* (2010)	5'CTACAATGGATGCCGAC3'	5'TTGACGAAGATCTTGCTCAT3'	33-49	1514-1533	1500bp	94º-5 min	94º-45s 55º-45s } **35** 72º-90s	72º-5min.
Brito *et al* (2011)	5'TATACTCGAATCATGATGAATGGAGGTCGACT-3'	5'-TTGACGAAGATCTTGCTCAT-3'	1287-1318	1514-1533	249bp	94º-1 min	94º-30s 37º-30s } **40** 72º-90s	72º-7min.
Qureshi *et al* *(*2011)	5'TTTGAGACTGCTCCTTTTG3'	5'CCC ATATAGCATCCTAC3'	587-605	1013-1029	443bp	94º-60s	94º-30s } **30** 45º-90s } 50º-20s } **5** 72º-90s } **5** 72º-60s } **30**	72º-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)

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Den- Denaturation; Ann- Annealing; Ext- Extension

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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'TTTGAGACT GCTCCTTTT3'	5'GCTTGAT GATTGGAA CT3'	5'AGAATG TTTGAGC CACGGCA 3'	5'TCAGGT GAAACCA GAAGTCC3 '	NR	NR	NR	NR	782	396	NR	95º-90s 45º-90s 72º-90s } 30s 60s 60s } 30s 90s 10m } 5 30 1	NR	95º-120s	95º-60s 52º-60s 72º-60s } **25**	72º 10min
Kulonen *et al* (1998)	5'GAAGCCTG AGATTATCG TGG3'	5'CCCTTCTA CATCAGTAC G3'	5'TGAGTA CAAGTAC CCTGC3'	5'GGAAC ATACATC GTCAGG3'	63-82	349-367	90-107	211-229	304	139	NR	NR**	NR	NR	NR	NR
Echeva-rria *et al* (2001)	5'AAGATGTG TGCCAACTG GAG3'	5'ATGTTTGA GCCAGGGC AAGA3'	5'TACTGC TTATGAG GATTGTT C3'	5'AAGAA CTTCGAG GAAGAG ATC3'	NR	NR	NR	NR	NR	NR	94º-2min	93º-60s 60º-60s 72º-60s } **30**	72º-5min.	94º-2min	94º-60s 50º-60s 72º-60s } **30**	72º 5 Min
Franka *et al* (2004)	5'GTAACACC TCTACAATG GA3´	5'AGTTTCTT CAGCCATCT C3'	5'GGATGC CGACAAG ATTGTAT 3'	5'CACATT TTGTGAG TTGTCA3'	57-75	1568-1585	73-92	633-651	1529	579	94º-5min	94º-40s 56º-40s 72º-60s } **35**	72º-7min.	94º-3min	94º-30s 60º-30s 72º-40s } **35**	72º 7 Min
Foord *et al* (2006)	5'ATGTAACA CCYCTACAA TG3'	5'CAGTTGG CACACATCT TGTG3'	5'AGATCA ATATGAG TAYAART AYCC3'	5'GTCATC AAAGTGT GRTGCTC 3'	55-73	641-660	139-163	617-636	605	497	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 40 1	72º-10m
Kasemp-imolporn	5'GTAACACC CCTACAATG	5'CAAAGAT CTTGCTCAT	5'GACATG TCCGGAA	5'GTATTG CCTCTCT	57-78	1508-1529	319-337	823-842	1473	524	94	94º 60º 72º } NR	72	94	94º 60º 72º } NR	72

et al (2006)	GATGC3’	GTTTGG3’	GACTGG-3’	AGCGGTG 3’												
Vazquez Moron *et al* (2006)	5’AARATNGT RGARCAYCA CAC3’	5’GCRTTSGA NGARTAAG GAGA3’	5·AARATG TGYGCIA AYTGGAG	5’TCYTGH CCIGGCT CRAACAT	538-557	892-911	574-593	814-833	374	260	94º-2min	93º-60s 53º-60s 72º-60s } **30**	72º-5min.	94º-2min	93º-60s 53º-60s 72º-60s } **30**	72 - 5min
Nadin Davis *et al* (2007)	5’AACACCTCT ACAATGGAT GCCGACAA3’	5’TTGTA/GGA T/CCAATATG AGTACAA3’	5’GGATTG AC(AG)AA GATCTTGC TCAT3’	5’CCGGCT CAAACATT CTTCTTA3’	59-84	1514 - 1586	135-56	876 896	1461	762	**NR**	94º-60s 55º-60s 72º-120s } **30**	+5s/ cycle	**NR**	94º-45s 55º-30s 72º-30s } **30**	**NR**
Zienius *et al* (2008)	5’GTAACACC TCTACAATG G3’	5’AGTTTCTT CAGCCATCT C3’	5’GGATGC CGACAAG ATTGTAT 3’	5’CTAAAG ACGCATG TTCAGAG 3’	57-74	1568-1585	73-92	472-491	NR	400	95º-5min	94º-40s 56º-40s 72º-60s } **35**	NR	95º-3min	94º-30s 60º-30s 72º-40s } **35**	72 - 10min
Muleyaa *et al* (2012)	5’CTACAATGG ATGCCGAC3’	5’GAGTCAC TCGAATATT GC3’	5’GACATG TCCGGAA GACTGG3’	5’GTATTGCC TCTCTAGCG GTG3’	66-82	1402-1419	319-337	823-842	1353	523	95º-5min	95º-60s 50º-60s 72º-60s } **35**	72º-5min.	95º-5min	95º-60s 50º-60s 72º-60s } **35**	72º-5min.

***** All nucleotides positions are based on genomic sequence of the reference PV (Pasteur Virus) strain sequence of Nucleoprotein gene (N) (GeneBank accession number M13215).

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Den- Denaturation; Ann- Annealing; Ext- Extension

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NR- Not Reported.

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'CAGAGACATATCTG/CCG/TG/TATGTG3'	5'GAGAAAGAGA/CTG/TCAAGAA/C/TA3'	5'CTTCAC/TCG/TACCA/TC/TC/TGTTCATCAT3'	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)CTACAATTG3'	5'CAATTCGCACACATTTTGTG3' + 5'CAGTTGGCACACATCTTGTG3'+ 5'CAGTTAGCGCACATCTTATG3'	5'ATGTAACACC(C/T)CTACAATTG3'	5'GTCATCAAAGTGTG(A/G)TGCTC3' + 5'GTCATCAATGTGTG(A/G)TGTTC3' + 5'GTCATTAGAGTATGGTGTTC3'	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 ATTTTCGAGACAGC3'	5'CCCATATAA CATCCAACAAAGTG3'	5'ATAGAGCAGATTTTCGAGACAGC3'	5'CCTCAAAGTTCTTGTGGAAGA3'	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* (2004)	5’ATGTAACAC TG3’	5’CARTTVGCR TGCC-3’	5’ATGTAACACCYCT AGTACCCGGC3’	5’GTCCCGAGTGAG TCATGCC-3’	55-74	641-660	55-74	447-465	606 bp	410 bp	95º-10min	94º-30s 55º-30s 72º-90s } **35**	72º-10min.	95º-10min	94º-30s 55º-30s 72º-1m } **25**	72º-10min.
													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’ATAGAGCAG ATTTTCGAGACAGC3’	5’CCCATATAA CATCCAACAAAGTG3’	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’TGACCATTCCARCARGTNG 3’	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*	5’ATGTAACACCYCTACAATG	5’CAATTCGCA	5’ATGTAACACCYCT	5’GTCATTAGAGTAT	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-10min.

(2008)	3’	CACATTTTG TG3’	ACAATG3’	GGTGTTC 3’ or 5’GTCCCGAG TGAGATCTT GA3’												
Coertse *et al* (2010)	5’ACGCTTAAC GAMAAA3’	5’GTRCTCCA RTTAGCRCA CAT3’	5’CACMG SNAAYTA YAARACN AA3’	5’GTRCTCCA RTTAGCRCA CAT3’	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’ATGTAACAC CYCTACAATG 3’	5’CAATTCG CACACATTT TGTG3’	5’ATGTAA CACCYCT ACAATG3’	5’GTCATCAA TGTGTGATGT TC3’	NR	NR	NR	NR	NR	NR	95º-15min	95º-20s 60º-30s 72º-30s } 10 95º-20s 52º-30s 72º-30s } 35 0	NR	94º-5 min	94º-20s 52º-20s 72º-30s } **35**	NR

***** All nucleotides positions are based on genomic sequence of the reference PV (Pasteur Virus) strain sequence of Nucleoprotein gene (N) (GeneBank accession number M13215)

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Den- Denaturation; Ann- Annealing; Ext- Extension

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NR- Not Reported.

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 **40** 60ºC-60s
	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	RABV D1 Rev	RABV D1	5’ATGTAACACCYCTACAAT	5’GCMGGRTAYTTRTAYTCATA	5’56FAM/CCGAYAAGAT	55 – 73	165-146	78 - 111	19	20	34	NR		

			Probe	G3’	3’	TGTATTYAARGTCAAKAATCAGGT/3BHQ_1-3’									
Nadin Davis *et al* (2009)	RABV D2 For	RABV D2 Rev	RAB D2 Probe	5’TRATGACAACYCACAARATGT3’	5’TGARCAGTCYTCRTARGC3’	5’56FAM/TAYGACATGTTTTTCTCYCGGATTGARCATC/3BHQ_13’	630 - 650	764-781	698-728	21	18	31	NR	95ºC - 2min	95ºC-15s 50ºC-60s } **45**
	RABV D3 For	RABV D3 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-13’	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 GAAACCTGTCGTGCCA-BHQ3	55-73	146-165	81-109 & 56-80	19	20	29 25	50º-30min	95º - 15min	94ºC-30s 55ºC-30s } **42**
	Jw12	N146-165	RabGT1-B-FAM & LacZCy5FAM	5'ATGTAACACCYCTACAATG3'	5'GCAGGGTAYTTRTACTCATA3'	6-FAM-CAGCAATGCAGTTYTTTGAGGGGAC-TAMRA & Cy5-TCCAGTCGG GAAACCTGTCGTGCCA-BHQ3	266-288	335-353	297-321 & 56-80	23	19	29 25			

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All nucleotides positions are based on genomic sequence of the reference PV (Pasteur Virus) strain sequence of Nucleoprotein gene (N) (GeneBank accession number M13215)

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Den- Denaturation; Ann- Annealing; Ext- Extension

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NR- Not Reported.

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 centres 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 group 2]). A sensitivity of 100% was obtained with the saliva sample when analyzed 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).

2.6.2 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 was 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.

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).

2.7 Postmortem confirmation of rabies

2.7.1 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).

Mc Coll *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-polymerase chain reaction (RT-PCR) and restriction endonuclease analysis (REA) to the rabies 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 (hnRT-PCR) protocol which is rapid and sensitive for the detection of rabies virus and rabies-

related 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 rabies-related viruses, 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 $3TCID_{50}$ 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 (RT-PCR), 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 genotype 1. 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), 902 nt cDNA 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 endonuclease 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, non 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 RT-PCR) 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 digoxigenin labelled 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 viral antigen 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.

2.7.2 Conventional approaches for post-mortem confirmation

2.7.2.1 Immunofluorescence

FAT is the widely used method for diagnosing rabies infection in animals and humans. FAT was first developed by Goldwasser and Kissling (1958) for the diagnosis of rabies. Kaplan and Koprowski (1973) reported that the "direct" FAT took the lead over all others for speed and accuracy. However, all fluorescent - negative specimens have been recommended to be tested in mice to maintain a constant check on FAT. Dean and Abelseth (1973) observed that combined Fluorescent - positive and mouse - negative specimens could be expected since the FA test detected inactivated as well as live antigen.

Howard (1977) described the fluorescent antibody test for the detection of rabies virus antigen in brain, salivary gland, cornea, lip, tactile follicle, and skin and the study indicated that these tissues were 100% effective for the diagnosis of rabies in naturally infected skunks. Umoh *et al* (1985) found that FAT could detect 90 per cent of cases which would have been picked up by the routine diagnostic systems and show up a substantial number of others which would have been missed by Negri body staining and mouse inoculation.

Kulonen (1989) described that routine diagnosis of rabies in Finland is performed by FAT on cerebral cortex, hippocampus, cerebellum and occasionally spinal cord tissues. Virus isolation in murine neuroblastoma cells and newborn mice are used as confirmatory tests.

Aubert (1982) and Barrat and Aubert (1995) suggested that FAT gives reliable results on fresh specimens within a few hours in more than 95–99% of cases. The sensitivity of the FAT depends on the specimen, the degree of autolysis, how comprehensively the brain is sampled, on the type of *lyssavirus* and on the proficiency of the diagnostic staff. Sensitivity may be lower in samples from vaccinated animals due to localization of antigen, which is confined to the brainstem.

Different workers employed FAT to determine viral antigen in different body parts e.g. in optic nerve (Shashenko *et al* 1985), hind limb peripheral nerve (Meyer *et al* 1986), in various body organs (Singh and Grewal 1998b, Singh 1999).

Meslin *et al* (1996) observed that fresh, frozen and glycerinated material might be examined with FAT and found that this test was fast, comparatively inexpensive and

more accurate than either the examination of films or sections by recommended procedures or mouse inoculation test.

Whitfield *et al* (2001) observed that the preferred method for routine diagnosis of rabies in fresh or frozen brain tissues is the Fluorescent antibody test.

Roehe *et al* (2002) optimized the direct fluorescent antibody test for rabies diagnosis and found that the combination that provided the greatest efficacy at direct fluorescent antibody test (DFAT) was attained when slides were fixed for 30 min in acetone at -20°C, the pre-adsorbing conjugate and uninfected mouse brain suspensions/infected mouse brain suspensions were incubated for 60 min at 37°C and subsequently layered onto impressions for 2 h at 37°C.

Bingham and Merwe (2002) reported that the thalamus, pons and medulla were the most reliable parts of the brain as they were positive in all cases tested by immunofluorescent antibody test.

Spilki *et al* (2003) reported first case of mixed rabies/ BHV-5 infection in calves. Rabies virus infection was confirmed by the direct fluorescent antibody test (FAT) as well as by mouse inoculation.

Milius *et al* (2004) investigated the prevalence of rabies during the period of 1993-2002 using the immunofluorescence method and the mouse inoculation test. Rabies was diagnosed in 35.73% of domestic and in 64.27% of wild animals where as Ondrejkova *et al* (2004) identified a *Lyssavirus* isolate from a bat using direct fluorescent antibody test (FAT), mouse inoculation test (MIT) and reverse transcriptase polymerase chain reaction (RT-PCR).

Ali *et al* (2006) conducted epidemiological studies of rabies in Sudan over the period 1992-2002 using Fluorescent Antibody Test (FAT) and found that most of the cases were from dogs and goats. Carrieri *et al* (2006) reported that in equine, greater amount of rabies viral antigen is present in the brainstem and cervical medullar tissues than in the hippocampus, cortical and cerebellar tissues using fluorescent antibody test (FAT) and virus-isolation laboratory tests.

Soun *et al* (2006) diagnosed rabies suspected case by using fluorescence antibody testing on nuchal skin biopsy specimen.

Menezes (2008) studied the space and time distribution of bovine rabies in Minas Gerais state, from 1998 to 2006 using the direct immunofluorescence test and concluded that a higher percentage of positive animals were from May to July. Teixeira *et al* (2008) reported that in Brazil, a total of 23,460 specimens were examined from 1985 to 2007 using the fluorescent antibody test (FAT) and mouse inoculation test (MIT). Rabies virus (RV) was detected in 739 specimens (3.1%), from which 656 (88.7%) were from cattle.

Aytekin and Mamak (2009) diagnosed rabies in a cow showing neurological signs using fluorescent antibody test. Smreczak *et al* (2009) described the first case of the isolation of the European bat *lyssavirus* type 1b in the serotine bat in Poland. Rabies was diagnosed by the fluorescent antibody test (FAT) as well as heminested RT-PCR.

2.7.2.2 Histopathological alterations

Negri (1903) described inclusions in the neurons of brains from rabid animal, which he regarded as typical of rabies virus infection. These Negri bodies consist of masses of ribonucleoprotein of rabies virus but not seen in all cases (Derakshan *et al* 1978).

Zimmer *et al* (1990) found no Negri bodies in brains of animal dying of rabies. However, Salem *et al* (1995) reported Negri bodies in purkinje cells of cerebellum of fetus of the pregnant dam that died of rabies. Sinchaisri *et al* (1992) reported absence of Negri body in CNS of mice experimentally infected with CVS strain of fixed rabies virus although antigen was detected in these mice by avidin-biotin peroxidase technique. Kat *et al* (1995) revealed eosinophilic Intracytoplasmic inclusions (Negri bodies) upon histological examination of 2 brain samples of African wild dogs supporting a diagnosis of rabies viral encephalitis. Negri body detection has been largely super seeded by the Mice Inoculation Test as very reliable and rapid Fluorescent Antibody Technique (Kaplan and Koprowski 1973).

Sullivan (1985) reported no gross changes in brain, ganglia and salivary glands in rabies. Baltazar *et al* (1986) found that gross lesions in experimentally produced rabies in sheep by using street rabies virus were non-specific. Dutta *et al* (1992) reported no gross lesions in experimentally inoculated virus in four groups except oedema of meninges in the mice. Singh (1999) and Archana (2001) revealed congestion and oedema in the brain of experimentally infected buffalo calves.

Macruz *et al* (1977) found encephalitis or myelitis in 20 rabid cows, induced by intramuscular inoculation, but no Intracytoplasmic inclusions were found.

Gonzalez and Stephano (1984) observed meningoencephalitis in 35 of 40 cases of rabies with Intracytoplasmic inclusion bodies.

Schulz (1986) reported non-purulent encephalitis confined to medulla oblongata in rabid cattle. Singh and Grewal (1998b) observed that pons, spinal cord, cerebrum, cerebellum and medulla oblongata had non-purulent encephalitis in experimentally infected buffalo-calves.

Singh (1999) observed histopathologically perivascular cuffing in pons, hippocampus and medulla oblongata at 30 DPI. Characteristic Negri bodies were observed on 60 DPI. Non-suppurative encephalitis was observed. Neuronal degeneration and necrosis of neuron were found at 30 and 60 DPI. Congestion and haemorrhages were also major alterations in the nervous tissues of infected buffalo calves. Bundza and Charlton (1988) revealed moderately extensive spongiform lesions that rarely affected basal ganglia or hippocampus in skunk's inoculated intra muscularly with street rabies virus. Spongiform lesions were characterized by less number of small vacuoles.

Green *et al* (1992) reported major histopathological findings in rabid horses, comprising diffuse lymphocytic perivascular cuffing with lymphocytes in the meninges, neuronal degeneration, neuronophagia, gliosis and malacia of gray matter of spinal cord. Hudson *et al* (1996b) revealed that the major histopathological findings in rabid horses were diffuse lymphocytic perivasicular cuffing, neuronal degeneration, neuronophagia, gliosis and malacia of gray matter of spinal cord.

Peixoto *et al* (2000) compared the sensitivity of three diagnosis techniques, microscopic examination of Negri bodies, fluorescent-antibody test (FAT) and mouse inoculation test (MIT) in 3,713 samples and observed that in equine rabid samples, only in few opportunities the Negri bodies could be observed. The absence of inclusion bodies and the longer incubation period for equine samples suggested that rabies pathogenesis studies for equine species are different. Url *et al* (2004) observed non-purulent pan encephalitis leading to the tentative diagnosis of rabies encephalitis in equines and confirmed by immunohistochemistry.

Stoltenow *et al* (2000) confirmed rabies in bison by the fluorescent antibody test. Intra cytoplasmic neuronal inclusions suggestive of Negri bodies in brain stem and hippocampus were also present. Jamadagni *et al* (2008) reported that there was significant detection of Negri bodies in cerebellum both in cattle (91.3%) and in buffaloes (85.1%), however, in case of cerebrum, Negri bodies were found more in buffaloes (65.95%) than in cattle (13.0%). Lahaye *et al* (2009) reported that Negri bodies are composed of the viral N and P proteins.

2.8 Comparison of different techniques

Rajamanickam *et al* (1994) compared the dipstick dot-ELISA with direct FAT for detection of rabies antigen and found that the dipstick dot ELISA test did not produce non-specific false positive results and was therefore specific and reliable.

Jayakumar *et al* (1995a) and Jayakumar *et al* (1995b) tested various specimens (400) with the dot ELISA technique and FAT and it was found that dot ELISA was a simple, inexpensive, rapid and highly sensitive method for rabies diagnosis.

Singh and Grewal (1998b) compared different diagnostic techniques to detect rabies virus antigen/antibody and reported that detection of neutralizing antibodies by using modified counter immuno-electrophoresis was the most sensitive technique followed by direct immunofluorescence; Seller's staining of Negri body, pleocytosis in cerebrospinal fluid, detection of rabies virus in nasal, salivary and rectal secretions and mice inoculation test. The least sensitive of all the diagnostic technique was the histopathological detection by Negri-body.

Silva *et al* (1999) tested central nervous system samples obtained from dogs with suspected rabies or distemper by Seller's and immunofluorescence techniques. Of 1610 samples, 374 (23.2%) were positive for rabies and 87 (5.4%) were positive for distemper.

Ratho *et al* (2001) compared the diagnosis of rabies among suspected human rabies encephalitis cases by Seller stain, fluorescent stain as well as mouse inoculation test. Out of 71 postmortem brain specimens, 26 were diagnosed as rabies positive. Negri bodies were demonstrated in 18 (25.4%) brain samples by Seller stain. Fluorescent antibody technique could detect rabies antigen in 21 (29.6%) samples.

Archana *et al* (2003) compared Fluorescent antibody technique (FAT), Seller's staining, mice inoculation test (MIT) and double-antibody sandwich ELISA (DAS-ELISA) to detect the rabies virus antigen in the calves and for their sensitivity of detection at 30 days post inoculation and concluded that DAS-ELISA is the most sensitive laboratory technique for rabies diagnosis.

Qureshi *et al* (2003) attempted to demonstrate the rabies virus/antigen in the saliva/brain tissue of affected/suspected/healthy animals belonging to different species, using agar gel precipitation (AGPT), fluorescent antibody (FAT) and mice inoculation (MIT). It was shown that out of the total 321 animals, 18 (5.0%) were positive for AGPT, 69 (21.49%) with FAT and 71 (22.11%) with MIT. FAT in combination with MIT was the most sensitive, reliable and quick method for diagnosis.

Chhabra *et al* (2007) evaluated in vitro isolation of rabies virus using mouse neuroblastoma cells (MNA). The sensitivity and reliability of in vitro procedure was performed in comparison with mouse inoculation test (MIT), the in vivo method of virus isolation, direct fluorescent antibody test (FAT) and Sellers staining. Of the 33 animal

brain samples tested, 24 (72.72%) were positive by MIT. Sensitivity of Sellers stain, FAT and rapid tissue culture infection test (RTCIT) was found to be 54.16, 100 and 91.6% respectively.

Durr *et al* (2008) evaluated the direct rapid immunohistochemical test (dRIT) in the Chadian National Veterinary Laboratory in N'Djamena by testing 35 fresh samples parallel with both the direct immunofluorescent antibody (DFA) test and dRIT. They found a 100% agreement of the dRIT and DFA in fresh samples ($n = 35$).

The information regarding correlation of molecular techniques with clinical syndrome for ante-mortem detection of rabies in animals is not available.

CHAPTER III

MATERIALS AND METHODS

3.1 Clinically suspected animals

In the present study, samples were collected for diagnosis of rabies from 20 rabies suspected animals presented to the Veterinary Clinics, GADVASU, Ludhiana, Punjab and Civil Veterinary Hospital from different districts of Punjab by applying molecular and conventional techniques over a period of eighteen months from July 2010 to December 2011. Out of 20 animals, the ante-mortem diagnosis of rabies was conducted on 11 saliva samples (3 buffaloes, 3 cows and 5 dogs) and 20 skin samples (7 buffaloes, 5 cows and 8 dogs) of rabies suspected animals (Table 5). Due to severe dehydration in some animals it was not feasible to collect the saliva samples from all cases. After clinical observation and collection of case history, a tentative diagnosis was made which was further substantiated by applying molecular approaches on different ante-mortem samples (skin and saliva).

3.2 Collection and processing of clinical samples

3.2.1 Saliva samples

Saliva was collected either in a sterilized vial directly (Fig. 1) or with the help of sterile syringe from oral cavity of animal. PBS was directly added in saliva sample to make 1:1 suspension. In case of soiled saliva samples, PBS was added and then centrifuged at 1500 rpm for 10 mins. The supernatant was collected and stored at -20ºC until further processing for extraction of RNA.

Table 5: Details of samples collected from clinically suspected animals

S. No.	Species	Age	Sex	Samples Collected
1.	Buffalo	2 yrs	F	Skin
2.	Dog	5 mths	F	Skin
3.	Cow	3 yrs	M	Skin, Saliva
4.	Dog	4 yrs	M	Skin, Saliva
5.	Buffalo	6 yrs	F	Skin
6.	Buffalo	4 yrs	F	Skin
7.	Dog	3½ mths	M	Skin, Saliva
8.	Buffalo	6 yrs	F	Skin, Saliva
9.	Dog	5 yrs	F	Skin
10.	Cow Calf	6 mths	F	Skin, Saliva
11.	Dog	2½ yrs	F	Skin, Saliva
12.	Cow calf	1 mths	F	Skin
13.	Cow	4½ yrs	F	Skin
14.	Buffalo	8 yrs	F	Skin, Saliva
15.	Dog	12 yrs	M	Skin, Saliva
16.	Dog	1 yrs	M	Skin
17.	Dog	7½ yrs	M	Skin, Saliva
18.	Buffalo	7 yrs	F	Skin
19.	Buffalo	6 yrs	F	Skin, Saliva
20.	Cow	1 yrs	F	Skin, Saliva

3.2.2 Skin biopsy samples

The skin biopsy samples with a minimum of 10 hair follicles were collected with the help of sterilized 3mm skin biopsy punch (Fig. 2). 500 mg of skin samples were cut into very small pieces with sterile scissors and then triturated with the help of sterilized sand in pestle and mortar using 4.5 ml PBS (pH-7.2) as a diluent to make 10% (w/v) suspension under sterile conditions in a laminar flow hood. The suspension was then centrifuged at 1500 rpm for 10 mins. The supernatant was collected and stored at -20˚C until further use.

3.2.3 Control

(i) **Positive control:** In the present study, anti-rabies vaccine (Rabipur) was used as positive control.

(ii) **Negative control:** The known negative samples were used as negative controls.

3.3 Extraction of RNA

3.3.1 Saliva samples:

Extraction of RNA from saliva samples were done by using QIAamp viral RNA mini kit (Qiagen, USA).

1. 560 µl of prepared buffer AVL containing carrier RNA was pipetted into a 1.5ml micro centrifuge tube.
2. 140 µl of saliva sample was added to the Buffer AVL–carrier RNA in the micro centrifuge tube and pulse-vortexed for 15 s.
3. Micro centrifuge tube containing mixture was incubated at room temperature (15–25°C) for 10 min.
4. Micro centrifuge tubes are briefly centrifuged to remove drops from the inside of

the lid.

5. 560 µl of ethanol (96–100%) was added to the sample, and mixed by pulse-vortexing for 15 s. After mixing, tubes were briefly centrifuged to remove drops from inside the lid.
6. Carefully 630 µl of the solution from step 5 was applied to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Cap was closed and centrifuged at 6000 × g (8000 rpm) for 1 min. QIAamp Mini column was placed into a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
7. QIAamp Mini column was carefully opened and step 6 was repeated with remaining lysate solution.
8. QIAamp Mini column was carefully opened and add 500µl of Buffer AW1 was added to the column. Cap was closed, and columns are centrifuged at 6000 x g (8000 rpm) for 1 min. QIAamp Mini column are then placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
9. 500µl of Buffer AW2 was added into QIAamp Mini column. Cap of column was closed and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min.
10. QIAamp Mini column was then placed in a clean 1.5 ml micro centrifuge tube. Old collection tube containing the filtrate was discarded. QIAamp Mini column was then opened and 60 µl of Buffer AVE was added into it. Columns are incubated at room temperature for 1 min and then Centrifuged at 6000 x g (8000 rpm) for 1 min. Micro centrifuge tubes containing RNA were stored at -80^{o}c for further use in nested RT-PCR and TaqMan real time PCR.

3.3.2 Skin samples

The tissue suspensions which were stored at -20ºC were taken out and thawed at room temperature to make a uniform solution.

1. To 400 µl of 10% tissue suspension samples, 20 µl 10% SDS solution and 20 µl Proteinase K (20 mg/ml) were added and tubes were incubated for 1-2 hrs at 37 ºC.
2. For obtaining sufficient RNA of a sample triplicates of each sample were made. For this fresh, clean, sterile, autoclaved eppendorf tubes (2 ml) were used and the whole procedure was carried under clean and sterile conditions in a laminar flow hood.
3. 1 ml Qiazol (Qiagen, USA) was added to each tube and was vortexed for 30 s.
4. Than 200 µl chloroform was added in each tube and subjected to vortex. Incubation for 5 mins at room temperature and then centrifuged at 14,000 rpm for 15 mins at 4ºC.
5. The top aqueous phase containing RNA was transferred into fresh, clean, sterile, autoclaved eppendorf tubes (1.5 ml). Upper aqueous phase is very viscous and care was taken during transfer of aqueous phase so that lower organic phase (containing Qiazol and chloroform) were not disturbed.
6. 500 µl Isopropanol was added to the aqueous phase and mixed gently and then incubated for 20 mins at room temperature and centrifuged at 12,000-14,000 rpm for 15 mins at 4ºC.

7. White or gel like pellet at the bottom of the tube was observed. The supernatant was discarded by gentle inversion when RNA pellet was intact, otherwise aspirated (when RNA pellet was loose).
8. The RNA pellet was washed with 75% ethanol i.e. 1 ml of 75% ethanol was added, vortexed and centrifuged @ 9000 rpm for 10 mins at 4ºC.
9. Finally the RNA pellet was air dried after inverting on blotting paper so that last traces of ethanol were lost. Care was taken so that pellet is not over dried or else dissolution becomes difficult.
10. Approximately, 20 µl of (Tris EDTA) TE buffer was added and the final product of each triplicate was pooled in one tube making the total volume 60 µl. Incubation for 10 mins at 55-60ºC was given to completely dissolve the pellet.
11. Eppendorfs containing RNA were stored at -80ºC until further use. The RNA was used both for nested RT-PCR and TaqMan real time PCR.

    - ❖ RNA concentration was measured using Nano Drop Spectrophotometer (Nanodrop Technologies, CA) in ng/µl and quality was checked as a ratio of OD 260/280.

3.4 Synthesis of cDNA

Total RNA extracted was converted into cDNA using high-capacity cDNA reverse transcription kit (Applied Biosystems, USA) as follows:

After thawing, all the components of the kit were mixed properly and were kept on ice. For first step, 10µl of total RNA extracted from each sample was taken into sterilised PCR tubes and 2µl of primer Rab N1 (30 pmol/µl) or random primers (used for internal control) were added into each tube, making a total volume of 12µl.

RNA	10.0 µl
Primer Rab N1 (30 pmol/µl)	2.0 µl
Total volume	12.0 µl

This mixture was centrifuge briefly and was incubated at 65°C for 10 min and was later snap cooled on ice. After that the following master mixture was prepared:

10X RT Buffer	2.0 µl
Multiscribe™ Reverse Transcriptase (50 u/ul)	1.0 µl
25X dNTP Mix (100mM)	0.8 µl
Nuclease free water	4.2 µl
Total volume	8.0 µl

These components were briefly centrifuged and 8 µl of above was added in PCR tubes containing RNA and Primer Rab N1 making a total volume of 20 µl. For c DNA synthesis cycling conditions were as below:

Temperature	Time	No. of Cycles	Remarks
25ºC	10 mins	1	Incubation
37ºC	120 mins	1	Reverse transcription
85ºC	5 mins.	1	Stopping the reaction

The resultant cDNA was used both for Nested RT-PCR and TaqMan real time PCR. cDNA concentration was measured using Nano Drop Spectrophotometer (Nanodrop Technologies, CA) in ng/µl and quality was checked as a ratio of OD 260/280.

3.5 Molecular approaches for Ante-mortem diagnosis

Two molecular approaches- nested RT-PCR and TaqMan real time PCR were applied on the various clinical samples at the Rabies Research cum Diagnostic Laboratory, Department of Veterinary Pathology and Research Laboratory, Department of Animal Biotechnology, GADVASU, Ludhiana, Punjab.

3.5.1 Nested Reverse Transcriptase-Polymerase Chain Reaction

3.5.1.1 Primers:

Primers based on N gene were used for first and second round Nested RT-PCR. The details are given below (Table 6).

Table 6: Primers used for Nested RT-PCR

Primer Name	Sequence	Gene	Position	Sense	Reference
Rab N1	5' GCTCTAG AAC ACC TCT ACA ATG GAT GCC GAC AA 3'	N	59-84	+	(Nadin Davis 1998), (Nagaraj *et al* 2006)
Rab N5	5' GGA TTG AC(AG) AAG ATC TTG CTC AT 3'	P	1514-1536	-	(Nadin Davis 1998), (Nagaraj *et al* 2006)
Rab Nfor	5' TTG T(AG)G A(TC)CA ATA TGA GTA CAA 3'	N	135-156	+	(Nadin Davis 1998), (Nagaraj *et al* 2006)
Rab Nrev	5' CTG GCT CAA ACA TTC TTC TTA 3'	N	876-896	-	(Nadin Davis 1998), (Nagaraj *et al* 2006)

3.5.1.2 Amplification protocol

1. Fresh master mix for first round of the PCR was prepared in PCR tubes on ice according to the following preparation:

Master Mix

Components	Volume/Reaction
10X PCR buffer	5.0 µl
10mM dNTP	1.0 µl
Primer Rab N1	1.0 µl
Primer Rab N5	1.0 µl

Taq DNA polymerase	0.5 µl
DEPC water	29.5 µl
cDNA	12.0 µl
Total	50.0 µl

The master mix was mixed thoroughly and carefully by vortexing for not more than 5 s, centrifuged briefly (10 s) to collect residual contents from the walls of the tube and stored on ice. Vortexing was done before adding the cDNA.

2. PCR tubes were placed in DNA thermal cycler and subjected to following conditions for the first round of the Nested PCR:

Temperature	**Time**	**No. of Cycles**	**Remarks**
95°C	2 min.	1	Initial Denaturation
95°C 55°C 72°C	1min. 1 min. 1min. 30s.	35	Denaturation Annealing Extension
72°C	5 min.	1	Final Extension

3. Fresh master mix for second round of the PCR was prepared in PCR tubes on ice according to the following preparation:

Master Mix

Components	**Volume/Reaction**
10X PCR buffer	5.0 µl

10mM dNTP	1.2 µl
Primer Rab Nfor	1.2 µl
Primer Rab Nrev	1.2 µl
Taq DNA polymerase	0.5 µl
Nuclease free water	35.9 µl
PCR product of first round	5.0 µl
Total	50.0 µl

4. PCR tubes were placed in DNA thermal cycler and subjected to following conditions for the second round of the Nested PCR.

Temperature	**Time**	**No. of Cycles**	**Remarks**
95°C	2 min.	1	Initial Denaturation
95°C 50°C 72ºC	1min. 1min. 1min.	35	Denaturation Annealing Extension
72°C	5 min.	1	Final Extension

3.5.1.3 Agarose Gel Electrophoresis:

3.5.1.3.1 Solutions and buffers

Stock solution of Tris Borate EDTA (TBE) buffer (10 x).

Tris base	108 g
Boric acid	55 g
EDTA (0.5M)	27.5 g
DW add to	1000 ml

Working solution of TBE buffer (0.5 x)

10X TBE	50 ml
DW add to	1000 ml

Agarose gel

Agarose	1.5 g
0.5x TBE	100 ml
Ethidium bromide (10mg/ml)	5 µl

3.5.1.4 Analysis of PCR products:

The PCR amplified products hence obtained were run by electrophoresis. 1.5% agarose was prepared in 0.5 x TBE in flask and was heated to dissolve agarose completely in buffer. Gel was cooled down to temperature of around 50^{o}C and ethidium bromide solution (stock conc. 10mg/ml) @ 5ul/100ml of gel was added into the gel to make the final concentration 0.5ug/ml. Gel was poured into a gel casting tray having comb of appropriate number and sizes hung into that and was allowed to solidify at room temperature.

Once the gel was solidified, comb was removed from the gel. Gel was placed in electrophoresis tank containing 0.5x TBE. PCR products and 6X loading dye (containing 0.03% bromophenol blue, 0.03% Xylene cyanole FF, 0.4% Orange G, 15% Ficoll 400, 10mM Tris-HCl (pH 7.5) and 50mM EDTA were mixed and were loaded in the wells with the help of micropipette. 5 ul of DNA ladder (100 base pair plus, Fermentas) was also loaded in one of the wells to access the size of PCR products.

The electrophoresis run was allowed for 30-45 mins at 80 volts. A positive control from anti- rabies vaccine was also run with the test samples in gel after RNA extraction by procedure described under 3.2.2.2.

Agarose gels were visualized under Geldoc (Alphaimager 3400 HP), photographed and analyzed with the same software.

3.5.2 TaqMan Real Time PCR

3.5.2.1 Primers:

All TaqMan primers and probes were designed by the Primer Express 3.0computer program (Applied Biosystems, Foster City, Calif.). Sequences were obtained by using the default settings of the program. For diagnosis of rabies virus samples, sequence was generated for the sets of sequences comprising N gene variant from a sequence alignment generated with the Bio Edit computer program. From this alignment, areas of relative conservation were selected as target regions for placement of the TaqMan primers and probes. These regions were used as input for Primer Express to generate the optimal primer and probe sequences according to the default settings. TaqMan primer and probe details are shown in (Table 7). All TaqMan probes were labelled at the 5' end with a fluorescent reporter dye (FAM) and at the 3' end with a quencher dye (TAMRA). Primer and probe concentrations were optimized according to the manufacturer's recommendations.

Table 7: Primers used for TaqMan real time PCR

Primer / Probe Name	Sequence	Gene	Length (nt)	Positions	Tmax (ºC)	Remarks
Primer 1F	5'-TTGACG GGAGGA ATGGAACT-3'	N	20	434-453	62	**Newly designed**
Primer 1R	5'-GACCGA CTAAAG ACGCAT GCT-3'	N	21	477-497	64	**Newly designed**
Probe 1Pr	5'-FAM- AGG GACCCCACT GTT-TAMRA-3'	N	15	458-472	48	**Newly designed**

3.5.2.2 Amplification protocol for TaqMan Real Time RT-PCR

1. Fresh master mix for the PCR was prepared in PCR tubes on ice according to the following preparation:

Master Mix

Components	**Volume/Reaction**
TaqMan Master mix	12.5 µl
Forward primer	1.0 µl
Reverse primer	1.0µl
Probe	1.0 µl
cDNA	2.0 µl
Nuclease free water	2.5 µl
Total	20 µl

2. 8 strip PCR tubes having reaction mixture was capped with 8 strip optically cleared flat caps were placed in ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and subjected to following temperature conditions:

- One cycle of reverse transcription at 50ºC for 2 mins.
- One cycle of denaturation at 95ºC for 10 mins.
- 40 cycles each of denaturation at 95ºC for 15 s, annealing at 60ºC for 1 min.

3.5.2.3 Analysis

After the PCR was completed, a threshold cycle number (*Ct*) was obtained corresponding to the PCR cycle number during which the fluorescence of the reaction rose above a threshold value statistically determined by the ABI prism software. The *Ct* values are inversely proportional to the log10 of the amount of template in the PCR. A difference of 1 *Ct* corresponds to a twofold difference in template amounts. A *Ct* value

less than the mean plus two standard deviations of the negative control wells was considered positive. A *Ct* value above 35 corresponds to no amplification.

3.6 Post mortem confirmation of rabies:

All the cases were followed up and confirmed by applying molecular and conventional techniques on brain samples.

3.6.1 Collection of Brain tissue:

Brain tissue samples were obtained after opening the skull of rabies suspected animals (Fig. 3). Half brain was preserved in formalin for histopathological analysis and half was stored at -20˚C until further use for molecular approaches.

Controls:

(i) **Positive control**: In the present study, anti-rabies vaccine (Rabipur) was used as positive control.

(ii) **Negative control:** The known negative samples were used as negative controls.

3.7 Molecular diagnosis:

3.7.1 Processing of Brain samples:

Brain tissues were triturated with the help of sterilized sand in pestle and mortar using PBS (pH 7.2) as a diluent to make 10% (w/v) suspension under sterile conditions in a laminar flow hood. 4.5 ml PBS was used for 500 mg tissue to make 10% brain suspension. The suspension was then centrifuged at 2500 rpm for 10 mins and stored at -20˚C until further use in molecular approaches.

Extraction of RNA and synthesis of cDNA from brain samples was done (as previously described under section 3.3.2 and 3.4). Two molecular approaches- nested RT-PCR and TaqMan real time PCR (as previously discussed under section 3.5.1 and

3.5.2) were applied on brain samples at the Rabies Research cum Diagnostic Laboratory, Department of Veterinary Pathology and Research Laboratory, Department of Animal Biotechnology, GADVASU, Ludhiana, Punjab.

3.8 Immunofluorescence:

The immunofluorescence was applied as diagnostic test because of its sensitivity, accuracy and speed as recommended by WHO (Meslin *et al* 1996).

1. Duplicate impression smears of 1 cm diameter on either ends of the labelled slides were prepared from cerebellum in case of large animals and from hippocampus in case of dogs. Control positive slides from known rabies positive case and control negative slide from normal, uninfected and unvaccinated animal was also prepared along the smear.
2. The impression smears were air-dried and fixed by immersing in coplin jar containing acetone at -20^{o}C for 30 mins.
3. A sufficient quantity (0.1 ml) of clarified conjugate (lyophilised, adsorbed anti-rabies nucleocapsid conjugate, Bio-Rad, France) was added on each smear and incubated at 37^{o}C for 30 mins in a moist chamber.
4. The slides were then washed in two successive phosphate buffers (PBS) baths for 5 mins each.
5. A few drops of glycerine buffer were added and covered with a cover slip.
6. The slides were examined using an AHBT3 - RFC reflected light fluorescence attachment (Olympus, Japan)

3.9 Histopathological alterations:

After fixation in 10% buffered formalin saline for 48 hours, the tissues were thoroughly washed in running water; dehydrated in ascending grades of alcohol and

acetone; cleared in benzene and embedded in paraffin at 58^{o}C. The paraffin embedded tissues were sectioned at five microns thickness and stained by haematoxylin and eosin (H&E) method (Lillie, 1965). Slides were examined by microscope, analysed and photographed (BX 61, Olympus Corporation, USA). The tissues were screened for the presence of Negri bodies, Perivascular cuffing, Neuronophagia, Gliosis, Congestion, Haemorrhage, Satellitosis and neuronal degeneration.

3.10 Correlation of molecular detection of rabies with clinical syndrome:

For the productive application of molecular approaches for detection of rabies in live animals in the field conditions, the detection of rabies by real time PCR was correlated with the observation of clinical symptoms in the suspected animals, since it is of paramount importance to establish the stage of disease progression viz. a viz. clinical syndrome, so that a correlation may be built for logical feasibility of employing molecular approaches for detection of rabies in live animals.

The clinical symptoms incorporated in the study were ranging from initial signs namely off feed, fever, change in behaviour, midterm signs namely salivation, Micturition and terminal signs namely paralysis and coma. Likewise, it was also assessed that whether absence of significant clinical symptoms had correlation with detection of rabies by molecular approaches.

3.11 Comparison of molecular technique for diagnosis of rabies virus RNA in skin samples:

The sensitivity of nested RT-PCR and TaqMan real time PCR was compared for ante-mortem diagnosis of rabies viral RNA in skin samples.

Since, FAT is recommended worldwide as a standard technique for diagnosis of rabies on neural tissue, after death of animal by World Health Organization (Hanlon *et al* 1999). So, nested RT-PCR and TaqMan pcr employed on skin samples were also compared with FAT for detecting the efficacy of these molecular techniques. The sensitivity of various tests applied was calculated using formulae:

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \text{ x } 100$$

3.12 Comparison of molecular technique for diagnosis of rabies virus RNA in saliva samples:

Sensitivity of diagnosis of rabies viral RNA in saliva samples by nested RT-PCR and TaqMan real time PCR was compared.

Since FAT was recommended gold standard test for diagnosis of rabies, so sensitivity of nested RT-PCR and TaqMan real time PCR applied for ante-mortem detection of rabies virus in saliva samples was also compared with FAT. The sensitivity of various tests applied was calculated using formulae:

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \text{ x } 100$$

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Clinically suspected animals

20 animals comprising of 7 buffaloes (all females), 5 cows (4 females, 1 male) and 8 dogs (3 female, 5 males) (Table 8) were incorporated in the present study. Mean average age of buffaloes was 5.57 yrs (range 2 yrs - 8 yrs), while mean average age of cows was 1.81 yrs (range 1 month – 4.5 yrs) and mean average age of dogs was 4.10 yrs (range 3.5 month - 12 yrs).

Table 8: Details of clinically suspected animals

Species	Age			Sex	
	≤2.5 yr	2.5-7.5yr	≥7.5yr	Male	Female
Buffalo	1	5	1	0	7
Cow	3	2	-	1	4
Dog	4	2	2	5	3

4.2 Samples from clinically suspected animals

4.2.1 Saliva samples

Collection of Saliva samples was attempted from 20 animals. Due to severe dehydration in some animals it was not feasible to collect saliva from 9 animals. 11 animals from which saliva was collected constitutes of 3 buffaloes, 3 cows and 5 dogs.

4.2.2 Skin samples

Skin biopsy samples were collected from all 20 animals comprising of 7 buffaloes, 5 cows and 8 dogs.

4.3 Analysis of nucleic acid

4.3.1 RNA

As per manufacturer's specification (Nano drop 1000 spectrophotometer Thermo scientific, USA), the 260/280 ratio of saliva samples was in the range of 1.71-1.89 and skin samples RNA was in the range of 1.70-1.89 (Table 9).

Further the concentration of RNA from saliva samples varied from 91.23-321.56 ng/µl and in skin samples varied from 100.17-744.40 ng/µl.

4.3.2 cDNA

As per manufacturer's specification (Nano drop 1000 spectrophotometer Thermo scientific, USA), the 260/280 ratio of saliva samples was in the range of 1.89-2.07 and skin samples cDNA was in the range of 1.87-2.12 and (Table 10)**.**

Further the concentration of cDNA from skin samples varied from 2021.80-4708.40 ng/µl and in saliva samples varied from 1956.60-4153.20 ng/µl.

Table 9: Analysis of extracted RNA

S. No.	Sample	Conc. (ng/µl)	260/280 ratio
1.	Saliva	104.34	1.76
2.	Saliva	318.08	1.88
3.	Saliva	91.23	1.77
4.	Saliva	251.80	1.81
5.	Saliva	95.46	1.83
6.	Saliva	201.15	1.77
7.	Saliva	265.43	1.79
8.	Saliva	267.65	1.85

9.	Saliva	99.05	1.77
10.	Saliva	125.35	1.71
11.	Saliva	321.56	1.89
12.	Skin	191.47	1.70
13.	Skin	161.57	1.81
14.	Skin	100.17	1.75
15.	Skin	178.40	1.75
16.	Skin	330.86	1.81
17.	Skin	454.60	1.86
18.	Skin	193.02	1.71
19.	Skin	422.01	1.89
20.	Skin	241.90	1.71
21.	Skin	512.20	1.81
22.	Skin	656.70	1.81
23.	Skin	242.70	1.86
24.	Skin	433.90	1.84
25.	Skin	744.40	1.81
26.	Skin	550.08	1.77
27.	Skin	198.30	1.82
28.	Skin	251.16	1.83
29	Skin	313.06	1.82
30.	Skin	222.28	1.82
31.	Skin	245.86	1.83

Table 10: Analysis of cDNA

S. No.	Sample	Conc. (ng/µl)	260/280 ratio
1.	Saliva	3736.01	1.92
2.	Saliva	4153.20	1.93
3.	Saliva	3903.91	2.04
4.	Saliva	2243.02	1.97
5.	Saliva	2373.45	1.89
6.	Saliva	1956.60	1.91
7.	Saliva	3946.10	2.07
8.	Saliva	2231.08	1.95

9.	Saliva	2649.70	2.07
10.	Saliva	2800.80	2.06
11.	Saliva	2859.60	1.99
12.	Skin	2261.00	1.87
13.	Skin	2746.20	1.97
14.	Skin	2753.20	1.89
15.	Skin	2488.60	1.90
16.	Skin	2068.90	1.95
17.	Skin	2408.50	1.96
18.	Skin	2021.80	2.04
19.	Skin	3113.30	1.99
20.	Skin	3653.30	1.87
21.	Skin	3135.00	1.87
22.	Skin	2773.20	1.91
23.	Skin	2766.80	1.96
24.	Skin	2712.00	2.04
25.	Skin	2652.00	1.91
26.	Skin	2621.80	1.99
27.	Skin	4181.70	2.02
28.	Skin	3905.60	1.93
29	Skin	4708.40	2.06
30.	Skin	3964.20	2.12
31.	Skin	3578.20	2.11

4.4 Molecular approaches for ante-mortem diagnosis:

4.4.1 Nested Reverse transcriptase polymerase chain reaction

Amplification with primers Rab N1 and Rab N5 yielded 1477 bp first round product. Nested pair of primers (Rab Nfor and Rab Nrev) used for amplification in second round yielded 762 bp product.

Nadin Davis (1998), Nagaraj *et al* (2006) & Kaw et al (2011) have also reported amplification with similar primers Rab N1 and Rab N5 with a yield of 1477 bp first

round product while amplification in second round with Rab Nfor and Rab Nrev yielded 762 bp product.

4.4.1.1 Saliva samples

By nested RT-PCR, viral RNA could be diagnosed in higher number (4/11) of saliva samples (Table 11) (Fig. 4 & 5) than Crepin *et al* (1998) and Nagaraj *et al* (2006), who reported detection of rabies virus in 11/37 and 6/21 respectively in saliva samples of human patients by conventional RT-PCR. However, Noah *et al* (1998) found rabies virus in (9/15) saliva samples by use of RT-PCR. Similarly, Wacharapluesadee and Hemachudha (2010) diagnosed rabies virus in (47/62) saliva samples by nucleic acid amplification based technique. Kaw *et al* (2011) confirmed rabies virus in (3/12) saliva samples of animals by nested RT-PCR which was less as compared with the present study.

Application of Nested RT-PCR on saliva samples for viral nucleic acid yielded a sensitivity of 66.67% which is more than the sensitivity of 30% reported by Crepin *et al* (1998) and 37% reported by Nagaraj *et al* (2006) who also used nested RT-PCR on human saliva samples. The sensitivity detected in present study was more than 37.5% as reported by Kaw *et al* (2011) who used nested RT-PCR on saliva samples of animals. However, Noah *et al* (1998) observed a higher sensitivity of >98% by the use of RT-PCR on saliva samples for ante-mortem diagnosis of rabies in human saliva samples. In another study, RT-PCR on saliva for viral nucleic acid detection yielded a sensitivity of 50-70% and a specificity of 100% (Madhusudana and Sukumaran 2008).

4.4.1.2 Skin samples

By nested RT-PCR, viral RNA could be diagnosed in higher number 9/20 (45.0%) in skin biopsy samples (Table 11) (Fig. 6 & 7) as compared to 4/11 (36.3%) observed by Kaw *et al* (2011). However, Macedo *et al* (2006) detected rabies viral RNA by use of RT-PCR technique in one out of two (50%) skin samples collected ante-mortem and six (75%) out of eight skin samples collected post-mortem. Noah *et al.* (1998) confirmed rabies viral antigen in higher number 10/15 (66.6%) human nuchal skin biopsy by RT-PCR.

Sensitivity of nested RT-PCR on skin samples was found to be 76.47% which was more than the sensitivity of 66.6% observed by Noah *et al* (1998) on human nuchal skin biopsy samples and Kaw *et al* (2011) who revealed sensitivity of 57.1% on animal skin biopsy samples. Another study revealed (77.7%) sensitivity with the use of RT-PCR on neck skin samples in human patients (Macedo *et al* 2006).

Table 11: Nested RT-PCR for ante-mortem diagnosis of rabies

S. No.	Species	Age	Sex	Skin	Saliva
1.	Buffalo	2 yrs	F	+	NA
2.	Dog	5 mths	F	-	NA
3.	Cow	3 yrs	M	+	-
4.	Dog	4 yrs	M	-	-
5.	Buffalo	6 yrs	F	-	NA
6.	Buffalo	4 yrs	F	-	NA
7.	Dog	3½ mths	M	-	-
8.	Buffalo	6 yrs	F	+	+
9.	Dog	5 yrs	F	-	NA
10.	Cow Calf	6 mths	F	+	-
11.	Dog	2½ yrs	F	+	-
12.	Cow calf	1 mths	F	-	NA
13.	Cow	4½ yrs	F	-	NA
14.	Buffalo	8 yrs	F	-	-
15.	Dog	12 yrs	M	-	-
16.	Dog	1 yrs	M	+	NA
17.	Dog	7½ yrs	M	+	+
18.	Buffalo	7 yrs	F	-	NA
19.	Buffalo	6 yrs	F	+	+
20.	Cow	1 yrs	F	+	+
		% Positivity		45%	36.36%

NA- not available, + Positive, - Negative

4.4.2 TaqMan Real time PCR

The samples in which threshold cycle number (*Ct*) values were found to be in the range of 20-35 were considered positive and above 35 were considered negative.

4.4.2.1 Saliva samples

By TaqMan real time PCR, viral RNA could be diagnosed in 6/11 (54.54%) saliva samples (Table 12) (Fig. 8) which was less as compared to 87% as detected by real time PCR on saliva samples of human patients by Saengseesom *et al* (2007). Nagaraj *et al* (2006) also found that 18/21 saliva samples positive by real time PCR.

TaqMan real time PCR on saliva samples for viral nucleic acid yielded a sensitivity of 80%. It is more as compared to Wacharapluesadee and Hemachudha (2010) who reported sensitivity of 75.8% from human saliva samples and with Saengseesom *et al* (2007), who obtained sensitivity of 75% on saliva samples by real time PCR.

4.4.2.2 Skin samples

By TaqMan real time PCR, viral RNA could be diagnosed in 11/20 skin biopsy samples (Table 12) (Fig. 9). A perusal of literature in the present study revealed that there is no published work on ante-mortem detection of rabies viral RNA in skin samples using TaqMan real time PCR in animals.

Sensitivity of TaqMan real time PCR on skin samples was found to be 86.67%. A perusal of literature in the present study revealed that there is no published work on ante-mortem detection of rabies viral RNA in skin samples using TaqMan real time PCR in animals.

Table 12: TaqMan real time PCR for ante-mortem diagnosis of rabies

S. No.	Species	Age	Sex	Skin	Saliva
1.	Buffalo	2 yrs	F	+	NA
2.	Dog	5 mths	F	-	NA

3.	Cow	3 yrs	M	-	-
4.	Dog	4 yrs	M	-	-
5.	Buffalo	6 yrs	F	-	NA
6.	Buffalo	4 yrs	F	-	NA
7.	Dog	3½ mths	M	-	-
8.	Buffalo	6 yrs	F	+	+
9.	Dog	5 yrs	F	-	NA
10.	Cow Calf	6 mths	F	+	+
11.	Dog	2½ yrs	F	+	+
12.	Cow calf	1 mths	F	+	NA
13.	Cow	4½ yrs	F	+	NA
14.	Buffalo	8 yrs	F	-	-
15.	Dog	12 yrs	M	+	-
16.	Dog	1 yrs	M	+	NA
17.	Dog	7½ yrs	M	+	+
18.	Buffalo	7 yrs	F	-	NA
19.	Buffalo	6 yrs	F	+	+
20.	Cow	1 yrs	F	+	+
		% Positivity		55%	54.54%

NA- not available, + Positive, - Negative

4.5 Postmortem confirmation of Rabies

Molecular viz. nested RT-PCR and TaqMan real time PCR and conventional techniques viz. immunofluorescence, histopathological alterations were applied on brain samples.

4.5.1 Post mortem confirmation of rabies by Molecular approaches

Nested RT-PCR and TaqMan real time molecular techniques were applied on brain samples for postmortem confirmation of rabies.

4.5.1.1 Nested RT-PCR

Out of 20 brain samples, 12 samples were positive by nested RT-PCR assay (Table 13) (Fig. 10 & 11). Sensitivity of nested RT-PCR was 92.85%. Similarly, Araujo *et al* (2008) found the sensitivity of 52% with nested RT-PCR on brain samples which is less than the sensitivity observed in present study. The sensitivity obtained in the present study with nested RT-PCR on brain samples was less as observed by Kamolvarin *et al* (1993) who concluded >98% sensitivity with nested RT-PCR and Romijn *et al* (2003) who detected rabies in 60 out of 72 brain samples by nested RT-PCR with a sensitivity of 86%.. However, Nadin Davis *et al* (2007) used nested RT-PCR for phylogenetic analysis of rabies virus isolates in India and confirmed rabies in 10 out of 37 (27%) suspected rabies cases which was less as compared to present study .

Table 13: Nested RT-PCR for post-mortem diagnosis of rabies

S. No.	Species	Age	Sex	Brain
1.	Buffalo	2 yrs	F	+
2.	Dog	5 mths	F	-
3.	Cow	3 yrs	M	+
4.	Dog	4 yrs	M	-
5.	Buffalo	6 yrs	F	+
6.	Buffalo	4 yrs	F	-

7.	Dog	3½ mths	M	-
8.	Buffalo	6 yrs	F	+
9.	Dog	5 yrs	F	-
10.	Cow Calf	6 mths	F	+
11.	Dog	2½ yrs	F	+
12.	Cow calf	1 mths	F	-
13.	Cow	4½ yrs	F	+
14.	Buffalo	8 yrs	F	-
15.	Dog	12 yrs	M	+
16.	Dog	1 yrs	M	+
17.	Dog	7½ yrs	M	+
18.	Buffalo	7 yrs	F	-
19.	Buffalo	6 yrs	F	+
20.	Cow	1 yrs	F	+
		% Positivity		60%

NA- not available, + Positive, - Negative

4.5.1.2 TaqMan Real time PCR

By TaqMan real time PCR, viral RNA could be detected in 13/20 brain samples (Table 14) (Fig. 12). TaqMan real time PCR when compared with FAT, revealed the sensitivity of 100%. The sensitivity obtained in present study with TaqMan real time PCR on brain samples was more than Hughes *et al* (2004), according to which about 5% (3/62) tissue samples were not detected by TaqMan real time PCR. Wacharapluesadee *et al* (2008) applied real time PCR on 143 brain samples and found that results were concordant with FAT which was similar as concluded by present study.

4.5.2 Post-mortem confirmation of rabies by conventional approaches

4.5.2.1 Immunofluorescence

Out of 20 cases, 13 (65%) cases were diagnosed positive using immunofluorescence on brain samples (Fig. 13 & 14). Out of 11 cases whose saliva samples are collected, 8 cases (72.73%) showed apple green fluorescence. Use of immunofluorescence technique for rabies diagnosis has been reported by several workers Howard (1977), Umoh *et al* (1985), Davis *et al* (1997) and Cortes *et al* (1979). Similarly, Tepsumethanon *et al* (1997), Meslin *et al* (1996), Singh and Grewal (1998a), Silva *et al* (1999), Singh (1999), Asha Rani (2001), Archana *et al* (2003) and Kaw *et al* (2011). Whitfield *et al* (2001) have observed that the preferred method for routine diagnosis of rabies in fresh or frozen brain tissues is the Fluorescent Antibody Test.

Table 14: TaqMan real time PCR for postmortem diagnosis of rabies

S. No.	Species	Age	Sex	Brain
1.	Buffalo	2 yrs	F	+
2.	Dog	5 mths	F	-
3.	Cow	3 yrs	M	+
4.	Dog	4 yrs	M	-
5.	Buffalo	6 yrs	F	+
6.	Buffalo	4 yrs	F	-
7.	Dog	3½ mths	M	-
8.	Buffalo	6 yrs	F	+
9.	Dog	5 yrs	F	-

10.	Cow Calf	6 mths	F	+
11.	Dog	2½ yrs	F	+
12.	Cow calf	1 mths	F	+
13.	Cow	4½ yrs	F	+
14.	Buffalo	8 yrs	F	-
15.	Dog	12 yrs	M	+
16.	Dog	1 yrs	M	+
17.	Dog	7½ yrs	M	+
18.	Buffalo	7 yrs	F	-
19.	Buffalo	6 yrs	F	+
20.	Cow	1 yrs	F	+
		% Positivity		65%

NA- not available, + Positive, - Negative

4.5.2.2 Postmortem confirmation of rabies by histopathology

Out of a total of 20 postmortem cases of different animal species in this study, 11 (55.0%) were found positive for rabies by demonstration of Negri bodies in Purkinje cells and in the neurons of hippocampus (Table 15) thus, histomorphology had 86.67 % sensitivity in comparison to FAT. Species-wise quantitative analysis of histopathological alterations was also done (Table 16).

In dogs, histomorphology findings confirmed Negri bodies in 2 out of 4 immunofluorescence positive cases (Fig. 15). Quantitative assessments of histomorphological alterations reported neuronal degeneration and gliosis in both histomorphological positive cases while, other histomorphological alterations viz.

Perivascular cuffing (Fig. 16), Neuronophagia (Fig. 17), Congestion, Hemorrhages, Satellitosis and Meningitis were detected in 1 case.

Histomorphological diagnosis of rabies on the basis of Negri body in cerebellum and hippocampus of rabid cows revealed 5/5 (100%) positive cases. Gliosis and Hemorrhage were observed in 4/5 cases, Neuronal degeneration, Congestion (Fig. 18) and Neuronophagia in 3/5 cases (Fig. 19), Perivascular cuffing in 2/5, Satellitosis in 1/5 cases (Fig. 20) and Meningitis in 1/5 cases.

Negri bodies were present in cerebellum of buffalo in all the 4 immunofluorescence positive cases. Neuronal degeneration was observed in all cases, while Perivascular cuffing, Neuronophagia and Hemorrhage were observed in 2/ 4 cases. Gliosis, Congestion, Satellitosis and Meningitis (Fig. 21) were observed in 1 case.

Table 15: Histopathological alterations in brain of rabid animals

Sr. No	Case No.	Species	Negri bodies	Perivascular cuffing	Neuronophagia	Neuronal Degeneration	Gliosis	Congestion	Haemo-rrhage	Satellitosis	Meningitis
1.	25 RL/10	Buffalo	+	+	-	+	+	+	+	+	+
2.	26 RL/10	Dog	-	-	-	-	-	-	-	-	-
3.	28 RL/10	Cow	+	+	+	+	+	-	+	-	+
4.	32 RL/10	Dog	-	-	-	-	-	-	-	-	-
5.	36 RL/10	Buffalo	+	-	+	-	-	-	+	+	-
6.	38 RL/10	Buffalo	-	-	-	-	-	-	-	-	-
7.	03 RL/11	Dog	-	-	-	-	-	-	-	-	-
										-	-
										-	-
										-	-
11.	14 RL/11	Dog	+	+	+	+	+	+	-	+	+
12.	21 RL/11	Cow	+	-	-	+	+	+	+	-	-
13.	23 RL/11	Cow	+	-	-	-	-	-	-	-	-
14.	25 RL/11	Buffalo	-	-	-	-	-	-	-	-	-
15.	27 RL/11	Dog	-	-	-	-	-	-	-	-	-

Sr. No	Case No.	Species	Negri bodies	Perivascular cuffing	Neuronophagia	Neuronal Degeneration	Gliosis	Congestion	Haemo-rrhage	Satellitosis	Meningitis
16.	32 RL/11	Dog	-	-	-	-	-	-	-	-	-
17.	35 RL/11	Dog	+	-	-	+	+	-	+	-	-
18.	36 RL/11	Buffalo	-	-	-	-	-	-	-	-	-
19.	37 RL/11	Buffalo	+	-	-	+	-	-	-	-	-
20.	38 RL/11	Cow	+	+	+	-	+	+	+	+	-
	Total		11/20	5/20	6/20	7/20	7/20	5/20	7/20	4/20	3/20

Table 16: Species-wise quantitative analysis of histopathological alterations
n= Positive cases

Histomorphological alterations	Species					
	Cow (n=5)		Buffalo (n=4)		Dog (n=2)	
	+ve samples	Percentage	+ve samples	Percentage	+ve samples	Percentage
Negri bodies	5	100	4	100	2	100
Perivascular cuffing	2	40	2	50	1	50
Neuronophagia	3	60	2	50	1	50
Neuronal	3	60	2	50	2	100
Gliosis	4	80	1	25	2	100
Congestion	3	60	1	25	1	50
Hemorrhage	4	80	2	50	1	50
Satellitosis	1	20	2	50	1	50
Meningitis	1	20	1	25	1	50

Negri bodies are considered pathognomic for rabies Negri bodies were detected in more in cows and buffaloes than in dogs, low reporting of meningitis could be attributed to complete peeling off of the meninges surrounding mid brainstem structure while collecting the specimen. From the present study, it was found that there were certain similarities and significant differences in the histopathological alterations in various nervous tissues of these species - that were often over looked. Negri bodies, the pathognomonic lesion of rabies are most consistently found in cerebellum, followed by cerebrum of buffaloes and cattle, and hippocampus followed by cerebellum of dogs.

Histopathological changes in cerebellum of rabid buffaloes revealed neuronal degeneration in 100% of samples (4/4) which is same as reported earlier by (Jamadagni *et al* 2008), while perivascular cuffing, Neuronophagia and hemorrhage were observed in 50% (2/4) of cases. Gliosis, congestion, Satellitosis and meningitis were observed in 25% (1/4)

case. Similar histopathological alterations have been qualitatively reported by (Singh 1999 and Archana 2001) and quantitatively reported by (Jamadagni *et al* 2008).

Negri bodies in cerebellum of rabid cattle were found to be higher (5/5) than earlier reports of (Arslan *et al* 2004) and (Lima *et al* 2005). Other histopathological changes revealed neuronal degeneration in 60% samples (3/5) and satellitosis in 20% samples (1/5) which is less as reported earlier by (Jamadagni *et al* 2008). Gliosis and hemorrhage were present in 80% samples (4/5). Congestion and neuronophagia were present in 60% (3/5), perivascular cuffing in 40% (2/5) cases, Satellitosis and meningitis was observed in 20% (1/5) cases. Similar histopathological alterations reported by (Pedroso *et al* 2009) in cattle.

Histopathology has 86.67% sensitivity in comparison to FAT on Brain tissue smears were found to be lesser as compared to study by (Arslan *et al* 2004) which reported sensitivity of 97.6%. Negri bodies were detected in all the cases (100%) in cattle and buffaloes which were found to be higher as compared with study by (Jamadagni *et al* 2008). Lima *et al* (2005) reported Negri bodies in 87% of the cattle cases. No Negri bodies were detected by (Macruz *et al* 1977) in cattle. Whereas, study conducted by (Singh and Grewal 1998a) and (Parveena *et al* 2003) reported that histopathological detection of Negri bodies was the least sensitive of all the diagnostic technique.

4.6 Clinical syndrome of rabid animals detected by molecular approaches

Signs and symptoms included in study was divided into three stages; mainly early phase characterized by off feed, fever, behavioural change, mid phase characterized by bellowing and Micturition and late phase characterized by difficult intake, nervous excitement (Fig. 22), salivation (Fig. 23), difficult standing and walking and paralysis (Fig. 24).

4.6.1 Nested RT-PCR

Most of the animals detected by nested RT-PCR assay reveal prodromal syndrome. Off feed was found in 10/11 (90.90%) cases, while fever was present in 7/11 (63.64%) cases, behavioural changes was observed in 3 cases. In 7/11 (63.64%) cases bellowing was observed, while frequent urination was found only in 2/11 (18.18%) cases. While late phase symptoms like difficult intake, salivation and difficulty in standing and walking was detected in 4/11(36.36%), 8/11(72.72%), 3/11 (27.27%) cases, while paralysis was observed in 2/11 (18.18%) cases (Table 17).

4.6.2 TaqMan real time PCR

Most of the animals exhibit early phase symptoms were detected by TaqMan real time PCR technique. Off feed was exhibited by 11/13 (84.61%) cases, behavioural changes was observed in 4/13 (30.76%) while fever was present in 8/13 (61.54%) cases respectively. Most of the animals found positive by real time PCR did not exhibit micturition, did not have proper vaccination schedule. While late phase symptoms like behavioural change and difficulty in walking was observed in 4/13 (30.76%) cases, while salivation & paralysis was observed in 10/13(76.92%) cases and 2/13 (15.38%) cases, respectively (Table 18).

Table 17: Clinical syndromes of rabid animals detected with Nested RT-PCR

S. No.	Species	Sex	AGE	Off feed	Fever	Behaviour change	Micturition	Bellowing	Difficult Intake	Difficult Standing, walking	Salivation	Paralysis
1.	Buffalo	F	2 years	+	+	-	-	+	+	-	-	-
2.	Cow	M	3years	+	+	-	-	+	-	+	+	-
3.	Buffalo	F	6years	+	+	+	-	+	+	+	+	+
4.	Buffalo	F	6 years	+	-	-	+	+	-	-	+	-
5.	Cow Calf	F	6 months	+	+	-	-	-	-	-	+	-
6.	Dog	F	2.5 years	+	+	+	-	-	+	-	+	-
7.	Cow	F	4.5 years	+	+	-	-	+	-	-	+	-
8.	Dog	M	1years	+	+	+	-	-	-	+	+	+
9.	Dog	M	7.5 years	-	-	-	-	-	-	-	-	-
											+	-
											-	-
											8/11	**2/11**

Table 18: Clinical syndromes of rabid animals detected with TaqMan Real time PCR

S. No.	Species	Sex	AGE	OFF feed	Fever	Behaviour change	Bellowing	Micturition	Difficult Intake	Difficult Standing, walking	Salivation	Paralysis

1.	Buffalo	F	2 yrs	**+**	**+**	**-**	**+**	**-**	**+**	**-**	**-**	**-**
2.	Cow	M	3yrs	+	+	-	+	-	-	+	+	-
3.	Buffalo	F	6yrs	+	+	+	+	-	+	+	+	+
4.	Buffalo	F	6 yrs	+	-	-	+	+	-	-	+	-
5.	Cow Calf	F	6 mths	+	+	-	-	-	-	-	+	-
6.	Dog	F	2.5 yrs	+	+	+	-	-	+	-	+	-
7.	Cow calf	F	1 mths	+	+	-	-	-	-	-	+	-
8.	Cow	F	4.5 yr	+	+	-	+	-	-	-	+	-
9.	Dog	M	12 yrs	-	-	+	-	-	-	-	+	-
10.	Dog	M	1yrs	+	+	+	-	-	-	+	+	+
11.	Dog	M	7.5 yrs	-	-	-	-	-	-	-	-	-
12.	Buffalo	F	6 yrs	+	-	-	+	+	+	-	+	-
13.	Cow	F	1 yrs	+	-	-	+	-	-	+	-	-
	Total			**11/13**	**8/13**	**4/13**	**7/13**	**2/13**	**4/13**	**4/13**	**10/13**	**2/13**

The perusal of literature reveals that the information regarding correlation of clinical syndromes with molecular approaches for detection of rabies in animals is lacking. So it was concluded that in absence of symptoms namely off feed, salivation and fever chances of false negative ante-mortem result by molecular approach increase. In other words, molecular ante-mortem detection should be attempted preferably in those animals that reveal early phase symptoms off feed, salivation, fever and behavioural change. Since this is an early analysis based on a few cases, therefore, further studies are recommended.

4.7: Sensitivity comparison of molecular and conventional techniques for diagnosis of rabies viral RNA in Saliva samples

Sensitivity of nested RT-PCR on saliva samples was found to be 66.67% whereas, sensitivity of TaqMan real time PCR on saliva samples was found to be 80.0 %.

In present study TaqMan real time PCR technique on saliva samples was 13.33 % more sensitive than nested RT-PCR on saliva samples, 6.67% and 12.85% less sensitive than histopathology and nested RT-PCR on brain samples and 20% less as compared to sensitivity of TaqMan real time PCR on brain sample and immunofluorescence (Table 19) (Fig. 25).

Table 19: Sensitivity comparison of molecular and conventional techniques for diagnosis of rabies viral RNA in saliva samples

Sample	FAT	Histopathology	Nested RT- PCR	Real time PCR

Brain	100 %	86.67 %	92.85 %	100 %
Saliva	NA	NA	66.67 %	80 %

NA- not available

4.8 Sensitivity comparison of molecular and conventional techniques for diagnosis of rabies viral RNA in skin samples

Sensitivity of nested RT-PCR on skin samples was found to be 76.47% whereas, Sensitivity of TaqMan real time PCR on skin samples was found to be 86.67%.

In present study sensitivity of TaqMan real time PCR technique on skin samples was 10.20 % more than nested RT-PCR on skin samples, concordant with histopathology and 5.18 % less than nested RT-PCR on brain samples and 13.33 % less as compared to sensitivity of TaqMan real time PCR on brain sample and FAT (Table 20) (Fig. 26).

Table 20: Sensitivity comparison of molecular and conventional techniques for diagnosis of rabies viral RNA in skin samples

Sample	FAT	Histopathology	Nested RT- PCR	Real time PCR
Brain	100 %	86.67 %	92.85 %	100 %
Skin	NA	NA	76.47 %	86.67 %

NA- not available

CHAPTER V

SUMMARY

The present study was conducted on 20 rabies suspected animals and diagnosis was made by the use of molecular approaches along with conventional techniques. As the virus is shed in all the secretions and present in nerve cells surrounding the base of hair follicle of the rabid animal, thus for ante mortem diagnosis samples like saliva, skin biopsy, were collected from 20 cases presented to the Veterinary Clinics, GADVASU, Ludhiana and CVH, different districts of Punjab. Nested RT-PCR and TaqMan real time PCR were used for the diagnosis of rabies from these samples, their percentage positivity and sensitivity was revealed. RNA was extracted from the samples and subjected to cDNA formation. Amplification with nested set of primers (Rab Nfor and Rab Nrev) yielded a 762 bp product. By nested RT-PCR, viral RNA could be detected in 36.36% (4/11) saliva samples and 45.0% (9/20) skin biopsy samples. The cycle threshold (*C*t) values ranging from 26 to 35 cycles were obtained. The (*C*t) value above 35 was considered negative. By TaqMan real time PCR, viral RNA could be detected in 54.54% (6/11) saliva samples and 55.0% (11/20) skin biopsy samples. The analysis of both the techniques revealed higher sensitivity of TaqMan real time PCR than nested RT-PCR as sensitivity of 86.67% was obtained with TaqMan real time PCR for skin biopsy samples and 76.47% with nested RT-PCR. Likewise sensitivity of saliva samples with TaqMan real time PCR and nested RT-PCR and was 80% and 66.67% respectively. Molecular approaches were also used for the post-mortem diagnosis of rabies in animals suspected of rabies as nested RT-PCR and TaqMan real time PCR were applied to the brain samples after RNA extraction and cDNA synthesis. Out of

20 brain samples, 12 were positive by nested RT-PCR, with a sensitivity of 92.85%. TaqMan Real time analysis revealed 65% (13/20) positive brain tissue with a sensitivity of 100%. The results were in concordance with the immunofluorescence test done on brain impression smears.

CONCLUSIONS

1. Skin offers greater sensitivity for ante mortem diagnosis of rabies as compared to saliva.
2. TaqMan real time PCR technique is more sensitive than nested PCR for ante-mortem detection of rabies from skin as well as saliva.

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VITA

Name of the student	:	Karan Bansal
Father's name	:	Mr. Raj Kumar Bansal
Mother's name	:	Mrs. Sunita Bansal
Nationality	:	Indian
Date of birth	:	24th February 1986
Permanent home address	:	Kothi No. 353, Model Town, Phase-1 Bathinda-151001, Punjab

EDUCATIONAL QUALIFICATION

Bachelor's degree	**:**	**B.V.Sc. & A. H.**
University Sciences	:	Guru Angad Dev Veterinary and Animal University, Ludhiana, Punjab.
Year of award	:	2009
OGPA/OCPA	:	6.88/10.00
Master's degree	**:**	**M.V.Sc. (Veterinary Pathology)**
OCPA	:	7.11/10.00
Year of award	:	2012