COMPARATIVE EVALUATION OF LATERAL FLOW ASSAY AND LOOP MEDIATED ISOTHERMAL AMPLIFICATION WITH ACETONE AND FORMALIN FIXATION BASED DIRECT FLUORESCENT ANTIBODY ASSAY FOR DIAGNOSIS OF RABIES IN ANIMALS

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Thesis submitted to the

KARNATAKA VETERINARY, ANIMAL AND FISHERIES SCIENCES UNIVERSITY, BIDAR

In partial fulfillment of the requirements

For the award of the degree of

MASTER OF VETERINARY SCIENCE

in

VETERINARY MICROBIOLOGY

By

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CERTIFICATE

This is to certify that the thesis entitled **"COMPARATIVE EVALUATION OF LATERAL FLOW ASSAY AND LOOP MEDIATED ISOTHERMAL AMPLIFICATION WITH ACETONE AND FORMALIN FIXATION BASED DIRECT FLUORESCENT ANTIBODY ASSAY FOR DIAGNOSIS OF RABIES IN ANIMALS"** submitted by **Ms. TAJUNNISA, M., ID. No. MVHK-1531** in partial fulfillment of the requirements for the award of degree of **MASTER of VETERINARY SCIENCE** in **VETERINARY MICROBIOLOGY** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, is a record of bonafide research work carried out by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles.

Place: Bengaluru

Date: August, 2017

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Affectionately Dedicated to

"My beloved Family

and

Teachers"

ACKNOWLEDGEMENT

*'**First of all, I thank almighty God, the most beneficent and merciful, for providing me this opportunity and granting me the capability to proceed successfully.***

*I must express my very profound gratitude to my father **Mushtakh Ahamed** , my mother **Syeda Gulazar**, my co-twin **Masooda** and my youngest sister **Masooma** for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.*

*Indeed the words are not sufficient to pay my sincere regards with immense pleasure and deep sense of gratitude to my guide **Dr. Shrikrishna Isloor**, Associate Professor, Dept. of Veterinary Microbiology, Veterinary College, Bengaluru for his wholehearted encouragement, unconditional support, utmost affection, meticulous supervision and most importantly the freedom for work and the space given to express the ideas, during this research is greatly acknowledged. I would not have been able to reach this stage without his constant and expertise technical support which have been the strong pillars for this work. I am very much thankful for him.*

*My words would fail to express my indebtness to **Dr. D. Rathnamma**, Professor and Head, Department of Veterinary Microbiology, Veterinary College, Bengaluru my advisory committee who was as keen as my guide, in my progress. Her interest in my welfare went a long way in bringing this task to a shape. I profusely thank her for rendering all sort of support from beginning to end of my study.*

*I was fortune enough to have advice, constructive suggestions and constant interest in my work throughout this study from **Dr. B. M. Veeregowda,** Associate Professor, Dept. of Veterinary Microbiology, Veterinary College, Bengaluru and member of my advisory committee. I would like to express my heartfelt thanks to him for his generous help, invaluable guidance and unceasing encouragement during my master's degree programme.*

I avail this opportunity to express my profound indebtedness and esteemed sense of heartfelt gratitude to ***Dr.Sharada, R,*** *Associate Professor (I/C) & Head, Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hassan and member of my advisory committee for her incessant encouragement, immense patience, subtle suggestions and friendly help in spite of her busy schedule towards the successful completion of my research. I confess that, it has been a great fortune and proud privilege for me to be associated with her during my master's degree programme.*

I thank Dr. ***Dr. P.T. Ramesh****, Professor & Head, Dept. of Veterinary Medicine, Veterinary College, KVAFSU, Hebbal, BengalurAssociate for his inspiring suggestions, throughout the course of my study.*

I am highly thankful to ***OIE-Twinned KVAFSU-Crucell-CVA-Rabies diagnostic Laboratory*** *where this work was carried out.*

I would also like to place on record my sincere thanks to ***Dr. S. Abdul Rahman*** *for his inspiring moral talks which made me to stand so today. I immensely thank* ***Lillan Orciari, Tony Fooks, , Ashley C Banyard, Richard Franka, David Selden*** *for their valuable suggestions and technical advice.*

I cordially extend my thanks to ***Dr. Satyanarayana Rao and Dr, Phaniraj*** *for their subtle creative suggestions and friendly help towards the successful completion of my research.*

I place my deep regards to ***Dr. Shesheer Kumar****, Managing Director and Director of RAS Lifesciences Pvt. Ltd. Hyderabad, for his guidance.*

I am extremely thankful to ***Dr. A.K, Santosh*** *for his constant support and help during my research. My sincere thanks to my colleagues,* ***Dr Suijth, S. Nath*** *and* ***Dr. Pannaga*** *for their help throughout my study.*

My warmest gratitude goes to ***Drs Chinmayee, Lekhsmi and Netra*** *for their support. I also acknowledge my seniors* ***Drs S.Y. Mukartal , Ramesha, C. B, Sheela, P, Sumathi, Sunil***

Kumar, NithinPrabhu, Kamal hassan, Anil Kumar , Shashikala, Sonali and Deepti for their suggestions and help one way or the other.

I am very much grateful to Mrs. Sheela, Mrs Jyothi, Mr. Mahadevappa, Mr. Murthy, Mrs. Nanjamma, Mr Suresh for their help and support rendered during the entire period of this study.

I have always been overwhelmed by the sacrifices, constant support and encouragement rendered by my beloved friend Mohmed Salman which will be remembered in rest of my life.

I deeply express my sincere thanks to my friends Sumaiya, Maneesha, Unnisa, Farha, Kubra, Poornima, who have been continuous inspiration, encouragement and affection that boosted up my morale during the period of study.

I wish to thank all those who helped me directly or indirectly and whom I could not mention here. I hope these tender words will carry message of thanks to all of them.

Bengaluru
August, 2014 *(TAJUNNISA, M.)*

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVATIONS / ACRONYMS

bp	Base pair
cm	Centimeter
^{o}C	Degree centigrade
DFA	Direct Fluorescent Antibody asaay
DNA	Deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside-5′ triphosphate
dRIT	direct Rapid Immunohistochemistry Test
EDTA	Ethylene Diamine Tetra acetic acid
et al.	*et alia*
etc.	*et cetera*
Fig.	Figure
gm(s)	Gram(s)
hr(s)	Hour(s)
IU	International unit
LFA	Lateral Flow Assay
μg	Microgram
μL	Microliter
mg	Milligram
mL	Milliliter
min.	Minutes
M	Molar
NaCl	Sodium Chloride
NFW	Nuclease Free Water
OD	Optical Density

%	Per cent
PCR	Polymerase Chain Reaction
psi	Pounds per square inch
pmol	Picomole
RNase	Ribonuclease A
rpm	Revolution per minute
sec	Seconds
TBE	Tris Borate EDTA
TE	Tris EDTA
U	Unit
UV	Ultra Violet
V	Volts
v/v	volume/volume
w/v	weight/volume

Introduction

I. INTRODUCTION

Rabies is considered to be a re-emerging zoonosis in many parts of the world, particularly in Asia, Africa and Latin America, where the disease is enzootic despite the availability of proven prevention and control tools. As a neglected zoonotic disease, rabies is present many parts of the world, with many deaths in human beings occurring in Africa and Asia in children younger than 15 years. The disease causes a severe and long-lasting societal and economic burden and the implications are especially apparent in poverty-stricken developing countries. Rabies has been endemic in India since time immemorial (Sudarshan *et al.,* 2007). This fatal viral encephalitis is transmitted to man by bites/licks/scratches from a rabid animal (WHO, 2005).

This disease is caused by rabies virus belonging to the genus *Lyssavirus* of the family *Rhabdoviridae.* Rabies virus (RABV) possesses a single-stranded, linear, non-segmented, negative-sense RNA approximately 12 kb in size. The rabies viral genome encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase (L). The highly conserved and abundant N protein, a key structural component of the viral ribonucleoprotien (RNP) core is essential for viral propagation which constitutes the main target for rabies diagnosis.

In India, rabies occurs primarily in the urban form, in which dogs play an important role as the reservoir and transmitter of the disease to humans and domestic animals. Dog rabies is widespread in India with Lakshadweep, Andaman and Nicobar Islands declared historically free (Sudarshan *et al.,* 2007).

Dogs are the principal reservoir for human rabies in India and are responsible for more than 99 per cent of human cases. Hence, controlling rabies in dogs and especially free-roaming (stray) ones, needs to be prioritized for control of human rabies (Meslin and Briggs, 2013). Shortage of resources and a limited public health infrastructure in many rabies endemic countries precludes data collection and analysis. Thus, rabies is regarded as under reported in many regions, due in part, to a lack of surveillance and laboratory infrastructure, confounded by cultural or social taboos. The absence of accurate data on disease incidence in turn tends to reduce rabies as a priority for policy makers and public health professionals.

There are new developments in rabies research including cost effective methods for detecting rabies virus in clinical samples and thereby enhancing the efforts towards the ultimate goal of rabies elimination.

Laboratory confirmation of rabies in animals is important for institution of post exposure prophylactic and suitable control measures. Different methods have been employed for the diagnosis of rabies which include Direct Fluorescent Antibody test (DFA), direct Rapid Immunohistochemistry (dRIT), Lateral Flow Assay (LFA), Reverse Transcription - Polymerase Chain Reaction (RT-PCR), Loop Mediated Isothermal Amplification Assay (LAMP) and Mouse inoculation test.

The DFA is the gold standard test for rabies diagnosis of rabies by the WHO (Dean *et al*., 1996; WHO, 2005). However, the requirement of expensive fluorescent microscope, laboratory facility and expertise limits the overall usage of the DFA in developing countries including India and hence limited to a few regional or state level

laboratories. Further, routine DFA fixation at -20˚C requires a deep freezer which may not be available in several laboratories. In addition, DFA involves fixation of the brain impression using chilled acetone which does not completely inactivate the rabies virus thereby posing a possible biohazard (Umoh and Blenden, 1981).

In this context, dRIT developed at Centers for Disease Control and Prevention (CDC), Atlanta, USA, is a simple test that requires no specialized equipment or infrastructure as in DFA and can be successfully performed on samples preserved in glycerol solution for 15 months or frozen for 24 months and in variable conditions of preservation (Lembo *et al.,* 2006). There was 100 per cent correlation between DFA and dRIT (Isloor *et al*., 2014 and Nithinprabhu *et al.,* 2014). Moreover, formalin fixation of the impression completely inactivates the rabies virus thus reducing the risk of biohazard involved (Velasco-Villa *et al.,* 2005 and OIE, 2011).

Recently, a novel rapid method is developed for the detection of RABV antigen from post-mortem samples based on the principles of immunochromatography (Kang *et al.,* 2007). This is becoming a popular diagnostic mainly due to its simplicity, specificity, sensitivity and most importantly rapidity without the need for specialized and costly equipments. This is being used for the rapid diagnosis of various infectious diseases (Al-Yousif *et al*., 2002; Kuroiwa *et al*., 2004 and Tsuguto *et al*., 2004) with an overall sensitivity of 96.9 per cent and specificity of 100 per cent when compared to DFA. It is a straight forward test that can be run under field conditions without the need of a microscope or electricity and yields results in five to ten minutes. This rapid immunodiagnostic test is an easy to use surveillance tool that can identify rabies positive

animals and help focus targeted control measures with the goal of reducing the rabies burden (Kristen *et al.,* 2014). One hundred per cent correlation between LFA and reverse transcription polymerase chain reaction (RT-PCR) was noticed in detection of Rabies virus in cell culture harvests (Sharada *et al.,* 2015).

Various versions of PCR targeting the N gene of the rabies virus have been developed both for detecting the fixed and the street RABV (Arai *et al*., 1997; Crepin*et al*., 1998; Black *et al*., 2000 and Gupta *et al*., 2001). Nucleotide amplification based techniques such as RT-PCR and real-time RT-PCR have exhibited higher sensitivity and specificity in the diagnosis of rabies and proven to be reliable methods (Smith *et al.,* 1972; Nadin-Davis *et al.,* 1994; Kissi *et al.,* 1995; Saengseesom *et al.,* 2007 and Dacheux *et al.,* 2008). These tests involve expensive equipments especially the thermal cycler which limits their usage and require at least 3-4 hours. Therefore, these tests can be done only at the regional laboratories and not in rural settings in developing countries such as India. Thus, there is a need for the development of specific, sensitive, rapid, cost-effective and user-friendly diagnostic tests which can be employed for the diagnosis of rabies.

Loop Mediated Isothermal Amplification (LAMP) offers an alternative DNA amplification method to the laboratory oriented PCR with high specificity, efficiency and without the need for thermal cycler. Amplification is achieved through the specific binding of two inner and two outer primers to the target sequence. The reverse transcription(RT) and LAMP reactions can be undertaken in a single tube at 40-45°C using a thermo stable reverse transcriptase, hence avoiding the step inherent in a RT-

PCR. At a constant temperature RNA/DNA could be detected within 30 minutes. The use of isothermal amplification has the benefit of reducing the technological requirements of thermal cycling used in RT-PCR. This in turn offers an opportunity, when linked with lateral flow devices, to develop surveillance protocols where testing can be done at the field or in less sophisticated laboratories (Notomi *et al*., 2000). The RT-LAMP developed can used at the field level to precisely identify the nucleic acid of RABV from the suspected clinical samples (Nithinprabhu, 2014).

Considering all the above, since acetone and formalin based fixation of brain impressions for diagnosis of rabies by DFA needs to be evaluated, the present study was taken up to compare lengthy acetone based tissue fixation with rapid formalin fixation and evaluate the efficacy of LFA and LAMP for diagnosis of rabies in animals with the following objectives.

1. To compare acetone and formalin based fixation of animal brain impressions in Direct fluorescent antibody assay for diagnosis of rabies in animals.
2. To evaluate Lateral flow assay and Loop mediated isothermal amplification with Direct fluorescent antibody test for rapid diagnosis of rabies in animals.

Review of Literature

II. REVIEW OF LITERATURE

Rabies is one of the most deadly infectious diseases, with a case-fatality rate of almost 100 per cent. The disease is reported from all continents apart from Antarctica; most cases are reported in Africa and Asia, with thousands of deaths recorded annually. However, the estimated annual figure of almost 60,000 human rabies fatalities is probably an underestimate (Fooks *et al.,* 2014). Rabies affects the central nervous system of warm blooded animals. Infected animals may not display all stages or may vacillate between clinical stages; however, abnormal behavior is the most consistent clinical sign of rabies in any animal (Rupprecht *et al*., 2001). Further, geographic distribution and host range of rabies virus genotype varies at the global level with region specific reservoir species genotypes (Yale *et al.,* 2014). Dogs are the principal reservoirs of rabies virus in developing countries and are responsible for majority (97%) of human infections. Other reservoirs and important vectors of rabies virus include wild and domestic *Canidae* such as wolves, foxes, coyotes, jackals, bats, cats, monkeys, skunks, raccoons and mongooses in different geographic locations.

2.1 History of rabies

Rabies is one of the oldest recognized diseases affecting humans and important zoonotic diseases in India. It has been recognized in India since the Vedic period (1500 – 500 BC) and is described in the ancient Indian scripture Atharvaveda, wherein, Yama, the mythical God of Death, has been depicted as attended by two dogs as his constant companions, the emissaries of death.

Extending way back to about 2300 BC, people in ancient Babylon have acknowledged the presence of this terrifying disease. A reference from the pre Mosaic Eshnunna code of Babylon stated that "if a dog is mad and the authorities have brought the fact to the knowledge of the owner; if he does not keep it in and it bites a man and responsible for his death, then the owner shall pay 40 shekels of silver". Aristotle in 4th century BC had stated that animals can contract rabies from the bite of a rabid dog, but erroneously declared that man did not get the disease (Kaplan and Koprowski, 1980). During the Renaissance, St. Hubert, one of the healing saints of France and Germany, was thought to employ his powers through a ring or a key that was heated red hot and used to cauterize the wounds inflicted by a rabid dog (Knipe *et. al.*, 2007).

In the first century AD, a Roman physician named Aulus Cornelius Celsus accurately described the disease and also stated "saliva as 'venomous' and the means of transmitting the disease". In the sixteenth century, Fracastoro strengthened the concept of rabies as a contagious disease. A scientific or experimental approach to rabies was delayed until 1793, when John Hunter suggested that the transmission of rabies should be studied by inoculating saliva from rabid animals and humans into dogs and attempts should be made to inactivate the 'poison' in the saliva (Zuckerman *et. al.*, 2009).

Early in time, when vaccines for rabies had not been discovered, people relied on natural means. For prevention, madstones (or moonstones) were carried as charms to ward off rabies. "The original amulets – apparently "hair balls" from the stomach of white deer (or their gallstones), gallstones of white cows, or any smooth white stones –

were used by the American frontiersmen and early settlers" (Baer, 1991). If bitten, the stones would then be placed on top of the wound for healing effects.

Other means of curing a person included cauterization, meaning to use heat to destroy tissue exposed from the bite wound. Various herbs and natural resources were also recommended to purify the wound site such as donkey's milk, child's urine, and 'stones' of a hedgehog (West and Geoffry,1972). Additionally, doctors favored submerging their patients underwater, sometimes even almost drowning them. The reason for this treatment was because people infected with rabies tend to exhibit a distinctive symptom called hydrophobia, where intense feelings of thirst and fear of water rage on. The doctors believed that confronting the patients with their fears and "baptizing" them in water would cleanse them of the disease (Willoughby *et al*., 2007). Since these treatments were not always successful, people resorted to combinations of these treatments. For example, Dr. Vaughan advised his patient to cauterize of the wound, filling it with gunpowder, and lighting it. These actions would, supposedly, allow the virus to discharge from the body.

Important research on treating rabies occurred in 1885, when Louis Pasteur grew "street virus" in laboratory animals and found he could reduce the virulence. Pasteur developed the first rabies virus vaccine using desiccated spinal cords from rabies infected rabbits. He used this vaccine for the first time on a nine year old boy, who had been bitten by a rabid dog, multiple times. The child, Joseph Meister, received a total of 13 inoculations and survived (Knipe *et al*., 2007). Pasteur's vaccine, with all its

modifications, became the accepted rabies prophylactic throughout the world in the early 20th century (Zuckerman *et al.,* 2009).

2.2 Etiology

The family *Rhabdoviridae* along with the families such as *Paramyxoviridae*, *Filoviridae* and *Bornaviridae* constitute the order of *Mononegavirales*. All the members of this order are negative sense single stranded RNA (Ribo Nucleic Acid) viruses (Mayo and Pringle, 1998). Three genera of animal viruses under the family *Rhabdoviridae* which include *Lyssavirus, Ephemerovirus* and *Vesiculovirus*. Currently, 14 viruses are affiliated to the genus *Lyssavirus* which includes Rabies virus, Australian bat lyssavirus, European bat lyssavirus type 1, European Bat lyssavirus type 2, Khujand virus, Aravan virus, Bokeloh bat lyssavirus, Irkut virus, Duvenhage virus, Lagos bat virus, Mokola virus, Shimoni bat virus, West Caucasian bat virus and Ikoma lyssavirus (WHO, 2013).

The viral genome encodes five viral proteins *viz.*, N (Nucleoprotein), P (Phosphoprotein), M (Matrix), G (Glycoprotein), and L (Viral polymerase) (Conzelman *et al.,* 1989).

All lyssaviruses are typically bullet-shaped, having helical ribonucleoprotein (RNP) core. The bullet shaped rabies virus is approximately 130-200 nm in length with an average diameter of 60-110 nm (Wunner, 2002). The viral negative sense single-stranded RNA genome bound by molecules of the nucleoprotein (N), phosphoprotein (P) and the polymerase protein (L), make up the helical ribonucleocapsid (RNP) of the virion. The virus envelope consists of a layer of matrix protein (M) surrounded by a lipid

membrane derived from the host cell. The glycoprotein (G) trimers are embedded within the lipid membrane of the virus (Wunner, 2002).

The nucleoprotein is the major protein of RNP core. The major function of nucleoprotein is encapsidation of the RNA genome and viral replicative intermediates (RI) to prevent digestion of these components by endogenous cellular ribonucleases (Tordo and Poch, 1998 and Wunner, 2002). It also plays a role in viral transcription and replication through its ability to promote read-through of the termination signals (Schneider *et al*., 1973 and Smith, 1989).

The Rabies virus (RABV) phosphoprotein is a key component of the RNA polymerase complex where it acts as regulatory protein in viral genome replication. When complexed with the N protein, the P protein directs the encapsidation of the viral RNA, and as a part of the RNA polymerase complex, functions as co-factor during transcription and replication of the viral genome by stabilizing the L protein and placing the polymerase (Banerjee, 1987 and Chenik *et al*., 1994)

The interaction of M protein with the cytoplasmic domain of the G protein facilitates budding of new virions from an infected cell (Finke *et al*., 2003). G-protein is the important structural protein of the virus that induces the formation of virus-neutralizing antibodies and confers immunity (Cox *et al*., 1977).

2.3 Host range

All mammals are believed to be susceptible to rabies. Natural hosts are usually members of *Canidae*, including dogs (Vaughn *et al.,* 1965), foxes, coyotes, wolves and

jackals; also cats (Blanton *et al.*, 2005), skunks, raccoons, mongooses (Everard and Everard, 1988), bats (Kuzmin *et al*., 2010) and other biting warm blooded animals. Brain and salivary gland suspensions of a bat suspected of rabies was inoculated into a group of mice and rabies virus was isolated from all animals in the group, rabies and its clinical signs in ferrets, guinea pig and rabbits was also reported by Lackay *et al.* (2008)

2.4 Epidemiology of the RABV

Rabies viruses are adapted to various animal species (acting as reservoir) on which they depend for their existence. Rabies virus perpetuation involves introduction into a susceptible host, most typically by transmission *via* an animal of the same species, and then the infection, dissemination, and multiplication of the virus within the host (Jackson and Wunner, 2002). Although most mammals are susceptible to infection by any lyssavirus, each viral strain is associated with and maintained by a reservoir host. Viral infection of a species that does not normally maintain that virus, referred to as a spill-over infection, occurs frequently but rarely resulting in spread within the new host (Nadin-Davis and Real, 2011). As for instance, although rabies is a zoonotic disease, human infections and deaths are an unfortunate consequence of biologic processes of virus maintenance in which humans play no significant role. Invariably, RABV is transmitted to a new susceptible host by direct bite through saliva. The process by which RABV circulates within diverse species of mammals causing serial infections is referred as the maintenance of transmission cycle of the virus. Since humans are rarely the source of virus for subsequent human infections, humans do not contribute to the maintenance of RABV. The reservoir for rabies is the animal pool that circulates RABV, with only occasion spills-over to humans (Jackson and Wunner, 2002).

De Serres *et al.* (2008) has defined three principal global areas of rabies. These areas are (1) countries with enzootic canine rabies (all of Asia, Latin America, and Africa); (2) countries in which canine rabies has been brought under control and wildlife rabies predominates (Western Europe, Canada and the United States); and (3) rabies-free countries (mostly islands, including England, Australia and Japan)

A national multicentric study of human rabies in India revealed that the annual incidence of human rabies was estimated to be 17,137 cases. The majority of the victims were male, adults, from rural areas and unvaccinated. The main animal responsible for bites were dogs (96.2%), most of which were stray dogs (Sudarshan *et. al.*, 2007). A survey in 2003 reported that the biting animal mainly responsible for human rabies death was dog (96.2%) of which majority strays (75.2%) followed by pet (11.1%); wild (3.5%); cat accounted for 1.7 per cent in India (Bhuyan, 2012). Ichhpujani *et al.* (2001) reported that dog bites caused maximum morbidity (92%), followed by monkey (3.2%), cat (1.8%) and fox (0.4%) in India. Most bites were unprovoked (64.3%) by stray animals (64.7%). A study conducted on 250 animal bite victims in Pune revealed that dog was the biting animal in 94.4 per cent cases, followed by cat (2.4%), Jackal (1.2%), mongoose (1.2%), monkey (0.4%) and horse (0.4%) (Shetty *et al.*, 2005). Andaman, Nicobar and Lakshadweep islands are regions of India free of rabies (Sudarshan *et al*., 2007). However, this report provides an overview of epidemiology of animal bites and retrospective information about rabies patients.

2.5 Demonstration of rabies viral antigens

Validated diagnostic tests capable of confirming the presence of rabies virus in clinical samples have improved the quality, accuracy and speed of rabies diagnosis in many national reference laboratories thereby supporting rabies control strategies with the global vision of dog rabies elimination in developing countries

2.5.1 Histological and histopathological examination

The Mann's and Sellar's method of demonstration were the two most popular methods of demonstration of these intra-cytoplasmic inclusions. Mann's and Sellar's method was compared by Andrade (1969) and elucidated that Sellar's staining method was easy and quicker. Generally, histological tests are performed on fixed material after a paraffin- embedding step, and the result of the test is obtained within 3 days. These techniques have the advantage that the laboratory equipment needed to perform them is inexpensive and any need to keep specimens cold after fixation is avoided. Negri Adlochi (1903) reported the mononuclear infiltration, perivascular cuffing and lymphocytic foci in the infected brain sections upon Hematoxylin and Eosin (H&E) staining along with the presence of intracytoplasmic, eosinophilic inclusions in rabies-infected tissues. These inclusions were later on referred to as the "Negri bodies" with acidophilic nature (Malovrh and Hostinik, 2005). These histological methods, especially the Seller's method, can no longer be recommended because they have very low sensitivity (Shankar, 2009).

2.5.2 Direct Fluorescent antibody test (DFA)

Direct fluorescent antibody assay was devised by Coons and Kaplan (1950). Goldwasser and Kissling (1958) demonstrated rabies viral inclusions for the first time. Mcqueen (1960) suggested that DFA was a rapid practical routine diagnostic procedure as he found that this test was in complete agreement with the results of mouse inoculation test with all 884 specimens tested from 23 different species of animals. The efficacy of DFA with histological examination and mouse inoculation was compared and found that both were equally sensitive and more accurate than histological examinations (Stone *et. al*., 1962 and Beauregard *et. al*., 1965). Currently, DFA is the most widely used method in diagnosing Rabies in humans and animals and is recommended by WHO and OIE (Neelufer *et al*., 2015)

The DFA detects rabies N antigen present in the rabies infected brain smear using a polyclonal or monoclonal N antibody which is coupled with a detector molecule (usually fluorescein isothiocyanate) most commonly fluorescein isothiocyanate. Anti-N protein monoclonal antibody usage increases the specificity (Neelufer *et al*., 2015). Bright apple green fluorescent particles of varying size can be observed within the neurons when observed under ultraviolet light in a fluorescence microscope,.

The DFA can be done on fresh the central nervous system tissues (brain) or on samples preserved by chilling, freezing, or on samples stored in a 50 per cent glycerol and saline solution. Partially decomposed brains and formalin fixed brains are not suitable for this test as it is very difficult to differentiate specific fluorescence due to N antigen from nonspecific fluorescence because of bacterial contamination. Furthermore,

the DFA had also been used to detect rabies infection in live animals and human beings, based on the demonstration of rabies virus antigen in corneal impression smear, (Schneider, 1975) and in skin biopsies (Smith *et al.*, 1972 and Blenden *et al.*, 1980).

The drawbacks of DFA includes the requirement of expensive fluorescent microscope and well-trained personnel (Malovrh and Hostinik, 2005). Two independent readers are necessary for routine rabies diagnosis (Robardet *et. al.*, 2011). Furthermore, the DFA involves fixation of the brain impression using chilled acetone which does not completely inactivate the rabies virus thereby posing a possibility of biohazard (Umoh and Blenden, 1981). Studies have demonstrated that intracerebral inoculation of acetone fixed tissues can cause disease in suckling mice (Umoh and Blenden, 1981). White and Chappell (1982) found that impression smears made out of brain samples suspected for rabies were fixed in acetone at -20 °C for 2, 4, 7, and 24 h and were further examined for virus viability. Tissues scraped from impression smears after acetone fixation were examined for virus viability in BHK-21/WI-2 cell cultures Scrapings from these smears contained infectious virus. The infectivity titers ranged from $10^{3.3}$/0.1 ml in suckling mice to $10^{5.1}$/0.1 ml in BHK-21/WI-2 cultures but acetone fixation at 50 °C for 30 min inactivated rabies virus without altering the outcome of DFA. An examination using the routine rabies direct fluorescent antibody test was performed on rabies or Eastern equine encephalitis positive mammalian brain tissue to assess inactivation of the virus. Neither the virus was inactivated with acetone fixation nor the routine test. Thus laboratory employees should treat all samples as rabies and when appropriate Eastern equine encephalitis positive throughout the whole procedure (Jarvis, 2016). More over , routine

DFA fixation at -20 ˚C requires a deep freezer which may not be available in several laboratories.

2.5.3 direct rapid immunohistochemistry (dRIT)

At the CDC, Atlanta, USA, dRIT has been developed to detect rabies virus using an immunoperoxidase technique (Niezgoda and Rupprecht, 2006). The principle of dRIT is based on the capture of rabies virus N protein antigen by biotinylated anti-nucleocapsid monoclonal antibodies and colour development by streptavidin-peroxidase and chromogen amino-ethly carbazole. There was 100 percent co-relation between DFA and dRIT (Chandrashekhara *et al*., 2014; Isloor *et al*., 2014 and Nithinprabhu *et al*., 2014). The test does not require a fluorescent microscope and ensures complete inactivation of RABV due to formalin fixation, making it user friendly and biologically safe. The dRIT has been validated as a field test for rabies surveillance and also on glycerol-preserved field samples (Lembo *et al.,* 2006). The dRIT is a reliable diagnostic method requiring only light microscope and less expensive (Durr *et al.,* 2008). These qualities make it ideal for testing under field conditions and in developing countries with limited diagnostic resources.

2.5.4 Lateral Flow Assay (LFA)

Lateral flow assay (LFA), also referred to as immune chromatographic assay is becoming popular as diagnostics because of its simplicity, specificity, sensitivity and most importantly rapidity without the need for specialized and costly equipments. Rapid Immunochromatographic Diagnostic Test (RIDT) is based on the principles of immunochromatography using a gold labeled monoclonal antibody (MAb). Purified anti-

N protein Mabs are immobilized in a test zone of the nitro- cellulose membrane, while purified goat anti-mouse IgG are immobilized in the control zone of the membrane to capture unbound MAb. The sample, once added in the sample pad, migrates through the gold MAb pad, the test zone and the control zone respectively. At the control line, antibodies that captures any particle are embedded and thereby shows that reaction conditions and technology worked fine.

Recently, these assays are being used for the rapid diagnosis of various infectious diseases (Al-Yousif *et al*., 2002; Kuroiwa *et al*., 2004 and Tsuguto *et al*., 2004). The RIDT might have great potential as a useful method for rabies diagnosis without the need for laboratory equipment (Markotter *et al*., 2009, Servat *et al*., 2012). Immunochromatographic test strips (Lateral Flow assay-LFA) are becoming popular in veterinary diagnosis including rabies due to its rapidity and ease of handling and performing (Tajunnisa *et al*.,2017).

The Anigen rapid immunodiagnostic test was evaluated using 80 clinical samples collected by US military veterinary units. . The rapid immunodiagnostic test had an overall sensitivity of 96.9 per cent and specificity of 100 per cent when compared to the direct fluorescent antibody test (Voehl *et al.,* 2014). Preliminary validation studies with a limited number of samples showed that the RIDT might have great potential as a useful method for rabies diagnosis without the need for laboratory equipment (Zhang *et al.,* 2009). Clinical samples like brain tissue, serum, saliva, blood of different animals can be used in LFA (Kang *et al.,* 2007). Monoclonal antibody based Lateral Flow Assay can

also be used for the detection of Rabies virus in saliva, which helps in early diagnosis of disease (Tajunnisa *et al*., 2016).

Sharada *et al.* (2015) employed Anigen rapid immunodiagnostic to confirm the propagation of Dr. Larghi's strain of rabies virus in BHK 21 cells through it's detection in cell culture harvest. In all, 25 infected (harvests from infected BHK 21 cells) and 15 uninfected controls (spent medium from uninfected BHK21 cells) were tested for the study. There was 100 per cent correlation between LFA and reverse transcription PCR in detection of Rabies virus in cell culture harvest.

The LFA was able to detect rabies virus antigen in 116 out of 118 DFA positive brain samples and detected 74 out of 77 DFA negative samples as negatives. Thus the diagnostic sensitivity and specificity of LFA were 98.3 per cent and 96.1 per cent respectively (Tajunnisa *et al*., 2017). It was capable of detecting recombinant nucleoprotein and showed sensitivity of 95.5 per cent (42/44) and specificity of 88.9 per cent (32/36) using brain samples from rabid dogs (Nishizono, 2008).

Lateral flow assay kits need neither cold chain for transportation nor complicated training for personnel (Nishizono, 2008) but when stored at 4 °C in a plastic bag with a desiccant they retained their specificity and sensitivity for at least 15 months, and strips stored at ambient temperature remained stable for 12 months. The ICT strip may therefore be useful for clinical laboratories lacking specialized equipment and for diagnosis in the field for rapid detection of rabies virus-specific antibodies (Wang *et al*., 2011).

2.6 Detection of rabies virus nucleic acid

With the feasibility of the molecular diagnostic techniques, it is possible to arrive at a diagnosis at the earliest and even aid in suggesting the post exposure prophylaxis to the exposed individuals. Molecular methods continue to show great promise for detection of Lyssaviruses worldwide (Woldehiwet, 2005). They are increasingly employed for molecular diagnosis and epidemiological investigations due to it's high sensitivity and specificity.

2.6.1 Polymerase Chain Reaction

Techniques like PCR have been used for the diagnosis of rabies using saliva and skin biopsy in humans (Crepin *et al.,* 1998). The RT-PCR for rabies diagnosis is considered to be a rapid and sensitive alternative technique compared with the previous rabies diagnostic methods (Sacramento *et al.,* 1991). The RT-PCR was also found to be effective in detection of RABV genome in samples from goat, sheep, cow, camel, fox and all the suspected cases of rabies (Araujo *et al*., 2008).The PCR involves expensive equipment with no field applicability and requires at least 3-4 hours of time. Therefore, PCR can be done only at the regional laboratories and not in rural settings in developing and under- developed countries. Thus, there is a need for development of specific, sensitive, rapid, cost-effective and user-friendly diagnostic tests which can be employed for diagnosis of rabies.

2.6.2 Loop-mediated isothermal amplification (LAMP)

Loop mediated isothermal amplification (LAMP) of DNA was described by Notomi *et al.*(2000). It is a simple, rapid, specific and cost-effective nucleic acid

amplification method when compared to PCR and involves nucleic acid sequence-based amplification, self-sustained sequence replication and strand displacement amplification (Mori *et al*., 2009).

The LAMP employs a strand-displacing *Bst* DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. It has a Non-Cyclic and Cycling Step**.** An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP at a constant temperature (40-45 °C). The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA. This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem–loop DNA structure. In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem–loop DNA and a new stem–loop DNA with a stem twice as long. The cycling reaction continues with accumulation of 10^9 copies of target in less than an hour. The final products are stem–loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity (Notomi *et al.,* 2000).

Loop mediated isothermal amplification has been employed for the detection of various pathogens at global level (Notomi *et al.,* 2000; Parida *et al.,* 2005; Venkatesan *et al.,* 2012). It is being used for the detection of Camelpox virus, Foot and Mouth disease,

Marek's disease, Malaria, *Entamoeba histolytica*, SARS coronavirus, *Mycobacterium tuberculosis* (TB), *Mycoplasma pneumoniae*, Legionella , Influenza type A virus, H5 influenza virus, and Human Papilloma virus (HPV). Of 17 NTDs (neglected tropical diseases) recognized by WHO, 14 were studied using LAMP *viz*., dengue, rabies, buruli ulcer, leprosy, Chagas disease, human African trypanosomiasis, visceral leishmaniasis, post-kala-azar dermal leishmaniasis, cysticercosis, echinococcosis, foodborne trematode infections, lymphatic filariasis, schistosomiasis, soil-transmitted helminthiases, and yaws. There are reports on application of LAMP for RABV detection (Saitou *et al.,* 2010; Hayman *et al.,* 2011; Venkatesan *et al.,* 2012 and NithinPrabhu, 2014).

The use of isothermal amplification has the benefit of reducing the technological requirements of thermal cycling used in PCR. A heating block or water bath is sufficient for conducting the reaction (Notomi *et al.,* 2000), disposable pocket warmers can also be used (Hatano *et al.,* 2010).

Materials and Methods

III. MATERIALS AND METHODS

3.1 General requirements

The glassware used in this study were of neutral glass of Corning Technologies, India Pvt, Ltd., Borosil India Ltd., or Scott Durham (Germany) make. The buffers and biochemical reagents were prepared in quartz glass double distilled water (M/s Borosil India Ltd.). The chemicals of Analar, Excellar or molecular biology grade were used for the preparation of various solutions and reagents. The buffers, reagents and other equipments were obtained from M/s. Invitrogen, Thermo Scientific, Fischer Scientific, Polyscientific, Bay Shore, NY; M/s. HiMedia, Mumbai and M/s. Sisco Research Laboratories Pvt. Ltd., Mumbai. All other chemicals used were of analytical grade obtained from standard manufacturers. Plasticware including syringes, microcentrifuge tubes, microtips and cryovials were procured from M/s Tarson Products Pvt. Ltd., Kolkata and Axygen Inc., USA.

3.1.1 Preparation of glassware and plasticware

The glassware used in this study were prepared by soaking them in detergent solution overnight. Next day, they were washed thoroughly in running tap water ten times, followed by rinsing in distilled water for twice. Then, the same were immersed in distilled water overnight. Finally, the air-dried glassware were packed and sterilized in hot air oven for one and half hour at 160 °C. The plasticware including microcentrifuge tubes and micropipette tips were sterilized by autoclaving at 15 psi at 121 °C for 15 min. All the glassware and plasticware used in the study were treated with one per cent diethyl

pyrocarbonate (DEPC) treated water and sterilized by autoclaving/hot air oven for making them RNAse free.

3.2 Collection and processing of rabies suspected samples

3.2.1 Post mortem and brain sample collection

Post mortem was carried out in the post-mortem hall of the Department of Veterinary Pathology, Veterinary College, KVAFSU Hebbal, Bengaluru, following the necessary precautions including the usage of personal protectives like heavy rubber gloves, face shield and lab gown. The head was restrained before opening the skull. The instruments were priorly disinfected by autoclaving and dipped in one per cent sodium hypo chloride.

The samples received from other locations/states were shipped to the laboratory under cold chain by the earliest route available. The tissue samples were stored at -80 $^{\circ}$C freezer without any preservatives in OIE Twinned KVAFSU-Crucell-CVA Rabies Diagnostic Laboratory, Veterinary College, Hebbal, Bengaluru until further processing.

3.3 Microscopy based confirmation of brain for rabies virus

Laboratory techniques intended for diagnosis of rabies are preferably conducted on fresh, composite brain tissue samples.

3.3.1 Direct fluorescent antibody assay (DFA)

3.3.1.1 Materials/reagents

- Microscopy slides (White frosted, Thermo Scientific Co.)
- Rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5600)
- Rabies negative control IgG-FITC conjugate (M/s Light Diagnostics, Cat # 5102).
- Normal Goat IgG-FITC conjugate (M/s Light Diagnostics, Cat # 5202)
- Evans Blue (0.5 % in PBS, Sigma, Product # E-0133)
- Acetone (M/s Sisco Research Laboratories Pvt. Ltd., Mumbai)
- Fluorescent microscope (AxioVert A1, M/s Carl Zeiss, Germany)

3.3.1.2 Conjugate titration

Commercially available antirabies FITC monoclonal antibody conjugate, negative control FITC conjugate and normal goat serum FITC conjugate mentioned above were used in the study. The working dilution of the conjugate was determined to optimize the exact dilution of the conjugate to be used. The serial two fold dilutions of the conjugate (e.g., 1:10, 1:20, 1:40 *etc*) was prepared for the newly opened vial containing conjugate. The impressions from known rabies positive and known rabies negative brains were made in duplicates for each of the dilutions and fixed in chilled acetone at -20 $^{\circ}$C for an hr. The slides were stained using standard protocol (Section 3.3.1.4). Each of the stained slides were read by two persons independently. The consensus of the last dilution providing crisp and high fluorescent staining with minimal background fluorescence was

considered as the end-point dilution of the reagent. The working strength of conjugate was kept at two steps more concentrated than the first dilution at which a fall-off in staining was observed. Once the conjugate dilution was made, the working solution of conjugate was prepared at the optimum dilution, filtered using 0.45µm filters and stored at 4 °C.

3.3.1.3 Counterstaining

To lower the background activity of the flourescent dye and to provide the contrast, Evans Blue (0.5 % in PBS, Sigma, Product # E-0133) was used as a counter stain, added to the working dilution of the conjugate. The stock solution (0.5 % Evans Blue in PBS) was aliquoted and stored at 4 ºC for up to six months. Tissue when stained with counter stain, notably appeared red, but care was taken that staining was not strongly red as to diminish the specific green fluorescence produced by the conjugate. In the present study, Evans Blue was used at a final concentration of 0.00125 per cent. The optimally diluted conjugates after addition of the counter stain were filtered using 0.45µm syringe filters.

3.3.1.4 DFA Protocol

The DFA was done essentially following the method as described by the CDC, Atlanta.

1. Touch impressions were made from brain tissue samples on microscope slides.
2. For each of the test samples, three impressions were made one each for the anti rabies nucleoprotein IgG- FITC conjugate (Millipore-Light Diagnostics, Rabies DFA III, Cat # 5600), negative control FITC conjugate (Millipore-Light Diagnostics, Cat

5102) and normal goat serum FITC conjugate (Millipore-Light Diagnostics, Cat # 5202).

3. A known healthy brain sample (as negative) was also included in the test as an internal control.
4. The smears were blotted onto paper towels to remove excess of moisture, tissue remains and the blood stains.
5. The smears were initially air dried for 5 min. before fixing in high grade chilled acetone (80 % v/v) either for an hr at -20 °C or overnight at 4 °C.
6. The fixed smears were briefly air dried to ensure that the acetone traces on it evaporated and were stained using 1:100 dilution of the above said FITC conjugates by incubating in a humid chamber at 37 °C for 45 min.
7. The smears were washed with 1X PBS for 5 min.
8. The wash step was repeated twice to remove excess stain.
9. The stained smears were observed under a fluorescent microscope (AxioVert A1, M/s Carl Zeiss, Germany).
10. Presence or absence of typical granular intra-cytoplasmic apple green fluorescence of aggregated nucleocapsids was used as a criterion in declaring positive and negative samples respectively.

3.3.3 The Modified DFA

In this modification, 10, 5, 2.5 and 1 per cent Neutral Buffered Formalin (NBF) were used as a fixatives instead of chilled acetone in modified DFA .

3.3.3.1 Materials / Reagents

- Microscopic slides (White frosted, Thermo Scientific Co.)
- 10, 5, 2.5 and 1 per cent NBF
- Tween 80
- Three per cent hydrogen peroxide
- Phosphate Buffered Saline
- Rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5600)
- Rabies negative control IgG-FITC conjugate (M/s Light Diagnostics, Cat # 5102).
- Normal Goat IgG-FITC conjugate (M/s Light Diagnostics, Cat # 5202)
- Evans Blue (0.5 % in PBS, Sigma, Product # E-0133)
- Fluorescent microscope (AxioVert A1, M/s Carl Zeiss, Germany)

3.3.3.2 Phosphate Buffered Saline (pH 7.2)

NaCl	:	8.00 g
Na_2HPO_4	:	1.15 g
KH_2PO_4	:	0.20 g
KCl	:	0.20 g
H_2O	:	1000 mL

Phosphate Buffered Saline (PBS) was sterilized by autoclaving at 121°C at 15 psi for 15 min.

3.3.3.3 Preparation of the wash buffer (TPBS, PBS with 1% tween 80)

Phosphate Buffered Saline (pH 7.2) : 990 mL

Tween 80 : 10 mL

3.3.3.4 Preparation of Neutral Buffered Formalin (10%, 5%, 2.5% and 1% NBF)

Available stock solution of formalin is 37- 40 per cent formaldehyde in aqueous solution and unbuffered. To make it 10, 5, 2.5 and 1 per cent formalin from stock formalin i.e. 1, 0.5, 0.25 and 0.1 part of the stock formalin was added to 9, 9.5, 9.75, 9.9 parts of distilled respectively. This makes an unbuffered formalin solution, which will have a pH of 3-4. To adjust to a neutral pH sodium phosphate buffer was used

- 100/50/25/10 ml Formalin (37- 40% stock solution)
- 900/950/975/990 ml Water
- 4g/L NaH_2PO_4 (monobasic)
- 6.5g/L Na_2HPO_4 (dibasic/anhydrous)

Or

- 100/50/25/10 ml Formalin (37-40% stock solution)
- 900/950/975/990 ml Water
- 9g NaCl
- 12g Na_2HPO_4 (dibasic/anhydrous)

3.3.3.5 Optimization of the modified DFA

The tissue impressions were fixed in different concentrations of formalin EDTA, by immersing slide in a beaker containing Tris EDTA and acetone at 50°C was also tried. Later, it was fixed in different concentrations of formalin. Optimization is summarized in Table 3.1.

Initially tissue impressions were fixed at 10 per cent NBF. To remove formalin effect and to unmask the RABV antigen, Tris EDTA was used by immersing the slide and warmed to 30°C for 30 seconds in microwave oven. Later treated with hydrogen peroxide and TPBS washes. Tissue impressions were fixed at different concentrations of 5, 2.5 and 1 per cent. NBF. Then impressions were stained with Rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100). Later, slides were examined under the fluorescent microscope at 200X and 400X magnification. Similar procedure was repeated with acetone at 50 °C as a fixatives.

Table 3.1 Optimization of the Modified DFA

Sl. No.	Reaction component/ Parameter (Unit)	Range
1.	Neutral Buffered Formalin	1%, 2.5%, 5%, 10%

3.3.3.6 Protocol

1. Touch impression were prepared from brain tissue samples on microscope slides.
2. For each of the test samples, three impressions were made one each for the anti rabies nucleoprotein IgG- FITC conjugate (Millipore-Light Diagnostics, Rabies DFA

III, Cat # 5600), negative control FITC conjugate (Millipore-Light Diagnostics, Cat # 5102) and normal goat serum FITC conjugate (Millipore-Light Diagnostics, Cat # 5202).

3. A known healthy brain sample (as negative) was also included in the test as an internal control.
4. The smears were blotted onto paper towels to remove excess of moisture, tissue remains and the blood stains.
5. The smears were initially air dried for 5 min.
6. Impressions were then fixed by immersing in varied concentrations of buffered formalin at room temperature for 10 min.
7. The slides were removed and dip-rinsed twice in wash buffer TPBS (PBS with 1 % Tween 80).
8. Further, the slides were immersed in three per cent H_2O_2 for ten min. to neutralize the endogenous peroxidases.
9. Later, the slides were removed and dip-rinsed twice in TPBS.
10. The slides were washed twice in 1X PBS.
11. The fixed smears were briefly air dried to ensure that the 2.5 per cent NBF traces on it evaporated and were stained using 1:20 dilution of the above said FITC conjugate by incubating in a humid chamber at 37 °C for 45 min.
12. The smears were washed with 1X PBS for five min.

13. The stained smears were observed under a fluorescent microscope (AxioVert A1, M/s Carl Zeiss, Germany).

14. Presence or absence of typical granular intra-cytoplasmic apple green fluorescence of aggregated nucleocapsids was used as a criterion in declaring positive and negative samples respectively**.**

3.3.4 Retrieval of rabies virus antigen in formalin fixed brain impressions from BHK-21 cells

The Baby Hamster Kidney–21 (BHK-21) cells were obtained from M/s. Ella foundation, Hyderabad and was propagated and stored in lyophilized state in LN_2 cryocans.

3.3.4.1 Materials / Reagents

- Lab-tek slide chambers
- NBF fixed tissue impressions from brain samples
- BHK-21 cells
- 10% Growth medium
- 70% chilled acetone
- 1x PBS
- CO_2 incubator (M/s ThermoScientific)

3.3.4.2 Growth Medium (GM)

Growth medium was prepared by adding 9.6 g Gibco Minimum Essential Medium (GMEM) powdered media to 970 ml of Milli-Q double distilled water. The GMEM did not contain sodium bicarbonate and hence 2.2 g of the same was added to the medium. To this, Penicillin (50 IU/ml), Streptomycin (50 µg/ml) (Gibco) and Kanamycin (100µg/ml) (Sigma) were added. The medium was mixed and filtered through positive pressure with membrane filter of size 0.22 µm. It was further supplemented with 10 per cent each of Foetal bovine serum (FBS, Gibco) and Tryptose Phosphate Broth (TPB, Himedia) to get a complete growth medium for cell propagation.

3.3.4.5 Protocol

- Routine touch impressions of primarily tested positive (VMC-559) and negative brain tissue (VMC-555) were made and fixed in varied concentrations NBF for 10 minutes at room temperature.
- Slides were allowed to air dry.
- Scrapings were taken from impressions made on slides and added to 400µl of 10 per cent GM (Tissue suspension)
- 200 µl of tissue suspension was added to Lab-tek slide chambers
- 100 µl of BHK-21 cell suspension was added to Lab-tek slide chambers containing 200 µl of tissue suspension
- Co-cultivation of virus with cells was carried out in 5% CO_2 incubator for 45 hours.
- After 45 hrs of incubation, contents were discarded.

- 100 µl of 70 per cent chilled acetone was added and kept for incubation at -20 °C for one hr.
- Contents were discarded and air dried for five min.
- Lab-tek slide chambers were stained using 1:20 dilution of the aforementioned FITC conjugate by incubating in a humid chamber at 37 °C for one hour.
- Contents were discarded and washed with 1X PBS twice.
- The stained slide chambers were observed under a fluorescent microscope (AxioVert A1, M/s Carl Zeiss, Germany).

3.4 Lateral Flow assay

The Lateral flow assay was performed using the Anigen Rapid Rabies Ag Test Kit of BIONOTE, Korea, as per the manufacturer's instructions detailed below.

3.4.1 Protocol:

- The brain tissue was mixed with equal quantity of the assay diluent in a micro centrifuge tube. A negative control (VMC 555, a brain tissue sample confirmed negative by DFA previously) was also simultaneously tested
- The test device was placed on a horizontal surface and four drops of the virus diluent mixture was added to the sample well.
- The results were read within five to ten min.
- Presence of two bands in the result window at position "T" (Test sample) and "C" (Control) indicated the presence of virus.

3.5 Nucleic acid based confirmation of rabies virus

3.5.1 Reverse Transcription-Loop mediated Isothermal amplification (RT-LAMP)

3.5.2 Isolation of total RNA

The total RNA was extracted from the brain tissues with TRIzol® reagent (Invitrogen, USA) following the manufacturer's instructions with slight modifications.

1) About 50 mg of brain tissue was added to 1mL of TRIzol® reagent for homogenization.
2) The brain tissue was homogenized by crushing them in between two applicator sticks/ sterile cotton swabs.
3) The homogenized sample was incubated for five min at ambient temperature to permit complete dissociation of nucleoprotein complex.
4) Then 200 µL of chloroform per mL TRIzol® reagent was added. The sample tube was securely capped and vigorously shaken manually for 15 sec and incubated at ambient temperature for three min.
5) The sample was centrifuged at 11,000 rpm for 15 min at 4 °C. Following centrifugation, the mixture separated into lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase.
6) The colourless upper phase was transferred to a fresh tube and RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol @ 500 µL per mL of TRIzol® reagent used for initial homogenization.

7) The sample was incubated at room temperature for ten min. and centrifuged at 11,000 rpm for 10 min. at 4 °C.

8) Then the supernatant was removed and RNA pellet was washed once with 75 per cent ethanol, adding at least 1 mL of 75 per cent (v/v) ethanol per mL of TRIzol® reagent used for initial homogenization.

9) The sample was vortexed and centrifuged at 6,000 rpm for 6 min. at 4 °C.

10) Then RNA pellet was briefly air dried for ten min.

11) Further, the RNA pellet was resuspended in 80 μL RNase free water (Genei, India) by passing the solution a few times through a pipette tip.

12) The resuspended RNA was incubated in water bath at 56 °C for 6 min.

13) Finally, the RNA was used either immediately or later after storage at -80 °C.

3.5.3 LAMP primers

In the present study, the primers designed by Muleya *et al.* (2012) were used (listed in Table 3.2.). These primers comprising of two outer primers were described as being forward outer primer (F3) and backward outer primer (B3). The inner primers were described as being forward inner primer (FIP) and backward inner primer (BIP).

3.5.4 Optimization of LAMP

The prototype LAMP conditions were optimized for six parameters, including varied concentrations of components as detailed in Table. 3.3 The negative controls (Orf Virus) was included for each LAMP run.

Table 3.2 : Details of the published oligonucleotide primers (HPLC-grade) for the RT-LAMP

Label	5' pos	3' pos	Length	Sequence
F3	363	382	20	GAAAAGGAGACAAGATCACC
B3	528	545	18	CCGGTGTTTTGTCCTGAT
FIP	383	460	44	CCTTGTCAGCTCCATGCCTCCCGGACTC TCTAGTGGAAAT
BIP	461	524	41	ACCCCACTGTCTCTGAGCATTGCTCAAC CTATACAGACTCA

Table 3.3 : Optimization of RT-LAMP

Sl. No.	Reaction component/ Parameter (Unit)	Range	Optimized concentration
1	Thermolpol Buffer (10x)	2, 2.5	2.5
2	$MgSO_4$ (in mM)	2, 3, 4 and 5	4
3	Betaine (in M)	0.8, 1.0 and 1.5	0.8
4	dNTP (in mM)	0.5, 1.0 and 1.5	1.0
5	Inner and outer primer ratio	1:4, 1:6, 1:8, 1:10	1:4
6	Assay temperature (in $^{\circ}C$),	42, 45, 61, 63	42
7	Incubation time (in min.)	60	60
8	RNA	2, 3, 4, 5	3
9	Reverse transcriptase (µl)	0.5	0.5
10	HNB Mm	200	200

The RT-LAMP reactions were optimized as described by Notomi *et al.* (2000) with slight modifications. The RT-LAMP reactions were carried out in a total of 25 µL reaction mixture containing 3.5 µL of RNAase-free water (M/s Amnion Biosciences,

Bangalore), 1 X ThermolPol Buffer (New England Biolabs, USA), 4 mM MgSO4 (New England Biolabs, USA), 0.8 M Betaine (Sigma Aldrich, Germany), 1mM dNTPs (New England Biolabs, USA), 20 picomoles of each HPLC-graded inner primers, FIP and BIP, 0.5 picomoles of each outer primers, F3 and B3, followed by the addition of 8 U of *Bst* polymerase (Large fragment, New England Biolabs, USA). Three µL of RNA template (approximately 100 ng/µL) was eventually added to the reaction mixture. Reaction mixtures were all prepared on an ice block. The protocol of LAMP is summarized in Table 3.8.

Table 3.4: The temperature and time duration in the RT-LAMP protocol

Sl. No.	Step in the protocol	Temperature (in °C)	Time duration (min.)
1	Amplification	42	60
2	Enzyme inactivation	95	2

3.5.5 Visual detection of the RT-LAMP products by application of hydroxy-naphthol blue (HNB)

The Hydroxy-naphthol blue (HNB) (Sigma Aldrich, USA, Cat # 219916), a metal chelating agent was incorporated in the RT-LAMP in order to visualize the amplified genomic target. Initially, optimum HNB concentration was determined by incorporating various dilutions of HNB (100, 150, 200 and 250 mM) in the reaction mixture. As the RT-LAMP progresses, accumulation of magnesium pyrophosphate resulting in turbidity. Thus, the colour changes from purple/deep blue to light blue. The amplification was confirmed by visualizing the change in colour from purple/deep blue to light blue (Parida *et al.,* 2009).

Results

IV. RESULTS

The present study was carried out to compare acetone and formalin based fixation of animal brain impressions in Direct fluorescent antibody assay for diagnosis of rabies in animals and to evaluate Lateral flow assay and Loop mediated isothermal amplification with Direct fluorescent antibody test for rapid diagnosis of rabies in animals. The results of the study are documented under the following sub headings.

4.1. Collection of samples

Preserved and freshly collected post-mortem brain samples (n=200) from rabies suspected animals from different species *viz*., Canine-178, Bovine-09, Equine-05, Feline-04, Bubaline-2, Bat-1 and Goral (Fig. 4.2) from different geographical locations of India like Karnataka (n=186), Manipur (n=08), Rajasthan (n=02), Jammu & Kashmir (n=1), Gujarat (n=1) and Maharashtra (n=1) were used in the study (Table 4.1 and Fig.4.1). Collected samples were shipped to the laboratory and were frozen as such without any preservatives at -80 °C.

All 200 brain samples were subjected to DFA and LFA. Fifty out of these 200 brain samples were subjected to Modified DFA and LAMP (Table 4.2).

4.2. Microscopy based detection of rabies virus

The collected samples were screened for rabies virus both by gold standard test DFA and by “modified” DFA.

Fig. 4.1. State wise collection of samples

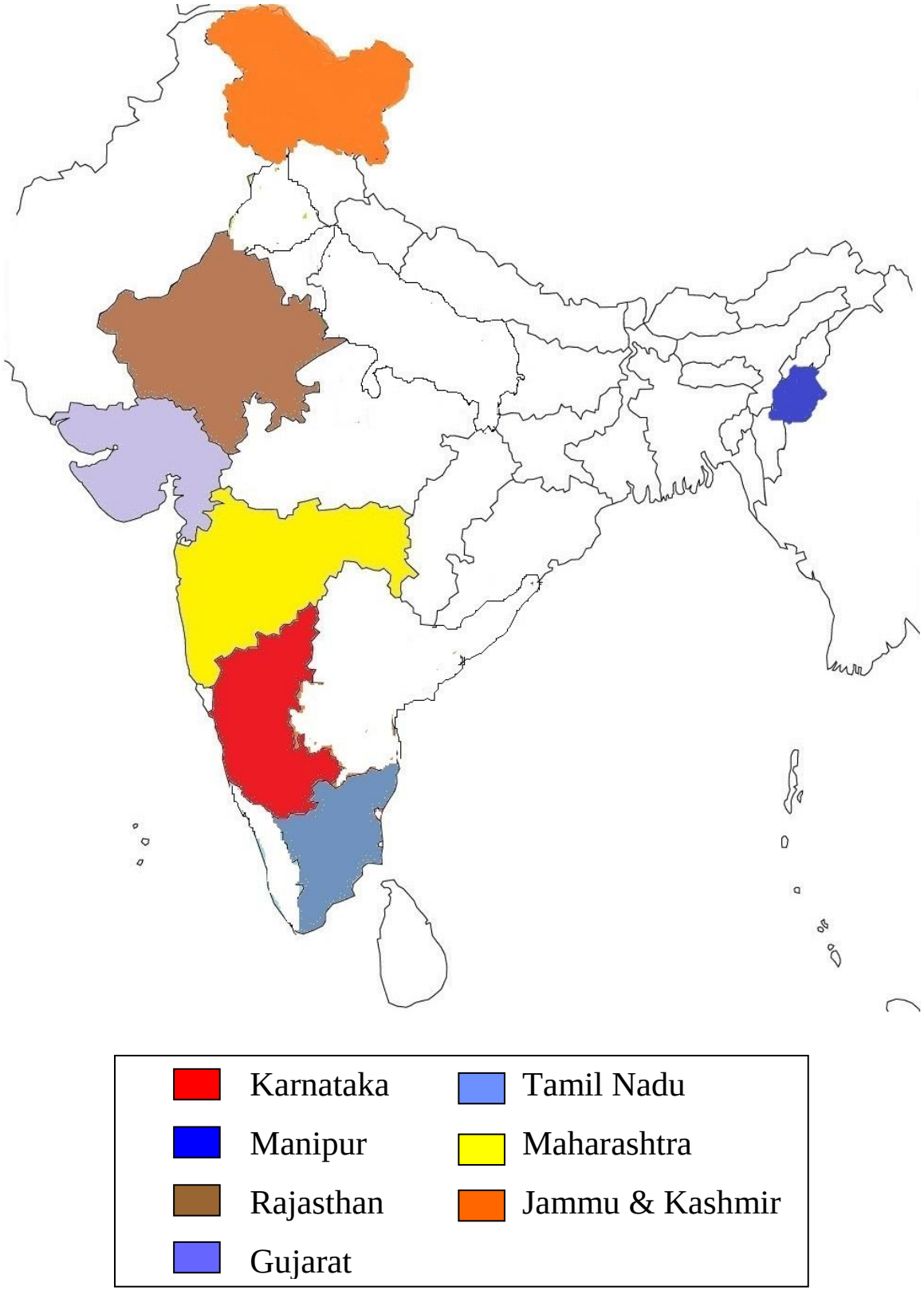


Fig. 4.2: Species wise collection of samples

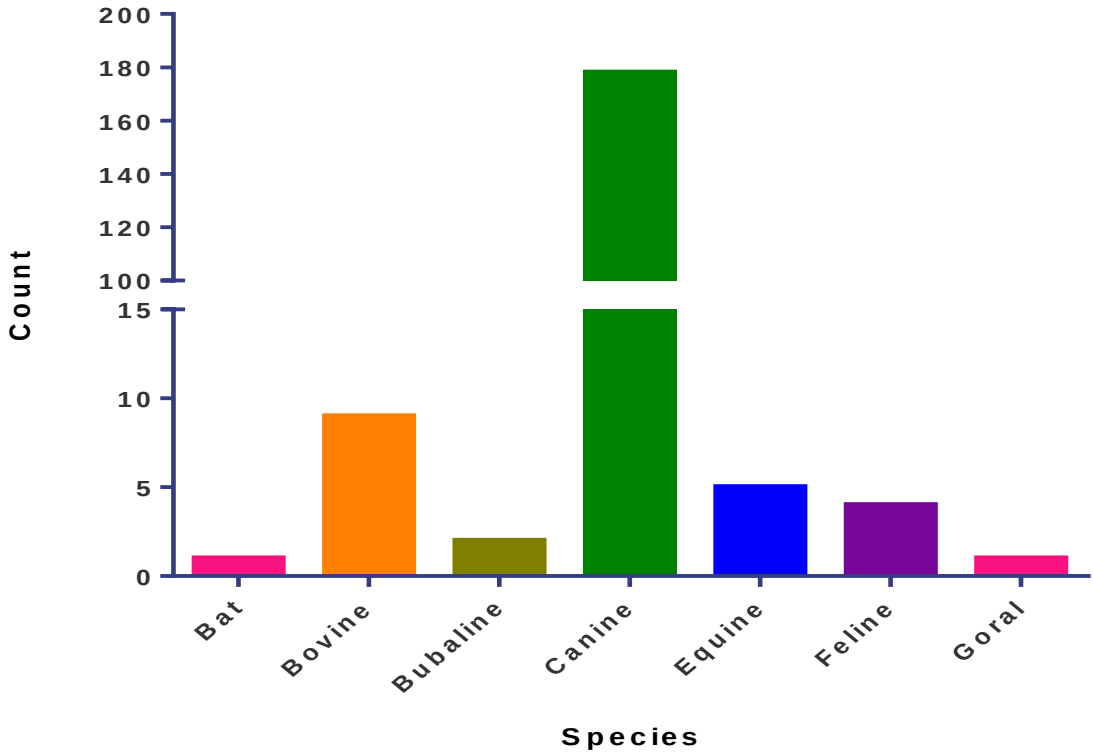
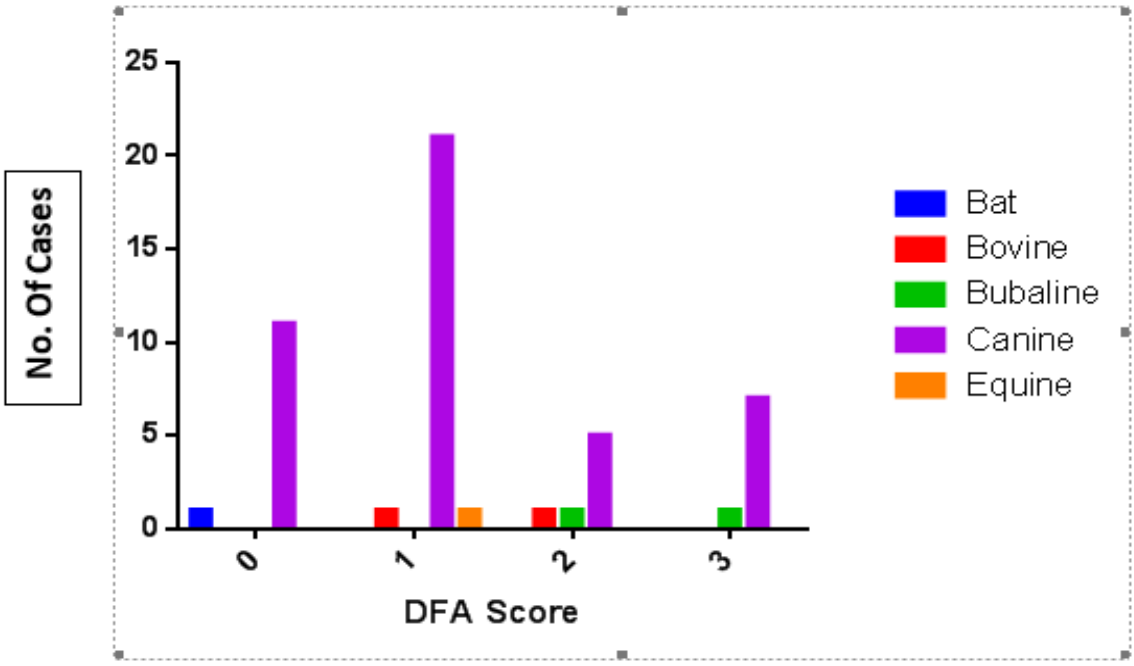


Fig. 4.3: Scoring of DFA results



4.2.1 Direct Fluorescent Antibody Test (DFA)

4.2.1.1 Conjugate titration

The titration for optimum dilution of the rabies virus anti-nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) was found to be 1:20. At this dilution, there was a intense and high fluorescence with minimal background. Beyond this dilution, there was a sudden fall in the fluorescent staining. It was however observed that there was considerable level of non-specific tissue fluorescence / auto-fluorescence, which made the interpretation ambiguous. This problem was overcome by using Evans blue as a counter stain at an optimized dilution of 0.000125 per cent. The counter staining not only reduced the ambiguity in the reading of the stained slides but it also created a good contrast by staining the background tissue.

Typically, tissue impressions from the brain stem and cerebellum were used for DFA. All 200 rabies suspected animal brain samples were subjected to DFA. The DFA protocols given by CDC Atlanta, USA was followed (Section 3.3.1.4). The slides were stained with Rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100), Rabies negative control IgG-FITC conjugate (M/s Light Diagnostics, Cat # 5102) (Plate 4.1), Normal Goat IgG-FITC conjugate (M/s Light Diagnostics, Cat # 5202) (Plate 4.2). The stained slides were examined under a fluorescent microscope at 400X magnificaiton. Samples were declared positive when bright apple green fluorescent particles of varying size were noticed either scattered or within the neurons (Plate 4.3), whereas, in the negative samples no such fluorescence was observed (Plate 4.4).

The analysis of data revealed that out of the 200 brain samples collected from rabies suspected animals from different geographical locations, 120 were positive by DFA out of 130 positives based on DFA and LFA. Of these 120 DFA positives, 109/178 Canines, 8/9 Bovines, 2/2 Bubalines and 1/5 Equines were found positive (Table 4.2). Scoring with respect to grade of positivity (0, +, ++ and +++) was done for DFA with respect to host species (Fig. 4.2).

4.2.2 The Modified Direct Fluorescent Antibody Test (DFA)

The brain stem was used to make tissue impressions for Modified DFA. All the 50 samples were subjected to modified DFA protocol as described in the Section 3.3.3.6. Initially tissue impressions were fixed in 10 per cent Neutral Buffered Formalin (NBF). Tris EDTA was used earlier to remove formalin effect and to unmask the RABV antigen. No fluorescence was seen after staining with Rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100). Later tried with hydrogen peroxide and TPBS washes. Acetone at 50 °C was used as a fixatives as detailed in Section 3.3.3.5. No fluorescence or less fluorescence found in highly positive brain sample declared by gold standard DFA (Plate 4.5 & 4.6). Later, tissue impressions were fixed at different concentrations of NBF (5%, 2.5% and 1%) (Plate 4.7 & 4.8). A good fluorescence was seen at 2.5 per cent NBF when compared to the other concentrations of formalin, besides100 percent inactivation of the virus when inoculated to BHK-21 cells. The stained slides were examined under a fluorescent microscope at 200X and 400X magnification. It was compared with the slides prepared from same impression, processed by gold standard DFA.

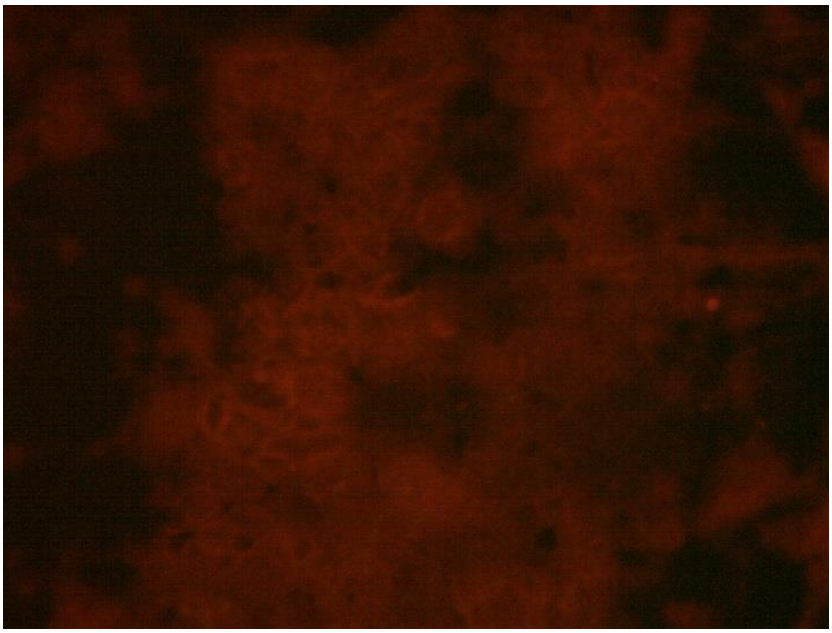


Plate 4.1: Rabid animal brain impression stained with the rabies negative control IgG-FITC conjugate (M/s Light Diagnostics, Cat # 5102) with counter stain (200X magnification)

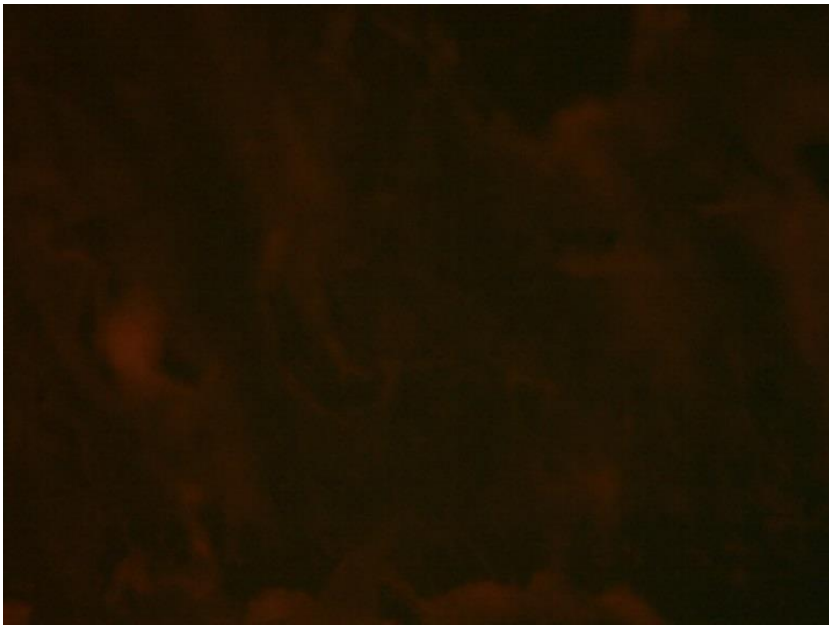


Plate 4.2: Rabid animal brain impression stained with the Normal Goat IgG-FITC conjugate (M/s Light Diagnostics, Cat # 5202) with counter stain (200X magnification)

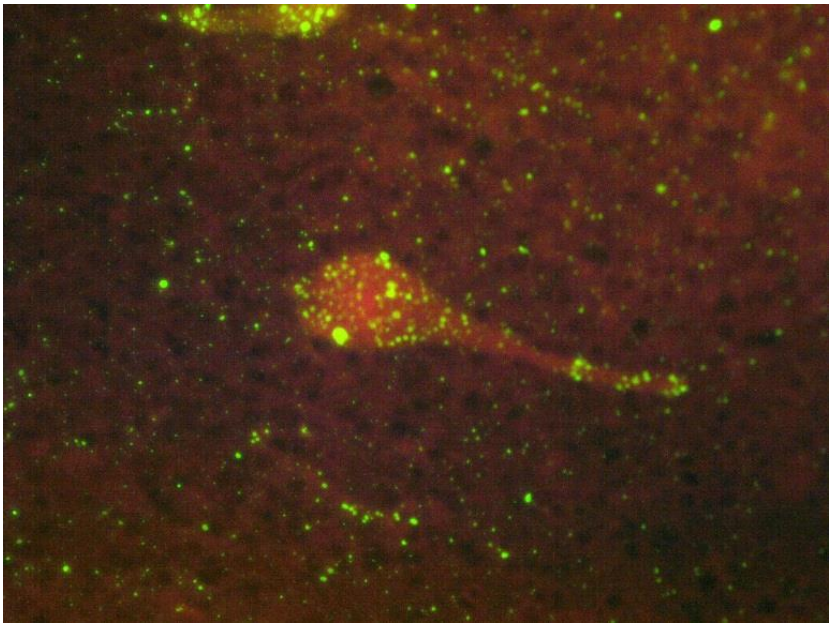


Plate 4.3: Rabid animal brain impression stained with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue) (200X magnification)

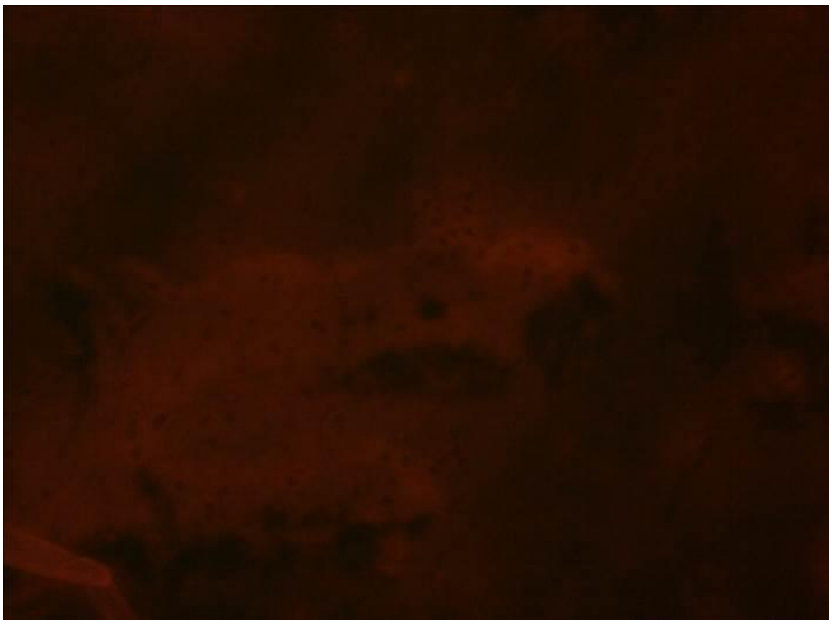


Plate 4.4: Non rabid animal brain impression stained with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (200X magnification)

Table 4.1 : State-wise details of the collected and the results of DFA vs LFA

Sl. No.	Sample No.	Region	Species	LFA	DFA
1	VMC-194	Karnataka	Bat	**Negative**	**Negative**
2	VMC-199	Tamil Nadu	Canine	Positive	Positive
3	VMC-270	Rajasthan	Canine	Positive	Positive
4	VMC-277	Rajasthan	Canine	Positive	Positive
5	VMC-320	Maharashtra	Canine	**Negative**	**Negative**
6	VMC-331	Jammu & Kashmir	Bubaline	Positive	Positive
7	VMC-346	Manipur	Canine	Positive	Positive
8	VMC-373	Manipur	Canine	**Negative**	**Negative**
9	VMC-381	Manipur	Canine	**Negative**	**Negative**
10	VMC-391	Manipur	Canine	Positive	Positive
11	VMC-400	Karnataka	Canine	Positive	Positive
12	VMC-401	Karnataka	Canine	**Negative**	**Negative**
13	VMC-402	Karnataka	Canine	Positive	Positive
14	VMC-403	Karnataka	Canine	Positive	Positive
15	VMC-404	Karnataka	Canine	**Positive**	**Negative**
16	VMC-405	Karnataka	Canine	**Negative**	**Negative**
17	VMC-406	Karnataka	Canine	Positive	Positive
18	VMC-407	Karnataka	Canine	**Positive**	**Negative**
19	VMC-408	Karnataka	Equine	Positive	Positive
20	VMC-409	Karnataka	Canine	Positive	Positive
21	VMC-410	Karnataka	Canine	**Negative**	**Negative**
22	VMC-411	Karnataka	Canine	**Positive**	**Negative**
23	VMC-412	Karnataka	Canine	Positive	Positive
24	VMC-413	Karnataka	Bovine	**Negative**	**Negative**
25	VMC-414	Karnataka	Canine	Positive	Positive
26	VMC-416	Karnataka	Canine	Positive	Positive
27	VMC-417	Karnataka	Canine	**Positive**	**Negative**
28	VMC-418	Manipur	Canine	**Negative**	**Negative**
29	VMC-419	Karnataka	Canine	Positive	Positive

Sl. No.	Sample No.	Region	Species	LFA	DFA
30	VMC-420	Karnataka	Canine	**Negative**	**Negative**
31	VMC-421	Karnataka	Equine	**Negative**	**Negative**
32	VMC-422	Karnataka	Canine	Positive	Positive
33	VMC-423	Karnataka	Canine	Positive	Positive
34	VMC-424	Karnataka	Canine	**Positive**	**Negative**
35	VMC-425	Karnataka	Canine	Positive	Positive
36	VMC-426	Karnataka	Canine	Positive	Positive
37	VMC-427	Gujarat	Bubaline	Positive	Positive
38	VMC-428	Karnataka	Canine	**Negative**	**Negative**
39	VMC-429	Karnataka	Canine	**Negative**	Positive
40	VMC-430	Karnataka	Canine	Positive	Positive
41	VMC-431	Karnataka	Equine	**Positive**	**Negative**
42	VMC-432	Karnataka	Canine	**Negative**	**Negative**
43	VMC-433	Karnataka	Canine	Positive	Positive
44	VMC-434	Karnataka	Canine	Positive	Positive
45	VMC-435	Karnataka	Canine	**Negative**	**Positive**
46	VMC-436	Karnataka	Canine	Positive	Positive
47	VMC-438	Karnataka	Canine	Positive	Positive
48	VMC-439	Karnataka	Canine	Positive	Positive
49	VMC-440	Karnataka	Canine	Positive	Positive
50	VMC-441	Karnataka	Canine	**Negative**	**Negative**
51	VMC-442	Karnataka	Canine	Positive	Positive
52	VMC-443	Karnataka	Canine	**Negative**	**Negative**
53	VMC-444	Karnataka	Canine	Positive	Positive
54	VMC-445	Karnataka	Canine	Positive	Positive
55	VMC-446	Karnataka	Canine	Positive	Positive
56	VMC-447	Karnataka	Canine	**Positive**	**Negative**
57	VMC-448	Karnataka	Canine	Positive	Positive
58	VMC-449	Karnataka	Canine	**Negative**	**Negative**
59	VMC-450	Karnataka	Canine	**Negative**	**Negative**

Sl. No.	Sample No.	Region	Species	LFA	DFA
60	VMC-451	Karnataka	Canine	Positive	Positive
61	VMC-452	Karnataka	Canine	Positive	Positive
62	VMC-453	Karnataka	Canine	**Negative**	**Negative**
63	VMC-454	Karnataka	Canine	**Negative**	**Negative**
64	VMC-456	Karnataka	Equine	**Negative**	**Negative**
65	VMC-457	Karnataka	Canine	Positive	Positive
66	VMC-458	Karnataka	Canine	**Negative**	**Negative**
67	VMC-459	Karnataka	Canine	Positive	Positive
68	VMC-460	Karnataka	Canine	**Negative**	**Negative**
69	VMC-461	Karnataka	Feline	**Negative**	**Negative**
70	VMC-462	Karnataka	Canine	Positive	Positive
71	VMC-463	Karnataka	Canine	**Negative**	**Negative**
72	VMC-464	Karnataka	Canine	Positive	Positive
73	VMC-465	Karnataka	Canine	**Negative**	**Negative**
74	VMC-471	Karnataka	Canine	Positive	Positive
75	VMC-481	Karnataka	Canine	Positive	Positive
76	VMC-482	Karnataka	Canine	Positive	Positive
77	VMC-483	Karnataka	Canine	**Negative**	**Negative**
78	VMC-484	Karnataka	Canine	**Negative**	**Negative**
79	VMC-485	Karnataka	Canine	Positive	Positive
80	VMC-486	Karnataka	Canine	**Negative**	**Negative**
81	VMC-487	Karnataka	Canine	**Negative**	**Negative**
82	VMC-488	Karnataka	Canine	**Negative**	**Negative**
83	VMC-489	Karnataka	Canine	Positive	Positive
84	VMC-491	Karnataka	Canine	**Negative**	**Negative**
85	VMC-492	Karnataka	Canine	Positive	Positive
86	VMC-493	Karnataka	Canine	Positive	Positive
87	VMC-494	Manipur	Canine	**Negative**	**Negative**
88	VMC-496	Karnataka	Canine	Positive	Positive
89	VMC-497	Karnataka	Canine	**Negative**	**Unfit for processing**

Sl. No.	Sample No.	Region	Species	LFA	DFA
90	VMC-498	Karnataka	Canine	**Negative**	**Unfit for processing**
91	VMC-499	Karnataka	Canine	Positive	Positive
92	VMC-500	Karnataka	Canine	Positive	Positive
93	VMC-501	Karnataka	Canine	**Positive**	**Unfit for processing**
94	VMC-502	Karnataka	Canine	**Negative**	**Negative**
95	VMC-503	Karnataka	Feline	**Negative**	**Negative**
96	VMC-504	Karnataka	Feline	**Negative**	**Negative**
97	VMC-505	Karnataka	Canine	Positive	Positive
98	VMC-507	Karnataka	Canine	**Negative**	**Negative**
99	VMC-508	Karnataka	Goral	**Negative**	**Negative**
100	VMC-509	Karnataka	Canine	Positive	Positive
101	VMC-510	Karnataka	Canine	**Positive**	**Unfit for processing**
102	VMC-511	Karnataka	Canine	Positive	Positive
103	VMC-512	Karnataka	Bovine	Positive	Positive
104	VMC-513	Karnataka	Canine	Positive	Positive
105	VMC-514	Karnataka	Canine	**Negative**	**Negative**
106	VMC-515	Karnataka	Canine	**Negative**	**Negative**
107	VMC-516	Karnataka	Canine	**Negative**	**Negative**
108	VMC-517	Karnataka	Canine	**Negative**	**Negative**
109	VMC-518	Karnataka	Canine	Positive	Positive
110	VMC-519	Karnataka	Canine	Positive	Positive
111	VMC-520	Karnataka	Canine	Positive	Positive
112	VMC-521	Karnataka	Canine	Positive	Positive
113	VMC-522	Karnataka	Canine	**Negative**	**Negative**
114	VMC-523	Karnataka	Canine	**Negative**	**Negative**
115	VMC-524	Karnataka	Canine	**Negative**	**Negative**
116	VMC-525	Karnataka	Canine	Positive	Positive
117	VMC-526	Karnataka	Canine	**Positive**	**Negative**
118	VMC-527	Karnataka	Canine	Positive	Positive
119	VMC-528	Karnataka	Canine	**Negative**	**Negative**

Sl. No.	Sample No.	Region	Species	LFA	DFA
120	VMC-529	Karnataka	Canine	Positive	Positive
121	VMC-530	Karnataka	Canine	**Negative**	**Negative**
122	VMC-531	Karnataka	Canine	**Negative**	**Negative**
123	VMC-532	Karnataka	Bovine	Positive	Positive
124	VMC-533	Karnataka	Bovine	Positive	Positive
125	VMC-534	Karnataka	Canine	Positive	Positive
126	VMC-535	Karnataka	Canine	**Negative**	**Negative**
127	VMC-536	Karnataka	Canine	Positive	Positive
128	VMC-537	Karnataka	Canine	Positive	Positive
129	VMC-538	Karnataka	Canine	**Negative**	**Positive**
130	VMC-539	Karnataka	Canine	**Positive**	**Negative**
131	VMC-540	Karnataka	Canine	**Negative**	**Negative**
132	VMC-541	Karnataka	Canine	Positive	Positive
133	VMC-542	Karnataka	Bovine	Positive	Positive
134	VMC-543	Karnataka	Canine	**Negative**	**Negative**
135	VMC-544	Karnataka	Canine	Positive	Positive
136	VMC-545	Karnataka	Canine	Positive	Positive
137	VMC-546	Karnataka	Canine	Positive	Positive
138	VMC-547	Karnataka	Canine	Positive	Positive
139	VMC-458	Karnataka	Canine	Positive	Positive
140	VMC-459	Karnataka	Canine	Positive	Positive
141	VMC-550	Karnataka	Canine	Positive	Positive
142	VMC-551	Manipur	Canine	**Negative**	**Negative**
143	VMC-552	Karnataka	Canine	**Negative**	**Negative**
144	VMC-553	Karnataka	Canine	Positive	Positive
145	VMC-554	Karnataka	Canine	Positive	Positive
146	VMC-555	Karnataka	Canine	**Negative**	**Negative**
147	VMC-556	Karnataka	Canine	Positive	Positive
148	VMC-557	Karnataka	Canine	Positive	Positive
149	VMC-558	Karnataka	Bovine	Positive	Positive

Sl. No.	Sample No.	Region	Species	LFA	DFA
150	VMC-559	Karnataka	Canine	Positive	Positive
151	VMC-560	Karnataka	Canine	**Negative**	**Negative**
152	VMC-561	Karnataka	Canine	Positive	Positive
153	VMC-562	Karnataka	Canine	Positive	Positive
154	VMC-563	Karnataka	Canine	Positive	Positive
155	VMC-564	Karnataka	Canine	Positive	Positive
156	VMC-565	Karnataka	Canine	Positive	Positive
157	VMC-566	Karnataka	Canine	**Negative**	**Negative**
158	VMC-567	Karnataka	Canine	Positive	Positive
159	VMC-568	Karnataka	Canine	**Negative**	**Negative**
160	VMC-569	Karnataka	Canine	**Negative**	**Negative**
161	VMC-570	Karnataka	Canine	Positive	Positive
162	VMC-571	Karnataka	Canine	Positive	Positive
163	VMC-572	Karnataka	Canine	Positive	Positive
164	VMC-573	Karnataka	Canine	Positive	Positive
165	VMC-574	Karnataka	Equine	**Negative**	**Negative**
166	VMC-575	Karnataka	Canine	Positive	Positive
167	VMC-576	Karnataka	Canine	**Negative**	**Negative**
168	VMC-577	Karnataka	Feline	**Negative**	**Negative**
169	VMC-578	Karnataka	Canine	Positive	Positive
170	VMC-579	Karnataka	Canine	Positive	Positive
171	VMC-580	Karnataka	Canine	Positive	Positive
172	VMC-581	Karnataka	Canine	Positive	Positive
173	VMC-583	Karnataka	Canine	Positive	Positive
174	VMC-584	Karnataka	Canine	Positive	Positive
175	VMC-585	Karnataka	Canine	**Negative**	**Negative**
176	VMC-586	Karnataka	Canine	**Negative**	**Negative**
177	VMC-587	Manipur	Canine	**Negative**	**Negative**
178	VMC-588	Karnataka	Bovine	Positive	Positive
179	VMC-589	Karnataka	Bovine	Positive	Positive

Sl. No.	Sample No.	Region	Species	LFA	DFA
180	VMC-590	Karnataka	Canine	Positive	Positive
181	VMC-591	Karnataka	Canine	**Negative**	**Negative**
182	VMC-592	Karnataka	Canine	Positive	Positive
183	VMC-593	Karnataka	Canine	Positive	Positive
184	VMC-594	Karnataka	Canine	Positive	Positive
185	VMC-595	Karnataka	Canine	Positive	Positive
186	VMC-596	Karnataka	Canine	**Positive**	**Negative**
187	VMC-597	Karnataka	Canine	Positive	Positive
188	VMC-598	Karnataka	Canine	Positive	Positive
189	VMC-599	Karnataka	Bovine	Positive	Positive
190	VMC-600	Karnataka	Canine	Positive	Positive
191	VMC-601	Karnataka	Canine	**Negative**	**Negative**
192	VMC-602	Karnataka	Canine	Positive	Positive
193	VMC-603	Karnataka	Canine	**Negative**	**Negative**
194	VMC-604	Karnataka	Canine	Positive	Positive
195	VMC-605	Karnataka	Canine	Positive	Positive
196	VMC-606	Karnataka	Canine	Positive	Positive
197	VMC-607	Karnataka	Canine	Positive	Positive
198	VMC-608	Karnataka	Canine	Positive	Positive
199	VMC-609	Karnataka	Canine	Positive	Positive
200	VMC-610	Karnataka	Canine	Positive	Positive

Samples were declared positive when bright apple green fluorescent particles of varying size were noticed either scattered or within the neurons (Plate 4.10 to 4.13), whereas, in the negative samples no such fluorescence was observed (Plate 4.9).

In all 50 samples were subjected to DFA and Modified DFA. The results were compared and analysed by Spearman's nonparametric correlation analysis. The "Correlation coefficient (Spearman r)" was found to be 0.98 ($P<0.001$) indicating very good positive correlation. Correlation Analysis of Modified DFA with DFA is detailed in Table 4.4 and scoring of Modified DFA provided in Fig. 4.4.

4.3 Retrieval of rabies virus antigen in formalin fixed brain impressions from BHK-21 cells

The presence or absence of virus in different fixatives (Tris EDTA, Acetone at 50 °C, 10%, 5%, 2.5%, 1% NBF) was confirmed by inoculating tissue scarping from DFA confirmed positive sample to BHK-21 cells. (Plate 4.14 to 4.19). Lab-tek slide chambers were used for the study (Plate 4.20 to 4.22). The same protocol followed as described in the Section 3.3.4.5 and stained with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue) (Plate 4.23 to 4.30). The tissue impressions which were fixed in 2.5 per cent NBF showed 100 per cent inactivation of virus in BHK-21 cells as indicator system (Plate 4.31). It reduces potential biohazard to laboratory personnel involved in handling the infected brain sample.

Table 4.2: Species wise positives by DFA and LFA

Species	Species Count	Total Positives	DFA Positives	LFA Positives
Bat	1	0	0	0
Bovine	9	8	8	8
Bubaline	2	2	2	2
Canine	178	118	109	117
Equine	5	3	1	2
Feline	4	0	0	0
Goral	1	0	0	0

Table 4.3: State-wise details of the samples collected and tested by DFA, Modified DFA, LFA and LAMP

Sl. No.	Sample No.	Region	Species	DFA	Modified DFA	LFA	LAMP
1	VMC-194	Karnataka	Bat	**Negative**	**Negative**	**Negative**	**Negative**
2	VMC-199	Tamil Nadu	Canine	Positive	Positive	Positive	Positive
3	VMC-270	Rajasthan	Canine	Positive	Positive	Positive	Positive
4	VMC-331	Jammu & Kashmir	Bubaline	Positive	Positive	Positive	Positive
5	VMC-373	Manipur	Canine	**Negative**	**Negative**	**Negative**	**Negative**
6	VMC-381	Manipur	Canine	**Negative**	**Negative**	**Negative**	**Negative**
7	VMC-391	Manipur	Canine	Positive	Positive	Positive	Positive
8	VMC-400	Karnataka	Canine	Positive	Positive	Positive	Positive
9	VMC-401	Karnataka	Canine	**Negative**	**Negative**	**Negative**	**Negative**
10	VMC-408	Karnataka	Equine	Positive	Positive	Positive	Positive
11	VMC-411	Karnataka	Canine	**Negative**	**Negative**	**Positive**	**Positive**
12	VMC-427	Gujarat	Bubaline	Positive	Positive	Positive	Positive
13	VMC-440	Karnataka	Canine	Positive	Positive	Positive	Positive
14	VMC-446	Karnataka	Canine	Positive	Positive	Positive	Positive
15	VMC-509	Karnataka	Canine	Positive	Positive	Positive	Positive
16	VMC-511	Karnataka	Canine	Positive	Positive	Positive	Positive
17	VMC-545	Karnataka	Canine	Positive	Positive	Positive	Positive

Sl. No.	Sample No.	Region	Species	DFA	Modified DFA	LFA	LAMP
18	VMC-546	Karnataka	Canine	Positive	Positive	Positive	Positive
19	VMC-551	Manipur	Canine	**Negative**	**Negative**	**Negative**	**Negative**
20	VMC-557	Karnataka	Canine	Positive	Positive	Positive	Positive
21	VMC-558	Karnataka	Bovine	Positive	Positive	Positive	Positive
22	VMC-561	Karnataka	Canine	Positive	Positive	Positive	Positive
23	VMC-562	Karnataka	Canine	Positive	Positive	Positive	Positive
24	VMC-567	Karnataka	Canine	Positive	Positive	Positive	Positive
25	VMC-568	Karnataka	Canine	**Negative**	**Negative**	**Negative**	**Negative**
26	VMC-571	Karnataka	Canine	Positive	Positive	Positive	Positive
27	VMC-572	Karnataka	Canine	Positive	Positive	Positive	Positive
28	VMC-573	Karnataka	Canine	Positive	Positive	Positive	Positive
29	VMC-579	Karnataka	Canine	Positive	Positive	Positive	Positive
30	VMC-580	Karnataka	Canine	Positive	Positive	Positive	Positive
31	VMC-581	Karnataka	Canine	Positive	Positive	Positive	Positive
32	VMC-585	Karnataka	Canine	**Negative**	**Negative**	**Negative**	**Negative**
33	VMC-586	Karnataka	Canine	**Negative**	**Negative**	**Negative**	**Positive**
34	VMC-588	Karnataka	Bovine	Positive	Positive	Positive	Positive
35	VMC-590	Karnataka	Canine	Positive	Positive	Positive	Positive
36	VMC-591	Karnataka	Canine	**Negative**	**Negative**	**Negative**	**Negative**
37	VMC-593	Karnataka	Canine	Positive	Positive	Positive	Positive
38	VMC-595	Karnataka	Canine	Positive	Positive	Positive	Positive
39	VMC-596	Karnataka	Canine	**Negative**	**Negative**	**Positive**	**Positive**
40	VMC-597	Karnataka	Canine	Positive	Positive	Positive	Positive
41	VMC-598	Karnataka	Canine	Positive	Positive	Positive	Positive
42	VMC-600	Karnataka	Canine	Positive	Positive	Positive	Positive
43	VMC-601	Karnataka	Canine	**Negative**	**Negative**	**Negative**	**Negative**
44	VMC-602	Karnataka	Canine	Positive	Positive	Positive	Positive
45	VMC-604	Karnataka	Canine	Positive	Positive	Positive	Positive
46	VMC-605	Karnataka	Canine	Positive	Positive	Positive	Positive
47	VMC-606	Karnataka	Canine	Positive	Positive	Positive	Positive
48	VMC-607	Karnataka	Canine	Positive	Positive	Positive	Positive
49	VMC-608	Karnataka	Canine	Positive	Positive	Positive	Positive
50	VMC-610	Karnataka	Canine	Positive	Positive	Positive	Positive

Plate 4.5: Rabid animal brain impression showing no fluorescence after fixing the slide in Tris EDTA (200X magnification)

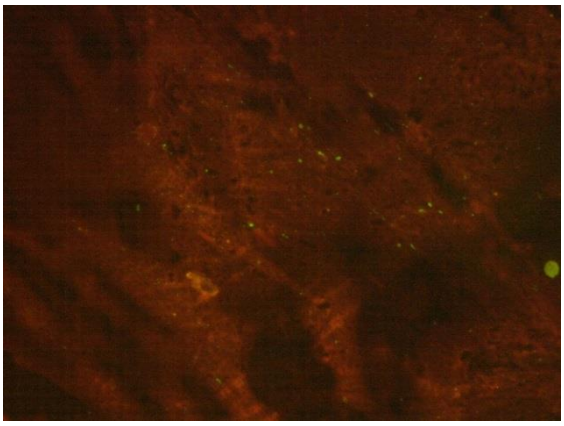


Plate 4.6: Rabid animal brain impression showing less fluorescence after fixing the slide in Acetone at 50 °C for 30 min (200X magnification).

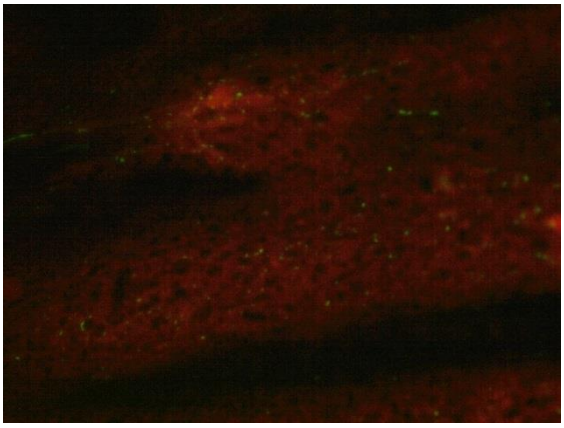


Plate 4.7: Rabid brain impression showing less fluorescence after fixing the slide in 10% Neutral Buffered Formalin (200X magnification).

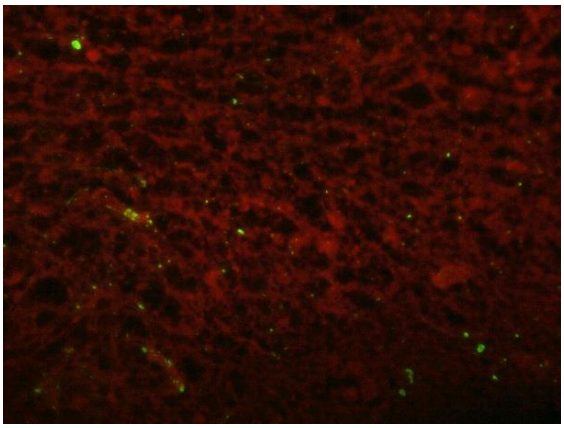


Plate 4.8: Rabid animal brain impression showing less fluorescence after fixing the slide in 5% Neutral Buffered Formalin (200X magnification)

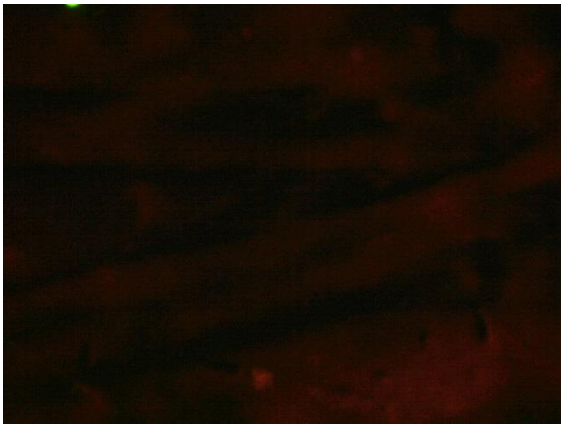


Plate 4.9: Non rabid animal brain impression showing no fluorescence after fixing the slide in 2.5% Neutral Buffered Formalin (200X magnification)

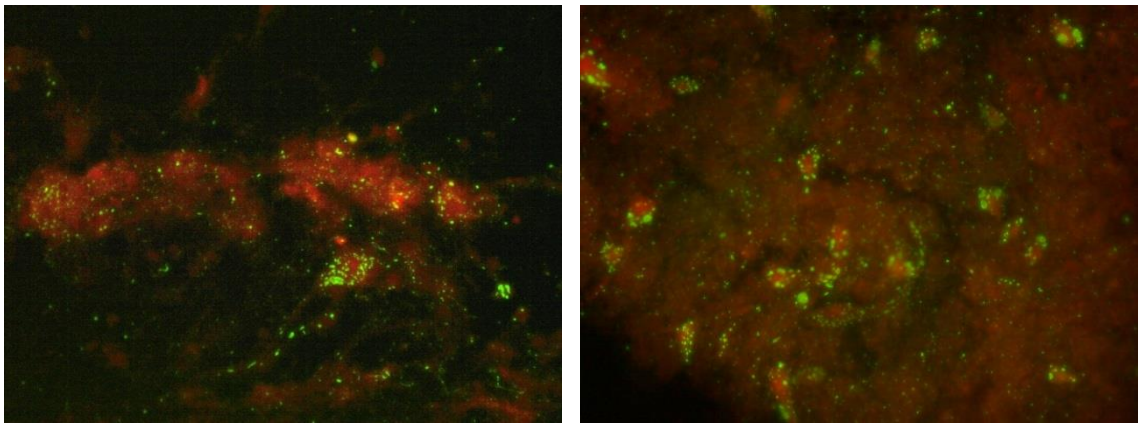


Plate 4.10: Rabid animal brain impression showing fluorescence (++++) after fixing the slide in 2.5% Neutral Buffered Formalin (Left) and chilled acetone (Right) (200X magnification)

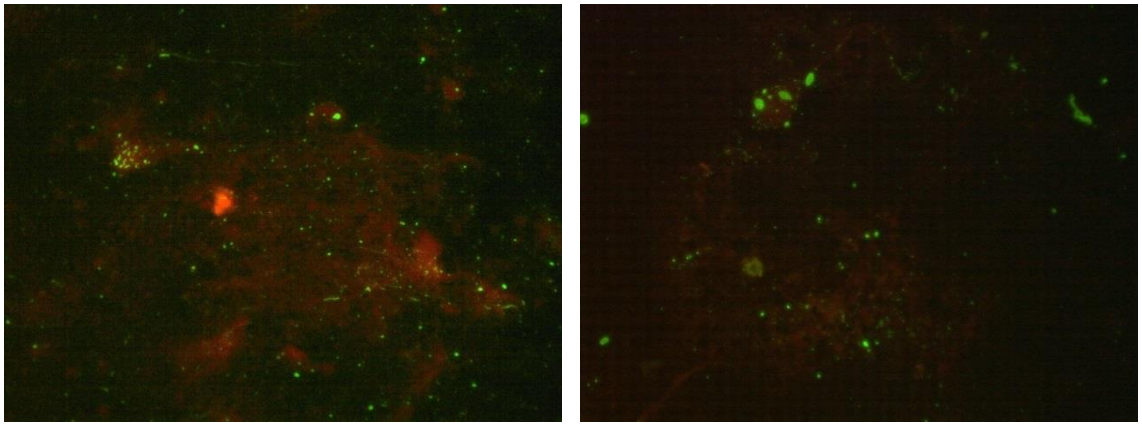


Plate 4.11: Rabid animal brain impression showing fluorescence (+++) after fixing the slide in 2.5% Neutral Buffered Formalin (Left) and chilled acetone (Right) (200X magnification)

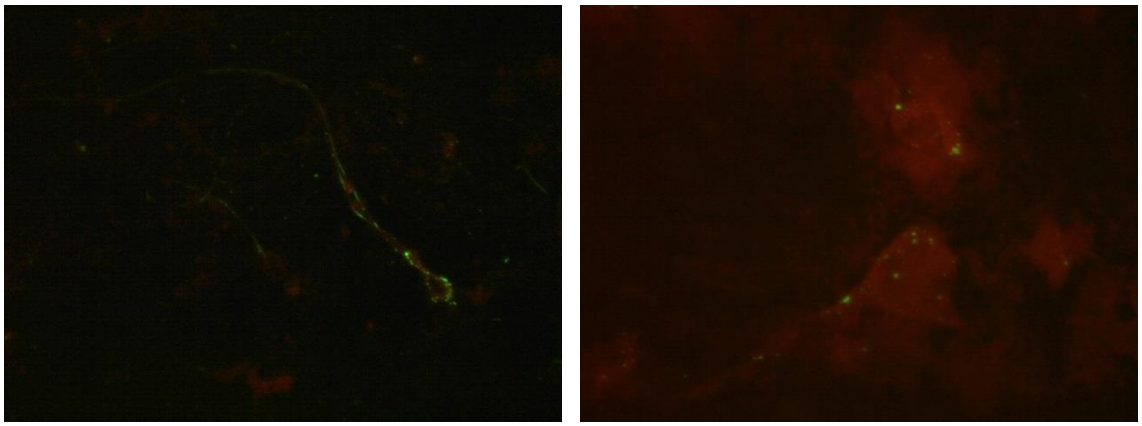


Plate 4.12: Rabid animal brain impression showing fluorescence (++) after fixing the slide in 2.5% Neutral Buffered Formalin (Left) and chilled acetone (Right) (200X magnification)

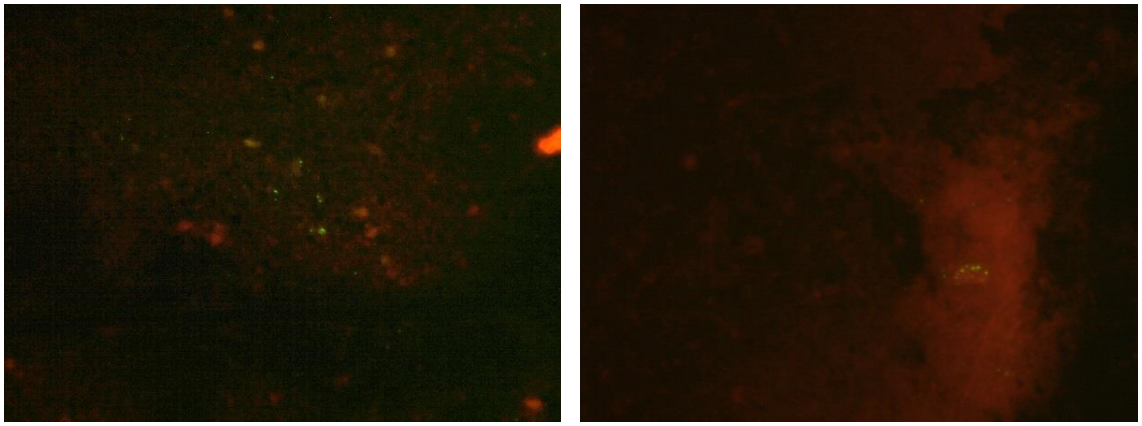
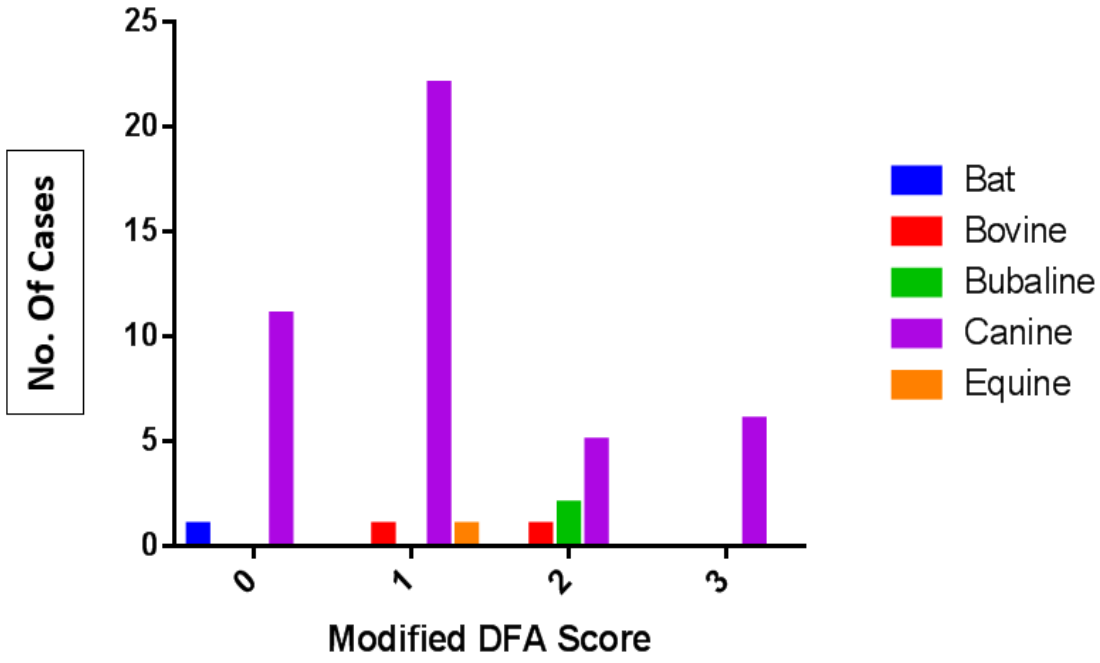


Plate 4.13: Rabid animal brain impression showing fluorescence (+) after fixing the slide in 2.5% Neutral Buffered Formalin (200X magnification)

Table 4.4: Spearman's nonparametric correlation analysis for DFA and Modified DFA

	DFA and Modified DFA
Spearman r	0.98
95% confidence interval	0.97 to 0.99
P value	-
P (two-tailed)	< 0.0001
P value summary	****
Exact or approximate P value?	Approximate
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	50

Fig. 4.4: Scoring of modified DFA results



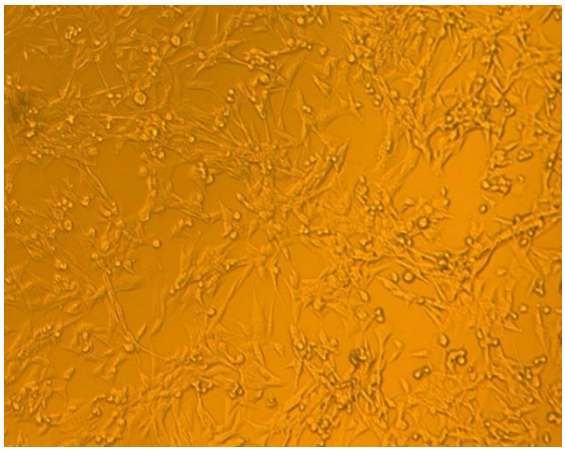


Plate 4.14: Cell control

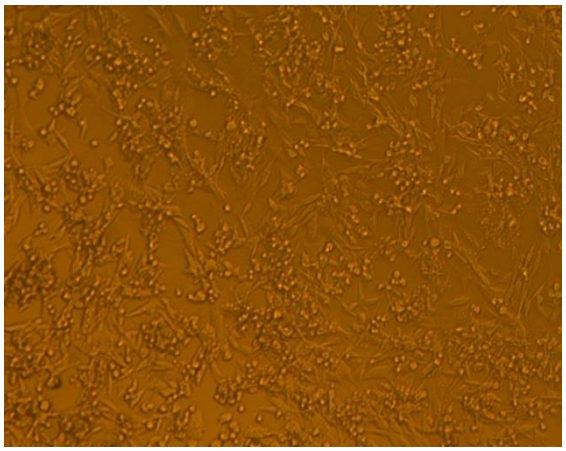


Plate 4.15: Positive control

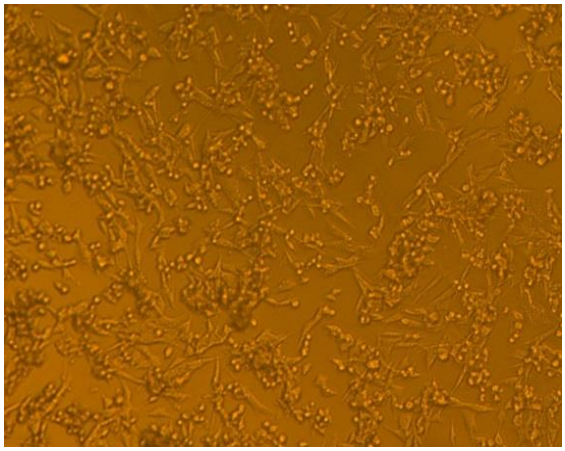


Plate 4.16: Negative control

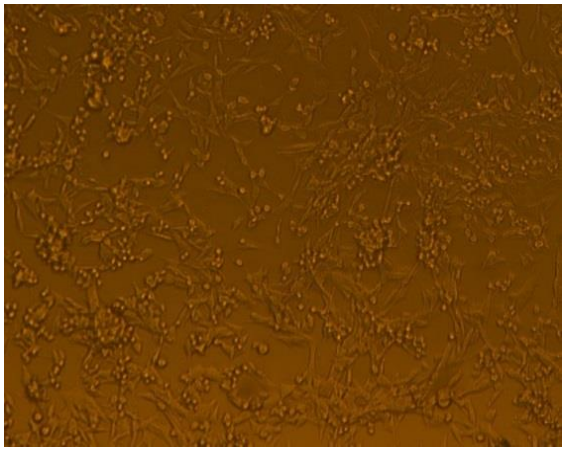


Plate 4.17: Acetone at -20 °C

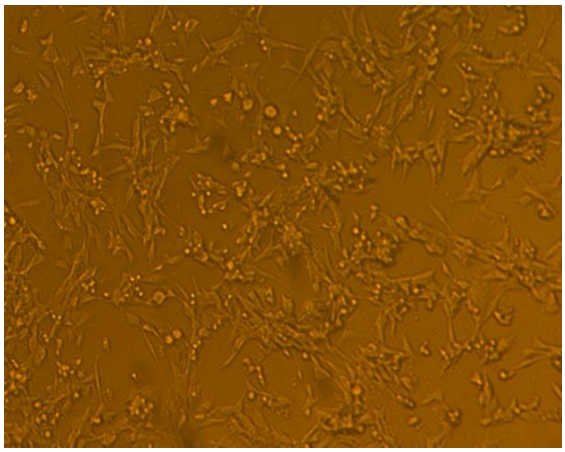


Plate 4.18: 2.5% NBF

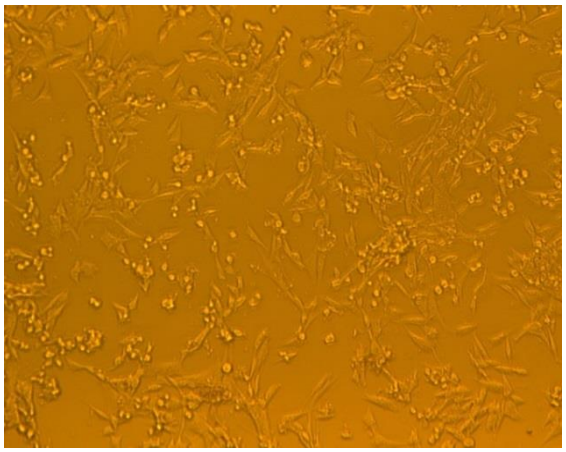


Plate 4.19: Acetone at 50 °C

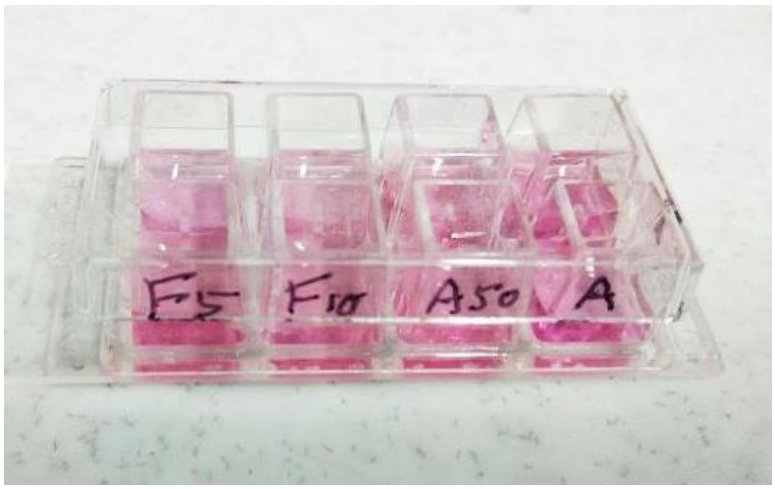


Plate 4.20: Lab-tek slide chambers; Day 1

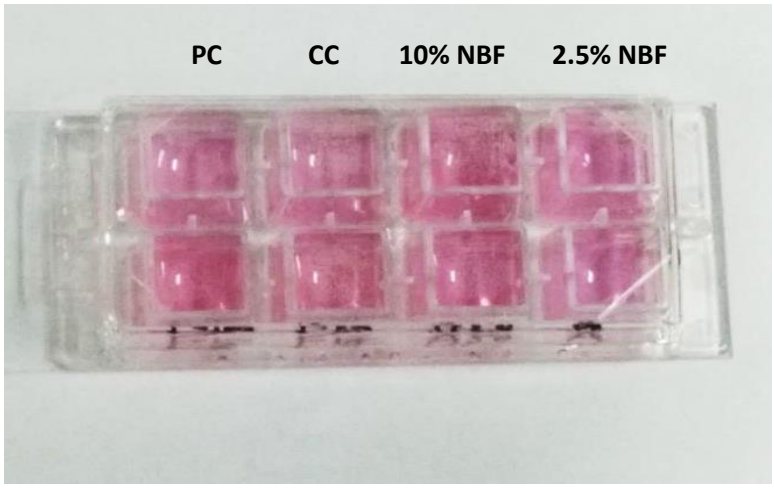


Plate 4.21: Lab-tek slide chambers; Day 1

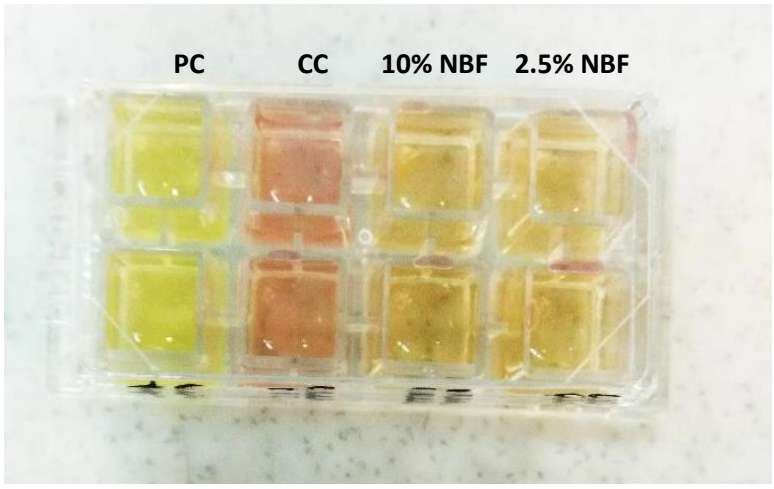


Plate 4.22: Lab-tek slide chambers; Day 2

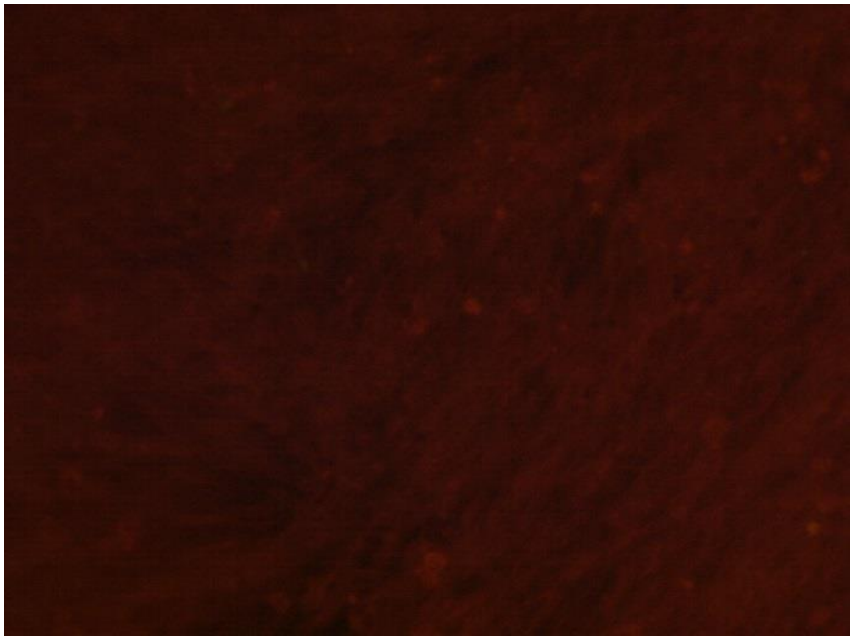


Plate 4.23: Cell control stained in Lab-Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue) (200X magnification)

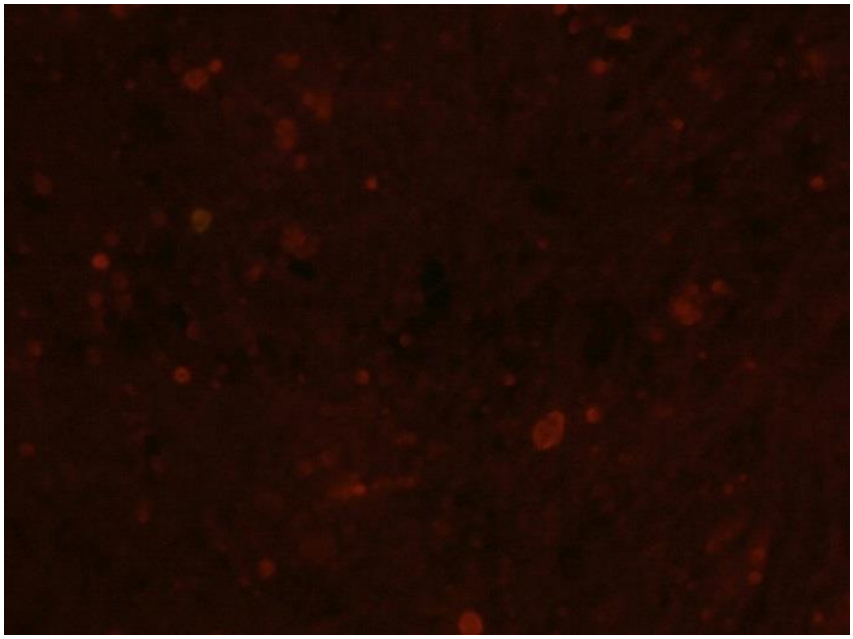


Plate 4.24: Negative control stained in Lab-Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue) (200X magnification)

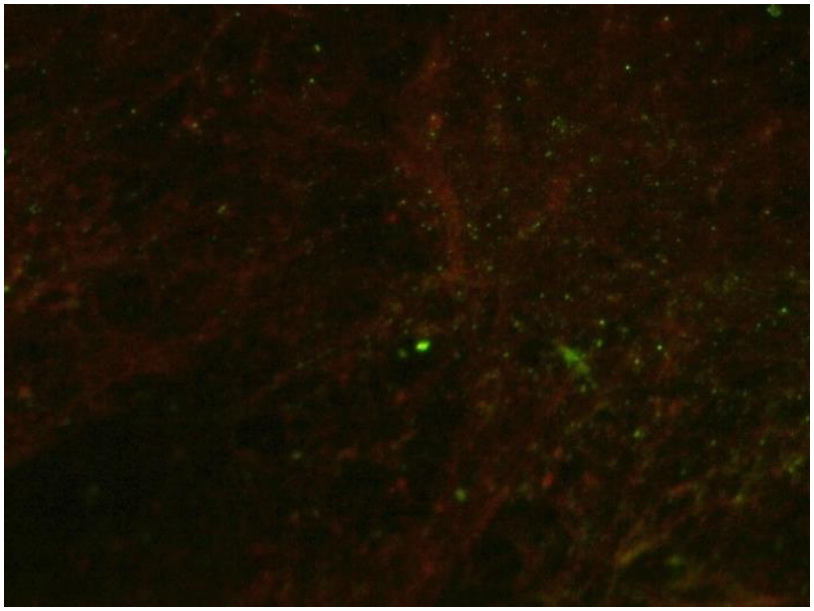


Plate 4.25: Positive control stained in Lab-Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue) (200X magnification).

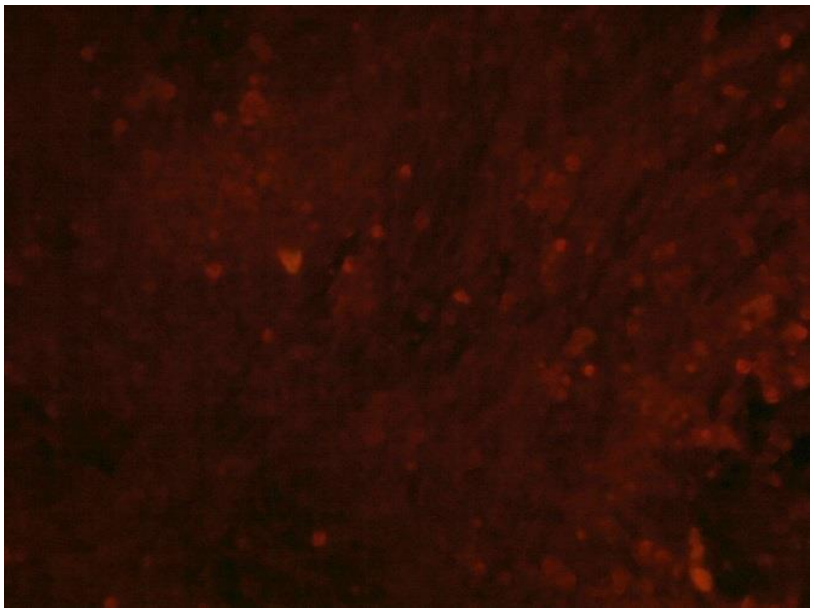


Plate 4.26: No fluorescence at 5 per cent NBF fixation in Lab-Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue) (200X magnification).

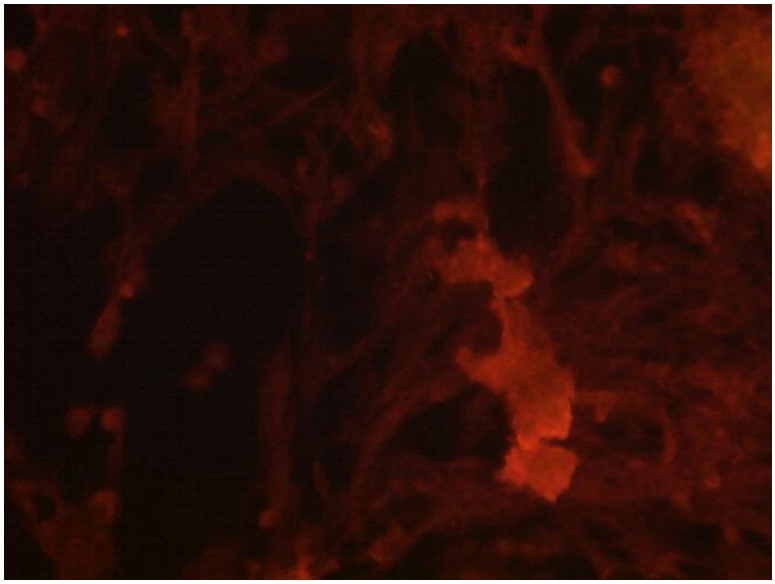


Plate 4.27: No fluorescence at 10 per cent NBF fixation in Lab-Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue) (200X magnification)

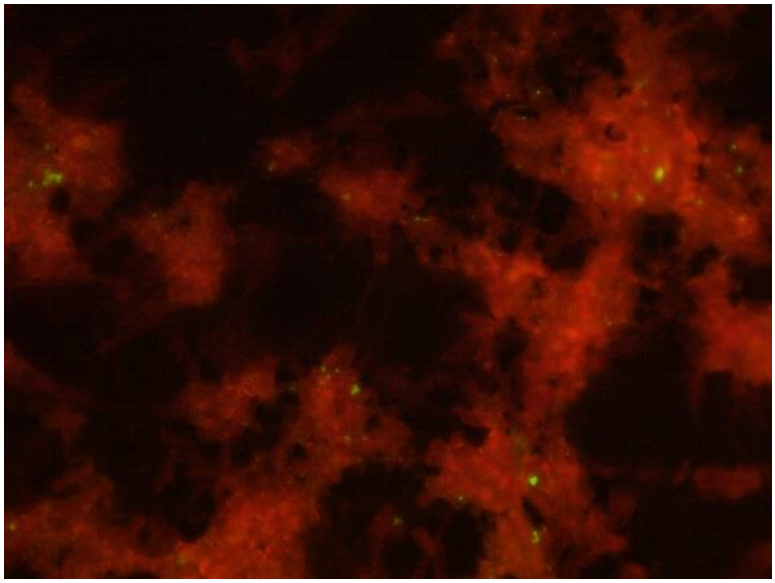


Plate 4.28: Presence of fluorescence at 1 per cent NBF fixation in Lab-Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue) (200X magnification)

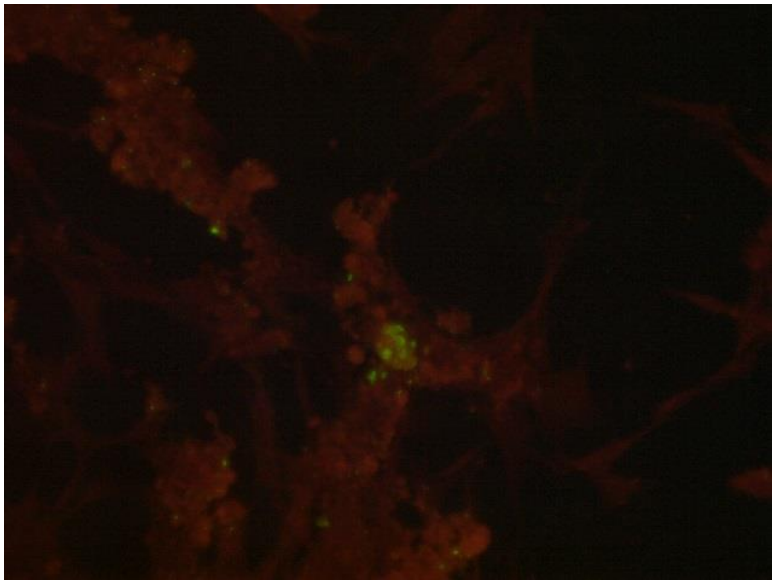


Plate 4.29: Presence of fluorescence at Acetone at 50 °C in Lab-Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue) (200X magnification)

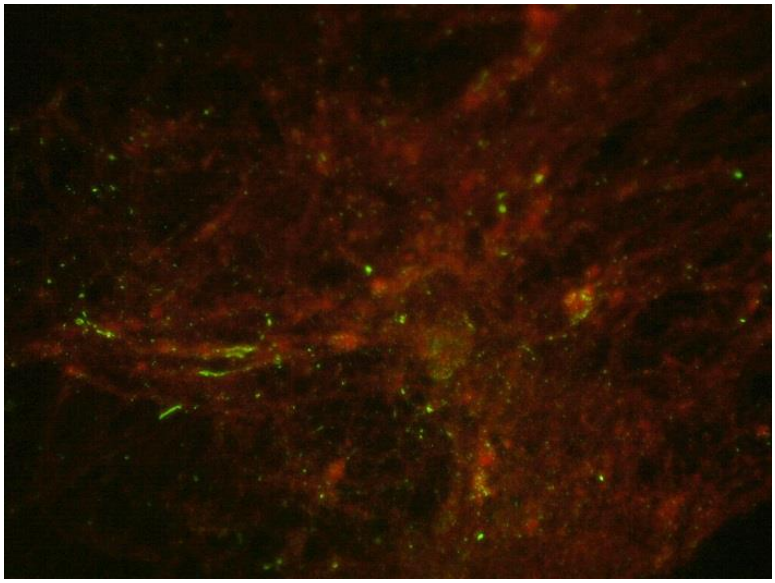


Plate 4.30: Presence of fluorescence at acetone at -20 °C in Lab-Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue) (200X magnification)

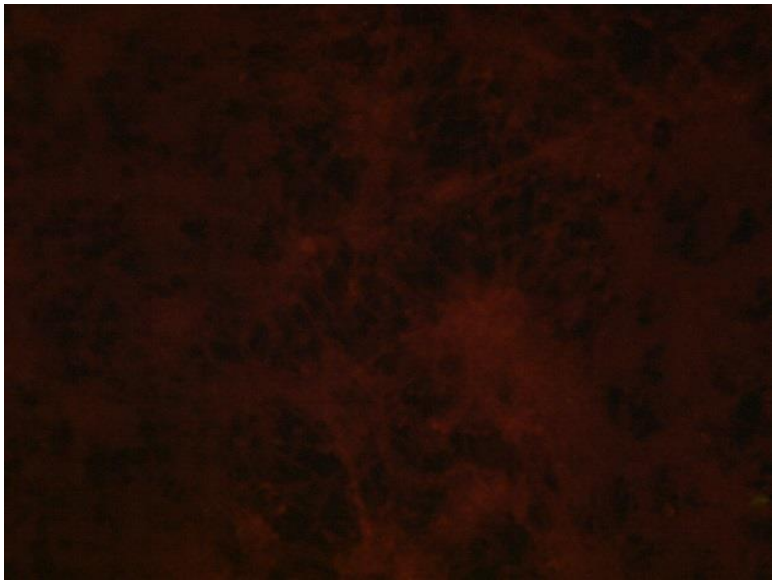


Plate 4.31: No fluorescence at 2.5 percent NBF fixation in Lab-Tek slides with the rabies virus anti nucleocapsid IgG-FITC conjugate (Rabies DFA III, Light Diagnostics, Cat # 5100) with counter stain (Evan's blue) (200X magnification)

4.4 Lateral Flow assay

The Lateral flow assay was performed using the Anigen Rapid Rabies Ag Test Kit of BIONOTE, Korea as per the manufacturer's instructions. The protocol is described in section 3.4.1**.** Around 0.5gm of brain was taken and mixed with assay diluent. A 50µl of tissue suspension was added on to the sample pad. Tissue suspension was made to flow through the nitrocellulose pad on a horizontal surface. After 5-10 minutes results were read and grading was done based intensity of the color developed in test line (Plate 4.32). In all, 200 samples were subjected to LFA (Table 4.1). The species wise analysis of data from LFA and DFA is detailed in the Table 4.2 and Fig. 4.3. Out of total 118 RABV positives, 117 were found positive by LFA, whereas, 109 were positive by DFA (Table 4.2). Some of DFA negatives were positive by LFA (Table 4.1).

All the samples (n=200) were subjected to LFA and DFA. The results were compared and analyzed by Spearman's nonparametric correlation analysis. The "Correlation coefficient (Spearman r)" was found to be 0.84 ($P<0.001$). Correlation Analysis of LFA with DFA is detailed in the table 4.5. The positive results were subjected to 2-way ANOVA with Tukey's multiple comparison test. Significant difference ($P< 0.05$) observed when LFA compared with DFA. The LFA scoring is detailed in the Fig. 4.6.

Table 4.5: Spearman's nonparametric correlation analysis for DFA and LFA

	DFA and LFA
Spearman r	**0.84**
95% confidence interval	**0.73 to 0.91**
P value	
P (two-tailed)	**< 0.0001**
P value summary	********
Exact or approximate P value?	**Approximate**
Significant? (alpha = 0.05)	**Yes**
Number of XY Pairs	**50**

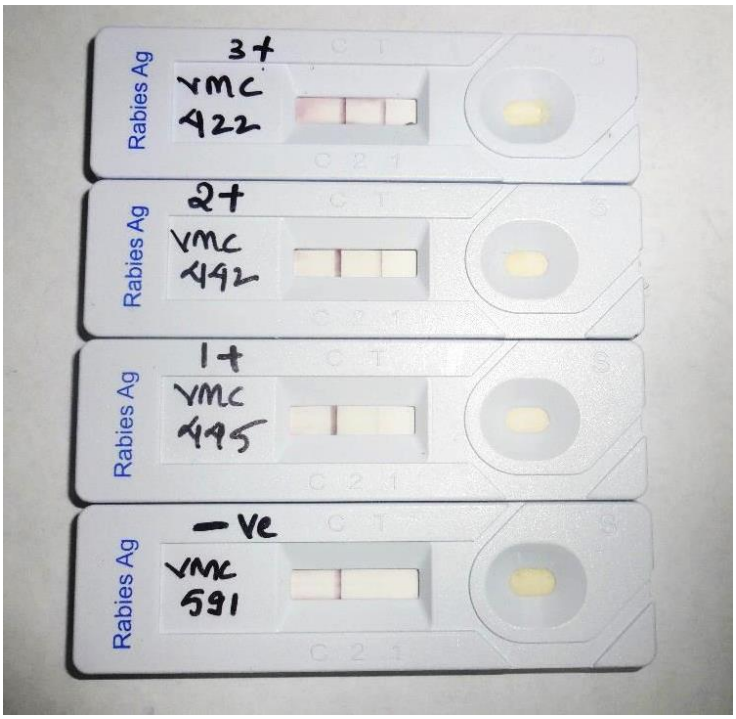


Plate 4.32: Grading of positivity by LFA

Fig. 4.5: Comparitive evaluation of LFA and DFA

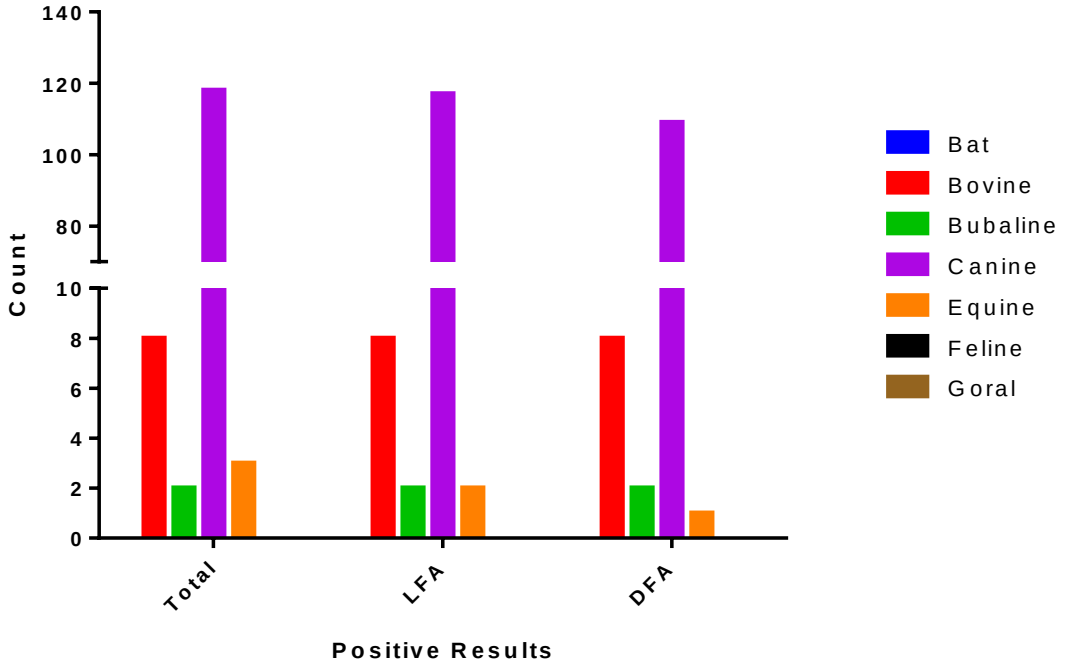
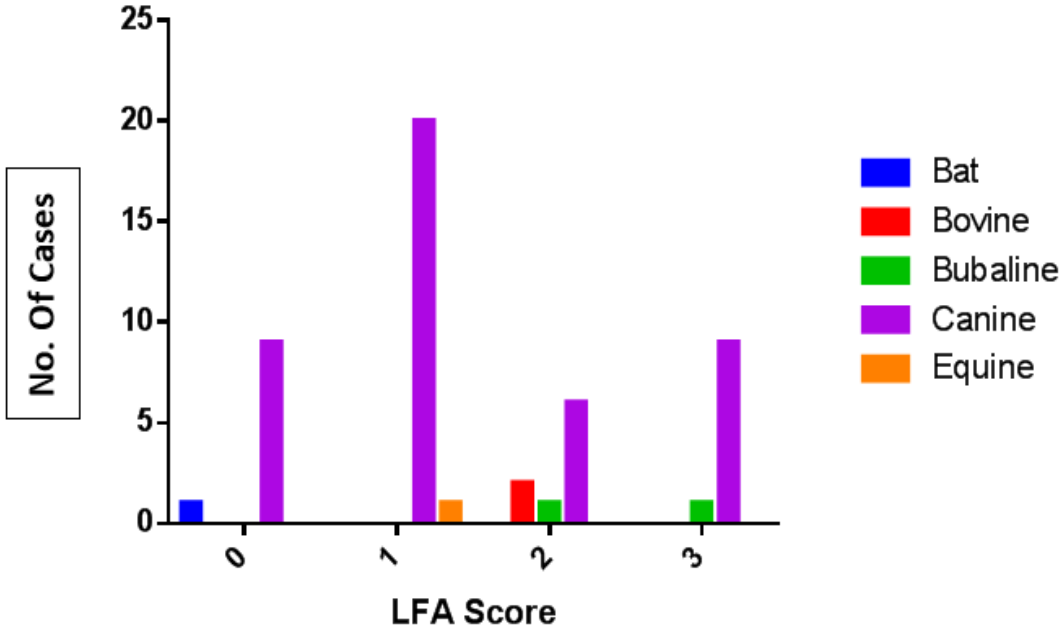


Fig. 4.6: Scoring of LFA results



4.5 Nucleic acid based confirmation of rabies virus

4.5.1 Reverse Transcription-Loop mediated Isothermal amplification (RT-LAMP)

4.5.1.1 Total RNA extraction from brain

The total RNA from the brain samples was extracted by using the Trizol reagent (Invitrogen) as per the manufacturer's instructions with slight modifications. The RNA extraction was done from 50 brain samples collected from animals suspected for rabies. Extracted RNA was used for amplification along with reverse transcriptase.

4.5.1.2 RABV-specific primers for RT-LAMP

The published primers used were designed on the basis of highly conserved regions were used (Muleya *et al.,* 2011). The RT-LAMP specific primers included a set of four primers comprising of two outer, two inner and that recognizes six distinct regions on the target sequence as shown in Table 3.2.

4.6.2 Optimized conditions for RT-LAMP

The LAMP was optimized as described by Notomi *et al*., (2000) with slight modifications. Reactions were carried out in 25 µL reaction mixture containing 3.5 µL of RNAase-free water (Amnion Biosciences), the reaction components were optimized to a final concentration of 1 X ThermolPol Buffer (New England Biolabs, USA), 4 mM $MgSO_4$ (New England Biolabs, USA), 0.8 M Betaine (Sigma Aldrich , Germany), 1mM dNTPs (New England Biolabs, USA). A twenty picomoles of each HPLC-graded inner primers, FIP and BIP, 5 picomoles of each outer primers, F3 and B3, in 1:4 ratio (Outer : Inner) followed by the addition of 8 U of *Bst* polymerase (Large fragment, New England Biolabs, USA). RNA template (3 µL of approximately 100 ng/µL) was eventually added

to the reaction mixture along with Reverse transcriptase (3 μL) (Table 3.3 and Plate 4.33). The temperature and time of incubation were also optimized by conducting a series of experiments. Finally, the reaction was carried out by incubation at 42 ^{O}C for 60 min. and terminated by heating at 95 ^{O}C for 2 min.

4.6.3 Visual detection of the RT-LAMP products by application of hydroxy-naphthol blue (HNB)

Hydroxy-naphthol blue was used in the RT-LAMP reaction for homogenous visualisation of the amplified genomic target. The final working concentration of 200 mM HNB in the reaction mixture was optimized to achieve desirable results. The HNB induced a colour change from purple in negative reactions to blue in positive samples while observed under visible light with naked eye (Plate 4.34). The results of the gel electrophoresis were in perfect agreement to the colour change (Plate 4.34).

4.6.4 Diagnostic sensitivity and specificity of LAMP

Diagnostic attributes of LAMP were determined using RNA from confirmed positive samples. Nuclease free water was used as a blank control. The probability to obtain negative results (exclusivity) was determined by testing the reaction against a non-RABV DNA such as that of Orf virus. The results showed that all the positive brain samples tested could be amplified by LAMP and its products were shown as the typical streak like bands in two per cent agarose gels (Plate 4.34). Three DFA negatives were found positive by LAMP (Plate 4.35). By contrast, none of the negative and blank controls were positive. All positive results confirmed by gel electrophoresis were also positive by HNB staining.

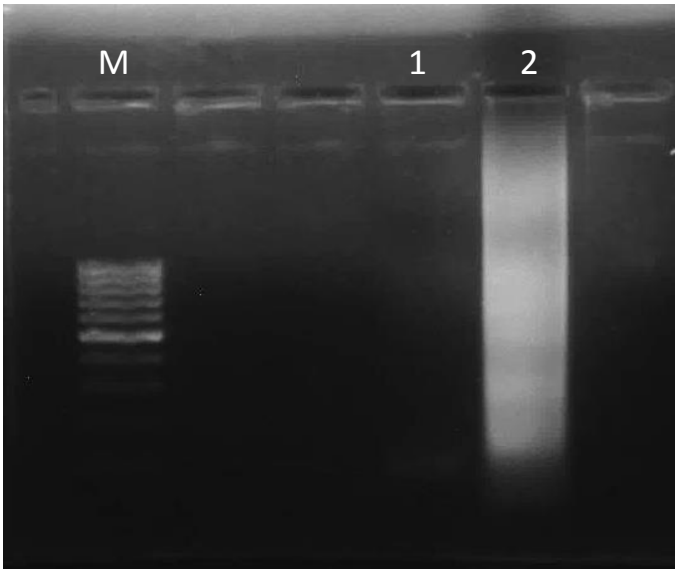


Plate 4.33: Optimization of template in LAMP

Lane M: 100 bp DNA ladder
Lane 1: LAMP with cDNA carried out at 42 ℃
Lane 2: LAMP with RNA & RT carried out at 42 ℃

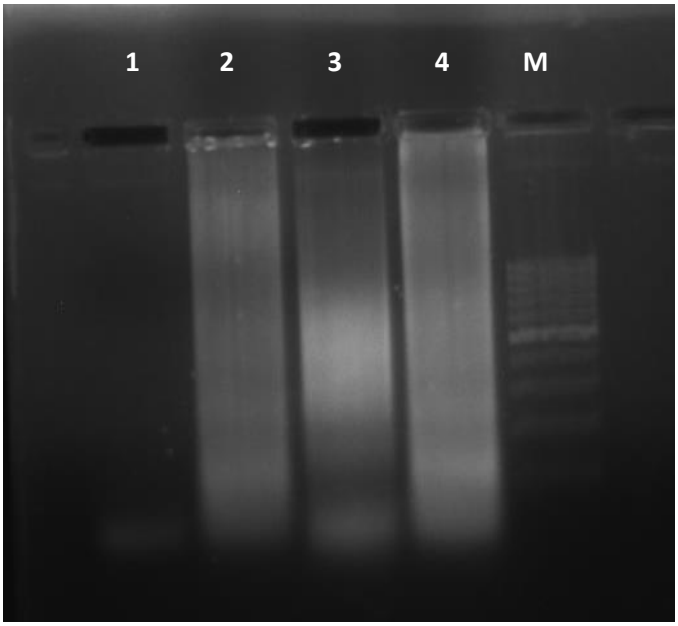
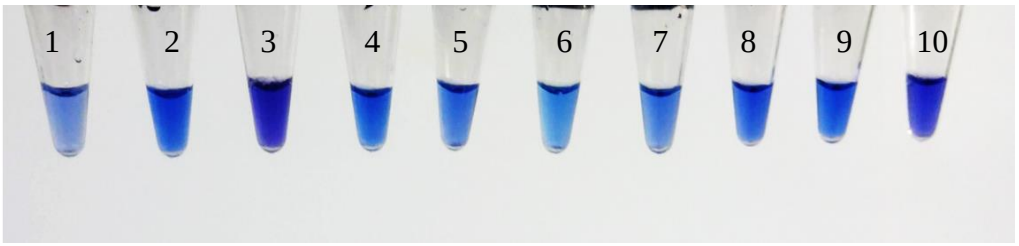


Plate 4.36: Results of DFA negative samples (n=3) found positive by RT-LAMP

Lane M: 100bp DNA ladder
Lane 1 : NTC
Lane 2: VMC -411- DOG-KARNATAKA
Lane 3 : VMC -586- DOG- KARNATAKA
Lane 4: VMC -596- DOG-KARNATAKA



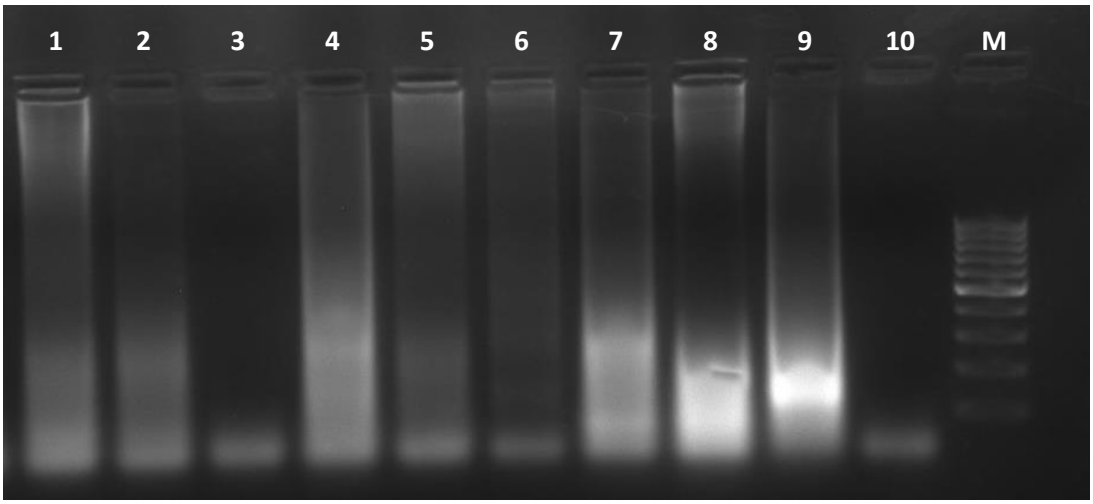


Plate 4.34 : Comparison of the electrophoretic patterns of the RT-LAMP reaction with the reaction carried out with the addition of the (HNB)

Lane M: 100bp DNA ladder
Lane 1 : VMC -199- DOG-TAMIL NADU
Lane 2 : VMC -511- DOG-KARNATAKA
Lane 3 : VMC -194- BAT-KARNATAKA
Lane 4 : VMC -391-DOG-MANIPUR
Lane 5 : VMC -331-BUFFALO-JAMMU & KASHMIR
Lane 6: VMC -558- CATTLE- KARNATAKA
Lane 7: VMC -427- BUFFALO-GUJARAT
Lane 8: VMC -546- DOG-KARNATAKA
Lane 9: VMC -589- RABV Positive sample
Lane 10: Non template control (NTC)

All the 50 samples subjected to DFA and LAMP were compared (Table. 4.3 and Fig. 4.7). The results were compared and analyzed by two way ANOVA with Sidak's multiple comparison test and no significant differences was found. Out of 50 samples, 41 were found to be positive . Sensitivity and specificity of LAMP was found to be 100 per cent and 75 per cent respectively.

4.6.4 Comparison of DFA, Modified DFA, LFA and LAMP

The samples (n=50) subjected to DFA, Modified DFA, LFA and LAMP revealed 38, 38, 40 and 41 to be positive respectively. Comparison of DFA, Modified DFA score, LFA is shown in the Fig.4.8. Scores were compared by 2-way multiple comparison ANOVA with Tukey's multiple comparison test. Each of the methods were complementary to each other.

Fig. 4.7: Comparison of DFA vs LAMP results

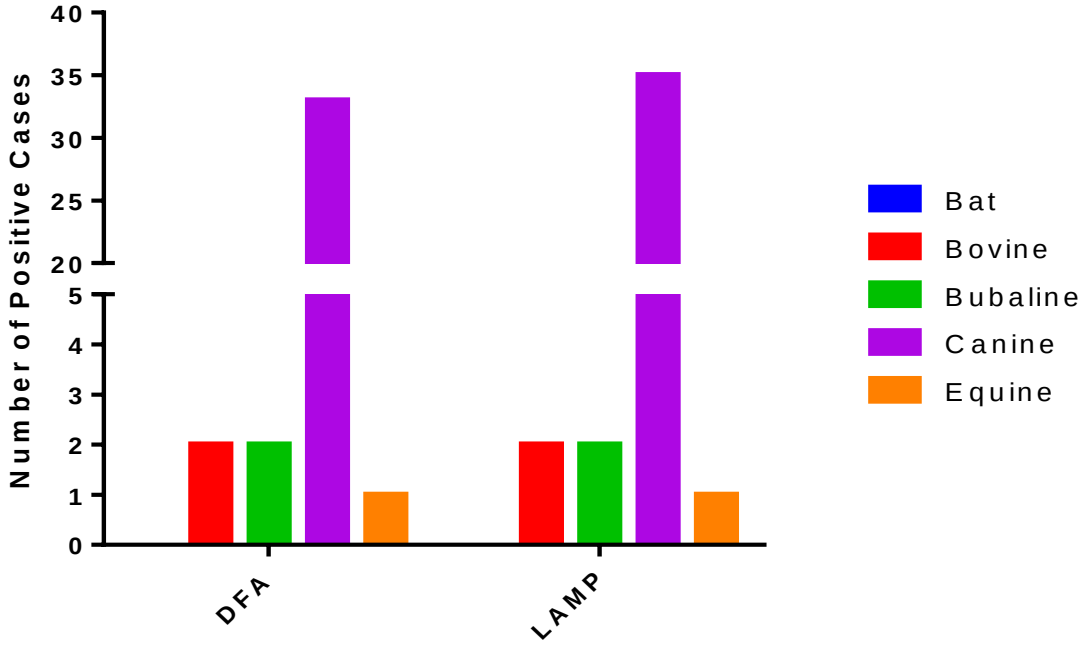
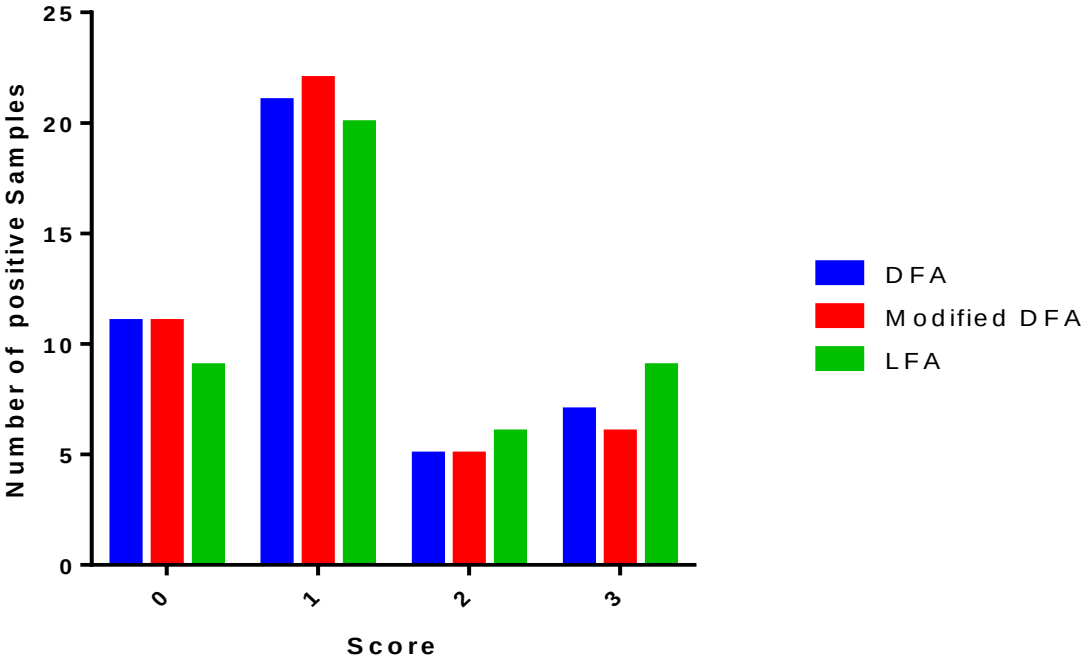


Fig. 4.8: Comparison of DFA, Modified DFA score and LFA



Discussion

V. DISCUSSION

Rabies is one of the ancient recognized diseases affecting humans and is the most important zoonotic diseases in India. It has been recognized in India since the Vedic period (1500–500 BC). Despite being a preventable disease due mainly to advances in science and technology, the age old scourge of rabies still accounts for an estimated 55,000 human deaths per year globally (Knobel *et al.,* 2005). India is endemic for rabies and has the highest prevalence in the world with 20,000 human rabies deaths reported annually (Sudarshan *et al.,* 2007). The vast majority of cases occur in developing countries, especially in Asia and Africa, and are related to endemic dog rabies where there is dog-to-dog transmission of the rabies virus and many animals and humans are at risk of exposure due to dog bites. In the background of WHO- OIE- FAO-GARC global goal of "Eliminating dog mediated human rabies by 2030", WHO-Strategic Advisory Group of Experts -Working Group on Rabies needs a rabies programmatic experience and evidence from India which is contributing 1/3rd human rabies (20,000 out of global 61,000) deaths. Global Alliance for Vaccines and Immunization (GAVI) also in need of evidence from India for possible human rabies vaccine investment in 2018. In view of this, WHO has entrusted APCRI a one year project during 2017 to assemble new evidence in support of dog-mediated human rabies elimination in India. One of the primary objectives of this project is to assimilate the existing data on and eventually conduct community and, Health care surveys on dog bite incidence in humans and the Veterinary survey on incidence of rabies in dogs through laboratory based surveillance preferably in the same settings compared high versus low dog vaccination coverage (the prevailing situation in the community).

5.1 Diagnosis of rabies

Diagnosis of rabies at the field level is based on the observation of clinical signs and is difficult especially in the paralytic form. The variable incubation period due to several factors is also a primary reason for difficulty field based diagnosis. Differential diagnosis also is difficult due to common sequelae resulting from various diseases including, transmissible spongiform encephalopathies, tetanus, listeriosis, poisoning, Aujeszky's disease and other viral nonsuppurative encephalitis which all exhibit overt nervous signs. Furthermore, paralytic rabies is often mistaken for Guillain-Barré Syndrome in humans (Hemachudha and Mitrabhakdi, 2000) and a few cases of dumb rabid animals may die without exhibiting any characteristic clinical symptoms emphasizing the importance of laboratory diagnosis. The facility for laboratory diagnosis and confirmation of rabies, be it in humans or in animals, is available in only a few institutions in India. This situation emphasizes the need of rapid, reliable and accurate diagnostic services which are key to effective surveillance efforts and for timely, ultimately enabling control of the disease.

5.2 Collection of samples

Brain tissue from suspected animals is considered to be the most reliable source/sample since the virus is neurotropic and can be found abundantly in the brain and other parts of the central nervous system. Brain stem and cerebellum are the tissue most reliably found to contain viral antigen because of retrograde pathogenesis as virus enters and leaves the brain through brain stem and cerebellum (Dietzschold *et al*., 2005 and Veera *et al*., 1997). Keeping this in view, the present study was carried out in the OIE

twinned KVAFSU-CVA-Crucell Rabies Diagnostic laboratory, Department of Microbiology, Veterinary College, Bangalore, using brain stem and cerebellum. These brain samples were collected from rabies suspected animals from different species (Dogs-178, Cattle-09, Horse-05, Cats-04, Buffalo-2, Bat-1 and Goral-1) (Fig. 4.1) from different states of India viz Karnataka (n=186), Manipur (n=08), Rajasthan (n=02), Jammu & Kashmir(n=1), Gujarat (n=1) and Maharashtra (n=1). The samples from Karnataka constituted the bulk of the samples since the laboratory is the state catering to the diagnostic confirmation of animal rabies. The sample size does not correlate to the incidence of the disease since previously tested brain samples from other diagnostic labs are submitted to this laboratory from other states for further confirmation. In India, dogs are considered the major reservoirs of rabies virus and act as the major source of transmission of infection to humans and other animals, hence maximum number of samples obtained from dogs were received and processed. The brain samples stored as such without any preservatives at -80 ^{o}C were found suitable for detection of viral inclusions, antigens and RNA.

5.3 Diagnostic tests employed for rabies

Direct Fluorescent antibody Assay (DFA) is presently considered to be the "Gold Standard" for RABV detection in tissues. The other methodologies include, Rabies Tissue Culture Infection Test (RTCIT), Reverse Transcription Polymerase Chain Reaction (RT-PCR), Mouse Inoculation Test (MIT), Lateral flow assay (LFA), direct Rapid Immunohistochemistry Test (dRIT) and Loop Mediated Isothermal Amplification (LAMP). In this study, all the above mentioned 200 samples were initially subjected to

DFA and LFA. Further, fifty of 200 samples were also subjected to modified DFA and LAMP.

5.4 Direct Fluorescent Antibody Assay (DFA)

Direct fluorescent antibody assay was devised by Coons and Kaplan (1950). Rabies viral inclusions were demonstrated for the first time by Goldwasser and Kissling (1958). The DFA is considered as the test of choice for quick laboratory confirmation of rabies and possesses high sensitivity and specificity (Rudd and Trimarchi, 1989).

In the present study, FITC conjugates of anti-N protein antibody IgG, rabies negative control IgG and normal Goat IgG were used for detection of RABV inclusions, non specific binding of antibodies to RABV inclusions and streptococcal G protein respectively in the brain impressions. 120 out of the 200 samples were positive by DFA. The DFA was carried out according to the protocol described by the CDC, Atlanta, USA. The impressions from all the 120 brain samples showed bright apple green fluorescent particles of varying sizes either scattered or within the neurons (Plate 4.3). Similar descriptions were reported by others Lembo *et al.* (2006); Durr *et al.,* 2008; Madhusudana *et al.* (2011); Chandrashekhara (2012); Isloor *et al.* (2014) and Nithinprabhu *et al.* (2014).

5.4 Modified Direct Fluorescent Antibody Assay (DFA)

The DFA has few limitations. The major drawback is that the acetone as fixative does not completely inactivate the infective virions, posing potential hazard to laboratory personnel (Velasco-Villa *et al.,* 2005; OIE, 2011 and Jarvis, 2016). Studies have

demonstrated that intracerebral inoculation of acetone fixed tissues caused disease in suckling mice (Umoh and Blenden, 1981 and Velasco-Villa *et al.,* 2005). But formalin inactivates the RABV (Velasco-Villa *et al.*, 2005, OIE, 2011) within 10 min and this property is explored in case of dRIT. Furthermore, chilled acetone fixation of tissue is time consuming and is dependent on availability of deep freezer since the brain impressions have to be fixed for at least one hour in -20^{o}C deep freezer. In view of these advantages of formaldehyde based fixation of brain impressions DFA was modified.

In this study, viability of virus in acetone and formalin fixed slides were checked by inoculating BHK-21 cell with scrapings from the impressions. There was 100 per cent inactivation of virus in formalin fixed slides (10%, 5% and 2.5%), whereas, about clear fluorescence was seen in acetone fixed slides when compared with positive control indicating incomplete inactivation of RABV. A similar study was performed by White and Chappell (1982) in impressions made from RABV fixed in acetone at -20 °C for 2, 4, 7 and 24 h and examined for viability of virus. Tissues scraped from impression smears after acetone fixation were examined for viability of virus in BHK-21/ Winster Institute-2 (WI-2) cell cultures. The infectivity titers ranged from $10^{3.3}$/0.1 ml in suckling mice to $10^{5.1}$/0.1 ml in BHK-21/ (WI-2) cultures. Scrapings from these smears contained infectious virus, thus it was concluded that acetone fixation at -20 °C does not inactivate rabies virus. Further, tissues scraped from acetone fixed rabies or Eastern equine encephalitis positive mammalian brain tissue impression were inoculated to mouse neuroblastoma C-1300 cells and incubated for 72 hrs in a 34 °C humidified incubator with 5% CO_2. It was found that the virus was not inactivated with acetone fixation, thus it

was adviced that laboratory employees should treat all samples as rabies and Eastern equine encephalitis positive throughout the whole procedure (Jarvis, 2016).

In the present study, initially several impressions of DFA confirmed brain samples were fixed with varying concentrations of NBF (10%, 5%, 2.5% and 1 %) for 10 minutes (Section 3.3.3.6). The criteria considered to select the particular concentration of formaldehyde for fixing the brain impressions were complete inactivation of the virus after fixing the impression without compromising with the fluorescence due to viral inclusions. If unbuffered formalin is used, the acidity can react with haemoglobin in the tissues to produce dark brown acid formaldehyde haematin precipitates, which complicate interpretation. The use of three percent H_2O_2 along with TPBS has an advantage of unmasking the antigen which is masked by the crosslinking of protiens after formalin fixation. It was observed that at 2.5 per cent NBF, there was complete inactivation of the virus and appreciable fluorescence of the viral inclusions based on three repetitions. All 50 brain samples which were subjected to DFA were also subjected to modified DFA. In both the tests 38 samples were found to be positive. This approach is completely biohazard free to the laboratory personnel and significantly minimized the fixation time from minimum 1hr to just 10 min. This is an important finding in view of the safe handling of brain impressions from rabid animals and rapid testing without compromising with the sensitivity and specificity of modified DFA.

5.5 Lateral Flow Assay (LFA)

The immunochromatographic lateral flow strip test is a one-step test. It has great potential as a useful method for rabies diagnosis without the need for laboratory

equipment (Zhang *et al.,* 2009). The LFA can also be used for the diagnosis using saliva, serum, blood (Kang *et al.,* 2007; Tajunnisa *et al*., 2016 and Sujith *et al*., 2017). Of the 200 samples subjected to LFA in the present study, 129 were found to be positive. Two of the DFA negatives found positive by LFA. This could be attributed to high sensitivity of LFA (Al-Yousif *et al*., 2002; Kuroiwa *et al*., 2004 and Tsuguto *et al*., 2004; Nishizono, 2008; Markotter *et al*., 2009, Servat *et al*., 2012; Voehl *et al.,* 2014; Sharada *et al*., 2015). The ability of LFA and RT-LAMP to detect additional positives as compared to DFA could be attributed to using homogenised tissue suspension in case of LFA and concentrated viral RNA in LAMP as against detection of only localised RABV inclusions in both the versions of DFA. In this study, the sensitivity and specificity of LFA was found to be 99.2 per cent and 98.5 per cent respectively. This diagnostic test is suitable for rabies screening at the field level, particularly in areas with a high prevalence of rabies and where the fluorescent antibody test can not be employed.

5.6 Reverse Transcription- Loop-mediated Isothermal Amplification (RT-LAMP)

The RT-PCR for rabies diagnosis is considered to be a rapid and sensitive alternative technique compared with the earlier rabies diagnostic methods (Sacramento *et al.,* 1991). The RT-PCR showed higher sensitivity and specificity both in ante-mortem and post-mortem diagnosis of the rabies virus in variety of clinical samples collected from human and livestock sources and are proven to be reliable diagnostic methods (Smith *et al.,* 1972; Nadin-Davis *et al.,* 1994; Kissi *et al.,* 1995; Saengseesom *et al.,* 2007 and Dacheux *et al.,* 2008). However, these tests are dependent on expensive equipments, restricting the application of these advanced tests either at local or field level in addition to the fact that it takes 3-4 hours to complete. Thus, there is a need for development of

specific, sensitive, rapid, cost effective and user-friendly molecular diagnostic tool which can be employed for confirmatory diagnosis of rabies. One such test is the loop mediated isothermal amplification (LAMP) (Notomi *et al.,* 2000), which was employed for the detection of various pathogens (Notomi *et al.,* 2000; Okafuji *et al.,* 2005 and Boldbaatar *et al.,* 2009).

In India, LAMP was developed for the detection of various pathogens (Parida *et al.,* 2005; Liu *et al.,* 2008; Dadas *et al.,* 2012; Divya *et al.,* 2012; Parthiban, *et al.,* 2012 and Venkatesan *et al.,* 2012) including rabies (Nithinprabhu, 2014). Several commercial kits were also developed for pathogens such as Leptospira, Mycoplasma and Human Immunodeficiency Virus (HIV) (Shesheer Kumar, 2012). However, there are only a few reports internationally on the development and application of LAMP for rabies viral genome detection (Boldbaatar *et al.,* 2009; Saitou *et al.,* 2010 and Muleya *et al.,* 2012). However, the LAMP assays employed in India and other countries for detection of rabies virus are using cDNA. This is time consuming and little tedious procedure. To overcome this, the present study RT-LAMP was standardized by directly using the rabies viral RNA itself as template instead of cDNA and thereby making the assay user-friendly and rapid.

5.7.1 Detection methods in LAMP

The LAMP reaction can be visualized by various means. First, the white turbidity of magnesium pyrophosphate can be visually observed by naked eye after a pulse spin to deposit the precipitate in the bottom of the tube (Parida *et al.,* 2009). Second, the amplified products can also be visualised in the presence of fluorescent intercalating dyes *viz*; ethidium bromide, SYBR Green I, Calcein, *etc*., by illuminating with a UV lamp.

Third, aliquots of LAMP amplified products can be electrophoresed on 2-3 per cent agarose gel, stained with ethidium bromide and visualised on a UV transilluminator. Finally HNB can be used to supplement the RT-LAMP reaction for homogenous visualisation of the amplified genomic target. The HNB is a metal chelating dye which is dependent on the Mg2+ concentration in the reaction mixture. As the LAMP reaction progresses, the accumulation of magnesium pyrophosphate resulting in turbidity in the suspension. Thus, the colour changes from violet to blue. In the present study, all the 50 samples were screened by LAMP using RNA as a template in presence of RT and thereby circumvets a step for preparation of cDNA making it much faster and saving reagents required in synthesis of cDNA to initiate RT-LAMP. In the earlier studies, cDNA was used as a template for LAMP (Nithinprabhu, 2014). The sensitivity and specificity of LAMP in this study was found to be 100 per cent and 75 per cent respectively.

The LAMP technique is a powerful gene amplification technique that is emerging as a simple and rapid diagnostic tool for early detection and identification of microbial diseases. The whole procedure is rapid and user friendly, as the amplification can be completed in about one hr under isothermal conditions (42 $^{\circ}$C), by incubating all the reagents in a single tube. Gene amplification products can be easily visualized by the naked eye and the advantages such as use of fluorescent dyes for better visibility, detection with a UV transilluminator and option of electrophoresis, staining and examination and/or use of HNB has enhanced its usefulness. The findings of the present study indicate that the LAMP is more sensitive than DFA as additional three positives were detected by LAMP. Furthermore, LAMP could be employed on those brain samples

which are unfit for testing by DFA. This assay could be further finetuned by designing the Loop primers for RABV and in turn improvise the profile.

Conclusion

In conclusion, the laboratory based confirmatory diagnosis of rabies in animals serves as the basis for the downstream post exposure prophylactic measures. However, one of the limitations of the currently employed gold standard DFA is that there is a biosafety concern with respect to handling of brain impression slides and the longer duration of fixing the slides in chilled acetone. In this context, the modified DFA is user-friendly in view of its bio safety and rapidity. Recently, yet another immunodiagnostic test based on immunochromatography principles (LFA) is becoming popular due mainly to it's simplicity and rapidity. The WHO has initiated through the network of laboratories to evaluate the performance of LFA in comparison with the standard DFA. Furthermore, molecular techniques based on the detection of nucleic acid content of RABV have been extensively carried out in view of their high sensitivity for diagnostic purposes and suitability for molecular epidemiology. The outcome of the present study reveals the statistically significant correlation between the DFA, modified DFA, LFA and LAMP. Hence, depending on the suitability and need, these tests can be employed for diagnosis of rabies in animals either individually or in combination at the laboratory or field level as operationally feasible.

Summary

VI. SUMMARY

The present study was undertaken with an objective of comparing acetone and formalin based fixation of animal brain impressions in DFA and to evaluate LFA and LAMP with DFA for rapid diagnosis of rabies in animals. Totally, 200 brain samples were collected from various domestic animals *viz*., Dog, Cat, Cattle, Buffalo, Horse, Bat and Goral. These samples were collected from different geographical locations / states of the country *viz*., Gujarat, Jammu & Kashmir, Karnataka, Maharashtra, Manipur, Rajasthan and Tamil Nadu.

All the 200 samples were screened for the presence of RABV by employing DFA and LFA. Then DFA was done as per the protocols described by the Centers for Disease Control and Prevention (CDC), Atlanta, USA. In all, 120 of the 200 samples were positive by DFA, whereas, 129 were positive by LFA. Fifty out of these 200 samples were subjected to modified DFA and LAMP. Of these 50 samples, 38 were positive by both gold standard DFA and modified DFA, whereas, 40 and 41 were positive by LFA and LAMP respectively.

The modified DFA was found to be more suitable as 2.5 per cent NBF fixative inactivated the RABV completely, thus eliminating the biohazard to the laboratory personnel involved. The fluorescence shown by positive sample fixed with 2.5 per cent NBF was comparable to the fluorescence shown by gold standard DFA. The time taken in modified DFA is less when compared to DFA. The results were compared and analysed by Spearman's nonparametric correlation analysis. The "Correlation coefficient (Spearman r)" was found to be 0.98 ($P<0.001$) indicating very good positive correlation.

The LFA was 100 per cent sensitivity compared to the gold standard DFA. This can be even used at the field level at the post-mortem site, where results are obtained within five minutes. The results were compared and analyzed by Spearman's nonparametric correlation analysis. The "Correlation coefficient (Spearman r)" was found to be 0.84 ($P<0.001$). The positive results were subjected to 2-way ANOVA with Tukey's multiple comparison test. Significant difference ($P< 0.05$) were observed when LFA compared with DFA

The nucleic acid amplification assay in isothermal condition *viz*; RT-LAMP of RNA was explored in combination with a detection that can be accomplished with the naked eye. A RT-LAMP assay targeting directly the RABV RNA, instead of cDNA was developed, targeting the conserved region of the RABV- N gene with incorporation of HNB dye. The results were compared and analyzed by two way ANOVA with Sidak's multiple comparison test and significant differences ($P<0.05$) was found. Out of 50 samples, 41 were found to be positive by RT-LAMP. Sensitivity and specificity of RT-LAMP was found to be 100 per cent and 75 per cent respectively. This single step approach is rapid compared to the conventional cDNA based LAMP and saves the additional reagents required for separate cDNA sysnthesis. In the present study, the RT-LAMP could detect 41 brain samples from rabies suspected animals as positive and the remaining brain tissues, which were also negative by DFA and LFA.

In conclusion, the comparative evalution of DFA, modified DFA, LFA and LAMP with respect to their performace based scoring in detection of RABV viral inclusion / antigen / RNA was made. In this approach, the scores were compared by 2-

way multiple comparison ANOVA with Tukey's multiple comparison test and each of the methods were complementary to the other.The outcome of the study indicated that the modified DFA can be used as it eliminates the biohazard with RABV and is rapid than the conventional DFA. Further, the LFA and LAMP were also found rapid, user-friendly and can be suitably employed at the field level to identify the RABV in the suspected clinical samples for the preliminary investigation.

Bibliography

VII. BIBLIOGRAPHY

AL-YOUSIF,Y., ANDERSON, J., CHARD-BERGSTROM, C. and KAPIL, S., 2002. Development, evaluation, and application of lateral-flow immunoassay Development, evaluation, and application of lateral-flow immunoassay (immunochromatography) for detection of rotavirus in bovine fecal samples. *Clin. Diagn. Lab. Immunol*., **9**: 723–724

ANDRADE, P. V., 1969. Fluorescent Antibody Technique in the Diagnosis of Rabies. *Arq. Hig. Sawde. Publ.*, **34(**119/20**)**: 51-61. Cited in *Trop. Dis. Bull.*, **67**: 949

ARAI, Y. T., YAMADA, K., KAMEOKA, Y., HORIMOTO, T., YAMAMOTO, K. and YABE, S., 1997. Nucleoprotein gene analysis of fixed and street rabies virus variants using RT-PCR. Arch. Virol., **142**: 1787-1796

ARAUJO, D. B., LANGONI, H., ALMEIDA, M. F. and MEGID, J., 2008. Heminested reverse- transcriptase polymerase chain reaction (hnRT-PCR) as a tool for rabies virus detection in stored and decomposed samples. *BMC, Res. Notes*, **1**: 17

BAER, G. M., 1991.The Natural History of Rabies. *In:* Boca Raton: CRC Press., *pp* 29

BANERJEE, A. K., 1987. Transcription and replication of rhabdoviruses. Microbiol reviews, **51**(1): 66-87

BEAUREGARD, M., BOULANGER, P. and WEBSTER, W. A., 1965. The use of fluorescent antibody staining in the diagnosis of rabies. *Can. J. Comp .Med. Vet. Sci.*, **29**(6): 141- 147

BHUYAN, P.J., 2012. Epidemiology, Prevention and Control of Rabies in India-A Review Study. *J. of Evolution of Medical and Dental Sciences*, **1**(3): 180-184.

BLACK, E.M., MCELHINNEY, L.M., LOWINGS, J.P., SMITH, J., JOHNSTONE, P.R. and HEATON, P.R., 2000. Molecular methods to distinguish between classical rabies and the rabies-related European bat Lyssaviruses. J. Virol. Methods, **87**:123–131

BLANTON, J. D., BOWDEN, N. Y., EIDSON, M., WYATT, J. D. and HANLON, C. A., 2005. Rabies Post-exposure Prophylaxis, New York, 1995–2000. Em. Infect. dis., **11**: 1921-1927

BLENDEN, D. C., FROST, G., WATCHENDORFER, J. N. and DORSEY, C., 1980. Identification of Rabies Virus Antigen in the Skin of Foxes. *J. Vet. Med. B.*, **27**(9-10): 698- 704

BOLDBAATAR, B., INOUE, S., SUGIURA, N., NOGUCHI, A., ORBINA, J. R., DEMETRIA, C., MIRANDA, M. E. and YAMADA, A., 2009. Rapid detection of rabies virus by reverse transcription loop-mediated isothermal amplification. *Jpn. J. Infect. Dis.*, **62**: 187–191

CHANDRASHEKHARA, N., RATHNAMMA, D., VEERESH, B. H., YATHIRAJ, S., SATYANARAYANA, M. L., VEEREGOWDA, B. M., SUGUNA RAO, ABDUL RAHMAN, S., SHRIKRISHNA ISLOOR, 2012. Application of reverse transcription polymerase reaction (RT PCR) for diagnosis of rabies in animals and its comparative evaluation with direct flouresecent antibody test (DFA) and direct immune histochemistry test (dRIT). *In*: Proceedings of APCRI conference, 6 to 8th July 2012, *pp* 22

CHANDRASHEKHAR, N., RATHNAMMA, D., ISLOOR, S., YATHIRAJ S, SATHYANARAYANA, M.L., SUGUNA RAO and VEEREGOWDA, B. M., 2014. Evaluation of a Direct Rapid Immunohistochemical Test (dRIT) in comparison with Fluorescent Antibody Technique (FAT) for Diagnosis of Rabies in Animals. *Frontier Journal of Veterinary and Animal Sciences*, **2**(2): 83-86.

CHENIK, M., CHEBLI, Y., GAUDIN, D., and BLONDEL, D., 1994. In vivo interaction of rabies virus phosphoprotein (P) and nucleoprotein (N): existence of two Nbinding sites on P protein. J. Gen. Virol., **75**: 2889-2896

CONZELMANN, K. K., COX, J. H., SCHNEIDER, L. G. and THIEL, H. J., 1989. Molecular cloning and complete nucleotide sequence of the attenuated rabies virus SAD B19. J. Gen. virol., **70**: 2063-2074

COONS, A. H. and KAPLAN, M. M., 1950. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of a fluorescent antibody. *J. Expt. Med.*, **91**: 1-13

COX, J. H., DIETZSCHOLD, B. and SCHNEIDER, L. G., 1977. Rabies virus glycoprotein II: Biological and serological characterization. Infection and Immunity, **16**(3): 754-759

CREPIN, P., AUDRY, L., ROTIVEL, Y., GACOIN, A., CAROFF, C. and BOURHY, H., 1998. Intravitam diagnosis of human rabies by PCR using saliva and cerebrospinal fluid. *J. Clin. Microbiol.*, **36**: 1117–1121

DACHEUX, L., REYNES, J. M., BUCHY, P., SIVUTH, O., DIOP, B. M., ROUSSET, D., RATHAT, C., JOLLY, N., DUFOURCQ, J. B., NARETH, C., DIOP, S., IEHLE, C., RAJERISON, R., SADORGE, C. and BOURHY, H., 2008. A reliable diagnosis of human rabies based on analysis of skin biopsy specimens. *Clin. Infect. Dis.*, **47**: 1410–1417

DADAS, R. C., DHANAVELU, M., PANDEY, A. B., SATHISH, B., SHIVCHANDRA, RAJAK, K. K. and VENKATESAN, G., 2012. Development of loop-mediated isothermal amplification (LAMP) assay, for rapid detection of peste des petits ruminants virus (PPRV) genome from clinical samples. *In*: XXVI Annual convention of IAVMI on Future of livestock health: a paradigm change to maximize productivity for economic gains.

DE, SERRES. G., DALLAIRE, F. and COTE, M., 2008. Bat rabies in the United States and Canada from 1950 through 2007: human cases with and without bat contact. *Clin. Infect. Dis*., **46**: 1329–1337

DEAN, D.J., ABELSETH, M, K. and ATANASIU, P.,1996. The fluorescent antibody test *In*: Laboratory Techniques in Rabies Edt. Meslin, F,X., Kaplan, M,M. and Koprowski, H., Edn. 2nd., *World Health Org*., *pp* 88–89

DIETZSCHOLD, B., SCHNELL, and KOPROWSKI, H., 2005. Pathogenesis of rabies, 2005, Microbiol. Immunol., **292**: 45-56

DIVYA, S., RAJA, A. and DHINAKAR RAJ, G. 2012. Rapid detection of Chikungunya viral genome by loop mediated isothermal amplification (LAMP). *In:* XXVI Annual convention of IAVMI on Future of livestock health: a paradigm change to maximize productivity for economic gains.

DURR, S., NAISSENGAR, S., MINDEKEM, R., DIGUIMBYE, C., NIEZGODA, M.K.I., RUPPRECHT, C.E., *et al.*, 2008. Rabies diagnosis for developing countries. *Trop*. *Dis.*, **26**: 206

EVERARD, C. O. and EVERARD, J. D., 1988. Mongoose Rabies. Rev. Infect. Dis., **4**: 604-610

FINKE, S., MUELLER- WALDECK, R., and CONZELMANN, K. K., 2003. Rabies virus matrix protein regulates the balance of virus transcription and replication. J. Gen. Virol., **84**: 1613-1621

FOOKS, A. R., BANYARD, A. C., HORTON, D. L., JOHNSON, N., MCELHINNEY, L. M., & JACKSON, A. C., 2014. Current status of rabies and prospects for elimination. *The Lancet.*, **384**(9951): 1389–1399

GOLDWASSER, R. A. and KISSLING, R. E., 1958. Fluorescent antibody staining of street and fixed rabies virus antigen. *Proc. Soc. Exp. Biol. Med.*, **98:** 219–223

GUPTA, P. K., SINGH, R. K., SHARMA, R. N., RAO, Y. U. B. and BUTCHAIAH, G., 2001. Preliminary report on a single tube, non interrupted Reverse transcription polymerase chain reaction for the detection of rabies virus in brain tissue. Vet. Res. Comm., **25**: 239-247

HATANO, B., MAKI, T., OBARA, T., FUKUMOTO, H., HAGISAWA, K., MATSUSHITA, Y., OKUTANI, A., BAZARTSEREN, B., INOUE, S., SATA, T. and KATANO, H., 2010. LAMP using a disposable pocket warmer for Anthrax detection, a highly mobile and reliable method for antibioterrorism. *Jpn. J. Infect. Dis*., **63**: 36–40.

HAYMAN, D. T. S., JOHNSON, N., HORTON, D. L., HEDGE, J., WAKELEY, P. R., BANYARD, A. C., ZHANG, S., ALHASSAN, A. and FOOKS, A. R., 2011. Evolutionary history of rabies in Ghana. *PLOS Negl. Trop. Dis.*, **5**(4): e1001

HEMACHUDHA, T. and MITRABHAKDI, E., 2000. Rabies. In: Infectious diseases of the nervous system. Edt. Johnson R.T., *pp* 401

ICHHPUJANI, R. L., BHARDWAJ, M., CHHABRA, M. and DATTA, K. K., 2001. Rabies control in Asia. In *Edt* Dodet, B and Meslin, F-X., Joph Libbey Eurotext, Paris. *pp* 45-60

ISLOOR, S., YATHIRAJ, S., SATYANARAYANA, M.L., VEERESH, B.H. and NITHINPRABHU, K., 2014. Advances in animal rabies research in India. *In:* Proceedings of International conference and 18th Asian regional meet of commonwealth veterinary association. 20-24th Feb 2014, *pp* 138

JACKSON A. C. and WUNNER W. H., 2002. Rabies, *Academic press. United States of America An Elsevier Science Imprint.*

JARVIS, J. A., FRANKE,M. A., DAVISA, A.D., 2016. Rabies Direct Fluorescent Antibody Test Does Not Inactivate Rabies or Eastern Equine Encephalitis Viruses. *J. Virol. Methods.*, **234**: 52–53

KANG, B., OH, J., LEE, C., PARK, B.K., PARK, Y., HONG, K., LEE, K., CHO, B., SONG, D., 2007. Evaluation of a rapid immunodiagnostic test kit for rabies virus. *J. Virol. Methods.*, **145**(1): 30–36

KAPLAN, M. M. and H, KOPROWSKI., 1980. Rabies Scientific American., **242**: 128–113

KISSI, B., TORDO, N. and BOURHY, H., 1995. Genetic polymorphism in the rabies virus nucleoprotein gene. *Virology*, **209**: 526-537

KNIPE, D. M., HOWLEY, P. M., GRIFFIN, D. E., LAMB, R. A., MARTIN, M. A., ROIZMAN, B. and STRAUS, S. E., 2007. Fields Virology 5th Edn. Philadelphia, PA: Lippincott Williams and Wilkins, *pp* 338

KNOBEL, D. L., CLEAVELAND, S., COLEMAN, P. G., FEVRE, E. M., MELTZER, M. I. and MIRANDA, M. E., 2005. Re-evaluating the burden of rabies in Africa and Asia. *Bull World Health Organisation*, **83**(5): 360-368

KRISTEN, M. VOEHL., 2014. Evaluation of a Rapid Immunodiagnostic Rabies Field Surveillance Test on Samples Collected from Military Operations in Africa, Europe, and the Middle East. Nucleic Acids Res: **28**(12): e63

KUROIWA, Y., NAGAI, K., OKITA, L., UKAE, S., MORI, T., HOTSUBO, T and TSUTSUMI, H., 2004. Comparison of an immunochromatography test with multiplex reverse transcription-PCR for rapid diagnosis of respiratory syncytial virus infections. *J. Clin. Microbiol*. **42**: 4812–4814.

KUZMIN, I. V., MAYER, A. E., NIEZGODA, M., MARKOTTER, W., AGWANDA, B. and BREIMAN, R.F., 2010. Shimoni bat virus, a new representative of the Lyssavirus genus. Virus Res., **149**:197-210.

LACKAY, S. N., KUANG, Y. and FU, Z. F., 2008. Rabies in small animals. Vet. Clin. North Am. Small Anim. Pract., **38**(4): 851-856

LEMBO, T., NIEZGODA, M., VILLA, A. V., CLEAVELAND, S., ERNEST, E. and RUPPRECHT, C. E., 2006. Evaluation of a direct, rapid Immunohistochemical Test for Rabies Diagnosis. *Emer. Infect. Dis*., **12**(2): 310-313

LIU, Z., HOU, J., BAKHEIT, M. A., SALIH, D. A., *et al*., 2008. Development of loop-mediated isothermal amplification (LAMP) assay for rapid diagnosis of ovine theileriosis in China. *Parasitol. Res*., **103**: 1407−1412

MADHUSUDANA, S.N., 2011. Structure and molecular biology of rabies virus. *In*: Rabies, Macmillan Medical Communications., *pp* 10-11

MALOVRH, T. and HOSTINIK, P., 2005. Diagnostics procedures in rabies. *Vet. Glasnik.*, **59**(1-2): 99-105

MARKOTTER, W., KUZMIN, I.V., RUPPRECHT, C.E and NEL, L.H., 2009. Lagos bat virus virulence in mice inoculated by the peripheral route. *Epidemiol. Infect.*, **137**:1155–1162

MAYO, M. A., and PRINGLE, C. R., 1998. Virus taxonomy- 1997. J. General. Virol., **79**: 649- 657

MESLIN, F.X., AND BRIGGS, D.J., 2013. Eliminating canine rabies, the principal source of human infection: what will it take?. *Antiviral.Res.* **98**: 291–96

MORI, Y. and NOTOMI, T., 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *J. Infect, Chemother.*, **15**: 62–69

MULEYA, W., NAMANGALA, B., MWEENE, A., ZULU, L., FANDAMU, P., BANDA, D., KIMURA, T., SAWA, H. and ISHII, A., 2012. Molecular epidemiology and a loop-mediated isothermal amplification method for diagnosis of infection with rabies virus in Zambia. *Vir. Res*., **163**(1): 160-168

NADIN-DAVIS, S. A., CASEY, G. A. and WANDELER, A. I., 1994. A molecular epidemiological study of rabies virus in central Ontario and western Quebec. *J. Gen. Virol.,* **75**: 2575–2583

NADIN-DAVIS S. A. and REAL L. A., 2011. Molecular phylogenetics of the lyssaviruses--insights from a coalescent approach. *Adv Virus Res.,* **79**: 203-238.

NEELUFER, M. S., ISLOOR, S., VEERESH, B. H., PALANIAPPAN, C., SEKHAR, B., RATHNAMMA, D., YATHIRAJ, S., CHANDRANAIK, B. M., DEEPTI, B. R., VISHAL. and MANJUNATH, REDDY., 2015. Standardization and application of rabies virus neutralizing antibody assay for assessment of vaccinal efficacy in dogs, In: Compendium of 17th national conference of APCRI, 4-5 th July 2015

NEGRI, A., 1903. Zur Aetiologie der Tollwuth: Die Diagnose der Tollwuth auf Grund der neueren Befunde. *Z. Hyg. Infektkrankh,* **44**: 519-540

NIEZGODA, M. and RUPPRECHT, C. E., 2006. Standard operating procedure for the direct Rapid immunohistochemistry Test for the detection of rabies virus antigen. National Laboratory Training Network Course, Atlanta: US Department of Health and Human Services, Centers for Disease Control and Prevention, *pp* 1-16

NISHIZONO, A., KHAWPLOD, P., AHMED, K., GOTO, K., SHIOTA, S., MIFUNE, K., YASUI, T., TAKAYAMA, K., KOBAYASHI, Y., MANNEN, K., TEPSUMETHANON, V., MITMOONPITAK, C., INOUE, S and MORIMOTO, K. 2008 A simple and rapid immunochromatographic test kit for rabies diagnosis. *Microbiol Immunol.,* **52**(4):243-9

NITHINPRABHU, K., 2014, Nucleoprotein Gene - Based Molecular Epidemiology Of Rabies Virus And Development Of Isothermal Nucliec Acid Amplification Assay for its Detection. M.V.Sc. thesis, Karnataka Veterinary Animal Fisheries Sciences University, India

NITHINPRABHU, K., VEERESH, B. H., NEELUFER, M. S., SHARADA, R., SWAPNA, A. S., MANOHARAN, S., RATHNAMMA, D., YATHIRAJ, S., SATYANARAYANA, M. L., PLACID E. D' SOUZA, ABDUL RAHMAN, S. and ISLOOR, S., 2014. A Multipronged Approach for Detection of Rabies Virus In Brain Samples And Complete Nucleoprotein Gene Based Phylodynamics. *In:* Proceedings of International conference and 18th Asian regional meet of commonwealth veterinary association, 20-24th Feb 2014, *pp* 138

NOTOMI, T., OKAYAMA, H., MASUBUCHI, H., YONEKAWA, T., WATANABE, K., AMINO, N. and HASE, T., 2000. Loop-mediated isothermal amplification of DNA. *Nucleic. Acids. Res*. **28**: e63

OIE, 2011. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds, and Bees), O.I.E., Paris.

OKAFUJI, T., YOSHIDA, N., FUJINO, M., MOTEGI, Y., IHARA, T., OTA, Y., NOTOMI, T. and NAKAYAMA, T., 2005. Rapid diagnostic method for detection of mumps virus genome by loop-mediated isothermal amplification. *J. Clin. Microbiol*., **43**: 1625–1631

PARIDA, M., HORIOKE, K., ISHIDA, H., DASH, P. K., SAXENA, P., JANA, A. M., ISLAM, M. A., INOUE, S., HOSAKA, N. and MORITA, K., 2005. Rapid detection and differentiation of dengue virus serotypes by a real-time reverse transcription loop-mediated isothermal amplification assay. *J. Clin. Microbiol.*, **43**: 2895–2903

PARIDA, M., SANNARANGAIAH, S., DASH, P. K., RAO, P. V. L. and MORITA, K., 2009. Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Rev. Med. Virol.*, **18**(6): 407-421

PARTHIBAN, M., SHALINI, K. S., KURUNCHI, DIVYA, C. and KUMANAN K., 2012. Loop mediated isothermal amplification for rapid detection of Fowl adenoviruses from field samples. *In*: XXVI Annual convention of IAVMI on Future of livestock health: a paradigm change to maximize productivity for economic gains.

ROBARDET, E., PICARD-MEYER, E., ANDRIEU, S., SERVAT, A. and CLIQUET, F., 2011. International interlaboratory trials on rabies diagnosis: An overview of results and variation in reference diagnosis techniques (fluorescent antibody test, rabies tissue culture infection test, mouse inoculation test) and molecular biology techniques. *J. Virol. Methods,* **177**(1): 15-25

RUDD, R. J. and TRIMARCHI, C. V., 1989. Development and evaluation of an *Invitro* virus isolation procedure as a replacement for the Mouse inoculation test in rabies diagnosis. *J. Clin. Microbiol.,* **27**: 2522-2528

RUPPRECHT, C. E., STOHR, K. and MEREDITH, C., 2001. Rabies. In: *Infectious diseases of wild mammals.* Edt. E. S. Williams and I. K.Barker, *Iowa State University Press,* Ames, Iowa, USA. *pp* 3-36

SACRAMENTO, D., BOURHY, H. and TORDO, N., 1991. PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. *Mol. Cell. Probe*, **5**: 229–240

SAENGSEESOM, W., MITMOONPITAK, C., KASEMPIMOLPORN, S. and SITPRIJA, V., 2007. Real time PCR analysis of dog cerebrospinal fluid and saliva samples for ante-mortem diagnosis of rabies. *Southeast Asian J. Trop Med. Pub. Heal.,* **38**: 53–57

SAITOU, Y., KOBAYASHI, Y., HIRANO, S., MOCHIZUKI, N., ITOU, T., ITO, F. H. and SAKAI, T., 2010. A method for simultaneous detection and identification of Brazilian dog- and vampire bat-related rabies virus by reverse transcription loop-mediated isothermal amplification assay. *J. Virol. Methods,* **168**: 13–17

SCHNEIDER, L. G., DIETZSCHOLD, B., DIERKS, R. E., MATTHAEUS, W., ENZMANN, P. J. and STROHMAIER, K., 1973. J. Virol., **11**: 748-755

SCHNEIDER, L. G., 1975. Spread of virus within central nervous system. *In*: The natural history of rabies, *Edt*. Baer, G. M., Academic Press, **1**: 199-301

SERVAT, A., PICARD-MEYER, E., ROBARDET, E., MUZNIECE, Z., MUST, K and CLIQUET, F., 2012. Evaluation of a Rapid Immunochromatographic Diagnostic Test for the detection of rabies from brain material of European mammals. *J .biologicals.,* **40**(1):61-6

SHANKAR, B.P., 2009. Advances in Diagnosis of Rabies. *Veterinary World,* **2**(2): 74-78

SHARADA, R., VEERESH, B.H., PALANIAPPAN, C., SEKHAR, B., RATHNAMMA, D., SATYANARAYANA, M.L., SURYANARAYANA, V. V. S., BALAMURUGAN, V. and ISLOOR, S., 2015. A novel approach through application of Lateral flow assay (immunochromatography) for detection of rabies virus in the cell culture harvest. *Commonwealth. Vet. J*., **31**(2): 5-8.

SHESHEER KUMAR, 2012. LAMP based kits for lab animal disease. *In*: Proceedings of National workshop Health monitoring in laboratory animals, IISc Bangalore, *pp* 26-28

SHETTY, R. A., CHATURVEDI, S. and SINGH, Z. 2005. Profile of animal bite cases in Pune. *J. of Communicable Diseases.*, **37**(1): 66-72

SMITH, J. S., 1989. Rabies virus epitopic variation: use in ecologic studies. Adv. Virus. Res. **36**: 215–253

SMITH, W. B., BLENDEN, D. C., FUH, T. H. and HILLER, L., 1972. Diagnosis of rabies by immunofluorescent staining of frozen sections of the skin. *J. Am. Vet. Med. Assoc*., **161**(11): 1495-1503

STONE, W. S., GOODRICH, W. O. and DEAN, D. J., 1962. The Fluorescein antibody test as a diagnosis aid in rabies. *Vet. Bull.,* **33** (6): 1956

SUDARSHAN, M.K., MADHUSUDANA, S.N., MAHENDRA, B.J., RAO, N.S., ASHWATH NARAYANA, D.H., ABDUL RAHMAN, S., MESLIN, F., LOBO, D., RAVIKUMAR, K and GANGABORAIAH., 2007. Assessing the burden of human rabies in India: results of national multi-center epidemiological survey. *Int. J. Infect. Dis*., **11**(1): 29-35

SUJITH, S. NATH., VEEREGOWDA, B.M., ISLOOR, S., RATHNAMMA, D., TAJUNNISA, M., SANTOSH, A.K., LEENA, G., and RAMESH, P.T., 2017. Ante-mortem *vis-a-vis* Post-mortem diagnosis of Rabies in dogs. *In*: XIX National conference of Association for Prevention and Control of Rabies in India: Towards elimination of human rabies by 2030, 8th -9th July 2017, *pp* 53

TAJUNNISA, M., ISLOOR, S., NETRA, B.T., RAMESH, P.T., RATHNAMMA, D., SUNILKUMAR, K.M., SANTOSH, A.K. AND SUJITH S. NATH., 2016. Ante-mortem vis-a-vis Post-mortem laboratory diagnosis of Rabies in animals – A case study. *In:* 25th National Conference of the Indian Virological Society and the International Conference: Global Prospectives in Virus Disease Management, 8th - 10th December, 2016, *pp* 206

TAJUNNISA, M., ISLOOR, S., GONGAL, G., RATHNAMMA, D., SUJITH S. NATH., SANTOSH, A.K., R. SHARADA, VEEREGOWDA, B.M., RAMESH, P.T., LEKSHMI J. DAS and CHINMAYEE, K. S., 2017. Comparative evaluation of Rapid immunochromatographic test and Monoclonal antibody based Direct Fluorescent Antibody assay for detection of Rabies virus. *In*: XIX National conference of Association for Prevention and Control of Rabies in India: Towards elimination of human rabies by 2030, 8th -9th July 2017, *pp* 53

TORDO, N. and POCH, O., 1998. Structure of rabies virus. In: CAMPBELL, J. B., CHARLTON, K. M., editors. Rabies; London. *pp*. 26-28

TSUGUTO, F., TERUO, O., TAKAO, O., MASAHIRO, I., SOICHI, N., MASATSUGU, C and OSAMU, N., 2004. Evaluation of a bedside immunochromatographic test for detection of adenovirus in respiratory samples, by comparison to virus isolation, PCR, and real-time PCR. *J. Clin. Microbiol*. **42**: 5489–5492

UMOH, J.V., and BLENDEN, D.C., 1981. Immunofluorescent staining of rabies virus antigen in formalin-fixed tissue after treatment with trypsin. *Bull. World. Health. Org*., **59**(5): 737–744

VAUGHN, J. B., GERHARDT, P. and NEWELL, K. W., 1965. Excretion of street rabies virus in the saliva of dogs. J. Am. Med. Assoc., **193**(5): 363-368

VEERA, T., LUMLERTDACHA, B., MITMOONPITAK, C., FAGEN, R. and WILDE, H., 1997. Fluorescent antibody test for rabies: Prospective study of 8987 brains. *Clin. Infect. Dis.*, **25**: 1459–1461

VELASCO-VILLA, A., ORCIARI, L. A., SOUZA, V., JUAREZ-LSLAS, V., GOMEZ-SIERRA, M., CASTILLO, A., FLISSER, A. and RUPPRECHT, C.E., 2005. Molecular epizootiology of rabies associated with terrestrial carnivores in Mexico. *Virus Res.*, **111**: 13-27

VENKATESAN, G., BALAMURUGAN, V., BHANUPRAKASH, V., AMIT KUMAR, YOGISHARADHYA, R., MUNISWAMY, K., MUTHUCHELVAN, D. and PANDEY, A.B., 2012. Development and evaluation of loop mediated isothermal amplification (LAMP) assay for rapid detection of Orf virus by simple visual detection methods. *In*: XXVI Annual convention of IAVMI on Future of livestock health: a paradigm change to maximize productivity for economic gains

WVOEHL, K.M., SATURDAY, G.A., 2014. Evaluation of a rapid immunodiagnostic rabies field surveillance test on samples collected from military operations in Africa, Europe, and the Middle East. *US.Army. Med. Dept. J. pp* 27-32

VOEHL, K.M., SATURDAY, G.A., 2014. Evaluation of a rapid immunodiagnostic rabies field surveillance test on samples collected from military operations in Africa, Europe, and the Middle East. *US.Army. Med. Dept. J. pp* 27-32

WANG , X., ZHANG, Y. Z., XIANG, C., ZIAO, Y., D., JIANG, R., XIAO, Q., HAO, Z. H., ZHANG, L., YU, Y. and FU, Z.F., 2011. Intracerebral Administration of Recombinant Rabies Virus Expressing GM-CSF Prevents the Development of Rabies after Infection with Street Virus *Virus Res.,* **121**: 179-188

WEST and GEOFFREY, P., 1972.Rabies in Animals and Man. Edn1st. Newton Abbot. Great Britain: David & Charles Limited.

WHITE, L. A., CHAPPELL, W, A.,1982. Inactivation of Rabies Virus in Reagents Used for the Fluorescent Rabies Antibody Test. *J. Clin. Microbiol*. **16**(2):253-6

WHO expert consultation on rabies. 2013. Technical Report Series, 2nd report. World Health Organisation, Geneva

WILLOUGHBY, R. E., TIEVES, K.S., HOFFMAN, G.M., GHANAYEM, N.S and RUPPRECHT, C. E., 2007. Survival after Treatment of Rabies with Induction of Coma. *The New England J.Med*., **24(**352**)**: 2508-2514

WOLDEHIWET, Z., 2005. Clinical laboratory advances in the detection of rabies virus. Clinica Chimica Acta, **351** (1-2): 49-63

WORLD HEALTH ORGANIZATION, 2005. WHO expert consultation on rabies, 1st report. Geneva, *pp* 1-88

WUNNER, W. H., 2002. Rabies Virus. In. Rabies, Edt. Jackson, A. C. and Wunner, W. h., Elsevier SCIENCE, London, *pp* 23-77

YALE, G., BHANU REKHA, V. and GAJENDRAGAD, M., 2014. Range of Rabies - A Review. *J. foodborne zoonotic dis*., **2** (1):1-9

ZHANG, G., GUO, J and WANG, X., 2009. Immunochromatographic lateral flow strip tests. *Methods Mol Biol.,* **504**: 169–83

ZUCKERMAN, A.J., BANATVALA, J.E., SCHOUB, B.D., GRIFFITHS, P.D., MORTIMER, P., 2009. Rabies and Other Lyssavirus Infections *Edn* $6^{t\ h}$., John Wiley & Sons., *pp* 329

Abstract

VIII. ABSTRACT

A study on brain samples of rabies suspected animals collected from different host species from seven Indian states was undertaken to compare acetone and formalin based fixation of animal brain impressions in DFA and to evaluate LFA and LAMP with DFA for rapid diagnosis of rabies in animals. In all, 200 samples were screened by DFA and LFA. Fifty out of 200 samples were subjected to modified DFA using 2.5% NBF as a fixative, LFA and LAMP. Out of 200 tested, 120 and 129 were found to be positive by DFA and LFA respectively. Out of fifty among these 200 samples, 38 were positive by both DFA and modified DFA, 40 by LFA and 41 by LAMP were found positive. The modified DFA was found to be more suitable as 2.5% NBF fixative inactivated the RABV completely, thus eliminating the biohazard to the laboratory personnel involved. The LFA can be even used at the field level at the post-mortem site, where results are obtained within 5 minutes. Furthermore, the RT-LAMP reaction can be carried out in one hr using RNA as a template which bypasses cDNA synthesis step and thereby saving time and reagents. The DFA and modified DFA results were analyzed by Spearman's nonparametric correlation analysis. The "Correlation coefficient (Spearman r)" was found to be 0.98 ($P<0.001$) indicating good positive correlation. Results of LFA and DFA were analyzed by Spearman's nonparametric correlation analysis and the "Correlation coefficient (Spearman r)" was found to be 0.84 ($P<0.001$). Sensitivity and specificity of LFA was found to be 99.2% and 98.5% respectively. The positive results subjected to 2-way ANOVA with Tukey's multiple comparison test revealed significant difference ($P< 0.05$) between LFA and DFA. Sensitivity and specificity of RT-LAMP was found to be 100% and 75% on comparison with DFA. The LFA and RT-LAMP were found rapid, user freindly and can be suitably employed at the field level to identify the RABV in suspected clinical samples.