FINAL REPORT

MAJOR RESEARCH PROJECT

Studies on *intra vitam* diagnostic approaches of Rabies in animals

Submitted to

ज्ञान-विज्ञान विमुक्तये

UNIVERSITY GRANTS COMMISSION

NEW DELHI

by

Dr Charan Kamal Singh

Principle Investigator

Senior Veterinary Pathologist-cum-Head

Department of Veterinary Pathology

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Ludhiana

Annexure - V

UNIVERSITY GRANTS COMMISSION

BAHADUR SHAH ZAFAR MARG

NEW DELHI – 110 002

Utilization certificate

Certified that the grant of Rs. 9,17,500 received from the University Grants Commission under the scheme of support for Major Research Project entitled **"Studies on *intra vitam* diagnostic approaches of Rabies in animals"** vide UGC letter No. **F No 41-188/2012** dated1st July 2012 has been fully utilized for the purpose for which it was sanctioned and in accordance with the terms and conditions laid down by the University Grants Commission.

PRINCIPAL INVESTIGATOR

(SIGNATURE OF THE REGISTRAR/PRINCIPAL STATUTORY AUDITOR

ज्ञान-विज्ञान विमुक्तये

Annexure – VIII

UNIVERSITY GRANTS COMMISSION

BAHADUR SHAH ZAFAR MARG

NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. **Project Report**: FINAL
2. **UGC APPROVAL NO. AND DATE F No 41-188/2012** Dated 1 July 2012.
3. **Period of Report from** 1st July 2012 **to** December 2016
4. **TITLE OF THE PROJECT Studies on *intra vitam* diagnostic approaches of Rabies in animals**
5. (a) **NAME OF THE PRINCIPAL INVESTIGATOR**: Dr Charan Kamal Singh,
   (b) Department: Department of Veterinary Pathology,
   (c) University: Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana
6. **Effective Date of Starting of the Project**: 1st July 2012
7. **Grant approved and expenditure incurred during the period of the report**:
   (a) Total Amount Approved: Rs 9,17,500
   (b) Total expenditure: Rs 9,07,672
8. **Report of the work done:** Final Report Attached

   i. **Brief Objective of the Project**: The objective of the project is to explore various aspects related to ante-mortem diagnosis of Rabies in animals. Accordingly, the present project shall compare the sensitivity of most significant body samples viz. saliva (secretion), urine (excretion), serum (body fluid) and skin (body tissues) for ante-mortem diagnosis of Rabies by most significant

laboratory techniques viz. PCR, Real time PCR, Immuno-pathological approaches thereby to ascertain most suitable secretion/ excretion body tissue for ante-mortem diagnosis in animals. Also, based on the findings of the present project, protocol of ante-mortem diagnosis of Rabies in animals shall be recommended.

ii. **Work done so far and results achieved and publications, if any, resulting from the work** (Give details of the papers and names of the journals in which it has been published or accepted for publication):

1. Pranoti Sharma, **C K Singh**, NK Sood, BS Sandhu, K Gupta and APS Brar. 2014. Diagnosis of rabies from brain: Comparison of histochemical and histopathological approaches. *Indian Journal of Veterinary Pathology* 38 9(4): 269-272.
2. Beigh A B, Sandhu B S, **Singh, C K**, Gupta K, Sood N K. 2015. Comparative evaluation of clinico-pathological, immuno-histochemical, and immunofluorescent techniques for diagnosis of rabies in animals. Comparative Clinical Pathology 23(6): DOI 10.1007/S00580-014-2057-9
3. Pranoti Sharma, **C. K. Singh** and Deepti Narang. 2015. Comparison of immuno-chromatographic diagnostic test with heminested reverse transcriptase polymerase chain reaction for detection of rabies virus from brain samples of various species. *Veterinary World* **8**(2): 135-138.
4. Pranoti Sharma, **C K Singh**, N K Sood, K Gupta, B S Sandhu and APS Brar. 2015. Immunohistochemical detection of rabies in dogs from skin. *Indian Journal of Canine Practice* **7**(1):86-90.
5. Ajaz Ahmad and **C. K. Singh**. 2016. Comparison of rapid immuno-diagnosis assay kit with molecular and immuno-pathological approaches for diagnosis of rabies in cattle. Veterinary World, **9**(1): 107-112 EISSN: 2231-0916
6. Akeel Bashir Beigh, Bhupinder Singh Sandhu, **CK Singh** and Naresh Kumar Sood. 2016. Diagnosis of rabies in buffaloes: Comparison of clinico-pathological, immuno-histochemical and immunofluorescent techniques. Buffalo Bulletin 35(3):331-345
7. Beigh A.B**.,** Sandhu BS, **Singh C.K**., Gupta K., Brar A.P.S., Sood N.K. 2016. Diagnosis of rabies in cattle: Comparison of clinico-pathological, immuno-histochemical and immunofluorescence techniques. Indian Journal of Veterinary Pathology **40**(1):1-7

8. Ajaz Ahmad and **C. K. Singh**. 2017. Sensitivity comparison of immuno-histochemical and histopathological approaches for diagnosis of Rabies in animals. International Journal of Current Microbiology and Applied Sciences 6(4): 400-405
9. Ajaz Ahmad and **CK Singh**. 2017. Efficacy study of immuno-histochemical approach for diagnosis of rabies in dogs. Journal of Animal Research, 7(2):265-269.

i) **Has the progress been according to original plan of work and towards achieving the objectives**: Yes. If not, state reasons: NA

ii) **Please indicate the difficulties, if any, experienced in implementing the project**: Nil

iii) **If project has not been completed, please indicate the approximate time by which it is likely to be completed.** NA

iv) **If the project has been completed, please enclose a summary of the finding of the study**: Summary attached. One bound copy of the final report of work enclosed.

v) **Any other information:** which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as

(a) Manpower trained: two MVSc students viz. Pranoti Sharma (Registration Number L-2011-V-70-M), Manoj Kumar (Registration Number L-2012-V69M) trained during the project.

(b) Ph. D. awarded: One Ajaz Ahmad Zargar (L-2012-V-16-D)

(c) Publication of results: 9

(d) Other impact: Advancement in knowledge and implementation of findings for ante-mortem diagnosis of rabies in animals.

Annexure – IX

UNIVERSITY GRANTS COMMISSION BAHADUR SHAH ZAFAR MARG NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. TITLE OF THE PROJECT: **Studies on *intra vitam* diagnostic approaches of Rabies in animals**

2. NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR: Dr Charan Kamal Singh, Department of Veterinary Pathology, GADVASU, Ludhiana

3. NAME AND ADDRESS OF THE INSTITUTION Guru Angad Dev Veterinary and Anima Sciences University, Ludhiana, Punjab

4. UGC APPROVAL LETTER NO. AND DATE **File No. F No 41-188/2012**.

5. DATE OF IMPLEMENTATION **1st July 2012**

6. TENURE OF THE PROJECT **3 years and 5 Months**

7. TOTAL GRANT ALLOCATED **Rs 10,10,000**

8. TOTAL GRANT RECEIVED **Rs 9,17,500**

9. FINAL EXPENDITURE **Rs 9,07,672**

10. TITLE OF THE PROJECT **Studies on *intra vitam* diagnostic approaches of Rabies in animals**

11. OBJECTIVES OF THE PROJECT

- To detect rabies in clinical secretions/excretions, body fluids and body tissues in live animals by employing PCR, Real time PCR, Immunopathological approaches.
- To establish the prevalence of rabies in various species of animals.
- To compare the sensitivity of PCR, Real Time PCR on saliva, skin and urine, ELISA and Fluorescence Polarization on serum & Immuno-fluorescence on skin biopsy detection as *intra vitam* diagnostic approaches of rabies in animals.
- To ascertain the most suitable secretion/ body tissue for *ante-mortem* diagnosis in animals.
- To correlate the detection of rabies antigen with the periodic quantification of rabies antibodies in the serum of the infected live animals as well as the symptoms exhibited by suspected animals.
- To recommend the species-wise protocol of *ante-mortem* diagnosis of Rabies in canine, feline and bovine.

12. WHETHER OBJECTIVES WERE ACHIEVED (GIVE DETAILS):

Yes, objectives were achieved. Details attached in Final Report.

13. ACHIEVEMENTS FROM THE PROJECT

1 TaqMan PCR is the most effective molecular approach for antemortem detection of rabies from skin.

2 Immunofluorescence on cryosections is most effective immunopathological approach for antemortem detection of rabies from skin.

3 Molecular approaches should be preferred over immunopathological approaches for detection of rabies from skin.

4 HnRT-PCR revealed higher sensitivity and accuracy while targeting L gene as compared to targeting N gene.

14. SUMMARY OF THE FINDINGS …….. (IN 500 WORDS)

In the present project, standardization of important molecular, immunological and immuno-pathological laboratory techniques for detection of rabies virus from saliva, urine and skin of living animals suspected for Rabies have been standardized and a protocol for *intra vitam* detection of rabies in animals recommended.

Various laboratory techniques have been tested for sensitivity, specificity and accuracy for detection of Rabies in live animals. These laboratory techniques include HnRT-PCR targeted at N gene, HnRT-PCR targeted at L gene, Taqman Real Time PCR, ELISA, Fluorescence Polarization Assay, Fluorescent Antibody Technique, Immunohistochemistry and Immuno-chromatographic test.

Out of the molecular and Immuno-pathological laboratory techniques employed for *intra-vitam* detection of rabies in saliva and urine of rabid animals, the highest sensitivity (84.62%) along with 90% accuracy and 100% specificity was obtained by employing Taqman Real Time PCR for detection of Rabies from saliva of infected animals.

And out of the molecular and Immuno-pathological laboratory techniques employed for *intra-vitam* detection of rabies in skin of rabid animals, Taqman Real Time PCR and HnRT-PCR targeted on L gene exhibited highest sensitivity (95.83%), accuracy of 97.05% and specificity of 100% for detection of Rabies from skin samples of rabid animals.

ELISA and Modified Counter-Immuno-Electrophoresis were standardized for quantification of anti-rabies antibodies in infected animals. And in another study, Florescence Polarization Assay and ELISA were compared and were found to be equally effective for estimation of anti-rabies antibodies in rabid animals. The symptoms shown by rabid animals could not be correlated with specific quantum of anti-rabies antibodies.

After testing various laboratory techniques comprising of molecular, immunological and immuno-pathological, and testing various clinical samples viz. saliva, urine skin and sera, it was r recommended to test skin samples of live animals suspected for Rabies by employing Taqman Real Time PCR or HnRT-PCR targeting L gene while sera samples may be tested with ELISA or Fluorescence Polarization Assay with caution due to intermittence of secretion of rabies virus while attempting *intra-vitam* diagnosis of Rabies in animals.

15. CONTRIBUTION TO THE SOCIETY Rabies is a cause of serious concern. Innumerable animals as well as more than 50,000 humans die annually of Rabies in the world and the majority of the mortalities occur in India. There is urgent need to control the deadly disease in animals. The findings of present project shall enable strengthening of Rabies control efforts by detection of Rabies in naturally prevalent animals in the society.

2. There are large number of animals, in India, that are harboring this deadly disease, at any given point of time. There is an urgent need to assess the prevalence of rabies antigen in the population of various species of domestic, community owned as well as stray animals. The tests standardized in the [project for sero-surveillance shall be highly significant to detect infected animals

3. There is urgent need to check the spread of Rabies from infected anima to non-infected animals and humans. Unless the animals in incubation are detected *intra vitam*, our rabies control efforts shall continue to be a step behind while the detection of such animals in incubation of the disease shall be a lot helpful to curb the spread of this deadly disease. The present resea5rch project has enabled to diagnose Rabies *intra-vitam* that shall control the spread of Rabies in society.

4. At present, diagnosis of rabies largely depends on the detection of rabies virus in brain tissue since no protocol for detection of rabies in live animals. The present project has recommended the considered protocol of intra-vitam diagnosis of Rabies in suspected animals.

16. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT: Yes. One PhD produced. Ajaz Ahmad Zargar (L-2012-V-16-D)

17. NO. OF PUBLICATIONS OUT OF THE PROJECT: 9 (Attached)

(Dr C K Singh)
(PRINCIPAL INVESTIGATOR)

1. Dr N K Sood : _____
2. Dr Deepti : _____
3. Dr B S Sandhu : _____

(CO-INVESTIGATORS)

(REGISTRAR/PRINCIPAL)

ज्ञान-विज्ञान विमुक्तये

Annexure 1

FINAL REPORT

TITLE OF THE PROJECT: **Studies on *intra vitam* diagnostic approaches of Rabies in animals**

INTRODUCTION:

Rabies is a cause of serious concern. Innumerable animals as well as more than 50,000 humans die annually of Rabies in the world and the majority of the mortalities occur in India. Hence, there shall be large number of animals, in India, that are harboring this deadly disease, at any given point of time. However, perusal of literature reveals that there is no significant effort so far for *intra vitam* detection of rabid cases in the general population of animals. There is an urgent need to assess the prevalence of rabies antigen in the population of various species of domestic, community owned as well as stray animals. It shall be highly significant to detect infected animals since these animals are the source of spread to other animals and humans. Unless the animals in incubation are detected *intra vitam*, our rabies control efforts shall continue to be a step behind while the detection of such animals in incubation of the disease shall be a lot helpful to curb the spread of this deadly disease.

Thus, *intra vitam* diagnosis of animals that bite such as dogs, cats, rats, bats and wild animals assumes even greater significance. Delay in diagnosis of rabies in such animals that continue to inflict bite wounds and render fast spread of rabies, also result in the need of more number of post-exposure vaccinations and/or consequential mortalities. Thus, *intra vitam* diagnosis in animals needs to be taken up at the earliest for detection of rabies especially in biting animals.

Rabies causes large number of moralities in productive animals, thus, a major cause of serious concern for the dairy farmers. Because of its fatality, it is the most dreaded zoonotic disease and of utmost concern to humans. Although India continues to report largest number of human deaths and innumerable number of animal deaths due to rabies, yet, no effort has been made so far to estimate the number of animals in incubation of this deadly disease so far even in a limited geographical area. In spite of the fact that, it is suspected that there always are a large number of

animals that harbor this deadly disease at any given point of time. Thus, there is an urgent need to assess the prevalence of rabies antigen in the population of various species of domestic, community owned as well as stray animals.

In the present project, standardization of all molecular and immunopathological techniques for detection of Indian strains of rabies virus from secretions (viz. saliva and milk), excretions (viz. urine) and tissues (viz. skin and hair follicles) of animals that are approachable in a living animal shall be standardized and a protocol for *intra vitam* detection of rabies in animals shall be established.

At present, diagnosis of rabies largely depends on the detection of rabies virus in brain tissue since no protocol for detection of rabies in live animals has been standardized by applying molecular and immunopathological techniques on various secretions and excretions of suspected animals that are relevant for detection of rabies in live animals. The project would, thus, analyze various molecular and immunopathological procedures of detection of Indian strains of rabies in urine, saliva, skin and hair follicle as applicable for different species. On comparing sensitivity and specificity of all the techniques, a hitherto unavailable protocol for diagnosis of rabies in live animals shall be established. The present attempts have so far not correlated the detection of rabies with duration of incubation of disease and presence or absence of symptoms. Further, various types of molecular/immuno-pathological approaches have to be comprehensively compared in a single study that has not so far been accomplished.

The major benefit of the project would be that a new standardized protocol would be established for diagnosis of rabies from secretions/excretions/non-nervous body tissues of live animals by detection of rabies by molecular (RT-PCR, qPCR) and immunopathological (Immunohistochemistry, immuno-fluorescence and Fluorescence Polarization) approaches.

Perusal of literature reveals that there is no standardized protocol for implementing *intra vitam* detection of rabies in the general population of animals. Moreover, there is an urgent need to assess the prevalence of rabies antigen in the population of various species of domestic, community owned as well as stray animals since these animals are the source of spread of rabies to other animals and humans. Thus, determination of the, *hitherto* unavailable, protocol of *intra vitam* diagnosis in animals needs to be taken up at the earliest.

The project would generate standardized procedures of detection of Indian strains of rabies in urine, saliva, skin and hair follicle as applicable to different species of animals with a special focus on dog which has been the cause of more than 90% spread of rabies in India resulting in the need of more number of post-exposure vaccinations and/or consequential mortalities. Thus, determination of the, *hitherto* unavailable, protocol of *intra vitam* diagnosis in animals needs to be taken up at the earliest.

OBJECTIVES OF THE PROJECT:

- To detect rabies in clinical secretions/excretions, body fluids and body tissues in live animals by employing PCR, Real time PCR, Immuno-pathological approaches.
- To establish the prevalence of rabies in various species of animals.
- To compare the sensitivity of PCR, Real Time PCR on saliva, skin and urine, ELISA and Fluorescence Polarization on serum & Immuno-fluorescence on skin biopsy detection as *intra vitam* diagnostic approaches of rabies in animals.
- To ascertain the most suitable secretion/ body tissue for *ante-mortem* diagnosis in animals.
- To correlate the detection of rabies antigen with the periodic quantification of rabies antibodies in the serum of the infected live animals as well as the symptoms exhibited by suspected animals.
- To recommend the species-wise protocol of *ante-mortem* diagnosis of Rabies in canine, feline and bovine.

Work Done to achieve the objectives:

OBJECTIVE No 1:

TO DETECT RABIES IN CLINICAL SECRETIONS/EXCRETIONS, BODY FLUIDS AND BODY TISSUES IN LIVE ANIMALS BY EMPLOYING PCR, REAL TIME PCR, IMMUNO-PATHOLOGICAL APPROACHES.

PART A of OBJECTIVE 1

DETECTION OF RABIES IN SALIVA/URINE IN LIVE ANIMALS BY EMPLOYING PCR, REAL TIME PCR AND IMMUNO-PATHOLOGICAL APPROACHES

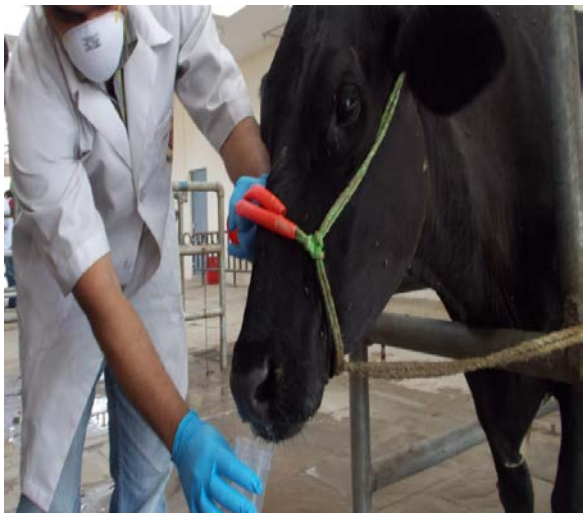
A study was conducted wherein saliva and urine samples were collected from 40 animals, clinically suspected for Rabies. These 40 animals comprised of 17 dogs, 8 buffaloes, 10 cattle, 2 rabbit, 2 mongooses and 1 mare.

Table 1: Details of clinically suspected animals

Species	**Age Group**		
	< 1.0 Year	**1.0-5.0 Year**	**> 5.0 Year**
Dog	8	5	4
Cow and Cow Bull	2	5	3
Buffalo	1	3	4
Mongoose	2	-	-
Mare	-	1	-
Rabbit	2	-	-
Total	15	13	12

Collection of Saliva samples

Saliva was collected directly in sterilized containers (Fig.1) or by swab method in sterilized containers from the rabies suspected animals. PBS was added in saliva sample to make 1:1 suspension. In case of offensive animal, soiled saliva samples were collected



centrifuged (1500 rpm for 8-10 minutes) and supernatant was used for study.

Fig. 1: Collection saliva sample in sterile container.

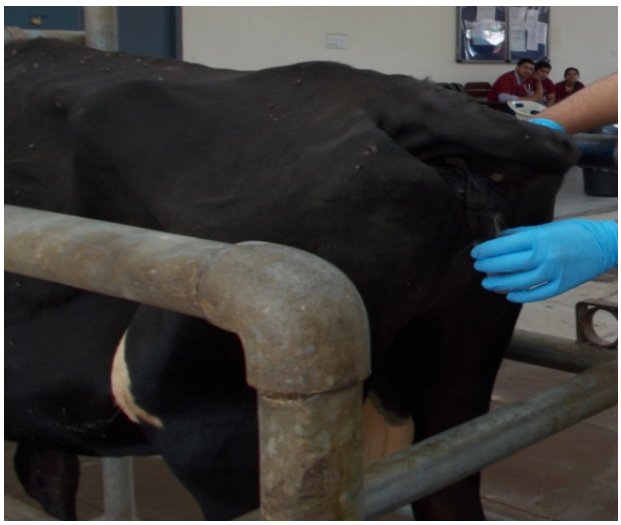


Fig. 2: Collection urine sample in sterile container

Collection of urine samples

Urine samples were collected directly from the rabies suspected animals, while animal was urinating (Fig.2), in sterilized vials. In case of offensive animal, soiled urine samples were collected centrifuged (1500 rpm for 8-10 minutes) and supernatant was used for study.

Control for saliva samples

1. **Positive control:** Positive saliva sample of known saliva positive case.
2. **Negative control:** The Confirmed negative saliva samples of known rabies negative case.

Control for urine samples

1. Positive control: Positive urine sample of known urine positive case.
2. Negative control: Confirmed negative samples of known rabies negative.

Storage of Saliva samples

Collected saliva samples were divided in two parts and one part was processed for RNA extraction and second part was stored in -80˚C (Ultra low temperature freezer, Haier, Biomedical).

Storage of urine samples

Collected urine samples were divided in two parts and one part was processed for RNA extraction and second part was stored in -80˚C (Ultra low temperature freezer, Haier, Biomedical).

MOLECULAR DIAGNOSIS: PCR and Real Time PCR

RNA Extraction from saliva and urine samples

Whole procedure of RNA extraction from saliva and urine was carried out under clean and sterile conditions in a class II Bio safety cabinet as under-

1. 400µlurine and saliva samplewas added in micro centrifuge tubes of 2.0 ml volume.
2. For sufficient output of RNA, triplicates of each sample were used.
3. 20µl of 10% SDS solution and 20µl of Proteinase K (20mg/ml) were added and tubes were incubated for 1.5-2 hrs at 37 ºC.
4. 1ml of Trizol (Invitrogen, USA) was added in each of these tubes and vortexed for 20-40 seconds.
5. These tubes were incubated for 5 minutes at room temperature.
6. 200µl of Chloroform was added in each tube and mixed by vortexing.
7. Mixture was incubated for 5 minutes at room temperature and then centrifuged at 14,000 rpm for 15 minutes at 4ºC.
8. The upper colorless aqueous phase containing RNA was carefully transferred into fresh, clean, sterile, Diethylpyrocarbonate (DEPC) treated, autoclaved micro centrifuge tubes (1.5ml) and care was taken during transfer of aqueous phase so that lower organic phase (containing Trizol and chloroform) was not disturbed

9. Isopropanol (500µl) was added to the aqueous phase and mixed gently with the help of pipetting.
10. Tubes were incubated for 20 mins at room temperature and centrifuged at 12,000-14,000 rpm for 15 min at 4ºC.
11. The supernatant was discarded by gentle inversion (when RNA pellet was intact) or aspirated (when RNA pellet was loose).
12. The RNA pellet was washed with 1ml of 75% ethanol, vortexed and centrifuged @ 9000 rpm for 10 min at 4ºC.
13. Supernatant was decanted without disturbing the pellet.
14. Ethanol was allowed to evaporate, while take care if pellet did not over dry.
15. RNA pellet was dissolved in 20µl of RNAse free water and final product of each triplicate sample was collected in single tube. Pellet was dissolved with repeated pipetting.
16. Pellet was incubated for 10 minutes at 55-60ºC to enhance complete dissolution of RNA.
17. Micro centrifuge tubes containing RNA were stored at -80ºC until further use.

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

Total extracted RNA was converted into cDNA using High-Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems, USA) as follows:

1. 10µl of total RNA from each sample was taken into PCR tubes and 2µl of random primers were added into each tube.
2. The sample RNA and Primer mixture was centrifuged briefly and was incubated at 65°C for 10 min and snap cooled on ice.
3. Master mixture was prepared as under:

S. No.	Reaction components	Volume
1	10X RT Buffer	2.0 µl
2	25X dNTP Mix (100mM)	0.8 µl
3	Multiscribe™ Reverse Transcriptase (50ug/ul)	1.0 µl
4	RNase Inhibitor	1.0 µl

5	Nuclease free water	4.2 µl
	Total volume	8.0 µl

4. Master mixture (8µl) was added to RNA-Primer mixture (12 µl) to make cDNA reaction mixture (20 µl).
5. The above cDNA synthesis mixture was run in thermal cycler as under:

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

The resultant cDNA of saliva and urine samples was used both for HnRT-PCR and TaqMan real time PCR.

Ante mortem diagnosis of rabies using molecular techniques

Heminested Reverse Transcriptase-Polymerase Chain Reaction

Primers

Primers targeted towards conserved blocks of L gene were used for first and second round HnRT-PCR. The details are given below (Table 2)

Table 2: HnRT-PCR primers targeting conserved blocks of L gene

Primer	Sequence	Gene	Position	Sense	Reference
PVO5 Forward primer 1Sround	5'ATGACAGACA ATTTGAACAA3'	L	7166-7185	+	Sharma (2013)
PVO9 Reverse primer	5'TGACCATTCC AGCAAGT3'	L	7485-7469	_	Sharma (2013)

1ST round					
PVO8 Reverse primer 2nd round	5'GGTCTGATCT ATCTGA3'	L	7400-7415	_	Sharma (2013)

Primers targeted towards nucleoprotein gene were used for first and second round HnRT-PCR. The details are given below (Table 3)

Table 3: HnRT-PCR primers targeting the Nucleoprotein gene

Primer	Sequences	Gene	Position	Sense	Reference
JW 12 Forward primer 1ST round	5'ATGTAACA CCCCTACAA TG3'	N	55-73	+	Sharma (2013)
JW 6 DPL Reverse primer 1ST round	5'CAATTGG CACACATTT TGTG3'	N	660-641	-	Sharma (2013)
JW 10 P Reverse primer 2nd round	5'GTCATCA GAGTATGG TGTTC3'	N	636-617	-	Sharma (2013)

Amplification protocol

1. Fresh reaction mixture for first round of the PCR was prepared in PCR tubes on ice tray according to the following protocol (Table 4)

Table 4: Reaction mixture components for primary HnRT-PCR

S. No.	Reaction mixture components (L gene assay)	Master mix components (N gene assay)	Volume
1	2X PCR master mix	2X PCR master mix	12.5 µl
2	Nuclease Free Water	Nuclease Free Water	8.5 µl
3	Upstream PrimerPV 05 (10pmol)	Upstream Primer JW 12	1.0µl

		(10pmol)	
4	Downstream Primer PV 09 (10pmol)	Downstream Primer JW 6 (10pmol)	1.0µl
5	Template (cDNA)	Template (cDNA)	2.0 µl
Total			25 µl

2. The reaction mixture was mixed thoroughly by vortexing, centrifuged briefly to collect residual contents from the walls of the tube.
3. PCR tubes were placed in DNA thermal cycler and subjected to following conditions:

Temperature	**Time**	**No. of cycles**	**Remarks**
94°C	3 min.	1	Initial Denaturation
94°C	30 s		Denaturation
56°C	45s	35	Annealing
72°C	40s		Extension
72°C	3 min.	1	Final Extension

4. Secondary PCR was performed using 2 µl of the primary PCR product as template. Fresh reaction mixture for second round of the PCR was prepared in PCR tubes on ice according to the following preparation (Table 5)

Table 5: Reaction mixture components for secondary HnRT-PCR

S. No.	**Reaction mix components**	**Master mix components**	**Volume**
1	2X PCR master mix	2X PCR master mix	12.5 µl
2	Nuclease Free Water	Nuclease Free Water	8.5 µl
3	Upstream Primer PV 05	Upstream Primer JW 12	1.0µl
4	Downstream Primer PV 09	Downstream Primer JW 10	1.0µl
5	Template (1st round PCR Product)	Template(1st round PCR Product)	2.0 µl

Total	25 µl

PCR tubes were placed in DNA thermal cycler and subjected to following conditions for the second round of the HnRT-PCR:

Temperature	Time	No. of Cycles	Remarks
94°C	3 min.	1	Initial Denaturation
94°C	30 s		Denaturation
56°C	45s	35	Annealing
72°C	40s		Extension
72°C	3 min.	1	Final Extension

Analysis of PCR products

The stock TBE buffer was prepared as follows

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

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

Working solution of TBE buffer (1x)

10X TBE	10ml
DW add to	90ml

1.0 gram of agar was added in 100 ml of 1x TBE to form 1.0% agar gel and it was heated to dissolve agar completely in buffer. Gel was cooled down to temperature of around 50°C and ethidium bromide solution (stock conc. 10mg/ml) was added to final concentration 0.5µg/ml of gel.

The molten gel was poured into holding chamber of gel electrophoresis apparatus and comb was inserted. Gel was allowed to solidify at room temperature for 20 min. Gel was placed in electrophoresis tank which filled with 1X TBE buffer and the gel was immersed in buffer and comb was removed.

Samples were loaded after mixing the PCR product with 6X loading dye (Thermo scientific) (5:1) in parallel with molecular weight marker 100bp plus DNA ladder (Tracklt 100 bp DNA Ladder INVITRIGEN, 0.1µg/µl). Electrophoresis was carried out for 45

minutes at 70 volts. The gel was visualized and photographed under Gel documentation system (Alpha Innotech, multi Image™ Light cabinet filter positions).

Analysis of RNA and cDNA: As per manufacture's specification (Nano-drop 100 Spectrophotometer Thermo Scientific, USA), the concentration and ratio 260/280 of samples was determined to check the quality and concentration of RNA and cDNA.

Diagnosis of rabies in saliva and urine by HnRT-PCR targeting L gene

Amplification of cDNA yielded 319 bp first round amplified product and 249 bp products in second round.

Diagnosis of rabies in saliva samples by HnRT-PCR targeting L gene

HnRT-PCR diagnosed rabies viral RNA in saliva of 20 (50%) animals.

Diagnosis of rabies in urine samples by HnRT-PCR targeting L gene

HnRT-PCR detected rabies viral RNA in urine of 17 cases.

Diagnosis of rabies in saliva and urine samples by HnRT-PCR Targeting N gene

Primers for first cycle yielded 606 bp product and primers for second cycle yielded 586 bp product of HnRT-PCR.

Diagnosis of rabies in saliva samples by HnRT-PCR Targeting N gene

Out of 40 suspected (Table 6) cases 19 (47.50%) cases were diagnosed positive for rabies in saliva samples.

Diagnosis of rabies in HnRT-PCR Targeting N gene on urine samples.

Out of 40 clinically suspected (Table 6) animals, 16 (40%) cases were diagnosed positive for rabies in urine samples.

Table 6: Diagnosis of rabies from saliva and urine samples by HnRT-PCR targeting L gene and N gene.

S. No.	Case No.	Species	HnRT-PCR L gene		HnRT-PCR N gene	
			Saliva	Urine	Saliva	Urine
1	47 RL12	Cow	–	–	–	–
2	53 RL12	Cattle Bull	+	+	+	+
3	54 RL12	Buff calf	+	+	+	+
4	6 RL13	Cattle	–	–	–	–
5	8 RL13	Buffalo	–	–	–	–
6	9 RL13	Cow	+	+	+	+
7	11 RL13	Buffalo	+	+	+	+
8	13 RL13	Dog	–	–	–	–
9	14 RL13	Dog	–	–	–	–
10	16 RL13	Dog	+	+	+	+
11	19RL13	Dog	+	+	+	+
12	20RL13	Dog	–	–	–	–
13	24 RL3	Cattle	+	+	–	–
14	28RL13	Dog	–	–	–	–
15	32RL13	Cow Bull	+	+	+	+
16	33RL13	Dog	+	+	+	+
17	37R13	Buffalo	–	–	–	–
18	41RL13	Dog	+	+	+	+
19	45RL13	Rabbit	–	–	–	–

S. No.	Case No.	Species	HnRT-PCR L gene		HnRT-PCR N gene	
			Saliva	Urine	Saliva	Urine
20	46RL13	Rabbit	–	–	–	–
21	47RL13	Equine	–	–	–	–
22	52RL13	Buffalo	–	–	–	–
23	53RL13	Dog	–	–	–	–
24	54RL13	Dog	–	–	–	–
25	56RL13	Cow	+	+	+	+
26	61RL13	Dog	+	+	+	+
27	3RL14	Buffalo	–	–	–	–
28	5RL14	Mongoose	+	–	+	–
29	6RL14	Dog	+	+	+	+
30	7RL14	Cow	+	+	+	+
31	8RL14	Dog	–	–	–	–
32	9RL14	Mongoose	–	–	–	–
33	10RL14	Buffalo Heifer	+	+	+	+
34	11RL14	Dog	+	–	+	–
35	12RL14	Cow Calf	–	–	–	–
36	13RL14	Dog	–	–	–	–
37	14RL14	Dog	+	+	+	+
38	16RL14	Cow	–	–	–	–
39	22RL14	Dog	+	+	+	+

S. No.	Case No.	Species	HnRT-PCR L gene		HnRT-PCR N gene	
			Saliva	Urine	Saliva	Urine
40	23RL14	Buffalo	+	_	+	_
Total positive cases			20	17	19	16

TaqMan Real Time PCR

TaqManReal-Time PCR was performed by Applied Biosystem's, Step one plus real time system in Department of Veterinary Microbiology, College of Veterinary Science, GADVASU, Ludhiana.

Primers

Table 7: Primers for TaqMan real time directed against N gene

Primer Name	Sequence	Gene	Length (nt)	Positions	Tmax (ºC)	Remarks
Primer 8F	5'-TTGACGGGAGGAA TGGAACT-3'	N	20	434-453	62	Bansal *et al*(2012b)
Primer 8R	5'-GACCGACTAAAGA CGCATGCT-3'	N	21	477-497	64	Bansal *et al* (2012b)
Probe 8Pr	5'-FAM-AGGGACCCC ACTGTT-TAMRA-3'	N	15	458-472	48	Bansal *et al* (2012b)

Amplification protocol for TaqMan real time PCR

TaqMan Real Time PCR reaction was run in 8 strip PCR tubes capped with optically cleared flat caps. Real-Time PCR reaction was prepared according to following:

Components	Volume
TaqMan Master mix	12.5µl
Forward primer(10 pmol)	1.0 µl
Reverse primer(10 pmol)	1.0µl

Probe(6 pmol)	1.0µl
Nuclease Free Water	2.5µl
Cdna	2.0 µl
Total	20 µl

All samples were run with endogenous control (18srRNA) to test sample integrity and verification of RNA extraction. Endogenous control reaction was prepared according to following.

Components	**Volume**
TaqMan Master mix(2x)	12.5µl
18srRNA 2X (primer-probe)	1 µl
Nuclease Free Water	4.5µl
cDNA	2.0 µl
Total	20 µl

PCR tubes having reaction mixture were subjected to following temperature conditions:

Stage	**Step**	**Temperature °C**	**Time**	**No. of Cycles**
I	Initial Denaturation	95	10.0 min	1
II	Denaturation	95	15 seconds	
	Annealing and extension	60	1 min	40
III	Final Extension	60	30 seconds	1

Analysis

After completion of PCR, critical threshold cycle number (C_T) was determined corresponding to the PCR cycle number at which the fluorescence of the reaction exceeded

a value determined to be statistically higher than the background by the ABI prism software.

The $C_T$values were inversely proportional to the log10 of the amount of template in the PCR. A difference of 1 $C_T$corresponded to twofold difference in template amounts. $C_T$value less than the mean plus two standard deviations of the negative control wells was considered positive. $C_T$value above 35 indicated to absence of amplification.

The resultant data was transferred to an Excel spread sheet and the resulting graphs were obtained.

Diagnosis of rabies in saliva and urine samples by Taq Man Real time PCR

Taq Man Real time PCR was applied in saliva (Table 8) and urine (Table 9) samples; C_T value below or equal to 35 was considered as positive amplification.

Diagnosis of rabies in saliva samples by Taq Man Real time PCR

TaqMan real time PCR detected rabies in saliva samples of 67.64% (22/40) cases.

Table 8: Diagnosis of rabies from saliva samples by Taq Man Real time PCR.

S. No.	Case No	Species	C_T value	Results
1.	47 RL 12	Cow	38.087	_
2.	53 RL 12	Bull	26.296	+
3.	54 RL 12	Buffalo	30.990	+
4.	6 RL 13	Cattle	39.258	_
5.	8 RL 13	Buffalo	40.000	_
6.	9 RL 13	Cow	30.931	+
7.	11 RL 13	Buffalo	26.129	+
8.	13 RL 13	Dog	34.088	+
9.	14 RL 13	Dog	39.931	_
10.	16 RL 13	Dog	27.671	+
11.	19 RL 13	Dog	28.970	+
12.	20 RL 13	Dog	39.568	_

S. No.	Case No	Species	C_T value	Results
13.	24 RL 13	Cattle	33.040	+
14.	28 RL 13	Dog	39.080	_
15.	32 RL 13	Bull	23.574	+
16.	33 RL13	Dog	26.726	+
17.	37R 13	Buffalo	34.031	+
18.	41 RL 13	Dog	28.091	+
19.	45 RL 13	Rabbit	39.878	_
20.	46 RL13	Rabbit	40.000	_
21.	47 RL13	Equine	39.427	_
22.	52 RL 13	Buffalo	40.000	_
23.	53 RL 13	Dog	36.431	_
24.	54 RL 13	Dog	38.973	_
25.	56 RL13	Cow	28.560	+
26.	61 RL13	Dog	26.129	+
27.	3 RL 14	Buffalo	38.847	_
28.	5 RL 14	Mongoose	32.088	+
29.	6 RL 14	Dog	32.930	+
30.	7 RL 14	Cow	22.350	+
31.	8 RL 14	Dog	39.058	_
32.	9 RL 14	Mongoose	38.764	_
33.	10 RL 14	Buffalo	30.990	+
34.	11 RL 14	Dog	29.671	+

S. No.	Case No	Species	C_T value	Results
35.	12 RL 14	Cow Calf	39.858	_
36.	13 RL 14	Dog	39.870	_
37.	14 RL 14	Dog	26.156	+
38.	16 RL 14	Cow	38.637	_
39.	22 RL 14	Dog	24.762	+
40.	23 RL 14	Buffalo	29.519	+
Total positive cases				22

Diagnosis of rabies in urine samples by Taq Man Real time

TaqMan real time PCR detected rabies viral genome in urine samples of 47.50% (19/40) cases (Table 9).

Table 9: Diagnosis of rabies from urine samples by Taq Man Real time PCR.

S. No.	Case No.	Species	Taq Man Real time PCR	
			Ct Values	Result
1.	47 RL12	Cow	39.272	_
2.	53 RL12	Bull	33.0141	+
3.	54 RL12	Buffalo	33.436	+
4.	6 RL13	Cattle	39.974	_
5.	8 RL13	Buffalo	40.000	_
6.	9 RL13	Cow	34.237	+
7.	11 RL13	Buffalo	30.248	+
8.	13 RL13	Dog	34.712	_
9.	14 RL13	Dog	39.459	_
10.	16 RL13	Dog	31.536	+
11.	19RL13	Dog	29.036	+
12.	20RL13	Dog	39.863	_
13.	24 RL3	Cattle	34.317	+
14.	28RL13	Dog	39.869	_
15.	32RL13	Bull	29.107	+
16.	33RL13	Dog	32.900	+
17.	37R13	Buffalo	34.784	+
18.	41RL13	Dog	34.260	+
19.	45RL13	Rabbit	40.000	_
20.	46RL13	Rabbit	40.000	_
21.	47RL13	Equine	39.715	_

22.	52RL13	Buffalo	40.008	_
23.	53RL13	Dog	38.143	_
24.	54RL13	Dog	39.164	_
25.	56RL13	Cow	30.109	+
26.	61RL13	Dog	32.091	+
27.	3RL14	Buffalo	40.000	_
28.	5RL14	Mongoose	40.000	_
29.	6RL14	Dog	31.110	+
30.	7RL14	Cow	26.550	+
31.	8RL14	Dog	38.143	_
32.	9RL14	Mongoose	39.479	_
33.	10RL14	Buffalo	32.994	+
34.	11RL14	Dog	26.726	+
35.	12RL14	Cow Calf	40.000	_
36.	13RL14	Dog	40.000	_
37.	14RL14	Dog	31.622	+
38.	16RL14	Cow	38.859	_
39.	22RL14	Dog	29.787	+
40.	23RL14	Buffalo	39.457	_
Total positive cases				19

IMMUNOPATHOLOGICAL DIAGNOSTIC APPROACHES ON SALIVA AND URINE SAMPLES:

A. FLUORESCENT ANTIBODY TEST on Saliva and Urine:

1. Two smears of saliva and urine were made on clean glass slides and smears were air dried.

2. Smears of saliva and urine were fixed in acetone at -20°C for 30 min.

Fluorescent Antibody Test on saliva and urine samples

1. Each Acetone fixed, air dried smear were circled with a wax pen.
2. A drop of conjugate (lyophilised, adsorbed anti-rabies nucleocapsid conjugate, Bio-Rad, France) was added on each smear and incubated at 37°C for 45 minutes in a moist chamber.
3. Slides were washed twice in phosphate buffer(pH 7.6) for 2 min.
4. A drop of buffered glycerol was added and covered with a cover slip.
5. The slides were examined using an AHBT3 - RFC reflected light fluorescence

The presence of distinct apple green fluorescence in the smear indicated positive reaction and absences of such fluorescence indicate negative reaction.

Diagnosis of rabies in saliva smears by **Fluorescent Antibody Test**

Out of 40 Saliva smears tested by FAT, 12 (30%) revealed apple green immunofluorescence (Fig. 3). Sensitivity, accuracy and specificity for Fluorescent Antibody Test on saliva smears were 46.15%, 60% and 100% respectively.

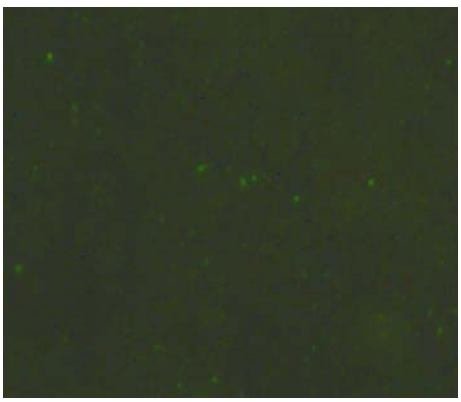


Fig. 3: Immunofluorescence test on saliva - diffused apple green fluorescence (40X)

Diagnosis of rabies in urine smears by Fluorescent Antibody Test

No urine smear of rabies suspected animals was found positive for FAT. This may be due to intermittence presence of rabies antigen in urine. Sensitivity, accuracy and specificity of FAT on urine was 0%, 35% and 100% respectively.

Table 10: Diagnosis of rabies in saliva and urine samples by Fluorescent Antibody Test.

S. No.	Case No.	Species	Fluorescent Antibody Test	
			Saliva	Urine

S. No.	Case No.	Species	Fluorescent Antibody Test	
			Saliva	Urine
1	47 RL12	Cow	_	-
2	53 RL12	Bull	+	-
3	54 RL12	Buffalo	+	-
4	6 RL13	Cattle	_	_
5	8 RL13	Buffalo	_	_
6	9 RL13	Cow	_	_
7	11 RL13	Buffalo	+	-
8	13 RL13	Dog	_	_
9	14 RL13	Dog	_	_
10	16 RL13	Dog	+	_
11	19RL13	Dog	_	_
12	20RL13	Dog	_	_
13	24 RL3	Cattle	_	_
14	28RL13	Dog	_	_
15	32RL13	Bull	+	_
16	33RL13	Dog	+	_
17	37R13	Buffalo	_	_
18	41RL13	Dog	+	_
19	45RL13	Rabbit	_	_
20	46RL13	Rabbit	_	_
21	47RL13	Equine	_	_
22	52RL13	Buffalo	_	_

S. No.	Case No.	Species	Fluorescent Antibody Test	
			Saliva	Urine
23	53RL13	Dog	_	_
24	54RL13	Dog	_	_
25	56RL13	Cow	+	_
26	61RL13	Dog	+	_
27	3RL14	Buffalo	_	_
28	5RL14	Mongoose	_	_
29	6RL14	Dog	_	_
30	7RL14	Cow	+	_
31	8RL14	Dog	_	_
32	9RL14	Mongoose	_	_
33	10RL14	Buffalo	+	_
34	11RL14	Dog	_	_
35	12RL14	Cow Calf	_	_
36	13RL14	Dog	_	_
37	14RL14	Dog	_	_
38	16RL14	Cow	_	_
39	22RL14	Dog	+	_
40	23RL14	Buffalo	_	_
Total positive cases			12	0

B. IMMUNOCHROMATOGRAPHIC TEST ON SALIVA AND URINE:

Antigen Test Kit was used which is a chromatographic immunoassay for the qualitative detection of Rabies. Following protocol was followed-

1. Sample containing swab was inserted into tube containing 1ml of assay diluent.
2. A swab sample was mixed nicely to extract antigen properly.
3. With the help of disposable dropper four drops of diluted sample was Added in sample hole of kit.
4. Presence of only one band within the control window indicates a negative result.
5. Presence of two color bands in test and control window, no matter which band appears first indicates a positive result.
6. If the purple color band was not visible within the control window after performing the test, the result was considered invalid.

Diagnosis of rabies in Saliva and urine Samples by Immuno-chromatographic Test.

Results of diagnosis of rabies in Saliva and urine Samples by Immuno-chromatographic Test are given under Table 11.

Immunochromatographic Test on Saliva and urine Samples

Rabies Antigen Test Kit (manufactured by BIONOTE) was used in present study for saliva and urine sample testing. The Rabies Antigen Test Kit is a chromatographic immunoassay for the qualitative detection of Rabies virus.

1. Fresh urine and saliva samples were collected with the help of swab.
2. In case of soiled samples, samples were centrifuged (1500 rpm for 8-10 minutes) and supernatant was used for test.

Diagnosis of rabies in Saliva Samples by Immuno-chromatographic Test.

Immunochromatographic Test kit was used for rabies diagnosis in saliva samples. Out 40 samples of 17 (65.38%) cases were positive for rabies (Fig. 4) with sensitivity of 65.38%, accuracy of 77.50% and specificity of 100%. The less sensitivity may be due to intermittent shedding of virus antigen in saliva; which might be below than the detection limit of kit.

A=Control negative B=Control positive C=Test positive
Fig. 4: Immunochromatographic Test on saliva samples

Diagnosis of rabies in urine samples by Immunochromatographic Test

All urine samples were negative for Immunochromatographic Test; it may be due to intermittent presence of rabies antigen in urine.

There was no published literature revealing use the of Immuno-chromatographic Test for detection of rabies from urine samples.

Table11: Diagnosis of rabies in saliva and urine samples by Immuno-chromatographic Test

S. No.	Case No.	Species	Immunochromatographic Test	
			Saliva	Urine
1.	47 RL12	Cow	_	_
2.	53 RL12	Bull	+	_
3.	54 RL12	Buffalo	+	_
4.	6 RL13	Cattle	_	_
5.	8 RL13	Buffalo	_	_
6.	9 RL13	Cow	+	_

S. No.	Case No.	Species	Immunochromatographic Test	
7.	11 RL13	Buffalo	+	_
8.	13 RL13	Dog	_	_
9.	14 RL13	Dog	_	_
10.	16 RL13	Dog	+	_
11.	19RL13	Dog	+	_
12.	20RL13	Dog	_	_
13.	24 RL3	Cattle	_	_
14.	28RL13	Dog	_	_
15.	32RL13	Bull	+	_
16.	33RL13	Dog	+	_
17.	37R13	Buffalo	_	_
18.	41RL13	Dog	+	_
19.	45RL13	Rabbit	_	_
20.	46RL13	Rabbit	_	_
21.	47RL13	Equine	_	_
22.	52RL13	Buffalo	_	_
23.	53RL13	Dog	_	_
24.	54RL13	Dog	_	_
25.	56RL13	Cow	+	_
26.	61RL13	Dog	+	_
27.	3RL14	Buffalo	_	_
28.	5RL14	Mongoose	_	_
29.	6RL14	Dog	+	_

S. No.	Case No.	Species	Immunochromatographic Test	
30.	7RL14	Cow	+	_
31.	8RL14	Dog	_	_
32.	9RL14	Mongoose	_	_
33.	10RL14	Buffalo	+	_
34.	11RL14	Dog	+	_
35.	12RL14	Cow Calf	_	_
36.	13RL14	Dog	_	_
37.	14RL14	Dog	_	_
38.	16RL14	Cow	_	_
39.	22RL14	Dog	+	_
40.	23RL14	Buffalo	+	_
Total positive cases			17	00

PART B of OBJECTIVE 1

DETECTION OF RABIES IN SKIN IN LIVE ANIMALS BY EMPLOYING PCR, REAL TIME PCR AND IMMUNO-PATHOLOGICAL APPROACHES

Another study was conducted to analyze the detection of rabies from skin of 34 animals clinically suspected for Rabies. The 34 animals comprised of 13 dogs (6 females and 7 males), 11 buffaloes (9 females and 2 males), 8 cattle (5 females and 3 males), 1 cat (female) and 1 horse (female).

Table 12: Details of clinically suspected animals

	Age	

Species	< 1.0 yr	1.0-5.0 yr	> 5.0 yr	Total
Buffalo	4	5	2	11
Cattle	1	3	4	8
Dog	7	2	4	13
Cat	1	-	-	1
Horse	-	-	1	1
Total	13	10	11	34

Collection, storage and processing of skin samples

Skin biopsies were collected (Fig. 5) with the help of sterilized 3mm skin biopsy punch (Fig. 6) from forehead or the neck region. Each sample weighed approximately 100mg and consisted of minimum of 10 hair follicles.

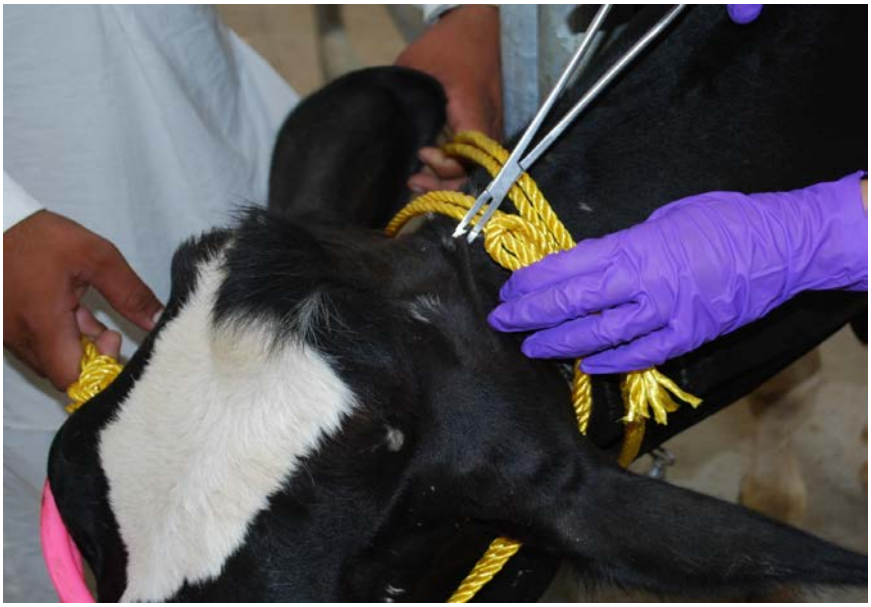


Fig. 5: Skin biopsy collection from nape of the neck region of rabies-suspected cow.

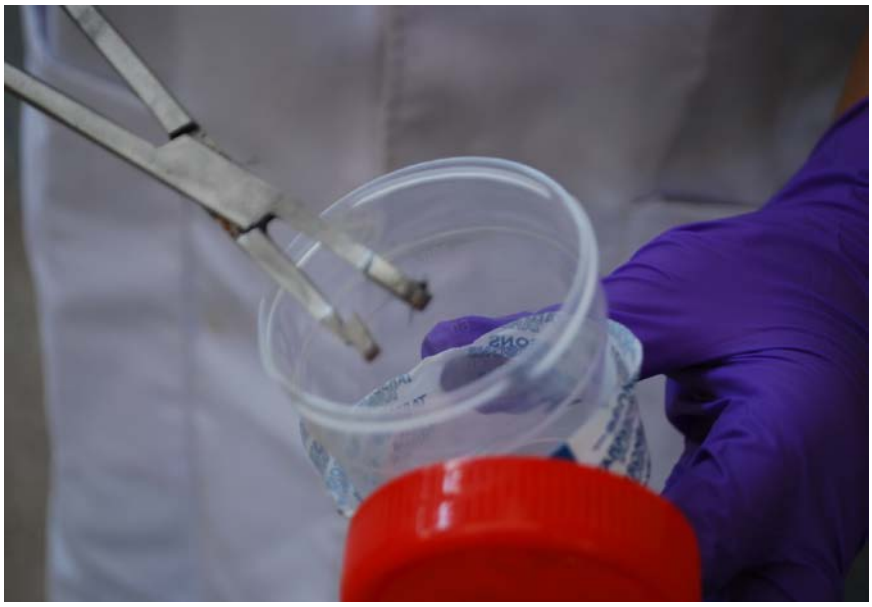


Fig. 6: Collection of punch biopsy.

Skin biopsy sample was cut into very small pieces with sterile scissors and placed in RNA*later* (ambion) for overnight at 4˚C then stored in -80˚C (Ultra low temperature freezer, Haier, Biomedical) for further molecular study.

One portion of the skin sample was kept at -80˚C for frozen section fluorescent

antibody test and remaining portion was stored in 10% neutral buffer formalin for immunohistochemistry.

Controls

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

(ii) **Negative control:** The confirmed negative samples of respective species were used as negative controls.

ANTE MORTEM DIAGNOSIS OF RABIES FROM SKIN USING MOLECULAR APPROACHES

Diagnosis of rabies was done ante mortem from skin using molecular approaches for viral RNA detection.

RNA Extraction from skin

Each skin sample stored in RNA*later* was thawed at room temperature**.** Skin (100 mg) was triturated with the help of sterilized sand, in pestle and mortar. One ml of PBS (pH-7.2) was used as a diluent to make 10% (w/v) suspension. The suspension was then centrifuged at 1500 rpm for 10 mins. The supernatant collected was used for RNA extraction by Trizol method**.** Whole procedure of RNA extraction from tissue suspension was carried out under clean and sterile conditions in a class II Biohazard safety cabinet as under;

1. Out of 1ml of 10% tissue suspension 400µl was collected in autoclaved micro centrifuge tubes. 20µl of 10% SDS solution and 20µl of Proteinase K (20mg/ml) were added and tubes were incubated for 1-3 hrs at 37 ºC.
2. After incubation 200µl of above suspension was collected in two diethylpyrocarbonate (DEPC) treated, autoclaved micro centrifuge tubes (2ml).
3. To each of these tubes, 1ml of Trizol (Invitrogen, USA) was added and was incubated for 5 minutes at room temperature.
4. 200µl of Chloroform was added to each preparation and mixed by inverting the tube multiple times till mixture appeared murky. Mixture was incubated for 5 minutes at room temperature and then centrifuged at 14,000 rpm for 15 minutes at 4ºC.
5. The upper colorless aqueous phase containing RNA was carefully transferred into fresh, clean, sterile, Diethylpyrocarbonate (DEPC) treated, autoclaved micro centrifuge tubes (1.5ml). The inter phase was fairly well-defined and care was taken during transfer of aqueous phase so that lower organic phase (containing Trizol and chloroform) was not disturbed.

6. Isopropanol (500µl) was added to the aqueous phase and mixed gently. Tubes were incubated for 20 mins at room temperature and centrifuged at 12,000-14,000 rpm for 15 min at 4ºC.
7. White or gel like pellet at the bottom of the tube was observed. The supernatant was discarded by gentle inversion (when RNA pellet was intact) or aspirated (when RNA pellet was loose).
8. The RNA pellet was washed with 1ml of 75% ethanol, vortexed and centrifuged @ 9000 rpm for 10 min at 4ºC. Supernatant was decanted without disturbing the pellet.
9. RNA pellet was air dried taking care that the pellet was not over dried.
10. RNA pellet was resolubilized in 20µl of (Tris EDTA) TE buffer and incubated for 10 min at 55-60ºC. Pellet was completely dissolved with repeated pipetting.
11. Micro centrifuge tubes containing RNA were stored at -80ºC until further use.

RNA concentration and quality analysis

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

cDNA synthesis

Total extracted RNA was converted into cDNA using High-Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems, USA) as follows:

6. 10µl of total RNA extracted from each sample was taken into autoclaved PCR tubes (250µl) and 2µl of random primers were added into each tube, making a total volume of 12µl.
7. The RNA Primer mixture was centrifuge briefly and was incubated at 65°C for 10 min and snap cooled on ice. Master mixture was prepared as under:

S. No.	Reaction components	Volume
6	10X RT Buffer	2.0 µl
7	25X dNTP Mix (100mM)	0.8 µl
8	Multiscribe™ Reverse Transcriptase (50ug/ul)	1.0 µl
9	RNase Inhibitor	1.0 µl
10	Nuclease free water	4.2 µl
11	Total volume	8.0 µl

8. Master mixture was briefly centrifuged to spin down the contents and to eliminate air bubbles.
9. Master mixture (8 µl) was added to PCR tubes containing RNA and Primer mixture, making total volume of 20 µl.
10. The above reaction mixture was run in thermal cycler and cycling conditions as under:

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

The resultant cDNA of skin sample was used both for hnRT-PCR and TaqMan real time PCR.

Heminested Reverse Transcriptase-Polymerase Chain Reaction

Primers

Primers targeted towards conserved blocks of L gene were used for first and second round HnRT-PCR. The details are given below (Table 13)

Table 13: Primers targeting conserved blocks of L gene

Primer	Sequence	Gene	Position	Sense
PVO5 Forward primer 1st round	5'ATGACAGACAATT TGAACAA3'	L	7166-7185	+
PVO9 Reverse primer 1st round	5'TGACCATTCCAGC AAGT3'	L	7485-7469	_
PVO8 Reverse primer 2nd round	5'GGTCTGATCTATC TGA3'	L	7400-7415	_

Primers targeted towards nucleoprotein gene were used for first and second round HnRT-PCR. The details are given below (Table 14)

Table 14: Primers targeting the Nucleoprotein gene

Primer	Sequences	Gene	Position	Sense
JW 12 Forward primer 1ST round	5'ATGTAACACCCCTACAATG3'	N	55-73	+
JW 6 DPL Reverse primer 1ST round	5'CAATTGGCACACATTTTGTG3'	N	660-641	-
JW 10 P Reverse primer 2nd round	5'GTCATCAGAGTATGGTGTTC3'	N	636-617	-

Amplification protocol

5. Fresh master mix for first round of the PCR was prepared in PCR tubes on ice according to the following preparation (Table 15)

Table 15: Master mixture components for Primary PCR

S. No.	Master Mix Components (L gene assay)	Master Mix Components (N gene assay)	Volume
1	2X PCR master mix	2X PCR master mix	12.5 µl
2	Nuclease Free Water	Nuclease Free Water	8.5 µl
3	Upstream Primer PV 05 (10pmol)	Upstream Primer JW 12 (10pmol)	1.0µl
4	Downstream Primer PV 09 (10pmol)	Downstream Primer JW 6 (10pmol)	1.0µl
5	Template (cDNA)	Template (cDNA)	2.0 µl
		Total	25 µl

6. The master mix was mixed thoroughly by vortexing, centrifuged briefly to collect residual contents from the walls of the tube and stored on ice.

7. PCR tubes were placed in DNA thermal cycler and subjected to following conditions :

Temperature	Time	No. of Cycles	Remarks
94°C	3 min.	1	Initial Denaturation
94°C	30 s		Denaturation
56°C	45s	35	Annealing
72°C	40s		Extension
72°C	3 min.	1	Final Extension

8. Secondary PCR was performed using 2 µl of the primary PCR product as template. Fresh master mix for second round of the PCR was prepared in PCR tubes on ice according to the following preparation (Table 16).

Table 16: Master mixture components for Secondary PCR

S.No.	Master Mix Components	Master Mix Components	Volume
1	2X PCR master mix	2X PCR master mix	12.5 µl
2	Nuclease Free Water	Nuclease Free Water	8.5 µl
3	Upstream Primer PV 05	Upstream Primer JW 12	1.0µl
4	Downstream Primer PV 09	Downstream Primer JW 10	1.0µl
5	Template (1st round PCR Product)	Template (1st round PCR Product)	2.0 µl
		Total	25 µl

PCR tubes were placed in DNA thermal cycler and subjected to following conditions for the first round of the HnRT-PCR:

Temperature	Time	No. of Cycles	Remarks
94°C	3 min.	1	Initial Denaturation
94°C	30 s	35	Denaturation

56°C	45s		Annealing
72°C	40s		Extension
72°C	3 min.	1	Final Extension

Analysis of PCR products

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

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

Working solution of TBE buffer (1x)

10X TBE	10ml
DW add to	990ml

1.0% agarose was prepared in 1x TBE in flask and was heated to dissolve agarose completely in buffer. Gel was cooled down to temperature of around 50°C and ethidium bromide solution (stock conc. 10mg/ml) was added to final concentration 0.5µg/ml of gel.

The molten gel was poured into holding chamber of submarine gel electrophoresis apparatus and comb was inserted. Gel was allowed to solidify at room temperature for 20 min. Gel was placed in electrophoresis tank which filled with 1X TBE buffer and the gel was immersed in buffer and comb was removed.

Samples were loaded after mixing the PCR product with 6X loading dye (Thermo scientific) (5:1) in parallel with molecular weight marker 100bp plus DNA ladder (Thermo scientific, 0.5µg/µl). Electrophoresis was carried out for 45 min at 70 volts. The gel was visualized and photographed under Gel documentation system (Alpha Innotech, multi Image™ Light cabinet filter positions)

RNA Concentration and Quality analysis

A 260/280 ratio of RNA extracted from skin samples, RNA was in the range of 1.80-1.98. Further the concentration of RNA from skin samples varied from 110.17-946.4 ng/µl (Table 17).

Heminested Reverse Transcriptase Polymerase Chain Reaction (HnRT-PCR)

HnRT-PCR Targeting L gene

Amplification with primers PV05 and PV09 yielded 319 bp first round product and primers PV05 and PV08 yielded 250 bp products (Fig. 7) in second round of HnRT-PCR.

By HnRT-PCR targeting the polymerase (L) gene, rabies virus RNA was diagnosed

in skin of 23 (67.64%) animals out of 34 clinically rabies suspected animals.

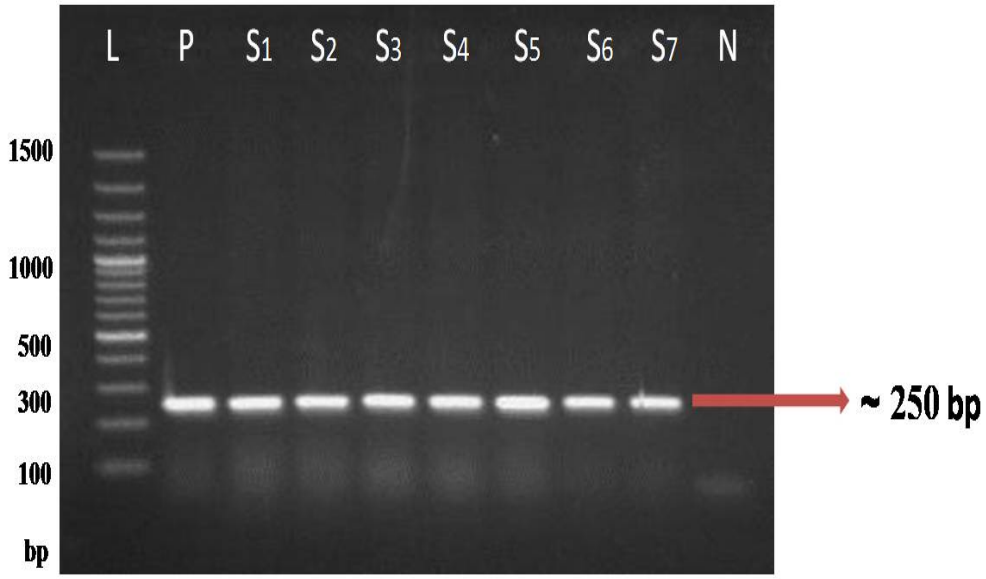


Fig. 7: PCR amplification of L gene of Rabies virus with ~ 250 bp amplicons

L- 100 bp plus DNA ladder (Thermo scientific, 0.5 µg/µl)

Lane P- Positive Control

Lane S1, S2, S3, S4, S5- Clinical Samples

N- Negative Control

Table 17: Analysis of extracted RNA

S. No.	Concentration (ng/µl)	260/280 ratio
1	501.34	1.93
2	374.21	1.88
3	110.23	1.91
4	309.7	1.81
5	964.2	1.83
6	774.2	1.8
7	383.9	2.13
8	267.65	1.85
9	908	1.88
10	125.35	1.82
11	321.56	1.89
12	191.47	1.75
13	161.57	1.81
14	120.17	1.9
15	178.4	1.78
16	330.86	1.81
17	454.6	1.86
18	193.02	1.63
19	422.01	1.89
20	241.9	1.9
21	512.2	1.81
22	656.7	1.88
23	242.7	1.86
24	161.57	1.63
25	130.17	1.89
26	178.4	1.88

27	330.86	1.81
28	454.6	1.91
29	193.02	1.78
30	422.01	1.9
31	241.9	1.88
32	512.2	1.81
33	656.7	1.81
34	242.7	1.86

Targeting N gene

In this study amplification with primers JW12 and JW6 yielded 606 bp first round product and primers JW12 and JW10 yielded 586 bp products (Fig. 8) in second round of HnRT-PCR.

Out of 34 clinically suspected animals, 20 (58.82%) were diagnosed positive for rabies virus RNA in skin biopsies using HnRT-PCR targeting N gene (Table18). Since there is dearth of studies on skin diagnosis by HnRT-PCR targeting N gene thus, for the sake of comparison, the results of HnRT-PCR in present study are being compared with studies on rabies diagnosis from skin samples using nested RT-PCR.

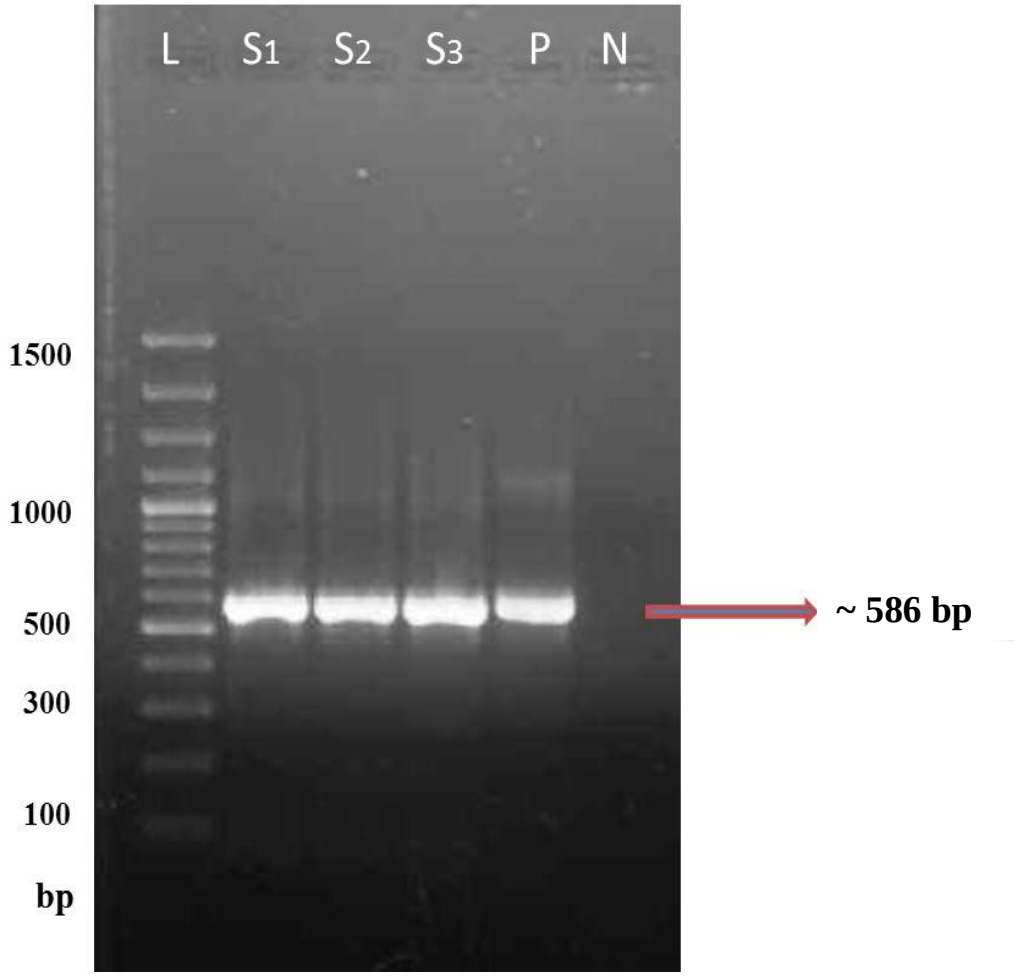


Fig. 8: PCR amplification of N gene of Rabies virus with ~ 586 bp amplicons

L- 100 bp plus DNA ladder (Thermo scientific, 0.5 µg/µl)

Lane P- Positive Control

Lane S1, S2, S3 - Clinical Samples

N- Negative Control

Table 18: Comparative result of HnRT-PCR targeting L gene and N gene for antemortem diagnosis of rabies from skin by HnRT-PCR

S. No.	Case No.	Species	HnRT-PCR L gene	HnRT-PCR N gene
	RL30/12	Dog	+	+
2	RL 32/12	Dog	+	+
3	RL 33 /12	Dog	-	-
4	RL 39 /12	Dog	+	+
5	RL 41 /12	Dog	+	-
6	RL 42/12	Buffalo	+	+
7	RL 43 /12	Buffalo	+	+
8	RL 44 /12	Cattle	-	-
9	RL 45/12	Cattle	-	-
10	RL 46 /12	Dog	-	-
11	RL 47 /12	Cattle	+	+
12	RL 48/12	Buffalo	-	-
13	RL 50 /12	Dog	+	-
14	RL 52 /12	Cattle	+	+
15	RL 53 /12	Bull	+	+
16	RL 54 /12	Buffalo	+	+
17	01-45/13	Buffalo	-	-
18	RL 2/13	Buffalo	+	+
19	RL 3/13	Buffalo	-	-
20	RL 4/ 13	Buffalo	+	+
21	RL 5 /13	Cat	-	-
22	RL 6 /13	Cattle	+	+
23	RL 8 /13	Buffalo	-	-
24	RL/9 13	Cattle	+	+

25	RL 10 /13	Cattle	+	-
26	RL 11 /13	Buffalo	+	+
27	RL 12/13	Dog	-	-
28	RL 13 /13	Dog	+	+
29	RL 14 /13	Dog	-	-
30	RL 16 /13	Dog	+	+
31	RL 19/13	Dog	+	+
32	RL 20/13	Dog	-	-
33	1885/13	Horse	+	+
34	RL 24 /13	Cattle	+	+
Total positive cases			***23***	**20**

TaqMan Real Time PCR

TaqMan Real-Time PCR was performed by Applied Biosystems, Step one plus real time system in Department of Veterinary Microbiology, College of Veterinary Science, GADVASU, Ludhiana

Primers

Primers directed against N gene were used

Table 19: Primers for TaqMan real time directed against N gene

Primer Name	**Sequence**	**Gene**	**Length (nt)**	**Positions**	**Tmax (°c)**	**Remarks**
Primer 8F	5'-TTGACGGGAGGAA TGGAACT-3'	N	20	434-453	62	Bansal *et al* 2012b
Primer 8R	5'-GACCGACTAAAGA CGCATGCT-3'	N	21	477-497	64	Bansal *et al* 2012b
Probe 8Pr	5'-FAM-AGGGACCCC ACTGTT-TAMRA-3'	N	15	458-472	48	Bansal *et al* 2012b

Amplification protocol for TaqMan real time PCR

TaqMan Real-Time PCR was performed on Step one plus Real Time System. The Real Time PCR reaction was carried out in 20 µl (Table 20) final volume and all the samples were run in triplicates.

All samples were also run with endogenous control (18srRNA) to test sample integrity and verification of RNA extraction. Fresh master mix for the PCR was prepared in PCR tubes on ice according to the following preparation:

Table 20: Master mixture constituents for TaqMan Real Time PCR

Components	Volume
TaqMan Master mix	12.5 µl
Forward primer (10 pmol)	1.0 µl
Reverse primer (10 pmol)	1.0µl
Probe (6 pmol)	1.0 µl
Nuclease Free Water	2.5 µl
cDNA	2.0 µl
Total	20 µl

Table 21: Endogenous control assay

Components	Volume
TaqMan Master mix	12.5 µl
18srRNA 2X (primer-probe)	1 µl
Nuclease Free Water	4.5 µl
cDNA	2.0 µl
Total	20 µl

8 strip PCR tubes having reaction mixture was capped with 8 strip optically cleared

flat caps were placed in ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and subjected to following temperature conditions:

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

Analysis

After completion of PCR, critical threshold cycle number (C_T) was determined corresponding to the PCR cycle number at which the fluorescence of the reaction exceeded a value determined to be statistically higher than the background by the ABI prism software.

The C_T values are inversely proportional to the log10 of the amount of template in the PCR. A difference of 1 C_T corresponds to a twofold difference in template amounts. A C_T value less than the mean plus two standard deviations of the negative control wells was considered positive. A C_T value above 35 corresponds to no amplification.

The resultant data were transferred in to an Excel spread sheet, and the resulting graphs were obtained.

Taq Man Real time PCR

All samples were tested positive with endogenous control real time PCR assay and C_T values were found to be in the range of 17.78 to 34.27 for skin samples (Table 22) and maximum number of C_T values was in between 29-32 class interval. The samples in which C_T values were found to be within 35 were considered positive and above 35 were considered negative corresponds to no amplification.

Out of 34 clinically suspected cases, 24 cases were confirmed to be rabid by post-mortem testing of brain tissues by FAT. TaqMan real time PCR could diagnose 23 cases, in live animals, out of 24 confirmed cases of Rabies, which were confirmed to be rabid, post-mortem, by FAT.

Table 22: C_T values in Taq Man Real time PCR assay for *intra-vitam* diagnosis of rabies

S. No.	Case No	Species	C_T value	Results
1.	RL 30 /12	Dog	32.12258	+
2.	RL 32 /12	Dog	26.47682	+
3.	RL 39 /12	Dog	24.08585	+
4.	RL 41 /12	Dog	31.45403	+
5.	RL 42 /12	Buffalo	21.8462	+
6.	RL 43 /12	Buffalo	30.32783	+
7.	RL 47 /12	Cattle	29.41935	+
8.	RL 50/12	Dog	34.27734	+
9.	RL 52 /12	Cattle	31.7329	+
10.	RL 53 /12	Buffalo	21.52078	+
11.	RL 54 /12	Buffalo	24.08585	+
12.	RL 2 /13	Buffalo	26.47682	+
13.	RL 4 /13	Buffalo	32.10932	+
14.	RL 6 /13	Cattle	17.14409	+
15.	RL 8 /13	Buffalo	25.77398	+
16.	RL 9 /13	Cattle	19.02316	+
17.	RL 10 /13	Cattle	33.88545	-
18.	RL 11 /13	Buffalo	30.9695	+

19.	RL 13 /13	Dog	28.73679	+
20.	RL 16 /13	Dog	26.62386	+
21	RL 19/13	Dog	30.96087	+
22	RL20/13	Dog	35.7601	+
23	885/13	Horse	17.7858	+
24	RL 24 /13	Cattle	22.8556	+

ANTE MORTEM DETECTION OF RABIES VIRUS ANTIGEN FROM SKIN BY IMMUNOPATHOLOGICAL APPROACHES

A. FLUORESCENT ANTIBODY TEST of SKIN

Frozen sections of skin were trimmed with fine scissors and were mounted on brass cryostat buttons with dermis upward with a commercial mounting medium (Microm HM 520, frozen section medium, Stephens scientific).

3. The cryostat buttons were placed into the cryostat chamber (Microm HM 520) and allowed to solidify at -20°C. Oblique sections 5-6 μm thick were cut with blade.
4. Two consecutive sections were placed on cleaned glass slides.
5. Five slides were prepared from each tissue block.
6. Slides were fixed in acetone at -20°C for 30 min.
7. Slides were washed twice in phosphate buffers (PBS) (pH 7.6) baths for 1 min each to fix the tissue and remove residual mounting medium.
8. The slides were air dried, and each tissue section was circled with a wax pen.
9. Clarified conjugate (0.1 ml) (lyophilised, adsorbed anti-rabies nucleocapsid conjugate, Bio-Rad, France) was added on each smear and incubated at 37°C for 45 minutes in a moist chamber.
10. Slides were washed twice in phosphate buffers (PBS) (pH 7.6) baths for 1 min each to fix the tissue and remove residual mounting medium
11. A few drops of glycerine buffer were added and covered with a cover slip.
12. The slides were examined using an AHBT3 - RFC reflected light fluorescence and initially done under low-power magnification to locate large and tactile hair follicles and then changed to higher magnification.
13. Examination was concentrated on the periphery of the germinal portion of the hair follicles, where the greatest concentration of nerve follicles is known to be located
14. The presence of distinct apple green fluorescence in the nerve plexus surrounding the follicle (nerve fibers are less common in other areas of the tissue) indicated positive reaction; the absences of such fluorescence indicate negative reaction.

Skin biopsy sample of rabid animals on FAT examination revealed characteristic apple green immunofluorescence in the cytoplasm of neurons around the base of hair follicles. Hair follicles showed diffused perifollicular fluorescence (Fig. 9).

B. IMMUNOHISTOCHEMISTRY OF SKIN

Sectioning of Tissue

Skin tissues which were fixed in 10% buffered formalin saline for 48 hours, were thoroughly washed in running water; dehydrated in ascending grades of alcohol and acetone; cleared in benzene and embedded in paraffin at 58°C.

For immunohistochemical studies 4-5µ thick paraffin embedded tissue sections were cut and mounted on Super frost/ Plus, positively charged microscopic slides (Fisher Scientific, USA). The slides were then kept on hot plate to melt the paraffin at 60° C for 30 minutes and stored till further use.

Polyclonal antiserum

Polyclonal antisera was obtained from rabies diagnostic lab, GADVASU, raised in chinchilla rabbits to be used as primary antibody in immunohistochemistry study. The purified antisera were tested by AGPT for presence of antirabies antibodies.

Protocol for Immunohistochemistry

i) Deparaffinisation

The sections were dewaxed and rehydrated by dipping in EZ-AR™ Common Solution (BioGenex Laboratories Inc., San Ramon, California, USA), and heating at 70° C for 10 minutes in EZ-Retriever ™ System (BioGenex Laboratories Inc., San Ramon, California, USA)

ii) Heat induced Antigen Retrieval

Heat induced antigen retrieval was performed in EZ-AR™ 3 Solution 10X Concentrated antigen retrieval solution, used in 1:10 dilution, at 90°C temperature for 10 min in EZ-Retriever ™ System (BioGenex Laboratories Inc., San Ramon, California).Following HIER the sections were allowed to cool and brought to room temperature. Three washings of 3 minutes each were given in PBS (pH 7.2-7.4). Sections were encircled with hydrophobic pen

iii) Determination of optimal antibody concentration for immuno-histochemical staining.

Optimal concentration of primary antibody was determined by making serial dilution viz. 1:50, 1:100, 1:200, 1:500 and 1:1000 in phosphate buffer saline (PBS) containing 1% bovine serum albumen (BSA) and subjecting them to standard immunohistochemical protocol. The dilution of primary antibody, which gave the best results, was used for further immunohistochemical staining.

iv) Immunohistochemical staining

Immunohistochemical staining was performed by using advanced immPACT™ DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame CA USA).

The endogenous peroxidase was quenched with a solution of 3% H_2O_2 in methanol for 15 min at room temperature in humid chamber, followed by thrice washing with PBS for 3 min each Nonspecific protein adherence was blocked using 5% BSA in PBS for 15 minutes.

The sections were incubated with ready to use normal horse serum-2.5% (Vector Laboratories, Burlingame CA USA) to block non-specific protein binding for 15 min at room temperature in humidified chamber.

Afterwards the sections were incubated with primary polyclonal rabbit anti-rabies antibody (1:1000 dilutions in PBS) antibody for 50 minutes at room temperature in humidified chamber. The sections were then given three washing in PBS for 3 minutes each and incubated in immPRESS™ reagent kit peroxidase universal anti-mouse/ rabbit Ig (Vector Laboratories, Burlingame CA USA) for 30 minutes at room temperature in a humidified chamber followed by three washings with PBS for 3 minutes each.

The antigen-antibody-peroxidase reaction was visualized by using freshly prepared 3, 3'-diaminobenzidine (DAB) solution (30 µl of DAB chromogen with 1 ml of DAB buffer (immPACT™ DAB Peroxidase Substrate Kit-Vector Laboratories, Burlingame CA USA) provided by the manufacturer and adding 5 µl of hydrogen peroxide).Sections were washed in distilled water for 5 minutes and counterstained with Gill's haematoxylin (Merck, Germany) for 30 seconds and washed in running tap water for 5 minutes.

Slides were dehydrated in ascending grades of alcohol, cleared in xylene, mounted in DPX and examined under microscope (BX 61, Olympus Corporation, Japan).

For antibody, a negative control was run by replacing primary antibody with PBS buffer.

Skin samples collected ante mortem from 34 clinically suspected animals were analyzed during the study. Among the 34 samples, 18 (52.94%) were found positive for the presence of rabies virus on the basis of antigen detection by immunohistochemistry in skin samples. Rabies virus antigens were detected in periphery of follicles (Fig.10), in nerve fibers surrounding hair follicle (Fig. 11).

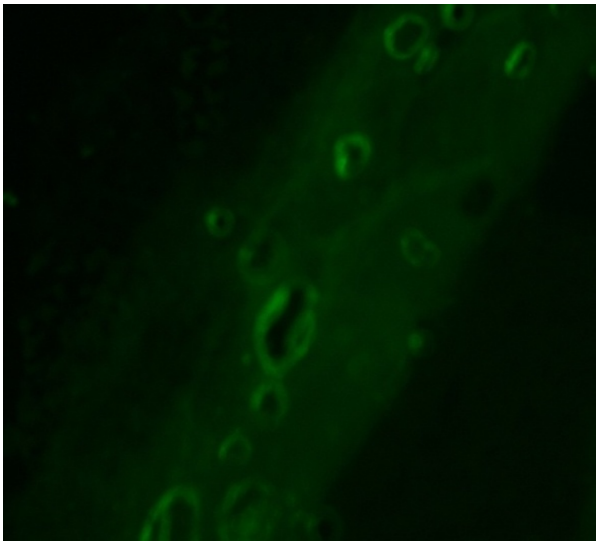


Fig. 9: Diffused fluorescence around hair follicles. FAT X 450

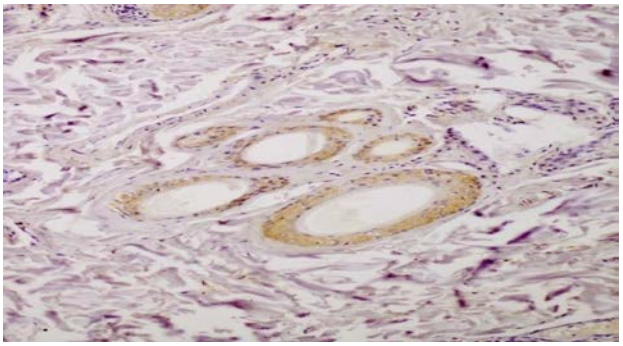


Fig. 10: Hair follicles in dog skin showing positive reaction.
IHC X 200

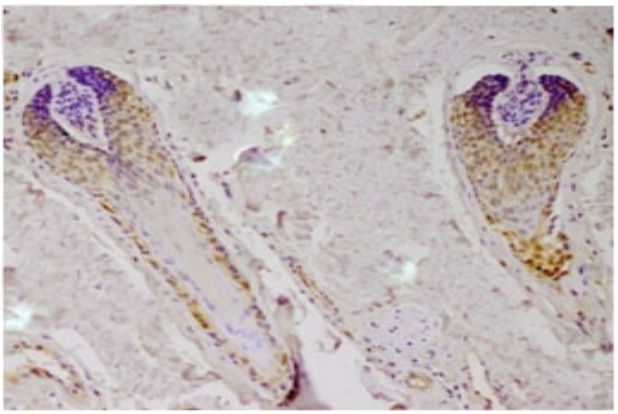


Fig. 11: Hair follicles in buffalo skin showing positive reaction.
IHC X 200

OBJECTIVE No 2:

TO ESTABLISH THE PREVALENCE OF RABIES IN VARIOUS SPECIES OF ANIMALS.

PREVALENCE OF RABIES IN ANIMALS

Naturally prevalent cases of Rabies in various animals that were suspected for Rabies based on history and clinical symptoms were referred to Rabies Laboratory of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana for confirmation of Rabies and to Veterinary Clinics for treatment. All such cases referred from throughout state of Punjab from July 2012 to December 2016 have been incorporated in the present study to assess the naturally prevalent cases of Rabies in various animals in the state.

Year 2012 (July to December)

S.No	Case No	Species	History	RESULT
1	34 RL 2012	Dog	No History	Positive
2	35 RL 2012	Buff Calf	No History	Positive
3	36 RL 2012	Dog	4 Persons bite	Positive
4	37 RL 2012	Dog	No History	Positive
5	38 RL 2012	Dog	No History	Positive
6	39 RL 2012	Dog	Dog bite 15 days back	Positive
7	40 RL 2012	Dog	No History	Negative
8	41 RL 2012	Dog	Paralysis	Positive
9	42 RL 2012	Buff Calf	No History	Positive
10	43 RL 2012	Buff Calf	No History	Positive
11	44 RL 2012	Cattle	No History	Negative
12	45 RL 2012	Cow	Head Pressing before death	Negative
13	46 RL 2012	Dog	Barking, Frequent Urinating	Negative
14	47 RL 2012	Cow	Bellowing	Positive
15	48 RL 2012	Buffalo	No History	Negative
16	49 RL 2012	Buffalo	Off feed 7 days	Negative
17	50 RL 2012	Dog	No History	Positive
18	51 RL 2012	Cow	Salivation	Negative
19	52 RL 2012	Cow	Salivation, Bellowing	Positive
20	53 RL 2012	Bull	Off feed Salivation	Positive
21	54 RL 2012	Buff Calf	Bellowing	Positive

Year 2013

1	01 RL 2013	Dog	Earlier bitten 2 Persons	Positive
2	02 RL 2013	Buffalo	Off feed 7 days	Positive
3	03 RL 2013	Buffalo	Blood from Mouth	Negative
4	04 RL 2013	Buffalo	Bellowing	Positive
5	05 RL 2013	Cat	NI History	Negative
6	06 RL 2013	Cow	Feed and water intake Nil	Positive
7	07 RL 2013	Dog	No history	Negative
8	08 RL 2013.	Buffalo	No history	Negative
9	09 RL 2013	cow	Bellowing, animal not sitting	Positive
10	10 RL 2013	cattle	Bellowing	Positive
11	11 RL 2013	Buffalo	Bellowing	Positive
12	12 RL 2013	Dog	Salivation	Negative
13	13 RL 2013	Dog	History of bite 4 month back	Positive
14	14 RL 2013	Dog	No history	Negative
15	15 RL 2013	Dog	Dog bite	Negative
16	16 RL 2013	Dog	Stray dog	Positive
17	17 RL 2013	Buffalo	Salivation	Negative
18	18 RL 2013	Cow	No history	Negative
19	19 RL 2013	Dog	3 person bitten	Positive
20	20 RL 2013	Dog	Salivation 2 days back	Positive
21	21 RL 2013	Dog	Salivation	Negative
22	22 RL 2013	Cow	No history	Negative
23	23 RL 2013	Dog	Salivation	Negative
24	24 RL 2013	Cow	Off feed	Positive
25	25 RL 2013	Dog	Behavior change	Negative
26	26 RL 2013	Dog	Behavior change	Negative
27	27 RL 2013	Dog	Off feed	Positive
28	28 RL 2013	Dog	No history	Negative
29	29 RL 2013	Dog	1 Person bitten	Positive
30	30 RL 2013	Bull	No history	Negative
31	31 RL 2013	Buffalo	No history	Positive
32	32 RL 2013	Cow calf	behavior change	Positive
33	33 RL 2013	Dog	5 person bitten	Positive
34	34 RL 2013	Dog	No history	positive
35	35 RL 2013	cow calf	Behavior change	Positive
36	36 RL 2013	Buffalo	No history	Negative
37	37 RL 2013	Buffalo	Bellowing	Positive
38	38 RL 2013	Dog	Bitten child	Negative
39	39 RL 2013	Cow	Blind	Negative
40	40 RL 2013	Dog	No history	Negative
41	41 RL 2013	Dog	2 Buffalo bitten	Positive
42	42 RL 2013	Dog	No history	Positive
43	43 RL 2013	Buffalo	No history	Negative
44	44 RL 2013	Dog	Stay dog bit a child	Positive
45	45 RL 2013	Rabbit	No history	Negative
46	46 RL 2013	Rabbit	No history	Negative

47	47 RL 2013	Equine	Head pressing since 3 days	Negative
48	48 RL 2013	Dog	Stray dog biting the animal	Negative
49	49 RL 2013	Cow	No history	Positive
50	50 RL 2013	Cow	No history	Negative
51	51 RL 2013	Cow	No history	Negative
52	52 RL 2013	buffalo	No history	Negative
53	53 RL 2013	Dog	Dog was bitten by rabies suspected dog	Positive
54	54 RL 2013	Dog	Attack of dog in the field	Negative
55	55 RL 2013	Cow	Bellowing	Positive
56	56 RL 2013	Cow	Paralysis	Positive
57	57 RL 2013	Buffalo	Head pressing and aggressive	Positive
58	58 RL 2013	Cow	No history	Negative
59	59 RL 2013	Cow	Suspected for rabies	Positive
60	60 RL 2013	Dog	Behavior change	Positive
61	61 RL 2013	Dog	Person bitten	Positive
62	62 RL 2013	Dog	Salivation	Negative

Year 2014

1	01 RL 2014	Dog	Off feed	Positive
2	02 RL 2014	Dog	Vomiting since 78 days	Negative
3	03 RL 2014	Bovine	off feed, head pressing	Negative
4	04 RL 2014	Dog	Behavior change	Negative
5	05 RL 2014	Mongoose	No history	Positive
6	06 RL 2014	Dog	Dog bite	Positive
7	07 RL 2014	Cow	Head shaking discharge from mouth	Positive
8	08 RL 2014	Dog	Dog was bitten by a stray dog	Negative
9	09 RL 2014	Mongoose	Mongoose was bitten by a dog	Negative
10	10 RL 2014	Buffalo	Off feed, behavior changes	Positive
11	11 RL 2014	Dog	Dog was bitten by a stray dog	Positive
12	12 RL 2014	Cow	High fever since one weak	Negative
13	13 RL 2014	dog	Off feed, salivation	Negative
14	14 RL 2014	Dog	Biting of animal and human	Positive
15	15 RL 2014	Cow	Off feed, salivation, behavior change	Positive
16	16 RL 2014	Cow	No water and feed intake	Negative
17	17 RL 2014	Dog	Off feed behavior change	Positive
18	18 RL 2014	Cow	Blood in urine	Negative
19	19 RL 2014	Cow	salivation, circling	Positive
20	20 RL 2014	Dog	Seizures	Positive
21	21 RL 2014	Cow	Bellowing	Positive
22	22 RL 2014	Dog	Dog was bitten by a stray dog	Positive
23	23 RL 2014	Buffalo	Off feed ,salivation, behavior change	Positive
24	24 RL 2014	Cow	No history	Negative

25	25 RL 2014	Dog	Off feed	Negative
26	26 RL 2014	Dog	History of dog bite	Positive
27	27 RL 2014	Buffalo	Sound from throat at the time of death	Negative
28	28 RL 2014	Dog	Off feed jump from 1st floor	Positive
29	29 RL 2014	Buffalo	Bellowing	Positive
30	30 RL 2014	Dog	One person bite, behavior change	Positive
31	31 RL 2014	Dog	2 Person bite	Positive
32	32 RL 2014	Buffalo	Aggressive behavior	Positive
33	33 RL 2014	Stray dog	No history	Positive
34	34 RL 2014	Dog	Before one month bitten by street dog	Negative
35	35 RL 2014	Dog	no intake of water from last 3 days	Positive
36	36 RL 2014	Dog	No history	Negative
37	37 RL 2014	Buffalo	Off feed behavior change	Positive
38	38 RL 2014	Dog	Sub normal temperature	Negative
39	39 RL 2014	Cow	Off feed	Negative
40	40 RL 2014	Street dog	Salivation, behavior change	Positive
41	41 RL 2014	Dog	No history	Positive
42	42 RL 2014	Cow calf	Calf was bitten by dog before 15 days	Positive
43	43 RL 2014	Dog	Off feed from 4 days	Negative
44	44 RL 2014	Dog	Furious	Positive
45	45 RL 2014	Dog	Suspected for rabies	Negative
46	46 RL 2014	Buffalo	Behavior change	Positive

Year 2015

1	01 RL 2015	Mongoose	One person bitten	Positive
2	02 RL 2015	Cow	Excessive salivation	Positive
3	03 RL 2015	Buffalo	Off feed, salivation	Negative
4	04 RL 2015	Dog	Off feed, circling	Negative
5	05 RL 2015	Dog	Foreign body	Positive
6	06 RL 2015	Cow	Off feed, salivation	Positive
7	07 RL 2015	Stray dog	one person bitten	Positive
8	08 RL 2015	Buffalo	Off feed, behavior change	Positive
9	09 RL 2015	Buffalo	Bellowing	Positive
10	10 RL 2015	Cow	Off feed, salivation	Negative
11	11 RL 2015	Buffalo	Animal recumbent	Negative
12	12 RL 2015	Buffalo	Blot since 5 days	Negative
13	13 RL 2015	Cow	Off feed, salivation	Positive
14	14 RL 2015	Buffalo	No history	Negative
15	15 RL 2015	Cow	Off feed, salivation	Positive
16	16 RL 2015	Buffalo	Bellowing	Positive
17	17 RL 2015	Dog	No history	Negative
18	18 RL 2015	Buffalo	Head pressing	Positive
19	19 RL 2015	Buffalo	No history	Positive
20	20 RL 2015	Cow	Off feed	Positive
21	21 RL 2015	Buffalo	Rabies suspected	Positive

22	22 RL 2015	Buffalo	History of dog bite	Negative
23	23 RL 2015	Cow	Salivation, frequent bellowing	Positive
24	24 RL 2015	Cow	Nervous signs	Negative
25	25 RL 2015	Buffalo	Nervous signs, head down	Negative
26	26 RL 2015	Dog	Rabies suspected	Positive
27	27 RL 2015	Cow	Off feed behavior change	Positive
28	28 RL 2015	Dog	No history	Positive
29	29 RL 2015	Dog	Off feed , salivation	Positive
30	30 RL 2015	Buffalo	Bitten by dog 1 year	Positive
31	31 RL 2015	Dog	Dog bite 15 days back	Negative
32	32 RL 2015	Cow	Off feed	Positive
33	33 RL 2015	Buffalo	No history	Positive
34	34 RL 2015	Cow	No history	Positive
35	35 RL 2015	Buffalo	Bellowing	Positive
36	36 RL 2015	Dog	No history	Positive
37	37 RL 2015	Bull	No history	Positive
38	38 RL 2015	Buffalo	No history	Negative
39	39 RL 2015	cow	Rabies suspected	Negative
40	40 RL 2015	Dog	Dog bite 1 week	Negative
41	41 RL 2015	Stray dog	Suspected for rabies	Positive
42	42 RL 2015	Buffalo	Mongoose bite	Positive
43	43 RL 2015	Mongoose	No history	Positive
44	44 RL 2015	Dog	No history	Positive
45	45 Rl 2015	Dog	Rabies suspected	Positive
46	46 RL 2015	Cow	Bellowing 4-5 days	Positive
47	47 RL 2015	Dog	Rabies suspected	Positive
48	48 RL 2015	Dog	No history	Negative
49	49 RL 2015	Bull	Not taking water	Positive
50	50 RL 2015	Dog	Suspected for rabies	Positive

Year 2016

1	01 RL 2016	Buffalo	Bellowing nervous sine	Positive
2	02 RL 2016	Dog	No history	Positive
3	03 RL 2016	Buffalo	No history	Positive
4	04 RL 2016	Buffalo	No history	Positive
5	05 RL 2016	Cow	Bellowing nervous sine	Positive
6	06 RL 2016	Buffalo	Bellowing	Positive
7	07 RL 2016	Dog	Dog bite 20 days	Positive
8	08 RL 2016	Buffalo	No history	Negative
9	09 RL 2016	Dog	history of biting owner	Positive
10	10 RL 2016	Buffalo	No history	Positive

11	11 RL 2016	Buffalo	Off feed, salivation, fever	Positive
12	12 RL 2016	Buffalo	Bellowing	Positive
13	13 RL 2016	Cow	Off feed	Negative
14	14 RL 2016	Dog	Behavior change	Negative
15	15 RL 2016	Dog	Off feed	Negative
16	16 RL 2016	Buffalo	No history	Negative
17	17 RL 2016	Cow	No history	Positive
18	18 RL 2016	Pig	No history	Positive
19	19 RL 2016	Cow	Off feed, salivation, fever	Positive
20	20 RL 2016	Dog	Off feed, Difficult intake	Positive
21	21 RL 2016	Buffalo	Bellowing	Positive
22	22 RL 2016	Dog	No history	Positive
23	23 RL 2016	Cow	Salivation, behavior change	Positive
24	24 RL 2016	Buffalo	Bellowing	Positive
25	25 RL 2016	Dog	swelling on face, open mouth	Negative
26	26 RL 2016	Buffalo	Off feed, salivation	Positive
27	27 RL 2016	Buffalo	No history	Positive
28	28 RL 2016	Cow	No history	Negative
29	29 RL 2016	Cow	Bellowing, off feed	Positive
30	30 RL 2016	Dog	No history	Positive
31	31 RL 2016	Cow	Salivation, fever	Negative
32	32 RL 2016	Dog	History of dog bite	Positive
33	33 RL 2016	Buffalo	No history	Positive
34	34 RL 2016	Cow	Behavior change, salivation	Negative
35	35 RL 2016	Buffalo	Dog bite on neck region	Positive
36	36 RL 2016	Dog	History of biting other dogs and people	Positive
37	37 RL 2016	Dog	Sudden death	Negative
38	38 RL 2016	Dog	Circling	Negative
39	39 RL 2016	Deer	Sudden death	Negative
40	40 RL 2016	Dog	No history	Positive
41	41 RL 2016	Dog	History of biting	Negative
42	42 RL 2016	Buffalo	Bellowing, circling, behavior change	Negative
43	43 RL 2016	Dog	No history	Positive
44	44 RL 2016	Mule	Bite history from stray dog	Positive
45	45 RL 2016	Buffalo	Reduced feed intake, difficult breathing	Positive
46	46 RL 2016	Dog	animal was getting treatment dog bite	Positive
47	47 RL 2016	Cow	Of feed, salivation, fever	Positive
48	48 RL 2016	Dog	Dog bitten before 2 month	Positive
49	49 RL 2016	Cattle	Off feed, salivation	Positive
50	50 RL 2016	Cow	Suspected for rabies	Positive
51	51 RL 2016	Cow	Off feed, salivation, behavior change	Positive
52	52 RL 2016	Cow	Off feed, salivation,	Positive
53	53 RL 2016	Bull	Off feed, salivation	Positive
54	54 RL 2016	Cow	Paralysis, Difficult intake, salivation	Positive
55	55 RL 2016	Buffalo	Dog bite and mandible fracture	Negative
56	56 RL 2016	Dog	Behavior change, recognized owner	Positive

57	57 RL 2016	Dog	Off feed, behavior change	Positive
58	58 RL 2016	Dog	Off feed, salivation	Positive
59	59 RL 2016	Dog	Stray dog aggressive, biting people	Positive
60	60 RL 2016	Buffalo	Salivation	Negative
61	61 RL 2016	Dog	Person bitten, off feed	Positive

During the period of study, 243 animals were suspected for Rabies based on their history and clinical symptoms. These animals included 108 dogs, 60 buffaloes, 60 cows, 4 mongooses, 3 cow bulls, two each of cats and rabbits and one each of horse, pig, mule, and deer. The animals were tested for Rabies, post-mortem, by Fluorescent Antibody Test wherein 150 animals were found positive for Rabies. The rabid animals included 68 dogs, 38 cows, 36 buffaloes, three mongooses, two cow bulls and one each of cat, pig and mule.

Thus rabies was found to be most prevalent in dogs, followed by cows and buffaloes. The details of 243 cases tested in the study are as under;

Year 2012 to 2016 (1st July,2012 to 31 Dec, 2016)

Species	Positive	Negative	Total
Dog	68	40	108
Buffalo	36	24	60
Cow Bull	2	1	3
Cow	38	22	60
Cat	1	1	2
Rabbit	0	2	2
Horse	0	1	1
Pig	1	0	1
Mongoose	3	1	4
Mule	1	0	1
Deer	0	1	1
Total	**150**	**93**	**243**

Year wise break up of all cases suspected for Rabies and tested by FAT post-mortem are as under;

Year 2012 (1st July to 31 Dec)

Species	Positive	Negative	Total
Dog	7	2	9
Buffalo	3	2	5
Cow Bull	2	0	2

Cow	2	3	5
Total	**14**	**7**	**21**

Year 2013

Species	**Positive**	**Negative**	**Total**
Dog	15	14	29
Buffalo	6	6	12
Cow Bull	0	1	1
Cow	10	6	16
Cat	0	1	1
Rabbit	0	2	2
Horse	0	1	1
Total	**31**	**31**	**62**

Year 2014

Species	**Positive**	**Negative**	**Total**
Dog	16	10	26
Buffalo	6	2	8
Cow	5	5	10
Mongoose	1	1	2
Total	**28**	**18**	**46**

Year 2015

Species	**Positive**	**Negative**	**Total**
Dog	11	5	16
Cow	10	3	13
Buffalo	12	7	19
Mongoose	2	0	2
Total	**35**	**15**	**50**

Year 2016

Species	**Positive**	**Negative**	**Total**
Dog	19	9	28
Buffalo	9	7	16
Cow	11	5	16
Pig	1	0	1
Cat	1	0	1
Deer	0	1	1
Mule	1	0	1
Total	**42**	**22**	**64**

OBJECTIVE No 3:

TO COMPARE THE SENSITIVITY OF PCR, REAL TIME PCR ON SALIVA, SKIN AND URINE, ELISA AND FLUORESCENCE POLARIZATION ON SERUM & IMMUNO-FLUORESCENCE ON SKIN BIOPSY DETECTION AS *INTRA VITAM* DIAGNOSTIC APPROACHES OF RABIES IN ANIMALS.

PART A of OBJECTIVE 3:

COMPARISON OF SENSITIVITY OF PCR, REAL TIME PCR ON SALIVA, URINE AND SKIN

In the study conducted on saliva and urine samples in 40 animals, clinically suspected for Rabies comprising of 17 dogs, 8 buffaloes, 10 cattle, 2 rabbit, 2 mongooses and 1 mare, the sensitivity, specificity and accuracy of molecular approaches viz. HnRT-PCR, TaqMan real time PCR as well as immunopathological approaches viz. Immunochromatographic Test and FAT was compared for ante mortem diagnosis of rabies in saliva and urine samples.

Since, FAT is recommended worldwide as a standard technique for diagnosis of rabies on neural tissue, after death of animal by World Health Organization, so, HnRT-PCR, TaqMan real time PCR and FAT employed on saliva and urine samples were compared with FAT on brain for detecting the sensitivity, specificity and efficacy of these molecular techniques.

The sensitivity, specificity and accuracy of various tests applied were calculated using formulae:

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

$$\text{Specificity} = \frac{\text{True negative}}{\text{False positive + True negative}} \times 100$$

$$\text{Accuracy} = \frac{\text{True positive + True negative}}{} \times 100$$

True positive + False positive + False negative + True negative

Sensitivity, Specificity and Accuracy of diagnosis of rabies in saliva samples by HnRT-PCR targeting L gene

HnRT-PCR diagnosed rabies viral RNA in saliva of 20 (50%) animals with 76.92% sensitivity, 100% specificity and 85% accuracy.

Sensitivity, Specificity and Accuracy of diagnosis of rabies in urine samples by HnRT-PCR targeting L gene

HnRT-PCR detected rabies viral RNA in urine of 17 cases with 65.39% sensitivity, 77.50% accuracy and 100% specificity.

Sensitivity, Specificity and Accuracy of diagnosis of rabies in saliva and urine samples by HnRT-PCR Targeting N gene

Primers for first cycle yielded 606 bp product and primers for second cycle yielded 586 bp product of HnRT-PCR.

Sensitivity, Specificity and Accuracy of diagnosis of rabies in saliva samples by HnRT-PCR Targeting N gene

Out of 40 suspected (Table 6) cases 19 (47.50%) cases were diagnosed positive for rabies in saliva samples with 73.08 % sensitivity, 82.50% accuracy and 100% specificity.

Sensitivity, Specificity and Accuracy of diagnosis of rabies in HnRT-PCR Targeting N gene on urine samples.

Out of 40 clinically suspected (Table 23) animals, 16 (40%) cases were diagnosed positive for rabies in urine samples with 61.54% sensitivity, 75% accuracy and 100% specificity.

Sensitivity, Specificity and Accuracy of diagnosis of rabies in saliva samples by Taq Man Real time PCR

TaqMan real time PCR detected rabies in saliva samples of 67.64% (22/40) cases with sensitivity of 84.62%, accuracy 90% and specificity 100%.

Sensitivity, Specificity and Accuracy of diagnosis of rabies in urine samples by Taq Man Real time

TaqMan real time PCR detected rabies viral genome in urine samples of 47.50% (19/40) cases (Table 9) with sensitivity of 73.08%, accuracy 82.50% and specificity 100%.

Sensitivity, Specificity and Accuracy of diagnosis of rabies in saliva smears by **Fluorescent Antibody Test**

Out of 40 Saliva smears tested by FAT, 12 (30%) revealed apple green immunofluorescence. Sensitivity, accuracy and specificity for Fluorescent Antibody Test on saliva smears were 46.15%, 60% and 100% respectively.

Sensitivity, Specificity and Accuracy of diagnosis of rabies in urine smears by Fluorescent Antibody Test

No urine smear of rabies suspected animals was found positive for FAT. This may be due to intermittence presence of rabies antigen in urine. Sensitivity, accuracy and specificity of FAT on urine was 00%, 35% and 100% respectively.

Sensitivity, Specificity and Accuracy of diagnosis of rabies in Saliva Samples by Immuno-chromatographic Test.

Immunochromatographic Test kit was used for rabies diagnosis in saliva samples. Out 40 samples of 17 (65.38%) cases were positive for rabies with sensitivity of 65.38%, accuracy of 77.50% and specificity of 100%.

Sensitivity, Specificity and Accuracy of diagnosis of rabies in urine samples by Immunochromatographic Test

All urine samples were negative for Immunochromatographic Test; it may be due to intermittent presence of rabies antigen in urine.

Table 23: Comparison of Sensitivity, Specificity and Accuracy of diagnosis of rabies in urine samples by molecular and immunopathological techniques

Test	**Urine / Saliva**	**Sensitivity**	**Specificity**	**Accuracy**
HnRT-PCR L gene	**Urine**	65.39%	100%	77.50%
	Saliva	76.92%	100%	85%
HnRT-PCR N gene	**Urine**	61.54%	100%	75.00%

	Saliva	73.08%	100%	82.5%
TaqMan Real time PCR	**Urine**	73.08%	100%	82.50%
	Saliva	84.62	100%	90%
Immunochromatographic Test	**Urine**	0%	100%	35%
	Saliva	65.38%	100%	77.5%
FAT	**Urine**	0%	100%	35%
	Saliva	46.15%	100%	60%

Molecular approaches are more sensitive as compared to immunopathological approaches for detecting rabies virus in saliva and urine samples. Between TaqMan Real time PCR and Hemi-nested PCRcomparison of sensitivity of detection of rabies reveals that TaqMan real time PCR is the more sensitive technique.

Sensitivity of detection of rabies in saliva samples with HnRT-PCR targeting L gene, HnRT-PCR N gene and TaqMan Real time PCR was 76.92%, 73.08% and 84.62% respectively while for urine samples sensitivity of HnRT-PCR targeting L gene, HnRT-PCR N gene and TaqMan Real time PCR was 65.39%, 61.54% and 73.08% respectively. Comparison of saliva and urine samples for detection of rabies with molecular techniques show the higher sensitivity for saliva samples. L gene was found to be more sensitive and accurate than nucleoprotein gene for detection of rabies virus. This might be due to highly conserved nucleotide blocks of L gene and lower threshold of detection of L gene.

PART B of OBJECTIVE *3*:

COMPARISON OF SENSITIVITY OF ELISA AND FLUORESCENCE POLARIZATION ON SERUM

Fluorescence Polarization Assay (FPA) and ELISA testing of sera samples: The Fluorescence Polarization Assay is a homogenous assay that uses fluorescence polarization technology for the detection of antibodies in serum. In principle, a tracer comprising of a

fluorophore conjugated to an antigen is added to a serum sample to make a mixture. The fluorescence polarization of the mixture is then measured. The presence of serum antibodies against the antigen is determined from the measured fluorescence polarization of the mixture, e.g., that it is higher than that of a control. In this study, vaccine (Rabipur) was used as an antigen. The vaccine was first autoclaved and then conjugated with fluorophore, FITC and run in Sephdex 50 Column. Elutes were collected and were screened in nanodrop. Fluorescence intensity was measured in all elutes. 20 ul of serum was added to 2 ml of 0.1M phosphate buffer, pH 7.0 containing 0.01% NaN_3, 0.15M NaCl and 0.5% lithium dodecyl sulfate. The diluted serum was incubated at ambient temperature for 2 minutes and a blank reading in a fluorescence polarization analyzer was taken. Tracer (Vaccine antigen conjugated with fluorescein isothiocyanate) 10 ul was added, mixed and incubated for two minutes after which a final reading was taken in the fluorescence polarization analyzer. DMSO was used as blank. Data was expressed in millipolarization units (mP).

A total of 26 sera samples collected from suspected cases of rabies and tested by ELISA, were compared for quantification of anti-rabies antibodies by Fluorescence Polarization Assay (FPA). The animals incorporated in the study included dogs (n=15), buffaloes (n=7) and cattle (n=4). The millipolarization (mP) units ranged from 40 mP to 289mP. A cut off of 103 mP could differentiate the positive and negative samples as all the samples positive by gold standard (FAT) were showing more than 103 mP values.

S. No.	Case No.	Species	Sex	Age	Optical Density	Titer (IU/µl)	ELISA Result	FPA (mP)	FPA Result	Post-Mortem FAT Confirmation
1	RN01-4787	Buffalo	F	5Y	0.122	-	-	77	-	ND
2	D12-14276	Dog	F	7Y	0.446	0.26	+	227	+	+
3	34 RL-15	Cow	F	2.5Y	0.376	0.183	+	230	+	+
4	D0-84	Dog	F	2Y	0.402	0.21	+	111	+	+
5	RN3-960	Buff. calf	F	10M	0.0167	-	-	42	-	ND
6	RN11-5380	Cow	F	3Y	0.0119	-	-	79	-	ND
7	D0-5286	Dog	M	4Y	0.374	0.181	+	265	+	+
8	RN05-1826	Cow	F	6Y	0.425	0.23	+	171	+	+
9	RN10-4787	Buffalo	F	10 Y	0.0164	-	-	40	-	-
10	RN02-458	Buffalo	F	4Y	0.325	0.128	+	289	+	+
11	DO-1272	Dog	F	1.5Y	0.355	0.160	+	132	+	ND
12	DO-2046	Dog	M	3Y	0.261	-	+	43	-	ND

S. No.	Case No.	Species	Sex	Age	Optical Density	Titer (IU/µl)	ELISA Result	FPA (mP)	FPA Result	Post-Mortem FAT Confirmation
13	D0-5747	Dog	F	5Y	0.0145	-	-	42	-	ND
14	DO-2047	Dog	M	4.5Y	0.448	0.302	+	103	+	+
15	RN05-2239	Cow	F	8Y	0.157	-	+	78	-	ND
16	DO-5174	Dog	F	4Y	0.109	-	-	73	-	ND
17	RN05-2153	Buffalo	F	7Y	0.449	0.264	+	119	+	+
18	DO-1540	Dog	F	6Y	0.346	0.155	+	110	+	+
19	DO-2016	Dog	M	14Y	0.424	0.236	+	122	+	+
20	RN04-1462	Buffalo	F	6Y	0.344	0.148	+	196	+	ND
21	DO-429	Dog	M	4Y	0.144	-	-	38	-	ND
22	DO-720	Dog	F	8Y	0.35	0.155	+	115	+	ND
23	DO-3548	Dog	F	3Y	0.347	0.152	+	135	+	+
24	DO -3207	Dog	M	1.5Y	0.0169	-	-	75	-	ND
25	03-1027	Buffalo	F	6.5Y	0.364	0.170	+	117	+	+
26	DO-1140	Dog	F	8Y	0.028	-	-	38	-	ND

ND= Not Done (Brain tissue not available)

Out of 26 sera samples of animals suspected for Rabies, wherein anti-rabies antibodies were tested by both ELISA and FPA, the brain tissue of 13 animals was available for confirmation of rabies by dFAT. These animals comprised of seven dogs, four buffaloes and two cows.

Anti-rabies antibodies could be detected in 12 out of 13 animals. And all 12 animals were confirmed for rabies by dFAT on post -mortem investigation. Further, detection of anti-rabies antibodies detected by ELISA and FPA complimented each other in efficacy in detection of anti-rabies antibodies by FPA and ELISA in rabid animals.

S. No.	Case No.	Species	Sex	Age	Optical Density	Titer (IU/µl)	ELISA Result	FPA (mP)	FPA Result	Post-Mortem FAT Confirmation
1	D12-14276	Dog	F	7Y	0.446	0.26	+	227	+	+
2	34 RL-15	Cow	F	2.5Y	0.376	0.183	+	230	+	+

S. No.	Case No.	Species	Sex	Age	Optical Density	Titer (IU/µl)	ELISA Result	FPA (mP)	FPA Result	Post-Mortem FAT Confirmation
3	D0-84	Dog	F	2Y	0.402	0.21	+	111	+	+
4	D0-5286	Dog	M	4Y	0.374	0.181	+	265	+	+
5	RN05-1826	Cow	F	6Y	0.425	0.23	+	171	+	+
6	RN10-4787	Buffalo	F	10 Y	0.0164	-	-	40	-	-
7	RN02-458	Buffalo	F	4Y	0.325	0.128	+	289	+	+
8	DO-2047	Dog	M	4.5Y	0.448	0.302	+	103	+	+
9	RN05-2153	Buffalo	F	7Y	0.449	0.264	+	119	+	+
10	DO-1540	Dog	F	6Y	0.346	0.155	+	110	+	+
11	DO-2016	Dog	M	14Y	0.424	0.236	+	122	+	+
12	DO-3548	Dog	F	3Y	0.347	0.152	+	135	+	+
13	03-1027	Buffalo	F	6.5Y	0.364	0.170	+	117	+	+

The animals that were confirmed to be rabid at post-mortem by FAT also revealed anti-rabies antibodies. The findings, therefore, suggest that in absence of history of vaccination, detection of anti-rabies antibodies could be considered for diagnostic purpose.

PART C of OBJECTIVE 3:

IMMUNO-FLUORESCENCE ON SKIN

FAT on frozen skin sections, detected rabies ante mortem, from skin biopsy samples with a sensitivity of 79.16% (Table 24). The difference in the sensitivities may be due to variables like the site of biopsy, number of sections examined and the amount of viral antigen present. The technique was highly specific (100%) and fairly accurate (85.29%) as compared to gold standard test.

Table 24: Summary of samples analyzed by FAT on frozen skin section and FAT on brain (gold standard) and calculation of sensitivity, specificity and

accuracy

Test	**Brain FAT Positive**	**Brain FAT Negative**	**Total**
FAT on frozen skin section Positive	19	0	19
FAT on frozen skin section Negative	5	10	15
Total	24	10	34
Sensitivity=19/19+5x100, Specificity=10/0+10, Accuracy=19+10/19+0+10+5			

IHC on formalin fixed skin samples

Sensitivity obtained for detection of rabies from skin using IHC was 75.00% (Table 25). IHC detected rabies from skin biopsy samples with an accuracy of 82.35% and specificity of 100%.

Table 25: Summary of samples analyzed by IHC on formalin fixed skin and FAT on brain and calculation of sensitivity, specificity and accuracy

Test	**Brain FAT Positive**	**Brain FAT Negative**	**Total**
IHC Positive	18	0	18
IHC Negative	6	10	16
	24	10	34
Sensitivity=18/18+6x100, Specificity=10/0+10, Accuracy=18+10/18+0+10+6			

Comparison of molecular and immunopathological techniques for diagnosis of rabies viral RNA in skin samples

On the basis of comparison of sensitivity, specificity and accuracy of detection of rabies from skin samples by molecular and immunopathological approaches, it was concluded

that TaqMan real time PCR was the most sensitive, specific and accurate. Skin biopsies of all animals diagnosed positive by brain FAT were also found positive with TaqMan real time PCR except one case; which may be due to inadequate level of virus load at the time of sampling.

Table 26: Comparative result of FAT and IHC on skin for *intra-vitam* diagnosis of rabies

S. No.	CASE No.	Species	FAT Skin	IHC Skin
1	RL30/12	Dog	-	-
2	RL 32/12	Dog	+	+
3	RL 33 /12	Dog	-	-
4	RL 39 /12	Dog	+	+
5	RL 41 /12	Dog	-	-
6	RL 42/12	Buffalo	+	+
7	RL 43 /12	Buffalo	+	+
8	RL 44 /12	Cattle	-	-
9	RL 45/12	Cattle	-	-
10	RL 46 /12	Dog	-	-
11	RL 47 /12	Cattle	+	+
12	RL 48/12	Buffalo	-	-
13	RL 50 /12	Dog	-	-
14	RL 52 /12	Cattle	+	+
15	RL 53 /12	Bull	+	+
16	RL 54 /12	Buffalo	+	+
17	01-45/13	Buffalo	-	-
18	RL 2/13	Buffalo	+	+
19	RL 3/13	Buffalo	-	-
20	RL 4/ 13	Buffalo	+	+
21	RL 5 /13	Cat	-	-
22	RL 6 /13	Cattle	+	+

23	RL 8 /13	Buffalo	-	-
24	RL/9 13	Cattle	+	+
25	RL 10 /13	Cattle	-	-
26	RL 11 /13	Buffalo	+	-
27	RL 12/13	Dog	-	-
28	RL 13 /13	Dog	+	+
29	RL 14 /13	Dog	-	-
30	RL 16 /13	Dog	+	+
31	RL 19/13	Dog	+	+
32	RL 20/13	Dog	-	-
33	#05-1885/13	Horse	+	+
34	RL 24 /13	Cattle	+	+

positive for rabies by FAT on frozen skin section.

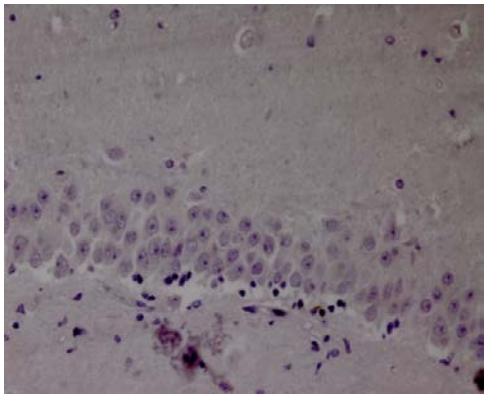


Fig. 22: Negative control of IHC – Section of hippocampus showing absence of reaction. IHC X400

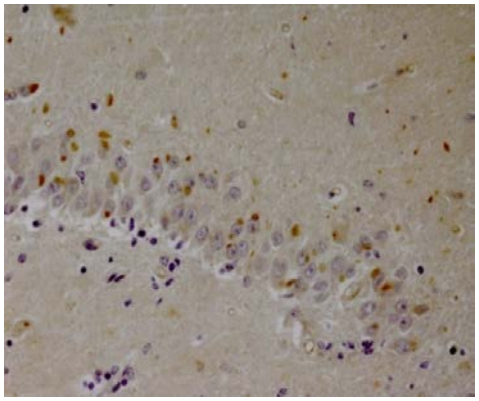


Fig. 23: Section of hippocampus of rabid dog showing sharply demarcated Negri bodies. IHC X 400

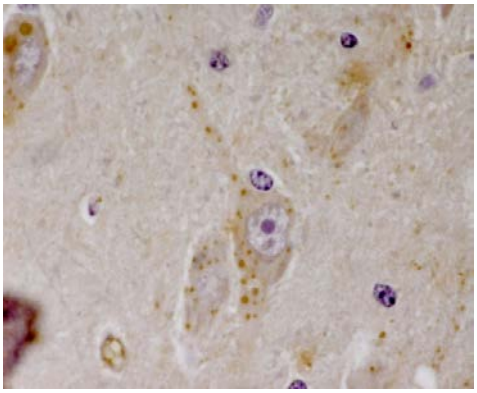


Fig. 24: Section of cerebellum of rabid cow showing multiple Negri bodies in the neuron. IHC-X 400

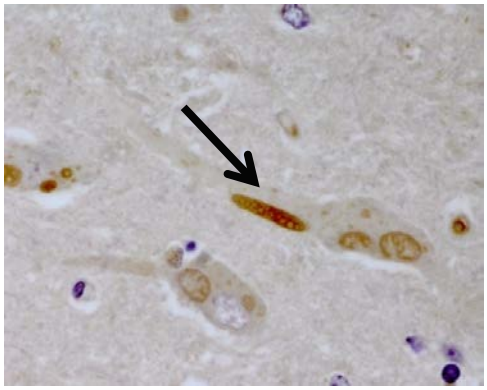


Fig. 25: Section of cerebellum of rabid cow showing internal bodies (arrow) within Negri bodies. IHC-X1000

Objective No 4:

TO ASCERTAIN THE MOST SUITABLE SECRETION/BODY TISSUE FOR *INTRA-VITAM* DIAGNOSIS IN ANIMALS.

PART A of OBJECTIVE 4

TO ASCERTAIN MOST EFFICACIOUS LABORATORY TECHNIQUE FOR *INTRA-VITAM* DIANOSIS OF RABIES

Efficacy of the gold standard FAT for *intra-vitam* diagnosis of Rabies

The gold standard FAT was tried for rabies diagnosis in secretion/excretion and non-nervous body tissues as under;

Diagnosis of rabies in saliva smears by **FAT**

Out of 40 saliva smears tested by FAT, sensitivity, accuracy and specificity for Fluorescent Antibody Test on saliva smears were 46.15%, 60% and 100% respectively.

Diagnosis of rabies in urine smears by FAT

Out of 40 urine samples tested, no urine smear of rabies suspected animals was found positive for FAT. This might be due to intermittent presence of rabies antigen in urine or due to incompatibility of FAT as a diagnostic approach for detection of rabies antigen from urine excretion. Sensitivity, accuracy and specificity of FAT on urine was 0%, 35% and 100% respectively.

Thus, it is revealed that the gold standard FAT in the nervous tissue for diagnosis of Rabies is rendered poor diagnostic approach once it is attempted on secretion viz. saliva/excretion viz. urine of rabid animals.

Further, FAT was also tried for diagnosis of Rabies from body tissue outside the nervous system. i.e. skin wherein FAT on frozen skin sections, detected rabies ante mortem, from skin biopsy samples with a sensitivity of only 79.16%.

Therefore, in order to ascertain the most suitable secretion/body tissue for *intra-vitam* diagnosis of Rabies in animals, it is pertinent to establish the most sensitive laboratory technique, other than FAT, that otherwise is the gold standard for post-mortem diagnosis of Rabies from nervous tissue.

The sensitivity, specificity and accuracy of various techniques need to be compared for detection of rabies from most feasible clinical samples from a live animals suspected for Rabies.

For diagnosis of Rabies in live animals, the most suitable secretion is saliva, the most

suitable excretion is urine and most suitable body fluid is serum and the most suitable body tissue is skin.

Thus, the sensitivity, specificity and accuracy of detection of Rabies by different laboratory approaches for detection of Rabies from saliva, urine, serum and skin shall have to be compared to arrive at the most suitable secretion/body tissue for *intra-vitam* diagnosis of Rabies in animals.

However, before considering any laboratory technique as a candidate approach for non-conventional detection of rabies from secretion/excretion and non-nervous system body tissue, it is important to assess such laboratory techniques in the brain tissue in comparison with the established gold standard in nervous tissue i.e. FAT.

Thus, various molecular and immunopathological laboratory techniques were tested for efficacy of diagnosis of Rabies from brain tissue in comparison with FAT.

A study was conducted on brain samples from 50 animals suspected for rabies. The animals comprised of 27 dogs, 11 cattle, 9 buffaloes, and 3 mongooses.

Different diagnostic approaches incorporated in the study were molecular viz. HnRT-PCR, TaqMan real time PCR and Immunopathological approaches viz. IHC and Immunochromatographic Test for comparison with FAT for postmortem diagnosis of rabies from brain tissues.

HnRT-PCR targeting N gene

Out of 50 brain samples collected from suspected animals, 30 samples were found positive by HnRT-PCR targeting N gene. Results obtained in this study were in 100% complete agreement to detection of Rabies by FAT from brain, wherein 30 animals out of 50 animals tested, were confirmed to be rabid.

HnRT-PCR targeting L gene

Out of 50 brain samples collected from suspected animals, 30 samples were again found positive by HnRT-PCR targeting L gene. Detection of rabies by this technique was also similar to the gold standard technique FAT on brain tissues.

TaqMan Real time PCR

All the FAT positive samples (n=30) were also diagnosed positive by TaqMan real time PCR.

Table 27: Diagnosis of rabies in brain tissues by TaqMan Real time PCR

S. No.	Case No	Species	C_T value	Results
1.	01 RL 14	Dog	28.546	+

S. No.	Case No	Species	C_T value	Results
2.	02 RL 14	Dog	37.693	–
3.	03 RL 14	Buffalo	36.604	–
4.	04 RL 14	Dog	39.893	–
5.	05 RL 14	Mongoose	21.000	+
6.	06 RL 14	Dog	22.600	+
7.	07 RL 14	Cow	18.959	+
8.	08 RL 14	Dog	36.778	–
9.	09 RL 14	Mongoose	39.497	–
10.	10 RL 14	Buffalo	19.860	+
11.	11 RL 14	Dog	39.005	–
12.	12 RL 14	Cow	29.825	+
13.	13 RL 14	Dog	37.250	–
14.	14 RL 14	Dog	15.564	+
15.	15 RL 14	Cow	21.221	+
16.	16 RL 14	Cow	39.531	–
17.	17 RL 14	Dog	27.760	+
18.	18 RL 14	Cow	37.972	–
19.	19 RL 14	Cow	18.730	+
20.	20 RL 14	Dog	19.914	+
21.	21 RL 14	Cow	33.872	+
22.	22 RL 14	Dog	23.969	+
23.	23 RL 14	Buffalo	27.497	+
24.	24 RL 14	Cow	39.834	–

S. No.	Case No	Species	C_T value	Results
25.	25 RL 14	Dog	38.712	–
26.	26 RL 14	Dog	34.707	+
27.	27RL 14	Buffalo	38.374	–
28.	28 RL 14	Dog	31.212	+
29.	29 RL 14	Buffalo	21.313	+
30.	30 RL 14	Dog	19.387	+
31.	31 RL 14	Dog	18.947	+
32.	32 RL 14	Buffalo	27.462	+
33.	33 RL 14	Dog	19.217	+
34.	34 RL 14	Dog	39.000	–
35.	35 RL 14	Dog	19.829	+
36.	36 RL 14	Dog	38.947	–
37.	37 RL 14	Buffalo	18.071	+
38.	38 RL 14	Dog	37.263	–
39.	39 RL 14	Cow	39.860	–
40.	40 RL 14	Dog	18.211	+
41.	41 RL 14	Dog	17.235	+
42.	42 RL 14	Cow	21.265	+
43.	43 RL 14	Dog	38.256	–
44.	44 RL 14	Dog	22.356	+
45.	45 RL 14	Dog	36.259	–
46.	46 RL 14	Buffalo	19.356	+
47.	01 RL 15	Mongoose	21.265	+

S. No.	Case No	Species	C_T value	Results
48.	02 RL 15	Cow	23.265	+
49.	03 RL 15	Buffalo	37.326	–
50.	04 RL 15	Dog	39.265	–

Sensitivity, Specificity and Accuracy of molecular approaches on brain samples

Out of 50 brain samples 30 samples were found positive by FAT, HnRT-PCR and TaqMan Real time PCR with sensitivity, specificity and accuracy of 100%.

Thus, the present study establishes that all three molecular approaches viz. HnRT-PCR targeting N gene, HnRT-PCR targeting L gene and Taqman Real Time PCR are equally effective as compared to FAT for diagnosis of Rabies from brain tissue.

Thus, molecular approaches are rendered suitable candidates for consideration as the most suitable laboratory technique for *intra-vitam* diagnosis of Rabies since FAT, which is a gold standard for diagnosis of Rabies from nervous tissue, has exhibited poor sensitivity for diagnosis of Rabies from secretion/excretion and body tissue outside nervous tissue.

Immuno-pathological laboratory techniques:

Immuno-pathological laboratory techniques used in the study included IHC and Immunochromatographic test:

IHC on Brain Samples

Out of 50 brain tissue samples examined from clinically suspected animals, 28 were diagnosed positive.

Immunochromatographic Test

Out of 50 suspected brain tissue homogenates 29 samples were detected positive for rabies on the basis of Immunochromatographic Test kit.

Sensitivity, Specificity and Accuracy of immunopathological approaches on brain samples

Immunochromatographic test kit:

In this study 96.60%, 100% and 98.03% sensitivity, specificity and accuracy was obtained with immunochromatographic test kit for rabies antigen detection in brain tissue.

Immunohistochemistry (IHC):

Rabies antigen was detected by IHC in 28 brain samples (out of 30 samples found positive by FAT) with 93.75% sensitivity, 100% specificity and 96.15% accuracy.

So this study reveals that molecular techniques, HnRT-PCR targeting N gene and L gene and TaqMan Real time PCR exhibit sensitivity, specificity and accuracy comparable to FAT in nervous tissue. And immunochromatographic test and IHC also reveal reliable degree of sensitivity, specificity and accuracy in comparison to post-mortem detection of rabies from brain tissues by FAT.

Table 28: Comparison of molecular, immunopathological and histopathological techniques for detection of rabies virus in brain samples.

Test	Sensitivity	Specificity	Accuracy
HnRT-PCR N gene	100%	100%	100%
HnRT-PCR L gene	100%	100%	100%
TaqMan Real time PCR	100%	100%	100%
FAT	100%	100%	100%
Immunochromatographic Test	96.60%	100%	98.03%
IHC	93.75%	100%	96.15%

PART B of OBJECTIVE 4

TO ASCERTAIN MOST EFFICACIOUS SECRETION/EXCRETION/BODY TISSUE FOR *INTRA-VITAM* DIAGNOSIS OF RABIES

URINE

With above studies, the sensitivity, specificity and accuracy of laboratory techniques for detection of Rabies has been established. However, this study has rendered most sensitive, specific and accurate laboratory techniques for detection of Rabies from the most convenient tissue i.e. nervous tissue. However, sensitivity of detection of Rabies RNA/antigen from body fluids/tissue shall reveal the most suitable secretion/excretion /body tissue for *intra-vitam* diagnosis of rabies

Sensitivity, specificity and accuracy comparison of molecular and immuno-pathological techniques for diagnosis of rabies from urine of rabid animals

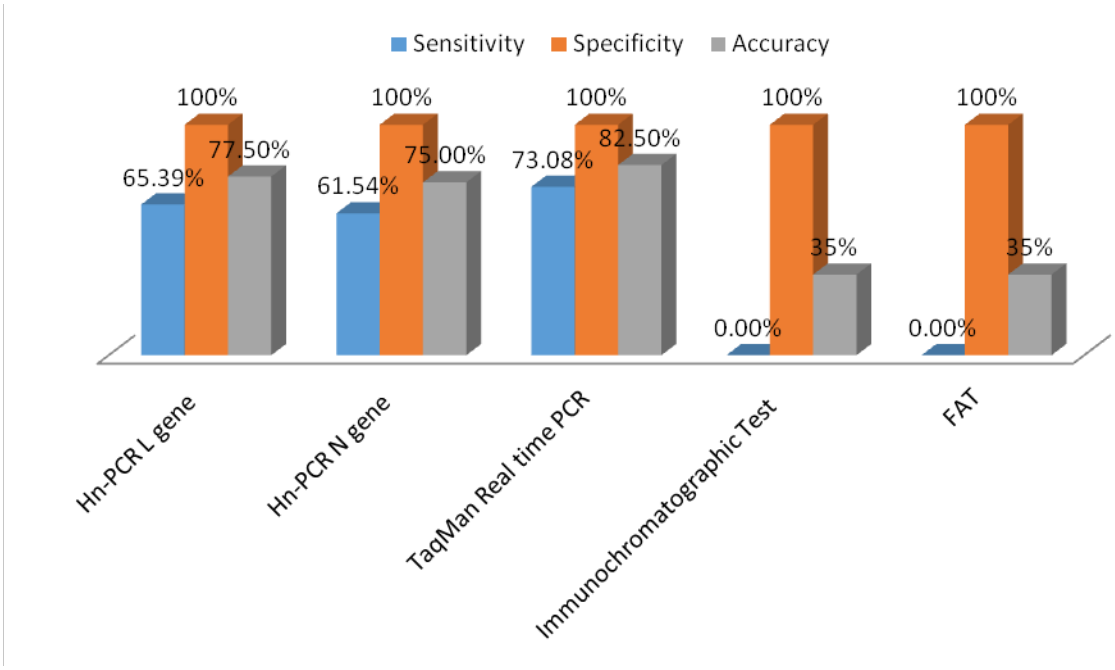


Fig. 12: Comparison of molecular and immunopathological techniques for detection of rabies in urine samples.

From detection of Rabies infection from urine of rabid animals, HnRT-PCR, Taqman Real time PCR, Immuno-chromatograhic test kit and FAT were tested for sensitivity, specificity and accuracy of detection of Rabies virus. Whereas all laboratory techniques exhibited 100% specificity (Fig. 12), Taqman Real time PCR could detect Rabies with highest sensitivity of 73.08% and accuracy of 82.5% followed by HnRT-PCR targeting L gene with sensitivity of 65.39% and accuracy of 77.5%.

Since even the highest sensitivity of Taqman real time PCR is short of a minimum sensitivity threshold of 90%, thus, urine cannot be considered as a suitable approach for *intra-vitam* diagnosis of Rabies.

SALIVA

From detection of Rabies infection from saliva of rabid animals, HnRT-PCR, Taqman Real time PCR, Immuno-chromatograhic test kit and FAT were tested for sensitivity, specificity and accuracy of detection of Rabies virus. Whereas all laboratory techniques exhibited 100% specificity, Taqman Real time PCR could detect Rabies with highest sensitivity of 84.62% and accuracy of 90% followed by HnRT-PCR targeting L gene with sensitivity of 76.92% and accuracy of 85% (Fig. 13).

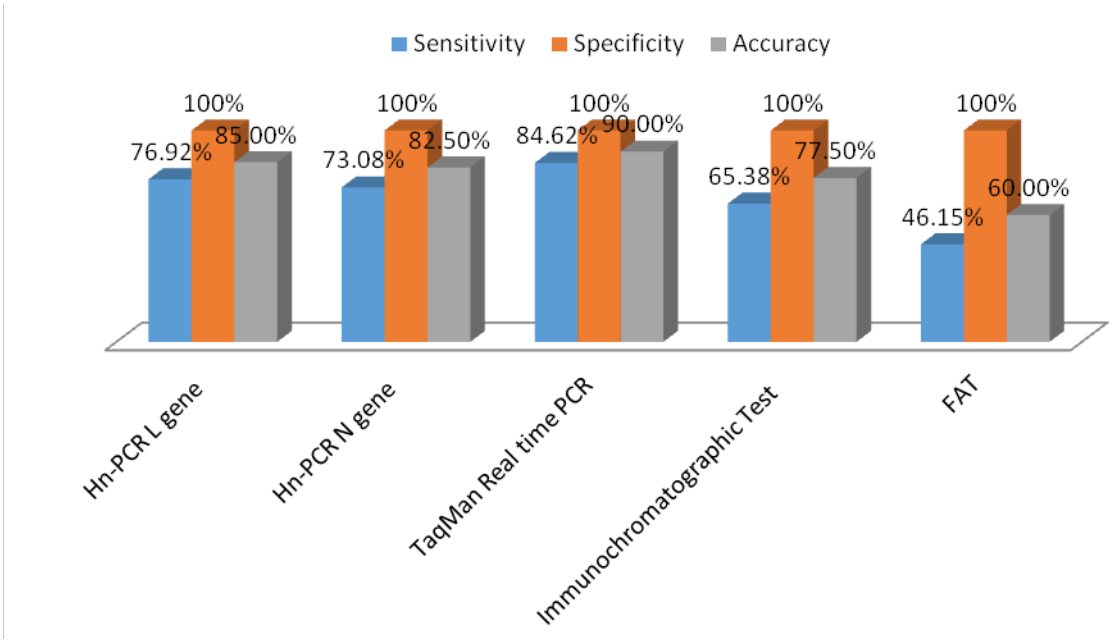


Fig. 13: Comparison of molecular and immunopathological techniques for detection of rabies in saliva samples.

Based on the studies conducted in this project, sensitivity of molecular and immuno-pathological techniques for diagnosis of rabies in saliva and urine samples is depicted hereunder (Table 29).

Table 29: Comparison of Sensitivity of diagnosis of rabies in in saliva and urine samples urine samples by molecular and immunopathological techniques

Sample	FAT	HnRT-PCR L gene targeted	HnRT-PCR N gene targeted	TaqMan Real time PCR	Immuno chroma tographic Test
Saliva	46.15%	76.92%	73.08%	84.62%	65.38%
Urine	0.00%	65.39%	61.54%	73.08%	0.00%

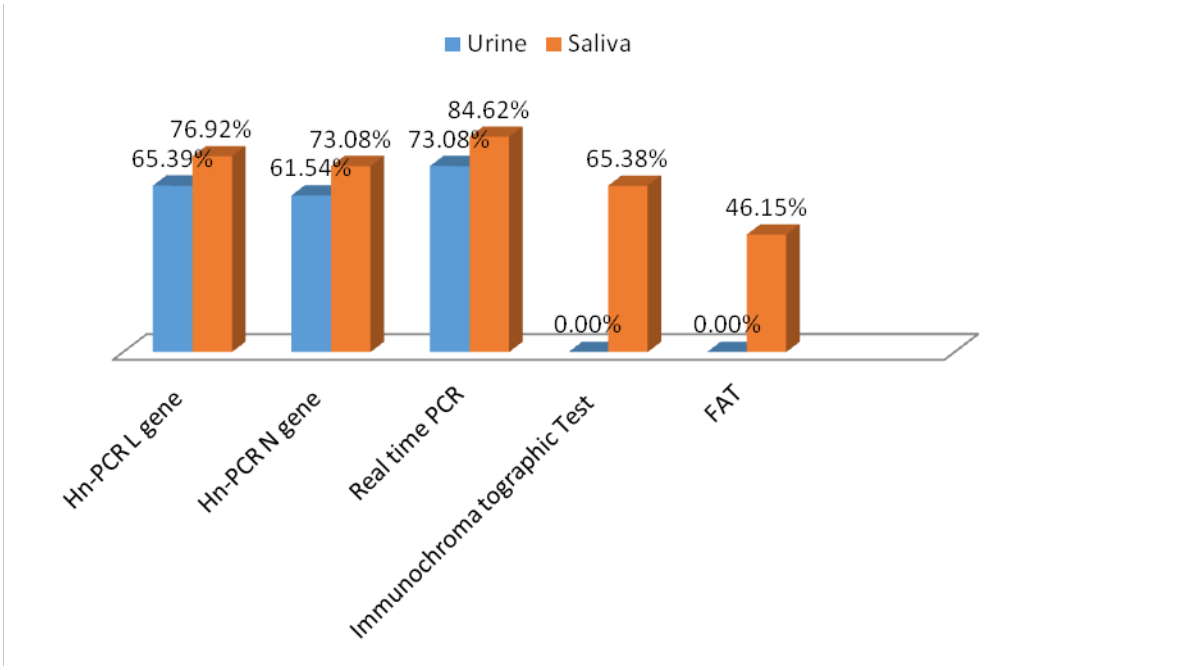


Fig. 14: Sensitivity comparison of molecular and immunopathological techniques for diagnosis of rabies in urine and saliva.

Although sensitivity of detection of Rabies is higher in saliva than in urine, yet it too falls short of the minimum 90% threshold sensitivity (Fig. 14) for the approach to be considered as a candidate for the suitable diagnostic approach for ante-mortem diagnosis of Rabies.

SKIN

HnRT-PCR targeting L Gene, HnRT-PCR targeting N Gene, and Taqman Real time PCR were tested as the most suitable molecular candidates for detection of Rabies from skin of live rabid animals.

HnRT-PCR targeting L gene on skin samples

HnRT-PCR while targeting the L gene, detected rabies from skin biopsy samples with a sensitivity of 95.83%, with an accuracy of 97.05% and specificity of 100%.

Table 30: Summary of samples analyzed by HnRT-PCR targeting L gene for calculation of sensitivity, specificity and accuracy for detection of Rabies from skin of rabid animals

Test	Brain FAT Positive	Brain FAT Negative	Total
HnRT-PCR L gene positive on	23	0	23

skin			
HnRT-PCR L gene negative on skin	1	10	11
Total	24	10	34
Sensitivity=23/23+1x100, Specificity=10/0+10, Accuracy=23+10/23+0+10+1			

HnRT-PCR targeting N gene

HnRT-PCR while targeting the N gene, detected rabies from skin biopsy samples with a sensitivity of 83.33%. Further, HnRT-PCR while targeting the N gene, detected rabies from skin biopsy samples with an accuracy of 88.23% and specificity of 100%.

Table 31: Summary of samples analyzed by HnRT-PCR targeting N gene and FAT, as gold standard and calculation of sensitivity, specificity and accuracy

Test	**Brain FAT Positive**	**Brain FAT Negative**	**Total**
HnRT-PCRN gene positive on skin	20	0	20
HnRT-PCRN gene negative on skin	4	10	14
Total	24	10	34
Sensitivity=20/20+4x100, Specificity=10/0+10, Accuracy=20+10/20+0+10+4			

TaqMan real time PCR

Sensitivity of TaqMan real time PCR for detecting of rabies from skin biopsy sample was 95.83% with an accuracy of 97.05 % and specificity of 100%.

Table 32: Summary of samples analyzed by Taq Man real time PCR and FAT, as gold standard and calculation of sensitivity, specificity and accuracy

Test	**Brain FAT Positive**	**Brain FAT Negative**	**Total**

TaqMan Real Time PCR positive on skin	23	0	23
TaqMan Real Time PCR negative on skin	1	10	11
Total	24	10	34
Sensitivity=23/23+1x100, Specificity=10/0+10, Accuracy=23+10/23+0+10+1			

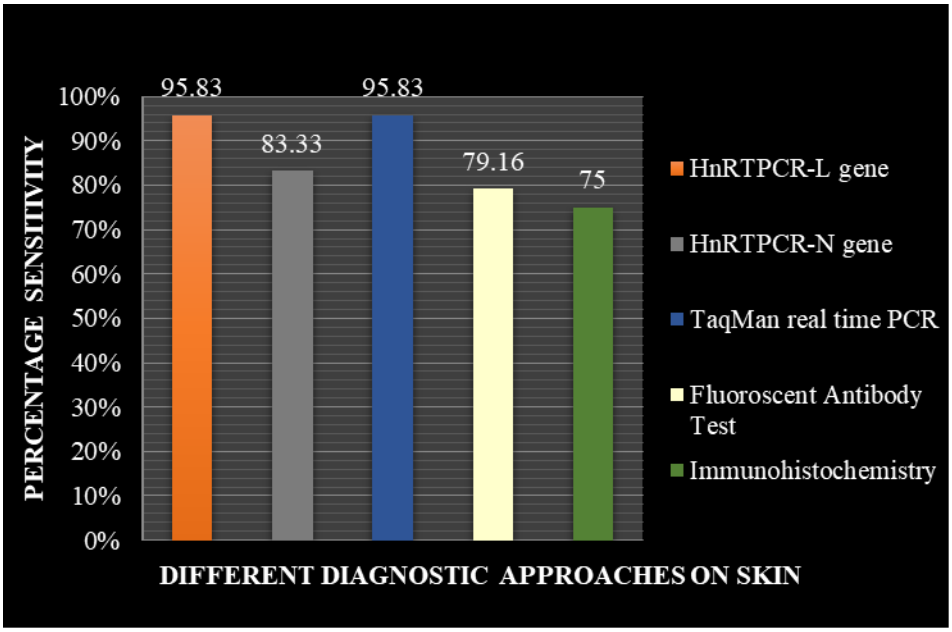


Fig. 34: Sensitivity comparison of molecular and immunopathological techniques on skin sample

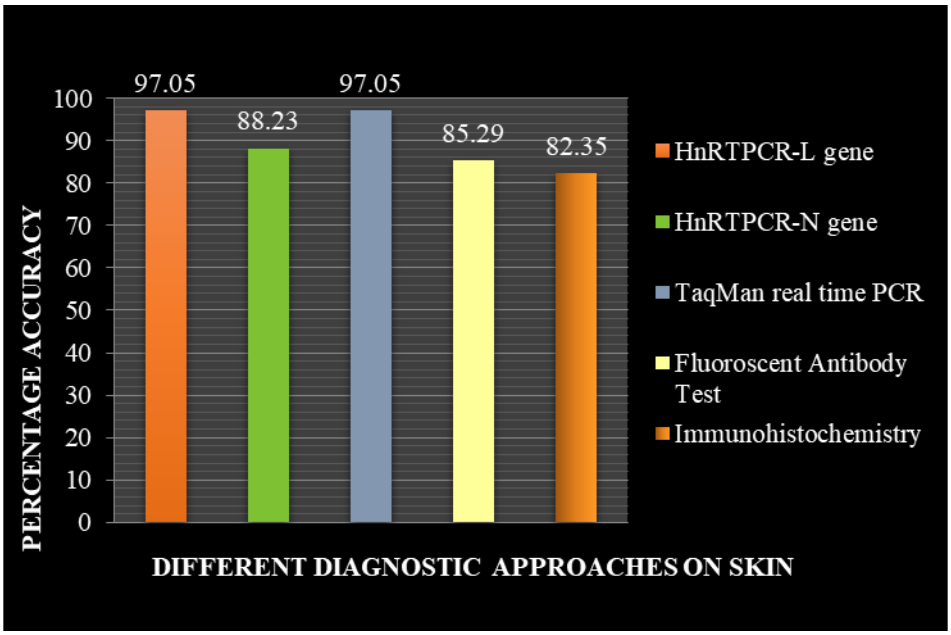


Fig. 35: Accuracy comparison of molecular and immunopathological techniques on skin samples

Table 33: Comparison of molecular and immunopathological techniques for detection of rabies viral RNA from skin of rabid animals

Test	Sensitivity	Specificity	Accuracy
HnRT-PCR L gene	95.83%	100%	97.05%
HnRT-PCR N gene	83.33%	100%	88.23%
TaqMan Real time PCR	95.83%	100%	97.05%
FAT	79.16%	100%	85.29%
IHC	75%	100%	82.35%

Thus, there are two diagnostic approaches when applied on skin of rabid animals could detect Rabies with more than 90% sensitivity. These diagnostic approaches are HnRT-PCR while targeting L gene and TaqMan real Time PCR. Both these molecular approaches match in sensitivity (95.85%), specificity (100%) and accuracy (97.05%) for diagnosis of rabies from skin biopsy samples of live animals suspected for rabies.

Based on the above findings of the present study, it is established that skin is the most (and only) suitable body tissue for *intra-vitam* diagnosis of Rabies. The two most suitable diagnostic approaches that can be applied on skin of suspected animal are Taqman real time PCR and HnRT-PCR targeting L gene.

OBJECTIVE No *5*:

TO CORRELATE THE DETECTION OF RABIES ANTIGEN WITH THE PERIODIC QUANTIFICATION OF RABIES ANTIBODIES IN THE SERUM OF THE INFECTED LIVE ANIMALS AS WELL AS THE SYMPTOMS EXHIBITED BY SUSPECTED ANIMALS.

PART A of OBJECTIVE *5*

QUANTIFICATION OF RABIES ANTIBODIES IN THE SERUM OF THE INFECTED LIVE ANIMALS

Sample Collection

The whole blood samples were collected from 32 unvaccinated, (16 dogs.10 buffalos, 6 cows) animals for detection of antibody titer against rabies antibody, presented at Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana. Blood was collected aseptically from the dogs by vein puncture from medial saphenous or femoral vein by using 22 gauge needles and from the rabies suspected large animals from Jugular vein puncture by using 16 or 18 gauge needle. Whole blood 2 to 3 ml was collected in AcCuvet disposable vials that contained gel and clot activator. The samples were kept at room temperature for 20-30 minutes and then, kept in refrigerator for 40–60 minutes at 4°C and after this centrifugation was done and serum was separated carefully to avoid haemolysis and stored at -20 °C in the deep freeze until analysis.

Antibody analysis

Protocol of Enzyme Linked Immunosorbent Assay (ELISA) by quantitative method

1. The ELISA test was performed by using the PLATELIA™ Rabies II kit ad usum Veterinarium as per the procedure described by the manufacturer Bio-Rad (92430 Marnes-La-Coquette France). Reagents were stored at 2–8 °C, and placed at room temperature for at least 30 minutes before use.

2. One micro plate was taken & required number of rows (R1) was unmasked from the protective packaging. Remaining rows were kept masked by protective packaging.
3. Then, carefully established the sample distribution and identification plan (Table 34)

Table: 34 Micro plate set-up for Quantitative assay

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	R3	S4	E1	E9	…	…	…	…	…	…	…	…
B	R3	S4	E2	E10	…	…	…	…	…	…	…	…
C	R4a	S3	E3	…	…	…	…	…	…	…	…	…
D	R4b	S3	E4	…	…	…	…	…	…	…	…	…
E	S6	S2	E5	…	…	…	…	…	…	…	…	…
F	S6	S2	E6	…	…	…	…	…	…	…	…	…
G	S5	S1	E7	…	…	…	…	…	…	…	…	…
H	S5	S1	E8	…	…	…	…	…	…	…	…	E80

4. The given control reagents namely R3, R4a and R4b as well as the unknown sera were diluted 1/100 in R6 reagent (Appendix I).
5. Quantification standards were prepared and the test was performed.
6. Transferred 100 µl of diluted samples, controls and quantification standards to corresponding micro plate wells according to the pre-established layout of the test.
7. Then, the micro plate was covered with adhesive film and pressed firmly all over the plate to ensure a tight sealing.
8. Incubated the micro plate that has already been sensitized with the rabies virus glycoprotein at 37±1 ºC for 60±5 minutes.
9. Prepared wash solution (R2) according to the given procedure.

10. The given concentrated horseradish peroxidase-conjugated protein reagent was diluted 1:10 in the wash solution reagent (R2) and prepared solution was known as conjugate solution (R7).
11. A negative (R3) and two positive (R4a and R4b) controls were tested in each run. The negative control was made a synthetic material and the positive controls were made of therapeutic rabies immunoglobulin in synthetic material. The positive controls calibrated against the WHO international standard for rabies immunoglobulin. R4b (4 equivalent units (EU)/µl) were used to establish a reference curve after successive two-fold serial dilutions (S5 = 2 EU/µl, S4 = 1 EU/µl, S3 = 0.5 EU/µl, S2 = 0.25 EU/µl, S1 = 0.125 EU/µl). The test was used as a quantitative (with the use of R3, R4a, R4b controls and preparation of the serial dilutions S1–S5 from R4b) method.
12. After 60 minutes, removed the adhesive film from micro plate and three wash cycles were performed by using a micro plate washer.
13. The plate was dried by inverting on the absorbent paper.
14. Distributed 100 µl of horseradish peroxidase-conjugated protein A (R7) in each well of micro plate and it was wrapped by adhesive film. Micro plate was incubated at 37±2 °C for 60±5 min.
15. Just before use; prepared the enzymatic development solution by mixing of reagent R8 and R9.
16. The incubated adhesive film was removed and five washings were given with the linked peroxidase conjugate solution.
17. The plate was dried by inverting on the absorbent paper.
18. After that, added 100 µl of freshly prepared enzymatic development solution reagent (R8 + R9) to each well away from direct light and then incubated the plate in dark by wrapping in a carbon paper at room temperature for 30 minutes.
19. The enzyme reaction was stopped by adding 100 µl of 1N sulphuric acid solution (R10) to each well according to the same sequence and same distribution rate followed for the addition of previous solution.
20. Thoroughly wiped the bottom of the plate and then optimal density was read at 450 nm by using ELISA reader (type PR3100, Bio-Rad) within 30 minutes after stopping the reactions. Serum titers were expressed in equivalent unit per millilitre (EU/ml).

Quantitative determination of antibody titre

In test included all standards and controls (R3, R4a and R4b, S5, S4, S3, S2 and S1) for each test run. The threshold value is equal to mean of two OD of the Quantification standards (S3). The S3 Quantification standard corresponds to the seroconversion threshold value 0.5 IU/µl. Quantification of anti-rabies antibodies in a sample is determined by comparing the optical densities (O.Ds) of the sample to standard curve. Serum titers were expressed as equivalent units per ml or IU/µl. Optical densities (O.Ds) mean values of the samples were situated between S1 (0.125EU/ml) and S6 (4 EU/ml). The standard curve was plotted. Optical densities (O.Ds) taken on vertical Y- axis & controls concentration taken on X – axis of standard curve. At the point of the intersection with the standard curve, drawn a vertical line to the X- axis, which gives corresponding value of titers of that sample in IU/µl.

Formula for conversion of OD value in to titer (IU/µl)

$$y = 0.9115x + 0.2083$$

(wherein y is OD and X is titer in IU/µl)

Counter Immuno Electrophoresis (CIE)

Hyper-immune sera

Liquid equine rabies immunoglobulin was procured from Central Research Institutes Kasauli, HP. Each ml of serum contained enzyme refined, purified and concentrated, immunoglobulin not less than 300 IU/µl of horses /mules hyper immunized against rabies for the specific neutralization of rabies virus.

Antigen

Purified inactivated rabies vaccine, prepared on VERO cells. Freeze-dried Powder after reconstitution, each 0.5 mL of reconstituted dose contains Rabies virus, Wistar rabies PM/WI 38 1503-3M strain (inactivated) ≥ 2.5 IU.

Antibody detection by CIE

Procedure: 0.9 per cent agarose gel was prepared in buffer (pH 8.2) (Appendix II). Each microscopic slide was layered with 3.5 ml of molten agarose and allowed to solidify at room temperature for half an hour. Two wells were punched in agarose bed with a distance of 0.3 cm from each.

Electrode compartments of electrophoresis chamber were filled with buffer.

The slide was placed on platform of electrophoresis apparatus and troughs were connected with Whatmanno.1 strips. 17µl of standard antigen was added on cathode side and 17µl of test serum was added on anode side. Electrophoresis was allowed to run at 5 mA for 2 hours. Then slide was removed from electrophoresis apparatus and incubated for overnight at 4°C.

Staining of gel: The slides were dried at 37°C by keeping a wet filter paper over them. After drying, the filter paper was removed and then slide was stained with 1 per cent coomassie brilliant blue stain (Appendix III) for 15 minutes and destained with destaining solution (Appendix IV).

Modified Counter Immuno Electrophoresis

Preparation of 0.9% agarose gel: Agarose gel (0.9%) (Appendix V) was prepared in buffer (pH 8.2). Each microscopic slide was layered with 3.5 ml of molten agarose and allowed to solidify at the room temperature for half an hour.

The sera samples were subjected to quantitative analysis of antibodies to rabies virus by employing MCIE as reported by Sehgal *et al* (1987). A serial double-fold dilution of antigen and double -fold dilution of equine antiserum was prepared and electrophoresis was carried out for each dilution to optimize the concentration of antigen and antiserum. The test sera samples were inactivated at 56°C for 30 minutes in water bath. Various dilutions of test sera starting from 1:2, 1:4, 1:8, 1:16 were prepared in PBS (pH 7.2) (Appendix IV). Each dilution of test serum was mixed with an equal volume of antigen (1:32) in dilution tube and incubated at 37°C for one hour in water bath. Serial final dilution of test serum i.e. 1:2, 1:4, 1:8: 1:16 and 1:32 which corresponded with the neutralizing antibody titre of 1:8, 1:16, 1:32, 1:64 and 1:128 (Sehgal *et al* 1987) mixed with an equal amount of antigen (1:32) were added to 6 mm wells by avoiding overflow of wells. Slides were placed in electrophoresis chamber and run for 45 minutes at 10 volts/slide.

After the run agar was removed from 3 mm wells and indicator serum (1:4) was added in these wells. Thereafter, electrophoresis was continued for another 2 hours at a constant current of 15mA/slide.

Staining of slides: The gels were dried at 37°C by keeping a wet filter paper over them. After drying the filter paper was removed and then slide was stained with one per cent Coomassie brilliant blue stain for 15 minutes and then destained with

destaining solution for 30-60 minutes. The slides were observed for precipitation lines after the run

Table 35: Details of sera samples collected from different animal species

S. No.	Case No.	Species	Sex	Age
1	RN01-4787	Buffalo	F	5Y
2	D12-14276	Dog	F	7Y
3	34 RL-15	Cow	F	2.5Y
4	DO-84	Dog	F	2Y
5	RN03-960	Buff. calf	F	10M
6	RN11-5380	Cow	F	3Y
7	DO-5286	Dog	M	4Y
8	RN05-1826	Cow	F	6Y
9	RN10-4787	Buffalo	F	10 Y
10	RN02-458	Buffalo	F	4Y
11	DO-1272	Dog	F	1.5Y
12	DO-2046	Dog	M	3Y
13	RN3-1024	Bullock	M	6Y
14	DO-5747	Dog	M	4 M
15	DO-2047	Dog	M	4.5Y
16	RN05-2239	Cow	F	5Y
17	RNDO-5174	Dog	F	4Y
18	RN03-1027	Cow	F	3.5Y
19	RN05-2153	Buffalo	F	4Y
20	DO-1540	Dog	F	6Y
21	DO-2016	Dog	M	14Y
22	DO-9646	Dog	F	1.5Y
23	RN04-1462	Buffalo	F	6Y
24	DO-429	Dog	M	4Y

25	DO-720	Dog	F	8Y
26	RN05-2155	Buffalo	F	5Y
27	RN03-1010	Buffalo	F	7Y
28	DO-3548	Dog	F	3Y
29	04-1464	Buffalo	F	3.5Y
30	DO -3207	Dog	M	1.5Y
31	RN03-1027	Buffalo	F	6.5Y
32	DO-1140	Dog	F	8Y

* RN=registration no of cow/buffalo in clinics, DO=case no. of dogs in clinics

Detection of rabies antibodies by ELISA

The present study was conducted on 32 unvaccinated animals of different species (16 dogs, 10 buffalos, 6 cows) presented at Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana from July 2015 to June 2016 and were tested for rabies virus specific antibodies by Enzyme-Linked Immunosorbent Assay (ELISA). Out of 32 cases 15 (46.8 %) revealed antibody titer varying from 0.128-0.302 IU/ µl. Out of these 16 dogs, 9 (56.25%) revealed antibody titer ranging from 0.152-0.302 IU/µl.

Similarly in the present study antibody titer was detected in unvaccinated buffaloes and cows. Out of 10 buffaloes, 4 (40%) revealed antibody titer ranging from 0.128-0.264 IU/µl. Out of 6 cows, 2 (33.3%) revealed antibody titer ranging 0.183 - 0.23IU/µl. None of the unvaccinated animals showed protective rabies specific antibody titer above 0.5 IU/µl.

Modified Counter Immuno Electrophoresis (CIE)

Standardization of antigen and antibody for Modified Counter Immuno Electrophoresis

A serial double-fold dilution of 1:2 1:4, 1:8 1:16 1:32, 1:64, 1:128 and 1:256 of antigen in PBS (pH 7.2-7.6, Appendix II) and different dilutions of 1:2 ,1:4, 1:8, 1:16, 1:32, 1:64 of equine antiserum was prepared and electrophoresis was carried out for each dilution to optimize the concentration of antigen and antiserum. Maximum dilution of antigen and antibody at which these samples revealed precipitation line was 1:32 and 1:4 respectively (Fig. 15a,b). There was loss in the quality of

identification of rabies antibody when antigen was diluted to 1:64.

Detection of rabies antibodies by CIE

CIE was conducted on 32 serum samples for the detection of rabies antibodies in different species of animals but precipitation line was not observed in any sample

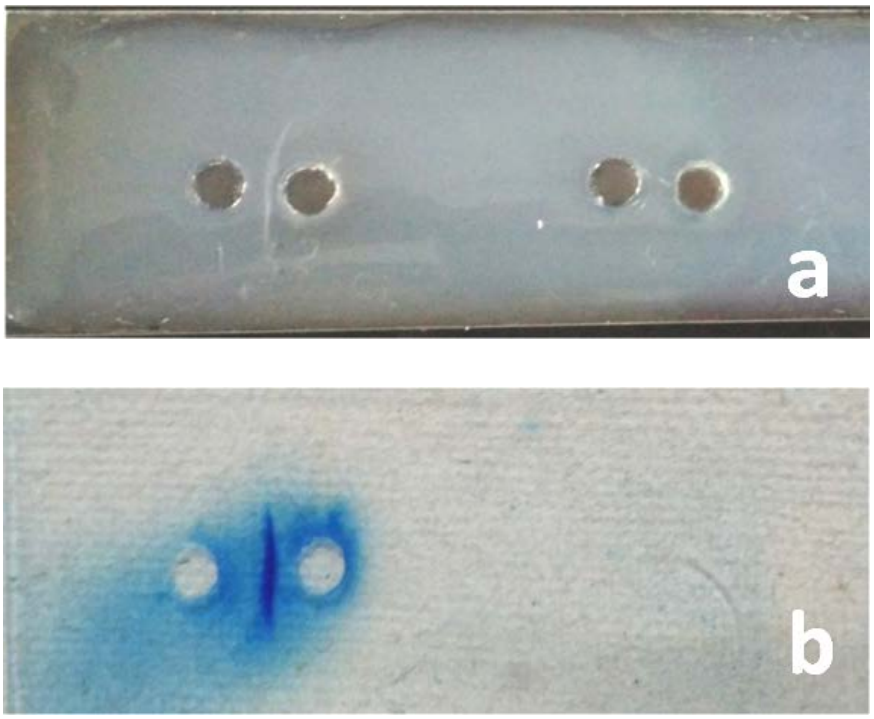


Fig. 15: Maximum dilution of antigen (1:32) and antibody (1:4)

(a) Before staining and (b) After staining

after overnight incubation and staining.

Modified Counter Immuno-Electrophoresis (MCIE)

Detection of rabies antibodies by MCIE

The corresponding sera (n=32) were subjected to MCIE for quantitative detection of antibodies to rabies virus at different dilution 1:2, 1:4, 1:8, 1:16 and 1:32 but none of them showed positive result after overnight incubation, staining and destaining.

In the present study, the antibody titer by ELISA in most of the cases was lower than 0.5 IU/µl. Thus, antibody titer might have not been detected by MCIE due to lower level of antibody titer in rabies suspected animals or may be due to some unknown factor.

Table 36: ELISA and CIE titers in unvaccinated animals

S. No.	Case No.	Species	Sex	Age	Optical Density	Titer (IU/µl)	ELISA	CIE
1	RN01-4787	Buffalo	F	5Y	0.122	-	-	-
2	D12-14276	Dog	F	7Y	0.446	0.26	+	-
3	34 RL-15	Cow	F	2.5Y	0.376	0.183	+	-
4	D0-84	Dog	F	2Y	0.402	0.21	+	-
5	RN3-960	Buff. calf	F	10M	0.0167	-	-	-
6	RN11-5380	Cow	F	3Y	0.0119	-	-	-
7	D0-5286	Dog	M	4Y	0.374	0.181	+	-
8	RN05-1826	Cow	F	6Y	0.425	0.23	+	-
9	RN10-4787	Buffalo	F	10 Y	0.0164	-	-	-
10	RN02-458	Buffalo	F	4Y	0.325	0.128	+	-
11	DO-1272	Dog	F	1.5Y	0.355	0.160	+	-
12	DO-2046	Dog	M	3Y	0.261	-	+	-
13	RN3-1024	Bullock	M	6Y	0.204	-	+	-

S. No.	Case No.	Species	Sex	Age	Optical Density	Titer (IU/µl)	ELISA	CIE
14	D0-5747	Dog	F	5Y	0.0145	-	-	-
15	DO-2047	Dog	M	4.5Y	0.448	0.302	+	-
16	RN05-2239	Cow	F	8Y	0.157	-	+	-
17	DO-5174	Dog	F	4Y	0.109	-	-	-
18	RN03-1027	Cow	F	3.5Y	0.158	-	+	-
19	RN05-2153	Buffalo	F	7Y	0.449	0.264	+	-
20	DO-1540	Dog	F	6Y	0.346	0.155	+	-
21	DO-2016	Dog	M	14Y	0.424	0.236	+	-
22	DO-9646	Dog	F	1.5Y	0.150	-	+	-
23	RN04-1462	Buffalo	F	6Y	0.344	0.148	+	-
24	DO-429	Dog	M	4Y	0.144	-	+	-
25	DO-720	Dog	F	8Y	0.35	0.155	+	-
26	RN05-2155	Buffalo	F	5Y	0.24	-	+	-
27	RN03-1010	Buffalo	F	7Y	0.0172	-	-	-

S. No.	Case No.	Species	Sex	Age	Optical Density	Titer (IU/µl)	ELISA	CIE
28	DO-3548	Dog	F	3Y	0.347	0.152	+	-
29	RN04-1464	Buffalo	F	3.5Y	0.0168	-	-	-
30	DO -3207	Dog	M	1.5Y	0.0169	-	-	-
31	03-1027	Buffalo	F	6.5Y	0.364	0.170	+	-
32	DO-1140	Dog	F	8Y	0.028	-	-	-
Percentage							46.8	0

*RN= registration no., + =Positive, -=Negative

Table 37: Antibody titer in dogs by ELISA (n=16)

Case No.	Titer (IU/µl)	ELISA
D12-14276	0.26	+
D0-84	0.21	+
D0-5286	0.181	+
DO-1272	0.160	+
DO-2046	-	-
D0-5747	-	-
DO-2047	0.302	+
DO-5174	-	-
DO-1540	0.155	+
DO-2016	0.236	+
DO-9646	-	-
DO-429	-	-
DO-720	0.155	+
DO-3548	0.152	+
DO -3207	-	-
DO-1140	-	-
Percentage		56.25

Table 38: Antibody titer in buffaloes by ELISA (n=10)

Case No.	Antibody Titer (IU/µl)	ELISA
RN01-4787	-	-
RN3-960	-	-
RN10-4787	-	-

RN02-458	0.128	+
RN05-2153	0.264	+
RN04-1462	0.148	+
RN05-2155	-	-
RN03-1010	-	-
RN04-1464	-	-
RN03-1027	0.170	+
Percentage		40

Table 39: Antibody titer in cows by ELISA (n= 6)

Case No.	Antibody Titer (IU/µl)	ELISA
34 RL-15	0.183	+
RN-11-5380	-	-
RN-05-1826	0.23	+
RN-3-1024	-	-
RN-03-1027	-	-
RN-05-2239	-	-
Percentage		33.3

Post mortem confirmation of rabies virus antigen

Fluorescent Antibody Test on brain tissue smears

Out of total 32 cases of different animal species 14 brain samples (7 dogs, 5 buffaloes and 2 cows) presented for post mortem, revealed congestion grossly (Fig. 16) were tested by FAT for rabies antigen detection, out of 14 cases 85.7%(12/14) were found positive for rabies. Characteristic apple green immunofluorescence (Fig. 17) was observed intra-cytoplasmic in neurons as well as in form of diffused fluorescence in the brain tissue smears.

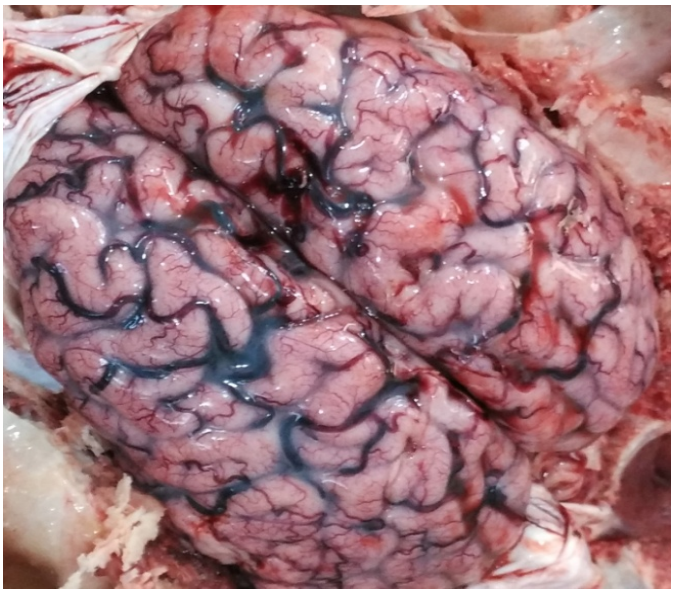


Fig. 16: Congestion, Hemorrhages and Edema in brain

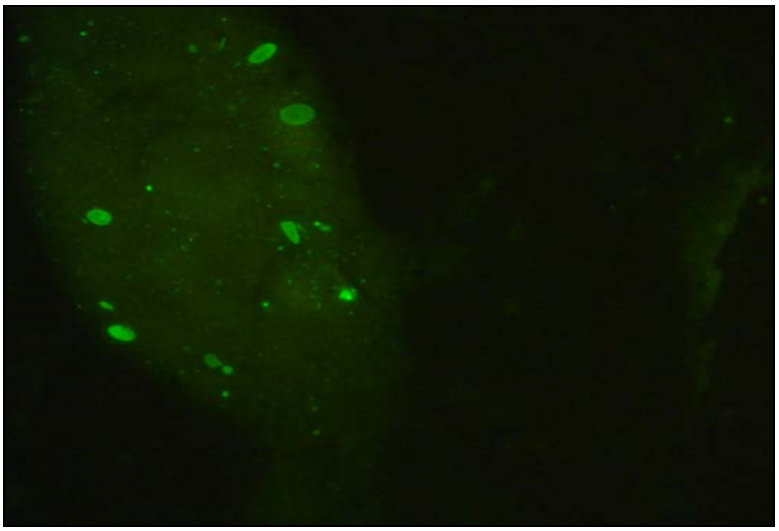


Fig. 17: Fluorescent Antibody Test (Brain smear)

PART B of OBJECTIVE *5*

SYMPTOMS EXHIBITED BY SUSPECTED ANIMALS.

Collection of case history

The whole blood samples were collected from 32 rabies suspected cases received from July 2015 to June 2016 at the Department of Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (GADVASU). The data regarding species, breed, habitat, age, sex was collected as per questioner prepared for the purpose.

Observation of clinical signs

The following clinical signs were observed and correlation with antibody titer was done.

- Off feed
- Hyper salivation
- Difficulty in standing/paralysis
- Fever
- History of biting /aggressiveness
- Difficult intake of food
- Not recognizing owner
- Behavioral change
- Circling
- Bellowing

Clinical signs in rabies suspected dogs

In the present study 85.7 % (14/16) rabies suspected dogs were found off feed followed by hyper-salivation in 75% (12/16) and43.7% (7/9) revealed biting behaviour/aggressiveness. Paralysis (Fig. 18) and difficult intake of food are also considered as characteristic symptoms of rabid dogs, which were observed in 62.5% (10/16) and 75% (12/16) cases, respectively. The present study revealed fever in 37.5% (6/16) cases. 43.7% (7/16) rabid dogs revealed behavioural changes and circling. 37.5% (6/16) dogs did not recognize their owner (Table 40 and Fig.19), hence these symptoms should be taken seriously while suspecting any case of dog for rabies.

Table 40: Clinical signs in rabies suspected dogs (n = 16)

Symptoms	No. of animals	Percentage
Off feed	14	85.71
Hyper salivation	12	71.42
Difficulty in standing/paralysis	10	62.5
Fever	6	37.5
History of biting/aggressiveness	7	43.75
Difficult intake of food	12	75
Not recognizing owner	6	37.5
Behavioural change	7	43.5
Circling	7	43.5

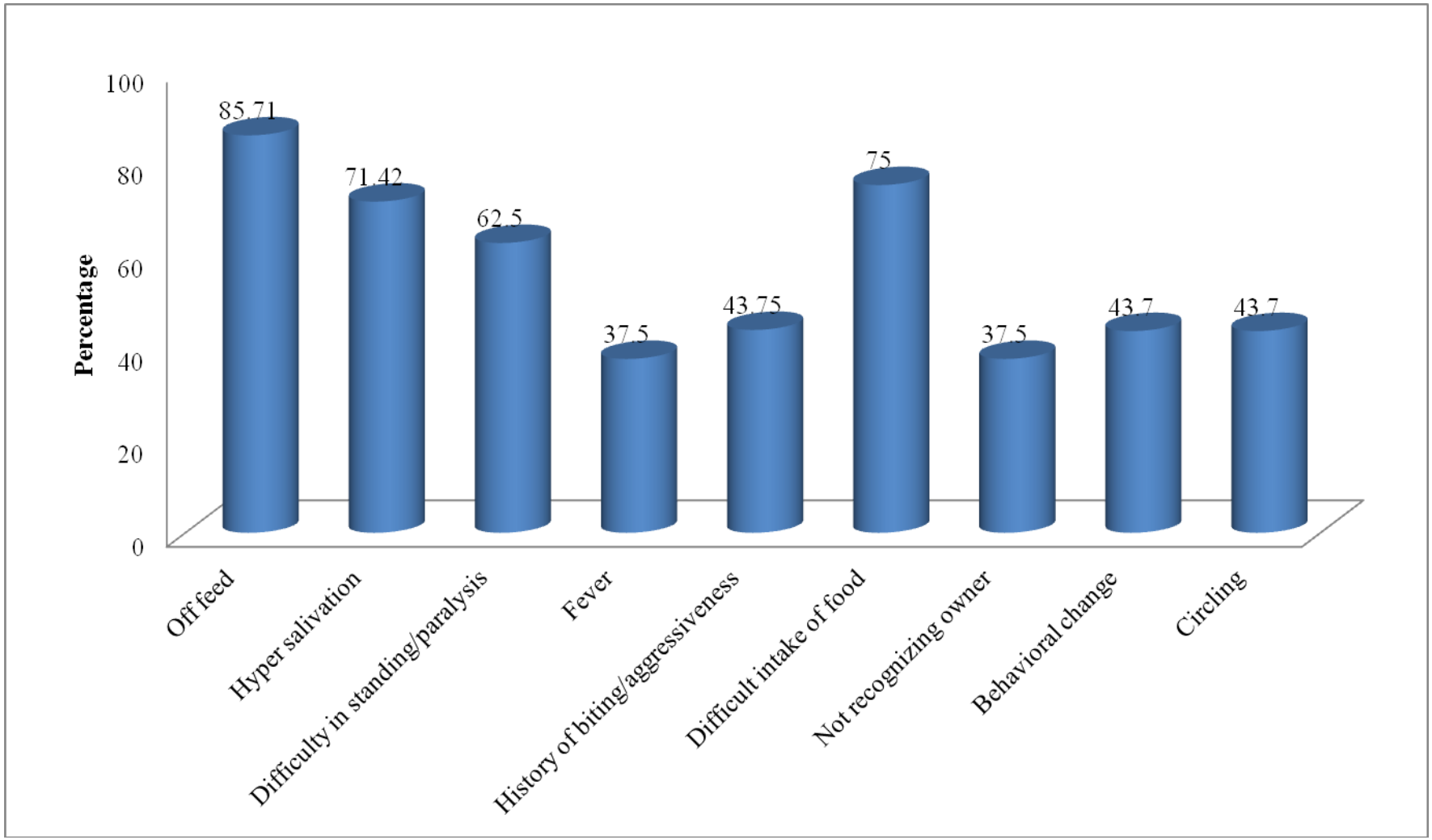


Fig. 19: Clinical signs in rabies suspected dogs

Fig. 18: Paralysis of jaw in rabid dog

Clinical signs in rabies suspected buffaloes

In case of rabies suspected buffaloes, anorexia was found in 80% (8/10) cases, followed by hypersalivation in 70% (7/10) cases. Whereas difficulty in standing/paralysis, pressing of head against hard objects (Fig. 20) and circling were reported in 50 % (5/10) cases, fever in 40% (4/10) cases. Difficult feed intake and bellowing were observed in 60 % (6/10) and 40% (4/10) cases respectively (Table 41 and Fig. 21).

Table 41: Clinical signs in rabies suspected buffaloes (n=10)

Symptom	No. of animals	Percentage
Off feed	8	80
Hyper-salivation	7	70
Fever	4	40
Not recognizing owner	2	20
Circling/Head pressing	5	50

Difficulty in standing/paralysis	5	50
Difficult intake of food	6	60
Bellowing	4	40
Behavioural change	5	50

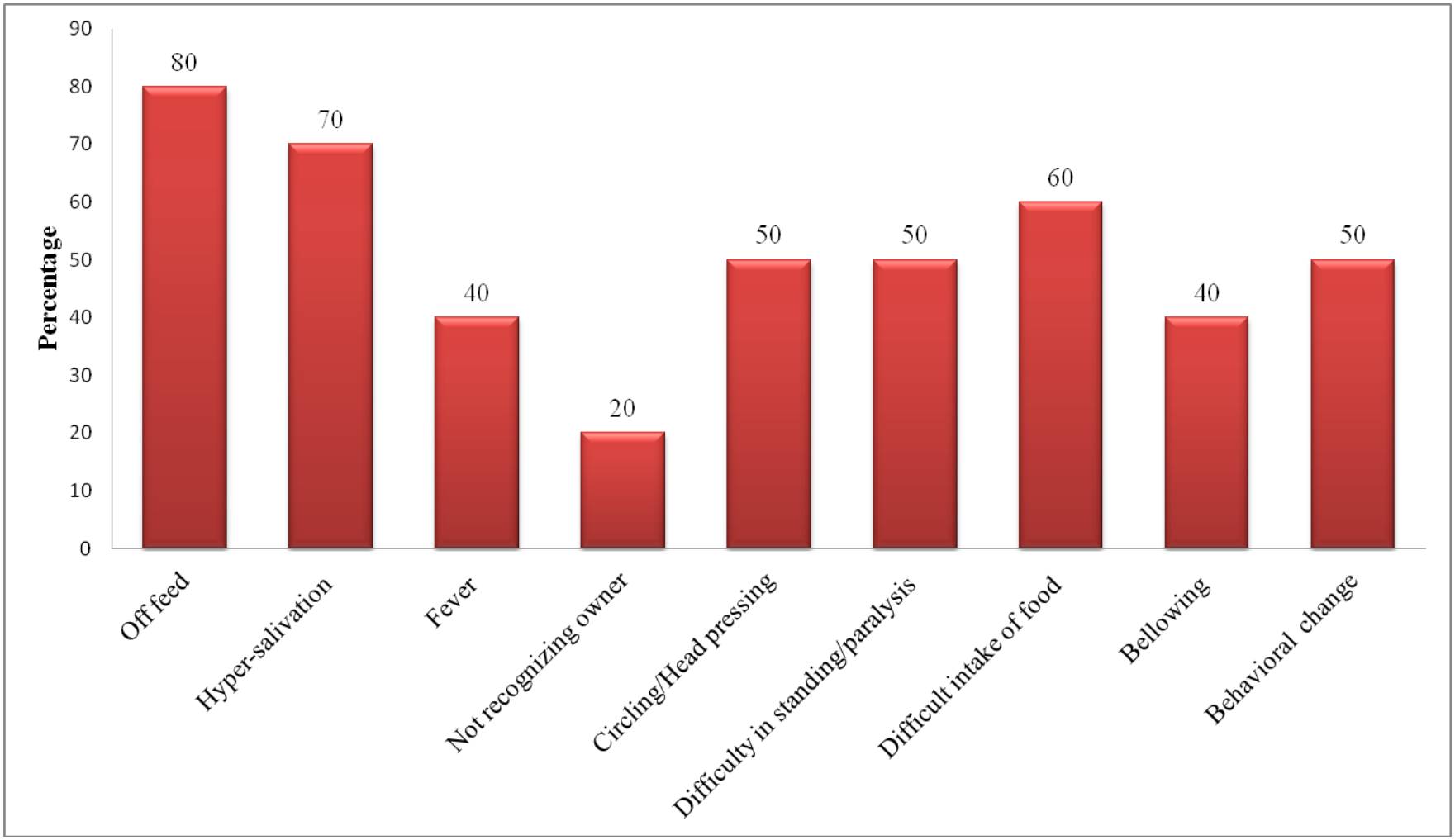


Fig. 21: Clinical signs in rabies suspected buffaloes

Fig. 20: Head pressing

Clinical signs in rabies suspected cows

In rabies suspected cows, anorexia, hyper-salivation (Fig. 22), difficulty in standing/paralysis (Fig. 23) and bellowing (Fig. 24) were observed in 66.6% (4/6) cases, followed by not recognized to owner, circling/head pressing & difficult intake of feed in 50 % (3/6) cases. However, fever was reported in 33.3% (2/6) cases (Table 42 and Fig. 25).

Table: 42 Clinical signs in rabies suspected cows (n = 6)

Symptom	No. of animals	Percentage
Off feed	4	66.6
Hyper-salivation	4	66.6
Fever	2	33.3
Not recognizing owner	3	50
Circling/Head pressing	3	50
Difficulty in standing/paralysis	4	66.6
Difficult intake of food	3	50
Bellowing	4	66.6
Behavioural change	3	50

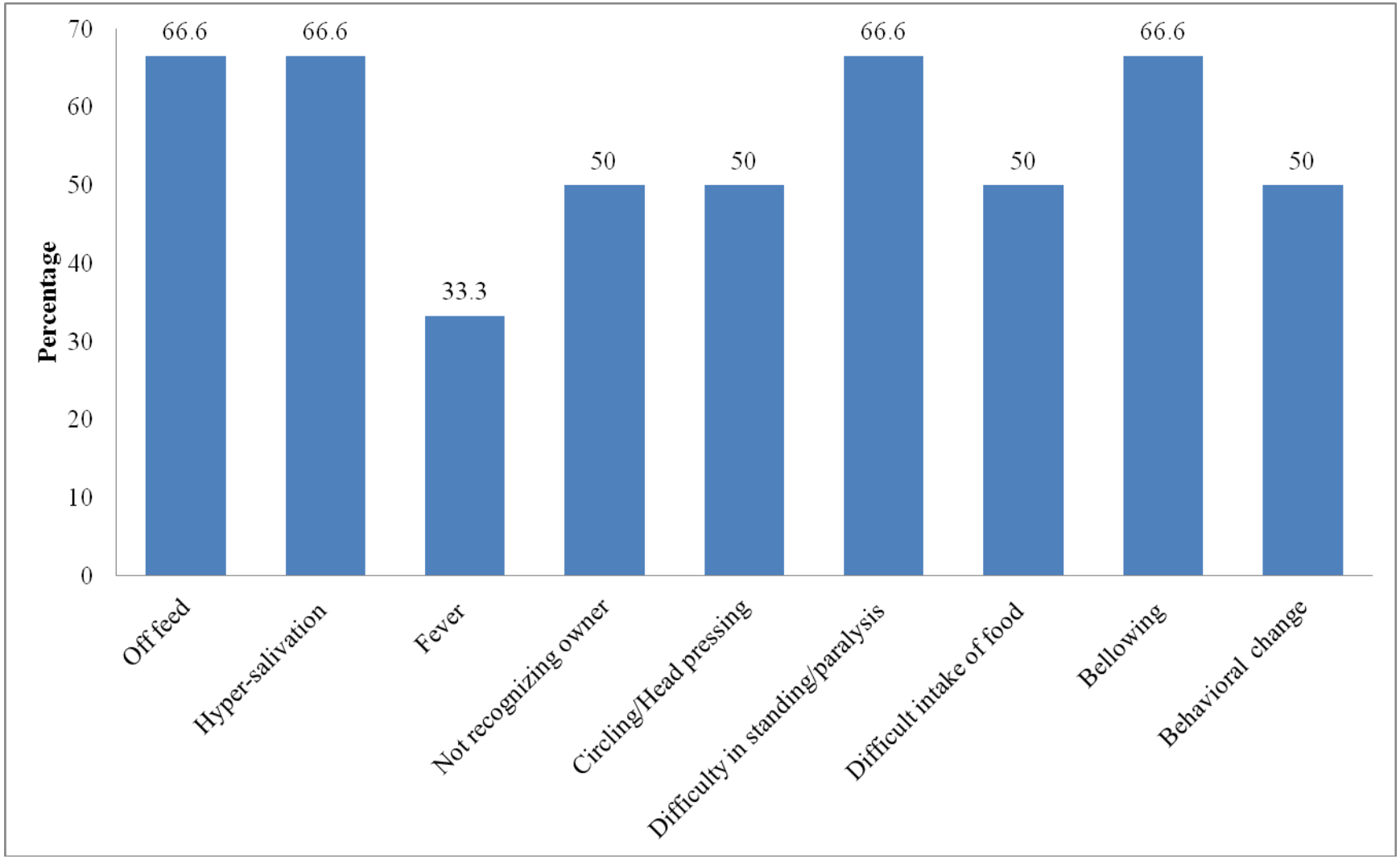


Fig. 25: Clinical signs in rabies suspected cows

Fig. 22: Hypersalivation in rabid Cow

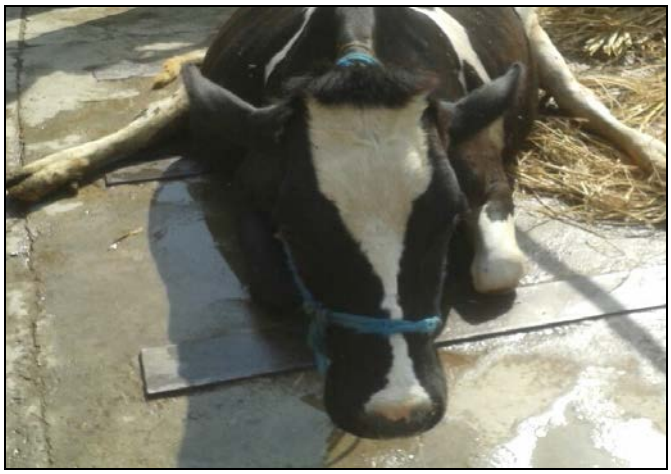


Fig. 23: Paralysis in rabid cow

Fig. 24: Bellowing in rabid calf

PART C of OBJECTIVE *5*

CORRELATION OF DETECTION OF RABIES ANTIGEN WITH RABIES ANTIBODIES, HISTOPATHOLOGICAL ALTERATIONS IN BRAIN AND SYMPTOMS EXHIBITED BY RABID ANIMALS

Correlation of clinical Signs with Antibody titer by ELISA in Dogs

Dogs with antibody titer varying from 0.152-0.160 IU/µl revealed hypersalivation, behaviour changes, history of biting, circling, difficult intake of feed, fever and not recognizing the owner. Whereas, Dog with antibody titer varying 0.21-0.302 IU/µl showed hypersalivation, behaviour changes, aggressiveness, circling, difficulty in standing and were found off feed (Table 43 and Fig.26).

Correlation of clinical signs with Antibody titer by ELISA in Buffaloes

Buffaloes with antibody titer varying 0.128-0.148 IU/µl showed hypersalivation, difficult intake of feed, difficulty in standing/paralysis, bellowing, fever. Whereas, buffaloes with antibody titer varying 0.170-0.264 IU/µ showed hypersalivation, not recognizing owner, behaviour changes, bellowing, circling/head pressing and were also found off feed (Table 44 and Fig. 27).

Correlation of clinical signs with Antibody titer by ELISA in Cows

Cows with antibody titer varying from 0.183-0.23 IU/µl showed behaviour changes, bellowing, circling and difficulty in standing/paralysis, followed by hypersalivation, difficult intake of feed and did not recognize their owner. Fever was not reported in both the animals (Table 45 and Fig. 28).

No significant correlation was observed in appearance of clinical signs and development of anti-rabies antibody titer in the infected animals.

Table 43: Correlation of clinical Signs with Antibody titer by ELISA in Dogs

S. No.	Antibody Titer (IU/µl)	Hyper salivation	Not recognizing owner	Behaviour change	History of biting /aggressiveness	Difficulty in standing/ paralysis	Circling/Head pressing	Off feed	Difficult intake of feed	Fever
1	0.26	+	+	+	+	+	+	+	+	-
2	0.21	+	+	+	+	-	+	+	+	-
3	0.23	+	+	+	+	-	+	+	+	-
4	0.160	+	-	-	-	+	-	+	+	-
5	0.302	+	+	+	+	-	+	+	-	-
6	0.155	+	-	-	-	+	-	+	+	-
7	0.236	+	-	-	-	+	-	+	+	-
8	0.155	+	+	+	+	-	+	+	+	+
9	0.152	+	-	+	+	-	+	-	-	-
Percentage		100	55.5	66.6	66.6	55.5	66.6	88.8	88.8	11.1

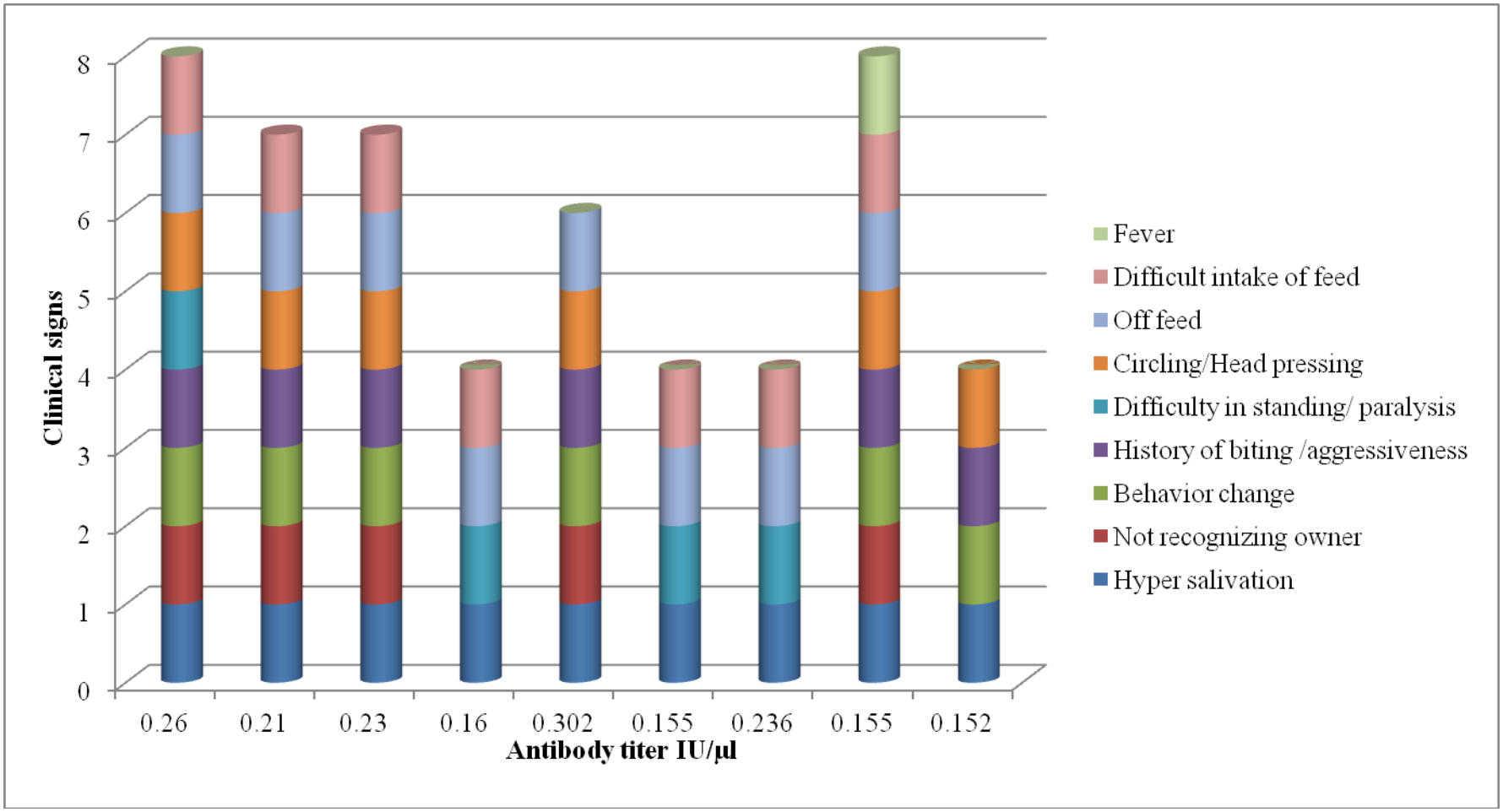


Fig. 26: Correlation of clinical Signs with Antibody titer by ELISA in Dogs

Table 44: Correlation of clinical signs with Antibody titer by ELISA in Buffaloes

S. No.	Antibody Titer (IU/µl)	Hyper salivation	Not recognizing owner	Behaviour change	Bellowing	Difficulty in standing/ paralysis	Circling/Head pressing	Off feed	Difficult intake of feed	Fever
1	0.128	+	-	-	-	+	-	+	+	-
2	0.264	+	+	+	+	-	+	+	-	-
3	0.148	-	-	-	-	+	-	+	+	+
4	0.170	-	-	+	+	-	+	+	+	-
Percentage		75	25	50	50	50	50	100	75	25

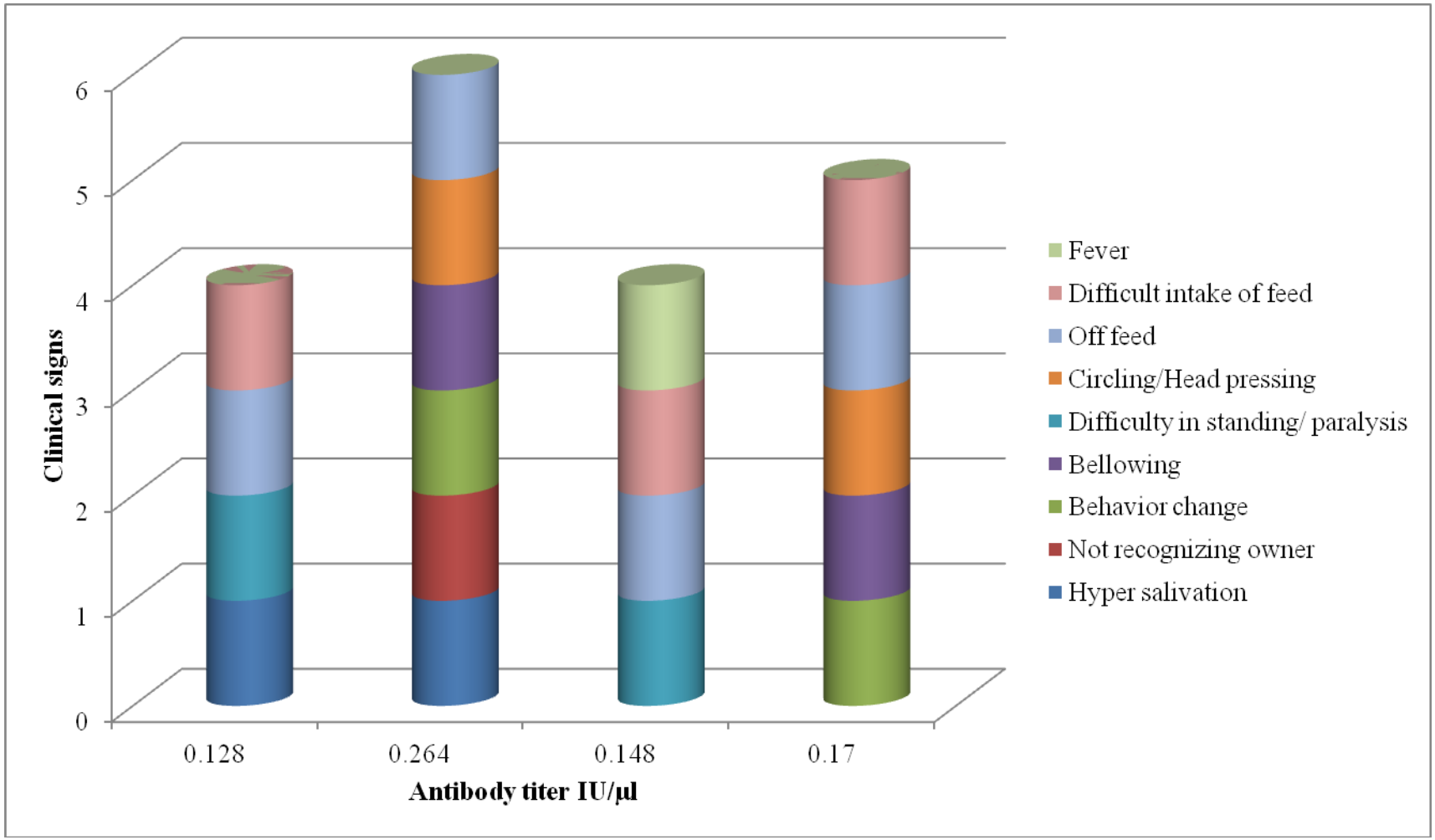


Fig. 27: Correlation of clinical signs with Antibody titer by ELISA in Buffaloes

Table 45: Correlation of clinical signs with Antibody titer by ELISA in Cows

S. No.	Antibody Titer (IU/µl)	Hyper salivation	Not recognizing owner	Behaviour change	Bellowing	Difficulty in standing/ paralysis	Circling/Head pressing	Off feed	Difficult intake of feed	Fever
1	0.183	+	+	+	+	+	+	+	+	-
2	0.23	-	-	+	+	-	+	+	+	-
Percentage		50	50	100	100	50	100	100	100	0

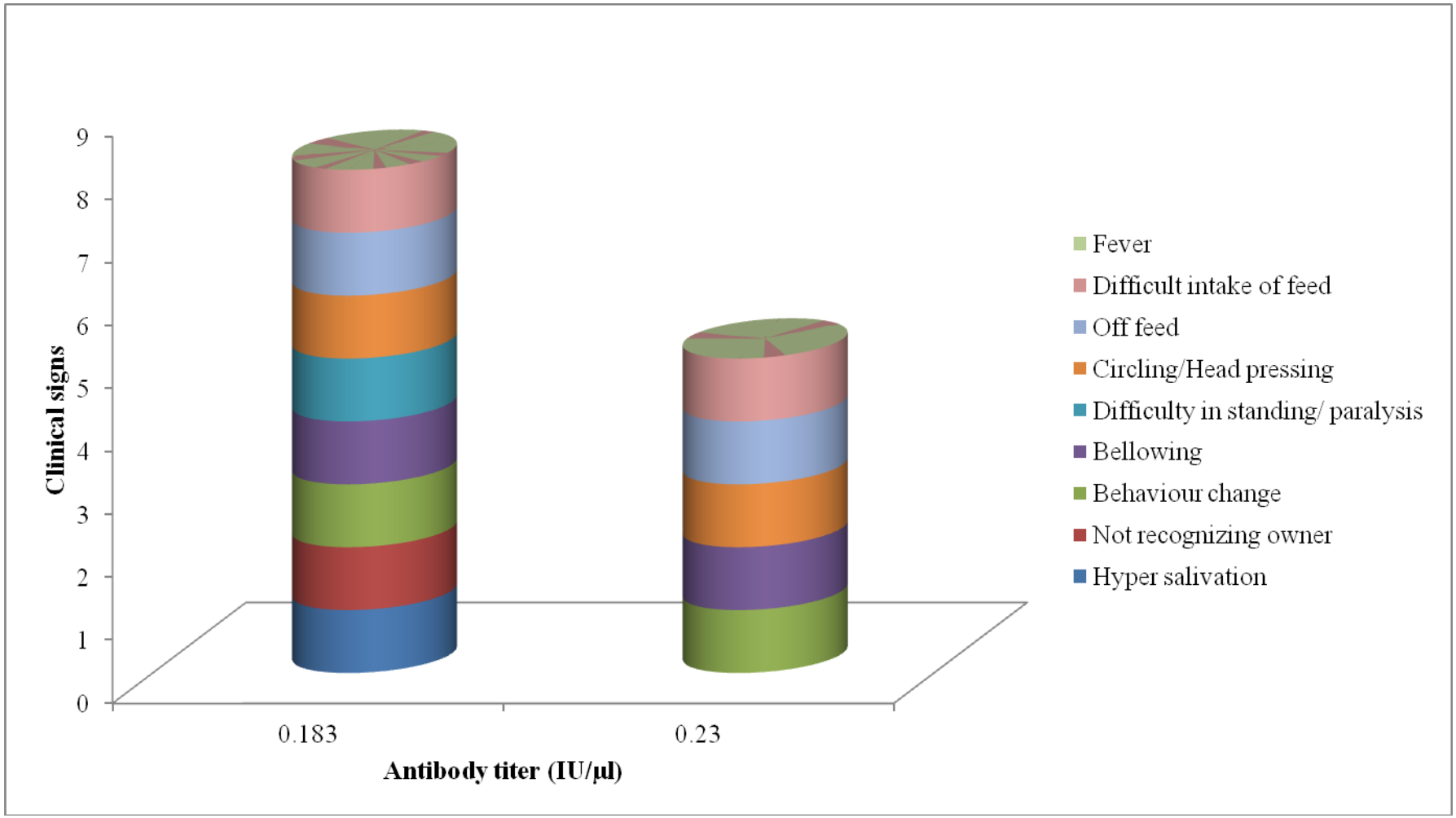


Fig. 28: Correlation of clinical signs with Antibody titer by ELISA in Cows

Objective No 6:

TO RECOMMEND THE SPECIES-WISE PROTOCOL OF *ANTE-MORTEM* DIAGNOSIS OF RABIES IN CANINE, FELINE AND BOVINE.

For establishment of protocol of ante-mortem diagnosis of rabies there were two major factors viz. the most suitable laboratory technique in different species of animals; the most suitable sample in different animal species.

The most suitable laboratory technique

Although the most suitable laboratory technique has been determined in earlier studies in this research project, however, with the perspective of comparing the laboratory techniques in various animals species, a study was conducted to test the different laboratory techniques in brain tissues of different animals species to observe any difference in detection of Rabies by the laboratory techniques in different species of animals.

Comparison of molecular and immunopathological techniques for diagnosis of rabies virus in brain samples of different species

Comparison of molecular and immunopathological techniques for diagnosis of rabies virus in brain samples of different species is given under (Table 46) and sensitivity, specificity and accuracy of molecular and immunopathological techniques for rabies diagnosis in brain samples in different species is given under (Table 47).

Table 46: Results of molecular and immunopathological techniques in brain samples of different species

Species	Total cases	FAT Positive	IHC Positive	HnRT PCR N gene Positive	HnRT PCR L gene Positive	TaqMan real time PCR Positive	Immunochroma tographic Test
Dog	27	16	15	16	16	16	16
Cattle	11	06	06	06	06	06	05
Buffalo	09	06	05	06	06	06	06
Mongoose	03	02	02	02	02	02	02

Table 47: Sensitivity, specificity and accuracy of molecular and immunopathological techniques for rabies diagnosis in brain samples in different species.

Species	Total	Brain FAT	Parameters	IHC	HnRT PCR N and L gene	TaqMan real time PCR	Immunochroma tographic Test
Dog	27	16	Sensitivity	94.11%	100%	100%	100%
			Specificity	100%	100%	100%	100%
			Accuracy	96.42%	100%	100%	100%
Cattle	11	06	Sensitivity	100%	100%	100%	85.71%
			Specificity	100%	100%	100%	100%
			Accuracy	100%	100%	100%	91.66%
Buffalo	8	06	Sensitivity	85.71%	100%	100%	100%
			Specificity	100%	100%	100%	100%
			Accuracy	91.66%	100%	100%	100%
Mongoose	03	02	Sensitivity	100%	100%	100%	100%
			Specificity	100%	100%	100%	100%
			Accuracy	100%	100%	100%	100%

In this study, it was revealed that Taqman Real Time PCR and HnRT-PCR revealed 100% sensitivity, 100% specificity and 100% accuracy in all species of animals. Therefore, these laboratory techniques can be used in all species of animals.
Immunochromatographic Test also revealed 100% sensitivity, 100% specificity and 100% accuracy in most species of animals except in cows where the sensitivity of detection of rabies even form brain tissue of cow fell to 85.71% and accuracy got decreased to 91.66%.
IHC exhibited 100% sensitivity, 100% specificity and 100% accuracy in cows and wild mongoose whereas efficacy of IHC fell in dog and buffalo.

The most appropriate clinical sample in different species of animals

SALIVA and URINE:

Saliva, urine, serum and skin were compared among different species of animals to determine the most appropriate clinical sample for ante-mortem diagnosis of Rabies in animal species.

Comparison of molecular and immuno-pathological techniques for diagnosis of rabies virus in saliva and urine samples of different species

Comparison of molecular and immuno-pathological techniques for diagnosis of rabies virus in saliva and urine samples of different species is given under Table 48 and sensitivity, specificity and accuracy of molecular and immuno-pathological techniques for rabies diagnosis in saliva and urine samples in different species is given in under Table 49.

Table 48: Molecular and immuno-pathological techniques for rabies diagnosis in saliva and urine samples in different species.

Species	Total cases	Brain FAT Positive	HnRT PCR L gene Positive cases		HnRT PCR N gene Positive cases		TaqMan real time PCR Positive cases		FAT (saliva and urine smear) Positive cases		Immuno-chromatographic Test	
			Saliva	Urine	Saliva	Urine	Saliva	Urine	Saliva	Urine	Saliva	Urine
Dog	17	12	9	8	9	8	10	9	5	0	8	0
Cattle	10	8	6	6	5	5	6	6	4	0	5	0
Buffalo	8	5	4	3	4	3	5	4	3	0	4	0
Mongoose	2	1	1	0	1	0	1	0	0	0	0	0
Rabbit	2	0	0	0	0	0	0	0	0	0	0	0
Horse	1	0	0	0	0	0	0	0	0	0	0	0

Table 49: Sensitivity, specificity and accuracy of molecular and immuno-pathological techniques for rabies diagnosis in saliva and urine samples in different species.

Species	Total	Brain FAT positive	Parameters	HnRT PCR L gene		HnRT PCR N gene		TaqMan real time PCR		FAT (saliva and urine smear)		Immuno-chromatographic Test	
				Saliva	Urine	Saliva	Urine	Saliva	Urine	Saliva	Urine	Saliva	Urine
Dog	17	12	Sensitivity	75.00%	66.67%	75.00%	66.67%	83.33%	75%	41.67%	00%	66.67%	00%
			Specificity	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
			Accuracy	82.35%	76.47%	82.35%	76.47%	88.24%	82.35%	58.82%	29.41%	76.47%	29.41%
Cattle	10	8	Sensitivity	75%	75%	62.5%	62.5%	75%	75%	50%	00%	62.50%	00%
			Specificity	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
			Accuracy	80%	80%	70%	70%	80%	80%	60%	20%	70.00%	20%
Buffalo	8	5	Sensitivity	80%	50%	80%	60%	100%	80%	60%	00%	87.50%	00%
			Specificity	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
			Accuracy	87.5%	75%	87.5%	75%	100%	87.50%	75%	37.5%	75.00%	37.5%

Mongoose	2	1	Sensitivity	50%	00%	50%	00%	50%	00%	00%	00%	50.00%	00%
			Specificity	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
			Accuracy	100%	50%	100%	50%	100%	50%	00%	50%	00%	50%

As determined in the earlier studies in the project, the detection of rabies from saliva and urine samples was possible with 100% specificity, however, with low sensitivity and unreliable accuracy, dependence solely on saliva and/or urine for ante-mortem diagnosis of rabies was not desirable, from any species of animal.

SERUM

Detection of rabies antibodies by ELISA

The study was conducted on 32 unvaccinated animals of different species (16 dogs, 10 buffalos, 6 cows) that were tested for rabies virus specific antibodies by Enzyme-Linked Immunosorbent Assay (ELISA). Out of 32 cases 15 (46.8 %) revealed antibody titer range varying from 0.12-0.30 IU/ µl. Out of these 16 dogs, 9 (56.25%) revealed antibody titer ranging from 0.15-0.30 IU/µl. None of the unvaccinated dogs showed protective rabies specific antibody titer.

Similarly in the present antibody titer was detected in unvaccinated buffaloes and cows. Out of 10 buffaloes, 4 (40%) revealed antibody titer ranging from 0.12-0.26 IU/µl. Out of 6 cows, 2 (33.3%) revealed antibody titer ranging 0.18 -0.23IU/µl. None of the unvaccinated animals showed protective rabies specific antibody titer above 0.5 IU/µl.

The study revealed that anti-rabies antibodies in sera samples of rabid animals were detected only in 46.8% animals of all species. Antibodies were detected in 56.25% dogs, 40% buffaloes and 33.3% cows.

Due to unreliable level of detection of anti-rabies antibodies in sera samples of rabid animals, this approach is not recommended for ante-mortem diagnosis of rabies in any species of animal.

SKIN

Further, skin was also tested for any variance in its suitability for ante-mortem diagnosis of rabies;

Comparison of molecular and immunopathological techniques for diagnosis of rabies viral RNA in skin samples of different species

In dogs out of nine true positive cases, eight cases were diagnosed by TaqMan real time PCR and HnRT-PCR targeting L gene. TaqMan real time PCR and hnRT-PCR L gene targeted detected rabies from skin biopsies sample with sensitivity of 88.88% and by an accuracy of 92.30%. Six cases were diagnosed positive by hnRT-PCR while targeting N

gene with sensitivity of 66.66% and accuracy of 76.20%. HnRT-PCR targeting L gene offers better sensitivity and accuracy than HnRT-PCR targeting L gene.

Six cases were diagnosed ante-mortem by immunofluorescence on cryosections of skin with an accuracy of 76.92% and sensitivity of 66.66%. Immunohistochemistry on skin could, detect rabies from skin in five cases with sensitivity of 55.55% and accuracy of 69.23%. Immunofluorescence of cryosections of skin is less time consuming and offers better accuracy and sensitivity than immunohistochemical detection of rabies from skin of dogs.

In cattle all six true positive cases, were diagnosed ante mortem using TaqMan real time PCR and HnRT-PCR targeting L gene. Thus both the technique offers high sensitivity (100%) and accuracy (100%) while detecting rabies from skin. However five cases were diagnosed positive using HnRT-PCR targeting N gene, FAT and IHC with sensitivity of 83.33% and an accuracy of 87.5%.

In buffaloes all 9 true positive cases were diagnosed positive ante mortem from skin biopsy using TaqMan real time PCR and HnRT-PCR both L gene and N gene assay. Immunofluorescence could detect rabies in 7 buffaloes with sensitivity of 87.5% and accuracy of 90.90%. IHC detected rabies in 6 cases with sensitivity of 75% and accuracy of 81.81%.

Table 50: Comparison of molecular and immunopathological techniques for detection of rabies viral RNA in skin samples in different species.

Species	Total	Brain FAT Positive	Parameters	HnRT PCR L gene targeted	HnRT PCR N gene targeted	TaqMan real time PCR	FAT	IHC
Dogs	13	9	Sensitivity	88.88%	66.66%	88.88%	66.66%	55.55%
			Specificity	100%	100%	100%	100%	100%
			Accuracy	92.30	76.92	92.30	76.92	69.23%
Cattle	8	6	Sensitivity	100%	83.33%	100%	83.33%	83.33%
			Specificity	100%	100%	100%	100%	100%
			Accuracy	100%	87.5%	100%	87.5%	87.5%
Buffalo	11	8	Sensitivity	100%	100%	100%	87.5%	75%
			Specificity	100%	100%	100%	100%	100%
			Accuracy	100%	100%	100	90.90%	81.81%

Taqman Real time PCR revealed highest sensitivity (100%), specificity (100%), and accuracy (100%) in cattle and buffalo. In case of dogs, the sensitivity fell to 88.88% which is slightly short than 90%. However, Taqman Real Time PCR exhibited maximum efficacy in for detection of rabies from skin of bovine species with 100% sensitivity, 100% specificity and 100% accuracy.

In addition, HnRT-PCR targeted for both L gene and N gene also exhibited highest efficacy of detection of rabies from skin of cattle and buffalo and was not recommended for usage in dogs.

FAT and IHC was found undesirable for ante-mortem diagnosis of rabies from skin of rabid animals of all species.

Thus, based on the findings of various studies in the present project, the protocol for ante-mortem diagnosis of Rabies in different animal species is recommended as under;

Buffalo:

In buffaloes, for ante-mortem diagnosis, skin biopsy should be tested by Taqman Real Time

PCR and /or HnRT-PCR targeted on N gene or L gene

Cow:

In cows, for ante-mortem diagnosis, skin biopsy should be tested by Taqman Real Time PCR and /or HnRT-PCR targeted on L gene

Dog:

In dogs, for ante-mortem diagnosis, skin biopsy should be tested by Taqman Real Time PCR. HnRT-PCR targeted on L gene should be used with caution