RESEARCH ARTICLE

Comparative Sensitivity of Polymerase (L) and Nucleoprotein (N) Genes of Rabies Virus

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Abstract

Ante-mortem diagnosis of rabies is highly desirable for veterinary as well as medical fraternity. Recently with advent of molecular approaches it has become feasible using saliva, skin and urine samples. However, there has been ambiguity about the comparative efficacy of molecular approaches while targeting different genes of rabies virus. Thus, present study was envisaged to diagnose rabies from skin samples using hemi-nested (Hn) RT-PCR. The sensitivity of Hn RT-PCR was compared by targeting nucleoprotein (N) gene and polymerase (L) gene of virus in rabies suspected animals. Skin biopsy samples were collected for antemortem diagnosis of rabies from rabies suspected animals presented to the Veterinary Clinics, GADVASU, Ludhiana (India) over a period of 12 months**.** FAT on brain tissue of the same animals was used as a reference method to establish true positive and true negative cases. Out of 34 skin samples, the sensitivity of molecular detection of rabies by L-gene primers was 95.83% and that of by N-gene primers was 83.33%, with accuracy of 97.05% and 88.23% while targeting L gene and N gene, respectively, whereas both the approaches detected rabies from skin samples with 100% specificity. Thus, Hn RT-PCR targeting L-gene is more sensitive than N-gene for antemortem detection of rabies virus RNA from skin samples.

Key words: Antemortem, Hemi-nested PCR, L gene, N gene, Rabies, Skin biopsy.

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Introduction

Rabies is a disease of grave concern in developing world and has the highest case fatality rate of any conventional infectious disease. India is the country with the highest burden of human deaths from canine rabies and a large free-roaming dog population (Hampson *et al.,* 2015), hence laboratory confirmed diagnosis is very essential for effective control and elimination of this zoonotic disease. Rabies is caused by *Lyssa virus* in Rhabdoviridae family (Pringle, 1991) and has five major genes arranged in conserved linear order 3′ as N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (glycoprotein), L (polymerase) 5′. WHO and OIE recommend FAT as gold standard test on brain sample for routine veterinary and human laboratory diagnosis of rabies, though test is feasible only after the death of the suspected patient. Antemortem diagnosis of rabies is possible with the advent of molecular approaches but the technique is still in juvenile stage in veterinary practice. Among all conventional molecular approaches hemi-nested RT-PCR (Hn RT-PCR) has proven sensitivity of detecting rabies virus from clinical samples. The centrifugal spread of rabies virus in course of disease localizes rabies virus in nerve ending surrounding hair follicles. Therefore, RT-PCR of nuchal skin biopsy specimens is recommended by the WHO for intravitam diagnosis of rabies in humans (Rupprecht *et al.,* 2018).

The nucleoprotein gene is widely used for molecular characterization and phylogenetic analysis of rabies virus (Tamura *et al.,* 2021), since it is one of the most conserved fractions in rabies virus. N gene RT-PCR on skin samples collected from domestic and wild animals yielded very promising results (Zieger, 2015) and hence recommended as an alternative to DFA testing on brain tissue for the post-mortem diagnosis of rabies. Dacheux *et al.* (2008) described highly reliable Hn RT-PCR protocol targeting polymerase (L) gene for intravitam diagnosis from nuchal skin biopsies in clinically suspected human cases. In another study on buffaloes N gene Hn RT-PCR diagnosed rabies antemortem with 100% sensitivity, specificity and accuracy (Sharma *et al.,* 2016). These studies represent variability in sensitivities

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of different molecular approaches while targeting different genes of rabies virus for intravitam rabies diagnosis. Thus, present study was envisaged to compare diagnosis of rabies from skin samples by employing Hn RT-PCR targeting two genes of Rabies virus, *viz.,* nucleoprotein (N) gene and polymerase (L) gene.

Materials and Methods

The study was conducted following approval of Institutional Animal Ethics Committee on 34 rabies suspected animals presented to Veterinary Clinics, GADVASU, Ludhiana from different districts of Punjab during the year 2012-13. Antemortem skin biopsies (34 samples) were collected from dogs (n=13), buffaloes (n=11), cattle (n=8), horse (n=1) and cat (n=1) using 3 mm biopsy punch from nape of the neck region. Skin biopsy was tested by applying Hn RT-PCR targeting N gene and L gene. For confirmation of diagnosis, brain samples were procured after postmortem examination of animals suspected for rabies. Flourecent antibody test (FAT on brain was used as gold standard test for comparing sensitivities of L gene and N gene Hn RT-PCR on skin samples.

10% (w/v) skin suspension was used for total RNA extraction by Trizol method. RNA concentration and quality analysis was done using Nano-Drop Spectrophotometer (Nanodrop Technologies, CA) in ng/µL as a ratio of OD 260/280. Total extracted RNA was converted into cDNA using High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, USA) using PCR cycling conditions 25˚C for 10 min, 37˚C for 2 h, 85˚C for 5 min and chilling on ice for 5 min in thermal cycler (Applied Biosystems). cDNA was subjected to PCR amplification using primers for L gene and N gene, respectively (Table 1). Primers targeted towards conserved blocks of L gene PV05 (10 pmol/µL) and PV09 (10 pmol/µL) were used in primary PCR and primers PV05 and PV08 (10 pmol/µL) were used in secondary Hn RT-PCR. In N gene Hn RT-PCR assay primers JW12 (10 pmol/µL) and JW 6DPL (10 pmol/µL) were used in primary PCR and JW12 and JW 10P (10 pmol/µL) in secondary Hn RT-PCR. Primary PCR was carried out in 25 µL of reaction volume using Go Taq green PCR master mix (Promega) and 2 µL of cDNA, subjected to PCR cycling conditions 94°C for 3 min followed by 35 cycles of 94 ˚C for 30s, 56 ˚C for 45s, 72 ˚C for 40s and final extension step of 3 min at 72 ˚C (Sharma *et al.,* 2015).

Primary PCR amplified product served as a template for secondary PCR amplification and same PCR cycling conditions were used. After death of same animals brain samples were collected, flourecent antibody test (FAT) on brain impression smears was carried out as per standard protocol using lyophilised, adsorbed anti-rabies nucleocapsid conjugate (Bio-Rad). The slides were examined using an AHBT3 - RFC reflected light fluorescence (Olympus) microscope. Nucleoprotein (N) gene Hn RT-PCR and polymerase (L) gene Hn RT-PCR employed on skin samples were compared with FAT on brain for detecting the efficacy of these molecular techniques.

The sensitivity, specificity and accuracy were calculated using formulae:

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

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

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

Results and Discussion

FAT examination of 34 brain samples established 24 cases as true positive for rabies on the basis of presence of characteristic apple green fluorescence in positive samples. For molecular detection work RNA extraction was performed on skin biopsies. The 260/280 ratio of RNA extracted from skin samples, was in the range of 1.80-1.98. Further, the concentration of RNA from skin samples varied from 100.27-900.4 ng/µL. Factors that may influence the quantity and quality of viral RNA are the stage of rabies infection, thickness of sample collected for testing and proper handling of samples. Nucleoprotein gene (N) Hn RT-PCR resulted in expected amplicon of 586 bp (Fig 1) from skin biopsies in second round of PCR, using first round product as template and primers JW12 and JW10. Studies with nucleoprotein gene primers also reported similar amplified product (Orlowska *et al.,* 2008; Sharma *et al.,* 2023). Polymerase (L) gene hemi-nested RT-PCR with second set of primers that is PV05 and

Table 1: Primers targeting the Polymerase gene (L) and Nucleoprotein gene (N)

Target	Primer	Sequence	Sense	Gene	Reference(s)
Poly-merase gene (L)	PVO5	5'ATGACAGACAATTTGAACAA3'	+	L	Dacheux *et al.* (2008)
	PVO9	5'TGACCATTCCAGCAAGT3'	_	L	Dacheux *et al.* (2008)
	PVO8	5'GGTCTGATCTATCTGA3'	_	L	Dacheux *et al.* (2008)
Nucleo-protein gene (N)	JW 12	5'ATGTAACACCCCTACAATG3	+	N	Orlowska *et al.* (2008)
	JW 6DPL	5'CAATTGGCACACATTTTGTG3'	_	N	Orlowska *et al.* (2008)
	JW 10P	5'GTCATCAGAGTATGGTGTTC3'	_	N	Orlowska *et al.* (2008)

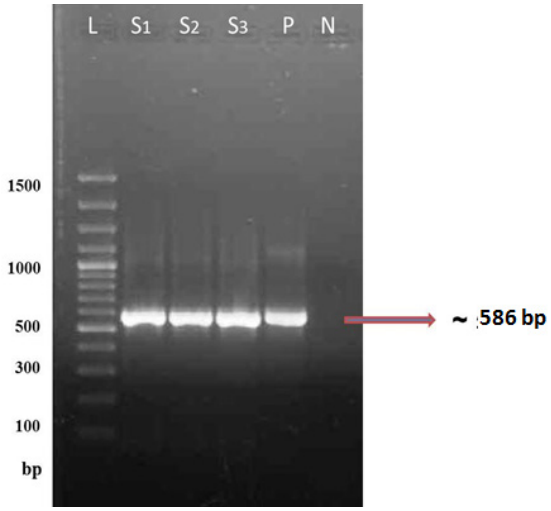

Fig 1: PCR amplification of N gene of Rabies virus with ~ 586 bp amplicons:
L- 100 bp plus DNA ladder (Thermo scientific, 0.5 µg/ µL)
Lane P- Positive Control, Lane S1, S2, S3, - Positive Samples;
N- Negative Control

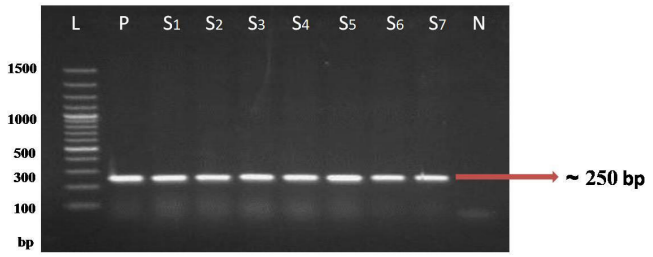

Fig 2: PCR amplification of L gene of Rabies virus with ~ 250 bp amplicons:
L- 100 bp plus DNA ladder (Thermo scientific, 0.5 µg/ µL)
Lane P- Positive Control; Lane S1, S2, S3, S4, S5, S6, S7- Positive Samples;
N- Negative Control

PV08 resulted in expected amplified PCR product of 250 bp (Fig 2). This was in accordance with previous studies on human skin biopsies (Dacheux *et al.,* 2008).

In present study, among the 34 clinically suspected rabies cases 24 cases were found true positive for rabies by FAT on brain samples. Out of 24 confirmed rabies positive cases, antemortem diagnosis using skin biopsies was successfully confirmed in 20 animals using N gene Hn RT-PCR and in 23 animals using L gene Hn RT-PCR. The sensitivity of detection of antemortem rabies from skin biopsy sample using N gene Hn RT-PCR was found to be 83.33%, whereas by L gene Hn RT-PCR it was 95.83%. The Hn RT-PCR protocol designed by Dacheux *et al.* (2008) revealed the sensitivity of 98% on human skin biopsies while targeting L gene of lyssa virus, whereas Reynes *et al.* (2011) by adopting similar approach obtained 88.88% sensitivity on animal skin biopsies, In present study, L gene exhibited higher sensitivity of 95.83% than results of Dacheux *et al.,* (2016) where L gene RT-qPCR resulted in 91.5% and 54.0% sensitivities, respectively, for skin and saliva samples of suspected animals. Nucleoprotein gene sensitivity in our study is greater than the result of previous antemortem study on human cases; RT-PCR of skin biopsy specimens collected from 10 patients that targeted the nucleoprotein gene resulted in a sensitivity of only 70% per sample and 77.7% per patient (Macedo *et al.,* 2006). N gene RT-PCR on skin samples obtained after death of the animals yielded a high sensitivity of 97.2% (35/36) and a specificity of 100% (31/31) (Zieger, 2015). However, nested PCR on antemortem skin biopsies (Bansal *et al.,* 2012) reported sensitivity of 76.5% by nucleoprotein gene. Sharma *et al.* (2023) successfully confirmed case of classical rabies virus infection from skin tissue specimens from nasolabial plate in dead cow adopting similar Hn RT-PCR approach targeting N gene. While comparing sensitivities of both genes it was found that the polymerase L gene is more sensitive than the nucleoprotein N gene and offers low threshold of detection. Specificity in detection of rabies was 100% in both assays.

Intravitam rabies diagnosis in 21st century has paved the way for reduction in rabies burden globally. Early diagnosis prevents further dissemination of virus, also helps in timely administration of post-exposure prophylaxis to contacts at risk and offers proper antiviral administration to confirmed cases. Skin offers higher sensitivity than fluid samples (Dacheux *et al.,* 2008) irrespective of stage of disease. It is very least invasive tissue and can be obtained with less difficulty compared to brain biopsies. In a recent study N gene Real Time PCR on suspected animal skin specimen demonstrated 98% sensitivity revealing good potential as an alternative sample to brain tissue for post-mortem rabies diagnosis in animals (Sujatha *et al.,* 2022).

Recommended ante-mortem diagnostic methods in disease investigation laboratories should have high sensitivity sufficient for early detection of a very low load of rabies viral RNA in biological samples and can be performed in short period of time. To our knowledge earlier no direct comparison between two genes sensitivity had been reported for antemortem diagnosis of rabies from skin samples of all major species of animals including cattle, buffalo, dog, cat and horse. Present study demonstrated higher accuracy of polymerase L gene Hn RT-PCR that is 97.05%, whereas 88.23% by nucleoprotein N gene Hn RT-PCR.

Conclusion

Our study emphasizes L gene Hn RT-PCR on nuchal skin biospies offers high sensitivities in all species of animals irrespective of their clinical presentation and stage of illness, thus can be recommended as standard routine test for antemortem rabies diagnosis in experienced animals' disease investigation laboratories. Both (L & N gene Hn RT-PCR) assays were highly specific indicate no false positive cases. Good sample collection and preparation can further increase success of intravitam molecular diagnosis of rabies.

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