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Efficacy Study of Immuno-histochemical Approach for Diagnosis of Rabies in Dogs

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ABSTRACT

Twenty seven dog brain tissues were examined for rabies infection by fluorescent antibody technique, immunohistochemistry and Hemi-Nested Reverse Transcriptase (HnRT-PCR). 16 (59.25%) dogs were confirmed to be rabid by FAT and HnRT-PCR assay while 15 samples were positive by immunohistochemistry (IHC). Sensitivities of HnRT-PCR and IHC were 100% and 94.11% respectively whereas both approaches detected rabies with 100% specificity. Rabies was diagnosed with accuracy of 100% by HnRT-PCR and 96.42% by IHC. It is, therefore, recommended that sensitivity, specificity and accuracy of IHC is comparable to advanced molecular approach viz. HnRT-PCR, thus, IHC could be relied upon for authentic diagnosis of rabies in dogs, in laboratories, where molecular approach is not feasible and/or facilities for immunofluorescence do not exist.

Keywords: Diagnosis, immuno-histochemical, rabies

Dogs happen to transmit more than 95% of rabies to other animals and humans in India and about 99% in Africa (Singh and Sandhu, 2007; WHO, 2005). Dogs, in such countries, roam around and mix freely with human population. Thus, dogs happen to be the species responsible for most spread of rabies from animals to humans. It is, therefore, often anticipated to rule out rabies in dogs (pets or stray) in case there has been provoked or unprovoked exposure of human beings. Dogs fall into four broad categories viz. pets (restricted and supervised); family dogs (partially restricted, wholly dependent); community dogs (unrestricted, partially dependent); and feral dogs (unrestricted, independent). Most dogs in India, perhaps 80%, would fall into the last 3 categories (Chaudhuri, 2005).

The diagnosis of rabies in dogs assumes great significance since, in developing countries, not many laboratories possess advanced molecular approaches, whereas, there are a few laboratories where requisite facilities for carrying out routine diagnosis of rabies with immunofluorescence exist. However, there are numerous laboratories that have facilities for histopathological procession of routine samples. Immunohistochemistry (IHC) is such a laboratory technique that is easily feasible in all the laboratories that perform histopathological analysis of tissue samples. The present study was, therefore, envisaged to compare the sensitivity, specificity and accuracy of IHC in comparison to advances molecular approach i.e. Hemi-Nested Reverse Transcriptase (HnRT-PCR) for analyzing feasibility of IHC for authentic diagnosis of rabies in dogs. IHC is a diagnostic technique which can demonstrate rabies antigen in fixed paraffin embedded tissue sections. In dog, the distribution of viral antigen was revealed either in granular form or as inclusion bodies in hippocampus cerebellum and cerebrum (Golbar, 2011). HnRT-PCR has been used to diagnose rabies virus (RABV) worldwide due to its sensitivity and immense versatility and can even be useful for examining paraffin-fixed archival and decomposed samples.

MATERIALS AND METHODS

Brain tissue samples of 27 dogs suspected for rabies were collected from post-mortem hall, Department of Veterinary

Pathology, GADVASU, Ludhiana, Punjab and different dairy farms of Punjab, India, between January 2014 and January 2015. All brain samples were stored at -20°C in the laboratory for further processing. Diagnostic tests like Fluorescent Antibody Test, immunohistochemistry and Hemi-nested reverse transcriptase PCR (HnRT-PCR) were carried out to detect the rabies virus infection. For immunohistochemistry, brain tissue samples were fixed and stored in 10% neutral buffered formalin for further analysis.

Fluorescent Antibody Test (FAT)

FAT was carried out on fresh brain sample following the standard protocol Meslin *et al.* (1996). Briefly, glass slides with impression smears of brain tissue were placed in coplin jar containing acetone and fixed at -4ºC for 1 hour. Positive slides from a known rabies positive case and negative slide from a normal and uninfected animal were used as positive and negative control, respectively. The slides were air-dried, incubated with lyophilized anti-rabies nucleocapsid conjugate (Bio-Rad, France) for 35 min at 37°C in a humid chamber and washed with Phosphate Buffered Saline (PBS) in 3 successive washes for 5-10 minutes. The slides were rinsed with distilled water, air-dried and a cover slip was mounted by adding buffered glycerol on the smear. The slides were visualized under an immuno-fluorescent microscope (Zeiss) for bright apple-green, round to oval bodies. Positive and negative controls were run together with the test specimens.

Immunohistochemistry

The paraffin embedded tissues were sectioned at 5µm thickness and sister sections were taken on Superfrost/ Plus, positively charged, microscopic slides (Fisher Scientific, USA) for each sample. Then, the sections were deparaffinized and rehydrated by immersing in 250 ml EZ-AR common solution at 70ºC for 10 minutes in EZ-Retriever R System V.2.1 and subsequent antigen retrieval was done in Citrate buffer (0.01 M, pH 6.0-6.2) at 95 ºC for 10 minutes and at 98 ºC for 5 minutes in EZ-Retriever R System V.2.1. Then, three washing were given in PBS buffer for 3 minutes each. The endogenous tissue peroxidases were inactivated by immersion of slides in 3% hydrogen peroxide solution in methanol for 15 minutes at room temperature in humid chamber followed by three washings with PBS buffer for 3 minutes each. On one section of each slide, primary polyclonal rabbit anti-rabies antibody 1:1000 dilution in PBS was added for 1 hour in moist chamber at room temperature. On the second section of each slide, PBS was added and no primary antibody was added so as to serve as a negative control. The sections were given three washing in PBS buffer for 3 minutes each, thereafter, incubated with polymer HRP (Super Sensitive label, One Step Polymer-HRPO Reagent) for 30 minutes at room temperature in moist chamber followed by three washing in PBS buffer for 3 minutes each. The antigen antibody- peroxidase reaction was developed with a freshly prepared 3, 3-diaminobenzidine (DAB) solution by mixing 2 drops of DAB chromogen with 1 ml of DAB buffer supplied by the manufacturer adding 5 ml hydrogen peroxide. Sections were washed in distilled water for 5 minutes and counterstained with Gill's haematoxylin.

Extraction of viral RNA

Total RNA was extracted directly from brain tissue using Trizol reagent (Invitrogen, USA), following the manufacturer's instructions with minor modifications. Briefly, 0.1 g brain tissue was homogenized with 1 ml Trizol and then 200µl chloroform (Ambion Life Technologies, USA) was added. After centrifugation of the sample at 10000 rpm for 15 min, the top aqueous layer was recovered and RNA was precipitated by adding 0.5 ml isopropanol. The sample was spun at 10000 rpm for 10 min, the liquid removed and the pellet washed with 1 ml of 75 % ethanol. The dried RNA pellet was dissolved in 50µl sterile RNase free water. RNA concentration was measured using Nano Drop Spectrophotometer (Nano drop Technologies, CA) in ng/µl and quality of RNA was checked as a ratio of OD 260/280 and stored at -80 ºC. RNA was converted into cDNA using High-Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems, USA).

Primary and Heminested amplification

Amplification of 2µl of the reverse-transcribed cDNA template was performed in a final volume of 25 µl; 12.5 µl 2XPCR mix (GoTaq Green Master Mix, Promega), 1.0 µl of each forward and reverse primer (JW12 and JW6) with 10 pmol concentration, and nuclease free water added to make final volume of 25 µl. The amplification was

performed in Thermal Cycler with cycling conditions as: initial denaturation at 94°C for 3 min, 35 cycles denaturation at 94°C for 30 s, annealing 56°C for 45 s and elongation 72°C for 20 s, and a final elongation was performed at 72°C for 3 min. For Hemi-nested PCR, similar quantities of the PCR mixture constituents except 2 µl of the primary PCR product as template and JW12 as forward and JW10 as reverse primer were used (Table 1). Thermocyclic conditions were kept same as that of primary PCR. PCR amplified products were visualized in 1% agarose gel electrophoresis after ethidium bromide staining for 586 bp amplicon specific for rabies virus.

STATISTICAL ANALYSIS

Calculation of sensitivity, specificity and accuracy

Sensitivity was calculated as [TP/(TP+FN)] × 100. Specificity was calculated as [TN/(TN+FP)] × 100. Accuracy was calculated as [TP+TN/(TP+FP+FN+TN)] × 100 wherein TP signifies true-positives; FN: false-negatives; TN: true-negatives and FP: false positives as determined by the reference assay i.e. FAT.

RESULTS AND DISCUSSION

A total of 27 dog brain samples collected on the basis of clinical suspicion of rabies were screened by FAT and 16 samples (59.25%) were found as true positive for rabies (Fig. A). All the positive samples (n=16) were also confirmed by HnRT-PCR. The samples positive for FAT were also subjected to immunohistochemistry and 15 samples (93.75 %) out of these 16 could also be detected by IHC. Sensitivities of IHC and HnRT-PCR were 94.11 % and 100 % respectively. Specificities of IHC and HnRT-PCR were found to 100% in both tests. Accuracy of IHC and HnRT-PCR were 96.42 % and 100 % respectively (Table 2).

The Direct Fluorescent Antibody test (DFA) is the gold-standard for rabies diagnosis from nervous tissue approved by World Health Organization (WHO) and Office International des Epizootics (OIE). The DFA is sensitive, specific and cheap (Dean *et al.* 1996). Bright apple-green, round to oval intracellular accumulations were observed (Fig. 1). Same findings were observed by Ehizibolo *et al.* (2009). IHC in formalin fixed paraffin embedded tissue sections was found to be a sensitive technique for detection of Negri bodies/Rabies antigen (Fig. 2) and, therefore, of immense value for retrospective studies. With IHC viral antigens were observed as fine granules in the cytoplasm of the neurons (Last *et al.* 1994), Gunawardena and Blakemore (2007), Rissi *et al.* (2004) which were not clearly visible with H & E staining. HnRT-PCR using a primer set that amplified the N gene of RABV was able to detect RABV in sixteen. Some isolates were not detected upto primary amplification was

Table 1: Oligonucleotide primers used for HnRT-PCR to diagnose rabies N gene

Primer	Nucleotide sequences (5´-3´)	Nucleotide position	Sense	Size of amplicon (bp)
JW 12	5'ATGTAACACCCCTACAATG3'	55-73	+	586
JW 6	5'CAATTGGCACACATTTTGTG3'	660-641	_	
JW 10	5'GTCATCAGAGTATGGTGTTC3'	636-617	_	

Table 2: Comparison of Sensitivity, specificity and Accuracy of immunohistochemistry and Heminested PCR

		IHC		HnRT-PCR		
		Positive	Negative	Positive	Negative	Total
FAT	Positive	15	01	16	00	16
	Negative	00	11	00	11	11
	Total	15	12	16	11	27
Sensitivity	94.11%			100 %		
Specificity	100%			100 %		
Accuracy	96.42%			100 %		

used. HnRT-PCR assay which is able to detect rabies virus (Fig. 3). Rabies diagnostic laboratories are increasingly faced with performing diagnostic procedures with brain tissue samples from animals. Most commonly, positive samples will be infected with an RV isolate of genotype 1. However, it is increasingly important to be certain that both RV and RRV isolates can be successfully detected. PCR detection of RV and RRVs has been described by a number of investigators McColl *et al.* (1993), Ermine *et al.* (1990). Although study in which HnRT-PCR was used has been published (Heaton *et al.* 1997) which had detect sixteen of the RV and RRV genotypes.

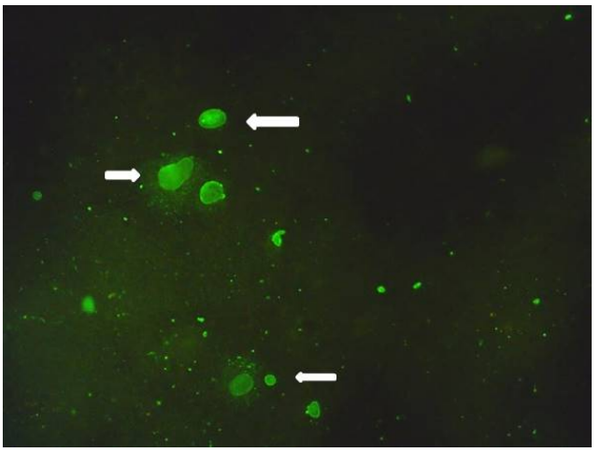

Fig. 1: Impression smear drawn from brain tissue showing diffused apple green fluorescence. Direct FAT. X40

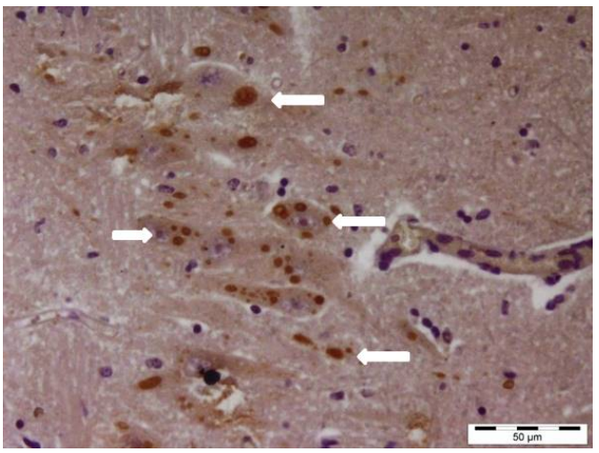

Fig. 2: Section of hippocampus of rabid animal showing sharply demarcated brown colored Negri bodies in the neurons (arrow) and brown colored small viral particles distributed in the stroma. IHC X 40

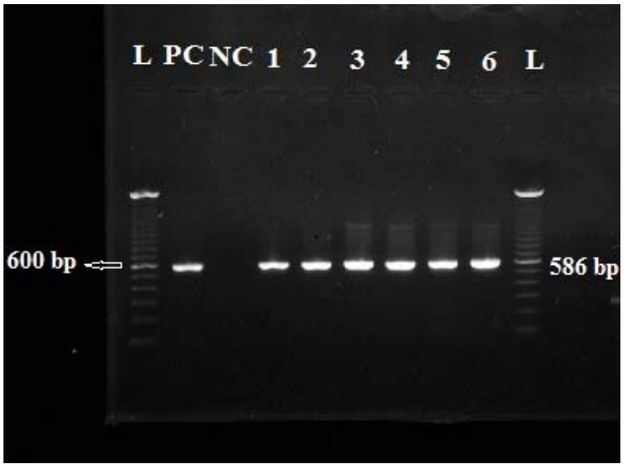

Fig. 3: Agarose gel (1%) stained with ethidium bromide. Lane L is the 100 bp ladder, NC is the negative control, PC is the positive control, S1 to S6 are the samples are positive by HnRT PCR

It is, therefore, recommended that sensitivity, specificity and accuracy of IHC is comparable to advanced molecular approach viz. HnRT-PCR, thus, IHC could be relied upon for authentic diagnosis of rabies in dogs, in laboratories, where molecular approach is not feasible and/or facilities for immunofluorescence do not exist.

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