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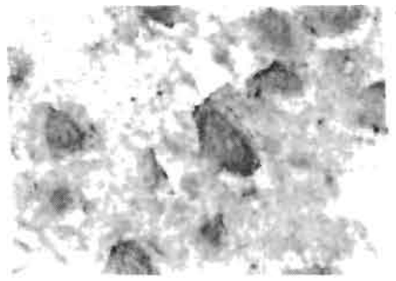

Figure 1. Hippocampus of dog, showing blue-coloured positive signals in the cytosol of neurons. *In situ* PCR, ×1000

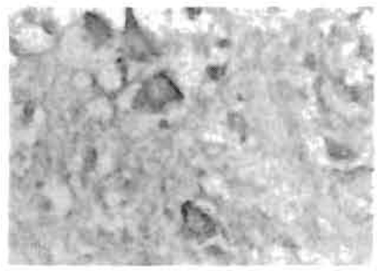

Figure 2. Hippocampus of dog, negative, showing no staining by *in situ* PCR, ×1000

Figure 3. Cerebrum of dog, showing blue granular positive signals in the cytosol of neurons. *in situ* PCR, ×800

DISCUSSION

This report describes the application of ISPCR for the detection and localization of rabies virus 'N' gene in paraffin embedded tissues. The FAT detects the 'N' protein while the ISPCR detects the 'N' gene mRNA coding for the 'N' protein. This could also explain the observation of the same sensitivities in both FAT and ISPCR. ISPCR has been demonstrated

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Detection of Rabies Virus Genes by *In-Situ* Polymerase Chain Reaction

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ABSTRACT

Rabies is diagnosed by FAT in the impression smears of brain tissues. In this study, an attempt was made to diagnose rabies using *in situ* polymerase chain reaction (ISPCR). A digoxigenin-labelled double-stranded probe specific for a portion of the 'N' gene of rabies virus was used. Positive signals were identified as blue dots in the intraneuronal and neuropil areas.

Keywords: *in situ* PCR, rabies diagnosis

Abbreviations: BSA, bovine serum albumin; DEPC, diethyl pyrocarbonate; dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosine triphosphate; DIG, digoxigenin; cDNA, complementary DNA; DNase, deoxyribonuclease; dTTP, deoxythymiidne triphosphate; dUTP, deoxyuridine triphosphate; FAT, fluorescent antibody technique; ISPCR, *in situ* polymerase chain reaction; NBT-BCIP, 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate; PCR, polymerase chain reaction; RNA, ribonucleic acid; RNase, ribonuclease; RT, reverse transcription; RT-PCR, reverse transcription–polymerase chain reaction; SSC, standard saline citrate; Tris, trimethoxyaminomethane

INTRODUCTION

Rabies is a disease caused by an RNA virus, belonging to the genus *Lyssavirus* of the family *Rhabdoviridae*, which can affect almost all mammals, including humans. The standard diagnostic test consists of direct fluorescent antibody test (FAT) on impression smears from fresh brain samples (Dean and Abelseth, 1973). This test remains the cornerstone of postmortem rabies diagnosis, but its sensitivity is reduced once the specimen starts to decompose. The polymerase chain reaction (PCR) technique is a rapid, sensitive and specific method that can be used as an alternative protocol for the routine diagnosis of rabies, as it is efficient even with highly, degraded samples (Heaton *et al.*, 1997). Despite PCR being a powerful and useful tool it has one major limitation, that it was not possible to correlate PCR results with the pathological features of tissue, because preparation for the PCR required disruption of the tissues to extract DNA or RNA from the sample. This problem can be overcome by employing *in situ* PCR (ISPCR) on the tissue samples. ISPCR can detect viral DNA or RNA in cells and in histological sections. ISPCR has three strengths for the detection of viral nucleic acids. First, it can combine PCR with the intracellular localizing ability of *in situ* hybridization; secondly, ISPCR provides information relative to the distribution of

the virus; and thirdly, ISPCR require only small samples. The ISPCR method to detect rabies virus RNA in mice, raccoons, dogs and human brain tissues and cells has been reported (Jackson *et al.*, 1989; Jackson and Wunner, 1991; Jackson, 1992; Jackson and Rintoul, 1992; Jayakumar *et al.*, 2003; Nadin-Davis *et al.*, 2003; Nuovo *et al.*, 2005). This paper describes a study in which an ISPCR procedure was developed for the detection of rabies virus gene.

MATERIALS AND METHODS

Twelve canine brain samples suspected of rabies were collected and impression smears of the hippocampus were subjected to FAT according to the method described by Dean and Abelseth (1973). The sections of hippocampus major, cerebrum and cerebellum were used for ISPCR.

Tissue processing

Tissues fixed in 10% formol saline (40% formaldehyde 100 ml, sodium chloride 9 g and distilled water 900 ml) were embedded in paraffin blocks. They were sectioned at 4 μm thickness and were mounted on silane-coated slides. The tissue sections were deparaffinized by immersing the slides twice in xylene for 5 min each. The slides were dehydrated with 100% ethanol for 5 min. Sections were digested with pepsin 2 mg/ml at room temperature for 30 min and were washed with diethyl pyrocarbonate (DEPC) water for 1 min to stop digestion. Slides were then dehydrated with 100% ethanol.

In situ PCR

Prior to ISPCR, sections were treated with 10–20 μl of RNase-free DNase. Sections were then covered with a piece of paraffin and incubated in a moist chamber at 37°C overnight. Subsequently, the DNase activity was destroyed by washing the slides with DEPC water for 1 min. Again the slides were dehydrated with 100% ethanol and air-dried.

One-step RT-ISPCR (cDNA synthesis and PCR amplification)

Reverse transcription (RT) PCR was carried out with reagents from Gene Amp following the manufacturer's instructions.

Primers

Rabies virus-specific primers set for nucleoprotein (N-gene) (Gibco-BRL) were used:

"N For 1": 5′-GCG GAT CCC ACC TCT ACA ATG GAT GCC G-3′ (29 bp)
"N Rev 2": TCC GGT ACC TTA TGA GTC ACT CGA ATA TGT CT-3′ (33 bp)

The nucleotide sequence number in the NCBI database was AF374721 (Dr R. Jayakumar, Indian street rabies virus isolate).

The expected size of PCR product using the above primers is 1380 bp. The digoxigenin (DIG) DNA labelling and detection kits (Boehringer Mannheim) were used following the manufacturer's instructions.

A master mix of 50 µl was prepared for PCR by adding the following reagents: 5XEZ buffer (1×), dGTP (300 µmol/L), dATP (300 µmol/L), dTTP (300 µmol/L), DIG 11 dUTP (1 mmol/L), rT^{th} DNA polymerase (5 units/50 µl), 25 mmol/L $Mn(OAc)_2$, forward primer (100 pmol), reverse primer (100 pmol) and nuclease-free water.

The reagents were mixed, centrifuged briefly, dispensed on to each tissue section, covered with the cover slip, and placed in an *in situ* block which was wrapped in aluminium foil. The PCR amplification was performed in a thermal cycler (PTC-100, MJ Research) using the following cycling parameters. Following an initial denaturation at 96°C for 3 min, further cycles included denaturation at 96°C for 2 min, annealing at 47°C for 1 min and extension at 74°C for 2 min. The annealing temperatures were increased to 52°C after 6 cycles and to 57°C after 5 cycles. Then 24 cycles were performed at 57°C, followed by a final extension at 74°C for 10 min.

Immunological detection of ISPCR products

Following PCR, the slides were washed with 0.2× standard saline citrate (SSC) containing 0.2% bovine serum albumin (BSA) at 60°C for 10 min. Washing was done with 0.1 mol/L trimethoxyaminomethane (Tris)–hydrochloric acid (HCl), pH 7.4, and 0.1 mol/L sodium chloride for 30 min, followed by incubation at 37°C with detection buffer containing anti-DIG alkaline phosphatase conjugate for 30 min. The slides were washed with 0.1 mol/L Tris-HCl (pH 8.0) twice and incubated with chromogen (4-nitrobluetetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, NBT-BCIP) solution for 1 h in a dark, humid chamber at room temperature. Colour development was stopped by washing sections with DEPC water, counterstained with nuclear Fast red stain and observed under a microscope.

RESULTS

Of the 12 cases examined (clinical data presented in Table I), 9 were positive by both FAT and ISPCR and 3 were negative by both the tests. Thus the sensitivities of ISPCR and FAT appeared similar. The sensitivity of ISPCR was good with 4 µm sections. The tissues treated with RNase-free DNase had lower backgrounds than untreated ones. Among different concentrations and incubation times of pepsin, optimal results were obtained with 2 mg/ml pepsin for 30 min. The areas showing blue dots were identified as positive (Figure 1). When no primers were added to the master mix, no positive signal was found in rabies-positive (Figure 2) or in negative tissues. In all positive cases signals were seen scattered around the nuclei of neurons in the hippocampus. The signals were also seen in the cytosol of neuronal cells (Figure 3) and in neuropil of cerebrum and cerebellum.

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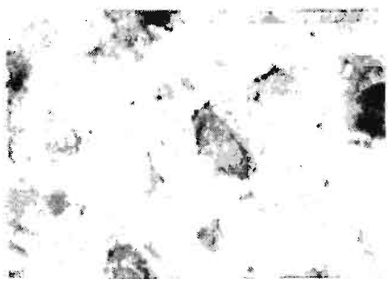

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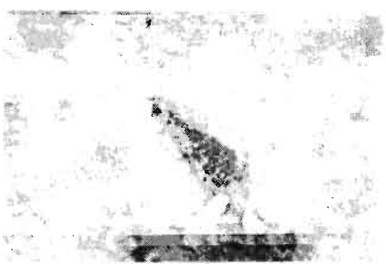

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to be useful in diagnosis of other diseases such as infectious bursal disease (Liu *et al.*, 2000).

During our experiments, amplification was less successful with thicker sections, and multiple cell layers were difficult to interpret. The strongest staining was obtained when the tissues were cut at 4 μm thickness. The brain tissues must be treated with DNase to prevent non-specific binding from cellular DNA (Liu *et al.*, 2000). In this study, digestion was carried with 10 units of DNase, which decreased non-specific binding background. Sensitivity decreased in sections of 5 μm or thicker because amplification was often less successful owing to the presence of multiple cell layers (Bagasra *et al.*, 1993).

Bagasra and colleagues (1993) stated that time and incubation temperature should be optimized for tissue sections. Viral proteins must be permeable to the RT-PCR reagents and probes for immune detection. Therefore, a permeabilization step is necessary. In this study, different concentrations and incubation times of pepsin were tested and optimal results were obtained with 2 mg/ml pepsin for 30 min. With too little digestion, the cytoplasmic and viral-shell proteins were not sufficiently permeable to primers and enzyme and the amplification was inconsistent. On the other hand, higher concentrations of pepsin and longer digestion times resulted in higher background and poor morphology. The present technique involved reverse transcription in ISPCR, where complementary DNA was synthesized *in situ* through a reverse transcription step followed by PCR (Nuovo *et al.*, 1993). With the incorporation of rT^{th} enzyme, the overall reaction was shortened and simplified. Similar results were reported by Gu (1995) and Nuovo (1997).

In this study, ISPCR detected the rabies gene in the same number of samples as did the FAT. Moreover, FAT is much more rapid, simpler and easy to perform than ISPCR for diagnostic purposes. The primers used in this study amplified a product of 1380 base pairs and it is matter of conjecture whether amplification of a shorter PCR product would have resulted in a more sensitive assay.

However, this observation does not discount the importance of ISPCR in rabies research. ISPCR can detect viral genes and provide information on its localization. With this, one can determine the cellular host range of the virus and the absolute number of cells that are infected. The cellular antigen can be detected because ISPCR is coupled with histology and immunohistochemistry. Despite these advantages, this technique is costly and time-consuming and needs skilled personnel and specialized equipment and hence can only be considered as a research tool in studies related to rabies virus. In a recent study Nuovo and colleagues (2005), while comparing Negri body detection, immunohistochemistry and IS-PCR, concluded that ISPCR for rabies virus was the most accurate test for the determination of viral load in rabies encephalitis.

REFERENCES

Bagasra, O., Seshamma, T. and Pomerantz, R.J., 1993. Polymerase chain reaction *in situ* intracellular amplification and detection of HIV-1 proviral DNA and other specific genes. *Journal of Immunological Methods*, **158**, 131–145

Dean, D.J. and Abelseth, M.K., 1973. The fluorescent antibody tests. In: M.M. Kaplan and H. Koprowski (eds), *The Laboratory Techniques in Rabies*, 3rd edn. (WHO, Geneva), 73–84

Gu, J., 1995. *In situ* PCR—an overview, (Eaton Publishing, Boston), 1–21

Heaton, P.R., Johnstone, P., McElhinney, L.M., Cowley, R., O'Sullivan, E. and Whitby, J.E., 1997. A hemi-nested PCR assay for the detection of six genotypes of rabies and rabies-related viruses. *Journal of Clinical Microbiology*, **35**, 2762–2766

Jackson, A.C., 1992. Detection of rabies virus mRNA in mouse brain by using *in situ* hybridization with digoxigenin-labelled RNA probes. *Molecular and Cellular Biology*, **6**, 131–136

Jackson, A.C. and Rintoul, A.E., 1992. Effect of post-mortem autolysis on the detection of rabies genomic RNA and mRNA in mouse brain by using *in situ* hybridization. *Molecular and Cellular Probes*, **6**, 231–235

Jackson, A.C. and Wunner, W.H., 1991. Detection of rabies virus genomic RNA and mRNA in mouse and human brains by using *in situ* hybridization. *Journal of Virology*, **65**, 2829–2844

Jackson, A.C., Reiner, D.L. and Wunner, W.H., 1989. Detection of rabies virus RNA in the central nervous system of the experimentally infected mice by using *in situ* hybridization with RNA probes. *Journal of Virological Methods*, **25**, 1–12

Jayakumar, R., Thirumurugan, K.G., Ganga, G., Sheela, K.P., Vijayarani, K. and Naina, A.M., 2003. *In situ* reverse transcription–polymerase chain reaction: a novel technique for detection of rabies virus RNA in murine neuroblastoma cells. *Acta Virologica*, **47**, 57–59

Liu, X., Giambrone, J.J. and Hoerr, F.J., 2000. *In situ* hybridization immunohistochemistry and *in situ* reverse transcription–polymerase chain reaction for detection of infectious bursal disease virus. *Avian Diseases*, **44**, 161–169

Nadin-Davis, S.A., Sheen, M. and Vandeler, A.I., 2003. Use of discriminatory probes for strain typing of formalin-fixed, rabies virus-infected tissues by *in situ* hybridization. *Journal of Clinical Microbiology*, **41**, 4343–4352

Nuovo, G.J., 1997. *PCR in situ Hybridization: Protocols and Applications*, 3rd edn, (Lippincott Raven, London), 1–9

Nuovo, G.J., Forde, A., Mac Connell, P. and Fahrenwald, R., 1993. *In situ* detection of PCR amplified HIV-1 nucleic acids and tumor necrosis factor cDNA in cervical tissues. *American Journal of Pathology*, **143**, 40–48

Nuovo, G.J., Defaria, D.L., Chanona-Vilchi, J.G. and Zhang, Y., 2005. Molecular detection of rabies encephalitis and correlation with cytokine expression. *Modern Pathology*, **18**, 62–67

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