LETTER-TO-THE-EDITOR

IN SITU REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION: A NOVEL TECHNIQUE FOR DETECTION OF RABIES VIRUS RNA IN MURINE NEUROBLASTOMA CELLS

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Rabies is the most dreaded disease that poses continuous challenge to control measures adopted all over the world, especially in developing countries like India where human rabies is often a serious problem. Although the diagnosis of rabies has improved over the last few years, laboratory facilities still play a major role. Molecular techniques, especially polymerase chain reaction (PCR) finds its application to many areas of diagnostics and also provides an increased sensitivity with less labor and time (*1*). Use of non-radioactive labels such as biotin and digoxigenin minimize the costs and also meet the safety concerns.

The objective of the present study was to assess usefulness of an *in situ* reverse transcription–PCR (ISRT-PCR) technique incorporating a digoxigen-labeled nucleotide into viral ribonucleoprotein as a means for identifying rabies virus antigen in murine neuroblastoma cells infected with street rabies virus isolates. The same has been compared for sensitivity with a direct fluorescent antibody test (FAT).

Rabies street virus isolates were multiplied in murine neuroblastoma cells (N2a) according to the method described by Bourhy *et al.* (*2*) with some modifications. Briefly, duplicate coverslip cultures were prepared from N2a cell suspensions of 4 x 10^5 cells per ml. Suspected brain samples were prepared as 20% suspensions in Eagle's Minimum Essential Medium (Life Technologies). The suspensions were clarified by centrifugation at 3000 x g for 10 mins at 4°C and 100 µl aliquot of the supernatants were used for infection of coverslip cultures. Duplicate infected cultures for each sample and one uninfected culture (negative control) were prepared. The cultures were incubated at 37°C for 24, 48 and 72 hrs. Then the coverslips were carefully removed, air-dried and subjected to indirect FAT and ISRT-PCR.

For the FAT with a rabies nucleocapsid antibody FITC conjugate the coverslips were fixed with acetone and processed as described earlier (*3*). For the ISRT-PCR the coverslips were fixed in methanol, air-dried and treated with pepsin (2 mg/ml) at room temperature for 30 mins. Subsequently they were washed with diethylpyrocarbonate (DEPC)-treated water (to inactivate pepsin) followed by absolute ethanol and air-dried. Twenty µl of a DNase digestion solution (10 µl of RNase-free DNase (1 U/ml; Qiagen) and 8 µl of 50 mmol/l Tris-HCl pH 7.5 with 5 mmol/l $CaCl_2$) per coverslip was added and were incubated overnight in a moist chamber at 37°C. The coverslips were then washed with DEPC-treated water and absolute ethanol and air-dried. An one-step RT-PCR was performed as described earlier (*4*) using the Gene Amp Ez One Step RT PCR Kit (Perkin Elmer). For the RT step a mixture (master mix, 50 µl) containing 1x Ez buffer, 300 µmol/l dGTP, 300 µmol/l dATP, 300 µmol/l dTTP, 300 µmol/l dCTP, 1mmol/l Dig-11-dUTP, 5U of recombinant *Thermus thermophilus* (rTth) DNA polymerase, 25 mmol/l manganese acetate, and 100 pmol/l rabies

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Abbreviations: DEPC = diethylpyrocarbonate; ISRT-PCR = *in situ* reverse transcription–PCR; FAT = fluorescent antibody test; PCR = polymerase chain reaction; rTth = recombinant *Thermus thermophilus*

nucleoprotein gene-specific primers (the forward primer 5'-GCG GAT CCC ACC TCT ACA ATG GAT GCC G-3' (29 bp) and the reverse primer 5'-TCC GGT ACC TTA TGA GTC ACT CGA ATA TGT CT-3' (33 bp)). The master mix was dispensed on a clean glass slide and the infected coverslips were placed over it. The glass slide was then placed in an *in situ* block, covered with aluminum foil and the RT-PCR was performed in a MJ Research Thermal Cycler. The cycling conditions were as follows. The RT step consisted of 96°C/1 min and 62°C/45 mins, while the PCR step consisted of 5 cycles of 96°C/2 mins, 47°C/1 min and 74°C/2 mins, 5 cycles of 96°C/1 min, 52°C/1 min and 74°C/2 mins, and 24 cycles of 96°C/1 min, 57°C/1 min and 74°C/2 min. Uninfected coverslip cultures and those incubated with the master mix without the primers served as negative controls. To address the possibility of non-specific labeling due to diffusion of the amplicons from the site of production to other regions, uninfected and infected coverslip cultures were mounted on the same slide and covered with the master mix and a single coverslip. Following PCR the coverslips were carefully removed, washed three times with a wash buffer (0.1 mol/l Tris-HCl pH 7.4 and 0.1 mol/l NaCl) at 37°C for 10 mins and incubated with the 1x Blocking Solution (Boehringer Mannheim) for 30 mins at 37°C. An anti-digoxigenin antibody-alkaline phosphatase conjugate was added to the blocking solution diluted 1:5000 and further incubated at 37°C for 1 hr. The coverslips were then removed carefully, washed three times with PBS, equilibrated with a detection buffer (100 mmol/l Tris-HCl, 10 mmol/l NaCl and 50 mmol/l $MgCl_2$ pH 9.5), charged with a substrate solution (4.5 µl of NBT, 3.5 µl of BCIP and 1 ml of the detection buffer) and incubated at room temperature in a dark place for color development. The latter was stopped by washing with water, counterstained with the Nuclear Fast Red, washed, dehydrated, mounted with permount and visualized.

For the ISRT-PCR the most optimum time and concentration of pepsin digestion of tissues was 30 mins and 2 mg/ml, respectively. The signal intensity decreased considerably if digestion was prolonged for more than 60 mins and the signal was absent if pepsin digestion was omitted. The cells treated with DNase had a lower background color than the untreated cells (results not shown).

ISRT-PCR produced an excellent cellular localization of signals. Parts A and B of the figure show purple color inclusions in the cytoplasm but not in the nuclei of infected cells. The absence of signals after cDNA synthesis alone indicated the necessity of the message amplification; this observation was confirmed when DNA polymerase was omitted from the PCR. The signals were first observed in the cells at 24 hrs p.i. No signals were observed in the uninfected cells and in coverslips without the primers in master mix. When virus-infected and uninfected coverslips were mounted on the same slide with the master mix positive signals were noticed only in the infected but not in the uninfected cells.

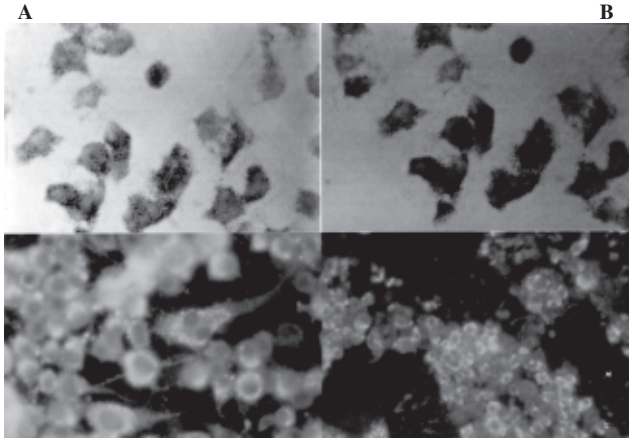

Fig. 1

The results of ISRT-PCR were compared with those of the gold standard FAT using a nucleocapsid antibody. Parts C and D of the figure show inclusions in the cytoplasm of infected cells. The 42 samples found positive by FAT gave also positive signals in ISRT-PCR. Parts A and B of the figure show similar inclusions, however, their intensity is stronger due to amplification of viral RNA. In general, the results obtained with ISRT-PCR were comparable with those of FAT.

A classical RT-PCR has been widely applied to clinical diagnosis of viral infections. However, extraction of viral RNA from a mixed cell population makes it impossible to identify cellular origin of the amplified signal. ISRT-PCR, in contrast, can detect a non-abundant mRNA and localize the signals to single cells; consequently, this technique can be also used to determine the frequency of gene expression. A combination of PCR and *in situ* hybridization could offer a powerful research and clinical tool for detection of the genes with low expression. A genomic target for PCR should be highly expressed and conserved at least in primer binding sites The need for sensitivity also suggests that the genes located close to the 3'-end of genome are suitable targets in negative-sense RNA viruses, as they are more intensively transcribed into mRNA owing to the decreasing transcription rate in the 3' to 5' direction. Therefore it is clear that the nucleoprotein gene, which fulfills both the criteria, should be the target in all the described diagnostic trials (*5*). Hence we used the primers for amplification of the rabies virus nucleoprotein gene in ISRT-PCR.

ISRT-PCR represents definitely an alternative for the radioisotope-based *in situ* hybridization as it is convenient

for handling, quicker and shows an excellent cellular localization of signals. An advantageous non-isotopic method of *in situ* hybridization employing digoxigenin-labeled RNA probes has already been reported for rabies (*6*). ISRT-PCR would allow for a routine and rapid detection of a low copy viral RNA. The key variable for the test is the protease digestion for which the optimal digestion time is to be determined. The one-step RT-PCR system allows for reproducible amplification and detection of low-copy RNA targets within few hours. Armed with the advantage of cellular localization of signals it is possible to determine the number as well as the percentage of cells that are infected.

References

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