NOTE Pathology

Optimization of In situ Hybridization Protocols for Detection of Feline Herpesvirus 1

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ABSTRACT. In situ hybridization (ISH) protocol including microwaving pre-treatment regimes was developed and compared with protease digestion as a pre-treatment regime for its effects on detecting feline herpesvirus 1 (FHV-1) in formalin fixed, paraffin embedded tissue. We found that optimum results were obtained using microwave pre-treatment. The results showed that the use of microwave irradiation would be recommended as a means of supplementing ISH methods, especially when using long-term formalin fixed, paraffin-embedded tissue.

KEY WORDS: FHV-1, in situ hybridization, microwave.

In situ hybridization (ISH) assay is a successful method for localization of specific viral nucleic acids inside individual cells with preservation of cell and tissue morphology, thus permitting a simultaneous assessment of the morphological alterations associated with the lesion [3]. For veterinary practitioners, formalin is the most common fixative to help retain nucleic acids but forms extensive cross-linkages in the process, hindering probe penetration [1]. The aim of this study was to develop a sensitive ISH protocol using a non-radioactive digoxigenin-labeled probe for the detection and localization of intracellular FHV-1 viral DNAs in formalin-fixed, paraffin-embedded sections.

The FHV-1 DNA probe was constructed by PCR, which was specific for 322-bp segment within the TK region, and labeled with digoxigenin after the amplification reaction. The FHV-1 specific PCR was performed as described previously [5]. After amplification, PCR product was purified using Wizard PCR preps (Promega Biotech, Madison, WI). Purified PCR product was labeled by random priming with digoxigenin by means of a commercial kit (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Cultured Crandell-Reese feline kidney (CRFK) cells were infected with FHV-1 and then harvested by trypsin treatment 2 days after incubation, resuspended in 3 ml of 10% neutral buffered formalin and were fixed for 12 hr, 24 hr, 3 days, 7 days and 14 days respectively. Fixed cells were centrifuged at 1,000 rpm for 5 min and each pellet was resuspended in 1 ml 2% liquid agarose at 65°C. Then, the reaction tube was centrifuged for 5 min at 1,000 rpm to concentrate the cells in the agar and the agar-cell pellet was solidified at 4°C for at least 1 hr. The agar cone was carefully taken out of the tube and embedded in paraffin using an automated tissue processor under standard conditions for surgical biopsies. Thereafter, paraffin sections were prepared on silane-coated slides (Sigma, St. Louis, MO). Sections were deparaffinized in xylene (2 × 10 min), taken through a graded series of ethanol (1 × 5 min in 100, 95, 75 and 50%) and washed in DEPC H2O (2 × 5 min). From this point sections were subjected to one of following two different pre-treatments: 1) Enzymatic digestion: sections were digested in 50, 100 or 200 µg/ml proteinase K (Roche) made up in phosphate buffered saline (PBS) for 30 min at 37°C. Digestion was halted by washing in PBS containing 2 mg/ml glycine (2 × 5 min). 2) Microwaving: sections were placed in Tris/EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and microwaved at full power on a rotating turntable, uninterruptedly for 5 min, 10 min and 15 min respectively (Amanda 850 W, U.S.A.). Sections were allowed to cool in the buffer for 15 min and DEPC H2O was added gradually over 5 min to reduce the temperature of buffer to room temperature. Sections were then washed in DEPC H2O (2 × 5 min). Hybridization and colorization was carried out in the same manner with the previous described for porcine epidemic diarrhea virus [2]. Thereafter, sections were counterstained with 0.5% methyl green. The development of a dark-purple precipitate at the enzyme site in positive cells was monitored by microscopic examination.

After ISH, qualitative analyses were performed for hybridization signal intensity and tissue morphology. The intensity results of each ISH protocols were presented in Table 1. As the fixation time was increased, the intensity was decreased. The highest signal intensity was observed in 12 hr-fixed cells. However, even in those sections, the signal was weak after digestion with 50 µg/ml proteinase K (Table 1). In case of proteinase K treatment, the highest signal intensities were observed when the sections were digested with 200 µg/ml proteinase K. However, digestion with 200 µg/ml proteinase K caused some tissue degradation and increased background staining. Digestion with 100 µg/ml proteinase K induce less non-specific signals and almost similar signal intensity as compared with digestion with 200 µg/ml proteinase K (Table 1). Microwaving with Tris/EDTA buffer produced high signal intensities, although 5-min microwaving induced less signal intensities. Also, microwaving had less effect on cellular morphology and background staining, comparing with proteinase digestion. By 10-min microwaving pre-treatment, ISH of the sections...
which fixed for 7 days showed positive signals with moderate intensities in the nucleus and cytoplasm as distinct areas of dark purple signals in a lot of cells (Fig. 1). However, the results of Proteinase K pre-treatment in the same sections showed weak positive signals (Table 1).

Table 1. Results of in situ hybridization with FHV-1-specific DNA probe

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>Time of formalin fixation</th>
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<tbody>
<tr>
<td></td>
<td>12 hr</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>50</td>
</tr>
<tr>
<td>(µg/ml)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>200</td>
</tr>
<tr>
<td>Microwave</td>
<td>5</td>
</tr>
<tr>
<td>(min)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>15</td>
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a) Signal intensity/morphology: –, negative; +, weak; ++, moderate; ++++, strong; and ++++, very strong. For tissue morphology the scores were: 0, no change; 1, some tissue disruption; 2, marked tissue disruption.

In this study, two pre-treatment regimes were compared in formalin fixed and paraffin embedded FHV-1-infected cells. Results were compared for the hybridization signal intensities and cellular morphology. We found that optimum results were obtained using 10-min microwave pre-treatment. The heat produced by microwaving disrupts cross-linkages formed during formalin fixation [4]. The major advantages with microwaving were lower background staining, preservation of tissue morphology, and shorter treatment time. This study suggested that microwaving is a suitable pre-treatment for in situ hybridization as compare with enzyme digestion using proteinase K for detection of FHV-1.

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REFERENCES


Fig. 1. In situ hybridization using 10-min microwaving pre-treatment with a 7-day fixed section. Positive signals were shown with moderate intensities in the nucleus and cytoplasm as distinct areas of dark purple signals in a lot of cells. NBT/BCIP colorization, methyl green counter-stain, × 400.