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Short Communication

Optimization of *in situ* hybridization assay using non-radioactive DNA probes for the detection of canine herpesvirus (CHV) in paraffin-embedded sections

Okjin Kim*

Department of Laboratory Animal Sciences and Center for Animal Resource Development, College of Medicine, Seoul National University, Seoul 110-799, Korea

Two non-radioactive probes using digoxigenin or biotin were developed for detecting canine herpesvirus (CHV) and compared for their sensitivities by *in situ* hybridization (ISH) in formalin fixed, paraffin embedded sections, which has been used routinely in veterinary fields. Sections of the CHV-infected cell preparation were subjected to several different ISH protocols using digoxigenin- or biotin-labeled probe respectively. Results were compared for the hybridization and background signal intensities. The best result was obtained by the optimized ISH protocol using digoxigenin-labeled probe for detection of CHV DNA. The optimized ISH assay, which developed in this study, may be a valid tool for the study of pathogenesis and diagnosis of CHV infection.

Key words: canine herpesvirus, digoxigenin, biotin, *in situ* hybridization

Canine herpesvirus (CHV) is a member of the alpha herpesvirus subfamily that can cause a severe hemorrhagic disease in neonatal pups as well as mild or subclinical respiratory infections in adult dogs [1]. Since its isolation, CHV has been identified in many countries and a worldwide distribution is presumed. Several studies in South Korea [5,9] and European countries [6,7], suggest a high prevalence of the virus among the dog population. As CHV is presumed to be widespread among the dog population and as the economic losses that breeding kennels may suffer after infection with CHV may be disastrous, it seems necessary to determine the CHV infection more exactly. Formalin-fixed, paraffin-embedded tissues have been used routinely in veterinary practice. For the study of pathogenesis and diagnosis of viral infection, *in situ* hybridization (ISH) assay and immunohistochemistry (IHC) have been used commonly. However, following previous reports, ISH is more sensitive and specific than IHC for the detection of viral infection in formalin-fixed tissues [3,4]. The aims of the present study were to develop an optimized *in situ* hybridization assay, which could be carried out reliably for diagnostic purposes and for study of pathogenesis using formalin-fixed, paraffin-embedded tissues with CHV infection.

The CHV DNA probes were constructed by PCR and labeled with either digoxigenin or biotin after the amplification reaction. The CHV specific PCR was performed as described previously [8]. After amplification, PCR products were purified using Wizard PCR preps (Promega Biotech, Medison, WI). Purified PCR products were labeled by either random priming with digoxigenin-dUTP (Roche) or Biotin-high prime (Roche) by means of a commercial kit according to the manufacturer's instructions. CHV infected cell preparation was devised as a tissue model for further work involving formalin-fixed, paraffin embedded tissues that are used routinely in the field of veterinary pathology. Madin Darby canine kidney (MDCK) cells were infected with CHV F-205 at amounts equivalent to between 10^3 and 10^7 $TCID_{50}$ and processed for paraffin-embedding as described previously [2]. Thereafter, paraffin sections were prepared on silane-coated slides (Sigma, St. Louis, MO). For ISH, sections were deparaffinized in xylene (2 × 10 min), taken through a graded series of ethanols (1 × 5 min in 100, 95, 75 and 50%) and washed in DEPC H_2O (2 × 5 min). Then, those sections were digested respectively in 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). After pre-treatment with proteinase K, all sections were subsequently washed in PBS (1 × 5 min) and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 min, and then hybridization was done for 3 hours or overnight at 45°C respectively. The

*Corresponding author
Phone: 82-2-740-8077; Fax: 82-2-763-5206
E-mail: kimoj@netian.com

Table 1. Comparison of different protocols for the detection of canine herpesvirus DNAs in paraffin sections by *in situ* hybridization

Labels[b]	Protease K[c]	Hybridization[d]	Titer of inoculated virus[a] ($TCID_{50}$)					
			0	10^3	10^4	10^5	10^6	10^7
Digoxigenin	100	3 h	-[e]	-	+	+	++	++
		Overnight	-	+	+	++	+++	+++
	200	3 h	-	-	+	+	++	++
		Overnight	-	+	+	++	+++	+++
Biotin	100	3 h	-	-	-	+	+	++
		Overnight	-	-	+	+	++	++
	200	3 h	-	-	-	+	++	++
		Overnight	-	-	+	+	++	++

[a]The assays were performed at 24 hours after viral inoculation
[b]Purified PCR products were labeled by either digoxigenin or biotin.
[c]Enzyme digestion was performed respectively in 100 or 200 μg/ml proteinase K
[d]Hybridization was done for 3 hours or overnight at 45°C respectively
[e]-; negative, +; weak positive, ++; moderate positive, +++; strong positive

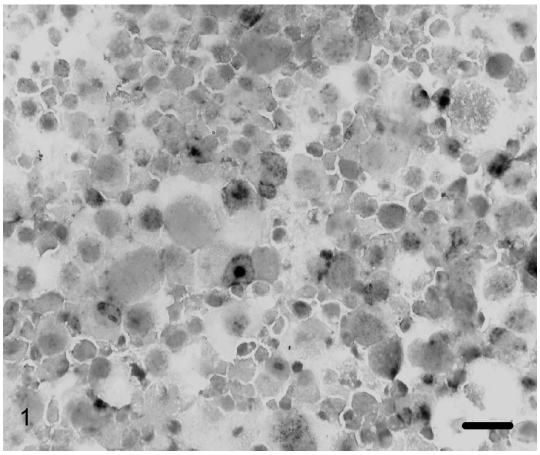

Fig. 1. Overnight hybridization with digoxigenin-labeled DNA probes. MDCK cells inoculated with 10^3 $TCID_{50}$ CHV. Some signals (arrows) are observed. NBT/BCIP colorization, methyl green counterstain, Bar = 50 μm.

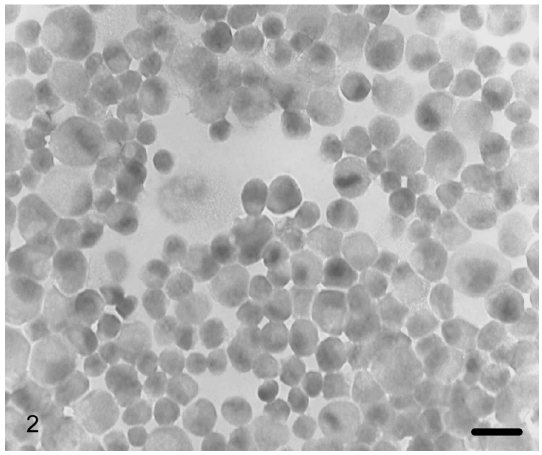

Fig. 2. Overnight hybridization with biotin-labeled DNA probe. MDCK cells inoculated with 10^3 $TCID_{50}$ CHV. No specific blue purple signals are present. NBT/BCIP colorization, methyl green counterstain, Bar = 50 μm.

formula of hybridization solution was described previously [3]. For detection of hybridization, sections were incubated with anti-digoxigenin conjugated with alkaline phosphatase (Roche) for digoxigenin-labeled probe and streptavidin conjugated with alkaline phosphatase (Roche) for biotin-labeled probe respectively, and then colorized with NBT/BCIP (Roche).

The results of ISH were presented in Table 1. It was revealed that the overnight hybridization protocol resulted in the increasing sensitivity as compared with 3 hours-hybridization protocol. Digoxigenin-labeled probe was capable of detecting CHV in MDCK cells infected with 10^3 $TCID_{50}$ using overnight hybridization protocol. However, biotin-labeled probe was able to detect CHV in MDCK cells infected with 10^4 $TCID_{50}$. By using digoxigenin-labeled probe, ISH of MDCK cells infected with 10^7 $TCID_{50}$ of virus resulted in strong positive signal in the nucleus and cytoplasm as distinct areas of blue purple signals in most cells. Cells infected with lower viral titers showed positive signals in correspondingly lower number of titer until 10^3 $TCID_{50}$ only a few cells per section were visibly positive (Fig. 1). In case of biotin-labeled probe, MDCK cells infected with 10^3 $TCID_{50}$ of virus could not be found any positive signals (Fig. 2). As changing proteinase K concentrations, there are no differences in the detection limit of ISH. 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 similar signal intensity as compared with digestion with 200 μg/ml proteinase K.

In this study, several ISH protocols, which were consisted of the changes of enzyme-concentrations, the time of hybridization and hybridization probes, were compared in formalin fixed and paraffin embedded CHV-infected cells. The optimum result was obtained using digoxigenin-labeled probe, 100 μg/ml proteinase K pre-treatment, and overnight hybridization. The practicality of digoxigenin-labeled probe is better than those of biotin-labeled probe in the hybridization assay for the detection of CHV. These results suggest that ISH assay using digoxigenin-labeled probe, which was optimized in this study, may be recommended for diagnosis of CHV in formalin-fixed tissues. The optimized ISH assay, which developed in this study, may be a valid tool for the study of pathogenesis and diagnosis of CHV infection.

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