# Improving Detection of Canine Distemper Virus in Formalin-Fixed, Paraffin-Embedded Tissues: Using *in situ* Hybridization with Integrated Optical Density to Give a Semi-Quantitative Assessment

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**KEY WORDS:** Canine distemper virus, *in situ* hybridization, integrated optical density

## ABSTRACT

Enhanced canine distemper virus (CDV) nucleoprotein RNA was applied to paraffin-embedded tissues from dogs with spontaneous CDV infections. In addition to proteinase K, the tissues were autoclaved in various solutions as a pre-treatment (Trilogy, TBS S3006, H-3301, and S1700) and then compared. The *in situ* hybridization (ISH) intensity was assessed using integrated optical density (IOD) and tissue morphology examinations. A combination of proteinase K digestion and autoclaving in a Trilogy solution resulted in optimal ISH signal enhancement of CDV RNA. This modified technique may be useful in retrospective viral studies.

## INTRODUCTION

Canine distemper virus (CDV) is a non-segmented, single-stranded negative RNA virus that belongs to the genus *Morbillivirus* within the family *Paramyxoviridae*. CDV is the most common viral cause of canine encephalitis (Appel 1970) and is typically detected by immunohistochemistry (IHC), immunofluorescence labeling, reverse transcription-polymerase chain reaction, clinicopathological findings, or a combination of these approaches (Beineke et al., 2009).

CDV RNA and viral transcription can also be detected in acute demyelinating lesions in formalin-fixed, paraffin-embedded

tissues with ISH (Muller et al., 1995; Gaedke et al., 1997; Hoyland et al., 2003; Engelhardt et al., 2005; D'Intino, et al., 2006; Zurbriggen et al., 1998; Vandevelde and Zurbriggen 2005). CDV RNA and viral transcription may also be detected in antigens that are not easily observed by IHC in chronic infection (Mitchell et al., 1991). For ISH, there are a variety of commonly used pre-treatments for the formalin-fixed paraffin-embedded tissue sections, including saponin (Yamawaki et al., 1993), diluted acids, detergents, alcohols, proteases (such as proteinase K, pronase, and pepsin) (Mcnicol and Farquharson 1997), sodium borohydrite (Gaedke et al., 1997), and boiling sections in citrate buffer in a microwave (Gaedke et al., 1997; Lan et al., 1996). Among the pre-treatments for ISH, the most common is the application of proteases.

However, problems with ISH persist because different types of tissues require different digestion conditions to achieve optimal results. Few reports have focused on optimizing the CDV RNA detection protocol in formalin-fixed, paraffin-embedded tissue sections. Furthermore, there are conflicting results from ISH optimization following proteolytic enzyme digestion and heat-induced antigen retrieval (HIAR) pre-treatments (Mcquaid et al., 1990; Gaedke et al., 1997; Kim and Chae 2003).

Thus, the intent of our study was to improve the ISH protocol and combine it with integrated optical density measurements (IOD) to provide a semi-quantitative and simple method for the detection of CDV RNA in formalin-fixed, paraffin-embedded tissues in retrospective studies.

## MATERIALS and METHODS

### Animals

Spontaneous CDV infections in eight dogs were previously confirmed and described by either RT-PCR, histopathology or by immunohistochemical labeling (Liang et al. 2007, 2011). The tissue samples obtained were fixed in 10% neutral buffered formalin within 36 h to 7 days of collection. The samples were then processed and embedded in paraffin, using a routine process.

### Probe Preparation

Total RNA was obtained from the field CDV-infected B95a cells (Liang et al. 2008) and isolated using an RNeasy Mini Kit (Qiagen). The cDNA probe was prepared with primers that amplify the CDV nucleoprotein (Frisk et al. 1999). The RT-PCR product (287 bp) was cloned using the PCR 2.1-TOPO TA cloning kit (Invitrogen). Digoxigenin (Roche)-labeled, double-stranded DNA probes were generated by PCR using Platinum Taq DNA polymerase (Invitrogen). The reactions were performed under the following conditions: 95°C for 10 min, 35 cycles at 95°C for 30 sec, 59.5°C for 30 sec, and 72°C for 30 sec, with a final extension for 10 min at 72°C. The resultant 287 bp DIG-dUTP-labeled dsDNA probe was then purified using a QIAquick® PCR purification kit (Qiagen) and analyzed on a 2 % agarose gel.

### Pre-treatment Before Pre-hybridization

From each of the formalin-fixed, paraffin-embedded tissues, we cut 6-µm thick serial sections. The sections were then deparaffinized, rehydrated, de-proteinized, and then treated with one of the following pre-treatments, either

- Digestion at 37°C for 15 min with proteinase K (20 µg/ml, Roche) in PBS (DEPC), with each sample being washed with 0.2% glycine and 2× SSC for 10 min and then pre-hybridized and hybridized; or
- Digestion at 37°C for 15 min with proteinase K (20 µg/ml, Roche) in PBS (DEPC), then washed with 0.2% glycine, immersed in 5% concentrated Trilogy stock (Cell Marque) in Q water (Milli-Q), and boiled for 15 min at 121°C in an autoclave (SA-252F, Sturdy Industrial, Taipei, Taiwan).

Next, the autoclaved sections were immediately transferred to a fresh 5% Trilogy solution in a second staining dish that had been pre-heated to 80°C in a 1450-W microwave oven (RE-C102; Sampo, Taipei, Taiwan). Then, the sections were left to

stand for 10 min at 80°C. The sections were then immersed in a 10% concentrated stock of Tris-buffered saline (TBS S3006; Dako Cytomation) in Q water at room temperature for 10 min, washed with 2× SSC, and then pre-hybridized and hybridized.

The pre-treatment c, d, and e samples were processed as previously described. However, the solutions used in both the autoclaving and microwaving were changed to:

- A 10% stock Tris-buffered saline (TBS S3006) in Q water,
- A 1% vector citrate-based stock antigen unmasking solution (H-3301, Vector Laboratories) in Q water, or
- A target-retrieval solution (S1700; Dako Cytomation). After the different autoclaving and microwaving pre-treatments (b, c, d, and e), the sections were immersed in a 10% concentrated stock of TBS S3006 solution in Q water at room temperature, and each sample was rinsed in 2x SSC for 10 min before being pre-hybridized and hybridized.

### *In situ* hybridization (ISH)

We used the pre-hybridization and hybridization protocol previously described by Chueh et al. (1999), which is in the supplemental section of this study.

### Immunohistochemistry (IHC)

We used the IHC protocol of Liang et al. (2007, 2011), which uses the Super Sensitive TM Non-Biotin HRP Detection System (BioGenex Laboratories, San Ramon, CA, USA). The primary antibody was mouse anti-CDV (MCA 1893, Clone DV2-12; Serotec, Kidlington, Oxford, UK).

### Positive Area Evaluation

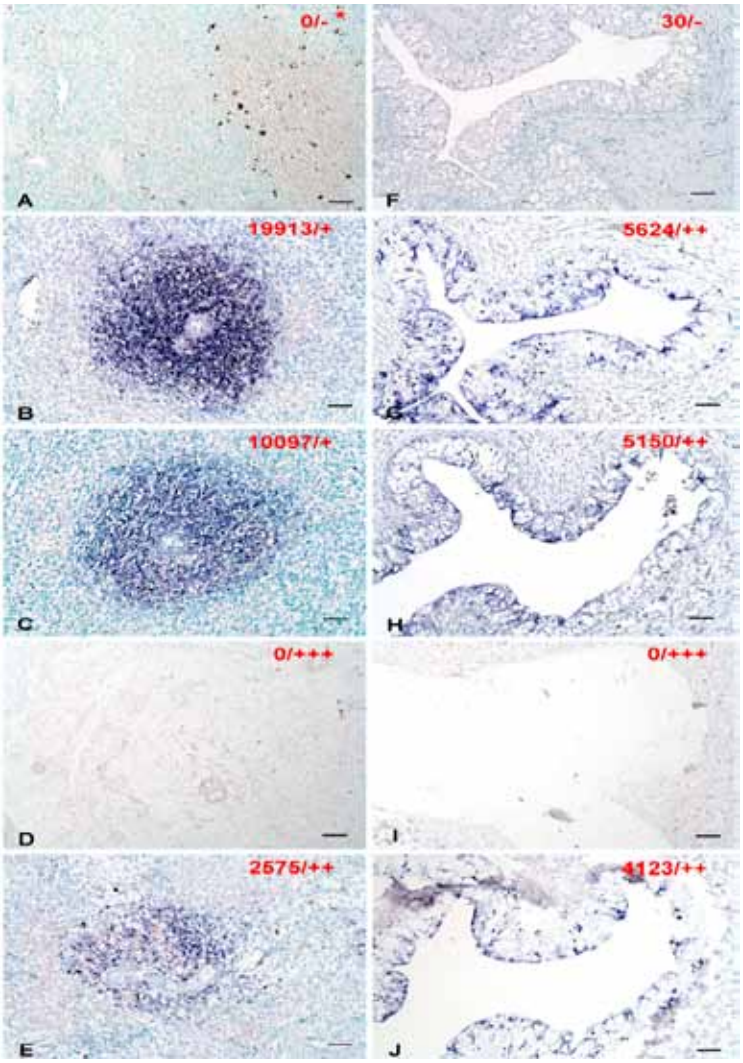
The ISH intensities of the five pre-treatments were assessed by estimating the area of the objects and the medium pixel intensity per object as the integrated optical density (IOD) of the positively stained areas. The image analysis program Image-Pro® Plus 6.0 (Media Cybernetic, NY, USA) is used to evaluate these parameters. For each tissue section, we calculated the IOD,

***Table 1.*** *Comparison of the five pre-treatments on major organ enhancement for the ISH detection of CDV RNA*

| Pretreatment methods | Spleen | | Urinary bladder | | Lung | | Cerebellum | |
|---|---|---|---|---|---|---|---|---|
| | Hybridization signal[a] | Tissue morphology[b] | Hybridization signal[a] | Tissue morphology[b] | Hybridization signal[a] | Tissue morphology[b] | Hybridization signal[a] | Tissue morphology[b] |
| 1.Proteinase K only | -, 0c | - | +, 30 | - | + | - | - | - |
| 2. Proteinase K with Trilogy (Cell Margue) | +++, 19913 | + | +++, 5624 | ++ | ++ | + | ++ | + |
| 3. Proteinase K with TBS (Dako, S3006) | ++, 10097 | + | ++, 5150 | ++ | ++ | + | ++ | + |
| 4. Proteinase K with citrate-based solution (Vector, H3301) | -, 0 | +++ | -, 0 | +++ | - | +++ | - | + |
| 5. Proteinase K with AR solution (Dako, S1700) | ++, 2575 | ++ | ++, 4123 | ++ | ++ | ++ | + | + |

*a Hybridization signal: -, negative; +, weak; ++, moderate; +++ strong*
*b Tissue morphology: -, no damage; +, mild damage; ++, moderate damage; +++, severe damage.*
*c: IOD score*

***Figure 1.*** *The effect of five pre-treatments on consecutive serial sections of CDV-infected spleen (A-E) and urinary bladder (F-J) were compared using hybridization signals and morphological tissue damage. The asterisk indicates "hybridization signal IOD score/tissue morphology evaluation". A negative hybridization signal with no morphological tissue damage was observed in spleen (A) and bladder (F) subjected to proteinase K pre-treatment only. Sections with mild morphological tissue damage showing the strongest hybridization signals, which were blue/purple to black in color, were observed in the splenic periarteriolar white pulp (B) and the cytoplasm of bladder mucosal epithelial cells (G). The proteinase K pre-treatment was combined with autoclaving in a Trilogy solution. Moderate hybridization signals with mild tissue morphological damage were noted in the spleen (C) and bladder (H). The proteinase K pre-treatment was combined with autoclaving in a TBS solution. A negative hybridization signal was present in the spleen (D) and bladder (I), in addition to a severe loss of splenic architecture. The proteinase K pre-treatment was combined with autoclaving in a H3301 solution. Moderate hybridization signals and tissue damage were observed in the spleen (E) and bladder (J). The proteinase K pre-treatment was combined with autoclaving in a S1700 solution. NBT/BCIP was counterstained with methyl green. Bar= 40 µm.*



which was equal to area × average optical density of positive staining. The average IOD scores were calculated from duplicate values in each section.

Tissue morphologies were graded on a semi-quantitative four-point scale: -, <10% to no damage; +, 10-25% mild damage; ++, 25-50% moderate damage; and +++, >50% severe damage.

## RESULTS

The pre-treatment process was conducted on a mock control using other canine sections without CDV infection and a reagent control, using serial sections of infected cases without the addition of the probe. No positive labeling was found, which directly contrast with the aforementioned observations made in the cases of infected sections where probes were used.

Consecutive serial tissue sections of the major CDV-infected organs, including the cerebellum, cerebrum, lung, urinary bladder, and spleen, were tested in parallel with the addition of different HIAR regimens. The results are summarized in Table 1 and Fig. 1, wherein it can be observed that the proteinase K pre-treatment, in combination with autoclaving and microwaving, enabled the detection of strong signals with very low non-specific backgrounds in spleen and urinary bladder tissues. The results corresponding to bronchiolar mucosa, cerebellar white matter, the 4th ventricle, and the cerebral cortex tissues were similar (data not shown). However, sections that were subjected to a combination of proteinase K pre-treatments and autoclaved in a Trilogy solution exhibited a stronger hybridization signal (Fig. 1B, 1G).

The ISH intensities of the five pre-treatments in the spleen tissue were assessed by the average IOD, wherein the corresponding average IOD scores were 0, 19913, 10097, 0, and 2575 (Fig. 1A-1E). Negative to strong hybridization signals, which were blue/purple to black in color, were observed in the splenic periarteriolar white pulp areas. In this retrospective study, splenic lymphoid depletion and necrosis with intranuclear inclusion bodies and other lesions were observed, which are characteristic of CDV infection.

The ISH intensities of the five pre-treated samples of urinary bladder were also assessed by IOD, wherein the average IOD scores were 30, 5624, 5150, 0, and 4123 (Fig. 1F-1J). Hyperplasia and ballooning changes with intranuclear and cytoplasmic inclusion bodies were also noted in the bladder mucosal epithelial cells (data not shown).

In terms of tissue morphology, tissue sections that had only been subjected to proteinase K pre-treatment exhibited intact and clear tissue outlines. However, the hybridization signal was rarely detected in tissues that did not undergo an autoclaving pre-treatment (Fig. 1A and 1F). The sections that were subjected to proteinase K pre-treatment, in combination with autoclaving in Trilogy solutions, exhibited mild losses in tissue architecture (Fig.1B and 1G).The sections that were subjected to proteinase K pre-treatment, in combination with autoclaving in TBS solutions, also exhibited mild losses in tissue architecture (Fig. 1C and 1H). However, the sections that were subjected to proteinase K pre-treatment, in combination with autoclaving in a H3301 solution, exhibited severe losses in tissue architecture without any ISH signal (Fig. 1D and 1I). The sections that were subjected to proteinase K and autoclaving pre-treatment in S1700 solution exhibited moderate losses in tissue architecture but had distinct signals (Fig. 1E and 1J).

## DISCUSSION

### Heat-induced Antigen Retrieval (HIAR)

In this study, through a combination of proteinase K digestion and autoclaving in a Trilogy solution, we found a relatively simple method that demonstrates an optimal ISH signal enhancement of CDV RNA.

Formaldehyde, as a 10% neutral buffered formalin, is the most widely used universal fixative because it preserves a wide range of tissues and tissue components.

However, attempts to extract usable DNA from formalin-fixed tissues for molecular biological studies have been variably successful (Srinivasan et al., 2002). The formaldehyde fixative initiates DNA denaturation (interchain hydrogen bonds break and bases unstack) at the AT-rich regions of double-stranded DNA, creating sites for chemical interaction. There are four interactions of formaldehyde with DNA. The first is an addition reaction. Formaldehyde is added to the nucleic acid base to form a hydroxymethyl (methylol) group (-CH2 OH). The second is a slower electrophilic attack of N-methylol on an amino base to form a methylene bridge between two amino groups. Thirdly, formaldehyde treatment can generate AP (apurinic and apyrimidinic) sites via hydrolysis of the N-glycosylic bonds, leaving free pyrimidine and purine residues. AP sites have a highly unstable cyclic carboxonium ion that hydrolyzes rapidly to yield 2-deoxy-D-ribose. Finally, formaldehyde may also cause slow hydrolysis of the phosphodiester bonds, leading to short chains of polydeoxyribose with intact pyrimidines. When compared to the DNA isolated from frozen tissues, formalin-fixed tissues exhibit a high frequency of nonreproducible sequence alteration (Srinivasan et al., 2002; Shi et al., 2001).

Previous attempts have used thermocycling (Kim and Chae, 2003), microwaving (Lan et al. 1996; Gaedke et al. 1997) or autoclaving (Relf et al. 2002) to enhance the ISH signal. Recent employment of HIAR has been shown to enhance the extraction of nucleic acid or increase the efficiency of subsequent ISH detection of a target sequence. The process of crosslinking makes probe access to the target sequence difficult. Therefore, tissues must be digested to improve probe access to the specific mRNA while minimizing mRNA loss and tissue morphology. Many similar digestion strategies have been employed to permeabilize fixed cells or tissues using acids, detergents, alcohols, and enzymes, such as proteinase K, pronase, and pepsin. However, this step remains problematic in that each tissue type requires a different set of digestion conditions (Mcquaid et al., 1990; Lan et al. 1996; Kim and Chae, 2003; Shi et al., 2001; Weise et al., 2005; Yamashita 2007). Heating cleaves inter- and intra-crosslinks in proteins and nucleic acids, and because a gel-like structure formed by the crosslinks is destroyed and the macromolecules are partially extracted, antibodies can easily penetrate into tissue sections, and the immunoreaction is greatly intensified (Yamashita, 2007).

The HIAR Trilogy solution is a novel product that has been shown to have good antigen retrieval effects for the immunohistochemical labeling of RNA viruses (Faoláin et al., 2005; Ward et al., 2006; Liang et al., 2007, 2011). However, due to the advantage of combining deparaffinization, rehydration, and the retrieval of antigens during pressure cooking, this solution has never been used in an ISH protocol. In this study, Trilogy solution was found to be ideal for the retrieval of RNA in comparison to other solutions.

The fact that the HIAR effect could be used as an approach to enhance the extraction of nucleic acids or to increase the detection efficiency of subsequent target sequences was not emphasized until recently (Shi et al., 2001; Kim and Chae, 2003). Trilogy solution is a novel product that combines deparaffinization, rehydration, and unmasking of antigens during pressure cooking (Faoláin et al., 2005). In recent reports, Trilogy solution has been shown to have good antigen retrieval effects for the immunohistochemical labeling of RNA viruses (Ward et al., 2006; Liang et al., 2007, 2011) or cancer diagnoses (Kuo et al., 2006), but has never been tried in the ISH protocol. This has led to the development of novel ISH labeling protocols that are especially important for retrospective studies.

The HIAR effect (Mcquaid et al., 1990; Kim and Chae, 2003) can greatly enhance the ISH signal and provides a simple detection method in formalin-fixed, paraffin-embedded tissues. However, the tissue damage caused by the retrieval solution needs to be taken into consideration. The pH value

of the antigen retrieval solution is another important factor (Shi et al., 2001; Ramos-Vara 2005). Some antigens will be retrieved only with high pH solutions, while in others, a wide range of pH values will suffice. With most antigens, HIAR with 0.01 M sodium citrate buffer (pH 6.0) will provide satisfactory results with good cell morphology (Ramos-Vara 2005). However, the pH values of the Trilogy (7.69), TBS (8.22), H3301 (9.14), and Dako S1700 retrieval working solutions (9.22) ranged from neutral to weakly basic in the present study. The S1700 and H3301 retrieval solutions had very similar pH values, but provided different results. The citrate-based Vector H3301 retrieval solution combined with the autoclave pre-treatment showed the most severe destruction of the tissue morphology.

The results demonstrated that the simple and modified combined pre-treatment of HIAR and autoclaving the tissue sections in antigen retrieval buffer and proteinase K digestion resulted in stronger hybridization signals than proteinase K digestion alone. It is also important to note that autoclaving in either Trilogy solution or TBS resulted in strong hybridization signals of major organs. These organs included the spleen, urinary bladder, and lung, all of which were infected by CDV with mild to no tissue morphological damage. Similar retrieval effects of autoclaving in Trilogy solution for ISH were also noted in cerebellar sections (data not shown). Because formalin is the most commonly used fixative in routine tissue fixation, our modified methods can be useful in retrospective and pathogenesis studies of CDV infections. However, most RNA viruses are low-copy infections, and thus, RT *in situ* PCR is often the best method available to detect the virus *in situ* (Nuovo, 1995). PCR will be used in the future to compare the results in this study.

**Proteinase K Effect**

In the beginning of this study, using proteinase K treatment only, the CDV RNA ISH signals were observed in positive immunohistochemical labeling sites in seven cases. However, the intensities and distribution of ISH signals were not as distinct and strong as those obtained following non-biotin, horseradish peroxidase-based IHC labeling (Liang et al., 2007). Therefore, we investigated if the CDV RNA decayed in formalin-fixed, paraffin-embedded tissues. The specificity of the ISH probe used in this study was validated by detecting CDV RNA in B95a cells infected with field Taiwanese CDV strains. In this study, 20 µg/ml of proteinase K was used for formalin-fixed, paraffin-embedded tissues.

In contrast, the final concentration of proteinase K used in the detection of CDV-infected B95a cells was 1 µg/ml. To facilitate probe access in ISH, sections are frequently treated with diluted acid, proteolytic enzymes and/or non-ionic detergents. While proteinase K is the most commonly used enzyme for ISH pre-treatment (Lewis and Wells, 1992; Mcnicol and Farquharson, 1997), the final working concentration used is variable, with previous studies using 1 µg/ml (Muller et al., 1995; Gaedke et al., 1997), 5 µg/ml (D'Intino, et al., 2006), 20 µg/ml (as in the present study), 300 µg/ml (Kim and Chae, 2003) and 1–4 mg/ml (Mcquaid et al., 1990). The concentration of proteinase K used is critical if morphology is to be preserved. The concentration used is dependent upon the specific tissue being evaluated, with the optimal proteinase K concentration of brain and lung being 0.25 mg/ml. The optimal concentration in kidney, liver, intestines, cervical, anal, and laryngeal sections is 0.5 mg/ml. While time is not generally critical for protease digestion in preparation for ISH, tissues can be destroyed if the proteinase K is too concentrated or if the tissues remain in solution too long, leading to unusable formalin-fixed tissues (Nuovo, 1995).

**Probe Effect**

The dsDNA probes for ISH have the disadvantage of not allowing one to distinguish between positive- and negative-stranded RNA. However, the digoxigenin PCR labeling method can still be used in the detec-

tion of new RNA viruses (Sritunyalucksana et al., 2006). DNA probes are still more frequently used, as they are relatively easy to produce in large quantities, they are the best characterized and they come in all sizes (Harvey and Schonau, 2006). Additionally, the advent of nucleic acid amplification techniques, such as PCR, has increased their availability greatly.

In this study, 50% formamide was added to the prehybridization and hybridization buffer, as higher concentrations did not improve the staining results and its omission resulted in negative staining (Gaedke et al., 1997). The CDV RNA-specific ISH signals in infected B95a cells were stronger compared to the formalin-fixed tissues using proteinase K treatment only. This observation may be explained partially by the fixation of the B95a cells in 4% paraformaldehyde. In the present study, 400 ng/ml of a dsDNA probe (287 bp) was used for formalin-fixed and paraffin-embedded tissues.

In contrast, the final concentration of the probe and proteinase K used in the detection of CDV-infected B95a cells was 100 ng/ml. However, the concentration of the dsDNA probe for the detection of CDV is variable, with previous reports using 170 or 500 ng/ml for 126 and 287 bp probes, respectively (Gaedke et al. 1997). The final working concentration of a CDV-specific RNA probe is also variable, with previous studies using 2 ng/ml (Engelhardt et al., 2005), 100 ng/ml (Gaedke et al., 1997), 1 µg/100 µl (D'Intino, et al., 2006), 10 µg/100 µl (Muller et al., 1995), and 1 mg/ml in muscle, heart, placenta and spermatozoa sections (Lewis and Wells, 1992). The same probe sequence (DNA-1) in our study still resulted in a 66% positive rate compared to either the shorter DNA-2 probe, an RNA probe or immunohistochemistry results shown in a previous study (Gaedke et al., 1997). CDV RNA was still detected in 60% of the samples by ISH, compared to 100% of the samples by *in situ* RT-PCR (Hoyland et al., 2003). To reduce this false negative rate in formalin-fixed, paraffin-embedded tissues, we combined HIAR techniques with proteinase K pre-treatment.

**IOD Evaluation**

The IOD, which has previously been successful when applied to analyze IHC staining (Xu et al., 2008), was used in the present study. This method provides a more objective and semi-quantitative evaluation of viral ISH labeling.

In summary, to simplify the tedious procedures that use enzyme digestion for ISH pre-treatment, which require adjustments in time, temperature, and concentration for different tissues, the present study has established a modified HIAR autoclaving technique. Most importantly, in addition to a fixed concentration of proteinase K digestion used for different tissues, this technique employed autoclaving in a Trilogy solution. Strong hybridization signals resulted in CDV-infected major organs, including the spleen and urinary bladder, with mild to no morphological tissue damage and a weak non-specific background. Similar retrieval effects of autoclaving in a Trilogy solution for ISH were also observed in cerebellar and lung sections (Table 1). This modified ISH method could be useful in the retrospective study of CDV and other viral infections.

## CONFLICT OF INTEREST STATEMENT

None of the authors of this paper have financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of the paper.

## ACKNOWLEDGMENTS

The authors greatly appreciate the financial support of the National Applied Research Laboratories, the valuable histological technical support from Miss Shiow-Ling Liao and Yi-Ying Chiu, and the helpful comments from Dr. Sarah L. Poynton, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

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