

Detection of porcine reproductive and respiratory syndrome virus in naturally infected pigs.

学位名	博士(獣医学)
学位授与機関	宮崎大学
学位授与番号	17601甲第21号
URL	<a href="http://hdl.handle.net/10458/5686">http://hdl.handle.net/10458/5686</a>

1 **Full Scientific Report**

2  
3 **Short Title: Biotinyl-tyramide-based PRRSV in situ hybridization**

4  
5 **Enhanced detection of porcine reproductive and respiratory syndrome virus in fixed**  
6 **tissues by in situ hybridization following tyramide signal amplification**

7 Nguyen Thi Trang, Takuya Hirai,<sup>1</sup> Pham Hong Ngan, Nguyen Thi Lan, Naoyuki Fuke, Keiko  
8 Toyama, Ryoji Yamaguchi

9  
10 From the Department of Veterinary Pathology, Faculty of Agriculture, University of  
11 Miyazaki, Miyazaki 889-2192, Japan (Trang, Hirai, Fuke, Toyama, Yamaguchi); Department  
12 of Veterinary Public Health (Trang, Ngan) and Department of Veterinary Pathology (Lan),  
13 Faculty of Veterinary Medicine, Hanoi University of Agriculture, Hanoi, Vietnam

14  
15 Corresponding Author:

16 <sup>1</sup> Takuya Hirai, Department of Veterinary Pathology, Faculty of Agriculture, University of  
17 Miyazaki Nishi 1-1, Gakuen-Kibana, Miyazaki 889-2192, Japan

18 Tel: +81-985-58-7272; Fax: +81-985-58-7272;

19 E-mail: [t-hirai@cc.miyazaki-u.ac.jp](mailto:t-hirai@cc.miyazaki-u.ac.jp)

1       **Abstract.** This study evaluated the sensitivity of biotinyl-tyramide-based in situ hybridization  
2 (TISH) methods by comparison with chromogenic in situ hybridization (CISH) and  
3 immunohistochemistry (IHC) methods. This study also determined the effect of fixative and  
4 fixation time on the detection of porcine reproductive and respiratory syndrome virus (PRRSV)  
5 in paraffin-embedded tissues. Lung samples were fixed in 4% paraformaldehyde (PFA) or 10%  
6 neutral buffered formalin (NBF) for various times before paraffin embedding. Of 30 paraffin-  
7 embedded lung samples fixed one day in either 4% PFA or 10% NBF, 18 (60%) were positive for  
8 PRRSV by nested reverse transcription-polymerase chain reaction (nRT-PCR). All 18 of these 18  
9 lung samples (100%) also were positive for PRRSV by TISH, but only 10 of these 18 specimens  
10 (56%) were positive for PRRSV by IHC and CISH. We demonstrated that TISH can detect  
11 PRRSV RNA in paraffin-embedded tissues after up to 90 days of fixation. PRRSV nucleic acids  
12 and antigens were better preserved in 4% PFA than in 10% NBF. Compared with CISH and IHC  
13 testing methods, TISH appeared to be more sensitive for the detection of PRRSV in paraffin-  
14 embedded tissues.

15       **Key words:** in situ hybridization; lung; porcine reproductive and respiratory syndrome virus.

16

17

1 Porcine reproductive and respiratory syndrome virus (PRRSV) emerged in North America in  
2 1987 and in Europe in 1991.<sup>14, 18, 20</sup> PRRSV is a small, enveloped, single-stranded RNA virus that  
3 contains 9 open reading frames (ORFs) and belongs to the family *Arteriviridae*, in the order  
4 Nidovirales.<sup>17</sup> *ORF5*, which encodes the major envelope protein, is often used for phylogenetic  
5 analysis because this locus is the most variable region of the PRRSV genome.<sup>13, 23</sup> Based on  
6 genetic, antigenic, and pathogenic analyses, PRRSVs are divided into 2 distinct classes  
7 corresponding to the European genotype (EU-type) and the North American genotype (NA-  
8 type).<sup>2, 10</sup> The disease is known to result in reproductive failure in sows and respiratory problems  
9 in piglets, and is considered to be one of the most important viral pig diseases. An accurate  
10 diagnosis is essential for confirming the disease and epidemiology on a particular farm, thereby  
11 permitting the implementation of suitable PRRSV control strategies and the eventual elimination  
12 of this financially devastating disease.

13 Paraffin-embedded (PE) tissues are commonly used for laboratory diagnosis, and they can be  
14 stored for long periods, easy to transfer among laboratories without transmission of disease  
15 agents. Immunohistochemistry (IHC), in situ hybridization (ISH), and nested reverse  
16 transcription polymerase chain reaction (nRT-PCR) can be used to detect PRRSV in PE tissues.  
17 To date, in situ hybridization (ISH) is the most commonly used method for localization of  
18 specific viral nucleic acid sequences inside individual cells while preserving cell and tissue  
19 morphology, thus allowing simultaneous assessment of the morphological alterations associated  
20 with lesions. However, chromogenic ISH (CISH) with nonradioactive probes has limited  
21 sensitivity when applied to low-copy-number nucleic acid sequences.<sup>16</sup> In recent years, biotinyl-  
22 tyramide-based ISH (TISH) methods have become widely used; TISH permits the detection of  
23 low-copy-number nucleic acids even in PE tissues.<sup>6-8, 21</sup>

1 Formalin is a commonly used fixative agent that inactivates most infectious agents and  
2 inhibits autolysis.<sup>9, 19</sup> Formalin fixation results in the cross-linking of tissue protein with DNA or  
3 RNA; over-fixation can reduce the sensitivity of IHC and ISH. For routine IHC and ISH,  
4 solutions of 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) are commonly  
5 used for fixation. However, there are no published comparisons of the effects of these 2 fixatives  
6 on the detection of PRRSV by TISH, CISH, and IHC. Thus, the objective of this study was to  
7 evaluate the sensitivity of TISH in comparison with IHC and CISH for the detection of PRRSV.  
8 We additionally examined the effects of fixative and fixation time on the detection of PRRSV in  
9 PE samples from naturally infected pigs.

## 10 **Materials and methods**

### 11 *Animals*

12 Thirty pigs, 70-150 days of age, were collected from 2 farms in the Miyazaki and Kagoshima  
13 Prefectures in Japan. Both farms had a persistent problem with respiratory disease in weaning  
14 piglets. Clinical signs varied from sudden death to chronic pneumonia with weight loss and slow  
15 growth. The mortality rate of weaning piglets was 15-30%. The pigs used for the present study  
16 had been euthanized because of respiratory signs including coughing and dyspnea. Lung samples  
17 were fixed for one day in either 10% NBF (at room temperature) or 4% PFA (at 4 °C). The 4%  
18 PFA was prepared one day before use. After fixation, lung sections were processed and  
19 embedded in paraffin by standard histologic procedures. PE lung tissues were examined for  
20 PRRSV by nRT-PCR, IHC, CISH, and TISH. To examine the effect of extended fixation on the  
21 sensitivity of TISH versus other direct detection assays, NBF- and PFA-fixed lung samples from  
22 one pig that tested positive for PRRSV by nRT-PCR, TISH, CISH, and IHC at 1 day fixation  
23 were maintained in these 2 fixatives for 7, 14, 21, 28, 60, 90, and 120 days before embedding.

### 24 *PCR from PE tissues*

1 RNA was extracted from PE samples using a commercial kit<sup>a</sup> in accordance with the  
2 manufacturer's instructions. Two PCR primer pairs were used for nested RT-PCR. The primers  
3 for nRT-PCR were designed as described previously.<sup>1, 22</sup>

#### 4 *Immunohistochemistry*

5 IHC staining was performed as previously described,<sup>5</sup> using monoclonal anti-PRRSV antibody  
6 SR30<sup>b</sup> as the primary antibody and the EnVision system<sup>c</sup> as the secondary antibody. Staining was  
7 visualized using the peroxidase stain diaminobenzidine (DAB) kit.<sup>d</sup>

#### 8 *In situ hybridization*

9 Antisense cRNA probes specific for PRRSV RNAs were synthesized as previously  
10 described.<sup>15</sup> Digoxigenin-labeled cRNA probes were prepared using a commercial labeling kit.<sup>e</sup>  
11 ISH for PRRSV was performed according to the method of Tanizaki et al. with slight  
12 modification.<sup>17</sup> ISH can be divided into 5 phases: section preparation, tissue unmasking,  
13 hybridization, post-hybridization washes, and detection. Briefly, deparaffinized sections were  
14 digested with 10 µg/mL proteinase K at 37<sup>0</sup>C, post-fixed in 4% paraformaldehyde, treated with  
15 0.1 N HCl, and acetylated with 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10  
16 minutes each. After treatment with 3% hydrogen peroxide for 1 hour, the sections were  
17 dehydrated and air-dried. Fifty µL of a hybridization solution<sup>f</sup> containing 50 ng cRNA probe for  
18 TISH and 200 ng cRNA probe for CISH was used and hybridized with the sections for 16-18  
19 hours at 50<sup>0</sup>C. After hybridization, the sections were immersed in 50% formamide/2X saline-  
20 sodium citrate buffer (SSC) for 1 hour at 60<sup>0</sup>C followed by rinsing in 1X buffer containing 1  
21 mmol/L Tris-aminomethane pH7.5, 0.5M NaCl, and 1 mmol/L EDTA (TNE) for 10 minutes at  
22 37<sup>0</sup>C. The sections were stringently washed sequentially in 2X SSC, 0.2X SSC, and 0.1X SSC  
23 for 40 minutes each at 60<sup>0</sup>C. CISH and TISH differed solely in the detection steps. For CISH,  
24 sections were incubated with 1.5% blocking reagents<sup>g</sup> for 30 minutes, then reacted with a 1:400

1 diluted anti-digoxigenin antibody.<sup>h</sup> For TISH, sections were incubated with 0.5% casein at room  
2 temperature for 10 minutes. Then biotinyl-tyramide signal was amplified by serial application of  
3 a 1:400 diluted anti-digoxigenin antibody,<sup>h</sup> 0.07 $\mu$ M biotinylated tyramide solution,<sup>7</sup> and 1:500  
4 diluted streptavidin antibody<sup>i</sup> for 15 minutes each at room temperature. After each incubation  
5 period, the sections were washed 3 times with TBS (2)-T (0.01 M Tris-HCl, pH 7.5, 150 mM  
6 NaCl) for 5 minutes. Finally, color was developed using the DAB<sup>j</sup> and counterstained with  
7 hematoxylin. Known PRRSV-positive and PRRSV-negative pig lungs were used as controls for  
8 IHC and ISH by replacing the primary antibody or probe with phosphate-buffered saline.

## 9 **Results**

10 At autopsy, multifocal areas of consolidation were disseminated throughout the lung lobes,  
11 and were accompanied by edema of the interlobular connective tissue in the cranial lobes with  
12 fibrinous adhesions to the thoracic wall. Bronchial lymph nodes were enlarged. Twenty-six of 30  
13 pigs (89%) had interstitial pneumonia and/or fibrinosuppurative bronchopneumonia with  
14 streaming degenerate alveolar leukocytes (oat cells). Lung samples were collected for bacterial  
15 culture, and *Pasteurella multocida* was detected in 15 pigs, *Mycoplasma* spp. in 6 pigs, and  
16 *Streptococcus suis* in 5 pigs. The pleura often had moderate numbers of neutrophils,  
17 macrophages, and abundant fibrin on the surface, with fibroplasia in chronic cases.

18 Among 30 autopsied pigs, PRRSV was detected in PE lungs of 18 animals (60%) by nRT-  
19 PCR. All 18 of these lung samples (100%) also tested positive for PRRSV by TISH. PRRSV was  
20 detected in the same 10 of these 18 lung tissues (56%) by both CISH and IHC. Consistent  
21 negative results were obtained from the remaining 12 PE tissues by TISH, CISH, and IHC. By all  
22 3 techniques, positive cells exhibited a dark-brown reaction in the cytoplasm. Distinct positive  
23 labeling was scattered throughout the alveolar septa. The positive signal intensity varied among  
24 histological structures in one section and among pigs. The PRRSV nucleic acids were located

1 within the cytoplasm of macrophages. Dark-brown cytoplasmic staining without background  
2 staining could be observed in infected macrophages. The intensity of PRRSV-positive cells in  
3 TISH was significantly higher than in CISH and IHC methods (Fig. 1). In 8 cases, IHC and CISH  
4 were not able to detect PRRSV, whereas TISH still showed clear positive signals. Positive signals  
5 were more often associated with acute lesions with necrotic macrophages versus subacute  
6 lymphohistiocytic interstitial pneumonia. Whatever the amplification procedure used, staining  
7 reactions were negative in the controls performed by omitting the specific viral probe.

8 In trials of prolonged fixation, RNA extracted from tissue fixed that had been stored in 4%  
9 PFA for up to 14 days or in 10% NBF for up to one day yielded positive results for both the first  
10 and second PCR (Table 1). PCR signals in PE lung tissues fixed in 4% PFA or 10% NBF for  
11 periods from 14 to 90 days yielded detectable positive staining only in the second PCR. Upon  
12 longer intervals of storage (90 days) in either of these fixatives, nominally PRRSV-containing  
13 tissues no longer yielded signal by the nRT-PCR assay. RNA accessibility was reduced with  
14 prolonged fixation, perhaps because of protein cross-linking. The accessible RNA obtained from  
15 fixed tissues was reduced with time, thus a reduction in TISH/ CISH labelling was observed.

16 By TISH, positive signals were detected in tissues stored for up to 90 days in either type of  
17 fixative. Lung samples fixed in 4% PFA showed strong positive signals without background  
18 staining at day 1 and mild background staining at 28 days of fixation (Fig. 2A, 2B). Moderate  
19 positive signals and mild background staining was seen in tissues stored for up to 90 days in 4%  
20 PFA (Fig. 2C). Tissue fixed in 10% NBF produced strong positive signals with mild to moderate  
21 background stain at 1 day and 28 days of fixation (Fig. 2D, 2E). Positive signals were strongly  
22 reduced in tissues stored for up to 90 days in 10% NBF (Fig. 2F). Fixation in 4% PFA increased  
23 positive signal intensity and reduced background staining compared to fixation in 10% NBF.  
24 IHC was strongly affected by prolonged fixation; weak positive signal was observed only up to



1 14 days fixation for tissues fixed in 4% PFA or in 10% NBF. After prolonged fixation for 120  
2 days, PRRSV was not detected in any lung tissues by any of the attempted techniques. Moderate  
3 to strong background staining was seen in tissue samples subjected to more than 14 days  
4 prolonged fixation.

## 5 **Discussion**

6 Many experiments have been performed to test methods that detect low-copy-number nucleic  
7 acids even in PE tissues. The application of the RT-PCR method for recovery of RNA from PE  
8 tissues has revolutionized the opportunity for diagnostic analysis of fixed tissues.<sup>9, 11</sup> Recently,  
9 ISH has been widely used for research and routine examination to detect target nucleic acids. The  
10 development of TISH provides potential detection of low- and single-copy nucleic acid  
11 sequences.<sup>6-8, 16, 21</sup> In the present study, we compared the analytical sensitivity of TISH with  
12 CISH and IHC methods for the detection of PRRSV in infected lung tissue.

13 Previous studies<sup>3, 4</sup> comparing RT-PCR, IHC, and CISH demonstrated that RT-PCR was the  
14 most sensitive method for PRRSV detection. In the present study, the addition of the tyramide  
15 signal amplification step via TISH significantly increased the sensitivity relative to CISH in the  
16 detection of PRRSV. We did not observe a difference in the sensitivity of PRRSV detection by  
17 nRT-PCR or by TISH in PE tissues. However, RT-PCR alone cannot be used to evaluate the  
18 severity of a histological lesion, because RT-PCR does not provide spatial or structural  
19 information. In contrast, TISH provides cellular detail and histological architecture so that the  
20 number of PRRSV-infected cells and lesions may be observed simultaneously in the same  
21 section. The TISH method increases the detection sensitivity in comparison with CISH method,  
22 while reducing background staining in the lung sections. The detection of false-positive signals is  
23 the most common problem encountered with ISH. However, the consistently negative results  
24 obtained in the present study with numerous negative controls indicate that false-positive

1 reactions were not a problem with TISH in this study. Thus, this method promises to be  
2 extremely useful for detecting low-copy-number nucleic acids such as those of PRRSV in  
3 chronically infected pigs, as well as rare viral RNA in other diseases.

4 Previous work has demonstrated that the choice of fixative and duration of fixation  
5 significantly influence the efficiency of RT-PCR, IHC, and ISH.<sup>9, 19</sup> In the present study, PRRSV  
6 was not detected by IHC in tissue samples fixed for more than 14 days in either 4% PFA or 10%  
7 NBF. Prolonged formalin fixation has been shown to lead to masking of epitopes because of  
8 cross-linking, which reduces or even abolishes antigen detection.<sup>19</sup> Moreover, the results in this  
9 study showed that the selection of fixative (NBF or PFA) strongly influenced the sensitivity and  
10 background staining as detected by ISH. Thus, prolonged formalin fixation had a significant  
11 influence on the detectability of pathogenic agent in PE tissues, consistent with the results of  
12 previous studies.<sup>9, 12, 19</sup>

13 A previous study<sup>9</sup> that compared fixation methods for preservation of morphology and RNA  
14 in PE tissue derived from cancer cell implantation models demonstrated that among the  
15 formaldehyde fixatives, 4% PFA was most effective as preserving mRNA. In the present study,  
16 we found that PRRSV RNA and antigens were better preserved in 4% PFA than in 10% NBF.  
17 The results in this study indicate that fixation in 4% PFA for fewer than 14 days provides better  
18 preservation of RNA and morphology. The reason for the difference between these 2 fixatives  
19 may be the purity of formaldehyde freshly prepared from PFA is superior to that of commercial  
20 stock, as well as the low temperature fixation used with 4% PFA.

21 In conclusion, TISH is useful for the detection of low-copy-number PRRSV nucleic acids.  
22 Additionally, fixation in 4% PFA provides better preserved RNA and reduced background  
23 staining compared to traditional 10% NBF fixation.

24

#### **Declaration of conflicting interests**

1 The authors declare no conflicts of interest

2

3

### **Funding**

4 This study was supported by a Japanese Society for the Promotion of Science (JSPS) Grant  
5 (No.23580425).

6

### **Sources and manufacturers**

- 7 a. RecoverAll™ total nucleic acid isolation kit, Life Technologies, Foster, CA.
- 8 b. Rural Technologies, Brookings, SD.
- 9 c. Dako REAL™ EnVision™, Dako, Glostrup, Denmark.
- 10 d. Nacalai Tesque, Kyoto, Japan.
- 11 e. DIG RNA labeling Kit, Roche Diagnostics, Mannheim, Germany.
- 12 f. Maxim Biotech, Rockville, MD.
- 13 g. Blocking reagents, Roche Diagnostics, Mannheim, Germany.
- 14 h. Anti-digoxigenin horse-radish peroxidase, Fab fragments antibody, Roche Diagnostics,  
15 Mannheim, Germany.
- 16 i. Peroxidase-Conjugated streptavidin, Dako North America, Carpinteria, CA.
- 17 j. Liquid DAB+ substrate chromogen system, Dako North America, Carpinteria, CA.

18

19

### **References**

- 20 1. Andreyev VG, Wesley RD, Mengeling WL, et al: 1997, Genetic variation and phylogenetic  
21 relationships of 22 porcine reproductive and respiratory syndrome virus (PRRSV) field  
22 strains based on sequence analysis of open reading frame 5. Arch Virol 142:993–1001.

- 1 2. Benfield DA, Nelson E, Collins JE, et al: 1992, Characterization of swine infertility and  
2 respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). *J Vet Diagn Invest* 4:127–  
3 133.
- 4 3. Benson JE, Yaeger MJ, Christopher-Hennings J, et al: 2002, A comparison of virus  
5 isolation, immunohistochemistry, fetal serology, and reverse-transcription polymerase  
6 chain reaction assay for the identification of porcine reproductive and respiratory syndrome  
7 virus transplacental infection in the fetus. *J Vet Diagn Invest* 14:8–14.
- 8 4. Cheon DS, Chae C: 2000, Comparison of virus isolation, reverse transcription-polymerase  
9 chain reaction, immunohistochemistry, and in situ hybridization for the detection of porcine  
10 reproductive and respiratory syndrome virus from naturally aborted fetuses and stillborn  
11 piglets. *J Vet Diagn Invest* 12:582–587.
- 12 5. Cheon DS, Chae C: 1999, Distribution of a Korean strain of porcine reproductive and  
13 respiratory syndrome virus in experimentally infected pigs, as demonstrated  
14 immunohistochemically and by in-situ hybridization. *J Comp Pathol* 120:79–88.
- 15 6. Evans MF, Mount SL, Beatty BG, Cooper K: 2002, Biotinyl-tyramide-based in situ  
16 hybridization signal patterns distinguish human papillomavirus type and grade of cervical  
17 intraepithelial neoplasia. *Mod Pathol* 15:1339–1347.
- 18 7. Kerstens HM, Poddighe PJ, Hanselaar AG: 1995, A novel in situ hybridization signal  
19 amplification method based on the deposition of biotinylated tyramine. *J Histochem*  
20 *Cytochem* 43:347–352.
- 21 8. Kubelka-Sabit KB, Prodanova ILj, Zografski GD, Basheska NT: 2008, In situ  
22 hybridization, with or without tyramide signal amplification, in evaluation of human  
23 papillomavirus status in early stage cervical carcinoma. *BJMG* 11:41–50.

- 1 9. Matsuda Y, Fujii T, Suzuki T, et al: 2011, Comparison of fixation methods for preservation  
2 of morphology, RNAs, and proteins from paraffin-embedded human cancer cell-implanted  
3 mouse models. *J Histochem Cytochem* 59:68–75.
- 4 10. Meulenbergh JJ, Hulst MM, de Meijer EJ, et al: 1993, Lelystad virus, the causative agent of  
5 porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV.  
6 *Virology* 192:62–72.
- 7 11. Mizuno T, Nagamura H, Iwamoto KS, et al: 1998, RNA from decades-old archival tissue  
8 blocks for retrospective studies. *Diagn Mol Pathol* 7:202–208.
- 9 12. Mostegl MM, Richter B, Dinhopf N, Weissenböck H: 2011, Influence of prolonged  
10 formalin fixation of tissue samples on the sensitivity of chromogenic in situ hybridization. *J*  
11 *Vet Diagn Invest* 23:1212–1216.
- 12 13. Nelsen CJ, Murtaugh MP, Faaberg KS: 1999, Porcine reproductive and respiratory  
13 syndrome virus comparison: divergent evolution on two continents. *J Virol* 73:270–280.
- 14 14. Rossow KD: 1998, Porcine reproductive and respiratory syndrome. *Vet Pathol* 35:1–20.  
15 Review.
- 16 15. Shin JH, Molitor TW: 2002, Localization of porcine reproductive and respiratory syndrome  
17 virus infection in boars by in situ riboprobe hybridization. *J Vet Sci* 3:87–96.
- 18 16. Speel EJ, Saremaslani P, Roth J, et al: 1998, Improved mRNA in situ hybridization on  
19 formaldehyde-fixed and paraffin-embedded tissue using signal amplification with different  
20 haptenized tyramides. *Histochem Cell Biol* 110:571–577.
- 21 17. Tanizaki Y, Sato Y, Oka H, et al: 2006, Expression of autocrine motility factor mRNA is a  
22 poor prognostic factor in high-grade astrocytoma. *Pathol Int* 56:510–515.

- 1 18. Terpstra C, Wensvoort G, Pol JM: 1991, Experimental reproduction of porcine epidemic  
2 abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad  
3 virus: Koch's postulates fulfilled. *Vet Q* 13:131–136.
- 4 19. Van Alstine WG, Popielarczyk M, Albrechts SR: 2002, Effect of formalin fixation on the  
5 immunohistochemical detection of PRRS virus antigen in experimentally and naturally  
6 infected pigs. *J Vet Diagn Invest* 14:504–507.
- 7 20. Wensvoort G, Terpstra C, Pol JM, et al: 1991, Mystery swine disease in The Netherlands:  
8 the isolation of Lelystad virus. *Vet Q* 13:121–130.
- 9 21. Yang H, Wanner IB, Roper SD, Chaudhari N: 1999, An optimized method for in situ  
10 hybridization with signal amplification that allows the detection of rare mRNAs. *J*  
11 *Histochem Cytochem* 47:431–446.
- 12 22. Yoshii M, Kaku Y, Murakami Y, et al: 2004, Polymerase chain reaction-based genetic  
13 typing of Japanese porcine reproductive and respiratory syndrome viruses. *J Vet Diagn*  
14 *Invest* 16:342–347.
- 15 23. Zhou YJ, Yu H, Tian ZJ, et al: 2009, Monoclonal antibodies and conserved antigenic  
16 epitopes in the C terminus of GP5 protein of the North American type porcine reproductive  
17 and respiratory syndrome virus. *Vet Microbiol* 138:1–10.

1 **Table 1.** Effect of fixative and prolonged fixation on detection of PRRSV by nRT-PCR, TISH,  
 2 CISH, and IHC in paraffin-embedded lung tissues.

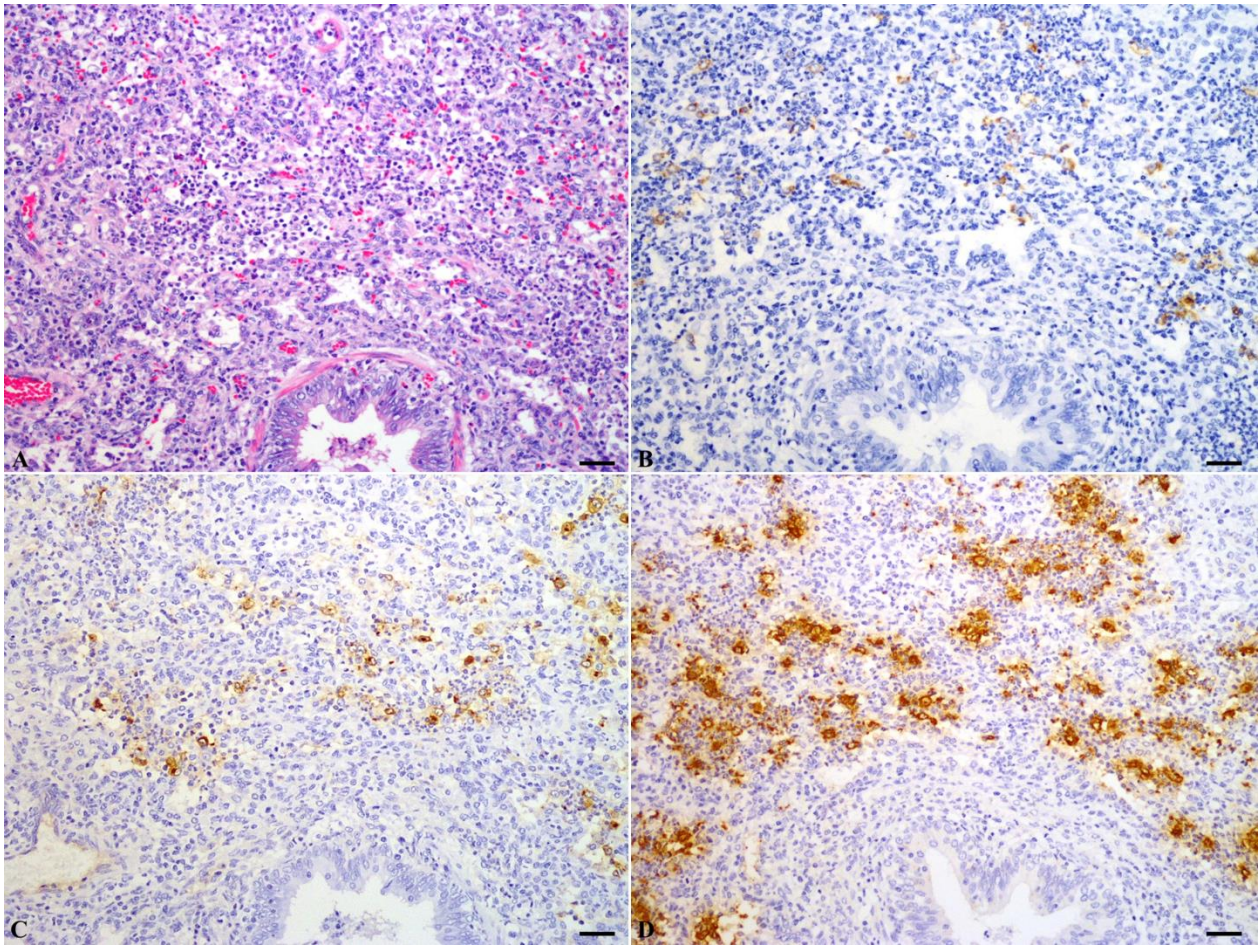
Fixative	Day of fixation	nRT-PCR*	TISH		CISH		IHC	
			Signal intensity <sup>†</sup>	Back ground staining	Signal intensity	Back ground staining	Signal intensity	Back ground staining
4% PFA	1	++	3	No	2	No	1	No
	7	++	3	No	2	No	1	No
	14	++	3	No	2	No	1	Yes
	21	+	3	No	1	No	0	Yes
	28	+	3	No	1	No	0	Yes
	60	+	2	No	1	No	0	Yes
	90	+	2	Yes	1	No	0	Yes
	120	-	0	Yes	0	Yes	0	Yes
10% NBF	1	++	3	No	2	No	1	No
	7	+	3	No	2	No	1	No
	14	+	3	No	2	No	1	Yes
	21	+	3	No	1	No	0	Yes
	28	+	3	Yes	1	No	0	Yes
	60	+	2	Yes	1	No	0	Yes
	90	+	1	Yes	0	Yes	0	Yes
120	-	0	Yes	0	Yes	0	Yes	

1 \* nRT-PCR: ++, sample tested positive at both first and second PCR reactions; +, sample tested  
2 positive at second PCR only.

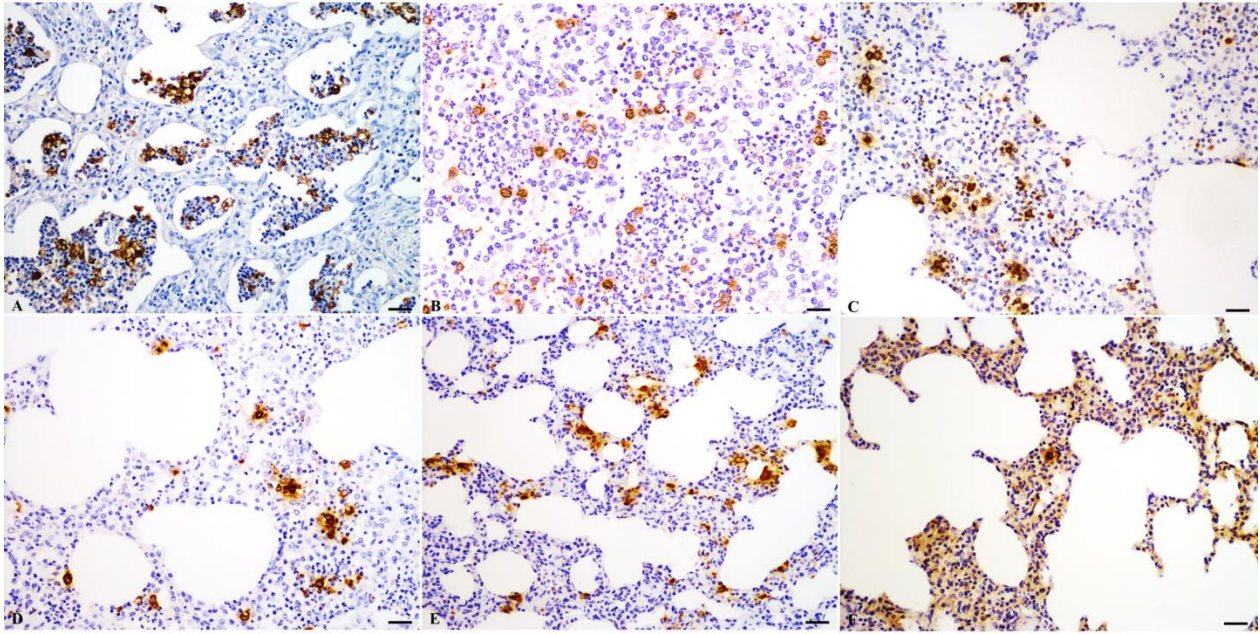
3 † Signal intensity (staining) was scored as 0 = none, 1 = weak, 2 = moderate, or 3 = strong.



1 **Figures and Legends**



2  
3 **Figure 1.** Serial sections of a lung tissue from a pig naturally infected with PRRSV. Lung sample  
4 was fixed in 4% PFA for one day. Positive signal was visualized as dark-brown staining in the  
5 cytoplasm of macrophages. **A.** H&E stain. Interstitial pneumonia and bronchopneumonia, with  
6 the thickening of alveolar septa. **B.** Signal detection by IHC revealed weak staining. **C.** Signal  
7 detection by CISH revealed moderate staining. **D.** Signal detection by TISH revealed strong  
8 staining. Bar = 20 $\mu$ m.



1  
2 **Figure 2.** Lung; pig naturally infected with PRRSV. Effect of fixation time and fixative on the  
3 detection of PRRSV by the TISH method. **A.** Lungs fixed in 4% PFA for 1 day. Strong positive  
4 signals without background staining. **B.** Lungs fixed in 4% PFA for 28 days. Strong positive  
5 signals with mild background staining. **C.** Lungs fixed in 4% PFA for 90 days. Moderate positive  
6 signals with moderate background staining. **D.** Lungs fixed in 10% NBF for 1 day. Strong  
7 positive signals with mild background staining. **E.** Lungs fixed in 10% NBF for 28 days.  
8 Moderate positive signal and strong background staining. **F.** Lungs fixed in 10% NBF for 90  
9 days. Weak positive signal and marked background staining. Bar = 20 $\mu$ m.

10