Immunohistochemical Detection of Porcine Reproductive and Respiratory Syndrome Virus Antigen in Formalin-Fixed, Paraffin-Embedded Tissues with Correlation to Clinicopathologic Data

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Abstract

Immunohistochemistry (IHC) was used in this study to detect the presence of Porcine Reproductive and Respiratory virus (PRRSV) antigen using monoclonal antibody (Mab SDOW17) in formalin-fixed, paraffin embedded tissues of lung, lymph node, heart, spleen, and kidney of pre-weaning to less than 90 day old pigs. Out of the 25 tissue samples examined from swine cases of the Philippine Animal Health Center (PAHC), Bureau of Animal Industry and College of Veterinary Medicine (CVM) Histopathology Laboratory, UPLB, 14 or 56% (14/25) were IHC positive for PRRSV antigen. Among the selected tissue samples, the PRRS virus was detected the most in the lymph nodes (64%) and lungs (40%), respectively, compared to other organs such as spleen (33%), kidney (28%), and the heart (17%). Only 43% (6/14) of cases coincided with the pathogenesis and clinicopathologic lesions of PRRS which are proliferative interstitial pneumonia, lymphoid follicular hyperplasia, and necrosis. PRRS positive cases in IHC were consistently found to have co-infections with viral and bacterial diseases. Since PRRSV has tropism for macrophages and destruction of these cells leads to immunosuppression, affected animals are vulnerable to secondary infections.

Keywords: Formalin-fixed, Immunohistochemistry, paraffin embedded tissue, piglets, Porcine Reproductive and Respiratory Syndrome, PRRS virus antigen

Introduction

Porcine Reproductive and Respiratory Disease Syndrome (PRRS) is an infectious viral disease that can cause significant morbidity and mortality in affected swineherds. The combined characteristics of being highly infectious and the potential of rapid spreading of this disease often lead to considerable economic losses especially on swine-raising countries worldwide. Affected pig populations primarily exhibit conditions of two overlapping clinical presentations namely, reproductive failure in breeding animals and respiratory distress in pigs of any age (Collins et al., 1992; Halbur et al., 1996; Cheon and Chae, 1999). Generally, the clinical signs and lesions are greater in younger and growing animals than in older ones. The uncomplicated signs of PRRS in nursery and growing pigs show signs of mild to moderate flu-like respiratory illness with minimal coughing (Halbur et al., 995a) while atypical manifestations of secondary bacterial diseases and co-infections of other respiratory viruses may complicate the case (Van Alstine et al., 1993; Teifke et al., 2001). Thus, clinical signs, complete history particularly in acute outbreaks, and microscopic lesions of primarily affected organs (e.g. lungs) are suggestive of PRRS infections but these are not pathognomonic. Hence, confirmation of the clinical disease to rule out other infectious diseases is necessary.

The routine use of immunohistochemistry (IHC) to detect PRRSV in the lung of nursery and grower/finisher pigs with respiratory disease

is commonly practiced since it has been reported to be highly specific (100%) and moderately sensitive (40 to >95%) (Halbur et al., 1996; Yaeger, 2002). In diagnosing PRRSV infection, IHC procedure utilized formalin-fixed paraffin-embedded (FFPE) tissue as the primary medium of choice because of its superior morphologic results and remarkable identification of the antigen in the tissues affected (Halbur et al., 1994; Larochelle and Magar, 1995; OIE, 2010). FFPE tissue preparations also provide an essential source for retrospective studies to detect diseases not detectable, even several decades ago, provided that protocols and adequate controls are critically administered (Ramos-Vara et al., 1999).

This study attempted to detect the presence of PRRSV antigen using immunohistochemical method in formalin-fixed paraffin-embedded tissues of diseased piglets (pre-weaning to early growing) submitted for histopathology from 1998 to 2012 and further aimed to demonstrate the PRRS virus as concomitant agent in the recorded diseases of examined pre-weaning to early growing pigs during that time.

Methodology

Case Selection

Twenty-five (25) case samples were selected from the archives of field cases submitted for laboratory examination from 1998 to 2012 where eleven (11) formalin-immersed tissue samples were acquired from PAHC, BAI, QC and fourteen (14) paraffin-embedded blocks samples were obtained from the Department of Veterinary Paraclinical Science, UPLB, College of Veterinary Medicine (CVM), Los Baos, Laguna. Selected paraffin-embedded tissue blocks were cut at about five (5) micrometers (μm) thick each and subsequently processed for immunohistochemistry. Duplicate samples were prepared for each processed tissue.

Representative tissue samples that served as the positive control was obtained from a piglet positive of porcine reproductive and respiratory syndrome case based on the reported history and clinico-pathologic records. Negative control tissue sections were prepared from the same tissue samples. The sections were incubated with phosphate buffer saline (PBS) pH 7.4 instead of primary antibody for antigen detection.

Immunohistochemistry

A commercial streptavidinbiotincomplex (ABC) immunoperoxidase assay using the corresponding primary monoclonal antibody (SDOW-17) to detect PRRSV was performed. The primary antibody (SDOW-17 mAb) was diluted in PBS as per manufacturers recommendation. The antibody was titrated into 3 different concentrations: pure, 1:100 and 1:500, to obtain the optimal final dilution to be used in the procedure. A 1:500 concentration was achieved for the final working dilution. An aliquot of pure concentrated monoclonal antibody was prepared into a ratio of two 1:10 and one 1:5 dilutions to make the final working dilutions of 1:500. With the use of adjustable pipettes, 100 microlitter (μL) aliquot of pure monoclonal antibody was diluted with 900 μL phosphate buffered saline [0.01M PBS] pH 7.4 to make one milliliter of 1:10 solutions. One part of 1:10 solutions was again diluted with 9 parts of PBS to make one milliliter of 1:100 concentration of solution. For the working dilutions of 1:500 concentrations, one part of the 1:100 dilutions was added with 4 parts of PBS. The method conducted was based on the process described by Halbur et al. (1994) using a slightly modified procedure particularly in the dilution and temperature adjustments for the incubation procedures.

For the immunohistochemical proper, 5 μm thick sections of lungs, lymph nodes, heart, spleen, and kidney were mounted on poly-L-lysinecoated glass slides. Mounted tissue sections were deparaffinized through heating in 60°C for one hour and through submerging into two washes of xylenes each in a five-minute interval, then rehydrated through a series of different alcohol concentrations, each in a 5 minute interval, respectively. Thereafter, antigen retrieval was performed using Protease XIV at 0.05% concentration in 0.05 M Tris buffer (pH 7.6) for 10 minutes at 37°C, then washed in a solution of TBS + 1% Tween (TBST) bath for 5 minutes. Endogenous peroxidase was also blocked with 3% hydrogen peroxide incubation for 30 minutes at room temperature and tissue sections were

washed with TBST bath for 5 minutes. Background blocking with 5% normal goat serum (NGS) for 30 minutes incubation at room temperature was done thereafter. Slides were treated with primary monoclonal antibody (SDOW-17) diluted in [0.01M] PBS pH 7.4 and incubated under improvised humidifying chamber at room temperature overnight or for 16 hours. After incubation, slides were rinsed and washed with TBST and TBST + 1% NGS each in a 5-minute interval. Tissue sections were then treated with secondary antibody (biotinylated goat anti-mouse linking antibody) diluted in [0.01M] PBS (1:200) pH 7.4, incubated for 30 minutes at room temperature, and washed following the primary antibody washes. Thereafter, peroxidase-conjugated streptavidin diluted in [0.01M] PBS (1:200) pH 7.4 was treated on the tissue sections, incubated for 40 minutes at room temperature, and subsequently rinsed in TBST bath solution for 5 minutes. DAB (3,3-diaminobenzidine tetrahydrochloride) substrate was then added on the tissue sections for 5-7 minutes and washed with distilled water for 5 minutes. Sections were counterstained with Mayers hematoxylin for 2 minutes then rinsed 10 times in distilled water. Slides were dehydrated with series of graded alcohols and 2 changes of xylenes in a 5-minute interval each. Sections were covered with mounting medium (Eukit) and a coverslip and examined under light microscopy. Tissue section in positive control was prepared from a known PRRS positive animal and treated with the primary antibody while the negative control was prepared from the same animal only with the omission of the primary antibody and replaced by PBS instead.

Histopathology Lesion Scoring (Krakowka et al., 2005)

Histological sections of lungs, lymph nodes, heart, spleen, and kidney were scored for histopathology lesions that uses a subjective grading system ranging from 0 to 4 where a score of 0 was assigned to sections that lacked the lesion in question; score of 1 was assigned to a lesion that is of minimal severity and present as 1 to 3 foci in the section; score of 2 was assigned to a lesion that is moderate in severity and multifocal in distribution; score of 3 was assigned to a lesion that

is severe and multifocally distributed with multiple confluent lesions; and a score of 4 was assigned to a lesion that is severe and diffusely distributed in the tissue section, respectively.

Immunohistochemistry Lesion Scoring (Halbur et al., 1996)

The intensity or distribution of viral antigen in each tissue sample from all pigs were scored subjectively on a scale of 0 to 4, where a score of; zero (0) was given to virus-negative tissue sections; one (1) was assigned to tissue sections with approximately 1-10 antigen-positive cells; two (2) was assigned to tissue sections containing approximately 11-30 antigen-positive cells; three (3) was assigned to tissue sections containing approximately 31-100 antigen-positive cells; and four (4) was assigned to tissue sections containing more than 100 antigen-positive cells, respectively.

Data Evaluation and Statistics

All data gathered were analyzed through descriptive statistics.

Results and Discussion

Of the 25 case samples processed, 14 (56%) tested positive for PRRS antigen in IHC of which six out of eleven (6/11) were from PAHC and eight out of fourteen (8/14) were from UPLB, CVM.

Table 1 shows the positive immunohistochemistry (IHC) results in selected clinical cases acquired from PAHC and UPLB-CVM. Out of the six (6) positive PAHC samples, three (3) cases were from pre-weaning pigs while three (3) cases were from pigs in the post-weaning to early growing phase. On the other hand, the eight (8) IHC positive samples from UPLB-CVM were from two (2) cases of pre-weaning and six (6) postweaning to early growing age pigs, respectively.

Immunohistochemistry (IHC) was used in this study to demonstrate the presence of Porcine Reproductive and Respiratory virus (PRRSV) antigen in a specific tissue of pigs with age ranging from day old to less than 90 days. The positive test results obtained in this study is indicated by

Table 1. Positive IHC Samples from Suspected PRRS Clinical Cases Acquired from PAHC and UPLB-CVM

CASE ORIGIN	PERCENTAGE AFFECTED BY AGE		TOTAL
	(1-30) days	(31-90) days	
PAHC	3/5 (60%)	3/6 (50%)	6/11(54%)
CVM	2/3 (67%)	6/11 (54%)	8/14 (57%)
Total	5/8 (62%)	9/17 (51%)	14/25 (56%)

the presence of virus antigen manifested as brown stain in the cytoplasm of specific infected tissues such as hypertrophied type 2 pneumocytes and alveolar macrophages scattered within the septal interstitium, histiocytes and endothelial cells lining blood vessels and lymphatics in the heart and kidney and within germinal centers, periarteriolar tissues and medullary cords in the lymphoid tissues where circulating macrophages are usually drained. These findings are similar with previous researches done by Halbur et al. (1994) and Halbur et al. (995b) where it was revealed that sixty-two percent of PRRSV positive were samples from animals in the pre-weaning age whereas 51% were at the early growing phase. It has also been reported that infection of PRRS at an earlier age leads to a more severe clinical disease outcome (Mengeling et al., 1995; Goyal, 1993; Rowland et al., 2003).

On the other hand, a negative IHC results does not necessarily indicate that the sampled animal is free of the virus. Although immunohistochemistry staining was performed in accordance to standard protocol, improper handling of tissues especially during formalin fixation to paraffin blocking could lead to obtaining false positive and false negative results as demonstrated by Yoon et al. (2003) and Ramos-Vara et al. (1999).

Table 2 shows the histopathology lesion scores in PRRS positive organs in relation to their histopathologic descriptions. The following were tested positive: 10 out of 25 lung samples, 9 out of 14 lymph node samples, 2 out of 12 heart samples, 7 out of 21 spleen samples, and 5 out of 18 kidney samples. The IHC results show that the lymph nodes (64%) and lungs (40%) demonstrated the PRRS virus antigen the most, which was then followed by the spleen (33%), kidney (28%), and heart (17%).

Histopathologic scores revealed that among the tested lung samples, 50% demonstrated very severe and diffusely distributed lesions, followed by a 30% minimal severity and 20% moderate severity of multifocal distributions. Lymph node samples also demonstrated both 29% moderate to very severe and multifocal to diffuse distributions with confluent lesions. In addition, t 71% of spleen samples demonstrated moderate severity with multifocal distributions of lesion; while 29% demonstrated minimal severity with lesions focally located in the tissue. The tested positive kidney samples demonstrated 60% lesions of minimal severity of which 1 to 3 foci were present in the tissue. The IHC positive heart samples also demonstrated 50% lesions of minimal severity and 50% with moderate severity that were multifocal in distribution. Of all the organ samples that tested positive, one lymph node sample and two kidney samples were scored 0 that described the lack of PRRS lesions.

Histopathology results show that among the affected organs resembling PRRS infected tissues, the lymph node demonstrated the highest lesion score that was described to be very severe and had diffusely distributed lesions in the tissue section examined. In the study by Rowland et al. (2003), it was demonstrated that in persistent infections of congenital PRRSV-infected pigs, the virus can be recovered in the tonsil and lymph node rather than in the typical lung tissue. It was further concluded that these tissues can support virus replication for an extended period of time. In the previous reports on the pathogenesis of acute PRRS infection, the PRRSV initially replicates in the tonsils following viremia. As the infection persists, it further replicates primarily within alveolar macrophages (primary predilection sites for viral replication) and other lymphoid (thymus,

Table 2. Histopathology Lesion Score and Corresponding Histopathologic Description in PRRS Positive Organ Samples Based on IHC

ORGANS	HISTOPATHOLOGY SCORES			HISTOPATHOLOGIC
IHC POSITIVE	1	2	3 - 4	DESCRIPTION
Lungs	3/10	2/10	5/10	Demonstrated minimal to very severe, multifocal to diffuse
(n=10)	(30%)	(20%)	(50%)	distributions with evidence of multiple confluent lesions
Lymph Nodes [a]	0/9	4/9	4/9	Demonstrated moderate to very severe, multifocal to diffuse
(n= 9)	(0%)	(29%)	(29%)	distributions with evidence of multiple confluent lesions
Heart	1/2	1/2	0/2	Demonstrated lesions of minimal to moderate severity with
(n=2)	(50%)	(50%)	(0%)	multifocal distributions
Spleen	2/7	5/7	0/7	Demonstrated lesions of minimal to moderate severity with
(n=7)	(29%)	(71%)	(0%)	multifocal distributions
Kidney [b]	3/5	0/5	0/5	Demonstrated lesions of minimal severity with 1 to 3 foci in the
(n=5)	(60%)	(0%)	(0%)	section

n = Total sample of tissue tested

[a] 1 IHC positive sample scored 0 in histopathologic scoring

[b] 2 IHC positive samples scored 0 in histopathologic scoring

spleen, tonsil, lymph nodes) and non-lymphoid (heart, aorta, kidney, liver, testes, salivary gland, intestine, brain, and stomach) organs of the affected pig (Halbur et al., 995b; Teifke et al., 2001; Halbur et al., 1994; Magar et al., 1993; Rowland et al., 2003).

In the present study, most of the lesions found in affected lungs were marked hypertrophy and hyperplasia of the pneumonocytes II, septal infiltration with mononuclear cells, accumulation of necrotic cell debris and mixed inflammatory cells in the alveolar spaces, and moderate perivascular accumulation of inflammatory cells. In lymphoid tissues, histopathology reading revealed marked follicular hyperplasia with foci of necrosis within germinal centers and in periarteriolar lymphoid sheaths. In the heart, few, small, and randomly perivascular foci of lymphocytes and macrophages with minimal focal histiocytic inflammation were found. The kidney revealed vascular lesions of marked swelling of arterial and venular endothelial cells, as well as focal, mild, non-supportive peri-glomerular and peritubular infiltrates in the renal cortices. These findings agree with the classical microscopic descriptions of PRRS infection in an experimented animal (Halbur et al., 1994; Cooper et al., 1997).

Figure 1 shows a microphotograph of a pig lung from PAHC clinical case with ID number PAHC-2725. The lesion describes a severely diffused chronic interstitial pneumonia with a corresponding score of 4. The lung tissue portrays
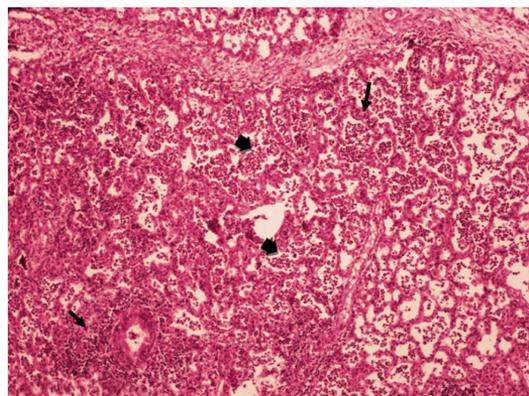
Figure 1. Pig Lung. Alveolar spaces are markedly infiltrated with macrophages, giant cells, and desquamated alveolar epithelium (thick arrows). The interalveolar septae is hardly distinguished because of infiltrating inflammatory exudates and hyperplasia of pneumocytes (thin arrows). 10X. H&E.

diffuse and extensive hypercellularity within the interalveolar septae and intra-alveolar spaces.

Table 3 shows the clinical signs manifested in IHC positive clinical cases. Eleven (79%) manifested signs of respiratory distress, 10 (71%) cases showed signs of mixed infections, 9 (64%) cases manifested general signs resembling a PRRS disease, and 6 (43%) cases showed clinical signs in the ear, eyes, and skin. Categories I, II, and III are the classic clinical signs often man-

ifested during a PRRS disease, while category IV is where mixed clinical signs are presented (i.e. lameness, severe diarrhea, and nervous signs) that are not typical of PRRS.

According to the recorded diagnostic notes of PRRS infection by Animal Health Australia (2006), clinical signs of infection usually occur within 48 days of exposure once a new host has been infected with the PRRS virus as a result of viral replication in mucosal, pulmonary, or regional lymphoid macrophages. In about 12 hours, the virus can reach regional lymph nodes and is systemically distributed to mononuclear cells, and tissue macrophages. PRRS suspected or infected pig shows varying clinical signs depending on the immune status of the herd and management factors. In an affected litter, variably sized, weak-born pigs can be observed, which may further manifest chemosis (excessive swelling of the mucus membrane lining the eyelids and surface of the eyes) especially in animals less than three weeks old. On the other hand, respiratory distress is obviously and critically severe in younger and growing animals, while pyrexia, lethargy, anorexia, chemosis, patchy dermal cyanosis, with respiratory infection of PRRS are seen in all ages of pigs (Collins et al., 1992; Halbur et al., 1996; Cheon and Chae, 1999).

Table 4 shows the comparison of post mortem lesions to PRRS positive organs. Ten lung samples that developed pneumonia have the following distribution of IHC scores: 4 (40%) with scores of 2, 3 (30%) with score of 1, 2 (20%) with a score of 4, and 1 (10%) with a score of 3, respectively. All 9 lymph node samples have a corresponding score of 4 while all heart samples have a score of 3. Three (60%) kidney samples have corresponding score of 4 while 2 (40%) have a score of 3. Seven spleen samples have the following scores: 3 (43%) with 1, 2 (29%) with 3, 1 (14%) with 2, and 1(14%) with 4, respectively.

PRRS is an infection with multi-systemic manifestations in pig, however, the gross lesions are remarkably observed in respiratory and lymphoid tissues. The findings of the present study coincided with the descriptions reported by Rossow (1998) where he described that the post mortem lesions are more severe in neonatal and pre-weaning pigs than in young growing pigs which sometimes are unremarkable, especially when not complicated by other systemic diseases. Regional lymph nodes particularly those in the cervical, cranial, thoracic and inguinal regions are most obvious at postmortem (Halbur et al., 995a). The classic macroscopic descriptions of PRRS infected lungs are mottled, tan and red, and failed to collapse, in which the cranioventral lobes are most affected, while the lymph nodes are moderately to severely enlarged and colored tan (Dietze et al., 2011).

Table 5 shows the results of IHC and clinical observations performed, together with, the recorded differential diseases for PRRS in the affected pre-weaning (1-30 days) to early growing (31-90 days) pigs. Of the 14 IHC tested positive cases, six (6) or 43% coincided with the clinical observations described.

Majority of the PRRS virus positive animals were burdened with systemic diseases that have both bacterial and viral in origin regardless of the age group affected. Teifke et al. (2001) reported that PRRS alone may have mild to subclinical signs that are usually non-detectable if not complicated by other respiratory or existing diseases, particularly of some bacteria and other viruses, that primary affect and compromise the respiratory system. The virus causes disease by infecting macrophages, compromising the cellular immune response, and damaging mucosal surfaces (Animal Health Australia, 2006). Thus, protective immunity against PRRSV infection is delayed and weak because of virus-mediated immune-modulation that can lead to virus persistence and severe secondary respiratory infections.

The manifested clinical signs may usually resemble those of other respiratory viral or bacterial pathogens affecting the same age range as described in this research (pre-weaning to early growing phase). In the respiratory and post-weaning form of the disease, it is often differentiated from other diseases such as swine influenza, enzootic pneumonia, proliferative and necrotising pneumonia, *Haemophilus parasuis* infection, haemagglutinating encephalomyelitis virus, porcine respiratory coronavirus, syncitial pneumonia and myocarditis, postweaning multisystemic wasting syndrome, and Nipah virus infection (Animal Health Australia, 2006). Moreover, the presence of PRRS virus infection, par-

Table 3. Clinical Signs Manifested in IHC Positive Cases

IHC results	Clinical Signs			
	Category I	Category II	Category III	Category IV
Positive	11	6	9	10
Negative	3	8	4	4
Total	14	14	14	14

Legend: Category I = respiratory distress; Category II ear, eyes and skin lesions; Category III General signs (sneezing/nasal discharges, laboured breathing/thumping/dyspnea, coughing/thumping); Category IV = mixed signs not typical of PRRS (i.e. epistaxis, diarrhea, joint lesions, etc.)

Table 4. Comparison of Postmortem Lesions to IHC Positive Organs

POST MORTEM CATEGORY OF LESIONS	ORGAN	IHC SCORES[A]			
		1	2	3	4
I - Pneumonia	Lungs *n=10*	3/10 (30%)	4/10 (40%)	1/10 (10%)	2/10 (20%)
II - Heart Lesion	Lymph Nodes n=9	0/9 (0%)	0/9 (0%)	0/9 (0%)	9/9 (100%)
III- Lymph Node Lesions	Heart n=2	0/2 (0%)	0/2 (0%)	2/2 (100%)	0/2 (0%)
IV Spleen Lesions	Spleen n=7	3/7 (43%)	1/7 (14%)	2/7 (29%)	1/7 (14%)
V Renal Lesions	Kidney n=5	0/5 (0%)	0/5 (0%)	2/5 (40%)	3/5 (60%)

[A] IHC numerical Scoring (refers to appendix 3b)

Table 5. Results of IHC and Clinical Observation with the Differential Diseases of PRRS in an Affected Age Group of Pigs

CASE NO	CASE ORIGIN	AGE (days)	PRRS CASES		OTHER DISEASES
			IHC	Clinical Observation	
1	PAHC 0443		+	+	Porcine Circovirus-Associated Diseases
2	PAHC 0344	1-30	+	+	Porcine Circovirus-Associated Diseases
3	PAHC 1848		+	+	Porcine Circovirus-Associated Diseases
4	CVM 04H24		+	N/A	N/A
5	CVM98H05		+	-	Hog Cholera
6	CVM 11H11		+	+	Greasy Pig Disease
7	CVM 09H14		+	-	Collibacillosis
8	CVM 06H48		+	-	Mycoplasmosis
9	CVM 01H12		+	N/A	N/A
10	CVM 2KH52	31-90	+	N/A	N/A
11	CVM 02197		+	N/A	N/A
12	PAHC 1292		+	+	none
13	PAHC 0321		+	-	Hog Cholera, Mycoplasmosis, and Collibacillosis
14	PAHC 2725		+	+	Porcine Circovirus-Associated Disease, Edema Disease, and Mycoplasmosis

Note: No. 13 tested positive while No. 2 tested negative to PRRS by FAT (Fluorescence antibody test) based on the given data from PAHC; N/A = Not Available

ticularly in non-breeding herds, may contribute to the porcine respiratory disease complex (PRDC) and also increases the severity of other diseases present in the farm such as streptococcal meningitis (Williamson, 2010). Therefore, when the clinical history and pathology is suggestive of PRRS, detection of viral antigens, viral genomic material, or isolation of virus from clinical specimens must be used to confirm the tentative diagnosis (Yoon et al., 2003).

Conclusions

Results showed that IHC can be used to detect PRRS antigen using SDOW-17 monoclonal antibody in formalin-fixed, paraffin embedded tissue even when tissues have been fixed for a long time. PRRS lesions of proliferative interstitial pneumonia and lymphoid follicular hyperplasia and necrosis in this study also coincided with the pathogenesis of PRRS. In the PRRS infection, the disease often occur with co-infection of both bacterial and viral in origins and this is shown in the recorded swine diseases at PAHC and CVM-UPLB among pre-weaning and post-weaning to early growing pigs.

Acknowledgements

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