UNIVERSITI PUTRA MALAYSIA

DETECTION OF AFRICAN SWINE FEVER AND CLASSICAL SWINE FEVER VIRAL ANTIGEN FROM FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUES OF WILD AND DOMESTIC PIGS

PHAN YONG WEI

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PHAN YONG WEI

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CERTIFICATION

It is hereby certified that we have read this project paper entitled “Detection of African Swine Fever and Classical Swine Fever Viral Antigen from Formalin-Fixed Paraffin-Embedded Tissues of Wild and Domestic Pigs”, by Phan Yong Wei and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

DR. NURUL IZZATI UDA ZAHLI

DVM (UPM), PhD (Miyazaki, Japan)

Lecturer,

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Supervisor)

ASSOCIATE PROFESSOR DR. OOI PECK TOUNG

DVM (UPM), PhD (Glasglow)

Associate Professor,

Faculty of Veterinary Medicine,

Universiti Putra Malaysia,

(Co-Supervisor)

DR. MICHELLE FONG WAI CHENG

DVM (UPM), PhD (UPM)

Lecturer,

Faculty of Veterinary Medicine,

Universiti Putra Malaysia,

(Co-Supervisor)

DR. AZLAN CHE' AMAT

DVM (UPM), PhD (Universidad de Castilla La-Mancha)

Lecturer,

Faculty of Veterinary Medicine,

Universiti Putra Malaysia,

(Co-Supervisor)

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ASF	African Swine Fever
ASFV	African Swine Fever Virus
BSA	Bovine serum albumin
CSF	Classical Swine Fever
CSFV	Classical Swine Fever Virus
DAB	Diaminobenzine
H&E	Hematoxylin and eosin
IHC	Immunohistochemistry
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PCV	Porcine Circovirus

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek.

PENGESANAN ANTIGEN VIRUS DEMAM BABI AFRIKA DAN DEMAM BABI KLASIK DARI TISU 'FORMALIN FIXED PARAFFIN EMBEDDED' BABI LIAR DAN DOMESTIK

Oleh

Phan Yong Wei

2023

Penyelia: Dr. Nurul Izzati Uda Zahli
Penyelia bersama: Profesor Madya Dr. Ooi Peck Toung, Dr. Michelle Fong Wai Cheng, Dr. Azlan Che' Amat

Demam babi Afrika (ASF) dan demam babi klasik (CSF) yang disebabkan oleh virus demam babi Afrika (ASFV), sejenis virus DNA daripada keluarga Asfaviridae dan genus Asfivirus, dan virus demam babi klasik (CSFV), sejenis virus RNA daripada genus Pestivirus dalam keluarga Flaviviridae masing-masing. ASF telah merebak di seluruh Asia sejak tahun 2018 dan dilaporkan pertama kali di Malaysia pada Februari 2021, manakala CSF adalah endemik di beberapa negara di Asia termasuk Malaysia. Kedua-dua penyakit ini telah memberi impak negatif kepada ekonomi Malaysia disebabkan oleh kadar kematian dan morbiditi yang tinggi dalam populasi babi

domestik. Kajian ini bertujuan untuk mengesan antigen virus ASF dan CSF dalam sampel tisu “Formalin-fixed paraffin embedded” (FFPE) dengan menggunakan teknik imunohistokimia (IHC) dan untuk mencirikan lesi histopatologi yang disebabkan oleh kedua-dua virus ini. Blok parafin dan slaid H&E limpa, tonsil, dan nod limfa dari sebanyak 17 ekor babi domestik dan 3 ekor babi hutan telah diambil, dan blok parafin tersebut diproses menjadi slaid IHC. Untuk IHC, antibodi utama dengan pencairan 1:1000 digunakan untuk ASF dan pencairan 1:100 digunakan untuk CSF. Keputusan IHC menunjukkan sel-sel positif antigen ASFV dalam 1/20 (5%) sampel dan sel-sel positif antigen CSFV dalam 4/30 (20%) sampel. Semua sampel positif CSF menunjukkan pengecilan limfoid dan 3/4 (75%) menunjukkan perdarahan. Kajian ini menunjukkan keperluan untuk petani memperkuatkan biosekuriti untuk mengelakkan jangkitan ASF dan CSF ke dalam ladang

Kata kunci: Babi, Demam babi Afrika, Demam babi klasik, Immunohistokimia, Histopathologi

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999- Project.

DETECTION OF AFRICAN SWINE FEVER AND CLASSICAL SWINE FEVER VIRAL ANTIGEN FROM FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUES OF WILD AND DOMESTIC PIGS

by

Phan Yong Wei

2023

Supervisor: Dr. Nurul Izzati Uda Zahli

Co-supervisors: Assoc. Prof. Dr. Ooi Peck Toung, Dr. Michelle Fong Wai Cheng, Dr. Azlan Che' Amat

African swine fever (ASF) and classical swine fever (CSF) is caused by African swine fever virus (ASFV), a DNA virus belonging to the family of Asfaviridae and genus Asfivirus, and classical swine fever virus (CSFV), a RNA virus belonging to the Pestivirus genus within the Flaviviridae family respectively. ASF has spread throughout Asia since 2018 and first reported in Malaysia in February 2021, while CSF is endemic in few countries in Asia including Malaysia. Both diseases have

negatively impacted the economy of Malaysia due to high mortality and high morbidity rate in domestic pigs. This study aimed to detect the ASF and CSF viral antigen in formalin-fixed paraffin embedded tissue (FFPE) samples by using immunohistochemistry (IHC) and to characterise the histopathological lesion caused by both viruses. Paraffin blocks and H&E slides of spleen, tonsil and lymph nodes of 17 domestic pigs and 3 wild boars are retrieved, and paraffin blocks are processed into IHC slides. For IHC, primary antibody of dilution of 1:1000 is used for ASF and dilution 1:100 is used for CSF. IHC revealed ASFV antigen positive cells in 1/20 (5%) samples and CSFV antigen positive cells in 4/30 (20%) samples. All CSF positive samples show lymphoid depletion and 3/4 (75%) shows hemorrhages. This study illustrates the need for farmers to strengthen the biosecurity to prevent transmission of ASF and CSF into the farm.

Keywords: Swine, African Swine Fever, Classical Swine Fever, Immunohistochemistry, Histopathology

1.0 Introduction

African swine fever (ASF) is a highly contagious, World Organisation for Animal Health (WOAH)-notifiable viral disease of wild and domestic pigs. The aetiology agent for ASF is the ASF virus (ASFV), a large double-stranded DNA virus and the only member of Asfarviridae family (Alonso et al., 2018). The main target cell of ASFV is monocyte and macrophages (Carrasco et al., 2002). ASFV is composed of more than 50 structural proteins, and is capable of producing more than 150 proteins in the infected cells, many of which are highly immunogenic (Dixon et al., 2012). ASFV enters the host mainly through the oral-nasal route. It can also be spread by other routes such as tick bites (Blome et al., 2013). ASF outbreak has been reported from multiple Asia countries since 2018, including China, Mongolia, Viet Nam, Cambodia, Democratic People's Republic of Korea, Lao People's Democratic Republic, Myanmar, The Philippines, Republic of Korea, Timor-Leste, Indonesia, Papua New Guinea, India, Malaysia, Bhutan, Thailand, Nepal and Singapore (FAO, 2023).

Classical swine fever (CSF), also known as hog cholera, is another important WOAH-notifiable contagious disease of wild and domestic pigs. CSF is caused by CSF virus (CSFV), a positive-sense single-stranded RNA virus that belongs to the Pestivirus genus within the Flaviviridae family (Smith et al., 2017). Generally, the acute form of ASF has a similar clinical and pathological picture as CSF (Moennig et al., 2003). CSFV has an affinity towards mononuclear cells (macrophages and dendritic cells) and endothelial cells (Summerfield et al., 2015). Pigs can be infected through the oronasal route, direct or indirect contact with infected pigs, through contaminated feed especially through swill feeding, and vertical transmission (Moennig et al., 2003). Insemination may also be a route of transmission as CSFV is also found in semen

(Floegel et al., 2000). CSF is endemic in many parts of Asia and the Pacific, including a recent outbreak in Korea and reemerging case in Japan (Ganges et al., 2020). Australia and New Zealand are currently listed as CSF-free members by WOAH (WOAH, 2022).

ASF and CSF are economically important diseases worldwide. This study will focus on the description of histopathological lesions and viral antigen distribution in wild and domestic pigs that are infected with ASF or CSF to allow a better understanding of pathological lesions of ASF and CSF. The information gathered will be valuable for future detection of ASF and CSF.

1.1 Situation in Malaysia

The first ASF case in Malaysia was reported in February 2021 in Sabah. Cases were then confirmed in Peninsular Malaysia in December 2021. Currently affected states include Melaka, Johor, Penang, Perak, and Sarawak. Positive cases were also found in wild boars in Negeri Sembilan and Pahang (FAO, 2023). Currently, no ASF vaccine is available in Malaysia.

1.2 Objectives

The study was conducted with the following objectives:

1. To detect the viral antigen of ASFV and CSFV using immunohistochemistry.
2. To determine the histopathological lesions of wild and domestic pigs infected with ASF and CSF.

1.3 Hypotheses

Null hypothesis 1: There are no CSFV and ASFV antigen detected.

Alternative hypothesis 1: There are CSFV and ASFV antigen detected.

Null hypothesis 2: Absent of ASFV in the domestic pigs and wild boars in Selangor.

Alternative hypothesis 2: Presence of ASFV in the domestic pigs and wild boars in Selangor.

1.4 Justification

There is limited description of histopathological lesions and viral antigen distribution in the natural cases of ASF and CSF in Malaysia. Hence, information from this study can aid in more effective detection of ASF and CSF through better understanding of histopathological lesions and viral antigens distribution.

2.0 Literature review

2.1 Clinical Form of CSF

CSF can be classified into acute, chronic and persistent forms (prenatal). The clinical forms may vary depending on the age of pigs, the virulence of CSFV, the hygiene status of the farm and the presence of secondary infections. Clinical forms of wild pigs and domestic pigs are similar (Shimizu et al., 2020).

2.1.1 Acute Form of CSF

The clinical signs of acute CSF include pyrexia, loss of appetite, gastrointestinal symptoms, general weakness, and conjunctivitis (Shimizu et al., 2020). Fever is usually higher than 40°C, but in adult pigs, the temperature may not exceed 39.5 °C (Moennig et al., 2003). After 2 to 4 weeks of infection, the pigs may show neurological signs and skin haemorrhages in different locations of the body. Neurological signs include incoordination, paresis, paralysis and convulsions, and is often referred to as

“typical” CSF sign. Pigs with acute form usually die after 10 to 30 days of infection (Blome et al., 2017)

2.1.2 Chronic Form of CSF

Pigs with inadequate immune response will develop the chronic form of CSF after infection occurs. Clinical signs are usually not specific, including ill, depressed, stunned in growth and diffuse dermatitis. The signs are generally less severe than the acute form, thus the pigs may live for months and shed high amounts of the virus constantly. However, the pigs will eventually die due to the infection (Blome et al., 2017).

2.1.3 Persistent (Prenatal) Form of CSF

Persistent form infection occurs through vertical transmission from the sow to the fetuses. The outcome of the infection may vary, which includes absorption, mummification of the fetus, stillbirth and abortion, depending on the stage of gestation when the infection occurs. Persistently infected piglets may be born if the infection occurs between days 50 and 70 of gestation. The piglets may appear healthy for a few months but eventually die due to the late -onset form of CSF, in which they shed high load of virus during that period (Blome et al., 2017).

2.2 Pathological Lesions of CSF infection

The pathological lesions of CSF infection may vary between different clinical forms, but the most commonly observed lesions include lymphoid depletion in spleen, lymph node and tonsils; histiocytic hyperplasia in spleen; cerebral haemorrhage; perivascular cuffing in the brain; renal erythrodiapedesis; urothelial vacuolation and degeneration and interstitial pneumonia (Izzati et al., 2021).

In acute cases, lesions more commonly observed are renal petechiae, splenic infarction, haemorrhages in the periphery of lymph nodes, perivascular cuffing in the brain, vascular lesions (vary from a slight thickening of the capillary wall to fibrinoid necrosis of arterioles and vasculitis) and lesions in the lymph nodes (oedema, proliferation of the reticuloendothelial elements, extensive haemorrhage, necrosis) (Robinson & Robinson, 2016)

In chronic cases, one of the characteristic lesions is button ulcer on mucosa of colon. (Robinson & Robinson, 2016)

In prenatal cases, common lesions observed are thymic atrophy, pale swollen lymph nodes and focal colonic mucosal necrosis. Surviving piglets with prenatal CSF infection are weak and stunted in growth, and usually die within 2-12 months. (Robinson & Robinson, 2016)

2.3 Pathogenesis of CSF infection

The pigs are normally infected through oronasal route, direct or indirect contact with infected pigs, through contaminated feed especially through swill feeding, and vertical transmission (Moennig et al., 2003). Insemination may also be route of transmission as CSFV is also found in semen (Floegel et al., 2000). After infection, CSFV primarily replicate in the tonsils, and subsequently spread to other lymphoid tissue (Liess B., 1987) . CSFV then spread to regional lymph nodes through the lymphatic vessels, and further replication takes place here. Viremia then occurs as CSFV spread through blood to secondary replication sites like spleen, bone marrow and visceral lymph nodes (Blome et al., 2017). CSFV has affinity towards mononuclear cells

(macrophages and dendritic cells) and endothelial cells. These activated macrophages and dendritic cells release high amount of IFN-a, leading to excessive apoptosis of lymphocytes (even unaffected B and T cells), causing severe lymphopenia and resulting in immunosuppression (Summerfield et al., 2015). Endothelial cells are also activated and pro-inflammatory and pro-coagulatory factors are increased. The endothelial cells are damaged, which contribute to hemorrhagic pathogenesis, causing widespread hemorrhage and thrombocytopenia (Bensaude et al., 2004).

2.4 Clinical Form of ASF

The clinical presentation of ASF vary depending on the virulence of the virus, the route and dose of infection, and host characteristics (Sánchez-Vizcaíno et al., 2015). ASFV strains are classified as highly virulent, moderately virulent and low virulent (Pan and Hess, 1984). ASF in pigs can be described as peracute, acute, subacute, or chronic form (Salguero, 2020).

2.4.1 Peracute Form of ASF

This form of ASF is typically caused by a highly virulent strain of ASFV, causing a very rapid clinical course. Pigs usually have high fever up to 42°C, loss of appetite and inactivity sudden death before any development of clinical signs (Salguero, 2020). Pigs usually die within 1-3 days after onset of clinical signs (Sánchez-Vizcaíno et al., 2015).

2.4.2 Acute Form of ASF

This form is caused by highly or moderately virulent strain of ASFV. Clinical signs include high fever (40-42°C), anorexia, lethargy, pigs huddling together. Bluish-purple

discolouration and petechial hemorrhages can be seen on the ears, abdomen, limbs, snout and perianal area (Salguero, 2020). Infected pigs usually show respiratory distress with nasal discharges due to pulmonary edema (Carraso et al., 2002). Other signs may include vomiting, diarrhea (sometimes melena) and abortion (Salguero, 2020). Infected pigs die in shock, usually 1 week after fever begins, with foam observed around the mouth and nose (Carrasco et al., 2002)

2.4.3 Subacute Form of ASF

This form is usually caused by moderately virulent strain of ASFV. The signs are similar to acute form of ASF, but normally less marked (Sánchez-Vizcaíno et al., 2015). However, the vascular changes (mostly hemorrhages and oedema) can be more intense than the acute form. Infected pigs die within 7-20 days, with mortality rate range from 30% to 70%. Pigs may recover within 3-7 weeks. Pigs may die at two different stages: in the intense thrombocytopenia/leukopenia phase or in the recovery phase. Recovery phase is when haemorrhages appear due to erythrodiapedesis by vasodilation, especially in young animals (Gómez-Villamandos et al., 2013).

2.4.4 Chronic Form of ASF

This form is usually caused by a low virulent strain of ASFV, and has no specific clinical signs (Arias et al., 2002). Affected pigs usually show necrotic lesions on the skin, arthritis, delayed growth, emaciation, lameness, respiratory signs, abortion and low mortality (Sánchez-Vizcaíno et al., 2015)

2.5 Pathological Lesion of ASF

In peracute form, pigs die rapidly with no lesions in organs. (Sánchez-Vizcaíno et al., 2015)

In acute form, affected pigs might show rounded edges and friable spleen with characteristic hyperaemic splenomegaly. Hemorrhages can be seen on lymph nodes, with petechial hemorrhages on kidney, mucosa of urinary bladder, endocardium, epicardium and pleura. (Sánchez-Vizcaíno et al., 2015)

In subacute form, pigs suffering from this form of ASF may display characteristics edema of gallbladder wall and bile duct as well as perirenal edema, ascites and hydropericardium. Similar to acute form, hyperemic splenomegaly, hemorrhagic lymph nodes and renal hemorrhages (more intense than in acute form) can be observed. (Sánchez-Vizcaíno et al., 2015)

In chronic form, affected pigs show no vascular lesions, but lesions caused by bacteria may be observed. These include fibrinous pleuritis, pericarditis, pleural adhesions, necrotic pneumonia, fibrinous arthritis/periarthritis and necrotic skin lesions, as well as necrotic areas on the tonsils and the tongue (Moulton et al., 1968)

2.6 Pathogenesis of ASF

ASFV enters the host mainly through oral-nasal route. It can also be spread by other routes such as tick bites. The pathogen then replicates in the tonsils and regional lymph node, and spreads to secondary replication organs through lymph and blood in 2 to 3 days (Blome et al., 2013) and eventually spreads to the other organs (Gomez et al., 2013).

2.6.1 Lymphoid Depletion

The main target cell of ASFV is monocyte and macrophages (Carrasco et al., 2002). Virus replication within the target cells can directly induce necrosis of cells, and virions are released by budding (Gómez-Villamandos et al., 1997). The action of replication in monocytes/macrophages also leads to the activation of the target cells, and secretion of proinflammatory cytokines increased (cytokine storm). These include IL-1, TNF-α, and IL-6. This mechanism then causes massive apoptosis of lymphocytes, leading to lymphoid depletion (Salguero et al., 2005)

2.6.2 Vascular changes

Hyperemic splenomegaly: Red pulp comprises a mesh of fibers and smooth muscle cells surrounded by a fixed population of splenic-cord macrophages. ASFV destroys macrophages, leading to the detachment of macrophages from smooth muscle cells, and inducing loss of intercellular junction. The basal lamina is then exposed, inducing the activation of the coagulation cascade, platelet aggregation, and fibrin deposition, leading to the accumulation of red blood cells within the splenic cords (Gomez-Villamandos et al., 2013)

Hemorrhages: The main factor contributing to hemorrhages is the phagocytic activation of capillary endothelial cells, accompanied by lysosome proliferation and the presence of phagocytosed cell debris, leading to endothelial hypertrophy and total occlusion of the capillary lumen and a severe increase in the intravascular pressure. The capillary then has loss of endothelial junction and give rise to hemorrhages (Gomez-Villamandos et al., 2013).

3.0 Methodology

3.1 Samples

Formalin-fixed paraffin-embedded (FFPE) blocks and H&E slides of the spleen, lymph node and tonsil from 17 domestic pigs and 3 wild boar samples were collected from year 2021-2023. 12 domestic pig samples were retrieved from pig farms around Tanjung Sepat, Selangor, and another 5 were retrieved from archived samples from Histopathology Lab FPV. The wild boar samples from around Selangor area were trapped and euthanized by Perhilitan. The FFPE blocks were then processed into a total of 32 slides for immunohistochemistry (IHC).

	Spleen	Tonsil	Lymph Node
Wild Boar 1	Nil	+	+
Wild Boar 2	+	+	+
Wild Boar 3	+	+	+
P1	+	+	+
P2	+	Nil	+
P3	+	+	+
P4	+	+	+
P5	+	+	+
P6	+	+	+
P7	+	+	+
P8	+	+	+
P9	+	+	+
P10	+	+	+
P11	+	+	+
P12	+	+	+
P13	+	Nil	+
P14	+	Nil	+
P15	+	+	+
P16	+	Nil	Nil
P17	Nil	Nil	+

Table 1: List of samples collected

\+ represents sample available; Nil represents sample unavailable

3.2 Preparation of Citrate Buffer Solution (0.1M, pH 6.0)

3.2.1 Materials

-Distilled water

-Sodium Citrate dihydrate

-Citric Acid

-0.1N HCL

3.2.2 Procedure

1. 800mL of distilled water is added into a 1L glass bottle.

2. 24.269g of Sodium Citrate dihydrate powder is weighed and added to the solution.
3. 3.358g of Citric Acid powder is weighed and added to the solution.
4. The solution is mixed well.
5. The solution is tested with a pH meter. 0.1N HCL is used to adjust the solution to pH 6.0.
6. Distilled water is added until the volume is 1L.

3.3 Immunohistochemistry (IHC)

The primary antibody used for ASF in this study is Alpha Diagnostic International Rabbit Anti-African Swine Fever Virus phosphoprotein p30 antiserum, which is specific for phosphoprotein p30 of ASF.

The primary antibody used for CSF in this study is WH303 monoclonal antibody (APHA Scientific), which is specific for CSFV E2 (gp53) glycoprotein.

The secondary antibody used for both CSF and ASF in this study is Nichirei Bioscience Inc. Histofine® Simple StainTM MAX PO (MULTI) mouse and rabbit primary antibody, which will bind on the primary antibody.

Dako DAB (3,3' Diaminobenzidine) is used as a chromogen. In the presence of HRP / peroxidase, DAB produces a brown precipitate that is insoluble in alcohol.

The antigen-positive cells will show cytoplasmic staining.

3.3.1 Materials

- Sigma Poly-L-Lysine solution, 0.1% (w/v) in H2O
- Citrate Buffer (pH6)
- 3% Hydrogen peroxide
- 1% Bovine Serum Albumin (BSA)
- Alpha Diagnostic International Rabbit Anti-African Swine Fever Virus phosphoprotein p30 antiserum
- Nichirei Bioscience Inc. Histofine® Simple StainTM MAX PO (MULTI) mouse and rabbit primary antibody
-WH303 monoclonal antibody (APHA Scientific)
- Dako DAB substrate buffer
- Dako DAB chromogen
- Hematoxylin
- Phosphate Buffered Solution (PBS)

3.3.2 Procedure

1. Formalin-fixed, paraffin-embedded tissue blocks are cut into thin sections.
2. The thin sections of samples are fished by using slides coated with Poly-L-Lysine.
3. The slides are left to dry overnight.
4. The slides are submerged into Xylene 1 and Xylene 2 for 5 minutes each.
5. The slides are then submerged into graded series of 100%, 95%, 80% and 70% alcohol for 5 minutes each.
6. The slides are washed with running tap water for 5 minutes.
7. Antigen retrieval is done by putting the slides immersed in citrate buffer pH6 into a microwave with a setting of 50W for 15 minutes.

8. The slides are allowed to cool down to room temperature.
9. The slides are then incubated with 3% hydrogen peroxide for 30 minutes at room temperature.
10. The slides are washed with PBS for 3 minutes for 3 times.
11. The slides are then incubated with 1% BSA for 30 minutes at room temperature.
12. The slides are washed with PBS for 3 minutes for 3 times.
13. Primary antibody (1:1000 dilution for ASFV; 1:100 dilution for CSFV) is then used to incubate the slides at 37°C for 1 hour.
14. The slides are washed with PBS for 3 minutes for 3 times.
15. Secondary antibody is then used to incubate the slides at 37°C for 30 minutes.
16. The slides are washed with PBS for 3 minutes for 3 times.
17. DAB chromogen is used to incubate the slides for 50 seconds.
18. The slides are then counterstained by using hematoxylin for 1 minute.
19. The slides are then washed under running tap water for 1 minute.
20. The slides are mounted.
21. The slides are observed under a light microscope.

4.0 Results

4.1 Histopathological Lesions

4.1.1 Lymphoid Depletion

Lymphoid depletion of lymphoid organs is described as a decrease in the number and size of follicles with few to no germinal centers or depletion of paracortical lymphocytes (Elmore, 2007). The lesion was observed in 68.42% (13/19) of the lymph node sample, 66.67% (12/18) of the spleen sample, and 33.33% (5/15) tonsil sample.

In short, 80% (16/20) of pigs have lymphoid depletion in at least one of the lymphoid organs.

Table 2 shows the summary of the presence of lymphoid depletion in each pig sample.

	Spleen	Tonsil	Lymph Node
Wild Boar 1	Nil	+	+
Wild Boar 2	+	-	-
Wild Boar 3	-	-	-
P1	+	+	+
P2	+	Nil	+
P3	+	+	+
P4	+	+	+
P5	+	-	+
P6	+	-	-
P7	+	-	+
P8	-	+	+
P9	-	-	+
P10	-	-	-
P11	-	-	-
P12	-	-	-
P13	+	Nil	+
P14	+	Nil	+
P15	+	-	+
P16	+	Nil	Nil
P17	Nil	Nil	+
Total	12/18= 66.67%	5/15= 33.33%	13/19= 68.42%

Table 2 : Result of presence of lymphoid depletion

+: Lymphoid depletion; -: Absence of lymphoid depletion; Nil: Sample unavailable

4.1.1.1 Spleen

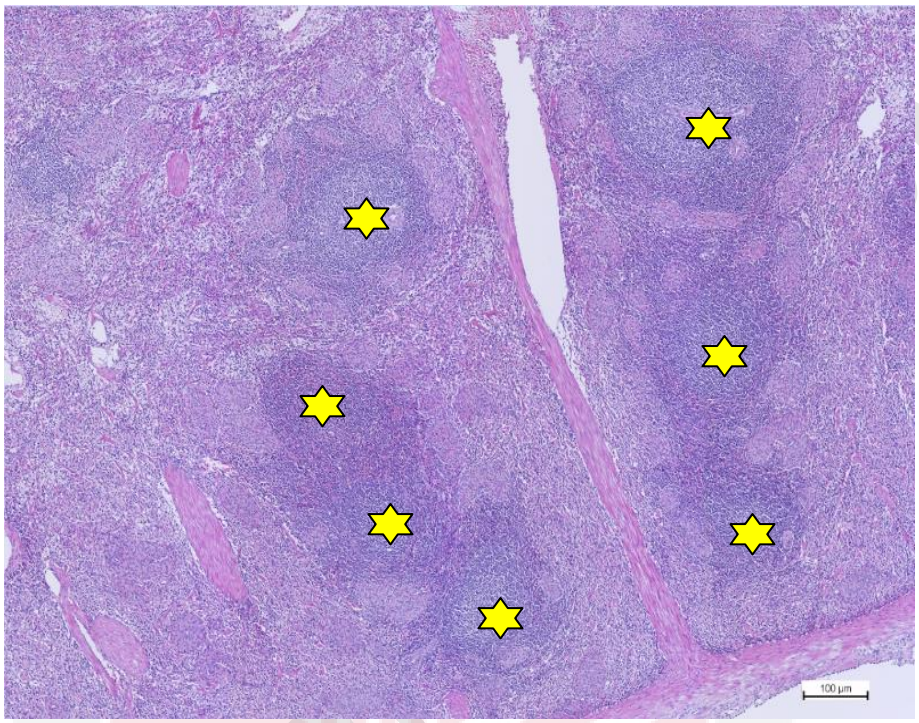

Figure 4.1.1.1.1 : Normal Spleen (40x) with numerous lymphoid follicle (asterisks)

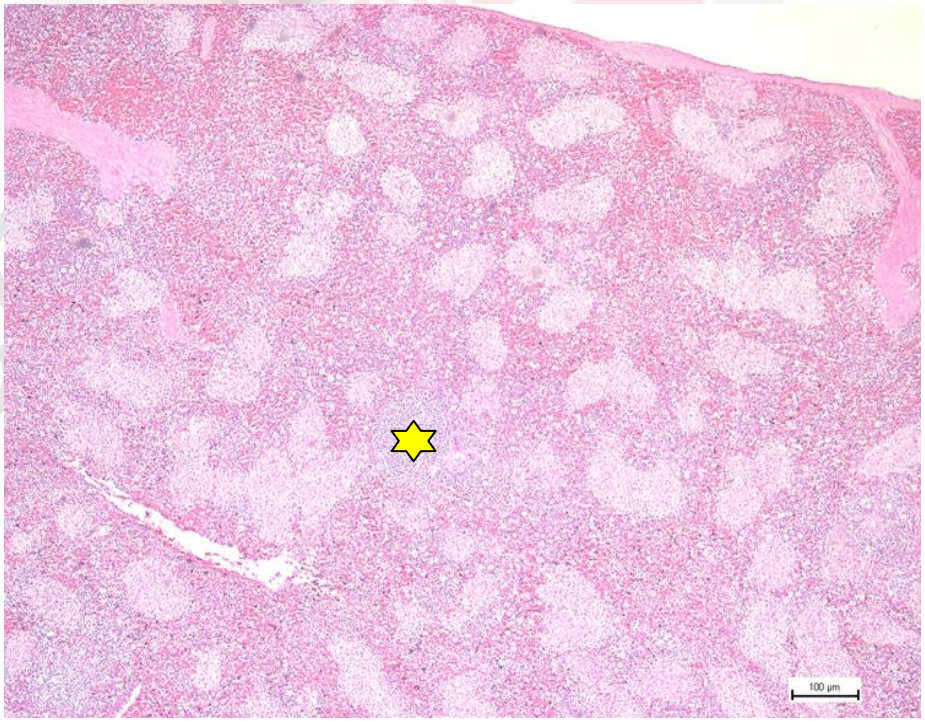

Figure 4.1.1.1.2 : Spleen of P5 (40x) with only single lymphoid follicle observed (asterisk)

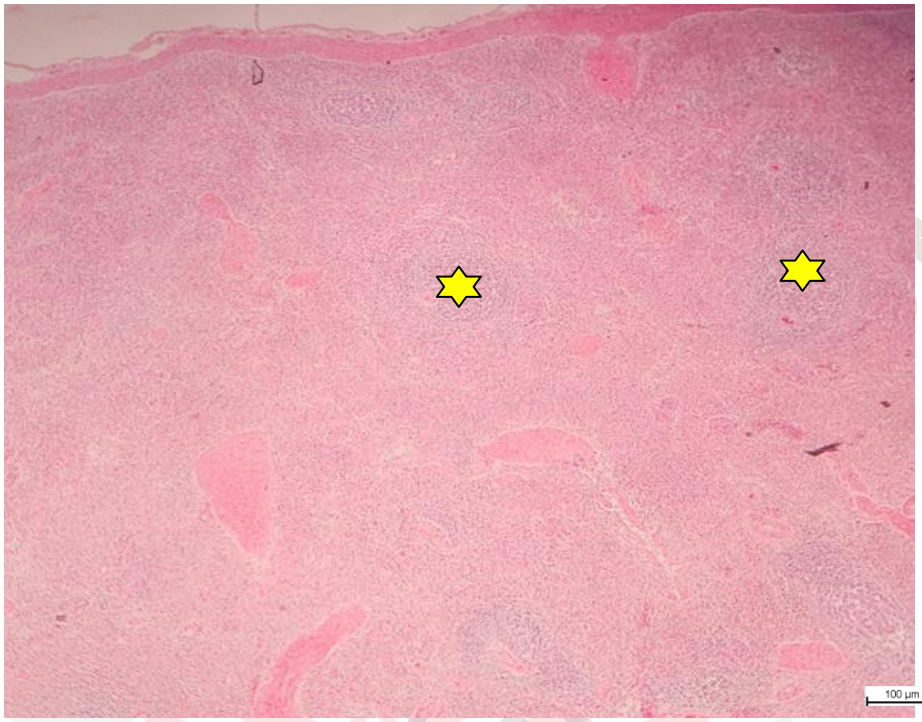

Figure 4.1.1.1.3 : Spleen of Wild Boar 2 (40x) with only few and small lymphoid follicle observed (asterisks)

4.1.1.2 Tonsil

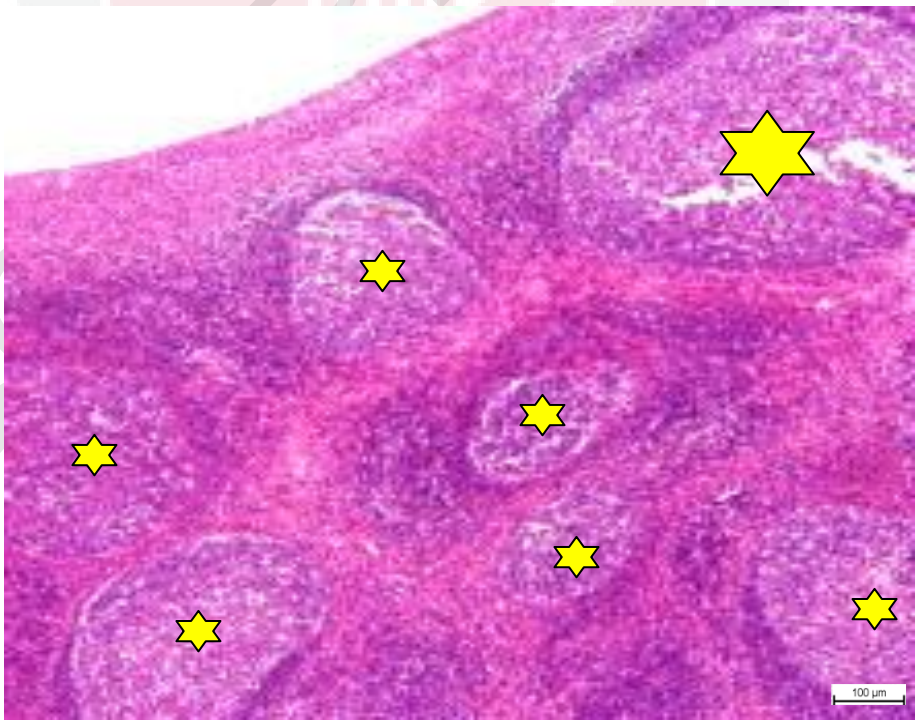

Figure 4.1.1.2.1 : Normal tonsil (40x) with numerous lymphoid follicle (asterisks)

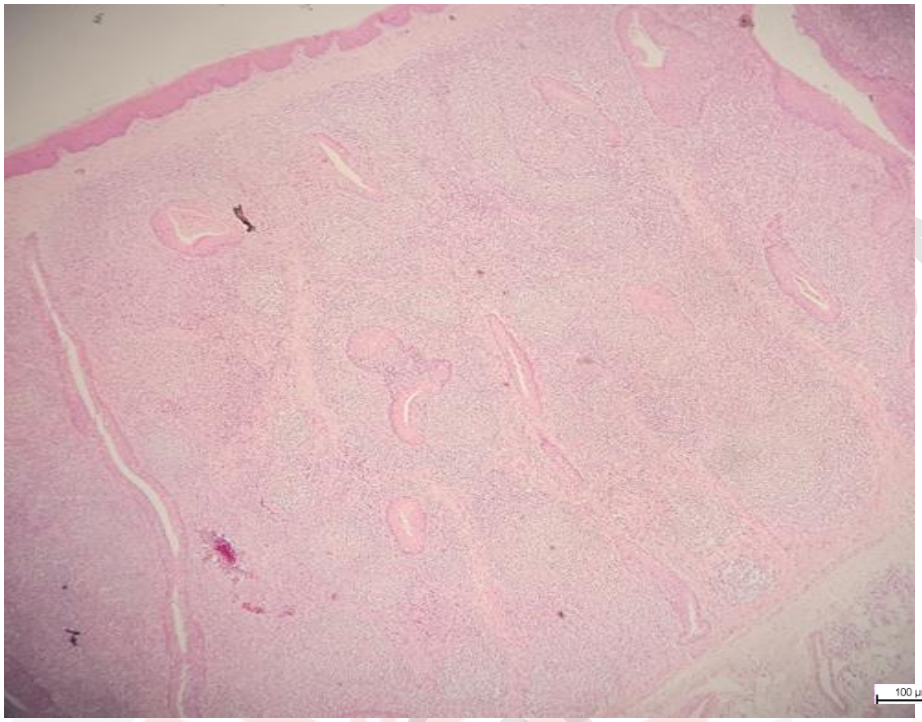

Figure 4.1.1.2.2 : Tonsil of P4 with loss of observable lymphoid follicle

4.1.1.3 Lymph Node

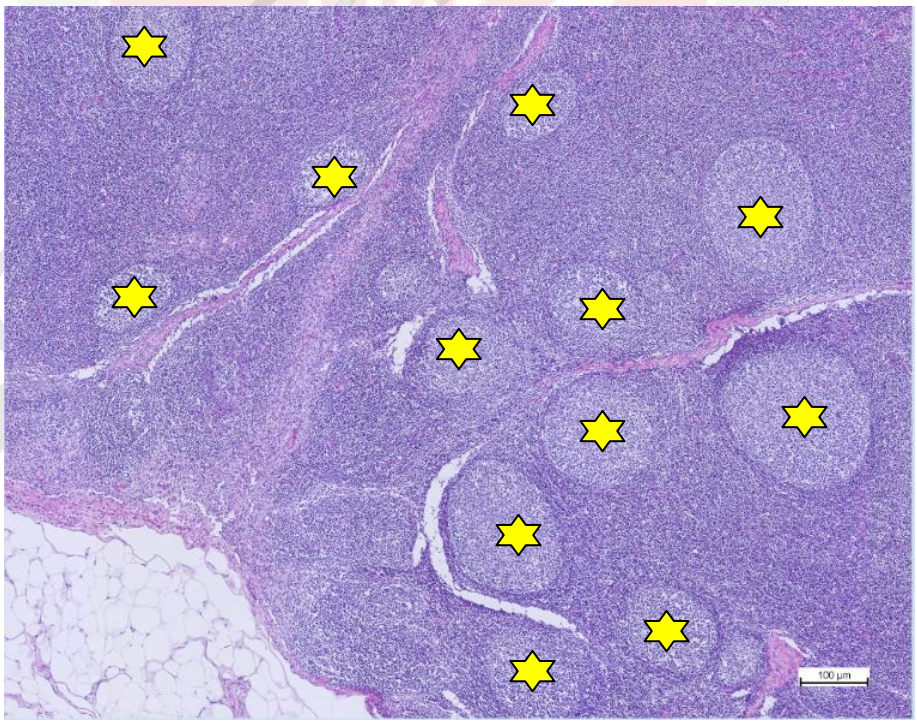

Figure 4.1.1.3.1: Lymph node of normal pig (40x) showing numerous lymphoid follicles (asterisks)

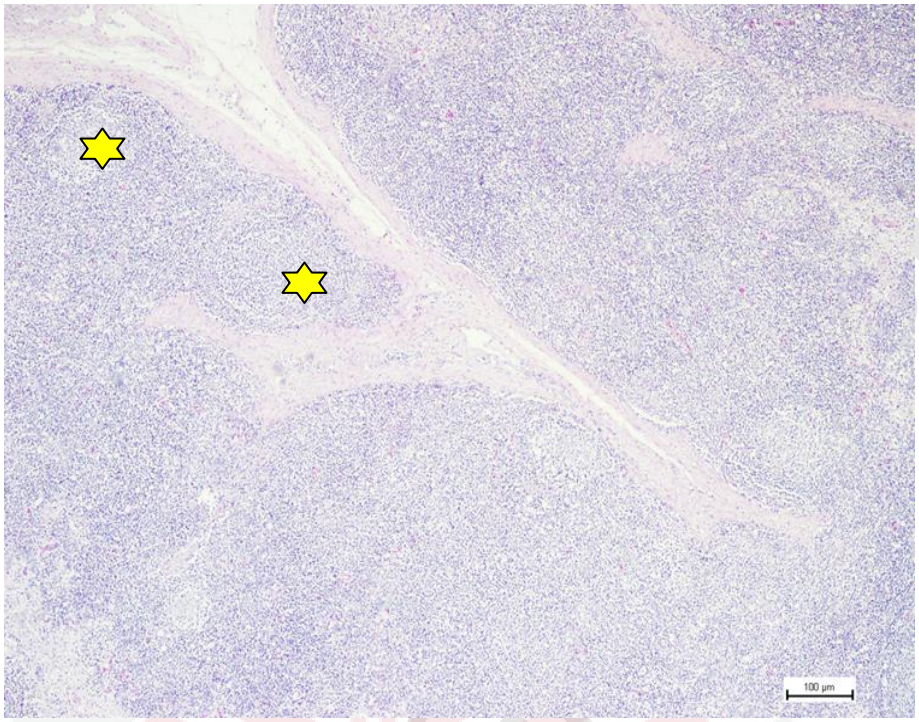

Figure 4.1.1.3.2 : Lymph node of Wild Boar 1 (40x) with only 2 small lymphoid follicles observed (asterisks)

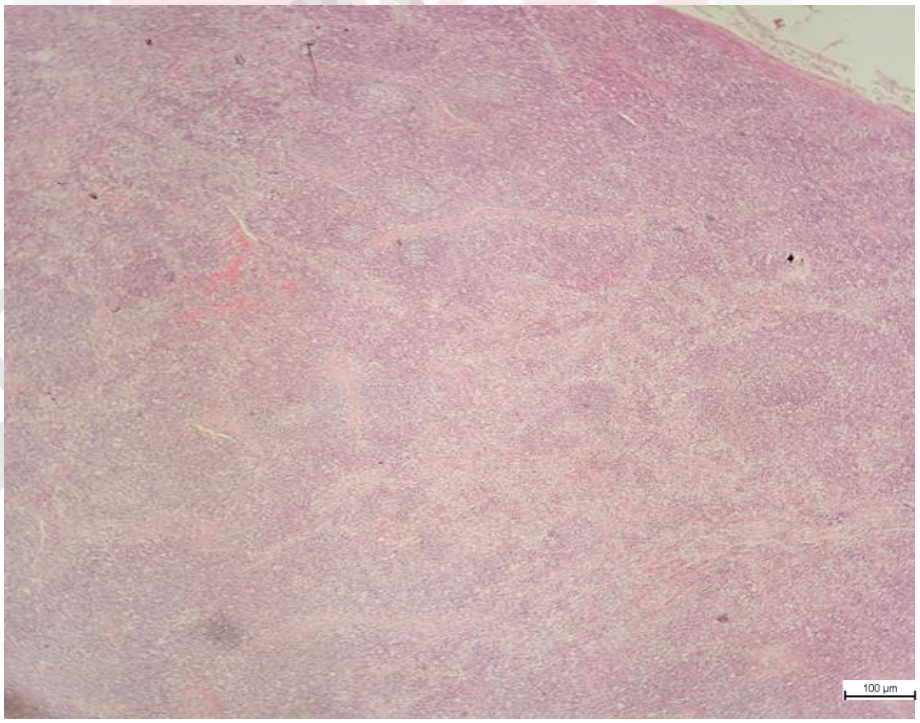

Figure 4.1.1.3.3 : Lymph node of P1 (40x) with absence of observable lymphoid follicles

4.1.2 Hemorrhages

Hemorrhages were less observed in the pig samples compared to lymphoid depletion. 3/18 (16.67%) of spleen, only 1/15 (6.67%) of tonsil and 3/19 (15.79%) of lymph node showing lesion of hemorrhage. In short, 6/20 (30%) of pigs have hemorrhage in at least one of the lymphoid organs.

Table 3 showed the summary of presence of hemorrhages in each pig sample.

	Spleen	Tonsil	Lymph Node
Wild Boar 1	Nil	-	-
Wild Boar 2	-	+	-
Wild Boar 3	-	-	-
P1	-	-	-
P2	-	Nil	-
P3	-	-	-
P4	-	-	-
P5	-	-	+
P6	-	-	-
P7	-	-	-
P8	-	-	+
P9	-	-	-
P10	-	-	-
P11	-	-	-
P12	-	-	-
P13	+	Nil	-
P14	+	Nil	+
P15	-	-	-
P16	+	Nil	Nil
P17	Nil	Nil	-
Total	3/18= 16.67%	1/15= 6.67%	3/19= 15.79%

Table 3 : Result of presence of hemorrhages

+: Presence of hemorrhages ; -: Absence of hemorrhages ; Nil: Sample unavailable

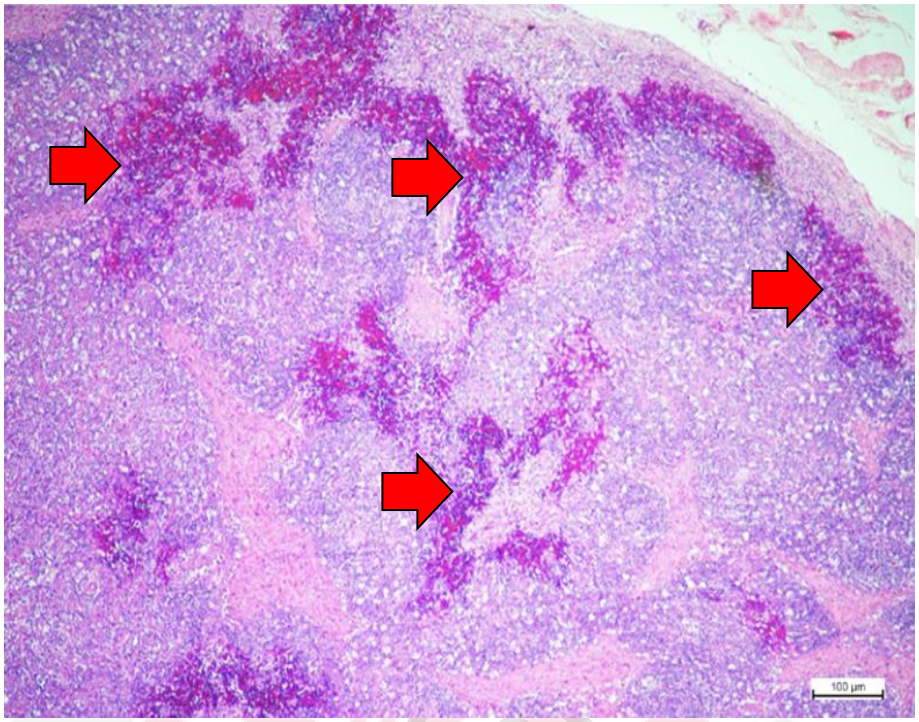

Figure 4.1.2.1 : Lymph node of P14 (40x) with presence of hemorrhages at sinus region (arrows)

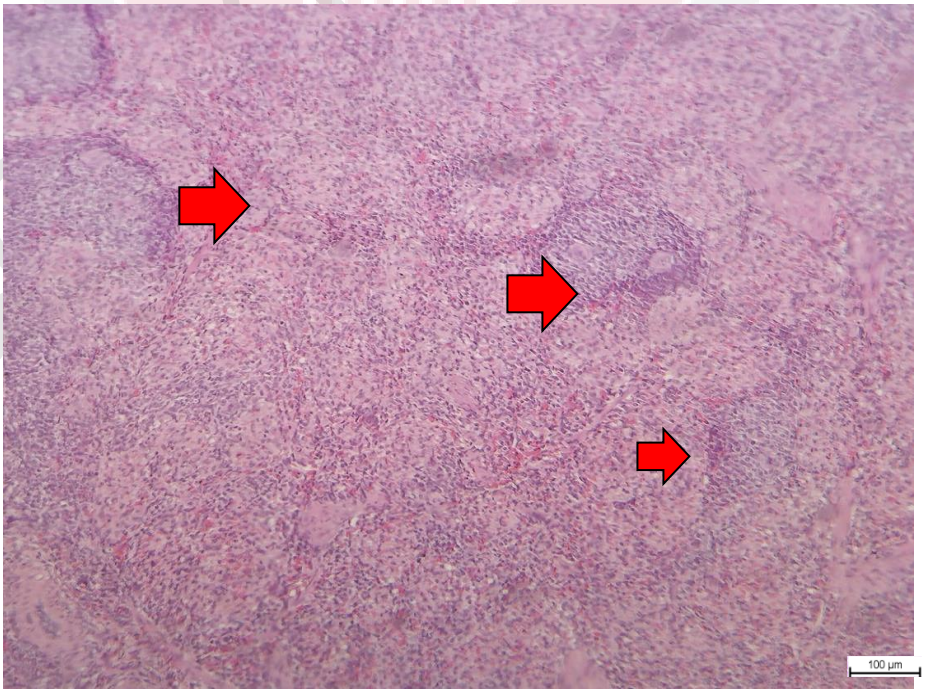

Figure 4.1.2.2 : Spleen of P14 (100x) with presence of hemorrhages (arrows)

4.1.3 Splenic Infarction

Splenic infarction is one of the characteristic lesions for CSF (Murcia et al., 2009). Only 1/20 (5%) of pig sample shows splenic infarction.

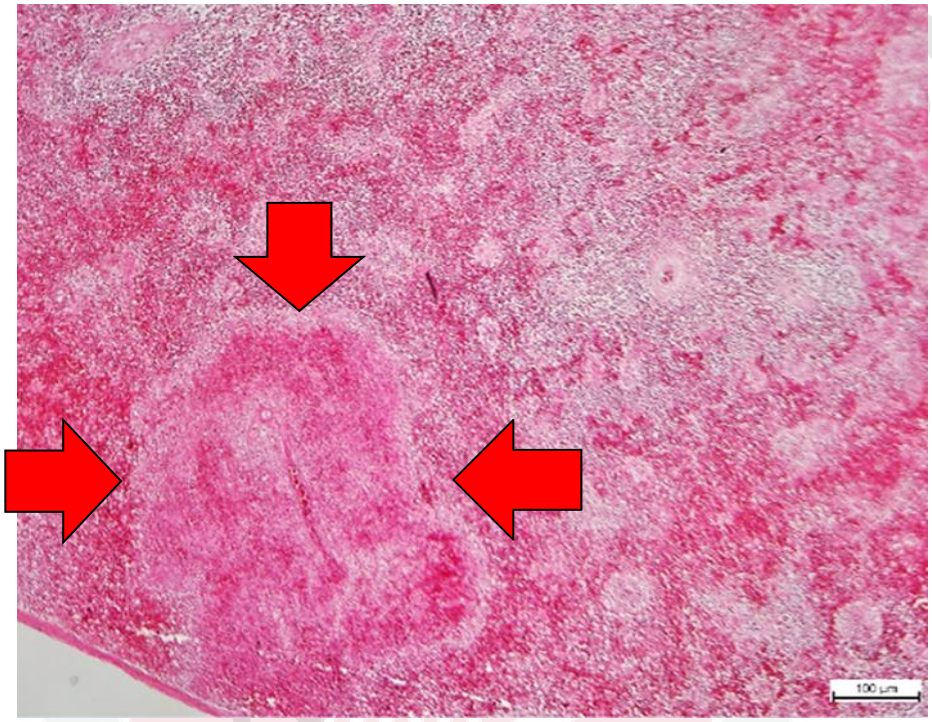

Figure 4.1.3.1 : Spleen of P16 (40x) with the presence of splenic infarction (arrows)

4.2 Immunohistochemistry

Overall, only 1/20 (5%) of pig samples were positive for ASF infection. Antigen-positive cells were observed in one of the wild boar samples. For CSF, 4/20 (20%) of pig samples were positive for CSF infection, where one of them is wild boar and the rest are domestic pigs.

Table 4 summarised the result of IHC.

	IHC	
	ASF	**CSF**
Wild Boar 1	-	+
Wild Boar 2	-	-
Wild Boar 3	+	-
P1	-	-
P2	-	-
P3	-	-
P4	-	-
P5	-	-
P6	-	-
P7	-	-
P8	-	-
P9	-	-
P10	-	-
P11	-	-
P12	-	-
P13	-	+
P14	-	+
P15	-	-
P16	-	-
P17	-	-
Total	**1/20= 5%**	**4/20= 20%**

Table 4 : Result of IHC

+: Positive; -: Negative

4.2.1 ASF

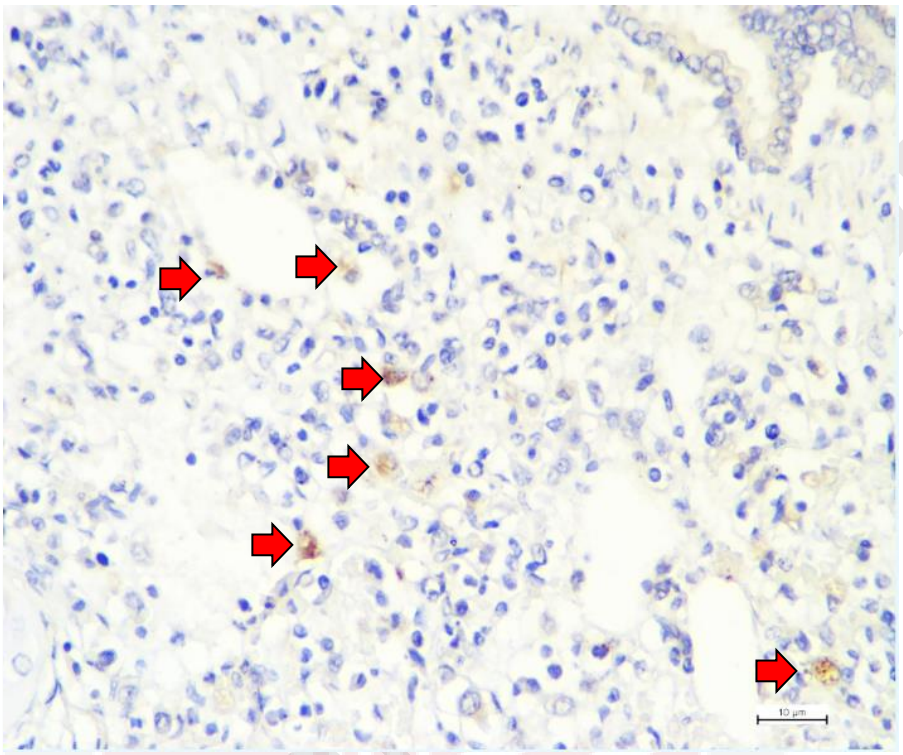

Figure 4.2.1.1 Positive control (lung) (400x) with numerous antigen-positive cells (arrows)

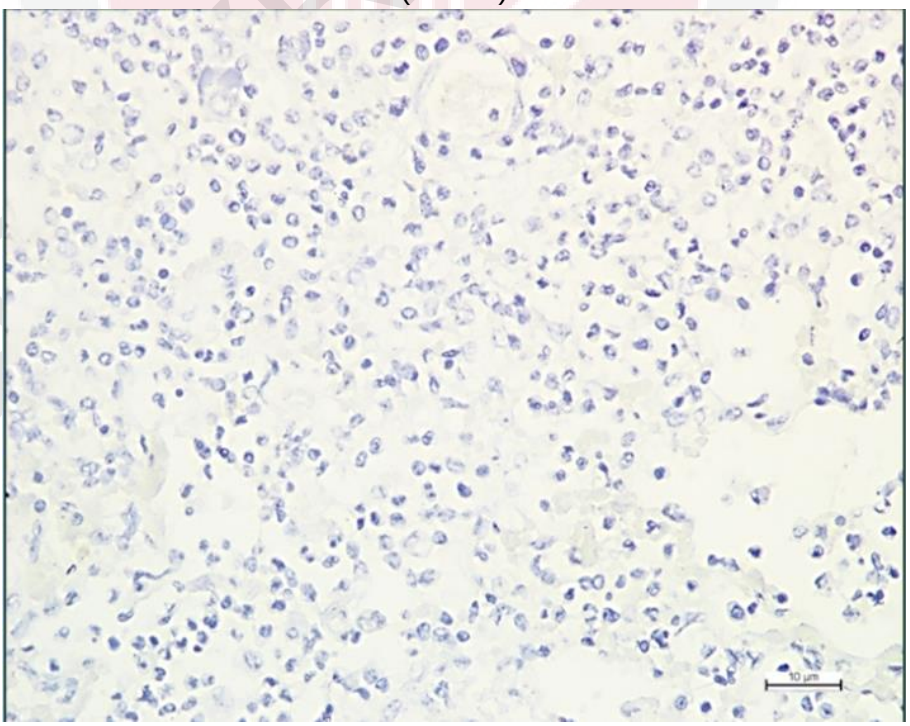

Figure 4.2.1.2 : Negative control (lung) (400x) with absence of antigen-positive cells

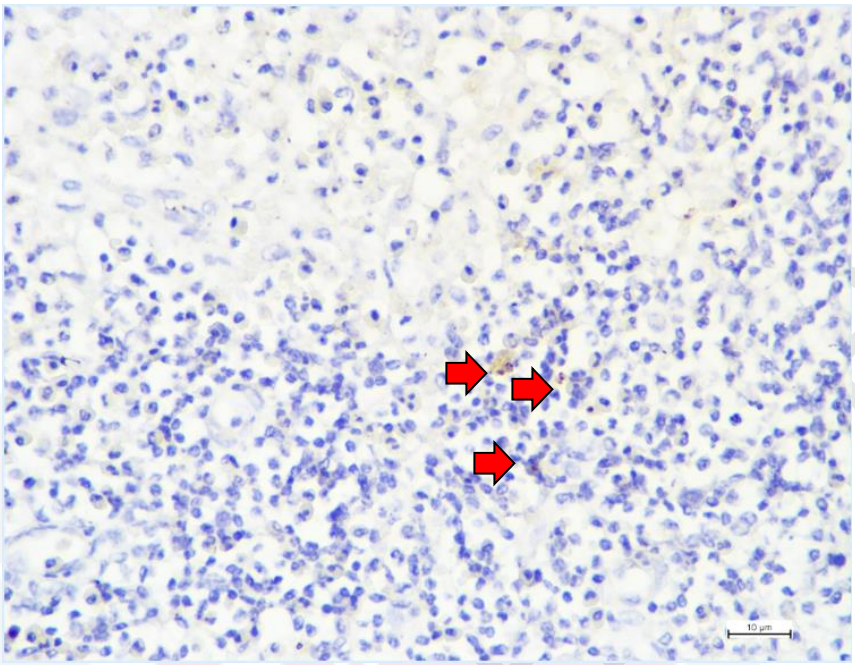

Figure 4.2.1.3 : Lymph node of Wild Boar 3 (400x) with few antigen-positive cells

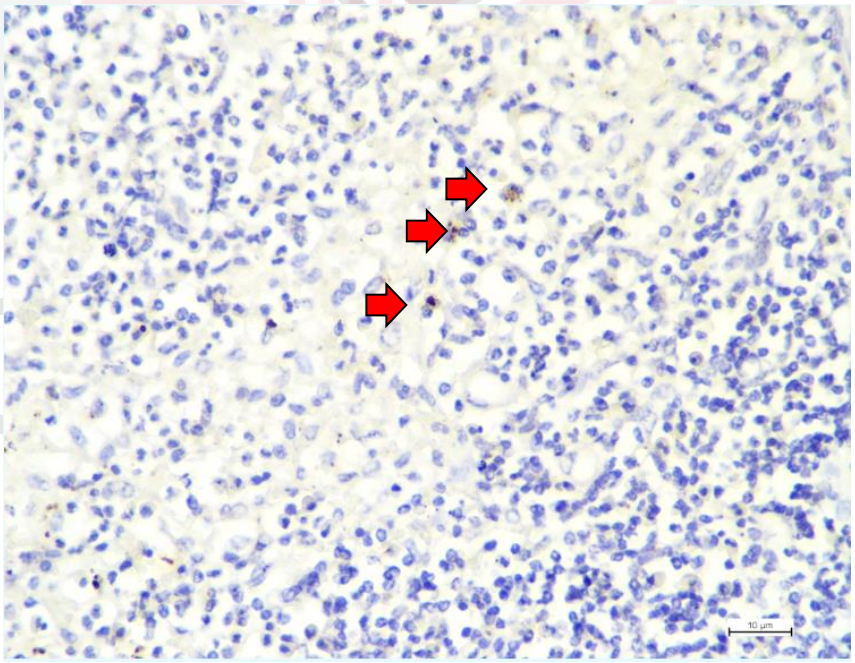

Figure 4.2.1.4 : Lymph node of Wild Boar 3 (400x) with few antigen-positive cells

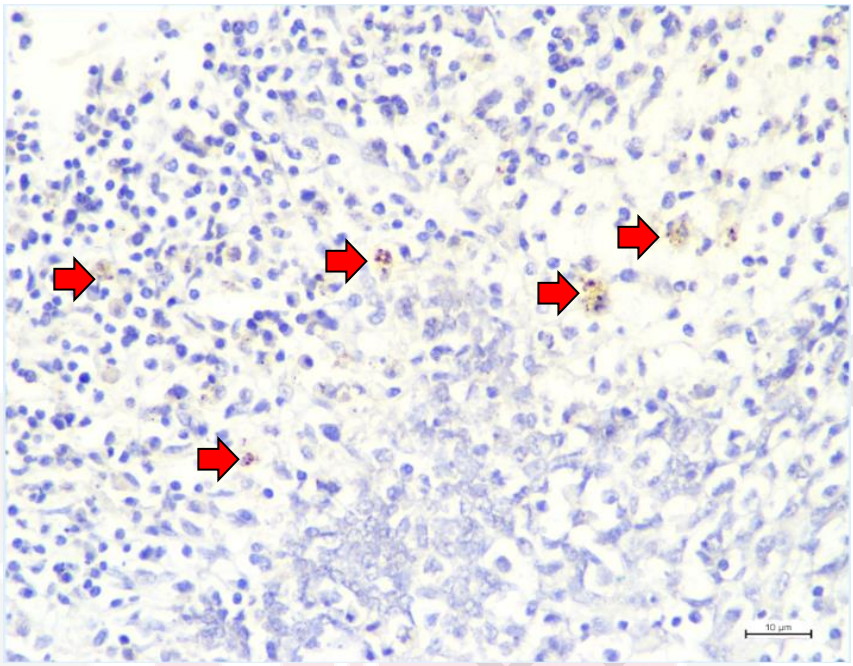

Figure 4.2.1.5 :Tonsil of Wild Boar 3 (400x) with few antigen-positive cells

4.2.2 CSF

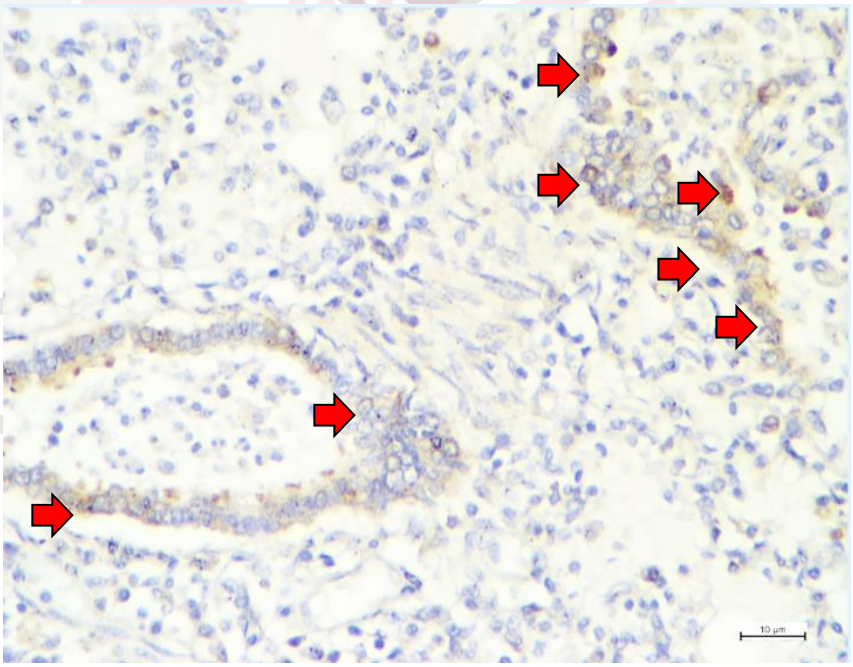

Figure 4.2.2.1 : Positive Control (lung) (400x) with numerous antigen-positive cells on epithelial cells (arrows)

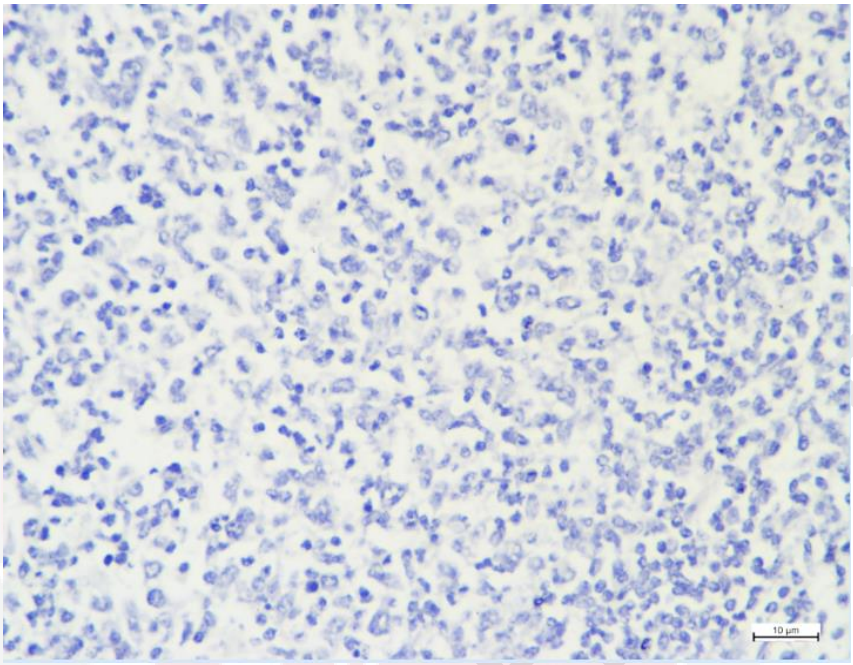

Figure 4.2.2.2 : Negative Control (lung) (400x)

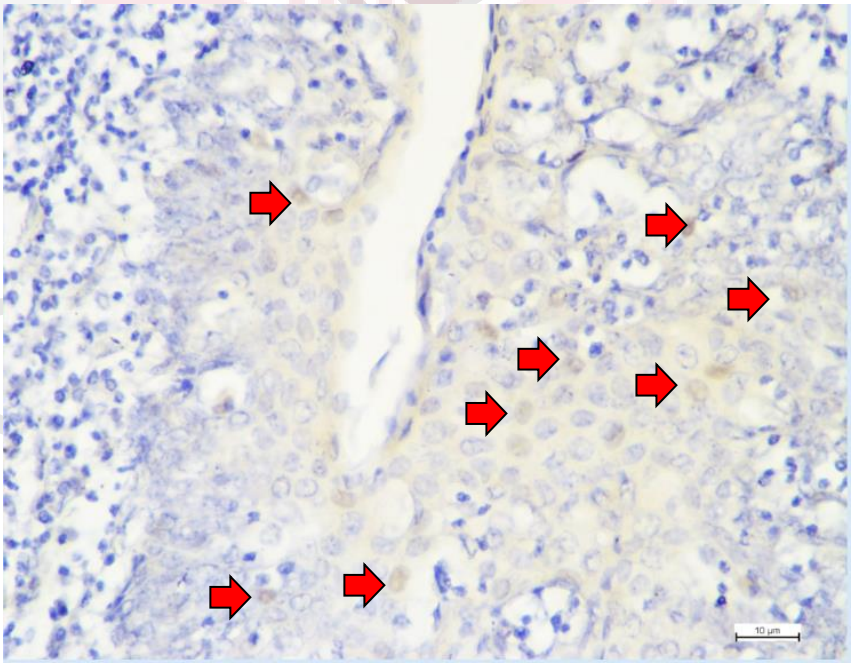

Figure 4.2.2.3 :Tonsil of Wild Boar 1 (400x) with numerous antigen-positive cells at tonsillar crypts

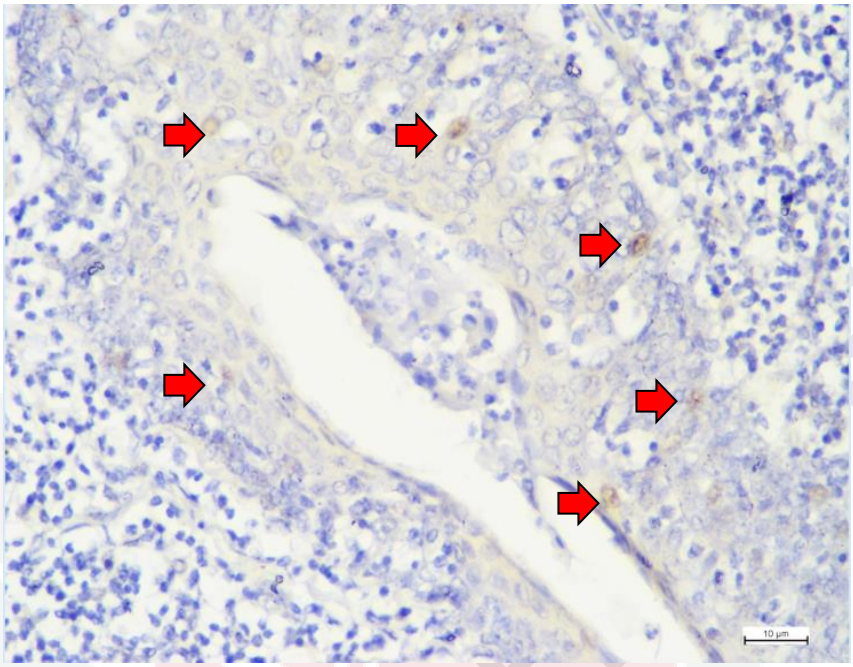

Figure 4.2.2.4 :Tonsil of Wild Boar 1 (400x) with numerous antigen-positive cells at tonsillar crypts

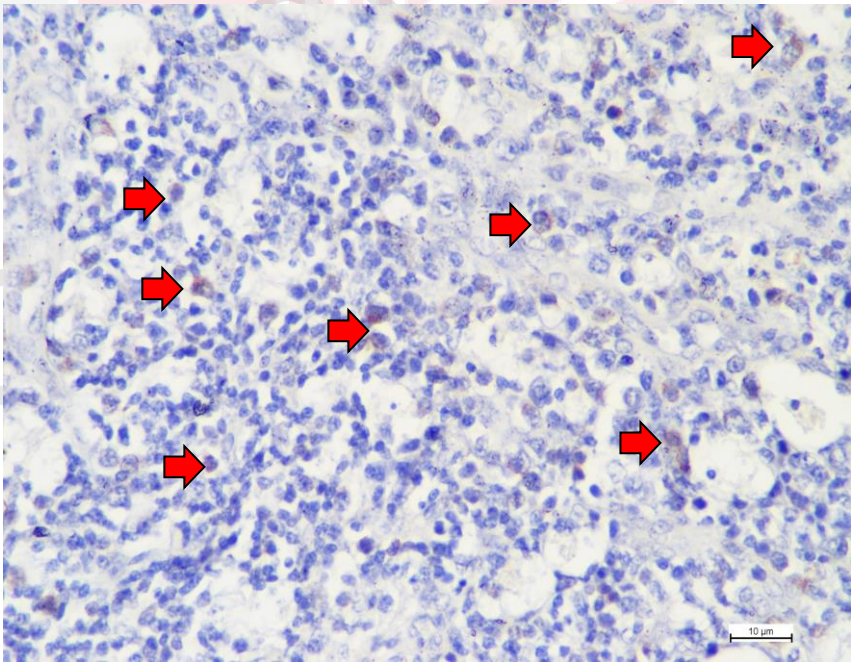

Figure 4.2.2.5: Lymph node of P14(400x) with numerous antigen-positive cells

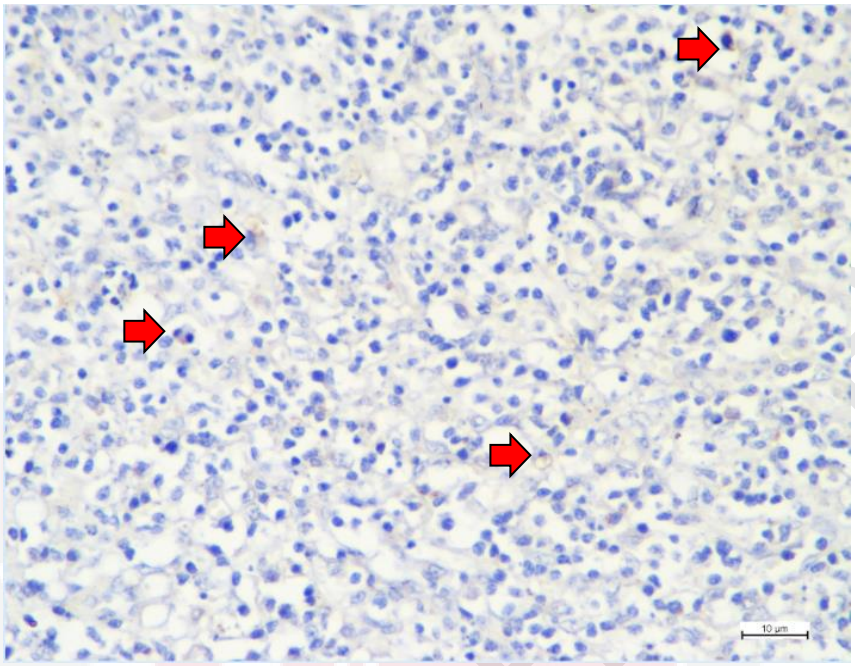

Figure 4.2.2.6: Spleen of P13(400x) with numerous antigen-positive cells

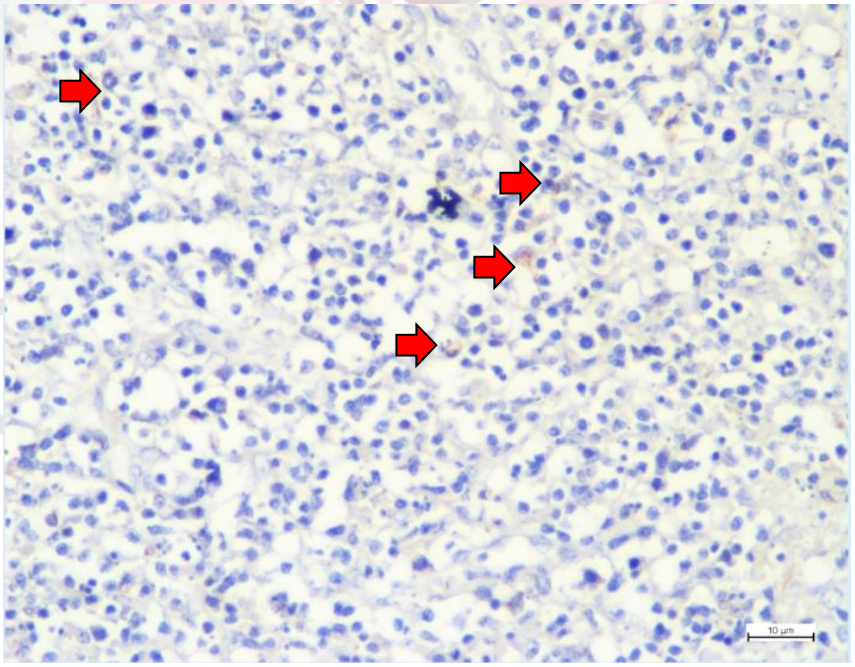

Figure 4.2.2.7: Spleen of P13(400x) with numerous antigen-positive cells

5.0 Discussion

5.1 Field Challenge

The results from this study indicates the possibility of an ongoing field challenge for the farmers in Malaysia. Malaysia is endemic for CSF, and despite the widely implemented vaccination program for CSF using attenuated live vaccine GPE (-) strain in Malaysia, the results still showed that four domestic pigs were positive for CSF. However, by investigating the history of the pigs, it is indicated that the four cases were just sporadic cases in respective farms without any actual outbreak reported. This has shown that the vaccination program played its role in achieving good herd immunity. These individual cases could be caused by failure of immune response towards CSF vaccination, which leaves the pigs vulnerable to CSF infection. In fact, one of the CSF-positive pigs, P14, had a history of CSF vaccination 2 weeks before succumbing to CSF. Several studies have shown the capacity of live attenuated CSF vaccine to provide early protection even at 1-day post-vaccination and the administration of a single dose (Graham et. al, 2012). Other study also shown that by using a live attenuated vaccine, both humoral and cellular immune responses will be elicited, and CSF virus-specific gamma interferon will be produced as early as 6 days post-vaccination (Suradhat, 2001). The cause of P14 being naive to CSF 2 weeks after vaccination remained unknown and further investigation must be done.

Possible causes of failure of vaccination include stress and the presence of maternally derived antibodies (MDA). It has been documented that pigs exposed to stressors will produce high levels of cortisol, and cortisol sometimes can inhibit lymphocytosis (de Groot et. al, 2001), thus causing inadequate immune response towards vaccination. MDA may interfere with the active humoral response

development of piglets, thus the timing of vaccination in piglets is crucial. Research has found that MDA has an effect towards CSFV vaccination with duration of more than 7 weeks, and declines steadily until the age of 10 weeks (Vandeputte et. al,). It is recommended to start CSF vaccination of piglets at the age of 7 weeks old.

5.2 Infection in wild boars

The result also showed that one wild boar was positive for CSF, and another one was positive for ASF. It is alarming as wild boar could act as a reservoir for both diseases, and it is an important factor in causing CSF and ASF outbreaks (WOAH, 2019). Therefore, the prevention of contact between domestic pigs and wild boars is significant. Various measures can be taken, such as strategizing farm locations, wild boar trapping and hunting, wild boar-proof fencing and more (Moennig, 2015). However, once the disease has spread to wild boars, the disease is hard to control. In South Korea, the government has put in great effort into controlling the disease through fencing, but to no avail (Jo, 2021). Similar outcome is seen in Lithuania (Mačiulskis, 2020).

Another measure that can be taken is implementing oral CSF vaccination of wild boar. In the early 2000s, the oral mass vaccination (OMV) of CSF in wild boar was supported by the European Communities, and many countries adopted the approach as part of the emergency plan. The countries include Germany, Luxembourg, France, Slovakia, Bulgaria, and Latvia (EFSA, 2008). In 2019, Japan also implemented the same approach after the re-emergence of CSF in 2018. The result was promising, as an increase in seroprevalence among wild boar and a decrease of CSF positive cases were observed after the implementation of OMV (Bazarragchaa et al., 2021).

5.3 Lymphoid depletion

Based on the results of this study, most of the samples (13/20, 65%) showed lymphoid depletion on lymphoid organs but were IHC negative for either ASF or CSF. Lymphoid depletion lesion is quite characteristic but not pathognomonic for ASF or CSF infection. Porcine Circovirus Type 2 (PCV2) infection in pigs will produce similar lesions at similar organs compared to ASF and CSF infection (Gillespie et al., 2009). It is not uncommon for domestic pigs to be infected with PCV2, as the virus is considered ubiquitous in the swine population (Tan et. al, 2022). Therefore, it is possible that the pigs might be infected with PCV2, and further molecular diagnosis like PCR can be done to confirm it. Diagnosis solely based on histopathology lesions in the case of ASF or CSF infection would be inappropriate.

Another explanation could be due to the age of the pig samples. The total number of lymphoid cells in a body depends on the size and the age of the animal (Pabst, 2020). Out of 13 samples that showed lymphoid depletion but were IHC negative, 11 were samples taken from juvenile pigs (except Wild Boar 2 and P1). The lymphoid follicle in juvenile pigs might not be fully mature yet, thus giving a false impression of lymphoid depletion when viewed under the microscope.

6.0 Conclusion

ASFV and CSFV antigens are detected through IHC in spleen, tonsil and lymph node. As for histopathology, there are significant histopathological lesions caused by CSFV, which are lymphoid depletion and hemorrhage. Thus, IHC and histopathology can be alternative methods to diagnose ASF and CSF in swine, as well as an additional confirmatory test in a farm that is confirmed with the ASF and CSF outbreak.

7.0 Recommendation

The sample size in future studies should be increased to at least 30 samples to have any statistical significance. Furthermore, PCR can be carried out as an additional test to validate the results of IHC to minimize false positives or false negatives. Sequencing can also be done to determine the current strain circulating in Malaysia. For better viral distribution study, other organs like lungs, kidney and brain can be retrieved in future studies.

8.0 References

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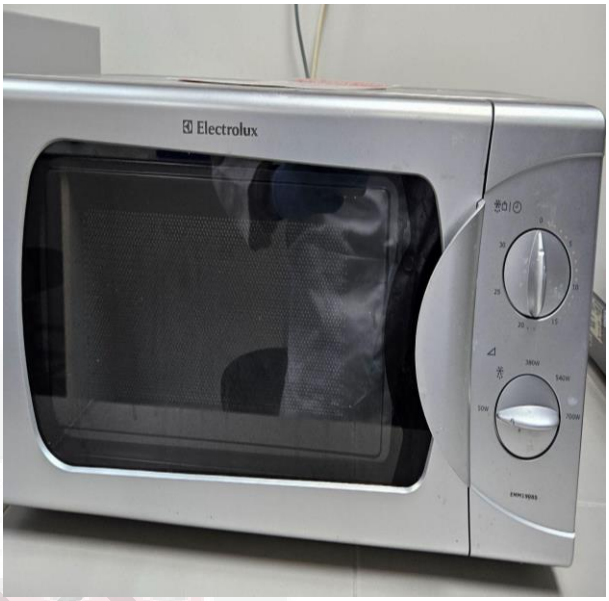
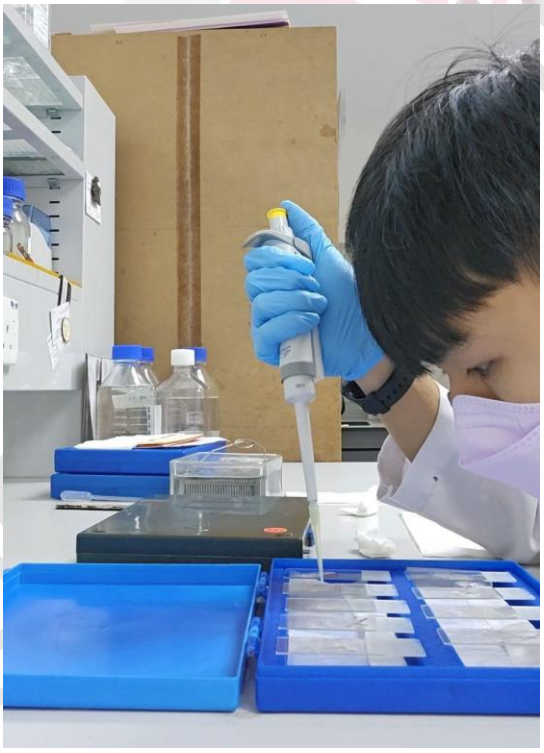
9.0 Appendices

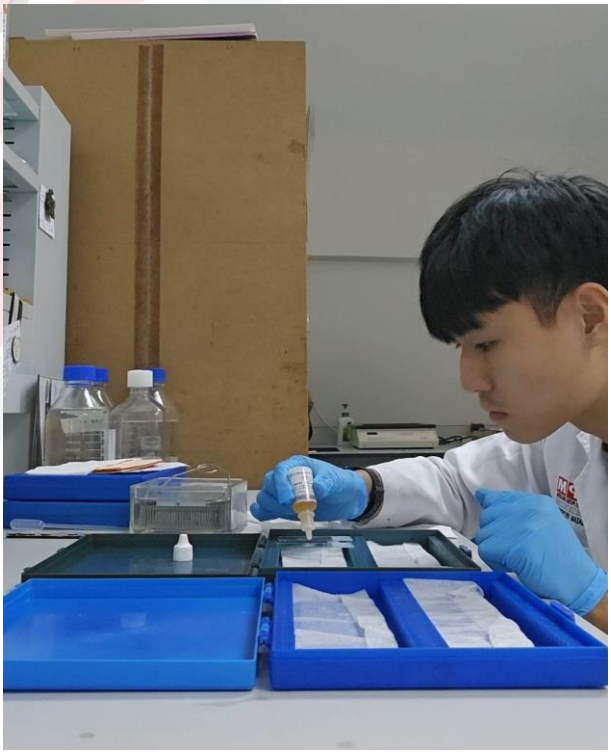
IHC steps



Microwave for antigen retrieval



Incubating slides with primary antibody



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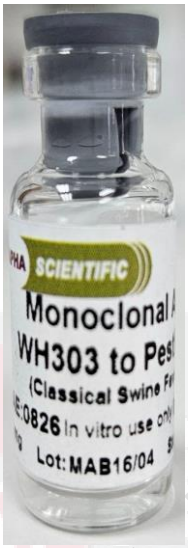
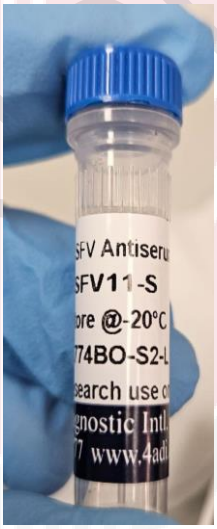


WH303 monoclonal antibody



Alpha Diagnostic International Rabbit Anti-African Swine Fever Virus phosphoprotein p30 antiserum

Nichirei Bioscience Inc. Histofine® Simple StainTM MAX PO (MULTI) mouse and rabbit primary antibody

Dako DAB substrate buffer &
Dako DAB chromogen