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FULL PAPER

Pathology

Experimental infection of pigs with different doses of the African swine fever virus Armenia 07 strain by intramuscular injection and direct contact

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ABSTRACT. We experimentally infected pigs with the African swine fever virus (ASFV) Armenia 07 strain (genotype II) to analyze the effect of different dose injections on clinical manifestations, virus-shedding patterns, histopathology, and transmission dynamics by direct contact. Each three pigs and four pigs were injected intramuscularly with 0.1 fifty percent hemadsorbing doses (HAD_{50})/ml, 10^1 HAD_{50}/ml and 10^6 HAD_{50}/ml of ASFV Armenia 07 strain, respectively. Each two of three pigs injected with 0.1 HAD_{50}/ml and 10^1 HAD_{50}/ml died by 10 days post inoculation. All pigs had a gross lesion of splenomegaly. Perigastric and renal lymph nodes were enlarged and resembled blood clots in nine of ten pigs. It was revealed that 0.1 HAD_{50}/ml of this ASFV was sufficient to infect healthy pigs by intramuscular injection and caused sub-acute lethal disease. For the transmission study, two 8-week-old pigs were injected intramuscularly with 10^3 HAD_{50}/ml of the same virus. Each of the experimentally inoculated pigs was co-housed with two 8-week-old naive pigs. All contact pigs exhibited clinical manifestations at 6 or 7 days after the experimentally inoculated pigs developed pyrexia. These findings suggest that this strain may spread slowly within a herd. Histologically, lymph nodes resembled blood clots were formed by severe blood absorption and followed hemorrhage result of disruption of the lymphoid sinus filling with absorbed red blood cells. The severity of the gross and histological lesions depended on duration after infection, regardless of the difference of injection doses in this study.

KEY WORDS: African swine fever, African swine fever virus, pig, quantitative PCR, transmission

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African swine fever (ASF), which is caused by the ASF virus (ASFV) of the genus *Asfivirus* within the *Asfarviridae* family, is a highly contagious disease with high mortality in pigs [2, 9, 24]. This disease was considered endemic in sub-Saharan Africa and on the Mediterranean island of Sardinia. However, in 2007, ASF was introduced into the Caucasus region, and it spread into Eastern Europe and the Baltic countries [5]. In 2018, it was found in China [2, 28], followed by Mongolia, Vietnam, Cambodia, Democratic People's Republic of Korea, Lao People's Democratic Republic, Myanmar, Philippines, Timor-Leste, and Indonesia in 2019 [1, 18]. ASF has become a major threat to pig industries in Europe and Asia, and the risk of introduction of the virus into ASF-free countries is increasing.

Pigs are infected with ASFV by contact with infected animals or virus-contaminated fomites, such as pork products and affected carcasses, or by tick bites [15]. ASFV transmission varies substantially across countries [15]. In Africa, ASFV circulates among bush pigs (*Potamochoerus*), warthogs (*Phacochoerus aethiopicus*), and soft ticks of the *Ornithodoros* species [5, 24]. In areas of the

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Caucasus, Eastern Europe, and the Baltic countries, European wild boars (*Sus scrofa scrofa*) and the practice of swill feeding play important roles in transmission [13]. Direct contact between naive and virus-infected pigs is considered the most effective mechanism of ASFV transmission [5, 15]. Experimental studies on the pathogenesis of the current ASFV genotype II strain have been described in several reports [4, 6, 11–13, 15, 16, 20, 21, 26, 27]. However, the pathogenesis of ASF has not been completely explained, and there are a limited number of histological studies on ASF; no detailed study on the histological distribution of histological lesions across the entire body of pigs in the early stage of ASF infection and on the histological description of the hemorrhagic lesion in ASF infection has been conducted. Experimental studies are required to further the understanding of the pathogenesis of the current ASFV genotype II strain [3].

To establish effective control measures against the currently circulating ASFV strain, we assessed the effect of different doses of injection, histopathology, distribution of ASF lesions across the entire body in the early stages of infection, and the transmission dynamics of the strain in domestic pigs by experimental infection with a virulent isolate of ASFV Armenia 07.

MATERIALS AND METHODS

Virus

The ASFV Armenia 07 strain (genotype II) used in this study was obtained from the World Organization for Animal Health (OIE) ASF reference laboratory (Universidad Complutense de Madrid, Spain). This strain showed marked virulence in pigs when injected intramuscularly [4]. The stock virus was propagated by infecting a 6-week-old Landrace–Large White–Duroc (LWD) pig. At 5 days post-injection (dpi), the infected pig was euthanized, and the spleen was collected. A supernatant of the 10% homogenate of the spleen was used as the inoculum in this study. A hemadsorption assay was performed with primary porcine alveolar macrophages (PAMs) for virus titration into the inoculum. Briefly, 1×10^5 cells of PAMs were seeded in each well of 96-well plates. Twenty-five microliters of 10-fold serial dilutions of the inoculum were injected into each well, and 20 µl of swine blood cells were added; dilutions were incubated for 7 days at 37°C in a 5% carbon dioxide/95% air incubator. Virus titers were visually examined for characteristic rosette formation, indicating hemadsorption reactions, and calculated as 50% hemadsorbing doses (HAD_{50}) according to the method of Reed and Muench [22]. Virus titers were 10^7 HAD_{50}/ml in the virus inoculum in this study.

Experiment 1: The effects of different doses.

Ten 8-week-old LWD pigs were used in this study. Three pigs (group 1: pigs 1 to 3) were injected intramuscularly with 1 ml of 0.1 HAD_{50}/ml of the virus inoculum and kept in room 1. Three other pigs (group 2: pigs 4 to 6) were injected intramuscularly with 1 ml of 10^1 HAD_{50}/ml of the virus inoculum and kept in room 2. Finally, four pigs (group 3: pigs 7 to 10) were injected intramuscularly with 1 ml of 10^6 HAD_{50}/ml of the virus inoculum and kept in room 3. All pigs were examined clinically at 10 dpi, and their rectal temperatures were recorded daily.

Experiment 2: Transmission by direct contact.

Six 8-week-old LWD pigs were used in this study. Two pigs (pigs 11 and 12) were kept separately in two animal rooms and injected intramuscularly with 1 ml of 10^3 HAD_{50}/ml of the virus inoculum. Two naive pigs were co-housed with each of the injected pigs from the day of injection (room 1: pigs 11, 13, and 14; room 2: pigs 12, 15, and 16). All contact pigs were examined clinically at 11 days post-contact (dpc), and their rectal temperatures were recorded daily.

After clinical assessment, the pigs were euthanized by injection of sodium pentobarbital and then subjected to necropsy examination. Dead pigs were immediately necropsied. These methods were conducted in animal rooms and laboratories in a high-containment facility of our institute in Kodaira, Tokyo. A humane endpoint was considered to have been reached when pigs markedly reduced play activity and lay down, justifying euthanasia on welfare grounds. These experiments were approved by the Animal Ethics Committee of the National Institute of Animal Health (authorization No. 17-004).

Detection of ASFV DNA by real-time polymerase chain reaction

Clinical samples of sera, heparin-anticoagulated whole blood, saliva, and feces were collected daily from all pigs. Nasal swabs were also collected daily. In experiment 2, clinical samples were collected firstly from contact pigs to prevent transmission by sampling. DNA was purified from whole blood, serum, 10% suspensions of nasal swabs, saliva, and feces using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Basel, Switzerland). Quantitative polymerase chain reaction (qPCR) was performed according to a previously described method, with minor modifications [17]. Briefly, PCR mixture was prepared in a volume of 20 µl containing a 2-µl DNA sample, 2× concentration of TaqMan Fast Advanced Master Mix (Applied Biosystems), 0.3 µM of sense primer (5′-CTG CTC ATG GTA TCA ATC TTA TCG A-3′) and antisense primer (5′-GAT ACC ACA AGA TC (AG) GCC GT-3′), and 0.15 µM of TaqMan probe (5′-FAM-CCA CGG GAG GAA TAC CAA CCC AGT G-TAMRA-3′). Primer pairs and probe are located within the *B646L* gene encoding major capsid protein P72. The qPCR amplification conditions were 50°C for 2 min, 95°C for 20 sec, followed by 45 cycles of 3 sec at 95°C and 30 sec at 58°C with fluorescence reading in the FAM channel at the end of each cycle.

Histopathology

Tissue samples for microscopy examination were collected from the tonsil, liver, spleen, kidney, heart, lung, stomach, small and large intestine, aorta, skin, brain, lymph nodes, thymus, urinary bladder, and skeletal muscle of each pig. Tissues were fixed in 10% neutral phosphate-buffered formalin, which was processed using routine procedures and embedded in paraffin wax. Sections were stained with hematoxylin and eosin (H&E).

RESULTS

Clinical signs, detection of ASFV DNA, and gross lesions in experiment 1

Rectal temperatures of the pigs are shown in Fig. 1A and clinical symptoms in the examined pigs are summarized in Table 1. In group 1 (min dose), two pigs (pigs 1 and 3) died at 10 dpi; the remaining pig (pig 2) had diarrhea at 9 dpi and was euthanized on welfare grounds at 10 dpi. All infected pigs developed high fever (>41°C) from 4 dpi and depression and loss of appetite from 6 dpi. In group 2 (low dose), two pigs (pigs 4 and 6) died at 6 and 8 dpi, respectively, and the remaining pig was euthanized on welfare grounds at 8 dpi. All infected pigs developed high fever (>41°C) from 3 dpi and depression and loss of appetite from 4 dpi. Pig 6 had diarrhea from 7 dpi. In group 3 (high dose), all infected pigs developed high fever (>41°C) from 2 dpi and depression and loss of appetite from 3 dpi. Pigs were euthanized at 4 or 5 dpi. In this experiment, six of ten pigs had severe fever (>42°C). Erythema or cyanosis was observed on the skin of three pigs (pigs 2, 3, and 6).

In group 1, viral DNA was detected in whole blood and serum samples from 4 and 5 dpi, respectively (Fig. 1B and 1C), nasal swabs from 5 dpi (Fig. 1D) and saliva of pig 3 from 7 dpi (Fig. 1E). In group 2, viral DNA was detected in whole blood and serum from 3 and 4 dpi, respectively (Fig. 1B and 1C), nasal swabs from 5 dpi (Fig. 1D), and saliva of pig 4 from 5 dpi (Fig. 1E). In group 3, viral DNA was detected in whole blood and serum samples from 1 and 2 dpi, respectively (Fig. 1B and 1C), nasal swabs from 3

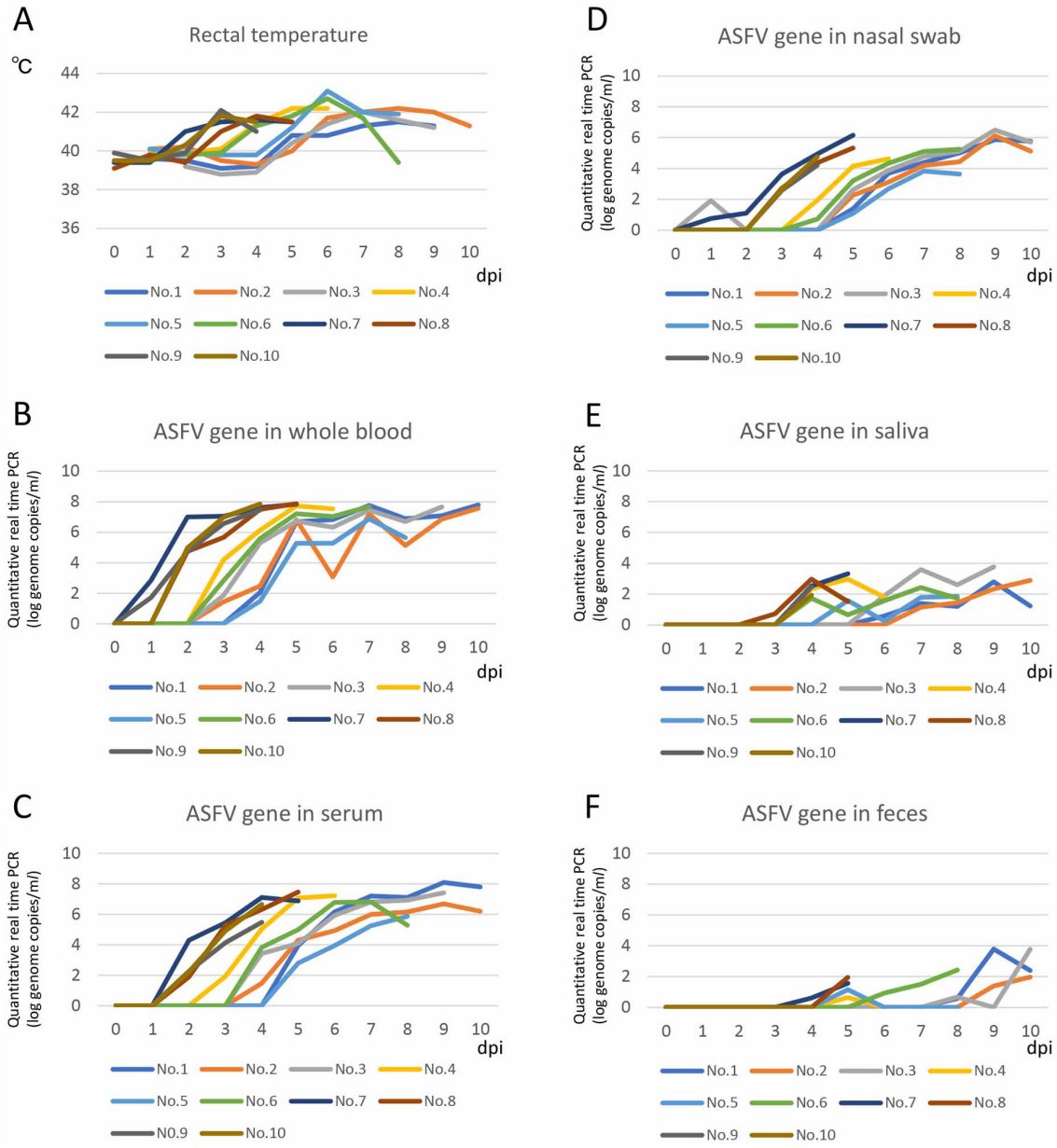

Fig. 1. Results of rectal temperatures and African swine fever virus (ASFV) gene detection in pigs inoculated with different doses of ASFV (experiment 1). (A) Rectal temperatures and ASFV gene copies in (B) whole blood, (C) serum, (D) nasal swab, (E) saliva, and (F) feces.

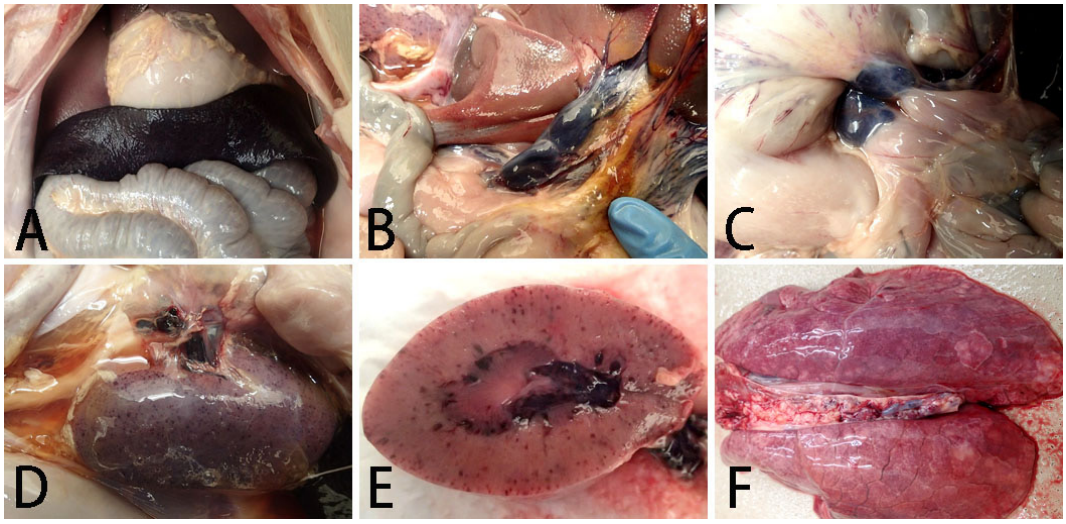

Fig. 2. Gross lesions in the (A) spleen, (B) hepatic lymph node, (C) celiac lymph node, the (D) surface of the kidney, (E) cut surface of the kidney, and (F) lung in pig 3 in experiment 1, inoculated with 0.1 fifty percent hemadsorbing doses (HAD_{50}) /ml of African swine fever virus and necropsied at 10 dpi. Hyperemic splenomegaly (A), the resemblance of perigastric lymph nodes to blood clots (B & C), petechial hemorrhages in the kidney (D & E), and pulmonary edema (F) are observed.

dpi (Fig. 1D) and saliva from 4 dpi (Fig. 1E). Viral DNA was eventually detected in the whole blood, serum, and nasal swab samples of all infected pigs. A marked increase in the copies of the ASFV genome was seen in samples of whole blood, serum, and nasal swab samples in all infected pigs over the experimental period (Fig. 1B–D). Viral DNA was detected in fecal samples from all pigs in group 1 and pig 6 in group 2, but it was not prominently detected in the other pigs examined in this study. (Fig. 1F).

Gross lesions observed in the examined pigs at necropsy are summarized in Table 1. All infected pigs in experiment 1 had severe hyperemic splenomegaly, which was characterized by a darkened, enlarged, and friable spleen (Fig. 2A). Almost all intra-abdominal lymph nodes resembled blood clots, except for pig 9. Among these lymph nodes, the perigastric, including the hepatic and celiac, lymph nodes (Fig. 2B and 2C) and renal lymph node demonstrated the most prominent lesions. Severe hemorrhage at the surface and cut surface of the kidney (Fig. 2D and 2E) were observed in all three pigs in group 1 and two of three pigs in group 2, but it was absent in the pigs in group 3. Hemorrhage in the tonsil was observed in two of three pigs in both groups 1 and 2, but it was not found in the pigs in group 3. Severe pulmonary edema was observed in the dead pigs (pig 1, 3, 4, and 6) (Fig. 2F). During necropsy, blood was serous, and the level of coagulation was low; blood did not clot in all examined pigs.

Clinical signs, detection of ASFV DNA, and gross lesions in experiment 2

Rectal temperatures of the pigs are shown in Fig. 3A and clinical symptoms in the examined pigs are summarized in Table 1. Inoculated pigs (pigs 11 and 12) developed fever (>40°C) from 3 dpi and depression and loss of appetite from 4 dpi. At 5 dpi, pig 12 exhibited high fever (42°C), lethargy, and diarrhea in the morning, and it died suddenly in the afternoon on the same day. Pig 11 developed high fever (>41°C) at 4 dpi and was euthanized on welfare grounds at 6 dpi.

Pigs 15 and 16, which were co-housed with pig 12, developed high fever (>41°C) from 9 dpc and had depression and loss of appetite at 11 dpc. Pigs 13 and 14 also had a fever (>40°C) at 11 and 10 dpc, respectively, but did not show any other clinical signs of disease during the experimental period. No erythema or cyanosis was observed on the skin of any of the pigs. All contact pigs were euthanized at 11 dpc based on the planned schedule.

In the inoculated pigs, viral DNA was detected in serum and whole blood samples from 3 dpi (Fig. 3B and 3C), nasal swabs from 4 dpi (Fig. 3D), and saliva from 5 dpi (Fig. 3E). In pigs 15 and 16, which were co-housed with infected pig 12, viral DNA was detected in whole blood and serum from 8 and 9 dpc, respectively (Fig. 3B and 3C); in pigs 13 and 14, which were co-housed with pig 11, viral DNA was detected in whole blood from 11 dpc (Fig. 3B). Viral DNA was eventually detected in the whole blood of all contact pigs; however, small quantities of amplicons could be detected in nasal swabs or saliva a few days earlier (Fig. 3B to 3E). A marked increase in the copies of the ASFV genome was seen in samples of whole blood, serum, and nasal swab samples in both injected and contact pigs over the experimental period (Fig. 3B to 3D). Viral DNA in fecal samples was not prominently detected in pigs examined in this study (Fig. 3F).

Gross lesions observed in the examined pigs at necropsy are summarized in Table 1. Two inoculated pigs (pigs 11 and 12) showed similar gross lesions which have been observed in inoculated pigs in the experiment 1.

In pig 15, severe hyperemic splenomegaly was observed, with almost all intra-abdominal lymph nodes resembling blood clots. Pig 16 also had hyperemic splenomegaly; however, only the hepatic, celiac, and renal lymph nodes resembled blood clots. Petechial hemorrhages in the kidney and gastrointestinal mucosa were observed in pigs 15 and 16. Pig 14 had hyperemic splenomegaly and petechial hemorrhages in the kidney and gastrointestinal mucosal membranes, and the hepatic, celiac, and renal

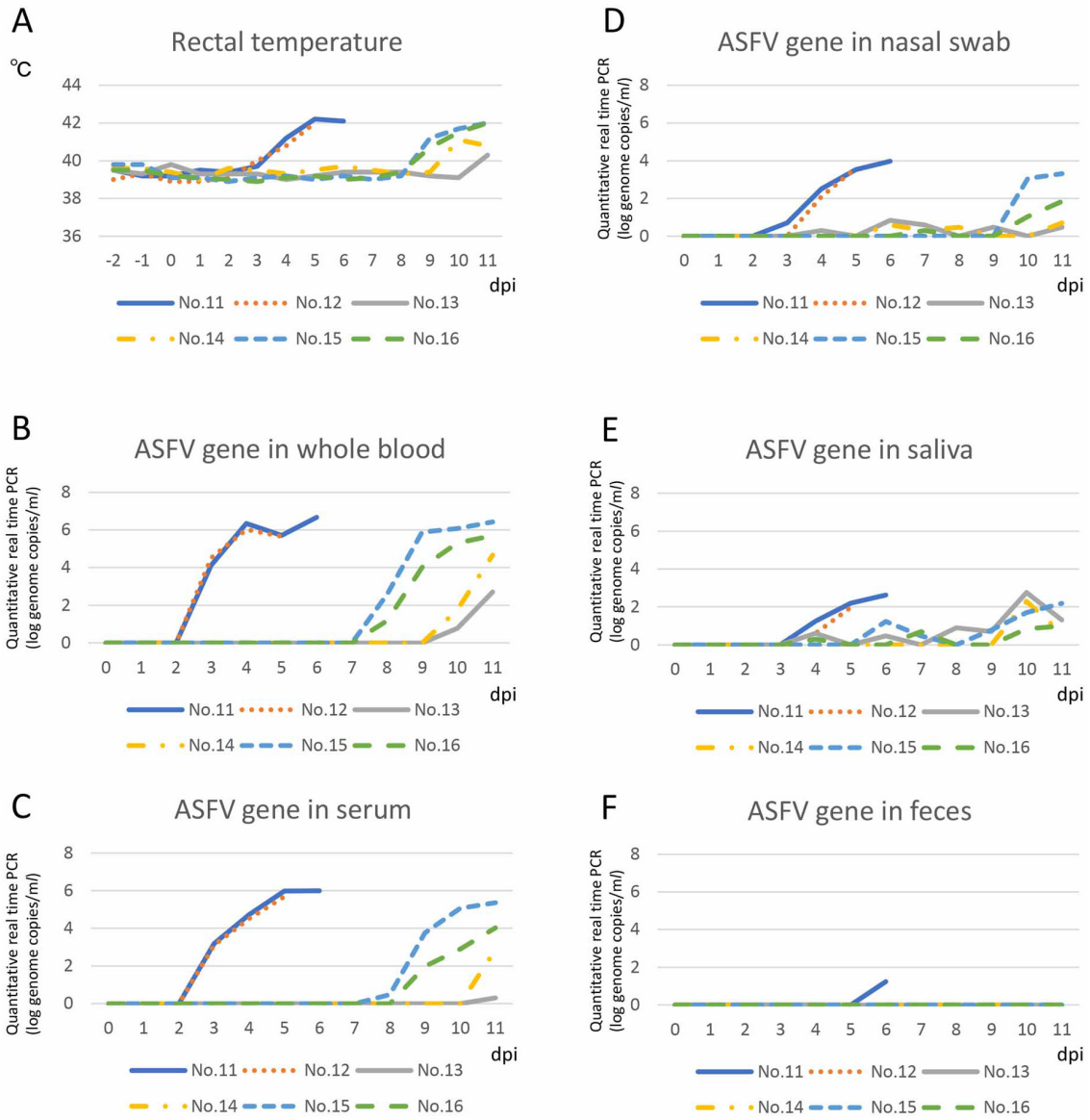

Fig. 3. Results of rectal temperatures and African swine fever virus (ASFV) gene detection in pigs kept together with the ASFV-infected pigs (experiment 2). (A) Rectal temperatures and ASFV gene copies in (B) whole blood, (C) serum, (D) nasal swab, (E) saliva, and (F) feces.

lymph nodes resembled blood clots. In pig 13, hyperemic splenomegaly and petechial hemorrhages in the gastrointestinal mucosa were observed; however, no lymph nodes resembled blood clots. No pulmonary edema or serohemorrhagic fluids in the thoracic or abdominal cavity was observed in any contact pigs, and no skin lesions were observed in any pigs in the study. During necropsy, blood was serous, and the level of coagulation was low; blood did not clot in most pigs, except for pig 13.

Histological examination

Histological lesions observed in the examined pigs are summarized in Table 2. Histologically, the most severe lesion was observed in pigs 1 and 3 in experiment 1, and the mildest lesion was observed in pig 9 in experiment 1 and pig 13 in experiment 2 in the examined pigs. In the pigs of group 3 in experiment 1, which were inoculated with a high dose of ASFV, histological lesions were not so severe, and the severity of histological lesions may increase proportionally with the duration of dpi.

The spleen in all examined pigs was hyperemic, and there was an expansion of the splenic sinus and splenic cord by filling of red blood cells and hemorrhage caused by structural disruption of the sinus in the red pulp. Severe atrophy of the white pulp caused by lymphocyte depletion and follicular necrosis was also present in all the spleens. In severe cases, almost all of the white pulp was absent, and the red pulp was replaced by accumulated red blood cells (Fig. 4A and 4B). Infarction was not found in the spleen.

Visceral lymph nodes showed blood absorption in the lymphoid sinus (Fig. 5A); the perigastric lymph nodes of the hepatic and celiac lymph nodes and the renal lymph node had severe blood absorption and hemorrhage following the disruption of a dilated sinus filling of red blood cells (Fig. 4C) in all infected pigs except two (pig 9 in experiment 1 and pig 13 in experiment 2). In the

Table 1. Summary of clinical signs and gross lesions found in pigs infected with African swine fever virus Armenia 07 strain

Experiment			1										2					
Group			1			2			3				Injected		Co-housed			
Pig No.			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Infectious route and doses			IM 0.1			IM 10^1			IM 10^6				IM 10^3		Direct contact			
Clinical signs	Died or euthanized (dpi)		D (10)	E (10)	D (10)	D (6)	E (8)	D (8)	E (5)	E (5)	E (4)	E (4)	E (6)	D (5)	E (11)	E (11)	E (11)	E (11)
	Fever		++	++	++	++	++	++	++	++	++	++	++	++	+	++	++	++
	Depression and loss of appetite		+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+
	Diarrhea		-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-
	Erythema or cyanosis on the skin		-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
Gross lesions	Splenomegaly		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Resemblance to blood clot	Perigastric lymph node	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+
		Renal lymph node	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+
		Mesenteric lymph node	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	-
	Hemorrhage	Gastro-intestinal mucosa	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
		Kidney	+	+	+	+	-	+	-	-	-	-	+	+	-	+	+	+
		Tonsil	+	-	+	+	-	+	-	-	-	-	-	+	-	-	-	-
	Pulmonary edema		+	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-
	Serohemorrhagic fluid in the abdominal cavities		+	+	+	+	-	+	+	+	-	+	+	+	-	-	-	-

IM: intramuscular inoculation. dose: HAD_{50}/ml. dpi: days post-injection. D: died. E: euthanized. +: positive, -: negative. Fever; ++: >41°C, +: >40°C.

Table 2. Distribution of histological lesions in pigs infected with African swine fever virus Armenia 07 strain

Organs	Histological lesion	Experimental 1										Experimental 2					
		0.1HAD_{50}/ml			10^1HAD_{50}/ml			10^6HAD_{50}/ml				Injected 10^3HAD_{50}/ml		Co-housed			
		No.1	No.2	No.3	No.4	No.5	No.6	No.7	No.8	No.9	No.10	No.11	No.12	No.13	No.14	No.15	No.16
		10dpi	10dpi	10dpi	6dpi	8dpi	8dpi	5dpi	5dpi	4dpi	4dpi	6dpi	5dpi	11dpc	11dpc	11dpc	11dpc
		D	E	D	D	E	D	E	E	E	E	E	D	E	E	E	E
Spleen	Lymphocyte depletion and follicular necrosis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Congestion in the venous sinus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Expanding hemorrhage in the red pulp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Celiac LN	Hemorrhage	+	+	+	ND	+	+	+	+	-	-	+	+	-	+	+	-
	Blood absorption	+	+	+	ND	+	+	+	+	-	+	+	+	-	+	+	+
	Lymphocyte depletion and follicular necrosis	+	+	+	ND	+	+	+	+	-	+	+	+	+	+	+	+
Hepatic LN	Hemorrhage	+	+	+	ND	+	+	+	+	-	-	+	+	-	-	+	-
	Blood absorption	+	+	+	ND	+	+	+	+	-	+	+	+	-	-	+	-
	Lymphocyte depletion and follicular necrosis	+	+	+	ND	+	+	+	+	-	+	+	+	+	+	+	+
Renal LN	Hemorrhage	+	+	+	ND	ND	+	+	+	-	-	+	+	ND	+	+	+
	Blood absorption	+	+	+	ND	ND	+	+	+	-	+	+	+	ND	+	+	+
	Lymphocyte depletion and follicular necrosis	+	+	+	ND	ND	+	+	+	-	+	+	+	ND	+	+	+
Mesenteric LN	Hemorrhage	+	+	+	ND	ND	-	-	-	-	-	-	-	-	-	-	-
	Blood absorption	-	-	-	ND	ND	-	-	-	-	-	-	+	-	-	-	-
	Lymphocyte depletion and follicular necrosis	+	+	+	ND	ND	+	+	+	-	-	+	+	-	+	+	+
Ileocolic LN	Hemorrhage	+	+	+	ND	ND	+	-	ND	ND	-	-	-	-	-	ND	ND
	Blood absorption	-	+	-	ND	ND	+	-	ND	ND	-	-	-	-	-	ND	ND
	Lymphocyte depletion and follicular necrosis	+	+	+	ND	ND	+	+	ND	ND	-	+	+	-	+	ND	ND
Tracheo-bronchial LN	Hemorrhage	+	+	+	ND	ND	ND	-	-	-	-	ND	-	-	-	-	ND
	Blood absorption	+	+	+	ND	ND	ND	-	-	-	-	ND	-	-	-	-	ND
	Lymphocyte depletion and Follicular necrosis	+	+	+	ND	ND	ND	+	+	-	+	ND	+	+	+	+	ND
Mandibular LN	Hemorrhage	+	+	+	ND	-	ND	-	-	-	ND	-	-	-	-	-	-
	Blood absorption	-	-	-	ND	-	ND	-	-	-	ND	-	-	-	-	-	-
	Lymphocyte depletion and follicular necrosis	+	+	+	ND	+	ND	+	+	-	ND	+	+	-	+	+	+
Lateral retro-pharyngeal LN	Hemorrhage	+	-	+	ND	ND	ND	-	-	-	-	-	-	-	ND	-	-
	Blood absorption	-	-	-	ND	ND	ND	-	-	-	-	-	-	-	ND	-	-
	Lymphocyte depletion and follicular necrosis	+	+	+	ND	ND	ND	+	+	-	+	+	+	-	ND	+	+
Cervical LN	Hemorrhage	+	-	+	ND	-	-	-	-	-	ND	-	-	-	-	-	-
	Blood absorption	-	-	-	ND	-	-	-	-	-	ND	-	-	-	-	-	-
	Lymphocyte depletion and follicular necrosis	+	+	+	ND	+	+	+	+	-	ND	+	+	+	+	+	+
Inguinal LN	Hemorrhage	+	+	+	ND	ND	ND	-	-	-	-	-	-	-	-	-	-
	Blood absorption	-	-	-	ND	ND	ND	-	-	-	-	-	-	-	-	-	-
	Lymphocyte depletion and follicular necrosis	+	+	+	ND	ND	ND	+	+	-	+	+	+	+	+	+	+

Table 2. Continued

Organs	Histological lesion	Experimental 1										Experimental 2					
		0.1HAD$_{50}$/ml			10^1HAD$_{50}$/ml			10^6HAD$_{50}$/ml				Injected 10^3HAD$_{50}$/ml		Co-housed			
		No.1	No.2	No.3	No.4	No.5	No.6	No.7	No.8	No.9	No.10	No.11	No.12	No.13	No.14	No.15	No.16
		10dpi	10dpi	10dpi	6dpi	8dpi	8dpi	5dpi	5dpi	4dpi	4dpi	6dpi	5dpi	11dpc	11dpc	11dpc	11dpc
		D	E	D	D	E	D	E	E	E	E	E	D	E	E	E	E
Popliteal LN	Hemorrhage	+	-	+	ND	ND	ND	+	-	-	-	-	-	-	-	-	-
	Blood absorption	-	-	-	ND	ND	ND	+	-	-	-	-	-	-	-	-	-
	Lymphocyte depletion and follicular necrosis	+	+	+	ND	ND	ND	+	+	-	-	+	+	+	+	+	+
Kidney	Hemorrhage	+	+	+	+	-	+	-	-	-	-	+	+	-	-	+	+
	DIC	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
Lung	Edema	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-
	Degeneration of vascular wall	+	+	+	+	+	+	-	+	-	-	+	+	-	-	-	-
	Hemorrhage with endothelial necrosis	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
Liver	Hemorrhage with endothelial necrosis	+	-	+	+	-	+	-	-	-	-	-	-	-	-	ND	-
Heart	Hemorrhage	+	-	+	ND	-	+	-	-	-	-	-	-	-	-	-	-
	Degeneration of vascular wall	+	+	+	ND	+	+	-	+	-	-	+	+	+	+	+	+
	Endothelial necrosis	+	+	+	ND	+	+	-	-	-	-	-	-	-	-	-	-
Gastro-Intestinal tract	Hemorrhage	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+
Tonsil	Hemorrhage	+	-	+	+	+	+	-	-	-	-	-	+	-	-	-	-
	Lymphocyte depletion and follicular necrosis	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Aorta	Degeneration of blood vessel wall	+	+	+	ND	ND	ND	-	+	-	+	+	+	-	+	+	-
	Endothelial necrosis	+	+	+	ND	ND	ND	-	+	-	-	+	+	-	-	-	-
Skin	Hyperemia	-	+	+	ND	ND	ND	-	-	-	-	-	-	-	-	ND	ND
	Endothelial necrosis	-	+	+	ND	ND	ND	-	-	-	-	-	-	-	-	ND	ND
Brain	Endothelial necrosis	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-

dpi: days post-injection, dpc: days post-contact, D: died, E: euthanized, LN: lymph node, ND: not done (the sample was not collected).

superficial lymph nodes, blood absorption in the sinus was not observed, and hemorrhage was only observed in the pigs of group 1 in experiment 1 (Fig. 4D), which were inoculated with 0.1 HAD$_{50}$/ml and underwent necropsy at 10 dpi, except for the popliteal lymph node of pig 7 in experiment 1 (The pig 7 had a injury at the hind leg). Lymphocyte depletion, necrosis of lymphocytes, and follicular necrosis were found in lymph nodes and tonsils in almost all infected pigs, except for three pigs (pigs 9 and 10 in experiment 1 and pig 13 in experiment 2). The lymph nodes with lymphocyte depletion showed prominent proliferation of reticular tissues and macrophages (reticulosis) (Fig. 5B and 5C).

In the kidney, severe hemorrhage in the medulla (Fig. 4E) and a mild multifocal interstitial hemorrhage in the cortex were observed. Glomerular capillary thrombosis was observed in only 4 pigs, which were severely affected and necropsied over 8 dpi in experiment 1 (Fig. 4F). Severe suppurative nephritis with bacterial colonies was observed in all the dead pigs. Seven of sixteen pigs did not show any histological lesions in the kidney.

In the lung, severe pulmonary edema characterized by interstitial proteinaceous edema and dilation of lymphoid vessels was observed in all dead pigs. Mild interstitial edema was found in pigs necropsied over 8 dpi in experiment 1. Hemorrhage with necrosis of endothelial cells was observed in four of five pigs over 8 dpi in experiment 1.

In the liver, focal to multifocal centrilobular hemorrhages with multifocal centrilobular sinusoidal dilation, capillary hyperemia, and necrosis of endothelial cells lining sinusoids were observed in dead pigs in experiment 1 (Fig. 4G and 4H). In the other pigs, including a dead pig in experiment 2, there was no prominent histological lesion, except for multifocal centrilobular sinusoid dilation with mild capillary hyperemia.

In the gastrointestinal tract, hemorrhage in the mucosal epithelium and submucosa was observed in twelve of sixteen pigs, and crypt herniation into the submucosa with lymphocyte depletion in the mucosa-associated lymphoid tissue was found in thirteen of sixteen pigs in this experimental study. Most severe intestinal lesions were found in the large intestine.

In the vascular system, edematous degeneration in the tunica media of the aorta (Figs. 4I and 5D) and arteries in the heart (Fig. 5E and 5F) and the lungs was observed from the early stage of infection. Necrosis of endothelial cells in the aorta was observed from 5 dpi, although endothelial damage in small and medium-sized blood vessels was observed only in pigs necropsied over 8 dpi (The aorta in pigs in group 2, and the heart in pig 4 in experiment 1 were not examined). Hemorrhage around blood vessels in the heart was seen in three of sixteen pigs. There was no vasculitis in blood vessels in any pigs examined.

There was no histological lesion in the other examined organs including the thymus, urinary bladder, and skeletal muscle in this study.

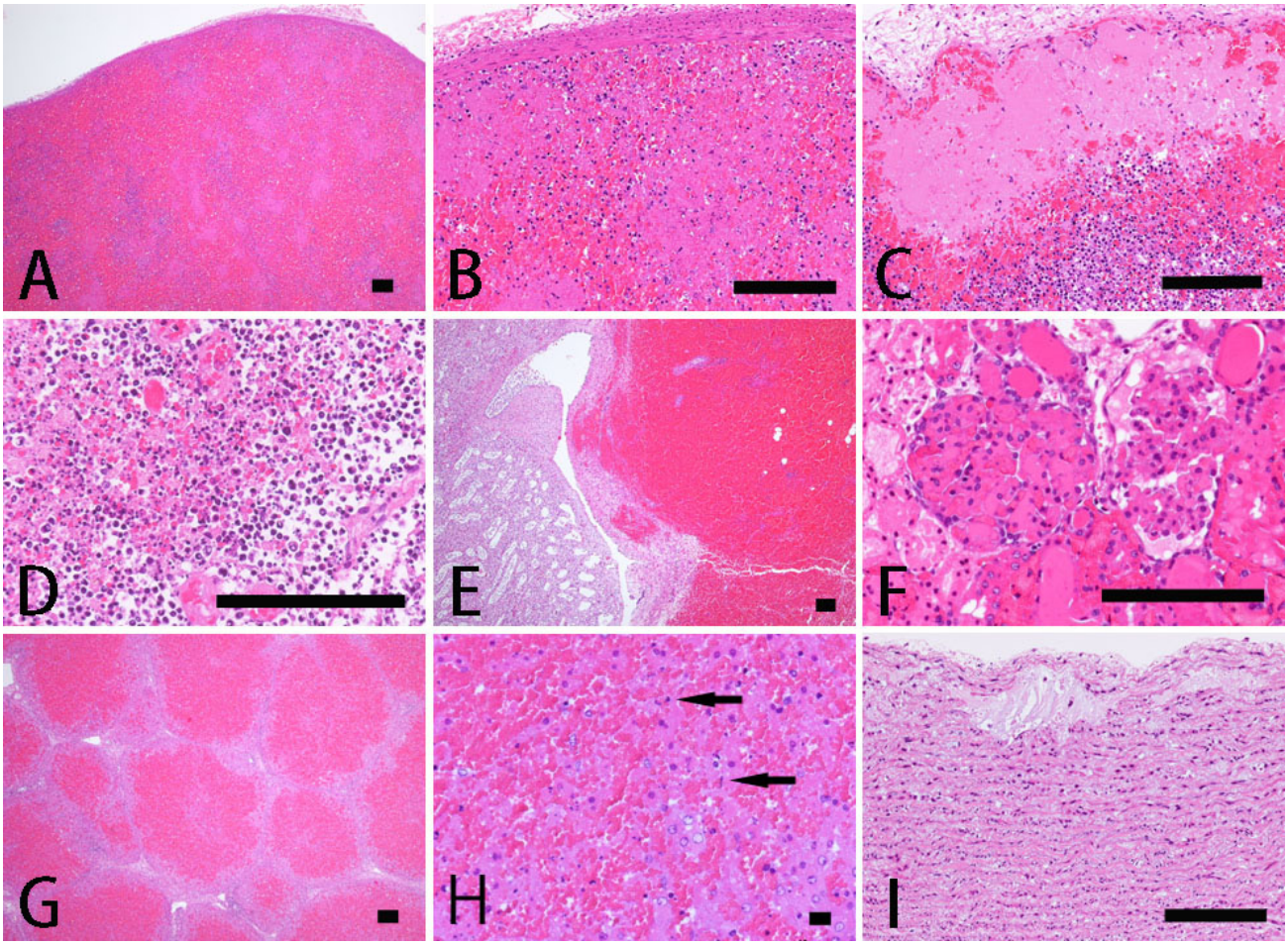

Fig. 4. Histological lesions in the (A and B) spleen, (C) hepatic lymph node, (D) inguinal lymph node, (E) medulla of the kidney, (F) cortex of the kidney, (G and H) liver and (I) aorta in pig 1 in experiment 1, inoculated with 0.1 HAD_{50}/ml of African swine fever virus and necropsied at 10 dpi (H & E). Bar=100 µm (A to G and I), 10 µm (H). A: Hyperemia. The red pulp is replaced by congested red blood cells and almost all the white pulp has disappeared. B: Structure of the spleen is disrupted. Almost all lymphocytes are necrotic. C: Severely dilated lymphoid sinus with hemorrhage. D: Hemorrhage with endothelial necrosis. E. Severe hemorrhage in the medulla. F: Glomerular capillary thrombosis. G: Multifocal centrilobular hemorrhage. H: Hemorrhage with sinusoidal dilation, capillary hyperemia, and necrosis of endothelial cells lining sinusoids (arrows). I: Severe edematous degeneration in the tunica media with endothelial damage in the aorta.

DISCUSSION

In experiment 1, it was revealed that 0.1 HAD_{50}/ml of the ASFV Armenia 07 strain was sufficient to infect healthy pigs by intramuscular injection. It caused lethal disease with characteristic hyperemic splenomegaly and perigastric and renal lymph nodes resembling blood clots, although the onset of fever and viral excretion were later than those in pigs injected with 10^1 HAD_{50}/ml and 10^6 HAD_{50}/ml of the virus. It has been reported that low doses of ASFV (10^2 and 10^1 HAD_{50}/ml) are sufficient to infect pigs and wild boars, especially those that are weak or runted, by the oronasal route; some of these animals did not present any clinical signs indicative of ASF and had almost no fever [21]. By intramuscular injection, the onset of the clinical signs of fever and depression and the detection of viral DNA were observed in all pigs, regardless of the difference in injection doses.

In this experiment 2, all contact pigs were infected with the Armenia 07 strain (genotype II) by direct transmission from the experimentally inoculated pigs. It was confirmed that ASF lesions of the spleen and perigastric and renal lymph nodes was induced by direct contact same as by intramuscular injection, although ASF lesions induced by direct contact were mild compared with those in pigs intramusculary injected with 0.1 HAD_{50}/ml of the virus. Contact pigs showed clinical manifestations 6 or 7 days after injected pigs exhibited pyrexia. It has been reported that ASF spreads slowly within a herd, and it is not as infectious as foot-and-mouth disease [10]. Our results suggest that ASF caused by the ASFV Armenia 07 strain spreads slowly within a herd, with high morbidity and mortality in infected pigs.

It has been confirmed that the differential diagnosis of ASF from other hemorrhagic diseases, such as classical swine fever, porcine dermatitis, and nephropathy syndrome, is difficult by clinical observation only [5, 7, 25]. Hyperemic splenomegaly and the resemblance of perigastric and renal lymph nodes to blood clots are considered pathognomonic of ASF [5, 19, 25]. In this

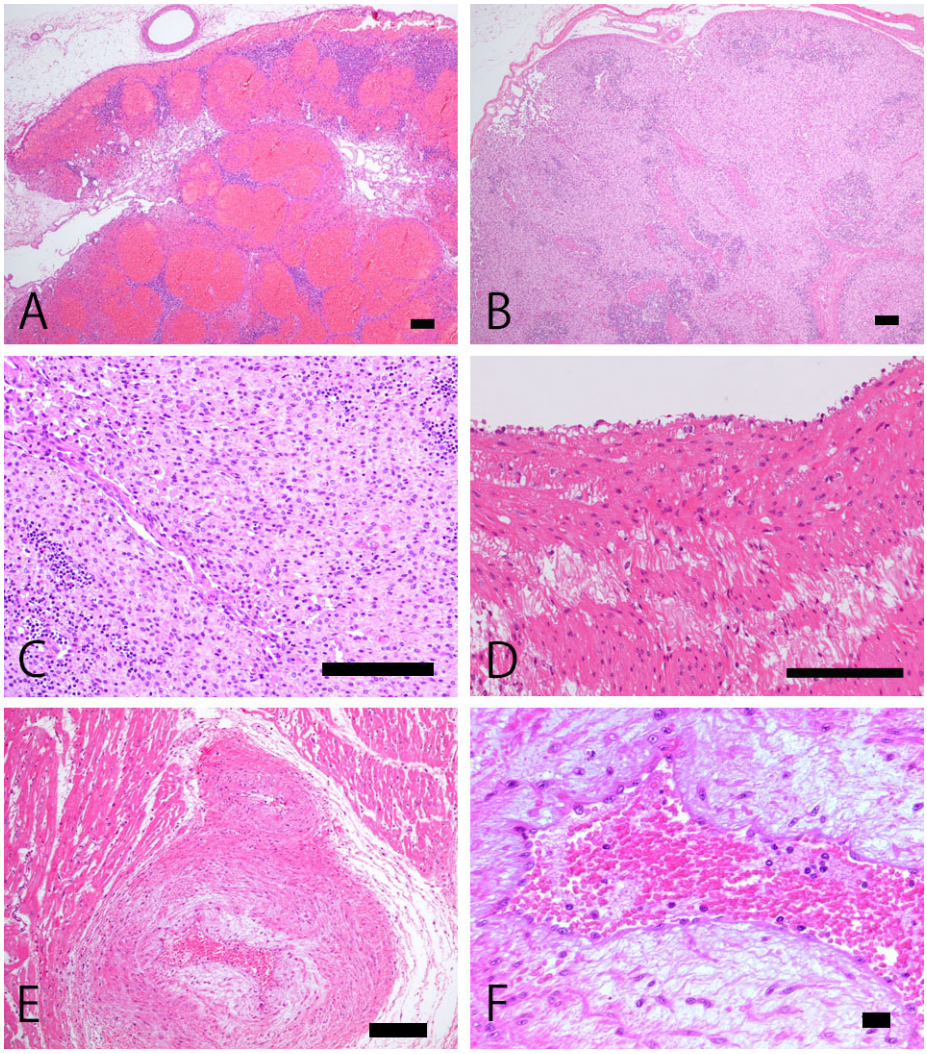

Fig. 5. Histological lesions at the early stage of African swine fever (ASF) infection in the (A) celiac lymph node, (B and C) cervical lymph node, (D) aorta and (E and F) artery in the heart in pig 11 in experiment 2 inoculated with 10^3 HAD_{50}/ml of ASFV and necropsied at 6 dpi. (H & E). Bar=100 µm (A to E), 10 µm (F). A: Severe blood absorption in the lymphoid sinus. B: Severe lymphocyte depletion. No blood absorption and hemorrhage. C: High magnification of the Fig. 5B. Proliferation of reticular tissues and macrophages. D: Moderate edematous degeneration in the tunica media with endothelial damage in the aorta. E: Severe edematous degeneration in the tunica media. F: High magnification of the Fig. 5E. Edematous degeneration. The nuclear of endothelial cells is not necrotic.

study, splenomegaly was observed in all examined pigs, including contact pigs and pigs injected with minute doses of ASFV. The resemblance of perigastric and renal lymph nodes to blood clots was also observed in nine of ten pigs examined in experiment 1 and five of six pigs examined in experiment 2 (Table 1). It has been suggested that the characteristic lesions in the spleen and perigastric and renal lymph nodes are notable in the differential diagnosis of ASF from other diseases. However, there were no gross lesions in the intra-abdominal lymph nodes of pig 9 in experiment 1 and pig 13 in experiment 2, which was euthanized on the day it developed a fever. The resemblance of lymph nodes to blood clots was also observed only in the perigastric and renal lymph nodes of pigs 14 and 16 in experiment 2, which were euthanized 1 and 2 days after exhibiting pyrexia, respectively. In experiment 1, the gross lesions in the pigs that were injected with 0.1 HAD_{50}/ml of ASFV and necropsied at 10 dpi were more severe than those in pigs that were injected with 10^6 HAD_{50}/ml of ASFV and necropsied at 4 or 5 dpi. The results of this study suggest that the resemblance of lymph nodes to blood clots may become apparent in the perigastric and renal lymph nodes, and gross lesions in other intra-abdominal lymph nodes may not be detected on examination of a pig at the time it develops a fever. In contrast, at necropsy or on examination of lethargic pigs, the characteristic lesions in the spleen and perigastric and renal lymph nodes can be considered pathognomonic of ASF. The results of the histological examination in this study suggest that the lesion in ASF may start from the spleen and spread to perigastric and renal lymph nodes. Thrombocytopenia has been reported in some ASFV-infected pigs [14], and decreased coagulability has also been observed during necropsy of pigs infected with ASFV Armenia 07 strain. Therefore, contagious coagulopathy in pigs may also be considered pathognomonic of ASF.

The pathogenesis of hemorrhage in ASF was previously reviewed [6, 14]. In ASF infection, infected macrophages can release

inflammatory mediators like TNF-alpha, IL-1alpha, and IL-6 [8, 23], which in turn cause severe injury to endothelial cells [11]. Direct ASF infection in endothelial cells was also found, although it has been reported only in the late stage of the disease [6, 11]. Hemorrhage without endothelial damage in ASF may be associated with severe angiectasia and an increase in vascular permeability [11]. It is well known that hemorrhage, with or without endothelial damage, presents in ASF [11, 14]. However, the histological process of the formation of hemorrhage in ASF has not been studied. In this study, histological lesions in the blood vessel wall of the aorta and arteries in the heart and lung were detected from the early stage of infection. This is the first report of histological lesions in the vascular wall in the early stage of ASF infection. The edematous change in the tunica media of the aorta and arteries in this study may have been caused by an increase in vascular permeability in ASFV infection [11]. Endothelial damage was also found in the aorta from 5 dpi, although necrosis of endothelial cells in arteries in the heart and lungs was found over 8 dpi. It was revealed that the characteristic gross lesion of hematoma-like perigastric lymph nodes followed severe blood absorption and hemorrhage resulting from the disruption of the lymphoid sinus filling with absorbed red blood cells from the early stage of infection. On the other hand, hemorrhagic lesions in the superficial lymph nodes were caused by damage to endothelial cells at 10 dpi. This hemorrhagic change with endothelial damage was also observed in the liver, heart, and lungs over 8 dpi. In this study, endothelial damage was not observed in the early stage of infection, except in the aorta. The results from this study suggest that hemorrhage in the perigastric lymph node and spleen in the early stage of infection may be caused by the disruption of the severely dilated sinus filling with red blood cells, and hemorrhage in later stage of the disease may be caused by endothelial damage.

It has been reported that the vascular changes observed in subacute forms of ASF, mainly hemorrhage and edema, are more intense than those reported in acute forms of the disease [14]. Gross and histological lesions in pigs infected with 0.1 HAD_{50}/ml of ASFV and necropsied at 10 dpi were more severe than those in pigs infected with 10^6 HAD_{50}/ml of the virus and necropsied at 5 dpi as well as those infected with 10^3 HAD_{50}/ml of the virus and necropsied at 5 or 6 dpi. The severity and distribution of histological lesions in pigs infected with 10^6 HAD_{50}/ml and 10^3 HAD_{50}/ml of the virus and necropsied at 5 dpi were similar. The results from this study also suggest that the severity of the gross and histological lesions may depend on duration after infection. In this study, there were no histological lesions in the thymus and the urinary bladder, which deviates from what was reported by a previous study [6, 25]. The lesions in those organs may develop depending on the virus strain, condition of the host, or the experimental design.

The study has used various types of samples from the infected pigs and has performed clinical, molecular (PCR) and pathological examination to confirm the detection of the virus in pigs and the dynamics of the virus over the time after inoculation or contact. In this study, the ASFV genome was detected in nasal swab and saliva samples of the contact pigs before they developed viremia. It has been reported that ASFV first replicates in the oropharyngeal region when pigs are infected by contact transmission, and it is excreted through the oral and nasal routes before systemic dissemination [21]. Experimental transmission dynamics for the current ASFV genotype II strain have been described in several reports [12, 15, 20, 21, 26, 27]. Similar infectious dynamics of direct transmission were demonstrated in this study. On the other hand, the ASFV gene was detected in the feces of all the pigs injected with 0.1 HAD_{50}/ml of the virus from 9 dpi and in one of three pigs injected with 10^1 HAD_{50}/ml of the virus from 8 dpi in experiment 1. The dynamics of transmission through feces may also be observed in the sub-acute stage of ASF by a low-dose infection in pigs. In this study, clinical samples were collected from the whole blood, serum, nasal swab, saliva, and feces. The ASFV genome was eventually detected in nasal swab samples as well as the whole blood and serum samples. Although the results in this study suggest that the whole blood is the most suitable sample for early diagnosis of ASF because ASFV genome was detected earlier and much more in the whole blood than in the other samples, the nasal swab sample may be also available for the diagnostics sample for ASF.

POTENTIAL CONFLICTS OF INTEREST. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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