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FULL PAPER

Pathology

Immunohistochemical analysis of the distribution of classical swine fever (CSF) viral antigen in boar-pig hybrids and pigs four weeks after infection

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ABSTRACT. We detected the classical swine fever virus (CSFV) antigen in three boar-pig hybrids (hybrids) and three pigs. All animals were experimentally infected with CSFV strain JPN/27/2019 to optimize diagnostic sampling and risk assessment of virus dissemination. Two hybrids died 17- and 19-days post-inoculation (dpi). The other animals were euthanized at 28 dpi. The detection of CSFV antigen at 28 dpi in epithelial cells of the apocrine sweat and sebaceous glands in the skin, salivary glands, mucosal epithelial cells in the rectum, and epithelial cells in the kidney and urinary bladder, suggests that CSFV persists in these tissues and spreads via sweat, saliva, feces, and urine for at least 4 weeks. These findings reveal that hybrids and pigs represent a high risk of virus dissemination four weeks after infection with CSFV strain JPN/27/2019. Prominent CSFV antigens were also detected in hair follicles of the skin. These results suggest that postmortem sampling of animal skin may be effective for CSF diagnosis and can be used to develop a rapid and easy diagnostic method using hair follicles.

KEY WORDS: boar-pig hybrid, classical swine fever, classical swine fever virus, immunohistochemistry, pig

Classical swine fever (CSF) is a highly contagious and economically devastating transboundary viral disease in swine and wild boars [1, 3, 11]. CSF is caused by the CSF virus (CSFV) of the *Pestivirus* genus and *Flaviviridae* family [11]. In 2007, the World Organization for Animal Health provided Japan with a CSF-free status. However, in September 2018, a CSF outbreak occurred in Gifu Prefecture, Japan, for the first time in 26 years [16]. From September 2018 to September 2021, 71 cases of CSF were confirmed in domestic pig farms, including 1 case in a wild boar farm [2]. Additionally, more than 3,200 cases of CSF were detected in wild boars in 24 prefectures in the central region of Japan [2]. CSFV isolated from a field case was classified as subgenotype 2.1d [14], which is characterized as moderately virulent in China [18].

A CSFV strain isolated from an outbreak in Japan in 2018 was less virulent compared to the highly virulent ALD strain [10]. Moderate-to low-virulence strains may be disseminated over a longer period than highly virulent CSFV strains [4, 5]. Outbreaks of CSF caused by low-virulence strains are more difficult to recognize, which facilitates their spread [4, 5]. These chronically infected pigs are important in the epidemiology of the disease because they serve as a source of infection in susceptible pigs [3]. Furthermore, CSFV is widely disseminated in wild boars in Japan, especially in prefectures where CSF outbreaks occur [2]. Evidence acquired during the course of recent CSF outbreaks in Japan strongly suggests that wild boars contribute to the spread of outbreaks [8]. Therefore, it is important to examine the pathogenesis of a recent Japanese CSFV in wild boars by conducting experimental infections to establish appropriate control measures and diagnostic laboratory assays [7]. Moreover, it is necessary to investigate the distribution of CSFV antigens in infected pigs and wild boars to optimize diagnostic sampling and risk assessment of virus dissemination.

Immunohistochemical detection of viral antigens in formalin-fixed, paraffin-embedded (FFPE) tissues, which detect and localize viral antigens within cells and tissues, serves as a simple and rapid method to study organ tropism, relationships between the virus and the pathogenesis of lesions, and virus shedding from tissues. In particular, it is necessary to determine the distribution of CSFV antigens in the salivary glands, large intestine, kidneys, and urinary bladder during virus shedding. In a previous study, the

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distribution of CSFV antigen in tissues from pigs infected with a field isolate of CSFV was analyzed from 4 days post-inoculation (dpi) to 18 dpi [6]. In addition, another study showed that CSFV antigen was detected in the tonsils, spleen, lymph nodes, kidneys, pancreas, ileum, lungs, and adrenal glands at 30 dpi in a pig infected with CSFV strain Kanagawa/74 [13]. To our knowledge, however, a detailed immunohistochemical analysis of the distribution of CSFV antigens across the entire body of CSFV-infected pigs beyond 18 dpi has not been conducted, and the existence of CSFV antigens in the tissues associated with virus shedding in the subacute and chronic stages of CSFV infection remains unclear. In this study, we used immunohistochemistry (IHC) to analyze the distribution of CSFV antigens in experimentally infected hybrids and pigs at 28 dpi to elucidate the suitable diagnostic sampling and virus dissemination risk.

MATERIALS AND METHODS

It is difficult to use wild boars as experimental animals because of the physical dangers they pose to researchers. Therefore, we used a boar-pig hybrid (hybrid) as a surrogate to investigate the pathogenesis of CSFV in wild boars. Three 8-week-old hybrids (crossbreed Duroc × wild boar × Duroc) (Nos. 4–6) and three 8-week-old pigs (crossbreed Landrace × Large White × Duroc) (Nos. 7–9) were inoculated orally with 1 ml of a $10^{6.5}$ 50% tissue culture infectious dose ($TCID_{50}$) of CSFV strain JPN/27/2019 [8]. The other hybrids (Nos. 1–3) were administered a single dose of the bait vaccine (RIEMSER Schweinepestoralvakzine, Riemser Arzneimittel AG, Greifswald-Insel Riems, Germany) 14 days before inoculation with the same dose of the JPN/27/2019 strain. The hybrids and pigs did not have detectable antibodies against *Pestivirus* before experimental infection [7], and their detailed clinical characteristics have been published [7]. Animals were determined to have reached a humane endpoint when they were dormant or unable to stand, and were euthanized by sodium pentobarbital injection [7].

FFPE tissue specimens used in this study were collected at 28 dpi from the skin of the ear and hind legs, salivary glands (submandibular and parotid), stomach, ileum, rectum, kidneys, and urinary bladder of all animals. Skin samples were collected from sites with and without erythema lesions. The skin from pig Nos. 7 and 8 and the rectum from hybrid No. 2 were not examined because these tissues were not collected at necropsy. According to recommendations from the manual of diagnostic tests for CSF [1], the tonsil, spleen, and lymph nodes (submandibular and parotid) were also collected and examined histologically and immunohistochemically.

Hybrids No. 6 and No. 5 died at 17 and 19 dpi, respectively [7]. Necropsy revealed severe pneumonia, severe multisystemic hemorrhage involving the lymph nodes, ileum, cecum, and cerebellum, and significant thymic atrophy. The surviving hybrids and pigs were euthanized at 28 dpi [7]. There were no prominent macroscopic lesions upon necropsy of each animal, except for thymic atrophy and mild hemorrhage in the lymph nodes. Published histological findings [7] are briefly summarized as follows: Necropsy revealed severe suppurative histiocytic bronchopneumonia with thrombus; severe necrotizing enteritis in the ileum and cecum; lymphadenitis; and hemorrhage with thrombus in the cerebellar cortex accompanied by meningitis. A rabbit anti-Salmonella O antiserum (NIAH, Tsukuba, Japan) detected gram-negative short bacilli in these lesions. In the other animals, prominent histological lesions were not detected, except for hemorrhage and follicular atrophy in the spleen and lymphoid tissues, and crypt herniation in the intestine. To examine the existence of other co-infectious agents, rabbit anti-porcine reproductive and respiratory syndrome virus (PRRSV) polyclonal antibody (NIAH, Tsukuba, Japan), rabbit anti-porcine circovirus Type 2 (PCV-2) polyclonal antibody (NIAH), and mouse anti-*Toxoplasma gondii* monoclonal antibody (Bio-Rad, Hercules, CA, USA) were used for IHC; however, positive antigens against these primary antibodies were not detected in the lung, intestine, and lymph nodes of any of the animals examined. The deaths of the hybrids were likely caused by simultaneous salmonellosis and CSFV infection, while sepsis associated with salmonellosis may have been induced by immunosuppression due to CSFV infection [7]. The Animal Care and Use Committee of the National Institute of Animal Health (NIAH) approved protocols using animal procedures before the initiation of this study (authorization number: 18-085). All experimental infections using live viruses were performed in a high-containment facility at the NIAH.

Deparaffinized sections were stained with hematoxylin and eosin (HE). For IHC, serial sections were processed using the Universal Immuno-enzyme Polymer method with Histofine Simple Stain MAX PO (Nichirei, Tokyo, Japan), according to the manufacturer's instructions. The samples were counterstained with hematoxylin prior to mounting. The primary antibody used in this study was an anti-CSF monoclonal antibody (WH303; APHA Scientific, Addlestone, UK) used at a 1:100 dilution. FFPE tissue specimens from three hybrids (Nos. 1–3), that were protected against CSFV infection by the bait vaccine were used as negative controls. FFPE tissue specimens from three normal, uninfected pigs (Nos. 10–12) were also used as negative controls.

RESULTS

In the skin, CSFV antigen was detected in the epidermis, hair follicles, sebaceous glands, and apocrine sweat glands (Fig. 1a) of all four infected animals, regardless of the presence of macroscopic erythematous lesions. In the sebaceous glands, CSFV antigens were detected in the acinar cells (Fig. 1b) and ductal epithelial cells. In the apocrine sweat glands, CSFV antigens were detected in secretory cells (Fig. 1b) and ductal epithelial cells. In the hair follicles, CSFV antigen was detected in the hair papilla, hair cortex, and outer root sheath (Fig. 1c). In the epidermis, CSFV antigen was detected in the epidermal cells of the stratum basale, stratum spinosum, and stratum granulosum (Fig. 1d). Some endothelial cells and fibroblasts in the dermis also expressed CSFV antigen (Fig. 1d).

Both pairs of submandibular and parotid glands of all infected animals showed similar CSFV antigen distribution. CSFV antigen was mainly detected in the ductal epithelial cells of the interlobular duct, intralobular duct, striated duct, and intercalated duct (Fig. 2a and 2b) in the salivary glands of all infected animals. Some endothelial and vascular smooth muscle cells were antigen-positive.

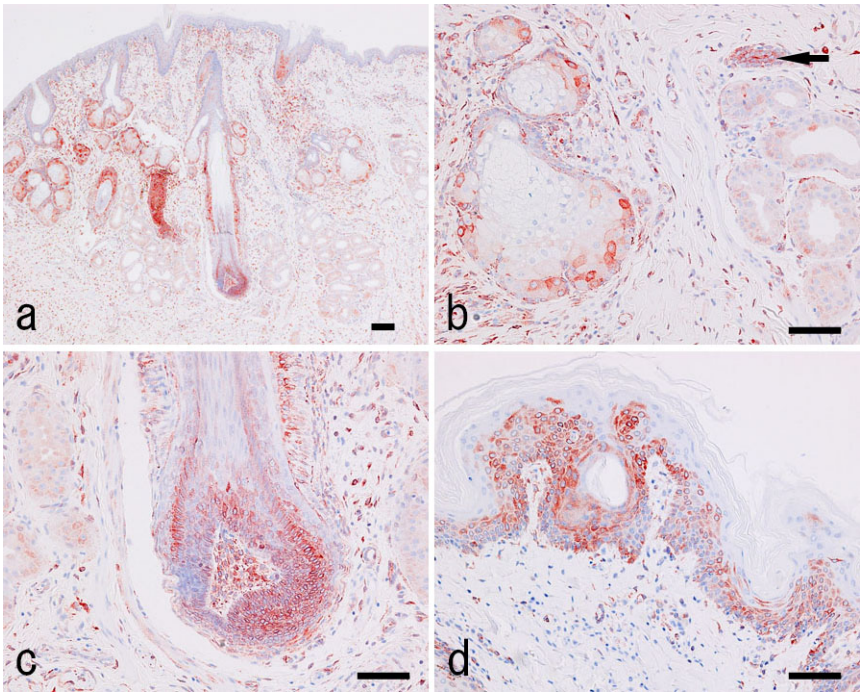

Fig. 1. Immunohistochemical detection of classical swine fever virus (CSFV) antigen at 28 dpi in the skin of pig No. 9 infected with CSFV strain JPN/27/2019. Immunohistochemistry (IHC). **a**: Skin. CSFV antigen is seen in the epidermis and epidermal derivatives. **b**: Sebaceous and apocrine sweat glands. CSFV antigen is detected in the sebaceous gland acinar cells and secretory and ductal epithelial cells (arrow) in the apocrine sweat gland. **c**: Hair bulb. CSFV antigen is detected in the hair papilla, hair cortex, and outer root sheath of the hair follicle. **d**: Epidermis. CSFV antigen is detected in epidermal cells of the stratum basale, stratum spinosum, and stratum granulosum. Antigen is also detected in fibroblasts in the dermis (a: Bar=100 µm, b–d: Bar=50 µm).

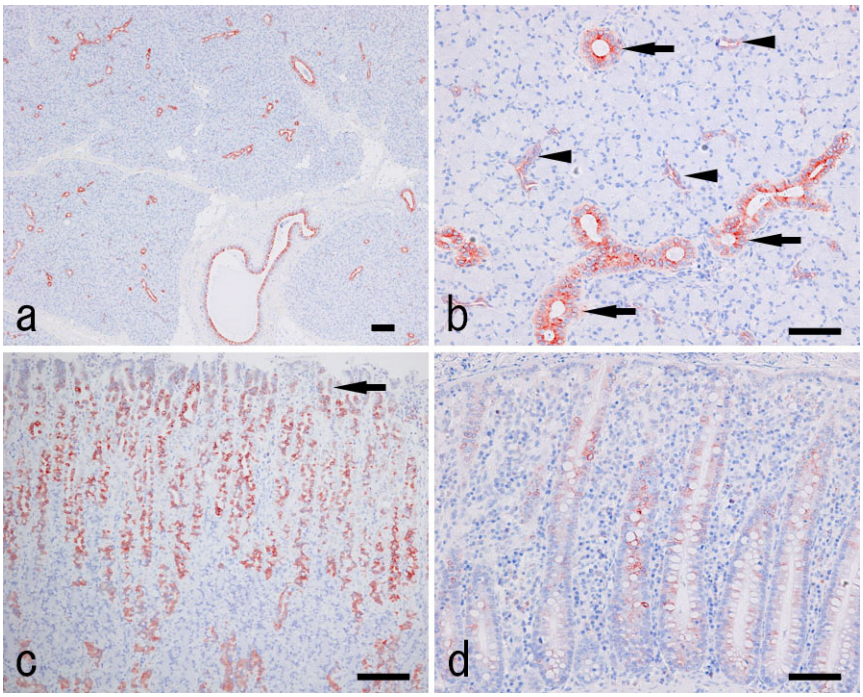

Fig. 2. Immunohistochemical detection of classical swine fever virus (CSFV) antigen at 28 dpi in the salivary gland (a and b), stomach (c), and rectum (d) of hybrid No. 4 infected with CSFV strain JPN/27/2019. IHC. **a** and **b**: Salivary gland (parotid gland). CSFV antigen is detected in the ductal epithelial cells of the interlobular duct, intralobular duct (arrows), and intercalated duct (arrowheads). **c**: Stomach. CSFV antigen in parietal cells of the mucosal epithelium. The gastric pit (arrow) is weakly positive, but the chief cells are negative. **d**: Rectum. CSFV antigen detected in the epithelium (a and c: Bar=100 µm, b and d: Bar=50 µm).

Although staining was very weak, CSFV antigen was detected in acinar epithelial cells in three of the six infected animals, except for hybrid No. 5, which died at 19 dpi.

In the stomach of all infected animals, CSFV antigen was detected in the parietal cells of the mucosal epithelium (Fig. 2c) and lymphatic follicles in the cardiac-gland region of the mucosa. Although the chief cells were negative, the gastric pit was weakly positive (Fig. 2c). In the ileum and rectum, CSFV antigen was detected in epithelial cells (Figs. 2d), macrophages in the mucosa-associated lymphoid tissue, endothelial cells, and vascular smooth muscle cells in all infected animals. Occasionally, smooth muscle cells of the intestinal wall were stained in all infected animals.

In the kidneys, CSFV antigen was detected in the cortex and medulla of all infected hybrids and pigs (Fig. 3a). In the cortex, CSFV antigen was detected in the distal convoluted tubule and collecting tubule, but not in the proximal convoluted tubule and renal corpuscles (Fig. 3b). In the medulla, CSFV antigen was detected in the collecting tubule, Henle's loop, papillary duct, and epithelial cells of the renal pelvis (Fig. 3c). Endothelial and vascular smooth muscle cells in some small to medial arteries were also positive. In the urinary bladder, CSFV antigen was detected in the transitional epithelial cells (Fig. 3d) in five of the six infected animals, and in the endothelial cells of all infected animals.

In the other organs examined, CSFV antigens were clearly detected in the tonsils, spleen, and lymph nodes of all infected animals. No histological lesions consistent with CSFV antigens were detected. CSFV antigen was undetectable in the negative controls (Supplemental Data 1).

DISCUSSION

CSFV antigens were strongly positive in the skin, salivary glands, rectum, kidney, and urinary bladder in hybrids and pigs approximately four weeks after inoculation with CSFV strain JPN/27/2019. In addition, CSFV antigens were detected in the tonsil, spleen, lymph nodes, and ileum. These findings demonstrate that these tissues are suitable diagnostic samples for CSF analysis. The CSF diagnostic test manual recommends collecting the tonsil, lymph nodes, spleen, ileum, and kidneys for diagnostic testing [1]. Furthermore, the results of this study suggest that the skin of the ear and salivary glands are also suitable for diagnostic testing because these tissues express CSFV antigen four weeks after infection. This is especially true for the salivary gland, which is a superficial organ located under the skin, as well as nearby sites, which are easily collected together with the submandibular lymph nodes. Usually, surveillance for CSF is conducted using dead wild boars; however, it is difficult to obtain diagnostic samples of internal organs, such as the spleen, ileum, and kidneys, from dead wild boars because many internal organ samples from carcasses are affected by severe postmortem changes. This study suggests that sampling of the superficial lymph nodes, salivary glands, and skin of the ear from dead wild boars may be effective for CSF diagnostics.

Delimitation of the lethal form of clinical CSF is based on the interval between infection and death. Peracute, acute, subacute, or

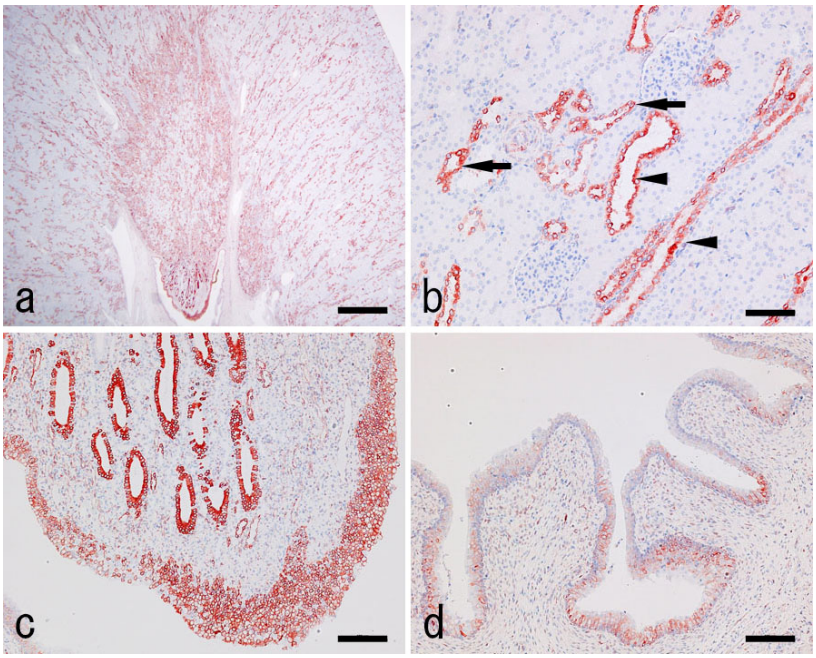

Fig. 3. Immunohistochemical detection of classical swine fever virus (CSFV) antigen at 28 dpi in the kidney (a to c) and urinary bladder (d) of pig No. 9 infected with CSFV strain JPN/27/2019. IHC. **a**: Kidney. CSFV antigen is strongly detected in both the cortex and medulla. **b**: Cortex of the kidney. CSFV antigen is detected in the distal convoluted tubule (arrows) and collecting tubule (arrowheads), but not in the proximal convoluted tubule and renal corpuscle. **c**: Renal papilla. CSFV antigen is detected in the collecting tubule, Henle's loop, papillary duct, and epithelial cells of the renal pelvis. **d**: Urinary bladder. CSFV antigen is detected in transitional epithelial cells (a: Bar=1,000 µm, b: Bar=50 µm, c and d: Bar=100 µm).

chronic illness is recognized when the interval is <10, 10–20, 21–30, or >30 days, respectively [4, 12]. CSFV antigens are diffusely detected in the skin during the acute course of CSF, and diagnosis is possible using skin punch biopsies [9, 15]. The results of the present study suggest that diagnosis is also possible using skin punch biopsies during the subacute or chronic stage of CSF infection, as well as in the acute stage. Diagnostic tests using hair biopsies are available for detecting animals persistently infected with bovine viral diarrhea virus (BVDV), which belongs to the same genus and family as CSFV [11, 17]. Our present findings strongly suggest that it may be possible to develop a rapid and easy diagnostic method using hair biopsies, as it was observed that hair follicles expressed readily detectable CSFV antigens.

Our findings showed that CSFV antigens were expressed by the ductal epithelial cells of the sweat and sebaceous glands in the skin, salivary glands, mucosal epithelial cells in the rectum, and epithelial cells in the kidneys and urinary bladder in a hybrid and three pigs at 28 dpi. This suggests that CSFV persists in these tissues, and that CSFV spreads from sweat, saliva, feces, and urine for a long time. Apocrine sweat glands and sebaceous gland acinar cells were occasionally immunopositive in the skin of pigs infected with the field-isolated CSFV Quillota strain until 18 dpi [6]. However, detailed cellular tropism of the CSFV antigen in the skin and the risk of virus dissemination from the skin have not been reported in CSFV-infected animals. In this study, using the CSFV JPN/27/2019 strain, prominent immunopositivity was detected in sweat and sebaceous glands in the skin of all animals examined. It is thought that there may be a high risk of virus dissemination for extended periods from the sweat of animals infected with the CSFV JPN/27/2019 strain. The findings in this study are valuable for establishing and improving control measures, diagnostic sampling and risk management for CSF caused by not only the recent Japanese CSFV isolate but also other CSFV strains.

CONFLICT 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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