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Antigenic characterization of classical swine fever virus YC11WB isolates from wild boar

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Classical swine fever (CSF), a highly contagious disease that affects domestic pigs and wild boar, has serious economic implications. The present study examined the virulence and transmission of CSF virus strain YC11WB (isolated from a wild boar in 2011) in breeding wild boar. Virulence of strain YC11WB in domestic pigs was also examined. Based on the severe clinical signs and high mortality observed among breeding wild boar, the pathogenicity of strain YC11WB resembled that of typical acute CSF. Surprisingly, in contrast to strain SW03 (isolated from breeding pigs in 2003), strain YC11WB showed both acute and strong virulence in breeding pigs. None of three specific monoclonal antibodies (7F2, 7F83, and 6F65) raised against the B/C domain of the SW03 E2 protein bound to the B/C domain of strain YC11WB due to amino acid mutations (^{720}K→R and ^{723}N→S) in the YC11WB E2 protein. Although strains YC11WB and SW03 belong to subgroup 2.1b, they had different mortality rates in breeding pigs. Thus, if breeding pigs have not developed protective immunity against CSF virus, they may be susceptible to strain YC11WB transmitted by wild boar, resulting in severe economic losses for the pig industry.

Keywords: B/C domain, *Classical swine fever virus*, *Sus scrofa*, virulence

Introduction

Classical swine fever virus (CSFV) belongs to the genus *Pestivirus* within the family *Flaviviridae* [10]. It is an enveloped virus with a single-stranded, positive-sense RNA genome that encodes a 3,898 amino acid (aa) polyprotein that undergoes co-translational and post-translational processing by cellular and viral proteases to yield four structural (C, E^{rns}, E1, and E2) and eight nonstructural (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [17]. Glycoprotein E2 is the most immunodominant protein and induces the production of neutralizing antibodies in infected pigs [21]. Four antigenic domains (A–D) have been identified in the N-terminal half of E2, which comprises two independent antigenic units: B/C and D/A [8,14,32]. Domain B/C, which comprises the 90 N-terminal residues, is responsible for the antigenic specificity of various CSFVs [20].

Acute classical swine fever (CSF) occurs mainly in young animals and is characterized by high fever, lack of appetite, conjunctivitis, and constipation; these symptoms are often followed by diarrhea, neurological signs, and hemorrhage of the skin and other organs, possibly accompanied by severe thrombocytopenia and leukopenia [15,25,27,30]. Host-virus interactions involve many factors (host age, genetic background, immune status, herd sanitary status, and strain virulence), and such interactions can lead to different clinical outcomes [11,19,26].

Since 2002, CSF in Korea has undergone an antigenic shift from genotype 3 to genotype 2 [29]. Recently, two virus isolates (strains YC11WB and PC11WB) were obtained from Korean wild boar captured during a campaign conducted for CSFV surveillance; these strains were identified as genotype 2.1b [29]. Comparative analysis of the nucleotide sequences of YC11WB Erns, E1, E2, and NS5B, along with those of a reference strain (SW03), revealed high sequence homology: 94.7% for the Erns genes, 94.4% for the E1 genes, 94.5% for the E2 genes, and 95.2% for the NS5B genes [3].

The aim of the present study was to compare the clinical signs, pathological lesions, viremia, virus shedding, and mortality among breeding pigs infected by strain YC11WB

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(isolated from wild boar) and strain SW03 (isolated from a breeding pig). Wild boar were challenged with strain YC11WB and contact-mediated transmission among animals was examined. The genetic differences between strains were also analyzed by using specific monoclonal antibodies (mAbs) targeting the B/C and D/A domains of the E2 protein.

Materials and Methods

Viruses

Strain YC11WB (GenBank accession KC149990) belongs to genotype 2.1b and was isolated from a Korean wild boar in 2011 [19]. Strain SW03 (belonging to epidemic genotype 2.1b), a representative virulent strain from Korea, was isolated from a breeding pig farm in 2003. Strain LOM (genotype 1) was derived from a low virulence strain and used to develop an attenuated live vaccine virus, which has been used in Korea for 40 years.

Cloning and expression of the E2 protein domains

Total RNA was purified from strain LOM by using an RNeasy kit (Qiagen, USA) and cDNA was amplified using the SuperScript III First-Strand Synthesis System (Invitrogen, USA). DNA fragments of the antigenic domains (B/C, D/A, and ABCD) were amplified and cloned into vectors pET30a or pRSET to construct pET30a-LOM-E2/BC, pRSET-LOM-E2/DA, and pET30a-LOM-E/2ABCD. These plasmids were then used to transform *Escherichia coli* BL21 (DE3), which was then cultured under the following conditions: 1 mM isopropyl β-D-1-thiogalactopyranoside (ITPG), 30°C, for 18 h (B/C protein); 0.5 mM IPTG, 37°C, for 5 h (D/A protein); or 1 mM IPTG, 25°C, for 18 h (ABCD protein).

B/C and D/A domain-binding mAbs

Fifteen mAbs specific for the E2 protein of the LOM strain were produced in-house or donated by Median Diagnostics (Korea), which manufactures veterinary diagnostic kits. The remaining mAb, WH303, which binds specifically to the D/A domain, was obtained from APHA (UK). The three virus strains (LOM, YC11WB, and SW03) were inoculated into porcine kidney-15 cells, which were then examined 3 days later using mAbs specific for the B/C or D/A domains to detect antigenic differences between strains. The B/C, D/A, and ABCD domains expressed by the pET30a-LOM-E2/BC, pRSET-LOM-E2/DA, and pET30a-LOM-E/2ABCD plasmids, respectively, were then examined by performing western blotting to identify the protein regions recognized by the mAbs.

Predicting the three-dimensional structure of the E2 protein

The sequences of the CSFV strains were obtained from GenBank via the National Center for Biotechnology Information (USA) website. The three-dimensional (3D) structures of the CSFV E2 proteins were predicted by using the RaptorX program [23]. The putative epitope within the E2 protein was predicted by using the Emini Surface Accessibility Prediction program [9] and BepiPred Linear Epitope Prediction [16].

Animals experiment using strains YC11WB and SW03

The test animals were separated into six experimental groups. Group 1, comprising two breeding wild boar, was used to verify the virulence of strain YC11WB. Group 2 comprised two breeding wild boar housed with the infected breeding wild boar from Group 1. These animals were used to examine contact-mediated transmission. Group 3 comprised two Landrace pigs, which were used to check the virulence of strain YC11WB. Group 4 comprised two Landrace pigs infected with strain SW03 to compare pathogenicity, used as positive control. Groups 5 and 6 comprised two breeding wild boar and two Landrace pigs, respectively (negative controls). All Landrace pigs and breeding wild boar were approximately 40 days old and 70 days old, respectively, and all were negative for anti-CSFV antibodies. All animals weighed about 14 kg (*n.b.*, the 30 day age difference between the Landrace pigs and the breeding wild boar is due to the fact that breeding wild boar grow slowly). Both virus strains were used at the same dosage in the challenge experiments ($10^{6.0}$ $TCID_{50}$/mL). Pigs were monitored daily until 21 days post-infection (DPI), mainly to record body temperature, and mortality was monitored. Pig samples used for virological, serological, hematological, and pathological analyses were collected at different intervals (2 or 3 days).

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay of samples

Samples (blood, nasal swabs, saliva, and feces) were collected from the experimental animals at 3, 5, 7, 9, 11, 14, 17, and 21 DPI. Necropsies were performed and tissue samples were obtained from 13 organs: tonsil, lung, heart, spleen, liver, mesenteric lymph node, kidney, ileum, cecum, inguinal lymph node, cerebrum, cerebellum, and spinal cord. Total RNA was extracted from all samples by using a micro-column-based QIAamp Viral RNA Mini Kit (Qiagen). The CSFV real-time RT-PCR assay was based on primers and probe developed at the Plum Island Disease Center. The nucleotide sequences of the primers and probe targeted the highly conserved 5′ untranslated region of the CSFV genome. The primer and probe sequences were as follows: TGCCCAAGACACACCTTAACC (forward primer), GGCCTCTGCAGCGCCCTAT (reverse primer), and FAM-TGATGGGAGTACGACCTG-MGBNFQ (probe) [5]. The thermocycling conditions for amplification were as follows: one cycle of RT at 50°C for 30 min and one cycle of heat activation at 95°C for 15 min, followed by 45 cycles of PCR amplification (heat denaturation at 94°C for 15 sec and extension at 56°C for 60 sec). Amplification was performed in

a thermocycler in standard mode and fluorescence data were collected in the FAM channel during the extension step (56°C). All real-time RT-PCR assays were performed in duplicate.

Survival rates upon infection with strain YC11WB

To examine viral pathogenicity in breeding pigs and breeding wild boar, animals (approximately 14 kg body weight) were intranasally inoculated with $10^{6.0}$ $TCID_{50}$/mL of CSFV strain YC11WB. Group 1 and 2 comprised seven breeding wild boar and seven Landrace pigs, all of which were negative for anti-CSF antibodies. The negative control Group 3 comprised seven Landrace pigs inoculated with phosphate-buffered saline. After inoculation with strain YC11WB, all animals were monitored daily for 25 days to observe mortality. All experiments were approved by an independent Animal Care and Use Committee (2012-354) and followed guidelines set down by the Animal and Plant Quarantine Agency.

Results

Specific B/C domain-binding mAbs

Western blot analysis revealed that seven mAbs bound to the B/C domain of the LOM E2 protein and eight (including WH303) bound to the D/A domain (Table 1). Interestingly, three mAbs (7F2, 7F64, and 6F65) that bound to SW03 E2 did not bind to YC11WB E2. Of the other eight mAbs, two (6H10 and 6H1) bound to the B/C and six (4C29, 6A92, 2-21-9, 45-43, 2-187, and WH303) bound to the D/A domains of the E2 proteins of both strains (Table 1).

Three-dimensional structure predictions for the E2 proteins

The best RCSB Protein Data Bank template [2] for the structural prediction of the two strains was 2YQ2, the envelope E2 glycoprotein from bovine viral diarrhea virus (BVDV) 1. When compared with SW03 E2, the amino acid (aa) sequences for the predicted 3D structures of YC11WB E2 differed at 13 sites: T692S, K720R, N723S, I745M, D786N, V854A, N855D, L871W, H868Y, T886M, A917V, A962G, and I972V (panel A in Fig. 1). The aa differences within the B/C domain of SW03 E2 were predicted to have strong effects on epitope-binding region 718–724, but only weak effects on epitope-binding region 718–722 within YC11WB (panel B in Fig. 1).

Clinical signs

Changes in the number of white blood cells among animals in experimental Groups 1–4 were striking. Leukopenia was noted in breeding wild boar and domestic pigs at approximately 5 to 6 DPI. The body temperature of animals in Groups 1 and 3 exceeded 40°C at 5 DPI, whereas those of animals in Groups 2 and 4 exceeded 40°C at 6 to 7 DPI.

Detection of CSFV in nasal swabs, feces, saliva, and blood

RT-PCR was performed to detect viremia and viral shedding in pigs after challenge with strains YC11WB or SW03. Virus were detected in blood samples from two breeding wild boar (W26 and W28) at 3 DPI, and another two (NW35 and NW32

Table 1. Reactivity of a panel of monoclonal antibodies with vaccine and virulent strains of classical swine fever virus (CSFV)

Domain	Anti-CSFV antibody	Western blot result			Indirect fluorescence assay (IFA) result		
		pET30a-LOM-E2/BC	pRSET-LOM-E2/DA	pET30a-LOM-E2/ABCD	LOM (genotype 1)	SW03 (genotype 2.1b)	YC11WB (genotype 2.1b)
B/C	6H10	+	−	−	+	+ + +	+ + +
	6H1	+	−	−	+	+ + +	+ + +
	7F2*	+	−	+	+	+ + +	−
	7F84*	+	−	+	+	+ + +	−
	6F65*	+	−	+ +	+ +	+	−
	1A39	+ + +	−	+ +	+ + +	−	−
	10A56	+ + +	−	+ + +	+ + +	−	−
D/A	4C29	−	+ +	+ + +	+ + +	+ +	+ +
	6A92	−	+ + +	+ + +	+ + +	+ +	+ +
	2-21-9	−	+ +	+ + +	+ + +	+ + +	+ + +
	45-43	−	+ +	+ + +	+ +	+ + +	+ + +
	2-187	−	+ + +	+ + +	+ + +	+ + +	+ + +
	4F69	−	+ +	+ + +	+ +	−	−
	4F41	−	+ +	+ + +	+ + +	−	−
	WH303	−	+ + +	+ + +	+ + +	+ + +	+ + +

*Antibodies that reacted only with strain SW03 and LOM. +, week; + +, moderate; + + +, strong; −, negative reaction.

in Group 2) wild boar at 3 and 5 DPI, respectively (Table 2). Analysis of blood, nasal, fecal, and salivary fluid samples from animals in Group 3 revealed that all pigs were positive at 5 DPI (Table 2). At 5 DPI, blood samples from pigs in Group 4 showed evidence of viremia and fecal samples showed evidence of viral shedding.

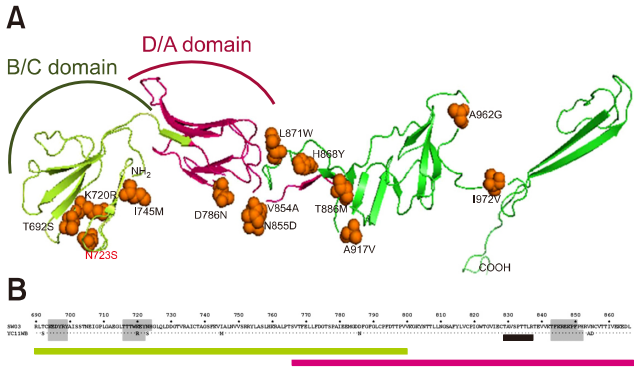

Fig. 1. Predicted structure of glycoprotein E2, and amino acid sequence similarities between the B/C and D/A domains of classical swine fever virus (CSFV) strains SW03 and YC11WB. (A) The best RCSB Protein Data Bank template for strains SW03 and YC11WB was 2YQ2 (BVDV1 envelope glycoprotein E2). (B) Amino acid sequence similarity between the B/C and D/A domains of strains SW03 and YC11WB. The B/C and D/A domains are marked with a green bar and a pink bar, respectively. Three linear epitope determinants identified by using antibody epitope prediction programs are shown in gray. The epitope region binding the WH303 mAb is shown as a black box.

CSFV RNA copy number in tissue samples

Results of RT-PCR of 13 tissue samples taken from wild boar W26 and W28 revealed between 1.49 and 6.87 $\log_{10}$ copies of CSFV RNA. Tissue samples from wild boar NW32 and NW35 in Group 2 (exposed to contact-mediated transmission from wild boar in Group 1) harbored between 1.06 and 6.38 $\log_{10}$ copies (Table 3). When Landrace pigs were infected with strain YC11WB, animals R110 and R112 in Group 3 harbored a high number of CSFV RNA copies in tonsil and lung tissues; however, SW03 RNA was rarely detected in cerebrum, cerebellum, spinal cord, and brain tissue samples from pigs SL44 and SL98 (infected with strain SW03) in Group 4 (Table 3).

Pathological and histopathological findings

The gross pathological findings for Groups 1 and 2 (breeding wild boar), and Group 3 (breeding pigs) infected with strain YC11WB comprised swelling and congestion of the spleen and the submandibular and mesenteric lymph nodes, hemorrhage of the skin, and local petechial hemorrhage of the bladder, gallbladder, and kidney. Histopathological examination of the spleen and lymph nodes from breeding wild boar (W26, W28, NW32, and NW35) revealed various lesions, including depletion of long-lived plasma cells, reticuloepithelial cell hyperplasia, and endotheliosis (data not shown). Meningitis and perivascular cuffing were observed in the cerebrum of four breeding wild boar (W26, W28, NW32, and NW35) and two breeding pigs (R110 and R112) infected with strain YC11WB, but were much less prominent in the cerebrum of pigs infected with strain SW03.

Table 2. Detection of viral RNA and virus shedding in *Classical swine fever virus*-infected pigs by reverse transcriptase-polymerase chain reaction

Group	1		2		3		4		5		6	
Cs	YC11WB		YC11WB		YC11WB		SW03		Non-treatment		Non-treatment	
Ps	Wild boar		Wild boar		Landrace pig		Landrace pig		Wild boar		Landrace pig	
Pn	W26	W28	NW32	NW35	R110	R112	SL44	SL98	W21	W22	L18	L22
0*	–	–	–	–	–	–	–	–	–	–	–	–
3	a	a	–	a	ac	c	–	–	–	–	–	–
5	abcd	abcd	a	bd	abcd	abcd	ac	ac	–	–	–	–
7	abcd	abcd	abd	bcd	abcd	abcd	abc	abcd	–	–	–	–
9	abcd	abcd	abc	ac	abcd	abcd	abcd	abcd				
11	abcd	abcd	abcd	a	–	abcd	abcd	abcd	–	–	–	–
14	abcd	abcd	abc	abcd	–	–	abcd	abcd	–	–	–	–
17	–	abcd	abcd	abcd	–	–	abcd	abcd	–	–	–	–
21	–	–	–	–	–	–	abcd	–	–	–	–	–
D	17	18	18	21	10	13		18				

*0 days post-infection. Cs, challenge virus strain; YC, YC11WB; SW, SW03; Ps, pig species; Pn, pig number; –, negative; a, blood; b, nasal swab; c, fecal; d, salivary fluid; D, death date [the figure in parentheses indicates the date of death (expressed as days post-infection)].

Table 3. Viral RNA copy number in tissue samples from pigs infected with classical swine fever virus strains

Group	Pig Number	Cs	Necropsy (DPI)	Organ												
				To	Lu	He	Sp	Li	ML	Ki	Il	Ce	LN	Ce	Cer	SC
1	W26	YC	17	2.17*	3.26	3.05	6.13	4.27	4.02	5.63	1.94	5.39	4.91	3.53	1.76	2.43
	W28	YC	18	6.87	3.75	4.64	3.89	6.03	3.52	4.57	4.11	5.87	2.04	1.85	1.49	2.36
2	NW32	YC	18	3.67	4.06	3.22	6.38	5.54	2.63	4.02	4.98	5.81	6.26	3.44	1.62	2.73
	NW35	YC	21	4.29	3.77	3.64	2.10	3.61	1.54	5.85	4.45	1.06	4.87	3.29	2.47	2.64
3	R110	YC	10	6.54	7.25	3.63	2.47	1.88	1.65	2.35	2.04	1.81	2.09	4.35	1.28	1.06
	R112	YC	13	5.05	5.69	3.48	4.81	−	−	−	2.57	3.60	1.48	1.03	2.05	1.45
4	SL44	SW	21	4.28	2.03	1.92	−	−	1.84	4.42	−	1.16	2.05	−	−	1.67
	SL98	SW	18	2.65	4.82	1.08	1.75	−	2.27	−	1.54	2.64	−	1.99	−	−
5	W21	−	21	−	−	−	−	−	−	−	−	−	−	−	−	−
	W22	−	21	−	−	−	−	−	−	−	−	−	−	−	−	−
6	L18	−	21	−	−	−	−	−	−	−	−	−	−	−	−	−
	L22	−	21	−	−	−	−	−	−	−	−	−	−	−	−	−

*Real-time polymerase chain reaction copy number. Cs, challenge strains; DPI, days post-infection; To, tonsil; Lu, lung; He, heart; Sp, spleen; Li, liver; ML, mesenteric lymph node; Ki, kidney; Il, ileum; Ce, cecum; LN, inguinal lymph node; Ce, cerebrum; Cer, cerebellum; SC, spinal cord; YC, YC11WB; SW, SW03; −, negative.

Animal survival rates

After challenge with strain YC11WB, the survival rate for Group 1 was 85.7% at 18 DPI, 57.1% at 20 DPI, 42.9% at 10 DPI, and 28.6% at 25 DPI (data not shown). The survival rate for Group 2 was 71.4% at 11 DPI, 57.1% at 13 DPI, 28.6% at 14 DPI, 14.3% at 15 DPI, and 0% at 16 DPI (data not shown). Strain YC11WB was fatal to breeding pigs at 11 to 16 DPI and to breeding wild boar at 18 to 25 DPI.

Discussion

In this study, we observed the aa differences between ABCD domain regions of the LOM vaccine strain and those of virulent CSFVs (SW03 and YC11WB) was particularly high (13.3%). These differences may have altered the topology of the mAb-binding sites in the D/A (mAbs 4F69 and 4F41) and B/C (mAbs 1A39 and 10A56) domains, thereby providing an explanation as to why these mAbs did not recognize the virulent CSFVs.

A previous study that examined the antibody responses of five overlapping synthetic peptides covering the antigenic domain B/C (aa 693−777) within envelope protein E2 showed that PV-BC1 (BC1: aa 693−716) elicited a potent protective response, whereas PV-BC3, PV-BC4, and PV-BC5 (BC3: aa 723−745; BC4: aa 741−760; BC5: aa 757−777) showed weaker activity [1]. Interestingly, three mAbs (7F2, 7F84, and 6F65) that bound to the B/C domain regions of strains LOM and SW03 in this study did not bind to that of YC11WB. The SW03 and YC11WB strains differed with respect to four aas: T692S, K720R, N723S, and I745M. Two epitope prediction programs (*i.e.*, Emini Surface Accessibility Prediction and Bepipred Linear Epitope Prediction) predicted the presence of a strong epitope at aa positions 718−724 within the B/C domain, a region harboring two of the aa substitutions. The topography of the mAb-binding sites within E2 may be flexible, provided that conformational integrity is maintained [29]. A recent study that compared the sequences of the Spain-01 and Margarita CSFV strains identified several aa substitutions in the E2-B/C domains [22]. Substitutions D723S, N725G, T738V, G761R, and S777N were observed at sites reported to be under positive selection pressure, suggesting that these changes may have a role in viral escape from neutralizing antibodies [24].

Various studies examining disease symptoms and CSF pathology have been undertaken in several countries in an attempt to characterize the virulence and clinical effects of different CSFV strains in pigs [12,13,18,19,22,28]. The overall aim of those studies is the global elimination of CSF; indeed, the USA, Canada, and Japan, as well as members of the EU, have eradicated this disease successfully. However, sporadic outbreaks of CSF that occurred in domestic pigs in Europe were linked to indirect or direct contact with wild boar [4]. Indeed, it is estimated that 59% of primary CSF outbreaks in Germany over a period of 10 years were caused by contact between wild boar and domestic pigs [6,7].

Korea has experienced many CSF outbreaks on breeding pig farms after the genotype 2 shift occurred in 2002: 13 in 2002, 72 in 2003, nine in 2004, five in 2005, two in 2006, five in 2007, seven in 2008, two in 2009, and one in 2013. All were attributed to strain SW03. In the present study, the observed mortality rate for SW03 during the 22 day period was 50%, and the

histopathological lesions observed in the organ tissue samples were less severe than those observed after infection by YC11WB. The YC11WB isolate produced more serious symptoms and higher mortality in breeding pigs than those from the SW03 isolate. However, anti-CSFV antibodies induced by vaccination would protect against all genotypes as the vaccine is derived from a single serotype.

Therefore, active disease surveillance and CSF vaccination should be undertaken regularly on breeding farms located at the base of mountains or near forest areas in Korea to prevent spillover transmission of CSF from wild boar to domestic pigs. In addition, farms should be surrounded by perimeter fences to prevent direct contact between wild and domestic animals.

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Conflict of Interest

The authors declare no conflicts of interest.

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