Vaccine 37 (2019) 3598–3604

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Assessment of the efficacy of an attenuated live marker classical swine fever vaccine (Flc-LOM-BE^{rns}) in pregnant sows

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ARTICLE INFO

Article history:
Received 6 December 2018
Received in revised form 20 April 2019
Accepted 24 April 2019
Available online 28 May 2019

Keywords:
Classical swine fever virus
Flc-LOM-BE^{rns}
Transplacental transmission
Pregnant sow
E^{rns} ELSIA

ABSTRACT

Here, we constructed an attenuated live marker classical swine fever (CSF) vaccine (Flc-LOM-BE^{rns}) to eradicate CSF. This was done by taking infectious clone Flc-LOM, which is based on an attenuated live CSF vaccine virus (LOM strain), and removing the full-length classical swine fever virus (CSFV) E^{rns} sequences and the 3′ end (52 base pairs) of the CSFV capsid. These regions were substituted with the full-length bovine viral diarrhoea virus (BVDV) E^{rns} gene sequence and the 3′ end (52 base pairs) of the BVDV capsid gene. Sows were vaccinated with the Flc-LOM-BE^{rns} vaccine 3 weeks before insemination and then challenged with virulent CSFV at the early, mid- or late stages of pregnancy. We then examined transplacental transmission to the foetuses. Piglets born to sows vaccinated with Flc-LOM-BE^{rns} did not show vertical infection, regardless of challenge time. In addition, CSFV challenge did not affect the delivery date, weight or length of the foetus. Pregnant sows inoculated with the Flc-LOM-BE^{rns} vaccine were anti-CSF E^{rns} antibody-negative and anti-BVDV E^{rns} antibody-positive. Challenge of pregnant sows with virulent CSFV resulted in anti-CSF E^{rns} antibody positivity. These results strongly indicate that differential diagnosis can be conducted between the Flc-LOM-BE^{rns} vaccinated animal and virulent CSFV affected animal by detecting antibody against BVDV E^{rns} or CSF E^{rns} gene. Therefore, the Flc-LOM-BE^{rns} vaccine may fulfil the function of differential diagnosis which required for DIVA vaccine.

1. Introduction

Classical swine fever (CSF) is a highly contagious multisystemic haemorrhagic viral disease of domestic and wild pigs that can have devastating effects on sustainable pig production worldwide [1]. This notifiable disease is caused by the classical swine fever virus (CSFV; genus *Pestivirus*, family *Flaviviridae*), which harbours a single positive-stranded RNA genome [2]. A CSF vaccine is available; this is an attenuated live vaccine that has been used worldwide for several decades. The benefits of this vaccine include high efficacy and safety, and low price. Indeed, several countries have used such vaccines (e.g., the C-strain or GPE$^-$ strain) as part of mandatory control programs that have eradicated CSF [3,4]. However, international trade in pigs vaccinated with conventional live attenuated vaccines is subject to restrictions because it is not possible to distinguish antibodies induced by vaccines from those induced by virulent strains of CSFV [5]. To overcome trade restrictions, the OIE (World Organisation for Animal Health) has attempted to distinguish between vaccine-inoculated and virus-infected pigs by inoculating animals with a CSF marker vaccine called DIVA (differentiating infected from vaccinated animals) [4]. However, this vaccine has several disadvantages, particularly with respect to early protection and protection against transplacental transmission [6]. Optimal marker vaccine candidates must show sufficient safety, high efficacy, definite DIVA function and marketability [4]. Recently, the CP_E2alf vaccine was chosen as a final candidate for an EU-funded research project aimed at improving tools and

Abbreviations: BVDV, bovine viral diarrhoea virus; CPE, cytopathic effects; CSF, classical swine fever; DIVA, differentiating infected from vaccinated animals; END, exaltation of Newcastle disease; DPI, days post-infection; ICS, immunocytochemical staining; NDV, Newcastle disease virus; nt, nucleotide; OIE, World Organisation for Animal Health; qRT-PCR, real time polymerase chain reaction; SNT, serological neutralisation antibody test; ST, swine testicular cell; $TCID_{50}$, 50% tissue culture infective dose; WPV, weeks post-vaccination; 5′ UTR, 5′ untranslated region.

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https://doi.org/10.1016/j.vaccine.2019.04.076
0264-410X/

strategies for preventing and controlling CSF [4]. Many studies of the CP_E2alf vaccine are related to construction, genetic stability, safety, efficacy, DIVA diagnostics and strategy design [7–12]. A chimeric Pestivirus CP7_E2alf marker vaccine candidate was developed using a CP7 bovine viral diarrhoea virus (BVDV) backbone that expresses the E2 glycoprotein of the Alfort/187 CSFV strain [7]. The CP_E2alf vaccine (produced by the Zoetis company under the name "Suvaxyn® CSF Marker") was licensed as the first live marker vaccine against CSF [4]. The LOM vaccine strain has been used as a live attenuated vaccine to eradicate CSFV in Korea since 1974; this vaccine is highly immunogenic in pigs and lasts for several decades [13,14]. In the future, CSF vaccine policy in Korea plans to replace the LOM vaccine with a live marker vaccine suitable for the international pig trade. As part of these projects, we have been developing candidate CSF live marker vaccines since 2009 [15]. The CSF live marker vaccine Flc-LOM-BE^{rns} was approved by five Korean animal veterinary vaccine companies in 2016 and 2017. This vaccine was developed by substituting the E^{rns} gene and partial capsid gene of BVDV into the Flc-LOM clone to yield the Flc-LOM-BE^{rns} vaccine. Here, we report construction and *in vitro* characterisation of the Flc-LOM-BE^{rns} virus vaccine, and assess its ability to prevent transplacental transmission in sows at different times post-insemination. In addition, the ability of differential diagnosis of vaccinated sows was examined by detecting antibodies against CSFV or BVDV E^{rns} protein.

2. Materials and methods

2.1. Construction of Flc-LOM-BE^{rns}

The pFlc-LOM clone comprises a pACYC177 plasmid harbouring unique restriction enzyme sites (*Cla* I, *Ngo* MIV, *Kpn* I, *Nru* I, *Srf* I, *Sac* II and *Xho* I), a T7 promoter, an ampicillin resistance site and LOM genome sequences [15]. The unique restriction enzyme sites *Cla* I and *Ngo* MIV are located at nucleotide (nt) positions 770 and 2440, respectively, and the region contains four genes: N^{pro} (nt 374–877), capsid (nt 878–1174), E^{rns} (nt 1175–1855) and E1 (nt 1856–2440) (supplemental Fig. 1A). A total of 1670 nt containing partial capsid and full-length E^{rns} genes between the *Cla* I to *Ngo* MIV sites were synthesised (Macrogen Inc., Korea). Amplicon synthesis was based on the LOM strain; however, synthesis of the 3′ end (52 base pairs) of the capsid gene and the full-length E^{rns} gene was based on BVDV type 1 (KD-26 strain) derived from Korea. The pACYC177 plasmid, including the Flc-LOM plasmid, was digested with *Cla* I and *Ngo* MIV, and then ligated with the synthesised amplicon (770–2440 bp) (supplemental Fig. 1B).

2.2. In vitro transfection of CPK cells and rescue of the Flc-LOM-BE^{rns} virus

The full-length Flc-LOM-BE^{rns} cDNA clone was linearised using the restriction endonuclease *Srf* I (12,298), which cuts at the 3′ end of the viral genome. Transfection of a cell line with linearised Flc-LOM-BE^{rns} was performed as described previously [15]. Briefly, authentic viral RNA was obtained by transcription from the T7 RNA polymerase promoter using a T7 MEGAscript kit (Ambion, USA). After digestion with TURBO DNase I (Ambion, USA), the transcripts were purified by lithium chloride precipitation and quantified spectrophotometrically (ND1000; Nano Drop Technologies, USA). Next, 2×10^5 CPK cells grown in 6-well tissue culture plates (TPP, Switzerland) were transfected with synthesised RNA (1.5 μg) using the Lipofectin reagent (Invitrogen, USA). Culture supernatants were collected 3 days later. Next, 1 ml supernatant was inoculated onto monolayers of CPK cells and the presence of infectious virus was determined by immunocytochemical staining (ICS) [16]. Culture supernatants containing virus were used for virus passage, measurement of replication kinetics and experimental infection of pigs.

2.3. In vitro characterisation of the Flc-LOM-BE^{rns} virus

CPK cells were infected with Flc-LOM-BE^{rns} and Flc-LOM, and titres were determined 72 h later by ICS using monoclonal antibody (mAb) 3B6 (MEDIAN Diagnostic Co. Cat No. 9011, Korea), which is specific for CSF glycoprotein E2, and mAb 4A4, which is specific for BVDV glycoprotein E^{rns}. The ability of CSFV to increase replication of Newcastle disease virus (NDV) in primary swine testicular (ST) cells, known as the exaltation of Newcastle disease (END) virus method, was evaluated as previously described [17]. Briefly, Flc-LOM and Flc-LOM-BE^{rns} were added to each well for 2 h at 37 °C. After 72 h, plates were inoculated with NDV Miyadera strain (10^6 PFU/ml) and cytopathic effects (CPE) were observed after 3 days. The growth kinetics of Flc-LOM and Flc-LOM-BE^{rns} in CPK cells were determined at 6, 12, 18, 24, 36, 48, 60 and 72 h by ICS using mAb 3B6 (specific for CSF glycoprotein E2).

2.4. Inoculation of pregnant sows with the Flc-LOM-BE^{rns} vaccine

To assess the efficacy of the Flc-LOM-BE^{rns} vaccine, 15 sows were purchased from Jeju Island (a CSF non-vaccinated region), which confirmed as negative for both CSF antibodies and antigen. Eleven sows were inoculated with the Flc-LOM-BE^{rns} vaccine ($10^{3.5}$ $TCID_{50}$/ml/dose) at 3 weeks prior to insemination. The remaining sows (n = 4) received a non-Flc-LOM-BE^{rns} vaccine as mock-vaccine control. After insemination, all 15 pregnant sows were divided into three groups. Each group was challenged with virulent CSFV at different stages of pregnancy: early (42 days), mid- (65 days) and late (90 days). The early, mid- and late groups comprised six, five and four sows, respectively. Of the six pregnant sows in the early group, five sows were received the Flc-LOM-BE^{rns} vaccine and one was inoculated with a non-Flc-LOM-BE^{rns} vaccine. The mid-group contained three vaccinated sows and two mock-vaccinated controls, and the late group contained three vaccinated sows and one mock-vaccinated control. All pregnant sows were challenged with a virulent CSFV strain (YC11WB) at a concentration of 10,000 LD_{50} via intramuscular inoculation. To detect shedding of the virulent YC11WB strain after challenge, faecal and nasal swabs were collected from 0 to 21 days post-infection (DPI). During the course of the experiment, pregnant sows were monitored daily for coughing, sneezing, appetite loss, movement and miscarriage or stillbirth. If pregnant sows died or gave birth to stillborn offspring, sows and foetuses were autopsied and 11 organs (tonsil, heart, kidney, lung, liver, spleen, small intestinal, large intestinal, submandibular lymph nodes, ileocecal lymph nodes and mesenteric lymph nodes) were examined to detect the presence of CSF antigen. To titre virus obtained from tissue samples, samples were diluted 10-fold and used to inoculate confluent monolayers of CPK cells. After 4 days of incubation, virus titres were calculated using the Reed and Muench formula and expressed as the 50% tissue culture infective dose ($TCID_{50}$) per gram. Blood samples obtained on the day of vaccination, at 2 weeks post-vaccination, on the day of insemination, on the day of challenge and on the day of farrowing were examined to detect serum neutralising antibodies. CSFV-specific neutralising antibodies were detected using a neutralising peroxidase-linked assay in accordance with the standards manual of the OIE [16].

2.5. Real-time PCR (qRT-PCR) of organ samples from pregnant sows and their offspring

The qRT-PCR was performed to detect the CSF antigen copy number in faecal and nasal swab samples after challenge inoculation.

The qRT-PCR was also used to identify vaccine virus shedding (in nasal and faecal samples) and to detect vaccine virus in the blood of pigs after inoculation with the Flc-LOM-BE^{rns} vaccine. The VDx® CSFV qRT-PCR (MEDIAN Diagnostic Co. Cat No. NS-CSF-31, Korea), which uses TaqMan probes, detects the CSFV 5′ untranslated region (5′ UTR) with high specificity; it does not detect BVDV or border disease virus, which also belongs to the *Pestivirus* genus. Briefly, the qRT-PCR program comprised the following steps: cDNA synthesis (50 °C, 30 min) and initial inactivation (95 °C, 15 min), followed by two step PCR comprising 42 cycles of denaturation (95 °C, 10 sec) and extension (60 °C, 60 sec).

2.6. Antibody ELISA

Seroconversion of pregnant sows before and after challenge with virulent CSFV was monitored using a serological neutralisation antibody test (SNT), a CSF E^{rns} ELISA and a BVDV E^{rns} ELISA. The VDPro® CSF Erns Ab b-ELISA (MEDIAN Diagnostic Co. Cat No. ES-CSF-05, Korea), a competition ELISA design to detect E^{rns} protein, also indicates the S/N value (>0.5 negative and 0.5 ≤ positive). The VDPro® BVDV Erns Ab i-ELISA (MEDIAN Diagnostic Co. Cat No. ES-BVD-01, Korea), which is based on the E^{rns} protein, provides an S/P ratio (≥0.6 positive and 0.6 < negative).

2.7. Safety of the Flc-LOM-BE^{rns} vaccine in pregnant sows

Eight sows were used to evaluate the safety of the Flc-LOM-BE^{rns} vaccine. One group (three sows) was inoculated with $10^{3.5}$ $TCID_{50}$/ml, and another (three sows) was inoculated with $10^{4.5}$ $TCID_{50}$/ml. Two sows were used as a negative control group. Clinical symptoms (fever, hypersensitivity, diarrhoea, suppuration or necrosis at the site of injection, abortion and any systemic response) were monitored daily after Flc-LOM-BE^{rns} vaccination. Blood samples were collected before vaccination, at 2 WPV, after parturition and from birth of piglets. Blood was tested for specific antibodies using the SNT, CSF E^{rns} ELISA and BVDV E^{rns} ELISA.

2.8. Examination for post-mortem

All challenged sows and piglets were subjected to necropsy to determine the presence of pathological lesions in different organs and tissues. Tissue samples were collected in 10% neutral buffered formalin and processed for microscopic evaluation using a double-blind protocol. ICS to detect CSFV was performed using mAb 3B6, which is specific for CSF glycoprotein E2.

2.9. Statistical analysis

All statistical analyses were performed using the GRAPHPAD PRISM software, version 5.03, for Windows.

3. Results

3.1. Sequence comparison between full-length genomic Flc-LOM and Flc-LOM-BE^{rns}

The Flc-LOM-BE^{rns} virus genome is 12,298 nucleotides in length, which is the same as the Flc-LOM virus [15]. Comparison of the complete nucleotide sequences of Flc-LOM-BE^{rns} with sequences deposited in the GenBank database (GenBank No. DI183602) revealed that the virus is most closely related to CSF strain LOM (98.1% sequence identity at the nt level; GenBank No. EU789580) and Alfort/187 (97.6% identity; GenBank No. X87939). Comparison of the full-length genomic Flc-LOM and Flc-LOM-BE^{rns} sequences revealed that the 3′ end capsid gene and the E^{rns} gene of the latter reside at nt positions (from 1123 to 1855) different from those in the former. The E^{rns} nt sequences of Flc-LOM-BE^{rns} showed high (99.5%) identity with those of BVDV type 1 (KD-26 strain) from Korea.

3.2. In vitro growth and recovery of infectious Flc-LOM-BE^{rns}

The 5′ end of the genome includes a *Sac* II restriction site and the T7 promoter sequence, which was placed immediately upstream of the viral genome and facilitates *in vitro* transcription of authentic viral RNA. A *Srf* I restriction site was introduced at the 3′ end of the viral genome to allow generation of authentic viral RNA by runoff transcription. The assembled full-length cDNA clone, called pFlc-LOM-BE^{rns}, was linearised by digestion with *Srf* I and used as a template for *in vitro* transcription by T7 RNA polymerase. CPK cells were transfected with the *in vitro* transcripts using a liposome-mediated method. About 50% of cells expressed envelope glycoprotein E2 at 72 h post-transfection. Supernatants collected from transfected cells contained infectious CSFV, as demonstrated by addition of supernatant to CPK cells. Culture supernatant recovered from first passage cells at 3 days post-inoculation consistently yielded a viral titre of $10^{4.5}$ $TCID_{50}$/ml. To confirm recovery of virus from cells infected with Flc-LOM-BE^{rns} transcripts, we used an ICS assay with mAb 3B6, which was raised against glycoprotein E2 of CSFV, and mAb 4A4, which reacts with the E^{rns} protein of BVDV (supplemental Fig. 2A). The cytoplasm-specific straining pattern of Flc-LOM-BE^{rns}-infected cells was identical to that of cells infected with the parental Flc-LOM virus (supplemental Fig. 2A).

3.3. A bio-marker of Flc-LOM-BE^{rns}

Flc-LOM and Flc-LOM-BE^{rns} induced NDV cytopathogenicity in primary ST cells, indicating that Flc-LOM was END phenomenon-positive and that Flc-LOM-BE^{rns} was END-negative (supplemental Fig. 2B). Analysis of the replication kinetics of Flc-LOM and Flc-LOM-BE^{rns} viruses in CPK cells revealed that they reached the highest titre at the same time point (72 h) (supplemental Fig. 2C).

3.4. Virus shedding by pregnant sows before and after CSFV challenge

At 2 WPV and 3 WPV, no shedding of the vaccine virus was detected in nasal and faecal swabs taken from 11 sows inoculated with Flc-LOM-BE^{rns} (Table 1). Virus-specific qRT-PCR results also revealed that nasal and faecal swab samples taken from five sows challenged during early pregnancy (42 days) were virus-negative up to 21 DPI (Table 1). By contrast, faecal and nasal samples taken from control sow C35 at 3–14 DPI contained high copy numbers (3.6–6.4 log_{10}) of virus, and sow C35 aborted the foetus at 12 DPI (Table 1). Most mid-gestation sows (65 days) inoculated with the Flc-LOM-BE^{rns} vaccine did not shed virus after challenge; however, negative control sows C39 and C40 shed viruses at 3–14 DPI and aborted the foetus at 8 DPI (C39) and 10 DPI (C40) (Table 1). Sows challenged at the late stage of pregnancy (90 days) did not shed virus, but control sow C36 harboured high viral copy numbers in faecal and nasal samples at 3–14 DPI and aborted the foetus at 13 DPI (Table 1).

3.5. Seroconversion in pregnant sows after and before CSFV challenge

The serum neutralising antibody titre (log_2) in the early pregnancy group significantly increased by 4–5 (average, 4.4) at 2 WPV, by 7–9 (7.8) at 3 WPV, by 8–10 (9) at 8 WPV and by 11–13 (12.2) at 18 WPV ($p < 0.05$) (Table 2). Pigs were inseminated at 3 WPV and challenged with virulent virus at 8 WPV. Fallowing time was at 18 WPV. Sows in the mid-pregnancy group showed an 8–9

Table 1
Detection of virus shedding after vaccination and detection of antigen copy number after challenge of pregnant pigs with classical swine fever virus.

Pregnancy (period)	Vaccination	Sow	Sample	RNA copies (log_{10}) at WPV*			RNA copies (log_{10}) at different days post-CSFV infection (DPI)								Stillbirth
				0	2	3	0	3	5	7	9	11	14	21	
Early (42 days)	Flc-LOM-BE^{rns} vaccinated	S5	Nasal	–	–	–	–	–	–	–	–	–	–	–	None
		S5	Faecal	–	–	–	–	–	–	–	–	–	–	–	
		S7	Nasal	–	–	–	–	–	–	–	–	–	–	–	None
		S7	Faecal	–	–	–	–	–	–	–	–	–	–	–	
		S20	Nasal	–	–	–	–	–	–	–	–	–	–	–	None
		S20	Faecal	–	–	–	–	–	–	–	–	–	–	–	
		S23	Nasal	–	–	–	–	–	–	–	–	–	–	–	None
		S23	Faecal	–	–	–	–	–	–	–	–	–	–	–	
		S38	Nasal	–	–	–	–	–	–	–	–	–	–	–	None
		S38	Faecal	–	–	–	–	–	–	–	–	–	–	–	
	Mock- vaccinated	C35	Nasal	–	–	–	–	–	3.6	5.7	5.8	6.4	5.9	Biopsy	12 DPI
		C35	Faecal	–	–	–	–	2.4	4.8	5.5	5.7	4.4	4.2		
Mid (65 days)	Flc-LOM-BE^{rns} vaccinated	S15	Nasal	–	–	–	–	–	–	–	–	–	–	–	None
		S15	Faecal	–	–	–	–	–	–	–	–	–	–	–	
		S16	Nasal	–	–	–	–	–	–	–	–	–	–	–	None
		S16	Faecal	–	–	–	–	–	–	–	–	–	–	–	
		S18	Nasal	–	–	–	–	–	–	–	–	–	–	–	None
		S18	Faecal	–	–	–	–	–	–	–	–	–	–	–	
	Mock- vaccinated	C39	Nasal	–	–	–	–	2.9	3.5	5.7	4.9	3.4	4.8	Autopsy (18 DPI)	8 DPI
		C39	Faecal	–	–	–	–	–	3.3	3.9	5.4	4.8	5.5		
		C40	Nasal	–	–	–	–	2.4	4.5	4.7	6.4	5.6	3.7	Autopsy(20 DPI)	10 DPI
		C40	Faecal	–	–	–	–	2.8	2.7	5.6	4.7	4.8	4.6		
Late (90 days)	Flc-LOM-BE^{rns} vaccinated	S14	Nasal	–	–	–	–	–	–	–	–	–	–	–	None
		S14	Faecal	–	–	–	–	–	–	–	–	–	–	–	
		S25	Nasal	–	–	–	–	–	–	–	–	–	–	–	None
		S25	Faecal	–	–	–	–	–	–	–	–	–	–	–	
		S26	Nasal	–	–	–	–	–	–	–	–	–	–	–	None
		S26	Faecal	–	–	–	–	–	–	–	–	–	–	–	
	Mock- vaccinated	C36	Nasal	–	–	–	–	2.6	3.7	4.8	4.5	5.9	4.1	Autopsy(17 DPI)	13 DPI
		C36	Faecal	–	–	–	–	2.3	3.8	5.4	4.4	5.7	3.9		

–: not detected.
* WPV: weeks post-CSF E^{rns} vaccination (one vaccine inoculation at 3 weeks prior to insemination).

Table 2
Seroconversion after CSF vaccination and virulence challenge.

Group	Sow	Pregnancy (period)	Seroconversion (SN titre/CSF E^{rns} ELISA/BVDV E^{rns} ELISA)*						
			0 weeks (vaccination)	2 weeks	3 weeks (insemination)	8 weeks (challenge)	11 weeks (challenge)	15 weeks (challenge)	18 weeks (farrowing)
Flc-LOM-BE^{rns} vaccinated	S5	114	1</0.74/0.28	4/0.62/0.74	7/0.76/0.88	9/0.72/1.14	NT**	NT	13/0.39/1.28
	S7	115	1</0.67/0.34	4/0.88/0.69	8/0.83/0.79	9/0.94/1.42	NT	NT	12/0.28/1.31
	S20	117	1<//0.88/0.17	5/0.75/0.71	9/0.64/1.31	9/0.73/1.27	NT	NT	11/0.43/1.09
	S23	115	1</0.74/0.14	5/0.86/0.84	7/0.71/0.91	8/0.69/0.84	NT	NT	13/0.25/1.24
	S38	115	1</0.85/0.38	4/0.94/0.82	8/0.85/0.85	10/0.65/1.21	NT	NT	12/0.36/1.16
	S15	114	1</0.78/0.25	4/0.68/0.77	8/0.67/1.48	NT	9/0.72/1.18	NT	12/0.42/1.37
	S16	113	1</0.63/0.22	4/0.81/0.83	7/0.71/1.02	NT	9/0.66/1.24	NT	12/0.32/0.94
	S18	113	1</0.82/0.41	5/0.93/0.74	8/0.78/0.74	NT	8/0.89/1.03	NT	14/0.39/1.51
	S14	112	1</0.93/0.19	5/0.76/0.81	8/0.72/1.21	NT	NT	9/0.68/1.17	14/0.48/1.41
	S25	114	1</0.88/0.24	5/0.85/0.93	9/0.64/1.34	NT	NT	8/0.84/1.19	11/0.45/1.07
	S26	115	1</0.75/0.21	6/0.91/0.87	9/0.74/1.05	NT	NT	8/0.71/0.89	12/0.37/1.23
Mock-vaccinated	C35	54	1</0.84/0.17	1</0.78/0.29	1</0.78/0.13	1</0.71/0.24	NT	NT	NT
	C39	73	1</0.69/0.24	1</0.86/0.41	1</0.66/0.21	NT	1</0.87/0.31	NT	NT
	C40	75	1</0.95/0.15	1</0.83/0.32	1</0.75/0.28	NT	1</0.72/0.17	NT	NT
	C36	103	1</0.71/0.26	1</0.95/0.19	1</0.87/0.34	NT	NT	1</0.75/0.23	NT

* SN titre (serological neutralising antibody titre: log_2)/CSF E^{rns} ELISA (SN value: >0.5 negative and 0.5 ≤ positive)/BVDV E^{rns} ELISA (SP value: ≥0.6 positive and 0.6 < negative).
** NT: not tested.

(average, 8.7 log_2) increase in serum neutralising antibody titre at 11 WPV and a 12–14 (12.7) increase at 18 WPV ($p < 0.05$). The SN titre (log_2) in sows in the late pregnancy group showed an 8–9 (8.3) increase at 15 WPV and an 11–14 (12.3) increase at 18 WPV ($p < 0.05$) (Table 2). Because sows were challenged during pregnancy, a high antibody seroconversion rate was detected at fallowing time. The CSF SNT revealed that sows were positive for neutralising antibodies at all times (2, 3, 8, 11, 15 and 18 WPV). However, they were negative (S/N value: >0.5) for antibodies specific for the CSF E^{rns} protein before challenge with virulent virus. The CSF E^{rns} ELISA showed that sows were positive (S/N value: <0.5) for anti-CSF E^{rns} antibodies after virus challenge (Table 2). Anti-BVDV E^{rns} antibodies appeared at 2 WPV and sows remained anti-BVDV E^{rns} antibody-positive after challenge with CSFV (Table 2).

3.6. Status of offspring at fallowing

Overall, the 11 sows challenged with virulent virus gave birth to 119 piglets (average, 10.9 per sow). Among these, 114 (average, 10.3 per sow) were born alive and five (average, 0.4 per sow) were stillborn (Table 3). The average weight of the 114 piglets born alive was 1568 ± 184.2 g, and the crown rump length (the length of the piglet from the top of the head to the bottom of the torso) was 32.1 ± 1.7 cm (Table 3). The average duration of pregnancy for the 11 sows was 114.2 days. The four control sows (C35, C39, C40 and C36) aborted between Days 8 and 13 (average, 10.7 days) post-virulent virus challenge. Three sows underwent autopsy immediately after death (at 18 DPI (C39), 20 DPI (C40) and 17 DPI (C36)), whereas sow C35 was biopsied at 21 DPI.

3.7. Transplacental transmission by pregnant sows

Autopsy and biopsy for four pregnant sows (controls) was performed at 17, 18, 20 and 21 DPI to examine virus titres in 11 organs. Virus titration revealed that sow C35 (early pregnancy), sows C39 and C40 (mid-pregnancy) and sow C36 (late pregnancy) showed high virus titres in 9 (C35), 8 (C39), 6 (C40) and 11 (C36) organs (Table 4). The qRT-PCR detected virulent virus in 4/10 foetuses (40%) from C35, 6/7 foetuses (85.7%) from C39, 11/11

Table 3
Status of offspring from pregnant sows after challenge with CSFV.

Group	Sow	Pregnancy (period)	Number of foetuses				Average weight (g)	Average length (C-R)
			Total	Alive	Dead	Mummified		
Flc-LOM-BE^{rns}-vaccinated	S5	114	11	11	0	0	1431.8 ± 237.5	33.3 ± 2.5
	S7	115	13	13	0	0	1501.5 ± 184.2	34.7 ± 2.1
	S20	117	15	14	1	0	1374.4 ± 125.6	32.3 ± 1.6
	S23	115	8	8	0	0	1462.5 ± 152.9	33.1 ± 1.9
	S38	115	4	4	0	0	1790.0 ± 261.4	34.8 ± 2.3
	S15	114	6	6	0	0	1868.3 ± 235.2	34.7 ± 1.7
	S16	113	13	13	0	0	1914.6 ± 141.3	34.9 ± 1.5
	S18	113	10	9	1	0	1746.7 ± 203.6	34.9 ± 2.5
	S14	112	15	14	1	0	1410.0 ± 166.2	30.9 ± 1.3
	S25	114	15	14	1	0	1247.1 ± 129.7	31.4 ± 2.2
	S26	115	9	8	1	0	1543.8 ± 226.1	30.3 ± 1.4
Mock-vaccinated	C35	54	10	0	10	0	–	–
	C39	73	7	0	7	0	–	–
	C40	75	11	0	11	0	–	–
	C36	103	18	0	18	0	–	–

–: not tested.

Table 4
Detection of classical swine fever antigen in the organs of sows and foetuses.

Group	Sow	No. of positive/total no. of foetuses (%)	CSF antigen-positive organs from sows (log_{10} $TCID_{50}$/g) and foetuses (no. of positive/total no. of foetuses)										
			To*	He	Ki	Lu	Liv	Sp	Si	Li	Ln.1	Ln.2	Ln.3
Flc-LOM-BE^{rns}-vaccinated	S5		–	–	–	–	–	–	–	–	–	–	–
		0/11 (0)	–	–	–	–	–	–	–	–	–	–	–
	S7		–	–	–	–	–	–	–	–	–	–	–
		0/13 (0)	–	–	–	–	–	–	–	–	–	–	–
	S20		–	–	–	–	–	–	–	–	–	–	–
		0/15 (0)	–	–	–	–	–	–	–	–	–	–	–
	S23		–	–	–	–	–	–	–	–	–	–	–
		0/8 (0)	–	–	–	–	–	–	–	–	–	–	–
	S38		–	–	–	–	–	–	–	–	–	–	–
		0/4 (0)	–	–	–	–	–	–	–	–	–	–	–
	S15		–	–	–	–	–	–	–	–	–	–	–
		0/6 (0)	–	–	–	–	–	–	–	–	–	–	–
	S16		–	–	–	–	–	–	–	–	–	–	–
		0/13 (0)	–	–	–	–	–	–	–	–	–	–	–
	S18		–	–	–	–	–	–	–	–	–	–	–
		0/10 (0)	–	–	–	–	–	–	–	–	–	–	–
	S14		–	–	–	–	–	–	–	–	–	–	–
		0/15 (0)	–	–	–	–	–	–	–	–	–	–	–
	S25		–	–	–	–	–	–	–	–	–	–	–
		0/15 (0)	–	–	–	–	–	–	–	–	–	–	–
	S26		–	–	–	–	–	–	–	–	–	–	–
		0/9 (0)	–	–	–	–	–	–	–	–	–	–	–
Mock-vaccinated	C35		6.4	4.5	–	3.6	5.7	6.8	4.5	6.7	6.1	–	6.1
		4/10 (40)	4/10	–	3/10	1/10	2/10	2/10	2/10	–	2/10	–	3/10
	C39		5.2	3.6	3.4	2.2	–	6.4	–	3.6	3.6	4.5	–
		6/7 (85.7)	6/7	2/7	3/7	2/7	1/7	3/7	2/7	1/7	1/7	–	3/7
	C40		6.1	3.1	3.1	4.7	–	5.9	–	–	–	5.3	–
		11/11 (1 0 0)	9/11	4/11	8/11	3/11	2/11	2/11	–	1/11	2/11	–	3/11
	C36		7.0	4.8	4.5	3.5	4.1	4.3	2.9	3.0	3.6	4.5	5.0
		14/18 (77.8)	14/18	3/18	4/18	3/18	2/18	5/18	2/18	1/18	1/18	1/18	4/18

–: not detected.
* To, tonsil; He, heart; Ki, kidney; Lu, lung; Liv, liver; Sp, spleen; Si, small intestine; Li, large intestine; Ln.1, submandibular lymph node; Ln.2, ileocecal lymph node; Ln.3, mesenteric lymph node.

Table 5
Safety of the Flc-LOM-BE^{rns} vaccine in sows.

Vaccination time	Vaccine concentration	Sow	Seroconversion (*SN titre/CSF E^{rns} ELISA/BVDV E^{rns} ELISA)/vaccine virus detection (RNA copies: log$_{10}$)			Clinical signs for sows	No. of births	No. of surviving suckling piglets
			0 weeks (vaccination)	2 weeks	After parturition			
2 weeks before insemination	$10^{3.5}$ TCID$_{50}$/dose	V10	1</0.82/0.29/–	5/0.76/0.71/–	8/1.06/1.02/–	–	14	12
		V11	1</0.87/0.31/–	4/0.68/0.82/–	7/0.93/1.26/–	–	10	10
		V12	1</0.75/0.18/–	6/0.83/0.79/–	8/0.86/0.93/–	–	13	11
	$10^{4.5}$ TCID$_{50}$/dose	V30	1</0.79/0.35/–	5/0.69/0.84/–	9/0.93/0.95/–	–	11	10
		V31	1</0.83/0.15/–	6/0.87/0.92/–	8/1.09/1.03/–	–	9	9
		V32	1</0.94/0.25/–	6/0.75/0.84/–	8/1.14/0.97/–	–	15	14
Negative control	No vaccine	C41	1</0.91/0.28/–	1</0.86/0.34/–	1</0.82/0.22/–	–	12	10
		C42	1</0.78/0.17/–	1</0.84/0.19/–	1</0.95/0.15/–	–	13	12

–: not detected. Clinical signs in sows were fever, hypersensitivity, diarrhoea, suppuration or necrosis at the site of injection, abortion and any systemic response.
* SN titre (serological neutralising antibody titre: log$_2$); CSF E^{rns} ELISA (SN value: >0.5 negative and 0.5 ≤ positive); BVDV E^{rns} ELISA (SP value: ≥0.6 positive and 0.6 < negative).

foetuses (100%) from C40 and 15/18 foetuses (83.3%) from C36 (Table 4). However, qRT-PCR revealed that all organs from 114 live piglets and all dead foetuses borne by pregnant sows inoculated with Flc-LOM-BE^{rns} were negative for virus. In addition, no specific gross or microscopic lesions related to CSFV were observed in 11 organ samples from vaccinated sows and their piglets (data not shown). However, lesions characteristic of CSF were observed in many tissues from control sows and their foetuses (data not shown). Grossly visible foetal lesions including ascites, hepatic nodularity, pulmonary hypoplasia, petechiation of the skin, microencephaly, hydrocephalus and cerebellar hypoplasia were observed. Moreover, petechial and ecchymotic haemorrhages, classical lesions of CSF, identified at several common sites including the epiglottis, bladder mucosa, cortex/pelvis of kidneys, gall bladder mucosa, lungs, heart, and the ileocecal junction. Most of control sows and their foetuses showing classical CSFV gross/microscopic lesions were also positive for virus gene detection and ICS (immunocytochemical staining) of organs.

3.8. Safety of vaccination in pregnant sows

To evaluate the safety of overdose vaccination, sows were inoculated with 1 dose or 10 doses of vaccine. No specific clinical symptoms were observed up until the time of birth, and all sows underwent normal delivery. The qRT-PCR did not detect vaccine virus in blood samples at 2 WPV or at the time of farrowing. Neutralising antibody titres averaged 5 (log$_2$) at 2 WPV and 8 (log$_2$) at the time of farrowing, and anti-BVDV E^{rns} antibody titres were positive throughout the experiment; however, anti-CSF E^{rns} antibodies were negative throughout the experiment (Table 5).

4. Discussion

A next-generation CSF marker vaccine candidate must be safe, show high efficacy, have DIVA potential and be marketable. Inoculation of pigs with the Flc-LOM-BE^{rns} vaccine induced production of anti-CSF E2 antibodies and anti-BVDV E^{rns} antibodies simultaneously, but no anti-CSF E^{rns} antibodies. Therefore, Flc-LOM-BE^{rns}-vaccinated pigs are anti-CSF E^{rns} antibody-negative and anti-BVDV E^{rns} antibody-positive. Pigs infected with virulent CSFV are anti-CSF E^{rns} antibody-positive and anti-BVDV E^{rns} antibody-negative. In addition, when pigs vaccinated with Flc-LOM-BE^{rns} were infected by virulent CSFV, they became anti-CSF E^{rns} antibody-positive and anti-BVDV E^{rns} antibody-positive. Therefore, the presence of anti-CSF E^{rns} antibodies is an important discriminatory marker required for a DIVA vaccine. In a previous study, pigs were challenged with two CSF marker vaccines (BAYOVAC CSF Marker and PORCILIS PESTI) at 2 WPV; however, two CSF E^{rns} kits (Ceditest and Chekit) revealed that the neutralising antibody titre was low and the anti-CSF E^{rns} antibody titre was negative [18]. Anti-CSF E^{rns} antibody-positive and -negative rates were similar at 7–15 days post-challenge; however, almost all pigs were positive at 42–49 days post-challenge. These results show that, when the CSF marker vaccine was inoculated twice, the anti-CSF E^{rns} antibody titre was positive at 50 days post-challenge [18]. Another study reported inoculation of a pregnant sow with a CSF marker vaccine (PORCILIS PESTI), resulting in detection of anti-CSF E^{rns} antibodies at 40 days post-challenge, although no anti-CSF E^{rns} antibody was present before challenge [19]. These results were confirmed in a study using the live CSF marker vaccine (CP7_E2alf), which was inoculated into sows; two kits (PrioCHECK CSF E^{rns} antibody ELISA and pig-type Erns antibody ELISA) failed to detect anti-CSF E^{rns} at 9 days post-challenge with moderately virulent CSFV [20]. However, all sows were anti-CSF E^{rns} antibody-positive at 44–47 days post-challenge. Interestingly, SPF pigs inoculated with the CP7_E2alf vaccine were positive for anti-CSF E^{rns} antibodies at 21 days post-challenge, but commercial pigs were anti-CSF E^{rns} antibody-positive at various times post-challenge [21]. In the present study, no anti-CSF E^{rns} antibody was present at the time of Flc-LOM-BE^{rns} vaccination, but pigs were anti-CSF E^{rns} antibody-positive at 3 weeks post-challenge. Detection of anti-BVDV E^{rns} antibodies after immunisation with Flc-LOM-BE^{rns} is an important key to differential diagnosis; however, not many studies have used the ELISA method to detect anti-BVDV E^{rns} antibodies. Our results showed that the level of anti-BVDV E^{rns} antibodies was significantly increased from 2 weeks after Flc-LOM-BE^{rns} vaccination regardless of vaccination doses (1 or 10 doses) compared to the control sows (no vaccination) ($p < 0.05$). We suggest that differential diagnosis based on levels of different antibodies is effective, although additional experiments are required to confirm the results.

The Flc-LOM-BE^{rns} live CSF marker vaccine provided complete protection to pregnant sows, regardless of pregnancy period. No piglets born to these sows harboured CSF antigens, and antibodies were detected in colostrum pre-ingestion. To evaluate the efficacy of CSF vaccines, it is very important to study not only prevention of horizontal transmission, but also vertical transmission from vaccinated pregnant sows to their offspring. As demonstrated by several groups [22–24], transplacental transmission of low virulence CSFV results in persistent infection of piglets. E2 subunit vaccines do not prevent virus transmission *in utero*; indeed, they only reduce transplacental CSF transmission [18,19,25]. A previous study suggests that all animals developed E2-specific neutralising antibodies 35 days after a first vaccination with the subunit marker vaccine Porcilis® Pesti, with titres ranging from 5.0 to 7.5 log$_2$; no anti-CSFV E^{rns} antibodies were detected by ELISA [19]. The study also showed that nine out of ten piglets in litters from vaccinated sows were protected from CSFV infection; however, the vaccine did not

protect 10% of piglets from viral infection when sows were challenged mid-gestation with the CSF strain Glentorf [19]. Vaccination with the CSFV E2 subunit vaccine reduces transmission of a moderately virulent strain of CSFV from the sow to the offspring [25]. Another study used a lentivirus-based gene delivery system to obtain a stable recombinant HEK 293 cell line for expression of E2 fused to porcine CD154 and then evaluated the ability of double vaccination with this novel subunit marker vaccine to prevent CSFV vertical transmission [26]. The results showed that 100% of foetuses from vaccinated sows were protected against vertical transmission of CSFV (challenge at approximate 91 days gestation after two inoculations). Only two (one tonsil and one thymus) out of 208 samples were positive for virus (Ct value, ~36), although these results may be due to detection of non-infectious viral particles [26]. A recent study demonstrated that pregnant sows and their foetuses were fully protected after a single dose of the DIVA vaccine "CP7_E2alf" [20]; vertical transmission of a relevant, moderately virulent CSF was prevented completely. The CP7_E2alf vaccine study suggests that, in the context of emergency vaccination, incomplete protection upon early challenge with highly virulent strains should still be taken into consideration [20]. The decision to vaccinate sows depends upon a risk assessment implemented by the relevant authorities during potential outbreak situations.

The END phenomenon is induced by inhibition of type I interferon production by Pestivirus-infected cells *in vitro*, which occurs via proteasomal degradation of cellular interferon regulatory factor (IRF)-3 by the viral autoprotease protein Npro [27]. Amino acid residues within the zinc-binding TRASH motif of Npro determine the characteristics of END phenomenon-positive and END phenomenon-negative CSFVs [27]. Another study shows that the cytopathogenicity of CSFVs lacking the END phenomenon is associated with accumulation of NS3 in serum-free cultured cell lines [28]. The novel DIVA vaccine (Flc-LOM-BE^{rns}) used in the present study was constructed by replacing the 3′ end part of the capsid protein and the full-length E^{rns} glycoprotein from the KD26 BVDV strain using a LOM CSFV backbone; this resulted in an END-negative strain, which is more stable. However, it is unclear why this END-negative phenomenon occurred.

In conclusion, the novel live CSF marker vaccine Flc-LOM-BE^{rns} protected foetuses from vertical transmission after challenge of pregnant sows with CSFV, irrespective of the pregnancy period (early, mid- or late). In addition, the Flc-LOM-BE^{rns} vaccine showed good safety, high efficacy and good differential identification of antibodies in serum, all of which fulfil the function of DIVA as required by the OIE.

Acknowledgements

This project was supported by a grant (Project Code nos. B-1543083-2017-18-02 and B-1543083-2019-20-01) from the Animal and Plant Quarantine Agency (QIA), Republic of Korea, 2017–2019.

Conflict of interest statement

The authors declare no competing interests.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.04.076.

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