Co-expression of the C-terminal domain of *Yersinia enterocolitica* invasin enhances the efficacy of classical swine-fever-vectored vaccine based on human adenovirus

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The use of adenovirus vector-based vaccines is a promising approach for generating antigen-specific immune responses. Improving vaccine potency is necessary in other approaches to address their inadequate protection for the majority of infectious diseases. This study is the first to reconstruct a recombinant replication-defective human adenovirus co-expressing E2 and invasin C-terminal (InvC) glycoproteins (rAd-E2-InvC). rAd-E2-InvC with 2×10^6 $TCID_{50}$ was intramuscularly administered two times to CSFV-free pigs at 14 day intervals. No adverse clinical reactions were observed in any of the pigs after the vaccination. The CSFV E2-specific antibody titer was significantly higher in the rAd-E2-InvC group than that in the rAdV-E2 group as measured by NPLA and blocking ELISA. Pigs immunized with rAd-E2-InvC were completely protected against lethal challenge. Neither CSFV RNA nor pathological changes were detected in the tissues after CSFV challenge. These results demonstrate that rAd-E2-InvC could be an alternative to the existing CSF vaccine. Moreover, InvC that acts as an adjuvant could enhance the immunogenicity of rAdV-E2 and induce high CSFV E2-specific antibody titer and protection level.

[Li H, Ning P, Lin Z, Liang W, Kang K, He L and Zhang Y 2015 Co-expression of the C-terminal domain of *Yersinia enterocolitica* invasin enhances the efficacy of classical swine-fever-vectored vaccine based on human adenovirus. *J. Biosci.* **40** 79–90] **DOI** 10.1007/s12038-014-9495-z

1. Introduction

Classical swine fever (CSF) is a highly contagious disease of swine and wild boar caused by *Classical swine fever virus* (CSFV) and is listed as a disease by the World Animal Health Organization (Moennig *et al.* 2003; Zhao *et al.* 2008). Vaccines and 'stamping-out' slaughter policy play an important role in controlling CSF (especially the recessive infection and persistent infection), which causes great economic losses to livestock (Moennig 2000). In recent years, the disease presented recurrence tendency in America, Asia, Europe, and other countries, and recurrence has been reported in some countries (such as Germany and Belgium) where CSF has been eradicated (Penrith *et al.* 2011). In China, the morbidity and mortality of CSF were significantly reduced because a live attenuated vaccine has been widely used. However, this vaccine was unable to discriminate between naturally infected pigs and vaccine-injected pigs. Therefore, developing a new type of swine fever marker vaccine is necessary.

CSFV is a small, enveloped, single-stranded, positive-sense RNA virus that belongs to the *Pestivirus* genus of

Keywords. Classical swine fever (CSF); E2; human adenovirus; invasin; vaccine

Published online: 11 January 2015

the Flaviviridae family. The RNA genome of CSFV is approximately 12.3 kb and is flanked by 5′ and 3′ non-translated regions (Meyers *et al.* 1996) and a large open reading frame that encodes a typical polyprotein of 3898 amino acids. The polyprotein undergoes co- and post-translational processing by cellular and viral proteases (Meyers and Thiel 1996; Meyers *et al.* 1996). The polyprotein processing generates four structural proteins of C, E^{RNS}, E1 and E2, and eight non-structural proteins of N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Stark *et al.* 1993; Van Rijn *et al.* 1994). In CSF vaccine, the envelope glycoprotein E2 is considered the major target of neutralizing antibodies (NAbs) during natural CSFV infection (Langedijk *et al.* 2001). E2 has also been used as the main component in designing CSFV marker vaccines, such as viral-vectored vaccines (Reimann *et al.* 2004; Voigt *et al.* 2007; Sun *et al.* 2010, 2013b;), subunit vaccines (Holinka *et al.* 2009; Leifer *et al.* 2009b), and DNA vaccine (Andrew *et al.* 2000; Ganges *et al.* 2005b; Tarradas *et al.* 2010b). Some of these vaccines confer varied protection to pigs against CSFV.

AdV vectors are versatile vaccine carriers and are capable of inducing strong CD4+ and CD8+ T cell antibody responses (Barefoot *et al.* 2008). The CSF vaccine, which uses HAdV-5 as a viral vector, obtains good immune protective effect in swine (Sun *et al.* 2010, 2013b) and proved that HAdV vectors are effective vehicles in delivering variety of agents to swine. The recombinant adenovirus (rAdV-E2) expressing only the glycoprotein E2 provides protection against virulent CSFV. However, the protection is unstable because some vaccinated pigs occasionally present clinical symptoms and pathological changes (Sun *et al.* 2010). Some strategies have been adopted to further optimize the efficacy of rAdV-E2. The strategies that are effective in enhancing the immune effect in vaccine design include co-expression of *E2* and E^{rns} genes, which induce virus-NAb and mount protective immunity in the natural host (Sun *et al.* 2012), transform vector to chimeric alphavirus replicon to increase the efficiency of adenovirus delivery (Sun *et al.* 2011), or combine with some cytokines (e.g., IL-2) that present adjuvant activity (Lin *et al.* 2005; Toubaji *et al.* 2007; Zhang *et al.* 2008).

Yersinia pseudotuberculosis invasin (Inv) protein is a 986-amino-acid outer-membrane protein encoded by the chromosomal *inv* locus (Anderson *et al.* 2006). It is a member of a large family of outer membrane proteins that are found in Gram-negative bacteria and is involved in promoting interaction with target host cells (Wong and Isberg 2005). Inv protein, which binds to β1-integrins of host cells, confers internalization into expression of proinflammatory cytokines by host cells. Inv protein also initiates signalling cascades, including activation of focal adhesion complexes, Rac1, mitogen-activated protein kinase, and NF-kB. When Inv is fused to antigen and coated to microparticles, it induces both CD4 and CD8 T cell responses, indicating the potential of Inv as a novel vaccination tool that confers both host cell uptake and adjuvant activity (Buhler *et al.* 2006; Autenrieth and Autenrieth 2008). Inv promotes the M-cell uptake in the gut by binding to β1-integrins, targeting delivery of antigen to the lymphatic tissues (Clark *et al.* 1998). This characteristic is used to enhance M-cell uptake of noninvasive *Shigella* or *Escherichia coli* for vaccination (Critchley-Thorne *et al.* 2006; Suzuki *et al.* 2006).

The C-terminal 192 amino acids of Inv (referred to as InvC) are sufficient for binding to β1-integrins and internalization (Rankin *et al.* 1992; Leong *et al.* 1995). Two domains of InvC that confer binding to β1-integrins (Leong *et al.* 1990) of M-cells are also sufficient for presenting the functions of full-length form. Given these phenomena, we propose that InvC is suitable as a delivery molecule and adjuvant similar to full-length Inv if used as a vaccine component. *E2* gene of CSFV Shimen strain and InvC by HAdV-5 was co-expressed, and its efficacy was evaluated in pigs. The adjuvant activity of InvC was evaluated by inducing high CSFV E2-specific antibody titer and protection of pigs against lethal challenge with virulent CSFV after inoculation of recombinant HAdV-5 co-expression of E2 and InvC (rAd-E2-InvC).

2. Materials and methods

2.1 *Reagents, cells and viruses*

Human embryonic kidney HEK 293 cell line was cultured in high-glucose Dulbecco's-modified Eagle's medium (DMEM) (Gibco, UK) containing 10% heat-inactivated fetal calf serum (Hyclone, China). Heparin (50 μg/mL; Sigma-Aldrich, USA) and antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin) were used to generate, propagate, and titrate recombinant adenoviral vectors from replication-defective human adenovirus human type 5 (rAd5). Neutralizing peroxidase-linked assay (NPLA) was employed to evaluate CSFV-specific NAbs, and CSFV isolation was conducted in pig kidney cell line PK-15. The virulent CSFV 'Shimen' strain was provided by the Control Institute of Veterinary Bioproducts and Pharmaceuticals (China). CSFV C-strain vaccine (lot number 2012003) is a commercially available vaccine manufactured by LvFang Biotech Co., YangLing, China.

2.2 *Construction of the recombinant adenovirus*

The primers for E2 are F1 (5′-ACGCGTCGACATGCGG CTAGCCTGCAAGGAAGATT-3′) (underlined sequences

indicate the restriction enzyme of SalI) and R1 (5AAGGAAAAA GCGGCCGCACCAGCGGCGAGTTGTTCTGTTAG-3′) (underlined sequences indicate the restriction enzyme of NotI), and the primers for InvC are F2 (5AAGGAAAAAAAGCGGCCGCGGATCCGGATC AGGTTCAGGGAG-3′) (underlined sequences indicate the restriction enzyme of NotI) and R2 (5CCCAAGCTT*AGCGTAATCTGGAACATCGTATGGGTA*TATTGCCAGCGCACAGAGCGGG-3′) (underlined sequences indicate the restriction enzyme of HindIII, and italicized sequences indicate the introduced HA tag). These primers were designed based on the CSFV gene sequence from the 'Shimen' strain (GenBank: AY775178.2) and Inv gene of *Y. pseudotuberculosis* (GenBank: HE80523 0.1) (graciously provided by Dr. Enqi Du, College of Veterinary Medicine, Northwest A&F University, China).

The CSFV E2 (nt 1 to 1119) and InvC (nt 1 to 606) genes were amplified from pMD-32a-E2 and pMD32a-InvC plasmids, respectively. The PCR products of CSFV E2 and InvC were ligated via T4 DNA ligase after digestion by NotI restriction enzyme, and then E2-InvC fusion was amplified by primers F1 and R2. rAd-Track-E2-InvC plasmid was constructed by inserting E2-InvC fusion genes into the adenoviral shuttle vector rAdTrack-CMV, which was double digested by SalI and HindIII restriction enzymes. The shuttle plasmid rAdTrack-E2-InvC was linearized by Pme I restriction endonucleases before undergoing homologous recombination with rAdEasy-1 in AdEasier-1 host bacteria BJ5183. The recombinant adenovirus backbone rAdEasy-E2-InvC DNA was identified by enzyme digestion, PCR, and DNA sequencing analysis and subsequently transfected into HEK293 cells for amplification and package of rAd-E2-InvC after linearization with the Pac I restriction endonucleases. The rAd-E2-InvC was propagated and titrated in HEK 293 cells.

2.3 *Western blot*

Western blot was conducted to analyse the expression of recombinant E2-InvC protein in HEK 293 cells infected by rAd-E2-InvC. The non-recombinant adenovirus rAd-CMV was treated as negative controls. After washing with PBS, the cells infected with rAd-E2-InvC were harvested by scraping and then lysed with protein lysis buffer (P0013; Beyotime, China). Cell lysates were centrifuged at 10,000*g* for 5 min at 4°C. Cell extracts were denatured and separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and were subsequently transferred to a polyvinylidene fluoride membrane (Millipore, USA). The membrane was blocked with 5% skim milk overnight in TBST buffer at room temperature. Subsequently, the membrane was incubated with anti-CSFV sera (diluted 1:100), which were used as the first antibody in the blocking liquid for 2 h. Rabbit anti-swine IgG (Ig-0309R; Sigma, St Louis, MO, USA) was used as the secondary antibody (diluted 1:1000). A mouse anti-porcine GAPDH antibody (LifeSpan Biosciences, USA) was used to determine porcine GAPDH protein. The protein bands were visualized by employing enhanced chemiluminescence methods according to supplier's instructions (Millipore, USA).

2.4 *Immunization and challenge*

Twenty 6- to 7-week-old cross-bred healthy piglets were used and randomly divided into four groups (A, B, C, and D), with five animals in each group. Prior to this experiment, all animals were double confirmed to be devoid of specific serum antibodies and antigens by using a commercially available CSFV antibody detection kit (IDEXX, USA) and conducting real-time reverse transcript-polymerase chain reaction (RT-PCR), respectively. Groups A and B were vaccinated with a dose of 2×10^6 $TCID_{50}$ of rAd-E2-InvC and rAd-E2, respectively, by intramuscular (i.m.) inoculation. All pigs were given a booster immunization after 2 weeks with the same vaccine, dose, and method of administration. The pigs in Group C were immunized with one-dose C-strain vaccine and served as the positive control. The pigs in Group D were injected with DMEM only. Finally, all animals were challenged with 1×10^3 $TCID_{50}$ of CSFV Shimen strain by i.m. injection 21 days after the second vaccination. Following the challenge, the pigs were monitored daily for clinical signs (anorexia, depression, shivering, hemorrhage, constipation, and diarrhea) of disease, and rectal temperatures were recorded. At 12 days post-challenge (DPC), all surviving pigs were euthanized.

2.5 *Blocking ELISA and NPLA*

Serum samples were collected from all pigs prior to vaccination and at weekly intervals after immunization and two-day intervals after the challenge. CSFV-specific NAbs in sera were tested by using an IDEXX HerdChek* CSFV antibody test kit according to the manufacturer's instructions and NPLA (Terpstra *et al.* 1984).

2.6 *Virus isolation*

Blood samples as well as nasal and fecal swabs were collected on day 0 after the challenge and every other day following the challenge until the pigs were euthanized for viral isolation. All samples were freeze-thawed three times in PBS and then centrifuged at 8000*g* for 5 min. The supernatant was used to inoculate 96-well microplates containing

growth cultures of PK15 cells in DMEM medium with 5% fetal bovine serum. After 96 h, the culture medium was discarded. The monolayer cells were air-dried, fixed for 2 h, and then incubated with anti-CSFV sera (diluted 1:100). The cells were washed and then incubated with rabbit anti-pig IgG/HRP antibody (diluted 1:200; BIOSS, China). Immunoreactivity of stained cell foci was detected under a light microscope. If the result was negative, the supernatant was blind passaged three times in cells and detected for CSFV again.

2.7 *Real-time RT-PCR*

After the pigs were sacrificed, samples from the spleen, kidney, mesenteric lymph nodes, and tonsil of each animal were collected and tested for CSFV antigen. The total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol and was subjected to DNase treatment with RNase-free DNase (Takara Bio, Dalian, China) to remove contaminated genomic DNA. The cDNA was synthesized via reverse transcription from 1 μg of total RNA by using RT primer mix (Takara, Dalian, China). Quantitative real-time PCR was carried out with a SYBR ExScript^{TM} RT-PCR kit (Takara, Dalian, China) according to the manufacturer's instructions. Reactions were performed in an iQ5 Real-Time PCR Detection System (Bio-Rad, USA) under the following conditions: 10 min at 95°C and 40 cycles of 5 s at 95°C, 10 s at 58°C, and 15 s at 72°C. The data were analysed according to the threshold (Ct) method.

3. Results

3.1 *E2-InvC expression in rAd-E2-InvC transduced HEK 293 cells*

The E2 and InvC genes were obtained from pMD-32a-E2 and pMD32a-InvC plasmids, respectively. After digested by NotI restriction enzyme and ligated by T4 DNA ligase, the E2-InvC gene was obtained by PCR and cloned into the adenoviral shuttle plasmid rAdTrack-CMV. The rAdEasy-E2-InvC DNA was generated by homologous recombination of rAdTrack-E2-InvC plasmid with rAdEasy-1 in AdEasier-1 host bacteria BJ5183. The linearized DNAs were transfected into 293 cells. The rAd-E2-InvC viruses were amplified in 293 cells. The E2-InvC protein expressed in 293 cells and infected with rAd-E2-InvC was confirmed by western blot using anti-CSFV serum. Approximately 87- and 50-kDa-specific bands were clearly visible in the rAd-E2-InvC- and rAd-E2-infected cell extracts, respectively. No specific bands were observed in the cell lysates of mock group and HAdV-5 group-infected cell extract (figure 1).

3.2 *Antibody production*

CSFV E2-specific antibodies were measured by NPLA and blocking ELISA following vaccination with rAd-E2-InvC, rAd-E2, CSFV C-strain, and DMEM. Table 1 shows that pigs immunized with rAd-E2-InvC, rAd-E2, and CSFV C-strain developed detectable antibody titers 2 weeks after the

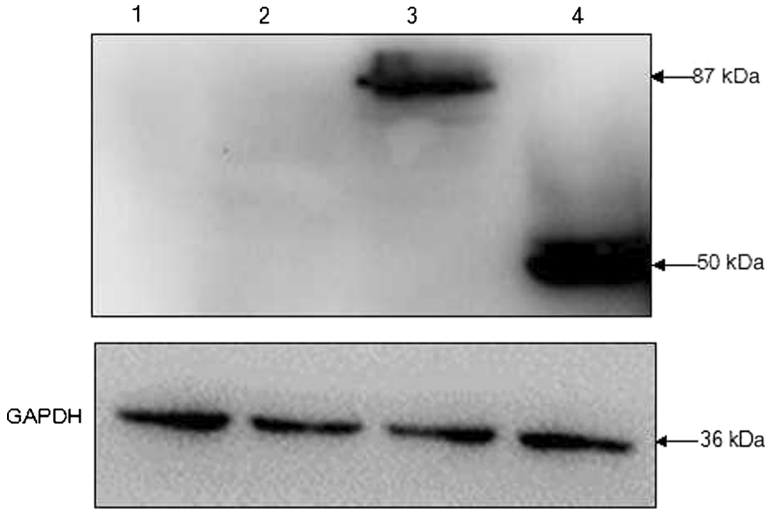

Figure 1. Western blot analysis of the E2 protein expression in rAdV-E2-InvC infected 293 cells. Lane 1, normal 293 cells lysates; Lane 2, lysates of 293 cells infected by HAdV-5; Lane 3, lysates of 293 cells infected by rAdV-E2-InvC; and Lane 4, lysates of 293 cells infected by rAdV-E2. The arrows indicate the bands of protein.

Table 1. Detection of serum neutralizing antibodies in immunized pigs

Groups	Vaccines	Pig no.	Days post vaccine					
			0	7	14	21	28	35
A	rAdV-E2-InvC	059	0	0	32	32	64	256
		080	0	8	16	32	128	128
		093	0	4	8	16	64	128
		108	0	4	16	64	256	512
		075	0	4	8	32	64	128
	mean			4.8	16	35.2	115.2	230.4
B	rAdV-E2	072	0	0	4	8	16	64
		069	0	4	8	16	32	64
		067	0	4	8	8	128	128
		150	0	8	16	64	128	256
		076	0	4	8	16	64	128
	mean			4	8.8	22.4	73.6	128
C	CSFV C strain	116	0	8	32	64	128	256
		084	0	4	16	32	256	512
		088	0	8	32	128	128	256
		073	0	16	64	64	128	128
		097	0	8	32	64	256	512
	mean			8.8	35.2	70.4	179.2	332.8
D	DMEM	071	0	<4	<4	<4	<4	<4
		113	0	<4	<4	<4	<4	<4
		082	0	<4	<4	<4	<4	<4
		127	0	<4	<4	<4	<4	<4
		118	0	<4	<4	<4	<4	<4
	mean		0	<4	<4	<4	<4	<4

Antibody titers were determined by NPLA as described previously (Terpstra *et al.* 1984). The antibody titers were expressed as the reciprocal of the highest serum dilution at which the infectivity of PK15 cells by neutralized 200 $TCID_{50}$ of CSFV was completely inhibited in 50 % of the wells.

prime immunization. CSFV E2-specific antibodies were markedly increased after the booster immunization. The CFSV E2-specific antibody titer in the rAd-E2-InvC group was significantly higher than that in the rAdV-E2 group, but was lower than that in the CSFV C-strain group after the booster immunization (figure 2). The pigs immunized with DMEM did not develop detectable antibody titers during the experiment (<4).

CSFV E2-specific antibodies were detected in rAd-E2-InvC (3/5), rAd-E2 (2/5), CSFV C-strain (4/5), and DMEM (0/5) groups two weeks after the booster immunization (table 2), with mean antibody blocking rates of 43.4%, 40.0%, 47.0%, and 5.8% (the cutoff of the assay was 40%), respectively. The levels of CSFV-specific antibodies produced by the four groups gradually increased (figure 3). Three weeks after the booster immunization, all rAd-E2-InvC and CSFV C-strain-vaccinated pigs seroconverted, with mean antibody blocking rates of about 52.2% and 56.2%, respectively. No significant difference was observed in the antibody level between the C-strain and rAd-E2-InvC, whereas a highly significant difference existed between the rAd-E2-InvC and rAd-E2 groups in most time points after the booster immunization and prior to challenge.

3.3 *Protection of immunized pigs from virulent challenge*

Twenty-one days after the last immunization with rAd-E2-InvC and rAd-E2 (35 days p.i.), all experimental pigs were inoculated intramuscularly with 1×10^3 $TCID_{50}$ dose of the CSFV 'Shimen' strain, which is lethal to susceptible pigs. Protective immunity was assessed by clinical signs, including body temperature, after challenge with virulent CSFV. No clinical symptoms were observed in pigs immunized with

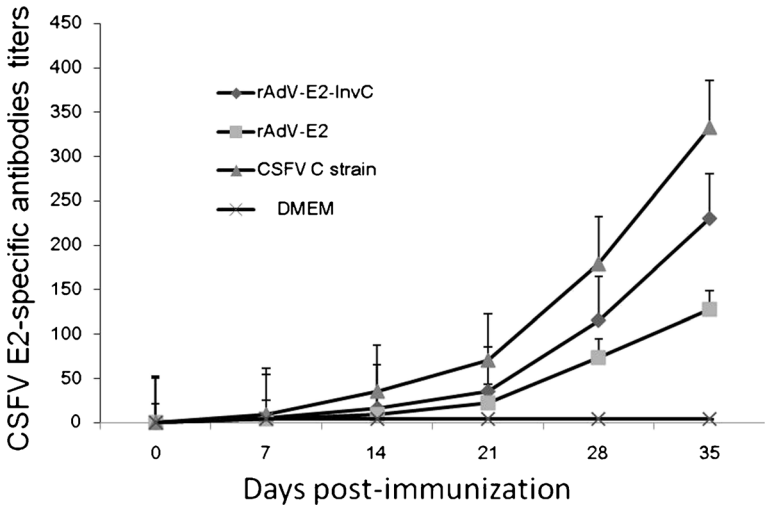

Figure 2. The dynamic changes of serum anti-CSFV antibody titer in immunized pigs with rAd-E2-InvC, rAd-E2, CSFV C-strain and DMEM as determined by NPLA. The values are presented as means ± S.D.

CSFV C-strain after the challenge. Rectal temperatures showed that all pigs in Group D developed a high febrile response by day 2 post-challenge, with temperatures above 40°C. Three pigs in the rAd-E2-InvC group exhibited fever 4 DPC, but returned to normal temperature one day later. Two pigs in the rAd-E2 group also had fever, and one pig recovered in 1 to 2 days. All pigs in Group D typically displayed clinical signs of CSF (i.e. inappetence, apathy, chill, prostration, incoordination, and constipation, followed by diarrhea, locomotor ataxia, and posterior paresis) with high fever (40.5–42°C) from 2 DPC until death. All the pigs in Group D died within 12 DPC, whereas the pigs in other groups survived.

Whole blood samples as well as nasal and fecal swabs were collected at 0, 2, 4, 6, 8, 10 and 12 DPC and used to isolate CSFV antigen. Viruses were detected in the DMEM group from day 4 until the animals died. CSFV was isolated in two pigs immunized with rAd-E2 at 9 (No. 072) and 8 (No. 069) DPC. However, viruses were not detected from the blood samples as well as nasal and fecal swabs of the pigs immunized with rAd-E2-InvC and CSFV C-strain at any time point (table 3). This result indicates that rAd-E2-InvC could effectively prevent virus shedding in the immunized animals.

At 12 DPC, all pigs were euthanized and subjected to pathological examination. None of the pigs immunized with rAd-E2-InvC and CSFV C-strain showed pathological changes. Two pigs immunized with rAd-E2 showed mild lesions, such as slight hemorrhages in the spleens, whereas the other pigs in this group were lesion-free. All the pigs in Group D showed severe pathological changes, including hemorrhages with necrotic foci in the tonsils, enlargement and hemorrhage of the lymph nodes, infarcts in the spleen, extensive petechiae in the kidney and bladder, and button-like ulcers in the ileocecal valve. CSFV was also detected from various organs, such as blood, spleen, kidney, intestines, lymph node mesenteric, and tonsil, of the pigs by real-time PCR (table 4). No viral RNA was noticed in pigs immunized with rAd-E2-InvC and CSFV C-strain. Viral RNA was detected in two pigs immunized with rAd-E2 at 9 (No. 072) and 8 (No. 069) DPC, and in all pigs vaccinated with DMEM (Group D).

4. Discussion

To develop a new marker vaccine that is safe and efficient and has the property of differentiating infected from vaccinated animals (DIVA) to prevent and control the CSF, numerous candidate vaccines surrounding CSFV or its structural proteins E2 and/or E^{rns}, which can induce virus-NAbs and offer protective immunity in the natural host, were investigated by various strategies and methods. These strategies include recombinant deletion vaccines based on live attenuated vaccines (Widjojoatmodjo *et al.* 2000; Maurer *et al.* 2005; Frey *et al.* 2006; Dong and Chen 2007), DNA vaccines (Ganges *et al.* 2005b; Li *et al.* 2007a; Li *et al.* 2007b), subunit vaccines (Hulst *et al.* 1993; Lipowski *et al.* 2000), peptide vaccines (Lipowski *et al.* 2000; Tarradas *et al.* 2011), chimeric vaccines (Leifer *et al.* 2009a; van

Table 2. Detection of serum antibodies to CSFV in immunized pigs using blocking ELISA

Groups	Vaccines	Pig no.	Antibody blocking rates (%)					
			0	7	14	21	28	35
A	rAdV-E2-InvC	059	5	14	22	33	38	45
		080	3	7	24	34	45	49
		093	8	12	33	45	51	62
		108	5	11	27	38	47	53
		075	8	7	23	29	36	52
	mean		5.8	10.2	25.8	35.8	43.4	52.2
B	rAdV-E2	072	5	7	26	34	39	47
		069	3	9	18	26	33	37
		067	8	13	35	40	46	54
		150	3	6	21	26	35	40
		076	7	11	32	38	47	53
	mean		5.2	9.2	26.4	32.8	40	46.2
C	CSFV C strain	116	5	27	36	48	59	67
		084	3	12	24	31	37	48
		088	8	21	33	40	46	63
		073	7	13	22	36	40	43
		097	6	19	34	42	53	60
	mean		5.8	18.4	29.8	39.4	47	56.2
D	DMEM	071	5	4	7	3	8	5
		113	3	7	5	8	4	6
		082	8	3	6	7	4	5
		127	3	5	5	9	6	6
		118	6	3	8	7	7	9
	mean		5	4.4	6.2	6.8	5.8	6.2

All serum samples were collected weekly and tested the anti-CSFV antibodies with the IDEXX HerdChek* CSFV Antibody Test Kit according to the manufacturer's instructions.

Gennip *et al.* 2000), and virus vector vaccines (Poxvirus vector (Hahn *et al.* 2001), Pseudorabies virus vector vaccines (Peeters *et al.* 1997), and adenovirus vector vaccines. These studies contribute greatly to the understanding of CSFV and provide many ideas and strategies for the development of CSFV marker vaccines. Among them, HAdV-5 has been widely used as a platform for vaccine delivery and gene therapy. The current vaccines used for CSF prevention are based on HAdV-5 expression of E2 by various strategies (Sanchez *et al.* 2008; Sun *et al.* 2013a, b). All of these vaccines could elicit NAb and could confer complete protection in pigs upon viral challenge. These vaccines also demonstrate that HAdV-5 could provide an effective vaccine delivery of a variety of agents to swine. Although vaccines with high efficacy have been developed, a more potent adjuvant with defined and appropriate properties is required to improve the immunogenicity of many antigens. Adjuvants, such as microbial proteins or carbohydrates, may activate antigen-presenting cells and induce specific immune responses (Pashine *et al.* 2005).

Inv protein is a high-affinity ligand for β1-integrins (Isberg and Leong 1990) and can exploit the β1-integrins of M cells for intestinal translocation (Schulte *et al.* 2000c) in the follicle-associated epithelium overlying the Peyer's patches (Grutzkau *et al.* 1990). Subsequently, Yersiniae colonize the Peyer's patches and may eventually distribute to lymph nodes, liver, lung and spleen (Autenrieth and Autenrieth 2008). The binding of Inv to β1-integrin receptors of epithelial cells induces activation of a signal cascade, involving Rac1, NF-κB and MAP kinases, and thereafter

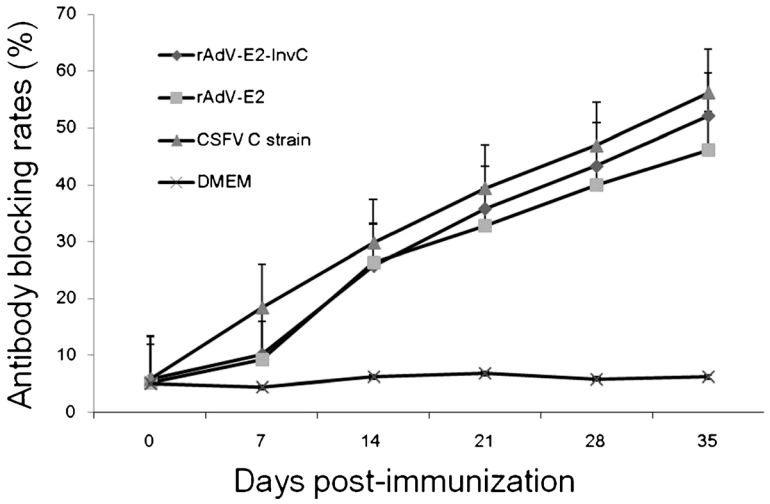

Figure 3. The dynamic changes of antibody blocking rates (%) in immunized pigs with rAd-E2-InvC, rAd-E2, CSFV C-strain and DMEM as determined by blocking ELISA (IDEXX HerdChek* CSFV Antibody Test Kit). The values are presented as means ± S.D.

Table 3. Virus isolation from blood samples, nasal swabs and fecal swabs in vaccinated groups following a challenge with the CSFV "Shimen" strain

Groups	Vaccines	Pig no.	Isolation of virus*																	
			Blood samples (days p.c.)						Nasal swabs (days p.c.)						Fecal swabs (days p.c.)					
			2	4	6	8	10	12	2	4	6	8	10	12	2	4	6	8	10	12
A	rAdV-E2-InvC	059	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		080	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		093	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		108	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		075	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B	rAdV-E2	072	−	−	−	−	+	+	−	−	−	−	+	+	−	−	−	−	+	+
		069	−	−	−	+	+	+	−	−	−	+	+	+	−	−	−	+	+	+
		067	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		150	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		076	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C	CSFV C strain	116	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		084	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		088	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		073	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		097	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D	DMEM	071	−	+	+	+	n.d	n.d	−	+	+	+	n.d	n.d	−	+	+	+	n.d	n.d
		113	−	+	+	n.d	n.d	n.d	−	+	+	n.d	n.d	n.d	−	+	+	n.d	n.d	n.d
		082	−	+	+	+	+	n.d	−	+	+	+	+	n.d	−	+	+	+	+	n.d
		127	−	+	+	+	n.d	n.d	−	+	+	+	n.d	n.d	−	+	+	+	n.d	n.d
		118	−	+	+	+	+	n.d	−	+	+	+	+	n.d	−	+	+	+	+	n.d

(−): Samples that resulted negative for virus isolation after three serial passages of the initial inoculums in PK-15 cells; (+): positive result; n.d., not done.

Table 4. Virus detected from blood, spleen, kidney, intestines, lymphonodi mesenterici and tonsil following a challenge with the CSFV "Shimen" strain

Groups	Vaccines	Pig no.	Detection of CSFV RNA					
			Blood	Spleen	Kidney	Tonsil	Lymphonodi mesenterici	Intestines
A	rAdV-E2-InvC	059	−	−	−	−	−	−
		080	−	−	−	−	−	−
		093	−	−	−	−	−	−
		108	−	−	−	−	−	−
		075	−	−	−	−	−	−
B	rAdV-E2	072	+	+	+	+	+	+
		069	+	+	+	+	+	+
		067	−	−	−	−	−	−
		150	−	−	−	−	−	−
		076	−	−	−	−	−	−
C	CSFV C strain	116	−	−	−	−	−	−
		084	−	−	−	−	−	−
		088	−	−	−	−	−	−
		073	−	−	−	−	−	−
		097	−	−	−	−	−	−
D	DMEM	071	+	+	+	+	+	+
		113	+	+	+	+	+	+
		082	+	+	+	+	+	+
		127	+	+	+	+	+	+
		118	+	+	+	+	+	+

Detection of viral RNA by real-time RT-PCR in the collected samples from immunized pigs after virulent CSFV challenge. (+) detectable; (−) undetectable

produces proinflammatory cytokines (Grassl *et al.* 2003; Schulte *et al.* 2000a) and exhibits immunological functions. Inv that acts as adjuvant can induce host cell binding, internalization, IL-8 responses (Schulte *et al.* 2000b), and significant CD4 and CD 8 T cell responses (Bühler *et al.* 2006). Inv is also used to enhance M-cell uptake of noninvasive *Shigella* or *E. coli* for vaccination (Critchley-Thorne *et al.* 2006; Suzuki *et al.* 2006). The C-terminal 192 amino acids of *Y. pseudotuberculosis* Inv (InvC) are sufficient for binding to β1-integrins and internalization (Rankin *et al.* 1992; Leong *et al.* 1995). Thus, we propose that InvC is suitable as a delivery molecule and adjuvant if used as a vaccine component.

In this study, InvC was used as a component of CSF-vectored vaccine based on HAdV-5 by co-expressing with CSFV E2 glycoproteins (rAd-E2-InvC). After the preliminary evaluation by PCR and Western blot, rAd-E2-InvC with 2×10^6 $TCID_{50}$ was administered intramuscularly two times to CSFV-free pigs at an interval of 14 days. No adverse clinical symptoms were observed in any of the pigs after vaccination. Moreover, neither CSFV RNA nor pathological changes were detected in the tissues after CSFV challenge. CSFV E2-specific antibody titer was significantly higher in the rAd-E2-InvC group than that in the rAdV-E2 group as measured by NPLA and blocking ELISA. These results agree with the data evaluated by CSFV C-strain vaccine to a certain extent. However, two of the five immunized pigs in the rAdV-E2 group showed short-term fever and viral replication and were unprotected from challenge with CSFV (in terms of both clinical signs and viremia). Compared with the rAdV-E2, the CSFV E2-specific antibody titer (figure 2), antibody blocking rate (figure 3), and protection level in the rAd-E2-InvC group were significantly higher than those in the rAdV-E2 group. As adjuvant, fusion of antigen to Inv can promote antigen-specific CD4 and CD8 T cell responses (Bühler *et al.* 2006). In this study, Inv was fused with the glycoprotein E2 of CSFV. Glycoprotein E2 is regarded as the most immunogenic of CSFV proteins and is mainly responsible for the induction of NAbs (Beer *et al.* 2007). In addition, the E2-specific CTL activity is

particularly evident 1 to 3 weeks after the challenge and plays an important role for complete immune defences (Rümenapf *et al.* 1991; Hulst *et al.* 1993; Ganges *et al.* 2005a; Tarradas *et al.* 2010a). When E2 fuses with InvC, both the CTL and NAbs may promote and produce complete protection against lethal challenge with highly virulent CSFV; however, the E2-specific CTL activity was not detected in this study.

rAd-E2-InvC is an E2-based DIVA vaccine and can be used to prevent swine from CSF. Considering the good immunization protection and the serological differentiation of vaccination from the wild-type CSFV infection using E^{rns}-based ELISA, this vaccine can be used as an alternative to the existing vaccine in controlling CSF and eradicating epidemic areas. Several experiments are still needed to comprehensively evaluate the adjuvant activity of InvC. Whether InvC can promote CSFV E2-specific cellular immunity, whether InvC can confer the escape of primary vector immunity for rAd-E2-InvC homologous booster vaccination, and whether InvC has better adjuvant activity than full-length Inv are all unknown. Results of the evaluation are expected to greatly promote the understanding of the mechanisms of InvC as a delivery molecule and adjuvant for vaccine.

In summary, this study is the first to introduce the C terminus of *Y. pseudotuberculosis* Inv (InvC) into CSF-vectored vaccine based on HAdV-5 (rAd-E2-InvC) by co-expressing with E2 glycoprotein. InvC significantly increased the E2-specific antibody and antibody blocking rate and provided complete protection of pigs against CSFV challenge; thus, InvC has the potential to be used as an antigen delivery vehicle and adjuvant for subunit vaccines or vectored vaccines.

Acknowledgements

This study was supported by the '13115' Science and Technology Innovation Major Special Project of Shaanxi Province (No.2010ZDKG-71). The authors appreciate the help of the staff from Yangling Lvfang Bio-engineering Co. Ltd. during the pigs' management and samples collection.

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MS received 24 January 2014; accepted 23 October 2014

Corresponding editor: SATYAJIT RATH