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Commercial E2 subunit vaccine provides full protection to pigs against lethal challenge with 4 strains of classical swine fever virus genotype 2

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ABSTRACT

Classical swine fever (CSF) still threatens the swine industry in China, with genotype 2 isolates of CSFV dominating the epizootics. In 2018 the first E2 subunit marker vaccine against CSFV (Tian Wen Jing, TWJ-E2®), containing a baculovirus-expressed E2 glycoprotein of a genotype 1.1 vaccine strain, was officially licensed in China and commercialized. To evaluate the cross-protective efficacy of TWJ-E2 against different virulent genotype 2 Chinese field isolates (2.1b, 2.1c, 2.1 h, and 2.2), 4-week-old pigs were immunized with the TWJ-E2 vaccine according to the manufacturer's instructions and then challenged with genotype 2 strains. A group vaccinated with the conventional C-strain vaccine was included for comparison. TWJ-E2 vaccinated pigs developed higher levels of E2 and neutralizing antibodies than those receiving the commercial C-strain vaccine. All TWJ-E2 and C-strain vaccinated pigs survived challenge without development of fever, clinical signs or pathological lesions. In contrast, all unvaccinated control pigs displayed severe CSF disease with 40–100% mortalities by 24 days post challenge. None of the TWJ-E2 and C-strain vaccinated pigs developed viremia, viral shedding from tonsils, E^{rns} protein in the sera, or viral RNA loads in different tissues after challenge, all of which were detected in the challenged unvaccinated controls. We conclude that vaccination of young pigs with TWJ-E2 provides complete immune protection against genotypically heterologous CSFVs and prevents viral shedding after challenge, with an efficacy at least comparable to that elicited by the conventional C-strain vaccine.

1. Introduction

Classical swine fever (CSF) represents one of the most devastating infectious diseases affecting the swine industry worldwide. The causative agent, CSF virus (CSFV), is an enveloped positive-sense RNA virus containing a 12.3-kb genome encoding a polyprotein of 3898 amino acids, which is processed by viral and cellular proteases into 4 structural proteins (Core, E^{rns}, E1 and E2) and 8 nonstructural proteins (N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Simmonds et al., 2017). Of the 3 envelope glycoproteins, the E2 protein is the major protective antigen inducing neutralizing antibody in the host (Lipowski et al., 2000), and has been selected as the most effective immunogen for development of subunit vaccines against CSFV (Hulst et al., 1993; Ahrens et al., 2000; Lipowski et al., 2000; Madera et al., 2016).

Prophylactic vaccination with modified live attenuated vaccine (MLV) is still the main strategy to control CSF in enzootic countries (Huang et al., 2014; Luo et al., 2014; van Oirschot, 2003). In China, extensive vaccination with MLV C-strain has largely controlled the CSF epizootics, but sporadic outbreaks still exist; however, CSFV field isolates are evolving under the pressure of vaccination into variants of genotype 2. Of these, the genotype 2.1 viruses currently predominating are genetically distant from the previously identified viruses of the 1990s (Ji et al., 2014; Gong et al., 2016a). Recently it was reported that CSF outbreaks caused by subgenotype 2.1 isolates occurred in C-strain vaccinated farms in China, with weaned pigs being the most affected populations due to inadequate protection by diminishing or low levels

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of maternal antibody (Hu et al., 2016; Xing et al., 2019). Most recently, our group found that one of the neutralizing antigenic epitopes in the C-strain E2 protein does not exist in any subgenotype 2.1 isolates (data not shown), which may be a reason why C-strain vaccination can allow partly escape of subgenotype 2.1 strains. Moreover, vaccination with the C-strain results in the inability to differentiate infected from vaccinated animals (DIVA). For the prevention, control and eradication of CSF in China, therefore, marker vaccines, together with serological DIVA assays, are necessary for screening and elimination of clinically healthy carrier sows which can spread the infection.

To date, three CSFV marker vaccines have been licensed in Europe: one live chimeric vaccine CP7_E2alf (Suvaxyn® CSF marker) carrying the E2 glycoprotein of CSFV "Alfort/187" in a bovine viral diarrhea virus type 1 backbone (CP7), and two E2 subunit vaccines *BAYOVAC CSF E2* from the Brescia strain (subgenotype 1.1) and Porcilis Pesti from the Alfort/Tübingen strain (subgenotype 1.1) (Blome et al., 2017; Reimann et al., 2004; Hulst et al., 1993; Ahrens et al., 2000). Serological DIVA assays, such as the *pigtype* CSFV E^{rns} Ab ELISA, are available to be accompanied with the application of these CSFV marker vaccines (Meyer et al., 2017). In 2018, the first Chinese CSFV E2 subunit marker vaccine, Tian Wen Jing (TWJ-E2®), was officially licensed and put on the market. This vaccine was developed by Tecon Biology Joint Stock Company Ltd (TECON), China, and utilizes the modified E2 glycoprotein of a subgenotype 1.1 vaccine C-strain as immunogen. It is produced in High Five insect cells in large scale fermenters using a baculovirus expression system. As part of the licensing process, the TWJ-E2 vaccine was shown to provide full protection against the highly virulent genotype 1.1 reference Shimen strain after two-dose vaccination, with the vaccinated piglets displaying no clinical signs, viremia or pathological lesions post challenge and with all being protected (Wang et al., 2018). In particular the maternal neutralizing antibody of 10-week-old piglets from two-dose TWJ-E2 vaccinated sows can still protect the pigs against Shimen strain (data not shown).

In the last decade, however, genotype 1-associated epizootics have largely disappeared, with the current CSFV isolates in China belonging to subgenotype 2.1, with subgenotypes 2.1b, c, and h being dominant (Gong et al., 2016a). Subgenotype 2.1b is widely spread in most regions of China, South Korea, Mongolia and Far East regions of Russia, and 2.1c is dominant in south China and neighboring southeastern Asian countries including Vietnam (Jiang et al., 2013; Gong et al., 2016a), while 2.1 h isolates mainly circulate in central and south China (Gong et al., 2016a). In addition, strains of the previously dominant subgenotype 2.2 of the 1990s in China still circulate in some south Asian countries, including India, Bangladesh, and Nepal (Tu et al., 2001; Sarkar et al., 2017; Singh et al., 2017; Postel et al., 2013). There are therefore significant differences in antigenicity between the currently prevalent strains of CSFV and the commercial TWJ-E2 vaccine, which raise questions about its efficacy against field isolates. The present study, testing the marker subunit vaccine against challenge with current virulent field strains of CSFV, was designed to address this issue.

2. Materials and methods

2.1. Vaccines and viruses

CSFV E2 subunit vaccine TWJ-E2® consists of an E2 glycoprotein, produced in insect cells with a baculovirus expression system. This marker vaccine was shown to provide complete protection against the highly virulent Shimen strain of CSFV belonging to subgenotype 1.1, and was licensed for commercialization by the Ministry of Agriculture and Rural Affairs, China, in March 21st, 2018 (License No.: 07040020180307-056). The TWJ-E2 used in this study was a commercial product, Lot No. 2018001. The C-strain vaccine used for comparison in the present study was also a commercial product, produced by the Guangdong Wens Dahuanong Biotechnology Co., Ltd (Lot No. 171816). Four CSFV field isolates belonging to subgenotypes 2.1b (JL23, 2015), 2.1c (GD23, 2017), 2.1 h (HuB12, 2015), and 2.2 (AH1, 1999) used for challenge were the same virus stocks previously utilized for virulence evaluation and characterized as moderately (GD23) or highly virulent (JL23, HuB12, and AH1) (Gong et al., 2019). The absence of extraneous bovine viral diarrhea virus (BVDV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus 2 (PCV2), porcine circovirus 3 (PCV3), porcine pseudorabies virus (PRV) and porcine parvovirus 1 (PPV1) in the virus stocks was determined as described previously (Gong et al., 2016b; Sun et al., 2018).

Table 1
Schedule of vaccination and challenge of experimental pigs.

Group		Vaccination[a]		Challenge
		0 DPV	21 DPV	35 DPV (0 DPC)
TWJ-E2 vaccinated group	E2/JL23	E2	E2	JL23
	E2/GD23	E2	E2	GD23
	E2/HuB12	E2	E2	HuB12
	E2/AH1	E2	E2	AH1
C-strain vaccinated group	C-strain/JL23	PBS	C-strain	JL23
Unvaccinated control group	JL23	PBS	PBS	JL23
	GD23	PBS	PBS	GD23
	HuB12	PBS	PBS	HuB12
	AH1	PBS	PBS	AH1
Unvaccinated/unchallenged group	Negative control	PBS	PBS	MEM

[a] TWJ-E2 and C-strain was immunized in pigs according to the manufacturer's instructions.

2.2. Animal experiments

Fifty 4-week-old pigs without CSF vaccination were randomly divided into 10 groups with 5 in each (Table 1). Each group was kept separately in different rooms within negative-pressure facilities and allowed free access to a standard diet and water. Before vaccination, the animals were observed daily for at least one week to ensure their healthy status, since only clinically healthy animals could be used. The pigs were further confirmed to be free of CSFV, BVDV, PRRSV, PCV2, PCV3, PRV and PPV1 by previously described methods (Gong et al., 2016b; Sun et al., 2018) and were also CSFV E2 antibody-negative as determined by the method described in section 2.3.

Pigs in the TWJ-E2 vaccinated groups were immunized intramuscularly (IM) by injection of two doses of TWJ-E2 (2 ml for each) 3 weeks apart, as per the manufacturer's instructions. The C-strain/JL23 group was immunized IM with one dose of vaccine (2 ml) as specified by the manufacturer. All groups were challenged IM with 2 ml ($10^{5.0}$ $TCID_{50}$) of one of the CSFV field isolates 2 weeks following the C-strain vaccination or the booster dose of TWJ-E2, as shown in Table 1. Pigs in an unvaccinated/unchallenged group (negative control) were injected with 2 ml placebo (minimum essential medium, MEM). After challenge, rectal temperature was taken daily and clinical and pathological scores were recorded as previously described (Mittelholzer et al., 2000; Floegel-Niesmann et al., 2003; Gong et al., 2019). Animal studies were approved by the Experimental Animal Use and Care Committee of the Academy of Military Medical Sciences.

2.3. Sample collection and analysis

Whole blood and sera were taken for hematologic, virologic and serologic examinations at 0 day post vaccination (0 DPV, first dose for TWJ-E2), 21 DPV (second dose for TWJ-E2, first dose for C-strain), 35 DPV (0 DPC) and every three days after challenge, until the end of the experiment at 24 DPC (59 DPV). Pharyngeal swab samples were concurrently collected with blood sampling during the challenge period and the cotton tips were transferred into vials containing 2 ml of MEM.

Upon necropsy, tissue samples of tonsil, lung, spleen, kidney, and lymph nodes were taken from moribund, dead, or euthanized pigs, and the supernatants of 10% tissue homogenate were prepared at 12,000 rpm for 10 min for detection of viral RNA loads and virus isolation.

Leucocyte counts were performed as previously described using a Mindray BC-2800Vet analyzer (Mindray Medical USA Corp, Mahwah, USA) (Gong et al., 2019). Viral RNA loads in whole blood, tissues, and tonsil swabs were determined by real time reverse transcription polymerase chain reaction (qRT-PCR) as previously described (Gong et al., 2019). For virus isolation, 100 µl of whole blood, supernatant of 10% tissue homogenate, or 200 µl of fluid squeezed from pharyngeal swabs were inoculated onto PK-15 cells, and indirect fluorescent antibody assay (IFA) with specific E2 mAb WH303 (Lin et al., 2000) and Alexa Fluor 488-conjugated Goat anti-mouse IgG (Life Technology, USA) was performed as previously described after incubation for 72 h (Gong et al., 2016b). The primary antibody WH303 was provided by Animal and Plant Health Laboratories Agency, Wey Bridge, United Kingdom.

Furthermore, CSFV E2 antibody and E^{rns} protein levels in blood were determined using the commercial CSFV Antibody Test Kit and CSFV-Ag/Serum kits (IDEXX, USA) according to the manufacturer's instructions. E^{rns} antibody was tested with the *pigtype* CSFV E^{rns} ELISA kit (QIAGEN, Germany). Neutralizing antibody titers in sera collected at 0 DPV, 35 DPV (0 DPC), and 59 DPV (24 DPC) were determined with homologous CSFVs in PK-15 cells according to the EU Diagnostic Manual (Anonymous, 2002; Floegel-Niesmann et al., 2009; Gong et al., 2019). In brief, serum samples diluted 1:5 with further 2-fold serial dilutions were prepared, followed by incubation with 100 $TCID_{50}$ homologous CSFVs for 1 h at 37 °C. PK-15 cells (2×10^4) were then added and incubation was continued for 72 h before performing IFA, and the neutralization titers were expressed as ND_{50}.

3. Results

3.1. TWJ-E2 vaccine produced complete clinical protection to pigs against lethal challenge with different CSFV genotype 2 field isolates

None of the experimental pigs showed any local or systematic reactions, including fever, after either TWJ-E2 or C-strain vaccination. Following challenge, none of the pigs in the four TWJ-E2 vaccinated groups developed fever and their rectal temperatures remained within the normal range (38.6–39.7 °C) during the 24-day observation period. One of the 5 pigs vaccinated with the C-strain developed a transient fever (40.4–41.5 °C) at 10–13 and 15 days post-challenge (DPC) but then recovered (Fig. 1A). In contrast, all 20 pigs in the 4 unvaccinated control groups developed fever (> 40 °C) following a 3–6 day incubation period, which lasted for the entire observation period. No clinical signs were observed in any of the TWJ-E2 or C-strain vaccinated pigs after challenge while all 20 pigs in the control groups displayed clinical signs after the 3–6 day incubation period. These included anorexia, mental depression, cough and dyspnea, reddened conjunctivitis, convulsions, hesitant gait, constipation and emaciation, with diarrhea and bleeding from the skin of the hips or hoofs of all four limbs occasionally being observed in some challenge control animals (Fig. 1B).

At 24 DPC all surviving pigs were euthanized for pathologic examination. Following challenge, none of the TWJ-E2 or C-strain vaccinated pigs developed pathological lesions, but all unvaccinated control pigs presented typical pathological changes, including pulmonary edema, hemorrhage or cellulosic exudation, swelling and hemorrhage of lymph nodes. Hyperemia and necrosis of the tonsils, petechiae on kidneys and pin-point hemorrhages in stomach and large intestines were observed in some animals.

Measurement of the body weights of the experimental pigs after challenge showed that the growth of all TWJ-E2 and C-strain vaccinated pigs remained uninfluenced by CSFV challenge, with gains of 40–56% over pre-challenge weights. There were no mortalities in the TWJ-E2 and C-strain vaccinated groups. In contrast, significant growth retardation occurred in unvaccinated control pigs, with the body weight of JL23, GD23, HuB12, and AH1 groups at 24 DPC increasing only 3.8%, 26.2%, 11.8%, and 0.3% respectively. Moreover, most challenged control pigs died of CSFV infection, with mortalities in the JL23, GD23, HuB12, and AH1 groups by 24 DPC of 100%, 40%, 80%, and 80% respectively.

Leukocyte counts after challenge revealed a rapid and transient decrease in some vaccinated pigs while still remaining within the normal range and with later full recovery (Fig. 2). In contrast, severe and long-lasting leukocyte depletion occurred in all unvaccinated control pigs post challenge: 35.7–51.9%, 19.1–64.2%, and 58.2–81.9% depletion at 3, 6, and 9 DPC (Fig. 2). From 15 DPC to the moribund stage, even larger numbers of leukocytes were depleted, with the highest reduction reaching 96% (Fig. 2).

3.2. TWJ-E2 vaccine elicited high levels of E2 and neutralizing antibodies

Since the TWJ-E2 vaccine uses a 2 dose regimen (at days 0 and 21) the E2 antibody response was determined using the IDEXX CSFV Antibody Detection Kit (IDEXX, Netherlands) at different time points after the second dose. As shown in Fig. 3, 13 of the 20 vaccinated pigs in the four groups (5 in E2/JL23, 3 in E2/GD23, 4 in E2/HuB12, and 1 in E2/AH1) developed E2 antibody after the initial vaccination, with all 20 animals becoming E2 antibody positive at 2 weeks post-boost with blocking rates of 73–92% (Fig. 3). Following a slight decline (5–25%) between 3–9 DPC, levels of E2 antibody in all 4 groups reached a peak at 12–15 DPC (Fig. 3). In the C-strain vaccinated pigs, only 1/5 was E2 antibody positive at 2 weeks post vaccination (*i.e.*, at 0 DPC). After challenge the E2 antibody response gradually increased, with all 5 pigs becoming positive by 12 DPC and reaching a peak at 21 DPC (74–85% blocking rates) (Fig. 3). However in the unvaccinated control groups, only 6 of the 20 pigs developed positive E2 antibody from 15 DPC, showing significant individual differences in these groups which led to intra-group deviations in antibody levels (Fig. 3). Interestingly, none of the 5 pigs in the JL23 challenge group developed an E2 antibody response.

Neutralizing antibody (NA) titers were determined as previously described (Floegel-Niesmann et al., 2009) with the results shown in Table 2. At 0 DPV pigs in all groups were neutralizing antibody negative. By 35 DPV (2 weeks following the booster dose, or at 0 DPC) all E2-vaccinated pigs had developed higher NA titers (80–640 ND_{50}) than those given the C-strain (< 5 to 7.5 ND_{50}) (Table 2); by 24 DPC however, all NA levels had markedly increased, reaching above 1000 ND_{50} (E2 groups) and 100 ND_{50} (C-strain group) (Table 2). In contrast most of the unvaccinated controls remained NA negative post challenge, with only 8 developing very low NA titers (Table 2).

3.3. E^{rns} antibody response in vaccinated pigs after challenge

Testing of E^{rns} antibody by the *pigtype* CSFV E^{rns} ELISA kit (QIAGEN, Germany) found positive titers in 10 of the 20 E2-vaccinated pigs (2 in E2/JL23, 1 in E2/GD23, 2 in E2/HuB12, and 5 in E2/AH1) and all C-strain vaccinated animals after challenge; however, the time of antibody onset differed between groups. For instance, 3 E2-vaccinated (1 in TE2/JL23 and 2 in TE2/AH1) and 3 C-strain vaccinated pigs developed stable E^{rns} antibody levels that were maintained from onset to the end of challenge period. Among the unvaccinated controls, E^{rns} antibody was present in 19/20 pigs from 9 DPC onward, with only one of the -/JL23 group remaining E^{rns} antibody free following challenge (Table 3). The onset of the E^{rns} antibody response was similar to that in the virulence evaluation of the CSFV field isolates (Gong et al., 2019), which were used as the challenge strains in this study.

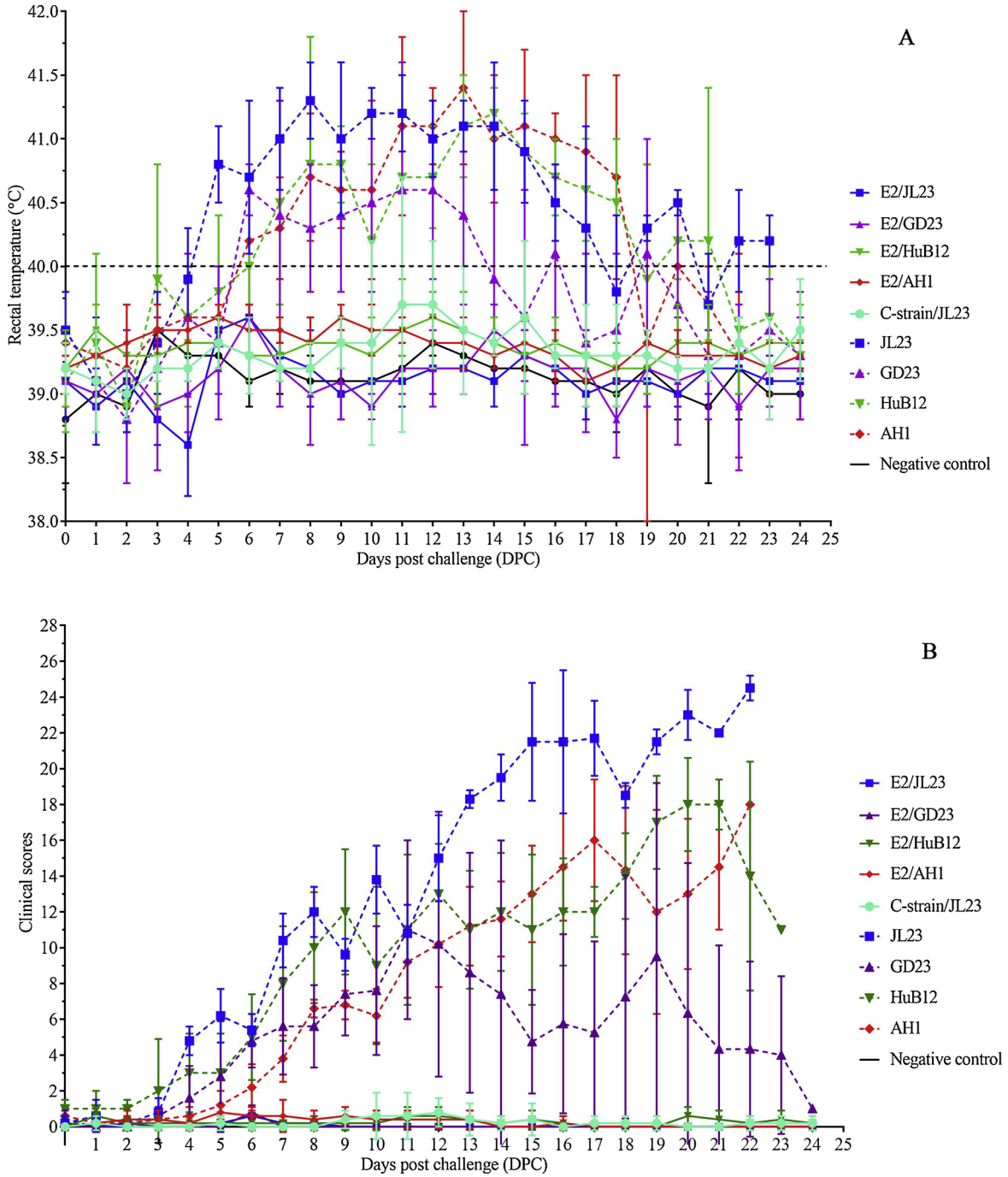

Fig. 1. TWJ-E2 vaccinated pigs showed no fever (A) or clinical signs (B) after challenge with CSFV field isolates. After challenge, rectal temperatures and clinical signs were recorded daily. Solid lines: TWJ-E2 vaccinated groups; dashed lines: unvaccinated controls.

3.4. TWJ-E2 vaccination eliminated the virus and prevented viral shedding after lethal CSFV challenge

Analysis of viral RNA in the blood, pharyngeal swabs and tissues by quantitative real time RT-PCR showed that all vaccinated pigs, as well as the unvaccinated/ unchallenged control, had only background amplification (Figs. 4A–C). In contrast, CSFV was detected in all challenged unvaccinated pigs in blood and pharyngeal swabs from 6 to 9 DPC to the end of challenge period (Fig. 4A, B) as well as in multiple tissues (Fig. 4C). Since 7 blood, 4 pharyngeal and 19 tonsil samples from challenged E2 and C-strain vaccinated groups had a slightly higher amplification of CSFV RNA than the negative control background, they were subjected to viral isolation in PK-15 cells as previously described (Gong et al., 2016b). All results by indirect fluorescent antibody assay (IFA) were negative. It appears, therefore, that immunization by either E2 or C-strain vaccine can eliminate CSFV from the body and prevent viral shedding.

The E^{rns} protein is secreted in considerable amounts into the serum of CSFV-infected pigs and the extracellular environment and is considered to be a marker of CSFV infection (Rümenapf et al., 1993; Gong et al., 2019). Using the CSFV Ag/Serum kit (IDEXX, Switzerland), E^{rns} was not detected in the serum of any challenged E2 and C-strain vaccinated pigs, but was present in all unvaccinated controls from 6 DPC onward (Fig. 5). There was a strong correlation between levels of viremia and serum E^{rns} protein during CSFV infection.

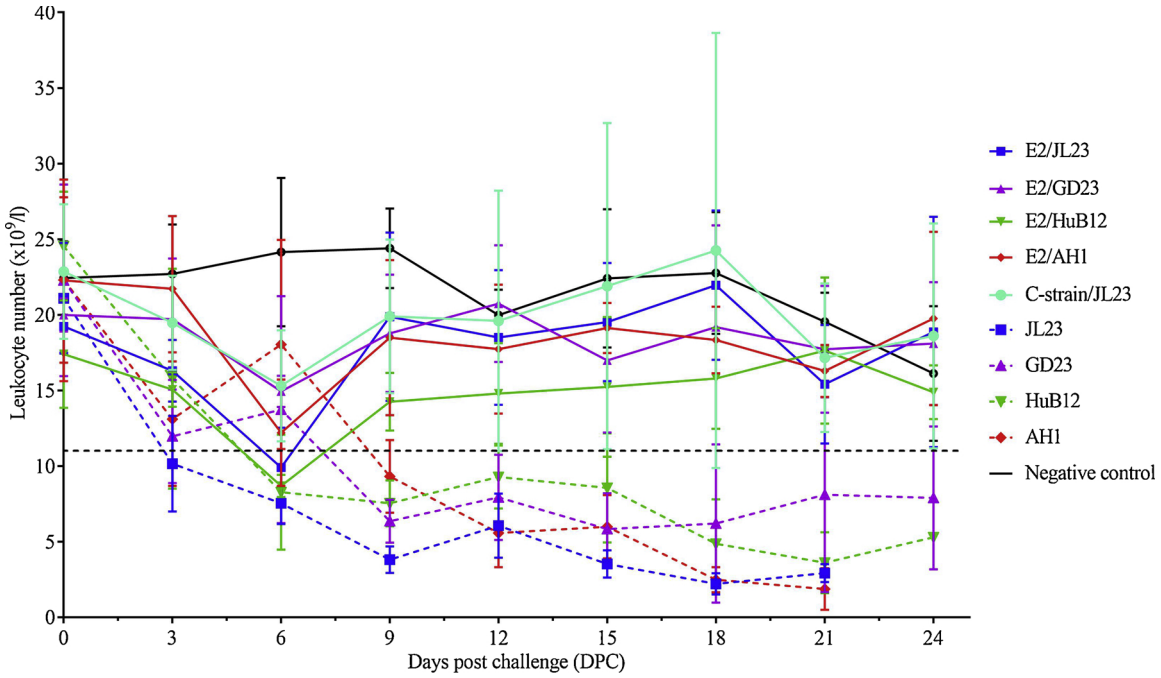

Fig. 2. Leukocyte counts in TWJ-E2 and C-strain vaccinated pigs. After challenge, leukocyte levels showed a transient decline in some TWJ-E2 vaccinated pigs at 3–6 DPC and then recovered from 9 DPC. Solid lines: TWJ-E2 vaccinated groups; dashed lines: unvaccinated controls.

4. Discussion

Vaccination with modified live vaccine C-strain is currently the most important and effective strategy for prevention and control of CSF in China, since it can induce complete immunity against various genotypes of CSF viruses (Huang et al., 2014). However, as a live attenuated vaccine, the efficacy of C-strain is influenced by multiple factors, including maternal antibody (Suradhat and Damrongwatanapokin, 2003; Suradhat et al., 2007; van Oirschot, 2003), cold chain storage and transportation, and co-infection with immunosuppressive pathogens (Suradhat et al., 2001, 2003, 2006, 2007; Huang et al., 2011, 2012). These variables may contribute to the sporadic outbreaks of CSF that still occur in C-strain vaccinated herds (Hu et al., 2016). Another major concern is that C-strain vaccination makes the DIVA strategy impossible. For this purpose, marker vaccines, together with discriminatory antibody diagnostic ELISAs, are necessary for CSF elimination and eventual eradication (Floegel-Niesmann, 2001). In addition to three CSF marker vaccines (CP7_E2alf, *BAYOVAC CSF E2* and Porcilis Pesti), which have been demonstrated to provide effective protection against genotypes 1.1 and 2.1 CSFVs (Hulst et al., 1993; Ahrens et al., 2000), more E2 subunit vaccine candidates have been developed by USA and Chinese research groups, including KNB-E2 and two other E2

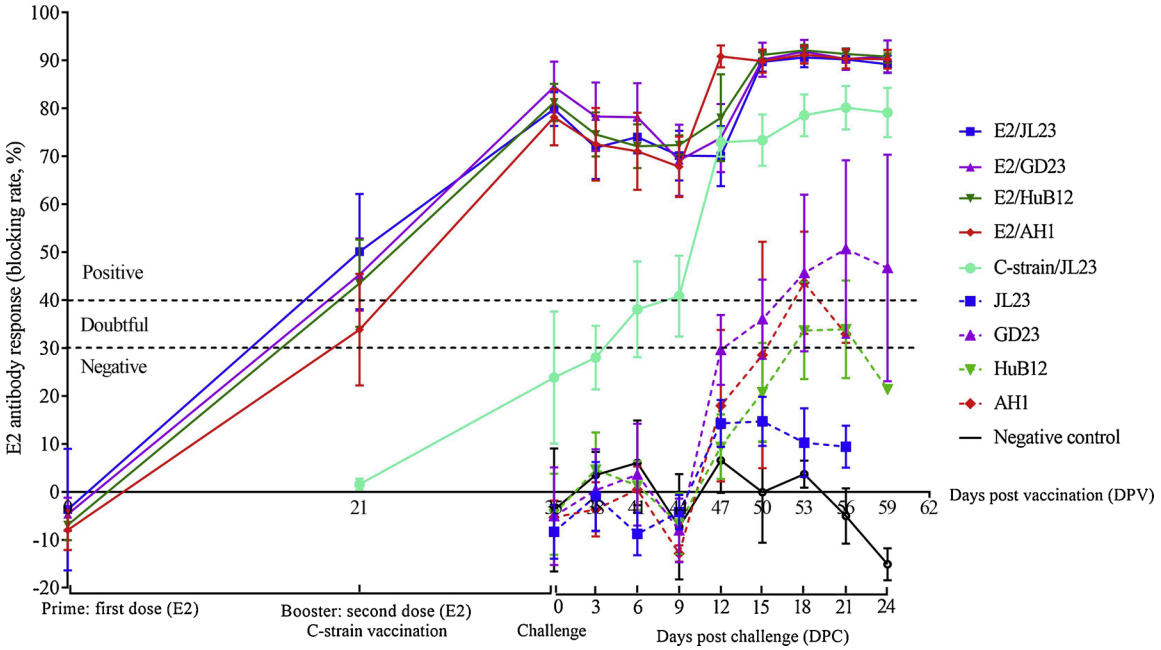

Fig. 3. Dynamics of E2 antibody in the vaccinated pigs during vaccination and challenge. TWJ-E2 vaccinated pigs developed high levels of E2 antibody after the booster vaccination. Positive cut-off value: 40%. Solid lines: TWJ-E2 vaccinated groups; dashed lines: unvaccinated controls.

Table 2
TWJ-E2 vaccinated pigs developed high levels of neutralizing antibody[a].

Group	Pig No.	0 DPV	35 DPV (0 DPC)	59 DPV (24 DPC)	Group	Pig No.	35 DPV (0 DPC)	50 DPV[b] (15 DPC)
E2/JL23	2324	< 5	120	3840	JL23	234	< 5	< 5
	2339	< 5	320	2560		260	< 5	< 5
	2348	< 5	160	3840		262	< 5	< 5
	2352	< 5	240	3840		264	< 5	< 5
	2359	< 5	320	2560		272	< 5	< 5
E2/GD23	2303	< 5	640	2560	GD23	232	< 5	7.5
	2323	< 5	480	1920		265	< 5	15
	2351	< 5	480	7680		268	< 5	10
	2354	< 5	120	1920		281	< 5	10
	2369	< 5	80	3840		285	< 5	5
E2/HuB12	2308	< 5	120	3840	HuB12	215	< 5	10
	2317	< 5	320	1280		233	< 5	7.5
	2343	< 5	160	2560		238	< 5	5
	2353	< 5	160	3840		244	< 5	< 5
	2355	< 5	320	5120		253	< 5	< 5
E2/AH1	2304	< 5	160	2560	AH1	275	< 5	< 5
	2356	< 5	160	15,360		288	< 5	< 5
	2361	< 5	80	2560		468	< 5	< 5
	2364	< 5	480	3840		473	< 5	< 5
	2367	< 5	240	7680		475	< 5	< 5
C-strain/JL23	2305	< 5	7.5	480	Negative control	13	< 5	< 5
	2306	< 5	< 5	160		20	< 5	< 5
	2346	< 5	7.5	160		37	< 5	< 5
	2349	< 5	< 5	320		45	< 5	< 5
	2350	< 5	< 5	480		46	< 5	< 5

[a] CSFV field isolate used for neutralization assay was the same as that used for challenge of both vaccinated and unvaccinated control pigs.
[b] Most pigs in the unvaccinated control groups were dead at 59 DPV (24 DPC), thus sera collected from these pigs at 50 DPV (15 DPC) were subjected to neutralization assay.

subunit vaccines derived from the C- and Shimen strains (subgenotypes 1.1) (Hua et al., 2014; Madera et al., 2016; Zhang et al., 2018), which have all been shown to be effective against challenge with genotype 1.3 field isolates or the Shimen strain, but have not been evaluated with the currently dominant CSFV genotype 2 field isolates in China. As the first E2 subunit vaccine licensed in China and the third worldwide, TWJ-E2 was known to provide complete protection against the genotypically homologous Shimen strain but, until the present study, it remained unknown whether it could confer effective cross immunity against different genotype 2 field isolates.

The four CSFV subgenotype 2.1 and 2.2 field isolates selected to investigate this concern as being representatives of the strains currently prevalent in China shared only 87.1–88.7% identity with the E2 protein of the TWJ-E2 vaccine. A separate study in our laboratory had categorized them as being highly virulent (3 strains) or moderately virulent (Gong et al., 2019) and these characteristics were confirmed in the present study (Fig. 1A, B). Nevertheless, all TE2-vaccinated pigs survived challenge with all 4 field strains without developing fever, clinical signs, pathological lesions, viremia or viral shedding. Serological tests showed that TWJ-E2 vaccinated pigs developed high levels of E2 and neutralizing antibodies, both of which being recognized as being markers of full protection against lethal challenge. As well, the C-strain vaccine also protected vaccinated pigs from challenge with the heterologous subgenotype 2 strain, thereby confirming the results of a previous study with sub-subgenotype 2.1b (Luo et al., 2017). In 1 of the 5 C-strain vaccinated pigs (No. 2350) its body temperature reached above 40 °C during the second week post challenge, but viremia, viral shedding and viral RNA in different tissues were never detected during the challenge observation period. Further testing showed that from 12 to 18 DPC the pig had very high leukocyte counts (32.7–49.2 × 10^9/l), which is significantly higher than the maximum threshold (22.0 × 10^9/l) noted in the manual of the Mindray BC-2800 Vet analyzer (Mindray Medical USA Corp, Mahwah, USA). The fever may therefore have been caused by factors other than CSFV infection.

The E2 protein is considered to be the major antigen eliciting production of protective NA against CSFV (Risatti et al., 2005). Following the two-dose vaccination with TWJ-E2, pigs developed high levels of E2 antibody and NA, although there was a transient decrease in E2 antibody levels for 3–9 days post challenge (Fig. 3). This may have been the result of some E2 antibody being used to neutralize the challenge virus in the vaccinated animals. In the C-strain vaccinated pigs, only low levels of E2 antibody and NA developed prior to challenge;

Table 3
E^{rns} antibody response induced by CSFV infection[a].

Group	DPC 0	DPC 3	DPC 6	DPC 9	DPC 12	DPC 15	DPC 18	DPC 21	DPC 24
E2/JL23	0/5	0/5	0/5	2/5	2/5	2/5	1/5	1/5	1/5
E2/GD23	0/5	0/5	0/5	0/5	1/5	1/5	0/5	0/5	1/5
E2/HuB12	0/5	0/5	1/5	0/5	1/5	0/5	0/5	2/5	2/5
E2/AH1	0/5	0/5	0/5	4/5	1/5	3/5	2/5	4/5	4/5
C-strain/JL23	0/5	0/5	1/5	4/5	4/5	4/5	4/5	5/5	4/5
JL23	0/5	0/5	0/5	0/5	4/5	3/4[b]	3/3[b]	2/2[b]	
GD23	0/5	0/5	0/5	1/5	4/5	5/5	4/4[b]	3/3[b]	3/3[b]
HuB12	0/5	0/5	0/5	0/5	1/5	5/5	4/4[b]	3/3[b]	1/1[b]
AH1	0/5	0/5	0/5	0/5	0/5	3/5	4/4[b]	2/2[b]	
Negative control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

[a] Criteria of positive E^{rns} antibody is S/P ≥ 0.5 (OD_{450}) based on the kit introduction, and the S/P values of the positive samples were.0.50–4.00.
[b] deaths were excluded.

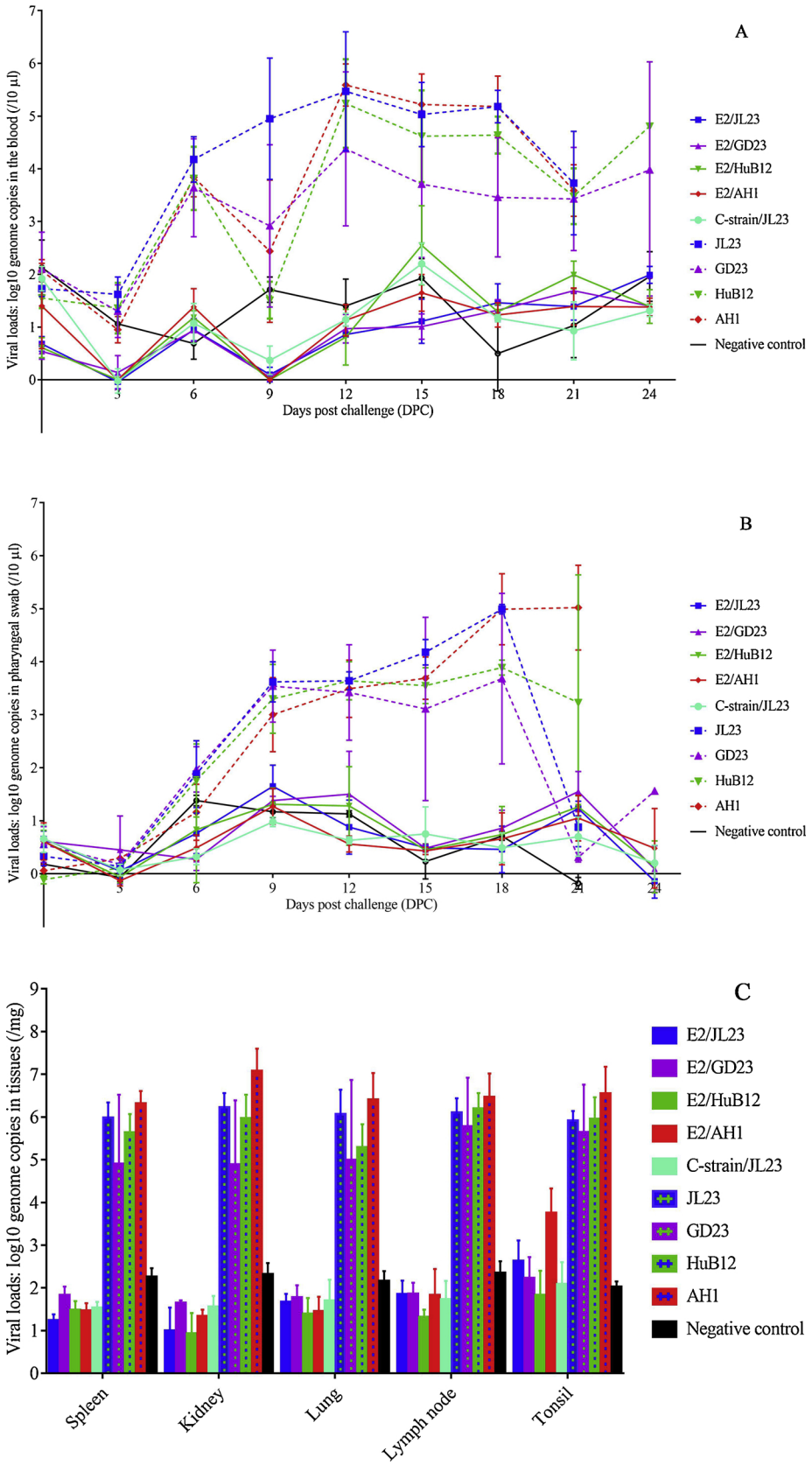

Fig. 4. TWJ-E2 vaccine eliminated the virus and prevented viral shedding after challenge. Viral RNA loads in anti-clotted blood (A), pharyngeal swabs (B), and tissues (C) were determined using qRT-PCR. Solid lines: TWJ-E2 vaccinated groups; dashed lines: unvaccinated controls.

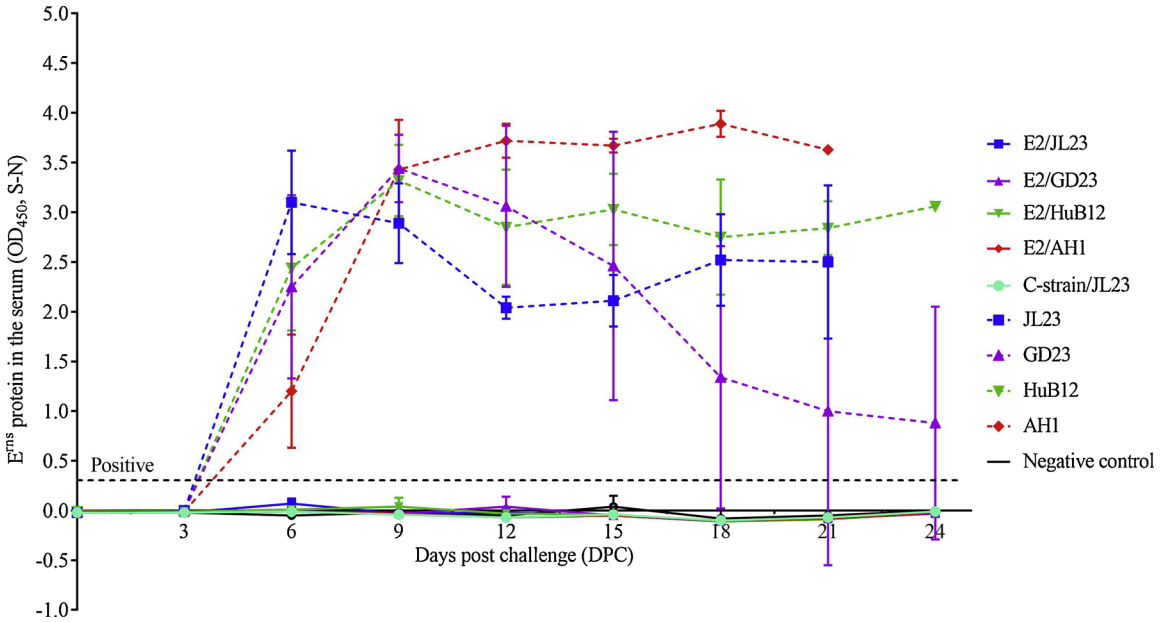

Fig. 5. Assay of E^{rns} protein in the serum of vaccinated, unvaccinated and negative control pigs. E^{rns} protein was not detected in any TWJ-E2 and C-strain vaccinated pigs after challenge, but was found in all unvaccinated control pigs from 6 DPC onward. Positive cut-off value: 0.3 (S—N). Solid lines: TWJ-E2 vaccinated groups; dashed lines: unvaccinated controls.

nevertheless, clinical symptoms of CSF disease were not observed in any of these pigs during the entire observation period (Fig. 1A, B). Early protection in the C-strain vaccinated pigs before appearance of NA might therefore have been mediated by a T-cell response resulting in production of $CD4^+CD8^+$ and $CD4^-CD8^+$ lymphocytes after C-strain vaccination (Ganges et al., 2008; Graham et al., 2012a; Pauly et al., 1995; Piriou et al., 2003; Suradhat et al., 2001, 2007; Van Oirschot, 2003). In addition, CSFV challenge could have served as a robust booster since much higher levels of NA developed in the vaccinated pigs after challenge. Vaccination with TWJ-E2 generated much higher levels of NA than with the C-strain (Table 2). This may be due to the large quantity of immunogen in the TWJ-E2 vaccine (> 30 μg of E2 protein per dose). However, because of insufficient induction of cellular immunity, the onset of robust immunity against CSFV challenge elicited by two-dose E2 subunit vaccine was much later than that induced by C-strain vaccine or live marker vaccine CP7-E2alf, which can prevent virus transmission within 5 or 7 days of vaccination (Graham et al., 2012b; Blome et al., 2017). A two-dose vaccination with the current TWJ-E2 is therefore unsuitable for emergency vaccination in CSFV-free countries. Furthermore, both E2 subunit vaccines, *BAYOVAC CSF E2* and Porcilis Pestis, have been previously reported to provide incomplete protection against vertical transmission (Depner et al., 2001). It remains to be determined if the recently developed TWJ-E2 vaccine can clear field viruses in vaccinated pregnant sows after challenge.

While previous studies of the CSF DIVA strategy have considered E^{rns} antibody to be the diagnostic target, the dynamics of its production have not been determined in pigs vaccinated with an E2 subunit vaccine (Lipowski et al., 2000; Hua et al., 2014; Madera et al., 2016; Zhang et al., 2018). In the present study we found that 19/20 unvaccinated challenge control pigs developed stable levels of E^{rns} antibody throughout the observation period (Table 3). In the vaccinated groups 10/20 TWJ-E2 vaccinated pigs developed E^{rns} antibody after challenge (2 in E2/JL23, 1 in E2/GD23, 2 in E2/HuB12 and 5 in E2/AH1 groups), but only 3 developed stable and persistent levels throughout, with the remaining 7 developing only an intermittent E^{rns} antibody response. E2-vaccinated pigs could possibly seroconvert to being E^{rns} antibody positive after contact with field CSFV, although it is unclear why some of the vaccinated/challenged pigs remained negative, at least when tested with the *pigtype* E^{rns} ELISA. One of the most likely reasons is insufficient sensitivity of the E^{rns} ELISA used, with the same shortcoming applying to other E^{rns} ELISA assays such as PrioCHECK CSFV E^{rns} (Schroeder et al., 2012). Furthermore, cross-reactivity with antibody raised against ruminant pestiviruses was observed upon using the *pigtype* E^{rns} ELISA (Meyer et al., 2017). These being the cases, more extensive studies are therefore required to assess the validity of the current E^{rns}-ELISAs, with development of more sensitive and specific assays being essential for accompanying the application of CSFV marker vaccines.

Development of E^{rns} antibody in E2-vaccinated pigs may be associated with the amount of E^{rns} protein produced. For instance, pigs in the AH1 challenge control group produced high levels of E^{rns} protein over the entire infection period (Fig. 4), while all pigs in the E2/AH1 vaccinated group developed E^{rns} antibody (Table 3). Although both E2/JL23 and C-strain/JL23 groups were challenged with the JL23 strain, their E^{rns} antibody responses differed: all C-strain vaccinated pigs but only 2 of the E2/JL23 group became E^{rns} antibody positive. This might be ascribed to the immune memory elicited by C-strain vaccine even though E^{rns} antibody was not detected in any C-strain vaccinated animal after vaccination during the 17 day observation period (2 weeks before challenge + 3 days before the vaccine immune response).

In conclusion, TWJ-E2 vaccinated pigs developed high levels of E2 and neutralizing antibodies, and completely protected pigs from lethal challenge by each of 4 heterologous (genotype 2) CSFV field strains. The resulting immunity was comparable to that induced by the conventional C-strain vaccine.

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