Indian Journal of Animal Sciences **88** (6): 651–654, June 2018/Article

Immunogenicity of a live attenuated classical swine fever vaccine in pigs under field conditions*

R SANGEETHA[1], K KUMANAN[2], K VIJAYARANI[3], A RAMESH[4] and P TENSINGH GNANARAJ[5]

Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu 600 007 India

Received: 27 July 2017; Accepted: 10 April 2018

ABSTRACT

A study was conducted for assessing the immunogenicity of live classical swine fever (CSF) vaccine in pigs under field conditions. Pre immunization blood samples and post immunization (PI) blood samples at 30, 60, 90, and 120 days post immunization (DPI) were collected. Th1 response was assessed by the expression of IFN-γ, IL-2 and IL-12, and serum antibody titres and IL-10 expression were studied for Th2 response. The mean±SE of IFN-γ gene response as fold change ($2^{\Delta\Delta Ct}$ values) at 30, 60, 90, and 120 days post immunization (DPI) were 9.61±0.65, 3.49±0.44, 2.22±0.407, and 3.12±1.35 respectively. IFN-γ response showed significantly increased fold change during PI period. Significant peak increase in the expression level was observed at 30 DPI. IL-2 gene response showed significantly increased fold change during PI (Mean expression at 30, 60, 90, and 120 DPI was 4.41, 2.61, 3.291 and 2.09 respectively) and peak increase was observed at 30 DPI. IL-12 response also showed significant increase during PI with a peak expression at 30 DPI (Mean fold changes at 30, 60, 90, and 120 days post immunization were 4.92, 2.97, 2.72, and 3.61 respectively). Maximum IL-10 expression was observed at 60 DPI (Mean fold change at 30, 60, 90, and 120 days post immunization were 3.364±0.723, 11.56±1.64, 4.73±1.01, and 2.599±1.05 respectively). Pre- and post-immunization antibody responses were monitored by enzyme linked immunosorbent assay. Antibody titres increased consistently from 30 days after primary immunization until 120 days. Montanide (gel 01) coupled PK-15 adapted live, CSV vaccine induced a good Th1 response followed by Th2 response.

Key words: Classical swine fever vaccine, Cytokines, ELISA, Pig, Real time qPCR

Classical Swine Fever (CSF) classified as a list A disease by Office International des Epizooties is an economically important disease of pigs causing heavy mortality in susceptible pigs worldwide (Xu *et al.* 2009) and the causative agent, CSF virus (CSFV) spreads by both direct and indirect methods (Paton *et al.* 2003). Infection with highly virulent CSFV strains leads to acute disease characterized by high mortality and morbidity, while moderate to low virulence strains induce a prolonged, chronic disease (Dong *et al.* 2013). Tamil Nadu has been found to be endemic to CSFV infection (Rathnapraba *et al.* 2012) and few isolates of CSFV recovered from local outbreaks have been characterized (Rathnapraba *et al.* 2013, Vadivoo *et al.* 2013).

Pigs reared under farm conditions are being immunized regularly with the lapinized (Oirschot 2003) CSFV vaccine. However, this vaccine is not freely available leading to the presence of an unvaccinated population even among the farm grown pigs. Moreover, the free ranging, community pigs which thrive by scavenging are never immunized. As a result, there exists always a susceptible population of unimmunized pigs. This has necessitated the farmers to look out for alternatives to the not so freely available, lapinized CSFV vaccine. PK-15 cell line, which is of pig kidney origin, supports the growth of CSFV. A candidate PK-15 adapted, live CSF vaccine has been developed at the Department of Animal Biotechnology, Madras Veterinary College, Chennai and limited immunization studies indicated its immunogenicity. The present study was aimed at assessing the immune response of the vaccine under field conditions with the ultimate idea of taking this vaccine to field use.

MATERIALS AND METHODS

Cells and virus: PK-15 cells (ATCC, USA) were cultured in DMEM medium, supplemented with 10% Pestivirus-free foetal bovine serum (FBS) at 37°C in 5% CO_2. The cells were infected with 10^5 $TCID_{50}$ of already adapted CSF virus and incubated in CO_2 incubator. At 5 days post infection (DPI), infected cells were subjected to 3 cycles of freezing and thawing for complete release of the CSFV from the infected cells. Cell lysate was clarified by centrifugation at 3,000 rpm at 4°C for 15 min and the virus containing

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*MVSc thesis work.

Present address: [1](drsangivet@gmail.com), [3]Professor and Head (ranikumanan65@hotmail.com), Department of Animal Biotechnology, Madras Veterinary College, Chennai. [2]Director (kumananrani@gmail.com), CAHS. [4]Vaccine Research Centre (VV) (ramesh.a@tanuvas.ac.in), [5]University Research Farm (tensinghgnanaraj.p@tanuvas.ac.in), MM Colony, Chennai.

supernatant was aliquoted in vials and stored at –20°C for further use. Presence of the virus in the cells was confirmed by fluorescent antibody virus neutralization (FAVN). Similarly, presence of virus in cell culture fluid was confirmed by RT-PCR.

Vaccine: A live attenuated cell culture vaccine developed at Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, using the PK-15 adapted strain was used in the present study. The vaccine candidate was developed after adapting the field CSFV for 45 times in PK-15 cells. Based on the preliminary dose trials, the dose was fixed as 10^7 $TCID_{50}$ (unpublished data). Thirty millilitres of CSFV infected cell culture fluid with a $TCID_{50}$ titre of 10^8 was mixed with 240 ml of serum free DMEM and 30 g of Montanide gel 01 (Seppic, France) to have a final concentration of 10% in the vaccine. Contents were mixed well at medium speed in a 500 ml conical flask kept over a magnetic stirrer for 15 min at 4°C. The resultant vaccine was stored at 4°C until used. Contents were mixed well before administration into animals.

Immunization of animals: Immunization of pigs was carried out as presented in Table 1 with the CSFV vaccine. Blood samples were collected from the immunized animals in vacutainer tubes with and without EDTA, prior to immunization and every month after immunization.

Isolation of peripheral blood mononuclear cells (PBMCs) and separation of serum: For lymphocyte isolation, blood samples were diluted with PBS (1: 1) and layered on Histopaque – 1077 (Sigma, USA) and the cells were spun at 3,000 rpm for 20 min at 4°C. PBMCs layer was carefully removed and washed twice with PBS. The cells were cultured and stimulated with the antigen and stored at –70°C for RNA isolation. Serum samples were separated by low speed centrifugation and stored at –20°C.

Table 1. Immunization schedule

No. of animals	Age	Place	Dose	Route
10	2–3 months	Farm 1 (Madavaram)	1 ml (10^7 $TCID_{50}$)	s/c
20	2–3 months	Farm 2 (Tambaram)	1 ml (10^7 $TCID_{50}$)	s/c
74	2–3 months	Farm 3 (Erode)	1 ml (10^7 $TCID_{50}$)	s/c

Isolation of RNA and cDNA synthesis: Isolation of RNA from PBMCs was done using Trizol-LS reagent (Sigma, USA). cDNA synthesis (reverse transcription) was done using random hexamers with initial concentration of 500 ng of total RNA from each sample using the high capacity cDNA synthesis kit (Applied Biosystems, USA). cDNA synthesis was carried out in a thermal cycler at 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and a hold at 4°C until used in qPCR.

Quantitative real time polymerase chain reaction: Quantitative real time PCR (qPCR) was carried out using Fast SYBR® Green (Eppendrof, Germany) reagent. Optimization of the qPCR for IFN-γ, IL-2, IL-12, and IL-10 genes along with endogenous control β-actin was carried out by employing different concentrations of primers (Table 2) as well as different annealing temperatures varying from 55°C to 60°C. Primers were designed by using the PrimerQuest tool of IDT. The following cycle conditions were used: initial denaturation at 95°C - 3 min, denaturation at 94°C - 10 sec, annealing at 57°C, extension at 72°C - 45 sec, final extension at 72°C - 10 min for 40 cycles. Annealing temperatures for IFN-γ, IL -10, β-actin was 60°C and for IL-2 and IL-12 was 55°C. After the run, data were saved and analyzed using Applied Biosystems StepOne™ Real time PCR software v2.0.

ELISA for antibody detection: Indirect ELISA for antibody detection was optimized using various coating concentrations of antigen as per the procedure of Moser *et al.* (1999) with minor modifications. Purified whole virus was used as antigen for coating plates at different concentrations for standardizing the ELISA. Polystyrene microplates (NUNC, Denmark) were coated overnight at 4ºC with 100 µl/well of 100 ng of antigen (in CBB buffer, pH 9.6). Plates were either used in the subsequent day or stored at –20ºC for further use. Before use, plates were washed three times with wash buffer (PBS with 5% Tween 20) and an appropriate dilution of each test serum (1:50) was added to each of two wells on the plate (100 µl/well). After an incubation period of 1h at 37ºC, the plates were washed three times as above and 100 µl of 1:2000 diluted rabbit anti-pig IgG peroxidase conjugate (Sigma, USA) was added to each well. Following further incubation of 1 h at 37ºC, the plates were again washed three times and 100 µl of ortho-phenylene-diamine - OPD substrate (Sigma, USA)

Table 2. Primers used in real time quantitative PCR (SYBR green chemistry)

Primers used		Primer sequence	Annealing temperature	Expected product size
IFN-γ	FP	AATGGAAAGAACAGTGGGAGG	60	118 bp
	RP	TTAAGATGAGGAAACAGCCCC		
IL-2	FP	GTGCACCTACTTCAAGCTCTAC	55	131 bp
	RP	TAAATGTGAGCATCCTGGAGAG		
IL-10	FP	TTCCCAACCAGCCTGCCCCACAT	60	90 bp
	RP	CGCCC1121ATCTGGTCCTTCGTTTGA		
IL-12	FP	CTCTTTCCGTTTTCCCCTCA	55	111 bp
	RP	TTTCCAAACATCACGCTTCAC		
β-ACTIN	FP	GGTTCTATTTGCTTTTCCCCAG	60	86 bp
	RP	ATACACCCACAGCACCTTATG		

was added. After 5 min of incubation, the reaction was stopped by adding 50 µl of 2M H_2SO_4 to each well. Plates were read in an ELISA reader at an optical density of 492 nm. The OD of each sera was expressed as the percentage optical density (OD %), which was calculated on the basis of the negative control serum in each microplate, in order to minimize variation between plates. The OD% was calculated as per the formula:

$$\text{OD \%} = \left(\frac{\text{OD test serum}}{\text{OD negative control serum}}\right) \times 100$$

The cut-off point was calculated on the basis of the arithmetic mean (m) of the OD% of 20 sera found negative for CSFV antibodies plus three standard deviations (s), thus Cut-off point = m(OD%) + 3s.

RESULTS AND DISCUSSION

Classical swine fever is one of the highly contagious disease of both domestic and wild pigs. The severity of CSF varies depending on the virulence of the virus and the immunological status of the host. Humoral immune response plays a vital role in inducing neutralizing antibodies and protection against a challenge by CSFV. Moreover, the role of different cytokines in CSF immunity has not been clearly understood (Tarradas *et al.* 2010). In this context, in the present study, apart from assessing the Th2 response, Th1 response in pigs following immunization was also studied by assessing the expression levels of Th1 cytokines like IFN-γ, IL-2 and IL-12.

Cell mediated immune response (Th1) following immunization with PK-15 adapted CSF vaccine was assessed by studying the expression of IFN-γ, IL-2 and IL-12 genes. IFN-γ, IL-2 and IL-12 genes with β-actin gene as endogenous control were amplified without any primer dimer and non-specific amplifications. Agarose gel electrophoresis revealed 118 bp, 131 bp, 111 bp and 86 bp PCR products of IFN-γ, IL-2, IL-12, and β-actin respectively.

Interferon-γgene response: IFN-γ response is considered as a good indicator of Th1 response (Ganges *et al.* 2008, Simon *et al.* 2013). The mean±SE of IFN-γ gene response as fold change ($2^{\Delta\Delta Ct}$ values) at 30, 60, 90, and 120 days post immunization (DPI) were 9.61±0.65, 3.49±0.44, 2.22±0.407, and 3.12±1.35 respectively. A significant increase in antigen specific IFN-γ response was noticed up to a period of 4 months after immunization with a peak response at one month after immunization (fold change of 9.61) indicating a very strong Th1 response in immunized pigs even under field conditions. The expression levels dropped at 2nd and 3rd month and a slight increase was noticed at 4th month. The results indicated that under field conditions, the live vaccine induced good cell mediated immunity characterized by the induction of CSFV specific IFN-γ response. Consistent levels of CSFV specific IFN-γ levels from 1 to 4 months might contribute in the control of the disease by early blocking of CSFV replication (Tarradas *et al.* 2010).

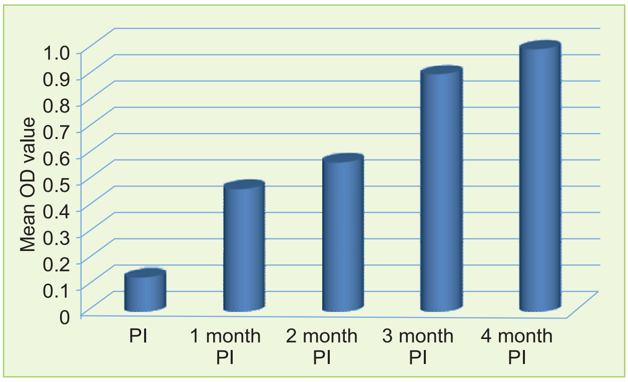

Fig. 1. Serum antibody response to live attenuated classical swine fever vaccine in immunized animals at monthly intervals.

IL-2 gene response: Mean expression as fold change ($2^{\Delta\Delta Ct}$) at 30, 60, 90 and 120 DPI was 4.41, 2.61, 3.291 and 2.09 respectively. As noticed in case of IFN-γ response, IL-2 expression showed a significantly increased fold change ($P<0.01$) during post immunization period and highest expression was observed at 30 DPI. However, IL-2 expression decreased during 2nd month and increased during the 3rd month which again got reduced during 4th month compared to the 3rd month.

IL-2 levels have also been reported to be associated with Th1 response. IL-2 stimulates a set of complex signal transduction pathways and mediates signaling through IL-2R, resulting in cell proliferation (Borca *et al.* 2008). As in the case of IFN-γ, a definite up regulation of CSFV antigen specific IL-2 response was also noticed in vaccinated animals at 1, 2, 3 and 4 months post immunization. However, the expression levels were high and low in alternating months covering the observation period of four months.

IL-12 gene response: Mean fold changes at 30, 60, 90, and 120 days post immunization were 4.92, 2.97, 2.72, and 3.61 respectively. Induction of functional Th1 response is dependent on cytokines such as IFN-γ and IL-12. Once secreted IL-12 can promote the development and maintenance of cell mediated Th1 responses. In our study, IL-12 (one of the Th1 type cytokine) was up-regulated in the immunized animals when compared to the pre immunization levels. It has been reported that the levels of IL-12 were high in the white blood cells of pigs following infection with CSFV (Sun *et al.* 2014) which could probably help the animal to mount a strong Th1 response to overcome the infection. In a similar way, in our study, a significant increase in the expression of IL-12 was noticed in immunized animals one month after immunization with the live vaccine with a mean fold change of 4.92. As in case of exposure to the virulent virus (Sun *et al.* 2014), a strong IL-12 response has been elicited when the animals were exposed to live attenuated CSF vaccine triggering a cell mediated immune response. However, as the months passed by, the expression levels of IL-12 decreased. Among the three Th1 response cytokines, expression of IFN-γ was strongest at one month post immunization followed by IL-

12 and IL-2.

IL-10 gene response: Humoral immune response (Th2) was assessed by studying IL-10 cytokine gene expression and serum antibody response. Agarose gel electrophoresis revealed 90 bp and 86 bp PCR products of IL-10, and β-actin respectively. IL-10 is reported to be a pleiotropic cytokine involved in a variety of immunological events like enhancement of B cell survival, proliferation and antibody production. The mean±SE of IL-10 gene response as fold change at 30, 60, 90, and 120 days post immunization were 3.364±0.723, 11.56±1.64, 4.73±1.01, and 2.599±1.05 respectively. In our study, IL-10, which is a pro Th2 response cytokine had a highly significant expression at 2 months after immunization with a mean fold change of 11.5. Considering the IL-10 function on B cell survival, it can be postulated that this cytokine may be involved in keeping B cell homeostasis after immunization and thereby sustaining the antibody production as has been reported earlier (Tarradas *et al.* 2010). The results indicated that a sustained expression of IL-10 would help in the survival of the B cells thereby maintaining the antibody production against CSFV. This finding further confirmed the immunogenicity of the live vaccine we had tested under field conditions.

Serum antibody response: Classical swine fever virus specific antibody titres are good indicators of humoral immune response. Pre immunization and post immunization (30, 60, 90 and 120 DPI) antibody titres were assessed in all the pigs. In contrast to an earlier report suggesting the absence of immune response to a live attenuated vaccine in pigs persistently infected with CSFV (Gonsalez *et al.* 2015), good seroconversion was noticed in immunized animals for the entire observation period of 120 days (Fig. 1) as has been observed when pigs were immunized with a live attenuated CSF virus vaccine with interferon-gamma adjuvant (Fan *et al.* 2016). The mean antibody titre peaked during the fourth month PI under field conditions. This again confirmed that the PK-15 adapted vaccine has induced good seroconversion in immunized animals and could confer good protective immunity against a field challenge of CSFV. Further studies are being taken up to assess the duration of the immunity under field conditions along with challenge studies.

It is concluded that, the live vaccine induced good humoral and cell mediated immune responses during the study period under field conditions and the results obtained in this study will help in large scale use of the vaccine in the field at farmer's door step.

ACKNOWLEDGEMENTS

The authors thank the Department of Biotechnology, Government of India, New Delhi for funding part of this work through the Network Project on Classical Swine Fever. The authors also thank the Dean, Madras Veterinary College; Dean, Faculty of Basic Sciences, TANUVAS; Director, Centre for Animal Production Studies, TANUVAS; Professor and Head, Department of Animal Biotechnology, MVC and Associate Professor and Head, VUTRC, Erode for the help and facilities provided.

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