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A Comparative Immune Response of Pigs Vaccinated with and without Adjuvanted Cell Culture Adapted Classical Swine Fever Virus Vaccine

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ABSTRACT

Background: Classical swine fever is a highly contagious economically devastating viral disease of pigs. In endemic country like India including NE States, vaccination is the best way for prevention and control. Although live attenuated vaccines are available, booster vaccination gives better protective immunity. Present study was designed to evaluate immunity and duration of immune response in cell culture CSF vaccine with and without incorporation of adjuvant.

Methods: In the present study, four groups of CSFV free pigs containing 6 pigs in each group were immunized as follows- with cell culture adapted whole CSFV vaccine, adjuvanted with montanide oil, primary CSFV vaccine with booster and without booster and control group as unvaccinated. Immune response was demonstrated by per cent inhibition in blocking ELISA.

Result: Vaccinated pigs elicited early antibody by 7th dpv which reached a peak at 30th dpv and maintaining high antibody titre upto 180dpv. Single oil adjuvanted whole vaccine effectively stimulated neutralizing antibody with 90-95% inhibition titre. In primary and booster vaccinated pigs, antibody titres showed significant difference. Oil adjuvanted vaccine was safe eliciting high neutralizing antibody titre with single vaccination till marketable age. Also a good comparison of all the four combinations of vaccines were obtained.

Key words: Adjuvanted vaccine, Blocking assay CSFV, ELISA,

INTRODUCTION

Classical swine fever (CSF) is a highly contagious WOAH listed viral disease of domestic pigs, wild hogs and pygmy hogs (Barman *et al*., 2012; Barman *et al.,* 2014). The disease is caused by a single-stranded, positive-sense RNA virus belonging to the Pestivirus genus in the Flaviviridae family (Lefkowitz *et al.,* 2018). India has experienced numerous CSF outbreaks due to its endemicity and North eastern region especially Assam is not an exception. It causes severity of disease and high mortality of pigs in affected areas (Lalremruata *et al.,* 2015). This has resulted in major economic losses in pig farming sector as it is the only livelihood source of the downtrodden people of the society.

Vaccination emerges as the most effective method to prevent CSF virus infection and control the disease in an endemic country like India. The traditional live attenuated C-strain of CSFV (sub-genotype 1.1), adapted as a lapinized vaccine, has been used to proactively manage CSF in pigs but it covers less than one percent of the total pig population failing to cater the need of pig farmers (Nath *et al.,* 2016). Cell culture-based vaccine can be an effective alternative approach to meetup animal ethical issues and can be produced in bulk. The incorporation of adjuvants in vaccine formulations, can enhance immune responses as well as the duration of immunity (Zhao *et al.,* 2023; Pulendran *et al.,* 2021). The detection of CSF virus from porcine is made rapid by the development of real-time TaqMan RT-PCR assay (Rout *et al.,* 2016).

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Therefore, the present study was designed in order to evaluate the development of immunity and duration of immune response in cell culture CSF vaccine with and without the incorporation of adjuvant.

MATERIALS AND METHODS

Ethical approval

The use of experimental animals in test procedure was approved by the Institutional Animal Ethics Committee of Assam Agricultural University, Khanapara, Assam, India. Experiments on animals were done with due care and

minimum pain and discomfort by the researchers.

Study area and source of vaccines

Lapinized C strain vaccine attenuated in PK-15 cell line upto 45 passage level in the Department of Veterinary Microbiology, College of Veterinary Science, Assam Agriculture University, Khanapara was used in this study during the period 2016-2019. Propagation of CSF virus in the PK-15 cell culture was confirmed by using sandwich ELISA as per the method described by Sarma and Sarma (1996) with slight modification as well as by single step Taqman probe based Real-time RT-PCR (Hoffmann *et al.,* 2005). Two different types of cell culture adapted CSF vaccine formulations were prepared. In one cell culture adapted CSFV vaccine containing $10^{3.0}$ $TCID_{50}$ per ml was used without incorporation of adjuvant. For preparation of adjuvanted vaccine. Montanide™ ISA R 71 VG (Seppic, France) a ready to use range of oil adjuvant was used.PK-15 adapted whole CSFV vaccine containing $10^{3.0}$ $TCID_{50}$ per ml was emulsified at a 1:1 ratio using montanide oil adjuvant. The vaccine and adjuvant each added in a sterile 5ml tubular glass container and injected back and forth several times with a 2ml glass syringe until properly mixed. Further sterility test for both adjuvanted vaccine and vaccine without adjuvant was done in10% blood agar plates incubated at 37°C for 48 hours and on Sabouraud's dextrose agar plates incubated at room temperature for 7-14 days for bacterial and fungal growth, respectively.

Immunization of pigs

Immune response of cell culture adapted CSF virus vaccine with and without adjuvant was studied in CSFV free 3 months old weaned piglets. A total of 24 numbers of piglets (Hampshire crossbred) devoid of CSF antibody were used for the experimental study. Animals were divided into 4 groups, each group consisting of 6 piglets (Table 1).

Assay of immune response

Blood samples were collected from immunized as well as unvaccinated control pigs before vaccination on 0 day and 7, 14, 21, 30, 60, 90 and 180 days post vaccination. Approximately 5-8 ml of blood was collected from anterior venacava and serum was separated for further tests. Humoral immune response in vaccinated animals was analyzed by indirect ELISA as per the method described by Sarma and Sarma, (1996) with modification and neutralization assay (IDEXX CSFV antibody ELISA Kit).

The presence of CSFV E2- specific antibodies in the serum samples of all the four groups were analyzed at different times after vaccination by blocking ELISA assay (IDEXX CSFV antibody ELISA Kit). The protocol of the test briefly, the IDEXX CSFV Ab assay utilizes microplates coated with CSFV antigen. Antibodies present in the test sample will block the binding of horseradish peroxidase conjugated CSFV specific monoclonal antibodies. The bound monoclonal antibodies are detected by a substrate reactive with horseradish peroxidase. The result was indicated by color development. The optical density was measured by a microplate reader at a single wavelength of 450 nm, or dual wavelengths of 450 nm and 650 nm. Color development was weak (positive result), when CSFV specific antibodies were present in the test sample. Color development was maximal (negative result) in the absence of specific antibodies. The blocking percentage of the sample was calculated from the optical density (OD450) obtained with the test sample and the OD450 of the negative control. The test sample was positive (antibodies were present) if it gives a blocking percentage ≥40%. The test sample was negative (antibodies were absent) if it gives a blocking percentage ≥30%. If the blocking percentage of the sample was > 30% and < 40%, retest the animal at a later date. If the sample was still doubtful after retesting, the result could be confirmed by serum neutralization test.

Detection of CSF vaccine virus in circulation

The live attenuated CSF vaccine virus was propagated and should remain in circulation at various day of post vaccination. The presence of the virus was detected in leucocyte by Taqmann based real time RT-PCR (Hoffmann *et al.,* 2005) at day 0, 14, 30, 60, 90 of post vaccination. A 6-8 ml peripheral blood was collected from each group in anticoagulant added vial and leucocytes were separated by density gradient medium Histopaque (Sigma-Aldrich) and CSF virus was detected by Taqman probe based RT-PCR all vaccinated groups.

RESULTS AND DISCUSSION

Study was conducted to evaluate the humoral immune response of CSF whole virus vaccine with adjuvant single dose, CSF whole virus vaccine without adjuvant single dose and with booster dose and unvaccinated as control group. The presence of CSF virus in the PK-15 cell culture was confirmed by sandwich ELISA where the cell culture virus

Table 1: Experimental design of CSFV vaccines in piglets.

Group	Type of vaccine	No. of piglets	Dose per animal	Route of administration
Group I	CSF whole virus vaccine with adjuvant single dose	6	$10^{3.0}$log $TCID_{50}$	I/M
Group II	CSF whole virus vaccine single dose	6	$10^{3.0}$log $TCID_{50}$	I/M
Group III	CSF whole virus vaccine with booster dose on 30th day	6	$10^{3.0}$log $TCID_{50}$	I/M
Group IV	Unvaccinated control (Sterile PBS)	6	2 ml	I/M

showed OD of 1.51 against the negative OD of 0.04. Li *et al* (2018) also performed ELISA for detection of and confirmation of CSF virus in serum samples of Tibetan pigs in Nyingchi area of Tibet, China making ELISA an appropriate detection assay. In single step Taqman probe based Real-time RT-PCR (Hoffmann *et al.,*2005), CT value exhibited at cycle 13.9. Quantitation of virus titre at 45th passage level of the whole CSFV vaccine adapted in PK-15 cell line was determined by immunoperoxidase test (IPT). $TCID_{50}$ value at 45th passage level was $10^{5.05}$ $TCID_{50}$. The resultant titre of the PK-15 adapted whole CSFV vaccine was adjusted to $10^{3.0}$ $TCID_{50}$ and was used for vaccination of pigs (Table 1). The present study was carried out to evaluate the humoral immune response of Classical Swine Fever vaccine using oil adjuvanted CSF whole virus vaccine, CSF whole vaccine without adjuvant single dose, CSF whole vaccine without adjuvant booster dose along with a control unvaccinated group.

The live attenuated PK-15 cell culture adapted whole CSFV vaccine developed in the Department of Veterinary Microbiology, Assam Agriculture University, Khanapara, using lapinized C-strain of CSFV used in this study also revealed presence of the CSF virus particle in vaccine in Sandwhich ELISA and single step Taqman probe based Real-time RT-PCR. Vaccination has been widely used to protect pigs from CSFV infection (Ji *et al.,* 2014). A number of live attenuated CSF vaccines have been developed by adapting and passaging in tissue culture. The C strain vaccine of CSFV has been regarded as one of the most effective vaccine (Coronado *et al.,* 2021). The efficacy of vaccine could be improved by incorporating adjuvants in the vaccine formulations (Zhao *et al.,* 2023; Pulendran *et al.,* 2021).

Post vaccination observation

All pigs of Group I immunized with Montanide oil adjuvant as well as other pigs in Group II and Group III, were found to be safe as no clinical alteration or allergic reaction was observed post vaccination. Although there was slight rise of rectal temperature (38.8-39.4°C) on 3rd and 4th day post vaccination in group I which was however within the normal range and animals maintained normal behavior. Rectal temperature in the remaining period until the end of the experiment in all animals in all groups were within the normal range. The Montanide oil adjuvanted vaccine preparation used for vaccination was found safe and compatible to the primary host. Clinical score assigned for evaluation of pre and post days vaccination status as per the method described by Everett and his team resulted in a score of 0 in pre vaccination days as well as at post day's vaccination in all groups of pigs. Further, there was no sero-conversion observed in control pigs which were kept in contact with vaccinated animals. The C-strain is generally considered to be very safe (Coronado *et al.,* 2021). In the present study, post vaccination observations in pigs like no clinical alteration or allergic reaction after immunization was supported with the findings of various workers (Klimka *et al.,* 2015; Aucouturier *et al.,* 2001) where cell culture-based CSF vaccine mixed with oil adjuvant was safe in immunized pigs. Slight rise in the temperature of the animal was observed but within the normal range and the animal exhibited normal behavior. This initial thermal reaction indicated efficient processing of vaccine antigens by immune associated cells particularly the macrophages and thus induced release of certain cytokines (Summer field *et al.,* 2015). The C-strain vaccine induces no disease in piglets or in pregnant sows, even when pigs have been immune-suppressed with corticosteroids (Tesmer *et al.,*1973). Mild fever with no local reaction at the site of application was also reported (Qiu *et al.,* 2005).

Antibody response

The presence of E2- specific antibodies in the serum samples of oil adjuvanted whole CSFV vaccine (Group I), CSF whole virus vaccine without booster (Group II), CSF whole virus vaccine with booster (Group III) and unvaccinated pigs (Group IV) were analyzed at different post vaccination period by blocking ELISA assay. In the test protocol following overnight incubation at 18-26°C yielded better OD than that of same day antigen coating procedure. The development of CSFV E2- specific antibodies in the serum samples of all vaccinated pigs from all the groups was assessed. It showed good level of antibody response detected in IDEXX blocking ELISA assay from day 7 to 180 dpv. In the CSFV vaccinated pigs a high blocking titre was maintained upto six months post vaccination period.

CSF oil adjuvanted whole virus vaccine

Serum samples collected from all the six animal of Group I showed no detectable level of CSFV antibody on the day of vaccination. The antibody titre at different days post vaccination of pigs vaccinated with the CSF whole virus vaccine is shown in Table 2. On the 30th day post vaccination, the mean of percent inhibition of CSFV antibody was 91.23±1.53. E2-specific antibodies appeared from 7th

Table 2: Mean ± SE of CSFV antibody per cent inhibition level in different groups of CSFV vaccinated pigs at different days of post vaccination detected by blocking ELISA.

Animal	Mean± SE of per cent inhibition at different day of post vaccination (dpv)							
group no.	0	7	14	21	30	60	90	180
Group I	24.82±1.53	80.17±2.23	83.63±1.57	86.65±2.45	91.23±1.53	89.22±1.53	79.52±1.89	67.68±1.66
Group II	23.33±1.23	78.67±2.02	83.63±0.61	84.6±1.16	88.73±2.01	81.1±1.98	59.38±3.29	22.38±0.44
Group III	23.67±1.14	77.33±1.58	83.26±0.56	85.05±1.06	81.53±2.06	90.4±2.13	81.88±1.08	60.71±3.09
Group IV	<20	<20	<20	<20	<20	<20	<20	<20

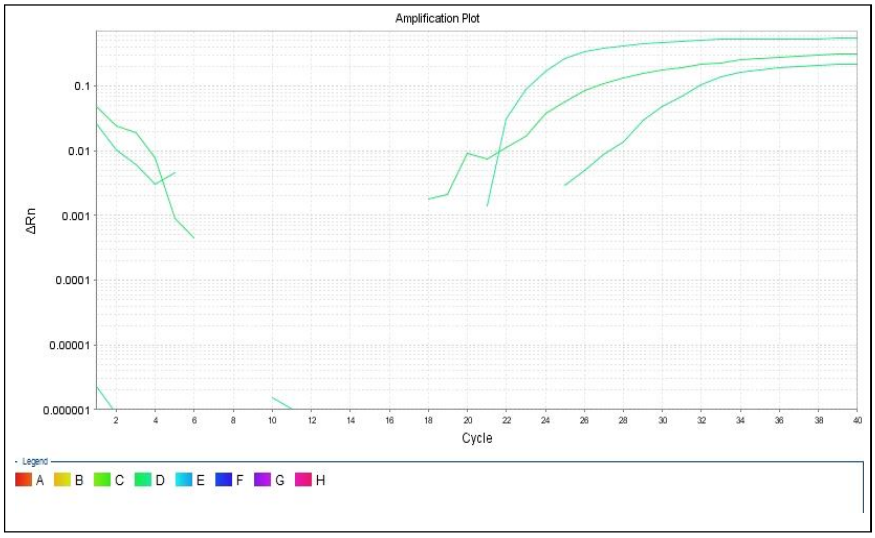

Fig 1: Representative samples from all groups found positive in qPCR showed Ct values 18, 21 and 25 for groups I, III and II respectively.

day itself after single vaccination with increasing trend till it reached the peak antibody titre at 30th day post vaccination (90-95% of inhibition titre). The inhibition titre was then found to decline steadily from 90thdpv (79.52±1.89) although high titre was maintained overall upto 180thdpv (67.68 ±1.66). The positive result obtained from E2 specific ELISA supports the major role of the glycoprotein E2 antibodies in the development of immunological response and eliciting high titers of neutralizing antibodies to CSFV as suggested by a group of researchers (Hulst *et al.,*1997; König *et al.,*1995). Single vaccination with oil adjuvants on the other hand showed promising protective immunity level with minimal side effects. Montanide ISA 50 V2 based vaccines have been safe and well tolerated and no lesions or serious inflammatory reactions have been documented in rabbits and mice (Leenaars *et al.,*1998), cattle (Shahzad *et al.,* 2019; P´erez Heredia *et al.,*2017; Ibrahim *et al.,* 2015; Pawar *et al.,* 2014), sheep (East *et al.,*1992) and poultry (Harrington *et al.,* 2009), safe and well tolerated in pigs (Suarez-Pedroso *et al.,* 2021). Early appearance of high neutralizing antibody titre in pigs vaccinated with oil adjuvanted vaccine can be a useful application for emergency vaccination in endemic areas. Detection of early antibody titre was also recorded (Nath *et al.,* 2016) in pigs vaccinated with cell culture vaccine without adding any adjuvant.

CSF whole vaccine without adjuvant single dose

Non-adjuvated cell culture adapted CSF primary vaccine induced detectable level percent inhibition antibody titre from 14th day post primary vaccination, which achieved peak level on 30th day (88.73±2.01) with 90-95% of inhibition titre in blocking assay. The mean value of antibody titre sharply declined after 30th day. Single dose vaccination did not induce protective titre at 7 dpv and was found partially protective at 10dpv. Detectable level of antibody titre persisted till 90 days. Single dose primary plain CSF vaccine without booster is less effective in comparison to other vaccine combination groups as it showed significant drop in titre at 180 day. However detectable level of antibody titre persisted till 90 days as the study carried out by some researchers (Khaund *et al.,* 2011).

CSF whole vaccine without adjuvant booster dose

Pigs receiving booster vaccine remained higher in antibody titre upto 90 days post vaccination with peak inhibition titre achieved on 60th day (90.4±2.13) and maintained it well till 180 days (60.71±3.09). Non-adjuvanted vaccine gives better immune response with a booster dose in comparison to CSFV vaccines without booster. Single vaccination only gives immunity till 3 months or so after which immunity level declines which when injected with a booster maintains protective immunity till marketable age (*i.e*. 6-8 months). Thus booster vaccination plays an important role. Pigs receiving booster vaccine remained higher in antibody titre upto 180 days post vaccination. This proved that booster vaccination plays an important role. Other studies also supported that with single vaccination no protective titre develops in vaccinated pigs till marketable age and need booster vaccination (Nath *et al.,* 2016).

Statistical analysis showed that there was significant difference($P<0.01$) between the vaccinated and unvaccinated groups of animal from the day 0 upto 90 days post vaccination, as unvaccinated group showed no rise in titre. Between vaccinated groups I, II and III upto 30 days no significant difference was observed. However at 180 day significant drop in titre was observed in single dose primary plain CSF vaccine without booster (Group II) but CSF oil adjuvanted whole virus vaccine (Group I) and CSF whole vaccine without adjuvant

booster dose (Group III) showed no significant difference as such (Table 2). Whereas Group IV animals which remained unvaccinated showed no increase or decrease in titre and served as the control group (Table 2). In the present study neutralizing antibodies were detected in oil adjuvanted and without adjuvant added CSF whole virus vaccine. A mean OD at 450 above 0.500 was considered negative control and a positive control blocking percent inhibition over 50%. Also CSF virus was detected in blood leucocytes upto 90 days post vaccination in qRT-PCR. This indicated that the vaccine virus was propagating and was able to induce antibody response in the experimental animals (Fig 1).

Neutralization assay has been an improved technique for detecting hog cholera virus (HCV) neutralizing antibodies (Laude *et al.,*1980). The NPLA test was used to determine the neutralizing antibody level after vaccinating pigs with recombinant CSF vaccine (rPAV-gp55) (Hammond *et al.,* 2000). In non-CPE producing virus like CSFV, neutralization assay was laborious and time consuming as it requires long procedures of cell culture techniques along with staining whereas blocking ELISA was found to be less laborious, rapid and advantageous. Blocking ELISA therefore can be an alternative assay technique to evaluate neutralizing antibody. Also it was reported that C-ELISA was comparable to NPLA and a useful tool for large scale screening and eradication programs for CSF and found that blocking ELISA is a reliable test for CSFV-specific antibodies, comparable to NPLA (kappa value 0.9) (Clavijo *et al.,*2001).

The propagation of CSF vaccine virus was detected by qRT-PCR in blood leucocytes collected upto 90 dpv. This indicated that the virus post vaccination was propagating. Studies made earlier reported tonsil as the target for vaccine virus replication wherein the vaccine virus persisted for more than 30 days post-vaccination (Ganges *et al.,*2008; Kaden *et al.,*2004). Another study also stated that vaccination with multivalent MLV vaccines in cattle resulted in high proportions of calves with PCR-positive results for viral respiratory pathogens in clinically relevant samples routinely collected from cattle undergoing respiratory disease (Cooper *et al.,*2010; Godinho *et al.,* 2007).

CONCLUSION

In context to the repeated vaccination and marginal rise of antibody titre in pigs vaccinated with non-adjuvanted CSF vaccine, present oil adjuvanted CSF vaccine can be a useful alternative to provide protective immunity till marketable age with single vaccination. Moreover, oil adjuvant retained viable CSF virus as depot for long period. Therefore, without booster vaccination present form of immunization schedule can reduce the handling stress and also minimize the cost of vaccination. However, selection of an ideal yet economic adjuvant for a vaccine needs further study.

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Conflict of interest

All authors declare that they have no conflict of interest.

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