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Purification of Cell Culture Adapted Swine Fever Virus by Sucrose Gradient Centrifugation

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In the present study the lapinized swine fever vaccine virus was propagated in the PK-15 cell line. Indirect fluorescent antibody test, indirect immunoperoxidase test and sandwich ELISA were employed for demonstration of the virus in cell culture. The comparative efficacy of the three tests was also evaluated. The cell culture adapted virus was concentrated and purified using polyethylene glycol (PEG) treatment and sucrose gradient centrifugation. A total of seven fractions could be collected out of which four fractions were found to be viable. The purified viable virus having $10^{5.25}$ $TCID_{50}$ per ml was successfully used for raising Swine Fever virus specific antibodies in rabbits and pigs.

Key words: ELISA, Lapinized SFV, PK-15, PEG, Sucrose gradient.

Classical swine fever (CSF) is an important disease in pigs caused by a small enveloped RNA virus, the CSF virus (CSFV), which belongs to the genus *Pestivirus* within the family *Flaviviridae*[1]. For a detailed biological and physico chemical evaluation of the virus its purification is necessary. Different workers have described methods for purifying the swine fever virus with preservation of appreciable infectivity[2-4]. But due to the intrinsic properties of the CSFV purification is somewhat tedious[3,5]. The present study primarily involved the concentration and purification of the cell culture adapted swine fever vaccine virus and subsequent assessment of the immunogenicity of the purified virus. Moreover, the comparative efficacy of Fluorescent Antibody Test (FAT), Immunoperoxidase Test (IPT) and Sandwich ELISA for detection of Swine Fever Virus (SFV) in cell culture was evaluated using both polyclonal and monoclonal antibodies.

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MATERIALS AND METHODS

Adaptation of lapinized CSFV in cell culture

Lapinized swine fever vaccine (Batch 7) procured from the Institute of Veterinary Biologicals, Government of Assam, Khanapara, Guwahati was used as the antigen model. The virus titre was increased by propagating in the PK -15 cell line, which was obtained from the National Centre for Cell Science (NCCS), Pune. The vaccine was reconstituted in 5ml sterilized distilled water and centrifuged at 3000 rpm for 15 minutes. The monolayers of PK 15 cells in tissue culture flasks were infected with the vaccine supernatant which had been filtered through a 0.2 micron membrane filter (Nalgene) and treated with cocktail antibiotic @ 20 ul/ml. The flasks were then incubated at 37°C in a CO_2 incubator for a period of 4-5 days and the virus was harvested by 2-3 cycles of freezing and thawing. Serial passages were done upto 7 passage level.

Detection of SFV in infected cell culture

The presence of the virus in the cells was detected by indirect FAT, indirect IPT and Sandwich ELISA.

Indirect IPT and Indirect FAT were performed as per the method described in the EU Diagnostic Manual[6] with slight modifications wherein the fixed infected cells were treated with 0.4% triton X-100 (in wash buffer) followed by 2% BSA (in wash buffer). Sandwich ELISA was performed as per the method described by Sarma and Sarma[7]. The titre of the virus in each passage was determined using monoclonal antibody based sandwich ELISA.

Furthermore, the cell culture fluid was tested by E2 specific nested RT-PCR done as per the EU diagnostic manual[6] with slight modifications.

Concentration and purification of the virus

For purification of the virus about one litre of cell culture propagated CSFV suspension was prepared. The virus suspension was initially concentrated and then subjected to gradient centrifugation[8]. In the present study the concentrated virus was run in 20% (v/v), 30% (v/v), 50% (v/v) and 70% (v/v) sucrose gradients (99.9% purity)[4].

For concentration, solid sodium chloride (NaCl) and polyethylene glycol (PEG) was added slowly, till a final concentration of 0.5 M NaCl and 7% PEG was achieved in the virus containing fluid. The solution was stirred gently for 20-30 minutes until the entire PEG had dissolved and then allowed to stand for a further 2 hours at 4°C to allow aggregation of the virus. The solution was then centrifuged at 10000 g for 30 minutes. The precipitate was distributed on the side of the centrifuge tube and the supernatant was removed using a pipette. The precipitate was resuspended in Glycine Tris NaCl EDTA (GTNE) buffer using a volume of about 1% of the initial virus containing fluid. Concentrated virus containing fluid was then purified by ultracentrifugation in sucrose gradients using 8 ml tubes (Quick seal, Beckman). Tubes were sealed and centrifuged for 1.5 hour (58,000 g) at 4°C using ultracentrifuge (Beckman Coulter Optima Max). Virus banding in gradients was observed as an opalescent layer and different fractions could be recovered from the tube by side puncturing. The ELISA titre was determined and viability of each fraction was studied in cell culture. The method of Karber was employed to determine the $TCID_{50}$ of the purified virus. The pooled gradient fractions from different runs were diluted with an equal volume of buffer to reduce the density and centrifuged at 4°C at 80000-100000 g for 2.5 hours. The supernatant was discarded taking care to drain as much as possible from the virus pellet. The virus was resuspended in GTNE buffer to give an opalescent suspension and stored at -50°C.

Immunogenecity of the purified virus

For assessing the immunogenecity, the purified virus was inoculated into rabbits and pigs. Two healthy rabbits (weighing about 500g) were injected intramuscularly with Freund's Complete Adjuvant (FCA) emulsified purified virus (0.89×10^4 $TCID_{50}$) in deep gluteal muscle. Subsequently booster doses were given with the virus, emulsified in Freund's Incomplete Adjuvant (FIA), on the 14th and 28th day post-primary injection using the same dose and route. The rabbits were bled after 7 days of last immunization. A similar schedule was followed in case of pigs. However, at the end of the schedule the pigs exhibited lower antibody titre and were subsequently given further booster doses with the plain antigen for four more occasions at 7 days interval. The antibody titre was determined by indirect ELISA performed as per the method described by Sarma and Sarma[9].

RESULTS AND DISCUSSION

The adaptation of the lapinized swine fever virus in PK-15 cells and detection of the viral RNAs in the infected cells by PCR has been reported earlier[10] and in this study also E2 specific nested RT-PCR results of the different passages showed swine fever virus specific band at 271 bp (Fig1) from the first passage itself. By Sandwich ELISA, a traceable titre was detected initially, which increased along with the passages and the highest titre (1:128) was obtained in the sixth and seventh passages. When stained with E2 specific monoclonal antibody a bright green fluorescence in FAT (Fig. 2) and an intense reddish brown colour in IPT (Fig. 3) was evident along with a distinct nucleus. Such a clear demarcation of the nucleus was not obtained with a polyclonal antiserum and a diffusion of the stain to a certain extent was observed (Fig. 4 and Fig 5). The better sensitivity and specificity of monoclonal antibody based assays in comparison to conventional ones has been reported earlier[11]. Moreover, the use of monoclonal antibody based indirect FAT and

indirect IPT facilitated specific and confirmatory detection of SFV in the PK 15 cells[12, 13, 14].

Virus aggregation by addition of PEG is gentle and usually results in lower loss of infectivity in comparison to direct ultracentrifugation[8] and 100 percent recovery of infectivity has been reported by using PEG for concentration of BVD virus[15]. In this experiment, the PEG concentrated virus exhibited a titre of 1: 640, showing a five fold increase from the titre of the initial virus containing cell culture fluid. After centrifugation in sucrose gradients altogether seven fractions could be collected, identified as F1, F2, F3, F4, F5, F6 and F7 (Fig.6). The fraction F1 was at the top and all the fractions could be collected by side puncturing conveniently. The fraction F5 was found to be the thickest band, measuring about 2mm thick and was observed almost at the middle of the tube length. Monoclonal antibody based ELISA as mentioned earlier was employed to determine the titre of the bands (Table 1). Fraction F5 possessed the highest titre (1:640) followed by F4 (1:160). Again F3, F4, F5 and F6 were found to be viable when inoculated into cell

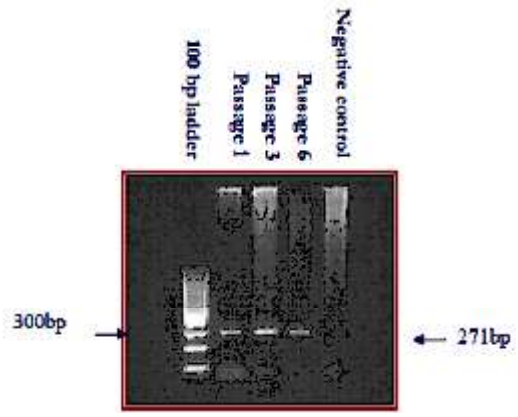

Fig. 1. Electrophoresis of nested product of E2 region of cell culture Adapted lapinized swine fever virus.

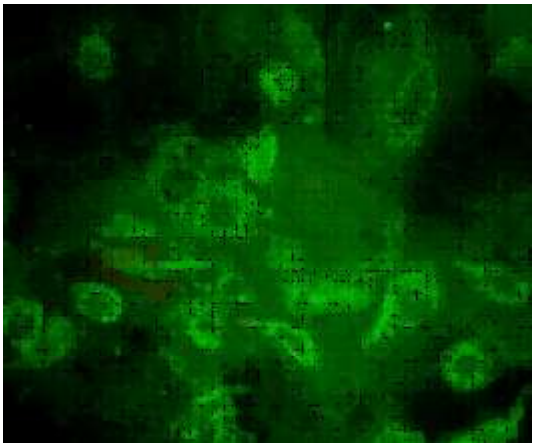

Fig.2. Lapinized SFV infected pk-15 cells stained with E2 specific Monoclonal antibody based indirect fat showing Brightgreen fluorescence in the cytoplasm after 72 hrs of infection (X400)

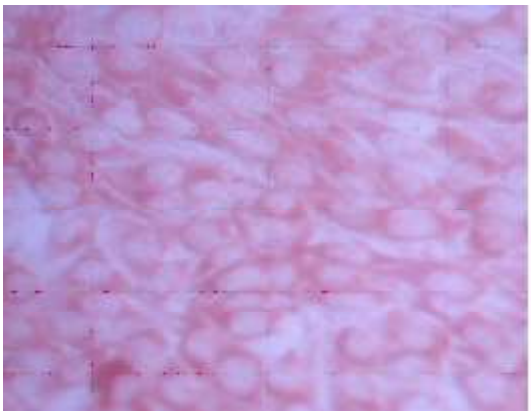

Fig. 3. Lapinized SFV infected pk-15 cells stained with monoclonal antibody based ipt showing red stained cytoplasm in ipt after 72 hrs of infection (X400)

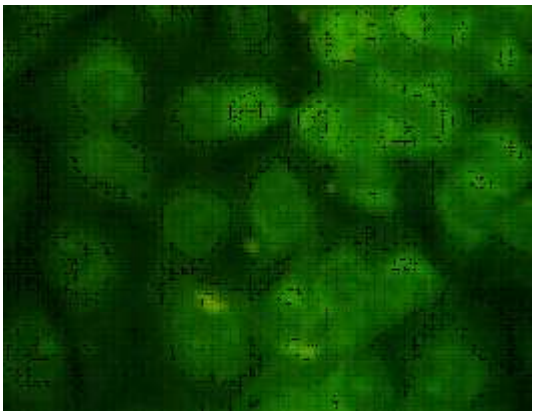

Fig. 4. Lapinized SFV infected pk-15 cells stained with Polyclonal antibody based indirect fat showing

culture (Table 1). In another study, following a similar scheme of purification for six strains of CSFV, the workers reported the collection of 12 fractions, the first fraction being at the bottom . They found that fraction 8 and 9 in five of the strains and fraction 3, 4, 9 and 10 in the ALD reference strain were found to be important[4]. In the present investigation, fraction F5 and then F4 were found to be of significance and were located at the middle and upper portion of the tube. A similar observation was also reported in case of bovine viral diarrhoea virus (BVDV) in which the upper bands were found to contain the majority of infectivity[16].

Sucrose gradients cause separation of molecules largely on the basis of size, density and molecular weight and so the lack of viability in particular fractions could be attributed to the absence of intact infective virions in them[17]. In glycerol/ Na K tartrate gradient purification of Brescia strain of CSFV it was observed that the first three fractions belonged to the high infectivity reaction group, while the other fractions had low infectivity reaction[18] and in sucrose or glycerol density gradients, Shope papilloma virus was separated into a highly infectious band and a less infectious lighter zone[19]. In this study, it was found that the fractions F1, F2 and F7 lacked viability in cell culture but were positive in sandwich ELISA.

The fraction F5 of different runs collected from various tubes were pooled and subjected to one round of ultracentrifugation. The precipitate was resuspended in GTNE buffer and used for further study. The titre of the virus was calculated by the method of Karber and was found to be log $10^{5.25}$ $TCID_{50}$/ml. The RT PCR results of the purified virus showed swine fever specific band at 271 bp .

The immunogenic property of the purified virus was evident in the findings, as an antibody titre of of 1:6400 and 1:1600 was obtained respectively in rabbits and pigs injected with the same. However, in pigs additional injections were required for acquiring an optimum antibody titre,

Table 1. Viability and ELISA titre of various sucrose gradient fractions collected after centrifugation

Properties	F1	F2	F3	F4	F5	F6	F7
Viability -	-	+	+	+	+	-	
ELISA time	1:10	1:80	1:80	1:160	1:640	1:40	1:20

Fig. 5. Lapinized SFV infected pk-15 cells stained with polyclonal antibody based IPT showing

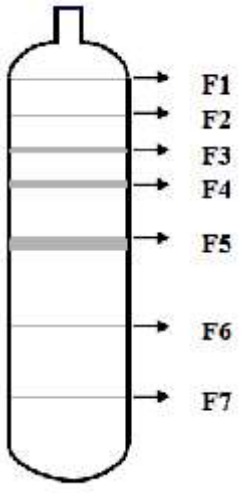

Fig. 6. Schematic diagram showing location of various fractions observed after sucrose gradient centrifugationnal antibody based indirect fat

reflecting that the dose of the virus used for inoculating the pigs was not optimum. The results of the present study therefore revealed that the swine fever virus purified by sucrose gradient centrifugation possessed both viability and immunogenicity.

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