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A Handy Method to Estimate Titer of Classical Swine Fever Virus in PK-15 Cells

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Abstract

Determination of virus titer is the key factor for development and quality control of classical swine fever (CSF) cell culture vaccines. Like any other non-cytopathic effect producing viruses, classical swine fever virus titration is done by immunological methods *e.g.* Fluorescent Antibody Test (FAT). These classical assays for the determination of Tissue Culture Infective Dose 50 ($TCID_{50}$) are time-consuming and require huge reagents. Hence, they are not very suitable for handling a large number of samples. An accurate and reliable technique for $TCID_{50}$ determination by FAT in cover-slip culture was optimized for titration of classical swine fever. Optimization of FAT in LAB TEK slide chamber could do away the tedious manipulation of cover-slip culture without affecting its final outcome. This newly optimized method of titration has shown 100% similar result to that of one full proof titration method of CSF virus in PK-15 cells in terms of fluorescent positive cells in wells and their fluorescent intensity.

Key words: Classical swine fever; Non-cytopathic; Virus titration; Titer; Immunofluorescence

Introduction:

Classical swine fever (CSF) is a re-emerging disease in many countries. The causative agent of CSF is classical swine fever virus which belongs to the genus *Pestivirus* of family *Flaviviridae* (Edwards et al., 2000; Postel et al., 2019). CSF has been eradicated from a number of countries (Sandvik et al., 2005; Ji et al., 2015) but its endemicity still prevails in parts of Asia and some other countries from South and Central America, and in parts of the Caribbean islands (Postel et al., 2013; Blome et al., 2017).Thus, endemic and re-emerging nature of CSF virus continues to threaten worldwide pork production and food security in developing countries (Saatkamp et al., 2000).

Despite the devastating nature, the disease is well controllable by vaccination in the endemic countries. For that, availability of efficacious and potent vaccine in the field settings is of farthermost important. Currently, as per requirement of any Pharmacopoea, each dose of CSF vaccine must contain at least 100 PD_{50} to pass the potency test (Indian Pharmacopoeia). To avoid challenge experiment of animals for vaccine potency evaluation, an alternate potency test based on the Fluorescent Antibody Virus Neutralization (FAVN) titer of vaccinated animal sera has been evaluated (Manu et al., 2023). To entirely exclude live animal use for the CSF cell culture vaccine evaluation, determination of virus titer in the finished vaccine product (virus titration) would help in quality control. Though most of the live virus vaccines are titrated by observing the cytopathic changes in cell culture produced by the virus, however with a few exceptions, the vast majority of CSF viruses are non cytopathogenic and do not produce visible cytopathic changes (Gallei et al., 2008; Ganges et al., 2020). Being a non-cytopathic effect (CPE) producing virus, virus titrations are done by immunological methods such as Fluorescent Antibody Test (FAT) or Immunoperoxidase Test (IPT) using specific hyper immune sera or monoclonal antibodies (Bouma et al., 2001). The assays were set up to quantify the infectious particle using antibody labeling of viral protein. The FAT is proven to be more sensitive and specific than IPT (Jafari et al., 2015; Zhang et al., 2017).This technique is based on the detection of infected cell at the single cell level using immune-detection of viral proteins by fluorescent microscope (Wang et al., 2020). To avoid pitfalls, virus infected cells must be clearly distinguished from the uninfected ones by emitting a fluorescent signal above the auto-fluorescent background. However, there are still further scopes to improve the detection method by either simplifying the existing FAT steps or by other measures which existed but not used for CSFV titration so far. So, the study is aimed at developing a suitable substitution of existing method/s which will be much faster, economical and easier to perform.

Materials and Methods:

Cells:

Porcine kidney-15 (PK-15) cells (ATCC, USA), free from mycoplasma and pestivirus contamination, maintained at Division of Biological Standardization, ICAR-IVRI, Izatnagar were used for the study. The cells were cultured in Eagle's Minimal Essential Medium (EMEM, HiMedia, India) supplemented with 10% pestivirus free foetal bovine serum (Invitrogen, USA) and 100X antibiotic and antimycotic solution (HiMedia,

India) and kept at 37°C under 5 % CO_2 tension and 100% relative humidity.

Virus:

Porcine kidney (PK)-15 cell culture adapted virus developed by adapting indigenous virulent isolate was used. This very high yielding vaccine strain, named as IVRI-CSF-BS and maintained in Division of Biological Standardization, ICAR-IVRI, Izatnagar was used in the study.

Previous method of titration:

Virus titration was performed in 24 well cell culture plate following protocol by Dhar et al., 2022. Cells were allowed to grow on sterile surface of cover-slips to be used in titration. Briefly, tenfold serial dilutions of vaccine virus were prepared and 100 µL of each virus dilution was transferred to the respective well keeping cell control and plate was incubated. After completion of incubation, cells were fixed and quenched to wane off auto fluorescence due to used fixative. After that addition of detergent acted as porogen helping Mab-FITC (diluted in blocking buffer) to reach viral protein inside cells. Finally stained cells were mounted on slides after washing to remove unbound conjugate.

New method of titration:

Lab-Tek^{TM} chambers were marked properly keeping cell control. PK-15 cells were subcultured and two hundred microliter of PK-15 cell suspension (1×10^5) cells were distributed per chamber (Figure 1). Fifty microliter of 10 fold serially diluted virus was added in respective wells keeping one cell control and mixed gently. Infected cells incubated for 72 h at 37℃, 5% CO_2 in a humidified chamber. Up on incubation spent media was discarded and washed and then developed based on modified FAT protocol. Cells were fixed with 100 µL of 3% para-formaldehyde. Then fixed cells were washed with PBS and treated with 100 µL of 50 mM NH_4Cl. After that all wells were treated with 100 µL of 0.01% Triton X-100 and washed three times with PBS. Then wells were incubated with 40 µL of 1:40 diluted MAb-FITC (MAb against CSFV-E2 protein,) for 30 minutes keeping in a moist chamber and again washed thrice. Finally sides of the chamber was removed and cover slip was mounted on respective wells.Cells were observed under fluorescent microscope and titer was calculated following Spearman, 1908 and Karber, 1931 method and results were recorded.

Results and Discussion:

FAT in Lab-Tek ^{TM}8-chamber slides was able to reproduce the inoculum titer exactly to that of the FAT titer in 24 well plate cover-slip culture. Using the old method obtained titer was $10^{7.5}$ $TCID_{50}$/mL. Newly optimized method of titration was showing 100% similar result in terms of fluorescent positive cells in wells and their fluorescent intensity. FAT in Lab-Tek ^{TM} chamber showed positive signal up to 10^{-6} virus dilution but no fluorescence was observed in 10^{-7} dilution of virus. Hence calculated titer following the optimized method in Lab-Tek ^{TM} chamber has also produced $10^{7.5}$ $TCID_{50}$ mL^{-1} (Figure 2).

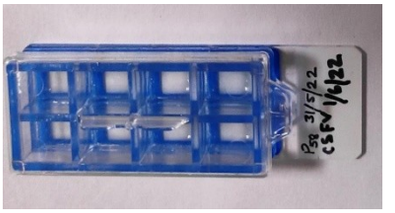

Figure 1: Lab-Tek^{TM} Chamber Slide System for growing cells on a standard microscopic slide for on-spot visualization after staining

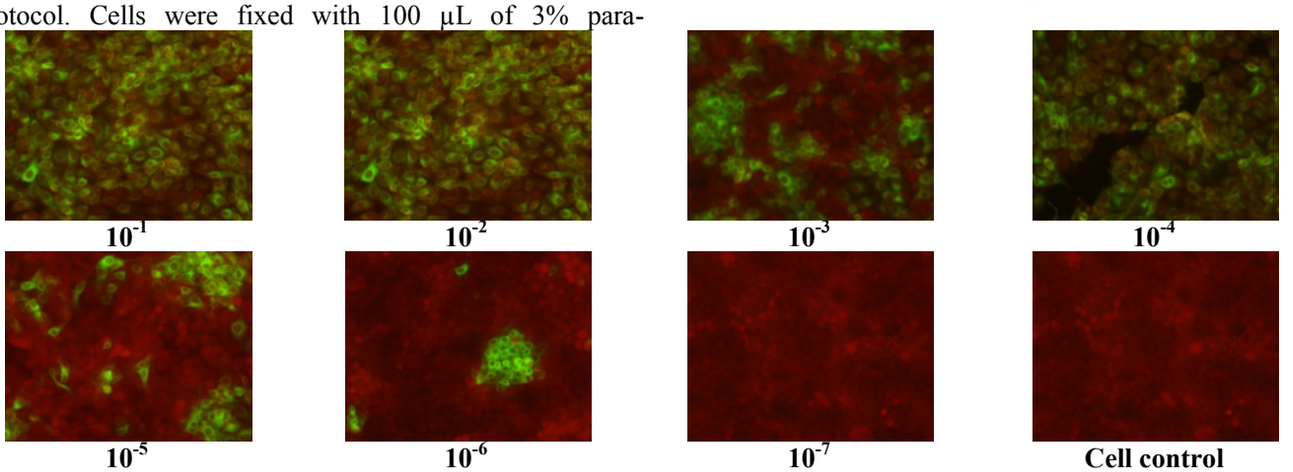

Figure 2: PK15 cells infected with CSF virus showing green fluorescence in cell cytoplasm. The infected and non infected cells in a Titration of the CSF virus in Lab-Tek ^{TM} slide chamber where fluorescence was found up to 10^{-6} dilution of virus. Socalculated titer was $10^{7.5}$ $TCID_{50}$/mL. 10^{-7} dilution of virus and cell control did not show any green fluorescence.

Producibility of CSF virus is measured by its demonstration of viral bodies inside cells and non-cytopathic nature of the virus added extra hardship in doing that. Growth of CSF virus in the cells is usually visualized by using immunological technologies with fluorescent or conjugated antibodies and virus titration is done by specific method like FAT or IPT (Bouma et al., 2001; Chander et al., 2014; Mahapatra and Dhar, 2021). There is always a need of alternatives which can ensure consumption of less sample and reagents, easy to perform and requires less effort and time and incurs less cost. A full proof method of titration of CSF cell culture vaccines employing FAT protocol in 24 well plate cover slip culture is in current use (Dhar et al., 2022). With the use of modified protocol, reagents and buffer consumption can be reduced to 1/4th. Development of FAT in Lab-Tek ^{TM} slide chamber could also do away the tedious manipulation of cover slip culture without affecting its final outcome. Though use of Lab-Tek ^{TM} slide chamber is higher cost demanding, it can be applied when easy handling is required by unskilled personnel.

Conclusion:

Any non-CPE viruses are difficult to work with and CSF virus was not an exception. Although FAT is gold standard for its detection and titration, the test is not without many disadvantages such as labour intensive, requiring serious cell culture interventions etc. Modified FAT protocol has successfully reduced the buffer consumption and FAT in Lab-Tek ^{TM} slide chamber could have successfully reduced the tedious manipulation of cover slips.

Conflict of Interests

The authors declare that they have no known competing interest.

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