Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 25, No. (2) : 2023 : 256-262

ISSN-0972-3005

DOI No.: http://doi.org/10.53550/AJMBES.2023.v25i02.012

EFFECTIVENESS OF CRYOPRESERVED CHICKEN EMBRYO FIBROBLAST CELLS FOR PROPAGATION OF DUCK ENTERITIS VIRUS AND FOWLPOX VIRUS

HIRAMONI SARMAH, SAMSUN NEHER, SOPHIA M. GOGOI*, NAYANMONIKONWAR, KEVISENUO EVALYN VIZO, PRANITA SHERPA, HIMASRI DAS, KANYAGA PARAMESHWARI M., N.N BARMAN AND D.P BORA

Department of Veterinary Microbiology, College of Veterinary Science, Assam Agricultural University, Guwahati 781 022, Assam, India

(Received 15 October, 2022; Accepted 11 December, 2022)

Key words: *Duck plague virus, Fowl pox virus, Chicken embryo fibroblast cells, Cryopreservation*

Abstract–Primary chicken embryo fibroblast (CEF) cultures are excellent model systems for *in-vitro* virus propagation. However, the cumbersome preparation process of CEF makes cryopreservation highly essential. Cryopreservation in Liquid Nitrogen liquid phase (-196 °C) or vapour phase (-156 °C) are frequently used methods. In the present study, efficiency of cryopreserved primary CEF cells for the propagation of Duck plague virus and Fowl pox virus was evaluated. The cells were preserved for different time periods and different cooling methodologies were employed in order to assess the effects exerted by these variations. The efficiency of the cells was compared based on cell viability/ revival rate, ability to form monolayer and $TCID_{50}$ of the propagated viruses. Cell count revealed viability of 75.60% and 68% post 2 weeks and 6 months of cryopreservation respectively following step freezing method. On the other hand, cells preserved using insulated cryo-containers exhibited 89% and 80% viability post 2 weeks and 6 months of cryopreservation respectively. Development of complete monolayer could be seen within 24 hours in all the batches irrespective of the cooling method used and the cryopreservation storage time. Infection with DPV and FPV yielded respective cytopathic effect from 48 hours onwards. CPE observed in the freshly prepared CEF and in those revived 2 weeks and 6 months post freezing, were similar in terms of typical characteristics and duration of appearance. From the findings of the present study it is evident that cryopreservation of CEF cells is a convenient and practical approach for virus propagation. Cryopreservation of primary cells offers a definite advantage over repeated culture preparation which is labour intensive, time consuming and dependent on the availability of embryonated eggs.

INTRODUCTION

Cells and tissue culture are extensively used techniques in the field of vaccine production, virus proliferation, drug development and cancer research. The process involves derivation of cells, tissues and organs from eukaryotic organisms and growing them *in vitro* under controlled environment mimicking natural conditions. Primary chicken embryo fibroblast cultures are excellent model systems as they are isolated straight from tissues and are unaltered. They find wide application in adaptation and propagation of various avian viruses like IBD, Fowl pox virus, Newcastle disease, Duck Enteritis Virus as well as for vaccine production (Wambura *et al.,* 2006; Audsley and Tannock, 2008). However, preparation of primary cultures is a cumbersome process, involving much time and precaution. In addition, the production cost is high as it requires large number of embryonated eggs, which is also non-ethical. Hence, in order to overcome these difficulties cryo-preservation of primary cells is highly essential. One of the frequently used methods to preserve culture cells for long termis cryopreservation in Liquid Nitrogen using its liquid phase (-196 °C) or vapour phase (-156 °C). However, it is essential to minimize the detrimental effects of freezing on cells due to ice crystals, dehydration, and alteration in the pH and

(*Assistant Professor)

osmotic changes (Mazur *et al*., 1972). To serve this purpose, a cryoprotective agent such as glycerol, dimethylsulfoxide (DMSO), or Ethylene glycol is added, prior to freezing, to the cell suspension to lower the freezing point (Doyong and Critser, 2000; Pegg, 2007). The cell suspension is then transferred as 1ml aliquots to cryovials and are subjected to slow cooling to -80 °C at an ideal rate of 1 °C/min (Jang *et al*., 2017). To attain this level of freezing, the cryovials are placed in containers which include conventional styrofoam boxes and iso-propanol filled containers to the more advanced controlled-rate freezers (Thompson *et al*., 2014). Once -80°C is reached, the vials are immediately transferred to Liquid Nitrogen storage. The cells can be preserved in this state for longer periods of time. The thawing process is another important factor when reviving cryopreserved cells. Generally the cells are subject to rapid warming to avoid recrystallization of ice which can cause further damage (Whaley *et al*., 2021). The present study was undertaken to evaluate the efficiency of cryopreserved CEF cells for propagation of two different avian viruses namely Duck enteritis virus or Duck plague virus (DPV) and Fowl pox virus (FPV).

MATERIALS AND METHODS

Preparation of Chicken Embryo Fibroblast culture

Embryonated chicken eggs of 4 to 5 days old were purchased from a reputable hatchery and incubated till 9-11 days old at 37 °C with regular turning daily.

The chicken embryo fibroblast cell culture was prepared as per the method described by Hernandez and Brown, (2010) with some modification. Briefly, 9-11 day old chicken embryos were taken, candled and those showing good development of the embryo with prominent blood vessels were selected. The eggs were swabbed with 70% alcohol and allowed to dry. The area of the air sac was marked and broken gently with the help of forceps. Then the embryo was taken out and put into a dish containing balanced salt solution. The extremities of the embryos along with the internal organs were removed and washed several times with Hank's Balanced Salt Solution (HBSS), so that the blood and other debris get removed. With the help of a pair of sterile curved scissors embryos were properly minced and washed with HBSS several times. Then the tissue pieces were transferred to a trypsinization flask containing a teflon-coated magnetic bar and sufficient amount of 0.25% trypsin was poured and trypsinized for 15 minutes in a magnetic stirrer at a moderate speed. The supernatant was discarded to remove the toxic factors released from the tissue fragments. Fresh trypsin solution was poured into and trypsinization continued for 10 min and the tissue fragments were allowed to settle. Then the cell suspension was passed through sterilized muslin cloth tied to the mouth of a beaker. The left-over tissue was further trypsinized twice as done before, 15 minutes each time and passed through muslin cloth. Then the cell suspension was centrifuged at 1000 rpm for 10 minutes. The cell pellet was retained and trypsin was poured off. Washing with balanced salt solution was done twice and finally with the growth medium.

Cryopreservation of cells

For preservation of primary cells two types of media were used: Media A-EMEM WITH 20% horse serum/20% foetal bovine serum (FBS). Media B-Media A with dimethyl sulfoxide (DMSO) 20%. The cell pellet was reconstituted in Media A and the viable cell concentration was determined in a haemocytometer and adjusted to 10^6-10^7/ml. The cell counting was performed using a haemocytometer using trypan blue (0.4%) exclusion method (Choi *et al*., 2007). Those cells which did not take up trypan blue were counted as viable cells. The cryovials were labelled and cell suspension was distributed in cryovial @ 500 µl/vial. Equal volume of Media B was added to all the vials. The suspension was mixed thoroughly. Two separate freezing methodologies were employed. One batch was transferred to a cryo-container half filled with isopropanol for slow cooling @ -1 °C / min and then stored at -80°C overnight, and ultimately immersed in liquid nitrogen (-196°C) for long term storage (Oyeleye *et al*., 2016). The other batch was first cooled to freezing temperature allowing release of the heat of fusion followed by further freezing up to below -40°C and finally to liquid nitrogen vapor phase temperature of <-150 °C (Gargi *et al*., 2015). To achieve this, the vials were first transferred to -4 °C for 2 hours, -20 °C for 2 hours, -80 °C for overnight and finally transferred to liquid nitrogen the following day. Comparison was made between both the batches of vials on the basis of the viable cell count and revival rate.

Revival of cryopreserved cell

Cryopreserved cells were taken out from liquid

nitrogen, thawed at 37 °C, suspended in 8 ml growth medium (EMEM+10% FBS) and incubated at 37 °C. Cell count was carried out using haemocytometer as mentioned before.

Susceptibility to virus infection

Revived cells were infected with 1ml of DPV vaccine virus/ 25 mm^2 flask and 1ml FPV/ 25 mm^2. Briefly, the monolayer was washed with HBSS and inoculated with antibiotic treated 1 ml virus inoculums per 25 cm^2 cell culture flask (Nunc, Denmark) and kept at 37 °C for 1 hour to allow proper adsorption of virus on to the cells. Then it was rinsed with Ca^{2+}, Mg^{2+} free PBS to wash away the un-adsorbed viruses. Then maintenance medium containing 5 percent foetal bovineserum was added and incubated at 37 R°C for 3-4 days. Un-inoculated monolayer served as control. The cell monolayer was microscopically observed for alterations in cell morphology for the next 3-4 days. The virus was harvested after appearance of cytopathic effect. The infected cell culture fluid was subjected to 3 cycles of freezing and thawing after which it was centrifuged at 3500 rpm for 10 minutes. The supernatant was then separated and stored at -30 °C for further use.

Confirmation by PCR

Viral DNA was extracted from the harvest by using the commercially available reagent TRIzol (Invitrogen, USA) in accordance with the manufacturer's instructions.

For detection of DPV, DNA polymerase gene *UL30* specific primers (OIE Terrestrial Manual, 2018) were used and the presence of FPV was determined by amplification of the *P4b* gene using a set of primers described by Lee and Lee (1997). The details of the primers and amplicon size are provided in Table 1. The amplified PCR products were electro phoretically analysed by performing agarose gel electrophoresis in 1X Tris Acetate EDTA (TAE) buffer with 1.7% Agarose (Amresco) containing ethidium bromide (10 mg/ml). Electrophoresis was carried out at 80V for 1hour and amplified product was visualized at 312 nm wavelength using UV transilluminator (DNR, Bio-imagingsystem, Minilumi).

Determination of $TCID_{50}$

$TCID_{50}$ was performed following the Virology laboratory manual (Burleson,Chambers and Wiedbrauk, 1992). CEF primary cells were cultured on 96 well micro plate and of 100 µl of growth media per well and incubated at 37 °C. After 24 hours, confluent monolayer was obtained. The growth media from the wells were discarded and then 100 µl of virus inoculum from different dilutions (10^{-1} to 10^{-6}) were added to all the wells of row 1-7 and row 8 was kept as control. The plate was incubated at 37 °C in the CO_2 incubator and monitored microscopically daily for 4-5 days. Development of CPE in all the wells were recorded. $TCID_{50}$ was calculated formula using Log $TCID_{50}$ = L-d (s - 0.5), Where L is $\log_{10}$ of the most concentrated virus dilution is log dilution factors is sum of proportion.

RESULTS AND DISCUSSION

Cell Viability and Revival

The cryopreserved cells were revived in two batches. One batch was revived after 2 weeks and another batch was revived after 6 months of cryopreservation. It was observed that the cells preserved by slow cooling presented more survival rate than those batches of cells preserved by step down cooling method. Cell count revealed viability of 75.60% and 68% post 2 weeks and 6 months of cryopreservation respectively following conventional step down cooling method. On the other hand, cells preserved following slow cooling method exhibited 89% and 80% viability post 2

Table 1.

Virus	Sequences	Product size	Reference
FPV	Forward primer:5′-CAGCAGGTGCTAAACAACAA-3′ Reverse primer: 5′-CGGTAGCTTAACGCCGAATA-3′	578bp	Lee and Lee (1997)
DPV	Forward primer: 5′-GAA-GGC-GGG-TAT-GTA-ATG-TA-3′ Reverse primer: 5′-CAA-GGC-TCT-ATT-CGG-TAA-TG-3′	446 bp	OIE Terrestrial Manual, 2018

week and 6 months of cryopreservation respectively. It was seen that 1 vial of cells can be divided in 1:2 ratio to seed two 25cm^2 cell culture flasks. Congruent results have been reported in many studies. Research has shown that the optimal cooling rate, to achieve higher survival rates of cryopreserved cells, is -1°C min^{-1} (Choi and Bischof, 2011; Naaldijk *et al.*, 2012). On the otherhand, Baboo *et al*., 2019, opined that employing very slow rates of cooling (0.1°C min^{-1}) has shown to significantly increase cell viability of thawed cryopreserved cells. The results of the present study in correlation with previously published reports clearly indicate that slow cooling is the better option for cryopreservation of cells in terms of revived cell viability. In this study DMSO was found to be an effective cryoprotective agent which can be substantiated by the findings documented in earlier studies. Baust *et al.*, 2009 reported that DMSO was more advantageous than glycerol for many cell types as it offered enhanced permeability capacity.In fact, DMSO is among the most commonly employed cryoprotective agent for the storage of stem cells (Ock and Rho, 2011). In another study concerning cryopreservation of Chicken embryo kidney cells, best viability was obtained when the concentration of dimethyl sulfoxide(DMSO) in the freezing medium was 20% (v/v) (Choi *et al.*, 2007)

Monolayer formation and Virus infection

Development of complete monolayer could be seen within 24 hours and the cells could be used for infection. Cytopathic effect could be observed from 48 hours onwards. CPE in the freshly prepared CEF, those revived after 2 weeks and 6 months post freezing, was similar in terms of the time taken and the characteristics. The time taken for initiation of CPE was 24 hours post infection and 72 hours for completion. It was characterized by rounding, vacuolation, shrinkage of cells and cell death. Uninfected cells did not show any changes. Extensive research on virus induced cytopathic effect in different cell line has been performed by many scientists. (Fig. 2, 3 and 4). In an earlier study carried out by Kumar and Ponnnoose, 1997, it was observed that DPV infection in CEF lead to vacuolation of cytoplasm and formation eosinophilic intranuclear inclusion bodies and clumping of cells. In an attempt to adapt DPV in CEF, Konwar *et al*., (2020) reported similar kind of CPE within 48 hrs of infection. Similarly, Doley *et al.* (2013) also reported congruent findings.

Confirmation by PCR

For detection of DPV adapted in CEF, this study reaffirms the protocol standardized by OIE terrestrial manual (2018) for detection of DP virus nucleic acid. The same gene DNA directed DNA polymerase was targeted by various workers because of the highly conserved nature of the gene (Xuefeng *et al*., 2008; Mondal *et al*., 2010). The targeted gene is non structural gene which have open reading frame (ORF) of UL-30 which play many roles like DNA replication, DNA replication-recombination and DNA cleavage-encapsidation (Li *et al*., 2009). The viral DNA was extracted directly from the cells yielded sufficient amount of viral DNA in pure form at a concentration of 80-120 ng/µl. The amplicon size was 446bp, there was no non-specific amplification or smearing (Fig. 5).

In a similar manner, the PCR based amplification of the 578 bp region of the highly conserved 4b gene of avipox virus has been previously described by other workers for diagnosis of FPV (Fig. 6). (Lee and Lee, 1997; Fasaei *et al*., 2014 and Zhao *et al*., 2014).

Determination of infective dose

To assay the infective dose of Duck Plague Virus $TCID_{50}$ was performed. $TCID_{50}$ of freshly prepared CEF cells was found to be $10^{6.45}$/ ml, but in case of CEF cells revived 2 weeks post freezing it was found to be $10^{6.00}$/ ml, on the other hand $TCID_{50}$of CEF cells revived after 6 months of freezing was recorded as$10^{5.49}$/ ml. In case of DPV, $TCID_{50}$ was found to be $10^{5.54}$/ ml, $10^{4.49}$/ ml, and $10^{4.00}$/ ml in freshly prepared CEF cells, CEF cells revived 2 weeks after freezing and CEF cells revived 6 months post freezing respectively (Fig. 1). Various workers used $TCID_{50}$ for studying the virulence of different viruses in different cultures. Aravind *et al*. (2015) reported comparable $TCID_{50}$ of Duck plague virus where $TCID_{50}$ was $10^{6.2}$ $TCID_{50}$/ml. Almost similar finding

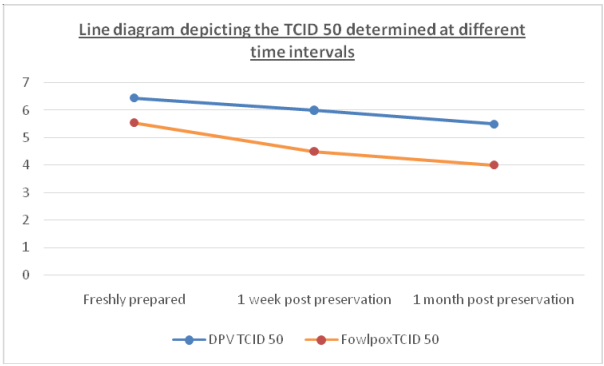

Fig. 1. Line diagram of $TCID_{50}$

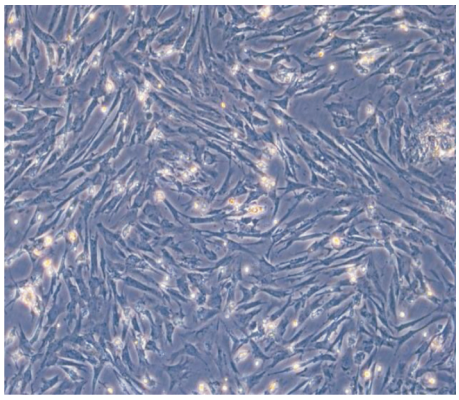

Fig. 2. Uninfected monolayer of CEF (100X)

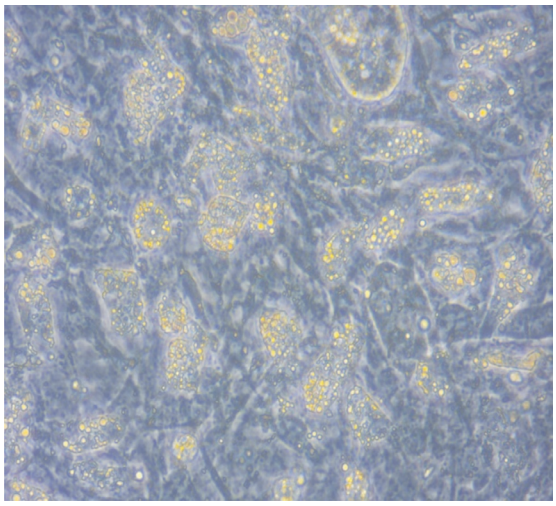

Fig. 3. DPV infected monolayer of CEF showing Vacuolation and rounding of cells (100X)

was also observed by Mondal *et al.* (2010). Dinh *et al.* (2004) observed $10^{7.2}$TCID/ml of DPV grown in CEF primary cell culture. In context of *Avipoxvirus*, different workers have reported the adaptation of Avipoxvirus in chicken embryo fibroblast cell culture (Balachandran *et al.*, 2012; Gilhare *et al*., 2015) and CPE consisting of rounding, vacuolation, degeneration of cells appearing as bunches of grapes, syncytia, detachment of cells, intracytoplasmic inclusions were common findings. In a study of isolation and attenuation of FPV by Sultana *et al*. (2019), $TCID_{50}$/ml was estimated at $10^{6.2}$/ml. In another study by Verma *et al*. (2015), $TCID^{50}$ of FPV reported to be $10^{4.6}$/ml which is comparable to the present study. The variation in $TCID_{50}$ observed by different workers mentioned above may be due to strain variation, passage level and different system used for propagation of the virus.

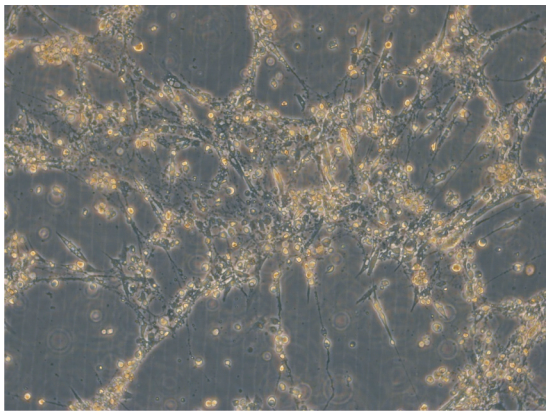

Fig. 4. FPV infected CEF cells showing thinning, clumping and detachment of cells a (100 X)

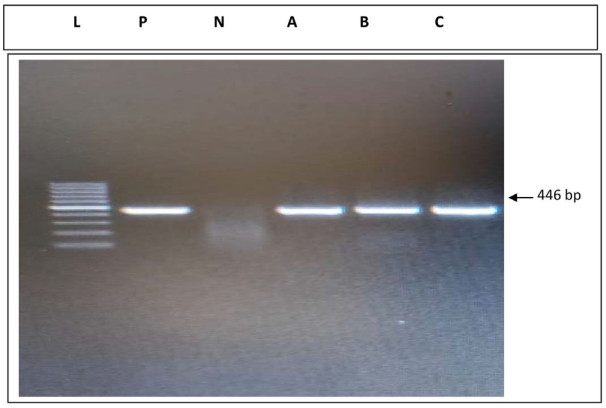

Fig. 5. L- 100 bp Ladder, P-Positive Control, N-Negative Control, A-DPV adapted in Freshly prepared CEF, B-DPV adapted in CEF revived 2 weeks post freezing, C-DPV adapted in CEF revived 6 months post freezing

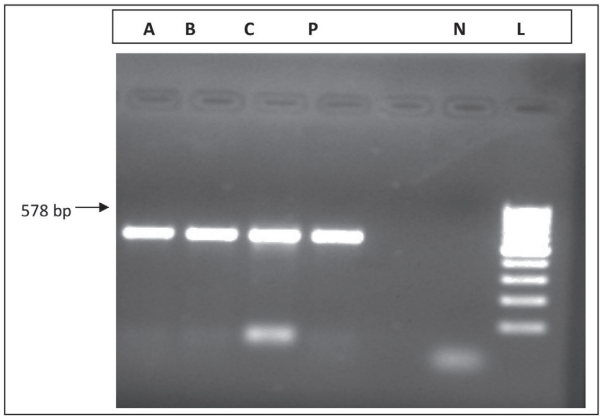

Fig. 6. L- 100 bp Ladder, P-Positive Control, N-Negative Control, A- FPV adapted in Freshly prepared CEF, B-FPV adapted in CEF revived 2 weeks post freezing, C-FPV adapted in CEF revived 6 months post freezing

CONCLUSION

Cryopreservation has been instrumental in expanding the scope of a variety of applications in biological science. Several factors are known to affect cryopreservation procedures while many more are yet to be unravelled. To surmount the shortcomings and to optimize cryopreservation techniques for different cell types and larger volumes, better understanding of the mechanisms involved in cryobiology is needed. This investigation brings to light the feasibility and practicability of cryopreserving chicken embryo primary cells for virus propagation. During the study period of 6 months, the efficiency of the cryopreserved cells was evident from the results obtained, which were comparable with that of freshly prepared cells. A single batch of chicken embryo fibroblast cells, therefore, could be preserved for maintaining a steady supply in the laboratory without the need to acquire embryonated eggs for each cell culture related activity.

Conflict of interests

The authors declare that no conflict of interests exists.

REFERENCES

Aravind, S., Kamble, N.M., Gaikwad, S.S., Shukla, S.K., Dey, S. and Mohan, C.M. 2015. Adaptation and growth kinetics study of an Indian isolate of virulent duck enteritis virus in Vero cells. *Microb. Pathog*. 78: 14-19.

Audsley, J.M. and Tannock, G.A. 2008. Cell-based influenza vaccines: progress to date. *Drugs.* 68(11): 1483-1491.

Baboo, J., Kilbride, P., Delahaye, M., Milne, S., Fonseca, F., Blanco, M., Meneghel, J., Nancekievill, A., Gaddum, N. and Morris, G.J. 2019. The Impact of Varying Cooling and Thawing Rates on the Quality of Cryopreserved Human Peripheral Blood T Cells. *Sci Rep.* 9(1): 3417. doi: 10.1038/s41598-019-39957-x. PMID: 30833714; PMCID: PMC6399228.

Balachandran, C., Pazhanivel, N., Prabhakar, T.G., Muugudas, V. and Prabhakar, P. 2012. Avipox virus infection in Rosella parakeet (*Platycercus*). *Journal of Advance Veterinary Research.* 2: 184-187.

Baust, J. G., Gao, D. and Baust, J. M. 2009. Cryopreservation: an emerging paradigm change. *Organogenesis.* 5(3): 90–96.

Burleson, F.G., Chambers, M.T. and Wiedbrauk, L.D. 1992. *Virology.* 12-TCID50 58-61, Editor (s): Burleson, FG., Chamber, MT. and Wiedbrauk, LD.Academic Press.

Choi, J. and Bischof, J. C. 2011. Cooling rate dependent biophysical and viability response shift with attachment state in human dermal fibroblast cells. *Cryobiology*. 63: 285–291.

Choi, J. W., Shin, E. K., Ha, S. H., Kim, H. A., Kim, Y. H., Kim, J. S. and Hahn, T. W. 2007. Optimal conditions for cryopreservation of primary chicken embryo kidney cells with dimethyl sulfoxide. *Molecular Biotechnology.* 35 (3): 237–241. https://doi.org/10.1007/BF02686009

Dinh, N.T.K., Dung, N.V., Hoangl, H.V., Hong, N.T.T., Dung, D.V., Trung, N.T., DinhTu, T., Huong, N.T.L., Nind, L., Zuhara Bensink, Z. and Spradbrow, P. 2004. Adaptation of duck plague virus to chicken embryo fibroblast cell culture for vaccine production, control of Newcastle disease and duck plague in village poultry Edited by Joanne Meers, Peter B. Spradbrow and Tran Dinh Tu.

Doley, M., Das, S.K., Barman, N.N. and Rajbongshi, G. 2013. Adaptation of vaccine strain of duck plague virus in chicken embryo fibroblast cell culture. *Indian J. Anim. Sci.* 83(9): 880-882.

Doyong, Gao and Critser, J.K. 2000. Mechanisms of Cryoinjury in Living Cells. *ILAR Journal*. 41(4):187–196.

Fasaei, N., Madadgar, B., Ghalyanchi O., Langeroodi, A. and Ghafari, M. M. 2014. Molecular detection and phylogenetic analysis of Avipoxvirus strains isolated from different bird species. *Iranian Journal of Veterinary Research.* 15:40-44.

Gilhare, V.R. Hirpurkar, S.D., Kumar, A., Naik, S.K. and Sahu, T. 2015. Pock forming ability of Fowl pox virus isolated from layer chicken and its adaptation in chicken embryo fibroblast cell culture. *Veterinary World*, EISSN; 2231-0916.

Hernandez, R. and Brown, D.T. 2010. Growth maintenance of Chick embryo fibroblasts (CEF). *Curr. Protoc. Microbiol*. 17:A.4I.1.8

Jang, T. H., Park, S. C., Yang, J. H., Kim, J. Y., Seok, J. H., Park, U. S., Choi, C. W., Lee, S. R. and Han, J. 2017. Cryopreservation and its clinical applications. *Integr Med Res.* 6(1): 12–18.

Konwar,N, Sarmah, H., Gogoi, S.M., Kevi,E.V., Bharali, A., Barman, N. N., Bora, D. P. and Das, S. K. 2020. Adaptation of wild strain of Duck plague Virus in cell culture system. *Ind. J. of Animal Research.* 54 (6): 716-722.

Kumar, Senthil and Ponnoose, K.T. 1997. Influence of Adaptation of the Vaccine Strain of Duck Plague Virus In: *Chicken Embryo Fibroblast on Its Immunogenicity.* Publisher: Mannuthy Dept. of Microbiology, College of Veterinary Science and Animal Husbandry.

Lee, H. and Lee, H. 1997. Application of the polymerase chain reaction for the diagnosis of Fowl pox virus infection. *J.Virol Methods.* 63(1-2): 113-119.

Li, Y., Huang, B., Ma X., Wu J., Li, F., Ai, W., Song, M. and Yang, H. 2009. Molecular characterization of the genome of duck enteritis virus. *Virology*. 391(2): 151–161.

Mazur, P., Leibo, S. P. and Chu, E. H. Y. 1972. A two-factor hypothesis of freezing injury: evidence from Chinese hamster tissue-culture cells. *Experimental Cell Research.* 71(2) : 345-355.

Mondal, B., Rasool, T.J., Ram, H. and Mallana, S. 2010. Propagation of Vaccine strain of Duck virus enteritis in a cell line of duck origin as an alternative production systemto Propagation in embryonatedegg. *Bilogicals*. 38 (2): 401-406.

Naaldijk, Y., Staude, M. V. and Stolzing, A. 2012. Effect of different freezing rates during cryopreservation of rat mesenchymal stem cells using combinations of hydroxyethyl starch and dimethylsulfoxide. *BMC Biotechnology.* 12: 49.

Ock, S. A. and Rho, G. J. 2011. Effect of dimethyl sulfoxide (DMSO) on cryopreservation of porcine mesenchymal stem cells (pMSCs). *Cell Transplant*. 20 (8) : 1231–1239.

OIE World Organisation for Animal Health, 2018. Duck virus enteritis. In: Manual of diagnostic tests and vaccines for terrestrial animals, OIE Terrestrial manual, Chap 2.3.7. World Organisation for Animal Health, Paris

Oyeleye, O.O., Ogundeji, S.T., Ola, S. I. and Omitogun, O. G. 2016. Basics of animal cell culture: Foundation for modern science. *Biotechnology and Molecular Biology Reviews.* 11(2): 6-16

Pegg, D.E. 2007. Principles of cryopreservation. *Methods Mol Biol*. 368: 39–57.

Seth, G. 2015. Recent advances in optimal cell banking of mammalian cells for biopharmaceutical production. *Pharmaceutical Bioprocessing.* 3: 35-43.

Sultana, R., Nazir, K.N.H., Rahman, M.T., Nipa, S.A., Rahman, M.M., Soma, S.S. and Rahman, M.B. 2019. Isolation and molecular detection of fowl pox and pigion pox viruses for the development of live attenuated vaccine seeds from the local isolates. *J. of the Bangladesh Agricultural Univ*. 17(2) : 211-219.

Thompson, M.L., Kunkel, E.J. and Ehrhardt, R.O. 2014. *Cryopreservation and Thawing of Mammalian Cells.* In: eLS, John Wiley & Sons, Ltd (Ed.). https://doi.org/10.1002/9780470015902.a0002561.pub2

Verma, R.K., Joshi, R.K., Joshi, N., Gangwar, A.K. and Shukla, S. 2015. Study of histopathological changes induced by fowl pox virus in Chorioallantoic membrane of developing chicken embryo and immunological characterization of the egg adapted and cell culture grown virus. *J. Pure and Applied Microbiol*. 9 (2): 1349-1354.

Wambura, P., Meers, J. and Spradbrow, P. 2006. Development of a Cell Culture Method for Quantal Assay of Strain I-2 of Newcastle Disease Virus. *Vet Res Commun.* 30: 689-696.

Whaley, D., Damyar, K., Witek, R.P., Mendoza, A., Alexander, M. and Lakey, J.R. 2021. Cryopreservation: An Overview of Principles and Cell-Specific Considerations. *Cell Transplantation*. 30. doi:10.1177/0963689721999617

Xuefeng, Q., Xiaoyan, Y., Anchun, C., Mingshu, W., Dekang, Z. and Renyong, J. 2008. The pathogenesis of the duck virus enteritis in experimentally infected ducks: A quantitative time- course study using Taq Man Polymerase chain reaction. *Avian Pathology*. 37(3): 307-310.

Zhao, K., He, W., Xie, S., Song, D., Lu,H., Pan, W., Zhou, P., Liu, W., Lu, R., Zhou, J. and Gao, F. 2014. Highly pathogenic fowlpox virus in cutaneously infected chickens, china. *Emerging Infectious Diseases*. 20: 1208-1210.