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Viral 2A-peptides mediate continuous transcription and self-cleavage of multiple heterologous genes in fowlpox virus vector

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ABSTRACT

Applicability of viral 2A-peptides in generation of multi-cistronic transcripts to deliver separate self-cleaved proteins is well established. However, the use of viral 2A-peptides in fowlpox virus vector construction to co-express multiple heterologous genes has not been explored. To evaluate the same, a recombinant transfer plasmid pJFWPVt was constructed through two intermediate plasmid constructs, pJF7F9 and pJFHNGFP. The construction of pJF7F9 involved cloning of F7 and F9 genes of FWPV into pCI-neo with modifications in the F7-F9 intergenic region. For the construction of pJFHNGFP, a synthetic DNA adapter consisting of one synthetic early late promoter ($P_{E/L}$), two viral 2A-peptides (P2A and T2A) and three multiple cloning sites (MCS1, MCS2 and MCS3) was synthesized chemically and was cloned into pUC19 to obtain pJFHNGFPi. Heterologous genes fusion (F) and haemagglutinin-neuraminidase (HN) of Avian Avulavirus-1 (AAv1) and marker gene AcGFP were cloned sequentially into MCS1, MCS2 and MCS3 of pJFHNGFPi to obtain pJFHNGFP. The insert ($P_{E/L}$-F-P2A-HN-T2A-AcGFP) in pJFHNGFP was cloned into pJF7F9 to obtain pJFWPVt, which upon transfection in FWPV infected chicken embryo fibroblast (CEF) cells resulted in fluorescence. This confirmed the expression of AcGFP and the continuous transcription ability of viral 2A-peptides. Further, western blotting of CEF pellet showed separate protein bands of F and HN protein at 66 kDa and 74 kDa respectively, which confirmed the self-cleaving ability of viral 2A-peptides. Herein, in FWPV vector construction, continuous transcription and self-cleaving ability of viral 2A-peptides in FWPV vector construction was confirmed. This warrants scope for future viral 2A-peptide based FWPV vector construction.

Keywords: Co-expression, Multi-cistronic transcription, Plasmid, Recombinant fowlpox virus, Self-cleaved protein, Viral 2A-peptide

Versatility of recombinant DNA (rDNA) technology led to the development of novel recombinant fowlpox virus (rFWPV) vaccines in veterinary research (Skinner *et al.* 2005). Greater part of rFWPV generation involves construction of recombinant transfer plasmid, that facilitates integration of heterologous genes into fowlpox virus (FWPV) through homologous recombination (Ball 1987). The construction of recombinant transfer plasmid involves appropriate organization of heterologous genes with promoters and translational enhancers to ensure proper expression. The design of recombinant transfer plasmid was modified from time to time as new research ideas came forth. One such idea was co-expression of multiple genes, which was approached earlier by using multiple uni-directional or single bi-directional promoters (Skinner *et al.* 2005). The use of multiple promoters caused promoter interference (Emerman and Temin, 1984), which demanded an alternative approach to co-express multiple genes. Currently, viral 2A-peptides have unwrapped the possibility of co-expression of multiple heterologous genes under a single promoter (Szymczak and Vignali 2005, Liu *et al.* 2017).

Viral 2A-peptides are small peptides,that facilitate multi-cistronic transcription of genes (*de* Felipe, 2002) and delivers individual cleaved proteins. Viral 2A-peptides function as cis-acting hydrolase elements (CHYSELs), to mediate "cleavage" between two proteins (*de* Felipe 2004). The cleavage site of viral 2A-peptides is a highly conserved consensus sequence in the carboxyl terminus (*de* Felipe *et al.* 2006), which consists of a glycine residue and a proline residue, between which self-cleavage is initiated (Donnelly *et al.* 2001). The self-cleaving efficiency is influenced by the Glycine-Serine-Glycine (GSG) linker present at the N-terminal of the viral 2A-peptides (Szymczak *et al.* 2004) and is observed only in eukaryotic system and not in prokaryotic systems (Szymczak and Vignali, 2005).

The viral 2A-peptides were initially identified in Foot and Mouth disease virus–F2A (Ryan and Drew, 1994) and later was also identified in *Thosea asigna* virus–T2A, Porcine-tescho virus – P2A (Kim *et al.* 2011) and Equine rhinitis A virus – E2A (Skinner *et al.* 2005). Among these four viral 2A-peptides, P2A and T2A are widely used as they possess high self-cleaving and translation ability

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(Szymczak and Vignali, 2005). Evaluation of viral 2A-peptides to co-express multiple genes were previously attempted in adenovirus (Lewis *et al.* 2015) and lentivirus (Ibrahimi *et al.* 2009) vectors. However, the use of viral 2A-peptides in co-expression of multiple genes in FWPV vector is yet to be explored.

Given the prospects of FWPV in veterinary vaccines (Skinner *et al.* 2005), exploration of the functionality of viral 2A-peptides in the same is necessary. Herein, two viral 2A-peptides—P2A and T2A were employed for co-expression of three heterologous genes—Fusion (F) gene of Avian Avulavirus-1, Haemagglutining-neuraminidase (HN) gene of Avian Avulavirus-1 and AcGFP gene, under a single synthetic early late promoter in chicken embryo fibroblast (CEF) cells.

MATERIALS AND METHODS

Virus strains, plasmids and cells: The Globivac FWPV strain (FWPV-G) of chicken embryo origin manufactured by Globion Pvt. Ltd. was used.

The plasmids, pCI-neo mammalian expression vector (Cat#1841, Promega, USA) and pUC19 vector (Cat#N3041S, NEB, USA) were used as base vectors. The plasmids pCI-AAv1F and pCI-AAv1HN constructed from D58 strain of Avian Avulavirus-1 (Kirubaharan and Palaniswami 2003) and maintained at this laboratory was used as template for amplification of AAv1F and AAv1HN genes respectively. The plasmid pCI-AcGFP developed from pAcEGFP vector (Cat# 632501, Clontech, Japan) and maintained at this laboratory was used as template for amplification of AcGFP gene.

Primary CEF cells from specific pathogen free embryonated chicken eggs were prepared and maintained in Dulbecco's minimum essential medium (Gibco, Life technologies; Cat # 11095–080) with 10% fetal bovine serum (HiMedia, India; Cat # RM 1112) as described by Freshney (2000).

DNA isolation: DNA was isolated from FWPV-G using QIAamp DNA mini kit (Cat#51304, Qiagen, Germany) as per manufacturer's instructions and was stored at –80°C until further use.

Primers: Primers used for amplification of gene inserts were designed at this laboratory using Primer3 software (Untergasser *et al.* 2012) and were validated using Oligoanalyzer software v2.1. Primers used for restriction free (RF) cloning were designed using the online software www.rfcloning.org (Bond and Naus, 2012). The details of the primer sequences, annealing temperature and product size are provided in Table 1.

PCR amplification: PCR amplification of gene inserts was done using Phusion high fidelity PCR kit (Cat# E0553S, NEB, USA) following manufacturer's instructions. In brief, the reaction mixture comprised of 10 ml of 5× Phusion HF buffer, 0.2 mM of dNTPs, 0.5 mM of each primer, 3% of DMSO, 1 unit of Phusion DNA polymerase and 10 ng of template in a final reaction volume of 50 ml. The reaction cycle involved initial denaturation at 98°C/ 30s followed by 35 cycles of denaturation at 98°C/10s, optimum annealing temperature (OAT) /10 s and extension at 72°C /30s/kb with a final extension at 72°C/5 min.

Molecular cloning procedures: All molecular cloning procedures including restriction digestion of plasmids and gene inserts, transformation of plasmids into competent cells and scaling-up of transformed bacterial cells were done as described by Sambrook and Russel, (2001).

Construction of recombinant transfer plasmid pJFWPVt: pJFWPVt was constructed through two intermediate plasmids namely pJF7F9 and pJFHNGFP. The schematic diagram showing the construction strategy of pJFWPVt is depicted in Fig.1.

Construction of pJF7F9 plasmid: For the construction of pJF7F9, F7 and F9 genes were amplified from FWPV-G using the primers specified by Boyle *et al.* (2004). During PCR, NheI and XhoI/AscI restriction enzyme (RE) sites

Table 1. Details of primer sequences, annealing temperature and product size

Gene/ Region	Primer sequence (5′ to 3′)[1] (F-Forward primer; R-Reverse primer)	Product size (bp)	Ta (°C)	Reference
F7	F: tac tca *GCT AGC* GAA ATA TTT AAT ACG ATG AA R: attctc *CTC GAG* tac gat *GGC GCG CC* CTA GTT GCG CTT CAG TAG TGA TAT TAG GTT GTT TAT ATA CAT ATT CTC G	879	58	Boyle *et al.* 2004 (RE sites added were modified for this study)
F9	F: tac gtc *CTC GAG* tta cat *GTC GAC* TTG TTA AAA AGG AAT TGA AAG AA R: acg ttt *GCG GCC GC* AAA CTC ATC TCC ACA AGG AAT GT	700	55	
AAv1F	F: aat gct *GGT ACC* ATG GGC TCC AGA CCT TCT ACC AAG R: act ggt *CCT GCA GG*C ATT TTT GTA GTG GCT CTC ATC TGA TCT AGA G TCT AGA G	1685	72	Designed for this study
AAv1HN	F: aaa ttt *CCA TGG* ATG GAC CGC GCC GTT AGC CA R: ggt taa *AAG CTT* GCC AGA CCT GGC TTC TCT AAC CCC GTC ATC	1755	72	
AcGFP	F: ccc ttt *TTA ATT AA* ATG GTG AGC AAG GGC GCC GA R: agc gat *GGG CCC* ctg agt *GTC GAC* TCA CTT GTA CAG CTC ATC CA	759	62	

[1]Italics, RE sequences; Lower case, Stuffer sequences; Upper case, Gene specific sequences.

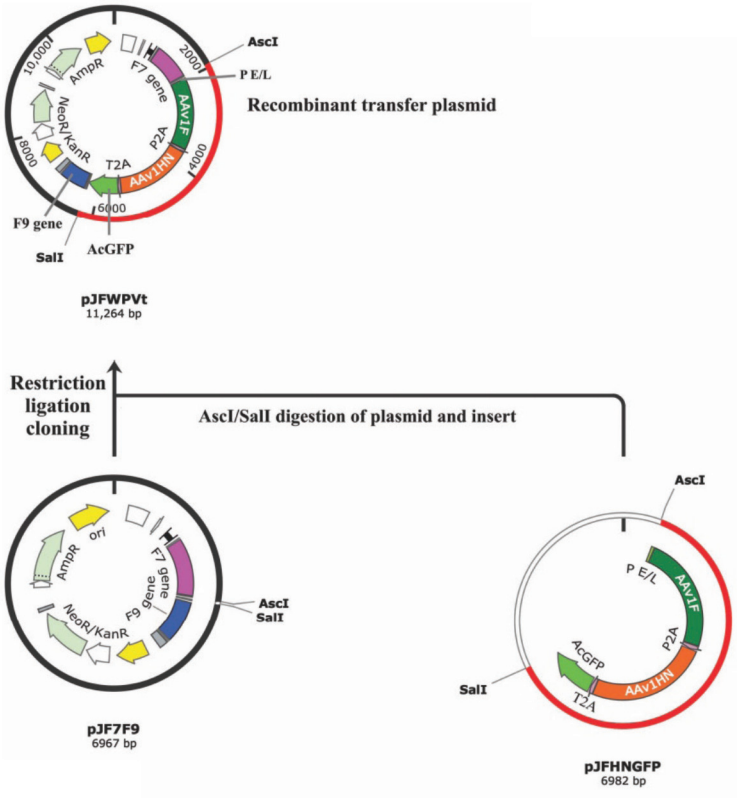

Fig. 1. Schematic diagram of construction of pJFWPVt recombinant transfer plasmid.

were added to 5′ and 3′ ends of F7 gene respectively. Whereas XhoI/SalI and NotI RE sites were added to 5′ and 3′ ends of F9 gene. The amplified F7 and F9 genes were linear ligated at XhoI RE site, thus resulting in the insertion of a unique multiple cloning site (MCS), AscI/XhoI/SalI, in F7–F9 intergenic region. The linear ligated product was cloned into NheI/NotI RE site of pCI-neo by restriction digestion method to obtain pJF7F9.

Construction of pJFHNGFP plasmid: For the construction of pJFHNGFP, a synthetic DNA adapter consisting of the following was synthesized chemically: AscI, synthetic early late promoter ($P_{E/L}$), KpnI/SbfI (MCS1), P2A, NcoI/HindIII (MCS2), T2A and ApaI/PacI (MCS3). The schematic diagram showing the synthetic DNA adapter is depicted in Fig. 2. This synthetic adapter was cloned by RF cloning method into pUC19 in two PCR steps. The primary PCR involved amplification of synthetic DNA adapter in Phusion High Fidelity PCR kit using RF cloning primers (F: 5′-*ACGTTGTAAAACGACGG CCAGT*TTCACAGGCGCGCCAAAA-3′; R: 5′-*GGAAAC AGCTATGACCATGATTAC GCC*CTGAGTGGGCC CAGCATT-3′). The reaction condition involved an initial denaturation at 98°C/10 s followed by 35 cycles of denaturation at 98°C/8 s, annealing at 60°C/20 s and extension at 72°C /30 s /kb with a final extension at 72°C/ 5 min. The resultant product or megaprimer was subjected to secondary PCR along with pUC19. The reaction mixture comprised 4 ml of 5X Phusion HF buffer, 0.2 mM of dNTPs, 3% of DMSO, 1 unit of Phusion DNA polymerase with 82 ng of megaprimer and 32 ng of pUC19 in a final reaction volume of 20 ml. The reaction condition involved an initial denaturation at 98°C/10 s followed by 15 cycles of denaturation at 98°C/8 s, annealing at 61°C/20 s and extension at 72°C/30 s/kb with a final extension at 72°C/5 min. Upon completion of the PCR reaction, 20 units DpnI enzyme was added directly to the reaction mixture, which was incubated at 37°C/ 2 h and inactivated at 80°C/ 20 min to obtain pJFHNGFPi.

The gene inserts AAv1F, AAv1HN and AcGFP were amplified from pCI-AAv1F, pCI-AAv1HN and pCI-AcGFP respectively using primers mentioned in Table 1. During PCR, KpnI and SbfI RE sites were added to the 5′ and 3′ ends of AAv1F gene; NcoI and HindIII RE sites were added to the 5′ and 3′ ends of AAv1HN gene; and PacI and SalI/ ApaI RE sites were added to the 5′ and 3′ ends of AcGFP gene. The AAv1F and AAv1HN genes were PCR amplified without the stop codon, and AcGFP was amplified with stop codon. The amplified gene inserts AAv1F, AAv1HN

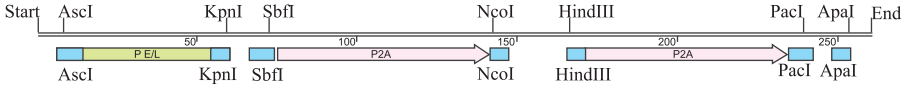

Fig.2. Schematic diagram of synthetic DNA adapter.

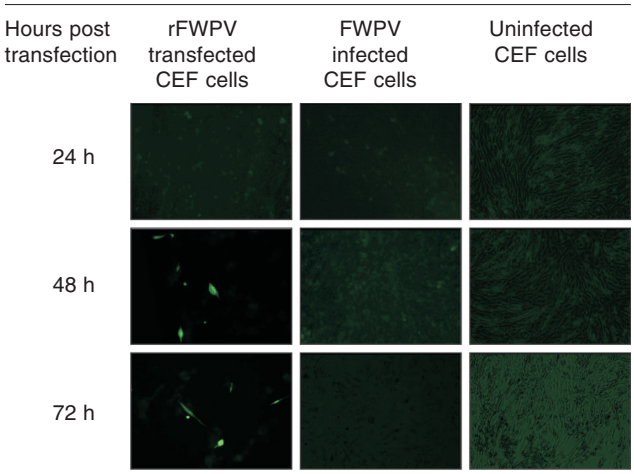

Fig. 3. CEF cells showing fluorescence at 48 h and 72 h post transfection.

and AcGFP were sequentially cloned into MCS1, MCS2 and MCS3 site of pJFHNGFPi respectively by restriction digestion cloning method to obtain pJFHNGFP.

Finally, the fragment AscI/SalI in pJFHNGFP encompassing the synthetic DNA adapter along with AAv1F, AAv1HN and AcGFP genes was released and cloned into AscI/SalI site of pJF7F9 by restriction digestion method to obtain pJFWPVt.

Transfection and homologous recombination: The recombinant transfer plasmid pJFWPVt was transfected using TransIT- LT1 transfection reagent (Cat#MIR2300, Mirus, USA) into CEF cells previously infected with FWPV-G at 0.1 multiplicity of infection (MOI) for 2 h. Uninfected CEF cells, FWPV infected CEF cells and plasmid only CEF cells were maintained as control. The cells were fixed using 4% para-formaldehyde and was observed for fluorescence at 24 h, 48 h and 72 h post-transfection.

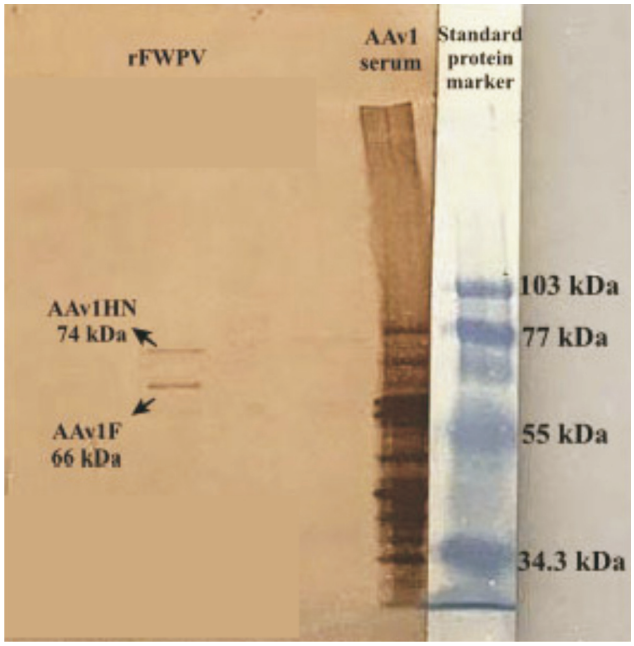

Fig. 4. Western blot assay showing AAv1F and AAv1HN protein expression.

Further, the transfected CEF cells were harvested using RIPA lysis buffer (Cat#89900, Thermo Scientific, USA) containing 0.1 ml of protease inhibitor cocktail buffer (Cat#8340, Sigma-Aldrich, Germany) at 24 h, 48 h and 72 h post-transfection. The cell lysates were analyzed for expression of AAv1F and AAv1HN genes using western blot assay as described by Sambrook and Russel (2001).

Western blot assay: The cell lysates were separated by electrophoresis in 10% sodium-dodecyl phosphate polyacrylamide gel with pre-stained low range SDS-PAGE marker (Cat#161-0305, Biorad laboratories, USA) as standard. The separated proteins were transferred onto 0.45 mm nitrocellulose membrane (Cat#1704158, BioRad laboratories, USA) at 1.3 A and 25 V for 30 min. The membrane was incubated with anti-AAv1 hyperimmune serum as primary antibody and anti-chicken IgY peroxidase antibody produced in rabbit (Cat# A9064, Sigma, USA) as secondary antibody. The protein bands were visualised using 3,32-Diaminobenzidine (Cat#D4293, Sigma-Aldrich, USA) and hydrogen peroxide.

RESULTS AND DISCUSSION

Ever since the establishment of the fact that FWPV can harbour and deliver multiple genes (Paoletti 1996), many studies were focussed on FWPV vector construction carrying multiple heterologous genes. It either involved the use of multiple uni-directional promoters (Chen *et al.* 2011) or single bi-directional promoter (Kumar and Boyle 1990, Du *et al.* 2015). Later, it was reported that the use of more than two promoters might cause promoter interference which would affect the efficiency of heterologous gene expression (Emerman and Temin, 1984). Therefore, continuous transcription of heterologous genes from a single promoter was considered as a better alternative. This could be facilitated either by Internal Ribosomal Entry Site (IRES) sequences or by viral 2A-peptides (Szymczak and Vignali 2005). Viral 2A-peptides are 60 to 66 bp in length (*de* Felipe 2002), that offers multi-cistronic transcription of heterologous genes and ensures individual protein expression of the same. Therefore, the present study is a pioneering attempt to explore the functionality of viral 2A-peptides in FWPV vector.

In the present study, transfection of CEF cells with recombinant transfer plasmid pJFWPV showed fluorescence at 48 h and 72 h post-transfection (Fig. 3). The fluorescence of CEF cells confirmed the expression of AcGFP gene and the activity of $P_{E/L}$ promoter, which initiated the transcription. The $P_{E/L}$ promoter is a synthetic early late promoter that offers increased promoter strength (Chakrabarti *et al.* 1997) and induces expression of heterologous genes throughout the replication cycle of FWPV (Moss and Earl, 2000). Further, AcGFP gene was cloned downstream of AAv1F, P2A, AAv1HN and T2A. Therefore, the expression of AcGFP gene validated the ability of P2A and T2A viral peptides to facilitate continuous transcription of heterologous genes placed downstream of the same. Western blot analysis of transfected CEF cell

lysates showed two separate protein bands of size 66 kDa and 74 kDa (Fig. 4), which confirmed the expression of AAv1F and AAv1HN genes respectively (Panda *et al.* 2004, Samal 2011). The AAv1F gene is expressed as a whole protein, F0 (66 kDa); or F0 is cleaved into F1 (55 kDa) and F2 (11 kDa) proteins (Panda *et al.* 2004). For the F0 protein to be cleaved in CEF cells, external protease is required (McGinnes *et al.* 2006). In this study, external protease was not provided to CEF cells, hence AAv1F gene is expressed as whole F0 protein of size 66 kDa. The AAv1F and AAv1HN proteins were at detectable levels only in the cell lysates that were collected at 72 h post transfection. Moreover, the separate bands of AAv1F and AAv1HN genes confirmed the self-cleaving ability of both P2A and T2A viral peptides (Szymczak *et al.* 2012).

Further, although the heterologous genes flanked between FWPV genes were cloned in pCI-neo mammalian expression vector, the CEF cells transfected with pJFHNGFP plasmid only showed neither fluorescence nor F / HN protein bands in western blotting. This confirms that cytomegalovirus (CMV) promoter of pCI-neo did not drive expression of the heterologous genes.

In conclusion, viral 2A-peptides are elements that facilitate continuous transcription of multiple heterologous genes, that were initiated by a single promoter. Although the utility of viral 2A-peptides have been validated in earlier studies, the same was not explored in FWPV vector construction. To the best of our knowledge, this is the first study that has evaluated the ability of viral 2A-peptides in FWPV vector construction. Also, this study serves as a foundation that allows development of 2A-peptide linked rFWPV based veterinary vaccines.

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REFERENCES

Ball L A. 1987. High-frequency homologous recombination in Vaccinia virus DNA. *Journal of Virology* **61**: 1788–95.

Bond S R and Naus C C. 2012. RF-Cloning.org: An online tool for the design of restriction-free cloning projects. *Nucleic Acids Research* **40**: W209–W213.

Boyle D B, Anderson M A A, Amos R, Voysey R and Coupar B E H H. 2004. Construction of recombinant Fowlpox Viruses carrying multiple vaccine antigens and immunomodulatory Molecules. *Biotechniques* **37**(1): 104–11.

Chakrabarti S, Sisler J R and Moss B. 1997. Compact, synthetic, vaccinia virus early/late promoter for protein expression. *Biotechniques* **23**: 1094–97.

Chen H Y, Cui P, Cui B A, Li H P, Jiao X Q, Zheng L L, Cheng G and Chao A J. 2011. Immune responses of chickens inoculated with a recombinant fowlpox vaccine co-expressing glycoprotein B of infectious laryngo-tracheitis virus and chicken IL-18. *FEMS Immunology and Medical Microbiology* **63**: 289–95.

de Felipe F P. 2002. Polycistronic viral vectors. *Current Gene Therapy* **2**: 355–78.

de Felipe F P. 2004. Skipping the co-expression problem: The new 2A "CHYSEL" technology. *Genetic Vaccines Therapy* **2**: 13.

de Felipe P, Luke G A, Hughes L E, Gani D, Halpin C and Ryan M D. 2006. Eunum pluribus: Multiple proteins from a self-processing polyprotein. *Trends in Biotechnology* **24**: 68–75.

Donnelly M L L, Hughes L E, Luke G, Mendoza H, Dam E, Gani D and Ryan M D. 2001. The 'cleavage' activities of foot-and-mouth disease virus 2A site-directed mutants and naturally occurring '2A-like'sequences. *Journal of General Virology* **82**: 1027–41.

Du S, Liu C, Zhu Y, Wang Y, Ren D, Wang M, Tan P, Li X, Tian M, Zhang Y and Li J. 2015. Construction and characterization of novel fowlpox virus shuttle vectors. *Virus Research* **197**: 59–66.

Emerman M and Temin H M. 1984. Genes with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism. *Cell* **39**: 459–67.

Freshney R I. 2000. *Culture of Animal Cells*: *A Manual of Basic Technique and Specialized Applications.* (4th Ed.). pp. 181–184. Wiley-Liss, New York.

Ibrahimi A, Velde G V, Reumers V, Toelen J, Thiry I, Vandeputte C, Vets S, Deroose C, Bormans G, Baekelandt V and Debyser Z. 2009. Highly efficient multi-cistronic lentiviral vectors with peptide 2A sequences. *Human Gene Therapy* **20**: 845–60.

Kim J H, Lee S R, Li L H, Park H J, Park J H, Lee K Y, Kim M K, Shin B A and Choi S Y. 2011. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. *PLoS One* **6**: e18556.

Kirubaharan J J and Palaniswami K S. 2003. Final report of the ICAR scheme on preparation and standardization of oral pellet vaccine against Newcastle disease.

Kumar S and Boyle D B. 1990. A Poxvirus bidirectional promoter element with early/late and late functions. *Virology* **179**: 151–58.

Lewis J E, Brameld J M, Hill P, Barrett P, Ebling F J P and Jethwa P H. 2015. The use of a viral 2A sequence for the simultaneous over-expression of both the vgf gene and enhanced green fluorescent protein (eGFP) *in vitro* and *in vivo*. *Journal of Neuroscience Methods* **256**: 22–29.

Liu Z, Chen O, Wall J B, Zheng M, Zhou Y, Wang L, Vaseghi H R, Qian L and Liu J. 2017. Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector. *Scientific Reports* **7**(1): 2193.

McGinnes LW, Pantua H, Reitter J and Morisson T. 2006. Newcastle disease virus: Propagation, quantification, and storage. *Current Protocols in Microbiology* **15**: 15F 12.

Moss B and Earl P. 2001. Overview of the *vaccinia* virus expression system. *Current Protocols in Molecular Biology* **3**: 1–5.

Panda A, Huang Z, Elankumaran S, Rockemann D D and Samal S K. 2004. Role of fusion protein cleavage site in the virulence of Newcastle disease virus. *Microbial Pathogenesis* **36**: 1–10.

Paoletti E. 1996. Applications of Pox virus vectors to vaccination: An update. *Proceedings of the National Academy of Sciences United States of America* **93**: 11349–353.

Ryan M D and Drew J. 1994. Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial polyprotein. *EMBO Journal* **13**: 928–33.

Samal S K. 2011. *The Biology of Paramyxoviruses.* Caister

Academic Press, USA.

Sambrook J and Russell D W. 2001. *Molecular Cloning*: *A Laboratory Manual.* (3rd Ed.). Cold Spring Habour Laboratory Press, New York.

Skinner M A, Laidlaw S M, Eldaghayes I, Kaiser P and Cottingham M G. 2005. Fowlpox virus as a recombinant vaccine vector for use in mammals and poultry. *Expert Review of Vaccines* **4**: 63–76.

Szymczak A L, Workman C J, Wang Y, Vignali K M, Dilioglou S, Vanin E F and Vignali D A. 2004. Addendum: Correction of multi-gene deficiency in vivo using a single "self-cleaving" 2A peptide-based retroviral vector. *Nature Biotechnology* **22**: 1590.

Szymczak A L and Vignali D A. 2005. Development of 2A peptide-based strategies in the design of multicistronic vectors. *Expert Opinion on Biological Therapy* **5**: 627–38.

Szymczak A L, Vignali K M and Vignali D A A. 2012. Design and construction of 2A peptide-linked multicistronic vectors. *Cold Spring Harbour Protocols* **7**: 199–204.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth B C, Remm M and Rozen S G. 2012. Primer3-New Capabilities and Interfaces. *Nucleic Acids Research* **40**: 1–12.