Veterinary Research 5 (4): 82-90, 2012
ISSN: 1993-5412

Characterization of Duck Plague Virus Tegument Protein VP22

[1]Long Jiang, [1, 2, 3]Anchun Cheng, [1, 2, 3]Mingshu Wang, [1, 2]Dekang Zhu,
[1, 2]Renyong Jia and [1, 2, 3]Xiaoyue Chen
[1]Avian Disease Research Center, College of Veterinary Medicine,
[2]Key Laboratory of Animal Diseases and Human Health of Sichuan Province,
Sichuan Agricultural University, Ya'an, 625014 Sichuan, China
[3]Epizootic Diseases Institute of Sichuan Agricultural University, Ya'an, 625014 Sichuan, China

Abstract: Duck Plague Virus (DPV) is a member of the Alphaherpesviridae subfamily and an important pathogen in domestic and wild ducks, geese, swans and other water fowl with migratory waterfowl contributing to spread between continents. VP22 is a structural protein assembled into the tegument compartment of the virion, highly conserved across the Alphaherpesviridae. The precise biological function performed by the VP22 tegument protein encoded by *UL49* gene in the virus life cycle remains to be elucidated. In this report, researchers aimed at elucidating the subcellular localization of VP22 in DPV infected primary duck embryo fibroblasts the recombinant VP22 protein and VP22-GFP fusion protein in DEF cells. Transcription characteristics of *UL49* gene was analyzed by RTFQ-PCR.

Key words: Alphaherpesviridae, virion, life cycle, fibroblasts, transcription

INTRODUCTION

The virion proteins of herpesviruses are arranged within three morphologically distinct components of the virus: the nucleocapsid the envelope and the tegument the region located between the capsid and envelope of the virus particle. VP22 is the protein product of the *UL49* gene which is expressed late in infection (Elliott and Meredith, 1992). In addition, VP22 is the most abundant tegument protein which contributing between 5 and 9% of the total mass of the virion and nearly 2000 copies in HSV-1 virions in terms of the number of molecules per virion particle (Heine *et al*., 1974). Numerous studies have suggested that the function of VP22 can be broadly divided into nuclear localisation (Ren *et al*., 2001) nucleolar localisation (Aints *et al*., 2001) chromatin binding (Elliott and O'Hare, 2000) DNA binding (Pinard *et al*., 1987) microtubule binding (Kotsakis *et al*., 2001; Elliott and O'Hare, 1998; Yedowitz *et al*., 2005) intercellular trafficking (Elliott and O'Hare 1997; Zheng *et al*., 2006) RNA binding (Sciortino *et al*., 2002) and protein delivery (Zhang *et al*., 2009; Saha *et al*., 2006; Lee *et al*., 2006; Zheng *et al*., 2005) and the application of transduction technology is increasing in gene therapy and immunization. The VP22 homologs are highly conserved across the Alphaherpesviridae (Ingvarsdottir and Blaho, 2010; Kelly *et al*., 2009) mostly within the carboxyl terminus of the protein suggesting that the carboxyl terminus may be important for structure or function (Ren *et al*., 2001).

Duck Plague Virus (DPV) is a member of the Alphaherpesviridae subfamily (Li *et al*., 2009) and an important pathogen in domestic and wild ducks, geese, swans and other water fowl with migratory waterfowl contributing to spread between continents (Kisary and Zsak, 1983; Davison *et al*., 1993; Keymer and Gough, 1986; Gough and Alexander, 1987; Wobeser, 1987). DPV VP22, a 253-amino-acid protein with a calculated minimal molecular mass of 27.8635 kDa (Jiang *et al*., 2010) encoded by the UL49 Open Reading Frame (ORF). The precise biological function of the VP22 tegument proteins in the virus life cycle remains to be elucidated.

MATERIALS AND METHODS

Viral strain, cell culture, infections and viral DNA isolation: The parental virus used in this study was DPV CHv strain provided by Key Laboratory of Animal Disease and Human Health of Sichuan Province. Primary Duck Embryo Fibroblasts (DEF) can be productively infected by DPV (Guo *et al*., 2009) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco)

Corresponding Author: Anchun Cheng, Avian Disease Research Center, College of Veterinary Medicine,
Sichuan Agricultural University, Ya'an, 625014 Sichuan, China

containing 10% Newborn Calf Serum (NCS) and penicillin-streptomycin at 37°C in a 5% CO_2 atmosphere. Infections were routinely performed at a Multiplicity of Infection (MOI) of 0.1. After 1 h, the inoculum was removed, fresh medium supplemented with 2% NCS was added and the cells were incubated at 37°C in 5% CO_2. When >80% of the cells showed cytopathic effects the culture of infected cells was subjected to three freeze-thaw cycles and stored at -80°C till use. Viral DNA was isolated from infected DEF cells as described previously (Cheng *et al*., 2006).

Expression and purification of VP22 protein: The full-length *UL49* gene used for this study was PCR amplified with the upstream primer 5'-GGATCCATGGCAA ATAGAATAGATCGACG-3' and the downstream primer 5'-AAGCTTTTATTGTGGTCTTCTTGAACTGTTA-3'. In-frame start and stop codons are indicated by boldface letters. BamH I and Hind III restriction sites which were introduced for convenient cloning are underlined. PCR products were cloned into the Multiple Cloning Site (MCS) of the pMD18-T vector (TaKaRa) producing a plasmid pMD18-T/UL49. Thereafter, the coding region of the VP22 open reading frame in pMD18-T/UL49 was confirmed by sequencing. The nucleotide sequence data reported in this study has been deposited in GenBank nucleotide sequence databases with the accession number EU195111. For recombinant protein production, a prokaryotic expression vector pET28a (+) (Novagen) was used. pMD18-T/UL49 was digested with BamH and Hind β and the UL49 sequense was inserted into the BamH/Hind β site of pET-28a (+) which is capable of producing recombinant protein with an N-terminal 6xHis tag. The result was designated pET28a/UL49 and transformed into *E. coli* BL21 (DE3) for recombinant protein production by Calcium Chloride Method. The transformed bacteria were selected by screening the colonies on LB media containing Kanamycin (Kan). The suspected colony was further analyzed by restriction enzyme digestion and PCR.

Overnight cultures were grown in the presence of 50 μg of Kan per mL at 37°C. The overnight culture was diluted 10 times with the same medium and incubated until Optical Density (OD_{600nm}) reached 0.6~0.8. Expression was induced by the addition of Isopropyl β-D-1-Thiogalactopyranoside (IPTG) 0.1 mM and incubation continued for a further 4 h. The cultures were then cooled on ice for 10 min and centrifuged at 6000 rpm for 10 min at 4°C. The bacteria pelleted and suspended in lysis buffer (50 mM sodium phosphate (pH 8.0) containing 300 mM NaCl; 0.1 mM Phenylmethylsulfonyl Fluoride (PMSF)). Lysozyme (1 mg mL^{-1}) was added and the samples incubated on ice for 30 min followed by sonication, insoluble material were removed by centrifugation (15,000 rpm for 10 min at 4°C). To reduce nonspecific protein binding, addition of 1 M imidazole to the supernatant to a final concentration of 50 mM imidazole, pH 8.0. The supernatant was 0.45 micron membrane filtered and then loaded onto a 5 mL Ni-NTA column (Qiagen) equilibrated in binding/wash buffer (50 mM sodium phosphate (pH 8.0) containing 300 mM NaCl, 50 mM imidazole). The column was then washed with binding/wash buffer and finally eluted with elution buffer (50 mM sodium phosphate (pH 6.0) containing 300 mM NaCl, 250 mM imidazole). Purified protein was lyophilized after dialysis in PBS then stored at -70°C until used.

Polyclonal rabbit anti-VP22 serum preparation and purification: For the production of the polyclonal rabbit anti-VP22 serum, a adult New Zealand white rabbit was inoculated four times with VP22 protein. For the first inoculation, purified VP22 protein (0.3 mg) was dialyzed against PBS, emulsified with an equal volume of Complete Freund Adjuvant (CFA) (Sigma, USA) and injected subcutaneously into a adult New Zealand white rabbit at six different sites of the back. Subsequently the rabbit was boosted three times with 0.5, 0.8 or 1.0 mg of VP22 protein emulsified with an equal volume of Incomplete Freund's Adjuvant (IFA) (Sigma, USA) at weekly intervals starting from 2 weeks after primary immunization. The rabbit was bled 7-10 days after the final booster then it was killed and the IgG fraction of the serum was isolated by ammonium sulfate precipitation as described previously with slight modifications (Zheng *et al*., 2005). Immune rabbit serum was heat inactivated at 56°C for 30 min. Then, using a DEAE-Sepharose column, the IgG fraction was purified by ion-exchange column chromatography according to the instructions of the suppliers and stored at -70°C until used.

Subcellular localization of VP22 protein in DPV infected primary DEF: In order to study the subcellular localization of VP22, coverslip cultures of DEF infected with DPV were washed in PBS and fixed with 3% paraformaldehyde at room temperature for 30 min at the time of harvest (6, 12, 24, 36, 48 and 72 h post-infection (hpi)). For VP22 detection, rabbit anti-VP22 polyclonal antibody and FITC-conjugated goat anti-rabbit secondary antibody were used. The cells were washed three times in PBS, permeabilized with PBS containing 0.2% Triton X-100, blocked for 1 h with 3% BSA and incubated with the rabbit anti-VP22 polyclonal antibody in blocking solution overnight at 4°C followed by three washes with PBS. FITC-conjugated goat anti-rabbit secondary antibody was diluted in the blocking solution and

incubated for 1 h at room temperature followed by three washes with PBS. For orientation purposes, DAPI (4',6-diamidino-2-phenylindol; Sigma, USA) to 1 μg mL^{-1} was added in the final incubation step to counterstain the nuclei of the cells for 5 min at room temperature. This was followed by three washes with PBS and visualization by a Nikon fluorescence microscopy (Nikon ECLIPSE 80i). The specificity of the reaction was confirmed by the failure of the anti-VP22 rabbit polyclonal antibody to react with uninfected cells and by the failure of preimmune serum to react with infected cells.

Transport activity of the recombinant VP22 protein: The DEF cells were grown on coverslips in six-well dishes in DMEM containing 10% NCS. Prior to the import assay (1 h) the medium was changed and the cells were washed three times in PBS. For each well, aliquots of purified VP22 (200 ng) proteins were added to prewarmed DMEM containing 1% NCS with a final volume of 2 mL. Then, the coverslip cultures were washed in PBS and fixed with 3% paraformaldehyde at room temperature for 30 min at the time of harvest (2, 4, 6, 8, 12, 24 and 36 h). The staining procedures were performed as Subcellular localization of VP22 protein in DPV infected primary DEF described.

Subcellular localization of VP22-GFP fusion protein in living and fixed DEF: The complete VP22 sequences were amplified by PCR using VP22-specific primers 5'-GTCG ACATGGCAAATAGAATAGATCGACG-3' and 5'-GG ATCCTGTGGTCTTCTTGAACTGTTA-3' with Premix PrimeSTAR® HS polymerase (TaKaRa). To facilitatethe expressionof GFP fusion proteins, stop codons of VP22 were removed. Hind III and BamH I restriction sites which were introduced for convenient cloning. PCR products were cloned into the MCS of the pMD18-T vector the resulting plasmids were designated pMD18-VP22. After digestion with BamH I and Hind III, PCR fragments were ligated into pEGFP-N1. The resulting plasmids were designated VP22-GFP. Then, the circularized plasmids were propagated in *Escherichia coli* DH5 α cells and purified using a small-batch plasmid DNA purification kit according to the manufacturer's instructions (Axygen). Thereafter, the coding regions of the VP22 open reading frame in VP22-GFP were confirmed by sequencing.

Subconfluent primary DEF cells in six-well plates were transfected with 4 μg of VP22 mutants per well by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 4 h of incubation the Opti-MEM mixture was replaced with DMEM containing 2% NCS. The cells were observed using a Nikon Eclipse TE2000-UInverted Microscope at 12, 24, 36, 48 and 72 h post-transfection. In order to study the subcellular localization of the VP22-GFP in fixed cells, coverslip cultures of DEF were washed in Phosphate-Buffered Saline (PBS) and fixed with 3% paraformaldehyde at room temperature for 30 min. The cells were washed three times in PBS, permeabilized with 0.5% Triton X-100 in PBS, followed by three washes with PBS. For orientation purposes, 1 μg mL^{-1} of DAPI was added in the final incubation step to counterstain the nuclei of the cells for 10 min at room temperature. This was followed by three washes with PBS and visualization by a Nikon ECLIPSE 80i fluorescence microscope at 12, 24, 36, 48 and 72 h post-transfection. The experiments were carried out in triplicate and repeated independently three times.

Real-Time Fluorescent Quantitative Polymerase Chain Reaction (RTFQ-PCR): The total RNA from DPV infected DEF cells was extracted at 1, 2, 4, 6, 8, 12, 24, 36, 48, 60, 72, 84 and 96 h post-infection (hpi) using RNAiso Plus reagent (TaKaRa) according to the manufacturer's instructions. The total RNA was quantified by optical density and the quality was evaluated by gel electrophoresis. Reverse transcription was performed with a PrimeScript® RT Reagent kit with gDNA Eraser (TaKaRa). Quantitative PCR was performed on a Bio-Rad iCycler and iQ5 2.1 Standard Edition Optical System Software. Data were analyzed by using the Delta-Delta Ct Method (Livak and Schmittgen, 2001). All samples were carried out in triplicate. For each sample the amount of target mRNA was normalized using endogenous β-actin mRNA. The following primer sets were used: UL49, 5'-TCCGCGC AATAACGAGA-3' (forward) and 5'-CCGAATGGGCCTT TATGT-3' (reverse) and β-actin, 5'-TACGCCAACACGG TGCTG-3' (forward) and 5'-GATTCATCATACTCCTGCTTGCTG-3' (reverse). Before the PCR, a mixture of the synthesized cDNA and SYBR® Premix Ex Taq™ II (TaKaRa) was incubated at 95°C for 30 sec. PCR was then carried out at 95°C for 5 sec and at 60°C for 30 sec for 40 cycles. Following RTFQ-PCR, homogeneity of PCR products were confirmed by the melting curve analysis then were electrophoresed on a 2.0 g L^{-1} agarose gel.

RESULTS AND DISCUSSION

Subcellular localization of VP22 protein in DPV infected primary DEF: Indirect immunofluorescence tests (Fig. 1) revealed that the VP22 antigens is detectable as early as 6 h postinfection, it exists predominantly in the cytoplasm and perinuclear region of DPV infected DEF in a diffuse fluorescent pattern early in infection then migrates to and accumulates in the nucleus at late stages of infection, virtually every cell in the culture exhibited bright nucleus

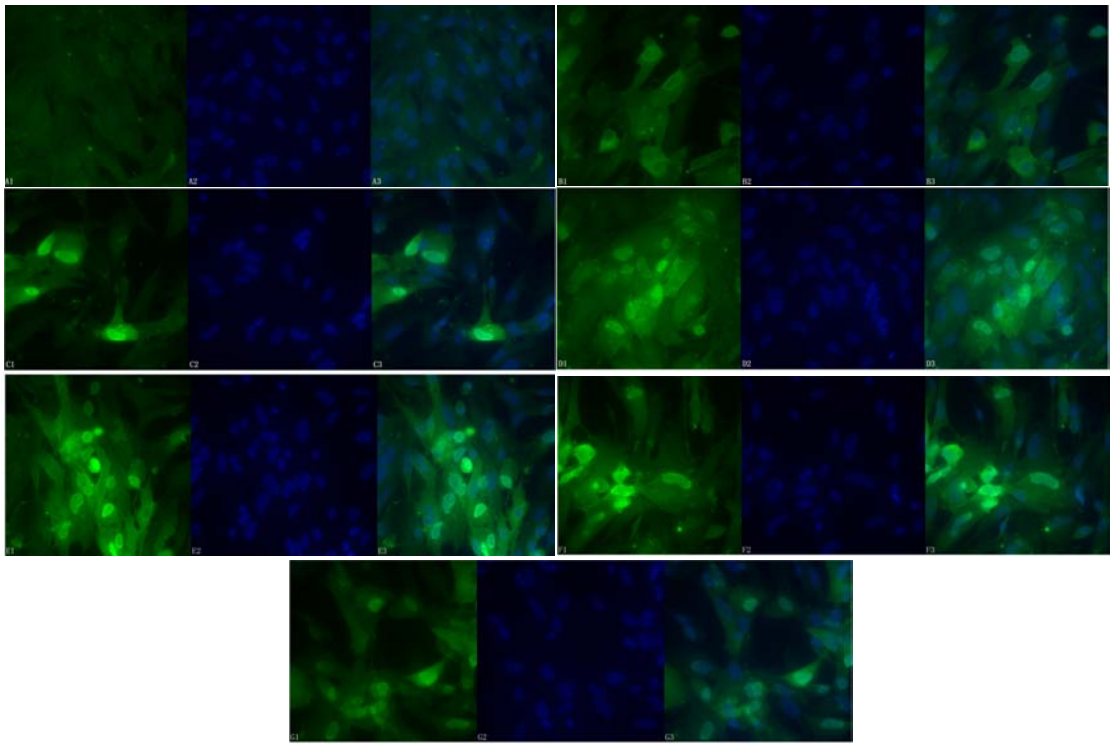

Fig. 1: Subcellular localization of VP22 in DPV infected primary DEF. A1, A2 and A3: uninfected DEF as negative control; B1, B2 and B3: DPV-infected cells at 6 h; C1, C2 and C3: DPV-infected cells at 12 h; D1, D2 and D3: DPV-infected cells at 24 h; E1, E2 and E3: DPV-infected cells at 36 h; F1, F2 and F3: DPV-infected cells at 48 h; G1, G2 and G3: DPV-infected cells at 72 h. After extensive washing, fixation and permeabilization, coverslip cultures were subjected to indirect immunofluorescence staining with rabbit anti-VP22 polyclonal antibody. Fluorescence microscopy was performed after incubation with FITC-conjugated secondary antibodies and staining of nuclear DNA with DAPI. Magnification 400x

fluorescence. VP22 found in infected cells is distributed in at least three distinct subcellular localizations which researchers define as cytoplasmic, diffuse and nuclear.

Transport activity of the recombinant VP22 protein: Results of immunofluorescence studies (Fig. 2) show that the VP22 recombinant protein can be detected in DEF after 1 h incubation in DMEM, exhibiting pronounced accumulation in the cytoplasm but failed migrates to and accumulates in the nucleus, virtually every cell in the culture exhibited bright cytoplasm fluorescence at 36 h. These results indicate that purified VP22 recombinant protein retains the import property.

Subcellular localization of VP22-GFP fusion protein in living and fixed DEF: The DPV VP22 derivatives was transfected into DEF cells as described in materials and methods and the subcellular localization pattern of the fusion protein were examined using a Nikon ECLIPSE 80i fluorescence microscope or a Nikon ECLIPSE 80i fluorescence microscope at 24, 36, 48 and 72 h post-transfection. Transfection of the parent vector, pEGFP-N1, resulted in a diffuse fluorescent pattern distributed throughout the cell. When VP22-GFP was transfected alone, VP22 firstly localized within the cytoplasm and a punctate speckled fluorescent pattern was observed then accumulated as helix-like pattern but the VP22a-GFP migrated into the nucleus and displayed a punctate nuclear pattern of fluorescence eventually. These patterns were observed at all subsequent time points (Fig. 3 and 4).

Transcription characteristics of DPV *UL49* gene: RNA was isolated from DPV-infected DEF cells at 1, 2, 4, 6, 8, 12, 24, 36, 48, 60, 72, 84 and 96 hpi using RNAiso Plus reagent then was reversed transcribed using PrimeScript® RT Enzyme, oligo dT primers and random hexamers. With gDNA Eraser, researchers can eliminate genomic DNA in just 2 min. After real time PCR amplification the Bio-Rad iCycler machine was programmed to do a melting curve in which the temperature is raised by a fraction of a degree

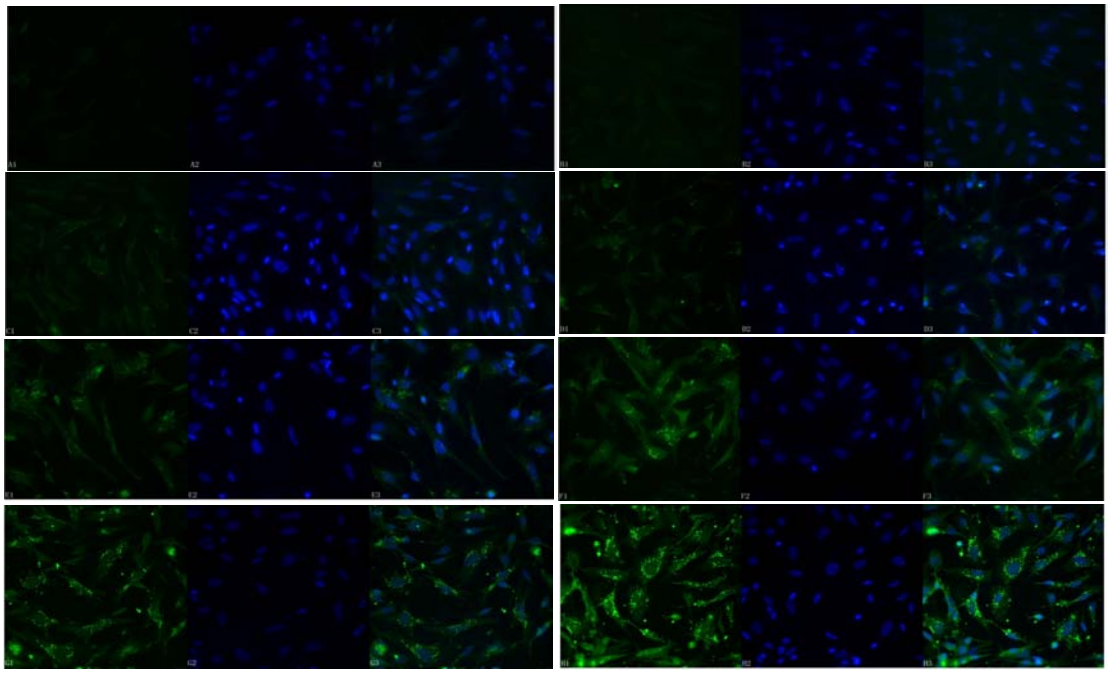

Fig. 2: Immunofluorescence of import of the recombinant VP22 protein after 200 ng application to tissue culture. The protein is taken up by the cell where it then localizes in the cytoplasm. A1, A2 and A3: normal DEF as negative control; B, C, D, E, F, G and H: 2, 4, 6, 8, 12, 24 and 36 h after VP22 protein was added in DMEM, respectively. After extensive washing, fixation and permeabilization, coverslip cultures were subjected to indirect immunofluorescence staining with rabbit anti-VP22 polyclonal antibody. Fluorescence microscopy was performed after incubation with FITC-conjugated secondary antibodies and staining of nuclear DNA with DAPI. Magnification 400x

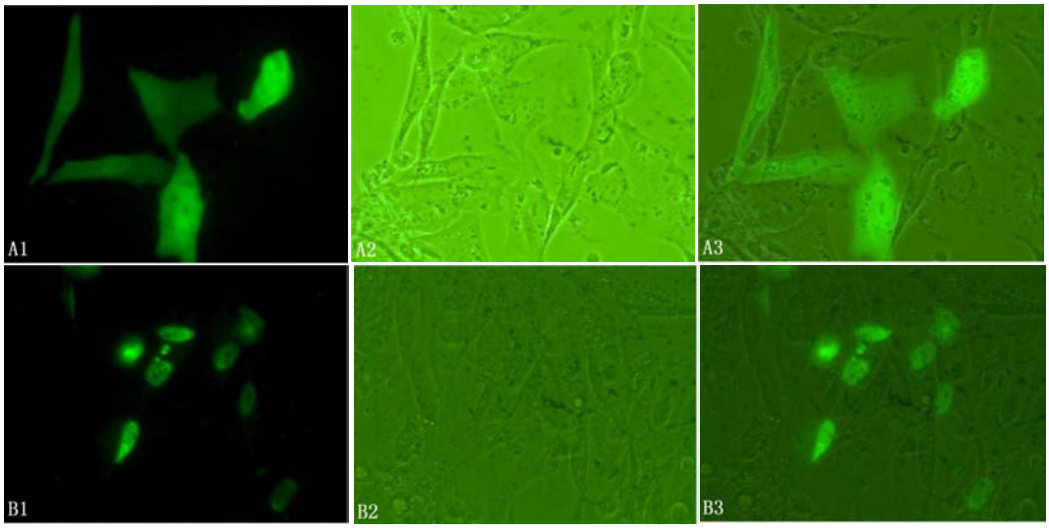

Fig. 3: Subcellular localization of VP22-GFP fusion protein in living DEF. Cells were seeded in 6 well plates at a density of 2×10^5 cells per well 24 h prior to transfection. Transfections were performed using Lipofectamine 2000 reagent (invitrogen) as recommended by the manufacturer. The cells were observed using a Nikon Eclipse TE2000-U Inverted Microscope at 36 h post-transfection. A1, A2, A3: EGFP-N1; B1, B2, B3: VP22-GFP. A3 and B3 are the merged images of A1 and A2, B1 and B2, respectively. Magnification 600x

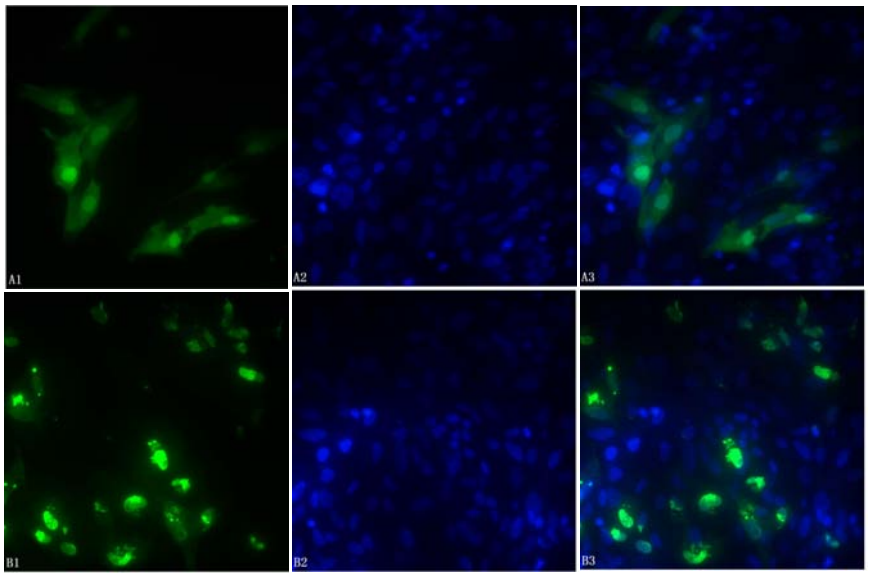

Fig. 4: Subcellular localization of VP22-GFP fusion protein in fixed DEF. Cells were seeded in 6 well plates on glass coverslips at a density of 2×10^5 cells per well 24 h prior to transfection. Monolayers of DEF grown on coverslips were transfectd with EGFP-N1 (A1, A2, A3), VP22-GFP (B1, B2, B3) using Lipofectamine 2000 reagent (invitrogen) as recommended by the manufacturer. At 36 h post-transfection, coverslips were washed, fixed, permeabilized, counterstained and observed using a Nikon ECLIPSE 80i fluorescence microscope. A3 and B3 are the merged images of A1 and A2, B1 and B2, respectively. Magnification 600x

and the change in fluorescence is measured. A melting curve was constructed for each primer pair to verify the presence of one gene-specific peak and the absence of primer dimmer. The Bio-Rad iCycler and iQ5 2.1 Standard Edition Optical System Software plots the rate of change of the Relative Fluorescence Units (RFU) with Time (T) (-d (RFU)/dT) on the Y-axis versus the temperature on the X-axis and this will peak at the melting temperature (Tm) (Fig. 5). Products of real time PCR amplification of the *DPV UL49* gene and *β-actin* gene were subjected to electrophoresis in a 2.0 g L^{-1} agarose gel (Fig. 6). As shown in Fig. 7, DPV UL49 mRNA can be detected as early as 1 h post-infection (hpi) the relative expression level was at a low level in the first 12 hpi then reached a peak at 84 hpi and then declined.

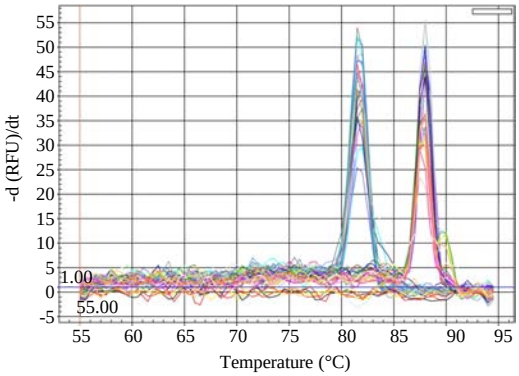

Fig. 5: Melting curve analysis of the UL49 and β-actin. The Bio-Rad iCycler and iQ5 2.1 Standard Edition Optical System Software plots the rate of change of the Relative Fluorescence Units (RFU) with Time (T) (-d(RFU)/dT) on the Y-axis versus the temperature on the X-axis and this will peak at the melting Temperature (Tm). The left peak represents the melting curve of *UL49* gene while the right peak represents the melting curve of *β-actin* gene

VP22, encoded by the *UL49* gene of Duck Plague Virus (DPV), its role is not yet known (Li *et al*., 2009). To study its subcellular localization the studies have focused on analyzing the localization of VP22 in DPV infected DEF the recombinant VP22 protein and VP22-GFP fusion protein. The results showed that VP22 in DPV infected DEF and VP22-GFP fusion protein exist in the cytoplasm early and migrates to and accumulates in the nucleus of DEF even at late stage, this finding may imply that VP22 can also localizes to the nucleus of DEF in the absence of other viral proteins. These studies have suggested dynamic trafficking properties of VP22 where the protein is capable of both cytoplasmic and nuclear accumulation. This parallels previous results on HSV-1 VP22 (Pomeranz and Blaho, 1999). Lundberg and Johansson

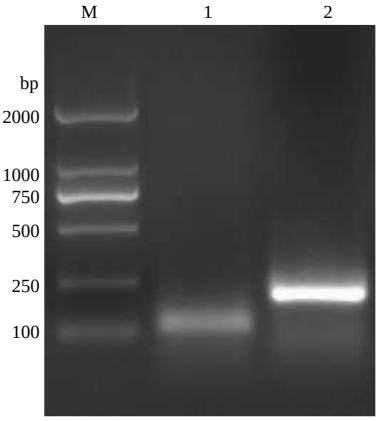

Fig. 6: Products of real time PCR amplification of the *UL49* and *β-actin* gene. Lane M: DL2000 DNA Marker; Lane 1: Products of amplification of a 116 bp fragment from the DPV *UL49* gene; Lane 2: Products of amplification of a 215 bp fragment from the duck *β-actin* gene

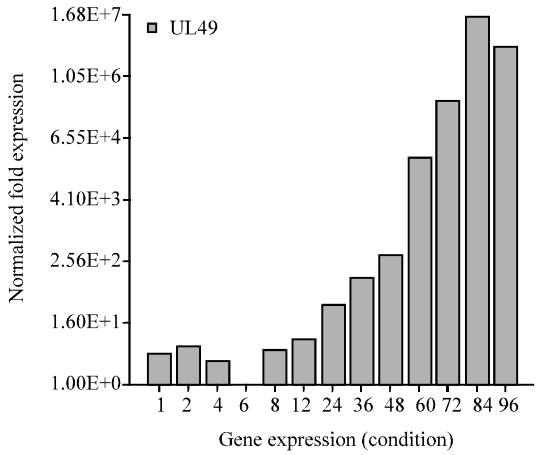

Fig. 7: Transcription characteristic of *UL49* gene from DPV-infected DEF cells. Quantitative PCR was performed on a Bio-Rad iCycler and iQ5 2.1 Standard Edition Optical System Software. Data were analyzed by using the Delta-Delta Ct Method. UL49 mRNA can be detected as early as 1 hpi

(2001) thought methanol fixation lead to the artificial import and nuclear localization of VP22, they expressed a fusion protein of HSV VP22 and GFP in *E. coli* but no fluorescence was detected in the nucleus even cells were incubated with purified VP22-GFP at 37°C for up to 24 h the cells exhibited nuclear GFP fluorescence only after methanol fixation and rehydration in PBS. However, researchers analysed the subcellular localization of the VP22-GFP both in living and fixed cells the study strongly supports import and nuclear localization of VP22 in living cells.

CONCLUSION

The recombinant VP22 protein only exists in the cytoplasm of DEF, virtually every cell in the culture incubated with VP22 exhibited bright cytoplasm fluorescence, this findingmay implythat VP22 may possess unusual trafficking properties. Furthermore the recombinant VP22 protein failed migrate to and accumulates in the nucleus of DEF may correlate with expression host. Kueltzo *et al*. (2000) studied the import of an N-terminal truncated VP22 protein, containing amino acids 159-301 that can be expressed in *Escherichia coli* and retain the ability of cellular import. Many studies showed that the herpesvirus VP22 homologs can be used as transport tool in protein transduction and the unusual capacity for intercellular trafficking can be observed not only in fixed cells but also in living cells (Elliott and O'Hare 1999; Phelan *et al*., 1998; Kalthoff *et al*., 2008; Zavaglia *et al*., 2003). Thus, its application is increasing in gene therapy and immunization.

In the attempt to elucidate transcription characteristics of DPV *UL49* gene, a reliable RTFQ-PCR protocol for transcriptional analysis was established. RTFQ-PCRis currently the most reliable method of quantifying transcripts. Researchers demonstrated that in DPV-infected DEF cells, UL49 mRNA can be detected as early as 1 hpi, peaked at 84 hpi and then declined. Researchers first established and validated a RTFQ-PCR Method which enables the early detection of DPV the method may beusedto diagnose DPV in future.

ACKNOWLEDGEMENT

The research was supported by grants from Changjiang Scholars and Innovative Research Team in University (PCSIRT0848).

REFERENCES

Aints, A., H. Guven, G. Gahrton, C.I. Smith and M.S. Dilber, 2001. Mapping of herpes simplex virus-1 VP22 functional domains for inter- and subcellular protein targeting. Gene. Ther., 8: 1051-1056.

Cheng, A.C., M.S. Wang, M. Wen, W.G. Zhou and Y.F. Guo *et al*., 2006. Construction of duck enteritis virus gene libraries and discovery, cloning and identification of viral nucleocapsid protein gene. High Technol. Lett., 16: 948-953.

Davison, S., K.A. Converse, A.N. Hamir and R.J. Eckroade, 1993. Duck viral enteritis in domestic muscovy ducks in Pennsylvania. Avian Dis., 37: 1142-1146.

Elliott, G. and P. O'Hare, 1997. Intercellular trafficking and protein delivery by a herpesvirus structural protein. Cell, 88: 223-233.

Elliott, G. and P. O'Hare, 1998. Herpes simplex virus type 1 tegument protein VP22 induces the stabilization and hyperacetylation of microtubules. J. Virol., 72: 6448-6455.

Elliott, G. and P. O'Hare, 1999. Intercellular trafficking of VP22-GFP fusion proteins. Gene. Ther., 6: 149-151.

Elliott, G. and P. O'Hare, 2000. Cytoplasm-to-nucleus translocation of a herpesvirus tegument protein during cell division. J. Virol., 74: 2131-2141.

Elliott, G.D. and D.M. Meredith, 1992. The herpes simplex virus type 1 tegument protein VP22 is encoded by gene UL49. J. Gen. Virol., 73: 723-726.

Gough, R.E. and D.J. Alexander, 1987. Duck virus enteritis in waterfowl. Vet. Rec., 121: 72-72.

Guo, Y., C. Shen, A. Cheng, M. Wang, N. Zhang, S. Chen and Y. Zhou, 2009. Anatid herpesvirus 1 CH virulent strain induces syncytium and apoptosis in duck embryo fibroblast cultures. Vet. Microbiol., 138: 258-265.

Heine, J.W., R.W. Honess, E. Cassai and B. Roizman, 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J. Virol., 14: 640-651.

Ingvarsdottir, K. and J.A. Blaho, 2010. Association of the herpes simplex virus major tegument structural protein VP22 with chromatin. Biochim. Biophys. Acta-Gene Regul. Mech., 1799: 200-206.

Jiang, L., A. Cheng, M. Wang, D. Zhu and R. Jia, 2010. Characterization of duck plague virus tegument protein vp22 and molecular evolution of herpesvirinae. 2010 3rd Int. Conf. Comp. Elect. Eng., 5: 38-42.

Kalthoff, D., H. Granzow, S. Trapp and M. Beer, 2008. The UL49 gene product of BoHV-1: A major factor in efficient cell-to-cell spread. J. Gen. Virol., 89: 2269-2274.

Kelly, B.J., C. Fraefel, A.L. Cunningham and R.J. Diefenbach, 2009. Functional roles of the tegument proteins of herpes simplex virus type 1. Virus. Res., 145: 173-186.

Keymer, I.F. and R.E. Gough, 1986. Duck virus enteritis (anatid herpesvirus infection) in mute swans (*Cygnus olor*). Avian Pathol., 15: 161-170.

Kisary, J. and L. Zsak, 1983. Comparative studies on Duck Viral Enteritis (DVE) virus strains in geese. Avian Pathol., 12: 395-408.

Kotsakis, A., L.E. Pomeranz, A. Blouin and J.A. Blaho, 2001. Microtubule reorganization during herpes simplex virus type 1 infection facilitates the nuclear localization of VP22, a major virion tegument protein. J. Virol., 75: 8697-8711.

Kueltzo, L.A., N. Normand, P. O'Hare and C.R. Middaugh, 2000. Conformational lability of herpesvirus protein VP22. J. Biol. Chem., 275: 33213-33221.

Lee, K.C., D.A. Hamstra, S. Bullarayasamudram, M.S. Bhojani and B.A. Moffat *et al*., 2006. Fusion of the HSV-1 tegument protein vp22 to cytosine deaminase confers enhanced bystander effect and increased therapeutic benefit. Gen. Ther., 13: 127-137.

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

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using Real-time quantitative PCR and the 2 (delta delta C (T) method. Methods, 25: 402-408.

Lundberg, M. and M. Johansson, 2001. Is VP22 nuclear homing an artifact?. Nat. Biotechnol., 19: 713-714.

Phelan, A., G. Elliott and P. O'Hare, 1998. Intercellular delivery of functional p53 by the herpesvirus protein VP22. Nat. Biotechnol., 16: 440-443.

Pinard, M.F., R. Simard and V. Bibor-Hardy, 1987. DNA-binding proteins of herpes simplex virus type 1-infected BHK cell nuclear matrices. J. Gen. Virol., 68: 727-735.

Pomeranz, L.E. and J.A. Blaho, 1999. Modified VP22 localizes to the cell nucleus during synchronized herpes simplex virus type 1 infection. J. Virol., 73: 6769-6781.

Ren, X., J.S. Harms and G.A. Splitter, 2001. Bovine herpesvirus 1 tegument protein VP22 interacts with histones and the carboxyl terminus of VP22 is required for nuclear localization. J. Virol., 75: 8251-8258.

Saha, S., S. Yoshida, K. Ohba, K. Matsui and T. Matsuda *et al*., 2006. A fused gene of nucleoprotein (NP) and herpes simplex virus genes (VP22) induces highly protective immunity against different subtypes of influenza virus. Virology, 354: 48-57.

Sciortino, M.T., B. Taddeo, A.P. Poon, A. Mastino and B. Roizman, 2002. Of the three tegument proteins that package mRNA in herpes simplex virions, one (VP22) transports the mRNA to uninfected cells for expression prior to viral infection. Proc. Natl. Acad. Sci. USA, 99: 8318-8323.

Wobeser, G., 1987. Experimental duck plague in blue-winged teal and Canada geese. J. Wildl Dis., 23: 368-375.

Yedowitz, J.C., A. Kotsakis, E.F. Schlegel and J.A. Blaho, 2005. Nuclear localizations of the herpes simplex virus type 1 tegument proteins VP13/14, vhs and VP16 precede VP22-dependent microtubule reorganization and VP22 nuclear import. J. Virol., 79: 4730-4743.

Zavaglia, D., M.C. Favrot, B. Eymin, C. Tenaud and J.L. Coll, 2003. Intercellular trafficking and enhanced *in vivo* antitumour activity of a non-virally delivered P27-VP22 fusion protein. Gene. Ther., 10: 314-325.

Zhang, C., A. Qin, H. Chen, X. Deng, Y. Su and K. Qian, 2009. MDV-1 VP22: A transporter that can selectively deliver proteins into cells. Arch. Virol., 154: 1027-1034.

Zheng, C., L.A. Babiuk and S.D.L. van den Hurk, 2005. Bovine herpesvirus 1 VP22 enhances the efficacy of a DNA vaccine in cattle. J. Virol., 79: 1948-1953.

Zheng, C.F., R. Brownlie, D.Y. Huang, L.A. Babiuk and S. van Drunen Littel-van den Hurk, 2006. Intercellular trafficking of the major tegument protein VP22 of bovine herpesvirus-1 and its application to improve a DNA vaccine. Arch. Virol., 151: 985-993.