Journal of Animal and Veterinary Advances 11 (9): 1512-1517, 2012
ISSN: 1680-5593

Immunogenicity of Foot and Mouth Disease Virus Type Asia 1 Protein VP1-2A Fused with a Multi-Epitope Expressed in *Pichia pastoris*

[1, 2, 3]Zhan-Bo Zhu, [3]Ying Xi, [1, 2]Bo Hu, [2]Chang Li, [2]Huijun Lu, [2]Kuoshi Jin,
[2]Jingqiang Ren, [1, 2, 3]Cunxia Liu, [2]Minglan Jin and [1, 2]Ningyi Jin
[1]College of Animal Science and Veterinary Medicine, Jilin University, 130062 Changchun, China
[2]Genetic Engineering Laboratory of PLA,
Academy of Military Medical Sciences of PLA, 130062 Changchun, China
[3]College of Animal Science and Veterinary Medicine,
HeiLongjiang Bayi Agricultural University, 163319 Daqing, China

Abstract: Foot and Mouth Disease (FMD) is a highly infectious and economically devastating disease of livestock. A genetically engineered vaccine against Foot and Mouth Disease Virus (FMDV) may be a good control measure for use during an epidemic. In this study, a secretory expression vector, pPIC9K-VP1-2A-CTE, containing a fusion of the bovine (FMDV) type Asia 1 *VP1-2A* gene and a multi-epitope CTB-TEpi (CTE) was constructed. After being linearized with Sal I, the recombinant plasmid was transformed into *Pichia pastoris* GS115 by electroporation. The fusion protein expressed in yeast was analyzed by SDS-PAGE and Western blot and immunogenicity of the fusion protein was assessed. The expressed recombinant VP1-2A and CTE protein elicited specific humoral and cellular immune responses in mice. Additionally, immunized guinea pigs were protected when challenged with the FMD Asia 1 virus.

Key words: FMDV, VP1-2A, CTE, *Pichia pastoris*, immunogenicity, China

INTRODUCTION

FMDV is a member of the family Picornaviridae and genus *Apthovirus*. The FMDV virion is composed of a single RNA strand and a protein capsid that is assembled from 60 units of four structural proteins known as VP1-VP4 (Sobrino *et al*., 2001). The predominantly exposed surface protein is VP1 which can induce neutralizing antibody (Wang *et al*., 2003; Shi *et al*., 2006). Additionally, serotype Asia 1 has three antigenic determinants on the G-H loop of VP1 (Fowler *et al*., 2010). A non-structural protein coded by the FMDV ORF, a cis-cleaving element can self-catalyze the separation of P1-2A and 2C. Hence, researchers can prepare a polyvalent vaccine or multi-vaccine by expressing major antigen genes simultaneously from the same or different viruses using the *2A* gene. Accordingly, the *FMDV VP1* gene and the self-cleaving element (VP1-2A) were selected as the major immunogens.

The non-toxic B subunit of Cholera Toxin (CTB) can specifically bind Monosialotetrahexosylganglioside (GM1) in karyocyte membranes and induce membrane configurational transformation. As a result, CTB can enter the cell and induce an immune reaction (Holmgren *et al*., 1993; Yamamoto *et al*., 2001). Thus, CTB is a desirable vector and preferable immunologic adjuvant for a vaccine. Cholera Toxin B subunit (CTB) is highly immunogenic when delivered mucosally and can act as a carrier to stimulate responses to linked antigens (Song *et al*., 2005). In one study, the fusion gene *CTB-VP1-2A-Epi* from FMDV O multi-epitopes was cloned into the pBC38C vector and transformed into *Bacillus subtilis*. *B. subtilis* expressed the recombinant protein which can induce cellular and mucous membrane immune responses (Holmgren *et al*., 1993).

Hu *et al*. (2011) reported a multi-epitope CTB-TEpiAs containing both the B subunit of the Cholera Toxin (CTB) and the TEpiAs epitope box. The epitope box derived from FMDV type Asia 1 contains epitopes of the structural protein VP1 which are antigen sites on VP1 (residues 40-60, 133-160 and 200-213) that were found via monoclonal antibody escape mutant studies and Th2 epitopes from VP4 and 3ABC.

In this study, we expressed the CTB and TEpi epitope box as an immunologic adjuvant in the *Pichia pastoris* expression system. The combination of the *VP1-2A* gene and the multi-epitope antigen box (referred to as CTB-TEpi or CTE) can enhance immunogenicity and has implications for the development of a novel FMD vaccine.

Corresponding Author: Ningyi Jin, College of Animal Science and Veterinary Medicine, Jilin University, 130062 Changchun, China

MATERIALS AND METHODS

Vector inactivated vaccines and animal: Cloning vectors pSK, pMD18-T-P1-2A-3C (serotype Asia 1) and pMD18-T-CTE (serotype Asia 1) were created and stored in the viral laboratory of the Academy of Military Medical Sciences of PLA. FMDV serotype O and Asia 1 bivalent inactivated oil adjuvant vaccine was purchased from the Lanzhou Biological Pharmaceutical Factory of China Animal Husbandry Industry Co., Ltd. Guinea pigs, each weighing between 400 and 500 g, female BALB/c mice that are 6-8 weeks old were purchased from Changchun Institute of Biological Products.

Plasmid construct: PMD18-T-P1-2A-3C amplified by the primer pairs shown previously and pMD18-T-CTE digested by Spe I/BamH I were used as sources for VP1-2A and CTE sequence for the expression vector, respectively. The two fragments were cloned into T vector and confirmed by sequencing and subcloning into pSK in the proper order. The filled vector pPIC9K was digested by EcoR I and Not I and ligated with the insert released from pSK with VP1-2A-CTE. The clone with the insert and frame was named pPIC9K-VP1-2A-CTE.

Expression of fusion proteins in *Pichia pastoris* GSll5 cell: Two plasmids, pPIC9K-VP1-2A-CET and pPIC9K were transformed into *Pichia pastoris* GS115 competent cells after being digested with Sal I. The cells were cultured in 50 mL BMGY at 28°C until OD_{600} reached 2.0-6.0. The pellets were resuspended in 100 mL BMMY after centrifugation and expression was induced at 28°C for a total 48 h with 1% methanol being added after 24 h of induction. The cells were then lysed by sonication on ice followed by centrifugation. The supernatant of interest was dialyzed and used for SDS-PAGE.

Western blot analysis: The cell lysate was added to 5× bromophenol blue loading buffer, boiled for 5 min and then subjected to SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane that was blocked with 5% milk protein in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) for 1 h at 37°C. The membrane was incubated with a 1:500 dilution of rabbit-anti-Asia 1 FMDV serum for 2 h at 37°C and washed in TBST. The washed membrane was incubated with HRP-conjugated goat-anti-rabbit IgG antibody in a blocking solution as described previously washed and developed using 10 mL NBT/BCIP for 5 min.

Immunization of mice: Female BALB/c mice aged 6-8 weeks old were divided into 5 groups (10 mice/group) and immunized with 30 μg of each of the following purified proteins: GS115/pPIC9K-VP1-2A-CTE, GS115/pPIC9K-CTE and GS115/pPIC9K. The remaining groups were immunized with 100 μL of FMDV serotype O and Asia 1 bivalent inactivated, oil adjuvant vaccine with PBS as the negative control. The mice were immunized three times after day 0 at 21 days intervals. The injected mixture was mixed with complete Freund's adjuvant at the first immunization and with incomplete Freund's adjuvant at the second and third immunization. Blood was collected from the tail vein of the mice and serum isolated from the blood samples was used for ELISA analysis. The mice were sacrificed 10 days after the last immunization (on day 52) and their lymphocyte were isolated for lymphopoiesis analysis and ELISPOT assay. All animals were treated under the international standards for animal welfare and the experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Chinese Academy of Military Medical Science, Changchun, China.

ELISA assay: FMDV Asia 1 antibodies in mouse serum were detected by ELISA using 96 well, flat-bottomed plates coated overnight at 4°C with the FMDV 1 MOI strain in 0.1 M carbonate/biocarbonate buffer (pH 9.6). After blocking with 5% Skim Milk Powder (SMP) diluted in PBS for 1 h at 37°C, the plates were washed with PBST three times and then incubated with a 1:50 dilution of the test sera in duplicate wells for one to one and a half hour at 37°C. Goat-anti-rabbit IgG peroxidase conjugate was added to the wells at a 1:1000 dilution and incubated for 1 h at 37°C. The plates were washed for 5 min with PBST three times. There is a substrate (orthophenylenediamine) added to the wells to elicit the color change reaction. The reaction was terminated by adding 2M H_2SO_4 and was read at 490 nm.

Lymphocyte stimulation test: Splenic lymphocytes from mice were isolated 10 days after the final immunization. Cells were plated in 96 well plates containing ConA (final concentration of 10 μg mL^{-1}) and inactivated FMDV (1 MOI) with no stimulus and experiments were carried out in triplicate. After incubation for 48 h at 37°C, 10 μL of WST was added to each well and absorbance was determined at 450 nm. The results were expressed as Stimulation Indices (SI) which is the ratio of the mean Count Per Minute (CPM) of the stimulated culture and the mean CPM of the control culture.

ELISPOT assay: The ELISPOT assay was executed according to the IFN-γ ELISPOT kit (Dakewe Biotech Co. Limited). Each group was represented by three stimulated wells and three control wells. About 50 μL of FMDV (1 MOI) was added to total volume 100 μL in the stimulated wells with 1×10^5 cells per well.

Cytokine detection: A cytokine ELISA detection kit (MABTECH AB, Sweden) that detects IL-2, IL-4 and IL-10 was used to evaluate serum from the immunized mice. The control group was tested using this kit as well and a standard curve was created.

Animal protection assay: About 4 groups consisting of five guinea pigs each were administered the following treatments: Group 1 (pPIC9K-VP12A-CTE), Group 2 (PIC9K-CTE), Group 3 (FMDV oil-vaccine at 1/10th of the cattle dose) and Group 4 (unvaccinated). Guinea pigs were immunized with prepared vaccines intramuscularly with 300 μg protein per guinea pig followed by a booster on day 21. About 35 days post-immunization, guinea pigs were challenged with 0.2 mL 250 Guinea Pig 50% Infective Dose ($GPID_{50}$) FMDV type Asia 1 by intraplantary inoculation in the left, back footpad. The animals were kept in biosecure isolated hutches and observed for the appearance of primary (at the site of inoculation) and secondary lesions (on the other foot pads) for 7 days post challenge. Lesions appearing on the left, back footpad were suggestive of partial infection whereas lesions on both back footpads were indicative of complete infection.

Statistical analysis: To determine whether immune responses among animal groups were significantly different, Student's t-test analysis was performed using Microsoft Excel Software and SPSS Version 10.0.

RESULTS AND DISCUSSION

Expression analysis of recombinant protein in *Pichia pastoris* GS115: Expression and immune response to the VP1 protein has been extensively studied (Shi *et al*., 2006; Su *et al*., 2007) though not all genes of interest are suited for the *Pichia pastoris* Expression System. Because of singal peptide catalyzed sites of α factor, Glu-Lys-Arg*Glu- Ala*Glu-Ala, existence of vector pPIC9K so, the expressed gene cannot contain any of these sites. In addition, proteins of interest cannot contain a PEST (Pro-Glu-Ser-Thr) sequence because this sequence is a substrate for Ca^{2+} activated proteolytic enzymes (Liu *et al*., 2005). Because different codons code for the same amino acid in *Pichia pastoris* in order to increase expression efficiency, rare codons are avoided to used for exogenous gene as far as possible. We found no limiting sequences or rare codons in the gene when analyzing the *VP1-2A-CTE* gene sequence; therefore the gene is compatible with the *Pichia pastoris* system. The positive clones used for inducing recombinant protein expression were confirmed by PCR using universal primers 5'-AOX1 and 3'-AOX1 from pPIC9K. The supernatant of the recombinant strain GS115/pPIC9K-VP1-2A-CTE lysate which was induced by methanol was analyzed by SDS-PAGE. The initial fusion protein was catalyzed into a 41.8 kDa CTE protein and a 26.5 kDa VP1-2A because of the self-catalyzing function of the 2A protein. The amount of the proteins of interest, VP1-2A and CTE was 10.7% of the total protein as shown by lamellar scanning of SDS-PAGE. Western blotting was used to identify the specific fusion protein in the lysate and was performed with anti-FMDV specific serum. Western blot results showed that the fusion protein demonstrates good immunogenicity (Fig. 1). The results of this study establish a foundation for further animal experiments and because the protein of interest is secreted into the culture supernatant and the isolation process is simple this study provides conditions for a larger scale operation.

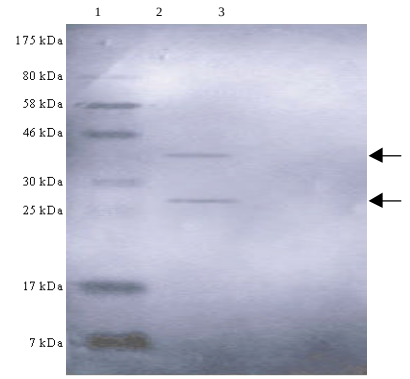

Fig. 1: Western blotting analysis of the target protein with anti-FMDV specific serum. Lane 1: protein molecular-mass markers. Lane 2: supernatant of the recombinant strain GS115/pPIC9K-VP1-2A-CTE. Lane 3: supernatant of control strains GS115/pPIC9K. The arrow indicate the expressed VP1-2A protein (about 26.5 kDa) and CTE protein (about 41.8 kDa)

Induction of humoral immune response: Immunogen, a mixture of purified VP1-2A, CTE protein and Freund's adjuvant can induce humoral and cellular immune responses as detected by indirect ELISA, MTT and ELISPOT in immunized mice. Antibodies were detectable 1 week after inoculation in mice immunized with GS115/pPIC9K-VP1-2A-CTE. The antibody levels steadily increased and peaked 2 weeks after the second immunization. There was a slight decline in antibody levels after the second immunization. The antibody levels in the GS115/pPIC9K-CTE group were higher compared to the PBS control and the GS115/pPIC9K negative group.

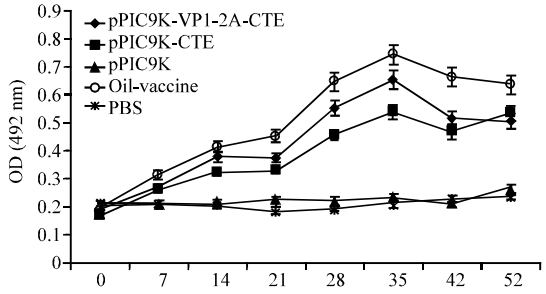

Fig. 2: Antibody titers to foot and mouth disease virus in mice after vaccination with different vaccines. Antibody titers were evaluated at eight points by Enzyme-Linked Immunosorbent Assay (ELISA). Blood samples were collected on days 7, 14, 21, 28, 35, 42 and 52. Mean antibody titers in mice immunized with PIC9K-VP1-2A-CTE and pPIC9K-CTE and FMDV oil-vaccine were significantly higher ($p<0.01$) than those immunized simply with pPIC9K and PBS

These results showed that fused proteins VP1-2A and CTE from Asia 1 stimulated the mice to produce specific antibodies (Fig. 2).

Induction of Cellular immune response in mice

T lymphocyte proliferation response in mice: T lymphocyte proliferation response and spleen T lymphocyte IFN-γ secretory responses were used to evaluate the FMDV-specific T cell response in immunized mice. Lymphocyte proliferation responses were evaluated in all tested groups by stimulation with Asia 1 FMDV (Fig. 3). SI indices were highest in the pPIC9K-VP1-2A-CTE vaccine group and the pPIC9K-CTE protein group and high in the group given the commercial vaccine. These groups had a remarkable advantage over the pPIC9K and PBS groups.

Specific IFN-γ responses in mice: Immune responses mediated by CD4+ are necessary for inducing antibodies against FMDV. CD4+ cells are the main source of IFN-γ production also. Recently, a positive correlation between IFN-γ production and vaccine-induced clinical protection has been demonstrated in cattle on the day of challenge (Oh *et al*., 2006). IFN-γ has antiviral activity against FMDV (Moraes *et al*., 2007; Summerfield *et al*., 2009; Zhang *et al*., 2002) and also promotes natural killer and macrophage activation which are likely to contribute to controlling FMDV replication and spread within the host (Summerfield *et al*., 2009). In this study, all immunized groups had spotting as detected in mouse splenic lymphocytes using the ELISPOT kit. There were more

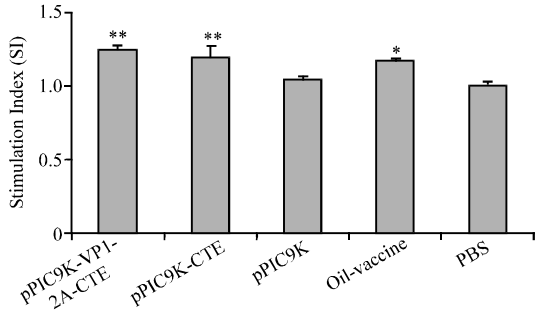

Fig. 3: The results of the mouse lymphocyte proliferation assay (Stimulation Index SI). The bars represent the proliferation response of splenic T lymphocytes stimulated with a live virus at day 52. The proliferation responses were expressed as Stimulation Index (SI) values from the mean±SD. *Compared with the pPIC9K and PBS groups, $p<0.01$. **Compared with the commercial vaccine group, pPIC9K and PBS, $p<0.01$

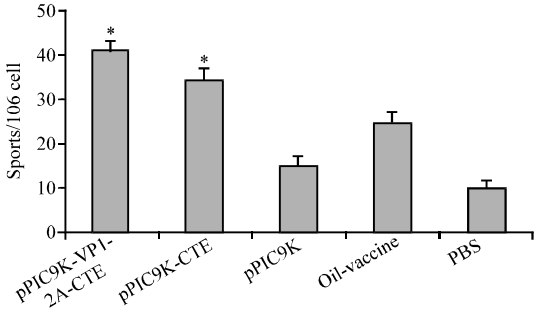

Fig. 4: Specific IFN-γ responses in vaccinated mice. On day 52, antigen-specific IFN-γ responses of mice were tested using IFN-γ ELISPOT with a live virus. The number of spots per 10^6 T lymphocytes are shown by the bars and presented as mean±SD. *Compared with the commercial vaccine, GS115/pPIC9K and PBS groups, $p<0.01$

spots with the GS115/pPIC9K-VP1-2A-CTE and GS115/pPIC9K-CTE immunization groups than with the commercial vaccine group. Additionally, there were more spots in these groups than with the PBS control and GS115/pPIC9K groups (Fig. 4).

Serum cytokine analysis of immunized mice: The profiles of cytokines in serum from mice immunized were examined by ELISA kit to further evaluate the protective mechanism. We detected cytokines in mouse serum 10 days after the last immunization. These results show that the concentration of cytokines from the two protein

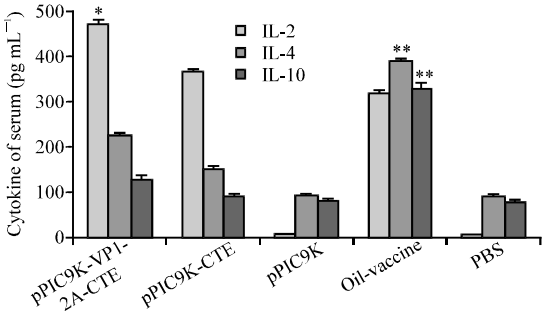

Fig. 5: Serum cytokine analysis of immunized mice. Concentrations of IL-2, IL-4 and IL-10 of mice are shown by the bars and presented as mean±SD. *Compared with IL-2 concentrations in the commercial vaccine, pPIC9K-CTE, pPIC9K and PBS groups, $p<0.01$. **Compared with IL-4 and IL-10 concentrations in the pPIC9K-VP12A-CTE, pPIC9K-CTE, pPIC9K and PBS groups, $p<0.01$

immunized groups were higher than the concentrations found in the PBS and GS115/pPIC9K control group and were lower than those found in the inactivated vaccine control group. Of these cytokines, the IL-2 levels in the pPIC9K-VP1-2A-CTE group are much higher than in the pPIC9K-CTE, pPIC9K, FMDV oil-vaccine and PBS groups. The IL-4 and IL-10 levels in the FMDV inactivated vaccine group are much higher than in the other groups (Fig. 5). Because the IL-2 and IFN-γ responses of splenocytes are associated with Th-1 responses, the findings indicate that the FMDV recombinant protein induced a cell-mediated response in mice.

Animal protection assay results: Guinea pigs were challenged with a virulent, guinea pig adopted, FMDV Asia 1/JL/05. The animals were observed for the appearance of lesions on the footpads for up to 10 days. All guinea pigs vaccinated with the commercial vaccine were protected completely from the homologous viral challenge (5/5) whereas 60% (3/5) of animals were protected in the pPIC9K-VP1-2A-CTE vaccine group and 20% (1/5) were protected in the pPIC9K-CTE group. All of the unvaccinated animals that were challenged developed lesions. The rate of protection was expressed as the number of guinea pigs with no lesions on either rear footpads/total number of guinea pigs.

CONCLUSION

In this study, using the Yeast Expression System, the VP1-2A and CTE protein was expressed in soluble forms. The expressed recombinant protein could elicit specific humoral and cellular immune responses in mice. To the knowledge this study is the first report of VP1-2A and CTE expression in the pPIC9K vector which could potentially be used for vaccination against FMDV.

ACKNOWLEDGEMENTS

This research was supported by grants from the National 863 Project of China (Grant No.: 2006AA10A204). The researchers thank P. Li and C.M. Tang for the assistance of cell culture and reagent preparation.

REFERENCES

Fowler, V.L., N.J. Knowles, D.J. Paton and P.V. Barnett, 2010. Marker vaccine potential of a foot-and-mouth disease virus with a partial VP1 G-H loop deletion. Vaccine, 28: 3428-3434.

Holmgren, J., N. Lycke and C. Czerkinsky, 1993. Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems. Vaccine, 11: 1179-1184.

Hu, B., C. Li, H. Lu, Z. Zhu and S. Du *et al.*, 2011. Immune responses to the oral administration of recombinant *Bacillus subtilis* expressing multi-epitopes of foot-and-mouth disease virus and a cholera toxin B subunit. J. Virol. Methods, 171: 272-279.

Liu, Z.M., H.L. Zhao, C. Xue, B.B. Deng and W. Zhang *et al.*, 2005. Secretory expression and characterization of a recombinant-deleted variant of human hepatocyte growth factor in *Pichia pastoris*. World J. Gastroenterol., 11: 7097-7103.

Moraes, M.P., T. de los Santos, M. Koster, T. Turecek, H. Wang, V.G. Andreyev and M.J. Grubman, 2007. Enhanced antiviral activity against foot-and-mouth disease virus by a combination of type I and II porcine interferons. J. Virol., 81: 7124-7135.

Shi, X.J., B. Wang, C. Zhang and M. Wang, 2006. Expressions of bovine IFN-gamma and foot-and-mouth disease VP1 antigen in *P. pastoris* and their effects on mouse immune response to FMD antigens. Vaccine, 24: 82-89.

Sobrino, F., M. Saiz, M.A. Jimenez-Clavero, J.I. Nunez, M.F. Rosas, E. Baranowski and V. Ley, 2001. Foot-and-mouth disease virus: A long known virus, but a current threat. Vet. Res., 32: 1-30.

Song, H., Z. Wang, D. Zheng, W. Fang and Y. Li *et al.*, 2005. A novel mucosal vaccine against foot-and-mouth disease virus induces protection in mice and swine. Biotechnol Lett., 27: 1669-1674.

Su, C., X. Duan, X. Wang, C. Wang, R. Cao, B. Zhou and P. Chen, 2007. Heterologous expression of FMDV immunodominant epitopes and HSP70 in *P. pastoris* and the subsequent immune response in mice. Vet. Microbiol., 124: 256-263.

Summerfield, A., L. Guzylack-Pirioua, L. Harwooda and K.C. McCullough, 2009. Innate immune responses against foot-and-mouth disease virus: current understanding and future directions. Vet. Immunol. Immunopathol., 128: 205-210.

Wang, J.H., C.M. Liang, J.M. Peng, J.J. Shieh and M.H. Jong *et al*., 2003. Induction of immunity in swine by purified recombinant VP1 of foot-and-mouth disease virus. Vaccine, 21: 3721-3729.

Yamamoto, M., J.R. McGhee, Y. Hagiwara, S. Otake and H. Kiyono, 2001. Genetically manipulated bacterial toxin as a new generation mucosal adjuvant. Scand. J. Immunol., 53: 211-217.

Zhang, Z.D., G. Hutching, P. Kitching and S. Alexandersen, 2002. The effects of gamma interferon on replication of foot-and-mouth disease virus in persistently infected bovine cells. Arch. Virol., 147: 2157-2167.