Acta virologica **57**: 429 – 434, 2013 doi:10.4149/av_2013_04_429

Expression of bovine Mx1 protein inhibits the replication of foot-and-mouth disease virus in BHK-21 cells

K. J. CAI[1,3], Q. L. MENG[1], J. QIAO[1*], J. HUANG[2], Z. C. ZHANG[1], G. C. WANG[1], J. W. WANG[1], C. F. CHEN[1]

[1]College of Animal Science and Technology, Shihezi University, Shihezi 832003, P. R. China; [2]Research Institute of Veterinary, Animal Sciences Academy of Xinjiang, Urumqi 830000, P. R. China; [3]Control and Diagnosis Center of Animal Disease of Urumqi City, XinJiang Urumqi 830063, P. R. China

Received April 19, 2013; accepted October 10, 2013

Summary. – Mx proteins belonging to the dynamin superfamily of large GTPases inhibit replication of a wide range of RNA viruses. In this study, we examined whether bovine Mx1 protein could interfere with the replication of foot-and-mouth disease virus (FMDV). For this purpose we established cloned BHK-21 cells expressing bovine Mx1 protein (BM1 cells) and infected them with FMDV serotype O. Cloned BHK-21 cells expressing neomycin resistance instead of Mx1 protein (BH1 cells) and original BHK-21 cells served as negative controls. The results showed that the expression of bovine Mx1 protein reduced viral yields by 90% and levels of viral VP1 mRNA by 60%. These findings correlated with a significant reduction of viral antigen detectable in infected cells by immunofluorescent assay. These results demonstrate that bovine Mx1 protein interferes with the replication of FMDV.

Keywords: foot-and-mouth disease virus; Mx1 protein; BHK-21 cells

Introduction

Foot-and-mouth disease (FMD) is an acute, febrile and highly contagious disease of cloven-hoofed animals, which influences not only the development of animal husbandry, but also the economy and foreign trade activities (Gibbens *et al*., 2001; Knowles *et al*., 2001). The etiological agent of FMD is FMDV, which belongs to the genus *Aphthovirus* of the family *Picornaviridae* (Brown, 2003). Current prevention and control measures include routine vaccination, restriction of animal movement, and slaughter. The FMD vaccines, although effective in preventing the disease, impose the risks of incomplete inactivation and the escape of virus from vaccine production laboratories (King *et al*., 1981; Barteling and Vreeswijk, 1991). In addition, the immune responses triggered by inactivated vaccines are short-lasting, and the vaccines are required to serologically match the prevalent strains of FMDV. Thus, it is imperative to develop new antiviral strategies against all FMDV serotypes.

Mx proteins are key components of the type I interferon (IFN)-induced defense responses against viruses. They belong to the dynamin superfamily, members of which are characterized by intrinsic GTPase activity (Kochs *et al*., 2002; Haller *et al*., 2007). The Mx protein was first discovered in studying the phenomenon that the inbred mouse strain A2G was resistant to mouse-adapted influenza A virus that was lethal to other inbred strains (Lindenmann, 1962). To date, Mx proteins have been found in mouse, human, cattle, sheep, fish and other species (Horisberger and Gunst, 1991; Gérardin *et al*., 2004). The numbers of Mx protein isoforms are between one and three in most species. These proteins possess biological activity to inhibit the replication of a variety of RNA viruses (Charleston and Stewart, 1993; Jin *et al*., 1999) and some DNA viruses (Gordien *et al*., 2001; Netherton *et al*., 2009), such as influenza A, measles virus, Thogoto virus, hepatitis B virus. Conserved structural

*Corresponding author. E-mail: xjmqlqj@163.com; phone: +86-993-205036.

Abbreviations: BM1 cells = cloned BHK-21 cells expressing bovine Mx1 protein; BH1 cells = cloned BHK-21 cells expressing neomycin resistance instead of Mx1 protein; FMD = foot-and-mouth disease; FMDV = foot-and-mouth disease virus; IFA = indirect immunofluorescent assay; IFN = interferon; Mx = myxovirus resistant

features of Mx proteins include the presence of tripartite GTPase domains in the N-terminal region, a dynamin signature, and a GTP effector domain containing a leucine zipper motif in the C-terminal region. The leucine zipper motif allows self-assembling of the protein into higher orders of structures that resemble rings and helical stacks of rings, which plays a key role in the antiviral activity (Melén *et al.*, 1992; Van der Bliek, 1999).

Recent studies have indicated that bovine Mx1 protein could inhibit the replication of vesicular stomatitis virus and rabies virus (Baise *et al.*, 2004; Leroy *et al.*, 2006). Since it was not known whether the Mx1 protein could interfere with the replication of FMDV, we decided to explore this issue in this study.

Materials and Methods

Virus and cells. Baby hamster kidney (BHK-21) cells were grown and maintained in Dulbecco′s modified Eagle′s medium (DMEM) supplemented with 10% fetal calf serum (FCS) plus 100 IU of penicillin and 100 mg of streptomycin (Gibco) per ml at 37°C in a 5% CO_2 – 95% air humidified atmosphere. BHK-21 cell clones stably expressing the bovine Mx1 and control cells expressing only the neomycin resistance gene were grown in DMEM essential medium supplemented as above and, in addition, with 2 mg of G-418 (Fermentas) per ml. BHK cells were used for infectious virus titration. Type O FMDV strain OS/99 was kindly provided by the Veterinary Institute of Xinjiang Academy of Animal Sciences.

Establishment of BHK-21 clones. Bovine Mx1 gene was amplified from peripheral blood lymphocytes (PBL) from Holstein cow by RT-PCR. PBL were treated with 1,000 U/ml of recombinant human IFN-α_{2b} (Intron-A, Schering, Kenilworth, NJ, USA) for 12 hrs to stimulate Mx mRNA expression before RT-PCR. The following primers (designed based on GenBank Acc. No. BTU88329) were used for bovine Mx1 gene amplification: 5′-ATGGTTCTTTCTGACTTGG-3′(F), 5′-TTCAGCCCGGGAACTTG-3′(R). The RT-PCR products were sequenced (NCBI Acc. No. JQ766265) and cloned into the expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA) between the *Kpn*I and *Xho*I restriction sites to generate the recombinant pcDNA3.1(+)/bovine Mx1 plasmid. Three clones, which constitutively expressed bovine Mx1, were selected after transfecting pcDNA3.1(+)/bovine Mx1 into BHK-21 cells, BM1 represented the mixture of the three clones. Two clones expressing only the neomycin resistance gene were selected as the negative control, BH1 represented the mixture of the two clones. To exclude the possibility that the antiviral activity is caused by the cytotoxic effect of bovine Mx1 expression, the effect of Mx1 protein on cell growth was assessed. Cells were seeded in six-well plates at the density of 10,000 cells per well. The cells were monitored by visual examination, and trypsinized and counted daily.

Infectious virus titration. To evaluate the ability of the bovine Mx1 protein to suppress the replication of the FMDV, a virus yield reduction assay was performed. About 1×10^6 BM1 or BH1 cells were infected with FMDV serotype O at a dose of 100 $TCID_{50}$. After 1 hr of infection, the monolayers of cells were washed three times and incubated with fresh media. Cultures were harvested at 6 hrs, 12 hrs, 24 hrs, 36 hrs, and 48 hrs post infection for the virus yield assay. The experiment was repeated three times.

Immunofluorescent assays. For FMDV detection, BHK-21 cells were infected with FMDV, medium was removed 18 hrs after infection, cells were washed in PBS and fixed with 4% (w/v) formalin in PBS (pH 7.4) for 30 min at 4°C. Cells were then permeabilized with ice-cold acetone-methanol (1:1) for 15 min at -20°C and incubated with pig anti-type O FMDV polyclonal antiserum (primary antibodies) for 1 hr in PBS containing 0.2% bovine serum albumin and 0.1% Triton X-100 at 37°C. After being rinsed three times with PBS, the cells were incubated with fluoresceinisothiocyanate (FITC)-labeled Protein A (SPA-FITC, second antibody) (Abcam, Cambridge, UK) for 1 hr at 37°C. For bovine Mx1 protein detection, BM1 and BH1 cells were fixed in 4% formalin, permeabilized with acetone-methanol (1:1), and stained with mouse anti-bovine antibody and fluoresceinisothiocyanate (FITC)-labeled protein A (Abcam). Coverslips were mounted using the Prolong Antifade kit (Molecular Probes), examined with an Olympus BX51 fluorescence microscope and photographed using the DP51 camera (Olympus). The mean fluorescence intensity was analyzed using a Thermo Scientific Fluoroskan Ascent CF fluorometer (Thermo Scientific, Waltham, MA) using 460-nm excitation and 510-nm emission filters.

Western blot analysis. To further confirm the expression of bovine Mx1, Western blot analysis was performed. Cellular proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millipore). The blots were incubated with mouse anti-bovine Mx1 antibodies (primary antibodies), followed by incubation with a horseradish peroxidase-conjugated goat anti-mouse IgG (secondary antibody) (1: 2,000) (Abcam, Cambridge, UK). The detection was carried out using a chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech). Mouse β-actin protein served as an internal control.

RT-PCR. In order to investigate the inhibitory effect of bovine Mx1 protein on FMDV genomic RNA, the relative levels of FMDV VP1 were measured at 6 hrs, 12 hrs, 24 hrs, 36 hrs, and 48 hrs post infection using real-time PCR. Real-time PCR analysis was performed on a Roche Light Cycle@480 sequence detector system (Roche, Mannheim, Germany) following the manufacturer′s instructions. The primers used for FMDV VP1 amplification were: 5′-TCAAG CCAAAGGAACAAGT-3′(F), 5′-TAGACGGTCGCTAAGACAC-3′(R) and for β-actin amplification were: 5′-ATTGTCACCAACTGGGACGATA-3′(F), 5′-TCT GGGTCATCT TTTCACGGTT-3′(R). Cycle threshold (Ct) values were normalized to β-actin, and relative levels of VP1 RNA were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

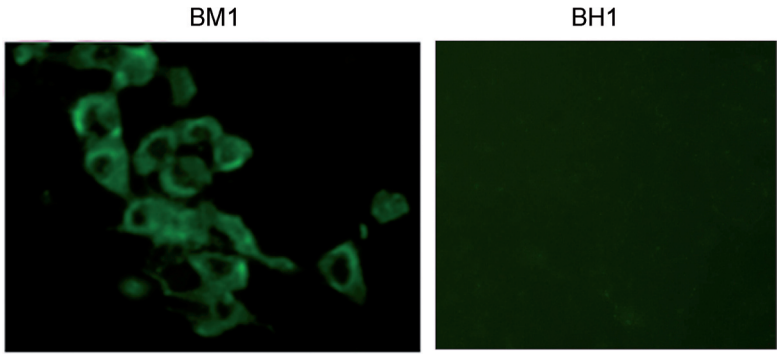

Fig. 1

Detection of bovine Mx1 protein in BM1 and BH1 cells by IFA

BM1 and BH1 cells were fixed in 4% formalin, were permeabilized with acetone-methanol (1:1), and detected using mouse anti-bovine antibody, and FITC-labeled Protein A.

To check for false positives, RT-free and no-template controls were run for each template. All samples were analyzed in triplicate.

Statistical analysis. All results are expressed as mean values ± standard deviation of the mean (SD). The statistical significance of the difference between mean values was determined by Student´s t-test at a significance level of $P \leq 0.05$. The experiments were carried out in triplicate.

Results

Stably transfected BHK-21 cells expressing bovine Mx1 protein

Clones of BHK-21 cells stably transfected with the plasmid pcDNA3.1(+)/bovine Mx1 were analyzed for Mx1 expression by indirect immunofluorescence assay (IFA) and Western blot. As shown in Fig. 1, the bovine Mx1 protein was detected by IFA. Western blot results showed that the dominant band had a molecular mass of 75 kDa, which is consistent with the predicted size of bovine Mx1 (Fig. 2). These results demonstrated that the clones constitutively expressing bovine Mx1 or the neomycin resistance gene were successfully established. BHK-21 cells are fibroblastic, growing in a monolayer, and having a well-defined tendency for parallel orientation. Expression of bovine Mx1 protein had no significant effects on the growth of the BM1 cells as compared to the BH1 cells (transfected with control plasmid) or parental BHK-21 cells (Fig. 3).

Bovine Mx1 protein expressed in BHK-21 inhibits FMDV replication

To evaluate the ability of the bovine Mx1 protein to repress the replication of the FMDV, a virus yield reduction assay was

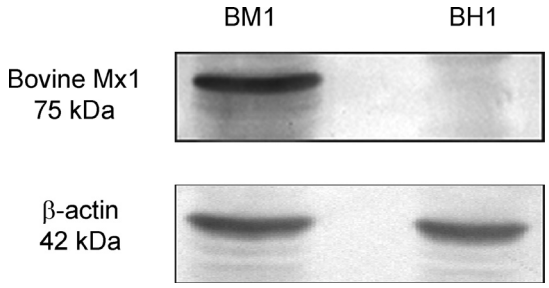

Fig. 2

Western blot analysis of bovine Mx1 protein in BM1 and BH1 cells

Cellular proteins were electrophoresed through SDS-PAGE, the blots were incubated with mouse anti-bovine Mx1 antibodies and horseradish peroxidase-conjugated goat anti-mouse IgG. Mouse-actin protein served as an internal control.

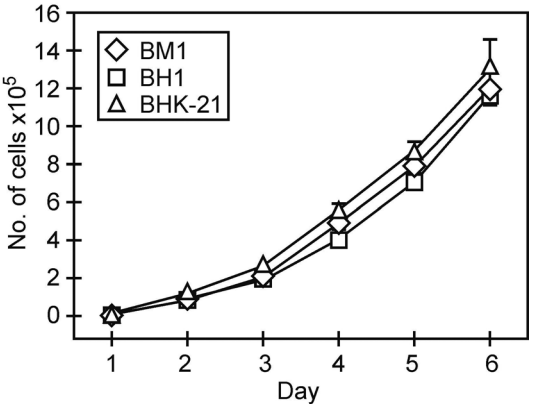

Fig. 3

Growth curves of BM1, BH1, and BHK-21 cells

BM1, BH1, and BHK-21 cells were seeded at the density of 10,000 cells per well of six-well plates, trypsinized, and counted daily. Bars show standard errors.

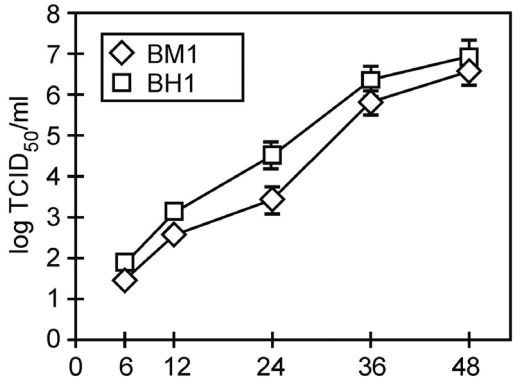

Fig. 4

FMDV growth in BM1 and BH1 cells

Cultures were harvested at various times post infection for the virus yield assay, and the virus titer was determined on BHK-21 cells by $TCID_{50}$. The experiment was repeated three times.

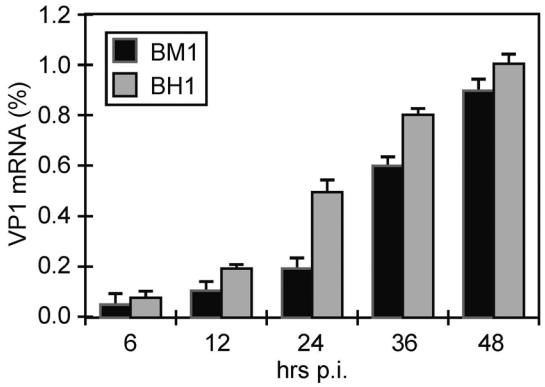

Fig. 5

FMDV VP1 mRNA levels in BM1 and BH1 cells

BM1 and BH1 cells were infected with 100 $TCID_{50}$ of FMDV, total RNA was extracted from the samples of the culture supernatants, and relative VP1 mRNA levels were determined by real-time RT-PCR.

performed. Results showed that the virus titers in BM1 cells infected with FMDV were lower than those in BH1 cells. At 24 hrs post infection, the virus titer in BM1 cells was about 10-fold lower compared to the control (P <0.05) (Fig. 4). Microscopic examination revealed that the cytopathic effect (CPE) on infected BM1 cells was delayed compared to BH1 cells, and the typical CPEs on BH1 cells infected with FMDV were more severe than those on BM1 cells.

In order to investigate the inhibitory effect of bovine Mx1 protein on FMDV genomic RNA, the relative levels of FMDV VP1 were measured at 6 hrs, 12 hrs, 24 hrs, 36 hrs, and 48 hrs post infection using real-time PCR. As shown in Fig.5, the expression of bovine Mx1 repressed viral replication at selected time points. At 6 hrs, 12 hrs, 36 hrs, and 48 hrs post infection, the relative levels of FMDV VP1 were decreased in BM1 cells by approximately 33%, 45%, 25%, and 10%, respectively, compared to BH1 cells. Especially at 24 hrs post infection, the FMDV VP1 was profoundly reduced in BM1 cells by about 60 % compared to BH1 cells (P <0.01). To confirm these results, IFA was performed 18 hrs after infection. Again, BM1 cells showed a dramatic reduction in viral replication (Fig. 6). The mean fluorescence intensity of BM1 cells (87 au) was significantly lower than that of BH1 cells (198 au) (P <0.01). Taken together, these data indicated that bovine Mx1 could interfere with the replication of FMDV.

Discussion

For the first time our results showed that the bovine Mx1 protein had an antiviral activity against FMDV. During innate immune responses against viral infection, IFN-α/β and IFN-λ induced by the virus infection generally play important roles in the first line of defense (Zhou *et al.*, 1999; Goodbourn *et al.*, 2000). They induce a number of proteins involved in antiviral actions, such as the 2′, 5′-oligoadenylate synthetase (OAS), the double-stranded RNA-dependent protein kinase (PKR), and the Mx proteins (Samuel, 2001). Studies have demonstrated that FMDV is highly sensitive to type I IFN, and the inhibition of FMDV replication involves two IFN-stimulated-gene products: OAS and PKR (Chinsangaram *et al.*, 1999, 2001; Zhou *et al.*, 1999; Monaghan *et al.*, 2004). This study revealed that bovine Mx1 induced by type I IFN might also contribute to the antiviral activity against FMDV.

The results of real-time PCR showed that the bovine Mx1 protein inhibited FMDV genomic RNA synthesis in a time-dependent manner. At 12 hrs and 24 hrs post infection, the relative levels of FMDV VP1 were profoundly decreased in BM1 cells by approximately 45% and 60%, respectively, compared to BH1 cells. At 36 hrs and 48 hrs post infection, the efficiency of inhibition was reduced to 25% and 10% respectively. The results of virus yield reduction assay also indicated that bovine Mx1 protein interfered with the replication of FMDV stronger at early times than at later times post infection.

The Mx proteins show antiviral activities through various mechanisms, depending upon the intracellular localization of the individual proteins and the virus species (Lee and Vidal, 2002). The process of FMDV infection is mainly composed of the following steps: early interaction of the virus with the cell, virus adsorption, penetration of the virus into the cell and virus particle uncoating, viral translation, transcription and genome replication, the plus-strand viral RNA encapsidation, and virion assembly to form mature and intact viruses. It would thus be interesting to clarify

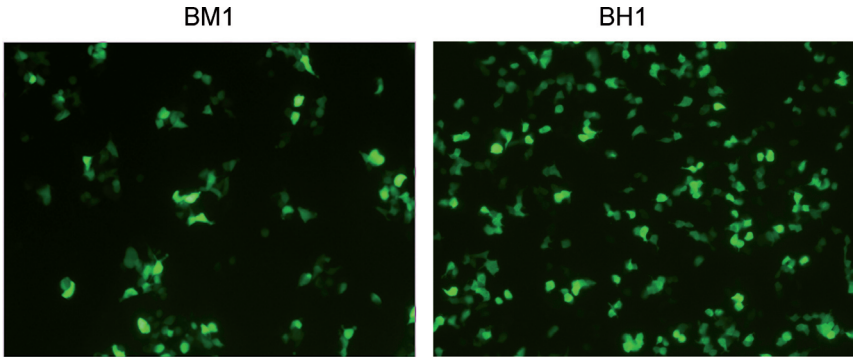

Fig. 6

Detection of FMDV in BM1 and BH1 cells by IFA

BM1 and BH1 cells were infected with FMDV serotype O. 18 hrs after infection, virus was detected using pig anti-type O FMDV polyclonal antiserum and FITC-labeled Protein A.

in a future study, which stage of the FMDV replication is interfered by bovine Mx1.

In conclusion, the present work demonstrated that the bovine Mx1 could interfere with the replication of FMDV at early times post infection *in vitro*. However, further studies are required to define the molecular mechanisms of bovine Mx1 action. Should this antiviral activity of bovine Mx1 be confirmed *in vivo*, a new therapeutic strategy would be developed for the prevention and control of FMD.

Acknowledgements. This study was supported by grants No. 2010CB530204 and No. 2010CB530206 from the State Basic Research Development Program of China (973 Program), No. 30960274 and No. 31260601 from the National Natural Science Foundation of China, and No. 2012BC006 from International Science and Technology Cooperation Program of Xinjiang Production and Construction Corps.

References

Baise E, Pire G, Leroy M, Gerardin J, Goris N, De Clerck K, Kerkhofs P, Desmecht D (2004): conditional expression of type I interferon-induced bovine Mx1 GTPase in a stable transgenic vero cell line interferes with replication of vesicular stomatitis virus. J. Interferon Cytokine Res. 24, 513–521.

Barteling SJ, Vreeswijk J (1991): Developments in foot-and-mouth disease vaccines. Vaccine 9, 75–88. http://dx.doi.org/10.1016/0264-410X(91)90261-4

Brown F (2003): The history of research in foot-and-mouth disease virus. Virus Res. 91, 3–7. http://dx.doi.org/10.1016/S0168-1702(02)00268-X

Charleston B, Stewart HJ (1993): An interferon-induced Mx protein: cDNA sequence and high-level expression in the endometrium of pregnant sheep. Gene 137, 327–331. http://dx.doi.org/10.1016/0378-1119(93)90029-3

Chinsangaram J, Piccone ME, Grubman MJ (1999): Ability of foot-and-mouth disease virus to form plaques in cell culture is associated with suppression of alpha/beta interferon. J. Virol. 73, 9891–9898.

Chinsangaram J, Koster M, Grubman MJ (2001): Inhibition of L-deleted foot-and-mouth disease virus replication by alpha/beta interferon involves double-stranded RNA-dependent protein kinase. J. Virol. 75, 5498–5503. http://dx.doi.org/10.1128/JVI.75.12.5498-5503.2001

Gérardin JA, Baise EA, Pire GA, Leroy MP, Desmecht DJ (2004): Genomic structure, organisation, and promoter analysis of the bovine (Bos taurus) Mx1 gene. Gene 326, 67–75. http://dx.doi.org/10.1016/j.gene.2003.10.006

Gibbens JC, Sharp CE, Wilesmith JW, Mansley LM, Michalopoulou E, Ryan JB, Hudson M (2001): Descriptive epidemiology of the 2001 foot-and-mouth disease epidemic in Great Britain: the first five months. Vet. Rec. 149, 729–743.

Goodbourn S, Didcock L, Randall RE (2000): Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J. Gen. Virol. 81, 2341–2364.

Gordien E, Rosmorduc O, Peltekian C, Garreau F, Bréchot C, Kremsdorf D (2001): Inhibition of hepatitis B virus replication by the interferon-inducible MxA protein. J. Virol. 75, 2684-2691. http://dx.doi.org/10.1128/JVI.75.6.2684-2691.2001

Haller O, Staeheli P, Kochs G (2007): Interferon-induced Mx proteins in antiviral host defense. Biochimie 89, 812–818. http://dx.doi.org/10.1016/j.biochi.2007.04.015

Horisberger MA, Gunst MC (1991): Interferon-induced proteins: identification of Mx proteins in various mammalian species. J. Virol. 180, 185–190. http://dx.doi.org/10.1016/0042-6822(91)90022-4

Jin HK, Takada A, Kon Y, Haller O, Watanabe T (1999): Identification of the murine Mx2 gene: interferon-induced expression of the Mx2 protein from the feral mouse gene confers resistance to vesicular stomatitis virus. J. Virol. 73, 4925–4930.

King AM, Underwood BO, McCahon D, Newman JW, Brown F (1981): Biochemical identification of viruses causing the 1981 outbreaks of foot-and-mouth disease in the UK. Nature 293, 479–480. http://dx.doi.org/10.1038/293479a0

Knowles NJ, Samuel AR, Davies PR, Kitching RP, Donaldson AI (2001): Outbreak of foot-and-mouth disease virus serotype O in the UK caused by a pandemic strain. Vet. Rec. 148, 258–259.

Kochs G, Haener M, Aebi U, Haller O (2002): Self-assembly of human MxA GTPase into highly ordered dynamin-like oligomers. J. Biol. Chem. 277, 14172–14176. http://dx.doi.org/10.1074/jbc.M200244200

Lee SH, Vidal SM (2002): Functional diversity of Mx proteins: variations on a theme of host resistance to infection. Genome Res. 12, 527–530. http://dx.doi.org/10.1101/gr.20102

Leroy M, Pire G, Baise E, Desmecht D (2006): Expression of the interferon-alpha/beta-inducible bovine Mx1 dynamin interferes with replication of rabies virus. Neurobiol. Dis. 21, 515–521. http://dx.doi.org/10.1016/j.nbd.2005.08.015

Lindenmann J (1962): Resistance of mice to mouse adapted Influenza A virus. Virology 16, 203–204. http://dx.doi.org/10.1016/0042-6822(62)90297-0

Livak KJ, Schmittgen TD (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408. http://dx.doi.org/10.1006/meth.2001.1262

Melén K, Ronni T, Broni B, Krug RM, von Bonsdorff CH, Julkunen I (1992): Interferon-induced Mx proteins form oligomers and contain a putative leucine zipper. J. Biol. Chem. 267, 25898–25907.

Monaghan P, Cook H, Jackson T, Ryan M, Wileman T (2004): The ultrastructure of the developing replication site in foot-and-mouth disease virus-infected BHK-38 cells. J. Gen. Virol. 85, 933–946. http://dx.doi.org/10.1099/vir.0.19408-0

Netherton CL, Simpson J, Haller O, Wileman TE, Takamatsu HH, Monaghan P, Taylor G (2009): Inhibition of a large double-stranded DNA virus by MxA protein. J. Virol. 83, 2310–2320. http://dx.doi.org/10.1128/JVI.00781-08

Samuel CE (2001): Antiviral actions of interferons. Clin. Microbiol Rev. 14, 778–809. http://dx.doi.org/10.1128/CMR.14.4.778-809.2001

Van der Bliek, AM (1999): Functional diversity in the dynamin family. Trends Cell Biol. 9, 96–102. http://dx.doi.org/10.1016/S0962-8924(98)01490-1

Zhou A, Paranjape JM, Der SD, Williams BR, Silverman RH (1999): Interferon action in triply deficient mice reveals the existence of alternative antiviral pathways. Virology 258, 435–440. http://dx.doi.org/10.1006/viro.1999.9738