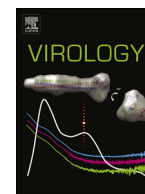




ELSEVIER

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

A partial deletion in non-structural protein 3A can attenuate foot-and-mouth disease virus in cattle

Juan M. Pacheco^{a,1}, Douglas P. Gladue^{a,c,1}, Lauren G. Holinka^a, Jonathan Arzt^a, Elizabeth Bishop^a, George Smoliga^a, Steve J. Pauszek^a, Alexa J. Bracht^b, Vivian O'Donnell^{a,c}, Ignacio Fernandez-Sainz^a, Paige Fletcher^a, Maria E. Piccone^a, Luis L. Rodriguez^a, Manuel V. Borca^{a,*}

^a Agricultural Research Service, U.S. Department of Agriculture, Plum Island Animal Disease Center, Greenport, NY, USA

^b Plum Island Animal Disease Center, APHIS, U.S. Department of Agriculture, Greenport, NY, USA

^c Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT, USA

ARTICLE INFO

Article history:

Received 26 April 2013

Returned to author for revisions

25 July 2013

Accepted 5 August 2013

Available online 6 September 2013

Keywords:

FMDV

Virus

Pathogenesis

3A

ABSTRACT

The role of non-structural protein 3A of foot-and-mouth disease virus (FMDV) on the virulence in cattle has received significant attention. Particularly, a characteristic 10–20 amino acid deletion has been implicated as responsible for virus attenuation in cattle: a 10 amino acid deletion in the naturally occurring, porciphilic FMDV O1 Taiwanese strain, and an approximately 20 amino acid deletion found in egg-adapted derivatives of FMDV serotypes O1 and C3. Previous reports using chimeric viruses linked the presence of these deletions to an attenuated phenotype in cattle although results were not conclusive. We report here the construction of a FMDV O1Campos variant differing exclusively from the highly virulent parental virus in a 20 amino acid deletion between 3A residues 87–106, and its characterization *in vitro* and *in vivo*. We describe a direct link between a deletion in the FMDV 3A protein and disease attenuation in cattle.

Published by Elsevier Inc.

Introduction

Foot-and-mouth disease (FMD) is an infectious viral disease that affects cloven-hoofed animals, including cattle, sheep, swine, goats, camelids and deer. Its wide host range and rapid spread make FMD an international animal health concern, since all countries are vulnerable to accidental or intentional trans-boundary introduction (Grubman and Baxt, 2004; Rowlands, 2003). The disease is caused by foot-and-mouth disease virus (FMDV), an *Aphthovirus* within the viral family *Picornaviridae* that exists as seven immunologically distinct serotypes: O, A, C, Asia 1, and South African Territories (SAT) type 1, SAT2 and SAT3. The viral genome consists of a single-stranded, positive-sense RNA of about 8200 nucleotides (nt). The open reading frame (ORF) encodes a single polypeptide that is post-translationally processed by virus-encoded proteases into four structural proteins (VP1 through 4) and eight non-structural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C and 3D) (Belsham, 1993). While the contribution of each of these proteins to virulence during infection of the natural host is not clear, the

role of non-structural protein 3A in virulence has been the focus of several studies.

FMDV 3A is a conserved protein of 153 amino acids (aa) in most FMDVs examined to date. Changes in 3A have been associated with altered host range as reviewed by Knowles et al. (2001) in the hepatoviruses (Graff et al., 1994a, 1994b; Lemon et al., 1991; Morace et al., 1993), rhinoviruses (Heinz and Vance, 1996) and enteroviruses (Lama et al., 1998). The 3A protein in FMDV is substantially larger than the 3A proteins of other picornaviruses being 50% larger than the 87 aa 3A of poliovirus (Kitamura et al., 1981). The first half of the 3A coding region, which encodes an N-terminal hydrophilic domain and a hydrophobic domain capable of binding membranes, is highly conserved among all FMDVs (Knowles et al., 2001). It was previously demonstrated that amino acid substitution Q44 to R in 3A was sufficient to give FMDV the ability to exacerbate virulence of FMDV strain C-S8c1 adapted to guinea pigs (Nunez et al., 2001). In FMDV, deletions in the C-terminal half of 3A has been associated with decreased virulence in cattle. Thus, FMDV strains that were attenuated through serial passages in chicken embryos for the development of vaccines in South America had reduced virulence in cattle and contained 19- to 20-codon deletions in the 3A coding region (Giraud et al., 1990). A deletion in the same 3A region consisting of 10 amino acids, was also observed (Beard and Mason, 2000) in the FMDV

* Correspondence to: Plum Island Animal Disease Center, USDA/ARS/NAA, P.O. Box 848, Greenport 11944-0848, NY, USA. Fax: +1 631 323 3006.

E-mail address: manuel.borca@ars.usda.gov (M.V. Borca).

¹ These authors have equally contributed to the results presented in this report.

isolate responsible for an outbreak of FMD in Taiwan in 1997 (O/TAW/97) that severely affected swine, but did not spread to cattle (Dunn and Donaldson, 1997; Yang et al., 1999). Thus, a region of the FMDV 3A protein located downstream of the hydrophobic domain is altered in O/TAW/97 virus and in the egg-adapted FMDVs. An association was therefore established between the presence of a specific deletion in this particular area of 3A and attenuation of virus virulence in cattle. However, this association was speculative, as these attenuated strains also contain numerous additional genome-wide amino acid substitutions when compared with virulent related or parental strains, and it cannot be ruled out that these other amino acid substitutions have some influence on the phenotype observed.

In the previous study by Beard and Mason (2000), the direct involvement of these deletions in the induction of attenuation was tested using reverse genetics with infectious clone chimeras. Chimeric viruses were created using the genomic backbone of FMDV strains virulent in cattle (A12 and O1Campos) containing the 3A protein of the porcineophilic O/TAW/97 isolate. When this chimeric virus was intradermally inoculated into the tongue of cattle, it produced less severe local lesions than parental A12 virus, yet still progressed to systemic FMD. Conversely, a chimeric virus harboring 3A of the A12 virus in the context of O/TAW/97 structural proteins produced severe local lesions in the inoculated tongue, yet failed to produce a systemic form of the disease. Thus, exchanging the 3A genomic area of parental virus strains altered the severity of lesions, but did not conclusively define virulence in cattle or the role of 3A. The possible contribution of genome-wide amino acid changes, in addition to the 3A deletions, to the observed patterns of virulence in cattle remained largely unclear. In fact, it should be noted that no complete genomic sequence was determined for the chimeric viruses constructed in that study.

To clarify the role of the 3A deletions in FMDV virulence in cattle, we developed a virus lacking 20 amino acids between residue positions 87 and 106 of 3A (O1CaΔ3A) using a full-length infectious cDNA clone derived from the FMDV O1Campos/Bra/58 strain (Borca et al., 2012) and evaluated its ability to replicate in cell cultures and its virulence profile in cattle and swine in comparison to its parental virus (O1Ca). Results demonstrated that while both viruses grow equally well in primary porcine cell cultures, O1CaΔ3A exhibited a significantly decreased replication ability in primary bovine cell cultures. Similarly, while O1Ca caused typical generalized disease in cattle and swine, O1CaΔ3A produced fulminant FMD in swine, but was completely attenuated in cattle. Therefore, this report presents direct evidence showing

that a deletion within the 3A protein is responsible for attenuation of a highly virulent FMDV strain in cattle.

Results

Generation and characterization of pO1Ca

The full-length cDNA clone pO1Ca was constructed by overlapping PCR fragments flanked by unique restriction enzymes as described previously (Borca et al., 2012). The full-length cDNA was preceded by the T7 polymerase promoter and two additional G residues to improve transcription efficiency and followed by a polyA tail of 15 residues and a unique EcoRV site. Plasmid O1CaΔ3A (pO1CaΔ3A) containing the deletion of amino acid residues between positions 87 and 106 in non-structural protein 3A (Fig. 1) derived from the infectious clone pO1Ca was constructed as described in Materials and methods.

RNA transcripts derived from pO1Ca or pO1CaΔ3A were used to transfect BHK-21 cells by electroporation, and then subjected to serial passage in LF-BK cells expressing the bovine integrin αVβ6 (LF-BK-αVβ6) (Larocco et al., 2013) to amplify the clone-derived viruses. Sequencing of the entire genome of recovered viruses (O1Ca and O1CaΔ3A) verified that they were identical to the parental DNAs, confirming that only the predicted mutations were incorporated into the viruses.

Cell tropism of viruses O1Ca and O1CaΔ3A assessed in primary cell cultures

To evaluate the species-specific tropism of viruses O1Ca and O1CaΔ3A, multistep growth curves were performed using different cell substrates to comparatively assess *ex vitro* growth characteristics (Fig. 2). Different cells were infected at a multiplicity of infection (MOI) of 0.01 using virus titers determined on LF-BK-αVβ6, the cells used to amplify the viruses. Samples were collected at regular intervals between 4 and 48 h post-infection (hpi).

Results demonstrated that viruses O1Ca and O1CaΔ3A exhibited similar growth characteristics and reached comparable titers in LF-BK-αVβ6 (Fig. 2A). The ability of these viruses to replicate in bovine and swine primary fetal kidney cell (FBK and FPK, respectively) cultures was assessed under similar experimental conditions as those described for LF-BK-αVβ6. The ability of either virus to replicate in FPK cell cultures showed a slight delay for O1CaΔ3A at 24 h although both viruses reached the same maximal titer at

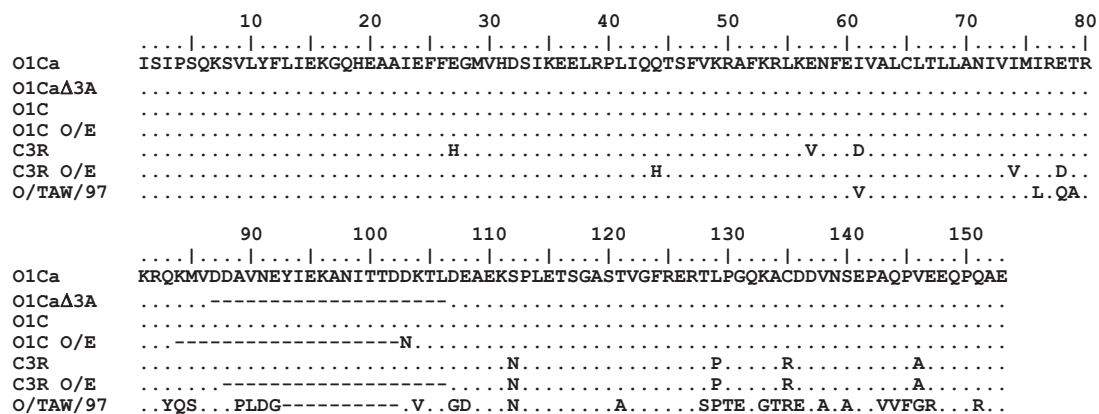


Fig. 1. Alignment of the amino acid sequence obtained from full length FMDV non-structural protein 3A from: O1Campos/Bra/58 (O1Ca) (Borca et al., 2012); O1Campos Bra/55 (O1C) (Parisi et al., 1985); O1Campos B/55 O/E (O1C O/E) (Parisi et al., 1985); C3 Resende (C3R) (Sagedahl et al., 1987); C3 Resende O/E (C3R O/E) (Sagedahl et al., 1987); O1 Taiwan 97 (O/TAW/97) (Beard and Mason, 2000); and the constructed virus used in this study O1Campos Δ3A (O1CaΔ3A).

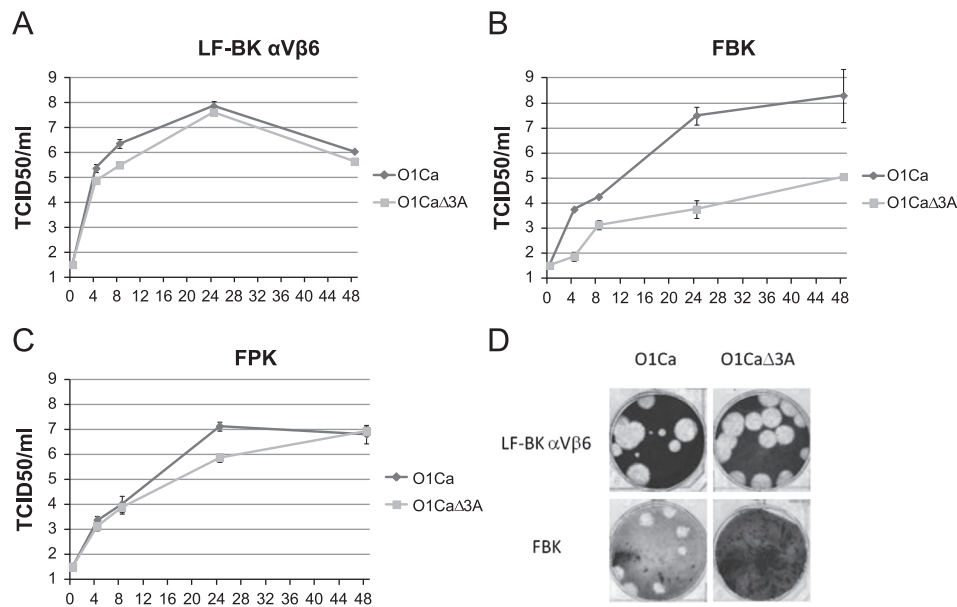


Fig. 2. Growth characteristics of FMDV O1Ca and O1Ca $\Delta 3A$. *In vitro* growth curves of O1Ca and O1Ca $\Delta 3A$ viruses (MOI 0.01) in LF-BK- $\alpha V\beta 6$ (A), FBK (B) and FPK (C) cells. Samples obtained at indicated time points were titrated in LF-BK- $\alpha V\beta 6$ cells and virus titers expressed as TCID50/ml. (B) Plaques corresponding to O1Ca and O1Ca $\Delta 3A$ viruses in LF-BK- $\alpha V\beta 6$ and FBK cells. Infected cells were incubated at 37 °C under a tragacanth overlay and stained with crystal violet at 48 hpi. Plaques from appropriate dilutions are shown.

48 h (Fig. 2C). However, O1Ca $\Delta 3A$ had substantially decreased its ability to replicate in FBKs when compared to virus O1Ca. Differences of approximately 1000-fold were observed in the titer values of samples harvested at 24 and 48 hpi (Fig. 2B).

The decreased ability of virus O1Ca $\Delta 3A$ to replicate in primary bovine cells was further analyzed in a standard plaque assay performed in LF-BK- $\alpha V\beta 6$ and FBK cell cultures (Fig. 2D). Results demonstrated that virus O1Ca and O1Ca $\Delta 3A$ both produced a similar large plaque size in LF-BK- $\alpha V\beta 6$ cells. However, while virus O1Ca produced smaller plaques in FBK cells than those produced in LF-BK- $\alpha V\beta 6$ cells, virus O1Ca $\Delta 3A$ was completely unable to produce any visible plaques. Thus, the deletion in 3A harbored by O1Ca $\Delta 3A$ virus determined the ability of the virus to replicate in primary cells of bovine origin but did not significantly affect replication in swine primary cells.

Assessment of FMDV O1Ca and O1Ca $\Delta 3A$ virulence in cattle

Virulence of both viruses, O1Ca and O1Ca $\Delta 3A$, in cattle was assessed utilizing an aerosol inoculation method developed in our laboratory (Pacheco et al., 2010a). This method was chosen since it appears to more closely simulate the natural route of infection as compared to the intradermolingual route. Three steers (each 300–400 kg) were infected with approximately 10^7 PFU/cattle of O1Ca or O1Ca $\Delta 3A$ virus, diluted in MEM, and infection and clinical signs were monitored daily as described previously (Pacheco et al., 2010a). Results demonstrated that the three animals (animals #72–74) inoculated with the O1Ca virus developed severe clinical FMD. Lesions typical of FMD appeared by 3 or 4 day post-infection (dpi) and reached the maximum clinical score (mouth and four feet affected with vesicles) by 4 or 5 dpi (Fig. 3). All three animals had fever (> 40 °C) from 3 to 7 dpi (results not shown). Viremia lasted 4–6 day, starting at 2 dpi. Virus shedding was detected in nasal swabs from all animals beginning at 1 dpi and was undetectable by 10 or 14 dpi; virus shedding was detected in oral swabs from 1 to 3 dpi until, at least, 10 dpi (Fig. 3).

Interestingly, cattle inoculated with O1Ca $\Delta 3A$ (animals #66–68) showed no clinical signs of FMD, including no hyperthermia, throughout the experimental period (Fig. 3). Neither viremia nor

virus shedding was detected in any of these animals at any sampling time after the inoculum virus was cleared. Additionally, no serum neutralizing antibodies nor antibodies to the non-structural 3ABC protein were detected throughout the experimental period (Table 1), which indicates very limited, if any, virus replication. Despite lack of detection of O1Ca $\Delta 3A$ from antemortem samples, infectious virus and viral RNA were detected in tissue samples of the pharyngeal area (dorsal soft palate, dorsal nasal pharynx and retropharyngeal lymph node) at 21 dpi in animals inoculated with either O1Ca or O1Ca $\Delta 3A$ virus (Table 1). Interestingly, while viral RNA was easily found in the interdental cleft skin in all animals infected with O1Ca, it was absent in these tissues from all animals inoculated with O1Ca $\Delta 3A$. These results clearly indicate that after aerosol inoculation with O1Ca $\Delta 3A$ virus there is only local replication in the bovine nasopharynx, with a clear absence of viremia, lack of spread to other organs and absence of clinical signs. Virus that was recovered from the dorsal soft palate of these O1Ca $\Delta 3A$ infected animals was amplified in LF-BK- $\alpha V\beta 6$ to obtain the complete sequence of 3A and all of the recovered viruses maintained the deletion in 3A. While animal 67 was identical to the O1Ca $\Delta 3A$ inoculum, point mutations occurred in animal 66 (G118D), and 68 (G118D, R54H).

Virulence assessment of FMDV O1Ca and O1Ca $\Delta 3A$ in swine

To examine the effect of the deletion in the 3A protein on virus virulence in swine, virulence studies were performed utilizing a direct-inoculation and direct-contact exposure methodology (Pacheco and Mason, 2010; Pacheco et al., 2012). Briefly, two animals were intradermally inoculated in the heel bulb with approximately 10^6 PFU/animal of O1Ca (Fig. 4) and after 48 h were placed into co-habitation with two naïve pigs. In a separate room, the same intradermal inoculation and comingling was performed with O1Ca $\Delta 3A$. All animals were monitored for the presence of clinical disease and sampled to characterize viral dynamics.

Both animals intradermally inoculated with O1Ca (#23 and #24) developed classic FMD (Fig. 4A). At 2 dpi animals became febrile, depressed, lame and developed lesions in the non-inoculated feet and mouth. Pig #24 was euthanized at 4 dpi due

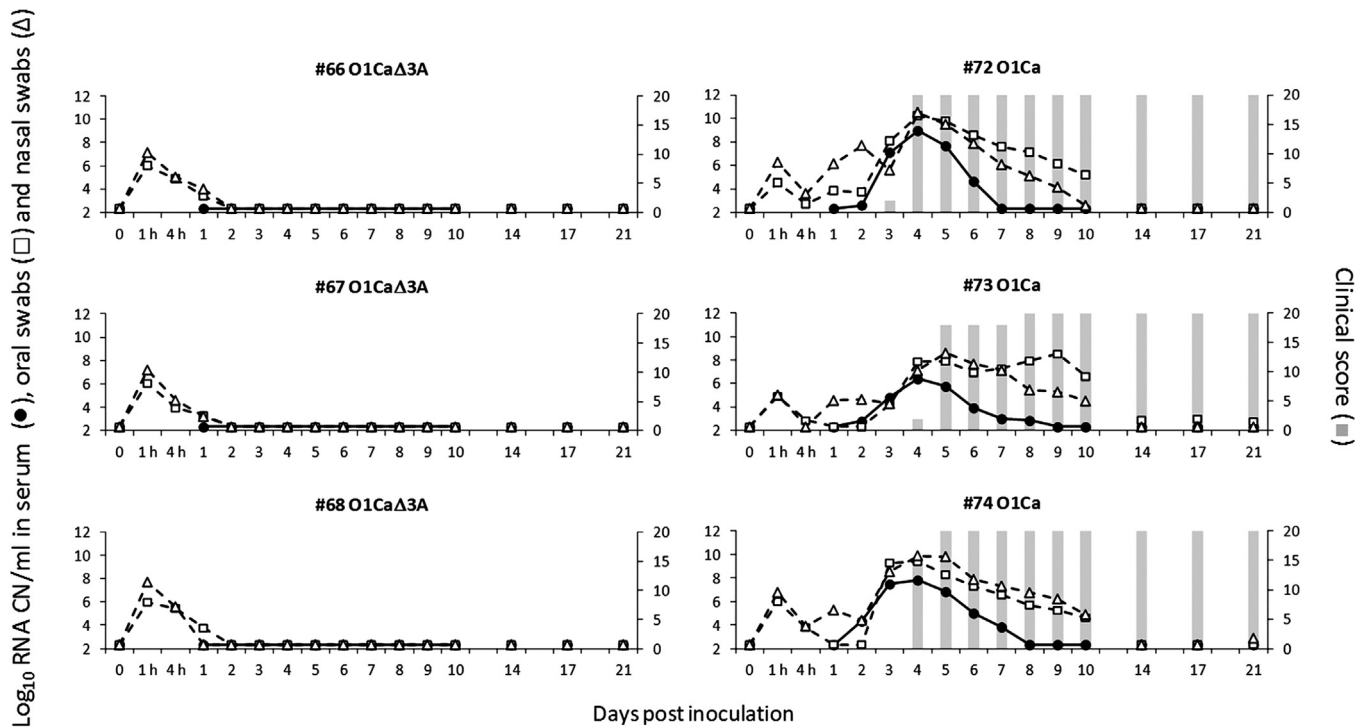


Fig. 3. Assessment of O1Ca and O1CaΔ3A virus virulence in cattle. Steers were inoculated with 10⁷ TCID₅₀ of either O1Ca (animals #72–74) or O1CaΔ3A (animals #66–68) as per the aerosol inoculation method. Presence of clinical signs (clinical scores), and virus yield (quantified as log₁₀ of viral RNA copies/ml of sample) in serum, oral and nasal swabs were determined daily during a 10 day observational period, and twice a week up to 21 dpi.

Table 1

Tissue-specific distribution of FMDV and viral RNA at 21 dpi in different tissues of cattle inoculated with either O1Ca or O1CaΔ3A virus.

Animal #	O1CaΔ3A			O1Ca		
	66	67	68	72	73	74
Dorsal soft palate	4.68 ^a /Pos ^b	4.30/Pos	3.64/Pos	2.93/Neg	3.98/Pos	4.08/Pos
Dorsal nasal pharynx	4.40/Pos	Neg/Pos	3.34/Pos	3.47/Neg	4.68/Pos	2.05/Neg
RPLN ^c	3.14/Pos	Neg/Neg	Neg/Pos	2.66/Neg	4.82/Neg	2.93/Neg
Interdigital cleft	Neg/Neg	Neg/Neg	Neg/Neg	3.96/Neg	2.98/neg	3.12/Neg
VNA ^d	< 0.9	< 0.9	< 0.9	3.6	3.6	3.6
3ABC antibodies ^e	Neg	Neg	Neg	Pos	Pos	Pos

^a Maximum RNA CN/mg (copy number, expressed as log₁₀, per mg of tissue) obtained from two different samples collected from the indicated tissue.

^b Virus isolation performed with the same two tissues used for RNA isolation above, on T-25 flask pre-seeded with LF-BK-αvb6 cells as previously described (Arzt et al., 2010).

^c RPLN=retropharyngeal lymph node.

^d VNA: titers of virus-neutralizing antibodies in sera titers were determined as previously described (Pacheco et al., 2010b).

^e Antibodies against FMDV 3ABC were detected using the prionics PrioCHECK FMDV NS ELISA.

to severe clinical disease. Viremia (detected by rRT-PCR) started at 1 dpi and peaked at 2 dpi coincident with onset of clinical disease. Virus was also detected by rRT-PCR in oral (tonsil) and nasal swabs of both animals starting as early as 1 dpi and peaking at 2–4 dpi.

Animals exposed by direct-contact (#25 and #26) developed disease with all classical symptoms described above and severe lesions in mouth and feet (Fig. 4C) At 3 day post challenge (dpc) both animals had clinical signs of congestive heart failure; on that day #25 died and #26 was euthanized due to respiratory distress. Real-time RT-PCR analysis demonstrated the presence of virus in blood as well as in oral and nasal swabs of both animals starting at 1 dpc.

Similar to what was observed in animals inoculated with O1Ca, both swine intradermally inoculated with O1CaΔ3A (#19 and #20) developed classic FMD, with severity and kinetics similar from the disease caused by O1Ca (Fig. 4B). Viremia and virus shedding also resembled that observed in O1Ca-infected animals. Animals infected by co-habitation (#21 and #22) developed a highly

virulent disease with the classical signs described above, including lesions in the mouth and feet (Fig. 4D). Progression of vesiculation and viral dynamics in swine caused by O1CaΔ3A did not significantly differ from those caused by the parental virus O1Ca, when pigs were inoculated intradermally or by co-habitation. Virus recovered from vesicles of the contact-inoculated animals was sequenced to confirm they maintained the deletion, and no other mutations in 3A were observed. These results clearly indicate that the effect of deletion within non-structural protein 3A does not significantly affect virulence in swine.

Discussion

The molecular basis of FMDV virulence during infection in natural hosts is incompletely understood (reviewed in references (Arzt et al., 2010; Mason et al., 2003a)). Although much research has been devoted to discovering virulence factors within the

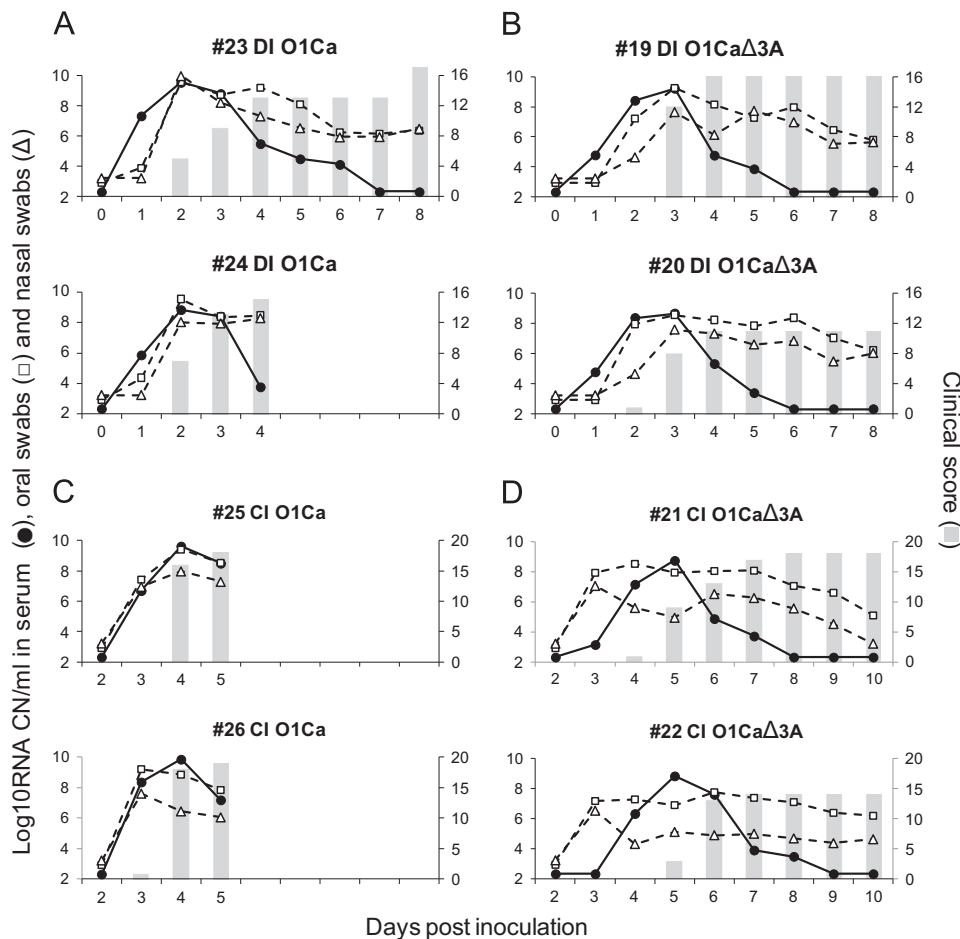


Fig. 4. Assessment of O1Ca and O1Ca Δ 3A virus virulence in swine. Two swine were intradermally inoculated into the foot bulb with 10^6 TCID₅₀ of either O1Ca (animals #23 and #24) or O1Ca Δ 3A (animals #19 and #20). After 48 hpi, inoculated animals #23 and #24 were put in co-habitation with naïve swine #25 and #26, while animals #19 and #20 were put into co-habitation with naïve swine #25 and #26. Presence of clinical signs (clinical scores) and virus yield (quantified as log₁₀ of viral RNA copies/ml of sample) in blood and nasal swabs were determined daily during an 8 day observational period after direct- or contact-inoculation. Animal #24 was humanely sacrificed at 4 dpi due to severe disease. Animals #25 and 26 were affected by FMDV induced cardiomyopathy. Day post inoculation indicates day of heel bulb inoculation of the first two pigs on each group.

FMDV genome, very few genomic determinants have been unquestionably identified. Besides the well established role of virulence in cattle and swine of the leader proteinase (Brown et al., 1996), the role of residue Arg56 in VP3 (Borca et al., 2012), the importance of virus structural proteins complex (Botner et al., 2011; Lohse et al., 2012) and the 3' untranslated region (Rodriguez Pulido et al., 2009), no other area of the virus genome has conclusively been associated with production of viral attenuation in the natural host.

FMDV non-structural protein 3A has been known to influence virulence since the report of an approximately 20 amino acid deletion within the central area of 3A found in two South American viruses adapted to growth in chicken embryos (residues 84–102 in O1Campos O/E, and residues 88–106 in C3 Resende/Brasil55) (Giraud et al., 1990, 1987; Sagedahl et al., 1987). These viruses were attenuated in cattle but not in swine. Further support came from the 1997 Taiwanese FMD outbreak strain (O/TAW/97) that devastated the country's pig industry, but did not spread to cattle (Dunn and Donaldson, 1997; Yang et al., 1999). Sequencing of O/TAW/97 indicated that the epidemic virus contained a 10 amino acid deletion between amino acid residues 93 and 102 of 3A, similar to the South American viruses described above (Beard and Mason, 2000). However, FMDV strain O/SKR/AS/2002, isolated from the South Korean outbreak of 2002, is phenotypically indistinguishable from O/TAW/97 in terms of its virulence pattern

(highly virulent in swine but fails to produce generalized disease in cattle). Interestingly, partial sequencing of O/SKR/AS/2002 demonstrated the presence of a full-length version of 3A without any of the deletions characteristic of the O1 PanAsia lineage (Oem et al., 2008), indicating that attenuated phenotype in cattle is not always associated with 3A deletions. Another PanAsia topotype strain, O/TAW/2/99, originally avirulent in cattle was highly virulent in cattle but only after undergoing two passages in swine. This strain harbors a full-length 3A and changes in virulence were attributed to other changes in the genome, and not to 3A deletions (Mason et al., 2003b).

Due to the aforementioned conflicting reports, it was difficult to establish a clear relationship between the presence of the deletion within the 3A protein and an attenuated phenotype in cattle. Genomic comparisons between viruses that have an attenuated phenotype in cattle and their putative parental viruses are quite difficult since the presence of the 3A deletion is always associated with several additional simultaneous mutations within the 3A genomic area as well as in many other areas of the virus genome.

Previous attempts to obtain direct evidence of the role of 3A in determining host specific virulence were inconclusive. In fact, there is only one report that used reverse genetics to explore the role of the 3A deletion in cattle (Beard and Mason, 2000). Specifically, Beard and Mason, investigated a chimeric virus

composed of the untranslated region and most of the non-structural proteins of A12, the structural proteins of O1Campos, and the carboxyl half of 3A along with the 3Bs and part of 3C of O/TAW/97. They determined that when this virus was intradermally inoculated into the tongue of cattle, mild local lesions appeared at inoculation sites, then progressed to systemic disease (Beard and Mason, 2000). Conversely, another chimeric virus composed of the untranslated region and the carboxy end of 2C along with 3A, 3Bs, 3C and the amino end of 3D of FMDV A12, and the structural proteins and carboxyl end of 3D from O/TAW/97, produced severe local lesions when intradermally inoculated in the tongue but failed to produce a systemic form of the disease. It is possible that the chimeric nature of the viruses constructed in their study masked the functional role of 3A. In addition, the strategy based on the swap of 3A areas between O/TAW/97 and A12 virus included, in addition to the deletion, at least 26 other substituted residues in 3A, as well as additional changes in other adjacent non-structural proteins as described above.

The current study is based upon a mutant virus that differs from its highly virulent parental virus exclusively in the particular genetic area under study, the 20 amino acid deletion between residues 87 and 106 of the non-structural protein 3A.

The role of this deletion in defining host range in cell culture remains controversial. As previously reported (Knowles et al., 2001), O/TAW/97 and closely related viruses obtained from swine in South East Asia since 1970, bearing a deletion between amino acid residues 93 and 102 of 3A, grew approximately 10- to 100-fold less in bovine-derived keratinocyte cultures than in cultures prepared from swine tissue. However, as reported by the same authors, viruses isolated before 1983 that have the same deletion grew very well in bovine cells (Knowles et al., 2001). On this basis, these authors proposed that the 93–102 deletion in this region of 3A cannot, alone, account for the inability to replicate in bovine cells, and that other mutations in the genome of this virus lineage, possibly generated in response to the 3A deletion, may be responsible for the host range of O/TAW/97. More recently, it was reported that an Asia 1 recombinant virus, developed by reverse genetics, containing the 93–102 amino acid residues deletion within 3A failed to replicate in primary bovine kidney cells while replicating as efficiently as its parental virus in the hamster cell line BHK or swine cell line PK15 (Li et al., 2010).

Our results indicate that the 3A deletion although does not significantly alter replication in primary swine cells, it severely affected virus yield in primary cells of bovine origin. The discrepancies between the complete lack of plaque formation by O1CaΔ3A virus on FBK cells virus versus the residual replication ability showed in the one step growth curve in those cells may be explained by utilizing LF-BK-αVβ6 as the substrate for determining titers in the growth curve assays.

The recovery of O1Ca and O1CaΔ3A from bovine nasopharyngeal tissues is consistent with previous works that have demonstrated tropism of FMDV for these regions in acute (Arzt et al., 2010; Pacheco et al., 2010a) and persistent (Zhang and Kitching, 2001) phases of FMD in cattle. It is intriguing that at 21 dpi similar levels of viral RNA were detected in the pharynx of cattle inoculated with either O1Ca or O1CaΔ3A. This indicates that O1CaΔ3A was able to infect bovine cells *in vivo* at the primary site of infection but failed to establish viremia, generalized in the infected animal and caused clinical disease. Sequencing of 3A in the recovered viruses demonstrated, in all three cases, that the 3A deletion remained unaltered. In addition, a single (G118D), and a double (G118D, R54H) amino acid substitution occurs in 3A of two of animals. We cannot rule out the possibility that these mutations may actually contribute to the attenuation caused by the 3A deletion. It is also possible that these point mutations were incorporated during the amplification of the clinical sample in

cell culture due to the low levels of virus that were recovered from the animals.

Our results demonstrate a direct correlation between the deletion of residues 87–106 of the 3A protein and the virus' ability to replicate in bovine primary cell cultures with no significant effect on replication in porcine cells. Importantly, this partial deletion of 3A led to a completely attenuated phenotype in cattle by aerosol inoculation with no significant effect on virulence for swine. Overall, these data provide evidence implicating the central region of FMDV 3A as a virulence factor in cattle and host range determinant of FMDV.

Materials and methods

Cell lines and virus

Baby hamster kidney cell line (BHK-21, ATCC, catalog number CCL-10) was used as previously described (Borca et al., 2012). A derivative cell line obtained from bovine kidney, LF-BK (Swaney, 1988), expressing the bovine αVβ6 integrin (LF-BK-αVβ6) (Larocco et al., 2013), and primary cell cultures of fetal bovine and porcine kidney (FBK and FPK, respectively) were grown and maintained in D-MEM containing 10% fetal calf serum. Growth kinetics and plaque assays were performed as described previously (Pacheco et al., 2010b). The FMDV strain O1Campos/Bra/58 was obtained from the Institute of Virology at the National Agricultural Technology Institute, Argentina (Borca et al., 2012).

Construction of the FMDV O1Ca full-length cDNA infectious clone and mutant O1CaΔ3A

The highly virulent FMDV strain O1Campos/Bra/58 was the template virus used to construct a cDNA infectious clone (IC). Construction of the pO1Ca full-length cDNA IC has been previously described in detail (Borca et al., 2012). pO1CaΔ3A is a derivative of pO1Ca that contains a 20 amino acid deletion (between residue positions 87 and 106) within 3A that was introduced by site-directed mutagenesis using full-length pO1Ca as a template using the Quick Change XL Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX) performed per manufacturer's instructions. Primers were designed using the manufacturer's primer design program (<https://www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Tool&SubPageType=ToolQCPD&PageID=15>). The primers used were 5'-caagaggcagaaaatggtggatgaggcggagaag-3' as the forward primer and 5'-cttcctcgctcatcaccattttctgctcttg-3' as the reverse primer. pO1Ca and pO1CaΔ3A were completely sequenced to confirm the presence of expected modifications and absence of unwanted substitutions.

Transfection and recovery of the infectious clone-derived viruses

Plasmid pO1Ca and its derivative, pO1CaΔ3A, were linearized at the EcoRV site following the poly(A) tract for RNA synthesis using the MegaScript T7 kit (Ambion, Austin, TX) according to the manufacturer's protocols. BHK-21 cells were transfected with these synthetic RNAs by electroporation (Electrocell Manipulator 600; BTX, San Diego, CA) as previously described (Borca et al., 2012). Briefly, 0.5 ml of BHK-21 cells at a concentration of 3×10^7 cells/ml in phosphate-buffered saline (PBS) were mixed with 10 μg of RNA in a 4-mm gap BTX cuvette. The cells were then pulsed once at 330 V, maximum resistance, and a capacitance of 1000 μF, followed by dilution in cell growth medium and attachment to a T-25 flask. After 4 h (h), the medium was removed, fresh medium was added and the cultures were incubated at 37 °C, 5% CO₂ for up to 24 h.

The supernatants from transfected cells were passed in LF-BK- α V β 6 cells until the cytopathic effect (CPE) appeared. After successive passages in these cells, virus stocks were prepared and the viral genome completely sequenced using the Prism 3730xl automated DNA sequencer (Applied Biosystems) as previously described (Piccone et al., 2010).

Viruses derived from plasmid pO1Ca and the derived mutant plasmid encoding the 3A deletion are referred to as O1Ca and O1Ca Δ 3A, respectively.

Comparative ability of viruses to grow in cells of different origin

Comparative growth curves between O1Ca and O1Ca Δ 3A viruses were performed in LF-BK- α V β 6, FBK and FPK cells. Preformed monolayers were prepared in 24-well plates and infected with the two viruses at MOI of 0.01 (based on TCID₅₀ previously determined in LF-BK- α V β 6 cells). After 1 h of adsorption at 37 °C the inoculum was removed and the cells were rinsed two times with ice-cold 145 mM NaCl, 25 mM MES (pH 5.5) to remove residual virus particles. The monolayers were then rinsed with media containing 1% fetal calf serum and 25 mM Hepes (pH 7.4) and incubated for 0, 4, 8, 24 and 48 h at 37 °C. At appropriate times post-infection, the cells were frozen at –70 °C and the thawed lysates were used to determine titers by TCID₅₀/ml on LF-BK- α V β 6 cells. All samples were run simultaneously to avoid inter-assay variability. Plaque assays in LF-BK- α V β 6 and FBK were performed as previously described (Pacheco et al., 2010b).

Virulence of O1Ca and O1Ca Δ 3A viruses in cattle and swine

Animal experiments were performed under biosafety level 3 conditions in the animal facilities at PIADC following a protocol approved by the Institutional Animal Care and Use Committee. Three steers (each 300–400 kg) were infected as per the aerosol inoculation method (Pacheco et al., 2010a) with approximately 10⁷ PFU/steer of O1Ca or O1Ca Δ 3A virus, diluted in MEM containing 25 mM Hepes. Different viruses were inoculated in different animal rooms. Rectal temperatures and clinical exam with sedation looking for secondary site replication (vesicles) were performed daily. Clinical score was based on presence of vesicles in the mouth and feet, with a maximum of 20 was performed as previously described (Pacheco et al., 2010a). Antemortem sample collection consisted of blood (to obtain serum), nasal and oral swabs collected daily up to 10 dpi, and at 14, 17 and 21 dpi. Postmortem sample acquisition, performed at 21 dpi, consisted of collection of tissues as previously described (Pacheco et al., 2010a) from dorsal soft palate, dorsal nasal pharynx, retropharyngeal lymph node and interdigital cleft.

Four 20 kg pigs were infected intradermally in the heel bulb (Pacheco and Mason, 2010) with 10⁶ PFU/pig of either O1Ca or O1Ca Δ 3A (two pigs per virus and one virus per room), diluted in MEM containing 25 mM Hepes. At 48 hpi, two naïve pigs were introduced to each room. The four animals remained cohabitating for the rest of the experimental period. For 8 day after inoculation, animals were clinically examined, including rectal temperature recordings and serum and nasal swab collections. Clinical score was based on severity of affected digits and/or snout with a maximum of 16 for direct inoculated animals (inoculated foot was not considered for scoring) and 20 for contact inoculated animals was performed as previously described (Pacheco and Mason, 2010).

After collection, clinical samples were aliquotted and frozen at ≤ –70 °C. One serum and one swab aliquot, collected each day from each animal, were used to perform viral titration by rRT-PCR as described previously (Pacheco et al., 2010a).

FMDV RNA quantitation by real time RT-PCR

FMDV real time RT-PCR (rRT-PCR) was performed as previously described (Pacheco et al., 2010a). RNA copy numbers per ml of fluids (antemortem samples) or per mg of tissue (postmortem samples) were calculated based on a O1Campos specific calibration curve developed with *in vitro* synthesized RNA obtained from pO1Ca. For this, a known amount of FMDV RNA was 10-fold serially diluted in nuclease-free water and each dilution was tested in triplicate by FMDV rRT-PCR. Ct values from triplicates were averaged and plotted against FMDV RNA copy number.

Acknowledgments

We wish to acknowledge Ethan Hartwig for assistance in processing samples. We also thank the Plum Island Animal Resource Unit personnel for their excellent assistance with the animal experiments. We also thank Melanie Prarat for the editing of this manuscript.

References

- Arzt, J., Pacheco, J.M., Rodriguez, L.L., 2010. The early pathogenesis of foot-and-mouth disease in cattle after aerosol inoculation. Identification of the nasopharynx as the primary site of infection. *Vet. Pathol.* 47, 1048–1063.
- Beard, C.W., Mason, P.W., 2000. Genetic determinants of altered virulence of Taiwanese foot-and-mouth disease virus. *J. Virol.* 74, 987–991.
- Belsham, G.J., 1993. Distinctive features of foot-and-mouth disease virus, a member of the picornavirus family; aspects of virus protein synthesis, protein processing and structure. *Prog. Biophys. Mol. Biol.* 60, 241–260.
- Borca, M.V., Pacheco, J.M., Holinka, L.G., Carrillo, C., Hartwig, E., Garriga, D., Kramer, E., Rodriguez, L., Piccone, M.E., 2012. Role of arginine-56 within the structural protein VP3 of foot-and-mouth disease virus (FMDV) O1 Campos in virus virulence. *Virology* 422, 37–45.
- Botner, A., Kakker, N.K., Barbezange, C., Berryman, S., Jackson, T., Belsham, G.J., 2011. Capsid proteins from field strains of foot-and-mouth disease virus confer a pathogenic phenotype in cattle on an attenuated, cell-culture-adapted virus. *J. Gen. Virol.* 92, 1141–1151.
- Brown, C.C., Piccone, M.E., Mason, P.W., McKenna, T.S., Grubman, M.J., 1996. Pathogenesis of wild-type and leaderless foot-and-mouth disease virus in cattle. *J. Virol.* 70, 5638–5641.
- Dunn, C.S., Donaldson, A.I., 1997. Natural adaptation to pigs of a Taiwanese isolate of foot-and-mouth disease virus. *Vet. Rec.* 141, 174–175.
- Giraud, A.T., Sagedahl, A., Bergmann, I.E., La Torre, J.L., Scodeller, E.A., 1987. Isolation and characterization of recombinants between attenuated and virulent aphthovirus strains. *J. Virol.* 61, 419–425.
- Giraud, A.T., Beck, E., Strebel, K., de Mello, P.A., La Torre, J.L., Scodeller, E.A., Bergmann, I.E., 1990. Identification of a nucleotide deletion in parts of polypeptide 3A in two independent attenuated aphthovirus strains. *Virology* 177780–783.
- Graff, J., Kasang, C., Normann, A., Pfisterer-Hunt, M., Feinstone, S.M., Flehmig, B., 1994a. Mutational events in consecutive passages of hepatitis A virus strain GBM during cell culture adaptation. *Virology* 204, 60–68.
- Graff, J., Normann, A., Feinstone, S.M., Flehmig, B., 1994b. Nucleotide sequence of wild-type hepatitis A virus GBM in comparison with two cell culture-adapted variants. *J. Virol.* 68, 548–554.
- Grubman, M.J., Baxt, B., 2004. Foot-and-mouth disease. *Clin. Microbiol. Rev.* 17, 465–493.
- Heinz, B.A., Vance, L.M., 1996. Sequence determinants of 3A-mediated resistance to antiviral drugs in rhinoviruses and enteroviruses. *J. Virol.* 70, 4854–4857.
- Kitamura, N., Semler, B.L., Rothberg, P.G., Larsen, G.R., Adler, C.J., Dorner, A.J., Emini, E.A., Hanecek, R., Lee, J.J., van der Werf, S., Anderson, C.W., Wimmer, E., 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. *Nature* 291, 547–553.
- Knowles, N.J., Davies, P.R., Henry, T., O'Donnell, V., Pacheco, J.M., Mason, P.W., 2001. Emergence in Asia of foot-and-mouth disease viruses with altered host range: characterization of alterations in the 3A protein. *J. Virol.* 75, 1551–1556.
- Lama, J., Sanz, M.A., Carrasco, L., 1998. Genetic analysis of poliovirus protein 3A: characterization of a non-cytopathic mutant virus defective in killing Vero cells. *J. Gen. Virol.* 79 (Pt 8), 1911–1921.
- Larocco, M., Krug, P.W., Kramer, E., Ahmed, Z., Pacheco, J.M., Duque, H., Baxt, B., Rodriguez, L.L., 2013. A continuous bovine kidney cell line constitutively expressing bovine alphaVss6 integrin has increased susceptibility to foot and mouth disease virus. *J. Clin. Microbiol.*
- Lemon, S.M., Murphy, P.C., Shields, P.A., Ping, L.H., Feinstone, S.M., Cromeans, T., Jansen, R.W., 1991. Antigenic and genetic variation in cytopathic hepatitis A virus variants arising during persistent infection: evidence for genetic recombination. *J. Virol.* 65, 2056–2065.

- Li, S., Gao, M., Zhang, R., Song, G., Song, J., Liu, D., Cao, Y., Li, T., Ma, B., Liu, X., Wang, J., 2010. A mutant of infectious Asia 1 serotype foot-and-mouth disease virus with the deletion of 10-amino-acid in the 3A protein. *Virus Genes* 41, 406–413.
- Lohse, L., Jackson, T., Botner, A., Belsham, G.J., 2012. Capsid coding sequences of foot-and-mouth disease viruses are determinants of pathogenicity in pigs. *Vet. Res.* 43, 46.
- Mason, P.W., Grubman, M.J., Baxt, B., 2003a. Molecular basis of pathogenesis of FMDV. *Virus Res.* 91, 9–32.
- Mason, P.W., Pacheco, J.M., Zhao, Q.Z., Knowles, N.J., 2003b. Comparisons of the complete genomes of Asian, African and European isolates of a recent foot-and-mouth disease virus type O pandemic strain (PanAsia). *J. Gen. Virol.* 84, 1583–1593.
- Morace, G., Pisani, G., Beneduce, F., Divizia, M., Pana, A., 1993. Mutations in the 3A genomic region of two cytopathic strains of hepatitis A virus isolated in Italy. *Virus Res.* 28, 187–194.
- Nunez, J.I., Baranowski, E., Molina, N., Ruiz-Jarabo, C.M., Sanchez, C., Domingo, E., Sobrino, F., 2001. A single amino acid substitution in nonstructural protein 3A can mediate adaptation of foot-and-mouth disease virus to the guinea pig. *J. Virol.* 75, 3977–3983.
- Oem, J.K., Yeh, M.T., McKenna, T.S., Hayes, J.R., Rieder, E., Giuffre, A.C., Robida, J.M., Lee, K.N., Cho, I.S., Fang, X., Joo, Y.S., Park, J.H., 2008. Pathogenic characteristics of the Korean 2002 isolate of foot-and-mouth disease virus serotype O in pigs and cattle. *J. Comp. Pathol.* 138, 204–214.
- Pacheco, J.M., Mason, P.W., 2010. Evaluation of infectivity and transmission of different Asian foot-and-mouth disease viruses in swine. *J. Vet. Sci.* 11, 133–142.
- Pacheco, J.M., Arzt, J., Rodriguez, L.L., 2010a. Early events in the pathogenesis of foot-and-mouth disease in cattle after controlled aerosol exposure. *Vet. J.* 183, 46–53.
- Pacheco, J.M., Piccone, M.E., Rieder, E., Pauszek, S.J., Borca, M.V., Rodriguez, L.L., 2010b. Domain disruptions of individual 3B proteins of foot-and-mouth disease virus do not alter growth in cell culture or virulence in cattle. *Virology* 405, 149–156.
- Pacheco, J.M., Tucker, M., Hartwig, E., Bishop, E., Arzt, J., Rodriguez, L.L., 2012. Direct contact transmission of three different foot-and-mouth disease virus strains in swine demonstrates important strain-specific differences. *Vet. J.* 193, 456–463.
- Parisi, J.M., Costa Giomi, P., Grigera, P., Auge de Mello, P., Bergmann, I.E., La Torre, J. L., Scodeller, E.A., 1985. Biochemical characterization of an aphthovirus type O (1) strain campos attenuated for cattle by serial passages in chicken embryos. *Virology* 147, 61–71.
- Piccone, M.E., Pacheco, J.M., Pauszek, S.J., Kramer, E., Rieder, E., Borca, M.V., Rodriguez, L.L., 2010. The region between the two polyprotein initiation codons of foot-and-mouth disease virus is critical for virulence in cattle. *Virology* 396, 152–159.
- Rodriguez Pulido, M., Sobrino, F., Borrego, B., Saiz, M., 2009. Attenuated foot-and-mouth disease virus RNA carrying a deletion in the 3' noncoding region can elicit immunity in swine. *J. Virol.* 83, 3475–3485.
- Rowlands, D.J., 2003. Special issue—foot-and-mouth disease—preface. *Virus Res.* 91, 1.
- Sagedahl, A., Giraudo, A.T., De Mello, P.A., Bergmann, I.E., La Torre, J.L., Scodeller, E. A., 1987. Biochemical characterization of an aphthovirus type C3 strain Resende attenuated for cattle by serial passages in chicken embryos. *Virology* 157, 366–374.
- Swaney, L.M., 1988. A continuous bovine kidney cell line for routine assays of foot-and-mouth disease virus. *Vet. Microbiol.* 18, 1–14.
- Yang, P.C., Chu, R.M., Chung, W.B., Sung, H.T., 1999. Epidemiological characteristics and financial costs of the 1997 foot-and-mouth disease epidemic in Taiwan. *Vet. Rec.* 145, 731–734.
- Zhang, Z.D., Kitching, R.P., 2001. The localization of persistent foot and mouth disease virus in the epithelial cells of the soft palate and pharynx. *J. Comp. Pathol.* 124, 89–94.