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Short Communication

In vitro surrogate models to aid in the development of antivirals for the containment of foot-and-mouth disease outbreaks

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ABSTRACT

Foot-and-mouth disease virus (FMDV) is a highly pathogenic member of the genus *Aphthovirus* (family *Picornaviridae*) that is only to be manipulated in high-containment facilities, thus complicating research on and discovery of antiviral strategies against the virus. Bovine rhinitis B virus (BRBV) and equine rhinitis A virus (ERAV), phylogenetically most closely related to FMDV, were explored as surrogates for FMDV in antiviral studies. Although no efficient cell culture system has been reported so far for BRBV, we demonstrate that infection of primary bovine kidney cells resulted in an extensive but rather poorly-reproducible induction of cytopathic effect (CPE). Madin–Darby bovine kidney cells on the other hand supported viral replication in the absence of CPE. Antiviral tests were developed for ERAV in Vero A cells employing a viral RNA-reduction assay and CPE-reduction assay; the latter having a Z' factor of 0.83 ± 0.07. The BRBV and ERAV models were next used to assess the anti-aphthovirus activity of two broad-spectrum antiviral agents 2′-*C*-methylcytidine (2CMC) and ribavirin, as well as of the enterovirus-specific inhibitor enviroxime. The effects of the three compounds in the CPE-reduction (ERAV) and viral RNA-reduction assays (BRBV and ERAV) were comparable. Akin to 2CMC, compound A, a recently-discovered non-nucleoside pan-serotype FMDV inhibitor, also inhibited the replication of both BRBV and ERAV, whereas enviroxime was devoid of activity. The BRBV and ERAV surrogate models reported here can be manipulated in BSL-2 laboratories and may facilitate studies to unravel the mechanism of action of novel FMDV inhibitors.

Foot-and-mouth disease (FMD) is arguably the world's most important transboundary animal disease, imposing constant threats to farmers' livelihoods and national economies. With an estimated annual global loss between US$6.5 and 21 billion (Knight-Jones and Rushton, 2013), FMD is ranked worldwide as the first and foremost priority among veterinary infectious diseases (Domenech et al., 2006). Current containment strategies (in Europe) include culling of entire herds of (mostly) healthy animals (Paton et al., 2009), leading to high costs and engendering public disapproval. The alternative control measure of emergency vaccination is hampered by several factors including serotype-dependency of the vaccine, the immunity gap (i.e., time lapse between vaccination and clinical protection) and prolonged waiting periods to recover the FMD-free status. Antiviral drugs may be employed for rapid and serotype-independent containment of viral outbreaks in livestock (Goris et al., 2008). Moreover, it has been demonstrated recently that a selective inhibitor of the pestivirus replication can reduce both the replication and spread of classical swine fever (CSF) virus in experimentally infected pigs (Vrancken et al., 2009a,b) in a manner that effectively contains CSF epidemics in densely populated livestock areas (Backer et al., 2013; Ribbens et al., 2012). This demonstrates the potential utility of antivirals in controlling livestock disease outbreaks.

However, the fact that FMDV can only be manipulated in high-containment laboratories complicates antiviral studies. This drawback could be circumvented by the use of surrogate, BSL-2 compatible viruses. Such approach has been, for example, employed successfully in the discovery and development of antivirals for viruses such as the smallpox and the hepatitis C virus (Buckwold et al., 2003; Smee, 2008). Besides FMDV, bovine rhinitis B virus (BRBV) and equine rhinitis A virus (ERAV) are two of the three other members of the genus *Aphthovirus* within the family *Picornaviridae* (Lauber and Gorbalenya, 2012). FMDV and ERAV

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share common physicochemical properties, nucleotide sequence, structural organization and molecular mechanisms of replication (Groppelli et al., 2010 and Hinton et al., 2000). Even the pathogenesis in the target host is more or less comparable (Lynch et al., 2013). BRBV, on the other hand, was previously classified as a member of the genus *Rhinovirus* because of similarities with other bovine rhinoviruses (Reed et al., 1971). Recent analyses on the genetic, phylogenetic and functional properties of BRBV demonstrate closer similarity to aphthoviruses (Hollister et al., 2008). Considering these similarities, aphthoviruses may be therefore employed to investigate the mechanism of inhibition by FMDV antivirals. We here report on the establishment of *in vitro* surrogate models for FMDV antiviral studies using ERAV and BRBV.

BRBV propagation is challenging owing to the lack of a reliable cell culture model. However, related bovine rhinoviruses have been cultured in bovine kidney cell lines at an optimal temperature of 33 °C (Lupton et al., 1980). To establish an efficient *in vitro* system for BRBV, primary cultures of cells were generated from the cortical tissue of an adult bovine kidney obtained from a slaughterhouse (hereafter referred to as primary bovine kidney or PBK cells) according to standard methods (Lindsey and Chow, 1969). Following three passages of BRBV strain EC-11 (ATCC VR-392) at 33 °C, 5% CO_2, cytopathic effect (CPE) was observed at 4 days post-infection (p.i.) (Fig. 1A). Viral replication was confirmed by a 1.2×10^5-fold upsurge in supernatant levels of BRBV RNA at 3 days p.i. using a reverse transcription quantitative PCR (RT-qPCR) with in-house designed BRBV-specific primers (Table 1 and Fig. 1B). Contrary to the expectation of viral adaptation, the susceptibility of the PBK cells to BRBV infection and CPE formation declined with increased viral passage numbers (data not shown). A similar phenomenon has been reported for FMDV infection in Mengeling–Vaughn porcine kidney cells (Dinka et al., 1977). Since the primary kidney cells are likely to be a heterogeneous mixture of various cell subtypes, isolation of stable clones may be required to generate cells with consistent susceptibility to BRBV.

Alternatively, Madin–Darby bovine kidney (MDBK) cells were found to support BRBV replication at 33 °C, 5% CO_2, although relatively low viral titers were measured in the supernatant

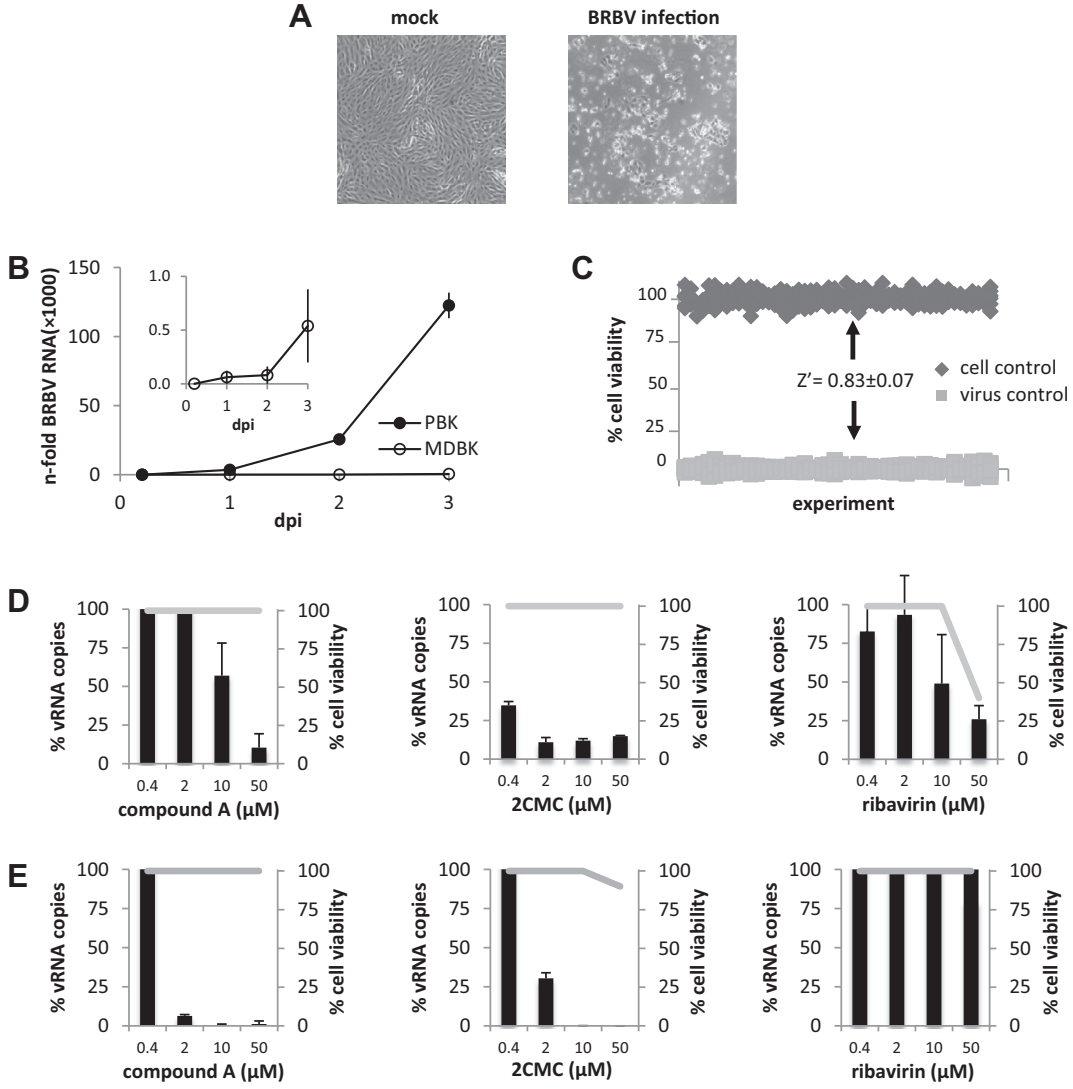

Fig. 1. Development of cell culture models and validation of *in vitro* antiviral assays for surrogate viruses for FMDV. (A) Microscopic image (40×) of PBK cells showing CPE induced by BRBV at 4 days post-inoculation (dpi). (B) Kinetics of BRBV replication in PBK (closed circles) and MDBK (open circles) cells. Inset illustrates kinetics in MDBK cells using a smaller *y*-axis scale. N-fold increase in BRBV RNA levels were calculated relative to levels at 5 h post inoculation. (C) Z' factor assessment of the ERAV antiviral assay in VeroA cells. Rate of cell viability in cell controls (dark grey diamonds) and virus controls (light grey squares) were determined by MTS assay for 53 independent experiments. The mean Z' factor and standard deviation are shown. (D) BRBV and (E) ERAV RNA reduction assay and cytotoxicity evaluation for various compounds. The bar graph (left *y*-axis) illustrates the percentage of viral RNA levels in compound-treated infected cells relative to virus controls. The line graph (right *y*-axis) depicts the rate of cell viability in the presence of compound alone. MDBK and Vero A cells were used for BRBV and ERAV assays, respectively.

Table 1
Sequences of primers and probes used for real-time RT-PCR.

Target	5′ to 3′ sequence		Reference
BRBV 5′UTR	Forward	AACTCGGTCTCTAGTATGACAGCCTAA	Designed in-house
	Reverse	GATCCCGGGTGTCACTTGTT	
	Probe	FAM-CCTCCAGGTACCCCGG-NFQ	
ERAV 5′UTR	Forward	AGCGGCKTGCTGGATTTTC	Lu et al. (2012)
	Reverse	CATYTGYCAGCTTGGTGACA	
	Probe	FAM-CGGTGCCATTGCT-MGB	
FMDV 3D	Forward	ACTGGGTTTTACAAACCTGTGA	Callahan et al. (2002)
	Reverse	GCGAGTCCTGCCACGGA	
	Probe	FAM-TCCTTTGCACGCCGTGGGAC-BHQ1	
FMDV 5′UTR	Forward	CACYTYAAGRTGACAYTGRTACTGGTAC	Reid et al. (2002)
	Reverse	CAGATYCCRAGTGWCICITGTTA	
	Probe	FAM-CCTCGGGGTACCTGAAGGGCATCC-BHQ1	

(~500-fold increase in viral RNA by 3 days p.i.) (Fig. 1B). Furthermore, no CPE was observed in these cell cultures (data not shown). Infection of confluent MDBK monolayers in 96-well plates with 5.2×10^8 BRBV RNA copy numbers yielded maximum viral RNA levels in the culture supernatant at 3 days p.i. again in the absence of CPE (data not shown). Hence, this inoculum was used in subsequent viral RNA-reduction assays.

ERAV is known to readily replicate in cell culture. Confluent Vero A monolayers were infected with $70 \times TCID_{50}$ (50% tissue culture infectious dose) per well of ERAV strain NM11/67 (a kind gift from Prof. Frank van Kuppeveld of the University of Utrecht, Netherlands) in 96-well plates at 37 °C, 5% CO_2 for three days. Following microscopic read-out of virus-induced CPE, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and the optical density (OD) of each well was read at 498 nm using a microplate reader. Cell viability was quantified by means of the formula:

$$100 \times (OD_{sample} - ODV_C)/(OD_{CC} - OD_{VC})$$

in which OD_{CC} and OD_{VC} represent the average OD of the cell and virus controls, respectively. ERAV infection resulted in 100% CPE following microscopic reading or 0% cell viability as determined by MTS at 3 days p.i. (Fig. 1C) and elevated levels of supernatant viral RNA as determined by an ERAV-specific RT-qPCR (Table 1 for primer and probe sequences) (data not shown). The Z' factor which constitutes a measure of statistical effect size to assess the quality of the assay for high-throughput screening purposes (Zhang et al., 1999), was calculated for 53 independent experiments using the formula:

$$1 - [3 \times (SD_{CC} + SD_{VC})/(OD_{CC} - OD_{VC})]$$

in which SD_{CC} and SD_{VC} represent the standard deviations of the cell and virus controls, respectively. A Z' factor of 0.83 ± 0.07 was obtained indicating an excellent assay for the screening of compound libraries (Fig. 1C). This is similar to the results previously obtained by Willems et al. (2011) for the suitability of the MTS assay to detect CPE induced by the FMDV reference strains O_1 Manisa, A Iran 11/96 and A_{22} Iraq 24/64 on BHK-21 cells.

Both *in vitro* models were next used to assess their potential as surrogate systems for FMDV in antiviral studies. To this end, known FMDV inhibitors as well as enterovirus-specific inhibitors were used. Only a few FMDV inhibitors have been reported in literature, most (if not all) of which demonstrate broad-spectrum activity (Goris et al., 2008). Ribavirin and 2′-*C*-methylcytidine [2CMC, which was originally developed as an HCV inhibitor (Sommadossi and La Colla, 2001)] have been previously reported as replication inhibitors of FMDV *in vitro* and also in severe combined immunodeficient mice for 2CMC (Goris et al., 2007; Lefebvre et al., 2013). Both compounds are known to exhibit pan-serotype FMDV activity, but ribavirin is less potent than 2CMC (Goris et al., 2007). Recently, we also identified a non-nucleoside analogue, compound A, as a selective inhibitor of *in vitro* FMDV replication. Compound A was active against a number of FMDV serotypes with O1 Manisa, A Iran 96 and C1 Noville being the most sensitive while SAT-1 ZIM 25-89 was not susceptible (Table 2). The activity of compound A against FMDV O1 Manisa in CPE-based assay was confirmed in virus yield and RNA reduction assays. Virus supernatants were titrated in SK6 porcine kidney cells and FMDV RNA levels were determined via RT-qPCR using two different primer sets targeting the 3D and 5′UTR regions of the viral genome (Table 1). Compound A effectively inhibited virus-induced CPE formation, reduced viral RNA levels by >98% and >99.98% at 20 and 40 μM, respectively, and reduced infectious virus titers at 48 h p.i. by 2.5 $\log_{10}$ and >3.8 $\log_{10}$ at 20 and 40 μM, respectively (Table 3). Compound A also proved inactive against a panel of non-aphthovirus picornaviruses that belong to the genus *Enterovirus* and *Cardiovirus* (Table 2).

Table 2
Effect of compound A and enviroxime on the replication of FMDV and a panel of other picornaviruses.

Compound	FMDV strain	EC_{50} (μM)	Other picornaviruses	EC_{50} (μM)
Compound A	O_1 Manisa	4.5	CVB3	>75
	A_{22} Iraq 24/64	22	CVB4	>75
	A Iran 11/96	5.9	HRV2	>75
	Asia1 Shamir	22	HRV14	>75
	C_1 Noville	0.89	Polio 1	>75
	SAT1 ZIM 25/89	>50	EV71	>75
	SAT2 ZIM 3/97	29	Echo 11	>75
	SAT3 ZIM 4/99	24	EMCV	>50
Enviroxime	O_1 Manisa	>1000 μg/ml	CVB3	0.77
			CVB4	0.47

CVB: Coxsackievirus; HRV: human rhinovirus; EV71: enterovirus 71; EMCV; encephalomyocarditis virus.

Table 3
Effect of compound A on virus yield and RNA reduction of FMDV O1 Manisa.

Compound A treatment (μM)	% CPE		% inhibition of viral RNA levels at 24 h p.i.[a]				$TCID_{50}$/ml determined at 48 h p.i.
			Extracellular		Intracellular		
	24 h p.i.	48 h p.i.	3D	5′UTR	3D	5′UTR	
0	~50	~100	0[b]	0[b]	0[b]	0[b]	4.8
10	0	~10	96.73	96.37	0	28.02	4.3
20	0	0	98.68	98.74	99.24	98.10	2.3
40	0	0	99.97	99.99	99.82	99.95	⩽1.0

[a] Relative to untreated virus control.
[b] CT values of the virus control were 27 and 25 for 3D and 5′UTR, respectively, for the extracellular fraction, and 28 and 30 for 3D and 5′UTR, respectively, for the intracellular fraction.

Table 4
Effect of selected compounds on the *in vitro* replication of FMDV, BRBV and ERAV.

	Virus	Cell line	Assay	Compound			
				Compound A	2CMC	Ribavirin	Enviroxime
EC_{50} (μM)	FMDV	SK6	MTS	0.89–35.85[a]	1.40-10[c]	350-1867[c]	>1000 μg/ml[b]
	BRBV	MDBK	vRNA	11 ± 4.53	0.47 ± 0.5	14 ± 3.14	>50
	ERAV	Vero A	vRNA	1.38 ± 0.01	1.64 ± 0.05	>50	>50
			CPE	1.13 ± 0.32	1.59 ± 0.08	>250	>50
			MTS	0.99 ± 0.37	1.44 ± 0.86	>250	>50
CC_{50} (μM)		SK6	MTS	>250	760[c]	>2000[c]	n.t.[d]
		MDBK	CPE	>50	>50	36.50 ± 2.00	>50
		Vero A	CPE	>50	>50	250 ± 0[e]	>50

[a] see Table 2.
[b] FMDV O1 Manisa.
[c] Goris et al. (2007).
[d] Not tested.
[e] $n = 4$.

Enviroxime, a broad-spectrum enterovirus inhibitor (Thibaut et al., 2011), was devoid of activity against FMDV (Table 2).

Cells infected with either BRBV or ERAV were treated with test compounds and the antiviral activity was evaluated at 3 days p.i. by quantifying (i) CPE microscopically and cell viability using the MTS method (ERAV only) and (ii) viral RNA levels in the supernatant (both BRBV and ERAV). In parallel, cytotoxicity was evaluated by microscopic read-out of cell morphology. EC_{50} and CC_{50} (50% cytotoxic concentration) values were calculated using logarithmic interpolation. Compound A, 2CMC and ribavirin resulted in a dose-dependent reduction of BRBV RNA levels (compound A and 2CMC did not result in any cytotoxicity at the concentrations tested, while ribavirin was cytotoxic at concentrations >10 μM) (Fig 1D). The EC_{50} values for inhibition of BRBV replication by compound A and 2CMC were comparable to the EC_{50} values reported for inhibition of the replication of various FMDV serotypes (Tables 2 and 4). Ribavirin proved somewhat more potent against BRBV than against FMDV. Enviroxime inhibited neither FMDV nor BRBV replication.

Both compound A and 2CMC resulted in a dose-dependent reduction of ERAV viral RNA without signs of cytotoxicity (Fig. 1E). This was corroborated by the CPE-reduction assays and cell viability assays based on MTS (Table 4). The EC_{50} values obtained did not depend on the assay method used, i.e., CPE or MTS (one way ANOVA p-value of 0.52 for 2CMC and 0.88 for compound A). Furthermore, the EC_{50} values were comparable to those reported for various FMDV serotypes. No inhibition of ERAV replication was observed for ribavirin and enviroxime (Table 4).

In conclusion, we here report for the first time on a cell culture model for BRBV. Furthermore, antiviral assays for BRBV and ERAV were established. As the CPE-based assay reported here is yet insufficiently reproducible, anti-BRBV assays are better carried out using the more laborious and expensive viral RNA reduction assay in MDBK cells. Further optimization of the *in vitro* CPE-based BRBV assay is planned for further antiviral studies. On the other hand, a simple yet robust ERAV antiviral assay was established using Vero A cells. The surrogate models presented here, in particular the ERAV model, may be useful tools to unravel the mechanism of antiviral activity of novel pan-aphthovirus inhibitors in a BSL-2 lab environment as the high-containment requirements for FMDV might hinder such investigations. This study identified a nucleoside and a non-nucleoside pan-serotype anti-FMDV compound (2CMC and compound A, respectively) which inhibit the replication of all members of the genus *Aphthovirus* with comparable efficacy. Whereas 2CMC has broad-spectrum activity, compound A appears to be exclusively active against aphthoviruses. This is the first report on an aphthovirus-specific inhibitor and thus underscores the potential use of the ERAV and BRBV models as surrogates for FMDV in mode-of-action studies.

Conflict of interest

A-M. Osiceanu, L.E. Murao, D. Kollanur, J. Swinnen, J. Neyts and N. Goris have a financial interest in Okapi Sciences NV.

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