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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

Ana-Maria Osiceanu [a], Lyre Espada Murao [a], Denny Kollanur [a], Jan Swinnen [a], Annebel R. De Vleeschauwer [b], David J. Lefebvre [b], Kris De Clercq [b], Johan Neyts [a,c], Nesya Goris [a,*]

[a] *Okapi Sciences NV, Ambachtenlaan 1, 3001 Heverlee, Belgium*
[b] *Unit of Vesicular and Exotic Diseases, Virology Department, CODA-CERVA, Veterinary and Agrochemical Research Centre, Groeselenberg 99, 1180 Brussel, Belgium*
[c] *Department of Microbiology and Immunology, Rega Institute for Medical Research, University of Leuven (KU Leuven), 3000 Leuven, Belgium*

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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

* Corresponding author. Tel.: +32 16 299726; fax: +32 16 299727.
E-mail address: nesya.goris@okapi-sciences.com (N. Goris).

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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

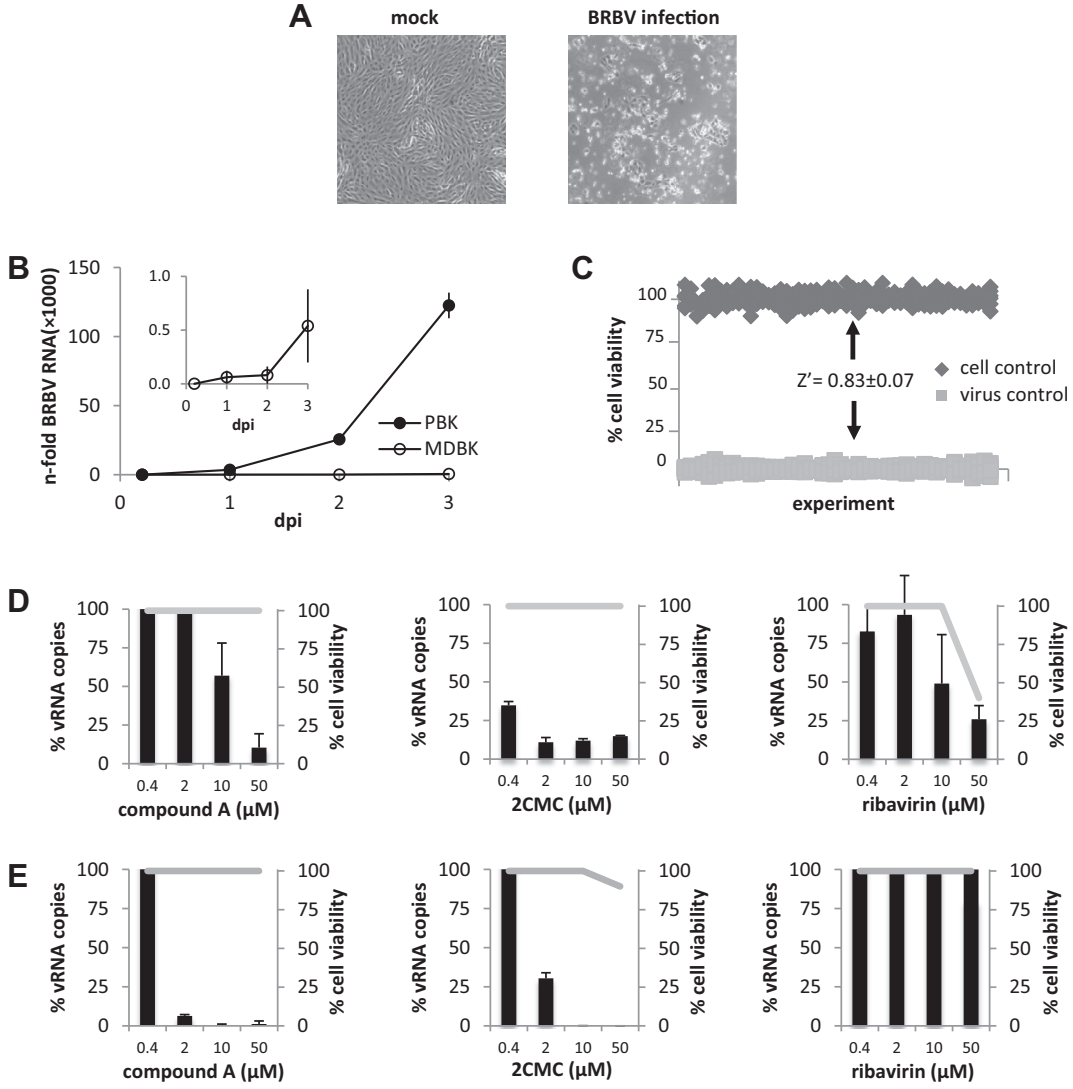

Fig. 1. Development of cell culture models and validation of *in vitro* antiviral assays for surrogate viruses for FMDV. (A) Microscopic image (40×) of PBK cells showing CPE induced by BRBV at 4 days post-inoculation (dpi). (B) Kinetics of BRBV replication in PBK (closed circles) and MDBK (open circles) 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.