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A direct high-throughput in Cell-ELISA for measuring infectivity of cytopathic and non-cytopathic bovine viral diarrhoea virus strains applied to the assessment of antiviral activity

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ABSTRACT

Low-cost high-throughput methods applicable to any virus strain are required for screening antiviral compounds against multiple field strains. Colorimetric cell-viability assays are used for this purpose as long as the viruses are cytopathic (CP) in cell culture. However, bovine viral diarrhoea virus (BVDV) strains circulating in the field are mostly non-cytopathic (NCP). An In Cell-ELISA aimed to measure viral infectivity by detecting a conserved protein produced during viral replication (non-structural protein 3, "NS3") was developed. The ELISA is performed without harvesting the cells, directly on the 96-wells culture plate. NS3 In Cell-ELISA was tested for its ability to assess BVDV-specific antiviral activity of recombinant bovine type I and III IFNs. Results correlated to those measured by qRT-PCR and virus titration. NS3 In Cell-ELISA was also efficient in estimating the IC50 of two compounds with different antiviral activity. Estimation of the 50% inhibition dose of each IFN using six BVDV strains of different biotype and genotype showed that CP strains were more susceptible to both IFNs than NCP, while type 2 NCP viruses were more sensitive to IFN-I. The In Cell-ELISA format using a detector antibody against a conserved non-structural protein can be potentially applied to accurately measure infectivity of any viral strain.

1. Introduction

Evaluating antiviral activity of interferons (IFNs) and other compounds requires cell culture-based assays for estimating the reduction in viral infectivity, usually measured as viral yield as a result of the antiviral treatment (Meager, 2002). Reduction of viral infectivity is estimated indirectly by determining virus titers (Familletti et al., 1981) usually computed in either focus-forming units (FFU/mL) (Yi, 2010) or through the calculation of the 50% tissue culture infective dose (Reed and Muench, 1938). These assays are labour-intensive. Supernatants from each antiviral treatment, commonly performed in serial dilutions in triplicate, are harvested and tested separately; thereby limiting the number of strains that can be screened.

Cytopathic effect reduction assays based on a colorimetric assessment of live cells are the high-throughput option for measuring infectivity. In these assays, the cells protected from the lytic effects by the antiviral compound are quantified by measuring coloured products produced by the metabolism of a chemical substrate by live cells in culture (Muller et al., 2017; Ramanathan et al., 2015). Viability of adherent cells can be measured using a crystal violet assay, as dead cells detach from the plate reducing the amount of crystal violet staining (Feoktistova et al., 2016). All these tests are useful provided the virus is lytic to the cultured cells. However, some viruses do not kill cells.

Up to date, there is not a high throughput assay to study the antiviral activity of compounds applicable to both cytopathic (CP) and non cytopathic (NCP) viruses, except qPCR than can be adapted to automation. PCR-based assays have been combined with immunological methods that rely on in cell-engineering (Lamontagne et al., 2013), while others have been modified to avoid genome extraction facilitating high throughput assessments (Virok et al., 2017). The cost of molecular

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assays is extremely high, especially when working at a research level.

Bovine viral diarrhoea virus (BVDV) is a highly-variable globally-disseminated pathogen accountable for several clinical syndromes that lead to major economic losses in the livestock industry. BVDV (family *Flaviviridae*, genus *Pestivirus*) is classified in two main species (type 1 and 2) based on genetic and antigenic differences (Yesilbag et al., 2017), and in two biotypes, CP and NCP, according to the capacity of virus infection to induce cell death in culture (Rumschlag-Booms et al., 2011). NCP biotypes are the most frequent in the field (Fulton et al., 2005; Ridpath, 2003) and epidemiologically relevant, as infection of pregnant animals with the NCP biotype may result in abortion, induce nonfatal teratogenic damage or lead to the birth of persistently infected (PI) calves that are born immunotolerant to the infecting BVDV strain and are lifetime carriers and shedders of the virus. PI animals can develop a fatal mucosal disease, with severe lesions in the gastrointestinal tract, when a CP variant emerges by mutation from the persisting NCP strain. CP strains are not relevant for BVDV evolution, because they cannot establish a chain of infections and do not cause persistent infections, but they are fatal for the PI host (Peterhans et al., 2010). Transient infections with NCP strains can cause a wide range of clinical manifestations, from mild or imperceptible signs to severe disease with high morbidity and mortality. BVDV infections also cause immunosuppression, the extent of which varies according to the virulence of the infecting strain (Malacari et al., 2018).

IFNs are currently used for treating virus infection in humans and small animals. Recent publications also show the potential use of IFNs for treating and preventing viral diseases in ruminants. Adenovirus-vectored bovine IFN-λ protected cattle against FMDV infection (Perez-Martin et al., 2012). Administration of type III IFN-λ as biotherapeutic agent can be effective for preventing and/or treating BVDV infection, as IFNs are functional even in the early stages of the intrauterine development (Lin et al., 2016), when the foetus is infected with BVDV. Type I interferon suppress the growth of CP and NCP BVDV in bovine blood mononuclear cells and primary bovine cell culture (Green et al., 1976). Recent studies show that bovines infected with either BVDV-1 or 2 produce high levels of acid-labile (type III) IFN (Reid et al., 2016).

The aim of this study was to develop a simple and reliable method to measure the antiviral activity of in house-produced recombinant bovine type I and type III IFN on different BVDV strains, based on the detection of a non-structural protein (NS3) which is highly conserved among pestiviruses and is produced only when the viral RNA is translated within the infected cell. NS3 is a true marker of viral infectivity as it has helicase/NTPase activity, which are absolutely essential for the synthesis of minus-strand RNA, and thus the production of infectious virus particles (Gu et al., 2000). The final goal of this work was to provide a step-by-step set-up protocol that could be easily applied to any other virus.

2. Materials and methods

2.1. Cells and virus

Madin-Darby Bovine Kidney (MDBK) cell line from the American Type Cell Collection (ATCC) was provided by the Institute of Virology (INTA) Argentina. Culture method and controls, including BVDV-free certification where detailed before (Cardoso et al., 2016). Primary foetal bovine kidney cells (RFBK cells) were prepared by the cell-culture unit of INTA using standardized procedures (Tami et al., 2003).

BVDV Singer and NADL reference strains were obtained from ATCC. All the other strains are part of INTAs virus repository and were used before (Cardoso et al., 2016; Franco Mahecha et al., 2011; Malacari et al., 2018; Odeon et al., 1999, 2009; Pecora et al., 2014). BVDV stocks were produced by infecting MDBK cells (Odeon et al., 2009). Titers were calculated as median tissue 50% culture infective dose ($TCID50\ ml^{-1}$) (Cardoso et al., 2016). Viral stocks (30 ml/each) were stored at -80 °C until use. Titers for each stock are shown in Table 2.

Foot and mouth disease virus (FMDV) A-24 Cruzeiro (Bucafusco et al., 2014) and Vesicular Stomatitis Virus New Jersey strain (VSV –NJ) (Capozzo et al., 2011) are part of INTA´s collection, and were grown in BHK-21 cells and MDBK cells, respectively.

2.2. Antibodies

Anti-NS3 mAb was kindly provided by Dr. Gerrit Keizer (Prionics AG, Zurich). The conjugated antibody was anti-mouse peroxidase labelled (KPL, Sera Care, Milford, MA). For direct immunofluorescent staining of BVDV-infected cells a polyclonal pig IgG anti-BVDV conjugated to FITC was used (VMRD, Veterinary Medical Research & Development, USA).

2.3. Interferon production

Full length sequences of bovine IFN-α and IFN-λ genes (GenBank accession NM_001017411 and HQ317919.1, respectively) were cloned into pcDNA-HisMaxA-vector (Invitrogen, Thermo Fisher Scientific Inc., USA). Plasmids were transfected into HEK293 T cells using Polyfect-Transfection Reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Transfections were performed with 2.4×10^6 cells in a 100 mm dish. Supernatants were harvested 2 days post-transfection and checked by western blot using a polyclonal goat anti-IL28B (dilution 1/750, Abcam, Cambridge, UK) or a polyclonal goat anti-IFNα (dilution 1/750, Abcam). IFN-stocks were prepared by pooling supernatants from 6 independent transfections (~40 ml each). Both pools were filtered (0.22 μm) and serial dilutions of these preparations (starting 1:4) were tested for their activity by the focus formation units reduction assay using vesicular stomatitis virus (VSV-NJ strain) as described before (Green et al., 1976). Detailed protocol is given below. Antiviral activity was determined as the reciprocal of the highest supernatant dilution that resulted in a 50% reduction in the number of plaques relative to the number of plaques in the untreated cells. Results were expressed as units of antiviral activity/ml (Green et al., 1976). Both IFNs were effective in reducing infectivity of VSV, BVDV and FMDV A24/Cruzeiro strains (Suppl. file 1). Sensitivity of FMDV A/24 Cruzeiro to our IFN-λ preparation was similar to values reported before (Diaz-San Segundo et al., 2011). Activity of each stock was 45 U/ml for IFN-λ and 40 U/mL for IFN-α. Aliquots of each stock were stored at −80 °C.

2.4. Focus Formation Unit Reduction Assay (FFURA)

Antiviral activity of the recombinant IFNs was determined by measuring the reduction in the number of viral foci. Briefly, MDBK-cells monolayers in 6-wells plates were pre-treated with 2-fold serial dilutions of the antivirals. Twenty-four hours later, supernatants were removed, and cells were washed and challenged with approximately 100 focus-formation units (FFU) of VSV, FMDV or BVDV CP strains for 1 h. The inoculum was then removed, washed with sterile PBS and overlaid with 2% low-melting point agarose. Incubation proceeded for 48 h at 37 °C, 5%CO2. Each treatment was performed in triplicate and each assay included two controls, virus alone (without IFN treatment) or untreated cell (without virus and IFN), also in triplicate. Plaques were visualized by staining with crystal violet. The base-line for negative control (untreated infected cells) was the mean of the viral foci number + SD in untreated wells. Then the percentage of foci reduction (FR %) compared to negative control was calculated as follows: FR (%) = (V-I) × 100/V. Where, V is the mean of the number of foci for negative control well (virus alone, without IFN) and I is the mean of the number of foci in IFN- treated wells. The number of FFU was proportional to the decrease in the IFN concentration. Results for FMDV A/24 Cruzeiro and BVDV singer strains are shown in Supplementary file 1.

2.5. Viability assessment

The metabolic activity of MDBK cells pre-treated with IFN-α or IFN-λ was measured with TACS® XTT Cell proliferation Assay Kit (TREVIGEN, Gaithersburg, MD) according to the manufacturer's instruction. OD values for mock-infected cells (using supernatants from cells transfected with empty plasmid) were computed as reference of viable cells. Dead cells were obtained by infection with BVDV-Singer strain at a multiplicity of infection (MOI) of 1 and also by growing the cells at 45 °C in serum-free media for 24 h. In some experiments cells were killed by performing an osmotic shock, washing the monolayer with desalted water.

A standard crystal violet staining method was also used in 96-wells plates as a measure of extension of cytopathic effect (Feoktistova et al., 2016). Assays were conducted in triplicate and the inhibitory dose 50 (ID50) was estimated by a nonlinear regression fitting of the data as the number of interferon units necessary to reduce cytopathic effect on MDBK cells by 50% compared to control infected and non-treated cells.

2.6. BVDV titration on MDBK cells

Titers of extracellular virus were obtained by micro-titter assay as previously described (Cardoso et al., 2016). Briefly, MDBK cells were grown in 96-well plates (Corning® Costar® Tewksbury, MA USA) to a confluence of 80% (10^4 cells per well). Serial fourfold dilutions of the tested samples were made in Dulbecco's Modified Eagle Medium (DMEM, Gibco) and 50 µl added to duplicate wells. Plates were incubated for 5 days at 37 °C, 5% CO2. Then, cells were fixed with 3% PBS-formaldehyde. Each well was treated with 0.1 M Glycine in PBS followed by 0.2% Triton X-100. Presence of BVDV was revealed with an anti-BVDV polyclonal antiserum conjugated to FITC (VMRD). Infected cells were observed in an inverted fluorescence microscope (Olympus IX71). Titers were calculated as median tissue 50% culture infective dose per millilitre (TCID50 ml^{-1}).

2.7. qRT-PCR

Virus RT-qPCR was performed using Pan Pestivirus primers 324–326 (Vilcek, 1994) to amplify a 288 pb fragment of the 5´UTR region of BVDV. RNA was extracted from supernatants of infected or mock-infected MDBK cells using High Pure Viral RNA Kit (Roche), according to the manufacturer's instructions. Reverse transcription was carried out with 300 ng of viral RNA, the antisense primer and Reverse Transcriptase (from Moloney murine leukaemia virus [M-MLV]; Promega, Madison, WI) under standard conditions. The qPCR was run using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, California, USA). A plasmid containing the 5´UTR 288 pb sequence of BVDV-NADL strain was used as reference standard. Samples and standards were run in triplicate in an iQ™5 Real-Time PCR detection system (Bio-Rad) and analysed using Bio-Rad iQ5 2.0 Standard Edition Optical System Software.

2.8. NS3 In Cell-ELISA

MDBK cells (1.5×10^3 cells/well; 70% confluent) seeded in a 96 wells plate were pre-treated with serial 2-fold dilutions of IFNs (~ 6 to 0.1 U/ml) for 24 h. After incubation, the IFN-containing media were removed, cells were washed with PBS 1X and infected with 50 µl of BVDV at a MOI = 0.05 for CP; and 0.001 for NCP strains. The MOI were pre-selected to yield an OD value of 1.10 ± 0.14 at 48 h post-infection (hpi). The inoculum was removed after 1 h incubation at 37 °C with gentle shaking and 200 µl of DMEM -2% foetal bovine serum was added to each well.

Each antiviral-treatment, infection and mock-infection controls were run in triplicate. Cells were fixed 48 hpi with PBS-4% formaldehyde (20 min), permeabilized with PBS-0.01% Triton X-100 (20 min) and blocked with Blocking Solution "BS" (PBS - equine serum 10%) for 90 min. Supernatants were then gently aspirated and the primary antibody (mAb Anti-NS3, Prionics) diluted 1:2000 in BS was added and incubated for 20 min at 37 °C. The reaction was revealed using an anti-mouse-peroxidase conjugate (1:1000 in BS; 30 min at 37 °C). After three washes with PBS, the substrate (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid, "ABTS") was added and incubated for 15 min at room temperature protected from light. The reaction was stopped with 0.5 M FNa and plates were read at 405 nm.

To account for differences in cell seeding density was normalized to the Janus Green staining dye. Briefly, after the In Cell assay protocol was completed, the plates were washed once with PBS1X and 1X Janus Green stain (SIGMA, San Luis, MO) was added (50 µl/ well). The plate was incubated for 5 min at RT. Then the dye was removed and after 5 washes the plate was read at 595 nm.

Once normalized, ABTS- OD values of each sample were subtracted from the mock-treated wells (corrected OD values) and then averaged (from triplicates). Each assay was repeated at least twice.

Protocols for antiviral activity of Thiosemicarbazone Derived from 5,6-Dimethoxy-1-Indanone (TSC) and Phenyl thiophene carboxamide derivative 12 (PTC12), kindly provided by Dr. Cavallaro and Dr. Alvarez, respectively; were performed as reported before (Castro et al., 2011; Pascual et al., 2018). Briefly, serial two-fold dilutions of TSC (from 10µM) were added to the cells after removal of the virus inoculum (BVDV-NADL strain, MOI = 0.05). Serial two-fold dilutions of PTC12 (from 10 µM) were added to the cells together with the virus (BVDV-VS253 strain, MOI = 0.001) and incubated for 72 h.

2.9. Data analysis

The 50% inhibitory dose of the tested IFNs was computed using the inhibition—response template in GraphPad Prism v5.0 (GraphPad Software, CA, USA). In this study, OD values were standardized in OD ~ 1 for infected control-wells, then, ID50 correspond to the units giving an OD ~ 0.5. The linear range of detection of this method enables the estimation of each ID50 value without performing an endpoint dilution.

Statistical analyses were carried out using GraphPad Prism v5.0. Spearman's correlation coefficient test was applied to measure the extent to which OD values estimated by In Cell-ELISA and readouts of other methods, tend to change together when MDBK cells were pre-treated with different concentrations of each IFN. Results of sensitivity of the different strains were compared by ANOVA 2-factor repeated measurements followed by Bonferroni multiple comparisons test. Paired-comparisons were performed using Mann-Whitney test. Confidence interval was 95%.

3. Results

3.1. Effect of recombinant interferon preparations in cellular viability

Serial dilutions of recombinant IFN preparations were added to MDBK cell monolayers and incubated for 24 h. At that time point, viability was tested by assessing the capacity of the cells to reduce XTT. A range of OD values was estimated for non-infected and mock-treated monolayers (OD: 0.9–1.2) and for BVDV-singer infected (MOI 1; 48 hpi), metabolically-stressed cells and blanks (OD < 0.22). Concentrations up to 45 U/ml of IFN-λ were not toxic on MDBK cells. The range was lower for IFN-α, as the highest concentration tested (non-diluted stocks) yielded OD values that were significantly lower than those measured for un-treated or mock-treated cells ($p < 0.05$). Based on this assay, a working range that did not affect MDBK cells´ viability for both IFNs (up to ~20 U/ml; Fig. 1) was selected.

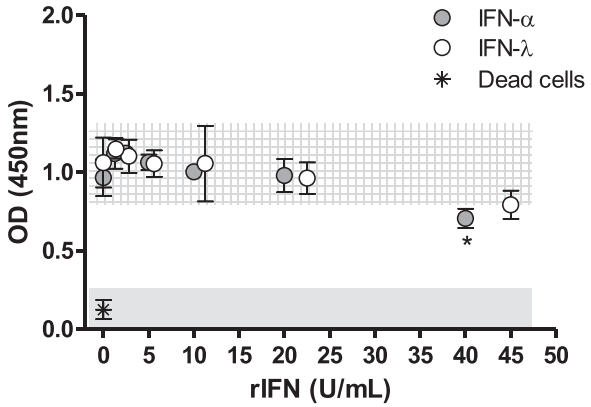

Fig. 1. Effect of rIFNs in cell viability. MDBK cells were treated with serial dilutions of recombinant IFNs and cellular viability was tested by assessing the capacity of the cells to reduce XTT. Range OD values for non-infected cells (0.9–1.2) and for BVDV Singer-infected or metabolically-stressed cells (< 0.22) are shaded in grey. Results are expressed as mean OD values ± SD. (*) Significant differences compared to OD values of mock-treated cells, $p < 0.05$.

3.2. Set up of the assay

Different conditions were assessed to set up the assay. MDBK cells seeded in 96 wells plates (1.5×10^3 cells/well) were infected (or mock-infected) with each BVDV strain at different MOI ranging from 0.05 to 0.00001 and incubated for 24, 48 and 72 h at 37 °C, 5% CO_2. For cytopathic strains infection was stopped always before cell detachment. After one wash with PBS, MDBK cells were fixed and permeabilized with PBS-triton 0.01%, and the ELISA followed using different combinations of blocking reagents, substrates and dilutions of both detector and revealing antibodies (detailed in Table 1). OD values for infected vs. mock-infected cells were computed for each condition. The differences between the OD yielded by infected and non-infected cells were higher when using ABTS than TMB substrate, and the highest range was found with the primary antibody diluted 1:2000. The secondary antibody dilution 1:750 and 1:1000 gave similar results; therefore the working dilution selected for future assays was 1:1000.

Infection dose and incubation times were also optimized for each individual virus strain to yield OD values ~1. In all cases this value was archived by incubating the virus for 48 h. Selected MOI were 0.05 for CP and 0.001 for NCP stains, which yielded OD values ranging from 0.96 to 1.24 (Table 2). The mAb was detected with an anti-mouse HPR conjugate. OD values of mock-infected cells were consistently low on distinct days and even when the assay was performed by different operator (data not shown). The coefficients of variation were always lower than 6% and all mock-infected OD values were below baseline.

The protocol was standardized in four steps: (1) definition of the MOI that yields an OD value = 1 (15% variation admitted), (2) infection of cell-monolayers at the defined MOI, (3) fix and permeabilize the cells; (4) ELISA and staining with janus green for normalization.

Table 1
Optimization of the NS3 In Cell-ELISA. In order to set up the assay, 12 conditions were tested combining different blocking reagents, substrates and dilutions of both detector and revealing antibodies. OD values (triplicates) were computed for mock-infected and infected wells and expressed as delta OD ± SD (ΔOD = OD values mock infected cells – OD infected cells). OD, optical density.

Condition	Blocking solution	Primary antibody (dilution factor)	Conjugate (dilution factor)	Substrate	ΔOD ± SD
1	0.5 %	500	750	TMB	0.327 ± 0.001
2	Casein		1000		0.298 ± 0.002
3		1000	750		0.362 ± 0.023
4			1000		0.420 ± 0.004
5		2000	750		0.450 ± 0.006
6			1000		0.408 ± 0.042
7	10 %	500	750	ABTS	0.995 ± 0.056
8	Normal		1000		0.938 ± 0.013
9	equine	1000	750		1.041 ± 0.099
10	serum		1000		1.005 ± 0.078
11		2000	750		1.382 ± 0.016
12			1000		1.187 ± 0.049

3.3. Correlation between In Cell-ELISA and the currently used tests

MDBK cells were pre-incubated with different concentrations of each IFN preparation before infection with BVDV singer (CP) or NY-93 (NCP) strains. Antiviral activity of each IFN was estimated using NS3 In Cell-ELISA, virus titration of supernatants, quantitation of viral RNA by qRT-PCR and colorimetric viability assessment (only for the CP strain). Correlation curves were built by plotting the outcome values estimated for each IFN dilution by the In Cell-ELISA and the different currently used assays (Fig. 2). Spearman´s analysis showed a significant correlation between the results obtained by the In Cell-ELISA and those estimated with all the other tests ($p < 0.05$) for both CP and NCP strains. There was a positive correlation between In Cell-ELISA results and the ratio of genome copy number or TCID50 ml^{-1} for both IFNs and strains ($r = 1$) and as expected, there was a negative correlation for the viability method for the cytopathic strain.

3.4. Interferon sensitivity assay

Due to the great diversity of BVDV, and the relevance of the NCP strains in the field, In Cell-ELISA was used to screen the individual sensitivity of BVDV strains of different genotype and biotype against both recombinant IFNs. Susceptibility to both IFNs was evaluated by In Cell-ELISA after infecting MDBK cell monolayers pre-treated with different concentrations of the recombinant IFN-α and IFN-λ preparations. IFN stocks were two-fold diluted, ranging from 0.17 to 5.4 U/ml for IFN-α and 0.35 to 5.6 U/ml for IFN-λ. Sensitivity curves for OD values obtained by In Cell-ELISA were built for each individual strain (Supplementary file 2). Due to the effect of the antiviral treatment, OD values adjusted to a typical inhibition-response curve. From these results the ID50 for both IFNs for each strain was estimated. The ID50 value represents the dose of IFN which reduces 50% the corrected OD in respect to that obtained by untreated-infected cells.

ID50 values show that all the BVDV strains tested were sensitive to both IFNs. Although, no pattern of sensitivity related to the virus genotype or biotype was found, differences were indeed detected between strains. Even within some strains (i.e. VS253, SINGER and 98–124) there were significant differences among ID50 values for each interferon, as shown in Fig. 3.

3.5. Application of NS3 In Cell-ELISA for estimating the activity of other antivirals

To extend our analysis, In Cell-ELISA was used to estimate the 50% inhibition concentration (IC50) of two compounds with different antiviral activity: TSC, an inhibitor of BVDV polymerase (Castro et al., 2011) and PTC12 that specifically interferes at the internalization step of virus entry (Pascual et al., 2018). Serial dilutions of TSC starting in 10 μM were added to the cells after adsorption of BVDV-NADL strain (MOI = 0.05). TSC was added together with the virus (VS253) and the incubation proceeded for 3 days. Each treatment was performed in triplicate. OD values measured for each antiviral concentration are depicted in Fig. 4.

IC50 values were estimated for both compounds and compared to those reported before evaluating reduction of cytopathic effect using crystal violet staining (Castro et al., 2011; Pascual et al., 2018). IC50 for

Table 2
Viral stocks and infectious dose used. Multiplicity of infection (MOI) for each BVDV strain estimated to yield an OD value of 1 in the In Cell-ELISA. N/A: not available.

BVDV strain	Genotype	Biotype	GenBank accession number	Virus stock (TCDI50/ml)	MOI	OD Value ± SD In Cell-ELISA, triplicates
Singer	1a	Cytopathic	DQ088995.2	2.08×10^7	0.05	0.99 ± 0.02
NADL	1a	Cytopathic	AJ133738	1.74×10^7	0.05	0.94 ± 0.03
98-204	1b	Non cytopathic	N/A	1.64×10^6	0.001	1.11 ± 0.05
NY1	1b	Non cytopathic	L32879.1/ AF145363.1	6.55×10^6	0.001	1.09 ± 0.12
NY93	2a	Non cytopathic	AF039173/ KR093034.1	6.55×10^6	0.001	1.07 ± 0.05
VS253	2a	Cytopathic	N/A	2.62×10^7	0.05	1.09 ± 0.13
98-124	2b	Non cytopathic	MH074881	1.31×10^7	0.001	1.22 ± 0.02

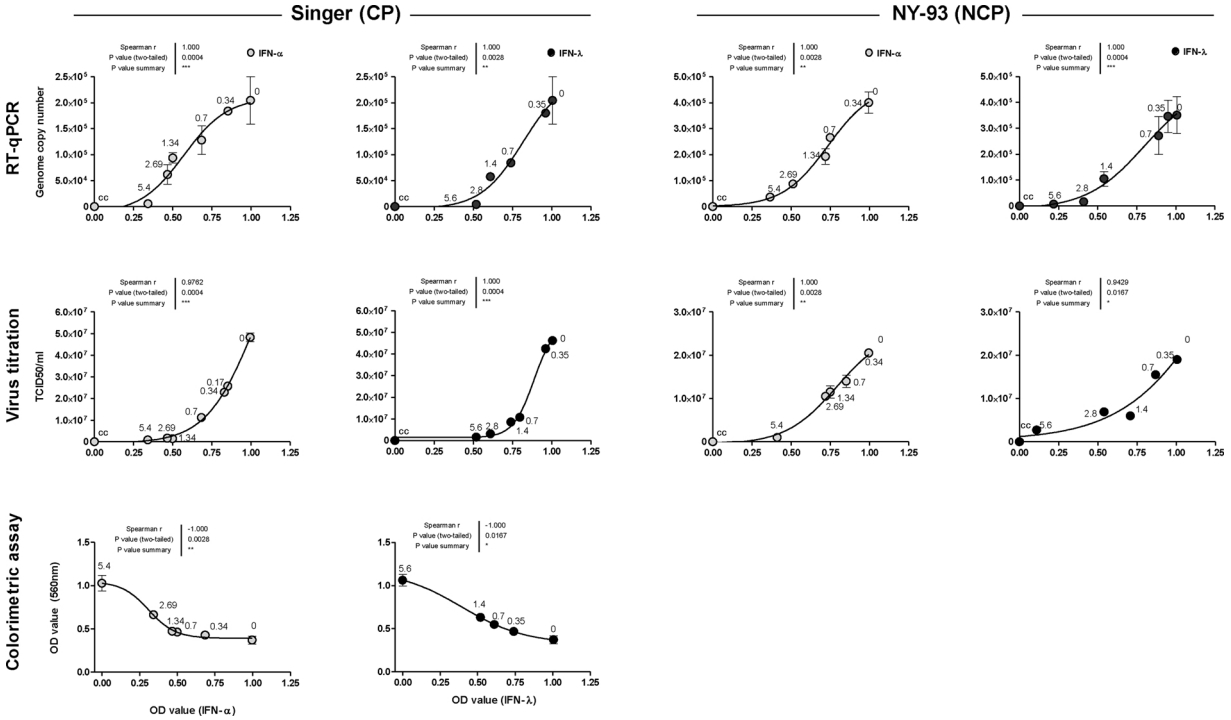

Fig. 2. Correlation between different antiviral activity tests. Antiviral activity of recombinant bovine IFN-α (grey circles) and IFN-λ (black circles) against BVDV-Singer and NY-93 stains was evaluated using the NS3 In Cell-ELISA. Results were compared to values measured by three currently-used methods: quantitation of viral RNA by qRT-PCR, virus titration and colorimetric viability assessment, as indicated. Spearman´s correlation coefficient and *P* values are depicted for each curve.

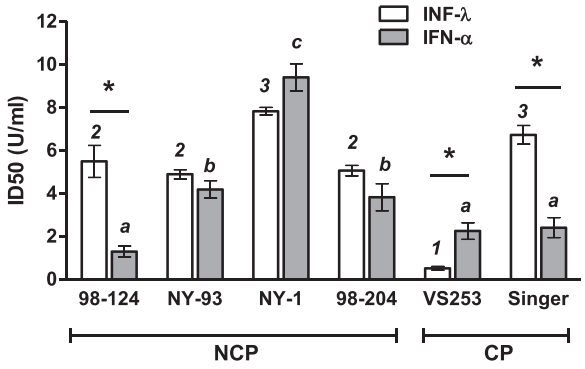

Fig. 3. Sensitivity of different BVDV strains to bovine IFN-α and IFN-λ ID50 values were plotted for NCP (98–124, NY-93, NY-1, 98–204) and CP (VS253, Singer) BVDV strains. Mean values ± SD of triplicate determinations are shown. Different letters and numbers above bars depict significant differences for IFN-α or IFN-λ, respectively ($p < 0.05$).
(*) Significant differences in ID50 values measured for each IFN within the same strain ($p < 0.05$).

PTC12-treated MDBK infected with VS253 was 0.0257 μM ± 0.004 by In Cell-ELISA, similar to the reported value (0.026μM ± 0.002). IC50 of TSC for BVDV-NADL strain was 1.75 μM using a colorimetric assay (Castro et al., 2011; Finkielsztein et al., 2008) and 1.91 μM ± 0.21 by In Cell-ELISA. Altogether, these results show that NS3 In Cell-ELISA can be used not only with IFNs but also with other anti-viral compounds.

4. Discussion

This study describes a novel approach for analysing antiviral activity by measuring the presence of a non-structural viral protein directly on the cell culture without harvesting the cells, applicable to any virus strain that can grow in cell culture regardless if it affects the cells' viability or not. This In Cell-ELISA produces an OD value that can be normalized to the total number of cells in the culture well, enabling comparisons between treatments. It is a direct high-throughput platform to assess viral infectivity, simple to perform and reliable, as results obtained using this method correlate with others currently used to measure viral infection.

Results obtained after treating MDBK cells with different IFNs using the NS3 In Cell-ELISA were initially compared with the corresponding readouts of a colorimetric assay, titration and genome copy number

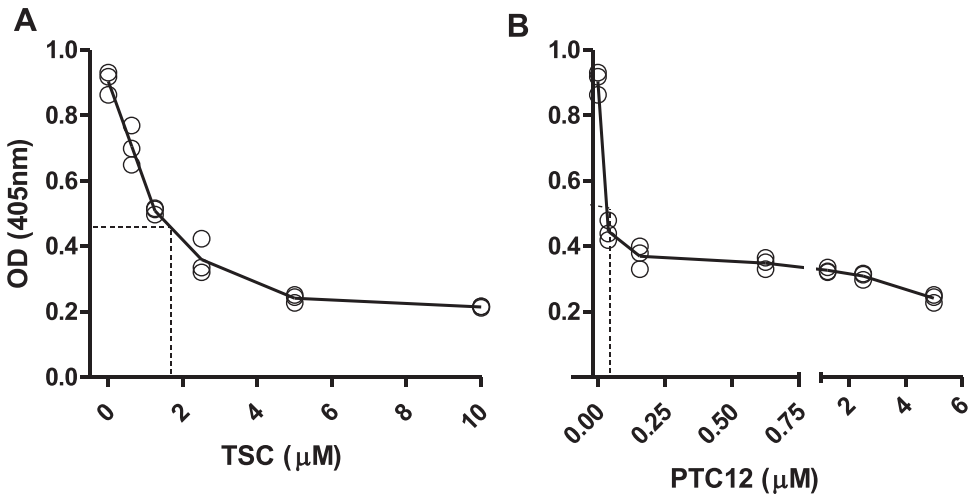

Fig. 4. Antiviral activity of TSC and PTC12. MDBK cells were treated with serial two-fold dilutions of TSC (A) or PTC12 (B) and infected with BVDV-CP strains. The graph depicts OD values of triplicates and the corresponding fitting curve. The inhibitory concentrations 50 (IC50) for each compound were estimated by a nonlinear regression fitting of the data as the compound concentration necessary to reduce cytopathic effect on MDBK cells by 50% compared to control infected and non-treated cells (indicated with dotted lines).

estimated by RT-qPCR, using reference cytopathic and non-cytopathic strains. In Cell-ELISA values correlated with those of the different currently-used tests, with positive correlation for titration and genome copy number and a negative correlation with the viable-cells colorimetric assay, as expected. The lowest correlation coefficient was observed between results produced by In Cell-ELISA and virus titration using Reed-Müench fifty per cent end-point method, probably due to the intrinsic variability of this assay (Brown, 1964). These results confirm that the ID50 computed by In Cell-ELISA or the other methods are similar.

NS3 In Cell-ELISA was efficient in estimating the IC50 of two compounds with different antiviral activity, TSC (Castro et al., 2011) and PTC12 (Pascual et al., 2018), producing similar values to those reported before for both compounds using a colorimetric method. These results indicate that the use of NS3 In Cell-ELISA is not restricted to IFNs. However, by detecting intracellular NS3, this assay measures infectivity as viral replication and may not be suitable for antiviral compounds that compromise viral assembly and/or budding.

All six BVDV strains tested were susceptible to both IFNs, with no evident association with the virus genotype or biotype. Interestingly, two NCP type-2 strains that differ in their virulence, 98–124 and NY-93 (Malacari et al., 2018), also differ in their susceptibility to INF-α, while their ID50 values were similar for IFN-λ. CP VS253 (type 2) was the most sensitive strain to both IFN treatments. This strain was also found more sensitive than a type 1 BVDV-NADL strain to another antiviral non-biological compound (PCT12), as reported recently by Pascual et al. (Pascual et al., 2018). Interestingly, VS253 is antigenically different to at least 12 strains, between reference, local and foreign isolates (Pecora et al., 2014), which may account for its particular sensitivity to antivirals. These findings may have implications when developing and optimizing antiviral treatments.

IFN-λ usually behaves as a more potent antiviral in vivo than in vitro (Ank et al., 2006). This effect was also uncovered when using IFN-λ to prevent FMDV infection in cattle (Perez-Martin et al., 2012), whereas in in vitro assays, this cytokine was always less effective than IFN-α, a fact confirmed in this study for FMDV A24/Cruzeiro strain in the FFURA (Supplementary file 1). Considering these results, the high sensitivity found against IFN-λ using the NS3 In Cell-ELISA for all six BVDV strains encourages us to pursue the use of recombinant bovine IFN-λ to prevent and/or treat BVDV infection in cattle.

NS3- In Cell-ELISA measures BVDV infectivity and can also be used to estimate the number of infectious particles in a sample. The assay was more sensitive for NCP than CP viruses as infection using CP strains needs to be stopped before cytopathic effect is developed. The high sensitivity in detecting NCP infectious virus can be valuable for controlling biological products (i.e. foetal bovine serum) and cell lines, as NCP-BVDV is considered an adventitious agent.

5. Conclusions

A novel method for measuring and analysing BVDV infection was developed and optimized. The In Cell-ELISA uses of an antibody against a non-structural conserved protein, enabling a direct detection of virus replication in the cell-culture plate and infectivity can be normalized to the number of cultured cells. This platform may be used with any virus strain that can grow in cell culture, regardless its effect on cell viability, and extended to any practical application requiring accurate measurements of viral infectivity.

Declaration of interest

none.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.07.010.

References

Ank, N., West, H., Bartholdy, C., Eriksson, K., Thomsen, A.R., Paludan, S.R., 2006. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. J. Virol. 80, 4501–4509.

Brown, W.F., 1964. Variance estimation in the reed-muench fifty per cent end-point determination. Am. J. Hyg. 79, 37–46.

Bucafusco, D., Di Giacomo, S., Pega, J., Juncos, M.S., Schammas, J.M., Perez-Filgueira, M., Capozzo, A.V., 2014. Influence of antibodies transferred by colostrum in the immune responses of calves to current foot-and-mouth disease vaccines. Vaccine 32 (48), 6576–6582.

Capozzo, A.V., Wilda, M., Bucafusco, D., de los, Angeles, Lavoria, M., Franco-Mahecha, O.L., Mansilla, F.C., Perez-Filgueira, D.M., Grigera, P.R., 2011. Vesicular Stomatitis Virus glycoprotein G carrying a tandem dimer of Foot and Mouth Disease Virus antigenic site A can be used as DNA and peptide vaccine for cattle. Antiviral Res. 92, 219–227.

Cardoso, N., Franco-Mahecha, O.L., Czepluch, W., Quintana, M.E., Malacari, D.A., Trotta, M.V., Mansilla, F.C., Capozzo, A.V., 2016. Bovine viral diarrhea virus infects

monocyte-derived bovine dendritic cells by an E2-glycoprotein-mediated mechanism and transiently impairs antigen presentation. Viral Immunol. 29, 417–429.
Castro, E.F., Fabian, L.E., Caputto, M.E., Gagey, D., Finkielsztein, L.M., Moltrasio, G.Y., Moglioni, A.G., Campos, R.H., Cavallaro, L.V., 2011. Inhibition of bovine viral diarrhea virus RNA synthesis by thiosemicarbazone derived from 5,6-dimethoxy-1-indanone. J. Virol. 85, 5436–5445.
Familletti, P.C., Rubinstein, S., Pestka, S., 1981. A convenient and rapid cytopathic effect inhibition assay for interferon. Methods Enzymol. 78, 387–394.
Feoktistova, M., Geserick, P., Leverkus, M., 2016. Crystal violet assay for determining viability of cultured cells. Cold Spring Harb. Protoc. 2016 pdb prot087379.
Finkielsztein, L.M., Castro, E.F., Fabian, L.E., Moltrasio, G.Y., Campos, R.H., Cavallaro, L.V., Moglioni, A.G., 2008. New 1-indanone thiosemicarbazone derivatives active against BVDV. Eur. J. Med. Chem. 43, 1767–1773.
Franco Mahecha, O.L., Ogas Castells, M.L., Combessies, G., Lavoria, M.A., Wilda, M., Mansilla, F.C., Seki, C., Grigera, P.R., Capozzo, A.V., 2011. Single dilution avidity-blocking ELISA as an alternative to the bovine viral diarrhea virus neutralization test. J. Virol. Methods 175, 228–235.
Fulton, R.W., Ridpath, J.F., Ore, S., Confer, A.W., Saliki, J.T., Burge, L.J., Payton, M.E., 2005. Bovine viral diarrhoea virus (BVDV) subgenotypes in diagnostic laboratory accessions: distribution of BVDV1a, 1b, and 2a subgenotypes. Vet. Microbiol. 111, 35–40.
Green, J.A., Stanton, G.J., Goode, J., Baron, S., 1976. Vesicular stomatitis virus plaque production in monolayer cultures with liquid overlay medium: description and adaptation to a one-day, human interferon-plaque. J. Clin. Microbiol. 4, 479–485.
Gu, B., Liu, C., Lin-Goerke, J., Maley, D.R., Gutshall, L.L., Feltenberger, C.A., Del Vecchio, A.M., 2000. The RNA helicase and nucleotide triphosphatase activities of the bovine viral diarrhea virus NS3 protein are essential for viral replication. J. Virol. 74, 1794–1800.
Lamontagne, J., Mills, C., Mao, R., Goddard, C., Cai, D., Guo, H., Cuconati, A., Block, T., Lu, X., 2013. Screening and identification of compounds with antiviral activity against hepatitis B virus using a safe compound library and novel real-time immune-absorbance PCR-based high throughput system. Antiviral Res. 98, 19–26.
Lin, J.D., Feng, N., Sen, A., Balan, M., Tseng, H.C., McElrath, C., Smirnov, S.V., Peng, J., Yasukawa, L.L., Durbin, R.K., Durbin, J.E., Greenberg, H.B., Kotenko, S.V., 2016. Correction: distinct roles of type I and type III interferons in intestinal immunity to homologous and heterologous rotavirus infections. PLoS Pathog. 12, e1005726.
Malacari, D.A., Pécora, A., Pérez Aguirreburualde, M.S., Cardoso, N.P., Odeón, A.C., Capozzo, A.V., 2018. In vitro and in vivo characterization of a typical and a high pathogenic bovine viral diarrhea virus type II strains. Front Vet. Sci. 5, 75.
Meager, A., 2002. Biological assays for interferons. J. Immunol. Methods 261, 21–36.
Muller, J.A., Harms, M., Schubert, A., Mayer, B., Jansen, S., Herbeuval, J.P., Michel, D., Mertens, T., Vapalahti, O., Schmidt-Chanasit, J., Munch, J., 2017. Development of a high-throughput colorimetric Zika virus infection assay. Med. Microbiol. Immunol. 206, 175–185.
Odeon, A.C., Kelling, C.L., Marshall, D.J., Estela, E.S., Dubovi, E.J., Donis, R.O., 1999. Experimental infection of calves with bovine viral diarrhea virus genotype II (NY-93). J. Vet. Diagn. Investig. 11, 221–228.
Odeon, A.C., Leunda, M.R., Faverin, C., Boynak, N., Vena, M.M., Zabal, O., 2009. In vitro amplification of BVDV field strains isolated in Argentina: effect of cell line and culture conditions. Revista Argentina de microbiologia 41, 79–85.
Pascual, M.J., Merwaiss, F., Leal, E., Quintana, M.E., Capozzo, A.V., Cavasotto, C.N., Bollini, M., Alvarez, D.E., 2018. Structure-based drug design for envelope protein E2 uncovers a new class of bovine viral diarrhea inhibitors that block virus entry. Antiviral Res. 149, 179–190.
Pecora, A., Malacari, D.A., Ridpath, J.F., Perez Aguirreburualde, M.S., Combessies, G., Odeon, A.C., Romera, S.A., Golemba, M.D., Wigdorovitz, A., 2014. First finding of genetic and antigenic diversity in 1b-BVDV isolates from Argentina. Res. Vet. Sci. 96, 204–212.
Perez-Martin, E., Weiss, M., Diaz-San Segundo, F., Pacheco, J.M., Arzt, J., Grubman, M.J., de los Santos, T., 2012. Bovine type III interferon significantly delays and reduces the severity of foot-and-mouth disease in cattle. J. Virol. 86, 4477–4487.
Peterhans, E., Bachofen, C., Stalder, H., Schweizer, M., 2010. Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extinction. Vet. Res. 41, 44.
Ramanathan, P., Zhu, J.J., Bishop, E.A., Puckette, M.C., Hartwig, E., Grubman, M.J., Rodriguez, L.L., 2015. A colorimetric bioassay for high-throughput and cost-effectively assessing anti-foot-and-mouth disease virus activity. Vet. Immunol. Immunopathol. 164, 74–78.
Reed, M.J., Muench, H., 1938. A simple method for estimating fifty percent end points. Am. J. Hyg. 27, 493–497.
Reid, E., Juleff, N., Windsor, M., Gubbins, S., Roberts, L., Morgan, S., Meyers, G., Perez-Martin, E., Tchilian, E., Charleston, B., Seago, J., 2016. Type I and III IFNs produced by plasmacytoid dendritic cells in response to a member of the flaviviridae suppress cellular immune responses. J. Immunol. 196, 4214–4226.
Ridpath, J.F., 2003. BVDV genotypes and biotypes: practical implications for diagnosis and control. Biologicals 31, 127–131.
Rumschlag-Booms, E., Zhang, H., Soejarto, D.D., Fong, H.H., Rong, L., 2011. Development of an antiviral screening protocol: one-stone-two-birds. J. Antivir. Antiretrovir. 3, 8–10.
Tami, C., Taboga, O., Berinstein, A., Nunez, J.I., Palma, E.L., Domingo, E., Sobrino, F., Carrillo, E., 2003. Evidence of the coevolution of antigenicity and host cell tropism of foot-and-mouth disease virus in vivo. J. Virol. 77, 1219–1226.
Vilcek, S., 1994. [Development of PCR tests for the detection of bovine herpesvirus-1, bovine respiratory syncytial viruses and pestiviruses]. Veterinarni medicina 39, 687–700.
Virok, D.P., Eszik, I., Mosolygo, T., Onder, K., Endresz, V., Burian, K., 2017. A direct quantitative PCR-based measurement of herpes simplex virus susceptibility to antiviral drugs and neutralizing antibodies. J. Virol. Methods 242, 46–52.
Yesilbag, K., Alpay, G., Becher, P., 2017. Variability and global distribution of subgenotypes of bovine viral diarrhea virus. Viruses 9.
Yi, M., 2010. Hepatitis C virus: propagation, quantification, and storage. . Current Protocols in Microbiology. Chapter 15, Unit 15D 1.