Development and assessment of a new bioassay for accurate quantification of Type I interferons induced by bovine respiratory viruses

Julie Lion, Gilles Meyer, Mariette Ducatez, Gilles Foucras, Adrien Lion

▶ **To cite this version:**

Julie Lion, Gilles Meyer, Mariette Ducatez, Gilles Foucras, Adrien Lion. Development and assessment of a new bioassay for accurate quantification of Type I interferons induced by bovine respiratory viruses. Journal of Immunological Methods, 2022, 504, pp.113256. 10.1016/j.jim.2022.113256 . hal-04008447

HAL Id: hal-04008447

https://hal.inrae.fr/hal-04008447

Submitted on 28 Feb 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

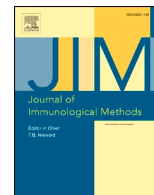
Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

Journal of Immunological Methods 504 (2022) 113256

Contents lists available at ScienceDirect

Journal of Immunological Methods

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



Development and assessment of a new bioassay for accurate quantification of Type I interferons induced by bovine respiratory viruses

Julie Lion, Gilles Meyer, Mariette Ducatez, Gilles Foucras, Adrien Lion *

IHAP, Université de Toulouse, INRAE, ENVT, Toulouse, France

ARTICLE INFO

Keywords:
Bioassay
Bovine type I interferon
Pathogen associated molecular patterns
Respiratory viruses

ABSTRACT

Type I interferon (IFN-I) plays a major role in antiviral and inflammatory processes of the infected host. In the bovine industry, the bovine respiratory disease complex is a major cause of economic and health problems. This disease is caused by interactions of pathogens, together with environmental and host factors. Several pathogens have been identified as causal agents of respiratory diseases in cattle. To better understand how primary infections by viruses predispose animals to further infections by pathogenic bacteria, tools to accurately detect antiviral and immunoregulatory cytokines are needed. To facilitate the detection and quantification of bovine IFN-I, we have established a new specific and sensitive bioassay studies in the bovine host. This assay is based on a Madin–Darby Bovine Kidney (MDBK) cell line that carries a luciferase gene under the control of the IFN-I inducible bovine Mx1 promoter. Specific luciferase activity was measured after stimulation with serial dilutions of recombinant bovine alpha and beta IFNs and human IFN-α. With this novel bioassay we have successfully measured IFN-I production in supernatant from MDBK cells after stimulation of Toll-like receptors (TLR3, TLR7 and TLR8) and RIG-I-like receptors (RIG-I and MDA5), after viral infection with bovine respiratory pathogens, but also in samples from infected calves. Finally, this new bioassay is an easy-to-use and low cost tool to measure the production of bovine Type-I Interferon.

1. Introduction

In response to bacterial or viral infections, hosts mount an innate immune response in which interferons play a major role. This response is induced by the detection of Pathogen Associated Molecular Patterns (PAMPs). Interferons (IFNs) are classified into three subgroups: types I, II, and III IFNs. Theses cytokines are mainly involved in antiviral activity, anti-proliferative activity, stimulation of cytotoxic T cells and modulation of the immune response (Pestka, 2007). Type I interferon (IFN-I) is the main cytokine in the development of antiviral and inflammatory processes in an infected host. Generally, the detection of viral PAMPs by Pattern Recognition Receptors (PRRs) lead to the induction of IFN-α and IFN-β (Randall and Goodbourn, 2008). The PAMPs from RNA viruses are mainly detected by the cytoplasmic receptors Melanoma Differentiation-Associated protein 5 (MDA5), the Retinoic acid-inducible gene (RIG-I), and the Toll-like receptors (TLRs) 3 and 7 (Randall and Goodbourn, 2008). Stimulation of these PRRs causes the activation of the transcription factors NFκB and interferon regulator factors (IRFs) that will lead to the production of several cytokines including IFNs. The binding of the IFN-I on the Interferon alpha/beta receptor (IFNAR) leads to rapid activation of Interferon-Stimulated Genes (ISGs), including myxovirus resistant (MX) genes, by different signaling pathways including the JAK/STAT (Janus Kinase/Signal transducer and activator of transcription), MAPK (Mitogen-activated

Abbreviations: BCoV, bovine coronavirus; BHV-1, bovine herpesvirus type 1; BT, Bovine Turbinate; BPIV-3, bovine parainfluenza type-3 virus; BRDC, Bovine respiratory disease complex; BRSV, bovine respiratory syncytial virus; BVDV, bovine viral diarrhea virus; CAT, chloramphenicol acetyltransferase; d.p.i, days post-infection; ELISA, enzyme-linked immunosorbent assay; hpi, hours post-infection; HRT-18G, Human Rectal Tumor; IDV, influenza D virus; IFN, interferon; ISGs, Interferon-Stimulated Genes; IRF3, Interferon Regulator Factor 3; IU, international units; JAK/STAT, Janus Kinase/Signal transducer and activator of transcription 1; MAPK, Mitogen-activated protein kinases; MDA5, Melanoma Differentiation-Associated protein 5; MDBK, Madin–Darby Bovine Kidney; MOI, multiplicity of infection; Mx, myxovirus resistant; NS1, non-structural protein 1; NS2, non-structural protein 2; PAMPs, Pathogen Associated Molecular Patterns; PI3K-AKT, (Phosphatidylinositol-3-kinase / Protein kinase B; PRRs, Pattern Recognition Receptors; ST, Swine Testis; TCID50, tissue culture infectious dose; TLR, Toll-like receptor.

* Corresponding author.
E-mail address: adrien.lion@envt.fr (A. Lion).

https://doi.org/10.1016/j.jim.2022.113256
Received 7 January 2022; Received in revised form 10 March 2022; Accepted 10 March 2022
Available online 15 March 2022
0022-1759/© 2022 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

protein kinases), NFκB and PI3K-AKT (Phosphatidylinositol-3-kinase / Protein kinase B) pathways (Hervas-Stubbs et al., 2011). However, it is well known that some viruses escape the IFN-antiviral response thanks to an IFN-antagonism function of specific viral proteins such as the nonstructural protein 1 (NS1) of influenza A virus (Randall and Goodbourn, 2008).

Bovine respiratory disease complex (BRDC) is a major disease of young cattle caused by interactions of pathogens, environmental and host factors. These pathogens include viruses and bacteria that are often found in combinations. The most prevalent respiratory viruses are bovine herpesvirus type 1 (BoHV-1), bovine respiratory syncytial virus (BRSV or bovine orthopneumovirus), bovine parainfluenza type-3 virus (BPIV-3), bovine coronavirus (BCoV) and sometimes bovine viral diarrhea virus (BVDV) and influenza D virus (IDV) (Grissett et al., 2015; Mitra et al., 2016; Zhang et al., 2019). The mechanisms by which bovine respiratory viruses modulate the IFN-I response were described for some respiratory viruses. Previous studies have indeed demonstrated that BRSV limits the IFN-I response thanks to nonstructural proteins 1 and 2 (NS1 and NS2) (Bossert et al., 2003; Valarcher et al., 2003). One of the mechanisms described shows that NS1 and NS2 block the activation of interferon regulator factor 3 (IRF3) in infected bovine cells (Bossert et al., 2003). The BVDV counteracts the IFN-antiviral response by the nonstructural proteins N^{pro} and the glycoprotein Erns (Alkheraif et al., 2017; Baigent et al., 2002; Chen et al., 2007; Hilton et al., 2006; Zürcher et al., 2014). It has been demonstrated that N^{pro} inhibits IFN-β activation by down-regulating the IRF3 pathway in BVDV-infected cells (Alkheraif et al., 2017). However, for IDV and BCoV, our knowledge of their ability to hijack the interferon system is very limited. The assessment of IFN-I activity according to the type of viral infection will highlight the potential of some of these viruses to inhibit the production of IFN-I and escape the innate immune response.

To measure bovine IFN-I, IFN-α or IFN-β, several tools are available like classical enzyme-linked immunosorbent assay (ELISA), antiviral assay or non-viral IFN assay. Several manufacturers have developed commercial ELISA kits to quantify bovine IFN-α and IFN-β. A single manufacturer's kit has so far been validated on the bovine model to detect protein in nasal and oral swabs (Cortese et al., 2017; Kroeker et al., 2020). So far, a non-viral IFN assay was used by others. Previous studies used the MDBK-t2 bioassay system, a stable cell line with the chloramphenicol acetyltransferase (CAT) gene under the control of human MxA promoter (Fray et al., 2001). The induction of MxA promoter by IFN leads to the production of the CAT protein, which is measurable by a commercial ELISA kit with anti-CAT antibody. Authors confirmed the specific response of this bioassay by using recombinant bovine IFN-α1. This tool has been successfully used to measure the production of IFN-I from bovine nasal fibroblasts and bronchoalveolar macrophages after BRSV infection (Valarcher et al., 2003). Nevertheless, the previous bioassay developed by Fray *et al* has drawbacks (Fray et al., 2001). Indeed, the CAT gene is under the control of a human MxA promoter in a bovine cell line. Authors tested the sensitivity of their method to bovine IFN-α but not to IFN-β. Moreover, the use of recombinant bovine IFN-α1, with a concentration calculated in international units (IU), as an external reference is prone to errors and precludes comparison between assays and experiments. Indeed, the international unit titer of commercial cytokines is determined *via* cytopathic inhibition assay on infected cells. Finally, this Mx/CAT reporter gene assay bioassay requires the use of a CAT ELISA which remains cumbersome and costly.

The present study reports on the development of a specific, sensitive, rapid,easy, and cheap method to measure the concentrations of bovine IFN-I in various biological fluids and culture supernatants. A luciferase reporter cell line was established by cloning the Firefly luciferase gene under the control of IFN-I inducible bovine Mx1 promoter. This tool has been used to quantify bovine IFN-I in medium of MDBK cells treated with specific agonists of PRRs or infected by different RNA viruses implicated in BRDC, as well as in nasal samples collected on IDV-infected calves.

2. Materials and methods

2.1. Cells

Madin–Darby Bovine Kidney cells (NBL-1, ATCC) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 4.5 g/l glucose (DMEM High Glucose), 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) and incubated at 37 °C in 5% CO2 atmosphere.

2.2. Virus

The influenza D virus strain D/bovine/France/5920/2014 was prepared in Human Rectal Tumor (HRT-18G) cells and titrated on Swine Testis (ST) cells using the tissue culture infectious dose 50 ($TCID_{50}$) method, as previously described (Salem et al., 2019). The bovine coronavirus strain BCoV/France/19-pool-LBA/2014 was isolated from young cattle with respiratory clinical signs and amplified on HRT-18G cells, as previously described (Salem et al., 2020). BCoV production was titrated on ST cells using the TCID50 method. The bovine respiratory syncytial virus isolate 3761 (BRSV-3761) was isolated from a nasal swab of a calf with respiratory clinical signs (Riffault et al., 2006). BRSV-3761 was amplified on Bovine Turbinate (BT) cells and titrated on MDBK cells by the TCID50 method (Riffault et al., 2006). The NADL strain of bovine viral diarrhea virus was amplified and titrated on MDBK cells, as previously described (Mendez et al., 1998).

2.3. Plasmid construction

As previously described, the genomic DNA derived from MDBK cells was used to amplify the Mx promoter by PCR with bMx1_F (5'-AGAGGTACCGTGGGGAGGACACTTGTTGT-3′) and bMx1_R (5'-AGACTCGAGCCGTCCCCAGCGCAGAGA-3′) primers containing the *Kpn*I and *Xho*I sites, respectively (Yamada et al., 2009). The PCR product was digested with *Kpn*I and XhoI enzymes and ligated into KpnI/XhoI sites of the pGL4.10 vector (Promega). The firefly luciferase gene under the control of bovine Mx promoter was digested by restriction enzymes KpnI and *Xba*I and inserted into KpnI/XbaI sites of the pcDNA3.1(+) vector. This vector was named pcDNA3.1(+)pBovMxLuc. The CMV promoter of the pcDNA3.1(−) plasmid was removed by *Bgl*II/BamHI digestion. After purification and self-ligation a pcDNA3.1(−) without CMV promoter was obtained and named pcDNA3.1(−)pCMVless. The pBovMxLuc reporter vector was obtained by the insertion of the fragment containing the firefly luciferase gene and the Mx promoter from the pcDNA3.1(+)pBovMxLuc into the pcDNA3.1(−)pCMVless by the restriction enzymes site KpnI and Bbs1.

2.4. Establishment of a stable cell line

The nucleofection of pBovMxLuc reporter vector was performed in MDBK cells by using the 4D-Nucleofector™ X Unit and the SF Cell Line 4D-Nucleofector™ X Kit L (Lonza) according to the manufacturer's instructions. Briefly, 100 μl of 4D nucleofactor solution containing 5×10^5 cells were mixed with 3 μg of pBovMxLuc reporter vector in nucleocuvette. After using the FS-100 program of the nucleofactor, the nucleofected cells were cultivated into 6-well plates with 400 μl of DMEM High Glucose supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml), and incubated at 37 °C and 5% CO2 for 24 h. After incubation, the medium was replaced with 3 mL of new medium containing 2.5 mg/ml of G418 (Gibco) and incubated for another 3 days. The Neomycin resistance gene of pBovMxLuc reporter vector confers resistance to the G418. The culture medium was replaced with fresh medium containing G418 and cells were re-incubated until the death of non-transfected cells. By repeating

serial dilution in 96-well plate with medium supplemented by 2.5 mg/ml of G418 (Gibco), 8 cell clones were amplified. To select the most relevant clone for the development of the bioassay, each clone was stimulated with 100 ng/ml of bovine IFN-α (Bio-Rad) and the luciferase signal was measured as described below. The clone F11 of MDBK-MxLuc with the highest ratio between the presence and absence of interferon was selected to establish the bioassay.

2.5. Reporter assay

IFN-I protein concentrations were determined by the measurement of IFN-I-related activity to induce luciferase expression in MDBK-MxLuc cells. Briefly, 1. 10^5 cells were seeded into 24 well plates with 1 ml of cell culture medium and incubated at 37 °C in 5% CO2 atmosphere for 24 h. The medium was replaced with 1 ml of a 1:5 dilution of sample in medium supplemented by 10% FBS. Standards were obtained by the use of recombinant bovine IFN-α and IFN-β (PBP019 and PBP032, respectively; BioRad) diluted from 6 pg/ml to 100 ng/ml, and the recombinant human IFN-α (PHP107Z, BioRad) diluted from 0.06 to 1000 IU/ml in medium with 10% FBS. Cells with diluted samples and standards were incubated 8 and 24 h. Cell culture medium was removed, and cells were lysed with 100 μl of Passive Lysis Buffer (PLB, Promega) at room temperature for 30 min. The quantification of the firefly luciferase activity was obtained after mixing 10 l of the lysate and 50 μl Luciferase Assay Substrate (Promega) into the wells of a 96-well opaque white plate and the use of CLARIOstar microplate Reader (BMG LABTECH). Relative bovine Mx promoter activation was calculated as a fold change of the normalized Firefly luciferase activity of the samples and standards to the control condition without interferon. The fold changes values and standard concentrations were log-transformed. The nonlinear regression curves of human IFN-α and bovine IFN-β correspond to the four-parameter dose-response curves calculated on GraphPad software (La Jolla) with at 95% confidence interval. The linear regression curve of bovine IFN-α was calculated on GraphPad software (La Jolla) with at 95% confidence interval. Relative sample concentrations were calculated by using the standard curve using recombinant bovine IFN-α, and according to the dilution factor.

2.6. Stimulation and infection of MDBK to stimulate IFN-I secretion

MDBK cells were stimulated in 24-well plates with poly (I:C) HMW (1 μg/ml), LyoVec Poly(I:C) HMW (500 ng/ml), R848 (1 μg/ml), "all from Invivogen", or infected by IDV (MOI = 1), BCoV (MOI = 1), BRSV (MOI = 0.5) or BVDV (MOI = 0.025). The poly (I:C) HMW, LyoVec Poly (I:C) HMW and R848 were used as agonists of TLR3, RIG-I/MDA5 and TLR7/8 pathways, respectively. At 2, 4, 8 and 24 h post-stimulation or -infection, supernatants were collected to quantify the IFN-I proteins using the MDBK-MxLuc bioassay system. In addition, cell lysate was recovered to quantify IFNA gene expression after mRNA extraction.

2.7. In vivo samples

In a previous study, 8 calves were inoculated by nebulization with 10^7 TCID50 of D/bovine/France/5920/2014 in 10 ml of DMEM medium and 5 control animals were inoculated with 10 ml of DMEM. Nasal swabs were collected daily to 21 days post-infection (dpi) in 1 ml of PBS and stored at −80 °C (Lion et al., 2021). Interferon production from nasal swabs of 4 control and 4 IDV-infected calves collected at 2, 7 and 14 dpi were analyzed in the present study.

2.8. RNA extraction and real time RT-PCR

Total RNA was extracted from stimulated or infected MDBK cells with NucleoSpin RNA kit (Macherey Nagel) according to the manufacturer's instructions. The real-time RT-PCR was performed using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad) and specific primers for IFNA, GAPDH, RPL19 and YWHA7 genes. The IFNA primers are: forward, 5'-CTGCTCTGACAACCTCCCAG-3′ and reverse, 5'-CTCTTCAGCACAGAGGGCTC-3′. The GAPDH primers are: forward 5'-ACAGTCAAGGCAGAGAACGG-3′ and reverse, 5'-GGTTCACGCCCATCACAAAC-3′. The RPL19 primers are: forward 5'-GCAGATCCGGAAGCTGATCA-3′ and reverse, 5'-GGTATCGTCTAAGCAGCCGG-3′. The YWHA7 primers are: forward 5'-CTACCGCTACTTGGCTGAGG-3′ and reverse, 5'-GAGAGCAGGCTTTCTCAGGG-3′. Relative expression (fold change relative to control) of the IFNA gene was calculated using the $2^{-\Delta\Delta CT}$ method with GAPDH, RPL19 and YWHA7 as the reference genes for normalization (Livak and Schmittgen, 2001).

3. Results

3.1. Optimization of incubation time for the bioassay

The incubation time required to activate the luciferase signal from the MDBK-MxLuc with a wide range of recombinant human IFN-α (rhuIFN-α) was first evaluated. A luciferase signal was measured after 8 and 24 h post-stimulation (Fig. 1). A luciferase signal was detected between 0.06 and 1000 IU/ml of rhuIFN-α for both incubation times. The nonlinear regression curves were obtained with 4 parameters. By comparison, after 24 h incubation, the variability amongst experimental replicates was lower than after 8 h. Therefore, an incubation time of 24 h was then validated and selected for further experiments when assaying bovine IFN references and unknown samples.

3.2. Determination of IFN-I standard curves

The recombinant bovine alpha and beta IFNs and human IFN-α were used to identify the best reagents for the external reference of our bioassay (Fig. 2). Luciferase induction was dose-dependent for each interferon tested, but the relations between intensity of luciferase signal and IFN concentration were different. The recombinant human IFN-α induced a signal above background between 0.06 and 1000 IU/ml, with a R^2 of the nonlinear regression curve of 0.99. The bovine IFN-α also induced a positive signal at concentrations between 0.006 and 100 ng/ml. Moreover, this cytokine had a linear regression curve with a $R^2 =$ 0.96 and a limit of detection of 11.8 pg/ml. The bovine IFN-β induced a positive luciferase signal between 0.006 and 100 ng/ml but the nonlinear regression curve, with an $R^2 = 0.99$, showed that the limit of detection was 77.6 pg/ml. Collectively, these data indicate that the MDBK-MxLuc is a sensitive assay to measure bovine IFN-I concentrations in culture medium.

3.3. Stability of the cell clone

To confirm the stability of the MDBK-MxLuc, bioassays were repeated using cells at different passages with human and bovine IFNs (Fig. 3). Regardless of the number of passages, cells emitted a luciferase signal after stimulation with 0.06 to 1000 IU/ml of recombinant human IFN-α (Fig. 3a). A plateau signal was found above 250 units for each cell passage. The R^2 regression coefficient was higher than 0.99 and remained stable, even after 25 passages. The cells were also sensitive to bovine IFN-α until passage 35 with $R^2 = 0.95$ (Fig. 3b). These results show that the established MDBK-MxLuc cell line was stable until at least its 35th passage.

3.4. Quantification of IFN-I from in vitro samples

To validate the usefulness of this bioassay, IFN-I levels were measured in supernatants of MDBK cells stimulated with various PRRs agonists or infected with viruses related to BRD (Fig. 4). The bovine IFNA gene expression was also quantified in cells from the same conditions.

Poly(i:c), lyovec-poly(i:c) and R848 treatments induced a very early

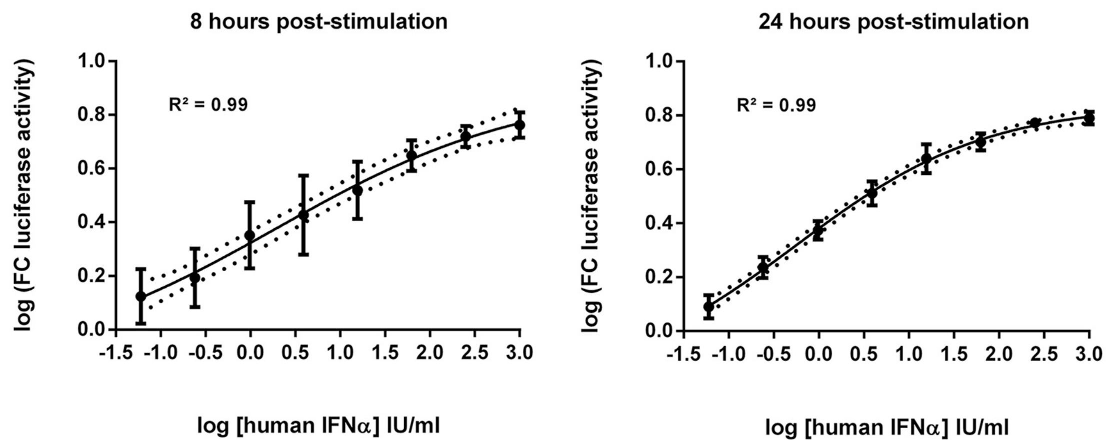


Fig. 1. The luciferase activity of MDBK-MxLuc cells was rhuIFN-α dose dependent after 8 and 24 h post-stimulation.
A total of 1×10^5 cells were seeded into 24 well plates and stimulated with 0.06 to 1000 IU/ml of recombinant human IFN-α for 8 h (left panel) or 24 h (right panel) before cell lysis and measurement of luciferase activity. The fold changes between the luciferase activity of the standard samples and the luciferase activity of the control sample were calculated. Results represent the mean fold changes values with the SEM and standard concentrations transformed in log. The nonlinear regression curve and the R^2 of the equation are represented.

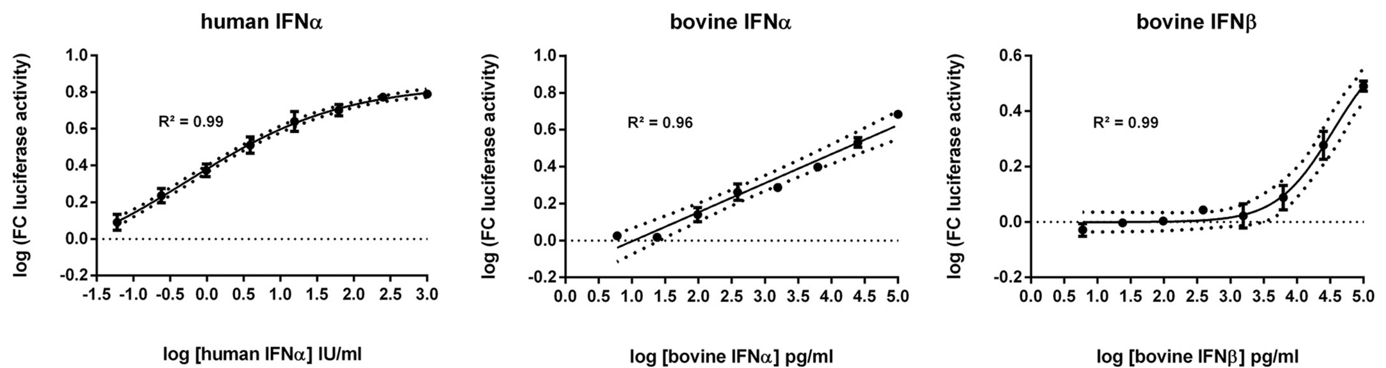


Fig. 2. The MDBK-MxLuc bioassay is sensitive to rbIFN-α and rbIFN-β.
A total of 1×10^5 cells were seeded into 24 well plates and stimulated with 0.06 to 1000 IU/ml of rhuIFN-α, or 6 pg/ml to 100 ng/ml of each rbIFN-α and rbIFN-β for 24 h before cell lysis and measurement of luciferase activity. Results represent the mean fold changes ± SEM. The regression curves and the R^2 are represented.

increase in IFNA gene expression, as expression was maximal at 2 h post-stimulation before a gradual decline (Fig. 4a). In comparison to the control condition, the fold increases were 35.7, 65.2 and 1.7 for poly(i:c), lyovec-poly(i:c) and R848, respectively. By using the MDBK-MxLuc bioassay and the bovine IFN-α standard curve, cytokine synthesis from supernatants was observed 2 to 24 h post-stimulation with a maximum level at 4 h. Thus the maximum IFN-I level was 141, 4877 and 0.4 ng/ml for poly(i:c), lyovec-poly(i:c) and R848, respectively. In comparison to the control condition, the fold increases were 1.4×10^3, 4.8×10^4 and 3.9 for poly(i:c), lyovec-poly(i:c), and R848, respectively. Protein levels remained high in the culture supernatants, even after 4 h, which can be explained by the interferon half-life. The time lag between the maximum levels of mRNA and protein was consistent with the time needed for protein synthesis upon gene trasncription.

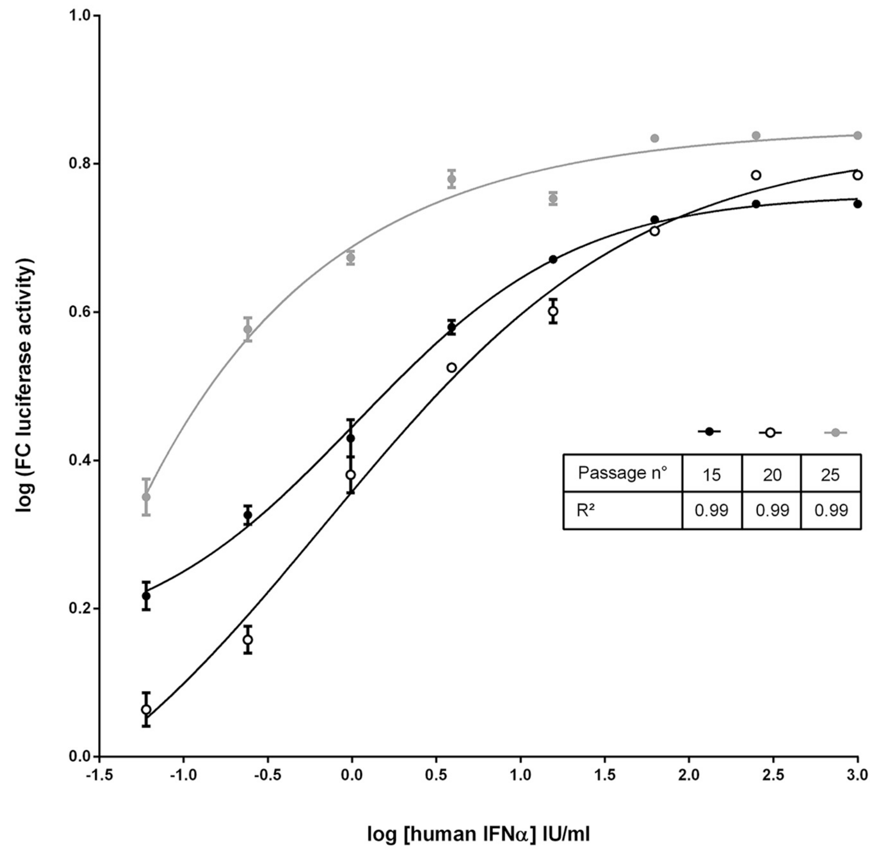
Using the same approach, mRNA (IFN-α) and protein (IFN-I) levels were measured from MDBK cells infected with several viruses (Fig. 4b). Compared to the control condition, viruses induced a rapid upregulation of IFN transcription. Indeed, all infections led to an overexpression of IFNA gene from 2 hpi, and to high levels of IFN-I secretion in supernatants at 4 hpi. In detail, the maximal fold increases of IFNA gene expression were 1.2 and 1.7 at 4 hpi for IDV and BCoV, respectively. BRSV and BVDV infections induced a strong overexpression of IFNA gene at 2 hpi with a fold change of 2.1 and 1.5, respectively. By using the MDBK-MxLuc bioassay system, at 4 hpi, the IFN-I concentrations were, 584, 820, 1092 and 832 pg/ml in the supernatant of MDBK cells infected by IDV, BCoV, BRSV and BVDV, respectively compared to 102 pg/ml in the uninfected control condition. As mentioned above, the time lag observed between the maximum levels of mRNA overexpression at 2 hpi and protein synthesis at 4 hpi was consistent with the time needed to translate mRNA into proteins.

3.5. Quantification of IFN-I from in vivo samples

After validation of this newly developed bioassay to quantify IFN-I production under *in vitro* conditions, the bioassay was confronted to *in vivo* samples to assess its performances as a tool to quantify type I IFNs in a variety of bovine samples (Fig. 5). To this end, IFN-I levels were quantified in nasal swabs collected on four control and four IDV-infected calves from a previous study (Lion et al., 2021). At 2 dpi, the levels of IFN-I were well above the detection limit for all IDV-infected calves with a range of 134 to 3298 pg/ml, while the lowest concentration was a control calf (#9238) with only 121 pg/ml. At 7 and 14 dpi IFN-I concentrations were strongly reduced, indicating that the production was transient. These data indicate that the MDBK-MxLuc bioassay is efficient at quantifying IFN-I from *in vivo* samples.

4. Discussion

In the present study, we developed a specific non-viral bioassay to quantify bovine IFN-I. The use of a gene reporter assay under the control



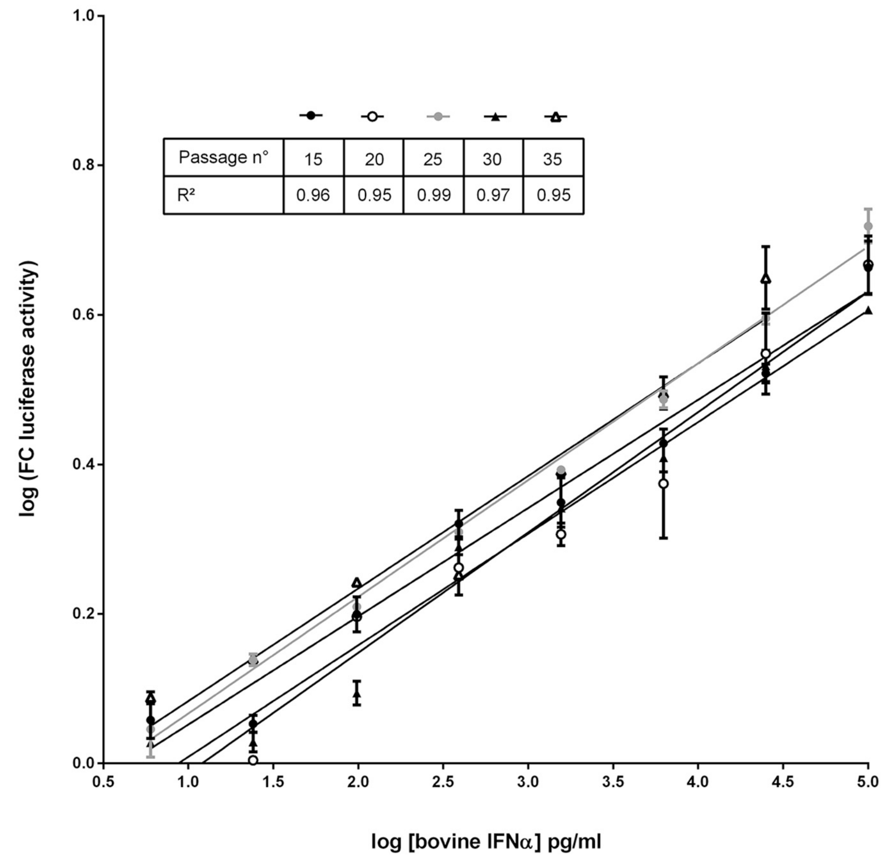


Fig. 3. The MDBK-MxLuc cell line is sensitive to IFN-α at least until passage 35.
Bioassays were performed every five passages of MDBK-MxLuc cells stimulated with 0.06 to 1000 IU/ml of rhuIFN-α (left panel) or 6 pg/ml to 100 ng/ml of rbIFN-α (right panel) during 24 h. The mean fold change activities ± SEM, the regression curves and the R^2 are represented for passages numbers 15 (black circle), 20 (white circle), 25 (gray circle), 30 (black diamond) and 35 (white diamond).

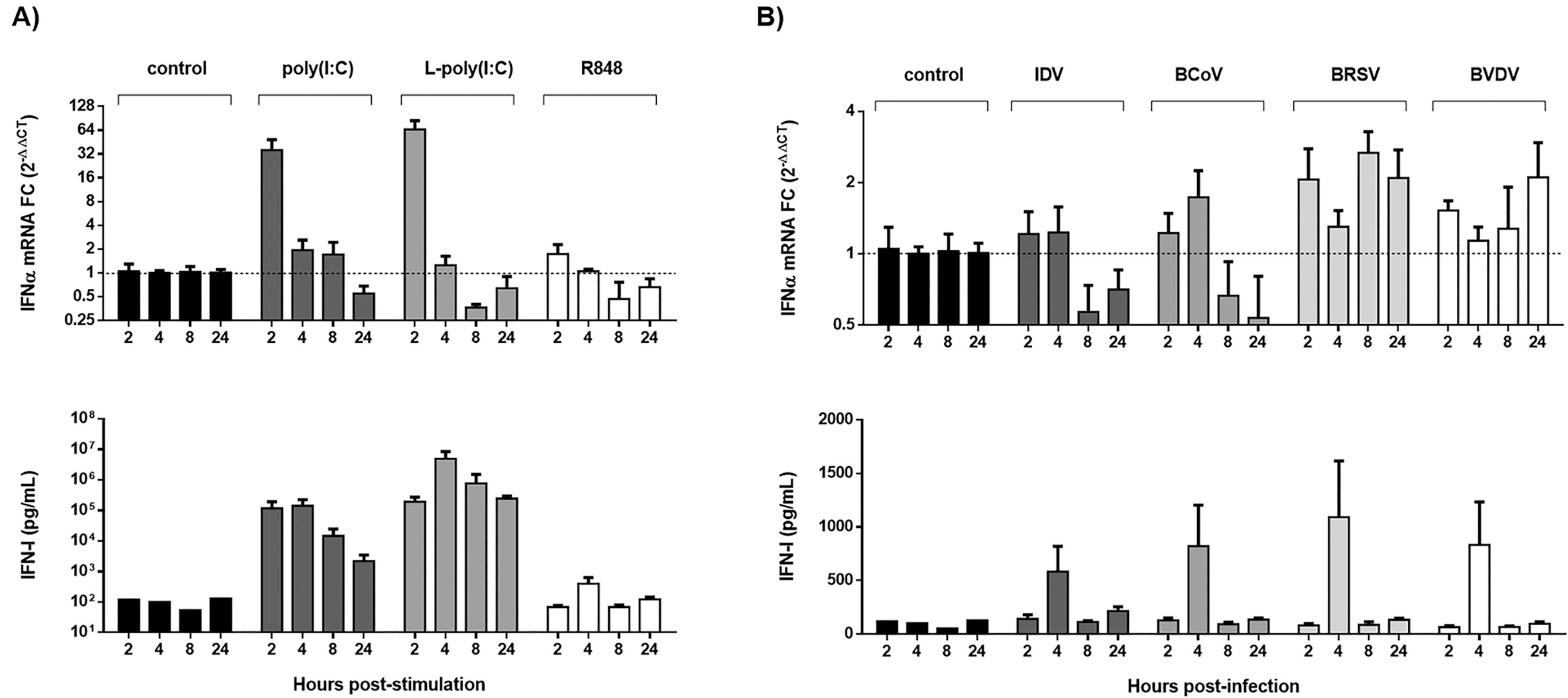


Fig. 4. Detection of IFN-I production from *in vitro* samples by the MDBK-MxLuc cells.
The MDBK cells were stimulated with poly(I:C), Lyovec-poly(I:C), R848 or infected by IDV, BCoV, BRSV or BVDV for 24 h. Total mRNA was extracted from cells to quantify the relative expression of IFN-α by real-time RT-PCR and the IFN-I protein level was measured by the MDBK-MxLuc bioassay with rbIFN-α as standard. (A) Data represent the mean ± SEM of relative IFNA gene expression (top panel) and the IFN-I protein level (bottom panel) from stimulated MDBK cells. (B) Data represent the mean ± SEM of relative IFNA gene expression (top panel) and the IFN-I protein level (bottom panel) from infected cells.

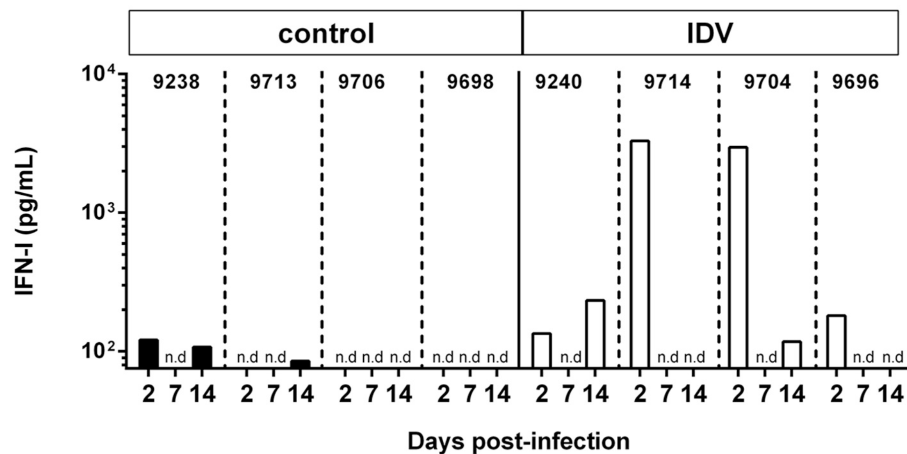


Fig. 5. Detection of IFN-I production from *in vivo* samples by the MDBK-MxLuc assay.
The nasal secretions of four control or IDV-infected calves were collected at 2, 7 and 14 days post-infection. The IFN-I protein level was measured by the MDBK-MxLuc bioassay with rbIFN-α as standard. Data represent the cytokine level of each animal.

of an ISG promoter including Mx to measure IFN-I production has already been described in other models (François et al., 2005; Lleonart et al., 1990; Schwarz et al., 2004). For bovine samples, Fray et al. had developed a MDBK-t2 bioassay system based on MDBK cells with the CAT reporter gene under the control of the human MxA promoter (Fray et al., 2001). However, this MDBK-t2 tool has the disadvantage to require an ELISA kit for the CAT protein quantification, which remains expensive. Moreover, the authors used the recombinant bovine rbIFN-α1 (Novartis) as an external reference which is expressed in international units per milliliter and cannot easily allow for comparison between laboratories and experimental conditions. This tool was previously used to quantify IFN-I from *in vitro* infection by BRSV (Valarcher et al., 2003).

The present model was established to be as sensitive as MDBK-t2 system, but easier to use and less expensive than previously available tool. To increase specificity of the model, the promoter that controls the reporter gene is the specific bovine *Mx1* promoter previously described (Yamada et al., 2009). To reduce the costs, the Firefly luciferase gene was placed under the control of the bovine *Mx1* promoter. Indeed, the total cost for the analysis of 96 samples with our newly developed MDBK-MxLuc bioassay is approximatively 4 times cheaper than when using the CAT-ELISA kit.

After transfection and cloning by limiting dilution, a cell clone was isolated and used to optimize and characterize the new bovine IFN-I bioassay system. As for the pre-existing MDBK-t2 bioassay, the MDBK-MxLuc was sensible to bovine IFN-α and this even after 35 cell passages. In the first model, authors determined a range of induction between 0.063 and 250 IU rbIFN-α/ml (Fray et al., 2001). The rbIFN-α (Novartis) used by the authors was not commercially available, but the use of rbIFN-α (Bio-Rad) confirmed the sensitivity of the MDBK-MxLuc in a dose-dependent manner and with a range of cytokine concentrations between 0.012 and 100 ng/ml. As for the previous model, the present new bioassay can also be used with samples obtained 8 to 24 h post-stimulation. However, the tested samples with low IFN-I concentrations were easier to test after 24 h of incubation. As demonstrated, the MDBK-MxLuc was also sensitive to rbIFN-β with concentrations from 0.078 to 100 ng/ml. Thus, this tool detects IFN-α and IFN-β with high sensitivity. It would be interesting to compare our model with the previous MDBK-t2 system. The replacement of the human promoter by the bovine *Mx1* promoter may improve the sensibility to detect bovine IFN-I. Indeed, while the nucleotide sequences of human and bovine *Mx1* promoters have been shown to be similar, the nature and location of the response elements such as interferon-stimulated response elements (ISREs), GC boxes and Sp1 sites are different (Thomas et al., 2006; Yamada et al., 2009).

To validate the use of MDBK-MxLuc as a new tool to quantify bovine IFN-I, it was assessed with biological samples from *in vitro* stimulation or infection but also from nasal swabs of experimentally infected calves. As expected, MDBK stimulation by PAMPs induced rapid IFN-α transcription from 2 h post-stimulation followed by the production of the protein detected by the bioassay from 4 h post-stimulation. As demonstrated in previous studies, TLR3, TLR7/8 and RIG-I/MDA5 pathways activation by agonists led to an increase in antiviral and pro-inflammatory responses (Carneiro et al., 2017; Chiba et al., 2012; Reid et al., 2021). We showed here for the first time that the activation of these pathways leads to IFN-I synthesis. The detection of IFN-α mRNA increase after MDBK infection by viruses was also confirmed by the quantification of IFN-I production with the bioassay. As mentioned above, these viruses involved in BRDC have already been demonstrated to induce IFN-I synthesis and/or transcription. As for bovine nasal fibroblasts and alveolar macrophages, we confirmed that BRSV infection increases both bovine IFN-α transcription and IFN-I synthesis (Valarcher et al., 2003). Also, we confirmed that BVDV induces IFN-I production from MDBK as previously described (Maldonado et al., 2020). We showed for the first time that BCoV can induce the production of IFN-I *in vitro*. Finally, we confirmed the overexpression of IFNA gene induced by IDV that was previously identified in cattle and mouse models but also in human cells (Oliva et al., 2019; Salem et al., 2019; Skelton et al., 2019). From the *in vitro* and *in vivo* samples, we also confirmed that IDV leads to IFN-I production as observed in human study (Skelton et al., 2019). Indeed, the authors observed that IDV increased IFN-β production in supernatant of A549 cells at 48 hpi with 200 pg/ml (Skelton et al., 2019). In contrast to a previous study where the authors failed to show variations in IFN-I production from nasal secretion of cows vaccinated with Bovi-Shield GOLD FP 5 L5 HB and ENVIRACOR® J-5 (Zoetis) by using an ELISA kit, we were able to demonstrate a strong and rapid IFN-I production in nasal cavities of IDV-infected calves.

In conclusion we have developed a bioassay for the quantification of IFN-I at a low cost, and with high sensitivity and ease of implementation to quantify bovine IFN-I cytokine from various biological samples.

Author contributions

JL and AL designed and carried out experiments; GM, MD, GF and AL wrote the manuscript and prepared figures. All authors reviewed the manuscript.

Declaration of Competing Interest

None.

Acknowledgments

This work was funded by the Ecole Nationale Vétérinaire de Toulouse (ENVT), project BQR-2020-ANIMEX. We thank Thomas Figueroa for his valuable scientific support.

References

Alkheraif, A.A., Topliff, C.L., Reddy, J., Massilamany, C., Donis, R.O., Meyers, G., Eskridge, K.M., Kelling, C.L., 2017. Type 2 BVDV Npro suppresses IFN-1 pathway signaling in bovine cells and augments BRSV replication. Virology 507, 123–134. https://doi.org/10.1016/j.virol.2017.04.015.

Baigent, S.J., Zhang, G., Fray, M.D., Flick-Smith, H., Goodbourn, S., McCauley, J.W., 2002. Inhibition of beta interferon transcription by noncytopathogenic bovine viral diarrhea virus is through an interferon regulatory factor 3-dependent mechanism. J. Virol. 76, 8979–8988. https://doi.org/10.1128/jvi.76.18.8979-8988.2002.

Bossert, B., Marozin, S., Conzelmann, K.-K., 2003. Nonstructural proteins NS1 and NS2 of bovine respiratory syncytial virus block activation of interferon regulatory factor 3. J. Virol. 77, 8661–8668. https://doi.org/10.1128/jvi.77.16.8661-8668.2003.

Carneiro, L.C., Bedford, C., Jacca, S., Rosamilia, A., de Lima, V.F., Donofrio, G., Sheldon, I.M., Cronin, J.G., 2017. Coordinated role of toll-like Receptor-3 and retinoic acid-inducible gene-I in the innate response of bovine endometrial cells to virus. Front. Immunol. 8, 996. https://doi.org/10.3389/fimmu.2017.00996.

Chen, Z., Rijnbrand, R., Jangra, R.K., Devaraj, S.G., Qu, L., Ma, Y., Lemon, S.M., Li, K., 2007. Ubiquitination and proteasomal degradation of interferon regulatory factor-3 induced by Npro from a cytopathic bovine viral diarrhea virus. Virology 366, 277–292. https://doi.org/10.1016/j.virol.2007.04.023.

Chiba, E., Villena, J., Hosoya, S., Takanashi, N., Shimazu, T., Aso, H., Tohno, M., Suda, Y., Kawai, Y., Saito, T., Miyazawa, K., He, F., Kitazawa, H., 2012. A newly established bovine intestinal epithelial cell line is effective for in vitro screening of potential antiviral immunobiotic microorganisms for cattle. Res. Vet. Sci. 93, 688–694. https://doi.org/10.1016/j.rvsc.2011.10.002.

Cortese, V.S., Woolums, A., Hurley, D.J., Berghaus, R., Bernard, J.K., Short, T.H., 2017. Comparison of interferon and bovine herpesvirus-1-specific IgA levels in nasal secretions of dairy cattle administered an intranasal modified live viral vaccine prior to calving or on the day of calving. Vet. Immunol. Immunopathol. 187, 35–41. https://doi.org/10.1016/j.vetimm.2017.04.003.

François, C., Bernard, I., Castelain, S., Charleston, B., Fray, M.D., Capiod, J.-C., Duverlie, G., 2005. Quantification of different human alpha interferon subtypes and pegylated interferon activities by measuring MxA promoter activation. Antimicrob. Agents Chemother. 49, 3770–3775. https://doi.org/10.1128/AAC.49.9.3770-3775.2005.

Fray, M.D., Mann, G.E., Charleston, B., 2001. Validation of an Mx/CAT reporter gene assay for the quantification of bovine type-I interferon. J. Immunol. Methods 249, 235–244. https://doi.org/10.1016/S0022-1759(00)00359-8.

Grissett, G.P., White, B.J., Larson, R.L., 2015. Structured literature review of responses of cattle to viral and bacterial pathogens causing bovine respiratory disease complex. J. Vet. Intern. Med. 29, 770–780. https://doi.org/10.1111/jvim.12597.

Hervas-Stubbs, S., Perez-Gracia, J.L., Rouzaut, A., Sanmamed, M.F., Le Bon, A., Melero, I., 2011. Direct effects of type I interferons on cells of the immune system. Clin. Cancer Res. 17, 2619–2627. https://doi.org/10.1158/1078-0432.CCR-10-1114.

Hilton, L., Moganeradj, K., Zhang, G., Chen, Y.-H., Randall, R.E., McCauley, J.W., Goodbourn, S., 2006. The NPro product of bovine viral diarrhea virus inhibits DNA binding by interferon regulatory factor 3 and targets it for proteasomal degradation. J. Virol. 80, 11723–11732. https://doi.org/10.1128/JVI.01145-06.

Kroeker, A.L., Smid, V., Embury-Hyatt, C., Collignon, B., Pinette, M., Babiuk, S., Pickering, B., 2020. Increased susceptibility of cattle to intranasal RVFV infection. Front. Veterinary Sci. 7, 137. https://doi.org/10.3389/fvets.2020.00137.

Lion, A., Secula, A., Rançon, C., Boulesteix, O., Pinard, A., Deslis, A., Hägglund, S., Salem, E., Cassard, H., Näslund, K., Gaudino, M., Moreno, A., Brocchi, E., Delverdier, M., Zohari, S., Baranowski, E., Valarcher, J.-F., Ducatez, M.F., Meyer, G., 2021. Enhanced pathogenesis caused by influenza D virus and mycoplasma bovis coinfection in calves: a disease severity linked with overexpression of IFN-γ as a key player of the enhanced innate immune response in lungs. Microbiol. Spectr. 9, e0169021 https://doi.org/10.1128/spectrum.01690-21.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Lleonart, R., Näf, D., Browning, H., Weissmann, C., 1990. A novel, quantitative bioassay for type I interferon using a recombinant Indicator cell line. Nat. Biotechnol. 8, 1263–1267. https://doi.org/10.1038/nbt1290-1263.

Maldonado, N., Fredericksen, F., Espiñeira, C., Toledo, C., Oltra, J., de la Barra, V., Fernandez, C., Salvador, J., Villalba, M., Olavarría, V.H., 2020. BVDV-1 induces interferon-beta gene expression through a pathway involving IRF1, IRF7, and NF-κB activation. Mol. Immunol. 128, 33–40. https://doi.org/10.1016/j.molimm.2020.09.018.

Mendez, E., Ruggli, N., Collett, M.S., Rice, C.M., 1998. Infectious bovine viral diarrhea virus (strain NADL) RNA from stable cDNA clones: a cellular insert determines NS3 production and viral cytopathogenicity. J. Virol. 72, 4737–4745.

Mitra, N., Cernicchiaro, N., Torres, S., Li, F., Hause, B.M., 2016. Metagenomic characterization of the virome associated with bovine respiratory disease in feedlot cattle identified novel viruses and suggests an etiologic role for influenza D virus. J. Gen. Virol. 97, 1771–1784. https://doi.org/10.1099/jgv.0.000492.

Oliva, J., Mettier, J., Sedano, L., Delverdier, M., Bourgès-Abella, N., Hause, B., Loupias, J., Pardo, I., Bleuart, C., Bordignon, P.J., Meunier, E., Le Goffic, R., Meyer, G., Ducatez, M.F., 2019. Murine model for the study of influenza D virus. J. Virol. 94 https://doi.org/10.1128/JVI.01662-19 e01662-19, /jvi/94/4/JVI.01662-19.atom.

Pestka, S., 2007. The interferons: 50 years after their discovery, there is much more to learn. J. Biol. Chem. 282, 20047–20051. https://doi.org/10.1074/jbc.R700004200.

Randall, R.E., Goodbourn, S., 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J. Gen. Virol. 89, 1–47. https://doi.org/10.1099/vir.0.83391-0.

Reid, C., Beynon, C., Kennedy, E., O'Farrelly, C., Meade, K.G., 2021. Bovine innate immune phenotyping via a standardized whole blood stimulation assay. Sci. Rep. 11, 17227. https://doi.org/10.1038/s41598-021-96493-3.

Riffault, S., Dubuquoy, C., Castagné, N., Baranowski, E., Charley, B., Eléouët, J.-F., 2006. Replication of Bovine respiratory syncytial virus in murine cells depends on type I interferon-receptor functionality. J. Gen. Virol. 87, 2145–2148. https://doi.org/10.1099/vir.0.82091-0.

Salem, E., Hägglund, S., Cassard, H., Corre, T., Näslund, K., Foret, C., Gauthier, D., Pinard, A., Delverdier, M., Zohari, S., Valarcher, J.-F., Ducatez, M., Meyer, G., 2019. Pathogenesis, host innate immune response, and aerosol transmission of influenza D virus in cattle. J. Virol. 93 https://doi.org/10.1128/JVI.01853-18.

Salem, E., Dhanasekaran, V., Cassard, H., Hause, B., Maman, S., Meyer, G., Ducatez, M., 2020. Global transmission, spatial segregation, and recombination determine the long-term evolution and epidemiology of bovine coronaviruses. Viruses 12, 534. https://doi.org/10.3390/v12050534.

Schwarz, H., Harlin, O., Ohnemus, A., Kaspers, B., Staeheli, P., 2004. Synthesis of IFN-β by virus-infected chicken embryo cells demonstrated with specific antisera and a new bioassay. J. Interf. Cytokine Res. 24, 179–184. https://doi.org/10.1089/107999004322917025.

Skelton, R.M., Shepardson, K.M., Hatton, A., Wilson, P.T., Sreenivasan, C., Yu, J., Wang, D., Huber, V.C., Rynda-Apple, A., 2019. Contribution of host immune responses against influenza D virus infection toward secondary bacterial infection in a mouse model. Viruses 11, 994. https://doi.org/10.3390/v11110994.

Thomas, A.V., Palm, M., Broers, A.D., Zezafoun, H., Desmecht, D.J.-M., 2006. Genomic structure, promoter analysis, and expression of the porcine (Sus scrofa) Mx1 gene. Immunogenetics 58, 383–389. https://doi.org/10.1007/s00251-006-0109-2.

Valarcher, J.-F., Furze, J., Wyld, S., Cook, R., Conzelmann, K.-K., Taylor, G., 2003. Role of alpha/Beta interferons in the attenuation and immunogenicity of recombinant bovine respiratory syncytial viruses lacking NS proteins. J. Virol. 77, 8426–8439. https://doi.org/10.1128/JVI.77.15.8426-8439.2003.

Yamada, K., Nakatsu, Y., Onogi, A., Takasuga, A., Sugimoto, Y., Ueda, J., Watanabe, T., 2009. Structural and functional analysis of the bovine *Mx1* promoter. J. Interf. Cytokine Res. 29, 217–226. https://doi.org/10.1089/jir.2008.0069.

Zhang, M., Hill, J.E., Fernando, C., Alexander, T.W., Timsit, E., van der Meer, F., Huang, Y., 2019. Respiratory viruses identified in western Canadian beef cattle by metagenomic sequencing and their association with bovine respiratory disease. Transbound. Emerg. Dis. 66, 1379–1386. https://doi.org/10.1111/tbed.13172.

Zürcher, C., Sauter, K.-S., Schweizer, M., 2014. Pestiviral E(rns) blocks TLR-3-dependent IFN synthesis by LL37 complexed RNA. Vet. Microbiol. 174, 399–408. https://doi.org/10.1016/j.vetmic.2014.09.028.