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Quantitative and rapid interference of bovine viral diarrhea virus BVDV/END⁻ and BVDV/END⁺ strains

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Summary. – Homologous interference *in vitro* is defined as the ability of primary viral infection to prevent secondary homologous superinfection. Non-cytopathic bovine viral diarrhea virus (ncp BVDV) has been classified according to the exaltation of Newcastle disease phenomenon (END) as END positive (E^+) and END negative (E^-) strains. Simultaneous inoculation of MDBK-SY cell monolayers with BVDV/E^- virus and a three log higher amount of BVDV RK13/E^+ virus, leads to acquisition of the BVDV/E^- feature of blocking Newcastle disease virus (NDV) infection in cells. BVDV/E^- strains, particularly at a high titre and MOI ≥1.25, can exert and impose their effects in BVDV/E^+ infected cells; however, if BVDV/E^- MOI is reduced to MOI below 0.625, the BVDV/E^+ effect can be restored leading to cytopathic effects (CPE) induction by NDV reciprocal to the titre of the BVDV RK13/E^+ strain. Moreover, blocking and prevention of induced CPE by NDV or vesicular stomatitis virus (VSV) occurs even when BVDV/E^- superinfects primary BVDV/E^+ infected cells, indicating a defective homologous interference between BVDV/E^+ and BVDV/E^- strains. Taken together, BVDV/E^- strains have a strong competitive potency and mediate a fast acting (i.e. within 60 min) influence against BVDV/E^+ activity. This may be relevant *in vivo* where BVDV/E^- and BVDV/E^+ combinations are frequently isolated from infected individuals.

Keywords: BVDV; END phenomenon; homologous interference; NDV; VSV

Introduction

Bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV) belong to the genus Pestivirus. The exaltation of Newcastle disease virus (END) phenomenon can be used for titration of non-cytopathic (ncp) CSFV as well as BVDV strains. Based on this characteristic, ncpBVDV strains are subclassified into: a) END positive (E^+) strains, which induce cytopathic effects (CPE) upon challenge with Newcastle disease virus (NDV), and b) END negative (E^-) strains, which competently inhibit CPE induction by heterologous viruses, such as NDV and vesicular stomatitis virus (VSV) in cell culture (Inaba *et al.,* 1963; Nishine *et al.,* 2014).

However, the genetic and functional differences between E^+ and E^- BVDV strains are not completely understood. Type I interferons are major components of the host innate immune response, and accordingly, many viruses have evolved mechanisms to modulate the host response during infection (Kozasa *et al.,* 2015). In contrast to E^- strains, E^+ strains possess the N^{pro} autoprotease which plays a role in blocking type I interferon (IFN1) induction, by preventing

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Abbreviations: BVDV = bovine viral diarrhea virus; CPE = cytopathic effects; END = exaltation of Newcastle disease phenomenon; ncp = non-cytopathic effects; NDV = Newcastle disease virus; VSV = vesicular stomatitis virus

synthesis of interferon regulatory factor 3 (IRF3), leading to subversion of host innate antiviral immune response (Gottipati *et al*., 2013). Linked to this mechanism, we observed the intrinsic difference of BVDV/E$^+$ and /E$^-$ strains active against heterologous viruses (NDV and VSV) in an *in vitro* culture system. Unexpectedly, we observed a strong influence by secondary BVDV/E$^-$ infection on primary BVDV/E$^+$ infected cells, which is considered as a one-way dominance in homologous interference between ncpBVDV strains. Homologous interference or superinfection exclusion *in vitro* is defined as the ability of primary infection to prevent secondary homologous virus superinfection. This has been described for various virus-host systems, such as pestiviruses (Qu *et al.,* 2001), and especially for BVDV (Kozasa *et al.,* 2015).

Materials and Methods

Cell culture and viruses. Madin-Darby bovine kidney (MDBK-SY) cells were grown and confirmed to be free of adventitious pestivirus (tested by RT-PCR) as previously described (Muhsen *et al.,* 2013a). MDBK-SY cells were seeded into 96-well flat-bottom plate microplates at a density of 2×10^5 cells/plate (~2×10^3cells /well). The cells were enumerated by using a 0.100 mm depth, improved Neubauer type haemocytometer (SLGC, Japan). BVDV strains (RK13/E$^-$, No.12 /E$^-$, Nose/E$^-$ and RK13/E$^+$) were propagated in MDBK-SY cells for virus stock preparation. No.12 /E$^-$ and Nose/E$^-$ were used as field strains for comparison with isolated RK13/E$^-$ tissue culture strain. NDV Miyadera strain and VSV New Jersey serotype were used as heterologous challenge viruses for BVDV/E$^+$ and /E$^-$ strains, respectively (Muhsen *et al.,* 2013b). The BVDV titre was calculated by endpoint dilution method, according to the property of END interference as previously described (Muhsen *et al.,* 2013a).

Mixture assay. RK13/E$^-$ strain was prepared with a titre of 10^5 $TCID_{50}$/ml. RK13/E$^+$ strain was prepared at titres of 10^5, 10^6, 10^7, and 10^8 $TCID_{50}$/ml. Equal volumes (50 µl) of RK13/E$^-$ strain and RK13/E$^+$ strain were mixed generating 4 different mixed virus concentrations (RK13/E$^-$and RK13/E$^+$): Mixture 1 (1:1), Mixture 2 (1:10), Mixture 3 (1:100), and Mixture 4 (1:1000). Two additional mixtures were prepared: Mixture 5 (1:1000) contains equal volumes of No.12/E$^-$ and RK13/E$^+$ strains, at titres of 10^5 and 10^8 $TCID_{50}$/ml, respectively. Mixture 6 (1:1000) contains equal volumes of Nose/E$^-$ and RK13/E$^+$ strains, at titres of 10^5 and 10^8 $TCID_{50}$/ml, respectively. Each of the six mixtures was prepared in 2-fold dilutions: x2, x4, and x8 (Fig. 1); followed by 10-fold dilutions from 10^{-1} to 10^{-8}, in parallel (data not shown). One hundred µl of diluted and undiluted mixtures were separately inoculated per well onto 1-day-old cultured MDBK-SY cells. Following five days incubation at 37°C in a 5% CO_2 humid incubator, cell monolayers were subsequently exposed to the NDV Miyadera strain. CPE development in cells was observed via light microscopy using an Olympus CKX41 inverted microscope (Olympus Corp, Japan).

Table 1. Blockage of VSV/NDV-induced CPE by BVDV/E$^-$ added 5 days after E$^+$ infection of MDBK-SY cells

		CPE appearance following superinfection of E$^+$-infected MDBK-SY cells with BVDV/E$^-$ at day 5		
		RK13/E$^-$	No.12/E$^-$	Nose/E$^-$
2-folds dilution	Undiluted virus	–[a]	–	–
	x 2	–	–	–
	x 4	+/-[b]	+/-	+/-
	x 8	+[c]	+	+
	x 16	+	+	+

[a]–: No CPE, [b]+/-: weak CPE induction, [c]+: CPE.

Two step interference assay. MDBK-SY cells were seeded together with 50 µl 10^5 $TCID_{50}$/ml of RK13/E$^+$ strain/well in a 96 well microplate. After five days of incubation, RK13/E$^+$ infected cell monolayers were exposed to 50 µl/well of BVDV/E$^-$ (RK13/E$^-$, No.12/E$^-$, and Nose/E$^-$) at a starting titre of 10^5 $TCID_{50}$/ml, and serial dilutions of x2, x4, x8, and x16 using EMEM, respectively (Table 1). After three days, cultures were challenged in parallel with NDV or VSV and then observed for CPE exhibition. VSV induces strong CPE not only in the E$^+$ infected culture, but also in the non-infected culture; therefore, blocking of VSV can only be observed in E$^-$ infected cells.

Results and Discussion

Inoculation of cell culture with equal amounts of pestivirus CSFV/E$^-$ and /E$^+$ leads to CPE exhibition after NDV challenge (Shimizu *et al.,* 1969), implying a potential inherent balanced strain activity between /E$^+$ and /E$^-$ (Inaba *et al.,* 1963). However, this does not appear to be the case in our study; instead, this process is dominated by the E$^-$ virus, with minor influence from BVDV/E$^+$ virus, in MDBK-SY cells. All cells exposed to diluted and undiluted (x2, x4, and x8) RK13/E$^-$ virus demonstrated CPE inhibition upon NDV inoculation, contrasting to cells exposed to RK13/E$^+$ virus which supported CPE induction. Interestingly, gradual increase of E$^+$ virus (up to 3 log greater) in mixtures did not abolish the E$^-$ induced inhibition of NDV-dependent CPE at the level of undiluted or x2 diluted mixtures. Incomplete CPE observation occurred at all x4 dilution mixtures; whereas all exposed cell mixtures at dilutions x8 demonstrated clear CPE by NDV (Fig. 1).

In both the mixture and two step interference assays, the maintenance of infective virus particle load of BVDV/E$^-$ strain was determined to be ~2,500/50 µl (titre $10^{4.7}$ $TCID_{50}$/ml in the x2 dilution). The number of exposed MDBK-SY cells was ~2 $\times10^3$cells/well in the 96-well plate. Taken together,

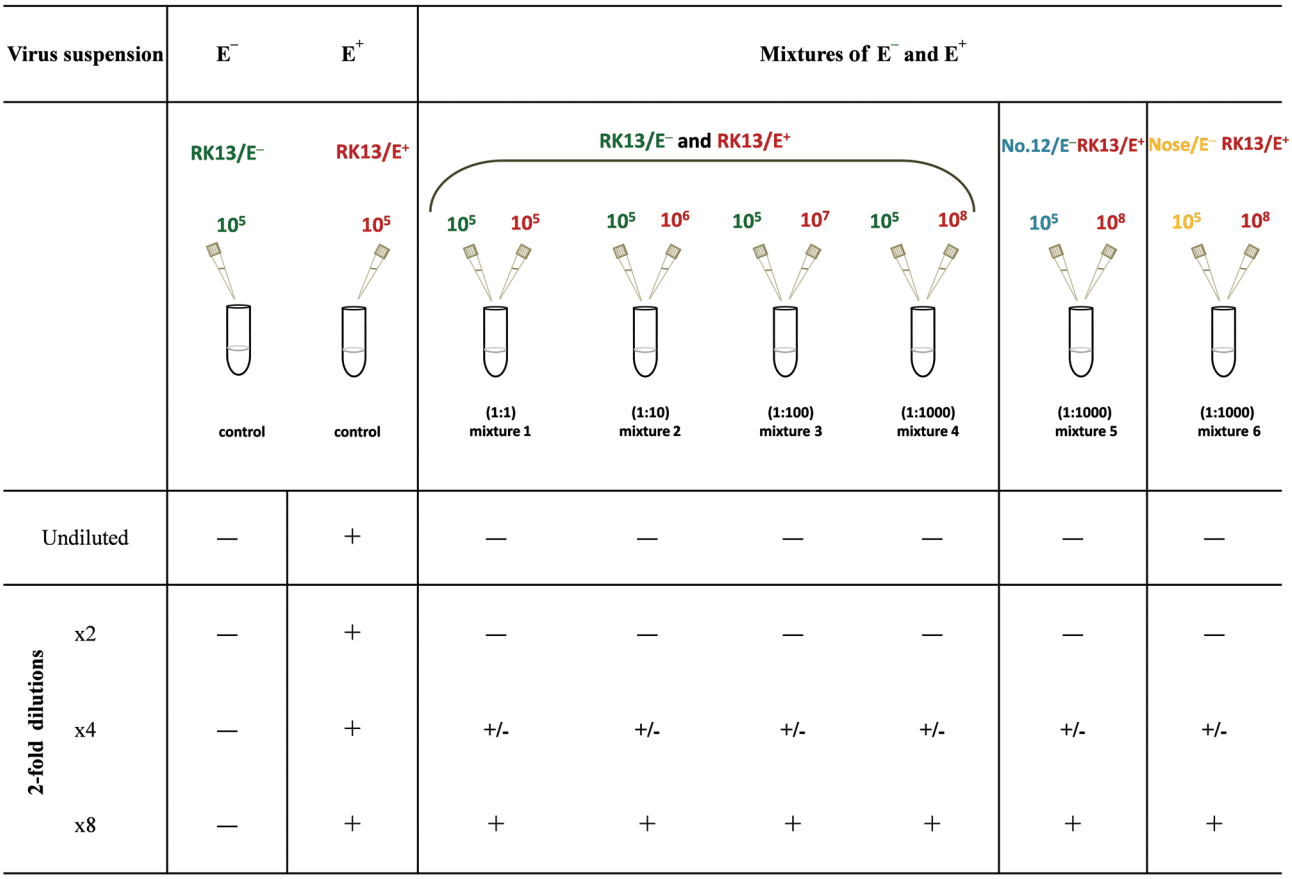

Fig. 1

Overview of the mixture assay

RK13/E⁺ infected MDBK-SY cells exhibited clear CPE (100%) upon NDV challenge (+). The E⁻ infected cells prevented any NDV-induced CPE (–). The weak/incomplete CPE indicates presence of E⁻ strain together with RK13/E⁺ strain in the exposed culture (+/–) at a dilution x4 of all mixtures. The BVDV/E⁻ (RK13/ E⁻, No.12/E⁻ and Nose/E⁻) strains stably blocked NDV-induced CPE, even in the presence of three log higher concentration of RK13/ E⁺ strain in the undiluted and diluted (x2) mixtures; whereas enhancement of NDV-induced CPE was clearly increased up to mixture dilution x8 without any indication of E⁻ function.

inoculation of BVDV/E⁻ strains (RK13/E⁻, No.12/E⁻, or Nose/E⁻), at a multiplicity of infection (MOI) ≥1.25, onto monolayers of normal or RK13/E⁺ infected cells (MOI 2,500 and 1,250 at undiluted and x2 dilution mixtures, respectively), forces the cells to acquire a BVDV/E⁻ status leading to competent and efficient interference against heterologous NDV- or VSV- induced CPE. However, if the BVDV/E⁻ MOI is reduced to <0.625, the BVDV/E⁺ effect can be restored, leading to CPE induction by NDV reciprocal to the titre of RK13/E⁺.

The required time for establishing a primary BVDV infection and blocking secondary infection in MDBK-SY cells has been reported to be one hour (Lee *et al.*, 2005). However, even when BVDV RK13/E⁺ primarily infects MDBK-SY cells, a strong dose-dependent effect of the secondary infection with undiluted or x2 diluted BVDV/E⁻ strain is evident. Delaying exposure of RK13/E⁺ (10^5 TCID50/ml) infected cells to the undiluted or x2 diluted BVDV/E⁻ (RK13/E⁻, No.12/E⁻, or Nose/E⁻) (10^5 or $10^{4.7}$ TCID50/ml, respectively) prevented CPE induction after challenge with VSV strain. A weak, incomplete CPE induction was observed at x4 dilution, whereas clear CPE occurred at x8 and x16 dilutions (Table 1). Identical results were obtained by using NDV instead of VSV as a hetero-challenge virus (Table 1). Surprisingly, shortening the interval time, between E⁻ virus superinfection and NDV or VSV challenge, from 3 days to 60 minutes did not render any alteration to the amount or type of exhibited CPE. We speculate that this subsequent, postponed phenomenon is based on a disturbance of the IFN type I pathway by ncpBVDV/END⁺ (Ruggli *et al.*, 2009). The IFN type I counteracting function of viral autoprotease N^{pro} protein in E⁺ pestiviruses has been recently demonstrated *in vitro* (Kozasa *et al.*, 2015) which might be the homologous interference loophole that BVDV/E⁻ may

exploit. With as little as one hour needed, probably linked to time-dependent IFN induction, BVDV/E$^-$ can develop its potent effect.

In conclusion, our study suggests that BVDV/E$^-$ strains, particularly at a high titre and MOI ≥1.25, can exert and impose their effects onto BVDV/E$^+$ infected cells, indicating a defective homologous interference between BVDV/E$^+$ and BVDV/E$^-$ strains. Moreover, BVDV/E$^-$ strains have a strong competitiveness and fast acting (within 60 min) influence against BVDV/E$^+$ activity. This may be relevant *in vivo* where BVDV/E$^-$ and BVDV/E$^+$ combinations are frequently isolated from infected individuals. Further investigation is required to clarify the underlying mechanism of BVDV/E$^-$ superinfection with regards to its ability of regulating INF type i induction and virus replication.

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References

Gottipati K, Ruggli N, Gerber M, Tratschin JD, Benning M, Bellamy H, Choi KH (2013): The structure of classical swine fever virus Npro: a novel cysteine autoprotease and zinc-binding protein involved in subversion of type i interferon induction. PLoS Pathog. 9, e1003704. https://doi.org/10.1371/journal.ppat.1003704

Inaba Y, Omori T, Kumagai T (1963): Detection and measurement of non-cytopathogenic strains of virus diarrhea of cattle by the END method. Arch. Gesamte Virusforsch. 13, 425–429. https://doi.org/10.1007/BF01244615

Kozasa T, Abe Y, Mitsuhashi K, Tamura T, Aoki H, Ishimaru M, Nakamura S, Okamatsu M, Kida H, Sakoda Y (2015): Analysis of a pair of END+ and END- viruses derived from the same bovine viral diarrhea virus stock reveals the amino acid determinants in Npro responsible for inhibition of type i interferon production. J. Vet. Med. Sci. 77, 511–518. https://doi.org/10.1292/jvms.14-0420

Lee YM, Tscherne DM, Yun SI, Frolov I, Rice CM (2005): Dual mechanisms of pestivirus superinfection exclusion of entry and RNA replication. J. Virol. 79, 2231–2241. https://doi.org/10.1128/JVI.79.6.3231-3242.2005

Muhsen M, Aoki H, Ikeda H, Fukusho A (2013a): Biological properties of bovine viral diarrhea virus quasispecies detected in the RK13 cell line. Arch. Virol. 158, 753–763. https://doi.org/10.1007/s00705-012-1538-x

Muhsen M, Ohi K, Aoki H, Ikeda H, Fukusho A (2013b): Competitive virus assay method for titration of noncytopathogenic bovine viral diarrhea viruses (END+ and END- viruses). J. Virol. Methods 188, 6–12. https://doi.org/10.1016/j.jviromet.2012.11.030

Nishine K, Aoki H, Sakoda Y, Fukusho A (2014): Field distribution of END phenomenon-negative bovine viral diarrhea virus. J. Vet. Med. Sci. 76, 1635–1639. https://doi.org/10.1292/jvms.14-0220

Qu L, McMullan LK, Rice CM (2001): Isolation and characterization of noncytopathic pestivirus mutants reveal a role for nonstructural protein NS4B in cytopathogenicity. J. Virol. 75, 10651–10662. https://doi.org/10.1128/JVI.75.22.10651-10662.2001

Ruggli N, Summerfield A, Fiebach AR, Guzylack-Piriou L, Bauhofer O, Lamm CG, Waltersperger S, Matsuno K, Liu L, Gerber M, Choi KH, Hofmann MA, Sakoda Y, Tratschin JD (2009): Classical swine fever virus can remain virulent after specific elimination of the interferon regulatory factor 3-degrading function of Npro. J. Virol. 83, 817–829. https://doi.org/10.1128/JVI.01509-08

Shimizu Y, Furuuchi S, Hayashi S, Kumagai T, Sasahara J (1969): Porcine kidney cell line persistently contaminated with a virulent swine fever virus. J. Gen. Virol. 4, 625–628. https://doi.org/10.1099/0022-1317-4-4-625