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Infection with the Noncytopathic BVDV-2 Strain 890 Prevents Replication of Superinfecting Cytopathic BVDV-1 RNA in BT and MDOK Cells

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Reports of nonhomologous recombination-events between persistently infecting and vaccinal strains of *Bovine virus diarrhea virus* (BVDV) indicate that mixed pestivirus infections occur in vivo and that bovine cells support replication of RNA from BVDV-1 and BVDV-2 (Ridpath and Bolin, 1995; Becher et al., 2001). BVDV type-specific molecular beacons were used to estimate the effect of the presence of two BVDV RNA genotypes on the RNA relative replication efficiency of each genotype by comparing Ct values obtained from the same reaction tube in a real-time RT-PCR. This was done in bovine and ovine cultured cells following simultaneous- or super-infection with different biotypes and genotypes of BVDV. The noncytopathic BVDV-2 890 blocked superinfection with the cytopathic BVDV-1 strain Singer in both BT and MDOK cells. The cytopathic BVDV strains greatly reduced the relative concentration of superinfecting but not co-infecting cytopathic and noncytopathic BVDV-2 RNA in BT cells. On the other hand, superinfection of Singer infected cells with BVDV-2 890 increased BVDV-2 890 RNA relative concentration in MDOK cells.

INTRODUCTION

Pestiviruses are a group of closely related small RNA viruses belonging to the family *Flaviviridae* (Becher *et al.*, 1999b). *Bovine viral diarrhea virus* (BVDV) genotype 1 (BVDV-1), BVDV-2, and *border disease virus* (BDV) are ruminant pestiviruses that cause primary enteric, respiratory, reproductive and immune system diseases in bovine, ovine, and wild ruminants (Tessaro *et al.*, 1999; Van Campen and Williams, 1995). The severity of the clinical signs depends on the virulence of the viral strain, host species (Tessaro *et al.*, 1999), physiological condition, age and immune status (Baker, 1995; Tessaro *et al.*, 1999).

Two *Pestivirus* biotypes exist, cytopathic (CP) and noncytopathic (NCP). In utero NCP *Pestivirus* infections that occurs prior to the development of the fetal immune system may result in the birth of persistently infected (PI) animals that are a continuous source of virus to herd-mates (Baker, 1995). Most PI calves are born to cows acutely infected during gestation (Wittum *et al.*, 2001). Mucosal disease (MD), a severe and often fatal condition, is caused by superinfection of the PI animal with a CP virus or by mutations of the persistently infecting NCP virus into a CP virus (Baker, 1995).

Modified live virus (MLV) vaccines are used in *Pestivirus* control programs. MLV vaccines have the potential to revert to virulence. Moreover, the clinical consequences of *Pestivirus* superinfection of animals are severe. RNA recombination

between a persisting *Pestivirus* and a vaccine strain generate CP viruses and induce lethal MD (Becher *et al.*, 2001). Nonhomologous recombinations with vaccinal strains of BVDV indicates that superinfection with *Pestiviruses* occurs in nature (Ridpath and Bolin, 1995). The incidence of superinfection events and the factors that influence the outcome of these events remain largely unknown.

Pestivirus RNA type-specific molecular beacons have been developed in our laboratory for detection of single or mixed type *Pestivirus* RNA from extracts of biological samples and tissue culture homogenates (Yousif *et al.*, 2002). Molecular beacons technology is currently used as a method of analysis of DNA sequences, and quantitation of virus loads during the course infections or drug therapy (Piatek, *et al.*, 1998). Multiplexing molecular beacons is possible because nucleotide mismatches in template sequences dramatically decreases the stability of the beacon-template hybrid is in the presence of (Tyagi *et al.*, 1998). Molecular beacon analysis of relative RNA levels during mixed *Pestivirus* infection may provide insight on some possible factors influencing nonhomologous recombination events in nature.

In this communication we investigated the effect of virus biotype, genotype as well as host cell origin on *Pestivirus* RNA replication during mixed infection events, simultaneous infection and superinfection. Comparison of the BVDV-1 and BVDV-2 RNA levels in cell extracts in mixed infections was done by comparing Ct values of RT-PCR utilizing pestivirus NS3-type-specific molecular beacons.

MATERIALS AND METHODS

Viruses and cells

NCP BVDV-2 890, CP BVDV-2 125 and CP BVDV-1 Singer were obtained from the NVSL, Ames, IA. Madin-Darby ovine kidney (MDOK) and bovine turbinate (BT) cells were obtained from the American Type Culture Collection (ATCC) (Rockville, Md.). Cells were cultured in minimum essential medium (MEM) (Gibco BRL, Rockville, Md.) supplemented with 10% irradiated-BVDV negative fetal bovine serum (FBS) (HyClone, Logan, Utah). The media also contained 100 IU/ml of penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone (Freshney, 2000). Cell cultures were split 1:3 when needed (confluency >95%) (Freshney, 2000). Mock-infected cultures were used as NS3 RT-PCR negative control. Mock-infected cultures were also screened for the presence of NCP virus contamination using the E^{rns}-based nested RT-PCR (Sullivan and Akkina, 1995). Cells were seeded in 24-well tissue culture plates at a concentration of $1X10^5$ cells/well 12 hr before infection.

Primer and Beacon design

Molecular beacons and the pestivirus NS3 universal primers (Integrated DNA Technologies, Inc., Coralville, IA) were designed based on *Pestivirus* sequence comparisons of the *Pestivirus* NS3 gene (Yousif *et al.*, 2002). Sequences of the primers and their polarities are as follows: SPTL (5'-CAAGGAGGGATCAGCTCGGTAGAC-3'), SPTU (5'-CTTCGGACACCTGAGTCGG-3'), (Yousif *et al.*, 2002).

The sequences of the two molecular beacons were: BVDV-1 TET beacon (5' TET-CCGACGAGAGTGGTTTGCCAAA

GCAACCGTCGG- DABCYL 3'). BVDV-2 FAM beacon (5' FAM-CCGACGTATGTGACACTATGGGC CCGTCGG- DABCYL 3').

Viral infection, RNA extraction and molecular beacon assay

Simultaneous infection and superinfection experiments were done in BT and MDOK cells using 1 MOI of each BVDV genotype according to the flow chart, Fig. 1. All treatment combinations were done in triplicates for each cell type, Fig 1. RNA from virus-infected cultures was extracted as indicated, Fig. 1, using the RNeasy Mini Kit (Qiagen, Valencia, California). Viral RNA extracts were quantitated spectrophotometrically. RNA was extracted 24 hr after simultaneous infection and 12 hr after superinfection of cells. RNA from each of the triplicate treatment well was pooled and tested in triplicates.

The total RNA amount used in the molecular beacons assay was fixed for each cell type. The RNA concentration from BT cells was 136 µg/ml while the total RNA concentration used from the MDOK cells was 56 µg/ml.

Two microliters of total RNA were added to each RT-PCR reaction mix containing 0.75 U AmpliTaq™ DNA polymerase (PE Applied Biosystems, Foster City, CA), 2 mM $MgCl_2$, 250 µM of dNTPs, 2 µM each PCR primer, 2.5 U/µl of the MuLV, RT and 1U/µl RNase inhibitor. In addition, the reaction contained 25 ng Type 1-TET beacon and 50 ng Type 2-FAM beacon. The volumes were adjusted to allow a final volume of 50 µl per reaction. Amplification was performed in ABI PRISM® 96-well reaction plates (Part No. 4314320, PE Applied Biosystems, Foster City, CA) sealed with optical adhesive cover (Part No. 4311971, PE Applied Biosystems, Foster City, CA). The ABI PRISM® 7700 sequence detection system (PE Applied Biosystems, Foster City, CA) was used. PCR plates were incubated 5 min at 50 ^{0}C, 10 min at 48 ^{0}C and 15 min 42 ^{0}C to allow cDNA synthesis. This was followed by a five-minute hold at 95 ^{0}C to inactivate the reverse transcriptase. Thirty-seven cycles of amplification were done using the following reaction conditions: template denaturation, 94 ^{0}C for 1 min., primer annealing, 61.5 ^{0}C for 1 min., and extension, 72 ^{0}C for 1 min. The final extension was at 72 ^{0}C for 10 min. For each reaction, the threshold cycle (Ct) indicates the cycle where the reported signal was higher than the cutoff point. Samples were considered positive when their normalized reporter value (ΔRn) exceeded that of the cutoff point indicated by the horizontal black bar above the X axis in the result plots. Within the same reaction well, comparisons between the relative replication efficiency of BVDV-1 and BVDV-2 RNA in the same RNA extracts were made by comparing the Ct values for amplification curves of each genotype's molecular beacon. Analysis was done using the Ct values (reflecting the relative amount of pestivirus RNA concentration in a given sample of total infected cell RNA) to avoid inconsistencies associated with RNA extraction and reverse transcription.

Mathematical representation of results

The effect of coinfection [simultaneous infection or superinfection] with a second pestivirus on the relative RNA concentration of a given *Pestivirus* after 12 hr was termed coinfection effect (CoE_{12}). CoE_{12} was calculated as follows: CoE_{12} = [(Ct of virus at 12 Hr of primary infection Ct of virus at 12

Hr of coinfection)-1]. CoE_{12} calculated following simultaneous infection and superinfection experiments is terms CoEsi and CoEsu, respectively. The results of CoE_{12} were plotted using Excel (Microsoft® Excel 97).

RESULTS

The effect of prior infection of BT or MBOK cells with BVDV-2 890 followed by superinfection with BVDV-1 Singer

Prior infection of BT or MBOK cells with BVDV-2 890 inhibited the superinfecting BVDV-1 Singer RNA replication. BVDV-1 Singer RNA was not detected 12 hr after superinfection of BT and MDOK cells that had received 1 MOI of BVDV-2 890 12 hr prior to superinfection, Fig. 2B. The Ct value given for BVDV-1 Singer results used for CoE_{12} calculation was 38 (indicating that it is below detection level). Interestingly, BVDV-2 890 RNA was below detection level in the MDOK infected cell extracts when used for primary infection or coinfection, Fig. 2A.

The effect of simultaneous infection with BVDV-2 890 and BVDV-1 Singer on the relative RNA concentration in MDOK and BT cells

Simultaneous infection with BVDV-2 890 did not inhibit BVDV-1 Singer RNA replication in MDOK and BT cells. BVDV-1 Singer RNA was detected in both BT and MDOK extracts 12 hr after simultaneous infection, Fig. 3B, Table A.1. BVDV-2 890 RNA was detected only in BT extracts, but not MDOK extracts, Fig. 3A, Table A.1. BVDV-2 890 RNA was below detection level even without a co-infecting virus.

The effect of prior infection with BVDV-1 Singer on the relative RNA concentration of superinfecting BVDV-2 890

Prior infection with BVDV-1 Singer reduced the relative RNA concentration of superinfecting BVDV-2 890 in BT cells and increased it to detectable levels in MDOK cells. BVDV-1 Singer RNA was detected 12 hr after superinfection with BVDV-2 890 (24 hr after primary infection) in BT and MDOK cells, Fig. 4B. BVDV-2 890 RNA relative RNA concentration, as expressed by Ct value, was lower, than that observed following primary infection of BT cells, Fig. 4A, Table A.1. Surprisingly, BVDV-2 890 RNA was detected in MDOK cells after 12 hr of superinfection, Fig. 4A, Table A.1.

The effect of prior infection with a CP BVDV on the relative RNA concentration of superinfecting CP in MDOK and BT cells

Prior infection with a CP BVDV reduced the relative RNA concentration of superinfecting CP BVDV below detection level in MDOK but not BT cells. BVDV-2 125 RNA was not detected in total RNA extracts 12 hr after superinfection of BVDV-1 Singer-infected MDOK cells, Fig. 5A. In BT cells, BVDV-2 RNA was low compared to primary infection results but was still detectable, Table A. 1. BVDV-1 Singer RNA was detected in both BT and MDOK cells 12 hr after superinfection (24 hr from the primary infection), Fig. 5B. The effect on the relative RNA concentration of superinfecting BVDV-1 Singer observed in cells previously infected with BVDV-2 125 was similar to that described above. The superinfecting CP virus RNA

replication was reduced in BT and inhibited in MDOK cells. Table A.1.

The effect of simultaneous infection with BVDV-1 Singer and BVDV-125 on the RNA replication efficiency of each viral RNA

Simultaneous infection with BVDV-1 Singer and BVDV-2 125 had no effect on the RNA replication efficiency of the individual viruses. Twelve hours following simultaneous infection with the CP viruses BVDV-1 Singer and BVDV-2 125, RNA levels comparable to levels of primary infection were detected, Fig. 6, Table A.1.

Mathematical representation of the co-infection effect (CoE12)

CoE12 following superinfection (CoEsu) or simultaneous infection (CoEsi) was represented on a scale from –0.6 to 0.6, Fig. 7. Simultaneous co-infection had little effect on the relative RNA concentration of both co-infecting viruses and was represented by shallow or flat cones. Fig. 7B. On the other hand, superinfection caused a reduction in the relative RNA concentration of superinfecting viruses and was indicated by deep cones pointing in the direction of the negative scale. Fig. 7A. The increase in the relative RNA concentration of BVDV-2 890 in MDOK cells was depicted by a cone pointing in the direction of the positive scale. Fig. 7A.

DISCUSSION

There are over 140 federally licensed BVDV vaccines in the US, yet the annual economic loss exceeds $70 billion dollars (Ridpath *et al.*, 2000). Genetic variability, persistent infections and interspecies transmission complicate BVDV control programs. Available BVDV vaccines provide the best protection against homologous but not heterologous strains of BVDV. This means that superinfection of animals and individual cells within these animals with another strain or BVDV genotype is possible. In addition, inadvertent superinfection of animals by veterinarians and owners can occur during vaccination of endemic herds with MLV vaccines. Mild Persistent infections are often missed using the current *Pestivirus* techniques (Ridpath *et al.*, 2000) and vaccination with MLV vaccines in BVDV suspect or confirmed herds is a common practice. Indeed, there are reports of genetic recombination between field isolates and vaccinal BVDV that occur *in vivo* (Ridpath and Bolin, 1995; Becher *et al.*, 2001).

BVDV-2 890 was selected for the experiment because many BVD viruses from persistently infected animals isolated in our laboratory are NCP BVDV-2, closely related to BVDV-2 890 (Yousif *et al.*, 2002). NCP viruses are also selected because they are the major source of contamination in biological materials of bovine origin. The cytopathic virus BVDV-2 125 was chosen because it was reported to act as a backbone for non-homologous recombination with BVDV-1 NADL (Ridpath and Bolin, 1995). The cytopathic BVDV-1 Singer was chosen because it is closely related to BVDV-1 NADL isolate that was reported to undergo non-homologous recombination with BVDV-2 (Ridpath and Bolin, 1995).

We used the NS3-type-specific molecular beacons developed in our laboratory (Yousif *et al.*, 2002) to determine the relative abundance of type 1 and type 2 *Pestivirus* RNAs during mixed-type *Pestivirus*

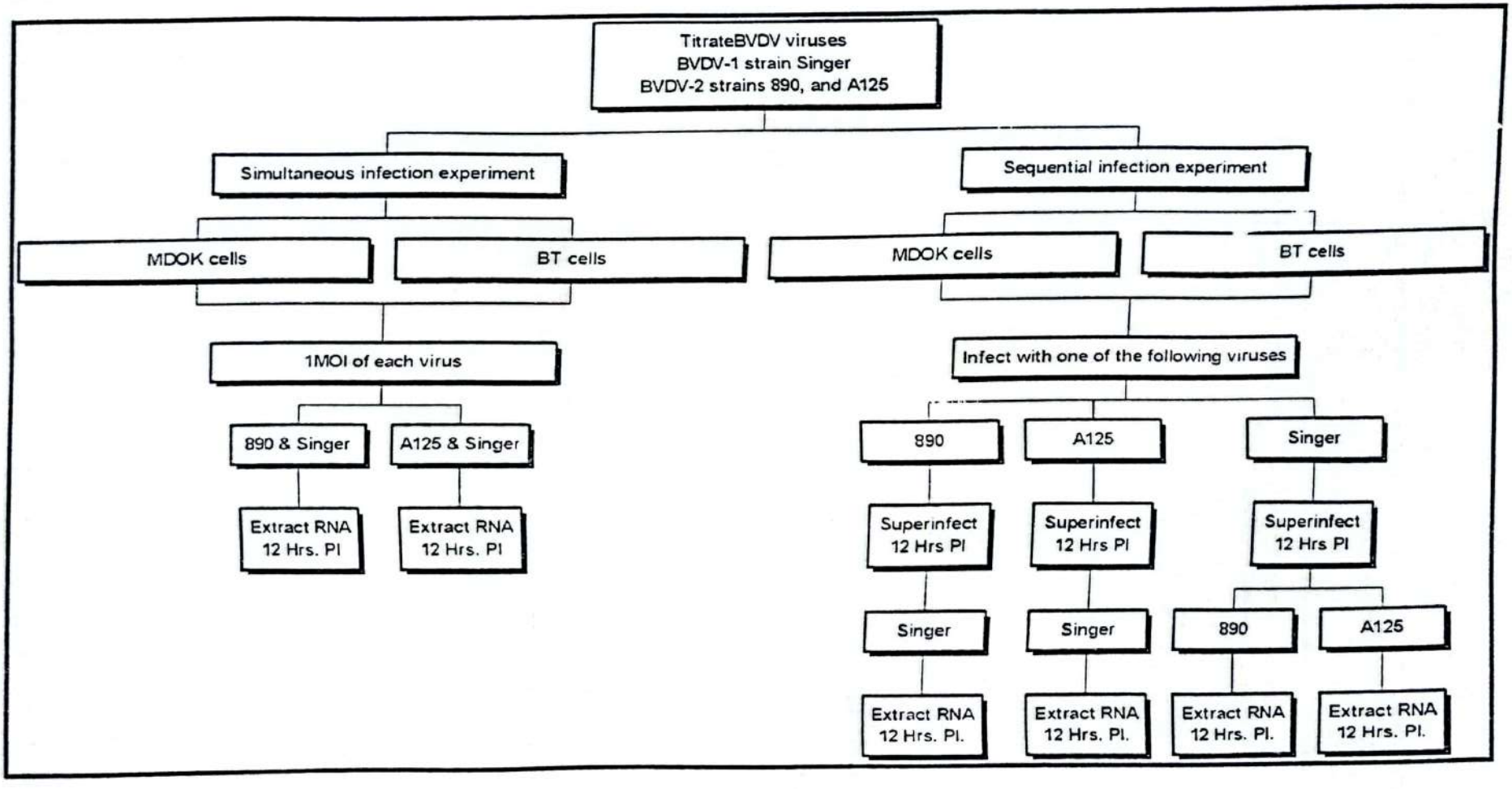


Fig. (1): The design of the mixed infection experiments

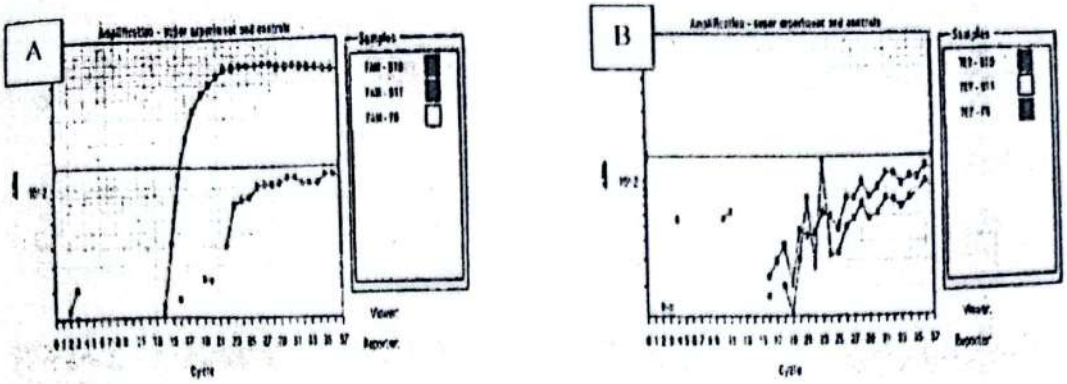


Fig. (2): Amplification plots of RNA extracts from cells sequentially infected with the NCP BVDV-2 890 followed by superinfection with the CP BVDV-1 Singer. B10 and B11 are the log amplification curves of pestivirus RNA extracted from MDOK and BT cells, respectively. A. FAM-labeled beacon detected BVDV-2 RNA in total RNA extracts. BVDV-2 890 RNA was extracted 12 hr after superinfection (24 hr after primary infection). BVDV-2 890 RNA was blow detection level in MDOK cells. B. TET labeled beacon detected BVDV-1 RNA. BVDV-1Singer RNA was not detected in total RNA extracts 12 hr after superinfection of BT cells.

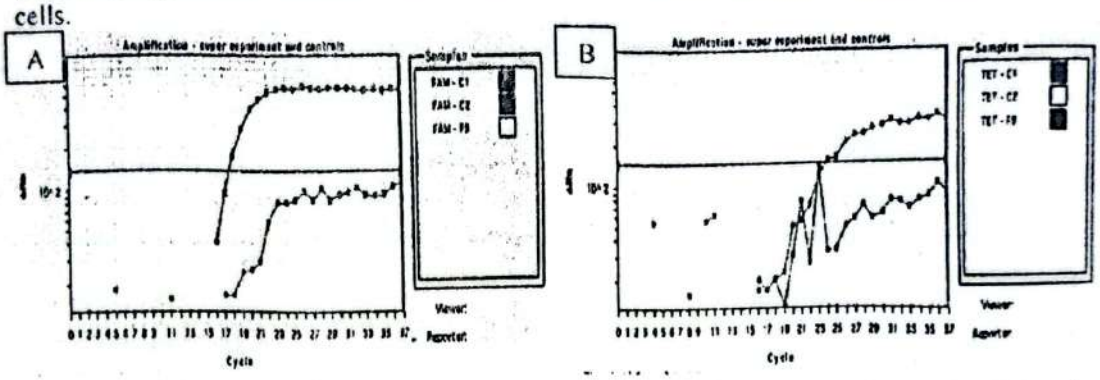


Fig. (3): Amplification plots of RNA extracts from cells simultaneously infected with 890 and Singer. C1 and C2 are the log amplification curves of pestivirus RNA extracted from MDOK and BT cells, respectively. RNA was extracted 12 hr after simultaneous infection. A. FAM-labeled beacon detected BVDV-2 RNA in total RNA extracts. BVDV-2 890 RNA was below detection level in MDOK cells but not in BT cells. B. TET labeled beacon detected BVDV-1 RNA. BVDV-1 Singer viral RNAs was detected in the total RNA extract from BT cells.

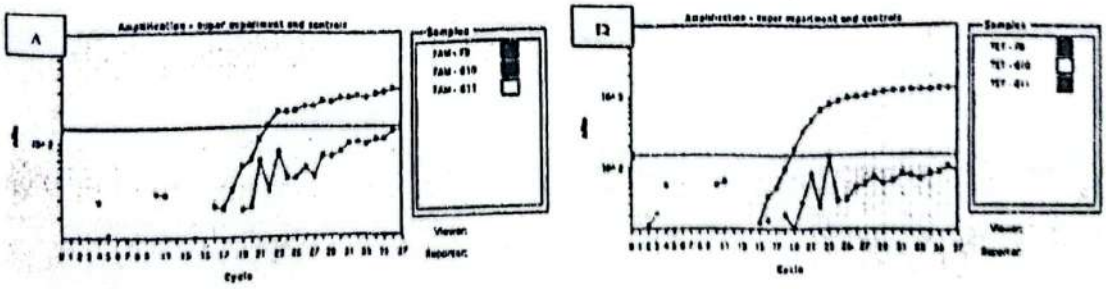


Fig. (4): Amplification plots of RNA extracts from cells sequentially infected with BVDV-1 Singer followed by superinfection with BVDV-2 890. G10 and G11 are the log amplification curves of pestivirus RNA extracted from MDOK and BT cells, respectively. A. FAM labeled beacon detected BVDV-2 RNA in total RNA extracts. BVDV-2 890 RNA was detected in both BT and MDOK cells. BVDV-2 890 RNA could not be detected in MDOK cells in primary or simultaneous coinfection, Fig. 2, Fig. 3. B. TET labeled beacon detected BVDV-1 RNA. BVDV-1 singer RNA was detected in both BT and MDOK cells 12 hr after superinfection (24 hr after primary infection).

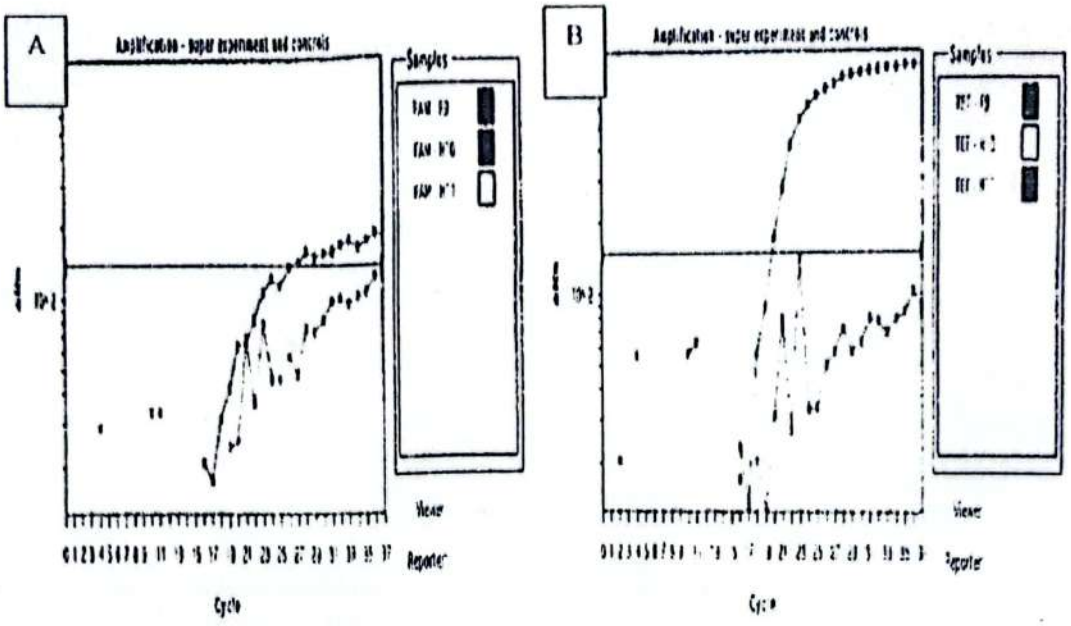


Fig. (5): Amplification plots of RNA extracts from cells sequentially infected with BVDV-1 Singer followed by BVDV-2 125. H10 and H11 are the log amplification curves of pestivirus RNA extracted from MDOK and BT cells, respectively. A. FAM labeled beacon detected BVDV-2 RNA in total RNA extracts. Prior infection with BVDV-1 Singer reduced BVDV-2 125 below detection level in MDOK cells, but not BT cells. B. TET labeled beacon detected BVDV-2 RNA. BVDV-1 Singer RNA was detected in extracts from both BT and MDOK cells 12 hr after superinfection.

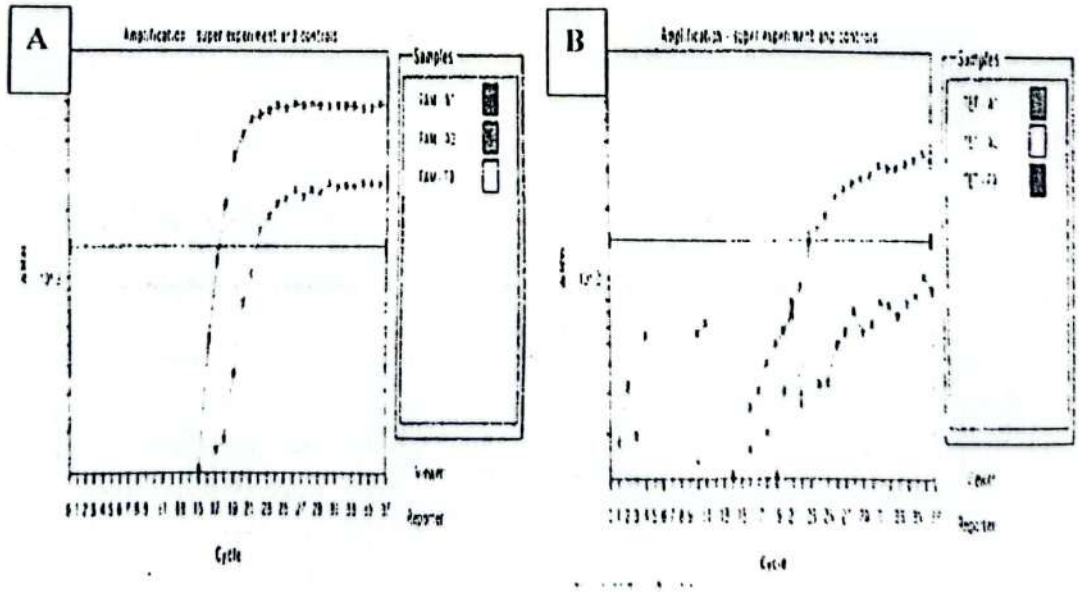


Fig. (6): Amplification plots of RNA extracts from cells simultaneously infected with A125 and Singer. A1 and A2 are the log amplification curves of pestivirus RNA extracted from MDOK and BT cells, respectively. A. FAM labeled beacon, detected BVDV-2 RNA in total RNA extracts. B. TET labeled beacon detected BVDV-1 RNA. Both A125 and Singer viral RNAs were detected in total RNA extracts from MDOK and BT cells following simultaneous infection.

Fig. 7A. Superinfection coinfection effect (CoEsu) on BVDV RNA replication in BT and MDOK cells

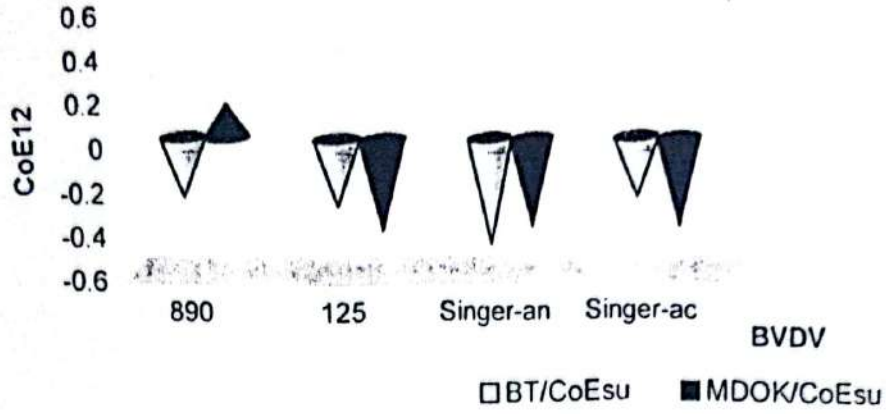
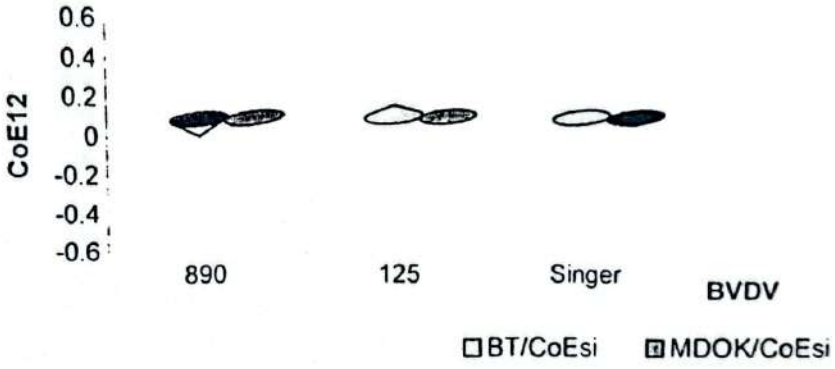


Fig. 7B. Simultaneous coinfection effect (CoEsi) on BVDV RNA replication in BT and MDOK cells



* Singer-an: CoE_{12} calculated from RNA extracts of NCP BVDV-2 890-infected cells after superinfection with BVDV-1 Singer.
* Singer-ac: CoE_{12} calculated from RNA extracts of CP BVDV-2 125-infected cells after superinfection with BVDV-1 Singer.

Fig. (7): The coinfection effect on relative BVDV RNA concentration in BT and MDOK cells. The effect of BVDV coinfection (CoE12) was calculated according to the following equation: CoE12 = [(Ct of virus at 12 Hr of primary infection/Ct of virus at 12 Hr of coinfection)-1]. CoE12 values calculated following simultaneous infection and superinfection experiments were terms CoEsi and CoEsu, respectively. Superinfecting virus RNA relative concentration in total RNA extracts is negatively influenced by the presence of a primary infection with CP or NCP BVDV in BT and MDOK cells, cones pointing towards the negative scale, Fig. 7A. In one case, primary infection with BVDV-1 890 has allowed RNA replication of the superinfecting BVDV-2 890 to increase to detectable levels, a cone pointing towards the positive scale Fig. 7A. Simultaneous coinfection effect values (CoEsi) were around 0.00 for both MDOK and BT cells, Fig. 7B.

Table 1. Threshold cycle (Ct) values from the pestivirus-NS3-type-specific molecular beacons assay

	BT cells			MDOK cells		
Infection type	Primary*	Simultaneous*	Superinfection	Primary*	Simultaneous*	Superinfection
BVDV-2 890	16	17.5	22	38	38	33
BVDV-2 125	18	17	26	22	22	38
BVDV-1 Singer	19.5	19.5	38/27♦	22.5	23.5	38

♦ The fist number is the Ct value for BVDV-1 Singer RNA after superinfection of BVDV-2 890-infected cells. The second number is the Ct value of BVDV-1 Singer RNA after superinfection of BVDV-2 125-infected cells.

* Recorded results are the mean of values obtained from the experiment when the difference of Ct values does not exceed 1.

infections. The experiment had two objectives. The first objective was to demonstrate concomitant BVDV-1 and BVDV-2 RNA replication during mixed infection. The second was to investigate the biotype and host cell type factors that influence this phenomenon. Comparing relative RNA concentrations of each virus in the same reaction well controlled the experiment and reduced false results. Our data showed that CP and NCP *Pestiviruses* proceed with vRNA replication in the presence of nonhomologous coinfection in both bovine and ovine cells. However, this is dependent on the time of co-infection, virus biotype and cell type.

Simultaneous co-infection had little effect on the relative RNA concentration of co-infecting viruses. However, when both BVDV-1 Singer and BVDV-2 890 viruses were inoculated simultaneously, both RNAs could be detected in comparable amounts in BT but not MDOK cells. This could be attributed to the relative speed/efficiency of BVDV-1 Singer RNA replication compared to BVDV-2 890 RNA replication in MDOK cells and the high demands on the cell machinery during the early stages of infection.

Superinfection usually reduced the relative concentration of the superinfecting virus, possibly due to competition on cell machinery and resources required for RNA synthesis. However, there was complete inhibition of the superinfecting BVDV-1 Singer RNA replication when BT cells were previously infected with BVDV-2 890. Cells becoming refractory to cytopathic *Pestiviruses* due to previous infection with NCP *Pestiviruses* have been reported with CSFV (Mittelholzer *et al.*, 1998). This could be another reason, besides the immune response, that persistently infected animals do not usually develop multi-strain or multi-type pestivirus infections. This phenomenon could have an evolutionary advantage from the virus standpoint. It helps the virus maintain a certain number of persistently infected animals in the environment to keep the cycle of infection. If superinfection were to be allowed more readily, PI animals would develop MD and become eliminated from the population more readily. This would be a loss from the viral standpoint because PI animals are a major source of infection to herd mates. This same phenomenon might also be partially responsible for the higher percentage of NCP BVDV field isolates.

Interestingly, BVDV-2 890 RNA levels were elevated to detectable

levels in ovine cells in the presence of prior BVDV-1 Singer infection. This finding could provide additional evidence to the danger of interspecies transmission of BVDV into the ovine hosts. Ovine cells may very well be better hosts for heterologous coinfection events and that may allow dangerous recombination events between the vRNAs of the coinfecting *Pestiviruses*. Investigation of the genetic makeup of the RNA products of superinfection experiments in ovine and ovine cells may provide clues on cellular factors that may facilitate or hinder such recombination events.

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