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Cytopathogenicity of local field strains of *Bovine Viral Diarrhoea Virus* isolated from apparently health buffaloes in Egypt

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The growth behaviour of BVDV field strains isolated from apparently health buffaloes in cell culture was studied. The isolated viruses were classified into 3 groups based on the onset and severity of cytopatheic effect (CPE). The cytopathogenecity of the isolates were different when these viruses were inoculated on BVDV-free and-infected cell lines. To study the discrepancies on the growth behaviour of the local isolates, the viruses subjected to further passages on MDBK cells infected with non cytopatheic (NCP) strain of BVDV which was homologues (pair) to their genotype (type I). After four passages on cell culture, the local isolates demonstrated enhancement in their CPE and virus replication as determined by virus titration assay. Propagation of these viruses in BVDV-free cells for five passages showed decrease in the severity of CPE with marked difference in virus titer when compared with those passaged in BVDV-infected cells. The present study, confirms that the cytopathogenicity of the local isolates of BVDV are directly related to the presence of a helper virus which could be NCP homologus or heterologus. After 12 passages in cell culture, the obtained BVDVs in the harvests of CP BVDV infected cells may represent naturally defecting interfering viruses as a result of recombination mechanism.

INTRODUCTION

According to their effects on tissue culture, BVDV strains can be differentiated into cytopatheic and non-cytopatheic isolates (Thiel *et al.*, 1996, Baker, 1987). at the molecular level the two biotypes differed in the production of P80 (NS3) which is only generated by cytopatheic BVDV (Donis and Dubovi, 1987), the N53 protein expressed in infected cells in accordance to the genetic rearrangement at region coding for the non-structural protein (NS2-3/P/25) either by introduce insertion at cleavage site (Tautz *et al.*, 1996) or the duplication of NS3/P80 region (Donis and Duboi, 1987 a.b.c, Haward *et al.*, 1987, Corapi *et al.*, 1988, Meyers and Thiel, 1996, Lambort *et al.*, 1997). The sequences coding for the NS3 is co-linear to the sequence coding for c-terminal 2/3 of the viral

polypeptide P125 (Meyers *et al.*, 1989, 1991, Qi *et al.*, 1992, Tautz, 1993).

BVDV is also classified into two genotypes BVD type I and BVDV type II based on phylogenic analysis of the 5'UTR (Pellerin *et al.*, 1994, Ridpath *et al.*, 1994).

Using a panel of MAbs against E2 epitopes, BVDV-type I strains classified into 5 subgroups while type II classify into only 2 subgroups (Baule *et al.*, 1997, Van Rijn *et al.*, 1997, Becher *et al.*, 1999).

The characteristic feature of *Pestiviruses* is the ability of the placental transmission of NCP-BVDV to infect the fetus at a particular stage of gestation, resulting in persistently infected state (McClurkin *et al.*, 1984). The *Pestivirus* genome is a positive stranded RNA molecule of about 12.5 kb containing single large ORF encoding approximately 4000 amino acids. 5` non-coding region of almost 400 nucleotides and 3` non-coding region of more than 200 nucleotides (Collett *et al.*, 1988a a,b,c, Meyers *et al.*, 1989, Moormann *et al.*, 1990). The virion of *Pestiviruses* consist, together with the RNA, of four structural proteins, the nucleocapsid C protein and the envelope glycoproteins E^{rns}, E1 and E2 (Thiel *et al.*, 1996). Currently, 11 pesti viral proteins have been identified as products of polyprotein processing, the proteins are arranged in order N^{pro}/C/E^{rns}/E1/E2/NS2/NS3/NS4A/NS4B/NS5A/NS5B (Collett *et al.*, 1988 a,b, Thiel *et al.*, 1996).

The aims of the present study were to study the growth behaviour of the local BVDVs isolated from buffaloes and the effect of NCP-BVDV strain (type-I) on the replication of super-infected buffalo CP-BVDV (type-I) in cell culture system.

MATERIALS AND METHODS

Viruses

Five local isolates of BVDV, which were isolated, antigenic and genomic characterized in our previous studies (Mohamed, *et al.*, 2004), were used in the study. The viruses were of cytopatheic type and designated as Cairo10/99, Cairo 12/99, Cairo 13/99, Seuz 60/99 and Seuz 68/99. All viruses were isolated from buffaloes. BVDV type I and type II (cytopatheic and non cytopatheic reference strains) were also included as controls in the study.

Tissue culture cells

A continuous cell line of MDBK (Madin Derby Bovine kidney cells). BVDV free, were provided by the National Veterinary Disease Laboratory, Ames Iowa, USA. The cells were used in the propagation and titration of the local isolates. Also other sources of MDBK cells, which were infected by NCP BVDV, were used in the study. These included cells obtained from local sources and also those obtained from Dept. of Veterinary Biological Science, South Dakota, USA. A continuous cell line of BT (bovine turbinate) cells obtained from Dept. of Veterinary Biological Science, South Dakota, USA were also used in the study.

Propagation and titration of local isolated of BVDV on MDBK cells

The BVDV local isolated were propagated in tissue culture flasks that were prepared as 80-90%

confluent. All viruses were inoculated on monolayer with adsorption time of 2 hours then serum free media was added and the flasks were incubated at 37 °C with 5% CO2 for 7 days with daily observation of CPE. Virus titration assay was carried out according to Frey and Liess (1971). The virus titer of each isolate was calculated as described by Reed and Munch, (1938).

RT-PCR

The assay was carried out as previously described (Hussein, 2001). Briefly, RT-reaction was applied on extracted viral RNA from the 5 local Egyptian isolates (from cell culture passages 5, 6 and 7) as well as reference strains of BVDV using random hexamers as primer sets during RT-reaction at 42°C/45 minutes incubation period, followed by three minutes heating at 94°C to inactivate RT enzyme. The PCR primer pair (UTR1 and UTR2) were designed according to BVDV-NADL published sequence of its viral RNA genome (Collett *et al.*, 1988 a,b,c). The primers amplify a specific stretch of 5`-UTR region of 288 bp, where the sense primer UTR1 located between 108-128 nt-sequence position and antisense primer UTR2 located in between 395-375 nt-sequence position of NADL viral genome PCR cycling profile composed of 35 cycles of 94°C for 1 minute denaturation, 56°C for 1 minute annealing, and 72°C for 1 minute polymerization Pre-PCR heating at 94°C for 1 minute, and final extension step at 72°C for 7 minutes. The products were analyzed on 2% agarose gels stained with ethiduim bromide and photographed with Polaroid camera.

Genotyping by Multiplex RT-PCR

Genotyping of local Egyptian BVDV isolates by multiplex PCR assay was carried out to genotyp these isolates to BVDV type I, BVDV type II, or *BDV* and to locate these viruses within the phylogenetic map of BVDVs. The genotyping PCR assay applied in the present study originally developed by Sullivan and Akkina, (1995) and used in our previous studies (Hussein, 2001, Hassanin *et al.*, 2003, Hussein *et al.*, 2003 and Abel Hafiez *et al.*, 2003). It depends on the use of pestvirus type specific primer cocktail during second round of the PCR assay. The primers designed according to published sequences of *Pestiviruses*, the first primer set depend on common sequence homology in all three ruminant pestviruses at E^{rns} coding region of the viral genomes (BVDV type I, BVDV type II and *BDV*). The second PCR depend on type specific primers, which anneal specifically to each of their corresponded genome (Sullivan and Akkina, 1995; Fulton *et al.*, 1999). The pesti-consensus fragment was 826 bp length amplified by gp 1 and gp 2 primer pair. The BVDV-type I fragment was 223 bp amplified by gp 2 and TS3 primer pair. The BVDV type II fragment was 448 bp hybridized by gp 2 and TS2 primer pair, while BVD fragment was synthesized of 566 nt nucleotide length and amplified by gp 2 and TS1 primer pair. The first round PCR cycling protocol counted a repetitive 30

cycles of denaturation, annealing and polymerization steps composed of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, respectively. Pre-PCR heating was applied at 94°C for 1 minute, while final extension step was at 72°C for 5 minutes. The second round of PCR assay (nested) aimed to differentiate ruminant *Pestivirus*es according to their genetic groups. It composed of 25 cycles, each as following: denaturation step at 94°C for 1 minute, annealing step at 50°C for 45 seconds and polymerization step at 72°C for 45 seconds. A pre-PCR heating was carried out at 94°C for 1 minute, while final extension step at 72°C for 7 minutes .

RESULTS

The growth behaviour of the local Egyptian isolates of BVDV on MDBK cell line which free from latent infection with NCP-BVDV

Five local isolates of BVDV, was successfully adapted and propagated on MDBK (BVD -free) cell line in small size tissue flasks till the passage (8), revealed CPE characteristic for BVDV. The CPE begin in the form of rounding and granulation of cells in scattered area of monolayer, followed by cellular darkness and starting of cells cluster formation, intracytoplasmic granules coalescent to form enlarging holes spanned by cellular filaments and surrounded by darkened irregular cells (Figure 1). Vacuolation increased gradually till involved more than 90% of cells with foamy appearance, till complete destruction and detachment of cells. By additional passages, the CPE was appeared earlier and destruction was observed in the sheet of cells in a shorter time. Based on their growth behavior, the local isolates were divided into three groups according to appearance, severity of CPE and time elapsed till complete detachment of inoculated monolayer. Group (1) included isolates designated Cairo 10/99 and Cairo 13/99 revealed CPE which was more severe in short duration compared with the others. CPE was delayed in appearance and continue with constant behavior till passage six. In the 7th passage generally a clear CPE was observed and begin of the detachment of the sheet was observed at day 7 (Table 1). Group (2) includes isolate designated Suez 68/99 local isolate of BVDV. Its CPE was moderate in severity, appearance and duration. The CPE was weak till 4th - 6th passages and increased gradually till it begin detachment of cells at passage 7 after 7 days post inoculation (Table 2). Group (3) includes local isolates designated Cairo 12/99 and Suez 60/99. Their CPE was weak than the 2 other groups and delayed in its appearance. It appear with constant weak behavior along 4th-7th passages (Table 3).

The propagated local isolates of BVDV on MDBK cells (BVDV free by PCR) were titrated to determine their infectivity rate. The $TCID_{50}$/ml was determined for each isolate (Table 4).

Table (1): Growth behavior results of the local Egyptian isolates of BVDV (Group 1) on MDBK cell line free from latent infection with NCP-BVDV

Passage No.	Days Post Inoculation						
	1	2	3	4	5	6	7
4	-	-	-	+	+	+ 2	+ 2
5	-	-	-	+	+	+ 2	+ 2
6	-	-	-	+	+ 2	+ 2	+ 3
7	-	-	+	2 +	+ 3	+ 4	+ 4

+: Begin of cell rounding. +2: Cell rounding and darkness. +3: Begin of cell groups. +4: Cluster formation and begin of detachment. +5: Cell detachment.

Table (2): Growth behavior results of the local Egyptian isolate of BVDV (Group 2) on MDBK cell line

Passage No.	Days Post Inoculation						
	1	2	3	4	5	6	7
4	-	-	-	+	+	+ 2	+ 2
5	-	-	-	+	+	+ 2	+ 2
6	-	-	-	+	+	+ 2	+ 2
7	-	-	+	+2	+ 2	+ 3	+ 4

+: Begin of cell rounding. +2: Cell rounding and darkness. +3: Begin of cell groups. +4: Cluster formation and begin of detachment. +5: Cell detachment.

Table (3): Growth behavior results of the local Egyptian isolates of BVDV (Group 3) on MDBK cell line free from latent infection with NCP-BVDV

Passage No.	Days Post Inoculation						
	1	2	3	4	5	6	7
4	-	-	-	-	-	-	+ 2
5	-	-	-	-	-	-	+ 2
6	-	-	-	-	-	-	+ 2
7	-	-	-	-	-	+ 2	+ 3

+: Begin of cell rounding. +2: Cell rounding and darkness. +3: Begin of cell groups. +4: Cluster formation and begin of detachment. +5: Cell detachment.

Table (4): Titration of the local isolates of BVDV on MDBK-BVDV free cells

	The isolated BVDV viruses				
Virus	**Cairo 10/99 isolate**	**Cairo 12/99 isolate**	**Cairo 13/99 isolate**	**Suez 60/99 isolate**	**Suez 68/99 isolate**
Titer/ml*	$10^{2.5}$	$10^{4.1}$	$10^{3.2}$	$10^{1.6}$	$10^{2.6}$

- Titers was calculated according to Reed and Muench (1938).

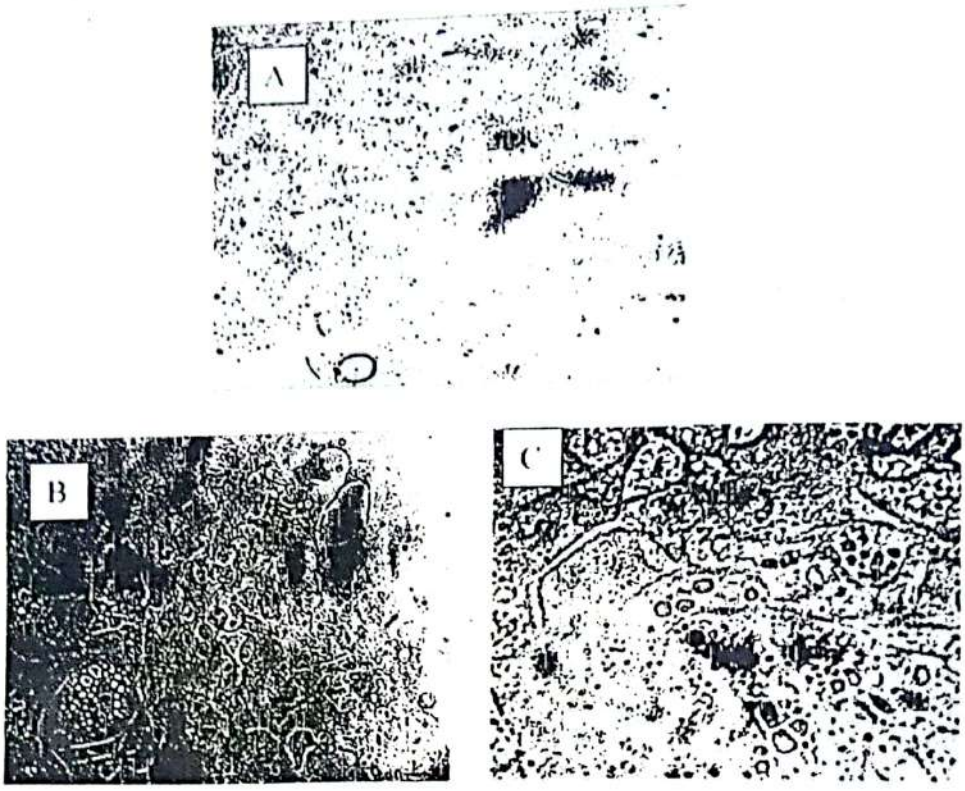

Fig. (1): Cytopathogenicity of the Egyptian Buffalo BVDV isolates. A. Control non-infected MDBK cells observed six days post-infection. B and C two representative degrees of CPE observed six days post infection.

Monitoring of the local Egyptian BVDVs in cell culture by RT-PCR assay using UTR1 and UTR2 primer pairs

The PCR amplified products of the five BVDVs local Egyptian isolates (Cairo 10/99, Cairo 12/99, Cairo 13/99, Suez 60/99, Suez 68/99) revealed strong visible band corresponding to the molecular weight marker band which confirmed primers and fragment specificity as visualized in 2% agar gel electrophoresis stained with ethiduim bromide (Fig.2). Control MDBK cells (BVDV free cells) were negative which indicated their freedom of latent NCP-BVDV contaminants.

Genotyping of local Egyptian BVDVs strains using primers cocktail located at the Erns region viral genome in an multiplex RT-PCR assay

The amplified PCR products were analyzed in 2% agar gel electrophoresis stained with ethiduim bromide, fragments were visible in specific molecular size band parallel an equal molecular weight marker band, which revealed primers and product specificity. The obtained results proved that the five Egyptian local BVDV isolates are of genotype I of *Pestiviruses* (BVDV-type I) . Control MDBK cells ere negative when utilized by RT-PCR

assay in the present genotyping assay.

Screening of cell culture cells by PCR assay utilizing UTR1 and UTR2 primer pairs for detection of NCP-BVDV

BT cells (obtained from South Dakota, USA) and MDBK cells (obtained from different sources including: South Dakota, USA, Vet. Serum and Vaccine Research Institute, Abbasia, Animal Reproduction Research Institute and Animal Health Research Institute) were used for the propagation of the isolated viruses between passages 8 to 12. RT-PCR assays were applied on the non inoculated cells to test for BVDV contamination amplifying a specific stretch of 5' UTR region of BVDV (288 bp). All BT and MDBK cells revealed strong visible band which confirmed primers and fragment specificity . All cells were positive for latent infection with NCP-BVDV.

Genotyping of NCP-BVDV of MDBK- and BT - latently infected cells

The infected MDBK cells with NCP-BVDV from Vet. Serum and Vaccine Research Institute, Animal Reproduction Research Institute, Animal Health Research Institute and South Dakota, USA were screened and genotyping of the contaminated NCR-BVDV utilizing multiplex-PCR assay using *Pestivirus* type specific primer cocktail during second round of PCR assay was applied. All MDBK cells tested found to be latently infected with NCP-BVDV type I(revealed a band of 223 bp). The BT cells were found to be latently infected with NCP-BVDV type II (revealed a band of 448bp).

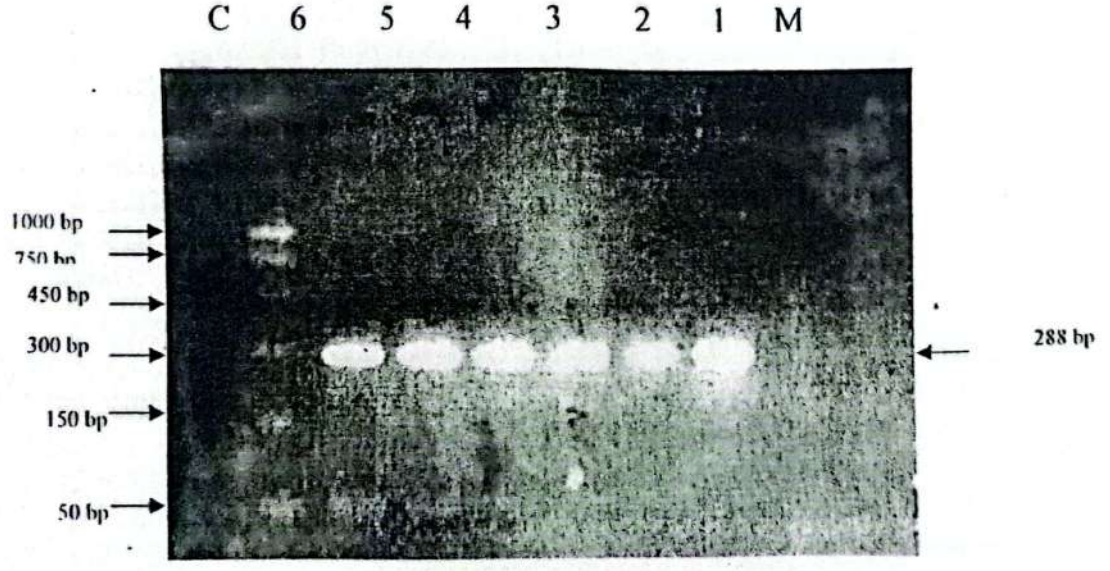

Fig. (2): PCR amplification products electrophoresed on 2% agarose gel, stained with ethidium bromide and photographed by Polaroid GelCam camera. M: molecular weight marker. Lanes 1 to 5 represent PCR amplification products of the five local buffalo BVDV strains. Lane 6 represents the BVDV control positive sample and lane 7 represents the negative control.

Growth behaviour of the local Egyptian isolates of BVDV on MDBK cell contaminated with NCP-BVDV

Previously, the 5 local isolates of BVDV were propagated on MDBK-BVDV free till the 7th passage. By the 8-12 passages the isolates were propagated on MDBK cell infected with NCP-BVDV. The growth behaviour of the isolates was greatly enhanced by propagation on infected MDBK cells with NCP-BVDV. The CPE appeared more earlier and destruct the sheet of cells in a shorter time. Table (5) summarized the CPE behavior of the local isolates on MDBK cells infected with NCP-BVDV

Titration of BVDV Egyptian local isolates on MDBK latently infected with NCP-BVDV

The propagated local isolates of BVDV were titrated at passage 12 on the MDBK cells latently infected with NCP-BVDV to determine their infectivity rates.

DISCUSSION

Mucosal disease is one of numerous disease processes induces by BVDVs. The mechanism of the disease require PI animal with NCP-BVDV followed by super infection with an appropriate CP-BVDV (Ripath *et al.*, 1991). It is well known that not every combination of NCP and CP-BVDV results in fatal disease and the interaction between CP and NCP that result MD is unknown (Bolin *et al.*, 1985a, b, Ripath *et al.*, 1991). From the data of several studies, the nearest hypothesis that CP-BVDV isolated from cattle with MD has been arisen by mutation of the persistent NCP-BVDV (Howard *et al.*, 1987, Ridpath *et al.*, 1991). Recent molecular studied have provided evidence for the involvement of CP viruses from NCP viruses by RNA recombination. Indeed in nature infection the origin of CP strains is almost certainly a mutation from NCP viruses.

In cell culture system BVDV isolates can be either CP or NCP. Remarkably, the production of NS3 correlate with cytopathogenicity. In NCP virus cleavage of 2/3 site of the genome does not occur and subsequently NS2-3 usually observed (Xue *et al.*, 1997). Recent work by Meyers et al. (1998) indicated the insertion of a sequence encoding light chain 3 (LC3) of microtubule associated protein in a *Pestivirus* genome has a connection with a cytopathogenicity and induction of lethal disease of cattle. Cleavage occurs directly down stream of LC3-encoded sequence and is not depended on NS3 serine protease (Meyers *et al.*, 1998).

Moreover, it was reported the identification of several types of cellular insertion and subsequently the evolvement of CP strains. Three types of cellular insertion have been reported to dates (Ubiquitin, part of cellular protein of unknown function CIns and LC3) (Becher *et al.*, 1996, Meyers *et al.*, 1991, 1996, 1998,

Table (5): The growth behaviour results of the local Egyptian isolates on BVDV on MDBK cell infected with NCP-BVDV

Group	Code No. of isolates	No. of passages	Days Post Inoculation						
			1	2	3	4	5	6	7
Group 1	Cairo / 10 Cairo / 13	8			+2	+3	+4	+5	
		9			+2	+3	+4	+5	
		10			+2	+3	+4	+5	
		11			+3	+4	+5		
		12		+3	+4	+5			
Group 2	Suez 68/99	8			+	+2	+3	+4	
		9			+	+2	+3	+4	
		10			+2	+3	+4	+5	
		11		+	+2	+3	+4	+5	
		12		+2	+3	+4	+5		
Group 3	Cairo 12/99 Suez 60/99	8				+2	+3	+4	
		9			+	+2	+3	+4	
		10			+	+2	+3	+4	
		11			+	+2	+3	+4	
		12		+2	+3	+4	+5		

+: Begin of cell rounding. +2: Cell rounding and darkness +3: Begin of cell groups. +4:Cluster formation and begin of detachment. +5: Cell detachment

behavior on cell culture was observed. A remarkable increase in the CPE of these viruses was occurred. Determination of the virus titer indicated and enhancement in the titer of these viruses. Such observation was previously recorded in which an enhanced replication of some viruses occurred when these viruses inoculated in cells infected with NCP-BVDV (Nakamura *et al.*, 1995). It should noted that these viruses (local BVDV) with enhanced CPE when inoculated again in MDBK cell line free from BVDV, astonishing behavior (suppression of CPE) was observed. Hence, the observed discrepancies in the CPE of these isolates in cell culture (BVD free and BVDV infected) confirm that the cytopathogenicity of these viruses are directly related to the presence of a helper virus. Such helper virus could be NCP homologous or heterologus (type I and type II) need to be addressed. The isolation of defective interfering particles has been reported by several researchers. In fact the obtained BVDVs in the harvests of CP BVDV infected cells (passage 12) in the current study may represent naturally defecting interfering viruses as a result of recombination mechanism.

Certainly, BVDV induce in cell culture many polypeptide during its replication. Different mechanisms induce alteration on the BVDV genome especially the cytopatheic strains which lead to cell lyses (Meyers *et al.*, 1998).

Molecular biology studies on BVDV especially those focused on cytopathogenicity of such viruses are of added interest since the generation of cytopatheic virus by point mutation from NCP virus can lead to lethal disease. Introduction of a set of mutations could affect the cytopathogenicity in cell culture and cytopathogenesis of the disease, thus and so understanding of the mechanism of cytopathogenicity and pathogenesis of *Pestivirus* is a hope for several scientist especially those interested in its human counter part (Hepatitis C virus).

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