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Bovine Viral Diarrhea Virus Propagates in Cell Culture after Inoculation of Inactivated Sera from Persistently Infected Calves

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Abstract: During routine diagnostic investigations on submitted serum and swab samples from three (n:3) calves in our laboratory for detecting BVDV antibody titers and antigen we noticed that successful virus propagation can occur from heat inactivated (in a water bath at 56° C for 30 min) 2 serum samples. For further confirmation of virus propagation in heat inactivated serum samples, 11 sera which were previously detected positive for BVDV were also investigated. As a result, from a total of 14 heat inactivated-field originated sera samples virus propagation was confirmed in 4 (28.5%) samples. This result suggests that heat inactivation of the serum is not eliminating the risk of pestivirus contamination, thus interfere the results of virus isolation and serological diagnostic methods including serum neutralization. Moreover relative resistance of BVDV to heat inactivation may lead to failure in complete inactivation of the virus in fetal calf sera which is an important contaminant for biologicals like cell cultures and attenuated live vaccines.

Key Words: BVDV, heat inactivation, propagation, resistance.

Persiste Enfekte Buzağıların İnaktive Edilmiş Serumlarından Bovine Viral Diyare Virusu İzolasyonu

Özet: Rutin laboratuvar çalışmaları doğrultusunda BVDV antikor ve antijen taraması istenen üç adet (n:3) buzağıya ait serum ve svab örneklerinin incelenmesi sırasında serum örnekleri su banyosunda 56° C'de 30 dakika süresince ısı inaktivasyonu tabi tutulmuş ancak 2 adet serum örneğinde virus çoğalmasının gerçekleştiği görülmüştür. Serumların ısı ile inaktive edilmelerine rağmen hücre kültüründe virus üremesinin gerçekleşmesini araştırmak amacıyla daha önceden BVDV pozitif olduğu bilinen 11 adet serum örneği de incelemeye alınmıştır. Sonuç olarak ısı inaktivasyonuna tabi tutulmuş saha menşeli 14 adet serum örneğinin 4 adedinde (%28,5) virus çoğalması tespit edilmiştir. Elde edilen bu veri serum inaktivasyon prosedürünün pestivirus kontaminasyonunu elimine etmede yetersiz kaldığını göstermiştir. Bu durum virus izolasyonu ve serolojik teşhis yöntemlerinin sonuçlarını olumsuz etkileyebilir. Ayrıca BVD virusunun ısı inaktivasyonuna karşı direnci, fötal dana serumlarında bulunabilen virusun tamamen inaktive olmasını engelleyebilmektedir. Buna bağlı olarak BVDV, hücre kültürü ve atenüe canlı aşı gibi biyolojik maddeler için ciddi risk haline gelmektedir.

Anahtar Kelimeler: BVDV, ısı inaktivasyonu, virus çoğalması, dayanıklılık.

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Introduction

Pestivirus infections cause diseases in ruminants worldwide with a huge economic impact. *Pestivirus* genus consists of bovine viral diarrhea virus (BVDV), border disease virus (BDV) and classical swine fever (CSFV), in the family of *Flaviviridae*. These are single stranded RNA viruses and cause clinical symptoms characterized by respiratory, enteric and reproductive disorders.

BVDV has two genotypes, BVDV-1 and BVDV-2 besides BDV has seven genetic clusters (BDV 1-7)[4]. These viruses have two biotypes called cytopathogenic (cp) and non-cytopathogenic (ncp), due to the effect on infected cells[6].

The ability of the virus to pass through the placenta during the first trimester of pregnancy can cause persistent infection (PI) (virus positive, antibody negative) in new born. These persistently infected calves can present a virus reservoir and shed virus through their life. In this circumstance, pestiviruses may be important contaminants for reagents of biological origin. Possible pestivirus contamination in commercial sera of bovine origin has been demonstrated long before[7].

During our routine work in the laboratory, successful virus propagation was occurred from heat inactivated serum samples. In this study the propagation of pestiviruses despite heat inactivation was investigated by increasing the number of the field samples.

Materials and Methods

The samples

Serum samples from 3 calves submitted to laboratory for seeking antibodies by serum neutralization assay were heat inactivated in a 2 ml plastic test tube in a water bath at 56° C for 30 min.

Because of no antibody existence and nascent clinical symptoms in these calves after serological investigation nasal swab samples were later collected for searching BVDV. Depends on obtained positive results, the possibility of persistent BVDV infection was suspected. After sampling calves were separated from the herd by the owner; hence there was no possibility to test the animals for confirmation of immunotolerant persistent infection by second sampling. Thus, initial (heat inactivated) serum samples of these calves were further examined by antigen ELISA, panpestivirus RT-PCR and virus isolation; despite heat treatment. At the end of the examination successful virus propagation was occurred.

For further confirmation of the virus propagation in heat inactivated serum samples, an experimental design was conducted on 11 sera previously detected positive for BVDV. Each of these samples (1,5 ml each) was divided into two tubes, one-half were inactivated at 56° C for 30 min. in water bath (treated group) and remaining half was not (untreated group).

Cell line

For the cell culture based methods Madin Darby bovine kidney (MDBK) cell line was used throughout the study. The cells were grown in Dulbecco's modified essential medium that was supplemented with 10% of heat-inactivated fetal bovine serum. The cell line and FBS were tested for the absence of pestivirus contamination by ELISA and IIPMA during the study.

Detection of pestivirus antigens by ELISA

According to the manufacturer's instructions a commercial pestivirus antigen ELISA kit (Herdcheck, Switzerland) which is developed to detect the E^{rns} protein of the pestiviruses was used.

Panpestivirus RT-PCR

From all the samples total viral nucleic acid extraction was performed by a commercial kit (Axygen, Canada). Synthesis of cDNA was carried out with a panpestivirus primer p324 (5'-ATG CCC WTA GTA GGA CTA GCA-3') using a cDNA synthesis kit (Biomatik, Canada). Panpestivirus RT-PCR was performed, with the primers p324 and p326 (5'-TCA ACT CCA TGT GCC ATG TAC-3')[10] according to the following protocol: 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min. Products (288 bp) were visualized on 1% agarose-ethidium bromide gels by electrophoresis.

Virus isolation

MDBK cell culture was prepared at the concentration of 100.000 cell/ml in 24-well plates for blind passages. The culture media was removed, and 200 µl of each sample (initial 3 samples, 11 untreated and 11 treated samples)

was inoculated into separate wells. After incubation for 1 hour at 37°C, 1 ml of DMEM (without FBS) was added to wells. The culture medium was replaced 24 hours later. Next 5 days, the cells were observed for cytopathic effects (cpe). At the end of the period, for revealing all the progeny virions, the cells were harvested by placing the plates in the deep freezer (-80°C). Harvested culture fluids were used for inoculation in the next step of blind passage which was repeated twice (total of 3 blind passages). To confirm the existence of ncp pestiviruses, an immunoperoxidase protocol was applied.

Indirect immunoperoxidase monolayer assay (IIPMA)

An IIPMA was applied in MDBK cell line to validate the ncp pestiviruses after blind passage process[1]. For the first step of IIPMA 24-well plates were placed in a fixation chamber that was heated to +80°C for 3 hours for fixation on the 3rd day of the last blind passage process. In the next step, 200 µl of O-D-glucopyranoside (Sigma, 75081-5G) was added to each well and incubated at room temperature for 10 minutes. The plates were rinsed 3 times with W-PBS solution after each incubation step. The primary anti-mouse monoclonal antibody 1/4/7[3] (obtained from Virology Institute, Justus-Liebig University) was diluted 1:40 in Tween-20 W-PBS and incubated for 90 minutes at +37°C. The wells were treated with a biotinylated anti-mouse antibody (Pierce, 31800) and then incubated with peroxidase labeled streptavidin-biotin complex (Pierce, 21124) at the same conditions for both steps. Finally with the addition of the substrate (3-amino-9 ethylcarbazole (Sigma, A5754), hydrogen peroxide and sodium acetate) at room temperature for 30 minutes the test was evaluated by checking for reddish-brown colored intracellular aggregates.

Results

All the serum samples (n:3) which were submitted from PI doubted calves detected positive for BVDV both by antigen ELISA and RT-PCR, meanwhile non-cytopathogenic virus propagation was detected by IIPMA only in two of these inactivated serum samples (Table 1).

At the end of the further examination on BVDV antigen positive samples selected among routine diagnostic serum samples; virus propagation was detected in 11 of the wells inoculated with the untreated sera as well as in 2 wells inoculated with heat inactivated sera (treated group).

As a result, from a total of 14 field originated sera samples positive for BVDV, four samples (28.5%) still produced positive result in IIPMA despite heat inactivation at 56° C for 30 min., indicating the resistance of the live virus to applied heat inactivation process.

Table 1. The result of serological examination, antigen (Ag) ELISA, RT-PCR, IIPMA of persistently infected calves serum and swab samples

Tablo 1. Persiste enfekte buzağıların serum ve svab örneklerinin serolojik incelemesi, antijen ELISA, RT-PCR ve IIPMA testlerinin sonuçları

	Serum*				Swab
	Antibody titer	Ag ELISA	Panpestivirus RT-PCR	IIPMA	Ag ELISA
Calve 1	0	+	+	+	+
Calve 2	0	+	+	-	+
Calve 3	0	+	+	+	+

*Sera were heat inactivated at 56°C 30 min
*Serum örnekleri 56°C 30 dk ısı inaktivasyonuna tabi tutulmuştur

Discussion

Possible resistance of BVDV against inactivation by heat was previously mentioned in experiments by laboratory strains. In one of those experiments 33% sorbitol was added for heat treatment at 60°C during 10 hours[8] and in the other experiment dry airflow was used for defined temperature, 95° C for 2 hours[9]. In the present study the samples were inactivated by using a 2 ml plastic test tube in a water bath at 56° C for 30 min. Though fail in the elimination of BVDV infectivity in laboratory conditions had been reported[7] our results further demonstrate the consistency of viral infectivity in diagnostic samples from PI animals.

In this study, virus propagation was confirmed in 4 (28.5%) of 14 field originated sera samples that were heat inactivated. This results suggest that heat inactivation of the serum is not eliminate the risk of pestivirus contamination, thus due to interference mechanism, this result may be important for cell culture based laboratory tests including serum neutralization and virus isolation applied onto diagnostic samples. Moreover the relative resistance of BVDV to heat inactivation may lead to failure in complete

inactivation of the virus in fetal bovine sera which is an important contaminant for cell cultures and attenuated live vaccines[2,5,11]. According to our practical experience regarding commercial FBSs a few bottles can produce positive results for pestivirus while the other bottles from the same lot are negative (data not shown). Thus, it is strongly suggested that each serum bottle to be used during vaccine preparation and cell culture applications should be tested one by one with a sensitive diagnostic method even if they are from the same lot.

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