CONTAMINATION OF COMMERCIAL BOVINE SERA, CELL CULTURE AND VACCINES BY BOVINE VIRAL DIARRHOEA VIRUS (BVDV) AS DETECTED BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

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SUMMARY

Bovine viral diarrhoea virus (BVDV) is a common contaminant in the commercial laboratory biological products. Numbers of commercial bovine serum samples, laboratory cell culture and commercial vaccines were screened for the presence of BVDV RNA by reverse transcription-polymerase chain reaction (RT-PCR). All commercial sera used have been certified by manufacturers as negative for BVDV. Two BVDV-specific primers were synthesised and used in RT-PCR to amplify viral cDNA of 300 bp in size. Materials tested in the study were obtained from several research institutes, general practitioner clinics, veterinary clinics, commercial serum suppliers and local cattle. Three out of eight imported-commercial bovine sera and fifteen out of 41 different samples of cell culture tested were found to be positive for BVDV. The virus was not detected in human vaccines, but two animal vaccines were found to be positive in the test. Surprisingly, none of serum samples obtained directly from local cattle was positive for BVDV. A significant number of cell culture samples tested was found to be positive for BVDV. This indicated that many laboratories may have been using BVDV-contaminated commercial bovine sera as well as propagating BVDV-contaminated cell culture. Thus there is a need to conduct the test regularly to ensure cell culture and relevant materials free from BVDV. This is crucial in the preparation of animal sera for tissue culture applications as well as virus vaccines to conform the generally accepted quality of immunobiologics.

Keywords: BVDV, diagnostic, RT-PCR, cell culture, bovine sera

INTRODUCTION

Bovine viral diarrhoea virus (BVDV) is an important pathogen of cattle that causes abortion and congenital disorders following exposures of early gestation pregnant females (Bolin, 1990). It causes fetal gastrointestinal disease in immunologically tolerant animals infected *in utero* (Baker, 1987). It may contribute to gastrointestinal and respiratory disease syndrome in post-natally exposed animals (Perdrizet *et al.,* 1987; Redostits and Littlejohns, 1988). The virus is closely related to swine fever virus and border disease virus of the genus *Pestivirus* which was recently classified as a separate genus within the family *Flaviviridae* (Carlsson, 1991; Paton *et al.,* 1992).

The BVDV strains varied from mild to highly pathogenic. The virus could be further classified as cytopathic or non-cytopathic type. Both types of BVDV can be easily propagated in most bovine cells. Among the commonly used cells were bovine embryonic skin and muscle, embryonic bovine kidney (Lee and Gillesple, 1957) and bovine testicular culture (Kniazeff *et al.,* 1965). Other species to include human intestinal cells (Kniazeff and Mc Clain,1963) and lamb testicular cells (Coggins, 1962). The non-cytopathic strains of BVDV do not give any cytopathic effects (CPE) to the cell, therefore, they are difficult to detect in cell culture. Infection of bovine cell cultures by non-cytopathic BVDV has particular significance for the production of vaccines (Fogh, 1973).

It was reported that both the cells and foetal calf serum, an essential growth factor of cell culture medium, are the main source of BVDV (Nuttall *et al.,* 1977). Contamination of commercial foetal bovine serum (Rossi *et al.,* 1980; Bolin *et al.,* 1991; Abraham, 1993), laboratory cell cultures (Nuttall *et al.,* 1977; Bolin *et al.,* 1994) and animal viral vaccines (Harasawa, 1994, 1995) with BVDV was previously reported. Among these contaminated animal vaccines are the live infectious rhinotracheitis vaccine (Tomaglia, 1968, Scott, 1973) and Aujeszky's disease vaccine (Vannier, 1985) and orf virus vaccine (Loken, 1991). Up-to-date, no data is available about the importance with regard to contamination of human viral vaccines with BVDV. However, some vaccines such as Measles virus vaccine and a potential respiratory syncytial virus vaccines are produced on bovine kidney cells grown in the presence of unheated commercial foetal calf serum (Wright *et al.,* 1971).

The detection of BVDV contamination in bovine sera, tissue cultures and vaccines was previously employed using transmission electron microscopy (TEM) (Mathiesen *et al.,* 1985), immunoperoxidase-linked monolayer assay (IPMA) (Potts *et al.,* 1989) and viral RNA oligonucleotide fingerprinting technique (Kelling *et al.,* 1991). However, these techniques may be too insensitive for detecting very low levels of BVDV (Bissey *et al.,* 1991). DNA dot blot and RT-PCR techniques were also employed with high sensitivity obtained (Brock, 1991; Hamel *et al.,* 1995).

This study was carried out to detect the presence of BVDV in some commercial foetal calf serum, tissue cultures and cell lines and viral vaccines. Serum samples from Malaysian cattle farms were also tested by using RT-PCR technique.

MATERIALS AND METHODS

Commercial bovine sera

Commercial fetal bovine sera, fetal calf serum and calf serum used in the study were obtained from various sources including several research laboratories.

Cell culture

Most of cell cultures used in the study were obtained from several laboratories from different research institues (Table 2).

Vaccine

Animal vaccines (canine distemper and feline rhinotracheitis vaccines) were obtained from veterinary clinics and general practiotioners. The names and properties of the vaccines used are listed in Table 3.

Serum samples from cattle

Serum samples used in the study were collected randomly from UPM's farm cattle and other small holder farms.

PCR primers

The primers used to amplify cDNA sequence of BVDV were designed based on the published genomic sequences of the virus by Collett *et al.* (1988), Renard *et al.* (1985) and Deng and Brock (1992). The sequences of the oligonucleotide primers used were 5' TAGCCATGCCCTTAGTAGGAC 3' and 5' ACTCC ATGTGCCATGTACAGC 3'.

BVDV-RNA extraction

Extraction of RNA was carried out before testing the presence of BVDV specific RNA in the samples as described by Sambrook *et al.* (1989). Two-hundred ml of sample was mixed with 200 ml of extraction buffer (0.2M Tris-HCl, 0.2M KCl, 0.03M $MgCl_2$ and 0.04% SDS) and 2 mg/ml of Proteinase K was added to the mixture. The mixture was incubated at 42°C for one hour. Eighty ml of 1M NaCl was added followed by addition of 480 ml buffer-saturated phenol. The mixture was vortexed and centrifuged at 5000-7000 rpm for five minutes. The top aqueous phase was transferred to a new tube. The solution was mixed with buffer-saturated phenol, vortexed and centrifuged as above. 480 ml of chloroform-isoamyl alcohol (24:1, v/v) was added to the supernatant. The mixture was mixed well and centrifuged as above. The top layer was transferred to a new tube. Again chloroform-isoamyl alcohol was added and centrifuged. Fourty ml of 3M sodium acetate was added to 360 ml of RNA extracts. 1 ml of cold absolute ethanol was added and mixed gently. The mixture was incubated at –70°C for two hours and centrifuged at 13,000 rpm for 15 minutes to pellet RNA. The ethanol was removed and RNA pellet was dried. RNA was suspended in 20 ml of 1M Tris-HCl buffer (pH 7.4) and kept at –20°C until use.

RT-PCR protocol

Firstly, the process of reverse transcription (RT) as carried using a master mix containing $MgSO_4$ (1.0 ml), 10xPCR buffer (2.0 ml), dNTP mix (0.5 ml), distilled water (4.5 ml), RNase inhibitor (0.5 ml), reverse transcriptase (0.5 ml) and RNA sample (1.0 ml) per reaction tube. This mixture was carefully mixed and allowed to stand at room temperature for 10 minutes. The mixture was incubated at 42°C for one hour, followed by denaturation at 99°C for five minutes and finally at 5°C for 5 minutes.

Following the above cycle, the PCR was carried out using a master mix containing DNA polymerase (0.5 ml), $MgSO_4$ (1.0 ml), primer 1 (0.1ml), primer 2 (0.1 ml), 10x PCR buffer (8.0ml) and distilled water (28.5ml) per reaction tube. This mix was added to the reverse transcription tube and mixed well. The mixture was then subjected to PCR cycles after heating at 95°C for three minutes. Thermal cycling was started with denaturation of the target DNA at 94°C for one minute, followed by annealing of the primers to the complementary region of BVDV strand at 55°C for one min. and extension at 72°C for one min. This cycle was repeated for 30 times.

Detection of amplified PCR product

Following amplification, approximately 25 ml of the PCR product was loaded into 1% agarose gel and electrophoresed at 30-50 volts for 3 hours. The amplified fragments were visualised in ultraviolet light following staining of the gel in 0.01 % ethidium bromide. DNA marker ranged between 100 bp and 1000 bp was used to estimate the molecular weight of the PCR product. A positive BVDV sample was added in one well for each gel as a positive control to detect the bands.

RESULTS

The types and number of positive and negative samples tested for BVDV contamination using RT-PCR technique are demonstrated in Table1. Only 20 samples out 76 (26.32%) samples were found positive whereas 56 samples (73.68%) were proved negative for BVDV. Electrophoresis of PCR product from commercial bovine sera revealed that three out of eight (37.5%) samples were positives for the presence of BVDV (Table 1).

The PCR product of tissue culture samples electrophoresed in the agarose gel revealed that 15 samples out of 41 (36.59%) samples were positives for the presence of BVDV. The types, sources and presence of BVDV in the tissue cultures tested are shown in Table 2.

Electrophoresis of PCR product from the vaccine tested revealed that two out of eight (25%) vaccines were positives for the presence of BVDV (Table 3). The results of the contamination of the vaccines tested with BVDV are illustrated in Table 3.

All serum samples collected from local cattle were found negative for BVDV when tested with RT-PCR (Table 1).

Table 1. BVDV contamination in various samples tested with RT-PCR

Type of sample	Total samples	Positives tested	Negatives (%) (%)
Commercial bovine sera	8*	3 (37.5)	5 (62.5)
Tissue culture	41	15 (36.59)	26 (63.41)
Vaccines	8	2 (25)	6 (75)
Local cattle sera	19	0 (0)	19 (100)
Total	76	20 (26.32)	56 (73.68)

* Number of samples

Table 2. Types, sources and results of tissue culture tested for BVDV contamination with RT-PCR

Cell line	Source	Presence of BVDV	Cell line	Source BVDV	Presence of
Red Cells	IW	+	ABC	IY	–
AP 61	IX	–	SP 2	"	–
MDCK	"	–	NS 1	"	–
C6/36	"	–	OPHIC 65	"	–
Vero	"	+	EPC 21	"	–
HEP-2	"	+	FHM 80	"	–
REF	IY	–	SPF CEF	"	–
MA 104	"	+	BT	"	–
BHK1	"	+	MC COY	"	–
CKK	"	–	L 3	"	–
CKT	"	–	NSO	"	–
ST 1	"	+	LT	"	+
MC COY	"	+	MDCK	"	+
MSB-1	"	–	HBC	"	–
CEK	"	–	KGPK	"	–
KTS	"	–	CRFK	"	+
FOM	"	+	RK	"	–
FC III	"	–	MDBK	"	+
SK2	"	+	SPV	"	–
FOS 3	"	+	Vero	"	–
HMLU	"	–			

IW, IX, IY research laboratories
+ positive for BVDV, - negative for BVDV

Table 3. Properties and results of vaccines tested for BVDV contamination with RT-PCR

Vaccine	Properties	Presence of BVDV
Polio Vaccine 1	Modified live virus	-
Polio Vaccine 2	Modified live virus	-
Measles Vaccine	Modified live virus	-
Canine distemper Vaccine	Modified live virus	-
Feline rhinotracheitis	Modified live virus	+
Aujeszky's disease Vaccine	Killed virus	-
Pseudorabies Vaccine	Killed virus	-
Swine Fever Vaccine	Live virus	+

+ Positive for BVDV; - Negative for BVDV

DISCUSSION

The results of this study show that contamination of commercial bovine sera, tissue cultures and vaccines is one of the common problems in the field of animal virology especially when vaccine production is considered. The results obtained are closely comparable to those of Smithies *et al.* (1975) and Kniazeff *et al.* (1975) for contamination of fetal calf sera (FCS) with BVDV. Beside BVDV, other viral contaminants could be isolated from commercial bovine sera include Parainfluenza virus 3 (PIV-3), Infectious bovine rhinotracheitis virus (IBRV) and enteroviruses. Methods for detecting BVDV in clinical samples rely on serial passage in cell culture and subsequent detection of the virus antigens in infected cells by immunofluorescence or immunoperoxidase staining. This technique is seemed to be laborious as the noncytopathic biotype of the virus grows slowly in cell culture and may require several passages before detectable amounts of the viral antigens are produced (Bezek *et al.,* 1988; Magar *et al.,* 1988; Potts *et al.,* 1989). Antigenic variation of BVDV, as an RNA virus, posed another obstacle on antibody-based detection of the virus (Ridpath *et al.,* 1991). This suggests that detection by DNA hybridization to nucleic acid based probes prepared from cDNA clones may also fail to detect some isolates of the virus. From these previous studies methods of screening of BVDV contamination in commercial FCS have not improved over the years.

The method used in this study (RT-PCR) is considered one of the recent and sensitive methods for detection of BVDV contamination in commercial calf serum. This method was previously employed by Hamel *et al.* (1995) who showed that the method is sensitive enough to detect very low levels of BVDV in FCS and thus may be suitable for routine screening of FCS in commercial settings. The protocol of RT-PCR technique adopted in this study was similar to that described by Schroeder and Balassu-Chan (1990) and Hertig *et al.* (1991) who described the use of reverse transcription followed by application of PCR to amplify specific fragments of BVDV genome. Therefore, similar results were obtained in this study.

RT-PCR method for detection of BVDV contamination in bovine sera, tissue cultures and vaccines was known to be more sensitive as compared to other methods. It was confirmed that this method is more than ten times sensitive in BVDV detection than virus isolation (Hooft van Iddekinge *et al.,* 1992). The contamination of tissue cultures and vaccines observed in this study may largely be attributed to the use of contaminated commercial sera. Furthermore, contamination of such sera gives a clear indication of improper screening that was performed by the companies to such sera. Therefore, quality control ought to be carried out in order to reduce usage of such contaminated bovine sera.

The PCR technique has been used to amplify BVDV isolates genome sequences of purified virion (Letellier *et al.,* 1999) or in serum samples collected from field animals (Schroeder and Balassu-Chan, 1990; Lopez *et al.,* 1991) and from milk (Radwan *et al.,* 1995; Drew *et al.,* 1999). We believe that our study supports the idea that the technique can be used to amplify BVDV genome sequences in commercial serum, tissue culture and vaccines.

This study is one of the first studies carried in Malaysia for screening of viral contamination purpose. The results of the study showed that the problem occurred similarly in the country. Since the incidence of BVD in cattle in Malaysia is not threatening, screening of these products especially bovine sera and vaccines ought to be carried out in order to avoid increased occurrence of the disease and the rapid and sensitive method of RT-PCR should be used to detect the contaminants in these products. The primers set used in this study can be used as a probe for detection of BVDV contamination in the laboratory biologics in the country.

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RINGKASAN

PENCEMARAN SERUM BOVIN KOMERSIL, KULTURA SEL DAN VAKSIN OLEH VIRUS CIRIT-BIRIT BOVIN YANG DI KESAN OLEH TINDAKBALAS RANTAIAN TRANSKRIPSI BERBALIK-POLIMERASE

Virus cirit-birit bovin (BVDV) merupakan pencemar biasa pada produk biologi makmal. Banyak sampel serum bovin komersil, kultura sel dan vaksin komersil telah di uji bagi kehadiran RNA BVDV dengan cara tindakbalas rantaian transkripsi berbalik-polimerase (RT-PCR). Kesemua serum komersil yang diuji telah di perakui bebas darpada BVDV. Dua primer BVDV telah disintesis dan di gunakan dalam ujian RT-PCR bagi memperbanyakkan cDNA virus yang bersaiz 300 bp. Bahan yang di uji diperolehi daripada beberapa institusi penyelidikan, klinik perubatan, kilinik veterinar, pembekal serum komersil dan lembu. Tiga daripada lapan serum bovin komersil yang di impot dan lima belas daripada 41 sampel kultura sel yang berlainan didapati positif kepada BVDV. Virus tidak dikesan pada vaksin manusia, tetapi dua vaksin haiwan adalah positif kepada BVDV. Tiada satu pun serum daripada lembu mengandungi BVDV. Ini menandakan banyak makmal telah menggunakan serum bovin komersil yang dicemari oleh BVDV dan juga menumbuhkan sel yang mengandungi BVDV. Oleh itu adalah perlu bagi menjalankan ujian secara kerap bagi memastikan kultura sel dan bahan yang berkaitan bebas daripada BVDV. Ianya penting dalam menyediakan serum haiwan bagi aplikasi kultura sel dan juga penyediaan vaksin bagi mematuhi kualiti umum bahan imunobiologi yang boleh diterima pakai.