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Detection of Bovine Viral Diarrhea Virus in some Vaccines, Sera and Cell Cultures

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Abstract

Bovine viral diarrhea virus (BVDV) causes contagious subclinical viral infections among dairy and beef cattle leading to economic losses worldwide. BVDV belongs to Family Flaviviridae, Genus Pestiviruses which is RNA viruses. It has two genotypes (I & II), each genotype has two biotypes, cytopathic (CP) and non-cytopathic (NCP) according to its cytopathology on cell culture. There is a risk factor from using bovine serum as a contaminant of the biological reagents and products prepared on cell cultures as vaccines. The study aimed to detect BVDV contamination in some biological agents such as (cell culture, serum and vaccines) using culture methods, ELISA and quantitate real time PCR. Samples consisted of 13 different attenuated and killed vaccines that are used in farm vaccination, five different serum samples that are used in cell culture and vaccines production in addition to three different cell culture samples that used in cell culture either for virus isolation or vaccines production. All samples inoculated on MDBK cells and examined for cytopathic BVDV revealed negative results. Cell culture and serum samples examined by ELISA technique also gave negative results. All samples were negative with real time PCR except one sample was positive. Comparing between all methods used, there was agreement between their results except in one sample that give positive result only by real time PCR. Therefore, we conclude that the chances of BVDV spread and contamination still there. The qRT – PCR is the most accurate method and can amplify a very little amount of virus. We also approve that all manufactured serum, vaccine samples were good as they were free from contamination with BVDV.

Keywords: BVDV, PCR, ELISA, Cell Culture, Serum, Vaccine.

Introduction

BVDV causes an acute contagious viral infection in dairy and beef cattle resulting in high morbidity, low mortality, diarrhea and buccal ulceration [1]. It is +ve ssRNA spherical particles [2]. Flaviviruses induce interspecies transmission, cross infection and in field infections [3,4]. Based on the nucleotide sequence of the virus and 5`UTR (Untranslated regions), there are two genotypes of the virus, type I and type II [5]. Each genotype yields cytopathogenic (CP) and non-cytopathogenic (NCP) biotypes [6]. All the BVDV strains are antigenically related with some serological differences [7] as they have common epitopes. Antibodies to one strain of virus should be protective against infection with the different strain [8]. BVDV induces leukopenia, salivation, nasal discharge, fever, diarrhea, dehydration, depression, anorexia, teratongenic defects, abortion, bovine respiratory disease complex and immune suppression [9]. BVDV can induce thrombocytopenia in young and adult cattle [10]. BVDV and mucosal disease (MD) are two clinically dissimilar conditions, although, both are caused by the same virus. BVD is an acute infection in susceptible cattle which may occur at any age in post-natal life [11]. MD is almost fatal but of low morbidity in cattle that have persistent BVD-MD virus acquired in the fetal stages and characterized by specific immune tolerance to the infection with virus strain and consequent lack of antibody [12]. Application of a vaccination protocol is important to reduce the risk of fetal infection in cow herd exposed to a viremic and virus shedding animals. Modified live vaccine (MLV) has inherent properties that might stimulate more complete protection against trans-placental infection [13].

Cell culture used in cellular and molecular biology includes the normal physiology and biochemistry of cells, the effects of drugs and toxic compounds on the cells and mutagenesis and carcinogenesis. It is used in drug screening and manufacturing of biological compounds (vaccines, therapeutic proteins). The presence of microorganisms can inhibit cell growth, kill

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cells and lead to inconsistent results [14]. Serum is commonly used as a supplement to basal growth medium in cell culture as it provides growth, attachment factors, iron transporters, vitamins, amino acids, lipids, carbohydrates, hormones and trace elements [15]. All MLV vaccines (Rota-coronavirus, bovine respiratory virus and rinderpest vaccines) produced in bovine cell cultures supplemented with serum from bovine source have risk to be contaminated with BVDV [16]. This work aimed to detect BVDV contamination in biological material such as sera, vaccines and cell cultures via isolation of BVDV on MDBK cells, detection by Ag ELISA, detection and identification of BVDV using quantitative real time PCR (qRT-PCR).

Material and Methods

Vaccine Samples

Commercial viral vaccines were purchased from Veterinary Serum and Vaccines Research Institute (VSVRI). They included BVDV+IBRV+PI-3V+BRSV vaccine (pneumo 4), inactivated vaccine that was examined in three animals, C.C. prepared sheep pox virus vaccine; attenuated vaccine and was examined in two animals. BEFV vaccine is attenuated vaccine and was examined in two animal, Camel pox virus vaccine is attenuated vaccine and was examined in two animals. Rota virus+ Corona virus vaccine is inactivated vaccine and was examined in one animal, RVFV vaccine is inactivated vaccine and was examined in one animal. PPRV vaccine is attenuated vaccine and was examined in one animal.

Table 1: Interpretation of QRT-PCR results

Interpretaon	Negative control	Positive control	Target
(+) Quantitative: calculate copy number	-	+	+
(-) Negative	-	+	-
Invalid	≤ 35	+	+/-
*	> 35	+	+/-
Invalid	+ / -	-	+/-

Table (2): Detection of BVDV in sera and cultured cells using ELISA:

Sample type	Sample A450	Sample A450 – Negative Control mean (S- N)	Interpretation
Calf serum (VSVRI)	0.104	0.01	Negative
Foetal bovine serum (E.C. Approved "Life Technologies in Germany")	0.125	0.031	Negative
New born calf serum (PAALABOR)	0.117	0.023	Negative
New born Calf serum (Sigma)	0.113	0.019	Negative
Equine serum (Sigma)	0.115	0.021	Negative
CEF cells (VSVRI)	0.132	0.038	Negative
MDBK cells (VSVRI)	0.136	0.042	Negative
Vero cells (Ahmed Abd El-Samie)	0.239	0.145	Negative

Negative control mean = 0.094 **Positive control mean = 1.422**

Serum

Sterile filtrated commercial serum samples were obtained from different companies, New born calf serum (PAA LABOR), Fetal bovine serum (Life Technologies in Germany), Calf serum (VSVRI), New born calf serum (Sigma), Equine serum (Sigma).

Cell culture

Samples included MDBK (VSVRI), Chicken embryo fibroblast cells (VSVRI), Vero cells (African green monkey kidney). It was grown on Hank's minimum essential medium (MEM), supplemented with New born calf serum (10%). Antibiotic solution: Penicillin G Sodium 100 IU/mL media,

Streptomycin Sulphate 100 µg/mL media, Mycostatin 100 µg/mL media. It was used for propagation and titration of the virus strains and for isolation of the virus. Isolation of BVD virus from the different samples was done on MDBK cells incubated at 37oC and 5% CO2. Samples were examined for the presence of cytopathic strains of the virus after three blind passages on MDBK cell as described by Cortese et al. [17].

Virus Strains

BVDV Type I CP (NADL) and NCP (NY1) biotype BVDV type-II CP (125 and NCP biotype (890) were kindly supplied from (Ahmed Abd El-samie H. Ali), Professor of Virology & Viral Immunology and Head of Virology Department, Faculty of Veterinary Medicine, Zagazig University. The viral strains were propagated and titrated on MDBK cells and were used as positive control in different tests.

Detection of BVDV by Enzyme linked immunosorbent assay (ELISA)

IDEXX BVDV Antigen Test Kit / Serum plus. Equation used in Calculation:

Calculation of negative control mean (NCx-):

$$NCx = \frac{NC1\ A450 + NC2\ A450}{2}$$

Calculation of positive control means (PCx-):

$$PCx = \frac{PC1\ A450 + PC2\ A450}{2}$$

Calculation of test samples:

$$S - N = Sample\ A450 - NCx$$

Samples with S-N values equal or less < 0.300 were considered negative for BVDV Ag. Samples with S-N values > 0.300 are classified as positive for BVDV Ag.

Molecular identification of BVDV by quantitative RT-PCR (qRT-PCR)

The RNA was extracted using Patho Gene-spine TMDNA/RNA Extraction Kit (iNtRON Biotechnology) according to the manufactured company.

Primer design™ gene sig® Kit (one step) for BVDV. primers and probe sequences kit have 100% homology with BVDV sequences on comprehensive bioinformatics analysis. Oligonucleotide primers and probes used in 5'UTR real time PCR.

Oligonucleotide primers and probes used for detection of BVDV: The probe was labelled at 5’ end with 6-carboxy fluorescein (FAM) reporter dye and labelled at 3’ end with 6-carboxy tetra methylrhodomine (TAMIRA) quencher dye. The primers and probes were synthesized by (Metabion,Germany). is F- 5’-GGG NAG TCG TCA RTG GTT CG-3’, R-5’- GTG CCA TGT ACA GCA GAG WTT TT-3’ and FAM-CCA YGT GGA CGA GGG CAY GC-TAMR as the probe [18]. The reaction was carried out using QuantiTect probe RT-PCR kits (catalogue No. 204443: ContainsA)

Positive control: For copy number determination and as a positive control for the PCR set up, the kit contains a positive control template. This can be used to generate a standard curve of BVDV copy number/Cq value. Alternatively, the positive control can be used at a single dilution where full quantitative analysis of the samples is not required.

A one step approach combining the reverse transcription and amplification in a single closed tube is the preferred method. Amplification conditions using oasig TM One Step or Precision TM One Step PLUS 2x qRT-PCR Master Mix. The conditions included reverse transcription for 10 min at 55°C, enzyme activation for 2 min at 95°C, denaturation for 10 sec at 95°C and data collection for 60 sec at 60°C. The interpretation of the results was carried out according to the criteria in Table 1.

Positive control template (red) is expected to amplify between Cq 16 and 23. The sample is positive with Cq> 35, sample must be reinterpreted based on the relative signal strength of the two results:

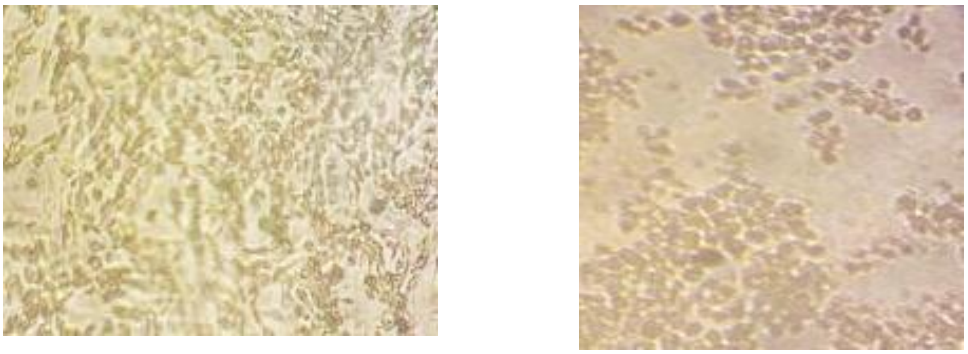
1-If sample amplifies >5Cq earlier than negative control then sample should be reinterpreted with the negative control verified as negative.

2- If the sample amplifies <5 Cq earlier than the negative control then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination.

Results

Isolation of BVDV on MDBK cell culture

Samples propagated on MDBK cell line revealed that no cytopathic effects in comparison with CP-BVDV strains (NADL & 125) after 72hours post inoculation were observed as shown in Figure 1a. The CP-BVDV inoculated in MDBK showed CPE characterized by elongation, vacuolation and granulation of the cytoplasm. Figure 1b.



1a 1b

Figure 1a: Normal MBDK cells showing confluent monolayer sheet. Figure 1b: MDBK cells inoculated with CP-BVDV showing characteristic CPEs such as vaculation, cytoplasmic granulation and detachment of cells.

Detection of BVDV by ELISA

For the assay to be valid, the difference (P-N) between the Positive Control means (PCx-) and Negative Control mean (NCx-) must be greater than or equal to 0.150 optical density (OD). In addition, the Negative Control mean (NCx-) must be less than or equal 0.250 OD. The presence or absence of BVD antigen in the sample is determined by the corrected OD value (S-N) for each sample. All the tested samples were negative for presence of BVDV antigen.

Samples with S – N values equal or less than 0.300 are negative.

Samples with S – N values greater than 0.300 are positive.

Quantitative RT-PCR (qRT-PCR)

Extracted genomic RNA was tested by Primer designTMGenesig® Kit for BVDV genomes. All samples except Vero cells give negative result by qRT-PCR even after 39 cycles (Figure 2). All examined vaccine and serum samples were BVDV free. MDBK and FBC were free also from contamination with BVDV as showed in Figure 2. Vero cells give positive result by qRT-PCR after 36 cycles compared with the negative control (Figure 3a). The positive sample was re-examined using another kit to approve its positivity and also give positive result after 28 cycles (Figure 3b).

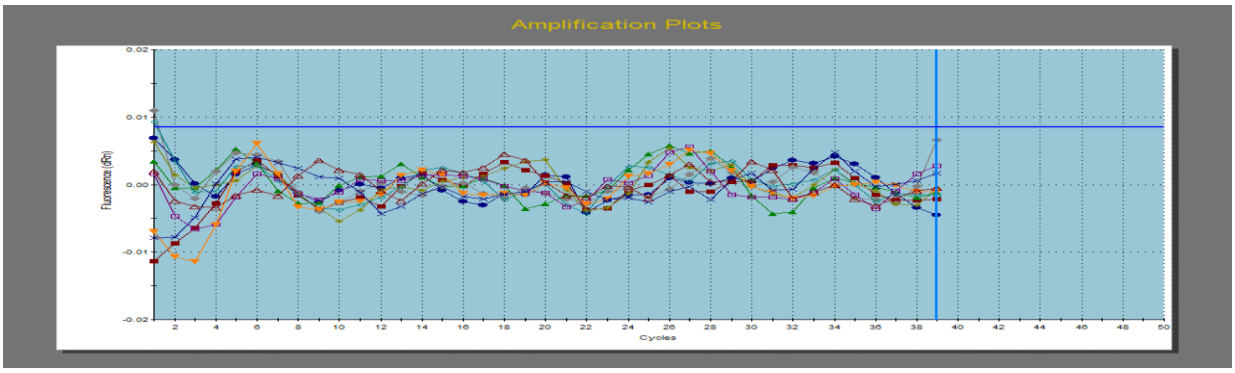


Figure 2: Amplification plots of negative samples for 5/ UTR qRT-PCR assay. TL = Thershold line (all curves below it considered negative).

Discussion

MDBK, bovine turbinate (BT) and VERO cells were susceptible to infection with BVDV [19], because, specific receptors for BVDV were identified on the plasma membrane of those cells. Multiplication of BVDV in VERO cells showed abortive attachment [20]. Infection of MDBK cells with BVDV induced endoplasmic reticulum stress mediated apoptosis [21]. Continuous propagation of the BVDV in MDBK cells is used for attenuation of BVDV for vaccine production [22]. Baby Hamster Kidney (BHK21) and porcine kidney (PKC) were not susceptible to BVDV [19].

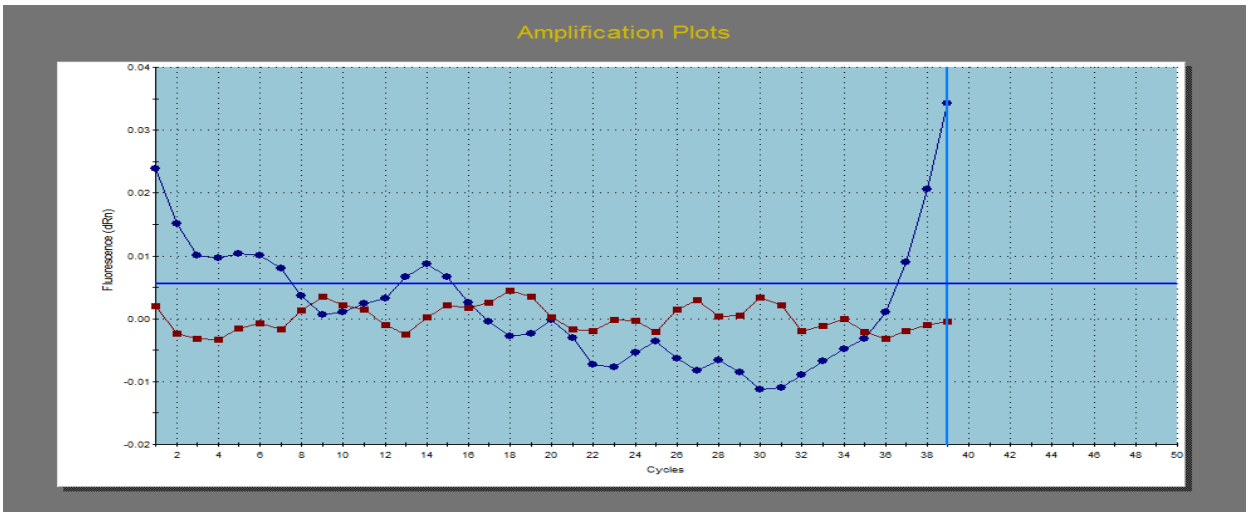


Figure 3a: Amplification plot of positive sample with negative control for 5/ UTR qRT-PCR assay. TL = Thershold line (curve above it consider positive and curve below it consider negative control).

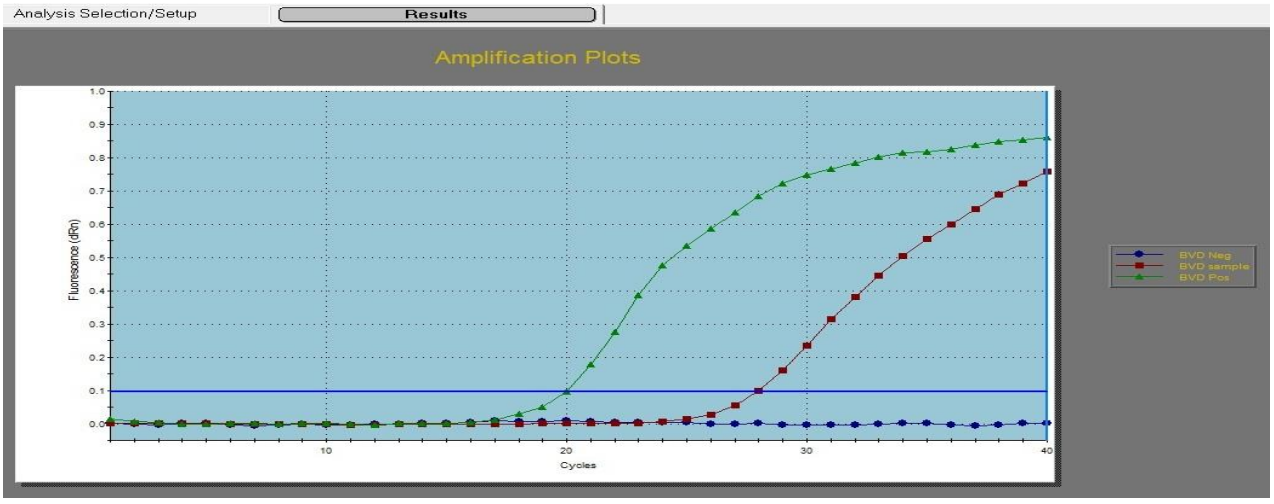


Figure 3b: Amplification plots of positive sample (Vero cell) compared with positive and negative control for 5/ UTR qRT-PCR assay. TL = Thershold line (curves above it considered positive compared with positive control and curve below it considered negative control).

BVDV is the most common contaminant agent to bovine serum, vaccine and cell culture production where the bovine serum is used [23]. Because contamination by BVDV changes the characteristic features of cell growth resulting in false-positive results when performing diagnosis of other viruses [24]. Cultural methods and immunological assays are used to detect extraneous BVDV in the biological products to assure that vaccines, sera and cell culture are free from contamination with this virus. Isolation of BVDV on MDBK cells revealed that all the samples were free from contamination with CP-BVDV. ELISA results showed that all the tested samples were negative for the presence of BVDV antigen as previously reported in a study to detect BVDV contamination in fetal bovine serum samples and some farm animal vaccines inoculated on MDBK [25]. Also, our findings agree with Webb et al. [26] who reported that ELISA is sensitive in detection of BVDV antigen as virus isolation. They also reported that it is objective, fast and work equally for both cytopathic and non cytopathic viruses. Jordon et al. [22] used ELISA in evaluating BVDV antigen during vaccine production. Quantitative Realtime polymerase chain reaction (qRT-PCR) is one of the recent techniques used for detection of BVDV through detection of its RNA using specific primers. The results revealed that all tested samples were free from contamination with BVDV-RNA except one sample. These results agree with Hassan [27] who detected BVDV in cell cultures and biological products. Moreover, Saliki et al. [28] proved that real-time PCR was 10-fold more sensitive than conventional RT-PCR. Our results agree with Zhang et al. [29] who used RT-PCR for detection of BVDV in cell cultures and reported that it was a specific and sensitive method. Our results are supported by the results of Belak and Ballagi-Pordany [30] who used it as a rapid, simple, specific and sensitive technique for the evaluation of live viral vaccines. Our results agree with Dang et al. [31] who used RT-PCR for identification of BVDV in serum and buffy coat. We agree also with Bock et al. [32] who reported that BVDV is not responsible for contamination of human measles, mumps and rubella vaccines. Our positive result agrees with Harasawa and Tomiyama [33] who reported that BVDV RNA was detected in 21 of 88 cattle, swine, human, rabbit, mouse, cat, sheep, monkey, and horse cell cultures tested (23.9%) by real-time and conventional PCR.

Conclusion

It be concluded from this study that the chances of BVDV spread and contamination still there. The qRT – PCR is the most accurate method and can amplify a very little amount of virus. We also approve that all manufactured serum, vaccine samples were good as they were free from contamination with BVDV.

Conflict of interest

The authors have no conflict of interest to declare.

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الملخص العربي

الكشف عن فيروس الاسهال الحموي البقري في بعض اللقاحات والامصال والخلايا النسيجية

ايمان مصطفى حافظ مصطفى- على عبد الرشيد على سلامه – عا دل محمد سليمان – محمد البكرى عبد الرحيم اسماعيل –
جميلات قطب فرج قطب- احمد عبد السميع حسن على
قسم الفيرولوجيا- كلية الطب البيطرى – جامعة الزقازيق

فيروس مرض الاسهال البقري الفيروسي يعتبر فيروس شديد العدوي التي تؤثر علي الابقار الحلوب والابقار المنتجة للحم يؤدي الي خسائر في الثروة الحيوانية التي تؤدي الي خسائر اقتصادية في جميع انحاء العالم

معظم العدوي بفيروس الاسهال البقري الفيروسي تكون في صورة تحت اكلينيكية. هناك عامل خطورة من استخدام المصل البقري حيث هناك امكانية واحتمال ان تكون ملوثة بهذا الفيروس. هذا يؤدي الي احتمالية تلوث المنتجات البيولوجية المشتقة من المصل البقري الذي يستخدم للزرع الخلوي والتي قد تؤدي الي تلوث المخزون الفيروسي بالاضافة الي المنتجات الحيوية مثل اللقاحات. هذه الدراسة الحالية وجهت للكشف عن الفيروس كعامل ملوث في بعض المنتجات البيولوجية مثل (المزارع الخلوية ، الامصال ، اللقاحات) باستخدام طرق الزرع الخلوي، اختبارالاليزا واختبار تفاعل انزيم البلمرة المتسلسل الكمي حقيقي الوقت .تم استخدام عينات عبارة عن ١٣ لقاح مختلف متنوع بين الحي والميت المستخدم للتحصين في المزارع ، خمسة عينات مختلفة من الامصال المستخدمة في الزرع الخلوي وانتاج اللقاحات، بالاضافة الي ثلاثة عينات مختلفة من الخلايا المستخدمة في الزرع الخلوي سواء لعزل الفيروسات او لانتاج اللقاحات.جميع العينات تم حقنها علي خلاياMDBK وتم فحصها للكشف علي فيروس الاسهال البقري الفيروسي المسبب للاعتلال الخلوي وكانت جميعها سلبية . تم فحص عينات الزرع الخلوي و عينات الامصال باختبار الاليزا وكانت جميعها ايضا سلبية . اختبار تفاعل انزيم البلمرة المتسلسل الكمي حقيقي الوقت تم اجراءه علي جميع العينات وجميع العينات كانت سلبية باستثناء عينة واحدة كانت ايجابية.بمقارنة جميع كل طرق الفحص المستخدمة ، وجد توافق بين نتائجهم باستثناء عينة واحدة التي اعطت نتيجة ايجابية فقط باستخدام اختبار تفاعل البلمرة المتسلسل الكمي . استنتجنا انه علي الرغم من انتشار العدوي تحت الاكلينيكية والسلبية لهذا الفيروس في المنتجات البيولوجية الا ان الطرق التشخيصية التقليدية لم تكشف عنه. لذلك استخدم تفاعل انزيم البلمرة المتسلسل الكمي حقيقي الوقت والذي اثبت وجود عينة موجبة وبذلك اثبت وجود الفيروس وهذا يثبت دقة مثل هذه الاختبارات في الكشف عنه.العينة الايجابية ممكن تكون نتيجة استخدام مصل ملوث بالفيروس اثناء التعامل مع الزرع الخلوي .ايضا ان زجاجات الامصال المصنعة للقاحات المستخدمة في هذه الدراسة خالية من التلوث بفيروس الاسهال البقري الفيروسي.