VIROLOGY

Contamination of Cell Cultures with Bovine Viral Diarrhea Virus (BVDV)

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The incidence of contamination of cell strains used in biological and virological studies and of fetal calf sera (FCS) manufactured by Russian and foreign companies used for cell culturing with noncytocidal bovine viral diarrhea virus (BVDV; *Pestivirus*, *Flaviviridae*) was analyzed. The virus was detected by reverse transcription PCR and indirect immunofluorescence with monoclonal antibodies to BVDV virion envelope glycoprotein in 25% of 117 cell strains and 45% of 35 tested FCS lots. The virus multiplied and persisted in a wide spectrum of human cell strains and in monkey, swine, sheep, rabbit, dog, cat, and other animal cells. The levels of BVDV genome RNA in contaminated cell cultures reached 10^2-10^3 g-eq/cell and in serum samples 10^3-10^7 g-eq/ml. These facts necessitate testing of cells and FCS for BVDV reproduced in cells without signs of infection detectable by light microscopy. The molecular mechanisms of long-term virus persistence in cells without manifestation of cell destruction are unknown.

Key Words: *cell strains; fetal calf serum; virus contamination; bovine viral diarrhea virus; reverse transcription–polymerase chain reaction (RT-PCR) and indirect immunofluorescence*

Sera from large animals (cattle, horse) serve as one of the main components of nutrient media for cell culturing. High incidence of numerous latent and acute viral infections (including those caused by pestiviruses, bovine reproductive and respiratory disease viruses, herpesviruses, adenoviruses, bovine leukemia viruses) in animal breeding centers of Russia and other countries of the world (Europe, America) necessitates special testing of the sera used as culture medium components [6]. Long-term culturing of cells and use of virus-contaminated sera (without proper testing of cells and sera for contaminating viruses) can lead to, in addition to "applied loss", erroneous conclusions in studies of the mechanisms of virus-cell interactions at the molecular and gene levels. In addition, the absence of regular testing of cells and preliminary analysis of commercial lots of sera (before use) can lead to animal virus contamination of vaccines for humans [14,15].

We studied a random sample of cell strains derived in different years from different cell culture collections in order to detect contamination with bovine viral diarrhea virus (BVDV; *Pestivirus*, *Flaviviridae*). The greater part of cell strains were from the collection of D. I. Ivanovsky Institute of Virology, included in the Catalogue of Russian Cell Culture Collection (1991) and European Catalogue of Human and Ani-

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mal Cell Strains (1993) and a part thereof. BVDV is highly contagious; it affects the mucosae and causes diarrhea, abortions, immune disorders, fetal transplacental infection; the disease can run an acute or chronic course [1]. The virus is highly prevalent in cattle breeding centers in Russia and in foreign countries [1,5,6]. Specific antibodies, virion proteins, and genome RNA serve as the markers for its detection in the sera. Genome RNA is a single-stranded RNA of positive polarity and is infectious [9].

Studies of recent years found BVDV in native cattle sera and in cell strains. The virus gets into cell cultures with cattle sera (including fetal and calf sera) used as components of nutrient media. In nature, the virus is presented by two genotypes [1,4,9], each has cytopathogenic (CPV) and non-cytopathogenic variants (non-CPV). The cells infected with BVDV CPV are usually discarded at the first passages; cell death develops by the apoptosis mechanism. The other variant of the virus usually causes chronic infection without apparent morphological disorders in the cells, this impeding its detection by nonspecific morphological methods.

The use of "pure" cell cultures is particularly important for studies of various aspects of cell biology. The use of standard tested components of culture media is also important for modern applied studies of stem cells [3]. We attempted this study in order to prevent the use of contaminated cells in culturing of viruses, including other viruses of the same family.

MATERIALS AND METHODS

The cells stored in liquid nitrogen (500,000/ml) were rapidly defrosted at 37°C, washed 3 times in 5-fold volume of medium 199 with 5-7-min centrifugation at 1500 rpm, counted in a Goryaev chamber, and lyzed for subsequent isolation of RNA. The cells were harvested with versene and chymotrypsin and precipitated at 4°C and 1500 rpm for 5 min. Precipitation/resuspending was repeated 3 times. Then 0.14 M NaCl (500 μl) and an equivalent volume of lyzing buffer from Vetbiochim kit were added to the cell precipitate.

Isolation of RNA and amplification of the virus genome were carried out as described previously [1].

The virus antigen was detected in cells fixed in cold acetone by indirect immunofluorescence (IIF) with specific mouse antisera and anti-species affine-purified FITC labeled IgG (Sigma). In some cases ultrathin sections of the cells were examined under electron microscope [2].

RESULTS

A total of 117 specimens of cell cultures (of different storage duration and from different sources, mainly from Cell Culture Collection of D. I. Ivanovsky Institute of Virology) and 35 serum specimens manufactured by the Russian and foreign companies were studied by RT-PCR. Our study was a part of cell culture quality control used in virological and biological studies.

The main types of analyzed cells were derived from various animals: simian continuous cell strains; human continuous cell strains (diploid strains: human fetal fibroblasts (HFF), human fetal lung (HFL) cells, M7 musculocutaneous cells, *etc.*); porcine continuous cell strains (SPEV, PS, PK-15); human cells not contaminated with HeLa cells (A-549, GL-6, CaCo, *etc.*); human HeLa and HeLa-like cells (HeLa, Hep2, *etc.*); human lymphoblastoid cells (Raji, Namalva, Daudi, L_{101}, *etc.*); mouse and rat cells (L_{929}, 3T3 BALB/c, *etc.*); hamster cells (BHK-21, HAK, BHK-BS, CHO-K1, *etc.*); cattle cells (MDBK, PT-80, PEK, TEB).

Human and simian cells. Of 31 studied strains (of different length of storage) of human cells, 25% were BVDV-contaminated (shown with an asterisk* in the text), the virus titers (in genome-equivalents, g-eq) varying from 10 to 1000 per cell.

The following cell strains were studied: *diploid* HFL, HFF, M7 cells, *human continuous cells not contaminated with HeLa cells*: A-549 (lung carcinoma), GL-6 (glioblastoma), T-24 (vesical tumor cells), HT29 (colorectal carcinoma), CaCo* (intestinal carcinoma), GM-639 (fibroblasts of a galactosemia patient), L_{41} (bone marrow cells from a leukemic patient); HEK* (human embryonal kidney cells); *human continuous HeLa and HeLa-like cells*: HeLa (carcinoma of the cervix uteri), Hep-2 (laryngeal carcinoma), RH (human fetal kidney), Chang conjunctiva, Chang liver, Huh-7* (human hepatoma cells); human lymphoblastoid cells: Raji – Burkitt's lymphoma, Namalva*, Daudi, P3H3(R-1)*, P3H3* – all lymphoma cells, L101* (blood leukocytes from a leukemic patient), T1387* (bone marrow T-lymphocytes), Molt-4 (T cells from a leukemic patient).

The BVDV virus was far less incident in primate cell strains of different length of storage. Virtually all the studied specimens of simian cells contained BVDV genome RNA (green monkey cells: BGM*, CV1, Vero, Vero E6*, Vero(B), 4647*, GMK*, BSC1*; rhesus macaque kidney cells: MA-104*, LLC-MK-2*, Frhk-4/R* (8 of 11 strains – 72%). It is noteworthy that no cytopathic effect was detected in any of the studied cell strains.

IIF analysis of cells. Detection of BVDV virion envelope glycoprotein in contaminated cell cytoplasm confirms virus multiplication in human and simian cells (Fig. 1, *a-e*). At the early stages of reproduction BVDV virus antigen was detected in the cytoplasm of morphologically intact infected cells, first in the perinuclear zone as diffuse or granular fluorescence. Fluorescence

then increased and involved the entire cytoplasm. Binuclear cells were found in 1-2% of Vero and MDCK cell samples with specific fluorescence; this could indicate cell fusion or incomplete mitosis phase (Fig. 1, *d*).

Fluorescence intensity varied greatly in different types of chronically infected cells. In other words, despite intensive accumulation of the virus in cells, no morphological changes in comparison with normal cells could be detected by light microscopy. By contrast, signs of cell death were detected in control cells infected with BVDV cytopathogenic variant on days 3-4: their shape changed, cytoplasmic incorpora-

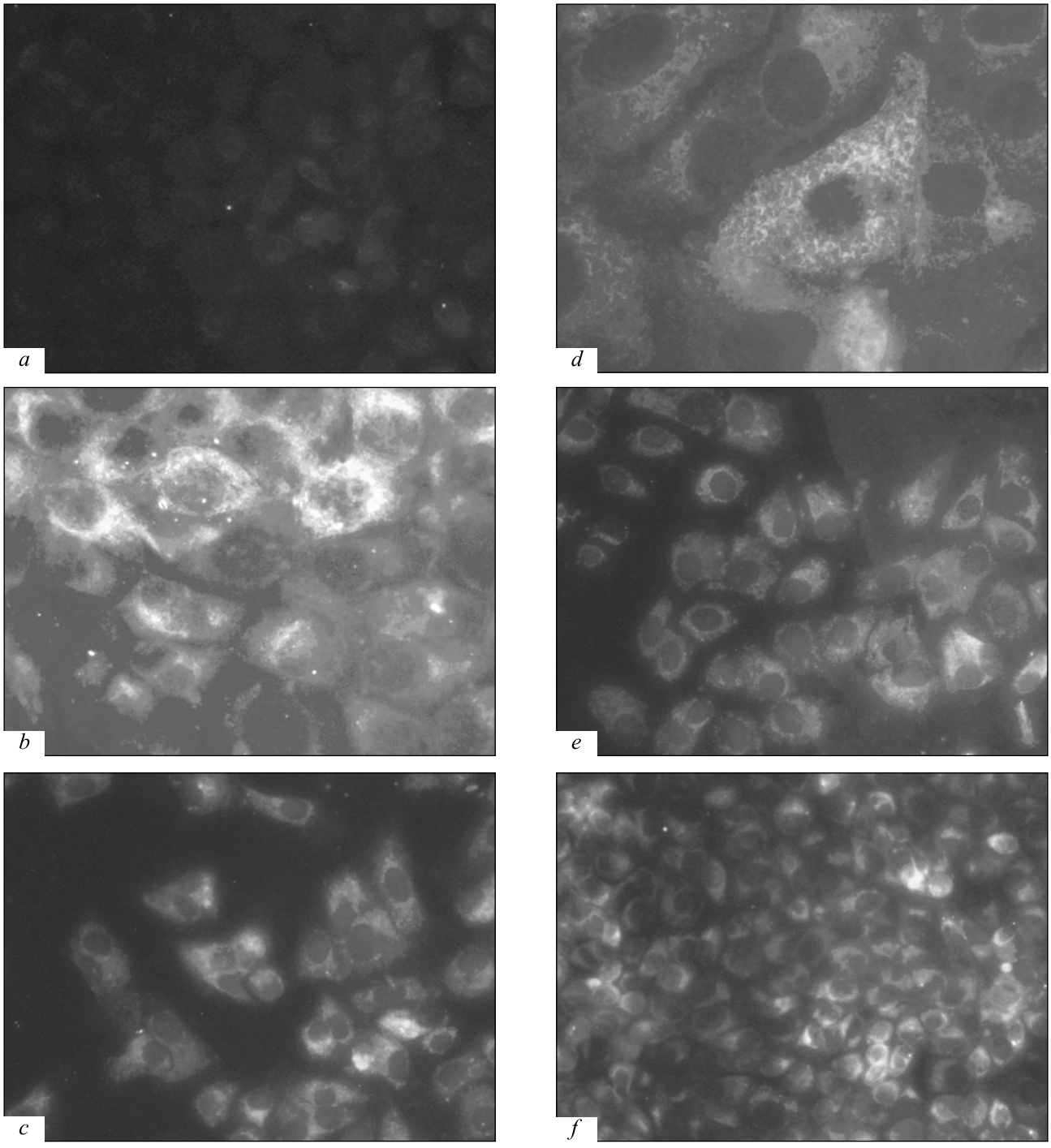

Fig. 1. Study of cells by IIF. *a*) control (not contaminated) Vero E6 cells, ×40; *b*) MDCK* cells. Diffuse fluorescence (distribution) of BVDV virion envelope glycoprotein in perinuclear zone; *c*) Vero* cells, growing culture, early stage of virus antigen accumulation in the cytoplasm; *d*) HEK* cells, ×100: "granular" fluorescence of cytoplasmic zone of the cells, a binuclear cell is seen; *e*) CV1* cells, growing culture, early stage of virus antigen accumulation in the cytoplasm; *f*) PS* cells, monolayer: diffuse fluorescence of virus antigen in the cytoplasm.

tions emerged, the nuclei shrank, and the cells were detached from the sublayer (the data not presented).

Hence, virus persistence in the studied human and simian cells was detected by three methods for detection of the virion components (IIF showed viral antigens, RT-PCR showed genome RNA, and electron microscopy showed the virions). These results indicated that BVDV infected numerous cell strains derived from humans. The virus multiplied and persisted in the respiratory and intestinal epithelium, in human fetal kidney cells, hepatoma cells, and white blood cells (T lymphocytes) affecting the same cells types as in BVDV infection of cattle associated with pathological changes in various tissues [5,6,11].

No data on clinical symptoms of BVDV infection in humans are available. However, the virus presumably infects humans and causes a disease with subsequent production of specific antibodies. Specific anti-BVDV antibodies were detected in blood specimens from veterinarian workers at cattle farms (T. I. Aliper, personal communication). Though no specific clinical symptoms of any kind in humans have been described until now [6], *human infection and asymptomatic carriership and even infection with manifest clinical symptoms are possible* .

All the studied cattle cells: MDBK (bovine kidney cells, stored since 31.01.05), PT-80 (calf kidney cells, 17.06.83), PEC (fetal calf kidney cells, 10.12.07), TEB (fetal calf testicles, 19.06.92) were contaminated with the virus, which was not surprising in view of high sensitivity of these cells to BVDV, specific symptoms related to cell and tissue involvement (diarrhea, reproductive disorders, transplacental penetration of the virus, and fetal involvement), and high prevalence in many countries.

The virus was also found in some cultures of liver cell strains: porcine (PS, SPEV, PPES), sheep (FLK), rabbit (RK, RK82), dog (MDCK), cat (CREK, CC-81). The virus was not found in any of the 7 rodent (rat, mouse, and hamster) cell strains, but this did not mean that BVDV could not infect these cells. The levels of BVDV virion RNA (genome-equivalents) varied significantly in different cells of different length of storage: from 10^2 to 10^8 per sample (Fig. 2). The virus was found in the cytoplasm by IIF used for detection of intracellular viral antigen (monoclonal antibodies to virion envelope glycoprotein E) (Fig. 1, *b-f*). Electron microscopy showed maturing virus particles in endoplasmic vacuoles (data not presented).

In order to clear out the possibility of BVDV virus penetration and time needed for its "fixation" in cell cultures of different origin (human, simian, dog) from contaminated serum (FCS), intact (not infected) Vero cells were cultured with 10% FCS containing BVDV virus. BVDV RNA was steadily detected in the cells after just 3-4 days of contact with this serum. The cells infected by this method produced the virus throughout subsequent passages.

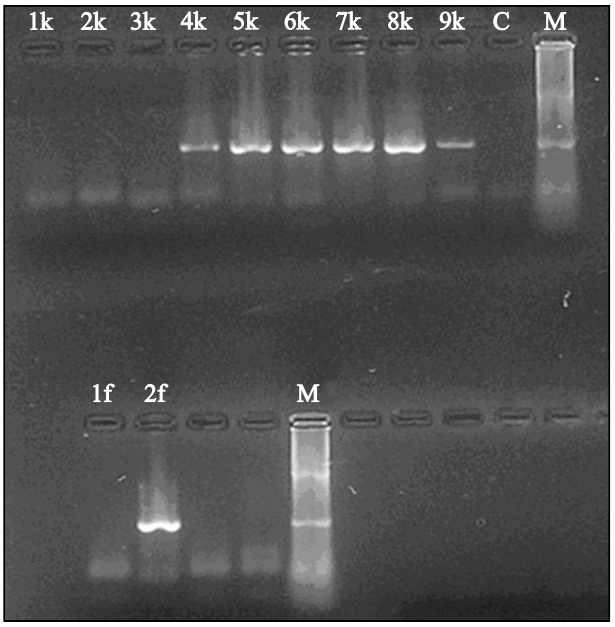

Fig. 2. Agarose gel electrophoresis of RT-PCR products in studies of cell cultures and fetal calf sera. 1k-3k: cell strains (HFL, HFF, M7) without BVDV; 4k-9k: contaminated cell strains (HEK, Huh-7, Vero E6, BGM, MA-104, CaCo). M: marker; C: negative control. 1f and 2f*: fetal calf sera.

Hence, the virus was detected in cell cultures of different origin: human, domestic animals (dog, cat), swine, cattle (adult animals and fetal tissue), the virus titers (in genome-equivalents) varying from 10 to 1000 per cell. This confirmed the possibility of virus penetration into the cell and indicated high receptor plasticity of the virus and the possibility of its reproduction in the above cells derived from tissues of different biological species. The results of genome RNA detection by RT-PCR correlated with the virus antigen detection by IIF and electron microscopy.

Analysis of contamination of commercial sera. A total of 35 sera of different lots from different companies were analyzed: Biolot (cattle), Furo (cattle), PanEco (FCS), Sigma (FCS), HyClone (FCS), Amimed (FCS). BVDV virion RNA was detected in a high percentage (>45%) of specimens. The levels of BVDV RNA in the serum samples varied from 10^3 to 10^7 g-eq/ml (Fig. 2). Heating of the sera at 56°C for 30, 45, or 60 min led to an appreciable reduction of the level of detectable BVDV RNA (by 3-5 log/ml), but if the level of virion RNA in the initial serum was very high (more than 10^6 g-eq RNA/ml), BVDV RNA was detected in the first dilutions even after the longest heating.

These results confirm the need of thorough testing of cells in experimental and practical studies.

The results indicate high prevalence of BVDV as a factor contaminating cell cultures and cattle sera intended for research and practical studies. This necessitates steady preliminary analysis of commercial lots of sera, as otherwise this virus can contaminate the new vaccine preparations for humans [12,14].

Cell cultures contaminated with BVDV used in our study are a convenient model for studies of molecular mechanisms of long persistence of the virus in cells without manifestation of cell destruction signs. The mechanisms of long persistence of contaminating virus without manifestation of morphologically detectable (by light microscopy) changes in cell strains remain unknown. Presumably, genetic information of the virus is retained in this case by the mechanism we described previously for flaviviruses, in particular, tick-borne and Japanese encephalitis viruses [7].

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