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PESTIVIRUSES, WHICH CONTAMINATE IMPORTED FETAL BOVINE SERUM, MAY BE A CAUSE OF THE GLOBAL SPREADING OF VIRAL DIARRHEA IN CATTLE — A MINI REVIEW

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Abstract

Pestiviruses are an important cause of economic losses in the dairy and beef industry. Diseases caused by them are common around the world with varying prevalence associated with the features of regional strategy of livestock including in Russia (A.G. Glotov et al., 2002; M.I. Gulyukin et al., 2013; J.F. Ridpath, 2010). The bovine viral diarrhea virus is considered as a prototype member of the genus *Pestivirus*, *Flaviviridae* family. Two distinct viruses designated as BVDV1 and BVDV2 cause the disease in cattle. A candidate member of the genus is BVDV3, the atypical and not classified pestivirus which shows high similarity to BVDV1 and BVDV2. The BVDV3 presence in the cattle population can compromise BVDV control or eradication (F.V. Bauermann, 2013). This virus requires special attention. BVDV was isolated from commercial lots of fetal bovine serum used for cell culture and biologicals, and is dangerous because of possible spread to new regions (H. Schirrmeier et al., 2004). Viruses of this genus are contaminants of fetal serum, continuous cell line cultures, human and animal vaccines, interferons, trypsin, embryos, stem cells, etc. (B. Makoschey et al., 2003; S.Q. Zhang et al., 2014). Because of globalization and rapid development of cell biotechnology in veterinary and human medicine, the demand for fetal bovine serum, which is a by-product of beef industry, is annually increasing (G. Gstraunthaler et al., 2013). OIE has established product quality standards and regulations according to which all the cell cultures intended to use must be tested for the absence of the virus and its RNA in some passages. Blood serum including fetal serum must be free of the virus and also of the specific antibodies thereto (OIE, 2015). These requirements should also apply to BVDV3. The lack of fetal bovine serum production in Russia creates the possible risk of lots from foreign manufacturers of questionable quality. Special scholar publications report on cases of contamination of different cell cultures and sera by noncytopathic BVDV strains in Russia (S.V. Alekseenkova et al., 2013). The live vaccines prepared using low-quality raw materials can be a potential source of virus for susceptible animals, and contaminated diagnostic antigens can cause false results of the study. Thence, more strict control is extremely important to prevent biological contamination of vaccines and other biologicals.

Keywords: pestiviruses, cattle, bovine viral diarrhea viruses, atypical pestivirus, fetal bovine serum, contamination

Pestiviruses are an important cause of economic losses in the dairy and beef industry. Diseases caused by them are common around the world, including Russia, with varying prevalence associated with the regional features of the cattle breeding strategy [1-3]. Viral diarrhea is caused by mucous membrane diseases in the bovine animals (BVDV — Bovine Viral Diarrhea Virus) and is considered to be a prototype member of the genus *Pestivirus* (*Flaviviridae*) family. Two distinct viruses designated as BVDV1 and BVDV2 cause the disease in bovine animals. The first one is common all over the world (recently, 21 subtypes — from 1a to 1u — are described) [4-7], and the second one is characterized by the limited

spread, in particularly, in USA and Canada [8, 9], South America (Brazil, Uruguay) [10, 11], in several European countries (Germany, Slovakia, Italy) [12-14], Asia (South Korea, Japan) [15, 16], and Mongolia [17]. The BVDV2 is divided into five subtypes (from 2a to 2e) [18]. A candidate member of the genus is a non-officially classified virus having several names (BVDV3, Hobi-like pestivirus, an atypical pestivirus) and showing high similarity to BVDV1 and BVDV2. The BVDV3 presence in the cattle population can significantly mitigate the effectiveness of BVDV control and pathogen eradication. The BVDV3 was for the first time secreted in 2004 from the fetal calf serum made in Brazil [21], and in furtherance was found in bovine animals in South America [22], Asia [23-25] and Europe [26, 27]. Representatives of *Pestivirus* genus are known as serum contaminants, continuous cell culture lines, vaccines for medical and veterinary purposes, interferon, trypsin, and other medicines for biotechnical researches and techniques, embryos, stem cells, etc. [19, 20]. Although BVDV as a biological product contaminant is well known since 1960s [28], role of the atypical virus is elucidated to a lesser extent.

Specific feature of the studied virus group is its ability to cause persistent fetal infection by only non-cytopathogenic biotype. Fetal infection takes place on day 40-125 of intrauterine growth, when fetal immune system is not formed yet [1]. It results in birth of immune tolerant calves serving as persistent source of pathogen for non-immune animals. Concentration of virus in blood of such individuals is high starting from the intrauterine growth, and they produce it during the entire life with all discharges and excreta. No specific antibodies are produced in persistently infected animals [3].

We have summarized information on contamination by fetal bovine serum (FBS) pestivirus. This fact should be accounted for due to the threat of pestivirus spread with the lots of imported FBSs, especially due to the growth of FBS consumption in cell technologies, biotechnology, pharmaceutics, and medicine.

Fetal serum. FBS is the most known and widely used additive to breeding grounds for initiation and increase of cell culture growth speed in mammals due to high concentration of biological substances in fetal blood [28]. There are still no other universal and effective cell growth stimulators. FBS is a natural mixture of factors required for cell sticking in substrata, their active growth, and proliferation [29]. In Russian scholar publications FBS is called a fetal calf serum, however during the last years such term was revised by several authors since serum is not produced in embryo period, but rather in the later fetal period.

The serum is produced in aseptic conditions from the fetal blood of randomly selected pregnant beef cows meant for slaughter [29]. Since animals of both sexes are freely pastured together in large-size fleets, cows often become pregnant. Cows are specially settled for production of fetal serum in Hungary, Baltic States, and, possibly, Czech Republic [30]. Usually, biomaterial is a 6-month foetus, but in fact foetus aged 3-months may be used. Usually, blood is collected by cardiocentesis, while in Uruguay, Brazil, and Australia blood in collected by centesis of umbilical or jugular vein [30]. Each lot of commercial fetal serum includes the material collected from different farms. As a result, the entire lot may be contaminated if it contains serum of infected animals [31].

The highest demand for FBS is in USA and Europe, where the largest share of FBS is produced, however, with the use of raw material supplied by Brazil, Argentina, Central America states, South Africa, Australia, and New Zealand [28]. Main exporters of finished products for cell culture at production of vaccines and preparations are USA, New Zealand, and Australia. Serum for research purposes is mainly supplied from the South America, South Africa, and

Brazil. Thus, in 2007 Brazil was the second beef meat producing and exporting country. Consequently, during the year 70 % of serum used in the European medical industry was from Brazil [32].

FBS market. Cell cultures are widely used in biopharmaceutical industry, growth of which, in its turn, promotes FBS production and sales. Since 2013 biopharmaceuticals became the largest and, according to forecasts, rapidly growing segment of cell culture market [33]. It is expected that by 2019 the global market of the cell culture based products (culture medium, serum, and reagents) in biotechnology, pharmacy, and medicine would grow by up to USD 4.1 billion [34]. Due to studies of human stem cells and their use for treatment of various malfunctions, it is expected that the market of products for cell cultures will grow up to USD 14.8 billion by 2019 (as compared to 2014, when such market volume comprised USD 6.0 billion) [35]. Nearly 500000 l of fetal bovine serum is produced annually all over the world requiring up to 1000000 calves. FBS sales grow [28], while the entire market is under control of several producers. For instance, in 2014 it was three American companies, the Thermo Fisher Scientific, Life Technologies Co. and Sigma-Aldrich, the aggregate share of sales of which comprised 80 %; buyers were mainly large biopharmaceutical companies. The first two of the above-listed firms accounts to 60 % of FBS market in USA and in the world [30].

In this context, it is important that FBS buyers around the world have reviewed their relations with the suppliers and have determined the strategy to lower the risks of possible FBS contamination by endotoxins, mycoplasmas, PrPsc, and viruses (in particular, pestiviruses) accounting for the qualitative and quantitative, geographic, and seasonal changes in FBS lots [36]. Regardless of the fact that veterinary specialists during studies select foetus only from animals suitable for human consumption, it is widely known that FBS is a potential source of many viruses. FBS contamination by bovine pestiviruses is known since 1960s, provided that BVDV is most prevalent due to its ability to trans-placental transmission with further persistence in immunologically immature foetus [37]. Due to the risk of virus contamination, it was strongly recommended to inactivate serum in addition to the direct virus testing with the use of validated and effective methods [38]. However, in recent years persistence of contamination was reported even after the recommended procedures.

To ensure FBS quality, representative samples of the unified lots are usually tested for sterility (bacteria, fungus), endotoxins, immunoglobulins, viruses, biochemical values and electrophoretic profiles. Afterwards, it is sterilized by filtration and may be treated by γ-rays or high temperatures. These procedures, as well as ultimate freezing, ensure the additional risk-free safety [39]. Premium quality values of FBS are low concentration of immunoglobulin, absence of viruses, and endotoxins. However, serum lots not always pass necessary testing or it is insufficiently effective. Following the protocol that combined cell culture method and detection of RNA pestiviruses, B. Makoschey at al. [37] had shown that 4 of 7 FBS lots were contaminated by infectious BVDV1 of non-cytopathogenic biotype.

H. Xia at al. [40], for the first time having demonstrated the contamination of commercial FBS of different geographic origin not only by BVDV1 and BVDV2, but also by the emergent BVDV3, had suggested that such viruses are much more widely spread than it was earlier assumed. Analysis of 33 FBS lots from 10 producers by reverse transcription polymerase chain reaction (RT-PCR) allowed detecting BVDV1 in 29 lots from 11 countries, BVDV2 in 11 lots from South America, and BVDV3 in 13 lots from America, Australia, Brazil, Canada, and Mexico. S.Q. Zhang at al. [31] had established that Chinese FBS medicines

from different regions of the country were contaminated by minimum one type of pestivirus (including BVDV1 and BVDV2).

BVDV3 virus. Although the origin and emergent properties of HoBi-like-virus are unknown, according to one of the hypothesis it originates from the South America, from where it was spread to other countries and other continents with contaminated biological products, such as fetal serum and vaccines [23]. As per F.V. Bauermann at al. [41], over 30 % FBS lots from the South America tested in the Europe were contaminated by such virus.

After PCR analysis of 26 archival FBS lots (years 1992-2013), having passed filtration and γ-raying [27], all of them was found to contain minimum one type of bovine pestivirus. BVDV1 was in 2 lots, BVDV2 was in 10 lots, and HoBi-like-virus was in 15 lots. Seven lots were produced in the South America, one lot was from Australia, and 7 lots had undefined origin. Based on results of philogenetic analysis, the identified virus was referred to the Brazil group. This virus was brought in Italy with FBS [2]

Upon examination of 90 series of commercial serum made in USA and pre-packed in Europe, the authors have reported that no virus was found and, thus, have concluded on no cross-country circulation thereof [42]. Nevertheless, part of lots contained BVDV: BVDV1 was found in 19 and BVDV2 was found in one lot out of 20 positive series based on results of the phylogenic analysis. Such fact implies the contamination possibility of FBS marked as US product, provided pre-sales treatment and packing, as well as probability of improper marking following such treatment and mixture with samples from other geographical regions of the world. It is alarming since lots marked as made in USA or Australia are present at FBS market, i.e. in countries, which according to the official data are free from the atypical bovine pestivirus.

Spread of atypical bovine pestivirus (as apart from BVDV1 and BVDV2) is possibly limited by several regions. As it was noted before, Hobi-like-virus was primarily secreted and characterized in 2004 in Germany by analysis of FBS lot collected in Brazil and pre-packed in Europe [21]. Isolate called D32/00_'HoBi' was considered to be prototype for the Brazil group of pestiviruses. Afterwards, several authors have identified its genetically varying subtypes with regional spread, in particular Thai subtype [42]. Afterwards, it was hypothesized on existence of the third, Indian group of strains [25]. There is an assumption on existence of the fourth group of virus secreted outside the Indian region, in particular in Italy [27]. Therefore, four genetic groups of BVDV3 (3a-d) are recently identified.

Available data confirm the need for constant updating and advancing the bovine virus identification methods, and for development of the rules of international trade in FBS and animals. In the past years, several technologies for lowering the quantity or inactivation of FBS viruses were tested. According to the Directive of the European Medicines Agency (EMEA), United Kingdom, BVDV was included in the list of viruses which could be used for quality verification of the inactivation procedure [30]. Impulse treatment of FBS by UV rays with wave length of 355 and 266 μm has a good effect [43]. In this respect, a device to perform 14 minute treatment with red LED (Light Emitting Diode, $\lambda = 627$ μm) and methylene blue in ultimate concentration of 1 μm effective against BVDV are proposed for use [41]. Nevertheless, risk of FBS contamination by pestiviruses remains real, and even weak contamination upon use of FBS as an additive to growing medium may result in infection of the cell cultures [30].

FBS use. Because of high FBS contamination by viruses, it is impossible to exclude the use of its virus-containing lots in large-scale production of vaccines [37]. Therefore, the virus must be inactivated in each lot under strict

control of the effectiveness of the procedure by laboratory tests. To this end, guidelines and rules of inactivation methods and relevant tests were developed. Recently, EME rules, which are applicable to production of medicines for veterinary and medicinal purposes, presuppose compulsory treatment by the approved methods [38]. BVDV test shall be one of the primary contamination assessment methods before and after the inactivation of virus. The ultimate lot of medicine shall be free from virus and antibodies [30].

Upon use of FBS in production of veterinary medicines, virus identification protocol presupposes at least three transits in sensitive cell culture and immunohistochemistry analysis with referent anti-BVDV monospecific antiserum (polyclonal or containing the pool of monoclonal antibodies). If virus is detected, titrating from the serum lot is performed to confirm that concentration of virus is sufficient for control of the performed inactivation with the use of validated tests and does not exceed 10^5-10^6 TCD_{50}/ml. Moreover, for testing purposes, it is proposed to use PCR with electrophoretic separation of products and in real time mode. Sensitivity and specificity of additional methods shall not be less than in standard tests. Besides, the producer shall be able to determine whether the detected RNA is infected. There is also a directive presupposing the methodology which should be used at suspicion to contamination of the finished serum lot by nuclein acids of virus [30] to identify whether virus infection factors are encoded by such consequences. To this end, double testing involving detection of virus by PCR (predominantly by semi-quantitative with internal control) and detection of contamination by virus in other tests are applied. Only laboratory tests are used upon identification of viral contamination of the live vaccines. To this end, the end product is inoculated in sensitive cells with conduction of at least three blind passages with further immunoperoxidase painting of monolayer, as well as by testing by immunofluorescence or PCR methods. In case of negative test results in vitro with confirmation thereof in PCR, no in vivo test is conducted, save for the exclusive circumstances.

Production of biological preparations for medical detection of virus requires methods to effectively detect both virus biotypes. Besides, immunofluorescent staining of cell culture monolayer with fluorescein-tagged antibodies (FA) is recommended. Direct PCR method is deemed to be less suitable for detection of infectious virus. At detection of contamination, it is estimated by quantitative methods (value shall be less that the established value for virus inactivation by valid methods). Upon detection of BVDV, serum shall be repeatedly tested with continued treatments until the negative result.

Cows used as donors of biomaterial for FBS production, shall be clearly established, and their state shall be fixed subject to EMEA regulations [37]. It is not recommended to carry out vaccination in such herds for prevention of any influence of post-vaccination antibodies.

Regardless of the numerous attempts to govern FBS safety, falsifications of products, from which honest suppliers and large number of serum consumers have suffered, take place [36]. From 2003 to 2011 one firm added bovine serum albumin, water and growth additives to FBS made in USA. Some lots of such serum (143 series, 280000 l) could be still sold around the world under other trademarks or trade names. In this regard, it is suggested that FBS market is not governed quiet effectively and falsification remains possible [28]. In this case, in addition to improper medicine composition, the producer could not be identified. Falsified product may contain BSA of mature animals from USA and/or mixture of fetal serum obtained from other sources in Canada, Argentina, Brazil, or Mexico. This recently reported illegal practice may significantly influence on the results and validity of scientific tests with cell and tissues cultures [28] and dis-

credit the global FBS market [30]. An issue of falsification of the geographical origin of FBS deserves special attention. As early as in 1994, it was reported on the sale of nearly 30000 l of serum from New Zealand around the world, however according to the official data only 15000 l of high quality products were annually prepared in this country [43]. Accordingly, the consumer may get a product, which by its geographical origin is not compliant with the official requirements and is produced in the region where infection status of donor fleets is less favorable and value of FBS is far lower [30].

It was suggested that several companies allow mixing the serum during production and transportation [40]. It results in breach of the equipment cleaning regulation or in infected lot among the lots from different producers. Moreover, FBS lots may be erroneously tagged by the country of origin different from the real one [39].

To ensure quality, it is necessary to take special care at confirmation of validity of the supplier information and to exercise duly care at operation with all suppliers of the preparation. Qualitative supply means that all values, including the history of origin, shall be properly documented, fully transparent, and confirmed by an independent auditor. Besides, other important information shall be also available. For instance, at detection of virus tests results depend on the inoculate volume, used cell culture line, number of passages, specificity of antibodies used in immunofluorescence test, correct selection of primers or probes for RT-PCR. It is also useful to indicate virus detection threshold in commonly used international units (for instance, number of infected particles or genome copies).

World Organization for Animal Health (OIE, France) issues a clear regulation subject to which all cell cultures prior to the targeted use shall be tested for BVDV in several passages. Blood serum, including fetal serum, shall be free from viruses, and also from specific antibodies [46, 47]. Nevertheless, cases of cell culture and serum contamination by BVDV strains of non-cytopathogenic type were described in Russia [48-50]. As a result, cultural live vaccines may become a source of viruses for sensible animals, and contaminated diagnostic antibodies may be a reason of invalid test results. Therefore, improved control aimed at prevention of biological contamination is highly important in production of vaccines and other biologicals [48].

Thus, because of globalization and modern cell biotechnologies in veterinary and medicine, a demand for fetal bovine serum (FBS) is annually increased. FBS contamination is still relevant and results from the increased demand, availability of unfair producers and sellers, incompliant marking of the products and lack of the unified FBS control methodology. In the extension of FBS market, existence of atypical pestiviruses requires special attention. They have been detected in the commercial blood serum pools used for cell culture and production of biological medicines, and represent a hazard due to ability to spread in new regions. Lack of FBS production in Russia opens the way for doubtful goods quality from unreliable manufacturers.

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