In: Advances in Medicine and Biology. Volume 95 ISBN: 978-1-63484-348-5
Editor: Leon V. Berhardt

Chapter 4

THE IMPACT OF BVDV PRESENCE ON FETAL BOVINE SERUM USED IN THE BIOTECHNOLOGY INDUSTRY

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ABSTRACT

Fetal Bovine Serum (FBS), a byproduct of the meat industry, is a valuable commodity for biotechnology. Due to growth factor richness, FBS is added to culture

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media to support proliferation of many cell lines *in vitro*. By virtue of inherent lot variability, careful quality control is essential to ensure performance and safety. Absence of viruses is among the desired features but BVDV-1, BVDV-2 and atypical strains are still isolated from FBS batches, despite guidelines and regulations for BVDV detection. Considering the steady increase in the demand for bioproducts and *in vitro* assays, ensuring strong adherence to guidelines and diffusion of existing criteria for FBS quality is mandatory. In this review we present implications of BVDV in cell cultures and bioproducts for human and animal health to encourage discussion.

FETAL BOVINE SERUM

Cell and tissue culture has become an indispensable tool in biomedical sciences and biotechnology [1, 2] and animal cell lines are widely used in the manufacturing of biological products [3]. Cell culture has applications in a large number of fields such as *in vitro* fertilization, *in vitro* diagnostics, biopharmaceutical and vaccine production, cancer research, drug screening and development, gene and cell therapy, tissue engineering, and toxicity testing [4-6].

As live cells in an organism obtain necessary nutrients from the blood and tissues that surround them, *in vitro* cell and tissue cultures demand the use of animal-derived products as culture medium constituents [1]. Animal serum derived from blood is an important source of growth factors for cell culture [7] and several types of sera, including adult bovine sera, newborn calf sera, calf sera, equine sera, and porcine sera, are commonly used [8]. Plasma from adult animals is generally not suitable for cell culture use, primarily due to its reduced levels of growth factors and also because of the accumulation of gammaglobulins (antibodies). Growth factors are at higher concentration in blood from fetuses and in newborn animals [7].

Fetal Bovine Serum (FBS) is the most common and widely used supplement of culture media and is added to allow or increase the growth of isolated mammalian cells [9]. FBS is a natural cocktail with most of the factors required for cell attachment, growth and proliferation, and is effective for most types of human, animal, and insect cells [10]. No other supplement has been found to provide the same degree and universality of cell growth stimulation. This effective stimulation comes from the abundance of blood-associated biochemicals responsible for the rapid cellular development inherent to fetal maturation [6].

FETAL BOVINE SERUM AS A BY-PRODUCT OF THE MEAT INDUSTRY

FBS, a commodity for the biotechnology industry [11], is a by-product of the beef industry, which drives its availability and exerts the strongest influence on the final product cost to the manufacturers of cell culture products [12].

Gstraunthaler describes how bovine fetuses, from which blood is drawn for FBS production [1], are obtained from pregnant cows sent to slaughtering. In massive herds of meat cattle, bulls and cows roam freely together and, as a result, many cows are pregnant at the time of slaughter. When a pregnant cow is discovered in the slaughter line, the fetus is

separated at the abattoir, and fetal blood is collected under aseptic conditions. Jochems reported that in Hungary, the Baltic States, and possibly in the Czech Republic and Slovakia, fetuses come from cattle that are deliberately made pregnant for the production of FBS and sent to slaughter [13]. In all other countries he comments that fetuses are derived from pregnant meat cattle. In Honduras fetuses are only derived from pregnant cull cows and in the USA fetuses are derived from both pregnant meat cattle and cull cows.

Bovine fetuses used for FBS harvest are usually at least 6 months of gestational age, though fetuses may be used as early as at 3 months gestation. The blood is usually obtained by means of a cardiac puncture (in most of FBS producer countries) but, alternatively, it may be harvested through umbilical vein puncture (as in Uruguay and Australia), or by puncture of the jugular vein (as in Brazil) [13]. Every batch of commercially available FBS is a mixture of collected raw serum material from different cattle farms [14].

FBS demand is highest in the US and Europe, which are the major producers, while the major sources of FBS are far away—in Brazil, Argentina, South Africa, Australia, New Zealand, and Central America [10]. FBS that is used to produce vaccines and drugs in cell culture mainly - comes from the US, New Zealand and Australia. The FBS used for research purposes, on the other hand, comes primarily from South America or South Africa, and Brazil in particular has been the major supplier of this product for many years. As the world's second-largest beef producer and the largest beef exporter in 2007, FBS from Brazil was available in almost unlimited quantities and, during this year, approximately 70% of the FBS used in European medical research came from Brazil [4].

THE FETAL BOVINE SERUM MARKET

Cell culture is one of the most important and widely used techniques for biopharmaceutical production and the growth in this industry will drive the FBS market. In 2013, biopharmaceuticals were the largest application segment of the cell culture market and they will be the fastest growing pharmaceutical product segment by 2017 [5]. It is expected that the global market for cell culture products (media, sera and reagents) for the biotechnology, pharmaceutical and medical markets will grow from nearly $3.2 billion in 2014 to nearly $4.1 billion by 2019 at a compound annual growth rate (CAGR) of 5.1% between 2014 and 2019 [15]. In addition, the global cell expansion market, with a focus on human stem cell research and the increasing usage of stem cells for treating various disorders, is expected to reach $14.8 billion by 2019 from $6.0 billion in 2014, growing at a CAGR of 19.7% from 2014 to 2019 [16].

It is estimated that approximately 500,000 L of FBS are sold per year, which means that more than 1,000,000 bovine fetuses have to be harvested. Plus, the numbers are still increasing [1, 10]. The market for FBS is highly concentrated. In 2014, it was controlled by three major players: Thermo Fisher, Life Technologies and Sigma-Aldrich, who controlled a combined share of more than 80% of the market with most of their customers being large biopharmaceutical companies. The acquisition of Life Technologies by Thermo Fisher in 2014 resulted in substantial anticompetitive effects in the cell culture media and sera markets by eliminating the close competition between them. Thermo Fisher and Life Technologies

today have together at least 60% share of sera market and 50% share of the cell culture media market, whether measured by US or worldwide sales [8].

FETAL BOVINE SERUM PRICE

As FBS is a commodity item whose price fluctuates considerably [7], its industry has experienced substantial changes in the few last years [17]. FBS is a unique product not only due to its composition but also because of its volatile pricing and its complex, regulated sourcing. There are many reasons for that complexity but the primary factor and main market-driver is the beef processing industry [6]. FBS supply is dictated by many factors including consumption of beef and dairy products, feed prices, environmental factors, such as drought, cattle import and export issues, governmental farm policies and the outbreak of diseases [10]. As beef production falls, so does FBS production. Lower supply, higher energy costs and a depreciated dollar all contribute to more expensive FBS [4].

Pricing becomes even more complicated when overlaid by the fact that some buyers can only use serum produced in certain geographies or require specific quality features. Siegel and Foster mentioned, for example, that serum originating from New Zealand and Australia is higher in price when compared to serum from the United States and Canada, while serum from these four countries is priced significantly higher than that from most South American countries [6]. They concluded that this price stratification results from the demand placed on FBS from preferred geographic locations by manufacturers of medicines who consume serum in large volumes. The most important step in assessing serum quality is to determine its intended use, according to good laboratory practices (GLP) in research or with good manufacturing practices (GMP) for human medicines, for example.

Häusl reported that until mid-2008, the prices for FBS in Europe remained stable [4]. However, he comments that this period of "unlimited" supply is over since the occurrence of a ban on imports of fresh meat from Brazil to the EU, which significantly reduced one of Brazil's most important meat markets. The number of animals being slaughtered fell and FBS production also declined. With low global stocks, distributors were forced to buy fresh FBS and confronted with a new situation - an unprecedented rise in the price of FBS. This author believes that the cost of this important material will continue to climb for several reasons. As expected, several suppliers increased FBS prices in the last few years. Gibco, the leading global supplier, communicated to consumers that it will be forced to increase FBS prices in 2015 to face the continuing rise in costs of raw materials needed for the production process [12]. This FBS producer considered as factors for these increases: the reduction in supply due to climatic events, economic conditions, and increased demand from life science and pharmaceutical customers.

In today's volatile market, it is critical that sera users worldwide thoroughly review their supply relationships and update sourcing and risk mitigation strategies [17] taking in account: (a) qualitative and quantitative, geographical and seasonal batch-to-batch variations; and (b) adverse factors, like endotoxin, mycoplasma, and prion proteins, or viral contaminants like Bovine Viral Diarrhea Virus (BVDV) [10].

BOVINE VIRAL DIARRHEA VIRUS IN FETAL BOVINE SERUM

Although governmental inspectors routinely assess the health of each culled cow and fetuses are collected only from those animals deemed fit for human consumption [6], it has long been known that FBS is a potential source of many viruses. Since the 1960s, FBS has been found contaminated with bovine pestiviruses [18] and BVDV is the most common of these due to the capacity of the virus to spread transplacentally and subsequently establish a persistent infection in the immunologically immature fetus [3, 19]. Due to the risk of viral contamination associated with the use of FBS, it is strongly recommended [20, 21], in addition to direct testing for viruses, to inactivate the serum by a validated and effective treatment. Despite these procedures, several authors have reported FBS contamination in recent years.

To ensure FBS quality, representative samples from pools are taken and subjected to a battery of tests including sterility (bacterial, fungal, and mycoplasmal), endotoxin detection, immunoglobulin (IgG) quantity, hemoglobin quantity, viral screening, biochemical panels, and electrophoretic profiles. FBS is then sterile-filtered and can be treated using gamma irradiation or heat-inactivation. These treatments, and final freezing, provide additional security in controlling potential adventitious agents such as viruses [6]. Premium quality FBS are those that contains low levels of haemoglobin, no viruses and no endotoxins [7].

Following a protocol that combines cell culture methods and detection of pestivirus RNA, Makoschey et al. indicated that four out of seven batches of FBS tested were contaminated with infectious BVDV [3]. All of the isolated strains belonged to the ncp biotype (which does not cause citopathic effects in cell culture), and most reacted with the BVDV-1 specific oligonucleotides, while a smaller number reacted with BVDV-2 specific oligonucleotides. A completely new pestivirus isolated from a batch of FBS was described by Schirrmeier et al. [22].

They proposed that the isolate D32/00_‘HoBi,’ isolated from SFT-R fetal sheep thymus cells grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS from batch 547 from Brazil, should constitute a novel sixth pestivirus species.

Recently, Xia et al. demonstrated for the first time that commercial FBS products of different geographic origins are contaminated not only with BVDV-1 and BVDV-2, but also with emerging pestiviruses, suggesting that is likely that atypical bovine pestiviruses have a much wider geographic distribution than previously described [18].

They analyzed 33 FBS batches from 10 manufacturers by RT-PCR and all of them showed at least one BVDV species. BVDV-1 was found in 29 batches from 11 countries, while BVDV-2 was found in 11 batches from South America and the atypical pestivirus (called HoBi-like virus or, in this study, BVDV-3) was found in 13 batches from America, Australia, Brazil, Canada, and Mexico.

Although the origin and emergence of HoBi-like viruses is unknown, one hypothesis is that the viruses originated in South America and were introduced to other countries and continents through contaminated biological products such as FBS and vaccines [23]. Unpublished data commented on by Bauermann et al. indicates that more than 30% of FBS batches originating from South America that are tested in Europe are contaminated with HoBi-like viruses [24].

The detection of an emerging pestivirus species, the "HoBi-like virus," in FBS labeled as US origin but packaged in Europe raised concerns that HoBi-like virus may have entered the United States. However, Bauermann et al., who isolated no such viruses from FBS originating in North America, affirm that there is no evidence of HoBi-like viruses, by virus isolation, in that region [9]. Since commercial batches of FBS come from animals of different origins, Zhang et al. proposed to investigate BVDV genetic diversity by determining the genotypes detected in FBS [14]. This study demonstrated that bovine serum products with different geographic origins in China are contaminated with at least one species of pestivirus including BVDV-1, BVDV-2, and a virus similar to Classical Swine Fever Virus (CSFV). Using RT-PCR and sequencing, they amplified similar 280 base pair (bp) segments from 20 batches of bovine serum and thirty-four different sequences from the 20 batches.

Some pathogen reduction technologies to inactivate viruses have been investigated in recent years for the elimination of viruses in serum. According to the guideline from EMEA on requirements and controls applied to bovine serum used in the production of immunological veterinary and medical products, BVDV is one of the viruses that may be used to validate inactivation procedures [20].

Pulsed UV treatment as Nd:YAG laser,has the potential to inactivate known and emerging viruses [19]. In its third and fourth harmonic with the wavelengths of 355 and 266 nm, respectively, the minimum doses for inactivation of BVDV suspended in FBS were 704 and 127 J/cm^2. Recently, a new device designated to inactivate viruses using Methylene Blue in a final concentration of 1 μM and illumination at 627 nm with red LED (Light Emitting Diode) for 45 minutes was demonstrated to be effective for inactivating BVDV [26].

This method showed its best results for enveloped model viruses and could inactivate and reduce their titer very close to approved commercial devices.

Recent studies support the successful use of of High Hydrostatic Pressure (HHP) application on FBS components for inactivation of BVDV (and other pestiviruses) with 220 MPa for 5 min at 25°C treatment [26]. Protein secondary structures were found to be unaffected but some changes were revealed in the lipoprotein structure and the virus strain lost its infectivity completely. These results indicate that HHP can be an adequate tool for BVDV inactivation while leaving structural and functional properties of serum and serum products unaffected. Despite all efforts, the risk of FBS contamination by BVDV is real. This fact combined with the use of FBS as a cell culture media supplement provide optimal conditions for BVDV infection in susceptible cell lines. Even low level FBS contamination is harmful, as using contaminated supplement to grow cells can result in the infection of the cells and amplification of the virus burden [27].

BOVINE VIRAL DIARRHEA VIRUS IN CELL CULTURES

Although genetically and antigenically diverse, BVDV type-1 and BVDV type-2 have in common the existence of two biotypes that are differentiated according to whether they induce death in cultured cells (cytopathic biotype, cp) or fail to induce cell death (non-cytopathic biotype, ncp) [28, 29]. Thus, cell cultures infected with noncytopathic BVDV do not display cytopathic effects and, although BVDV particles accumulate inside host cells, no morphological changes can be observed by light microscopy. The cell infection will only be

detected with additional testing. Bianchi et al. performed a comprehensive characterization of biotypes, genetic factors and antigenic factors of 20 BVDV isolates from Rio Grande do Sul, Brazil between 2000 and 2010 [30].

In this study, most isolates (19 or 95%) belonged to the non-cytopathic biotype and only one isolate (5%) had a mixture of ncp and cp viruses. This biotype profile is certainly reflected in commercial FBS, increasing the chance that cell culture infections pass unnoticed.

As mentioned by Flores and Donis, cultured bovine cells, as either primary or continuous cell lines, are invariably very susceptible to BVDV infection *in vitro* [31], but BVDV contamination has been detected in cells of several species and origins. Even prestigious cell banks like the American Type Culture Collection (ATCC) cannot be considered free of risk. Due to their importance for biological research, 41 cell lines from the repository of this institution were tested in 1994 for contamination with noncytopathic BVDV. While no BVDV viral particles were found in swine cell lines, all the others (e.g., sheeps, goats, rabbits and cats) were contaminated, especially the bovine cell lines [32].

It is noteworthy that, in spite of this result, swine cell lines were already reported as infected with BVDV in other studies [33-36]. While monkey cell lines, such as LLC MK2, were shown to be variably susceptible, the Vero (African green monkey kidney) cell line was proven capable to efficiently replicate BVDV [32].

The results of virulence and other processes involving host-cell interactions can be affected by BVDV contamination and makes questionable any conclusion at cellular and molecular levels. Noncytopathic infections with BVDV can compromise research and commercial use of cultured cells [37] and may pose serious risks for activities such as *in vitro* assays, virology diagnosis and vaccine production [38].

CONTAMINATION OF VACCINES AND OTHER BIOTECHNOLOGICAL PRODUCTS

Several biotechnological products, including vaccines, recombinant peptides and expressed proteins, are developed using mammalian cell cultures maintained with FBS supplementation. For this reason, these products are susceptible to contamination by BVDV, among other agents, and can pose a risk to animal or even human health. Considering vaccines, which are the major biotechnological product that can harbor BVDV as a contaminant, a previous infection of cultured cells with this virus can interfere with the growth of the vaccine virus and lead to a product harboring a significant dose of the contaminant. The effects of vaccine contamination may differ according to the circumstances. The nature of the vaccine (attenuated or inactivated), the titer of the contaminant, its degree of inactivation and its pathogenicity would play a significant role in the consequences of a contamination. Practical effects of a vaccine contamination with BVDV might be infection (clinical or sub-clinical) of the recipient or a serological response to the contaminant [39-41]. Furthermore, as BVDV is associated with immunosuppression and acts in synergy with other pathogens [42], it would allow the emergence of new infections on the herd. Also, the vaccination of pregnant heifers with live vaccines contaminated with ncp BVDV strains can result in persistently infected fetuses [43], malformations, fetus death [44] and Mucosal

Disease development in calves [45], which can increase the spread of the virus throughout the flock.

The economic losses derived from BVDV infection were previously described, especially for the Netherlands and Italy, in 1999. Falcone et al. described that in 1999, March 9, a rapid alert was activated in all European Union Member States concerning the occurrence of serious BVD-like symptoms, following a Bovine Herpesvirus 1 (BoHV-1) vaccination [46]. RT-PCR, sequencing, virus isolation in susceptible cells, antigenic characterization with monoclonal antibodies, and experimental infection of susceptible animals were used to assess the risk that the vaccine contamination had posed. The expected immunity was not reached by these animals, which eventually succumb to other infections due to the immunosuppressive effect of BVDV [46, 47]. In order to ensure that BVDV-free vaccines are produced, the authors comment that it is essential to rely on valid tests and to develop effective quality assurance programs, stage by stage, throughout the manufacturing process. Bruschke et al. report that BVDV could be isolated from seven out of 82 vaccine batches of BoHV-1 that were on the Dutch market in February 1999 [48]. Six batches tested positive for BVDV-1 and one tested positive for a type of BVDV-2 that induced clinical signs in susceptible animals. On 23 February 1999, the Dutch Animal Health Service advised all Dutch veterinary practitioners to postpone vaccination against BoHV-1 immediately. This was the day before severe disease problems were diagnosed on four of twelve dairy farms after vaccination with the same batch of the BoHV-1 marker vaccine. Barkema et al. described the outbreak of BVD caused by BoHV-1 contaminated vaccine [49]. Morbidity was high in 11 out of the 12 farms and, on five farms, more than 70% of the animals became ill. All animals were culled between 32 and 68 days after vaccination when an agreement was set with the manufacturer of the vaccine. On only one farm, no symptoms could be detected. This was the third outbreak of BVD in cattle after administration of a contaminated vaccine in the Netherlands and the authors will discuss possible actions to prevent this kind of vaccine contamination.

Still dealing with contaminated vaccines for cattle, Schirrmeier et al. and Bauermann et al. proposed that the atypical pestivirus D32/00_'HoBi' (sometimes called BVBV-3), which was first described in FBS originating from South America, was introduced to other countries and continents through contaminated biological products such as FBS itself and vaccines [22, 27].

Several articles report problems with BVDV contamination in veterinary vaccines. Harasawa detected the presence of adventitious pestivirus by RT-PCR in live virus vaccines against Akabane disease, Ibaraki disease, infectious bovine rhinotracheitis, porcine parvovirus, transmissible gastroenteritis, and Japanese encephalitis [50]. They concluded that pestivirus RNA or pestivirus particles in the fetal bovine serum used to grow the host cells used to prepare the bovine and swine viral vaccines are the likely sources of contamination. In attenuated vaccines against Transmissible Gastroenteritis Virus (TGEV), where BVDV was shown after 68 passages, the presence of both viruses can lead to clinical disease and lesions similar to those observed in infections with TGEV alone, with the co-infection being even more severe. Other examples of infections aggravated by the presence of BVDV are bovine coronavirus (BCV), Salmonella sp. and bovine respiratory syncytial virus (BRSV) [51, 52]. In China, an abundance of bovine sera has been used in the production of live vaccines against PRRSV, PRV and CSFV in pig herds [53]. In a recent review, Tao et al. cited several reports and described some unpublished data about BVDV infection in pigs because of the use of contaminated vaccines [54]. Zhang et al. suggested that this is the reason why both pig

and cattle have the same predominant BVDV sub-genotypes in China [14]. BVDV-contaminated vaccines can also result in the induction of antibodies that might interfere with serological surveillance programs for the eradication of hog cholera [3].

As an important biotechnological process, *in vitro* embryo production also commonly uses FBS for washings or as a rinsing media, during *in vitro* maturation and in pre-implantation embryo culture. Contamination of FBS is a worrying and critical issue [55] and many reproduction center laboratories uses heat-inactivated or gamma radiation sterilized serum to avoid that contamination risk [56]. Lindberg et al. and Drew et al. reported the use of contaminated FBS in embryo transfer medium [57, 58]. In the first report, receptors developed high BVDV antibody titers following contaminated embryo implantation, while in the second one the resulting calf was diagnosed as persistently infected with an exotic BVDV strain.

Although there are no available data on clinical symptoms of BVDV infection in humans, some researchers believe that this hypothesis cannot be ruled out because (i) the virus can replicate and persist in cell strains derived from humans, such as respiratory and intestinal epithelium, human fetal kidney cells, hepatoma cells and T lymphocytes, (ii) blood specimens from veterinarian workers at cattle farms were shown to contain specific anti-BVDV antibodies [32, 59], and (iii) even a small number of infectious BVDV particles represents an enormous risk for the safety of the final products [3]. Further, since noncytopathic biotypes of BVDV are capable of incorporating cellular RNA from their host into their genomes, pestivirus contamination would raise another issue with regard to the safety of live virus vaccines produced in continuous cell lines, which are potentially oncogenic [60]. This author concluded that it is important to avoid the risk of contamination of human viral vaccines and recommended to check for the presence of adventitious pestiviruses by PCR complemented by other tests such as culture methods or immunological assays.

The examination of five monovalent or combined live vaccines against measles, mumps and rubella for pestivirus contamination by RT-PCR lead to the conclusion that the FBS used to grow the host cells and for the preparation of the vaccines was the source of contamination [61]. BVDV RNA was also detected by RT-PCR in 33% of 36 human vaccine lots [56]. All positive results were caused by the mumps component from a single manufacturer and the authors concluded that FBS is most likely the source of BVDV contamination in live viral vaccines. The authors, however, reasoned that RT-PCR detection of BVDV contamination in FBS used for vaccine production does not appear to be an immediate concern to human health. Furthermore, they indicate that gamma-irradiation of FBS destroys BVDV particles and is also effective in preventing the presence of BVDV RNA in vaccines.

Human recombinant interferons (IFNs) were examined for the presence of pestivirus RNA by RT-PCR and researchers found that, from 46 samples, 30.4% were contaminated with RNA derived from BVDV genotypes I, II and III with incidence rates of 86%, 7%, and 7%, respectively [62]. These findings, according the authors, do not mean that IFNs or live virus vaccines that showed a positive reaction by PCR were all contaminated with infectious pestiviruses. Therefore, this survey only suggests that a trace amount of BVDV RNA may exist in interferon products throughout the world and it may be harmless to the recipients.

Regarding BVDV contamination of biotechnology products for human health, there are still some issues to be considered as, for example, the mechanism of interaction between BVDV and HIV infections and the synergistic effects with other opportunistic pathogens

[42]. So, the use of processes the that requires FBS like cell therapy must be also exercised with caution in immunosuppressed patients.

Taking into account the generally accepted proposition that (i) most, if not all, FBS batches are contaminated with BVDV (or the BVDV genome) and (ii) even using the most sensitive methods for detection of BVDV, a contamination with that virus can never be completely excluded [3], the possibility of infection of cultured cells by BVDV present in FBS is a real and constant risk. In order to avoid the consequences related to these findings, some researchers were involved in the selection of cell lines resistant to this virus. Although many cell lines are susceptible to the virus, it is known that some lineages are refractory to infection with BVDV such as MRC-5 cells [56]. The cell line CRIB, which is resistant to BVDV infection, has been derived from the MDBK bovine kidney cell line and was obtained by selection and cloning of cells surviving infection with a highly cytolytic BVDV strain [31]. Three other cell lines resistant to BVDV were obtained from canine (MDCK), swine (PK-15) and rabbit (RK-13) parental cell lines by expanding and cloning cells that survived lytic infection with BVDV [38]. Their resistance is probably due to a block in viral entry and these new cell lineages will be useful for many purposes, and will minimize the risk of BVDV contamination.

The reported incidence of bioproduct contamination by virus carried by FBS, largely due to BVDV, and the increasing number of *in vitro* assays and biotechnological products made available for animal and human health in the last years forced the industry to change certain practices and the regulatory authorities to develop more stringent and detailed requirements [41]. Thus, for this industry, the testing of BVDV presence on ingredients, cell substrates and final products is a prerequisite to ensure purity, safety and efficacy [63].

GUIDELINES AND REGULATIONS ON FBS USE

Although we do not strongly agree with the statement from Siegel and Foster that the risk profile of the use of FBS in research typically involves negligible risk of adverse effects [6], we do agree with them in that there is a big potential risk in the use of serum for cellular therapy products, vaccines, and biopharmaceuticals directed for use on animals and humans. They considered that these applications require additional risk management strategies focused on quality testing and geographic source verification. It is worth considering that, with the increasing demand for new biotechnological products and validated methodologies for *in vitro* evaluation of product toxicity, the risk to health for animals and humans involved in research is not negligible. Thus, it is necessary that the quality and safety of FBS is guaranteed if it is to be used in research and in *in vitro* assays.

Given the high prevalence of BVDV in batches of FBS, it is not feasible to exclude all lots tested positive for BVDV from use, especially in large scale processes like vaccine production [3]. Thus, virus inactivation methods can be applied to serum lots and their effectiveness should be monitored by laboratory tests. For this, guides and regulations determine the validation of viral inactivation methods and tests.

The current guidelines of the European Medicines Agency (EMEA) applied to the production of immunological veterinary medical products [20] and in the manufacture of human biological medical products [21] prescribe that FBS has to be treated according to a

validated method that effectively inactivates viruses. BVDV is one of the viruses that may be used for the validation of the inactivation procedure and a check for BVDV must be included first to determine the level of BVDV contamination before inactivation and later to confirm the inactivation performance. These tests could only be omitted if no virus is detected before the inactivation treatment. No virus should be detected in the final serum batch. Recent alterations in the guideline from EMEA applied to the manufacture of human biological medical products [21] included testing requirements for BVDV and anti-BVDV antibodies present in the guideline applied to the production of immunological veterinary medical products from this agency [20].

For BVDV detection in immunological veterinary products by isolation protocol [20], three passages of the inoculated cells are necessary and an immuno-cytochemical technique is applied to the cells with an anti-BVDV reference monospecific serum (polyclonal or a pool of monoclonal antibodies). If isolated, the virus has to be titrated directly from the bovine serum batch to ensure it is below the level that has been shown to be effectively inactivated in the validation tests for inactivation treatments. In addition to the recommended tests, other validated methods such as PCR and RT-PCR are mentioned as additional options to increase the probability of detecting viral contaminants. If such tests are used, they should be demonstrated to have a sensitivity and specificity at least equivalent to the conventional tests. In addition, the manufacturer should be able to clarify whether any nucleic acid detected originates from infectious particles. To detect BVDV antibodies, validated techniques are also listed.

In addition, another current guideline describes the procedure to be followed when a finished vaccine batch is under suspicion of contamination with BVDV nucleic acids [64]. In this case, it is essential to determine if the detected sequences came from infectious particles or from non-infective remnants. In this situation, a double test has to be carried out: (i) diagnosis of BVDV contamination by PCR (preferably a semi-quantitative RT-PCR with an internal control) and (ii) diagnosis of BVDV contamination by other tests. In principle, only *in vitro* tests should be used to detect BVDV contamination of live vaccines. It is indicated that the finished product has to be inoculated into sensitive cells, with at least three passages of the cells inoculated and an additional immunoperoxydase assay, an immunofluorescent assay or a PCR. If the result of the *in vitro* test is negative, either as first choice test or as confirmation of PCR, an *in vivo* test should be avoided, unless justified in very exceptional cases.

For BVDV detection during the manufacture of human biological medical products, the assay required [21] should be suitable to detect cp as well as ncp BVDV strains and staining of cell cultures with fluorescent antibody (FA) is recommended. Direct RT-PCR is considered to have a limited value in the detection of infectious virus. The level of contamination, if present, should be quantified and must be below the level that has been shown to be effectively inactivated in the validation tests for the inactivation treatments. If BVDV is detected, the serum must be re-tested for infectious virus after any inactivation/removal step(s) and used only if no infectious virus is detected. For donor herd health, EMEA also states that the herds should be well defined and documented [21]. It is recommended not to vaccinate these herds against BVDV in order to prevent any impact of vaccine-derived antibodies on the herd health control strategy.

In the United States of America, the licensees for veterinary biological products must conduct tests and report the results to the Animal and Plant Health Inspection Service

(APHIS) and this agency can determine if the products are eligible for release. The APHIS, from the United States Department of Agriculture (USDA), administers and enforces the Virus-Serum-Toxin Act [65], under which a veterinary biological product must be shown to be pure, safe, potent, and efficacious before a veterinary biological product license may be issued. The regulations in 9 CFR part 113.47 [66] prohibit the release of biological products prior to the completion and reporting of tests identified in the filed Outline of Production or in the Standard Requirements. According to 9 CRF 113.47, all cells shall be tested for BVDV [66]. The established test for its detection is the fluorescent antibody technique (FA). If the material under test shows any evidence of specific viral fluorescence, it is unsatisfactory and may not be used. If specific fluorescence is absent in the positive control monolayers or if the fluorescence in the positive or negative control is equivocal, or both, the test shall be declared inconclusive and may be repeated. In this condition, if the test is not repeated, the material under test shall be regarded as unsatisfactory for use in the production of biologics.

APHIS is now proposing to amend the regulations in order to bring them up to date with current industry standards and to better define the terminology used when reporting the results of tests performed on veterinary biological products [67]. The proposed changes would clarify when the results of a prescribed test can be reported as "satisfactory, "unsatisfactory," "inconclusive," or can be designated as a "no test." These changes will improve communication between regulators and products' licensees regarding their test results.

A program aimed to harmonize technical requirements for veterinary product registration, VICH, was officially launched in April 1996. Its full title is the International Cooperation on this Harmonization and it involves EU, Japan and USA. In that program, the ability of the manufacturing process to remove or inactivate viruses should be demonstrated according the strategy described in the ICH Harmonized Tripartite Guidance "Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin" [68, 69].

Despite the many efforts to regulate the safety of FBS, the first mass recall in history for adulterated product occurred recently [17] and affected a large number of sera suppliers and users. From 2003 to 2011, a producer added adult bovine serum albumin (BSA) of United States origin, water, and cell growth promoting additives to its products. As some of those FBS lots, comprised of 143 batches with approximately 280,000 L, may still be sold under other brand names or other labels, Gstraunthaler et al. believes that the FBS market is only loosely regulated [10], creating the opportunity for abuses to occur until now. In addition to the FBS composition problem, this episode led to errors of origin attribution. The adulterated product may contain adult BSA of United States origin and/or may contain FBS from other sources including Canada, Argentina, Brazil or Mexico. These recently reported malpractices might have a substantial impact on the results and the scientific outcome of thousands of cell and tissue culture experiments [10] and affect the credibility of the FBS industry in the marketplace [70].

Moreover, the question about geographical origin of FBS is not a novelty. In 1994, it was reported that around 30,000 L of "New Zealand" serum was sold worldwide while only 15,000 L of high-quality FBS were collected annually in New Zealand [71]. Jochems alerts that the consumer may be saddled with a product which is not of the geographical origin he desired because the true geographical origins of these sera have a less favorable bovine disease status and lower prices [13]. In a study of BVDV diversity in China, the data obtained by Xia et al. suggested that (i) mixing of raw serum materials of different origin might have

occurred at certain companies, intentionally or unintentionally, during manufacturing steps or (ii) the transport, manufacturing, processing, mixing, inactivation, gamma irradiation, sterile filtration or centrifugation facilities were not cleaned enough after producing one batch, so a small amount of raw sera collected from one location was mixed with sera collected from another location, and even such a small amount of residue sera tests positive by the highly sensitive real-time RT-PCR method. Another possibility is that the FBS batches may be mistakenly labeled with a country of origin different from their real origins [18].

With the objective to provide guidance to manufacturers, suppliers, customers and regulatory agencies by standardizing the most critical serum quality and integrity assessment tests, the International Serum Industry Association (ISIA) organized strict guidelines for product quality testing and simplified their documentation in an appropriate Certificate of Analysis on a suggested form that is attached to the FBS product [72]. ISIA members are also encouraged to move towards Virus Testing - Bovine Virus Diarrhea Tested N/A 9 CFR and to adhere to companies' certifying programs to ensure traceability and product quality.

In their recent article [6], Siegel and Foster emphasized the importance of "exercising extra vigilance in confirming the integrity and authenticity" of information provided by a supplier and performing "due diligence in vendor qualification of all serum suppliers." Integrity of supply means here that all aspects of product quality and traceability are well-documented, validated by independent audit, and completely transparent.

NEW CHALLENGES IN FBS QUALITY ASSURANCE

There are several methods for detecting BVDV infection mainly applied to animal diagnostics. Saliki et al. provide an excellent review on the matter [73]. Another known reference is the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals published by the OIE (World Organization for Animal Health) [74]. However, the methods to be used to detect BVDV in FBS should be chosen judiciously to bring useful information to the certification of each batch.

Although some assays for the detection of adventitious agents (such as mycoplasma and viruses) are made by FBS providers, the form of its execution or the way the results are expressed does not always meet customer needs. As noted earlier, there is regulation for the production of vaccines and biopharmaceuticals for use in animal or human health, but not for other purposes such as *in vitro* assays or even for biomedical research. In this situation, the consumer should directly request that the manufacturer rest for that particular microorganism or even specify the tests to be done so that the FBS matches the consumer's needs. Another option is to perform these tests in the consumer's own laboratory or at specialized companies. This would raise the cost of the FBS for small laboratories that would need to dedicate resources and efforts to this action.

To create an overview of the tests presented in certificates of products currently available in the Brazilian market, in 2014, we analyzed the documentation that accompanied 35 lots of FBS (from 6 suppliers) available to small laboratories. It was possible to identify the declared origin for 34 lots - Brazil (29), New Zealand (3) and USA (2). In one lot, the origin was not identified. With respect to the tests for detecting possible contamination by BVDV, little information was available. In the documentation of ten lots, no reference to the tests done to

detect BVDV was found. Among the remaining batches (25), the following tests were cited: (i) virus neutralization (VN) (6 lots), (ii) VN and ELISA for antigen detection (ELISA-Ag) (8 lots), (iii) RT-PCR, VN and ELISA-Ag (2 lots), (iv) viral isolation (1 lot), (v) viral isolation followed by immuno-fluorescence (IF) (3 lots), (vi) virus isolation with IF and VN (2 lots), and (vii) Viral Isolation, RT-PCR, Ag ELISA and VN (3 lots). In addition, a ncp BVDV strain was isolated from one batch after 10 passages in MDBK cells, even though the batch was previously tested for virus isolation by the supplier. With these results, not all lots would meet the requirements set in some guidelines or rules since infective particles would not be distinguished from non-infective particles, as recommended. In addition, we judge that there is other useful consumer information missing. In an isolation testing, for example, the result is influenced by the volume of inoculum, the cell line used, the number of passages, and the specificity of the antibody used in IF or of the primers and probes used for RT-PCR. Besides this information, it would be important, for each test, to know the limit of detection (LOD) of the method in well established units, such as infective particles or number of copies, for example.

Considering the above findings, we believe that there should be a greater effort to improve current guidelines and regulations and that quality control of these raw materials be expanded to other activities, such as cell therapy, alternative methods, and to other *in vitro* assays and research also. Although the latter are considered, by some, less noble or less demanding activities, they have enormous repercussions on the safety of new products and on tests used in healthcare.

REFERENCES

[1] Gstraunthaler G. Alternatives to the use of fetal bovine serum: serum-free cell culture. *Altex*, 2003; 20(4): 275-281.

[2] Kokate, Jalapure, Hurakadle. Textbook of Pharmaceutical Biotecnology. Haryana, India: Elsevier; 2011. Chapter, 5: *Animal Cell and Tissue Biotecnology;* p.55-73.

[3] Makoschey B, van Gelder PT, Keijsers V, Goovaerts, D. Bovine viral diarrhoea virus antigen in foetal calf serum batches and consequences of such contamination for vaccine production. *Biologicals* 2003 Sep; 31(3): 203-208.

[4] Häusl, P. European Biotechnology - Life Sciences and Industry Magazine Fetal Bovine Serum running short [Internet]. 2008 October 20. Available from: http://www.european-biotechnology-news.com/news/messages-archive/archive/article/fetal-bovine-serum-running-short.html.

[5] Market Research Reports. "Cell Culture Market by Equipment (Bioreactor, Incubator, Centrifuge), by Reagent (Media, Sera, Growth Factors, Serum Free Media), by Application (Cancer Research, Gene Therapy, Drug Development, Vaccine Production, Toxicity Testing) - Global Forecast to 2018" [Internet]. 2014 July 01. Available from: http://www.rnrmarketresearch.com/cell-culture-market-by-equipment-bioreactor-incubator-centrifuge-by-reagent-media-sera-growth-factors-serum-free-media-by-application-cancer-research-gene-therapy-drug-development-vacci-market-report.html.

[6] Siegel W, Foster L. Fetal Bovine Serum: *The Impact of Geography*. *BioProcess. J*. 2013; 12(3): 28-30.

[7] MLA. Recovery of Specific Proteins and Enzymes from Blood and Offal Part 2: Growth Factors - PPI - Processing & Product Innovation [Internet]. 2001 July. Available from: http://www.meatupdate.csiro.au/infosheets/Recovery%20of%20Specific%20Proteins%20and%20Enzymes%20from%20Blood%20and%20Offal%20Part%202.pdf

[8] FTD. Analysis of agreement containing consent order to aid public comment in the matter of thermo fisher scientific inc., file no. 131-0134 [Internet]. 2014 January 31. Available from: https://www.ftc.gov/system/files/documents/cases/140131thermofisher analysis.pdf.

[9] Bauermann FV, Flores EF, Falkenberg SM, Weiblen R, Ridpath JF. Lack of evidence for the presence of emerging HoBi-like viruses in North American fetal bovine serum lots. *J. Vet. Diagn. Invest*. 2014 Jan; 26(1): 10-17.

[10] Gstraunthaler G, Lindl T, van der Valk J. A plea to reduce or replace fetal bovine serum in cell culture media. *Cytotechnology* 2013 Oct; 65(5): 791-793.

[11] Census. United States Census Bureau. Schedule B 2015 - Section VI - Chapter 30 Pharmaceutical Products [Internet]. 2015 January 1. Available from: https://www.census.gov/foreign-trade/schedules/b/2015/c30.html.

[12] Gibco. Fetal bovine serum 2015 FBS market update [Internet]. 2014 February 12. Available from: https://www.lifetechnologies.com/content/dam/LifeTech/global/life-sciences/CellCultureandTransfection/pdfs/FBS-Market-Update-Flyer-Global-FLR.pdf.

[13] Jochems, CEA. The Use, Trade and Harvest of Livestock Sera. Animal Writes Issue [Internet]. 2000 September 09. Available from: http://www.reocities.com/rainforest/1395/aro000920.html.

[14] Zhang SQ, Guo BTL, Wang FX, Zhu HW, Wen YJ, Cheng S. Genetic diversity of bovine viral diarrhea viruses in commercial bovine serum batches of Chinese origin. *Infect. Genet. Evol*. 2014; 27: 230-233.

[15] BCC Research. Global Markets for Media, Sera and Reagents in Biotechnology / "Highlights" and "Toc & Pricing"- Chapter 1: INTRODUCTION [Internet]. 2015 March. Available from: http://www.bccresearch.com/market-research/biotechnology/media-sera-reagents-biotechnology-markets-report-bio014h.html.

[16] Market Research Reports. "Cell Expansion Market by Product (Reagent, Media, Serum, Bioreactors, Centrifuge), Cell Type (human, animal), Application (Stem Cell Research, Regenerative Medicine, Clinical Diagnostics), End User (Hospital, Biotechnology, Cell Bank) – Forecast to 2019" [Internet]. 2014 Oct 29. Available from: http://www.reportlinker.com/p02462980-summary/Cell-Expansion-Market-by-Product-Reagent-Media-Serum-Bioreactors-Centrifuge-Cell-Type-human-animal-Application-Stem-Cell-Research-Regenerative-Medicine-Clinical-Diagnostics-End-User-Hospital-Biotechnology-Cell-Bank-Forecast-to.html.

[17] Davis D, Hirschi SD. Fetal Bovine Serum: What You Should Ask Your Supplier and Why. *BioProcess. J*. 2014; 13(1): 19-21.

[18] Xia H, Vijayaraghavan B, Belák S, Liu L. Detection and identification of the atypical bovine pestiviruses in commercial foetal bovine serum batches. *PLoS One 2011*; 6(12): e28553.

[19] Daryany MKA, Hosseini SM, Raie M, Fakharie J, Zareh A. Study on continuous (254 nm) and pulsed UV (266 and 355 nm) lights on BVD virus inactivation and its effects

on biological properties of fetal bovine serum. *J. Photochem. Photobiol. B.* 2009 Feb 9; 94(2): 120-124.

[20] EMEA. Revised Guideline on requirements and controls applied to bovine serum used in the production of immunological veterinary medical products [Internet]. 2005 November 9. Available from: http://www.ema.europa.eu/ docs/en_GB/document_ library/Scientific_guideline/2009/10/WC500004575.pdf.

[21] EMEA. Guideline on the use of bovine serum in the manufacture of human biological medicinal products [Internet]. 2013 May 30. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/06/WC500143930.pdf.

[22] Schirrmeier H, Strebelow G, Depner K, Hoffmann B, Beer M. Genetic and antigenic characterization of an atypical pestivirus isolate, a putative member of a novel pestivirus species. *J. Gen. Virol*. 2004 Dec; 85(Pt 12): 3647-3652.

[23] Ståhl K, Kampa J, Alenius S, Persson WA, Baule C, Aiumlamai S, Belák, S. Natural infection of cattle with an atypical 'HoBi'-like pestivirus--implications for BVD control and for the safety of biological products. *Vet. Res*. 2007 May-Jun; 38(3): 517-523.

[24] Bauermann FV, Harmon A, Flores EF, Falkenberg SM, Reecy JM, Ridpath JF. *In vitro* neutralization of HoBi-like viruses by antibodies in serum of cattle immunized with inactivated or modified live vaccines of bovine viral diarrhea viruses 1 and 2. *Vet. Microbiol*. 2013 Sep 27;166(1-2): 242-245.

[25] Elikaei A, Sharifi Z, Hosseini SM, Latifi H, Musavi HMK. Inactivation of model viruses suspended in fresh frozen plasma using novel methylene blue based device. *Iran J. Microbiol*. 2014 Feb; 6(1): 41-45.

[26] Ceylan C, Severcan F, Ozkul A, Severcan M, Bozoglu F, Taheri N. Biophysical and microbiological study of high hydrostatic pressure inactivation of Bovine Viral Diarrhea virus type 1 on serum. *Vet. Microbiol.* 2012 Jan 27; 154(3-4), 266-271.

[27] Bauermann FV, Ridpath JF, Weiblen R, Flores EF. HoBi-like viruses: an emerging group of pestiviruses. *J. Vet. Diagn. Invest*. 2013(a) Jan; 25(1): 6-15.

[28] Ridpath JF. BVDV genotypes and biotypes: practical implications for diagnosis and control. *Biologicals* 2003,Jun; 31(2): 127-131.

[29] Peterhans E, Bachofen C, Stalder H, Schweizer M. Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extinction. *Vet. Res*. 2010 Nov-Dec; 41(6): 44.

[30] Bianchi E, Martins M, Weiblen R, Flores EF. Genotypic and antigenic profile of bovine viral diarrhea virus isolates from Rio Grande do Sul, Brazil (2000-2010). *Pesq. Vet. Bras*. 2011 Ago; 31(8): 649-655.

[31] Flores EF, Donis RO. Isolation of a mutant MDBK cell line resistant to bovine viral diarrhea virus infection due to a block in viral entry. *Virology* 1995 Apr 20; 208(2): 565-575.

[32] Bolin SR, Ridpath JF, Black J, Macy M, Roblin R. Survey of cell lines in the American Type Culture Collection for bovine viral diarrhea virus. *J. Virol. Methods* 1994; 48(2-3): 211-221.

[33] Wellemans G, Van Opdenbosch E. Presence of bovine viral diarrhoea (BVD) virus in several cell lines. *Ann. Rech. Vet*. 1987; 18: 99-102.

[34] Potts BJ, Sawyer M, Shekarchi IC. Peroxidase-labeled primary antibody method for detection of pestivirus contamination in cell cultures. *J. Virol. Methods* 1989; 26: 119-124.

[35] Ridpath JF, Bolin SR, Katz, J. Comparison of nucleic acid hybridization and nucleic acid amplification using conserved sequences from the 5' noncoding region for detection of bovine viral diarrhea virus. *J. Clin. Microbiol.* 1993; 31(4): 986-989.

[36] Gombold J, Karakasidis S, Niksa P, Podczasy J, Neumann K, Richardson J, Sane N, Johnson-Leva R, Randolph V, Sadoff J, Minor P, Schmidt A, Duncan P, Sheets RL. Systematic evaluation of in vitro and in vivo adventitious virus assays for the detection of viral contamination of cell banks and biological products. *Vaccine* 2014 May 19;32(24):2916-2926.

[37] Givens MD, Stringfellow DA, Dykstra CC, Riddel KP, Galik PK, Sullivan E, Robl J, Kasinathan P, Kumar A, Boykin DW. Prevention and elimination of bovine viral diarrhea virus infections in fetal fibroblast cells. *Antiviral Res*. 2004 Nov; 64(2): 113-118.

[38] Dezengrini R, Weiblen R, Flores EF. Selection and characterization of canine, swine and rabbit cell lines resistant to bovine viral diarrhea virus. *J. Virol. Methods* 2006; 137: 51-57.

[39] Bolin SR, Matthews PJ, Ridpath JF. Methods for detection and frequency of contamination of fetal calf serum with bovine viral diarrhea virus and antibodies against bovine viral diarrhea virus. *J. Vet. Diagn. Invest*. 1991; 3: 199-203.

[40] Bolin SR, Ridpath JF. Prevalence of bovine viral diarrhea virus genotypes and antibody against those viral genotypes in fetal bovine serum. *J. Vet. Diagn. Invest*. 1998; 10: 135-139.

[41] Pastoret PP. Human and animal vaccine contaminations. *Biologicals* 2010; 38(3): 332-334.

[42] Giangaspero M, Vacirca G, Morgan D, Baboo KS, Luo NP. Antibovine viral diarrhoea virus antibodies in adult Zambian patients infected with the human immunodeficiency virus. *Int. J. STD AIDS* 1993; 4: 300-302.

[43] Palomares RA, Marley SM, Givens MD, Gallardo RA, Brock KV. Bovine viral diarrhea virus fetal persistent infection after immunization with a contaminated modified-live virus vaccine. *Theriogenology* 2013 May; 79(8): 1184-1195.

[44] Giangaspero M, Vacirca G, Harasawa R, Büttner M, Panuccio A. Genotypes of pestivirus RNA detected in live virus vaccines for human use. *J. Vet. Med. Sci*. 2001; 63: 723-733.

[45] Potgieter LND. Infectious Diseases of Livestock. 2 ed. Cape Town: Oxford University Press; 2004. Chapter Bovine viral diarrhea and mucosal disease; vol 2, p.946-69.

[46] Falcone E, Tollis M, Conti G. Bovine Viral Diarrhea disease associated with a contaminated vaccine. *Vaccine* 1999; 18: 387-388.

[47] Polak MP, Zmudzinski JF, Jacobs JG, Langeveld JP. Atypical status of bovine spongiform encephalopathy in Poland: a molecular typing study. *Arch. Virol*. 2008; 153(1): 69-79.

[48] Bruschke CJ, Paal HA, Weerdmeester K. Detection of bovine virus diarrhea virus in a live bovine herpes virus 1 marker vaccine. Tijdschr *Diergeneeskd* 2001 Mar 15; 126(6): 189-190.

[49] Barkema HW, Bartels CJ, van Wuijckhuise L, Hesselink JW, Holzhauer M, Weber MF, Franken P, Kock PA, Bruschke CJ, Zimmer GM. Outbreak of bovine virus diarrhea on Dutch dairy farms induced by a bovine herpesvirus 1 marker vaccine contaminated with

bovine virus diarrhea virus type 2. *Tijdschr Diergeneeskd.* 2001 Mar 15;126(6): 158-165.

[50] Harasawa R. Adventitious pestivirus RNA in live virus vaccines against bovine and swine diseases. *Vaccine* 1995; 13: 100-103.

[51] Wray C, Roeder PL. Effect of bovine virus diarrhoea– mucosal disease virus infection on salmonella infection in calves. *Res. Vet. Sci.* 1987; 42: 213-218.

[52] Woods RD, Kunkle RA, Ridpath JE, Bolin SR. Bovine viral diarrhea virus isolated from fetal calf serum enhances pathogenicity of attenuated transmissible gastroenteritis virus in neonatal pigs. *J. Vet. Diagn. Invest*. 1999; 11(5): 400-407.

[53] Fan XZ, Ning YB, Wang Q, Xu L, Shen QC. Detection of bovine viral diarrhea virus as contaminant in classical swine fever virus live vaccine with RT-PCR. *Chin. J. Vet. Med*. 2010; 46: 8-10.

[54] Tao J, Liao J, Wang Y, Zhang X, Wang J, Zhuu G. Bovine viral diarrhea virus (BVDV) infections in pigs. *Vet. Microbiol*. 2013; 165: 185–189.

[55] Perry GH. Risk assessment of transmission of bovine viral diarrhea virus (BVDV) to abattoir-derived in vitro-produced embryos. *Theriogenology* 2007; 68: 38-55.

[56] Studer E, Bertoni G, Candrian U. Detection and Characterization of Pestivirus Contaminations in Human Live Viral Vaccines. *Biologicals* 2002; 30: 289-296.

[57] Lindberg A, Ortman K, Alenius S. Seroconversion to bovine viral diarrhea virus (BVDV) in dairy heifers after embryo transfer. In: 14th international congress on animal reproduction, 2000 July 2–6, vol. I p.250.

[58] Drew TW, Sandvik T, Wakeley P, Jones T, Howard P. BVD virus genotype 2 detected in British cattle. *Vet. Rec.* 2002; 151(18): 551.

[59] Harasawa R, Hikiji K, Takada Y, Mizusawa H. Detection of adventitious pestivirus in cell cultures by polymerase chain reaction using nested primers. *Tissue Cult. Res. Commun*. 1993; 12: 215-220.

[60] Harasawa R. Comparative analysis of the 5' non-coding region of pestivirus RNA detected from live virus vaccines. *J. Vet. Med. Sci.* 1994; 56: 961-964.

[61] Harasawa R, Tomiyama T. Evidence of pestivirus RNA in human virus vaccines. *J. Clin. Microbiol*. 1994; 32: 1604-1605.

[62] Harasawa R, Sasaki T. Sequence analysis of the 5' untranslated region of pestivirus RNA demonstrated in interferons for human use. *Biologicals* 1995; 23: 263-269.

[63] Levings RL, Wessman SJ. Bovine viral diarrhea virus contamination of nutrient serum, cell cultures and viral vaccines. *Dev. Biol. Stand*. 1991; 75: 177-181.

[64] EMEA. Guideline on the procedure to be followed when a batch of a vaccine finished product is suspected to be contamined with Bovine Viral Diarrhoea (BVD) Virus [Internet]. 2008 March 13. Available from: http://www.ema.europa.eu/docs/en_GB/ document_library/Scientific_guideline/2009/10/WC500004558.pdf.

[65] APHIS/USDA. Virus-Serum-Toxin Act 21 USC 151-159 et. seq. – Chapter 5. Viruses, Serums, Toxins, Antitoxins and analogous products [Internet]. 1985 December 23. Available from: http://www.aphis.usda.gov/animal_health/vet_biologics/publicati ons/ vsta.pdf.

[66] GPO-US. Detection of extraneous viruses by the fluorescent antibody technique [Internet]. 2014 January 01. Available from: http://www.gpo.gov/fdsys/pkg/CFR-1998-title9-vol1/pdf/CFR-1998-title9-vol1-sec113-47.pdf.

[67] APHIS/USDA. A Proposed Rule by the Animal and Plant Health Inspection Service on 05/30/2014 - Proposed Rule APHIS-2013-0034, RIN 0579-AD86, Viruses, Toxins, and Analogous Products; Standard Requirements; Addition of Terminology to define test results [Internet]. 2014. Available from: https://www.federalregister.gov/articles/2014/05/30/2014-12551/viruses-serums-toxins-and-analogous-products-standard-requirements-addition-of-terminology-to-define.

[68] ICH. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use - Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin - Q5A(R1) [Internet]. 1999 September 23. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5A_R1/Step4/Q5A_R1__Guideline.pdf.

[69] VICH. Guidance for Industry specifications: test procedures and acceptance criteria for new biotechnological /biological Veterinary Medicinal Products [Internet]. 2006 June 14. Available from: http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/ucm052475.pdf.

[70] ISIA. ISIA position with regard to the PAA/ GE Healthcare product hold [Internet]. 2013 July. Available from: http://www.serumindustry.org/documents/Market-PAA.pdf.

[71] Hodgson J. To treat or not to treat: that is the question for serum. Nature *Biotechnology* 1995, Apr; 13(4): 333-343.

[72] ISIA. Standardization of fetal bovine serum quality assessment and reporting definitions and sample certificates included - White paper on Definitions and QC Standardization [Internet]. 2008 April. Available from: http://www.serumindustry.org/documents/ISIAStandardizationdocument081.pdf.

[73] Saliki JT, Dubovi EJ, Laboratory diagnosis of bovine viral diarrhea virus infections Bovine viral diarrhea virus diagnostic philosophy. *Vet Clin Food Anim*. 2004; 20: 69–83.

[74] OIE. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals - Chapter 2.4.8. Bovine Viral Diarrhoea [Internet]. 2015 May. Available at http://www.oie.int/fileadmin/Home/eng/Health_standards/ tahm/2.04.08_BVD.pdf.