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The Pervasiveness of Bovine Viral Diarrhea Virus in Commercial Bovine Serum

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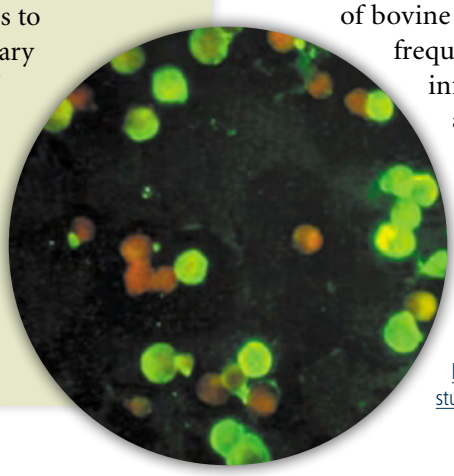
The procurement of lots of bovine serum that are free of infectious bovine viral diarrhea virus (BVDV) and of neutralizing antibodies to BVDV can still be problematic for manufacturers of biologics. For cell-culture based applications requiring the use of bovine serum as a cell culture medium supplement, the BVDV issue has plagued the industry for decades. Has there been any improvement over the years? The literature from the past four decades has been reviewed to answer this question. There is some evidence that the frequency of detection of infectious BVDV in commercial bovine serum lots has decreased in recent years. There are, however, insufficient data for making conclusions in the case for neutralizing antibodies to BVDV. There are several complementary approaches for mitigating the risk of introducing infectious BVDV into a manufacturing process. These include eliminating the use of serum, pre-treatment of the serum by the vendor or end-user, or treatment of media formulated with the serum to inactivate any BVDV that might be present.

Introduction

One of the animal-derived materials (ADM) most commonly utilized for cell culture and for production of biologicals manufactured using cell cultures is bovine serum (most typically, fetal bovine serum and less commonly, calf serum). There is an inherent risk of introduction of adventitious contaminants (especially viruses and mollicutes) associated with the use of cell culture media containing serum. In fact, of the viral contaminants that have been isolated from biologics bulk harvests (including REO type 2, Cache Valley virus, epizootic hemorrhagic disease virus, and vesivirus 2117), most are believed to have been introduced via bovine serum.[1] Another potential viral contaminant resulting from bovine serum use is the pestivirus bovine viral diarrhea virus. The risks associated with the use of bovine sera, as a result of the relatively frequent contamination of this ADM with infectious BVDV, have been described in a review by Plavsic.[2] Contamination of recombinant interferon with genomic RNA for BVDV[3], and the relatively frequent finding of



BVDV-infected cells evaluated using immunofluorescent antibody staining – *Courtesy of University of Saskatchewan* http://homepage.usask.ca/~vim458/virology/studpages2007/Chad_Jan_Amy/home.html

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genomic RNA for BVDV in live viral vaccines[4-6] have been attributed to the use of bovine serum in these manufacturing processes.

The regulatory requirements pertaining to the use of bovine-derived raw materials for manufacturing biologics (*e.g.*, 9 CFR 113.47[7]) contain specific instructions relating to the detection of BVDV contamination. EMA regulations[8] require that bovine sera be tested not only for infectious BVDV, but also for the presence of neutralizing antibodies to BVDV. The latter are of concern, since their presence could theoretically interfere with the detection of this virus in release (suitability) testing performed for the serum. Testing performed for release of lots (batches) of bovine serum by the vendor or by the end-user typically involves a sample size of less than 300 mL. In many cases, this sample is drawn from a single container. As the lots of commercial serum may represent relatively large pools (*i.e.,* 1000–2000 L) derived from many individual donor animals, there is a possibility of non-homogeneous contamination of the lot (batch) due to a relatively low level contamination from small numbers of infected donors included in the pool. This is one of the reasons usually given for the fact that a serum lot can be tested and found to be negative for infectious BVDV during release testing performed by the serum vendor, and yet can subsequently be found positive for infectious BVDV during release testing performed by an end-user.

How frequently is infectious BVDV detected in commercially available bovine serum? What percentage of serum lots contains neutralizing antibodies to BVDV? Is genomic RNA for BVDV invariably found in bovine serum? These questions have been addressed by various authors over the past four decades.[9-34] In the present review, we have assembled this information for the convenience of the reader. In addition, we have evaluated the data in order to identify any potential trends in detection frequency that may have occurred during the time period covered by the review.

Detection of Infectious BVDV

Perhaps the earliest systematic attempt to determine the frequency of virus contamination of commercial batches of bovine sera intended for use as a supplement to cell culture medium was the 1972 study of Molander *et al.*[9] In this early study, the detection endpoints used included viral cytopathic effect (CPE) in bovine and human cells, hemadsorption of guinea pig erythrocytes, and interference with infectivity of spiking viruses. The detector cells used in the study were not evaluated for viral antigen using immunofluorescence (IFA) techniques. The importance of this fact on the relevance of the results obtained (in particular, the relatively low frequency of detection of BVDV) will be discussed shortly.

By 1975, studies using electron microscopy had described the common detection in commercial batches of bovine serum of 50 nm virus-like particles presumed to be bovine viral diarrhea virus.[10] Studies reported by King and Harkness[11] and Kniazeff and co-workers[12] that same year described the finding of a non-cytopathic BVDV in bovine sera. It subsequently became clear that non-cytopathic BVDV is by far the predominant isolate and this has remained the case over the past three decades (Table 1, on the following page). It is suspected, but not proven, that cytopathic BVDV arises within infected animals as a result of a mutation in non-cytopathic BVDV[17] (and discussed in[2]). Methods for detecting infectious BVDV in sera that have been developed and used subsequent to this discovery have employed the use of IFA staining for BVDV viral antigen (which detects both the cytopathic and non-cytopathic strains of BVDV) in addition to observation of CPE (which is only relevant for the cytopathic strain of BVDV).

As shown in Figure 1, the frequency of detection of infectious BVDV in bovine serum intended for cell culture use has varied over the years. Within a few years of the initial use of the IFA method for screening

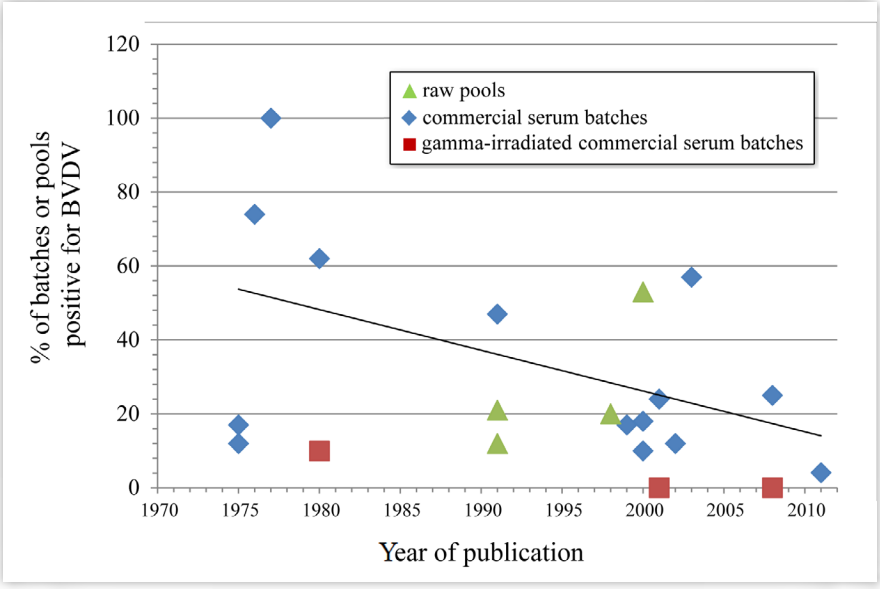

FIGURE 1. Frequency, by publication year, of infectious BVDV detection in FBS pools. For illustrative purposes, the linear regression line for detection in non-irradiated commercial serum is shown.

TABLE 1. Detection of infectious BVDV, neutralizing antibodies to BVDV, and BVDV genomic RNA in bovine serum.

FBS Sample*	*n*	% Infectious BVDV Positive	% Non-Cytopathic†	% Antibody Positive	% RT-PCR Positive	Year of Publication	Reference
Commercial lots	16	0%**	N/A	Not tested	Not tested	1972	[9]
Raw pools	10	10%**	0%	Not tested	Not tested	1972	[9]
Commercial lots	6	17%	100%	Not tested	Not tested	1975	[11]
Commercial lots	51	12%	Not reported	Not tested	Not tested	1975	[12]
Commercial lots	19	74%	≥95%	Not tested	Not tested	1976	[13]
Commercial lots	5	100%	100%	Not tested	Not tested	1977	[14]
Commercial lots	21	62%	100%	Not tested	Not tested	1980	[15]
γ-irradiated commercial lots	9	10%	100%	Not tested	Not tested	1980	[15]
Abattoir samples	147	Not tested	N/A	27%	Not tested	1987	[18]
Commercial lots	190	47%	98%	Not tested	Not tested	1991	[19]
Raw pools	1068	21%	99%	13%	Not tested	1991	[19]
Raw pools	154	12%	"most"	2%	Not tested	1991	[20]
Commercial lots	6	Not tested	N/A	Not tested	100%	1996	[22]
γ-irradiated commercial lots	1	Not tested	N/A	Not tested	100%	1996	[22]
Commercial lots	15	Not tested	N/A	Not tested	100%	1997	[24]
Raw pools	1000	20%	98%	13%	Not tested	1998	[25]
Commercial lots	13	Not tested	N/A	Not tested	46%	1999	[26]
Commercial lots	6	17%	Not reported	Not tested	67%	1999	[26]
Commercial lots	20	10%	Not reported	Not tested	Not tested	2000	[27]
Commercial lots	165	18%	Not reported	Not tested	Not tested	2000	[27]
Raw pools	1877	53%	Not reported	Not tested	Not tested	2000	[27]
Commercial lots	102	24%	100%	61%	Not tested	2001	[28]
γ-irradiated commercial lots	31	0%	N/A	55%	Not tested	2001	[28]
Commercial lots	22	12%	Not reported	Not tested	95%	2002	[29]
Commercial lots	7	57%	100%	Not tested	86%	2003	[30]
Commercial lots	4	25%	Not reported	Not tested	75%	2008	[31]
γ-irradiated commercial lots	1	0%	N/A	Not tested	0%	2008	[31]
Commercial lots	33	Not tested	N/A	Not tested	100%	2011	[32]
Commercial lots	49	4.1%	100%	98%	57%	2011	[33]
Commercial lots	35	Not tested	N/A	Not tested	>45%	2012	[34]

*The FBS samples tested represented commercial lots, raw pools destined for use in commercial lots, or γ-irradiated commercial lots.
†The percentage of the BVDV isolates detected that were non-cytopathic
**An assay for the non-cytopathic strains of BVDV was not performed in this study.

sera, reports of detection of infectious BVDV in 74–100% of batches evaluated were being published.[13, 14] Did this increase in detection frequency reflect improvements in sensitivity and/or specificity of the immunological reagents being used? It is likely that optimization of the reagents played some, but perhaps not a primary, role in this increase, as intra-lab results collected over nearly a decade[21] (presumably reflecting a single methodology) displayed a marked variability in results year-by-year, as shown in Figure 2. Over the more extended time period from 1975 to the present, the variability in frequency of detection of infectious BVDV in commercial serum has persisted, although an apparent downward trend in frequency is suggested by the data (Figure 1). This might be expected on the basis of efforts on the part of serum vendors to more carefully pre-screen samples from individual fetuses for the presence of BVDV prior to pooling.

Detection of Neutralizing Antibodies to BVDV

As early as 1974, investigators began to evaluate levels of neutralizing antibodies to various viruses in commercial bovine serum batches.[35] This was an extension of work suggesting that the high γ-globulin levels found in some commercial serum lots might be associated with adulteration of post-colostral serum (containing maternal antibodies).[36] Since this time, it has become clear that antibodies to BVDV in fetal bovine serum may also arise as a result of an *in utero* BVDV infection after a certain age of fetal development.[37] Due to concerns over the possible presence of neutralizing antibodies to BVDV and the potential for interference in the virus assay, methods for detecting and quantitating these have been developed and employed. As mentioned above, the evaluation of bovine sera (intended for use in the manufacture of human biological medicinal products) for the presence of neutralizing antibodies to BVDV, and for potential impact on the detection of BVDV using cells grown in medium containing the sera, is an EMA requirement.[8]

A number of reports describing the frequency of detection of neutralizing antibodies to BVDV in abattoir pools or commercial serum batches (including γ-irradiated batches) have appeared in the literature over the past three decades.[18-21, 25, 28, 33] The variability in detection of neutralizing antibodies has been high, ranging from 2–98% of lots (Table 1). This may be a reflection of differences in sensitivity of the methodologies and to the genotypic specificities of the neutralization endpoints used.[25, 33] The antibody titers detected, when reported, have ranged from 1:2 to ≥1:256. A possible increase in detection frequency by year of publication is suggested by the data (Figure 3), although the *n* of 6 precludes making conclusions regarding any trends. This, in our opinion, represents an area of investigation that deserves more attention.

As might be expected, the frequency of detection of neutralizing antibody to BVDV is similar in non-irradiated bovine serum batches and serum batches that have been γ-irradiated (Table 1, Figure 3).[28]

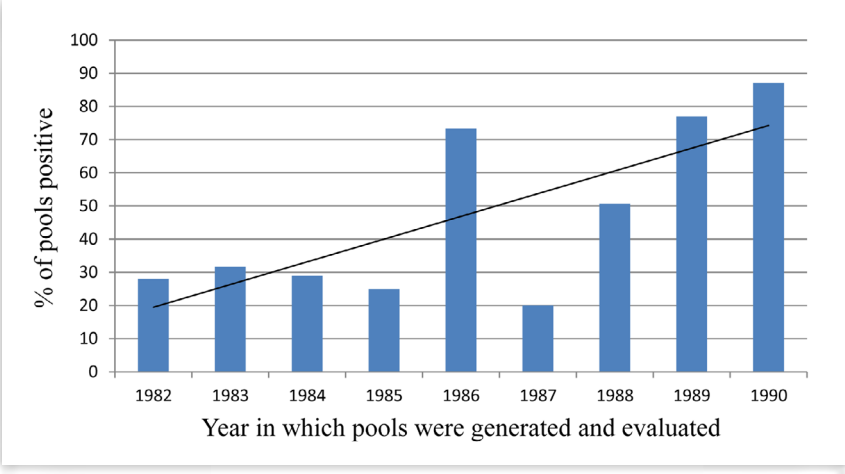

FIGURE 2. Frequency, by year of evaluation, of detection in 1 L abattoir FBS pools (each derived from no more than two bovine fetuses) of infectious BVDV or neutralizing antibodies to BVDV.[21] For illustrative purposes, the linear regression line is shown.

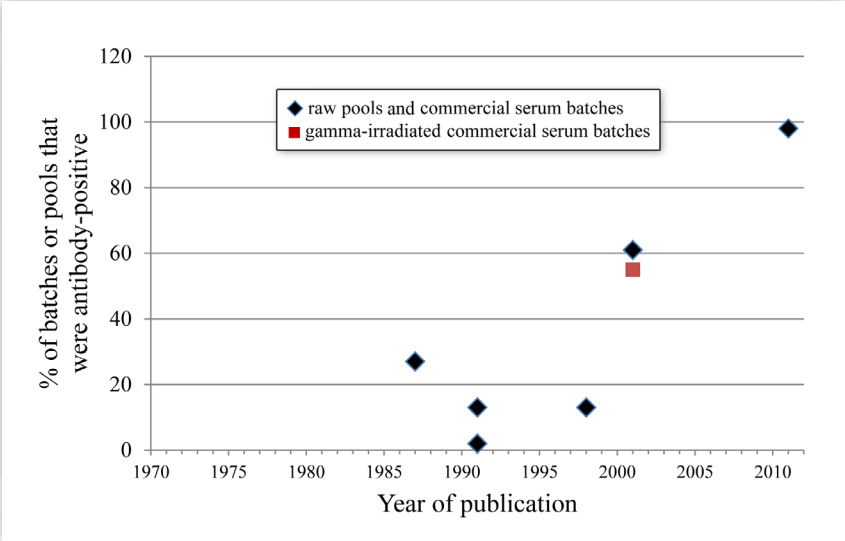

FIGURE 3. Frequency, by publication year, of neutralizing antibody detection against BVDV in FBS pools.

Detection of BVDV Genomic RNA

In the mid-1990s, reports of the use of reverse transcriptase-polymerase chain reaction (RT-PCR) for evaluating bovine sera for genomic RNA encoding BVDV began to appear.[22, 24] While the technology is useful for detecting genomic material for BVDV species, it has also been used to determine the genotype of the BVDV present (*i.e.*, type I or type II).[22, 24, 30-32] The results have indicated that the predominant type detected in bovine serum is BVDV type I, in agreement with evaluations using type-specific antibodies.[25] During the time period covered by this review, RT-PCR results have indicated that a high percentage of commercial fetal bovine serum batches contain detectable genomic RNA for BVDV (Table 1; Figure 4). This percentage has ranged from as high as 100% to as low as 45%.

The detection of genomic RNA for BVDV in fetal bovine serum is not necessarily cause for rejecting a batch of serum for cell culture use. As typically performed, testing of sera by RT-PCR can determine whether or not genomic RNA for BVDV is present in the sera but cannot determine whether that RNA is associated with infectious BVDV. Experiments may be designed in such a manner that RT-PCR results may be used to evaluate sera for infectious BVDV. For instance, Jennings[26] described the use of RT-PCR conducted on serum before and after a 21-day culture period in BVDV-free MDBK cells per the 9 CFR 113.53 method.[38] In this protocol, the detection of BVDV RNA following the 21-day culture period is considered to reflect the presence of infectious BVDV in the bovine serum. The more typical use of RT-PCR evaluation of bovine serum is the exclusion of BVDV RNA-positive individual sera from larger pools, and as described above, for obtaining more detailed information on the genotype of a contaminant.

The frequency of detection of BVDV genomic RNA in serum should not impact be impacted by γ-irradiation. Yanagi *et al.*[22] detected BVDV RNA in 100% of the commercial FBS lots evaluated, including six non-irradiated and one γ-irradiated lot. On the other hand, Liu *et al.*[31] described the detection of BVDV RNA in three of four lots (75%) of bovine serum, but not in the single lot of γ-irradiated serum evaluated. An *n* of 1 for γ-irradiated serum in each case is not sufficient to allow conclusions. A more thorough study of γ-irradiated bovine serum lots for BVDV RNA using RT-PCR is, in our opinion, warranted.

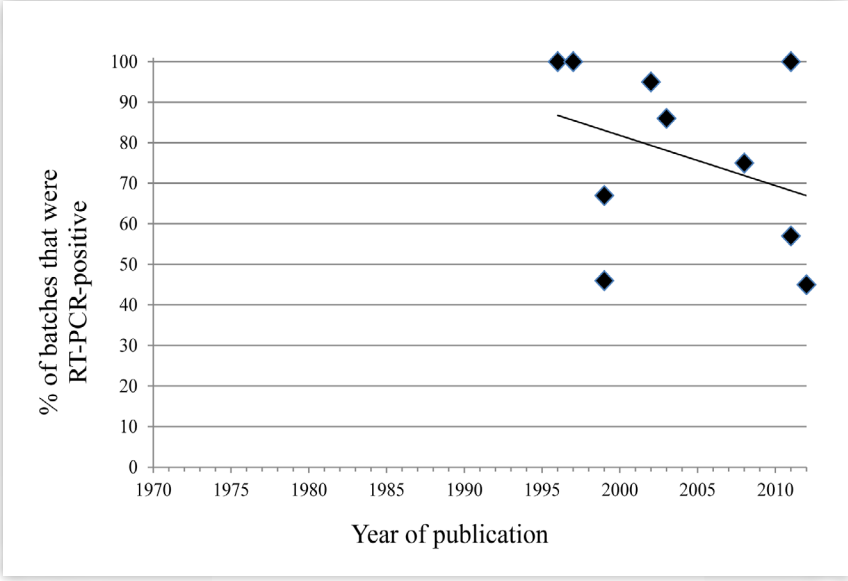

FIGURE 4. Frequency, by publication year, of detection of genomic RNA for BVDV in commercial FBS batches. For illustrative purposes, the linear regression line is shown.

How Can Manufacturers Mitigate the Risk of BVDV Contamination?

The risk of introduction of BVDV into biologicals (recombinant proteins, antibodies, vaccines) has been associated primarily with the use of bovine sera. Reducing, replacing, or eliminating the use of this ADM therefore represents an effective risk mitigation approach. In cases where bovine sera must be used, manufacturers are expected to subject the raw material to risk-mitigation treatments.[8, 39] In the past, the treatments evaluated for inactivating BVDV contaminating high-risk animal-derived materials have included heat, pH adjustment, γ-irradiation, and UV-C irradiation.[8, 11, 15, 16, 39-44]

Heat inactivation of serum intended for use as a cell culture supplement is often performed (typically 56 °C for 30–45 minutes) in order to inactivate-complement. It has been shown that this treatment also inactivates BVDV.[11, 15, 16, 40] In fact, Danner *et al.* found that 4.9 $\log_{10}$ of BVDV were inactivated by 30 minutes of heating at 56 °C.[40] However, the subsequent detection of infectious BVDV by other authors in some lots of sera subjected to this treatment[11, 16] may reflect variability in the treatment process control or residual viable (infectious) BVDV. This implies that heat inactivation under these conditions is not robust enough to assure total mitigation of this risk, especially in cases of high viral load. Another type of heat treatment that is being considered for viral risk mitigation currently is high-temperature short-time (HTST) treatment. For instance, HTST (16 seconds at 72 °C) has been shown to inactivate > 5.4 $\log_{10}$ of BVDV in protein-containing solutions.[41] At sufficiently high temperatures, this strategy should

therefore be useful for inactivating BVDV in culture media formulated with BVDV-contaminated bovine serum.

Adjustment of the pH of process solutions potentially contaminated with BVDV was evaluated as a means of mitigating risk of viral contamination. For instance, samples of fetal bovine serum spiked with BVDV were adjusted to relatively high pH (~11) or low pH (~3) and then held for 2 hours at room temperature. Under these conditions, 4.4–7.0 $\log_{10}$ reduction in titer of infectious bovine rhinotracheitis virus and parainfluenza type 3 virus were observed.[42] Unfortunately, this treatment resulted in only 0.5–1.3 $\log_{10}$ reduction in titer of BVDV, possibly indicative of study limitations (sampling, virus aggregation, *etc.*) or a high level of resistance of this enveloped virus to pH adjustment.

Historically, γ-irradiation has been applied to animal sera for viral risk mitigation. From a recent survey of the literature, a consensus inactivation constant of 0.198 $\log_{10}$ reduction per kGy irradiation in frozen serum has been obtained for BVDV.[43] On the basis of this value, exposure of frozen serum to 25–45 kGy γ-irradiation would be expected to result in at least 5 $\log_{10}$ reduction in BVDV titer. Ultraviolet radiation in the C range (UV-C; λ max 248–254 nm) has also been demonstrated to effectively inactivate BVDV when spiked into bovine serum. For instance, irradiation of spiked serum at 100 mJ/cm^2 resulted in 8 $\log_{10}$ reduction in BVDV titer.[44]

Several of these inactivation approaches, including HTST and UV-C treatment, should be capable of operating in a flow-through manner for large volume process additives such as liquid serum and serum-containing media. These technologies are applicable to liquids, while γ-irradiation is typically performed only on frozen materials (*e.g.*, serum or trypsin) in order to limit the adverse impacts associated with the indirect effects of that radiation.

Although viral safety testing of animal serum represents a risk mitigation strategy for BVDV, it is not a robust approach. Testing of animal-derived materials used as serum is required (9 CFR 113.47[7] and 113.53[38]), yet the testing that is typically performed by raw material vendors and by the end-users has limited value as a risk mitigation strategy for BVDV. The reality is that raw material contamination is not typically homogenously distributed throughout the entire lot, the infections can be of relatively low titer, detection of the virus can be blocked

by neutralizing antibodies, and only small amounts of a given lot are sampled for testing. Manufacturers should not, therefore, consider a negative result from such testing as evidence that the entire lot of the raw material is free of infectious BVDV. While chances of a false positive are low for this testing, the probability of false negatives must be viewed as relatively high. As mentioned previously, this is the primary reason that a batch of serum may be released as BVDV-free by the serum vendor yet still may be found to contain infectious BVDV in confirmatory testing performed by the end-user.

Discussion

There are three potential sources of information on the frequency of detection of infectious BVDV, neutralizing antibodies to BVDV, and genomic RNA for BVDV in commercial bovine serum. These sources are: the scientific and trade literature, contract testing laboratory experiences, and the experience of the animal serum industry.

In this paper, we have surveyed the literature for reports addressing these questions. While much attention in the literature has been paid to the presence of infectious BVDV, more work needs to be done in the areas of neutralizing antibodies and genomic RNA including the presence of these in γ-irradiated bovine sera.

Another potential source of information on these topics is contract testing laboratories, which test many lots of bovine serum (including irradiated serum) for the presence of viruses each year. It would be particularly useful if the recent testing experiences of these organizations could be compared to the results summarized herein. Anecdotal reports would appear to indicate that non-cytopathic BVDV is still being detected in a higher percentage of non-irradiated lots of bovine serum than has recently been reported in the literature (Figure 1). In addition, the year-by-year results of testing of bovine sera for BVDV neutralizing antibodies would represent an important addition to the rather minimal information currently available in the public domain. It would be beneficial to obtain more information on this topic so that the apparent increase in detection of neutralizing antibodies suggested by the results in Figure 3 could be confirmed or refuted.

The last source of information on these topics is the bovine serum industry itself. This industry has been a major source of information in the past on the efficacy of adventitious agent inactivation approaches and the impact of such treatments on serum performance.

This literature survey and Figure 1, in particular, appear to indicate a downward trend, year-by-year, in the frequency of detection of infectious BVDV in commercial fetal bovine serum. Further reduction can be expected following pre-treatment of the serum with inactivating technologies such as γ-irradiation. There is not sufficient data in the published literature to make conclusions on potential trends in frequency of detection of neutralizing antibodies to BVDV in commercial fetal bovine serum (including γ-irradiated serum). The limited data in Figure 3 indicate that, if anything, the frequency of detection of neutralizing antibodies to BVDV may be on the rise.

The implication of a positive finding of neutralizing antibody in a serum lot is dependent on the titer detected. Very low titers (*e.g.,* 1:4 or lower) may reflect non-specific neutralization and may not result in significant interference with BVDV detection in an infectivity assay. To determine significance, end-users may be required to conduct specific testing.[8, 39] In this testing a constant amount of undiluted serum is mixed with a known amount of diluted BVDV stock, incubated, and inoculated onto permissive detector cells, along with appropriate controls. The amount of virus blocked by the serum (indicated by a drop in viral titer) is determined at the end of cell culture incubation. The EMA guidance document[8] does not specify acceptable limits for the amount of virus being neutralized, and it is left to serum users to determine the acceptance criteria. More than 2 $\log_{10}$ of virus neutralization in this assay is generally considered as significant (in the context of assay variability) within the scientific community.[45]

As discussed above, the acceptability of bovine serum for use in manufacturing biologics may depend on demonstrating both absence of infectious BVDV and absence of neutralizing antibodies to BVDV. Because of this, obtaining acceptable lots of bovine serum can still be problematic for end-users. The experience, reported by Kozasa *et al.*[33], of finding only one lot out of 49 serum lots tested to be free of both infectious BVDV and neutralizing antibodies to BVDV is probably atypical. Notwithstanding this result, the difficulty of finding acceptable bovine serum (in terms of BVDV status) together with the inherent risk in introducing BVDV and other viral and mollicute contaminants into a biologics manufacturing process through use of bovine serum have combined to drive the industry toward use of serum-free medium.

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