

Bovine viral diarrhea virus (BVDV): Epidemiologic concerns relative to semen and embryos

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Abstract

Artificial insemination and embryo transfer are used commonly in cattle production and exchange of germplasm between populations of cattle. If properly monitored, assisted reproductive techniques can be used to prevent the spread of infectious agents. However, these techniques potentially represent unnatural routes for transmission of diseases. Bovine viral diarrhea virus (BVDV) is broadly distributed among the world's populations of cattle. Fluids, gametes and somatic cells from infected animals are likely contaminated with the virus. Thus, use of semen or embryos from infected animals could result in spread of BVDV. This paper provides an overview of the risks of transmitting this virus by AI or production and transfer of embryos and summarizes the precautions needed to prevent such transmissions of disease from occurring.

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1. Introduction

Because of its broad distribution among populations of cattle and association with cells and fluids from infected animals, bovine viral diarrhea virus (BVDV) represents a potential problem in applications of assisted reproduction. Contaminated semen from acutely or persistently infected bulls can transmit the virus to inseminated cattle [1,2]. It is also possible that semen from bulls with localized persistent testicular infections might infect inseminated cattle [3]. Further, although embryo transfer has never been proven to result in transmission of BVDV, based on relatively recent reports in the literature, there is some potential for the virus to be spread via the transfer of either in

vitro- or in vivo-derived bovine embryos. This paper is intended to provide an overview of the potential for transmission of BVDV via AI and three generations of embryo technologies. In addition, essential precautionary measures are summarized.

2. Bovine viral diarrhea virus

Bovine viral diarrhea virus, a single-stranded RNA virus, is found in cattle and other ruminants worldwide [4–6]. The presence of BVDV in other domestic species such as sheep or wild species such as whitetail deer might or might not be relevant to the epidemiology of the disease in cattle [7]. Infections with the virus, and associated enteric, reproductive and respiratory disease are economically important in cattle [8]. There are two genetically distinct genotypes (1 and 2) and biotypes (cytopathic and noncytopathic) of the virus [4,9]. Both acute and persistent infections occur in cattle, with the latter developing as an outcome of fetal exposure

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before the development of immune competence [4]. In addition, prolonged testicular infections have been described in bulls after exposure to noncytopathic strains of the virus [10]. Acutely infected cattle are an important temporary source of virus for new infections, whereas persistently infected cattle are lifetime reservoirs of BVDV [4,5]. The role of prolonged testicular infections in the epidemiology of the disease has yet to be completely defined [10].

Since BVDV exhibits poor survival in the environment, direct contact is the primary natural method for transmission of the virus [4,8,11,12]. Persistently infected cattle continuously shed large quantities of virus in saliva, nasal mucous, tears, milk, feces, urine and vaginal mucous [5]. In addition, fluids, gametes and other cells derived from these cattle represent challenging sources of contamination when these ‘materials of animal origin’ are used in embryo production and transfer [13–18].

Because of the negative impact on the health, well being and productivity of cattle and due to the inconsistency with which vaccines have been able to control spread of infections, several countries and territories have initiated eradication programs for BVDV [5,19–22]. Further, there is strong support for development and implementation of programs to eradicate BVDV from cattle in the USA [23]. In the face of increased efforts to control and ultimately eradicate BVDV from given geographic locations, it is logical that the use of semen and embryos for exchange of germplasm will be emphasized. Further, it is certain that international markets for semen and embryos will demand that semen and embryos are certifiably BVDV-free. Thus, it is imperative that risks of transmission of the virus via semen and embryos are fully understood and appropriate quality assurances are used.

3. Epidemiologic impact of assisted reproductive techniques

Environmental change often contributes to a shift in the critical balance between health and disease within a population of individuals [24]. Over the past 60 years, AI and three generations of embryo technologies have been developed and applied at various levels [25,26]. Each of these assisted reproductive techniques has represented a potentially significant change in the animal production environment with the potential to increase or decrease the spread of infectious diseases within and between populations of cattle. It is estimated that worldwide over 200×10^6 doses of bovine semen are prepared annually for artificial insemination [27]

(www.fao.org). Further, the first two generations of embryo technologies (in vivo- and in vitro-embryo production) are also widely used in commercial cattle production. The International Embryo Transfer Society reported that, in 2003, approximately 694,000 in vivo-derived embryos of cattle were transferred to recipients worldwide. Also, in 2003, approximately 331,000 bovine embryos from IVF were produced and over 106,000 IVF embryos were transferred worldwide [28]. In addition, the industry in North America was reported to have increased by more than 10% as compared to the previous year, and North America was responsible for producing and transferring approximately 44.7% of the total number of in vivo-derived embryos [28]. This level of domestic and international activity could facilitate broad distribution of any pathogens that might be transmitted even occasionally via semen or embryos.

4. Semen

Semen can be cryopreserved, stored indefinitely, shipped over broad geographic areas, and used to produce offspring by artificial insemination or by use for in vivo or in vitro embryo production [29]. Whereas cryopreservation and shipment of semen provide alternate opportunities for pathogen transmission, this should not occur if advantage is taken of convenient critical control points in semen production and distribution.

4.1. Risk of transmitting BVDV via cryopreserved semen

Viral contamination of semen can result from four distinct types of infections with BVDV: (a) persistent infection of bulls due to in utero exposure to the virus, (b) acute infection of bulls due to exposure after development of immune-competence, (c) prolonged testicular infection of bulls due to an enduring infection of testicular tissue after acute infection, and (d) persistent testicular infection due to unknown viral exposure.

Persistently infected bulls have exhibited normal growth and development and have previously gained access into AI centers due to desirable growth and performance characteristics [30]. These bulls may exhibit normal concentration, motility and morphology of spermatozoa [31]. Persistently infected bulls shed large quantities of BVDV in semen ($10^{7.6}$ CCID₅₀/mL) which can survive processing and cryopreservation [31]. This contaminating virus will consistently infect susceptible, inseminated heifers and cows and

occasionally may result in production of persistently infected calves [31,32]. It is noteworthy that semen from bulls persistently infected with BVDV may produce excellent first service conception rates in seronegative heifers [32]. Virus in the semen of persistently infected bulls is associated with sperm and cannot be separated from viable sperm using swim up techniques, glass wool filtration, glass bead filtration, or centrifugation through Percoll gradients [33]. Thus, semen from persistently infected bulls creates a risk of viral transmission via AI, in vivo embryo production, and in vitro embryo production.

Acutely infected bulls can shed BVDV in semen from 2 to 20 days after infection [34]. The concentration of BVDV in these contaminated semen samples (5–75 CCID₅₀/mL) is much less than detected in semen of persistently infected bulls [34]. This viral contaminant may infect approximately 5% of susceptible inseminated heifers [2]. Resulting acute infections can cause proliferation of BVDV within a herd which results in new persistent infections of developing fetuses [2]. Thus, acute infection of bulls creates a narrow temporal window for transmission of small quantities of virus on a limited basis to susceptible semen recipients. Unfortunately, recent research has demonstrated that acute infections of seronegative peri-pubertal and post-pubertal bulls can result in prolonged testicular infections [10,35]. In a prolonged testicular infection, virus replicates within the seminiferous tubules and is protected by the blood–testis barrier from humoral and cell-mediated immunity [36]. These prolonged testicular infections can result in very low quantities of BVDV in semen for prolonged intervals. The viral contaminant can be detected using RT-nPCR [36], virus isolation after ultracentrifugation of semen samples [35], or intravenous inoculation of seronegative calves [36]. The viral contaminant in these semen samples cannot be detected using routine virus isolation techniques [10,35]. Whereas preliminary studies indicated that insemination or natural breeding with this semen does not consistently result in transmission of BVDV, research needs to be completed to clarify the potential for viral transmission via semen from bulls with prolonged testicular infections.

A single seropositive, nonviremic bull has been reported with a persistent testicular infection of unknown origin [37]. Over a period of 11 months, this bull consistently produced semen from which virus could be isolated using routine virus isolation techniques [37]. The semen contained very low quantities of virus but did exhibit the potential to infect seronegative heifers via AI using cryopreserved semen [3]. When the

bull was euthanized, BVDV could only be isolated from testicular tissue [37]. This case appears to be unique and speculation indicates that the bull may have been infected with BVDV during puberty or around the time of maturation of the immune system in utero [38].

4.2. Preventing transmission of BVDV via cryopreserved semen

Whereas the risk of transmitting BVDV via cryopreserved semen is apparent, the safeguards to prevent transmission of BVDV via semen are not extraordinarily difficult to accomplish. The World Organization for Animal Health (OIE) outlines recommended biosecurity measures for semen in Appendix 3.2.1 of their Terrestrial Animal Health Code [39] (www.oie.int). In the USA, Certified Semen Services (CSS; a subsidiary of the National Association of Animal Breeders to establish industry self regulation) describes minimum disease-control requirements for cryopreserved semen produced for artificial insemination [40] (www.naab-css.org). The OIE recommended standards require bulls to be free of BVDV in blood on entry with ongoing testing for BVDV and anti-BVDV antibodies during semen collection procedures. When admitting bulls through an isolation and quarantine procedure, groups of animals should be assembled, tested and cleared based on all-in and all-out management after assurance that BVDV is not circulating within the group, based on serologic evidence. The OIE recommended standards require seropositive bulls to produce semen free of BVDV using virus isolation testing. To meet CSS requirements, bulls must be nonviremic on entry and maintain the nonviremic status. If bulls test seronegative, no semen testing is required. If bulls are seropositive, virus isolation attempts from semen must fail to detect BVDV. Both OIE and CSS guidelines will prevent transmission of BVDV due to persistent infections, acute infections, and persistent testicular infections. Although these testing protocols will not detect bulls with prolonged testicular infections, transmission of BVDV due to this type of infection remains to be demonstrated.

5. In vivo embryo production and transfer

The first generation of embryo technologies to be developed is so called ‘in vivo embryo production’. The original techniques were developed over half a century ago [41]. The basic procedures which currently are used widely in cattle production include, superovulation of donors, nonsurgical embryo collection, embryo

cryopreservation and nonsurgical transfer of embryos to synchronized recipients [42]. For some time, our working hypothesis has been that the intact zona pellucida of an *in vivo*-derived bovine embryo would protect it from infectious agents and that proper treatment of zona-intact embryos prior to cryopreservation and/or transfer was likely to reduce the risks that naturally occurring pathogens would be transmitted to recipients [43].

5.1. Risk of transmitting BVDV via in vivo-derived embryos

As relates to the relative risk of transmitting BVDV by transfer of an *in vivo*-derived embryo, this virus is currently listed as a ‘category 3’ agent [39] (www.oie.int). “Category 3 diseases or pathogenic agents are those for which preliminary evidence indicates the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer according to the IETS Manual, but for which added *in vitro* and *in vivo* experimental data are required to substantiate the preliminary findings”. The placement of BVDV into ‘category 3’ was based on a 1982 study [44] validating the efficacy of embryo washing procedures for removal of a cytopathic strain of BVDV. Later *in vivo* studies supported conclusions from the original *in vitro* research by producing five BVDV-free calves through transfer of washed or trypsin-treated, *in vivo*-derived embryos from persistently infected cows [45–48]. Despite these multiple reports of embryo transfer to produce BVDV-free calves, a more recent *in vitro* study using noncytopathic isolates of BVDV indicated that embryo washing procedures might not be equally effective for all isolates of BVDV [49]. Additional research in our laboratory indicates that some embryos artificially exposed to specific high affinity isolates of BVDV might retain a small amount of infectious virus even after proper washing or trypsin treatment [50]. Efforts to quantify virus associated with individual, transferable embryos are currently underway [51], but it remains to be determined if the quantity of virus associated with individual embryos might be sufficient to infect a synchronized embryo recipient.

Despite the large number of *in vivo*-derived, bovine embryos that are transferred annually worldwide, only two reports have seemingly linked transmission of BVDV to embryo transfer. In the first report, multiple recipients developed anti-BVDV antibody titers indicative of infection after receipt of embryos that were imported from another country [52]. In the second

report, a calf resulting from transfer of an imported embryo was diagnosed as persistently infected with an exotic strain of BVDV [53]. In both reports, the use of contaminated fetal bovine serum in embryo transfer media was mentioned as a possible source of the virus. Although the source of the virus could not be defined in either case, these reports serve to highlight the need to consider ‘materials of animal origin’ used in embryo collection and transfer as well as donor, embryo and recipient health when trying to produce embryo transfer progeny that are free of BVDV.

5.2. Preventing transmission of BVDV via in vivo-derived embryos

The World Organization for Animal Health (OIE) outlines recommended biosecurity measures for *in vivo*-derived embryos in Appendix 3.3.1 of their Terrestrial Animal Health Code [39] (www.oie.int). Three methods for producing specific-pathogen-free embryos are available [54,55]. Ensuring that donor and recipient animals are free of and unexposed to BVDV or any other pathogen will provide assurance that embryo transfer progeny will be specific-pathogen-free provided ‘materials of animal origin’ such as fetal bovine serum used in media are also pathogen free. Embryo processing (i.e. washing or trypsin treatment) will certainly reduce the level of contamination should embryos be exposed to the virus [43–47]. However, it is unclear if embryo processing will reduce contamination below the threshold of an infective dose for susceptible recipients if embryos have been exposed to high affinity strains of the virus. Based on current knowledge, it is recommended that: (1) embryos be washed or trypsin-treated, (2) materials of animal origin used in embryo handling be free of BVDV, and (3) to the extent possible, both donors and recipients should be protected against exposure to or infection with BVDV.

6. In vitro embryo production and transfer

The second generation of embryo technologies is so called ‘*in vitro* embryo production’. Most of the components of bovine IVF embryo production have been developed and refined over the past 30 years [56]. The basic procedures in current clinical use include transvaginal aspiration and IVM of oocytes, *in vitro* capacitation of spermatozoa, IVF of matured oocytes, and IVC of embryos to the stage of blastocyst [57]. This technology represents a potentially significant change in animal breeding affording opportunities for infectious agents to enter, amplify and be transmitted

to susceptible recipients. However, these procedures which require considerable time and expertise in the laboratory, can easily be subjected to quality assurances that can effectively prevent the entry and transmission of specific pathogens such as BVDV.

6.1. Risk of transmitting BVDV via in vitro-derived embryos

All of the ‘materials of animal origin’ used in IVF embryo production in cattle have been shown to contain BVDV when they originate from infected animals. These materials include ovary, follicular fluid, cumulus cells, oocytes, uterine tubal cells and serum [58]. The hazard is enhanced by the facts that many of these materials originate in the abattoir [14,59,60], and many cattle infected with BVDV are asymptomatic [5]. Cattle with persistent BVDV infections are not only often asymptomatic, but they serve as reservoirs for large quantities of noncytopathic biotypes of the virus [5]. Since most of the field isolates of BVDV are noncytopathic, they can cause unapparent continuous infections in cell lines that could be used for co-culture with embryos [61,62]. Because somatic cells used in IVM, IVF and IVC are susceptible to infections with BVDV, a small amount of viral contamination can be amplified during the course of embryo production resulting in exposure of embryos to large quantities of infectious virus by the end of IVC.

As with in vivo-derived embryos, it was originally hypothesized that the zona pellucida of IVF embryos would protect the developing embryo from infections, and in fact, in vitro exposure studies have shown that an intact zona pellucida is an effective barrier to pathogens which is seldom penetrated [63]. However, the procedures recommended by the IETS for treatment of in vivo-derived embryos [42] are not as effective for IVF embryos since several pathogens including BVDV have been shown to readily adhere to IVF embryos and remain even after washing or trypsin treatment [64].

While BVDV remains readily associated with exposed IVF embryos [65,66], it is not clear whether or not the amount of infectious virus that remains associated with individual embryos after washing or trypsin treatment might constitute an infectious dose for susceptible recipients when delivered via the intra uterine route. However, some preliminary studies have been conducted. In one study in our laboratory [67], zona-intact, Day-7 embryos were artificially exposed to a noncytopathic, genotype 1 strain of BVDV, washed and individual embryos were transferred in to primary cultures of uterine tubal cells which were devoid of

BVDV-neutralizing antibodies. After 2 days of co-culture, the susceptible cells were not infected by the embryo-associated virus. In a similar subsequent study [68], the quantity of the same isolate of BVDV that was associated with groups of 5 or 10 embryos could infect uterine tubal cells when the embryos were sonicated. Yet similar groups of zona-intact, developing embryos did not infect the cells after 3 days in co-culture. We concluded that insufficient quantity of virus and reduced infectivity of virus as well as antiviral influence of the developing blastocysts might have all been contributing factors to failure of the embryo-associated virus to infect the cells. In a third study [69], two genotype 1 and two genotype 2 strains were evaluated for their ability to replicate and associate with embryos during IVF embryo production and for the ability of embryo-associated virus to infect co-cultured cells. All four strains of BVDV were capable of replication in the uterine tubal cells and remained associated with washed embryos. However, embryo-associated virus seldom infected uterine tubal cells in subsequent co-cultures regardless of the strain.

Thus, the collective results of these studies were inconclusive. Whereas multiple strains of BVDV can replicate during IVF embryo production and remain associated with developed embryos, there appeared to be barriers to in vitro transmission of embryo-associated virus to susceptible cells. Resolution of the issue will require the transfer of exposed embryos to susceptible recipients and monitoring the recipients and the developing fetuses for infection with BVDV. Alternatively, the embryo-associated virus could be quantified and the minimum intrauterine infective doses could be determined.

6.2. Preventing transmission of BVDV via in vitro-derived embryos

The World Organization for Animal Health (OIE) outlines recommended biosecurity measures for in vitro-derived embryos in Appendix 3.3.2 of their Terrestrial Animal Health Code [39] (www.oie.int). Establishment of detailed, universal, sanitary precautions for the production of IVF embryos is difficult because of the variability in procedures that are used by different labs, because of the tendency for pathogens to adhere to the zona pellucida and because many of the raw materials originate from abattoirs [70]. Conversely, it is relatively easy to install critical quality assurances. Essential precautions for preventing the introduction and transmission of BVDV should include: (a) screening of ‘materials of animal origin’ (e.g. serum) for the

virus; (b) ensuring that abattoir-origin materials are only collected from abattoirs that slaughter under inspection and are processed in an area of the laboratory which is segregated from other procedures (e.g. IVF, IVF, and IVC); (c) washing of oocytes and washing or trypsin treatment of embryos [42]; (d) adhering to laboratory protocols designed to prevent cross contamination between batches of oocytes and embryos [70]; (e) quality assurance tests (e.g. virus isolation or RT-PCR) on selected materials such as follicular fluid, somatic cells, or media and/or other cells from IVF, IVF and IVC.

An additional safeguard that might be used in the future is the addition to media of antiviral substances to inhibit the replication of any BVDV that might be introduced in relatively small quantities and otherwise amplifies in the system. Antiviral substances are not currently marketed for this purpose. However, research in our lab has demonstrated that novel aromatic cationic compounds can effectively inhibit replication of BVDV at extremely small concentrations which are not cytotoxic [71]. In addition, we have demonstrated that the use of one of these compounds affects neither cleavage or development rates of embryos *in vitro*, nor post transfer pregnancy rates and development *in vivo* [72,73] or reproductive capacity of female progeny through at least one calving cycle [74].

7. Somatic cell nuclear transfer

The third generation of embryo technologies includes somatic cell nuclear transfer (SCNT). This technique for embryo production involves maturation and enucleation of oocytes, insertion of somatic cell nuclei into the oocytes, activation, and IVC to the blastocyst stage [75]. This technology represents a dramatic change in the animal production environment, affording the opportunity to mass produce highly desirable genotypes including some which might be genetically modified to enhance normal or initiate unnatural production characteristics [75].

7.1. Risk of transmitting BVDV via embryos produced through somatic cell nuclear transfer

The same hazards that are associated with *in vitro* embryo production apply to SCNT, with added caveats. The zona pellucida is either broken or removed, and the donor nuclei are harvested from cell lines. Often these cell lines are cultured for many weeks through many cell cycles to amplify their numbers and, in some cases, to alter their genetics. This time in culture could allow

noncytopathic BVDV to be introduced with animal-origin materials (e.g. fetal bovine serum) and amplified. This proposed scenario has actually been documented in two reports. In one report, 5 of 39 fetal cell lines that were being used in cloning research were shown to be infected with a noncytopathic strain of BVDV, and sequence comparison of cDNA from one lot of fetal bovine serum that had been used to supplement culture media verified that it was the source of the BVDV [76]. In another study, it was noted that a particular fetal fibroblast cell line which had been used in cloning research seemed to be associated with a greater than usual amount of embryonic death and fetal resorption [77]. After subsequently determining that the cell line had been inadvertently infected with noncytopathic BVDV, they terminated the existing pregnancies from this cell line and discovered that the fetuses were all persistently infected with BVDV. Obviously, this indicates that a scenario such as this might result in the birth of cloned, persistently infected calves. Although additional reports are not available, the potential for this technology to create animals that can serve as reservoirs for BVDV has been demonstrated.

7.2. Preventing transmission of BVDV via somatic cell nuclear transfer

The World Organization for Animal Health (OIE) outlines recommended biosecurity measures for micro-manipulated embryos in Appendix 3.3.3 of their Terrestrial Animal Health Code [39] (www.oie.int). These recommendations are general because of a lack of available research reports on the subject. However, since much of the technology overlaps and the hazards are very similar, it is reasonable to apply the same precautions that are recommended for *in vitro* embryo production. In addition, since the zona is broken or removed in SCNT, it is desirable to screen oocyte donors for infection with BVDV. Finally, it is imperative that sera used in media and cell lines used as karyoplasts are confirmed to be free of BVDV, and cell lines should be handled and propagated using precautions designed to prevent cross contamination and subjected to regular, rigorous efforts to confirm that BVDV has not been introduced [76].

8. Summary

Development and applications of assisted reproductive techniques constitute environmental changes with the potential to influence the distribution of pathogens among cattle. Although there is a clear potential for the

use of semen and embryos to provide unnatural routes for transmission of BVDV, in reality, the use of appropriate precautionary measures has effectively prevented this from happening. However, the use of these and other emerging reproductive technologies (e.g. spermatogonial transfer) [78] should be subjected to continuous scrutiny. Ultimately, the ethical and technical excellence of those responsible for assessing risks and implementing appropriate control measures will determine whether health or disease will prevail among populations of cattle in which assisted reproduction is used [79].

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