

Relative risks and approaches to biosecurity in the use of embryo technologies in livestock

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Abstract

Embryo technologies have been integrated into production systems for a variety of livestock species. As relates to transmission of infectious diseases, our working hypothesis has been that use of embryo transfer for distribution of germ plasm within and between herds and flocks is likely safer than the movement of postnatal animals. Indeed, research and experience generally have been supportive of this hypothesis. However, the relative risks of transmitting infectious agents via embryo transfer vary among donor species. Further, different methods of producing embryos appear to present different risks. This paper provides a comparative overview of the risks of transmitting infectious diseases via transfer of both in vivo- and in vitro-derived embryos in common domesticated livestock species. Also discussed are universal approaches to biosecurity in embryo production and transfer.

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1. Introduction

Prior to the advent of assisted reproductive techniques, improvement or replenishing of bloodlines was accomplished through the movement of post natal animals [1]. In the middle of the last century, AI became an option [2]. More recently, embryo technologies have evolved, providing a more humane alternative to the transportation of post natal animals for exchange of germ plasm [1,2]. A statistical report published by the International Embryo Transfer Society for 2005 indicated an increase in embryo transfer activity in cattle as well as geographically widespread use of the technology in other species [3]. According to that report, record

numbers of in vivo- ($n = 612, 178$) and in vitro-derived ($n = 265, 991$) bovine embryos were transferred worldwide, with 45% of the in vivo-derived embryos transferred in North America and 48.6 and 47.9% of the in vitro-derived embryos transferred in South America and Asia, respectively. Furthermore, embryos were transferred worldwide in a variety of other species including sheep ($n \sim 25,000$), goats ($n \sim 7000$), cervids ($n \sim 330$), swine ($n \sim 30,000$), and horses ($n \sim 14,000$).

It is imperative that we continue to monitor and maintain an awareness of the epidemiologic potential of current and emerging embryo technologies. Whereas experience and experimental evidence has indicated a low potential for transmission of infectious pathogens via in vivo-derived bovine embryos, some research reports seem to indicate a greater tendency for transmission of pathogens via in vivo-derived embryos of other species [4]. Further, research with in vitro-derived embryos seems to indicate a greater hazard than with in vivo-derived embryos [4]. In this paper, we

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provide a comparative overview of the risks associated with the transfer of embryos in various domesticated species. Also provided is a summary of generic approaches to biosecurity in embryo transfer that are applicable regardless of the species.

2. Estimating biohazards associated with the use of embryo transfer

The term ‘biohazards’ is used within the context of embryo transfer to encompass all factors relating to the potential for introduction and transmission of pathogens [5]. Our initial awareness of the relative safety or biohazards associated with the transfer of embryos was developed through research. Definitive risk assessments and development of appropriate biosecurity measures incorporate the cumulative findings of well designed studies [6–8]. The donor species, type of embryo (in vivo or in vitro-derived), and specific pathogens that are studied generally are dictated by a need to know, which is established by the demand to use these technologies in breeding and germ plasm exchange in the given donor species. It is important to recognize that information gained with a specific pathogen using a specific type of embryo in a specific donor species cannot be extrapolated to other pathogens, types of embryos and donor species [6]. In this section, we will provide an overview of different approaches to the conduct and interpretation of embryo-pathogen research which generally are applicable to all species. Specific examples will be used for illustrative purposes. Additional examples and detail can be found elsewhere [4,5,9,10].

2.1. Strategies used for in vivo-derived embryo-pathogen research

Many pathogens of livestock have been evaluated to determine if in vivo embryo production and transfer might prevent or contribute to their spread [11]. Generally, the working hypothesis has been that proper handling of zona pellucida-intact (ZP-I) embryos is likely to reduce the spread of pathogens that might be associated with donor animals [4–6]. Four recognized strategies have been used in this research [6]. The first and second methods, involve collection of ZP-I embryos from pathogen-free donors, artificially exposing them to high doses of pathogen in vitro (representing a worse-case scenario), washing or trypsin treating them [12], and then assaying them for pathogen using in vitro tests (method 1), or alternately, transferring the embryos to disease-free recipients and monitoring the recipients and their progeny for infection (method 2).

The third and fourth methods involve collection of ZP-I embryos from diseased (acute or convalescent) donors, washing or trypsin treating them [12], and then assaying them for pathogen using in vitro tests (method 3), or alternately, transferring the embryos to disease-free recipients and monitoring the recipients and their progeny for infection (method 4).

Important, additional considerations that are often evaluated in incidental research are the potential for introduction of pathogens via materials of animal origin used in media for embryo collection and handling (e.g. serum) and the health of recipient animals [13–15]. Unfortunately, these potential sources for introduction of pathogens might be easily overlooked as we focus on embryos as the primary concern.

2.2. Conclusions from in vivo-derived embryo-pathogen research

Annually, the Research Subcommittee of the International Embryo Transfer Society (IETS) Health and Safety Advisory Committee reviews the published results of research and field studies relating to the epidemiologic consequences of the use of in vivo-derived embryo transfer in livestock (comprehensive summary available at www.iets.org). All diseases and pathogenic agents which have been studied within the context of in vivo-derived embryo production and transfer have been placed into one of four categories as follows [11]:

“Category 1 diseases or pathogenic agents are those for which sufficient evidence has accrued to show that the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer according to the IETS Manual.”

“Category 2 diseases are those for which substantial evidence has accrued to show that the risk of transmission is negligible provided the embryos are properly handled between collection and transfer according to the IETS Manual, but for which additional transfers are required to verify existing data.”

“Category 3 diseases or pathogenic agents are those for which preliminary evidence indicates that 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 additional in vitro and in vivo experimental data are required to substantiate the preliminary findings.”

Table 1

Diseases or pathogens for which there is sufficient evidence that risk for transmission through transfer of in vivo-derived embryos is negligible if embryos are properly handled between collection and transfer [11,12]

Disease or pathogen	Embryo donor species	Recommended handling
Bluetongue	Cattle	Washing
Bovine spongiform encephalopathy	Cattle	Washing
<i>Brucella abortus</i>	Cattle	Washing
Enzootic bovine leukosis	Cattle	Washing
Foot-and-mouth disease	Cattle	Washing
Infectious bovine rhinotracheitis	Cattle	Trypsin treatment
Aujeszky's disease (pseudorabies)	Swine	Trypsin treatment

“Category 4 diseases or pathogenic agents are those for which studies have been done or are in progress, that indicate: (1) that no conclusions are yet possible with regard to the level of transmission risk; or (2) the risk of transmission via embryo transfer might not be negligible even if the embryos are properly handled according to the IETS Manual between collection and transfer.”

The pathogens currently listed in category 1 are found in Table 1. An up-to-date list of the diseases or pathogenic agents in each of the other categories is found in Appendix 3.3.5 of the Terrestrial Animal Health Code [11] (www.oie.int). It must be emphasized that total reliance on embryo handling (i.e. washing or treatment) can only be recommended, based on the accumulated results of comprehensive supporting data which generally results from a combination of in vitro and in vivo studies.

2.3. *Brucella* and in vivo-derived embryos

Brucella abortus was typical of the original pathogens selected for study in embryo-pathogen research because it was subject to national control and eradication programs in cattle and because the organism was found in the reproductive tract [9,16]. This organism is currently in category 1 for cattle because of the accumulation of in vitro and in vivo research data indicating that transmission via in vivo-derived bovine embryos is highly unlikely [17]. Early research with this pathogen was designed to determine if the organism could persist in the reproductive tract of normally cycling and superovulated cows [17]. In one study, *Brucella* was not found in the uterine flush fluid from 20 normally cycling, naturally infected cows, although it was isolated from their udder secretions [18]. In a second study, 67 ova and flush fluids from 15 superovulated, *Brucella*-infected cows were all culture negative [18]. To further test the hypothesis that

Brucella would not persist in the uterus of infected cattle through the multiple estrous cycles that are necessary for superovulation, flush fluids were collected weekly from five cows that had aborted *Brucella*-positive fetuses [19]. In this study, *Brucella* was never isolated after 41 days post abortion, nor was it ever isolated after the second estrous cycle post abortion.

The probability of *Brucella* persisting in the uterus of a normal cycling cow was further tested by culturing cervical mucus twice weekly from 16 artificially infected heifers that were bacteremic during the period of sampling [20]. *Brucella* was only isolated from one heifer (during the bacteremic phase of her infection). This heifer was in estrus shortly after the positive sample was taken, and all subsequent samples were culture negative, including three collected during her extended bacteremic phase of infection. Finally, the effects of superovulation on *Brucella* infections in the uterus of 11, post-abortion cattle was evaluated [21]. Before post-abortion return to estrus, *Brucella* was isolated from flush fluids from 6 of the 11 cows. However, flush fluids collected after superovulatory treatments were always negative. Thus, the accumulated results indicated that the exposure potential for embryos collected from superovulated, *Brucella*-infected cows would be, at best, very low. In fact, this was supported by subsequent research in which 169 ZP-I, 18 zona pellucida-free and 5 zona pellucida-defective embryos collected in 27 nonsurgical collections from superovulated, *Brucella*-infected cows were culture negative as were all of the flush media from these cows [22].

Also, the interaction of *Brucella* and 415 ZP-I, bovine embryos, was studied through in vitro exposure studies with the result that washing was universally effective for removal of the organisms [23–25]. In addition, the survivability of *Brucella* that were exposed to antibiotics or embryo cryopreservation techniques were tested with the results that simply freezing and thawing caused a 64% reduction in viability of the

organisms and the addition of antibiotics (100 units of penicillin, 100 µg streptomycin, and 0.25 mg amphotericin B per mL) caused a 99.9% reduction in viability [26].

Finally, the demonstration of the low potential for exposure of transferrable embryos that are collected from *Brucella*-infected cows, as well as the cumulative evidence that embryo handling procedures would prevent survival and transmission of *Brucella abortus* with embryos, were validated through two field trials. In these latter studies, a total of 45 embryos were collected from *Brucella*-seropositive cows and transferred to *Brucella*-negative recipients [27,28]. All recipients and offspring remained free of the disease. Thus, based on the accumulated evidence from numerous studies, *Brucella abortus* in cattle qualified for inclusion in category 1.

Brucella can also infect other species (e.g. sheep, goats and swine) in which the epidemiology and pathogenesis can be very similar to those in cattle [16]. Therefore, it is tempting to try to extrapolate what we know in cattle to these other species. However, the danger of extrapolation is demonstrated by the limited embryo-pathogen research that has been done with *Brucella* in sheep. Contrary to what was found in cattle, mechanical washing of ZP-I, in vivo-derived, ovine embryos after in vitro exposure to *Brucella abortus* was not totally effective for removing the organism in the absence of antibiotics [29]. Further, *Brucella ovis* could not be removed by washing with or without antibiotics [30,31], and 4 of 7 recipient ewes seroconverted after transfer of embryos that had been exposed to *Brucella ovis* in vitro [32].

2.4. Transmissible spongiform encephalopathies (TSE) and in vivo-derived embryos

Bovine spongiform encephalopathy (BSE) in cattle is the most recent disease to be placed in category 1. This disease, which was first reported in the United Kingdom, caused an extraordinary epidemic with serious consequences to international trade in cattle and bovine products including embryos [33]. Motivated by the desire to resume embryo exports from BSE affected countries like the United Kingdom and to develop a means to salvage valuable genetics if significant, natural transmission of BSE from cow to cow were ever shown, a comprehensive effort was undertaken to evaluate the efficacy of recommended embryo-handling protocols [12] for preventing the transmission of the agent of BSE [34]. The study truly represented a worse case scenario in that embryos were

collected from 167 cows with clinical signs of BSE, and these cows were inseminated with semen from 13 bulls, seven of which also were displaying clinical signs of BSE. Viable and nonviable embryos collected 7 days after insemination were washed 10 times according to IETS recommendations [12]. Of these, 587 of the viable embryos were individually transferred into 347 recipients (some recipients were used for more than one transfer), and 266 live calves were born of which 54% had both a BSE-positive sire and dam. The design was to monitor all recipients for clinical signs of BSE for 7 years after receipt of embryos and to monitor all offspring for 7 years from birth. The brains of any recipients and offspring that died early as well as those that survived for 7 years were examined for signs of BSE (histopathology, PrP immunohistochemistry, and electron microscopy for scrapie-associated fibrils). All were negative. Additionally, 1020 non-viable embryos were sonicated and assayed using intracerebral mouse inoculation which were monitored for up to 700 days before their brains were examined. They were all negative. Thus, the conclusion was that washed, ZP-I, bovine embryos are unlikely to harbor BSE infectivity even at a stage of disease in the donors in which infectivity was believed to be the highest [37].

Scrapie in sheep is another TSE which also has been studied extensively. However, scrapie in sheep is currently in category 2, indicating that additional transfers are required to confirm that embryo processing [12] will efficiently prevent transmission of the disease via transfer of in vivo-derived, ovine embryos. Analysis of the situation with scrapie is complicated by questionable methodology, design (e.g. embryos were unwashed or insufficiently washed) and conclusions presented in some reports [35,36]. Conversely, several seemingly well designed studies do seem to indicate that in vivo-derived ovine embryos that are washed according to IETS recommendations [12] are unlikely to transmit the scrapie agent to recipients or offspring [37–39]. Regardless, the difficulty in drawing definitive conclusions about categorization of scrapie points to the need to use sound methodology in the design, conduct and assessment of research and field trials.

2.5. Interactions of pathogens with in vivo-derived embryos from different donor species

Much of the original embryo-pathogen research which was conducted in cattle led to the general belief that pathogens would not easily adhere to the zona pellucida, and washing (or washing with the enzyme trypsin) would be an effective method to control spread

Table 2

Isolation of foot-and-mouth disease virus (FMDV) and bluetongue virus (BTV) with zona pellucida-intact, in vivo-derived, embryos after in vitro exposure to the viruses, washing and in vitro assay

Donor species	Virus	No. of embryos	Virus pos./total	Reference
Cattle	FMDV	263	0/263	[40,41]
Swine	FMDV	124 9 Groups of 8	4/124 2/9	[40]
Cattle	BTV	120	0/120	[42]
Sheep	BTV	22	22/22	[43]

of disease [12]. However, when in vivo-derived embryos of other species were evaluated, it became clear that at least some pathogens were more likely to adhere to the zonae of these species after artificial (in vitro) exposures [10]. For example, washing [12] was shown to be effective for removal of both foot and mouth disease virus (FMDV) and bluetongue virus (BTV) after artificial exposure of ZP-I, bovine embryos [40–42]. However, in similar in vitro exposures, FMDV remained associated with a small proportion of porcine embryos, and BTV remained associated with a very high proportion of ovine embryos (Table 2) [40,43]. Of course, these were artificial exposures with all assays conducted in vitro.

Conversely, a trend in research involving cattle, sheep, and goats has been that in vivo-derived, ZP-I embryos from pathogen-exposed donors do not transmit disease to recipients or offspring when properly treated [12], regardless of the nature of the interaction after artificial (in vitro) exposures [4]. For example, as stated above, BTV remained associated with a very high proportion of ovine embryos after artificial (in vitro) exposure [43], yet ZP-I embryos from BTV-infected ewes bred by either uninfected or infected rams did not transmit infection to recipient ewes or their offspring

when the embryos were washed 10 times prior to the transfers [43,44]. In fact, there is experimental evidence that transfer of either porcine or ovine, ZP-I, washed/trypsin treated embryos from donors infected with a variety of pathogens are unlikely to transmit the agent to recipients or offspring (Table 3). The message here is that in vitro exposure studies provide important preliminary evidence about the efficacy of recommended embryo handling procedures [12]; however, the ultimate test of transmission or non-transmission should be determined from transfers from infected animals.

2.6. Assessing risks associated with transfer of in vitro-produced embryos

It was reported that 265,991 bovine, IVF embryos were transferred worldwide in 2005 [3]. However, the number of IVF embryos transferred worldwide in other livestock species is much lower. None was reported in sheep, a few hundred were reported in goats ($n \sim 300$), and somewhat larger but still relatively small numbers were reported in swine ($n \sim 19,000$) [3]. All of the activity in goats and swine was reported in Korea [3]. Clearly, the major activity is in cattle, thus, the focus here will be on risks and solutions in cattle.

Sources of pathogens in IVF embryo production include gametes, fluids associated with gametes, somatic cells used in IVM, IVF and IVC; and any supplements to media that originate from animals [5]. Existing hazards are compounded by the fact that many of these materials often originate from an abattoir [5]. Low to moderate levels of bovine herpesvirus-1 (BHV-1) and bovine viral diarrhoea virus (BVDV) have been repeatedly found when raw materials used in IVF laboratories are tested [53–57]. Since both BHV-1 and BVDV have worldwide distribution among populations of cattle and are found in reproductive tissues, they have been the focus of most of the IVF-embryo-pathogen

Table 3

Infective status of recipients and offspring after transfer of in vivo-derived embryos which were collected from seropositive or infected donors and processed using methods recommended by the International Embryo Transfer Society [12]

Donor species	Pathogen	Transfer of disease to recipient or offspring?	References
Swine	Hog cholera virus	No	[45]
Swine	Porcine parvovirus	No	[46]
Swine	Pseudorabies virus	No	[47,48]
Swine	Swine reproductive and respiratory disease virus	No	[49]
Swine	Swine vesicular disease virus	No	[50]
Sheep	Bluetongue virus	No	[43,44]
Sheep	Scrapie	No	[36–38]
Sheep	<i>Chlamydia psittaci</i>	No	[51]
Sheep	Sheep pulmonary adenomatosis	No	[52]

research [58]. In fact, semen, oocytes, follicular fluid, cumulus cells, uterine tubal cells and serum collected from animals actively infected with either of these viruses are likely to be contaminated [5,58,59]. Furthermore, detection of infected cattle with active infections is complicated by the fact that many infections are asymptomatic [59,60]. Thus, unless they are specifically tested, the infectious status of materials from these animals will not necessarily be known. Finally, it is noteworthy that conditions in IVM, IVF and IVC are all likely to be conducive to replication of these viruses. Thus, a low level of contamination could be amplified such that embryos are exposed to high quantities of virus by the end of IVC [5].

As was the case with in vivo-derived embryos of cattle, our initial working hypothesis was that the intact zona pellucida of IVF embryos would protect them from infectious agents and proper handling of embryos would be likely to reduce the spread of any pathogens that they might encounter [5]. However, several pathogens (i.e. BTV, BHV-1, FMDV, BVDV) associated more readily with the zona pellucida of IVF embryos than with the zona pellucida of in vivo-derived embryos [4,61–64], and the presence of some viruses (i.e. BHV-1, BVDV, infectious epizootic hemorrhagic disease virus) reduced rates of oocyte maturation, fertilization and/or embryo development [65–68].

2.7. Risks associated with exposure of embryos to pathogens after transfer to recipients

Risks of exposure of the embryo or fetus to pathogens after transfer are often overlooked. Obviously, the health of embryo-transfer progeny is heavily influenced by the health of the recipient [69]. Thus, management of recipients should ensure that they are vaccinated against or that precautions are taken to prevent exposure to any disease that can affect the conceptus throughout gestation causing early embryonic death, abortion or birth of infected offspring. Pathogens that might cause termination of pregnancy and methods to protect pregnant cattle (including recipients) have been reviewed elsewhere [70]. However, three specific pathogens of cattle are noteworthy because of their ability to cross the placenta, infect the fetus and potentially result in the birth of infected offspring. These agents are BVDV, *Neospora caninum* and *Mycobacterium avium* ssp. *paratuberculosis* [71–73]. Since infections with each of these pathogens could be asymptomatic in both recipients and offspring, efforts to control them should be proactive rather than reactive.

3. Approaches to biosecurity in the use of embryo transfer

As stated, the relative risks of transmitting infectious agents via embryo transfer vary among donor species, and different methods of producing embryos appear to present different risks as well. In this section, we describe options which can be used regardless of donor species or type of embryo. The specific biosecurity measures that are applied in a given circumstance must take into account our knowledge about the epidemiology and pathogenesis of the given pathogen, the availability of suitable diagnostic tests, regulatory concerns, experience, and results of relevant embryo-pathogen research.

3.1. Biosecurity in transfer of in vivo-derived embryos

Biosecurity recommendations for international movement of in vivo-derived embryos can be found in Appendix 3.3.1 of the Terrestrial Animal Health Code of the World Organization for Animal Health (OIE) [74] (www.oie.int). Certainly, these recommendations are applicable to domestic embryo transfers as well. Three strategies are available to prevent transmission of pathogens from donors to recipients with in vivo-derived embryos. These include donor testing, embryo processing and recipient testing [5,58,75,76].

Processing embryos (i.e. washing or trypsin treatment) according to standards recommended by the International Embryo Transfer Society [12] is the preferred method, because it is easy in the field, inexpensive, and requires relatively little time. Further, it can be a broad spectrum approach to disease control in that the same procedure can protect against transmission of a number of different pathogens. However, total regulatory reliance on this method can only be supported when the validating research has been done for the pathogens of concern and with the specific donor species.

When specific data are not available to validate embryo processing, alternate methods are donor or recipient isolation and testing. Provided specific tests are available for the pathogen of concern, donors can be isolated and tested at or around the time of embryo collection and at a later time after weeks or months have elapsed to encompass normal incubation periods of the disease. Of course, during this elapsed time, embryos would be held in “quarantine” in the cryopreserved state. Similarly, isolation of recipients and testing them after receipt of embryos and possible testing offspring

after they are born is a method which uses the recipients and offspring as sentinel animals [75]. Disadvantages of either donor or recipient testing are expense, length of time required, and the need to conduct multiple tests if multiple pathogens are a concern.

Regardless of the strategy for ensuring that transferred embryos are (were) specific pathogen free, additional assurances may be required to ensure that materials of animal origin used in embryo production, handling and transfer are free of infectious agents [14,77], and to ensure that semen is free of specific pathogens [78]. Further, testing flush fluids and nonfertile or degenerated embryos for presence of specific pathogens might be required under certain circumstances for international movement of embryos [12]. Finally, managing risks should include establishing strict protocols for acquisition, testing and management of recipients [5,13].

3.2. Biosecurity in transfer of *in vitro*-derived embryos

Biosecurity recommendations for production and transfer of *in vitro*-derived embryos can be found in Appendix 3.3.2 of the Terrestrial Animal Health Code of the World Organization for Animal Health (OIE) [74] (www.oie.int). Compared to *in vivo*-derived embryos, strategies for quality assurance in dealing with *in vitro* embryos are not as easily defined because of: (1) the high degree of variability in techniques used by different laboratories, (2) the relative ease with which infectious agent associate with the zona pellucida of IVF embryos, and (3) that many of the materials of animal origin used in IVF embryo production originate from the abattoir [5]. However, production procedures are conducted in sophisticated laboratories with the clear capability to apply appropriate controls [77,79]. Critical controls should include: (1) testing of all raw (e.g. gametes and somatic cells) or processed (e.g. fetal bovine serum) materials of animal origin for contaminating agents, (2) washing of oocytes and washing or trypsin treatment of developed embryos [12], (3) the appropriate use of antimicrobial substances, perhaps including antiviral agents [80,81], (4) tests for contaminating microorganisms of samples of media/cells from IVM, IVF and IVC cultures, and (5) establishment of minimal sanitary standards for not only laboratories but also the abattoirs from which raw materials are collected [74,79]. Whereas isolation of infectious agents from raw materials or cultures in progress would be complicated by the need to identify and submit samples to a reliable diagnostic laboratory,

use of 'in house' polymerase chain reaction assays might be a desirable alternative [82]. Certainly, use of this technology is well within the capability of the sophisticated personnel required for IVF laboratories, and could be used where rapid results are needed. Specific details and caveats associated with each of these critical controls are detailed elsewhere [5].

4. Summary

Embryo transfer has been used worldwide for several decades in multiple livestock species, and the number of embryos transferred annually has increased in recent years to well over 500,000 [3]. In light of these numbers, it is reassuring that reports implicating embryo transfer in disease transmission are extremely rare [5]. This record is due in part to the innate safety of the procedures [75] and in part due to the ethical and technical excellence of those who apply the techniques [1]. It is imperative that we continue to monitor and maintain an awareness of the epidemiologic potential of current and emerging embryo technologies. It is equally important to understand that lessons learned through field experience and through research with one embryo technology in one donor species should not be extrapolated to other embryo technologies or to other species without verification. Up-to-date information on risks associated with the use of embryo technologies and strategies for biosecurity in production and transfer of embryos in livestock is readily available to all either from the World Organization for Animal Health in their Terrestrial Animal Health Code (on line at www.oie.int) or from the International Embryo Transfer Society in their Manual [1] or in their comprehensive database of published information on embryo-pathogen interactions (on line at www.iets.org).

While we remain vigilant, we should continue to encourage the use of embryo technologies because of the economic, animal welfare and disease control advantages which have long been established [75].

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