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Sanitary control in bovine embryo transfer How far should we go? A review

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SUMMARY

Embryo transfer is a globally executed technique which, when properly done, has both economic and sanitary advantages. International guidelines are available to prevent infection of the embryo with pathogens, both originating from the donor animals as from the environment. This manuscript describes the bacteria, viruses, protozoa, fungi and prions that are of major concern in the context of embryo transfer in cattle. In addition, the actual scientific knowledge on these pathogens is evaluated in terms of the current international and national guidelines and legislation.

Keywords: *Bacterial diseases; Belgian legislation; Bovine reproduction; Cattle diseases; Disease transmission; Embryo transfer; EU legislation; Fungal diseases; Infectious diseases; Legislation; Prevention of infection; Prion diseases; Protozoal diseases; Reproductive technology; Sanitary measures; Veterinary public health; Viral diseases.*

Introduction

The first successful embryo transfer in cattle leading to the birth of a live calf has been performed more than fifty years ago in Wisconsin, USA, by Elwyn Willet (3). The description of the technical details of this milestone achievement in reproductive technology shows that many hurdles had to be taken in order to enable embryo and recipient to survive this transfer. First of all, the recipient received the 8-cell embryo through a midline incision under general anaesthesia, a surgical method which yielded considerable risk for the mother but which was practised until in the late seventies non-surgical transfer methods were developed (72). Back in the fifties, the holding medium for the embryo was maternal blood serum, which was heat-treated to prevent any toxic effects on the embryo. Nowadays, commercial sterile flushing and holding media are available, which have been designed to supply the optimal nutrients for the embryo. And last but not least, at that time it was common practice to transfer the embryo to the uterine horn contra-lateral of the corpus luteum. Today, we know that this technique may have significantly reduced the chances of the embryo to survive, since the interferon-tau or trophoblast protein it is producing may not reach a sufficiently high concentration in the systemic circulation to allow maternal recognition of pregnancy, resulting in embryonic demise. Fortunately, the first embryo transfer apparently succeeded to prevent luteolysis to occur and did overcome this hazard, leading to a viable pregnancy and to the birth of a heifer calf called Prima (3).

Detailed practical instructions to enable veterinarians to prevent any disease transmission by means of embryo transfer have been published in the manual of the International Embryo Transfer Society (64). In addition to these worldwide accepted guidelines EU legislation prescribes the sanitary conditions to which embryo collection and transfer should adhere (20,23,24,25,26). This legislation is transcribed in national legislation, also in Belgium (45).

It is the purpose of this manuscript to review the current scientific knowledge on infections taking place during embryo transfer in cattle and evaluate its coherence with the actual international and national guidelines and legislation.

Risk of disease transmission via embryo transfer of a specific pathogen originating from the donor

Since the fifties, the scientific knowledge about bovine embryo transfer and the technical possibilities have extremely improved. Superovulation has considerably increased the number of embryos to a mean of 5-6 transferable embryos per donor, the high embryo survival rates after cryopreservation have made donor-recipient synchronization

virtually redundant and both flushings and transfers are currently entirely being performed under farm conditions. The economic advantages of embryo transfer for cattle breeders are mainly situated in the possibility to increase the offspring of a valuable dam ten to fifty fold, to store valuable embryos frozen for future use and to commercialise the genetic potential of their herd, since exporting embryos is much cheaper and more considerate for animal welfare than exporting live animals. This is reflected by the fact that in 2003, over 500,000 *in vivo*-derived embryos were transferred worldwide, of which 94,617 were transferred in Europe, including 4,640 in Belgium (66). In Belgium, a mean of 6.1 embryos were collected per non-surgical flushing in the year 2004 (Table 1), one percent of a total of 4,615.

In addition to economical benefits, embryo transfer can be considered as a particularly safe and effective means of preventing the spread of many pathogens in the international movement of genetic material provided that proper sanitary collection, handling and transfer techniques are used, and uniform procedures for embryo identification and record keeping are followed to ensure that health certification corresponds to the appropriate embryos (64,67,68).

In what follows, an overview is given of the current scientific knowledge on bacteria, both originating from the donor animal as from the environment, viruses, protozoa, fungi and prions that may interact with the oocyte or the early embryo and therefore mean a risk for its development.

Bacteria

Obviously, in a normal, healthy animal, the embryo develops without the presence of any micro-organism. However, in septicemic animals with bacteria that have a tropism for the genital tract, embryonic infection may occur. Also while manipulating the embryo during transfer, bacteria may come in contact with the zona pellucida or with the solutions used. For instance, *Leptospira* spp. may cross the channels in the zona pellucida, thus reaching the embryo. In addition, *Leptospira*, *Brucella*, *M. paratuberculosis*, *Mycoplasma* and *Streptococcus* spp. may adhere tightly to the zona pellucida in such a manner that intensive washing cannot remove them. Some of these tight associations have only been demonstrated *in vitro* (Table 2), and the probability that this occurs *in vivo* is not known (e.g. *E. coli* O9:K99 and *Leptospira*). Commercial media used for recovering of embryos therefore contain antimicrobials.

Pathogens originating from the donor

To estimate the risk that the bacterial agents listed in Table 3 may present for the Belgian sector, information on the occurrence (exposure), insight in the pathogenesis and incubation times of the infectious agents and the available tests are needed (Table 4). In what follows, a brief description of the available information is given.

- Brucellosis is characterised by contagiousness linked to the presence of large numbers of organisms in reproductive fluids and milk (56). However, it was observed that *Brucella abortus* bacteria were not adsorbed on the zona pellucida after experimental contamination (42). In Belgium, until the end of the eighties, *Brucella abortus* was a main cause of abortion in cattle. However, Belgium is officially free from bovine brucellosis since 25 June 2003 according to the decision 2003/467/EC (22). Therefore, the risk to transfer bovine brucellosis through embryo transfer is negligible in Belgium.

- *Mycobacterium bovis* causes nodular granulomas (tubercles). Although lesions are most commonly found in the lymph nodes of the head and thorax, lungs, intestines, liver, spleen, pleura, and peritoneum, lesions may also occur in the ovaries. Mostly, the course of the disease is chronic. In 2002 to 2005, less than 10 cattle herds were found to be infected by *Mycobacterium bovis* (27). Therefore, the risk to transfer bovine tuberculosis through embryo transfer must be considered as negligible in Belgium.

- *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) causes an intestinal granulomatous infection, initially located in the small intestine and its lymph nodes. At a later phase, lesions also occur in the large bowel and *M. paratuberculosis* may finally be found throughout the body. Cattle may be infected by ingestion of *M. paratuberculosis* from the contaminated environment, and infection can spread vertically to the foetus and through semen. In 2000, the true herd prevalence of *M. paratuberculosis* infection in Belgium was estimated at 6%, but due to the relatively low test specificity, this figure must be considered with caution (9). Cows with subclinical

Johne's disease however do not seem to transmit the disease by embryo transfer when the embryos have been washed as recommended by the International Embryo Transfer Society (IETS) (4,49).

- *Campylobacter fetus* subspecies *venerealis* infection causes infertility, early embryonic mortality, and abortion. Healthy carrier bulls with contaminated prepuces represent the natural reservoir. During natural mating, but also during artificial insemination, the bacterium may pass on to the cow. *Campylobacter fetus* subsp. *fetus* is less pathogenic than *C. fetus* subsp. *venerealis*, but may be isolated from aborted foetuses as well. In Belgium, *Campylobacter fetus* has not been isolated during the last 5 years.

- Various *Leptospira* serovars (e.g. *pomona*, *grippotyphosa*, *hardjo*) may infect cattle and reside in liver and kidneys. These animals excrete *Leptospira* with their urine. Chronic infections with *Leptospira* spirochetes may cause abortion, stillbirth or birth of weak offspring. Species involved are, amongst others, *L. borgpetersenii* serovar *hardjo* (*hardjobovis*) and *Leptospira interrogans* serovar *hardjo* (type *hardjoprajitno*). Both a serological test (agglutination with living *Leptospira* cultures for the detection of specific antibodies) and an antigen detection (immunofluorescence) test are available. However, only old data on the prevalence of *Leptospira* infections in Belgian cattle is accessible. One study from 1989 on dairy herds distributed over the country showed 9.2% of herds seropositive for serovar *hardjo* (16).

- *Hemophilus somnus* (current name: *Histophilus somni*) is less frequently encountered in cattle (mainly young bovines). Generally, respiratory disorders (e.g. coughing, nasal discharge, salivation) are present in cattle before neurological disorders (thrombo-embolic meningo-encephalitis) and not necessarily in the same animals (57). Embryos from *H. somnus*-infected heifers survived in culture media for a significantly shorter time than embryos from control heifers (39). *Hemophilus somnus* may have effect on ovarian activity (40) and may adhere to zona pellucida-intact embryos (70).

- The intracellular parasite *Chlamydophila abortus* (formerly called *Chlamydia psittaci* serovar 1) infects cattle via the oral route, via inhalation or via direct inoculation of the urogenital tract during mating or artificial insemination. Subclinical infection as well as abortion may occur. *C. abortus* has been demonstrated in the bull's semen and has been associated with reduced fertility (15). Several studies report high levels of anti-*Chlamydophila* antibodies in cattle (38), as well as the detection of *C. abortus* in the vaginal tract (14). Culture of *Chlamydophila* is labour-intensive and requires inoculation of embryonated chicken eggs or cell lines.

- *Escherichia coli* O9:K99 are enterotoxigenic germs, causing diarrhoea mainly in young calves, but may be found in older animals also. It is known that K99 positive *E. coli* may firmly adhere to the zona pellucida of embryos (47). A contamination of embryos with K99 positive *E. coli* during prelevation and washing is possible. In Belgium, approximately 150 to 300 *E. coli* strains isolated from diseased cattle are tested annually for virulence sequences by means of PCR. About 5% of the strains are K99 positive (non published results).

- *Mycoplasma* species have been linked to respiratory disease, mastitis and infertility (1,46,55). *Mycoplasma bovigenitalium* has been isolated from the reproductive tract of cows with vulvovaginitis and infertility (2). However, in this survey conducted in Great Britain between 1990 and 2000, only 8 isolates of *M. bovigenitalium* were found on a total of 1413 isolates, whereas *M. bovis*, which is more frequently associated with respiratory disease, accounted for 735 of the cases (52%) (2). In general, *Mycoplasma* is not considered to be a major pathogen. Moreover, their culture is laborious and expensive, which may explain why veterinarians may not easily send samples to the laboratory for isolation. In Belgium, the only data which are available on the prevalence of *Mycoplasma* describe the isolation of six *Mycoplasma* species in healthy cattle (16% positive) and cattle with respiratory disease (65-78% positive) (69), but there are no recent data on the prevalence of *Mycoplasma* and *Ureaplasma* in cattle with infertility problems.

Environmental pathogens

It is well recognized that semen and embryo collection procedures as well as semen and embryo processing and cryopreservation are not sterile techniques. Both semen and embryos can be associated with potentially pathogenic bacterial

agents. It has been shown that microbial contamination may be present in liquid nitrogen, frozen semen and embryos (5). Thirty-two bacterial and one fungal species have been identified in the latter study (among them *Enterobacteriaceae* and *Stenotrophomonas* species), but the source of the bacterial infection was not identified and may probably have resulted from laboratory contamination rather than genuinely being present within the sample (i.e. resulting from the donor). *Stenotrophomonas maltophilia* has been reported to be detrimental for embryo development in vitro (65) but data on *in vivo* effects are lacking.

Samples from flushing and washing media and degenerated embryos and unfertilized oocytes which have been analysed by the Veterinary and Agrochemical Research Centre for bacterial contamination in the period from 2000 to 2004 are represented in Table 5. Because of only a few numbers of reports and the change in Agar media used since 2003, the interpretation of these results is difficult.

The current legislation on quality control in Belgium is presented in Annex 1. Samples in which bacteria were identified may be indicative for the infection status of the animal. Since there is no indication that the corresponding animals showed clinical signs, the results suggest that the farm and handling conditions (circumstances in which the embryos were processed) were not optimal. However, these laboratory results are not conclusive to estimate the risk for disease transmission. Even if the bacteria are originating from the reproductive tract of the cow, their pathogenic significance is probably limited. In literature there are no data available on the correlation between vaginal microflora and infertility in cattle, but similar research in dogs has shown that bacterial culturing of vaginal swab specimens from bitches without signs of genital disease is of little value (8,28).

Viruses

From the data presented in table 3 it is clear that sufficient evidence exists that the risk for disease transmission of the following viruses is negligible provided that the embryos are properly treated: enzootic bovine leucosis, foot-and-mouth disease, bluetongue and infectious bovine rhinotracheitis (IBR) or bovine herpesvirus-1. Bovine viral diarrhoea virus (BVDV) however requires further investigation. BVDV is an economically significant pathogen with a worldwide distribution among cattle populations. It is known to be associated with semen, ovaries and serum of infected cattle. Despite early studies indicating that it was safe to transfer embryos from BVDV infected cattle, some doubt has risen on the safety related to embryo transfer with BVDV infected cattle. It has been shown that BVDV is present in oocytes of persistently infected cattle (29) and in bovine oocytes after acute infection (34). However, despite the fact the BVDV antigen has been detected in developing oocytes, no report so far has shown BVDV associated with zona-pellucida intact embryos that have been washed according to the IETS protocol (62). Moreover, the few calves that have been produced from infected cows have been BVDV free (62). It is not clear whether the oocytes from infected cows indeed are developmentally competent. If this is not the case, the risk for disease transmission should be negligible. Further research should address the oocyte quality and also the efficiency of IETS washing procedure after *in vitro* exposure of embryos to representative field isolates of type I and type II non-cytopathic BVDV (62).

Protozoa and fungi

It is generally accepted that adverse effects on embryos are indirect such as placental lesions or late foetal lesions (e.g. *Tritrichomonas fetus*, *Neospora caninum*, *Aspergillus* sp., *Toxoplasma gondii*) (36,37,52).

Prions

The great majority of cattle in the British BSE epidemic acquired infection by consumption of contaminated feed (73). Despite this, the maternal transmission of BSE was demonstrated in an experimental model using transgenic mice (12). Whether the latter takes place vertically (i.e. via the embryo or across the placenta before parturition), or horizontally after parturition is unknown. The results of several field epidemiological studies in cattle in the UK suggest a rate of maternal risk enhancement of approximately 10% in the offspring of dams within 12 months of the onset of clinical signs of BSE (18,74). This finding was not confirmed according to the Belgian data (e.g. 58). A recent model study that took into account all data on the epidemic curve in the UK has estimated the current risk to be around 2% with a 95%

confidence interval that includes the zero value (J.W. Wilesmith, personal communication). An explanation may be that the maternal transmission only played a role when the exposure to BSE risk was very high (50).

If the time lapse between parturition and the onset of clinical symptoms is longer than 12 months, the rate of maternal transmission is reduced. Whether infectivity is transferred directly before birth or after birth by a variety of mechanisms (e.g. calf infection by contaminated material, environmental contaminated with blood, faeces, infected feed, etc.) is uncertain and should be further investigated (21). There is no scientific data to support the hypothesis that infected calves are unduly sensitive to infection on a genetic basis. It appears that there is no enhanced risk of the development of BSE in the offspring of sires that developed BSE. It is therefore unlikely that semen constitutes a risk factor for BSE transmission (21). Preliminary results suggest an extremely low risk of transmission (95% confidence interval: 0-1.5%) of BSE using embryo transfer. These results are consistent with maternal transmission being mediated later in the pregnant period either during or following birth of the animal. Recent studies published by Wrathall and coworkers (75), indicate that embryos are unlikely to carry BSE infectivity even if they have been collected at the end-stage of the disease, when the risk of maternal transmission is believed to be highest. Transmission of BSE by artificial insemination is unlikely for semen derived from BSE-affected bulls early in their incubation period. For these reasons, transmission via embryos is unlikely provided IETS protocols are followed (21).

Risk of disease transmission via embryo transfer of a specific pathogen originating from the materials of animal origin

It is widely recognized that the use of material of animal origin carries a risk to introduce infectious agents into the recipient of the contaminated material (11). It is essential to find alternatives to the use of materials of animal origin, but until satisfactory replacements have been developed it is necessary that the used materials are safe and free of infectious agents. An overview of how to validate the efficiency of virus clearance in biotechnological products such as serum and hormones has been published more than ten years ago. The authors emphasized that evidence of viral clearance must be obtained in all stages of purification and adequate virus removal and/or inactivation must be proven (71).

Bovine foetal serum

Commercial batches of bovine foetal serum are often contaminated with various bacteria and viruses, such as BVDV, BoHV-1 and bovine para-influenzavirus-3 (PI-3). Probably, the assumption has to be made that all commercial lots of foetal bovine serum are potentially contaminated with at least BVDV (11). Even a certificate stating that a batch is free from BVDV is not reliable, since the performed analysis does not allow identifying small amounts of virus by direct infection of cells. Serum can be made virus free by chemical inactivation by means of binary ethylenimine and by gamma irradiation (30 kiloGray) (19). Such methods have been shown to be effective in terms of virus inactivation, but may have some effects on hormone activities (41) or on other proteins present in the serum. Virus inactivation with beta-propiolactone may not be equally efficient in all blood products and must hence be tested independently in individual conditions (60). Serial ultrafiltration through 40 nm porosity filters has been advocated to reduce viral contamination to a minimum level but it may be necessary to ask for additional treatment to make the sample safe. Recently, EU regulators have formalised their position on issuing guidelines on the use of bovine serum during manufacture (53).

Bovine serum albumin

The risk of infectious agents in BSA is less than in whole foetal bovine serum. Nevertheless, these products should be equally tested and declared free of pathogens. Alternatives which may replace BSA during bovine embryo culture and freezing, can either be macromolecules of non-animal origin such as vegetal peptones (30) or chemicals with surfactant properties (48).

Hormones

Embryo transfer requires different natural and synthetic hormones for the synchronisation or induction of oestrus and for superovulation of the donor females. The prostaglandins are the most frequently used hormones to synchronise females presenting a cyclic activity. Numerous molecules are presently synthesized by the pharmaceutical

industry. The synthetic progestagens as implants and the progesterone included in the silastic, the inert polymer of the vaginal spiral, can also be used for the synchronisation or induction of oestrus. One injection of oestrogen ester is frequently combined with these treatments in order to stimulate the ovary. The treatments to induce a superovulation are aimed to increase the number of follicles on the ovaries at around superovulation. At first, Pregnant Mare Serum Gonadotrophin (PMSG) (current name - equine chorionic gonadotrophin, eCG) extracted from mare serum was used. The most important disadvantage of this gonadotrophin lies in its long half life and in its bifunctional activity, leading to unreliable superovulatory responses. More recently, pituitary extracts, with predominantly FSH-activity, have been used for superovulation with a significant increase of the results in term of the number and the quality of the embryos. Presently, all these substances are commercially available with official registrations at the national commissions of drugs. These registrations permit to reduce the risk of contamination by a biological agent. Moreover, the inocuity of batches of those preparations in terms of prion transmission can be verified with an appropriate testing procedure (13).

Enzymes

Trypsin should be tested and declared free of *Mycoplasma* and viruses. We have found no data on the possible contamination of trypsin with pathogens. As for serum and BSA, commercial alternatives for animal-derived trypsin are being tested for use in bovine embryo manipulation, such as recombinant trypsin-like proteases including TrypLE (43) and Trypzean (59). Although some results were promising, more data are needed before any conclusion can be drawn about the safety and efficacy of these products.

Antiviral compounds

Pretreatment of embryos with antiviral compounds has been investigated as a possible approach to prevent virus transmission via embryo transfer. Adding an antiviral agent to culture medium could provide an integral safeguard by preventing the replication of cytopathic and noncytopathic, type one and two BVDV that could have been introduced accidentally (33). Several antiviral compounds have been shown to be effective against BVDV and non-toxic for bovine embryos in vitro, such as DB606 (31) and BPIP (44). Transfer of DB606-treated embryos led to the birth of normal calves (33) and the female calves developed to heifers with a normal reproductive capacity (32).

Media and antibiotics

The exact composition of most commercial media such as ViGro Holding Plus is not made available to the general user. The manufacturer only states that it is a HEPES-buffered complex salt solution, with a protein content provided by 0.4% certified and export cleared BSA. The solution also contains growth factors, amino acids, co-factors, vitamins and antibiotics (penicillin, streptomycin, kanamycin and gentamicin).

Current tests

Current testing, as imposed by the Belgian legislation, is focusing on the flushing and washing fluids and media. Obviously, it is beneficial for sanitary, economic and practical reasons to consider the sanitary conditions of the donor, or even the population to which the donor animal belongs, in order to evaluate the risk of transmission of infectious agents.

As an example, for the viral infections BoHV-1 and BVDV, it is helpful to direct the efforts on the individual status of the donor cow and on the status of its herd. Indeed, the virological tests on embryos are very time consuming and should be done systematically in order to be efficient. Furthermore, their sensitivity is probably quite low. Therefore, it is recommended to select only BoHV-1 seronegative donor cows or cows that have tested negative against glycoprotein gE. Alternatively, the donor cow should originate from a BoHV-1 free herd or a herd controlled for BoHV-1 infection and which has an approved IBR control programme. Concerning BVDV, the donor should have been tested individually for the absence of persistent infection, by a validated RT-PCR or antigenic test.

As for the diseases or infectious agents listed in Table 3, the currently used analytical methods are mentioned in Table 4.

Legal and recommended sanitary measures

In 1920, the transit of zebus through Belgium unintentionally stood at the origin of a new international sanitary legislation. The animals originated from India and were shipped for Brazil

via Antwerp's harbour. These animals caused multiple outbreaks in Belgium of rinderpest by infecting cattle that were housed in the same quarantine stables and that were later sold at the cattle market places of Ghent and Brussels. This economical and sanitary catastrophe had an important consequence at international level. Twenty-eight countries or territories agreed in 1924 to create the "*Office International des Épizooties*" in Paris, which recently has changed its name to World Organisation for Animal Health (WOAH) and which is until our days managing the world animal health information system. To prevent the occurrence of future epidemics, Member Countries were asked to notify to the WOAH the main animal diseases. The list of notifiable diseases, which includes zoonoses, can be consulted at its website (78).

This historical example clearly illustrates the importance of certification of the sanitary status, as organised under the auspices of the WOAH.

International recommendations

The official sanitary control of in vivo derived embryos destined for international transport is covered by the Terrestrial Animal Health Code of the World Organisation of Animal Health (80). The aim of this control is to make sure that a number of organisms, which could be pathogenic for embryos, are controlled and that transmission of infection to recipient animals and their offspring is avoided. The recommendations described in the WOAH code are based on the International Embryo Transfer Society (IETS) (76) handbook published in 1998. According to the available knowledge, the Research Subcommittee of the IETS has categorized a number of microbial agents in order to estimate the risk for their transmission via in vivo collected and properly treated embryos. The categories with agents infectious for cattle are listed in Table 3. Infections of which most evidence is available that their risk of transmission may be neglected under the condition that embryos are handled following the instructions as described in the IETS handbook, belong to category 1. The list should be used as guidance when evaluating the risk for disease transmission via embryo transfer.

The transfer of in vivo obtained embryos is a method for transplantation of animal genetic material that, if correctly done, is only associated with a limited risk for transmission of microbial agents. Concerning risk assessment, and independently of the animal species, the WOAH sanitary code, article 3.3.1.5, (81) identifies three levels that determine the risk associated with the technique of embryo transfer:

- The first concerns infections not listed in the IETS category 1 and deals with the probability an embryo gets infected. This probability depends on [i]the zoosanitary situation of the country or region, [ii]the sanitary situation of the herd and of the donor animals from which the embryos are taken, and [iii]the virulence of the microbial agent.

- The second level concerns the application of the sanitary measures as described in the IETS handbook. These procedures concern the washing, treatment and analysis of embryos (separate rinsing of embryos that originate from different donor animals, sufficient number of washings, use of single-use material, eventual use of trypsin in order to inactivate certain viruses, examination of the zona pellucida of each embryo).

- The third level also concerns infections not listed in the IETS category 1 and concerns the following measures: [i]surveillance of the donor animals and herds from which they originate as for the outbreak of infectious diseases, taking into account their incubation periods, and [ii]examination of embryo-collection (flushing) fluids and non-viable embryos, or other samples such as blood, for presence of specified disease agents.

As for embryo transfer, Stringfellow (61) argued that certification on the health of transferred embryos relies on specific tests conducted on the donor animal, appropriate washing or trypsin treatment of the embryos (Table 6), and testing of the samples associated with the embryo collection procedure.

Justification for testing samples of collection fluid and washes lies in the fact that it gives some indication of the pathogens to which embryos have been exposed in the uterus and to the efficacy of the washing procedure (61). It may be worthwhile to test embryo samples when the donor is seropositive for a particular disease, but it would be unnecessary to test these samples if donor males and females were shown to be free of disease (61).

It may be worthwhile to focus on serological or other testing of the donor animals instead of collecting fluids from all flushes for future analysis, because this approach may lead to more consistent results than sample testing for various reasons.

The description of the general guidelines on the collection and processing of embryos is available (79). Working with disease and pathogen free donor animals and pathogen free media and with sterile plastic or glass recipients is certainly the best approach to prevent inadvertent disease transmission via assisted reproductive technology.

Belgian legislation

The continuous introduction of new technologies requires new legislation: the development of embryo transfer techniques during the eighties prompted the Belgian government in 1992 to enact a regulation concerning the sanitary control for bovine embryo collection and transfer teams (Royal Decree of January 23, 1992 and its modifications (77). This legislation lists measures that are necessary in view of Good Veterinary Practice such as conditions for approval of an embryo collection and transfer team and official measures concerning quality control during embryo collection and transfer (Annex 1), conditions relating to the collection, processing, storage and transport of embryos by the approved embryo collection and transfer teams, conditions applying to donor animals, identification of consignment, identification and registration of embryo collection and transfer teams, conditions of animal health certification.

Meticulous and strict implementation of the above regulation, although susceptible to improvement, is time-consuming and costly but aims at the early detection of viral or bacterial contaminants, and therefore at an embryo transfer where the risk for infection is maximally reduced.

The Royal Decree specifies that donor animals have to be derived from herds that are officially free from brucellosis, tuberculosis and enzootic bovine leucosis, when the embryos are subject to intra-community trade. No specifications of other important bovine pathogens such as BoHV-1 and BVDV are mentioned. However, these viruses may cause persistent infections in cattle and therefore may escape the attention of the herd owner or the veterinarian.

Taking into account these WOAH recommendations, it is reasonable to consider the following levels of qualification:

- Qualification of the country or region (considering the existing surveillance programmes).
- Qualification of the herds origin where donor animals originate from (considering the existing surveillance programmes).
- Qualification of the donor animals based on the absence of clinical symptoms and / or favourable analytical results.
- Qualification of the embryos on the basis of laboratory analysis of flushing and washing fluids, and of non-viable and degenerated embryos. This examination also serves as quality control for the manipulations as described in the IETS handbook: a bacterial contamination of the rinsing liquids, but absence of contamination of the embryos themselves, is indicative for non-septic work.

The Scientific Committee of the Federal Agency for the Safety of the Food Chain advised to adapt the existing Belgian legislation according to these considerations.

General conclusions

The *in vivo* recovery of embryos from cattle may represent a risk in terms of infection with pathogenic bacteria, viruses, protozoa, fungi or prions. Obviously, these pathogens may originate from the donor animal, the fluids and media used for recovery of the embryos, and from the environment.

Due to well established, globally accepted sanitary measures during handling, the risk of pathogen transmission may be significantly reduced. However, in order to improve the confidence and quality of the manipulations and to further reduce the possible contamination with pathogens, the donor should be selected from countries, regions or herds that have been shown to be free of specific pathogens. If this information is not available, the donor should be tested individually. Officially accepted tests that appear in the manual of diagnostic tests and vaccines for terrestrial animals (82) maximally guarantee the absence of specific pathogens. Also fluids, media and other material should be certified free of pathogens. The use of antibiotics is also a generally accepted procedure in this context. Finally, specific effort should go to the aseptic environment and clean handling during *in vivo* recovery.

It should be noted that professional workers (farmers, veterinarians and officials of the veterinary services) should adhere to a professional scientific awareness about the veterinary public health aspects of the bovine embryo transfer.

Table 1. Estimation of the number of embryos per collection in Belgium in 2004 (Source: Federal Agency for the Safety of the Food Chain.

Number of collections	Number of donors	Number of embryos	Number of embryos per collection
1	370	2,087	5.70
2	105	1,326	6.40
3	34	641	6.30
4	9	307	8.60
5	6	200	6.70
9	1	54	6.00
Total	525	4,615	6.10

Table 2. Zona-intact bovine embryos exposed to bacteria, washed and then assayed. Adapted from Stringfellow and Givens (62).

Pathogen	Embryos exposed	Embryos positive for pathogen*, expressed in %	Ref.
Brucella abortus	96	0 (0-3)	42, 63
Haemophilus bovis	38	10 (13-43)	70
Mycoplasma bovis	111	111 (97-100)	6, 51
Mycoplasma bovigenitalium	49	49 (94-100)	51
Mycobacterium paratuberculosis	20	6 (12-54)	54
Ureaplasma diversum	26	26 (89-100)	10

**95% confidence interval (binomial exact).*

Table 3. Diseases or infectious agents in cattle listed according to the risk for their transmission via in vivo derived embryos. Update International Embryo Transfer Society (IETS), 1998.

Disease category*	Disease or infectious agent
Category 1: Diseases or disease agents for which sufficient evidence has accrued to show that the risk of disease transmission is negligible provided that the embryos are properly handled between collection and transfer.	Enzootic bovine leucosis Foot-and-mouth disease virus (cattle) Bluetongue virus (BTV) *Brucella abortus* Bovine herpesvirus 1 (BoHV-1) (trypsin required) Bovine spongiform encephalopathy (BSE) agent
Category 2: Diseases or disease agents for which substantial evidence has accrued to show that the risk of disease transmission is negligible provided that the embryos are properly handled between collection and transfer, but for which additional transfers are required to verify existing data.	None
Category 3: Diseases or disease agents for which evidence indicates that the risk of disease transmission is negligible provided that the embryos are properly handled between collection and transfer, but for which additional *in* vivo and in vitro experimental data are required to substantiate the preliminary findings.	Bovine immunodeficiency virus Rinderpest virus (RV) Bovine viral diarrhea virus (BVDV) *Histophilus somnus*, formerly *Haemophilus somnus* *Mycobacterium paratuberculosis* *Neospora caninum*
Category 4: Diseases or disease agents on which preliminary work has been conducted or is in progress, that indicate: 1. that no conclusions are yet possible to the level of transmission risk; or 2. the risk of transmission via embryo transfer may not be negligible even if the embryos are properly handled according to the IETS-manual between collection and transfer.	Akabane virus (AV) Bovine anaplasmosis Bovine enterovirus (BEV) Bovine herpesvirus-4 (BHV-4) *Chlamydia psittaci* *Escherichia coli* O9:K99 *Leptospira borgpetersenii* serovar *hardjobovis* *Mycobacterium bovis* Parainfluenza-3 virus *Trichomonas foetus* *Ureaplasma-Mycoplasma* species Vesicular stomatitis virus (VSV)

**The classification of infectious diseases is based on the availability of more (category 1) or less (category 4) scientific evidence, that the transmission of disease represents a risk for the embryo.*

Table 4. Specific pathogens of bovine embryos

IETS Classification [a]	Pathogen (according to IETS list)	Occurrence in Belgium (b)	Incubation duration	Available diagnostic test(s) in function of the considered unit			
				Herd	Animals	Flushing media	Degenerated and unfertilized oocytes
4	*Rickettsieae* (agents of Anaplasmosis)	-	14-15 days (Donatien and Lestoquard, 1936)	serology	blood smears and staining, PCR	isolation	isolation
1	*Brucella abortus*	officially free	some weeks to some months	serology, milk ring test	isolation, skin test, serology, g-IFN	isolation	isolation
[3] [c]	*Campylobacter fetus* [subspecies *venerealis*]	+	some months (also subclinical cases)	-	vaginal mucus or preputial mucus for cultivation or serology	isolation, IFT	isolation
4	*Chlamydia psittaci* [current name: *Chlamydophila abortus (immunotype 1)*]	?	some months (also subclinical cases); less known for cattle than for sheep and goats	serology	serology, detection of antigen	antigen detection	antigen detection
1	Bovine spongiform encephalopathy	+	mean of 4-5 years	-	post-mortem rapid test	-	-
4	Enterovirus	?	?	?	RT-PCR	?	?
4	*Escherichia coli* O9:K99	+++	? (Otoi et al., 1993)	isolation on faeces	isolation on faeces	isolation	isolation
1	Foot-and-mouth disease virus	-	2 - 8 days	clinical examination, serology	serology, isolation	?	?
1	Bluetongue virus	-	5-20 days	clinical examination			
3	*Haemophilus somnus* [current name: *Histophilus somni*]	+++	1-4 weeks	clinical examination	?	isolation	isolation
4	Bovine herpesvirus-4	+	?	serology	serology, isolation	?	?
4	*Leptospira borgpetersenii* serovar *hardjobovis*	+	4-12 weeks (Bielanski and Surujballi, 1998)	serology	serology (micro-agglutination test, ELISA)	isolation, IFT	isolation, IFT
4	*Leptospira* spp.	+++		serology	serology		
1	Bovine leukemia virus	officially free	4-10 years	serolgy and milk test (ELISA)	immunodiffusion test, ELISA		
4	*Mycobacterium avium* subspecies *paratuberculosis*	++++	mean of 2-4 years	serology, skin test, isolation, PCR on tank milk	serology, skin test, isolation, PCR	isolation, PCR	isolation, PCR
4	*Mycobacterium bovis*	officially free	some months	skin test	skin test, g IFN	isolation	isolation
4	*Ureaplasma / Mycoplasma*	+++	subclinical cases (Hartman et al., 1964)	serology (ELISA), isolation	isolation	isolation	isolation
3	*Neospora caninum*	++++	3-15 days	serology (ELISA, IFAT)	serology, isolation	isolation	PCR
3	Rinderpest virus	-	3-5 days	ELISA	ELISA, SN, PCR	?	?
1	Bovine herpesvirus-1	++++	2-4 days	clinical examination, ELISA	clinical examination, ELISA, isolation	isolation	isolation
4	Vesicular stomatitis virus	-	1-21 days	clinical examination	isolation, ELISA, SN		
4	Akabane virus	-	?	-	-		
3	Virus de l'immunodéficience bovine	-	3-5 years	serology	serology		
3	Bovine viral diarrhea virus	++++	5-7 days	clinical examination, serology, isolation	SN (coupled sera), isolation, PCR	isolation	isolation
4	Para-influenza-3 virus	++++	4-10 days	serology	serology, isolation	isolation	isolation

(a) International Embryo Transfer Society classification (disease categories 1 to 4 are explained in the first column of Table 3).

(b) ?: unknown; -: never encountered; +: rare; ++: occasionnaly encountered; +++: less frequently encountered; ++++: frequently encountered.

(c) The IETS list mentions "Campylobacter fetus (sheep)".

ELISA: enzyme-linked immunosorbent assay.
g INF: gamma interferon test.
IF: immunofluorescence test.
IFAT: indirect immunofluorescence antibody test.
PCR: polymerase chain reaction.
RT-PCR: real time polymerase chain reaction.
SN: seroneutralisation test.

Table 5. Bacteriological results of the examination of the flushing and washing media and degenerated embryos and unfertilized oocytes which have been analysed in Belgium (years 2000-2006).

Year	Number of reports	Number of analysed samples	Reports with favourable results[1] Number	Percentage (95% CI[2])
2000	5	116	4	80 (28-99)
2001	17	247	14	82 (57-96)
2002	19	155	13	68 (43-87)
2003[3]	24	259	3	12.5 (3-32)
2004	19	116	6	32 (13-57)
2005	16	105	7	44 (20-70)
2006	13	64	8	62 (32-86)
Total	113	1062	55	48 (37-59)

[1] *A favourableresult is considered when the bacteriological examination gives a result equal or less than 5 colony forming units. If the quality control for bacteriological official procedure (cf. Annex I) is not respected, a new badge of samples must be sent by the considered embryo transfer team to the authorized laboratory for re-analysis.*

[2] *95% confidence interval (binomial exact).*

[3] *Year where the agar used to screen the samples was optimalized (using Columbia agar with 10% sheep's blood instead of simple Columbia agar).*

Table 6. Essential requirement for proper washing and trypsin treatment of embryos. Adapted from the International Embryo Transfer Society, 1998

Requirements for proper washing of embryos	Requirements for trypsin-treatment
Only embryos from a single donor washed together	Transfer embryos through five washes in PBS with 0.4% BSA and antibiotics
Ten or fewer embryos washed at a time	Expose to sterile trypsin [1:250] at a concentration of 0.25% for 60 to 90 seconds
Only zona-intact embryos washed	Transfer embryos through five additional washes in PBS with 2% serum and antibiotics
Only embryos free of adherent material washed	
Minimum of ten washes	
Use a new sterile micropipette each time embryos are moved from one wash to the next	
Regulate volumes so that each wash is at least 100-fold dilution of previous wash	

Annex 1. Quality control which is required for the official recognition of the embryo transfer team (Ministry of Small Enterprises, Trade and Agriculture, 1992)

Chapter IV : Quality control

Article 6:
Samples of flushing media [A], of washing media [B] and of degenerated and unfertilized oocytes [C] which have been obtained while performing embryo transfer activities will be transferred on a yearly basis by the team to a certified diagnostic laboratory for investigation according to stipulations in addendum III. Costs will be charged to the team.

Addendum III:
1. Sampling
- Flushing fluids : must be kept in a sterile recipient
- Washing fluids : media of three last washes of embryos from a single donor must be mixed and preserved
- Degenerate embryos and unfertilized oocytes of a single donor animal must be put together, washed ten times and preserved

2. Preservation of samples
All aforementioned samples must be kept in liquid nitrogen at a temperature of -196°C

3. Organisation of quality control
- Each year
- By an authorized laboratory
- Random sampling among all embryo harvests

4. Interpretation of the results
- Quantitative bacteriological analysis for [A] and [B]:
  - Less than 5 CFU (colony forming units) : 0
  - Between 5-100 CFU : +
  - Between 100-1000 CFU : ++
  - Between 1000-10 000 CFU : +++
  - More than 10 000 CFU : ++++
- Qualitative bacteriological analysis for [A] and [B]
- Qualitative analysis for *Mycoplasma* for [A] and [B]
- Virological analysis for BoHV1 and BVDV for [B] and [C]
- Results of the analysis *

  Favourable result :
  - mixture of three washes [B] has to be sterile
  - no virus may be present in washing fluid [B] and embryos [C]
  - some contamination in the flushing fluid [A] is acceptable but not systematically

  All other results are unfavourable and will require a second analysis, which will lead in case of confirmation of the unfavourable results, to suspension of the licence of the collection and transfer team

**On the basis of above mentioned bacteriological and virological criteria.*

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