Table 4. Specific OIE requirements for international distribution of semen with a negligible risk of infecting other animals or humans with pathogens transmissible by semen (OIE, 2012).

| Diseases | Semen |
|---|---|
| Bovine Brucellosis (BB)* | • semen from an AI center: testing program with the buffered Brucella antigen (rose Bengal test; RBT) and complement fixation tests (CFT);<br>• semen not issued from an AI center: country or zone free from BB; or herd officially free from BB, no clinical sign of BB on the day of collection of the semen and animals subjected to a RBT with negative results over the 30 days prior to collection; or herd free from BB, no clinical sign of BB on the day of collection and animals subjected to RBT and CFT with negative results during the 30 days prior to collection. |
| Bovine Genital Campylobacteriosis (BGC) | • donor animals have never been used for natural service; or have only mated virgin heifers; or kept in an establishment or AI center where no case of BGC has been reported; culture of semen and preputial specimens for the presence of the causal agent of BGC proved negative. |
| Bovine Tuberculosis (BT)* | • donor animals without any sign of BT on the day of collection of the semen and either: kept in an AI center free from BT in a country, zone or compartment free from BT and which only accepts animals from free herds in a free country, zone or compartment; or negative results to tuberculin tests carried out annually and kept in a herd free from BT. |
| Bovine Tuberculosis (BT) in farmed cervidae* | • no sign of BT in any species on the day of collection of the semen; and either: herd free from BT in a country, zone or compartment free from BT of farmed cervidae, and which only accepts animals from free herds in a free country, zone or compartment; or negative results to tuberculin tests carried out annually and were kept in a herd free from BT. |
| Blue Tongue Virus (BTV) | • animals kept in a BTV free country or zone for at least 60 days before commencement of, and during, collection of the semen; or subjected to a serological test between 21 and 60 days after the last collection, with negative results; or subjected to an agent identification test on blood samples collected at commencement and conclusion of, and at least every 7 days (virus isolation test) or at least every 28 days (PCR test) during semen collection, with negative results. |
| Bovine Viral Diarrhea (BVD) | • animals subjected to a virus isolation test or a test for virus antigen, with negative results. Only when all the animals in pre-entry isolation have had negative results, may the animals enter the semen collection facilities;<br>• animals subjected to a serological test to determine the presence or absence of BVD antibodies. Only if no seroconversion occurs in the animals which tested seronegative before entry into the pre-entry isolation facility, may any animal (seronegative or seropositive) be allowed entry into the semen collection facilities;<br>• if seroconversion occurs, all the animals that remain seronegative should be kept in pre-entry isolation until there is no more seroconversion in the group for a period of 3 weeks. Serologically positive animals may be allowed entry into the semen collection facilities;<br>• animals negative to previous serological tests should be retested to confirm absence of antibodies. Should an animal become serologically positive, every ejaculate of that animal collected since the last negative test should be either discarded or tested for virus with negative results. |
| Contagious Bovine Pleuropneumonia (CBPP)* | • from CBPP free countries, zones or compartments**: donor animals without clinical sign of CBPP on the day of collection of the semen; kept in a CBPP free country since birth or for at least the past 6 months;.<br>• from CBPP infected countries or zones: no clinical sign of CBPP on the day of collection of the semen; animals subjected to the CFT for CBPP with negative results, on two occasions (interval between each test from 21 to 30 days, the second test within 14 days prior to collection); isolated from other domestic bovidae from the day of the first CFT until collection; kept since birth, or for the past six months, in an establishment where no case of CBPP was reported during that period, and that the establishment was not situated in a CBPP infected zone; AND EITHER: not been vaccinated against CBPP; OR vaccinated using a vaccine complying with the standards described in the TM not more than 4 months prior to collection. |

| | |
|---|---|
| Enzootic Bovine Leucosis (EBL)* | • donor bull resident at the time of semen collection in an EBL free herd; and if less than 2 years of age, the bull came from a serologically negative 'uterine' dam; or bull subjected to diagnostic tests for EBL on blood samples on two occasions with negative results (first test at least 30 days before and the second test at least 90 days after semen collection). |
| Foot and Mouth Disease (FMD) | FMD free countries<br>• with no clinical signs of FMD on the day of collection of semen and for the following 30 days;<br>• animals kept for at least 3 months prior to collection in an FMD free country or zone without vaccination or a FMD free compartment;<br>FMD infected countries<br>• no clinical sign of FMD on the day of collection of the semen;<br>• animals kept in an establishment where no animal had been added in the 30 days before collection, and that FMD has not occurred within 10 kilometers for the 30 days before and after collection;<br>• have not been vaccinated and were subjected, not less than 21 days after collection of the semen, to tests for antibodies against FMD virus, with negative results; or had been vaccinated at least twice (last vaccination not more than 12 and not less than one month prior to collection);<br>• no other animal present in the artificial insemination center has been vaccinated within the month prior to collection;<br>• the semen subjected, with negative results, to a test for FMDV infection (if donor animal vaccinated within the 12 months prior to collection); stored in the country of origin for a period of at least one month following collection, and that during this period no animal on the establishment showed any sign of FMD. |
| Infectious Bovine Rhinotracheitis/ Infectious Pustular Vulvovaginitis * (IBR-IPV) | • Fresh semen: IBR/IPV free herd at the time of collection of the semen;<br>• Frozen semen: IBR/IPV free herd at the time of collection of the semen; or donor animals in isolation during the period of collection and for the 30 days following collection and subjected to a diagnostic test for IBR/IPV on a blood sample taken at least 21 days after collection of the semen, with negative results; or if unknown serological status of the bull or positive serology, an aliquot of each semen collection subjected to a virus isolation test or PCR, performed in accordance with the TM, with negative results. |
| Lumpy Skin Disease* (LSD; caused by group III virus, type Neethling) | • from LSD free countries (cattle and water buffaloes): no clinical sign of LSD on the day of collection of the semen; kept for at least 28 days prior to collection in an LSD free country;<br>• from countries considered infected with LSD: no clinical sign of LSD on the day of semen collection (SC) and for the following 28 days; kept in the exporting country for the 28 days prior to SC, in an establishment or AI center where no official case of LSD during that period, and establishment or AI center was not situated in an LSD infected zone; and either: vaccinated against LSD (28 to 90 days before SC and thereafter vaccinated annually); or tested with negative results using a serum neutralization test (SNT) or an indirect enzyme-linked immunosorbent assay (ELISA) for LSD on the day of first or up to 90 days after last SC; or stable seropositivity (not more than a two-fold rise in titre) on paired samples (tested side by side) to indirect ELISA or SNT carried out in quarantine, 28-60 days apart (first sample taken on the day of first SC). |
| Trichomonosis* | • donor animals never been used for natural service; or have only mated virgin heifers; or kept in an establishment or AI center where no case of trichomonosis has been reported; animals subjected to a direct microscopic and culture of preputial specimens with negative results. |

*For each listed disease, Veterinary Authorities of importing countries should require the presentation of an international veterinary certificate attesting that the semen was collected, processed, and stored in conformity with the provisions of Chapters 4.5 and 4.6. **Compartment means an animal subpopulation contained in one or more establishments under a common biosecurity management system with a distinct health status with respect to a specific disease or specific diseases for which required surveillance, control, and biosecurity measures have been applied for the purpose of international trade.

Table 5. Assessment of the sanitary risk for *in vivo* derived embryos using scientific approaches.

| | *In vivo* infection | *In vitro* infection |
|---|---|---|
| *In vivo* embryo transfer | • naturally infected donor or insemination with infected semen;<br>• Embryos transferred to recipients. | • experimentally spiked embryos/semen;<br>• embryos transferred to recipients. |
| *In vitro* embryo washing | • naturally infected donor or insemination with infected semen;<br>• embryo status analyzed after washing procedure described in IETS Manual. | • experimentally spiked embryos/semen;<br>• embryo status analyzed after washing procedure in IETS Manual. |

Concerning transmission risk via ET, the IETS HASAC Committee reviews scientific publications on an annual basis and updates a complete set of more than 400 references, which can be consulted on their website (www.iets.org). In the bovine species, 89 potential embryo pathogens have been investigated (Thibier, 2011). All diseases and pathogenic agents have been placed into one of four categories based on the amount of research indicating the likelihood of disease control through the use of embryo transfer (Table 6). For category 1 diseases, risk of transmission of a given disease from donor to recipient via an embryo is negligible, providing biosecurity measures described for handling embryos, material disinfection, and animal health requirements (semen, donor, and recipients ) as described in the IETS Manual have been respected (Stringfellow, 2010; Thibier, 2011).

New questions have been raised regarding trypsin treatments: Al Ahmad *et al*. (2012) compared a treatment standard (TS) comprised of phosphate-buffered saline (PBS), 0.4% BSA (five washes of 100 fold dilution for 10 sec each), followed by two treatments with 0.25% trypsin in Hank's solution (45 sec each), and then PBS 0.4% BSA again (five times for 10 sec). The four other washing procedures all included the same first and last washing steps with PBS but without BSA (five times for 10 sec) and with PBS 0.4% BSA (five times for 10 sec), respectively. The intermediate step varied for each washing procedure, with other trypsin treatments (longer time, twice for 60 sec) or hyaluronidase treatments in order to eliminate Blue tongue virus (BTV) from *in vitro* infected goat embryos: only two trypsin treatments of 60 sec each was effective in removing BTV from the embryos.

*Legal and sanitary measures applied to in vivo derived embryos*

Practical guidelines have been published in the Manual of the International Embryo Transfer Society in order to provide risk management procedures ensuring the safety of ET (Stringfellow, 2010). Since these guidelines have been adopted by the OIE, they are well accepted and implemented worldwide (OIE, 2012; Chapter 4.7). In Europe, legislation prescribes the sanitary conditions to which embryo collection and transfer should comply. The Council Directive 89/556/EEC of 25 September 1989 describes animal health conditions governing intra-community trade in and importation from third countries of embryos derived from the bovine species. The legislation defines sanitary and biosecurity requirements including donor females, environmental and handling conditions, and semen used for donor insemination.

- Sanitary requirements

In addition to OIE recommendations (Table 7), EU legislation includes the following requirements: donor cows must have spent the previous 6 months within community territory or in the third country of collection in a herd officially tuberculosis and brucellosis free, enzootic bovine leucosis free (or no clinical signs of enzootic bovine leucosis during the previous 3 years); and where no clinical signs of infectious bovine rhinotracheitis/infectious pustular vulvo-vaginitis have been observed during the previous year.

- Environmental and handling conditions

Both the OIE Terrestrial Code (Chapter 4.7) and Directive 89/556 include biosecurity measures based on a team approved by competent authority (government or local veterinary authorities), supervised by a team veterinarian responsible for all team operations (health status of donor cows, appropriate disease control measures with handling and operating on donors, disinfection, and hygiene procedures). Team personnel should be adequately trained in the techniques and principles of disease control. High standards of hygiene should be practiced to preclude the introduction of disease.

Procedures, facilities, and equipment are verified through regular official inspections (at least once a year) regarding embryo collection, process, and manipulation of embryos at a permanent site or mobile laboratory, storage of embryos as well as activity records.

The testing of samples can be requested by an importing country to confirm the absence of pathogenic organisms that may be transmitted via *in vivo* derived embryos (see Table 9), or to assess the quality control of the collection team together with washing procedures. Specimens may include degenerated embryos, embryo collection fluids, and a pool of the last washes of the embryos. In the French regulations, this testing procedure is performed annually in a central laboratory

and represents a prerequisite of renewal of approval together with a favorable report from the official inspection (France, 2008).

- Sanitary controls of semen used in embryo transfer

The safety of semen is another critical point and international regulations include requirements regarding ejaculates being used for assisted reproduction techniques (Wrathall *et al*., 2006). With regard to semen that is used to produce embryos for international trade, batches of frozen semen are selected from bulls located in accredited AI Centers in the majority of cases. Such bulls are normally certified negative for acute, epidemic diseases such as foot-and-mouth disease, and chronic diseases such as brucellosis, tuberculosis, leptospirosis, campylobacteriosis, and trichomonosis. For international trade, some countries request that bulls are certified negative for enzootic bovine leukosis, infectious bovine rhinotracheitis, and bovine viral diarrhea. (Bielanski, 2006; Wrathall *et al*., 2006).

Table 6. Diseases or infectious agents in cattle listed by IETS according to the risk for their transmission via *in vivo* derived embryos (OIE, 2012).

| Disease category | Disease agent |
|---|---|
| Category 1:<br>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. | Bluetongue<br>Bovine spongiform encephalopathy<br>*Brucella abortus*<br>Enzootic bovine leukosis<br>Foot and mouth disease<br>Infectious bovine rhinotracheitis: trypsin treatment required |
| Category 2:<br>Substantial 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, but for which additional transfers are required to verify existing data. | None |
| Category 3:<br>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. | Bovine immunodeficiency virus<br>Bovine viral diarrhea virus<br>Rinderpest virus<br>*Campylobacter fetus (subs. veneralis)*<br>*Haemophilus somnus*<br>*Mycobacterium paratuberculosis*<br>*Neospora caninum* |
| Category 4:<br>No conclusions are yet possible with regard to the level of transmission risk, or 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. | Akabane<br>Bovine anaplasmosis<br>Bovine herpesvirus-4<br>Enterovirus<br>Lumpy skin disease<br>Vesicular stomatitis<br>*Chlamydia psittaci*<br>*Escherichia coli 09:K99*<br>*Leptospira borgpetersenii serovar hardjobovis*<br>*Mycobacterium bovis*<br>Parainfluenza-3 virus<br>*Trichomonas foetus*<br>*Ureaplasma and Mycoplasma spp.* |

*Requirements applicable to in vitro produced (IVP) embryos*

*Assessment of disease transmission via in vitro produced embryos*

*In vitro* embryo production entails the completion of three biological steps that are now well established in cattle: oocyte maturation, *in vitro* fertilization (IVF), and embryo culture. The following factors have hindered progress toward the establishment of recognized sanitary procedures for IVP embryos.

The zona pellucida of intrafollicular oocytes appears to differ from that of ovulated ova. This structural difference might be associated with differing resistance to adherence to or penetration of the zona pellucida by

infectious agents (Marquant-LeGuienne *et al*., 2010). Thus, simple extrapolation from sanitary procedures described for *in vivo* derived embryos is not advised.

Oocytes may be collected either from ovaries of slaughtered animals or by ovum pick-up, which involves ultrasoud-guided transvaginal aspiration of oocytes from ovarian follicles. In the first instance, the sanitary status of the slaughtered females is not well defined, which increases sanitary risks associated with IVP. In the case of ovum pick-up, control of the sanitary status of the donor cow is greater (Table 7). Regardless of the source of oocytes, one should consider incorporating special precautions into protocols for IVP of embryos. Any biological product used in the recovery of gametes, sperm, and oocytes or embryos, dilution, *in vitro* maturation of oocytes, and washing or storage is potentially a source of contamination. Indeed, contamination of slaughterhouse oocytes with BVDV and BHV-1 has been reported (Marquant-LeGuienne *et al*., 2000; Galik *et al*., 2002).

Table 7. Potential sources of pathogen transmission related to *in vitro* embryo production.

| Origin | Slaughterhouse oocytes | OPU oocytes |
|---|---|---|
| Donor, Ovaries, Oocytes | • randomly collected ovaries;<br>• unknown health status of the donor animals (risk of clinical or subclinical diseases);<br>• pool of oocytes during transportation to the IVF laboratory, and then during IVM, IVF, and IVC treatment. | • ovaries are collected from ovaries of well identified animals;<br>• health status of donor females well known;<br>• oocytes can easily be treated separately if necessary. |
| Semen | • sperm fraction used for in vitro fertilization (seminal plasma removal via different methods as "swim up" or Percoll gradient centrifugations);<br>• use of cryopreserved spermatozoa to achieve a high rate of fertilization. | |
| Environment, Media | • pathogens present in serum or media (containing animal derived products, use of cell lines) used for the in vitro maturation (IMV), fertilization (IVF), culture or handling of embryos;<br>• added during the manipulation of embryos (collection of the oocytes, washing, culture, or transfer of embryos). | |

Moreover, risk assessment should not be extrapolated from *in vivo* to *in vitro* produced embryos, or from one pathogen to another (Thibier, 2011). This was illustrated by Bielanski *et al*. (2009) in an experiment comparing two BVDV biotypes (NY-1 *vs*. PA-131) added to bovine IVP embryos, treated according to IETS recommendations and then transferred to recipients (Table 8). The proportion of seroconverted recipients differed between the two viruses. Even in the "worst-case" strain, term pregnancies resulted in seronegative calves, demonstrating that risk of disease transmission to offspring and recipients remains low. Another recent experiment reported that approximately 20% of embryos still remained infected following the IETS-recommended 10-sequential wash procedure, after exposure *in vitro* to BVDV type 2 (strain PA-131; Lalonde and Bielanski, 2011).

Thus, the following suggestions have been made in France regarding the prevention of contamination of IVP embryos from the donor side (Fig. 2) as well as environment and media: washings of IVP embryos (IETS recommendations), addition of synthetic compounds in the media, use of controlled cell lines, certification for companies and products, re-testing biological products before use (mainly for BVDV and Mycoplasma sp.), and media heating (56°C/30 min).

Table 8. Comparison of subtypes of bovine viral diarrhea virus with *in vitro*-produced embryos (Bielanski *et al*., 2009).

| Type of non cytopathic BVDV | NY-1 | PA-131 |
|---|---|---|
| Number of pregnancies/number of transfers | 20/33 | 25/61 |
| Percentage of seroconversions in recipients | 0% | 51.4% |
| Number of seroconversions in offspring | 0 (18 full-term calves) | 0 (2 full-term calves) |
| Virus isolation tests on non-transferred embryos (%) | 25% | 28% |

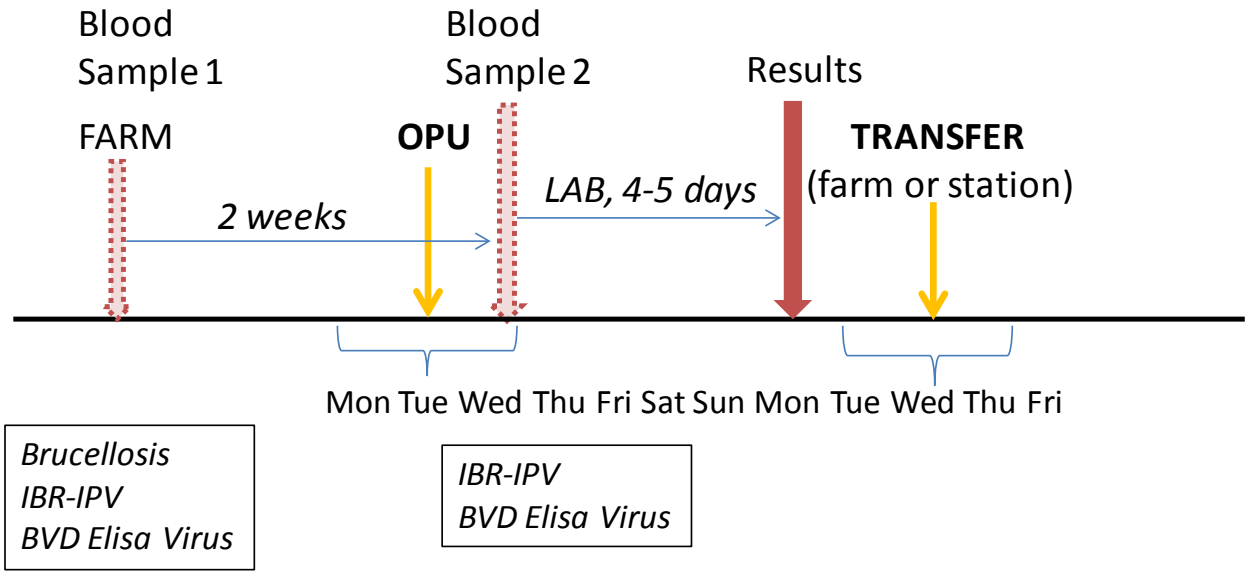


Figure 2. Additional sanitary controls recommended in France and voluntarily applied to donor cows before Ovum Pick Up (OPU) on farms.

*Legal and sanitary measures applied to in vitro produced embryos*

Practical guidelines have been published in the Manual of the International Embryo Transfer Society (IETS) in order to provide risk management procedures ensuring the safety of herds using IVP (Marquant-LeGuienne *et al*., 2010). Ideally, quality-assurance procedures should be outlined for buildings, staff, biological materials, and *in vitro* manipulations. The general plan of quality assurance should include adequate documentation including general procedures, operating modes with details of specific laboratory procedures and traceability documents (Marquant-LeGuienne *et al*., 2010). The international and national legislations define sanitary and biosecurity requirements including donor females, environmental and handling conditions, and semen used for donor insemination.

Since adopted by the OIE, biosecurity measures have been implemented and accepted worldwide (OIE, 2012; Chapter 4.7). According to these recommendations, embryos should be produced by a team approved by a national sanitary authority and under supervision of a team veterinarian. When oocytes are collected from ovaries of slaughtered animals, the slaughterhouse should be inspected regularly by official veterinary authorities. In addition, IVP embryos should be washed using techniques shown to be effective for *in vivo*-derived embryos in the IETS Manual. As in the case of *in vivo* derived embryos, donor cow status is described in the Terrestrial Code, which distinguishes clearly between recovering oocytes from live donors and from slaughterhouse ovaries (Table 9; OIE, 2012; Chapter 4.8). In Europe, legislation prescribes the sanitary conditions to which embryo collection and transfer should comply. The Council Directive 89/556/EEC of 25 September 1989 describes animal health conditions governing intra-community trade in and importation from third countries of IVP embryos of the bovine species.

Table 9. Diseases specific OIE recommendations for the importation of bovine embryos (OIE, 2012).

| Disease* | *In vivo* derived embryos | *In vitro* produced embryos/ova |
|---|---|---|
| Bovine Brucellosis (BB)* | | • donor females kept in a country or zone free from BB; or kept in a herd officially free from BB (tests as prescribed in Chapter 1.3); oocytes fertilized with semen meeting the conditions referred to in Chapters 4.5 and 4.6. |
| Blue Tongue Virus (BTV) | | • donor cows kept in a BTV free country or in a seasonally free zone or in a vector-protected establishment (in BTV infected countries) for at least the 60 days prior to, and at the time of, collection of the embryos;<br>• donor cows subjected to a serological test between 21 and 60 days after collection, with negative results;<br>• donor cows subjected to an agent identification test taken on the day of collection, with negative results. |
| Foot and Mouth Disease (FMD) | | FMD free countries<br>• no clinical sign of FMD at the time of collection of the oocytes;<br>• donor kept at the time of collection in a FMD free country or zone with or without vaccination or a FMD free compartment;<br>• embryos produced in zones with vaccination and destined for an FMD free country or zone without vaccination or an FMD free compartment: no vaccination of donor and negative results to tests for antibodies against FMD virus; or vaccinated at least twice (last vaccination not less than one month and not more than 12 months prior to collection); no other animal present in the establishment vaccinated within the month prior to collection. |
| Bovine Tuberculosis (BT) in cattle or farmed cervidae* | • no sign of BT during the 24 h prior to embryo collection in the herd of origin; and either donor originated from a herd free from BT (cattle or farmed cervidae) in a country, zone or compartment free from BT; or kept in a herd free from BT (cattle or farmed cervidae), and subjected to a tuberculin test for BT with negative results during an isolation period (30 days) in the establishment of origin prior to collection (COL). | |
| Contagious Bovine Pleuropneumonia (CBPP)* | • from CBPP free countries, zones or compartments: donor animals without clinical sign of CBPP on the day of collection of the embryos/oocytes; kept in a CBPP free country since birth or for at least the past 6 months; oocytes fertilized with semen meeting the conditions of Article 11.8.8;<br>• from CBPP infected countries or zones: no clinical sign of CBPP on the day of COL of the embryos/oocytes; donor subjected to the CFT for CBPP with negative results, on two occasions (21 to 30 days between each test, 2nd test within 14 days prior to COL); isolated from other domestic bovidae from the day of the first CFT until COL; kept since birth, or for the past 6 months, in an establishment where no case of CBPP was reported, and that the establishment was not situated in a CBPP infected zone; AND EITHER: not been vaccinated against CBPP; OR vaccinated using a vaccine complying with the standards described in the TM not more than 4 months prior to COL; oocytes fertilized with semen meeting the conditions of Article 11.8.9. | |
| Lumpy Skin Disease (LSD; caused by group III virus, type Neethling)* | • from LSD free countries (embryos/oocytes of cattle and water buffaloes): donor animals without clinical sign of LSD on the day of COL of the embryos/oocytes;<br>• from countries considered infected with LSD (embryos/oocytes of cattle and water buffaloes): no case of LSD has been reported during the 28 days prior to COL in the establishment; and no clinical sign of LSD on the day of COL; and either: vaccinated against LSD between 28 days and 90 days before first embryo/oocyte COL and thereafter vaccinated annually; or tested with negative serological results (SNT or indirect ELISA) for LSD on the day of embryo/oocyte COL or up to 90 days after last collection; or showed stable seropositivity on paired samples tested side by side to indirect ELISA or SNT carried out in quarantine (28–60 days apart, one sample on the day of COL). | |

*For each listed disease as well as Enzootic Bovine Leucosis (EBL) and Infectious Bovine Rhinotracheitis/Infectious Pustular Vulvovaginitis (IBR-IPV), Veterinary Authorities of importing countries should require the presentation of an international veterinary certificate attesting that the embryos/ova have been collected, processed, and stored in conformity with the provisions of OIE Chapters 4.7, 4.8, and 4.9.

## Conclusion

Since 40 years, billions of embryos and semen straws have been distributed around the world. This fact is reassuring that reports implicating germplasm in disease transmission are extremely rare. The high degree of biosecurity measures under official approval and the professionalism of ET teams and the good practices of the AI industry ensures germplasm movement with negligible risk of disease transmission using gametes and embryo based biotechnologies. While emerging diseases threaten international trade, increased use of *in vitro* embryo production and micromanipulation and pre-implantation diagnoses necessitate the updating of specific guidelines and related research work.

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