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Il rischio nascosto: trasmissione di microrganismi patogeni mediante Embryo Transfer bovino.

The hidden risk: transmission of pathogens by means of Embryo Transfer in cattle.

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Riassunto:

L'embryo transfer (E.T) è una tecnica ormai nota e di ampio e crescente utilizzo nel mondo dell'allevamento bovino. Nel corso degli anni tecnologie sempre più innovative sono entrate nella prassi di campo, e con esse la necessità di valutare materiali e metodi che possano risultare cruciali per l'efficienza produttiva di processo e critici per la trasmissione di patogeni. L'emergenza di nuovi microrganismi, come anche l'introduzione in aree indenni di ceppi virali tramite prodotti di origine animale, ha portato negli ultimi decenni ad una maggior attenzione e consapevolezza nel trasporto di materiali potenzialmente a rischio. La movimentazione di embrioni, per trasferire nello spazio e nel tempo materiale genetico, risulta essere economicamente vantaggiosa e più sicura da un punto di vista sanitario che non il trasporto di animali vivi. Il commercio globale di embrioni è aumentato significativamente negli ultimi anni, e data la crescente domanda si è reso necessario una valutazione accurata degli eventuali patogeni che potessero essere trasmessi tramite E.T. Ciò ha portato alla redazione di protocolli standard da eseguire per la metodica in-vivo, mentre per la metodica in-vitro non ci sono linee guida precise e la stessa valutazione del rischio di trasmissione è stata banalmente sottovalutata, consigliando trattamenti analoghi a quelli per la metodica in-vivo per eliminare i microrganismi adesi alla zona pellucida. Studi hanno però dimostrato differenze morfologiche e di sviluppo in embrioni prodotti in-vitro piuttosto che in-vivo; prima tra cui la dimensione dei pori nella zona pellucida e la loro incapacità di obliterarsi in seguito alla formazione dello zigote. Inoltre, nella metodica in-vitro, diversi patogeni come: Bovine Viral Diarrhea Virus (BVDV), Bovine Herpesvirus-1(BoHV-1), Bovine Herpesvirus-4 (BoHV-4), Blue tongue virus (BTV), *Chlamydophila spp., Coxilella burnetii, Histophilus somnus, Leptospira spp., Mycobacterium avium sub.Paratuberculosis (MAP), Mycoplasma spp. e Tritichomonas foetus* possono aderire tenacemente alla zona pellucida e persistere anche dopo i lavaggi con Dulbecco's phosphate buffered saline (dPBS) e tripsina consigliati da *International Embryo Transfer Society* (IETS). Un tema spesso poco considerato è il ruolo della ricevente nella buona riuscita di un programma di embryo transfer, quindi l'ottimizzazione dell'efficienza di processo passa anche attraverso una selezione e una gestione accurata della ricevente e non solo della donatrice. Alimentazione, stato nutrizionale, sanità dell'apparato riproduttore, nonché una accurata gestione del peri-partum influiscono pesantemente

sulla futura fertilità e quindi sulla probabilità che l'attecchimento embrionale possa avvenire con successo. L'obiettivo di questo lavoro è di fornire una visione ampia del processo di produzione di embrioni tramite l'utilizzo e la comparazione delle diverse tecniche disponibili, saggiando problematiche della donatrice della ricevente e del toro, e dando ampio spazio alla discussione della trasmissione di patogeni tramite la metodica in-vitro. Il tutto è stato nella seconda parte del trattato adattato nello specifico ai diversi microrganismi presi in esame

Abstract

The embryo transfer is a worldwide and growing technique employee in cattle husbandry. Over the years, more e more new technologies have been applied in the field and along with them the need to evaluate methods and materials which can be crucial in order to enhance the system's efficiency and the risky for the transmission of pathogens. The emergence of new microorganisms and the introduction in free areas of viral strains by means of animal products, increased over the last few decades the watchfulness and awareness regarding the shipment of hazard materials. Moving embryos appears to be the safer (from a sanitary stand point) and cheaper way to transport genetic material around the world than shipping live animals. Recently, the world trade market of embryos has raised tremendously, the increasing demand led to an accurate evaluation of all those pathogens which may be transmitted by E.T. Has a result, standard protocols for the in-vivo technique has been drawn up, conversely, for the in-vitro technique there are no guidelines yet, indeed, the evaluation of risk transmission has been trivially overlooked, overlapping the results ad methods from in-vivo technique to IVP. Studies demonstrated that in-vitro produced embryos slightly differ from embryos obtained with in-vivo technique. The dimensions of pores in the ZP of IVP embryos are greater compared with the ones present in-vivo derived embryo's surface, moreover with IVP the ZP pores do not obliterate after fertilization leading to a possible penetration and transmission of some pathogen. In addition, microorganisms like: Bovine Viral Diarrhea Virus (BVDV), Bovine Herpesvirus-1(BoHV-1), Bovine Herpesvirus-4 (BoHV-4), Blue tongue virus (BTV), *Chlamydophila spp., Coxilella burnetii, Histophilus somnus, Leptospira spp., Mycobacterium avium sub.Paratuberculosis (MAP), Mycoplasma spp. and Tritichomonas foetus* have the tendency to adhere strongly to the surface of embryos obtained by IVP, and washing procedure with

Dulbecco's phosphate buffered saline (dPBS) and trypsin suggested by *International Embryo Transfer Society* (IETS). fails to remove them. A topic which is not much considered is the influence of the recipients to obtain a good E.T outcome. In order to achieve high results, the selection have to account for both donor and recipient. Nutrition, cow condition, uterine health, as well as a good management around calving, heavily impact the future fertility and the likelihood that the embryo's implantation will successfully happen. The goal of this work is to provide a wide overview on the embryo's production, by:-describing and comparing different techniques,-discussing donors, recipients and bulls issues, -and indeed a deep analysis about the transmission of pathogens in IVP. In the second part of the text, the all discussion has been adapted to each microorganism took into account.

General introduction

The eternal attempt to control the reproductive parameters of domestic animals and man can be traced back 5000 years to Egypt, where Pharaohs tried to create Apis bulls by positioning cows in open fields during storms in an attempt to have them struck by lightning (1). In more recent times, Walter Heape performed the first mammalian embryo transfer (ET) transferring rabbit embryos into a surrogate doe in 1890 (2). The first recorded successful transfer and live birth from an embryo derived from cattle was in 1951 by Willett et al (3) at the University of Wisconsin. It was not until the late 1960s and early 1970s that commercialization of in vivo ET technology in cattle began to flourish, and significant research inroads regarding collection and transfer of bovine embryos began (4). Initially surgical collection and transfer of embryos were widely applied, by the mid-late 70s less invasive, and more rapid and efficient methods started to take place. In late 80s the non-surgical technique become available also for transferring embryos into recipients (4). The transition from surgical to nonsurgical transfers was slowed by 2 main problems. The first is that the pregnancy rate using surgical transfer techniques was significantly higher initially than nonsurgical in certain operations (4). The second impediment to this transition was the lack of useful tools to accomplish the transfer. The first ET gun that was introduced in North America in 1984 to 1985 (5). Therefore, the introduction of new technologies, along with infertility issues of surgical technique which affected more or less 10% of donors (6), and the achievement of good results in embryos quality and quantity with non-surgical technique; led to the final transition to the use of non-invasive methods for transferring embryos. A major development in the ET world was the ability to cryopreserve bovine embryos and to keep them indefinitely. In 1973, Wilmut and Rowson were the first to cryopreserve bovine blastocysts, using dimethyl sulfoxide (DMSO), and subsequently establishing a day 42 pregnancy in a recipient cow after thaw (7). This kind of technology had been and has today a great importance in order to preserve and to ship embryos for long time and distances, moreover, cryopreserve embryos allows brings new genetics in different countries and not less important, moving embryos result in great economic value.

The embryo transfer (E.T) is a technique which by collection of one or more embryos from a donor cow, allows to transfer embryos into one or more different recipients (8). The exchange of genetic material between countries or even within a country was initially

limited to live animals and frozen semen. However, moving embryos appears to be the safer (from a sanitary stand point) and cheaper way to transport genetic material around the world. Therefore, is not a surprize noticing that embryo market is increase tremendously over the past 10 years. The transmission of pathogens by means of embryo transfer can happen through: semen, oocytes, embryos, donor/recipient heath status, and environmental condition (9). Anyhow, there are little scientific studies regarding pathogens transmission by E.T, moreover, emergence of new diseases and the increase importance of new techniques to produce embryos such as in vitro produced (IVP), need a better assessment of risks and critical points in order to improve the efficiency and safeness of this practice. The will of this work is to summarize the present knowledge regarding embryos technology with a particular regard to infectious disease.

Since the beginning, the E.T practice has taken into account only the donor, below a brief explanation of how this selection has been carried on, further, in next chapters many topics here mentioned will be discuss in detailed and will include recipient, bull and the role of other players which can affect the result of the E.T.

Donor selection is focus on choosing animals with superior genotype of phenotype characters. Nowadays, genetic indexes are generally well evaluated and use to foresee which cow will be destinated to undertake the E.T process. Anyhow, cows can be chosen for many reasons: -genetic value -market request -owner will. Other criteria which influence the selection are: reproductive performance, conformation, body condition score (BCS), stage of lactation, age, number of calving, days open, environment, and endocrine factors (10). When a E.T fails or the result is poor (low harvesting rate/ few viable embryos), the causes that lead to this negative result can be summarized in three categories: 1-owner 2-technician or veterinarians who performed the E.T 3-the animal (11). The first category to analyse is represented by the owner. An E.T program is deeply affected by the health and physical condition of donors and recipients (12), therefore the owner is the one how is in charge of handling this issues, influencing positively or negatively the animals condition and as a result the final outcome of the E.T program. There are many underestimated problems, for instance: the use of frozen semen from bulls with low fertility and the use of sex semen. In 2013 the viable embryos rate collected from donors inseminated with sex semen were 25,1%, on the other hand, the use of conventional semen produced 55.95% of

viable embryos (13). Thus, education is an important vehicle in order to increase awareness on the use of new technologies and to optimize the E.T efficiency (11). The second group is about technicians and veterinarians. In this case, critical points are represented by: inexperience of the stuff, MOET protocols badly structured, lack or inadequate equipment, low technological level. Indeed, education is the best way to minimize the negative influence of the technicians (11). Last group is the animal herself; the increase number of data available can help to evaluate the clinical story and the performance of the donor. Has been proved that less than average embryos production can be related to both owner and technicians. The understanding of different responses between *Bos taurus* and *Bos indicus*, heifers and mature cows, as well as for beef and dairy breeds, is paramount in order to choose MOET protocols and E.T programs that can optimise the embryo production in each category (4). Manipulation of the estrous cycle to enhance estrus, correcting physical abnormalities of the reproductive tract, and nutritional counselling with the owner of that BCS 2.0 cow with a calf at her side will not only enhance the owner's own education but also prevent wasting a great deal of time and increase the practitioners success rate (10).

In-vivo technique and Superovulation program:

In this chapter the part of the in vivo technique will be describe with a particular reference to the application of different MOET procedure and how they affect reproduction performance and embryos quality. The in vivo E.T is composed by several steps.

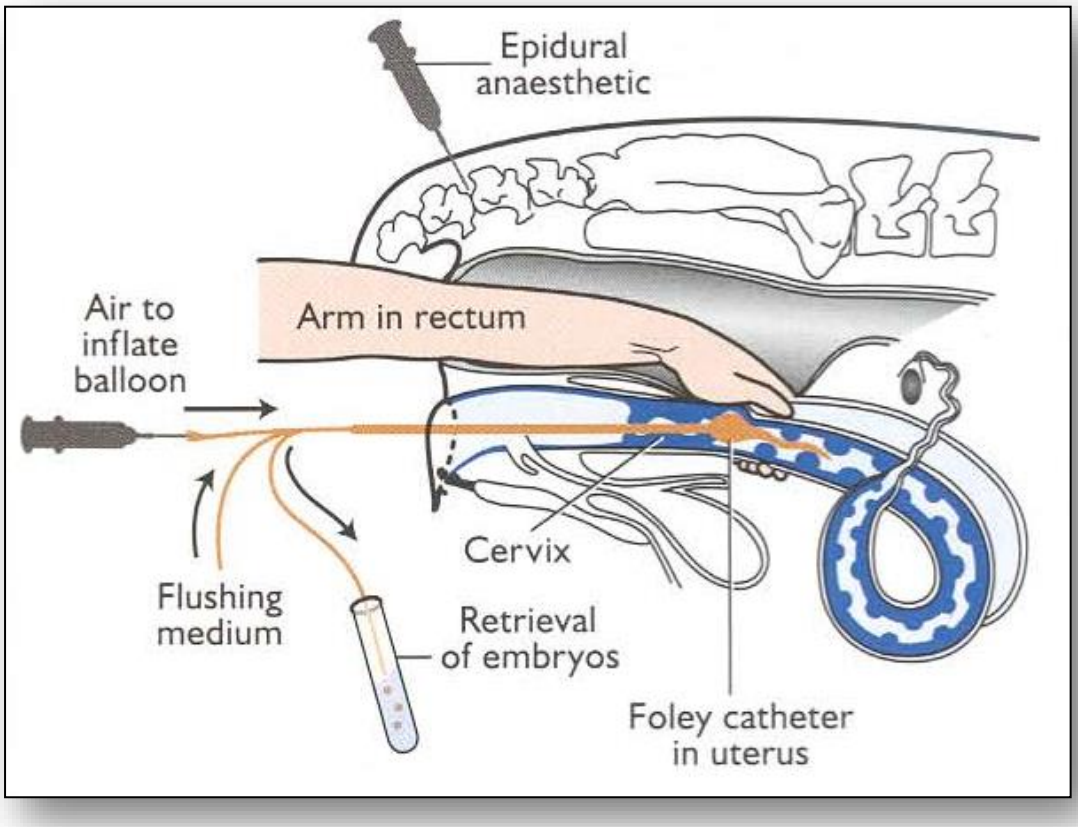
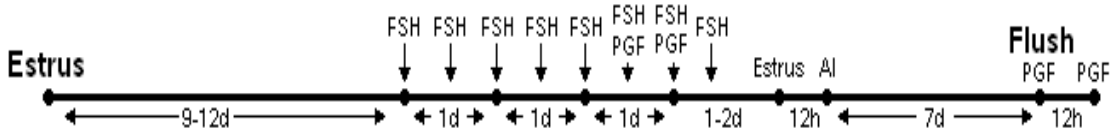


Figure 1 framework of embryos collection performed in a donor by syringe technique. Senger, P.L., Pathways to Pregnancy and Parturition, 2003. pp 299

After a preliminary part where donors and recipients are selected, synchronisation and eventually MOET protocol is set up, the E.T process can take place. As follow, a cow is superovulated and inseminated, embryos are collected 7 days after by uterine flushing which can be perform by "gravity" technique or by means of a syringe (figure 1). Afterwards, embryos are evaluated under stereomicroscope and prepared for be transferred into recipients and/or be frozen. In the next part, will be discuss the efficiency and relationship of molecules combination and techniques apply in MOET protocols.

Back in the days, before FSH would be available, the first MOET procedure was set up using equine chorionic gonadotropin (eCG) (14). This hormone has FSH and LH like activity in

cattle causing multiple ovulation after the first injection. Its main drawback is a long half life (roughly 50 hours) which is way longer than the one own by the other two gonadotropins (15). A long persistency in the blood stream and therefore a long activity in the ovaries can lead to asynchrony ovulations and the persistence of big non-ovulatory follicles. Moreover, since eCG is highly antigenic it causes therefore, is not possible to use the hormone again before these immunoglobulins dying out the bloodstream. The first FSH product available was of pig. The commercial products available on the market nowadays are variably contaminated by LH and commercialized under the name of Pluset and Folltropin. In Folltropin is more purified; the LH: FSH ratio is approximately 0.12, whereas in Pluset the LH: FSH ratio is 1.0. The effect of LH in the superovulation response is not totally clear. Some author found that high LH contamination impairs the ovarian response to superstimulation, resulting in decreased yield of viable embryos (16). The origin of the deleterious effects of excess LH is considered to be an untimely maturation of the oocytes (17). Remarkably, low LH levels are well tolerated during the follicular growth but exceeding a certain threshold may cause adverse effects to the follicle and oocyte (18). Recently a study (19) found no difference in the success rate of superovulation with Folltropin or Pluset when evaluated as the number of transferable embryos or the quality of embryos recovered. The total number of recovered structures was higher for Pluset, due to a higher proportion of UFO; an earlier study of Kelly et al. supports the same result (20). No morphologic differences as well as embryo viability after transfer have been observed in embryos produced by the two FSH products. Nonetheless, Ferré (21) used low doses of Folltropin and Pluset to stimulate follicular growth before ovum pick-up (OPU). In this study Folltropin was superior compared with Pluset or eCG, both of which have relatively high LH activity, in terms of collected and viable oocytes, cleavage, and embryonic development. Another interesting aspect is the different oocytes maturation using a product with a higher or lower concentration of FSH. Indeed it has been thought that LH triggers oocytes development, as a result, a study conducted by Kelly et al. (20) proved that time interval from induction of luteolysis with prostaglandin until the first insemination was longer for Folltropin-treated donors than Pluset-treated donors.



The onset of the superovulation protocol in donors cannot be set before 45 days after calving. First at all, the donor has to cycle normally, with the presence of active corpus luteum (CL) responsive to prostaglandin; between day 9 up to 14 days since last ovulation. Suine FSH has a short half-life (5 hours), thus, it is necessary to inject it twice a day (22). Many MOET protocol has been set over the years, the most common one consists in 8-9 or 10 injections (usually decreasing dose) in 4-5 days. At the and PGF2α is provided in order to regress the CL and inducing estrus and ovulation. In order to gather a good outcome, the cow has to cycle normally, indeed, a normal estrus cycle can last 18 to 26 days, and, it is the luteal phase that influence the most its length. Hence, according with the duration of the follicular growth and the development pattern of the dominant follicles, only 20% (4 or 5 days) of the estrus cycle can be available for the onset of the protocol, which has to correspond with the emergence of a new follicular wave. As a consequence, the other 80% of the cycle do not lead to a good superovulatory response, because of the presence of a dominant follicle (23) (24). (25) and (26) found that this event reduce the protocol's efficiency by 40 to 50%. According with Sali, there is a broad variability in response to MOET protocol in terms of ova's rate among cows, the author underlined the fact that some cows do not respond to any superovulatory protocol, assuming that a possible genetic component may interfere with the lack of treatment response (27). Recently, striving for improving the success rate ed the embryo's quality, leads to the application of progesterone devices such as the *controlled internal drug release* (CIDR). This device ensures a flexibility in the onset of the superovulation protocol, since it can be apply at any time of the estrus cycle. The most important tools to induce the emergence of a new follicular wave consist in physical or pharmacological suppression of the dominant follicle. This allows the early release of FSH and the emergence of a new follicular wave in a fix time, without the need to monitoring the estrus or waiting for narrow window at the middle of the cycle (28). There are 3 methods for synchronise the emergence of a new follicular wave (24): the follicular ablation, use of estradiol 17B and the use of GnRH (29). The use of estradiol 17B is forbitten in Italy, EU ad US, but in other countries such as Canada is widely used. The follicular ablation consists in the transvaginal aspiration ultrasound-guided of all the follicles greater of 5mm (23) (28) (24), or just the two biggest follicles (30). Removing the dominant follicles we have no more the negative effect of inhibin towards small follicles, and therefore the pool of small follicles become responsive to FSH, they start

growing and as a result a new follicular wave emerge after one (31) or two days (30). Many studies found that the follicular ablation allows to harvest a greater number of oocytes/embryos compared with the conventional protocol (32) (33), anyhow, it is higher the number of UFO and degenerated embryos obtain with the follicular ablation. As a result the number of transferable embryos is comparable with the conventional E.T, even though with follicular ablation the treatment can be started at anytime of the estrus cycle, moreover follicular ablation is much more accurate leading to a fine correspondence with the beginning of new follicular wave and therefore exceeding the superovularory response (28), (23), (34), (30), (35), (36). According with Shaw and Good, the findings mentioned above proved that the aspiration of the dominant follicle increases the ovulatory rate, anyhow, the author claimed that embryos quality is influenced by factors which are not dependent on follicular dominance (37). Kim et al found no difference in the number of transferable embryos comparing the two methods. The increase number of oocytes depends on the result of follicular ablation: animals in which follicular ablation is performed, show an early follicular growth, and therefore at the one set of the pharmacological treatment there would be more follicles ready to be stimulated (33). A similar study of Hill and Kuehner gathered a higher number of transferable embryos (8,9 vs 6) in cows which undertook a follicular ablation compared with another pool of cows treated with conventional system (38). In addition also (36) (39) and (32) obtained similar result comparing total number of embryos harvested, on the other hand the rate of viable embryos remained the same. The GnRH protocol is probably the gold standard nowadays. It has been proved that the use of GnRH results in great reproduction efficiency (50 to 60% in time AI) (40). Moreover, combination of GnRH and CIDR allows to remove the dominant follicle and to recruit a new follicular wave as efficiently as it is the estradiol 17B (41). The GnRH is a polypeptide which working at iphotalamic level triggers the release of FSH and LH. The latter influence the growth of follicles greater than 0,8 cm inducing ovulation and luteinization of the dominant follicle. Providing progesteron with some progestinic device cause the regression of the dominant follicle and the beginning of a new follicular wave (35). The GnRH prevents the display of a spontaneous estrus during the superovulatory treatment up to the injection of PGF2α, furthermore, it is possible to gather the same superovulatory response of the conventional protocol, but with the advantage of starting the protocol at anytime of the estrus cycle (42). However, the only use of GnRH is not

advisable for the asynchrony of the follicular wave emergency; since different moment of the estrus cycle causes different ovulatory responses (43) (31), indeed, there is a lower embryos harvested. According with (35), we have outcomes that crash together also by using progesesteron alone. compared with follicular ablation. But is not just the superovulaory treatment that influences the final outcome in terms of embryos harvested per flushing. Many authors found that the age of the donor and the season can deeply affects the final result. (44) (45) and (46) in their conclusions, they underline how the ovulatory and embryo rate was higher in winter: Roth claim that this event is likely to occur in case of heat stress experienced by animals during the summertime and that along with lower milk production, also reproduction performance is negatively affected (47). Heat stress has a direct and deleterious effect on follicles in short and long term. Heat stress can compromise the growth of both small antral follicles and medium size follicles, causing a drop of inhibin release and an increase quantity of FSH in the bloodstream, as a result a early follicular wave emergency extend the follicle dominance length. Two or three estrus cycles are needed in order to recover for the heat stress. (48) reported conflicts in many studies results regarding how seasons affects reproduction. Gordon underlined that the reasons of this conflicts may not be always clear because of complexity of nutritional and environmental factors associated with every particular season. In the chapter “Cow management and uterine health” will be discuss in detailed how age environment, and health issues affects donors and recipients fertility (for both heifers and mature cows).

In Vitro Technique

The sanitary considerations were initially limited to in vivo produced embryos from a number of domestic species. However, with the rapid practical application of in vitro embryo production, it is necessary to include also these types of embryos in the discussion of sanitary precautions and risk of transmission of diseases.

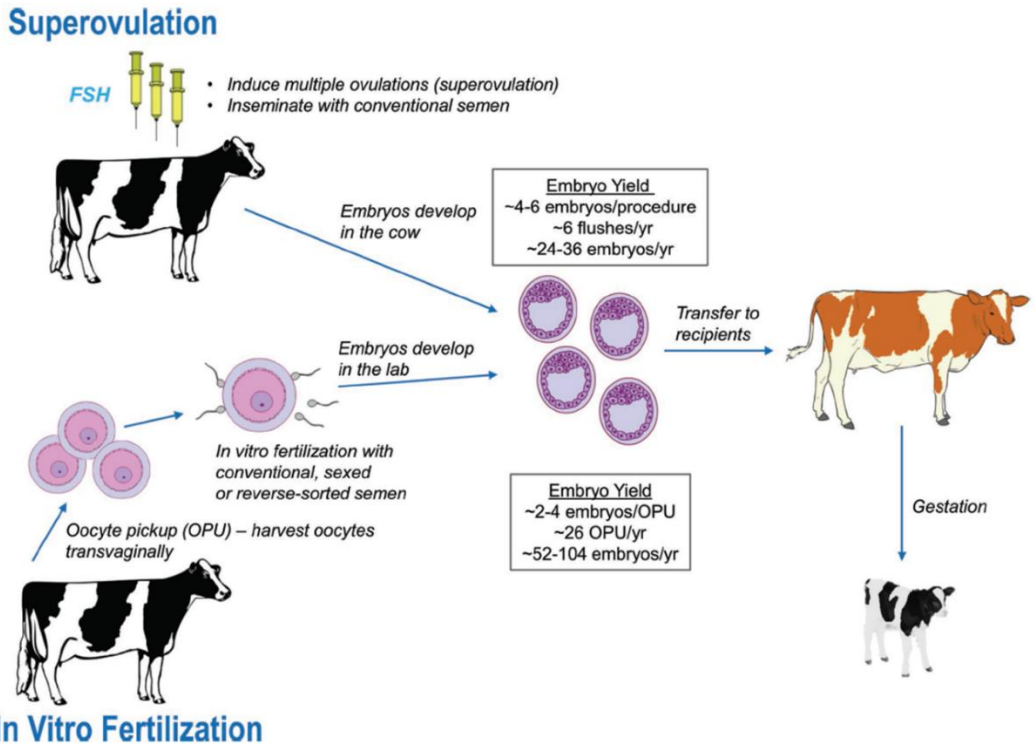


Figura 1. Comparison of the predominant methods to produce embryos for transfer in cattle. Superovulation involves treatment of cows with follicle stimulating hormone (FSH) to induce multiple ovulations. Note that although fewer transferrable embryos are typically produced from a single OPU procedure than for superovulation, OPU can be performed more frequently (49)

The in vitro embryo production (IVP) consists of the use of immature oocytes for the production of embryos. The laboratory procedure is divided into three sequential stages called in vitro maturation (IVM), in vitro fertilization (IVF), and in vitro culture (IVC). Oocytes can be harvest by ovum pick-up (OPU) or by ovaries in the slaughter house. Approximately 90% of the oocytes recovered by OPU reach metaphase II with the expulsion of the first polar corpuscle when passing through the IVM stage. But, which 80% are fertilized and begin to cleave, and only 25% to 40% of probable zygotes reach the stage of Blastocyst at day 7 of the culture stage (CIV). The effectiveness of the production is evaluated from the final rate of blastocysts, which is calculated on day 7 of the culture when the embryos can

be transferred to the recipient or cryopreserved

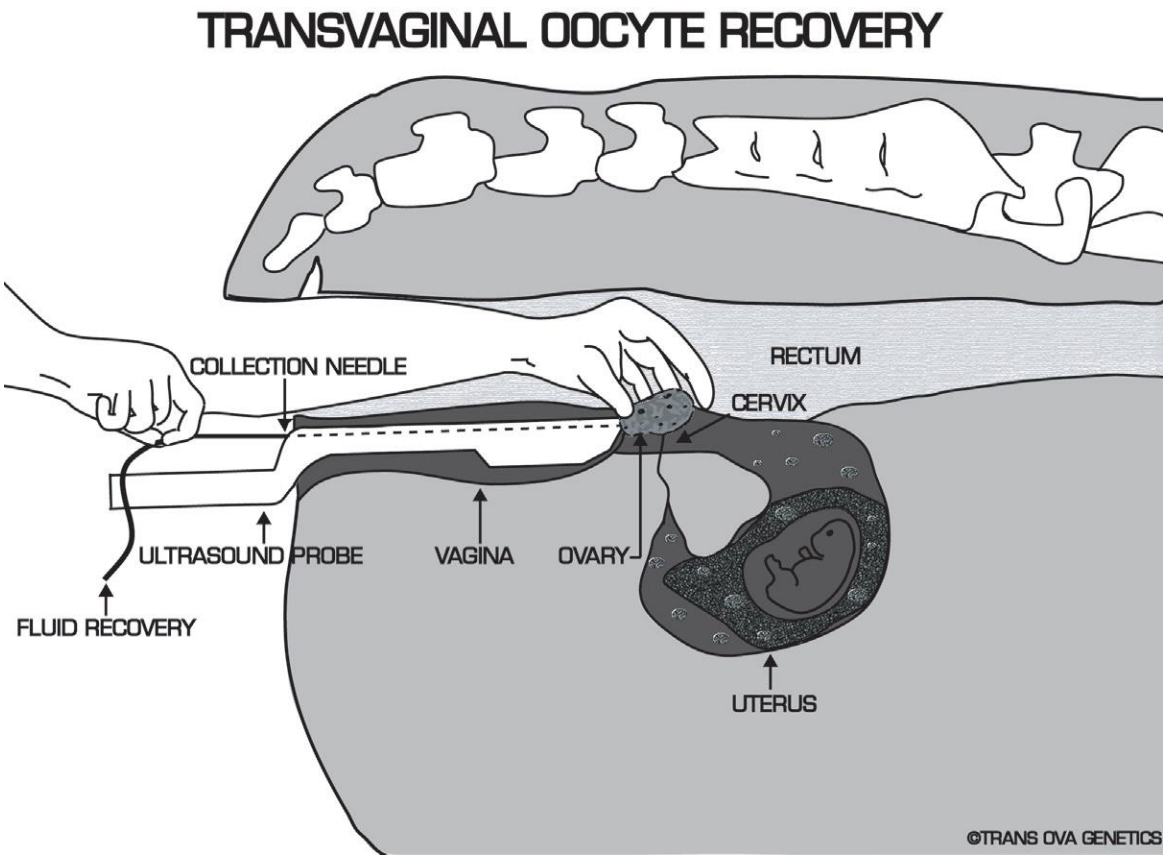


Figure 3 OPU procedure; oocytes collection can be done up to 100 days of pregnancy by transvaginal route (50)

In donors, oocytes can be collected every 12/18 days up to 100 days of pregnancy using OPU technique. With OPU, oocytes are collected from the ovaries of identified donor females with a well-defined health status and oocytes can easily be treated separately if necessary. On the contrary, ovaries from the slaughterhouse are randomly collected, so that the donor females may present a potential risk of clinical or subclinical disease (51).

Harvesting follicles from slaughter house's ovaries: Oocyte collection is usually performed by dissection, aspiration or slicing of ovaries. Aspiration of the antral follicle is the most widely adopted recovery technique because of its speed of operation and low risk of contamination, but the oocyte recovery rate is poor (averaging 30–60%). The follicle dissection technique generally has the highest oocyte recovery rate of up to 100% but has a high risk of contamination. Typically, the cumulus-oocytes complexes (COCs) are washed two or three times in different dishes, despite the lack of a standard protocol for washing oocytes before IVM.

Dissimilarity between in vivo and in vitro produced embryo and final considerations:

1-most of embryos obtained from IVP are transferred fresh not frozen. However, approximately 18% of the IVP embryos transferred worldwide in 2014 were frozen-thawed which is double that reported from the previous year (52). 2-IVP embryos are much more stage sensitive and conception is more likely to fail if it is not performed in the proper time, 3-pores on the ZP are bigger and they are not prone to close after IVF procedure (figure 10). Indeed, Pathogens attached strongly to the surface of embryos produced by IVP compare with in vivo technique.

4-In the end, at the time of fertilization, in vitro matured oocytes are still surrounded by cumulus cells, and contrary to in vivo ovulated oocytes no additional oviductal glycoproteins are present in the ZP. Thereafter, pathogens such as BVDV and BHV-1 can bind to the ZP and penetrate into the oocyte during fertilisation, leading to a lower fertilisation and development rates along with risk of transmission by E.T.

5-Additional sanitary risks associated to in vitro culture may originate from contaminated media which are supplemented with serum, Bovine Serum Albumin or other biological products. There are therefore many troubles to face with IVP, this is particularly true since there is also no set procedure or recommendation for allocating harvested oocytes into groups for the IVP process. In general, it should be noted that the relevant EU legislation covering livestock embryos does incorporate recommendations made by the IETS and the OIE. However, there is an important omission: the EU does not seem to differentiate between risks associated with in vivo-derived embryos, which for most disease agents are negligible if processed by IETS/OIE recommended protocols, and the risks with in vitro-produced embryos, which, compared with the in vivo type, can be greater.

Semen

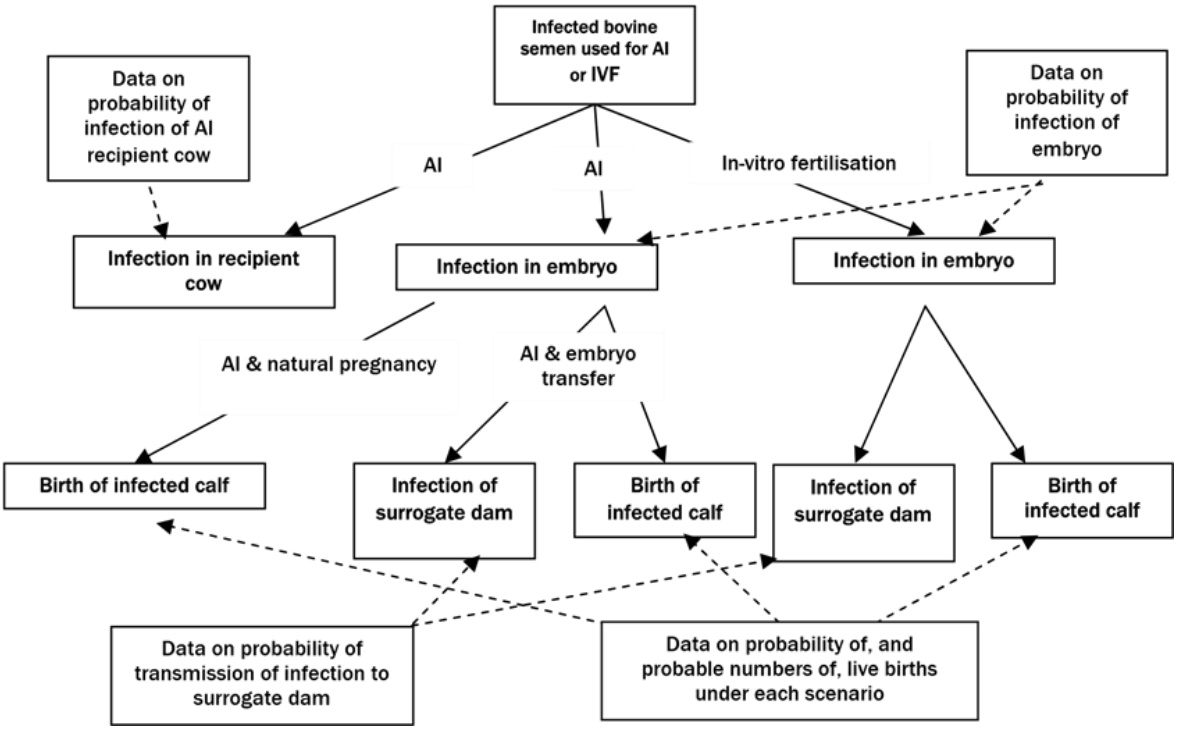


Figura 4: Risk pathway from insemination with infected semen to infection in semen or embryo recipient (53)

It is well known that service sires can have a very profound effect on conception rate following natural service or AI. Embryos quality is deeply affected also by semen (54). A support of it, a study of Court and Colas showed that AI bulls with low fertility have higher rates of embryonic loss than do bulls with high fertility (55). Stroud et al presented data showing that semen quality was directly related to fertilization rate in superovulated donors (56). Ultimately, there is a strong relationship between semen quality and percentage of excellent-quality embryo. Moreover, the use of sex semen can be tricky since the conception and fertilization rate are lower compared with conventional straw. By the way, sex semen is the first choice in IVF, whereas it is generally preferred to use conventional semen in in-vivo E.T. Talking about infectious disease, sperm can carry may pathogens which may lead to transmit disease to recipient or directly infect the embryo. In the chapter “pathogens” for each micro-organism is discuss the role of semen in the transmission of the disease.

Enviroment

There are many critical points others than semen, donor recipient and embryo to examined. In order to extend embryo viability, to preserve it by cryopreservation or to produced embryos in artificial condition, the use of substances and technological aids are paramount to obtain good outcomes. There are many risks associated with the application of animal products in embryo's media or culture that may carry pathogens and infect embryos during production; the second part of the chapter will be discuss in detailed the main biological risk associated with E.T protocol. Regarding technological aids, using plastic or other materials which enter in contact with embryos during the E.T process or stocking, may impaired embryo viability. As an example, in the US by the end of 1983 and the beginning of 1984 the conception rate in recipient was strongly declined. In some case the conception rate in Holstein heifers dropped from 71% in 1983 to 53% in 1984 (57). Later, everything has been delighted; the use of syringes sterilised by gamma ray irradiation, had released toxic substances that had a detrimental effect in embryo viability, results were shown in IETS conference in 1986 (58).

Biological risks:

Fetal calf serum (FCS) is a common ingredient in the various media used during the in vitro process, being commonly used in pre-IVM washes, in the IVM media, and in the IVC media. However, contamination of FCS is common and a serious cause for concern. BVDV for instance, can readily infects the developing bovine fetus, where, after replicating in several fetal cell types, the virus can be found cell-free in the FCS. The serum may also contain BVDV antibodies that can shade the virus and escape its detection by isolation. Nonetheless, samples were always positive to the reverse transcription nested polymerase chain reaction (RT-Npcr) (59). Irradiation of FCS is recommended for reducing low levels of viral contamination difficult to detect by conventional screening methods. The OIE recommends gamma-irradiation at 25 kgrays, but this may not remove all virus. On the other hand an excess gamma irradiation of bovine serum albumin solution can cause disruption of the ordered structure of protein molecules as well as degradation, cross-linking and aggregation of polypeptide chains (60), it may also cause fragmentation or aggregation of other proteins in FCS. Fetal calf serum(FCS) has been widely used in the past

for embryo collection an cryopreservation. Nowadays FCS has been worldwide replaced with other media such as BSA and polyvinyl alcohol.

Bovine serum albumin (BSA): Serves as a macromolecular substitute in media for oocyte maturation, fertilization and early embryo culture. It provides essential embryotrophic and sperm capacitation functions (61). Production of BSA involves heating (65–75.8C for more than 3 h) to denature non-albumin protein (62). The heat process destroys viruses and other potential pathogens. Therefore, BSA can be consider a safe media free from contaminants.

Somatic cell for in vitro culture: To culture zygotes to blastocyst stage, fertilized oocytes are co-cultured with a suspension of bovine. oviductal epithelial cells or other somatic cells or synthetic media mimicking these oviductal cells, and incubated in droplets for 7–8 days under oil at 39,8°C in 5% CO2 in humidified air. Although a wide variety of somatic cells can be used as co-culture cells in the IVC phase, monolayers of co-culture cells prepared from the cumulus cells previously stripped from the oocytes prior to IVF is common practice. Pathogens, if present, may adhere to or infect co-culture cells or contaminate the culture media. Oviducts of several animal species, e.g. rabbits, sheep and mice, can be used in bovine IVC systems to avoid contamination or infection with pathogens infectious to cattle. There is no report of pathogens indigenous to other animal species infecting the embryos. Pathogens infecting or contaminating the culture may grow and proliferate during IVC, depending on resistance to antibiotics, presence of antibodies, and suitability of the culture system for pathogen growth within the IVC timeframe (63). Avoiding the use of somatic cells from donors of unknown disease status is advisable. However, when use of abattoir-derived co-culture cells is necessary, testing follicular fluid and subsets of cultured cells for the pathogens of concern is recommended (64).

Conclusion: Essential procedure for preventing the introduction and transmission of diseases should include:

1. Screening of ‘materials of animal origin’ (e.g. serum)
2. Ensuring that abattoir-origin materials are only collected from abattoirs that slaughter under inspection and are processed in an area of the laboratory which is segregated from other procedures (e.g. IVM, IVF, and IVC)

3. Washing of oocytes and washing or trypsin treatment of embryos
4. Adhering to laboratory protocols designed to prevent cross contamination between batches of oocytes and embryos
5. Quality assurance tests (e.g. virus isolation or RT-PCR) on selected materials such as follicular fluid, somatic cells, or media and/or other cells from IVM, IVF and IVC.
6. An additional safeguard that might be used in the future is the addition to media of antiviral substances to inhibit the replication of any BVDV that might be introduced in relatively small quantities and otherwise amplifies in the system

cow management and uterine health

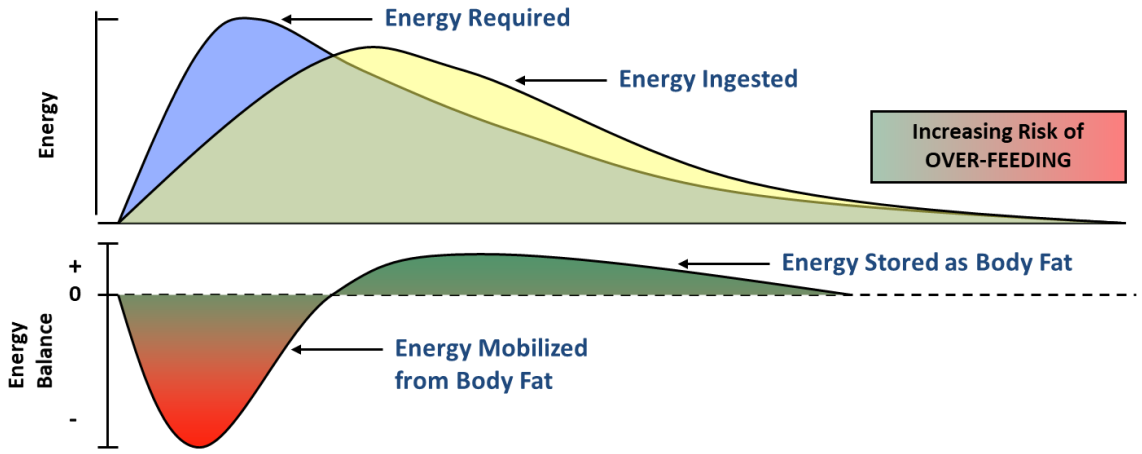
In 2013, the average of embryos flushed per cow were 6.8 (13). The number of transferable embryos in the last 30 years has not -been changed, for both beef cattle (65) and dairy cows (66). Interestingly, 45% of donors (dairy cows) in 1983 had seven years (36) or more, while today the use of heifers is widespread. Also the conception rate in dairy cows has drastically diminished during last decades (67). Retrospective studies analysed the efficiency of four different ET programs over 20 years, the final outcome was no significant differences in embryos harvested per flushing (68). Therefore, it seems likely that the lack of improvement is bound to reproductive disorders and infertility instead of technology inefficiency. Moreover, in the past the recipient's role has been often underestimated and not much attention has been payed to evaluate this category of animals. Since the good outcome of E.T program depends also on the recipient health and general condition, it is obvious that cows which are going to receive an embryo have to be accurately chosen (69). For both donors and recipients, disorders that may take place within 2 months from the beginning of the ET program, should be carefully evaluated. Since synchronisation and MOET program should start from 45 days after calving, disorders that pop up in the transition period can deeply affect fertility and therefore the good result of the ET program. Factors that delay uterine involution are important because completion of involution is associated with fertility (70).The factors that delay involution include dystocia, hypocalcaemia, retained placenta, metritis, and endometritis (71). The following chapter will discuss: nutritional, metabolic and uterine health with a particular reference to disorders in transition cows that can result in reduction of fertility and reproduction performance.

1-Metabolic disorders:

metabolic stress compromises the ability of animals to respond sufficiently to pathogens, this may result in persistence of infections and chronic inflammation such as endometritis or metritis.

Nutrition. Negative energy balance (NEB): Dairy cattle are under metabolic stress after parturition, with reduced

concentrations of nutrients and changes in metabolic hormones, including reduced abundance of glucose, glutamine and insulin-like growth factor 1 **(72) (73)**

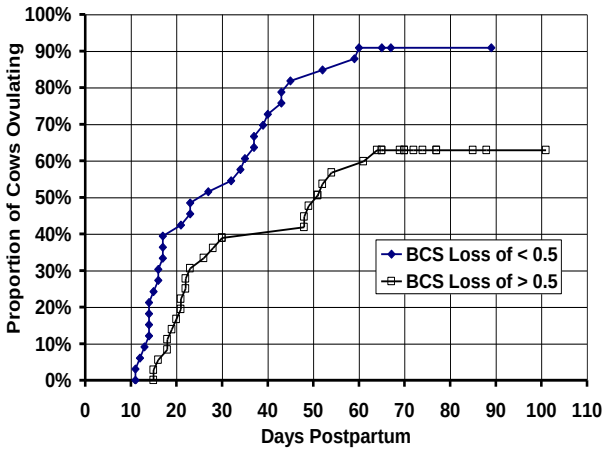


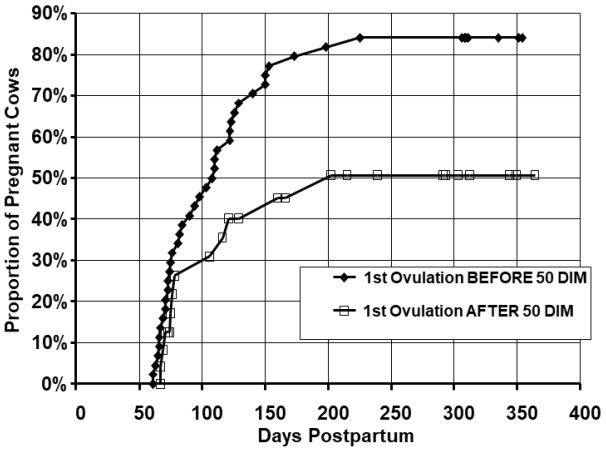
Negative energy balance related to dry matter intake DMI during early lactation is the major nutritional link to low fertility in lactating dairy cows. Moreover, NEG delays recovery of postpartum reproductive function and exerts carryover effects (BCS, oocytes, uterus) that reduce fertility during the breeding period. High milk production and NEB results in tissue mobilization and BCS loss, thus, greater is the BCS loss during early lactation, lower is the fertility at insemination time. Santos et al showed how BCS changes after calving impact in conception rate and pregnancy loss.

	Pregnancy %	Odds Ratio	Pregnancy loss, %
≥ 1 unit	28	Referent	20.5
< 1 unit	37	1.4	**14.5**
No Change	42	**1.7**	**10.7**

Figure 5 Relationship between: BCS points loss after calving to AI, and conception and pregnancy loss (74)

A support of it, studies demonstrated that NEB and BCS loss delays first ovulation and is related to poor fertility and increased risk of culling





High fatty acids mobilization directly affects the follicular development, causing a deficiency of glucose and high levels of NEFA inside the follicular antrum (75). Along with follicles, also embryo development is compromised (75). Negative energy balance may impair the inflammatory response and clearance of bacteria from the endometrium, leading to chronic endometritis (76).

Hypocalcemia: The onset of lactation causes a severe and rapid drain on blood calcium required to produce milk. If this blood calcium is not replaced as rapidly as it is reduced via bone calcium release (resorption) or intestinal absorption of calcium, cows will become hypocalcemic with some developing clinical milk fever. Subclinical hypocalcemia is considered when Ca serum level drop under 5.5-8.0 mg/dl, Subclinical hypocalcemia can be very frequent within a herd and affects up to 75% of periparturient cow. Negative effects in productivity and reproduction performance are often hidden, but they can compromise for long time fertility and milk production. Martinez et al found that cows with low blood calcium concentration (below 8.6mg/dl) during days 1 to 3 post calving had:-increase of neutrophil function -increase NEFA and BHBA concentrations in bloodstream -increase incidence in metritis and endometritis -lower subsequent reproductive performance (77). In another study accomplished by Chapinal et al in 55 herds in US and Canada found that cows with level serum of calcium <8.6 mg/dl during the first week post calving had: -2.4 higher rate of displacement abomasum (DA). -3.8 kg/d less milk. -30%decrease in conception rate at 1st service (78). Others troubleshot liked to hypocalcemia are: -increase risk of dystocia 6,5 times -increase the risk of retain placenta of 3,2 times -increase the risk of ketosis of 8,9 times and increase the risk of mastitis of 8,1 times (79). Moreover, subclinical hypocalcemia had a negative effect on return of ovarian function during the voluntary waiting period and decreased the odds of pregnancy at first service (80)

Ketosis: Ketosis is a metabolic state in which low level of glucose in the blood stream pulls the production and release of ketone bodies in the blood stream in order to sustain the energy demand.

High NEFA's before calving associate with:

1. ~1.5 X ↑ risk of retained placenta (81)
2. ~2-3 X ↑ risk of subclinical ketosis
3. ~2.2 X ↑ risk of metritis (> 0.37 mEq/L) (82)
4. ↑ risk of DA: ~ 2 X (> 0.27 mEq/L) (82)

5. 19% ↓ risk of pregnancy within 70 days of VWP (> 0.27 mEq/L) (82)

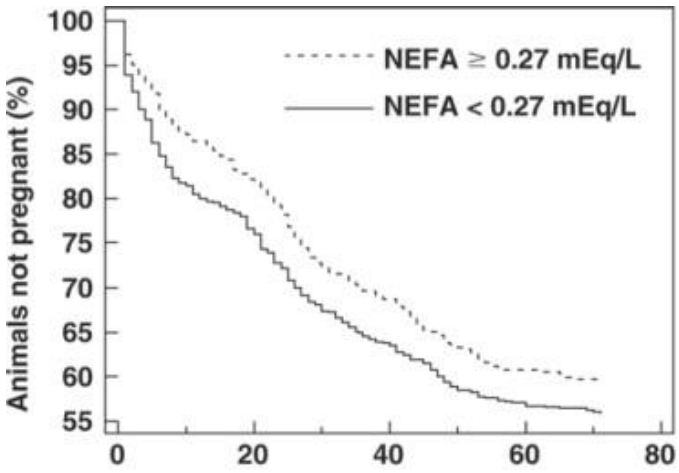


Figure 6 lower conception rate in cows with higher NEFA prior to calving (82)

Marr et al found that nonovulatory cows during early lactation had higher plasma NEFA, higher plasma BHBA, higher liver Triglycerides (TG) content. In contrast ovulatory cows had lower ratio of liver TG to plasma NEFA (83). Subclinical ketosis in post-partum, when BHBA is above 1,2 mmol/L causes increase of disease incidence and decrease the pregnancy rate (82). In conclusion; ketosis badly affects reproduction performance and can influence the oocytes quality and pregnancy rate.

2-Uterine health

Uterine disease reflects a disturbance of the normal postpartum period, which usually lasts about 40 days, and is defined as the time between parturition and completion of uterine involution (84). After parturition, four concomitant events need to be completed before cows are likely to be able to conceive again: uterine involution, regeneration of the endometrium, return of ovarian cyclic activity, and the control of pathogenic bacteria in the uterus (84) (85).

Retained placenta: soon after calving cows have to shed placental membranes within 2 to 12 hours. If it takes longer than 12 hours, it is called a retained placenta. Incidence in a herd varies from 4.0-16.1% (86). Leukocytes chemotactic factor is found in placentomes of cows with normal placental separation. The lack of white blood cells reactivity causes the retained placenta. The increase in the number of microorganisms in the uterus causes inflammation, decreased milk yield, reduce conception rate and in turn extends calving

interval and reduces fertility.

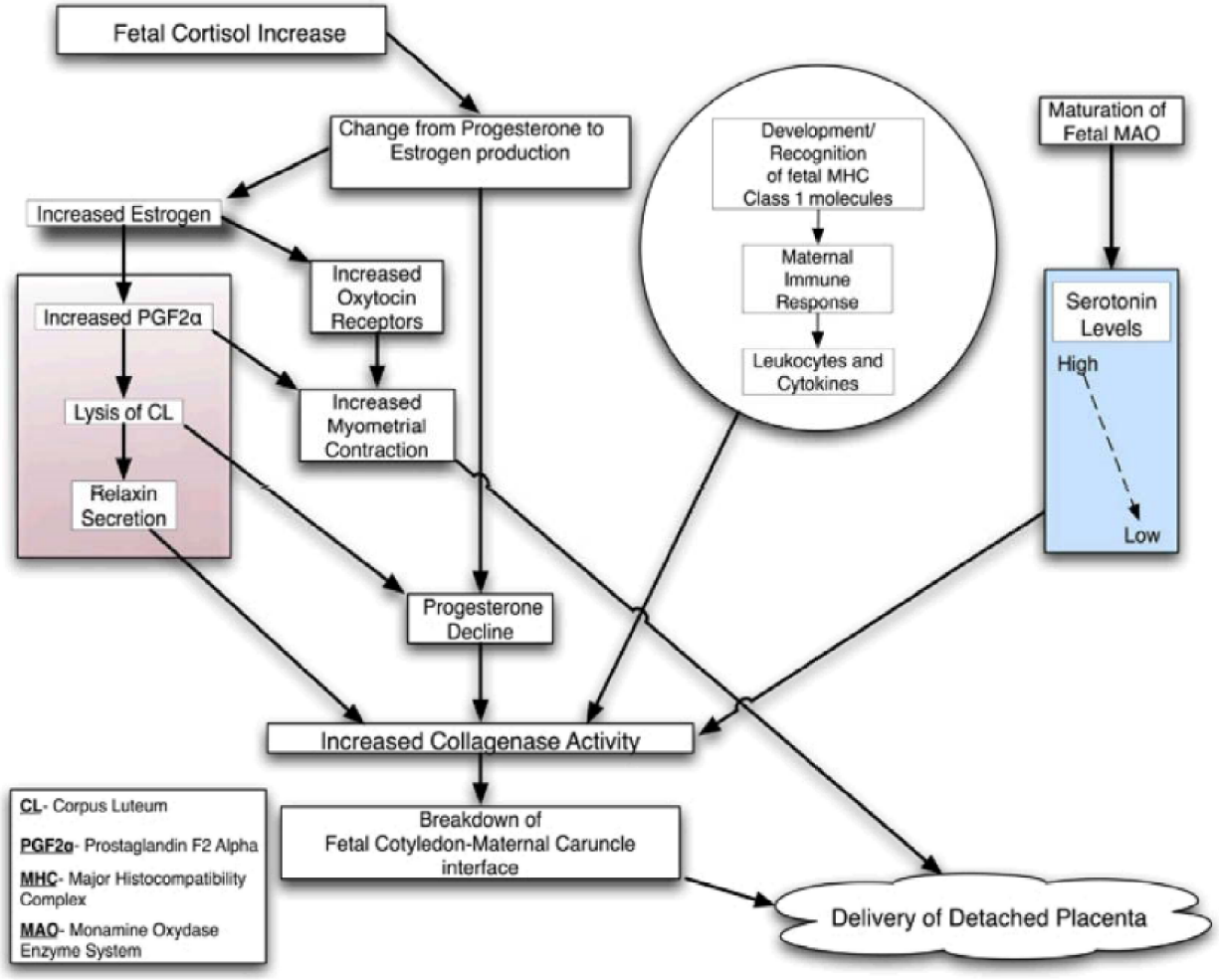


Figure 7 Physiologic processes leading to the detachment of the placenta in dairy cows. (86)

In order to reduce the incidence should be evaluated the main factors which influence the RP rate:

- Mechanical factors: difficult birth (dystocia), twins, stillborn, abortion.
- Nutritional factors: mineral and vitamin deficiency, low levels of vitamin E in the blood stream increase the risk of retained fetal membrane (86). low levels of calcium in blood.
- Management factors: stress, obesity; Negative energy balance(NEB) is associate with high NEFA prepartum as a result RP is more likely to occur.
- Infectious diseases: Brucellosis, Leptospirosis, IBR, BVD.

Metritis and endometritis:

Before parturition the uterine lumen is sterile and if bacterial invasion occurs, there is usually resorption of the fetus or abortion (87). During parturition, the physical barriers of the cervix, vagina and vulva are compromised providing the opportunity for bacteria to ascend the genital tract from the environment as well as the animal's skin and faeces (84). Bacterial infections of the endometrium can cause uterine disease and leading to decrease of productivity and fertility (88). The incidence of metritis varies between breed, country and herd, but in a study from 97,318 cows in the USA, the incidence of metritis, including retained placenta, was 21% (89). Another study reported that 16.9% of 1,865 cows were affected in Canada (90). Overall the incidence of clinical metritis is considered to be around 10 to 20%. Metritis usually occurs within 10 days after calving. It is characterised by an enlarged uterus and watery, red-brown fluid to viscous, off-white, purulent uterine discharge, which often has a fetid odour. The severity of disease is categorised by signs of health (as shown in figure n.8). -Grade 1: animals with an abnormally enlarged uterus and a purulent uterine discharge, but without any systemic sign of ill health. -grade 2: animals with an abnormally enlarged uterus and purulent uterine discharge, with addition sign of systemic illness such as decreased milk yield, dullness, and fever. -grade 3 sometimes called puerperal metritis or toxic metritis, uterus is abnormally enlarged, purulent discharge with signs of toxaemia. There are evidence that postpartum metritis caused subfertility by increasing the time to first insemination by 7.2 days, reducing conception rate to first insemination by 20%, and increasing the calving to conception interval by 18.6 days (91).

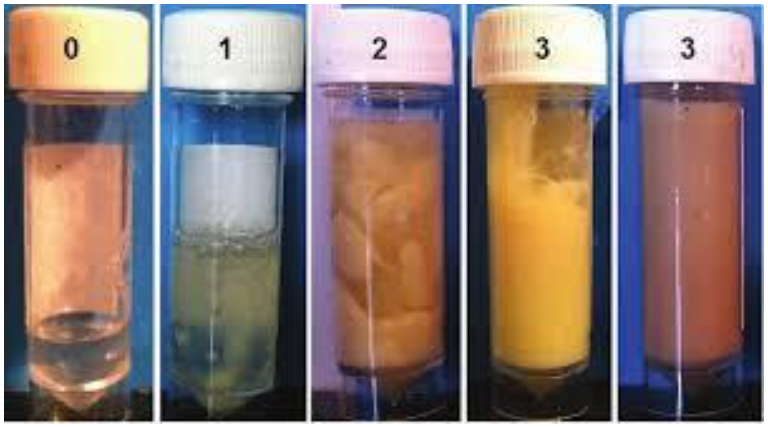


Figura 8 grading scheme for clinic endometritis (439)

Clinical endometritis is defined as endometrial inflammation occurring 21 days or more post parturition without sign of illness, and associated with presence of a purulent discharge detectable in the vagina. (85) The importance of subclinical endometritis has

emerged over the last 15 years, with the realisation that cytological evidence of inflammation of the endometrium is associated with reduced fertility (92) (93). Subclinical endometritis is characterized by inflammation of the endometrium that results in a significant reduction in reproductive performance in the absence of signs of clinical endometritis. Subclinical disease is defined by the proportion of polymorphonuclear neutrophils (PMNs) exceeding operator-defined thresholds, usually about 5% of cells in samples collected by flushing the uterine lumen or by endometrial cytobrush, in the absence of clinical endometritis, about 35 to 40 days post-partum (85) (94). The cause of subclinical endometritis is not yet clear, and may include resolving bacterial infections, immune-pathology without pathogenic bacteria, or even aberrations of postpartum tissue regeneration and repair. A direct negative effect of subcinical endometritis on embryo quality and survival has already been described (95), and associated with lower conception rates. In addition, results from various studies suggest that subclinical endometritis may be associated to altered patterns of prostaglandin E_2 and $F_{2\alpha}$ synthesis (96) (97) which could compromise luteal function and pregnancy.

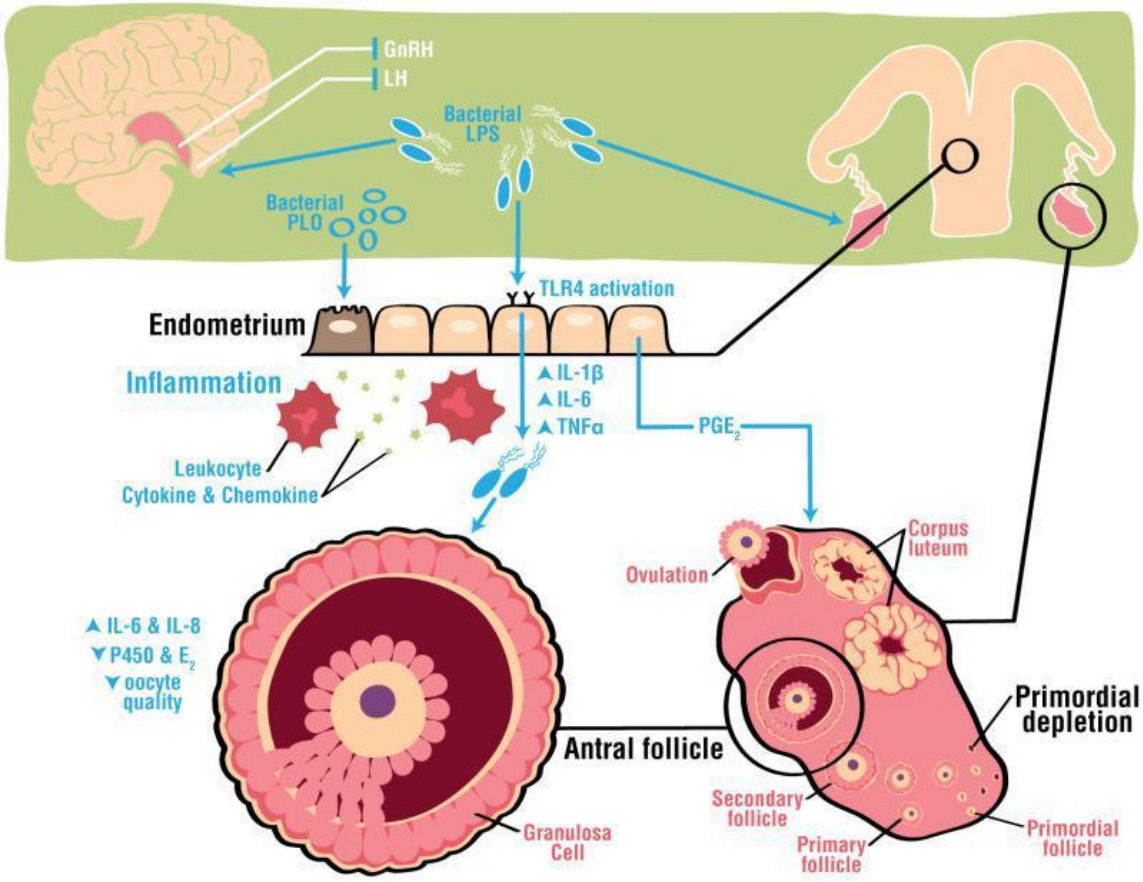


Figure 9 Uterine infection links with infertility in dairy cows (98)

Furthermore, Clinical endometritis increased the interval to first insemination by 11 days, and delayed conception by 32 days, compared with animals that did not have endometritis (99). An important observation for mechanisms that perturb fertility, is that postpartum uterine infection also impacts fertility after resolution of the clinical disease (99). Several mechanisms may underlie the wider effects of uterine infection on fertility, beyond the tubular genital tract. First, there is evidence that bacterial infections disrupt the endocrine signalling in the hypothalamic pituitary-gonadal axis, and the secretion of gonadotrophins (100). Secondly, uterine infections disrupt ovarian follicle growth and function, with smaller and less steroidogenic ovarian follicles (101). Finally, uterine infections may reduce oocyte quality, with increased rates of meiotic arrest and germinal vesicle breakdown failure (102). Examination of the effect of uterine infection on hypothalamic and pituitary function has focussed on the role of endotoxin, which is a component of Gram-negative bacterial cell walls. Indeed, endotoxin can be absorbed from the postpartum uterine lumen into the peripheral circulation and plasma endotoxin concentrations are increased in cows with spontaneous postpartum uterine infections (103) (104). Spontaneous uterine infection does not appear to alter the first postpartum transient increase in FSH concentration and follicle wave emergence (105). Endotoxin inhibits pulsatile LH secretion from the pituitary, suppressing hypothalamic GnRH secretion and reducing the pituitary responsiveness to endogenous or exogenous GnRH pulses. However, in some animals, endotoxin blocks the pre-ovulatory increase in peripheral plasma estradiol concentration even in the face of normal LH pulsatility (106). This observation suggests that there may also be direct effects of endotoxin on the ovary. Oocyte development lasts about 120 days, between the primordial follicle stage to ovulation of a cumulus-oocyte complex. Thus, in cows inseminated 60 to 120 days post-partum, the oocytes that are ovulated may have been exposed to pathogen molecules and inflammatory mediators throughout the postpartum period, if the animal had uterine disease. Thus, uterine disease can deeply compromise not only the health condition of the affected cow but also the quality of oocytes\embryos harvested the offspring genetic potential (71).

3-Heifers

As mentioned above heifers have been using more frequently compared with the past. The advantage of this practise is a higher conception rate (from 10% to 23% more) compared with cows (5). Heifers are not affected to all those metabolic and reproductive issues that usually afflict cows in lactation. On the other hand implanting an embryo into a heifers can hide some problem especially in beef, since most of cross-breed genetic lines are not selected for calving ease and therefore using those embryos in heifers may be a risk. Moreover, colostrum and milk production is fairly less in primiparous compared with multiparous cows. This aspect can be a trick especially in the beef industry where calf rearing is closely dependent on the mother supply. As a result the genetic potential of the offspring can be compromised also by poor energetic supply. Multiparous cows between 3 and 6 years are considered the most eligible category for receiving embryos since they have a better production of colostrum and milk, a well-known clinical history and they can be selected for docility (69). Anyhow, in order to choose good recipient also heifers have to be carefully evaluated (clinical story, vaccinations, genetic). The rearing management is paramount in order to obtain animals in good shape for the moment in which they will go through the MOET program or they will receive an embryo. Thus, excellent calf and heifer management ensure to get animals ready to be bred at right time with great fertility. In order to optimize fertility and milk yield heifers should be inseminated and get pregnant when they reach 55-60% of the mature body weight around 15 months of age. And furthermore, at calving the body weight should be 82-85% of the mature body weight. Doing so we avoid to over condition these animals decreasing the risk of dystocia and metabolic imbalance after calving, enhancing fertility and milk yield.

Embryo

An embryo is an organism resulting from the fertilization of an oocyte with a sperm cell, and which is in the first stage of development. Once embryos are collected, they are morphologically evaluated by means of a stereomicroscope, and finally grade according with stage of development and embryo quality (as shown in table n.1) before be transferred into a recipient or be frozen. Visual observation of embryos is very important for differentiate unfertilized ova (UFO) from embryos, to detect eventual abnormality in the embryo integrity and evaluate the stage of development (107).

Stage of development	
Code	**stage**
1	Unfertilized
2	Two- to 12-cell
3	Early Morula
4	Morula
5	Early blastocyst
6	Blastocyst
7	Expanded blastocyst
8	Hatched blastocyst
9	Expanded hatched blastocyst

Quality of embryos	
code	**Description**
1	Excellent or good
2	Fair
3	Poor
4	Dead or degenerating

Tabella 1 stage of development and embryo quality assessment according with IETLS classification (108)

The bovine embryo's diameter is around 150 and 190 µm, the size doesn't change from zygote's formation to blastocyst stage. Fertilization happen into the oviduct and embryos reach the uterus not before the day 5 after heat. Therefore, flushing in donor is performed between day 6 ½ e il 7 ½ after estrus, at this point we expect to collect embyos in a stage of development between stage 4 (morula) and stage 6 (expanded blastocyst) (12). Big difference in pregnancy rate in embryos collected between days 5 to 8 post heat has not been observed (57) (109). Rather than that, has been demonstrated a great decrease in pregnancy rate associated with a progressive thinning up to hatching of the zona pellucida (57). Age and stage of development are closely liked, indeed, fresh embryos

(in vivo-derived) from stage 4 to stage 7 have greater pregnancy rate compared with embryos in stage 8 (57). Whereas, embryos obtained in vitro produced (IVP), seem to be more stage sensitive. As a result, in IVP embryos the conception rate is higher transferring embryos in a more advance stage of development (expand blastocyst) than using embryos in morula or early blastocyst stage (110) (111). Embryos are made of a glicoprotein outter layer called zona pellucida (ZP) which embraces the cytoplasmatic membrane underneath. Inside, the blastomeres organised themselves in a structure called inner cell mass (ICM) and trophoblastic cells. The ICM will turn into a fetus whereas the trophoblast will constitute the placenta and other embryo's accessory structures. As follow a detailed description of zona pellucida and its interaction with pathogens is provided.

The zona pellucida (ZP) is a barrier against viruses and other microorganisms. For embryos with an intact ZP, viral infection of the embryo is unlikely to occur. However, the virus may stick to the ZP and, in this case, International Embryo Transfer Society (IETS) washing procedures in combination with trypsin treatment are mandatory. A caveat is the fact that currently more and more types of embryos are becoming available for transfer and scientific data cannot be extrapolated from one species to another (112). The bovine ZP is a 12µm-thick acellular matrix that surrounds the oocyte and the early embryo. It is composed of three major sulfated glycoproteins generally designated as ZP1, ZP2 and ZP3 (113). The ZP becomes progressively thinner during embryo develop until the blastocyst hatched around the 9th day after fertilisation. A complex fibrous network containing numerous pores is the matter which composed the ZP, its acellular nature and the lack of

cellular receptor avoid the penetration and the attachment of pathogens.

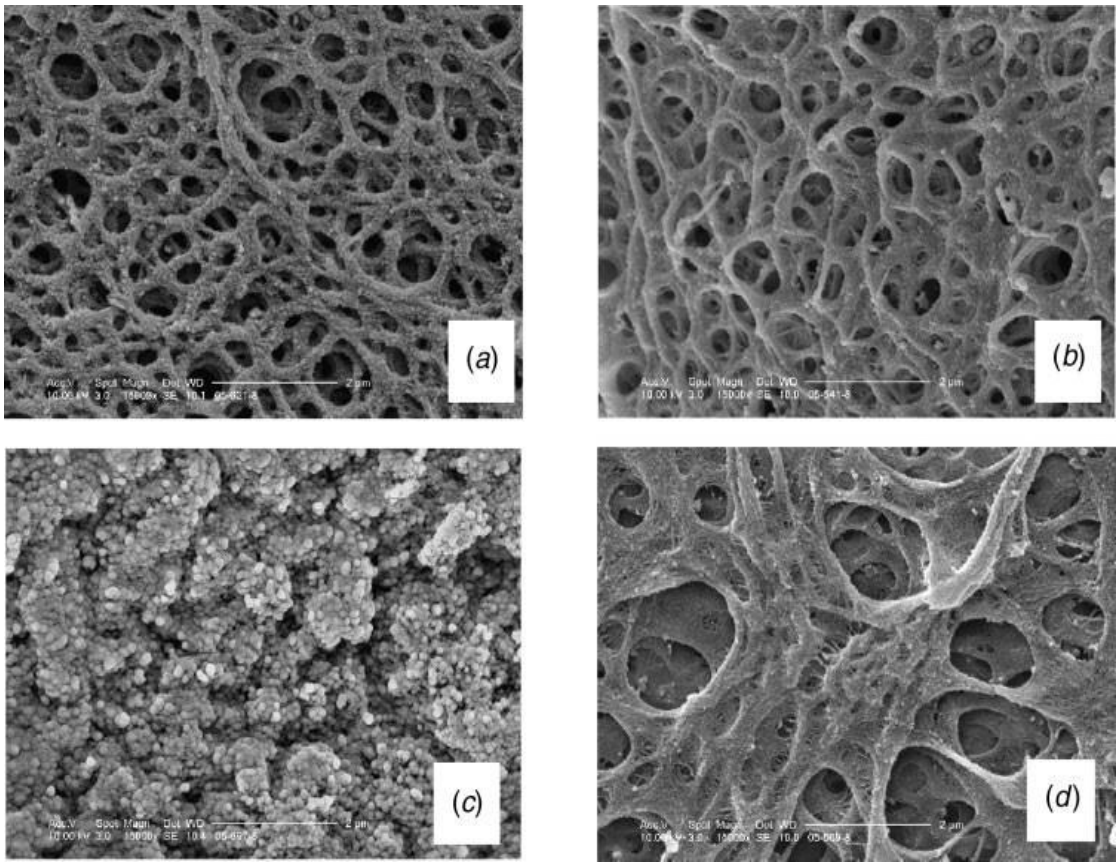


Figura 10 Scanning electron microscopic images of bovine zygotes (a, b) and blastocysts (c, d), derived in vivo (a, c) and produced in vitro (b, d). Note the increased pore size in the in vitro-produced blastocysts compared with the zygote stage and the granular material covering in vivo-derived blastocysts. (112)

The pores present in the matrix are communication channels used by granulosa cell and oocyte prior to ovulation. Later, the structural changes of the ZP tend to obliterate these pores (figure 10), although not completely (115). Viruses can reach the cellular membrane of the embryo, either by passing through the ZP or because the ZP has been lost, removed or damaged at the time of infection. A specific receptor for the virus must be present on the cellular membrane of the cell to be infected (116). The absence of one or more receptors on embryo cells will prevent entry of the virus into the cell. Finally, the virus needs an active intracellular mechanism in order to replicate its genome and to produce viral proteins. Indeed, small pathogens, mainly viruses, can potentially penetrate the ZP through its pores before, during or after fertilization. Certain RNA viruses, especially *Oncornavirus*, have been shown to penetrate the ZP, particularly before or during fertilization, and appear to be vertically transmitted (112). Regarding cattle embryo this event seems to be unlikely due to the resistance of the ZP against pathogens penetration.

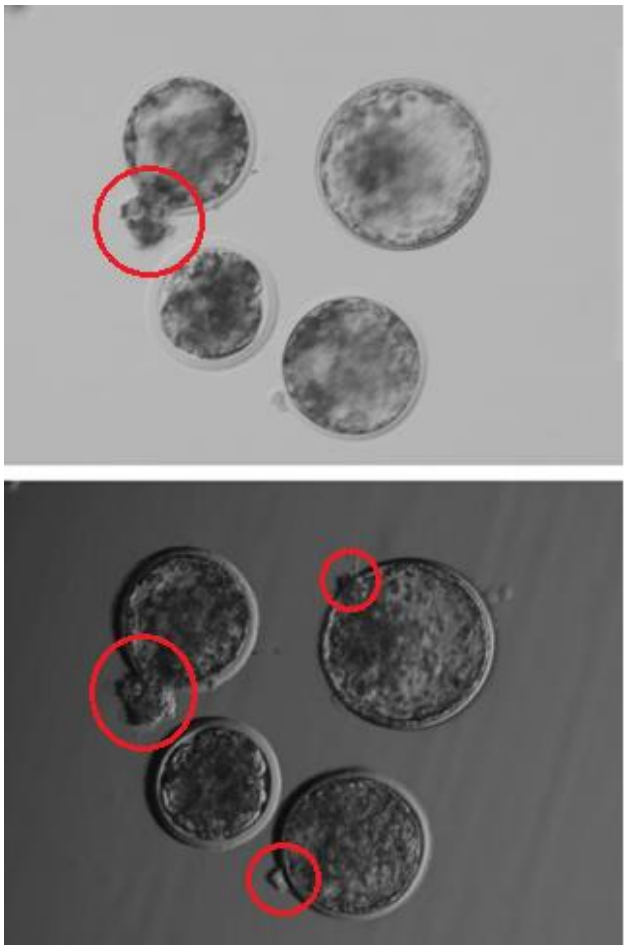


Figura 11 Trophoblastic cells extruded through the zona pellucida of very expanded bovine blastocysts may not be visible with conventional light microscopy (a), but can usually be seen when viewed by differential interference contrast microscopy (b). (112)

A study found Bovine Viral Diarrhea Virus (BVDV) inside the oocyte of Persistently Infected (PI) cattle, suggesting that this is a disease that could be vertically transmitted. On the other hand, Brock et al, have shown that in vivo embryos from PI cows can be effectively washed free of BVDV and can be safely transferred to recipients without seroconversion (117). The possibility of the embryo becoming infected after fertilization seems to be less likely due to the changes in the ZP after fertilization in vivo, additional proteins become associated with the ZP at that time. Interestingly, this does not hold true for some enteroviruses of 20-30nm in diameter that can readily penetrate the ZP of mouse morula. This finding suggests that factors, with regard to ZP-pathogen interaction, other than size, seem to be important, since not all similar size viruses can readily cross the ZP. A possible explanation for penetration of pathogens at more advanced stages of development arises from a publication of Gonzales et al, who showed that there are cytoplasmic extensions of the trophectoderm cells through the ZP prior to hatching (118). Recently has been observed that expanded blastocysts show protrusion of trophoblastic cells and/or microvilli through the ZP (figure 11), which cannot be seen with a conventional light microscope; the use of differential interference contrast microscopy or polarisation microscopy can be beneficial in this respect (112). An additional question mark emerged when analysing horse embryos has been discovered that the trophectoderm can penetrate through the ZP and releases an outer embryonic coat which hasn't the same characteristics as ZP (112). If penetration does occur, degeneration of the embryo is the likely outcome with most pathogens. The importance of integrity of the ZP is highlighted by the

International Embryo Technology Society (IETS) (119) recommended protocol for successful removal of most bovine pathogens from the ZP of in vivo produced embryos. According to the Society, only ZP intact embryos should be washed and treated with trypsin and the ZP integrity should be confirmed before and after washing (120). Bear in mind that viruses and bacteria can become firmly attached to the ZP, as it is showed (table 1), microorganisms have different affinity to the ZP (121). The exact mechanism that underlies the interaction between pathogens and the ZP is poorly understood. The irregular surface of the ZP may entrap virions and bacteria or pathogens can enter the external pores and become entrapped further inside the ZP (122). Bacteria can only stick to the ZP due to their size. Brucella abortus for instance, was not isolated from ZP-intact or ZP-free groups of bovine embryos after 10 sequential antibiotic-free washings but all groups containing ZP defective embryos were positive (123). Similarly also viruses such as BVDV and BHV-1 should not be able to penetrate an intact ZP and reach the embryonic cell (115), even though the risk that virions can be embedded in the outer layer of ZP exists (122). Treatments to remove pathogens from ZP surface are several. The first and still in use consists in washing the embryos at least 10 times in a buffer solution such as Dulbecco's phosphate buffered saline (dPBS). Washing the embryos allow to reduce/eliminate the embryo's contamination and to remove the donor's fluids present in the flushing; useful for diseases such as Bovine Leukosis which are mediated by presence of somatic infected cells in the flushing. Indeed, this procedure lead to produce seronegative calves from seropositive BLV donor cow. IETS recommends this protocol in their guidelines (108), etablishing 10 washes each of which is dPBS 100-fold diluted. Some pathogens such as BHV-1, BHV-4, Vescicular Stomatitis Virus(VSV), H.*somnus* and U. *diversum*, are firmly attached to the ZP, so only washing is not sufficient. Other treatments have been developed consist in the use of trypsin and antibiotics. Trypsin treatment is performed 10 times, intercalated with the standard wash, this allow to remove viruses such as BHV-1, BHV-4 and VSV. Trypsin probably works digesting some proteins exposed in the virus envelope or changing the receptor conformation present in the ZP surface. Anyway, has been proved that trypsin treatment erodes the ZP; since trypsin is a small globular protein (23-24 kDa), of approximately 4nm (124) which may enter through ZP pores. However, substantial data gathering over many years assure scientists that any adverse action of trypsin on the embryo itself is negligible. *Mycoplasma spp.*, H.*somnus* and U. *diversum* are not sensitive

to trypsin treatment (108), so antibiotics addition is required. Conversely, pathogens like BHV-1 cannot be removed completely by standard washing and trypsin treatment in IVP embryos (125). This fact underlines how the recommendation of IETS for removing certain pathogens from in-vivo produced embryos may sometimes not be adequate in vitro produced embryos (122). The addition of antibiotics in the holding media is useful to control bacteria contamination and prevent transmission of bacteria diseases to the embryos and recipient. Has been proved that pathogens such as *H.somnis* can be efficiently eliminated by using broad spectrum antibiotics in the holding media during IVP embryo procedure (126). Concerning viruses contamination and transmission; the use of monoclonal antibodies has been tested for inactivating BHV-1 in oocytes exposed during IVM operations. This procedure yielded 88% of the embryos free of the virus whereas all the controls were positive (127).

Table2: *Characteristics of the association between several viruses and the zona pellucida of bovine embryos (121)*

Pathogens	**Association**
Parainfluenza-3 (PI-3)	None
Bovine enterovirus	None
Foot and mouth disease virus (FMDV)	Loose
Akabane virus	Loose
Bovine leukemia virus (BLV)	Loose or none
Bluetongue virus (BT)	Loose
Bovine viral diarrhea virus (BVDV)	Loose
Bovine herpes virus type 1 (BHV-1)	Firmly attached
Bovine herpes virus type 4 (BHV-4)	Firmly attached
Vesicular stomatitis virus (VSV)	Firmly attached

Table 3:*Characteristics of the association between several bacteria and the zona pellucida of bovine embryos*

Pathogen	**Association**	**Reference**
Brucella abortus	Not attached or loosely attached	(123)
Haemophilus somnus (H. somuns)	Attached	(126)
Ureaplasma diversum	Attached	(128)
Leptospira	Attached	(129)
Mycoplasma bovis/ bovigenitalum	Attached	(130)
Mycobacterium paratuberculosis	Attached	(131)
Campylobacter fetus subsp venerealis	Attached	(132)

As follow, the list of pathogens with recommendations regarding the risk of disease transmission via in vivo derived embryos. Based on the conclusions of the IETS, the following diseases and pathogenic agents are categorised into four categories, which applies only to in vivo derived embryos (108). There is no standard procedure for in-vitro technique that can ensure a zero/low risk of transmission. Therefore, in pathogen's chapter will be discuss also this subject.

1. Category 1

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 in accordance with the Manual of the IETS.

The following diseases or pathogenic agents are in category 1:

– Bluetongue (cattle)

– Bovine spongiform encephalopathy (cattle)

– Brucella abortus (cattle)

– Enzootic bovine leukosis

– Foot and mouth disease (cattle)

– Infection with Aujeszky's disease virus (pigs): trypsin treatment required

– Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis: trypsin treatment required

– Scrapie (sheep).

2. Category 2

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

The following diseases are in category 2:

– Bluetongue (sheep)

– Caprine arthritis/encephalitis

– Infection with classical swine fever virus.

3. Category 3

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 in accordance with the Manual of the IETS, but for which additional in vitro and in vivo experimental data are required to substantiate the preliminary findings.

The following diseases or pathogenic agents are in category 3:

– Atypical scrapie (not a listed disease)

– Bovine immunodeficiency virus (not a listed disease)

– Bovine spongiform encephalopathy (goats) (not a listed disease of goats)

– Bovine viral diarrhoea virus (cattle)

– Campylobacter fetus (sheep) (not a listed disease of sheep)

– Foot and mouth disease (pigs, sheep and goats)

– Haemophilus somnus (cattle) (not a listed disease)

– Infection with rinderpest virus (cattle)

– Maedi-visna (sheep)

– Mycobacterium paratuberculosis (cattle)

– Neospora caninum (cattle) (not a listed disease)

– Ovine pulmonary adenomatosis (not a listed disease)

– Porcine circovirus (type 2) (pigs) (not a listed disease)

– Porcine reproductive and respiratory disease syndrome (PRRS)

– Swine vesicular disease (not a listed disease).

4. Category 4

Category 4 diseases or pathogenic agents are those for which studies have been done, or are in progress, that indicate:

that 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 in accordance with the Manual of the IETS between collection and transfer.

The following diseases or pathogenic agents are in category 4:

– African swine fever

– Akabane (cattle) (not a listed disease)

– Bovine anaplasmosis

– Bluetongue (goats)

– Border disease (sheep) (not a listed disease)

– Bovine herpesvirus-4 (not a listed disease)

– Chlamydia psittaci (cattle, sheep)

– Contagious equine metritis

– Enterovirus (cattle, pigs) (not a listed disease)

– Escherichia coli 09:K99 (cattle) (not a listed disease)

– Infection with equid herpesvirus 1 (Equine rhinopneumonitis)

– Infection with equine arteritis virus

– Leptospira borgpetersenii serovar hardjobovis (cattle) (not a listed disease)

– Leptospira sp. (pigs) (not a listed disease)

– Lumpy skin disease

– Mycobacterium bovis (cattle)

– Mycoplasma spp. (pigs)

– Ovine epididymitis (Brucella ovis)

– Parainfluenza-3 virus (cattle) (not a listed disease)

– Parvovirus (pigs) (not a listed disease)

– Q fever (Coxiella burnetii)

– Scrapie (goats)

– Tritrichomonas foetus (cattle)

– Ureaplasma and Mycoplasma spp. (cattle, goats) (not a listed disease)

– Vesicular stomatitis (cattle, pigs) (not a listed disease).

Pathogens assessment

virus

1. Bovine Viral Diarrhea Virus (BVDV)

Introduction: Bovine viral diarrhea is disease of cattle and other ruminants that is caused by the bovine viral diarrhea virus (BVDV). BVDV is a member of the Pestivirus genus, family *Flaviviridae*. It is considered to have 3 genotypes: BVDV-1, BVDV-2, BVDV-3 and many sub-genotypes. Bovine diarrhea virus (BVDV) causes a variety of economically important enteric, respiratory and infertility problems. For that reason, several countries have eradicated the disease and some others have schemes in progress to achieve freedom from it. The risk assessment of transmitting BVDV by transfer of an in vivo-derived embryo has been evaluated by IETS This virus is currently listed as a 'category 3' agent. ''Category 3 diseases or pathogenic agents are those for which preliminary evidence indicates the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer according to the IETS Manual, but for which added in-vitro and in-vivo experimental data are required to substantiate the preliminary findings''

Semen: BVDV can be transmitted by natural services or artificial insemination (AI), indeed the use of infected semen results in a higher fertilization failure and early embryonic mortality (133). Noncytopathic strains are of particular concern since semen and embryo with BVDV can lead to the birth of animals persistently infected (133). It has been demonstrated that some bulls, despite being seropositive but non-viremia, can shed the virus in the testes and spread disease by AI (134) (135). In a study accomplish by Bielanski et al, seronegative cows have been bred with contaminated semen. Viremia has been detected in the donors around day 7 after insemination, even though seroconversion occurs only 3 to 5 weeks after AI. A proportion of the flushed embryos resulted contaminated (133). Nonetheless all embryos when washed according to IELS and OIE guidelines, do not cause transmission to recipient or their offspring.

Donor: Has been proven the presence of BVDV antigens within the ovarian stroma in ovaries collected from persistently infected (PI) heifers (136). On the other hand, Brock et al, have shown that in vivo embryos from PI cows can be effectively washed free of BVDV

and can be safely transferred to recipients without seroconversion (117). In experimentally infected cows, BVDV was found in: follicles, follicular fluid and oocytes (137).The presence of the virus has been also proven in acutely infected animals, causing a reduction in ovarian functionality and overall fertility (138). In infected cows the virus shed inside macrophage-like cells and stromal cells in the ovarian cortex for up to 8 days post infection, moreover in the study in object oophoritis have been shown throughout the 60 days study period (139). This suggest a possible chronic infection also in non-persistent infected cows, leading to a reduction of fertility and a possible risk in virus transmission. We have to underline the fact that virus antigen has also been detected in ovaries of cows immunised with a modified live BVDV vaccine (140). Interestingly, follicular fluid of immunized cows can contain BVDV-neutralising antibody (141), it seems that antibody is able to protect cumulus cells and oocytes from infection, as survey by Galik et al. (142).In the trail virus isolation on 55 follicular fluid pooled samples from slaughterhouse ovaries failed to detect BVDV, ostensibly because of presence of antibody (142). Tests using RT-PCR detected only one sample positive for one serotype. Each sample of follicular fluid contained sufficient antibody to neutralize large quantities of BVDV1 and BVDV2. The authors concluded the low level of detectable BVDV to be due to the presence of neutralising antibodies in the follicular fluid. They also noted that identifying the virus in 1.8% (1 of 55) of samples was statistically consistent with other studies reporting that the prevalence of BVDV in pooled abattoir-derived material ranged from 0.9 to 12%.

Recipient: In a study of Gard JA et. al. embryos collected by a cow free from BVDV were placed into transfer media containing BVDV (SD-1; type 1a) and transferred into the uterus of 10 seronegative recipients. As a result, all the recipients seroconverted within 15 days. At day 30 after the transfer 6 of 10 heifers were pregnant, however 30 days later only 1 was still pregnant and the fetus was nonviable and positive for BVDV. Therefore, recipients receiving contaminated embryos can result in viremia and seroconversion after transfer (143). The virus also infects ovaries of acutely infected cows, affecting ovarian function and reducing fertility (144). Failure to conceive, early embryonic loss and stillbirth can all sequelae of infection (145). Whether infection in recipient occurs in mid-gestation, it may result in the birth of calves that are congenitally deformed or persistently infected.

IVP: Ovaries may be infected as a result of infection in the cow or be contaminated by handling in the abattoirs. Transmission of BVDV should be carefully assess in IVP technique since the procedure recommended today do not ensure a biological zero risk. Moreover, is not completely clear why pathogens including BVDV are more likely to adhere in ZP of embryos produced by IVP than in vivo derived. Yet, IETS standard washing procedure(common to both practices) was ineffective in removing BVDV from the embryos, even after a brief exposure to the virus (146) (147). Even regular washing of oocytes/zygotes during the IVP process, denuding the zygote of cumulus cells after IVF, and washing embryos according to IETS procedures did not always remove the virus from embryos derived from acutely infected cows, even when the co-culture of oviductal cells were kept BVDV-free (148). Gard J.A et al. tried to determine the amount of bovine viral diarrhea virus associated with single in vivo-derived and in vitro-derived embryos following the recommendation of IETS. Both the batches have been artificially exposed for 2h to $2x10^{(5-7)}$ cell culture infected dose (CCID(50))/ml of SD-1 (a noncytopathic, type 1a strain of BVDV), and then washed according to international Embryo Transfer Society (IETS). 27% of in vivo-derived embryos were positive for virus meanwhile 42% of in-vitro derived embryo were positive. Therefore, although many embryos were positive for virus, there were limited numbers of copies, thereby posing doubt regarding their potential for contamination following embryo transfer (149). Another experimental trail using two non-cytopathic biotypes, either NY-1 (type 1) and PA-131 (type 2), washed 10 times, and transferred into recipients free of BVDV and its antibody, found a potential risk for transmission of the BVD by the means of in vitro technique. Embryos can carry the virus and infect the recipient, causing sometimes viremia and seroconversion in these animals. Furthermore, embryo vitality and the end term pregnancies decrease dramatically compared with the control. Transferred embryos exposed to type 2 BVDV were associated with a titer of virus sufficient to induce a systemic serological response in 45% recipients after the first ET attempt. Moreover, transfer of the embryos exposed to the PA-131 strain caused more pregnancy failures (51% vs. 30%) and resulted in more seroconversions among recipients (23% vs. 0%) than the NY-1 strain. However, all term pregnancies resulted in calves free of both virus and antibody. (150). Despite multiple reports of embryo transfer to produce BVDV-free calves, in vitro study using noncytopathic isolates of BVDV indicated that embryo washing procedures might not be equally effective for all isolates of BVDV

(151). Some embryos artificially exposed to specific high affinity isolates of BVDV might retain a small amount of infectious virus even after proper washing or trypsin treatment. Hazard materials that have to be consider for transmission of BVDV during the IVF process include: ovary, follicular fluid, cumulus cells, oocytes, uterine tubal cells and serum (114). Because somatic cells used in IVM, IVF and IVC are susceptible to infections with BVDV, a small amount of viral contamination can be amplified during the course of embryo production resulting in exposure of embryos to large quantities of infectious virus by the end of IVC. The use of antiviral products (such as DB606) added in IVC can efficiently avoid proliferation of BVDV and don't interfere with embryo develop; a study of Givens et al (152) demonstrated normal development of bovine zygotes after they were exposed to 0.4mM DB606 during 7 d of IVC; resulting heifers had normal characteristics during puberty, breeding, gestation, and lactation. Fetal calf serum can contaminate the IVP process. Heat treatment was not sufficiently effective in removing infectious BVDV. In a study of Gard JA et. al. (already reported above) embryos collected by a cow free from BVDV were placed into transfer media containing BVDV (SD-1;type 1a), and transferred into the uterus of 10 seronegative recipients. As a result all the recipients seroconverted within 15 days. At day 30 after the transfer 6 of 10 heifers were pregnant, however 30 days later only 1 was still pregnant and the fetus was nonviable and positive for BVDV. Therefore, recipients receiving contaminated embryos can result in viremia and seroconversion after transfer (143). Despite the large number of in vivo-derived, bovine embryos that are transferred annually worldwide, some reports liked transmission of BVDV to embryo transfer (153) (154). In both reports, the use of contaminated fetal bovine serum in embryo transfer media was mentioned as a possible source of the virus. Although the source of the virus could not be defined in either case, these reports highlight the need to consider 'materials of animal origin' used in embryo collection and transfer as well as donor, embryo and recipient health when trying to produce embryo transfer progeny that are free of BVDV. (155).

Conclusion: transmission of BVDV by embryo transfer is controversial; in the E.T process there are many steps which can be a potential source of infection. Semen is a risk for donors and decrease the fertilization rate even though it seems unlikely to lead to the birth of infected calf. Donors can shred the virus in many organs including ovaries, antibodies can

mask the presence of the virus and increase the risk of transmission with a particular regard to non-cytopathic strain. Moreover, also live vaccine can persist in these organs and eventually be transmitted. Whether carefully managed embryos from PI cow can be transfer without affecting recipients and calf. Nonetheless, embryos can carry the virus which and be transmitted in recipients, resulting in seroconversion and high pregnancy failure, apparently the offspring is BVD naïve at birth. The in-vitro technique presents many hazards, is not easy to assess the risk carried by 'material of animal origin' employed in the procedure, anyhow infected media or tissues can lead to embryo's contamination. Oocytes collected from slaughterhouse are of more concern, adding antiviral such as DB606 in culture media seems to protect embryos from infection, in spite of this, more studies and research need to be done in order to develop safe guidelines for both in-vivo and in-vitro techniques.

2.IBR:

Introduction:The Bovine herpesvirus type 1 (BoHV-1) is a dsDNA virus equipped with envelope. It belongs to *Varicellovirus* genus, subfamily *Alphaherpesvirinae*, family *Herpesviridae*. BoHV completes its life cycle inside the cell where typical inclusion can be found at nucleus level, whether or not they are associated with formation of giant cells. The role of door opener is not just fulfilled by a direct damage towards the host but also by the means of immunosuppression. BoHV-1 can induce apoptosis of many immune cells, affects the functionality of major histocompatibility complex (MHC) and interferes with the release of interferon y (INFy). Indeed, BHV has many mechanisms to evade the hosts' immune systems involved in both innate immunity and adaptive immunity. After primary infection of BoHV-1, the latent infection is quite often found in the cranial and dorsal root ganglion of the cow, until the infection reactivate again also after many years from the primary infection or the last fresh outbreak (156) (157). BoHV-1 is classified in list B by OIE, among transmissible diseases with an important social and economic impact. Moreover, there are 3 genotypes: BoHV1.1 responsible for rhinotracheitis, conjunctivitis and abortion, BoHV1.2a has been associated to vesicle or pustule-producing infections of the reproductive tract known as "infectious pustular vulvovaginitis" (IPV) or balanoposthitis (IPB) as well as other forms of reproductive failure and BoHV-1.2b which seems to be the less virulent type (158),according with Miller it doesn't cause abortion after experimental

infection (159). Some BoHV-1 strains appear to have more affinity for the reproductive tract than others (160). Although epidemiological data strongly suggest that BoHV-1.1 and -1.2 strains differ in the induction of clinical disease, the basis for such differences in the relationship host/parasite remain unclear (161) (162). Both subtypes are able to infect respiratory and genital tract of cattle, however, it has been suggested that each genotype is better adapted to either respiratory (BoHV-1.1) or genital (BoHV-1.2) tract (163) (161) (162). Even though they have different tropism, the transmission happens through the same ways. The main mode of disease transmission is direct nose-to-nose contact between an infected and a susceptible animal. This is made possible because of the virus sloughing off into the mucus. Aerosols have to be exhaled, sneezed, or coughed from an infected animal during viral shedding in order for transmission to occur (164). Transmission also originates from: contaminated semen through use of live breeding or AI; bulls that have been affected genitally may shed the virus in their semen. By infected mucus from genital tract and by vertical transmission after mother's viremia. Beside in the U.S.A. has been proved the transmission by means of ticks bite (*Ornithodorus coriaceous)* infected with BoHV-1 (165). Viruses antigenically related to BoHV-1 have also been isolated from several ruminant species including red deer, reindeer, mule deer, pronghorn antelope and wildebeest. Buffalo and wildlife may play an important role in the maintenance of the infection (166)

Semen: BoHV-1 infection in bulls leads to replication of the virus at the preputial level, as a consequence, contamination of sperm occurs. According with (167) the foreskin is the main replicative site of the virus rather than testicles parenchyma, epididymis and seminal vesicles. The excretion with the semen is high titer, up to 10^8 TCID50 $ml_{-1.}$ After primary infection in bull, virus can be intermittently recovered in the sperm (168). Reactivation is often unpredictable and associated with stressful events (169) (170) such as: shipping (171), superinfection by IP-3 virus (172) or *Dictyocaulus viviparous* (173), use of corticosteroids. Moreover, have been reported cases where spontaneous sporadic reactivation has not been followed by clinical signs (174) (175). Infected bulls can spread the virus for few days or for many week (167). Both fresh and frozen infected semen represent a real risk (176); the virus survives in the sperm over 1 year after it has been frozen (temperature -176°C) (177). In order to avoid the spread of virus in the population,

bulls in AI centres should be certify IBR free. The most sensible test to identify infected, excreted bulls is represented by PCR in the semen instead of isolation (178). The transmission is possible by natural service and artificial insemination, infected bulls can spread the virus and infect others animals by semen or by direct contact with other animals (179). Once the bull is infected, it is considered to shred and excrete the virus for ever. Common international legislation for bulls in AI centres imposed the use of IBR seronegative animals. Introduction of young animals from infected farms into AI centres may lay several traps. It is possible an early latency in calves drinking colostrum of seropositive cows and in which infection occurs during or after calving. The presence of neutralizing antibody in the colostrum protects calf from viremia and do not allow the develop of an immune response, however infection can happen and the virus can hide into the host since protected antibodies are washed out from the bloodstream. Therefore, the calf can be infected and don't develop an active immune response. Herpesvirus can shred into the host and come out after a long time, as a consequence animals after be tested at six months of age (before be shipped to the centre), may show neither the presence of antibodies and virus in the bloodstream, hence the recognition of infected calves can failed. Emblematic was the case of ANABIC S. Martino in Colle (PG), were a seronegative calf had been introduced into the AI centre and after reactivation of the virus, the pathogen has been spread to all bulls in the building causing the culling of many high genetic value bulls. Furthermore, Kupferschmeid et al. reported seroconversion of cattle in Switzerland after AI with imported semen from a donor bull that was seronegative at the time of semen collection (180).

Donor: Semen contaminated with BoHV-1 can cause in the donor vulvovaginitis (IVP), inflammation of the cervix and endometritis with abundant vaginal discharge. Whether BoHV-1 infection becomes established after a cow is inseminated with contaminated semen depends on the properties of the virus strain and the amount of virus in the semen straw. With a regard to the use of infected semen, it can lead to reproductive and health issues in inseminated cows such as: endometritis, low conception rate and short estrus length (181) (160) (182). The presence of the virus has been proved in ovaries, corpus luteum and cervix of heifers experimentally infected (183). IBRV has been detected from the 4th day after infection in blood, nasal and vagina discharge in heifers recruited in similar

trail. Ovariohysterectomy (OHE) in infected animals confirmed the presence of virus in the ovaries, the histological exam showed presence of focal necrosis, infiltration of mononuclear leukocytes and haemorrhagic areas (184). Studies in Australia showed that when cows were inseminated with doses higher than $10^{5.3}$ $TCID_{50}$ they invariably became infected (181), (185) but only 6 of 25 cows seroconverted with doses below 200 $TCID_{50}$ (174). In another study two seronegative cows were superovulated and inseminated with semen seeded with $TCID_{50}$ $10^{4.5}$/0.1 ml IBRV. One cow produced 2 non-developing virus free embryos, the other failed to conceive. Nearly all the reproductive organs contained significant amount of virus (186).

Recipient: Several studies have been carried out in which in vivo-derived, zona pellucida-intact embryos have been collected from BoHV-1 infected donor cows approximately 7 days after fertilisation. Some of the cows had been experimentally infected by intrauterine or intranasal inoculation at or close to the time of fertilisation while others were naturally infected or seropositive embryo donors. The collected embryos were either tested for the virus in vitro or transferred into seronegative recipients. In other instances, in vivo-derived embryos were exposed to BoHV-1 in culture, washed with or without trypsin washes (as per IETS Manual protocols), then assayed for presence of the virus. In all these studies, provided that trypsin washes were included in the protocol, the results, so far as presence of virus on the embryos, or transmission to recipients and their offspring, were concerned, were negative (9). In big survey embryos were collected from dairy herds in the U.S.A. where approximately 95% of the cattle were seropositive from IBRV (based on 89 sera sampled from 89 donor cows). These cows yielded 750 embryos which were shipped to France and 600 of them were transferred to seronegative heifers. Two hundred and fifty calves were born. None of the recipients seroconverted, although the embryos were not trypsin-treated (187). Also Singh et al (188) after experimental infection of donors, collection and washing of embryos; transferred embryos into 49 seronegative recipients. The survey ended up with the birth of 20 live calves, 5 stillbirths and 1 abortion. All calves and recipients have been seronegative throughout all the trail. We can claim that IETS and OIE procedures if strictly follow can effectively avoid transmission of BoHV-1 by embryo transfer.

IVP: Introduction: Other experiments to evaluate the risks of BoHV-1 transmission have been done with in vitro fertilised (IVF) by means of infected semen and/or exposure of oocytes to the virus at the time of fertilisation. Guerin et al. studied the effect of BoHV-1 on groups of oocytes that were exposed to the virus during maturation and fertilisation, then washed 10 times before being tested for presence of virus. The virus appeared to have no effect on oocyte maturation but significantly reduced the IVF rate to 65%, compared to 85% in controls. It also led to an increased level of sperm decondensation abnormalities (49% as compared to 4% in controls). The authors concluded that BoHV-1 was not only absorbed onto the gametes but it also impaired their ability to undergo IVF, possibly due to an effect on sperm penetration or an interaction with the intracellular fusion mechanisms (189). Ten years later Vanroose et al (190) found a 60% reduction in numbers of sperm (previously exposed to BoHV-1) bound to the zona pellucida compared to the control. They also noticed some mononuclear antibodies (anti-gC and anti-gD) interfered with the inhibition of sperm-zona binding that was caused by the virus. The authors suggested that molecules on the surface of the sperm cell plasma membrane may act as receptors for the BoHV-1 glycoproteins gC and gD, and, when sperms have viruses attached to their surface in this way, their ability to attach to the zona pellucida is impaired. The same researchers found more recently evidence for the involvement of gC and gD in the virus-sperm interaction. According with the autor purified gC and gD decreased sperm-zona binding in a dose-dependent way with gC being more effective than gD. These results indicated that BoHV-1 inhibits bovine sperm-zona binding by interacting with spermatozoa. The binding of BoHV-1 to a spermatozoon is mediated by the viral glycoproteins gC and gD, and therefore seems to be comparable with the mechanisms of BoHV-1 attachment to its natural host cell (191). Although it is evident from these experiments that BoHV-1, if present in semen, has a tendency to bind onto the plasma membrane of the spermatozoa and to inhibit their ability to fertilise oocytes, there is no suggestion that infected sperm might penetrate through the zona pellucida and thereby lead to infected embryos (192). In one In-vitro trial of Singh et al; 63% of embryos ZP-I were exposed to 10^6 to 10^8 $TCID_{50}$/ml of IBRV (NADL strain) and then washed, the result was that the virus were retained, although the embryonic development was not affected. (193). When Bielanski and Dubuc added 10^6 $TCID_{50}$/mL BoHV-1 to the embryo production system at fertilisation they saw only a small trend towards retarded embryonic development but were able to isolate

the virus from all the embryos despite the fact that their post-culture processing included more than the IETS Manual recommended number of washes (194). In a later experiment Bielanski and Dubuc used oocytes from both experimentally and naturally infected donor cows and found again that the IVF systems became infected, as also did the embryos. The rate of embryo development and the proportion of morphologically normal (transferable blastocysts) produced in the infected IVF system were significantly reduced in this instance (195). Moreover, despite washing and trypsin treatment to IETS-recommended standards, the blastocysts produced were shown to be infected and probably had the potential for disease transmission. After further studies Bielanski et al. (196)concluded that, compared with in vivo-derived embryos, in vitro-produced embryos have a greater propensity to carry BoHV-1 after experimental exposure to the virus, and are more difficult to disinfect by means of the trypsin treatment protocol recommended in the IETS Manual (9). Lately a study of Makarevich AV et al found that BoHV-1 can compromise preimplantation and development of embryos produced in vitro, according with the study the ZP may not be enough to prevent virus-induced damage, unless trypsin washing is performed (197). Addition of antiviral agents to in vitro embryo production systems could provide meaningful disease control options. Phosphonoformic acid has been used to treat human cytomegalovirus retinitis and acyclovir-resistant herpes simplex virus infections (198), and has been shown to inhibit in vitro replication of BoHV-1 strains (199) (200). At 400mg/mL, phosphonoformic acid completely inhibited replication of the virus. However, the proportion of embryos developing to blastocysts in vitro decreased, and the number of cells/blastocyst was lower than in the untreated embryos (200). Lactoferrin from bovine milk (10 mg/mL) also inhibited IBRV-1 on MDBK cells. When lactoferrin (10, 5, or 2.5 mg/mL) was added to IVC medium, the nucleated cell count of treated embryos was not affected, but there was a significant decrease in blastocyst development (201). Interferon tau appeared to inhibit BVDV replication, but it did not decrease BoHV-1 (Colorado strain) replication on MDBK cells (202). Babiuk et al also suggested that recombinant bovine IFN-alfa1 does not have a direct antiviral effect on BHV-1 (203).

Conclusion: The recommendations of IETS for in vivo technique can ensure a low risk of transmission, as long as sperm free from IBRV is used for AI, and accurate washing (with or without trypsin) is done. More complicated seems to be the situation for in vitro production

embryo where the risk of transmission doesn't seem negligible. In the case of which, sperm form infected or unknown animals need to be used, treatment of BoHV-1-infected semen with gamma globulins from hyperimmune serum can neutralise the virus and reduce the risk of viral transmission via AI without affecting fertility (204). An opportunity for the future, would be the develop of specific monoclonal antibody (such as anti-gC) to bound the virus and therefore increasing fertility rate and the process safety. Antiviral products for IVP system that can prevent replication of IBRV-1 without interfering with the embryo develop has not been found yet.

2.1 IBRV-4: Bovine herpesvirus 4 (BoHV-4) is increasingly considered as responsible for various problems of the reproductive tract. The virus infects mainly blood mononuclear cells and displays specific tropism for vascular endothelia, reproductive and fetal tissues. Rather than being a pathogen by itself, BoHV-4 may cooperate with other infectious agents. Indeed, in 75% of cases of BoHV-4 isolation, bacteria, fungi or other viruses were also identified (205) (206) (207). It is responsible to cause metritis after replication in the endometrium and inducing cytopathic effect (208). Abortion, vaginitis and infertility are other consequences of BoHV-4 infection, therefore it can affect the E.T success reducing the likelihood to obtain good quality embryos (from donors) and the establishment of a pregnancy in the recipient. Stringfellow et al demonstrated that IBRV-4 could adhere to the bovine ZP after in vitro exposure. Nonetheless trypsin treatment has shown to be effective for insuring freedom of ZP-I ova from the virus (209). Donofrio et al confirmed that the virus is not infective for embryonic cells, except in case of zona pellucida breakage (210). Recently, however, epidemiological studies suggest its impact on reproductive performance, and its presence in various sites in the reproductive tract highlights its potential transmission in transfer-stage embryos (211). In bulls BoHV-4 can cause orchitis and be associated with azoospermia. Virus was also identified in the sperm of healthy bulls: viral DNA was isolated from spermatozoa and from leucocytes (212). The sperm thus appears as a potential carrier of viral transmission. Nevertheless, of the 50 bulls tested in a sperm collection facility in Serbia, 18 presented anti-BoHV-4 antibodies (36%), but the virus was identified in a single sperm sample and only by nested PCR (whereas conventional PCR gave a negative result) (213). Not much has been published about the potential danger of

BoHV-4 in embryo transfer, further research is advertised in order to assess the real risk carried by this pathogen.

2.2 BoHV-5: the most studied bovine herpesvirus is the type 1, which is known to cause reproductive and respiratory issues. Type 5 has been detected in bull semen and aborted foetuses but before the study of Silva-Frade C. et al presence of the virus in oocytes and embryos hadn't been recorded. The trail consisted in the evaluation of the effects of: infected fetal bovine serum, infected sperm and infected embryos. In conclusion BoHV-5 infected gametes were transmissible to the embryo during in vitro development. Zygote infected 1 d after fertilization showed compromised development, as a result, BoHV-5 has the potential to be a pathogen with economic consequences (214).

3. Bluetongue Virus

Introduction: Bluetongue virus (BTV) is an arthropod-borne virus infecting domestic and wild ruminants. Infection in cattle is commonly asymptomatic and characterised by a long viraemia. Animal-to-animal transmission is mainly achieved by biting midges of the genus *Culicoides* but direct transmission (transplacental or sexual) has also been observed. Clinical expression of the disease consists in reproductive disorders such as abortion, stillbirth and fetal abnormalities. BTV has a diameter of 68-70 nm (215), thus is very unlikely that BTV may cross the ZP since only molecules of 50 nm in diameter could pass through it (115). In addition, studies with BTV-1, BTV-10, BTV-11, BTV-13 and BTV-17 have also shown that ZP-intact bovine embryos could not be infected in vitro (216) (217) (218). In contrast, it has been shown that ZP-free are susceptible to infection with BTV-11 and BTV-17 in vitro, with virus replication and cytopathic effects on the embryos (219).

Sperm: The transmission through AI was proved in the 1984 after insemination (with infected semen containing BTV-17) of sensitive cows, some of them had viremia (217). With a regard to BTV-8, despite its presence in bull sperm was detected, transmission by AI has not been shown yet. Although BTV-8 infection had no effect on sperm volume and concentration, sperm motility was reduced. Moreover, malformed sperm in both; fresh and thawed semen from BTV-positive animals was above the 20%, in conclusion infection with BTV-8 transiently impaired semen quality in bulls (220). To prevent shipment of semen containing BTV, Chapter 8.3 of the OIE terrestrial animal health code recommends that

semen should be collected from bulls which show no clinical signs of bluetongue on the day of semen collection and have been – for at least 60 days before commencement of and during collection of semen (a) kept outside a restricted zone, (b) protected against viral vectors or (c) kept during the seasonally vector-free period in a BT seasonally free area. Bulls may also be subjected to diagnostic tests with negative results to provide assurance of uncontaminated semen: (a) lack of detected antibodies to the BTV group at least every 60 days throughout the semen collection period and between 28 and 60 days after the final collection for this consignment, or (b) virus isolation from blood samples collected at commencement, at least every 7 days during, and at conclusion of semen collection for this consignment with negative results or (c) PCR test on blood samples collected at commencement, at least every 28 days during, and at conclusion of semen collection for this consignment with negative results (221).

Donors: If donors receive infected sperm, have viremia and become infected, embryos on the other hand, if washed according with IETS, don't become infected nor carry the virus (222). From the previous study emerged also that the virus was resistance to freezing, and therefore semen could be a long distance vehicle of transmission.

Recipients: forty-eight, day 6-8 ZP-I embryos, collected from 17 BT-viremic (serotypes 10,11,13,17,18) dams inseminated with BTV-negative semen and transferred into 48 BTV-seronegative recipients. There were 4 stillbirths and 10 live births. All calves and recipients remained seronegative for BTV. Calves and recipients sampled for BTV isolation were also negative (216). A similar study in the U.S involved many heifers which were infected with BTV-11 by exposure to bites from *Culicoides*. During the acute phase embryos were collected, washed,

and transferred into seronegative recipients. At the end, all recipients and their offspring remained BTV free (223). Many other studies testing different serotypes have been done; the final outcome were for all of them the birth of non-viremia antibodies free calves, likewise the recipient remained seronegative (as reported in table 4). There is only one study of Schlafer et al which report seroconversion in heifers receiving embryos from BTV-11 infected dornors (186).

Species	BTV	Passage history	Diagnostic test(s) used	Donor	Recipients	Seroconversion	Virus detection	Outcome 1	Outcome 2	Reference
Cattle	BTV-10, 11, 13 and 17	Passages at least once in chicken embryo and twice in cell culture	Serology and virus isolation	20 donors during the peak of viraemia	39 recipients	None of the recipients seroconverted	No virus was detected in any of the recipients	Virus antigen not detected in 63 embryos and oocytes recovered from viraemic donors	Pregnancy rate (21/39): no difference between recipients and controls	(216) (224)
Cattle	BTV-11	Low passage tissue culture virus	Serology and virus isolation	3 infected donors	3 recipients	All three recipient heifers seroconverted at day 35	No virus detected, except in vaginal swab at day 7 in a recipient	No virus detection in embryos	-	(186)
Cattle	BTV-17 and 18	Whole blood passage for BTV-17 Passage in mouse and steer for BTV-18	Serology and virus isolation	10 infected donors	28 recipients	None of the recipients seroconverted	No virus was detected in any of the recipients	BTV-free calves could be obtained from infected dams by embryo transfer	-	(225)
Sheep	BTV-11	Four serial sheep passages	Serology and virus isolation	18 infected donors	27 recipients	None of the recipients seroconverted	No virus was detected in any of the recipients	BTV-free calves could be obtained from infected dams by embryo transfer	Pregnancy rate (11/27)	(218)
Sheep	BTV-10 and 11	Propagated or adapted cell culture	Serology and virus isolation	14 infected donors	6 recipients	None of the recipients seroconverted	No virus isolated from flushing fluids in ECE	The transmission of BTV did not occur when embryos were collected from viraemic donors and washed	Pregnancy rate (4/6)	(226)

								according to the IETS protocol		

Tabella 4 Studies on the potential transmission of BTV through transfer of embryos. EFSA Journal 2011;9(5):2189

IVP: BTV did not penetrate the zona pellucida (ZP) or attach to it when day 5 to 7, ZP-I embryos were exposed to 102-107 pfu/ml (serotype 10) for 1-24 hours, washed and then assayed. Nor was the embryonic development of these embryos affected by exposure to the virus (193). In another study twenty-one 6-7 days old bovine embryos from BTV seronegative cows were placed in a bovine turbinate cell culture containing 0,75ml of media which was seeded a few minutes later with serotype 11 BTV. After 18-24 hours in cell culture, nine unwashed embryos were processed for and examined by EM; all exposed embryos had numerous particles on the surface of the zona pellucida, but none were observed within the embryo (227). In another experiment, Langston et al. exposed a total of 77 zona pellucida-intact embryos (in vitro) to between 8 × 105 and 2 × 108 plaque-forming units per millilitre of BTV-17 for 2 h. The embryos were then washed according to the IETS protocols except that 12 rather than 10 washes were given. Trypsin treatment was not used. BTV was recovered from all the groups of washed embryos and from almost all the washing fluids. Consequently the authors concluded that a protocol slightly more rigorous than that proposed by the IETS, shown to be effective for removal of BTV from in vivo-derived embryos, was ineffective for removal of the virus from in vitro-produced embryos (228).

3.1.BTV-8: Since the first publications in the late 70s when scientists hypothesised that BTV transplacental infection could result in the birth of immunotolerant animals, in the 1993 and then in 2006 this event turned apart. A similar enquiry popped up together with BTV-8 emergence. As a result, De Clercq et al. demonstrated that BTV serotype 8 can cause a transplacental infection of developing fetuses that results in the birth of virus-positive, specific antibody-negative calves. However, a month later calves were not maintaining a detectable persistent infection (229). Moving forward, Vandaele L. et al trayed to evaluate the effect of BTV-8 in hatched bovine blastocysts. They found a retarded development of

infected blastocysts compared with control blastocysts, and there was significantly more apoptosis. Consequently, the author believes that problems with herd fertility during a BTV-8 epidemic may not be exclusively caused by BTV-8 induced maternal effects in the cows (such as fever), but potentially may also be attributed to direct infection of early embryos in utero. (230). In goats embryo BTV-8 shows a strong tendency to remain associated with the utter part of the embryo in both ZP free and ZP intact embryos. The virus could not be removed by the washing procedure recommended by the IETS for bovine embryos. This persistence of BTV after washing ZP intact embryos makes the embryo and embryo transfer procedure a potential means of transmitting the virus, and disease, to recipient goats (231). Some concern for BTV-8 regard the possibility of transmission not just by vectors or vertical route as proved by many authors (232) (229) (233) (234) but also by direct contact, possibly through the ingestion of infected placentas (235). Also colostrum spiked with BTV-8 infected blood led to oral BTV-8 infection of a calf (236). However horizontal transmission has been described also for other serotypes, such as BTV-26 in goats (237) and BTV-1 in red deers (238). Transplacental transmission of BTV is considered of little to no importance in endemic areas. However in free areas it can be a vehicle to introduce the pathogen as happened in northern Ireland with the introduction of an in utero infected calf (239). A new BTV introduction leads to the implementation of a series of rules and measures to contain the BTV outbreak. These measures seriously impact livestock farming and severely disrupt international trade of animals and animal derived materials (240).

Conclusion: concerns regarding the persistent infection of a seronegative, post-pubertal bull that consistently or sporadically produces semen contaminated with BTV appear to lack substantial scientific support. BTV is classified by IELTS in 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 in accordance with the manual of the IETS. Nonetheless, in IVP the virus is not completely wash out as it happens with in-vivo technique. In addition, BTV-8 attached very strongly on goat ZP resulting in a possible transmission to recipient, no study has been done to evaluate BTV-8 behaviour towards cattle embryos. Anyhow, in-vitro technique and the pathogenesis of BTV-8 need to be further investigated. No studies has

been done to assess the eventual transmission in recipients by means of IVP and/or BTV-8 infected embryos.

4.Schmallenberg Virus (SBV)

Introduction: This virus was unknown before it popped up in 2011 in Europe causing outbreaks in many countries. The first identification was done in November 2011 in Germany. Afterwards congenital malformation was reported in newborn lambs in the Netherlands linked to the presence of the virus. Subsequently up to March 2012, Belgium, Germany, United Kingdom, France, Luxembourg, Italy and Spain reported acute infections of adult ruminants or malformed SBV-positive offspring were detected, and high seroprevalences were seen in adult ruminants in the core regions in The Netherlands, Germany and Belgium. Characteristics: It is a ssRNA virus included in the Simbu serogroup of the *genus Orthobunyavirus, Bunyavirus family* SBV is most closely related to viruses of *Sathuperi species* such as Australian Douglas virus (241) (242), moreover SBV has a number of biological properties that are similar to those of Akabane virus. For this reasons Alkabane virus has been used as a model for describing some of SBV feature. SBV is consider a vector borne disease since the main way of transmission seems to be by Culicoides biting midges. Transovarial SBV-transmission in Culicoids has not been observed. Vertical transmission is also possible; in pregnant cattle and sheep, the virus can infect multiple organs of the un-borne foetus and this infrequently leads to malformations. Schmallenberg virus was detected in semen and embryos from SBV-infected cattle and sheep, respectively (243). A short viraemia of only 5–6 days occurs during the acute phase of the infection in adult animals.

Semen: This virus can be detected in seminal plasma early in acute infections (which may clear without serial positive semen samples) and be associated with the seminal cell fraction in serial positive samples weeks after seroconversion (244) (245). A single insemination dose of semen can contain SBV sufficient to infect naïve cattle through experimental subcutaneous injection though transmission of SBV by natural breeding or artificial insemination remains to be demonstrated (246). Ponsat et al. in a experimental study found that positive semen batches from SBV infected bulls could provoke an acute infection in IFNAR$^{-/-}$ mice (245). Lack of studies had led to overlap results which were conducted for Alkabane disease. Speaking of Alkabane, a trial of Parsonson et al suggested

no risk of transmission by means of semen (247). Furthermore, in another experiment Singh et al found no evidence that Akabane virus could infect the developing embryo, in addition washing techniques were considered to be a safe approach to ensuring that Akabane virus was not inadvertently transmitted by embryo transfer (248). However, the risk for transmission of SBV by insemination of dams with SBV-containing semen remains to be evaluated since no official trails have been done to evaluate the effective transmission by semen. No informations are available regarding embryo's collection, and other risk factors. According to the OIE, semen should only be collected from clinically healthy animals (221). For other vector-borne diseases, such as bluetongue virus, transmission via semen collected from viremic animals could be possible (217) even though unlikely. The OIE recommends that mitigations for bluetongue virus, when applied to SBV, should provide sufficient assurance of safety for semen regarding SBV, because the infective period of SBV is shorter than that of bluetongue virus (249).

Embryo: The OIE states that the viremic period for SBV in adult animals is very short and that embryos should be collected from clinically healthy animals. SBV affects embryos and fetuses in a manner similar to that of Akabane virus, so safety measures applicable to Akabane virus should be implemented. Risk from seronegative donor animals is negligible. Animals should be seronegative for 21 days after the collection (249). As a clarification; Akabane virus is classified under the category 4 (diseases or pathogenic agents for which studies have been done or are in progress that indicate that either 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 between collection and transfer),in conclusion not enough studies on *Bunyavirus family* ensure no risk of transmission by semen and embryo.

Geo-political trade: The emergence of the infection had a major impact on international trade of susceptible animals and animal products such as semen and embryos. Because the risk pathways for semen and embryos may be similar to those of Akabane virus and bluetongue virus, shipments of bovine germplasm collected in EU countries were blocked in 2011 (250). Cattle semen trade has been restricted in several countries, in terms of percentage of total semen trade, most of the trades happens within the EU (2010: 73.4 % and 2011: 82.8 %). For the semen trade outside of the EU (2010: 26.6 % and 2011: 17.2 %),

around 60 % of those are trade with countries imposing restrictions, representing for 2010 a 15.1 % of the total EU semen trade and for 2011 10.9 %. A decline between 11 and 26 % of the semen doses have been observed from previous years compared to 2012, as for the pure-bred breeding animals, the export value dropped 20 % in 2012 with respect to 2011. Additional restrictions on the import of embryos and semen of ruminants were, for example, imposed by the USA, Mexico and Japan. However, based on the updated OIE factsheet on SBV (251), the EU is of the opinion that SBV does not deserve to be treated differently from Akabane virus, which causes a disease that is neither OIE listed nor notifiable in the EU nor subject to specific OIE standards or restrictions, although it is endemic in many areas of the world (252).

Exotic viral disease

1.Rift Valley Fever (RVF):

Introduction: The rift valley fever is a zoonotic disease of domestic ruminants and humans caused by an arbovirus belonging to the *Phlebovirus genus* (*family Bunyaviridae*). It causes high mortality rates in newborn ruminants, especially sheep and goats, and abortion in pregnant animals. Human infection by the RVF virus (RVFV) may result from mosquito bites, exposure to body fluids of livestock or to carcasses and organs during necropsy, slaughtering, and butchering (253). The pathogenesis of the disease varies depending on the animal species and age. Transplacental infection has been reported (254). In pregnant ewes, abortions are frequent, ranging from 5% to 100%, moreover, newborn lambs, kids and calves frequently develop an acute form of the disease with high mortality (up to 100%).

Transmission: RVFV is transmitted by a broad range of mosquito species. *Aedes* mosquitoes preferably feed on domestic and wild ruminants, who act as amplifier of RVFV, leading to expansion of disease (255). Rodents and bats have also been suggested to be somehow involved in the cycle (256). Infections in humans due to direct contact with infected tissues have been documented; however, there is no evidence of direct transmission of RVFV between humans or between animals (257) (254). Finally, wild ruminants may play a role

in the epidemiology of RVF in areas where their population density is high (258). Vector: The bite of infected mosquitoes is the main transmission mechanism of RVF in ruminants during inter-epizootic periods. Many mosquito species were found to be infected by RVFV, belonging to seven genera of which *Aedes* and *Culex* are considered as the most important from the point of view of vector competence. In mosquitoes, transovarian RVFV transmission has been observed in *Aedes Mcintoshi*. It appears to be a likely phenomenon in several other species, including the widespread *Ae. vexans* species complex. In some of these *Aedes* species, infected, diapaused eggs may survive in dried mud during inter-epizootic and/or dry/ cold periods (259). Several potential RVFV vectors are present in the EU, therefore, there is almost no doubt that many species in the EU, e.g. *Culex pipiens*, would be competent vectors for RVF (260). Many epidemiological concerns arise from this species current distribution in Europe: Albania, Bosnia and Herzegovina, Croatia, Italy (including Sicilia and Sardinia), south eastern continental France and Corsica, limited areas of Germany (north of the Alps), Greece, Monaco, Montenegro, the Netherlands (green houses), San Marino, Slovenia, eastern Spain, southern Switzerland, and the Vatican city (261). A risk assessment has been done by EFSA for the four regions in the union (northern, eastern, southern and western Europe) in 2017. The final outcome was: "After entry, the risk assessment model estimated the level of transmission to be moderate in the four regions. The probability of establishment was estimated to be very high/high in all four regions of the EU. The overall rate of introduction of RVFV (being the combination of the rate of entry, the level of transmission and the probability of establishment) was estimated to be very low; hence, the extent of spread and potential impact after introduction was not assessed".

Conclusion: Europe has the potential to become an infective area due to several reason; presence of competent vectors and sensible animals, moreover climate changes are supporting mosquito's persistence and proliferation in environment which indeed could cause the introduction and adaptation of other species from Africa continent. Migratory flows from Africa to Europe is another risk factor that could lead to the pathogen's introduction, as well as importation of infected ruminants (which is probably the greatest hazard). Thanks to sanitary restriction is not possible to import livestock and animal by products from areas which are not free from RVF and other diseases. The first large

epidemy was reported in Egypt between 1977 and 1978, where 600 people died. In the first decade of the 21th century the main outbreaks were recorded in: Saudi Arabia and Yemen (2000), Tanzania Somalia and kenya (2006) Sudan and Mayotte(2007) Morocco south Africa and Madagascar (2008) Turkey(2009). More recently also Nigeria(2011) Iraq(2012) Chad and Tunisia (2014) Niger(2016) were affected by the epidemic. Looking at the distribution of the disease overtime, is clear that the presence of outbreaks in countries close to Europe could represent a great hazard. Nowadays according with EFSA the risk to introduce RVFV is low, nonetheless monitoring the situation is highly recommended for the future. There is no evidence that direct contact transmission plays a significant role in the transmission of RVF. In susceptible species, the virus can be transmitted in utero to the fetus (262). RVFV has also been found in semen (263). Because transmission can occur through direct contact with blood and other body fluids, it is hypothetically possible that animals could become infected through contact with an aborted fetus or placental membranes which do contain virus, though the relative importance of this transmission mechanism is not well understood (264).

2.Lumpy Skin Disease(LSD):

Introduction: Lumpy skin disease virus (LSDV), a member of the genus *Capripoxvirus* within the *family Poxviridae*, is a double-stranded DNA virus. It causes disease in cattle, characterized by firm, circumscribed skin nodules, necrotic plaques in the mouth and nares, fever and generalized lymphadenopathy (265). Due to pneumonia, infertility reduced feed intake and a harmful effect on the quality of hides, economic losses associated with the disease can be substantial (266). According to the current information available, there is no evidence about differences in virulence of the different LSDV strains. The severity of the

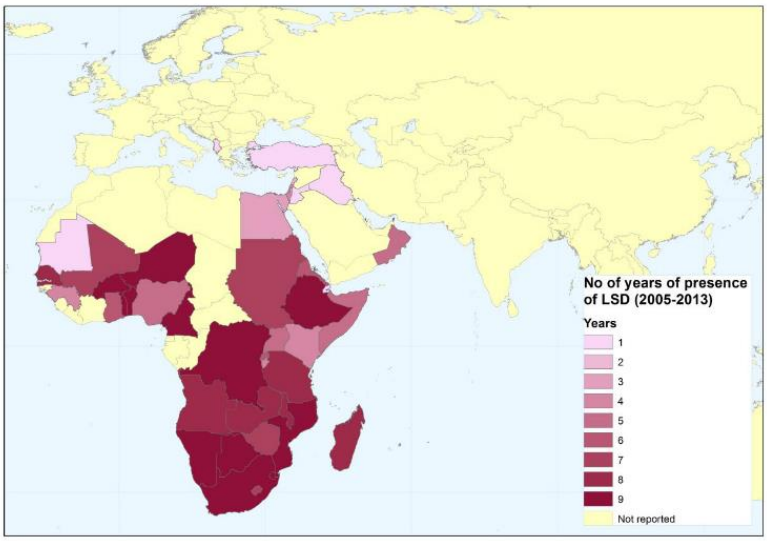


Figure 12 Number of years of presence of LSD in different countries as reported to OIE for the period 2005–2013

disease depends mainly on the host immune status, breed, production stage and age. Although endemic to sub-Saharan Africa, lumpy skin disease (LSD) has the potential to spread to other parts of Africa, the Middle East and Europe (267). Transmission routes are many. The most important one is mediated by vectors: blood-sucking arthropods such as stable flies (*Stomoxyscalcitrans*), mosquitoes (*Aedes aegypti*), and hard ticks (*Rhipicephalus, Amblyomma* and *Ixodes* species). Mechanical transmission has been reported, worldwide fly (*S. calcitrans*) is suspected to be the most important vector of the disease. Nonetheless, also mosquitoes and ticks have the potential to carry and transmit the virus. Recently also non-biting flies have been included, by feeding on the carcasses of cattle having recently died of LSD or were culled due to LSD, thereby taking up the virus from open skin lesions or body fluids containing high virus titers (268).In some ticks *Rhipicephalus appendiculatus* and *Amblyomma hebraeumalso* intra-stadial transmission has been reported (269). In the last decade also direct and vertical transmission have been analysed.

Semen: Irons et al. in 2005 found virus particles in semen of experimentally infected animals for up to five months. They also noticed a drastic deterioration in semen quality during the acute phase of the infection, which recovered before the end of the period of virus excretion (270). Afterwards, other trails showed that LSDV is not limited to specific fractions of the ejaculate and the testes, and that the epididymides was most profoundly affected. virus was not sperm or blood associated, therefore the ejaculate was likely to have been contaminated with virus that was being shed during emission from necrotic lesions in the genital tract (271). In another study though, the virus was associated with sperm in 9% of the samples (272). Finally; transmission via contaminated bovine was experimentally demonstrated by (273),consequently, artificial insemination and natural mating should be considered as risk factors for transmission. Interestingly, live virus could be detected in semen for up to 42 days post infection, and viral DNA was detected up to 159 days post infection (270). Transmission by means of infected semen was demonstrated for the first time in a trial lead by Annandale C.H et al (273); Eleven young beef heifers, naive to LSDV, were synchronized using an Ov-Synch protocol and inseminated on Day 0 with fresh semen spiked with a field strain of LSDV. Three of the seven animals tested positive for viral DNA in blood between 10 and 17 dpi. By the end of the trial,

seroconversion was demonstrated in eight out of the 11 heifers inseminated with semen spiked with LSDV. Is important to notice that after vaccination, and vaccinated animals did not shed vaccine virus in the semen (274).

Interestingly OIE recommendations for the importation of semen from infected countries are that donors should be kept in an establishment or artificial insemination centre in which no case of lumpy skin disease has been reported for the 28 days prior to collection and that the donors remained free from lumpy skin disease for 28 days after semen collection. However, has mentioned before lumpy skin disease virus may be present in semen for at least 42 days after infection and that viral DNA may be present for at least 159 days (270). Therefore the OIE recommendations may be considered inadequate. In addition, in AI centres, semen should be tested directly by virus isolation or by PCR (270). Alternatively in donors LSD can be isolated for 6 months prior to semen collection. There are no OIE recommendations regarding embryos

Embryo: In the same study mentioned above, also embryos were harvested by 2 infected heifers, these embryos were found positive for LSDV DNA by PCR (273). The risk posed to in vitro fertilization (IVF) procedures was also demonstrated. It was established that the virus was sperm-associated in 9% of spermatozoa, and that the early blastocyst contained viral DNA (272). In both cases none of embryos were transferred into recipients, therefore, the risk of transmission by embryo is still unknown due to lack of studies.

Risk factors: According to animal health EU legislation (EC Regulation No 206/20104) the import of live cattle from third countries such as northern African and Middle Eastern countries to EU is forbidden; therefore, there should be no movement of live cattle across the border. Nevertheless, EFSA in a report claimed that: “some discrepancies were noted when commercial data (Eurostat) were compared with veterinary border checks (TRACES system). For example, some movements of live cattle were registered in Eurostat in 2011–

2013 and these are displayed on the map below (Figure n.13)" (275).

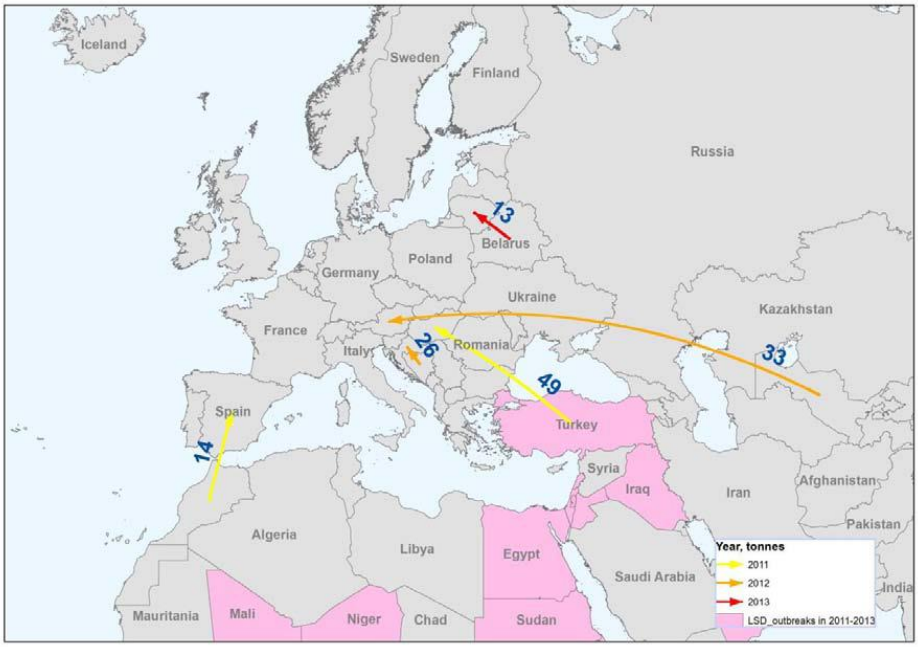


Figure 13 Trade movements of live cattle from some northern African and Middle Eastern countries towards MSs in 2011–2013 and related amounts LSD-affected countries in 2011–2013 are highlighted (275).

LSD is endemic in most African countries. Since 2012–2013 though, LSD has been spreading on an unusually large scale throughout Middle and Near Eastern countries including Turkey, where it is now considered endemic. Moreover, In 2015, the lumpy skin disease virus spread throughout the Russian Federation. Following a modified stamping-out campaign, the disease re-emerged with a greater incidence across 16 regions of Southern and Central Russia. A total of 313 outbreaks were reported to OIE (276). Indeed, epidemiological study of LSD outbreaks in Russia, three cases were identified that occurred more than 800km away from the outbreak epicentre, suggesting vehicle-assisted transport of infected animals (276). LSD situation

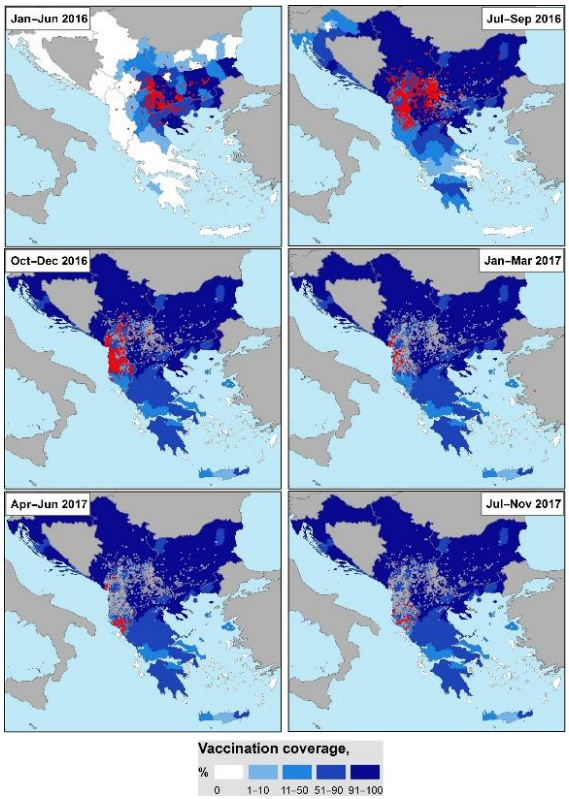


Figure 14 outbreaks of LSD in the Balkan 2016-2017 (277)

between 2016 and 2017 shows that 7,483 LSD outbreaks were reported in the Balkan region in 2016 with 12,330 affected animals, while only 385 outbreaks with 850 affected animals were notified in 2017. These were mainly in Albania (379 outbreaks), in areas where vaccination was not completed and where the cattle population was most susceptible, and very few in Greece (two outbreaks) and the former Yugoslav Republic of Macedonia (four outbreaks). This reduction in the number of outbreaks reported in 2017, particularly in Bulgaria, Serbia, Montenegro and Kosovo where none was reported, provides field evidence of the effectiveness of the mass vaccination campaigns conducted at regional level (277). Anyway, a stepwise spreading of LSDV towards Europe should worry authorises, carefully evaluated should be the mechanisms that enhance the spread of the virus. There is lack of information regarding semen and embryo handling, as well as safety procedure for recipients, thus, in order to face new epidemy, down the road would be highly recommended to improve knowledge and awareness about LDSV.

BACTERIA

1-*Brucella abortus*

Introduction: Bovine brucellosis is one of the most important zoonotic diseases worldwide, and is of particular significance in developing countries (as show in picture n.15). The disease, which results in serious economic losses due to late term abortion, stillborn, weakly calves and infertility. Brucellosis is caused by Gram negative bacteria of the

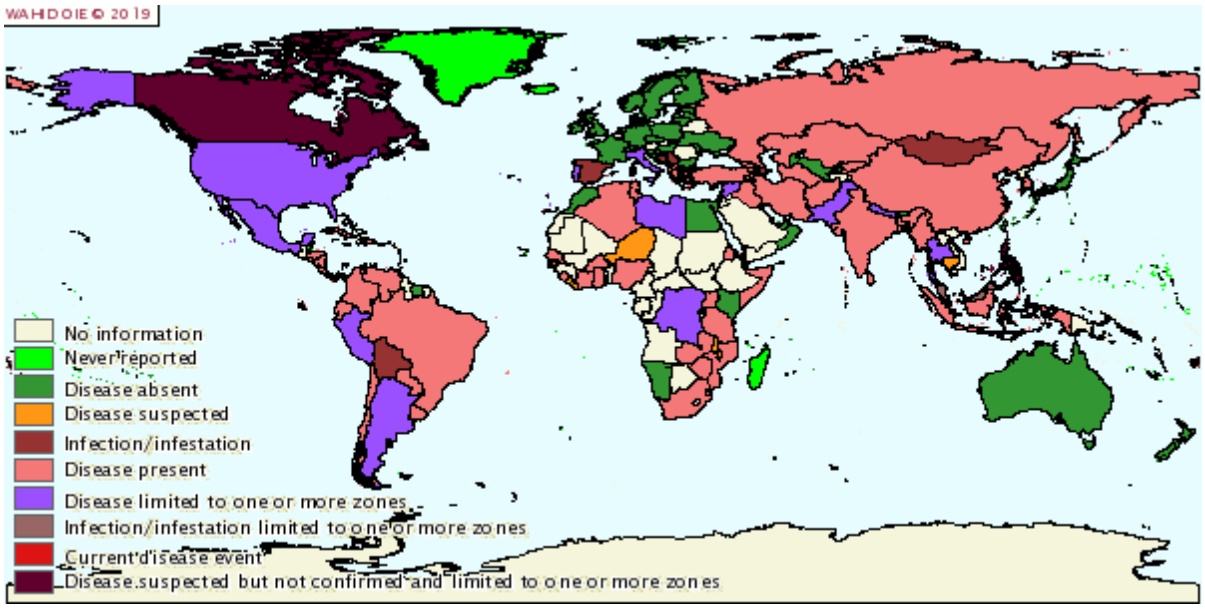


Figure 15 distribution of Brucella in the world (440)

genus *Brucella*, which are facultative intracellular coccobacilli that belong to the α2-Proteobacteriacea family. Species are *Brucella melitensis, Brucella abortus, Brucella suis, Brucella ovis, Brucella canis, Brucella Neotomae* and *Brucella Maris.* Transmission can happen by means of oral route: contamination of the udder during milking and contact with heavily contaminated placenta and aborted fetuses are considered the main source of infection for humans (direct contact) and other animal hosts. Secondary, venereal transmission of the disease occurs due to infected male or contaminated semen. Moreover, fetuses can be infected vertically, and surviving, becoming a latent carrier.

Semen: An agent that is transmissible through semen is *Brucella abortus*, which causes orchitis and may be associated with epididymitis and inflammation of accessory sex gland (278). However, many animals are asymptomatic carriers, which is why cattle breeders do not see brucellosis as a cause of infertility and decline in reproduction rates. Nonetheless Brucella can be shed with semen and infect recipient. The infection of healthy cows through

artificial insemination (AI) with contaminated semen may be more frequent than through natural mating. This is because the semen is deposited directly in the uterus, which contains few antibodies and defence cells, and is the preferred site for bacteria, thus making AI an important transmission route and an efficient form of dissemination of the disease in cattle herds (279).

Donor: According with Stringfellow et al superovulatory treatment is not likely to reactivate the release of Brucellae into the uterine lumen during the period when embryos are normally collected (280). Furthermore, embryo and ova collected from infected donors at 100 days or greater postpartum or post abortion are not likely to harbour Brucella (281)

Recipient: Embryos collected by chronically seropositive cows were placed into seronegative recipient without be washed previously. All recipient and offspring were tested periodically, all samples turn out to be negative (282). *Brucella*-induced latent infections are transmitted from infected dams to offspring either during pregnancy or perinatally (usually through milk). Latently infected animals are apparently healthy and show negative responses in the indirect immunological diagnostic tests, being thus very dangerous epidemiologically. Latent infections have been reported in up to 10% of the offspring born to *B. abortus* infected cattle (283) (284).The duration of the latent status until the animals develop the disease is highly variable, usually showing an abortion during their first or second pregnancy (283) (284). *B. abortus* has a strong tropism to the uterus during the last trimester of gestation, due to high concentrations of erythritol and steroid hormones. Erythritol favours bacterial survival since it can be metabolised by *B. abortus* as a source of carbon and energy (285).

IVP: Results showed that the zona pellucida-intact can effectively protect embryo from brucella's penetration, and IETS washing procedure efficiently clean out brucella after in vitro exposure (286) (287). Nonetheless brucella seems to have a deleterious effect on embryonic development (287). Other insight; the use of antibiotics resulted in a 99,9% reduction in viability of the organism (288). The use of Glycerol protects brucella during freezing and thawing if antibiotics are not added (288).

Conclusion: Overall, embryo transfer can be considered a safety technique to break Brucella's transmission. Sperm can be a source of infection, nowadays most of the breeding

are done by AI and according with a report of the Ministry of Brazilian Agriculture (279), there is a higher risk to spread the disease by this way compared with natural mating. However, the vast majority of AI centres in Europe are certified Brucella-free. Since all E.T protocols use artificial insemination, the risk is negligible. According with researches the use of infected cows as donors can be done with no hazard, if embryos are washed following IETS recommendations. The recipient status is the main worry since in endemic areas both infected cows or seronegative latently infected animals can abort in late stage of pregnancy due to imminent infection or Brucella re-activation. Pregnancy loss deeply affects the E.T efficiency and the overall reproduction performance in the herd.

2-Campylobacter fetus venerealis

Introduction: Venereal Campylobacteriosis, a widespread bacterial disease associated with both bovine infertility and abortion. In livestock, there are two species of Campylobacter fetus relevant: Campylobacter *fetus subspecie fetus* and Campylobacter *fetus subspecie venerealis* (289). The species Campylobacter *fetus venerealis* resides exclusively in the genital tract of cattle. Whereas *Campylobacter fetus fetus* usually inhabits the intestine, but due to an ascending genital infection or even venereal route, it can migrate to the genital tract via (290).

Semen: The primary mode of transmission of *T. foetus* and *C. fetus venerealis* is coitus; however, they can survive in raw and processed bull semen, which makes them transmissible via artificial insemination (AI). Campylobacter fetus venerealis is sexually transmitted and it do not cause disease in the bull (291) (292). This organism resides on the epithelium of the preputial cavity of infected bulls; with increasing age, the epithelial crypts of the prepuce become deeper, providing a microaerophilic environment that supports replication of these. The incidence of infection is higher among bulls over five years of age, and this may be attributed to the deeper epithelial crypts in the prepuce and penis of older bulls which allow the pathogen to survive and to grow more readily. *C. fetus* is transmitted to female cattle at natural or artificial service and causes vaginitis, cervicitis, endometritis and salpingitis. Infertility can last at least ten months. Persistence of *C. fetus* infection in

the bovine genital tract may be due to successive (or intermittent) changes in superficial antigens of the organism (293).

Donor and Recipient: Adverse effects of *C. fetus venerealis* in cows is characterized by genital infection, which can cause abortion (294). Natural infection with *C.fetus venerealis* in cows is associated with reproductive failure, irregular estrus, transient infertility, and in pregnant cows, embryonic or fetal death (295) (296). Moreover, experimental intrauterine and cervicovaginal infection with *C. fetus venerealis* in cows provoked varying grades of genital inflammation, including vaginitis, cervicitis, endometritis, and salpingitis (297). Occasionally, postcoital pyometra can result from uterine infection. A humoral immune response commonly clears infections of the female reproductive tract within 90 d (298)

IVP: In a study of Bielanski, *C. fetus* had no detrimental effect on fertilization rate nor in early embryo development in conditions used for the in vitro production (IVP) (299).

Conclusion: Campylobacteriosis can negatively affect fertility and uterine environment causing genital inflammation which can lead to reduction in conception rate and in embryo's quality and quantity harvested in donors. Similarly, in infected recipient embryo's development can be compromised and abortion can occur in late gestation. Moreover, bulls can develop a life-long infection and therefore acting as a vehicle for transmission. No studies have been done in order to assess transmission by means of embryo transfer.

3-Chlamydia:

Introduction: *Chlamydophila spp.* genus Chlamydia, in the *family Chlamydiaceae*, is an intracellular bacterium comprehensive of eleven species and responsible for causing: abortion and other urogenital tract infections, pneumonia, conjunctivitis, enteritis, polyarthritis, encephalomyelitis, and mastitis. The most important species for cattle's infectious are: *C. abortus, C. pecorum* and *C.psittaci*. Abortion occurs in pregnant infective animals between the sixth and the eighth months of gestation, particularly among heifers in their first pregnancy. Placentitis is the most consistent and striking pathologic feature which has been reported after experimental infection (300). Abortus is also known to cause zoonotic infection in humans, where the greatest threat is to pregnant women and results in spontaneous abortion (301). Association between chlamydia seropositivity and abortion

has been proved in livestock (302), whereas lately several studies have shown that the CFT (complement fixation test) lacks of sensitivity and specificity, due to cross reactivity between different chlamydial species (301) (303). One study, in naturally infected calves, demonstrated that only eight of thirteen animals were serology positive, despite all animals demonstrating positive results for *C. pecorum* and/or *C. abortus* by PCR, at one or more anatomical sites throughout the observation period (304). The lack of association between chlamydial shedding and seropositivity has also been documented in other studies in cattle (305) (306). *Chlamydia spp*. commonly inhabits the gastrointestinal and reproductive tracts (307) (308) with faecal shedding and infection typically occurring at around three months of age in sheep and genital infection reported as early as 2 weeks of age in calves and in unmated heifers (307) (308). Subclinical infections are generally thought to be harmless, several studies over the last 10 years have emerged to suggest that these asymptomatic chlamydial infections in dairy calves may contribute to a variety of pathological features including clinically silent respiratory infections (309) and an overall reduction of performance and growth (310). In adult a reduction of milk yield (311), vaginitis (307) and infertility (305)have been observed. As a result, also subclinical infections can cause a great economical loss.

Semen: Chlamydiaceae can cause infection of the reproductive tract of the bull (312). These gram-negative intracellular pathogens can be present in semen and survive cryopreservation. Venereal route of transmission might also be possible, with other studies reporting *C. pecorum* present in the semen and reproductive organs of healthy bulls (306) (312)and in experimentally infected rams (313). Moreover Kauffold et al. found no correlation between serological data and PCR of semen, preputial washing samples or faeces (306). In addition, *C.abortus* and *C.psittaci* have shown to adhere in sperm (as shown in figure n.16) and to cause a reduction in sperm fertility (314). There are no data regarding the likelihood to successfully carry the infection to a recipient by AI in

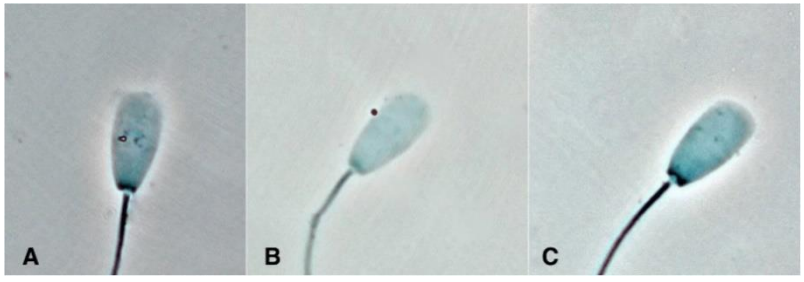


Figura 16 Light microscopical evaluation clearly confirms binding of C. abortus and psittaci to bovine spermatozoa. The orange particles represent chlamydial particles (a. C. abortus; b. C. psittaci). negative control (c) (314)

cattle, similarly also in sheep the transmission of *C. abortus* by sperm is doubted. Probably the transmission is possible in sows inseminated with sperm containing *C.suis* (315). Interestingly venereal infection is the classical route for the transmission of *C. trachomatis* in humans, furthermore there is a study indicating that, after natural infection of man, *C. trachomatis* penetrates the sperm, preferentially their heads, and can also proliferate within the spermatozoa as indicated by the presence of reticulate bodies (316). In cattle neither penetration and replication inside the sperm has been proven.

Donor: In a study of Bowen et al. sperm infected with *C.Psittaci* was used to bred synchronized heifers, embryos harvested seven days after did not show Chlamydial inclusion. In the same study the author claim that is the uterine inflammation the dominant factor in bovine female infertility caused by *C. abortus* and not direct infection of the fertilized egg (317). Subclinical effect of chlamydial infections should be considered, which have significant repercussions in economic terms and may play an important role in uterine health and fertility.

Recipient: Twelve embryos obtained by five ewes artificially exposed to *C.psittaci* were collected and after washing according with IETS recommendation, transferred into seven disease-free recipients. Both offspring and recipients remained free from infection (318).

IVP: According with recent studies *C. abortus* adheres to the ZP as well as the early embryonic cells of in vitro produced bovine embryos after in vitro infection, and that the standard washing protocol recommended by the IETS fails to remove it (319). Likewise, caprine embryos after in vitro infection show to adheres to and/or penetrates the ZP, and that the standard washing protocol recommended by the IETS is unable to remove it (320).

Conclusion: *Chlamydia spp.* causes many reproductive issues including abortion. Reproductive failure (especially in subclinical infection) has not been assess. Nonetheless sperm seems to be a carrier for the pathogen, vertical transmission has been proved but the impact in the recipient and how the sperm can affect embryo develop has not been assessed. Washing procedure recommended by IETS are not effective for cleaning out the microorganism with particular regard to embryos obtained by IVP. *Chlamydia psittaci* is classified by IETS in category 4, the lack of scientific opinion about the possible transmission

of Chlamydia by embryo transfer require more studies and field trial to evaluate this possibility.

4-*Coxiella burneti*

Introduction: *Coxiella burnetii* is a zoonotic obligate intracellular bacterium that has an almost worldwide distribution, a Gram-negative intracellular bacterium that has been reported in a broad range of host species, it causes the Q fever. In domestic ruminants, Q fever is thought to cause major clinical manifestations are abortions and stillbirths. Infected females shed *C. burnetii* mainly through birth or abortion products, as well as in feces and milk. studies focused on Q fever and abortion concurrently conclude that *C. burnetii* is an infrequent cause of abortion in cattle (321) (322).

Semen: *C. burnetii* has been detected in bull semen, suggesting that sexual transmission may occur (323), moreover after intravaginal artificial infection in cows, bacteria has been started shredding in urine after few days (324), These findings have strongly suggested the possibility of sexual transmission of the disease, especially in view of the fact that the infections were recognised after artificial insemination of the animals. In a study using mice revealed that *C. burnetii* was shed to semen from the urogenital tract, they were bound to the surface of spermatozoal cells, mainly to their heads, suggesting specific adhesion. Bacteria shed to semen were transmitted to female mice by sexual contact (325).

Donor: *C. burnetii*, ever since the first studies has been done, has been worldwide considered to cause abortion, placentitis, infertility, and other reproductive disorders in ruminants. Cabassi et al in 2006 found a correlation between abortion and seropositivity in dairy cows. However, in recent times, surveys found no solid evidence to support the hypothesis that *C. burnetii* is responsible for disorders such as subfertility, endometritis/metritis, or retained fetal membranes in any kind of domestic animal species. Epidemiological study based on 287 cases of abortion and 1318 age matching controls suggests that the abortion risk is not influenced by presence of maternal antibodies (326). Indeed, Ruiz-Fons et al. (327) did not find a significant difference in prevalence of *C. burnetii* antibodies in beef cattle herds with a recent history of abortion and those without. Several studies have used PCR for this purpose, but *C. burnetii* in infected animals is most of the time excreted in the fetal membranes, birth fluids and vaginal mucus, therefore this

method is unreliable and PCR most likely overestimates the importance *of C. burnetii* as an abortifacient (328). Abortion storms causes by *Neospora caninum* is more likely to occur in herds where antibodies against *C. burnetii* are present, rather than in seronegative herds (329). It is probable that an increased abortion rate is due to *N. caninum* rather than *C. burnetii*; in cattle infect, acute infection for *C. Burnetii* is usually asymptomatic.

Recipient: The aim of the present study was the detection and quantification of Coxiella Burnetii DNA in the flushing media (oviducts and uterine horns) and genital tract of non-pregnant goat from 20 goats chosen at random from 86 goats originating from 56 different breeding herds in south/west France. The serological prevalence rate of C.burnetii in the study population was 70,3%. The DNA of Burnetii was identified using conventional PCR in the flushing media from the oviducts and uterus in 8/20 goats (40%) and in genital tract tissues (oviducts, uterus and ovary) in 5/29 goats (25%). This study clerly shows for the first time that media used to flush the oviducts or uterine horns, collected using the standard embryo harvesting technique in goats, are susceptible to infection with *C.burnetii*. The 16 conventional PCR-positive samples were media varied from $2.9*10^4$ to $7.5*10^6$ bacteria per flushing medium, while the bacterial load of the tissue samples varied from $1.0*10^2$ to $1.5*10^5$ bacteria per mg of tissues. The infection of genital tract flushing media and tissues is a risk factor for the transmission of *C. burnetii* from donor to recipient during embryo transfer or the embryo and fetus when gestation is pursued to them (330). Anyhow, there is no specific study regarding recipients and embryo transfer accomplish in cattle, therefore, it has been possible just to repot study results from other species.

IVP: In a trail where embryos collected from goats were exposed to artificial infection in a medium containing 10^9 *C. burnetii* CBC1 (IASP, INRA Tours) for 24 hours; results showed that, *C. burnetii* remain strongly attached to early embryonic cells and the ZP of caprine embryos isolated from in vivo–fertilized goats after in vitro infection. Washing procedure recommended by the IETS for bovine embryos failed to remove it. *C. burnetii* was found in all batches of ZP-intact and ZP-free infected embryos after 10 successive washes (331). Some year after, another study confirmed that *C.Burnetii* stuck strongly to the external part of the zona pellucida of in vitro produced caprine embryos without deep penetration and that the 10 washings protocol recommended by IETS is unable to eliminate the bacteria from in vitro-produced goat embryo (332). Pathogenic agents such as *Coxiella burnetii* may bind to the ZP of an in vitro-produced bovine embryo and could penetrate the outside pores, making the embryo a means of transmitting the disease. These studies showed clearly that adherence to the ZP depends on the structure of the ZP and on the outer membrane of the bacteria, and that transmission by embryo transfer is possible.

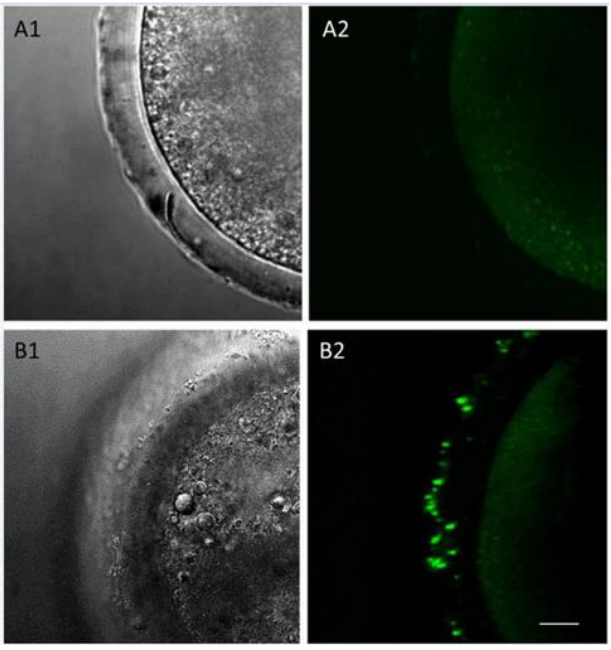


Figure 17 Immunofluorescent detection of Coxiella burnetii in vitro produced goat embryos after in vitro infection with 109C. burnetii/ml for 18 h.Coxiella burnetii was localised at the surface of the embryo, in the external part of the zona pellucida of the contaminated (332)

Conclusion: *C.burnetii* induced in-herd epidemics of this complete expression of reproductive failure have been reported for sheep and goats, but not for cattle. There is no solid evidence to support a hypothesis of *C. burnetii* causing disorders such as subfertility, endometritis/metritis, or retained fetal membranes in any kind of domestic animal species. There is no experimental evidence to support that *C. burnetii* causes abortion in cattle. In fact, a recent study (333) showed that seropositive shedding cows had better reproduction than non-infected cows. Also infection by means of semen is in doubt since there are no so many scientific study a support to this thesis. In the end there is no specific study regarding embryo transfer transmission risk accomplish in cattle, therefore, it has been possible just

to repot study results from other species. *C.Burnetii* is classify in category 3 by IELTS, anyway, since there is inconsistency in results seeing *C.Bunetii* as cause of abortion and infertility, in the future it may be needed reconsider the role of *C.Burnetii* as cause of reproductive disorders in cattle.

5-*Histophilus somnus:*

Introduction: *Histophilus somni* (formerly *Haemophilus somnus*) is a gram-negative, fastidious pleomorphic coccobacillus of the family Pasteurellaceae. Earlier investigations have shown that *H. somni, Haemophilus agni,* and Histophilus ovis represent the same species, and recent analysis of genes of strains supports the allocation of this species to a novel genus within the family Pasteurellaceae as *Histophilus somni*. Histophilus somni is an important pathogen that may produce four different kinds of diseases: respiratory disease, thromboembotic meningoencephalopathy (TEM), arthritis and reproductive disorders. Histophilus somni is a common inhabitant of the genital tracts of male and female cattle. The organism can be routinely isolated from the mucosal surfaces of the urogenital tract of normal healthy cattle in the absence of any macroscopic lesions (334).

Semen: The organism colonises the genital tract of the bull and can be isolated from semen, this may well be a source of infection of cows and heifers (335). Good hygiene and the use of combinations of antibiotics should control infection after AI. In addition, testicular degeneration, orchitis, epididymitis in bulls have been reported.

Donor: In a study of Kaneene et al a pool of cows were superovulated, inseminated and exposed to *H.somni* by intrauterine infusion. Embryos were recovered 8 days after and evaluated. The result were that *H.somni* had a detrimental effect on early bovine embryos (336), similar result were obtain by Thomson et al after in vitro exposure (126). There is one report suggesting that *H. somni* could be transmitted via embryo transfer and causing the death of transferred embryos (337)

Recipient: Reproductive failure caused by *H. somni* can occur either via systemic hematogenous dissemination (abortion) or by ascending route from vagina (338). *H. somni* causes vulvovaginitis, endometritis, cervicitis and abortion (339) (340). However, under natural conditions, the pathological significance of the isolation of *H. somni* from vaginal

discharges is difficult to interpret. *H. somni* is isolated at higher rates from inflamed uterus and cervixes than from normal organs. The fact that the pathogen is also isolated from normal reproductive tracts implies that these strains cannot always be associated with disease. *H. somni* persists in the cervico-vaginal area from periods ranging from 8 to 87 days postinoculation, even in the presence of humoral response (340). The main site of bacterial persistence is unknown; however, the major vestibular gland is considered a significant reservoir *of H. somni* (341). The detrimental effect of *H. somni* on embryo development was proposed as a possible cause of infertility in subclinically infected cows (342). Experimental abortion after intravenous inoculation of *H. somni* demonstrated that the bacterium is able to reach the pregnant uterus and placenta by hematogenous dissemination (343). *H.somni*-induced abortion was also reproduced by intra-amniotic inoculation and the lesions observed in placenta resembled the vascular changes associated with bacterial septicemia (344).

IVP: *H.somni* adheres after in vitro exposure and remained attached also after washing with dPBS and tripsin (126).The use of antibiotics in the flushing and holding media, can effectively eliminate the pathogen from bovine embryos.

Conclusion: *H.Somni* can be isolated from the reproductive tract of normal bulls and be present in semen (345). Although the organism is sensitive to antimicrobials, it is not known if transmission via processed semen would result in infection of susceptible cows (298). Moreover *H.somni* has a deleterious effect towards early embryonic development when infection is experimentally apply. There is no information regarding the possibility of infection of the recipient by embryo, *H.somni* shows to attach very strongly on ZP surface and standard washing with dPBS and trypsin are ineffective. Good practice includes the use of antibiotics in order to reduce the risk transmission to the recipient. In infected cows the uterine environment can be compromised along with others multi-organs disorders, thus, early pregnancy loss or abortion can occur.

6-*Leptospira spp*

Introduction: Leptospirosis is a worldwide zoonotic disease of domestic animals and wildlife. It is caused by a spirochete bacteria classified under the Leptospira, of which are

approximately 17 species. The clinical signs may be acute, subacute, or chronic and is usually associated with two serovars, Pomona or Hardjo. Transmission of *Leptospira spp*. in cattle can happen in an indirect way through contact with contaminated water or soil, and directly through sexual contact (346).

Semen: The presence of *Leptospira spp.* in semen of infected bulls was demonstrated naturally and experimentally, indicating the possibility of bovine leptospirosis transmission by natural coition or by artificial insemination (347) (348).*Leptospira spp*. can be isolated also from the genital tract of subclinical bulls and transmitted in semen (349).

Donor Recipient: *Leptospira spp.* has major economic impact in farm animals which is associated with abortion, stillbirth and birth of weak neonates, with a high death rate. Looking into the embryo transfer technique; despite the presence of the *Leptospira spp*. in the reproductive tract of donor animals and the association of leptospiral DNA with uterine stage embryos, the transmission of the disease is unlikely to occur by transfer of in vivo produced embryos in cattle (350). On the other hand, in recipients; abortion usually occurs several weeks after septicaemia. Placenta degeneration may be present or not. Abortion can happen from 4 months on of pregnancy. Anyhow, is more likely to occur in the second half of gestation (351).

IVP: The presence of serovar hardjo in the IVF system had no detrimental effect on fertilization rates or on embryonic development to the blastocyst stage (352). The study proposed by Bielanski and Surujballi revel that it was possible to obtain transferrable embryos from oocytes recovered from infected donors and from oocytes exposed to Leptospira in vitro (352). Some years later Bielanski and Surujballi have *observed Leptospira hardjo* in the pores, matrix and channels of ZP and in the embryonic cells, indicating the ability of these pathogenic agents to attach the ZP or penetrate into the embryos (353). The organism could not be isolated from IVF embryos that were produced from oocytes exposed in vitro to Leptospira and cultured in medium supplemented with penicillin and streptomycin. In contrast, leptospires were isolated from IVF embryos that were produced from in vitro exposed oocytes cultured in medium free of antibiotics therefore the authors suggest that the use of culture medium supplemented with antibiotics would be advisable to prevent risk of transmission. (352). Regulations established by IETS for IVP could be reviewed and possibly redefined, because the effectiveness of the treatment may depend

not only on the pathogen species, but also its virulence as well as its concentration and the action of the treatments on the type of pathogen (354).

Conclusion: Infected bulls can carry the pathogens and transmit it to cows by AI or natural service. *Leptospira spp.* can strongly attached on embryo ZP penetrating partially or completely the outer glycoprotein layer. There are no studies to support a possible transmission by means of embryo. Nonetheless, recipient can experience abortion during pregnancy and infection can occur throughout many ways including sex contact. In IVP antibiotics should be added in media culture in order to decrease risk of embryo's infection and transmission in recipient. According with IELS Leptospira spp. is classify in category 4: diseases or pathogenic agents are those for which studies have been done, or are in progress, that indicate: that 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 in accordance with the Manual of the IETS between collection and transfer.

7-*Mycobacterium avium subsp paratuberculosis*

Introduction: *M. avium* subsp. *paratuberculosis* (MAP) belongs within the family *Mycobacteriaceae* and is part of the *M. avium* complex. MAP is a small, acid-fast, facultatively anaerobic bacillus, with characteristic slow growth and known to have a distinct clumping morphology. MAP has a lipid-rich hydrophobic cell wall composed of long chain mycolic acids. This cell wall structure enables survival in the environment outside of the ruminant host. MAP is the etiological agent of paratuberculosis in ruminants, also referred to as Johne's disease (JD). Infected animals may have systemic infection resulting in the presence of MAP cells within tissues (355) and in faeces. The primary route of transmission is the faecal–oral route (356). Faecal contamination of the udder or the calving environment is a main risk factor for neonatal infection (357). MAP can be also excreted in milk/colostrum (358) and shedding depends on the severity of MAP infection and lactation stage with higher risk observed in early than mid or late lactation (359). In utero transmission can also occur and the risk increases with infection stage (360). MAP can also be excreted in semen but data on transmission via semen are sparse (361).

Transmission is considered to occur primarily from cows to calves, which are considered most susceptible. Calf-to-calf transmission has been described (362), and cow-to-cow transmission may take place but remains mainly undetected because delayed exposure results in lower incidence of detectable cases (363). In utero infections occur in cattle (364) (360) and have been reported in goats as well (365). Further, transmission by semen and embryo are debated.

Semen: Semen contamination can be liked to: -infection associated with bacteriological localisations in the genital tract or -preputial contamination linked to an infection associated with bacteriological localisations in the intestinal tract.

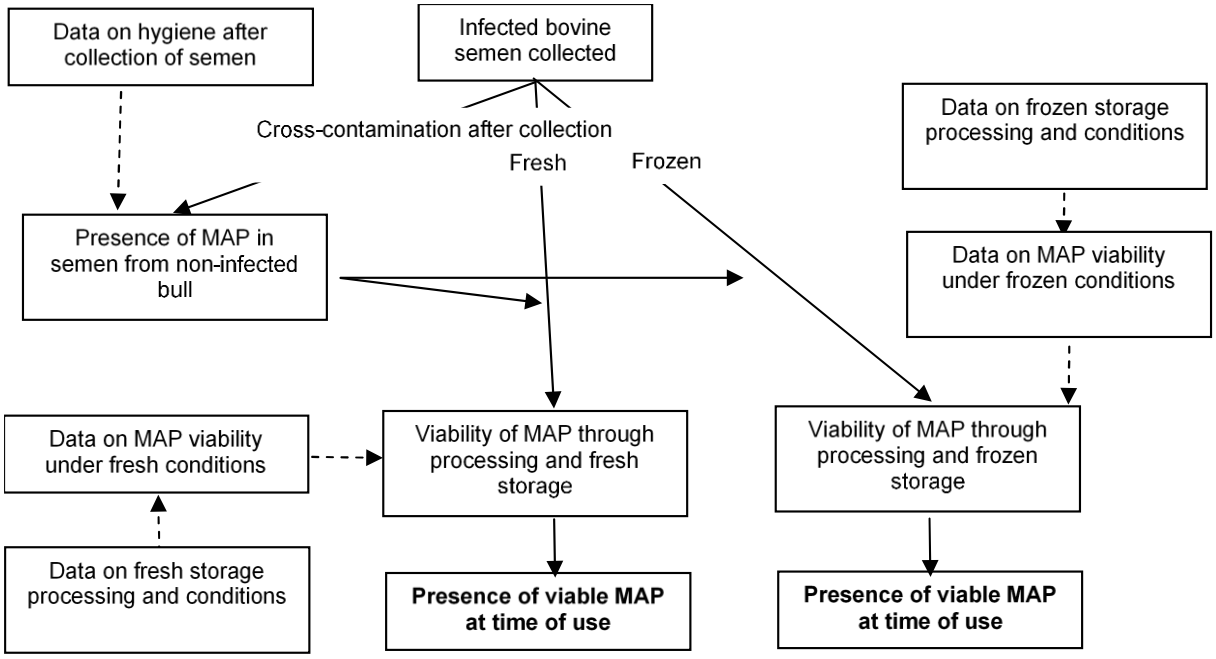


Figura 18: Risk pathway from collection of infected semen to use for insemination (53)

Genital localizations were reported in accessory sex glands (seminal vesicles, prostate gland, bulbo-urethral gland) or in the testis or epididymidis MAP has been isolated from the reproductive organs and semen of bulls (366) (367) (368). Most of these isolations have been from bulls with mild or severe clinical MAP but isolation of MAP in semen has been also demonstrated despite the absence of clinical signs (369). The organism is capable of surviving antibiotics and cryopreservation (298).

Donor: Apparently using donors which shred Map should not be vehicle for transmit the disease. Conversely in utero transmission can also occur and the risk increases with infection stage (360). Back in the days Lawrence suggested the possible infection of ovaries

in advance stage of the disease (370), proposing a possible vertical transmission by means of infected oocytes. Moreover, also reproductive performance is badly affected, in fact pregnancy rate has been shown to decrease in dams in advance stage of infection (371). In cows artificially exposed by intrauterine route, MAP was isolated from the uterine body and horns, suggesting a potential for MAP to survive in the uterus and to move to adjacent lymph nodes. Similarly, the intrauterine route was investigated as a means of infection by inoculating three cows with Map at the time of AI (372). One cow shed Map in faeces from 5 months post exposure. This cow was the only one to conceive but aborted at 8 months gestation and MAP was recovered from liver, spleen, mesenteric lymph node and intestine of the foetus.

Recipient: A pool of embryos were collected by subclinical infected cows and transferred into recipient. Offspring and recipient remained free for all the trial's length. MAP were not present in any of Embryos batches after washing procedures, contrary samples collected from the donors (uterine flushes/mucus) were positive (373).

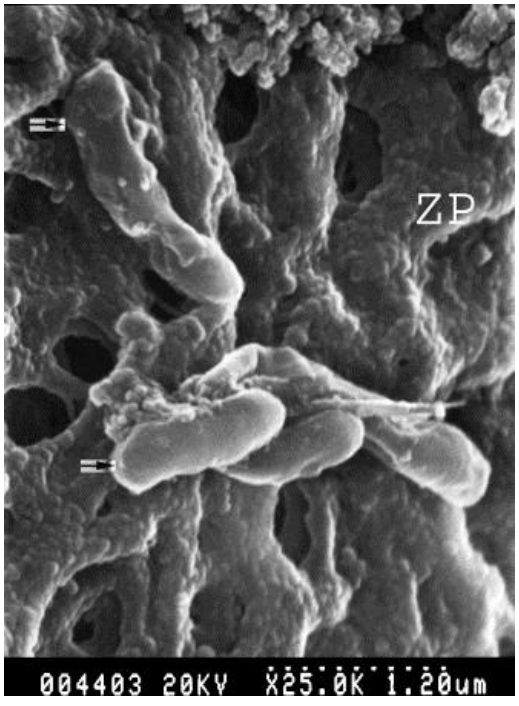


Figure 19 Scanning electron micrograph of a bovine blastocyst with a cluster of Mycobacterium paratuberculosis (arrows) adhering to the zona pellucida (ZP) (Bielanski, Algire, Randall, & Surujballi, 2006)

IVP: Exposure to MAP in vitro for 3 h had no apparent effect on the development of the embryos (373). Embryos collected from two uninfected heifers were fertilized in vivo and exposed to MAP in vitro; after sequential washing as recommended by IETS, all of these embryos were positive on culture and PCR (373). Similarly, IVF embryos exposed in vitro to MAP and then washed as recommended by IETS were tested in batches. Fifty-one percent of the embryo batches tested positive on culture and 28.1% were positive on the PCR as remarkably depicted in figure n.19, where bacteria on the embryo's surface are displayed by electronic microscope. Furthermore, A pool of embryos obtained by IVF were transferred into recipient and calves and dams tested several times for 5 years. None

recipient and offspring become infected (373). In another study frozen IVP embryos derived from cows with subclinical Johne's disease were processed and tested. No Map was detected in any embryos or media (374).

Conclusion: In the available literature, there are no reports on the transmission of Map (or other pathogenic mycobacteria) by embryo transfer to recipients and offspring due to natural infection. There is just one study where after artificial infection, Map was found in many tissues in the aborted foetus (372). It is indeed not enough to assume a possible infection by vertical route. Finally, semen can carry the pathogen, but no information was found either on the possibility, or probability, of the specific transmission route in question. Collectively, it can be assumed that despite the embryo's exposure to the pathogen in utero or in vitro, the sequential washing procedure established by IETS apparently eliminated or effectively reduced the bacterial load associated with the zona pellucida. It should be noted that a high number of both IVF and in vivo-fertilized embryos exposed to MAP in vitro remained positive when tested in vitro. Therefore, more information is required in order to surely rule out the possibility of transmission by AI and embryo transfer.

8-*Mycoplasma spp:*

Introduction: Organisms in the *Mycoplasma spp* genus belong to the class Mollicutes which are characterized by their lack of cell wall, low G + C content (23%–40%) and small genome size (0.58-1.4 Mbp). In recent years, more than 20 species of *Mycoplasma*, *Ureaplasma and Acholeplasma* have been isolated from cattle with different diseases. All those species have been referred to as the Mycoplasmas. *Mycoplasma bovis* is currently recognized as one of the most important and frequently isolated *Mycoplasma* species associated with disease in cattle worldwide (375). *Mycoplasma spp*. can cause several diseases in cattle including mastitis, arthritis, pneumonia, otitis media and reproductive disorders. Reproductive disorders are associated with: vulvovaginitis, infertility, endometritis, dystocia, however these manifestations are less consistently reported compared with pneumonia, mastitis and arthritis.

Semen: Mycoplasmas have the ability to travel with or on sperm cells to the fertilization medium according with Bielanski et al (376). *Mycoplasma bovis* has been isolated in

commercial semen (377), with *M. bovis* positive semen reported to cause alterations in the fertilization process leading to infertility (378). In an experimental study, infected semen was the source of *M.bovis* infection in two dairy farms (379), causing mastitis in animals which have been bred during the trial.

Donor: In vitro exposure of bovine embryos to *Mycoplasma bovis* and *Mycopasma bovigenitalium* lead to the adherence of these organisms to the zona pellucida-intact (380). Moreover, washing standard procedures along with trypsin do not remove mycoplasma. Most of the antibiotic treatments have no effect against the pathogen (381), high dose tylosin appears to remove efficiently Mycomplasma and doesn't affect embryo's development (382). Also kanamycin eliminates *M. bovis* with no embryotoxic effects (383).

Recipient: Infection, lesions, and clinical significance of *M. bovis* in genital disease of cattle are less described and include experimentally induced or naturally occurring chronic endometritis, suppurative salpingitis, infertility, and abortion (384) (385) (386). Experimental and field studies have revealed that there is a vertical transmission of *M. bovis* infection from the infected cow to the fetus or horizontal from the dam to the newborn calf to young cattle (387) (388). The presence of the agent in blood, amnion and cervical mucus immediately after parturition, the endometrium of mastitic cows, and organs and abomasal content of viable fetuses suggest the possibility of hematogenous dissemination and vertical transmission of *M. bovis* to the newborn calf (385) (387). There are few reports of *M. bovis*–induced abortions and isolation of *M. bovis* from aborted fetuses and joints from neonatal animals (389) (390) (391). Intrauterine infections with *M. bovis* in viable calves resulting in pneumonia or delayed occurrence of polyarthritis are rarely reported (389) (391).

IVP: It would appear that mycoplasmas interact in the same manner with in vivo and in vitro-produced embryos in contrast to some viral and bacterial agents (392). The lack of cell wall, a small diameter (0.3 to 0.8 pm), and the presence of cytoplasmic projections in *Mycoplasma spp* all make possible a close association with host cells, subsequently causing difficulty in detaching them by multiple washing from the intact-ZP of bovine embryos (376). Bielansky et al. performed in-vitro fertilization with semen experimentally infected with *Mycoplasma bovis* or *Mycoplasma bovigenitalium* and showed that these pathogens can be transmitted through the IVF system and therefore infect the embryos (376).

Furthermore, their experiments showed that the supplementation of the media used for in vitro culture with standard antibiotics and the washing procedure of the embryos as recommended by IETS were not effective in rendering IVF embryos free from *M. bovis* and *M. bovigenitalium*. It has been recognized that *M. bovis* and *M. bovigenitalium* have the ability to colonize the reproductive tract and to produce severe salpingo-oophoritis. Consequently, these microorganisms can be isolated from the oviducts of slaughtered cows (393). Therefore, another possibility of introducing *Mycoplasma spp.* inadvertently into the IVF system can happen by means of infected serum or oviductal cells used in embryo co-culture.

Conclusion: It remains to be established if the concentration of Mycoplasma associated with the embryo is sufficient to transmit the pathogen to recipient through embryo transfer and to induce the clinical sign of the disease. Even though introduction of *M. bovis* into a herd via semen appears to be rare, semen has to be taken into account as a source of infection, and precautions need to be taken, especially in areas free of *M. bovis*, as well as in high-biosecurity herds. Global trade in semen may spread *M. bovis* to a new country or area. The antibiotics used in semen extenders should be re-evaluated to obtain *M. bovis*-free semen or tested *M. bovis*-free semen should be available (379). Study of transmission risk should be applied also for embryo transfer technique, since according with IELS, *Mycoplasma spp.* is classify in category 4. Therefore, there are no solid data that can clearly assess a risk for transmission by E.T.

Parasites

1.*Neospora caninum*:

Introduction: *Neospora caninum* is protozoa, identified for the first time in 1989. Neosporosis is considered to be one of the main causes of abortions in bovines in various regions of the world. The main transmission route in bovines is vertical or endogenous, being transmitted from mother to fetus. This route may cause abortions or lead to the birth of calves chronically infected or with neurological symptoms. Cows may also have reproductive defects, such as estrus repetition and infertility (394).

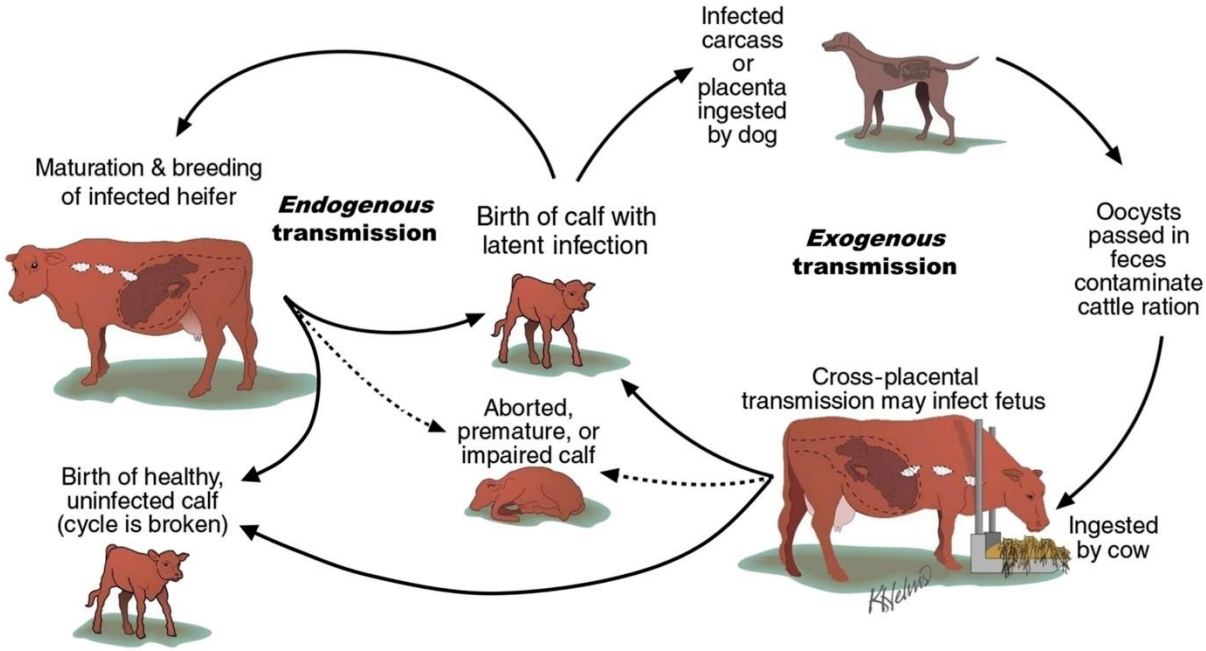


Figura 20 Framework Transmission of Neospora caninum.Artist: Kerry Helms (395)

Semen:*Neospora caninum* has been detected in semen, studies indicate that the possibility of venereal transmission is very low to non-existent (396).

Donor: The zona pellucida is an effective barrier against infection by Neospora (397). There is however a controversy whether reproduction performance is compromised in infected animals. According with Lopez et al *N. caninum* infection does not affect the fertility of high-producing dairy cows (398). In contrast two independent studies found that seropositive cows required a greater number of inseminations per conception than seronegative cows (399) (400) and therefore performance were poorer in seropositive

cows. On the other hand in another trail no significant difference was observed between the quantity of embryos obtain from seropositive and seronegative donors, and the rate of pregnancy of the recipients (401) (402).

Recipient: (401) and (403) showed that embryo transfer is a safe and effective technique for the control of vertical transmission, as embryos from positive donors break the cycle of congenital infection when transferred to negative recipients. However, when recipients are *N. caninum* positive, the fetus is infected regardless of donor status. In the study of Baillargeon et al, the rate of vertical transmission was 75% when the embryos were implanted in positive recipients (401). This indicates that the calves of *N. caninum* positive recipients are also positive.

IVF: The adhesion of *N. caninum* tachyzoites to preimplantation embryos was investigated in in-vitro studies by Bielanski et al. In vitro fertilized embryos were exposed to tachyzoites of *N. caninum* in culture with a monolayer vero cells. Using transmission electron microscope, dividing tachyzoites of the parasite were observed within trophoblastic cells of hatched blastocytes only when the zona pellicula was absent (404). The results of that study implicated that with an intact zona pellicula, preimplantation embryos are protected by the zona pellicula against *N. caninum* invasion.

Conclusion: The transfer of embryos being a safe technique to avoid vertical transmission of *N. caninum* when negative recipients are used. Prior serological diagnosis in recipient cows is of crucial importance when the transfer of embryos is routinely done.

2.*Tritrichomonas foetus*

Introduction: *Tritrichomonas foetus* is a flagellate protozoan that lives in oxygen-poor environments, such as the bovine reproductive tract. *Tritrichomonas foetus* belongs to the family *Trichomonadidae*, order *Trichomonadida*. This protozoan is found in the urogenital mucosal surface of males and females, causing bovine trichomoniasis, an infectious disease with venereal transmission, which causes infertility and abortion in cattle. This disease has worldwide distribution and is endemic especially in regions with poor sanitary control or where the use of natural mating for reproduction is extensive. *T. foetus* is physically associated with the epithelium lining the urogenital cavities of cattle. In bulls, the parasite

can be detected in the preputial cavity and urethra. In cows, it inhabits the vagina and uterus, in which the parasite can inhibit the attachment of embryo or rupture its membranes after attachment, leading to abortion.

Semen: *Tritrichomonas foetus* persistently and asymptomatically colonizes the epithelium of the prepuce, penis, and occasionally the urethral orifice of bulls. *T. foetus* was detected (immunohistochemistry) on preputial epithelial surfaces (five of 24 bulls) and penile crypts (14 of 24 bulls) but not in the penile or prostatic urethra, seminal vesicles, prostate, or epididymis (405). It appears that *T. foetus* is restricted to mucosal surfaces and is incapable of invading tissues. However, epithelial cells could react with *T. foetus* antigens in bulls and interact with stromal antigen-presenting cells (406). *Tritrichomonas foetus* is sexually transmitted diseases that do not cause disease in the bull (291) (292). Infection of bulls with *T. foetus* is considered to have limited or no effect on male fertility because *T foetus* does not inhabit the male urethra (407) (405) and its presence in semen is rare (407). Notwithstanding, when bovine sperm (1×10^6 cells) were exposed to *T.foetus* (1×10^6 organisms) *in vitro* (30 minutes to 6 hours at 37 °C), they were damaged or killed (408). However, in natural infections, this cytotoxic effect of *T. fetus* would be greatly reduced because trichomonas are rarely detected in semen (407), and mature bulls often ejaculate as many as 6×10^9 sperm per coitus. *T. foetus* appears to be resistant to the organism, or it does not induce pathogen-specific inflammation in bulls because it primarily inhabits the lower genital tract for prolonged intervals without causing clinical symptoms.

Donor and Recipient: In contrast to bulls, infection with *T. foetus* in cows provokes genital inflammation, including cervicitis and endometritis (409) (410). Moreover, genital infections in heifers were limited to 13 to 28 weeks (411). In pregnant cows, fetal death occurs in the first trimester (412) or later (413). In one experimental study, genital inflammation and pregnancy loss occurred after 7 weeks of infection (412). Furthermore, cows infected with *T. foetus* induced a detectable antigen-specific antibody response in the vagina. Intravaginal inoculation of nonpregnant heifers with *T.foetus* (7×10^6 organisms) induced *T.foetus* cell-specific IgG1 and IgA antibodies in vaginal secretions at 7 to 9 weeks (411), with IgA persisting for 24 weeks after infection or until genital clearance (414). However, the parasite can evade

the immune system's response causing long-term infections. The pathogenic mechanisms that cause embryonic or fetal death have not been understood fully yet. *T. foetus* initially adheres to and infects the vagina, causing vaginitis, and then invades the uterus and grows in fetal membranes producing placentitis, detachment, and death of the embryo, by direct action of the protozoa or from the effects of the inflammation (415) (416). The inflammatory process can vary between acute and chronic, characterized by accumulation of neutrophils, macrophages, lymphocytes, and occasionally plasma cells. The inflammatory reaction that develops in the host, causing changes in the uterine environment, and cytotoxicity mediated by lymphokine, has been suggested as possible mechanism. After abortion, retained placenta with membranes can result in chronic catarrhal or purulent endometritis and may result in permanent sterility (412) (416).

IVP: In vitro experiments, *T. foetus* was added to embryo cultures both pre- and post-fertilization to mimic the hypothetical possibility of natural infection of oocytes and embryos from zygotes to hatched blastocyst stages which take place during the passage of embryos through the reproductive tract. From this study appears that *T. foetus* has no detrimental effect on the fertilization and development of IVF embryos, the parasite had no ability to penetrate the ZP but it can adhere on it (417). The potential risk of transmission of trichomoniasis by IVF embryos seems to be unlikely due to the limited survival of the parasite in IVF culture conditions (417). However in another in vitro study, interaction between *T. foetus* and oocytes caused damage and apoptosis in cow's reproductive cells (418). *T. foetus* is not affected by trypsin washing (419)

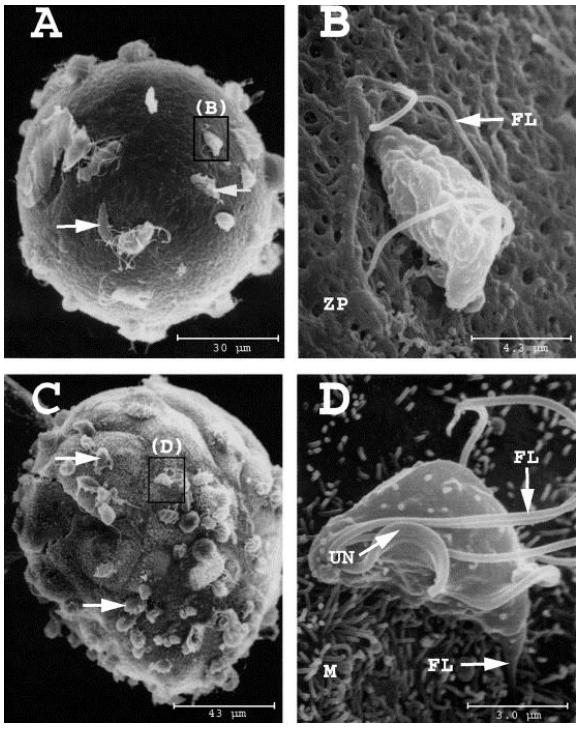


Figure 21 (A) Scanning electron micrographs of bovine blastocyst showing Tritrichomonas foetus (arrows) adhering to ZP (1100). (B) Higher magnification from (A), ZP: zona pellucida; Fl: flagella (7700). The parasite's posterior end is embedded in the ZP. (C) Multiple parasites (arrows) attached to the surface of hatched embryo (770). (D) Higher magnification from (B), M: trophoblastic microvilli (11,000). The three anterior flagella, flagellum undulating membrane (UN) and the posterior flagellum buried in microvilli are clearly visible. (417)

Conclusion: Bulls sperm can be a source of infection for donors even though fertilization and early embryo development doesn't seem to be affected. *T.Foetus* can potentially cause severe inflammation in genital tract of donor and recipient, thus it can compromised the E.T success. Moreover, *T. foetus* seems to have also a direct deleterious effect towards embryos and oocytes, however, this event is still controversial. IETS classify *T.foetus* in category 4 therefore no conclusions are yet possible with regard to the level of transmission risk since no research has been done regarding the transmission by means of embryo transfer.

Other agents

1. Bovine spongiform encephalopathy (BSE)

Introduction: (BSE) is a fatal neurodegenerative disease, caused by a prion, that mainly affects cattle. Other ruminants, cats, nonhuman primates and humans are occasionally affected. BSE is a member of the transmissible spongiform encephalopathies (TSEs), a group of neurodegenerative disorders caused by prions, infectious proteins that appear to replicate by converting a normal cellular protein into copies of the prion. (420). The cellular protein, which is called PrPc, is found on the surface of neurons. Pathogenic isoforms of PrPc are designated PrPres (The 'res' refers to the proteinase K-resistant nature of prions, compared to normal PrPc). PrPSc or PrPTSE are other names for this protein. Prions that cause different diseases (e.g. BSE or scrapie) are considered to be different strains of PrPres. In addition to the 'classical' BSE prion, at least two atypical BSE prions can be found in cattle. One has higher molecular mass fragments than classical BSE and is called 'H-type' BSE or H-BSE; the other has a lower molecular mass and is called 'L-type' BSE or L-BSE. The disease caused by the latter organism has also been termed 'bovine amyloidotic spongiform encephalopathy (BASE).' Atypical BSE prions are thought to represent additional strains of BSE. (420) Transmission BSE is usually transmitted when an animal or human ingests tissues containing the BSE prion. Young animals may be particularly susceptible: some studies suggest that most cattle become infected with BSE during the first six months of life. (420)

Semen: Epidemiological evidence and transmission studies suggest that BSE is not transmitted in milk, semen or embryos. (421) (420)

Donor and Recipient: Semen from 13 bulls, eight with clinical bovine spongiform encephalopathy (BSE), was used to artificially inseminate (AI) 167 cows with clinical BSE, and their resultant embryos were collected non-surgically seven days after AI. The viable and non-viable embryos with intact zonae pellucidae were washed 10 times then frozen. After transferring into negative recipient 266 live offspring were born, 54.1% of those had a BSE-positive sire and a BSE-positive dam. The recipients were monitored for clinical signs of BSE for seven years after the transfer, and the offspring were monitored for seven years after birth. All animals gathered in the trial remain BSE-negative. It is concluded 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 (422). In contrast a cohort experiment which involved 273 offspring of clinically BSE-affected cows, and 273 offspring from unaffected cows were monitoring during seven-year. As a result, 42 offspring from the BSE group developed the disease or had histological lesions post mortem, as against 13 in the control group; a statistically significant ($p < 0.0001$) excess risk of 10.6% for the occurrence of BSE in the offspring of cows which had had clinical BSE themselves (423). The BSE cases in the control group were ascribed to the fact that offspring in both groups would almost certainly have been exposed to BSE-infected feed prior to imposition of the government ban on inclusion of ruminant protein in ruminant feed (424). All the calves in the BSE group were born within 13 months of the onset of BSE in their dams, and a large majority within five months, so the data provide little insight into the risk of maternal transmission more than six months before the onset of disease in the dam. It was assumed, however, that the risk might increase towards the end of the incubation period and in the clinical phase (423).

Conclusion: The transmission of BSE by semen is negligible. Also embryo transfer appears to be safe. Problems may be associated with the recipient: in fact, there is no evidence that BSE is transmitted horizontally between cattle; however, there is an unexplained increase in the risk of BSE among the offspring of infected animals (423). In one study, calves seemed to be more likely to develop BSE when the dam was in the later stages of infection (i.e., nearer to the onset of clinical signs). These observations have led to speculation that vertical transmission might be possible in cattle. If this occurs, it seems to be rare, and the route is unknown. (420).

Final Conclusion

In the last decades the average of embryos flushed per cow were 6.8. Even though technologies developed tremendously the rate hasn't changed, it seems likely that the lack of improvement is bound to reproductive disorders and infertility instead of technology inefficiency. Many studies have been done regarding uterine health, cow's management and metabolic issues that can substantially affect the fertility of affected animals. More attention is being payed to cows which are going to undertake the E.T procedure, including not just the donor but also recipients. Is slowly arising the awareness that if animals are not in excellent condition, they will never produce good amount and quality embryos, likewise embryo will never implant in a ill uterus (where for instance a endometritis is proliferating). Since most of the cows which are chosen for an E.T are within 100 days in milk (DIM), problems during calving can impaired for long time normal ovarian functionality, delaying the return to cyclicity and affecting the quality of oocytes released. In a similar way also the uterus has to regress and the uterine environment become adequate for hosting a new life. In summary, each event that can negatively impact against the cow health may affect fertility and therefore embryo's harvesting rate (for donors) and pregnancy rate (for recipients). The sperm is another potential source of infection, using only sperm from certify AI stations can reduce the risk of pathogens transmission, even though is good to remember that certify AI centres are free only for some pathogens. Moreover, sperm quality can be compromised by other factors not always related with infections, but which can lead to lower fertilization rate and as a result a lower viable embryos and more UFO. It is important to bear in mind that the use of sex semen can result in a lower conception rate and that its use can be enhance in healthy heifers. For IVF sex semen is discourage, meanwhile reverse sorted semen has higher fertilisation rate and better final outcome. The shipment of embryos is today a one of the most economical and safe ways to move genetic material around the world. Nevertheless, there are many things which has not been assessed yet: IVP for instance, is a developing technique which is becoming more and more meaningful, on the other hand there are very little information regarding transmission of pathogens by IVP. Studies demonstrated that embryos produced in this way slightly differ from embryos obtained with in-vivo technique. The dimensions of pores in the ZP of IVP embryos are greater compared with the ones present in-vivo derived

embryo's surface, moreover with IVP the ZP pores do not obliterate after fertilization leading to a possible penetration and transmission of some pathogen. In addition, both virus and bacteria have the tendency to adhere strongly to the surface of embryos obtained by IVP, and washing procedure normally applied in-vivo fails to remove them. With a regard to IVP, many recent studies to evaluate the transmission of pathogens has been performed in-vitro and never applied in the field. Therefore, there is lack of substantial scientific support to assess the eventual transmission of these microorganisms, and wheatear or not, their presence associated with the ZP is a real risk factor. It appears to be sure that materials applied for collection and cryopreservation as well as media or other animal products employee in the IVP, can be a source of infection for embryos leading to damage or impair embryo's development. The Fetal calf serum has a high biological hazard, and often samples have been resulted positive for non-cytopathic strains of BVDV; recently, its use is shifting towards alternative and more safe media such as BSA and polyvinyl alcohol. Oocytes and other animal products harvested from slaughterhouses can hide many troubles and represent a great risk since there is no sanitary information regarding the animals where these organs come from. Interestingly, there is no sanitary guidelines telling how to handle oocytes harvested at the slaughterhouse and embryos produce during IVP. Therefore, promote a uniform method to process these materials should be advisable along with studies in the field for the assessment of transmission of pathogens by E.T, with a particular regard to in-vitro technique. Some studies tried to face these challenges testing molecules which have been added in the culture media during IVP procedure. Antiviral such as DB606 in culture media seems to protect embryos from BVDV infection, similarly adding hyperimmune serum in culture media bounds BoHV-1 very efficiently preventing virus transmission. Furthermore, broad spectrum antibiotics block proliferation and kill bacteria like *Histophilus somnus, Leptospira spp.,* conversely, the supplementation of the media used for in vitro culture with standard antibiotics and the washing procedure of the embryos as recommended by IETS were not effective in rendering IVF embryos free from *M. bovis* and *M. bovigenitalium*. For other microorganism such as: Bovine Herpesvirus-4 (BoHV-4), Blue tongue virus (BTV), *Chlamydophila spp., Coxilella burnetii, Mycobacterium avium sub.Paratuberculosis (MAP),* and *Tritichomonas foetus* it is known that they can adhere to the ZP of IVP embryos also after washing nevertheless any effort has been made until this moment in order to find a protocol that can effectively eliminate them from

culture media. In the end, embryos can be a vehicle for pathogens transmission and infect recipients. In this world trade market, shipping embryos can hide many traps; whether animal selection, collection and embryo's handling are not performed properly it can lead to introduce potential pathogens in a herd or in area considered free for those microorganisms. Since movimentation of animals and genetic material is under sanitary restriction, the introduction of biological hazards in new area can affect the economy and the animal welfare of a whole country. Es an example the epidemy of BTV and Shallenberger blocked the transport of live animals and drastically diminished the shipment of sperm and embryos from infected areas. As a consequence, whether embryos are handled properly and if the transmission risk is negligible, the export of embryos would be preserved, without interrupting or breaking trade agreements between countries. Thus, down the road more effort should be made in order to assess the potential transmission of each pathogen and the creation of standard procedure for the in-vitro production.

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