European Food Safety Authority

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SCIENTIFIC OPINION

Scientific Opinion on Risk of transmission of TSEs via semen and embryo transfer in small ruminants (sheep and goats)[1]

EFSA Panel on Biological Hazards[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

An assessment of the risk of transmission of Transmissible Spongiform Encephalopathies (TSEs) via semen and embryo transfer in small ruminants (sheep and goats) was performed. The TSE agents considered were Classical scrapie, Atypical scrapie and Bovine Spongiform Encephalopathy (BSE). Because of the lack of specific data for goats the assessment was carried out mainly using data obtained in sheep and, because of the similarities of TSE pathogenesis between sheep and goats, the assessment was also considered to be valid in goats. According to the data currently available, the risk of TSE transmission associated with semen and embryos collected from Classical Scrapie incubating sheep and goats ranges from negligible to low but the data are insufficient to conclude that such a risk is negligible. Because of the similarities between Classical scrapie and BSE pathogenesis in small ruminants, this statement is also to be considered valid for BSE. The lack of knowledge of the pathogenesis and anatomical distribution of the Atypical scrapie agent within affected animals hampers the possibility to provide an assessment of its transmission risk via semen or embryos. Due to the use of animal-derived hormones and surgical devices for artificial insemination and embryo transfer procedures there is an inherent but unquantifiable risk of iatrogenic TSE transmission associated with these practices.

KEY WORDS

Transmissible Spongiform Encephalopathies (TSEs), small ruminants, semen, embryo transfer, transmission risk

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1 On request from the European Commission, Question No EFSA-Q-2009-00620, adopted on 10 December 2009.

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3 Acknowledgement: the Panel wishes to thank the members of the Working Group on Risk of transmission of TSEs via semen and embryo transfer in small ruminants (sheep and goats) for the preparation of this opinion: Olivier Andreoletti, James Foster, Jean Luc Gatti, James Hope, Ciriaco Ligios, Anthony Wrathall.

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SUMMARY

Following a request from the European Commission, the Panel on Biological Hazards (BIOHAZ) was asked to deliver a scientific opinion on the Risk of transmission of Transmissible Spongiform Encephalopathies (TSEs) via semen and embryo transfer in small ruminants (sheep and goats).

Regulation (EC) No 999/2001[4] of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain TSEs sets the specific restrictions on the placing on the market, export and import of the semen and embryos of the ovine and caprine animals.

Three articles were recently published[5,6,7] as regard to the TSE transmission risk through artificial insemination (AI) and embryo transfer (ET) techniques in small ruminants. These articles suggest that this risk would be very low or negligible.

In the light of these new data the European Commission (EC) requested EFSA to provide a scientific opinion concerning the risk of transmission of TSEs via semen and embryo transfer of small ruminants (sheep and goats).

The TSE agents considered in the assessment were: Classical scrapie, Atypical scrapie and Bovine Spongiform Encephalopathy (BSE). The risk assessment was mainly performed using data obtained in sheep. Because of the lack of specific data in goats, and because the high similarities of TSE pathogenesis between sheep and goats, this assessment was considered to be also valid in goats.

The BIOHAZ Panel considered all available scientific information related to TSE transmission via semen and embryos in small ruminants.

The Panel concluded that the risk of TSE transmission associated with semen and embryos collected from Classical Scrapie incubating sheep and goats ranges from negligible to low. However, data are insufficient to conclude that such a risk is negligible. Because of the similarities between Classical scrapie and BSE pathogenesis in small ruminants, these conclusions are also to be considered valid for BSE.

The BIOHAZ Panel considered that at this stage, the assessment of the risk of transmission by semen or embryos collected from sheep or goats affected by Atypical scrapie is not possible because of a lack of knowledge on the pathogenesis and anatomical distribution of the Atypical scrapie agent within affected animals.

Furthermore, it was highlighted that there is an inherent but unquantifiable risk of iatrogenic TSE transmission that is associated with artificial insemination and embryo transfer procedures (use of animal-derived hormones and surgical devices).

The Panel noted that absence of reliable figures on the annual number of artificial inseminations and embryo transfers performed in small ruminants in the European Union (EU) Members States hampers the quantitative assessment of the potential impact of an artificial insemination and embryo transfer transmission risk on TSE prevalence in the EU small ruminant population.

4 Regulation (EC) No 999/2001 of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. European Community, OJ L 147, 31.5.2001, p. 1-40.

5 Sarradin P, Melo S, Barc C, Lecomte C, Andreoletti O, Lantier F, Dacheux JL and Gatti JL, 2008. Semen from scrapie-infected rams does not transmit prion infection to transgenic mice. Reproduction, 135, 415-8.

6 Wrathall AE, Holyoak GR, Parsonson IM and Simmons HA, 2008. Risks of transmitting ruminant spongiform encephalopathies (prion diseases) by semen and embryo transfer techniques. Theriogenology, 70, 725-45.

7 Low JC, Chambers J, McKelvey WA, McKendrick IJ and Jeffrey M, 2009. Failure to transmit scrapie infection by transferring preimplantation embryos from naturally infected donor sheep. Theriogenology,72, 809-16.

The BIOHAZ Panel recommended further assessing the Classical scrapie and BSE transmission risk associated with small ruminants semen and embryos for infectivity, using highly sensitive animal models, in semen and embryos collected from a statistically significant number of TSE infected animals bearing susceptible PrP genotypes at different stages of the disease and with different TSE agents. Moreover, specific data about pathogenesis and anatomical distribution of the Atypical scrapie agent should be generated. Investigations should include small ruminant males and females at different stages of the reproductive cycle.

The Panel further recommended the promotion of procedures to limit the risk of iatrogenic transmission of TSEs associated with ET and AI. In particular, the replacement of ruminant-derived hormones by recombinant proteins should be considered.

The BIOHAZ Panel advised that a database recording the AIs and ETs performed every year in the EU should be established.

Finally the Panel emphasised that homozygous and heterozygous ARR rams and ewes as donors and recipients would minimise the risk of Classical scrapie and BSE transmission that could be associated with reproductive technologies. Similarly, once clarified and if validated, resistant-genotype he-goats and she-goats as donors and recipients would minimise the risk of Classical scrapie and BSE transmission that could be associated with reproductive technologies.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies sets the specific restrictions on the placing on the market, export and import of the semen and embryos of the ovine and caprine animals. Recently several new scientific articles have been published in relation to the transmission of transmissible spongiform encephalopathies (TSEs) via semen or embryos of small ruminants.

According to the article "Semen from scrapie-infected rams does not transmit prion infection to transgenic mice" by Pierre Sarradin et al., published in *Reproduction* (2008) 135, ram semen did not transmit infectivity to scrapie-susceptible transgenic mice with the VRQ-allele of the sheep prion (PRNP) gene under the experimental conditions, which leads to the suggestion that artificial insemination and natural mating have a very low or negligible potential for the transmission of scrapie in sheep flocks.

The review "Risks of transmitting ruminant spongiform encephalopathies (prion diseases) by semen and embryo transfer techniques" by A. E. Wrathall et al., published in *Theriogenology* 70 (2008), provides an update on information relevant to the potential risks of transmission of TSEs via semen and embryo transfer in domesticated ruminants.

It is concluded that transmission of classical scrapie by embryo transfer in sheep is very unlikely if appropriate embryo handling precautions are taken.

The study "The role of the pre-implantation embryo in the vertical transmission of natural scrapie infection in sheep" by J. C. Low et al. (provisionally accepted to *Theriogenology*, its pre-publication copy will be provided to EFSA) indicated that the pre-implantation embryo did not act as a carrier of scrapie.

In the light of these new data the risk related to the transmission of TSEs via embryos or semen of small ruminants should be re-assessed.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The Commission requests EFSA, based on these new scientific publications, of which two are enclosed to this letter, to provide a scientific opinion concerning the risk of transmission of TSEs via semen and embryos of small ruminants (goat and sheep).

Clarification on the Terms of Reference

After discussion with the requestor it was agreed to modify the terms of reference as reported here below:

> "*The Commission requests EFSA, based on these new scientific publications, of which two are enclosed to this letter,* ***and on other available scientific data****, to provide a scientific opinion concerning the risk of transmission of TSEs via semen and* ***embryo transfer*** *of small ruminants (goat and sheep).*"

ASSESSMENT

1. Introduction

1.1. Approach to the assessment

The TSE agents considered in this assessment are: Classical scrapie, Atypical scrapie and Bovine Spongiform Encephalopathy (BSE). Scrapie is a disease of ovine and caprine animals caused by a variety of TSE agents harbouring different biological properties that are still incompletely characterised, rather than by one specific transmissible entity. 'Classical scrapie' and 'Atypical scrapie' are operational rather than purely biological terms (Benestad et al., 2008; EFSA Panel on Biological Hazards, 2005, 2008b; Saegerman et al., 2007).

This risk assessment was mainly performed using data obtained in sheep. Because of the lack of specific data in goats, and because of the high similarities of TSE pathogenesis between sheep and goats, this assessment was considered to be also valid for goats.

1.2. Transmission of Transmissible Spongiform Encephalopathies in small ruminants

1.2.1. Classical scrapie and BSE Transmission in small ruminants

Both Classical scrapie and BSE are infectious diseases of small ruminants for which susceptibility is influenced by polymorphisms on the gene (PrP) encoding for PrP protein (EFSA Panel on Biological Hazards, 2006).

In sheep, the major polymorphisms associated with susceptibility or resistance are codons 136 (A or V), 154 (R or H) and 171 (R, Q or H) (Clouscard et al., 1995; Hunter et al., 1996). VRQ/VRQ, ARQ/VRQ and ARQ/ARQ genotype animals are considered as the most susceptible to Classical scrapie, whereas homozygous or heterozygous AHQ and heterozygous ARR animals only show a marginal susceptibility. AHQ allele carriers as well as ARQ/ARQ sheep were described to be the most susceptible genotype to experimental BSE, while VRQ/VRQ were reported to be of lower susceptibility. ARR/ARR sheep are considered to be strongly resistant to both Classical scrapie and cattle BSE agents (oral experimental exposure) (Hunter, 1997; Hunter et al., 1996).

In past opinions, the EFSA Panel on Biological Hazards has fully endorsed a breeding policy favouring the selection of sheep carrying the ARR allele as a way of reducing the risk of animal to animal transmission of TSEs and any potential risk of transmission to humans (EFSA Panel on Biological Hazards, 2006, 2008a)

In goats other PrP polymorphisms (e.g. I/M142, H/R154, R/Q211, D/S146 and Q/K222) could also impact on individual susceptibility to these TSE agents (Barillet et al., 2009; EFSA Panel on Biological Hazards, 2009a, 2009b; Gonzalez et al., 2009; Vaccari et al., 2006).

Classical scrapie and experimental BSE transmission have been mainly investigated in sheep, and only limited data are available for goats.

It is commonly accepted that natural contamination with Classical scrapie in affected flocks mainly occurs around birth (materno-lateral transmission[8]) and that placenta, which can accumulate large amount of prions in incubating animals, plays a major role in this process (Andreoletti et al., 2002; Pattison and Millson, 1961; Race et al., 1998; Tuo et al., 2002). More recently, colostrum and milk were described to contain infectivity and their capacity to transmit disease to suckling lambs was demonstrated (Konold et al., 2008; Lacroux et al., 2008).

Materno-lateral transmission was also observed in two independent experiments that were performed using sheep orally infected with cattle BSE agent (Bellworthy et al., 2005; Lantier et al., 2008) and in a research project by Andreoletti and colleagues[9]. While these experiments remain limited, they established the proof of concept of the possible inter-individual transmission in BSE infection context.

Scrapie transmission from ewe to lamb is likely to occur as a peri- and/or post-natal event, although the precise timing remains unknown.

Contamination with Classical scrapie was reported in sheep that were introduced into an infected flock after they had reached adulthood (Hourrigan et al., 1979; Hourrigan, 1988). The efficacy of such transmission appeared to be lower in older animals than in younger animals. The origin of such contamination remains unclear and both inter-individual horizontal transmission or environmental sources could be at their origin.

The role of the environment as a source of contamination has never been unambiguously demonstrated. However, converging evidence strongly supports its participation in TSE contamination of small ruminants. The policy for eradication that was applied in Iceland since 1947, with the recording of new contamination after stamping out infected flocks and reflocking with scrapie-free animals, strongly support the implication of environment in Classical scrapie transmission (Georgsson et al., 2006).

Scrapie incubating ewes' placentas that are released at lambing appear as a major source for environment contamination. More recently, PrP^{Sc} was detected in kidney of sheep with different genotypes (Ligios et al., 2007; Siso et al., 2006). To date no PrP^{Sc} or infectivity have been detected in the urine of sheep. However, in both Chronic Wasting Disease (CWD) (in wild cervids) (Haley et al., 2009) and scrapie in hamster (Gregori et al., 2008), infectivity was detected in urine and urine from affected sheep remain suspect to be a source of environmental contamination. Similarly, PrP^{Sc} has been detected in the salivary gland of sheep (Vascellari et al., 2007) and infectivity was evidenced in saliva of cervids affected by CWD (Mathiason et al., 2006), which support the hypothesis of a potential spreading of scrapie agents in small ruminants through this secretion. Once shed into the environment TSE agents have been shown to resist to degradation over long periods, in particular in clay rich soil (Genovesi et al., 2007; Johnson et al., 2006; Wiggins, 2009).

Iatrogenic TSE transmissions in sheep have been reported on several occasions. In the UK, tissues (brain, spinal cord and spleen) from young sheep were used to produce three batches of a formalin inactivated vaccine against 'louping-ill'. Several thousand of sheep were vaccinated and scrapie appeared two and a half years later amongst sheep vaccinated with one of the batches, and on some farms over 35% of the animals were affected (Gordon, 1946). In Italy several thousands of sheep and goats were sub-cutaneously vaccinated against *Mycoplasma agalactiae*. This vaccine was produced using homogenised, filtered ovine brain, mammary gland and lymph nodes (Capucchio et al., 1998).

8 Materno-lateral transmission: the spread of infection from the dam to her offspring horizontally in the immediate post-parturient period via milk, saliva, faeces etc... This definition excludes vertical transmission. Modified from Wrathall AE, Holyoak GR, Parsonson IM and Simmons HA, 2008. Risks of transmitting ruminant spongiform encephalopathies (prion diseases) by semen and embryo transfer techniques. Theriogenology, 70, 725-45.

9 EU funded research project reference QLK-CT-01309 - 'BSE in sheep' – Program Coordinator: Dr. Olivier Andreoletti.

Of a total of over 1,000 goats and 1,000 sheep on three farms, 18.5% of the goats and 1.15% of the sheep developed scrapie.

More recently the presence of both BSE and scrapie infectivity in blood from symptomatic and asymptomatic infected sheep was established (Houston et al., 2000; Hunter et al., 2002). Invasive surgery and the use of medical instruments are known to cause iatrogenic transmission of Creutzfeldt-Jakob Disease in humans and this discovery of a prionaemia (blood-borne TSE infection) in sheep has stimulated similar concerns about current veterinary practices.

1.2.2. Atypical scrapie transmission in small ruminants

Atypical scrapie was first reported in Norway (Benestad et al., 2003) and the estimation of the prevalence of this TSE agent in the EU sheep population is directly linked to the implementation of the active TSE surveillance in small ruminants. Today the prevalence of Atypical scrapie is estimated to be 1 per 10,000 tested small ruminants/year (Fediaevsky et al., 2008).

PrP genetic sensitivity to Atypical scrapie is different from that observed in Classical scrapie and BSE. While a strong over risk to develop Atypical scrapie is associated with AF141Q and AHQ alleles, wild type ARQ/ARQ and VRQ/VRQ animals seem to be at lower risk. Strikingly ARR allele carriers (both homozygous and heterozygous) can develop the disease (Arsac et al., 2007; EFSA Panel on Biological Hazards, 2006; Moreno et al., 2007; Moum et al., 2005).

The transmissibility of the Atypical scrapie agent is clearly established in both rodent models (transgenic animals expressing the ovine PrP gene) and sheep (Le Dur et al., 2005; Simmons et al., 2007). However, the contagiousness of Atypical scrapie is still debated. The analysis of the data collected through the active surveillance program (Fediaevsky et al., 2009a; Fediaevsky et al., 2009b) seems to indicate that the capacity of Atypical scrapie cases to contaminate other sheep under field conditions is low and possibly nil. However, the description of several cases in sheep that originated from the same flock (Konold et al., 2007; Onnasch et al., 2004; Simmons et al., 2009) coupled with our lack of knowledge of the pathogenesis of Atypical scrapie, the distribution of prions within an affected animal and the sensitivity of tests to detect pre- or sub-clinical stages of this disease means that we cannot be certain that this disease is non-contagious in all natural circumstances.

1.3. Artificial Insemination (AI)

1.3.1. AI use in EU small ruminants.

Artificial insemination (AI) in sheep and goats is a major tool for:

(i) Genetic progress in breeding schemes: production traits in particular in dairy sheep and goats, disease resistant traits (e.g. ARR PrP allele in sheep)…

(ii) Zootechnic management of flocks: insemination out of the breeding season to meet the needs in milk production and help producers meeting out-of-season demands of customers and industry (Mapletoft and Hasler, 2005).

In small ruminants and other species, AI is also used for breed and genetic diversity conservation purpose.

There are no available figures allowing an accurate estimation of AIs carried out in small ruminants at international level. In EU (about 95 million sheep and 13 million goats[10]) it also remains very difficult to estimate the importance of AI in sheep and goats since not all countries possess databases or dedicated organisations to collect the data. It can however be assumed that AI, because of its intrinsic technical constraints and cost in field flocks, is mainly of interest in dairy sheep and goats (high added value products).

Partial data are available for countries like Spain (about 40,000 inseminations) and Italy (15,000 insemination mainly in Sardinian population) through a report made to the ICAR organisation[11]. In France (about 8 million of sheep and 1 million of goats[10]) about 80,000 goats and more than 800,000 ewes are inseminated each year. In the UK the number of sheep that are artificially inseminated annually is approximately 30,000, and a high proportion (about 80%) of these are inseminated by laparoscopy.

Despite the incompleteness of the figures, it can be assumed that in the EU more than one million AIs in small ruminants are performed each year.

1.3.2. Technical aspects on AI in small ruminants

1.3.2.1. Semen collection

Semen from he-goats and rams is collected using artificial vagina. Several types of artificial vagina are available but all follow the same general design: a tubular inner liner, usually made of latex, surrounded by a water jacket encased in a harder outer shell. Ejaculated semen is collected in an attachable collection tube either made of glass or plastic. During collection days, these devices are routinely re-used with different animals after a simple drying/washing.

Collected semen can be used either as fresh semen or after cryopreservation.

Ram semen is mainly used as fresh semen, which for instance is used in almost all the inseminations carried out in France. However, the situation is very variable among the countries. When using fresh semen the insemination should be performed within 24 hours after collection. Semen (between 1 to 1.5 ml with concentration ranging between 2 to $10x10^9$ sperms/ml) is directly diluted in boiled skimmed milk kept at 35 °C with addition of antibiotics (milk extender). Temperature is then decreased to 15 °C before aliquoting sperm in 0.25 ml straw ($1.2\text{-}1.5x10^9$ sperms per ml, about $300\text{-}400x10^6$ sperms per straw) and storage at 15°C.

Different procedures are available to cryopreserve the ram sperm. However, since cryopreservation strongly decreases the fertility performances of exo-cervical insemination the cryopreserved ram semen is usually delivered through intra-uterine laparoscopic insemination (surgical invasive procedure), which obviously limits the scale of use of this procedure. Before freezing, sperms are diluted with egg yolk lactose media, cooled at 4°C and then glycerol is added. Sperm is then conditioned in 0.25 ml straw (40 to $100x10^6$ sperms per straw) and frozen in liquid nitrogen (N_2).

In he-goats since the seminal plasma is deleterious for the sperm preservation, it should be eliminated. Semen is diluted to $400x10^6$ sperms/ml after collection in a Krebs-Ringer-Glucose solution and centrifuged at 500-600xg. This is repeated twice and the sperm is then diluted in the milk extender. It could be used for insemination with fresh semen after dilution and straw conditioning. Alternatively

10 Source Eurostat (http://epp.eurostat.ec.europa.eu/portal/page/portal/eurostat/home), Statistics Database, data category: Livestock statistics, herd structure (Table "food_in_pagr1").

11 ICAR: International Committee for Animal Recording (http://www.icar.org/). Some data on artificial inseminations in sheep can be found at the following link: http://www.waap.it/sheep_enquiry/.

the solution is cooled to 4°C, glycerol is added and then the straws (0.2 ml containing 100 million sperm) are frozen in N_2.

1.3.2.2. Synchronisation of oestrus

In dairy goats and ewes, treatment for induction and synchronization of ovulation consists of a progestagen delivered by vaginal sponge, followed by an Equine Chorionic Gonadotropin (eCG) or Porcine Follicule-Stimulating Hormone (p-FSH), a pituitary extracted hormone, injection (Fatet et al., 2008). eCG (also named PMSG from Pregnant Mare's Serum Gonadotropin), obtained from mare's serum, is a convenient and largely used hormone for the induction of ovulation and is necessary for out-of-season breeding and AI.

1.3.2.3. Insemination

In sheep, most inseminations are carried out with fresh semen after hormonal induction of oestrus and mean fertility ranges from 60 to 70%.

In she-goats, oestrus and ovulation are induced by hormonal treatment sometimes in conjunction with photoperiodic treatment, and cryopreserved semen is used for AI. This protocol provides a kidding rate of approximately 65%.

In ewe and she-goats insemination is routinely intravaginal (exo-cervically but close to uterus for sheep and almost in uterus for goats). In ewes, it is difficult to pass the cervix with the insemination syringe and using a speculum. The syringe uses in general a metallic pestle that slides in a metallic tube where the straw is inserted. The pestle pushes the sperm from the straw.

When AI is intra-uterine, the semen (generally for frozen semen in sheep) is deposited directly in the uterus by laparoscopy, which means a surgical approach of the insemination. In most cases (mainly in the field) the material's disinfection procedures are designed for bacteria and viruses destruction and are poorly or not efficient towards prions.

1.3.3. AI and infectious disease transmission risk

A number of sheep and goat infectious diseases can be transmitted between animals *via* the venereal route or by the use of semen in commercial AI. These diseases can affect fertility causing inflammatory changes in the reproductive tract or lead to systemic disease. Viral environmental pathogens can contaminate semen during collection (Sellers, 1983) in addition to those (e.g. Border Disease virus) that may be present in the semen within the germ cell (Sellers, 1983). Several bacterial diseases can be transmitted by semen like *Mycobacterium paratuberculosis subsp. avium* in sheep (Eppleston and Whittington, 2001) or *Brucella ovis* and *Brucella melitensis* (Amin et al., 2001).

Different sanitary regulations exist concerning the collection, the AI centres and the trade of semen at the EU, international and national levels.

At EU level, the general animal health requirements governing intra-community trade in and imports into the Community of semen, ova and embryos of the ovine and caprine species are laid down in Council Directive 92/65/EEC of 13 July 1992 (Council of the European Communities, 1992). This Directive harmonises:

- the health conditions which such semen, ova and embryos must satisfy for the purposes of intra-Community trade or importation to the Community from third countries;
- the conditions for approval of semen collection centres and embryo and ova collection teams.

At international level, the OIE has edited official notes on sanitary control of semen production in order to:

1. Maintain the health of animals on an artificial insemination centre at a level which permits the international distribution of semen with a negligible risk of infecting other animals or humans with pathogens transmissible by semen;

2. Ensure that semen is hygienically collected, processed and stored.

These files can be found at :

www.oie.int/eng/normes/en_chapitre_1.4.5.pdf and
www.oie.int/eng/normes/mcode/code2005/en_chapitre_3.2.1.htm

The application of the disease control recommendations provided by OIE strongly reduces the risk of viral and bacterial pathogen transmission (Givens and Marley, 2008). The OIE Terrestrial Code prescribes the way of cleaning and disinfection (70° ethyl or 98–99° isopropyl alcohol, ethylene oxide or steam) of, e.g., the artificial vagina.

OIE sanitary recommendations for AI reduce efficiently the risk of infectious disease transmission. However, considering the particular characteristics of TSEs (long asymptomatic incubation period, difficulty to establish the infectious status of individuals) such procedures alone may not prevent recruitment of TSE infected rams and he-goats as semen donors.

1.3.4. Conclusions

- Despite the relatively small (by comparison to the EU small ruminant population) number of AIs that are performed each year in the EU, the existence of even a low TSE transmission risk associated to AI could result in a considerable number of TSE cases.

- Semen collection and insemination procedures may pose a risk of infectious disease transmission. The OIE recommended procedures to minimise disease transmission risk by AI may not in themselves be effective for TSE agents.

1.4. Embryo Transfer (ET)

1.4.1. ET importance in small ruminants

The main commercial use of ET in the livestock industry is for the selection and rapid proliferation of genetically valuable animals. Other advantages include reduction of the risks of disease transmission (bacterial, viral and parasitary) compared with the movement of adult breeding animals or semen. Use of embryo transfer also eliminates much of the cost of long-distance transport of post-natal animals, and the need for quarantine.

In species such as sheep and goats where embryo freezing is effective, cryobanking of gametes and/or embryos can provide long-term insurance against loss of genetically valuable animals and rare breeds.

Recent data for world ET activity are shown in Table 1 but, according to the author, they are incomplete.

Table 1: Data for world embryo transfer activity in sheep and goats in 2006, 2007 and 2008 (please note that according to the author these data are incomplete). From Thibier, M. (2007, 2008, in press in 2010).

Year	Species	Transferable embryos collected	Embryos fresh	Transferred frozen	Total transferred
2006	Cattle	777,747	314,706	356,005	670,711
2006	Sheep	56,519	24,293	18,966	43,259
2006	Goat	23,826	7,966	16,423	24,389
2007	Cattle	763,467	281,740	296,137	577,877
2007	Sheep	25,421	9,769	2,365	10,483
2007	Goat	2,434	1,110	113	1,223
2008	Sheep	18,828	4,793	433	5,226
2008	Goat	3,141	824	278	1,102

Data in Table 1 are those supplied by practitioners and national embryo transfer societies around the world and are for numbers of in vivo-derived embryos only. Unfortunately, some countries which are known to carry out many embryo collections and transfers supply little or no data, so the table gives an incomplete picture. In comparison with cattle, numbers of embryos produced by *in vitro* fertilisation for commercial use (as distinct from for research) in sheep and goats can be considered insignificant.

1.4.2. Embryo collection

Collection of embryos in sheep and goats is usually preceded by oestrus synchronisation and superovulation, and then by natural mating, or laparoscopic AI at the synchronised oestrus. In contrast to the usual procedure in cattle, where embryos are collected non-surgically using catheters manipulated manually (per rectum) into the genital tract (Christie, 1996), embryo collection in sheep and goats involves either full-scale surgical laparotomy, or most commonly, laparoscopy which is a less invasive surgical procedure.

Oestrus synchronisation is normally achieved by use of intravaginal devices (sponges or other soft polyurethane/plastic appliances) impregnated with slow-release synthetic progestagens inhibiting ovarian activity. To overcome limitations caused by seasonal breeding in sheep and goats, photoperiodic conditioning or courses of the (synthetic) hormone melatonin may be used. Withdrawal of progestagen is normally followed by synchronous growth of follicle(s) and 'rebound' into oestrus within 2 to 4 days. For superovulation, however, injections of follicular stimulating hormone (FSH) are given to coincide with decline of the progestagen and to stimulate growth of many more ovarian follicles and ovulations.

While the steroid hormones are available as synthetic analogues, thereby avoiding the use of tissue extracts, the gonadotrophins are a different matter because they cannot be readily synthesised, so the FSH, with its key role in superovulation, is obtained from biological sources. Several FSH preparations are commercially available but the most effective and widely used are extracts from the pituitary glands of pigs or sheep. Due to their disease risks, extracted pituitary hormones have been replaced by recombinant products in human medicine. However, similar recombinant FSH products are not available commercially for use in animals.

Insemination, especially if frozen semen is used, is performed by laparoscopy, as described above (section 1.3.2.3). Five or six days after insemination, when embryos have descended into the uterus

and developed to the morula or blastocyst stage, but are as yet unhatched from the zona pellucida, they are collected (flushed) from the uterine cavity. Embryo collection entails full surgical laparotomy with exteriorization of ovaries and uterus, or laparoscopy using similar instruments to those used for AI (McKelvey, 1999; McKelvey et al., 1986). In addition to the actual laparoscope, various surgical instruments, normally made of stainless steel, are used to make incisions, grasp the relevant parts of the reproductive tract, and afterwards to suture the wounds. A small Foley catheter made of silicone rubber, latex or plastic is inserted via an incision into the uterine lumen. The flushing medium is then injected via the catheter into the uterine lumen and the embryos are flushed back via plastic tubing into a collection flask.

For the collection and processing of embryos a fluid medium is used which consists of buffered saline with low levels of blood protein (e.g. foetal calf serum or bovine serum albumen) to maintain embryo viability and to prevent the embryos from sticking together. Using a microscope, the embryos are picked out from the uterine flushings and examined to establish their developmental stage, integrity and viability.

For purposes of disease control, embryos with an intact zona pellucida are usually washed ten times, as recommended in the Manual of the International Embryo Transfer Society (IETS), and are sometimes also treated with trypsin (a proteolytic enzyme from porcine or bovine pancreas) to ensure certain viruses will be removed, if present (Stringfellow, 1998). Embryos for freezing are passed through solutions of a cryoprotectant (e.g. glycerol), aspirated into plastic straws (0.25ml), cooled in a freezing apparatus and then stored in a liquid nitrogen refrigeration tank.

1.4.3. Transfer of embryos

Recipients are usually treated with progestagens to ensure oestrus synchrony between the maternal reproductive cycle and the developmental stage of the embryo. Methods for synchronisation are similar to those already described, but, except in the case of out-of-season breeding, induction of ovulation by use of FSH (such as eCG or pituitary gonadotrophin) may not be necessary. Fresh (i.e. unfrozen) embryos can be loaded directly into straws in the original collection medium and transferred into recipients immediately or at most within a few hours of their collection. Frozen embryos are usually thawed by passing through dilutions of glycerol or sucrose in buffered saline to remove the cryoprotectant. They are then loaded into new straws for transfer into the recipient(s).

Embryos are transferred into the recipients by penetrating the uterine wall then inserting them directly into the uterine cavity. This involves laparotomy under general anaesthesia, or use of the laparoscopic method under general or local anaesthesia. Laparoscopy is commonly used to transfer embryos into recipients. The transfer procedure resembles that used for AI in sheep and goats, i.e. the straw or pipette containing the fresh or frozen-thawed embryo(s) is used to insert them into the uterus.

1.4.3.1. Advanced reproductive technologies

In addition to AI and the collection and transfer of *in vivo*-derived embryos (i.e. embryos collected a few days after *in vivo* fertilisation), a number of other artificial reproductive technologies have been developed. These include the collection and *in vitro* maturation of of oocytes, *in vitro* fertilisation (IVF), and *in vitro* culture of the resulting zygotes to the morula or blastocyst stage. Embryos of this type are referred to as *in vitro*–produced (IVP) embryos and these can form the basis for additional reproductive technologies such as cloning and transgenesis. In cattle large numbers of IVP embryos are produced and transferred commercially. However, few IVP embryos are used for commercial transfer in small ruminants, and those that are produced are mostly for research or to create transgenic animals that yield pharmaceutical proteins in their milk or serum for medical use. Description of the advanced reproductive technologies and the TSE transmission risks they pose are beyond the remit of this mandate.

1.4.4. ET and disease transmission risks

Sanitary protocols such as the official registration of embryo collection teams, embryo washing, and veterinary certification have been established by the IETS (IETS, 1998) and are advocated by the Office International des Epizooties (OIE) to reduce the risk of transmitting infectious diseases by ET.

Due to the properties of the zonae pellucidae of IVP embryos, which seem to make them 'sticky', they are less amenable to pathogen removal by washing than *in vivo*-derived embryos (Booth et al., 1999; Langston et al., 1999; Marquant-Le Guienne et al., 1998; Stringfellow and Wrathall, 1995; Trachte et al., 1998). The potential for pathogen exposure during oocyte collection, IVF and *in vitro*-culture is further increased by batch production methods, and by the use of many substances of animal origin, including cell cultures, which are routinely used (Bielanski, 1998). Most laboratories collect oocytes weekly but culture to the morula/blastocyst stage can take up to nine days, so there may be some overlap between batches, with attendant risks of introducing new infections into ongoing batches. The advanced technologies such as *in vitro* embryo production (IVP), cloning and genetic modification (transgenesis), tend to carry higher risks simply because they involve prolonged culture and/or complex instrumentation, and often require substantial use of biological materials.

In general the risk of transmitting infectious diseases associated with embryos that have been collected in accordance with the sanitary protocols advocated by IETS and OIE are considered to be low.

TSE in small ruminants have unique features such as long silent incubation period and widespread distribution of infectivity in the host. Moreover, TSE agents themselves have high abilities to resist to decontamination or treatment usually applied to destroy bacteria or viruses.

In this context, depending on the scrapie status and aspirations of the importing country, region or flocks, the OIE and IETS proposed procedures for TSE risk prevention (see Appendix A). These measures, which intend to reduce at minimum TSE transmission risk, are not compulsory and their application is up to users and their final objectives

1.4.5. Conclusions

- The current number of ETs in small ruminants carried out annually remains limited compared to those in the cattle sector. This limits the potential number of TSE cases that could occur if any TSE transmission risk is associated with ET.

- Since ET is recommended as a method to limit/avoid introduction and geographical spread of infectious diseases, the occurrence of a single TSE transmission by ET (like the introduction of the disease in an otherwise “scrapie free” area) could have major impact if it should occur.

2. Risk of transmitting TSEs via AI in small ruminants

Data related to the presence of TSE agents in male reproductive tract or in semen are rare. Publications in this field are mainly related to rams and only involve Classical scrapie. No elements related to BSE in small ruminants or Atypical scrapie are available, which is a major limit to this risk assessment.

2.1. Cellular and abnormal PrP in male genital tract and semen

Cellular prion protein (Prp^{C}) is widely expressed in male seminal tract in human, bovine, ovine, mouse and hamster (Ecroyd et al., 2005; Ecroyd et al., 2004; Fujisawa et al., 2004; Gatti et al., 2002; Shaked et al., 1999). In rams large amounts of PrP^{c} are found in spermatozoa and genital tract fluids. In sperm a large part of PrP^{c} is present under soluble form in the seminal plasma but a fraction of the

PrP^{c} protein is found within the sperm cytoplasmic droplet (a cytoplasmic remnant shed from sperm as a large vesicle after ejaculation), the epididymosomal vesicles (small vesicles of about 100 nm present in this fluid and derived from epithelial cells from the epididymis and accessory glands) and also under a micellar form where it is associated with lipids and hydrophobic proteins (Ecroyd et al., 2005; Ecroyd et al., 2004; Gatti et al., 2002).

Only two published studies reported investigations on the presence of PrP^{Sc} in ram semen, comprising seminal plasma and spermatozoa, and epididymal fluid (Gatti et al., 2002; Sarradin et al., 2008).

PrP^{Sc} was investigated in (i) cauda epididymal fluid from two scrapie-infected VRQ animals and one ARR from the same flock used as a control and (ii) the seminal plasma from three scrapie-infected VRQ animals and three ARR animals from the same flock. The PrP was first immunoprecipitated from the cauda fluid and the seminal plasma due to the presence of high concentration of protease inhibitors in these fluids that interfered with the direct use of proteinase K treatment. The immunoprecipitated PrP was then treated by proteinase K, and no PrP^{Sc} could be observed. This technique is used to demonstrate the presence of PrP^{Sc} in the brain from an infected sheep. Then this first observation, although limited, indicated that the fluid from the reproductive tract of ram did not contain detectable amount of PrP^{Sc}. Meanwhile, the absence of detectable PrP^{Sc} cannot warrant a lack of infectivity (in particular in biological fluids) (Lacroux et al., 2008; Lasmezas et al., 1997).

2.2. TSE infectivity in male genital tract and semen

Attempts to detect prion infectivity in male genital organs or semen are few.

Semen from one scrapie affected ram was tested by bioassay in a natural host (Palmer, 1959). The semen was diluted (1/5) before subcutaneous injection in two 21 days old lambs. The animals were then observed during 30 months without occurrence of clinical signs of TSE or neuropathological signs of scrapie at culling. Drawing conclusions from this experiment is very difficult since:

- The PrP genotype of recipients was unknown;
- A single donor ram was tested;
- The observation period of recipients remained too short to ensure the animals would not have developed scrapie.

Testes and seminal glands from scrapie-infected rams were tested by heterologous bioassay (C57Bl6 mice) and no transmission was observed (Hadlow et al., 1982). However, heterologous species bioassay includes the passage of a species barrier which limits the sensitivity detection limits. In the same experiment, no transmission was observed with skeletal muscle, blood or salivary glands, which were later reported to be PrP^{Sc} positive and/or infectious using Tg mice model that over-express the ovine PrP gene (Andreoletti et al., 2004; Vascellari et al., 2007; WHO, 2006). Consequently, these negative results have to be considered with great caution.

More recently Sarradin et al. (2008) reported an attempt to detect infectivity in ram semen (seminal plasma and spermatozoa) by bioassay in transgenic mice (Tg338) over-expressing the ovine VRQ prion protein. This mouse model is considered to be highly permissive to most of Classical scrapie agents (Vilotte et al., 2001). The rams belonged to a flock which was naturally infected with Classical scrapie (Langlade flock). The tested samples (n=3) included an animal at the terminal phase of the disease. None of the mice inoculated with 20 µl of semen (containing about 10^{8} spermatozoa, one quarter of the quantity used in insemination) developed scrapie in the time frame of the experiment (up to 749 days post inoculation).

This experiment brings some interesting elements however, its design strongly limits their significance. Indeed, the number of tested samples is extremely low. Moreover, if brain homogenates from the animals belonging to the same flock were tested, no titration of these scrapie isolates was provided, which precludes the possibility to quantify the infectivity detection limit for the tested semen samples.

Studies carried out in mice transgenically engineered to organ-specific chronic inflammation with development of granuloma or ectopic lymphoid follicles demonstrated that such inflammatory process allows the accumulation of prions in affected tissues (kidney, pancreas and liver). A similar phenomenon has been reported in sheep which developed mammary ectopic follicles following Maedi viral infection, resulting, in case of scrapie co-infection, into PrP^{Sc} accumulation in the mammary gland and shedding in milk (Lacroux et al., 2008; Ligios et al., 2005).

In natural hosts PrP^{Sc} deposition has been observed in chronic inflammation histologically characterized by development of ectopic lympho-follicular structures (Hamir et al., 2006; Lacroux et al., 2008; Ligios et al., 2005). To date, other common chronic inflammations such as granuloma, which is characterized by aggregations of activate macrophages, are reported to accumulate PrP^{Sc} only in Tg mice.

Considering these data, inflammatory changes affecting the reproductive organs of male, particularly when showing ectopic follicles, could promote the shedding of prion infectivity in genital fluids.

A number of infectious agents can result in inflammation, including granulomatous inflammation, of ram and he-goat genital tract or organs (Cerri et al., 1999; Doherty, 1985; Ladds, 1993; Palfi et al., 1989). However, recommended OIE sanitary procedures for AI centres should allow detection and discarding of affected animals, since several of these inflammatory conditions (orchitis or epididimitys) give rise to semen improper for AI.

2.3. AI as a TSE associated risk factor

Only one epidemiological study, aimed at identifying the risk factors and flock management practises that are associated with Classical scrapie risk, investigated the potential role of AI use. According to this study AI was not significantly associated to Classical scrapie introduction risk in flocks (Philippe et al., 2005). Despite its value, it is clear that conclusions from this single study, which is based on case-control design and on retrospective investigations in flocks, must be considered with caution.

To date, there is no publication describing experiments in which AI procedures with semen collected in infected animals have been tested for their ability to transmit scrapie. The only results that are available were produced in two independents experiments carried out in the early eighties in USA by Foote et al. These experiments remained unpublished (Wrathall, 1997).

In these experiments semen from orally-challenged rams (using SSBP-1 scrapie) was collected by electro-ejaculation and then pooled to inseminate ewes. Lambs born from this AI and their mothers were then followed up during a period ranging from 2 to 5 years. While no transmission could be observed in ewes and offspring, no clear conclusions can be drawn from these experiments since:

- The proportion of sperm donor rams that developed the disease was low;
- The genotypes of recipients ewes and born lambs was unknown;
- The number of involved individuals was limited.

2.4. Iatrogenic TSE transmission risks associated to AI

2.4.1. Use of animal-derived hormones

In humans about 200 cases of iatrogenic Creutzfeldt-Jakob Disease (CJD) arose from the use of pituitary hormones (growth hormone and gonadotrophins) which were extracted from what were presumably infected human cadavers (Brown et al., 2006). In small ruminants, abnormal PrP^{Sc} and infectivity presence in pituitary gland of Classical scrapie incubating ewes is established. The presence of BSE and Atypical scrapie infectivity in the pituitary gland has not been established but is highly probable.

Hormones are traditionally used for AI procedures. They are mainly extracted from mare (eCGH) or porcine (pFSH), which are two species in which natural TSEs remain currently unreported. However, in a context where (i) production sources and origin of the animal-derived hormones remain difficult to establish, (ii) ovine-derived hormones are commercially available (FSH), the use of such hormones should be considered to be associated to a potential risk of TSE transmission. The use in farm animals of recombinant protein rather than animal-derived products (as in humans) would avoid such a risk.

2.4.2. AI surgical procedure

Iatrogenic transmissions of CJD by surgery resulted in more than 200 known cases. They arose mainly from transplants of dura mater from cadavers that were subsequently shown or suspected of having had CJD (Brown et al., 2006). Smaller numbers have resulted from transplantation of eye tissues (cornea and sclera) (Duffy et al., 1974; Maddox et al., 2008) and from use of contaminated neurosurgical instruments or intracerebral electrodes.

More recently, the discovery of the presence of infectivity in blood and lymphoid tissues of variant-CJD (v-CJD) incubating patients, but also in some peripheral tissue of sporadic-CJD (s-CJD) patients raised new concern about the potential TSE transmission risk that could be associated to surgical devices. While decontamination procedures commonly used are highly efficient for destruction of viruses and bacteria, their efficacy on prions remains limited. In small ruminants, TSE agents dissemination in organism of incubating individuals is very similar to what reported in human v-CJD cases (blood, lymphoid tissues, etc…). Surgical instruments and laparoscopes used for intrauterine and possibly for extra-cervical insemination should so be considered as potential sources of iatrogenic transmission of TSE. Such iatrogenic transmission risk remains unquantifiable.

2.5. Conclusions

- Data related to the TSE transmission risk associated with AI or semen in small ruminants are sparse. All were produced in the context of Classical scrapie infection. Each study considered individually is of limited significance.

- When taken together the available data suggest that the risk of TSE transmission associated with semen collected from Classical scrapie incubating rams and he-goats ranges from negligible to low. However, data are insufficient to conclude that such a risk is negligible.

- Because of the similarities between Classical scrapie and BSE pathogenesis in small ruminants, the previous statement should also to be considered valid for BSE

- Absence of data related to semen collected from small ruminants infected with Atypical scrapie precludes the assessment of the risk of transmission of this disease via semen in sheep and goats.

- Some of the AI procedures are associated with an inherent but not quantifiable iatrogenic TSE transmission risk (use of animal-derived hormones and surgical procedures for intrauterine insemination).

3. Risk of transmitting TSEs via ET in small ruminants

3.1. Abnormal PrP and Infectivity in female genital tract and embryos

In small ruminants, the presence, from early pregnancy, of the Classical scrapie agent in a gravid uterus from incubating sheep and goats has been established since the early sixties (Pattison and Millson, 1961). The main structure accumulating the TSE agent in a gravid uterus is the placenta and more precisely the foetal part of the placentome (trophoblast).

There are consistent results supporting a lack of detectable PrP^{Sc} in the non gravid uterus and in uterine regions which are not involved in placentomes during pregnancy (Andreoletti et al., 2002; Tuo et al., 2002). Data related to TSE infectivity in ovary and (non gravid) uterus wall are very limited (Hourrigan et al., 1979; Hourrigan, 1988). However, they indicate a probable absence or low infectivity titer in these tissues. All these data are related to Classical scrapie, and no specific data on BSE and Atypical Scrapie are available.

There are no available results concerning abnormal prion protein or presence of infectivity in embryos collected from TSE incubating small ruminants. The lack of data on that particular aspect represents a major limitation to the development of this risk assessment.

3.2. TSE transmission via ET

All the studies conducted to investigate the potential development of TSEs in offspring born from ET share a common design:

- The collection of embryos from TSE incubating or affected ewes/goats;
- The transfer of the embryos into recipient ewes/goats;
- The monitoring of ET offspring and their surrogate mothers;

3.2.1. Classical scrapie

There are a number of studies that were carried out to investigate the potential for Classical scrapie transmission by embryo transfer in sheep. Details of these are given in Appendix B.

3.2.1.1. Historical data

In the 1980s two separate groups, one in the USA (Foote et al., 1993), and another in Scotland (Foster et al., 1993; Foster et al., 1996; Foster et al., 1992) started to investigate the possibility of TSE transmission by ET. In both cases, embryos were collected from donor ewes or goats which had been inoculated with scrapie but which had not developed clinical disease.

In the experiments conducted by the US group scrapie cases were not identified in sheep and goats born as the result of embryo transfer. However, these experiments were carried out at a time when the influence of PrP gene polymorphisms on TSE susceptibility was unclear, and retrospective studies revealed that a significant proportion of the inoculated donor ewes were not susceptible to the disease. Moreover, there were several other caveats (number of animals included, duration of the monitoring

period, absence of clear TSE status at death) which limit the significance of these results (Foote et al., 1993; Maciulis et al., 1992).

The experiments conducted by the Scottish group were carried-out at a time when genetic susceptibility to scrapie was better established but the results obtained were complex. In two experiments the occurrence of scrapie was reported in a proportion of the susceptible genotype offspring born from the embryo transfers, which suggested that transfer of embryos from Classical scrapie incubating ewes into apparently healthy recipient could result in transmission of the disease into the offspring. However, these results are very controversial and are still debated (see Appendix B).

The flock in Scotland from which the experimental animals were obtained had an endemic natural scrapie infection, albeit of a different strain than the inoculum (SSBP-1) given to the donor ewes prior to collecting their embryos. Some embryo transfer offspring in the second (follow-up) study developed the disease despite the fact that their true mothers, i.e. the donors of embryos, had not been inoculated with scrapie. This indicates that at least some of the cases in offspring were caused by the endemic scrapie agent. Further investigations (Foster et al., 1996; Hunter et al., 1996) indicated that the clinical manifestations and brain lesions found in the majority of the scrapie-affected embryo transfer offspring were characteristic of the incriminated endemic scrapie agent rather than of the SSBP-1 that had been inoculated into the embryo donors. It was originally thought that the neuropathological profiles in two out of the ten scrapie cases observed in these offspring could have been consistent with SSBP-1.

It is impossible to draw definitive conclusions from the Scottish group's experiments as they stand. Nevertheless, despite their weaknesses and their controversial nature, they cannot be simply discarded. Consequently, it is advisable that bioassay strain typing should be carried out using the available biological material in order to assess the exact nature of the scrapie agent (natural endemic scrapie vs SSBP-1) that developed in the two suspect offspring.

Embryo transfer experiments to investigate Classical scrapie transmission in goats were performed by Foote and colleagues in the USA but few details were ever published. Methods were generally similar to those used in their sheep work (cited above) and the donors (Spanish breed goats) were inoculated orally with the same sheep-passaged scrapie inoculum prior to embryo collection. None of the embryo transfer offspring or their surrogate dams developed scrapie during observation periods of 5 years (Holyoak personal communication, cited in Wrathall et al., 2008).

3.2.1.2. Newly published studies

More recently two studies related to the Classical scrapie transmission in sheep by ET were published. In both studies the donors of the embryos were affected with natural scrapie and embryos were collected at different stages of the incubation period (Low, 2008; Low et al., 2009; Wang et al., 2002; Wang et al., 2001).

In both studies:

- PrP genetic susceptibility of both donors and recipients ewes was considered;
- Particular attention was paid to the TSE status of recipient ewes;
- TSE status of animals born from embryo transfer experiment was checked at culling.

No transmission was observed in any of the animals born from ET. However, because of the limited number of animals that can reasonably be included in experiments of this kind, the statistical significance of the studies remains limited. The more significant experiment concluded that scrapie

transmission risk via the transferred pre-implantation embryos could be as high as 9.1% (IC 95%) (Low et al., 2009).

3.3. BSE and Atypical scrapie

Only one experiment of embryo transfer from BSE infected small ruminants has been performed. Eleven donor goats had been challenged with BSE 13 months prior to embryo collection and ten developed scrapie-like clinical signs between 18 and 42 months after challenge (Foster et al., 1999). Embryos were washed and transferred into 29 recipients and a total of 37 offspring was born. Some of these offspring died due to inter-current disease but the rest remained healthy and without clinical signs or pathological lesions of BSE for up to five years after birth (J. Foster, personal communication cited in Wrathall et al. (2008)).

Despite its interest this experiment suffers from several weaknesses which limit its significance, These are:

- The number of animals included and the duration of the monitoring;
- The absence of knowledge related to the PrP gene polymorphism (in donor- recipient goats and their offspring);
- The methodologies used for establishing the BSE infectious status of the animals.

To date, there are no available data concerning the risk of transmitting Atypical scrapie via embryo transfer.

3.4. Iatrogenic risks of TSE transmission associated with embryo transfer procedure

3.4.1. Use of animal derived hormones

In humans about 200 cases of iatrogenic Creutzfeldt-Jakob Disease (CJD) arose from the use of pituitary hormones (growth hormone and gonadotrophins) which were extracted from what were presumably infected human cadavers (Brown et al., 2006). In small ruminants, abnormal PrP^{Sc} and infectivity presence in pituitary gland of Classical scrapie incubating ewes is established. Presence of BSE and Atypical scrapie infectivity in the pituitary gland has not been established but is highly probable.

FSH derived from ovine pituitary gland is used for super-ovulation in ewes in order to obtain large number of embryos and during the procedure of transfer to prepare the recipient female. This hormone can be obtained purified or as crude extract from different chemical or biological companies. Although suppliers are regulated for such products (using mainly New Zealand animals as a source of biological materials), the origin of the tissues or animals from which the hormones are extracted remains difficult to control. Little information is available on the method and different steps of purification of these hormones (if any) or if they are only crude extracts. In advanced reproductive technologies, some of these extracts are used also in cell cultures systems.

Considering the uncertainties in term of tissues used for production of ovine-derived FSH, the use of this product should be considered a potential TSE risk. A switch towards the use of recombinant FSH, as in human medicine would avoid such a risk.

3.4.2. ET Surgical procedure

Iatrogenic transmissions of CJD by surgery resulted in more than 200 known cases. They arose mainly from transplants of dura mater from cadavers which were subsequently shown or suspected of having had CJD (Brown et al., 2006). Smaller numbers have resulted from transplantation of eye tissues (cornea and sclera) (Duffy et al., 1974; Maddox et al., 2008) and from use of contaminated neurosurgical instruments or intracerebral electrodes.

More recently the discovery of the presence of infectivity in blood and lymphoid tissues of variant-CJD (v-CJD) incubating patients, but also in some peripheral tissue of sporadic-CJD (s-CJD) patients raised new concern about the potential TSE transmission risk that could be associated to surgical devices. While decontamination procedures commonly used are highly efficient for destruction of viruses and bacteria, their efficacy on prions remains limited. In small ruminants TSE agent dissemination in the organism of incubating individuals is very similar to what reported in human v-CJD cases (blood, lymphoid tissues etc). Surgical instruments and laparoscopes used for intrauterine and possibly for extra-cervical insemination should so be considered as potential sources of iatrogenic transmission of TSE. Such iatrogenic transmission risk remains unquantifiable.

3.5. Conclusions

- The presence of infectivity in embryos collected from TSE infected small ruminants has never been directly assessed.
- Several experiments have been carried out to assess the risk of transmitting Classical scrapie by embryo transfer in small ruminants. Each study considered individually is of limited significance.
- When taken together the available data suggest that the risk of TSE transmission associated with embryos collected from Classical scrapie incubating ewes and she-goats ranges from negligible to low. However, data are insufficient to conclude that such a risk is negligible.
- Because of the similarities between Classical scrapie and BSE pathogenesis in small ruminants, the previous statement should also to be considered valid for BSE.
- Absence of data related to ET from Atypical scrapie affected small ruminants precludes the assessment of the risk of transmission of this disease via embryo transfer in sheep and goats.
- Embryo transfer procedures are associated with an inherent but unquantifiable iatrogenic TSE transmission risk (use of animal-derived hormones and surgical procedures for intrauterine ET).

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

- Based on the data currently available the risk of TSE transmission associated with semen collected from Classical scrapie incubating rams and he-goats ranges from negligible to low. However, data are insufficient to conclude that such a risk is negligible.
- Based on the data currently available the risk of TSE transmission associated with embryos collected from Classical scrapie incubating ewes and she-goats ranges from negligible to low. However, data are insufficient to conclude that such a risk is negligible.

- Because of the similarities between Classical scrapie and BSE pathogenesis in small ruminants, the two previous statements are also to be considered valid for BSE.

- At this stage the assessment of the risk of transmission by semen or embryos collected from sheep or goats affected by Atypical scrapie is not possible because of a lack of knowledge of the pathogenesis and anatomical distribution of the Atypical scrapie agent within affected animals.

- There is an inherent but unquantifiable risk of iatrogenic TSE transmission associated with the artificial insemination and embryo transfer procedures (use of animal-derived hormones and surgical devices).

- The absence of reliable figures on the annual number of artificial inseminations and embryo transfers performed in small ruminants in the EU Members States hampers the quantitative assessment of the potential impact of an artificial insemination and embryo transfer transmission risk on TSE prevalence in the EU small ruminant population.

RECOMMENDATIONS

- In order to further assess the Classical scrapie and BSE transmission risk associated with small ruminant semen and embryos, infectivity should be assayed, using highly sensitive animal models, in:

    - Semen collected from a statistically significant number of TSE infected males bearing susceptible PrP genotypes at different stages of the disease and with different TSE agents.

    - Embryos collected from a statistically significant number of TSE infected females bearing susceptible PrP genotypes at different stages of the disease and with different TSE agents.

- Specific data about pathogenesis and anatomical distribution of the Atypical scrapie agent should be generated. Investigations should include small ruminant males and females at different stages of the reproductive cycle.

- Procedures to limit the risk of iatrogenic transmission of TSEs associated with embryo transfer and artificial insemination should be promoted. In particular the replacement of ruminant-derived hormones by recombinant proteins should be considered.

- A database recording the artificial inseminations and embryo transfers performed each year in the EU should be established.

- In sheep homozygous and heterozygous ARR rams and ewes as donors and recipients would minimise the risk of Classical scrapie and BSE transmission that could be associated with reproductive technologies.

- Similarly, once clarified and if validated, resistant-genotype he-goats and she-goats as donors and recipients would minimise the risk of Classical scrapie and BSE transmission that could be associated with reproductive technologies.

DOCUMENTATION PROVIDED TO EFSA

1. Letter (ref. n. SANCO E.2/MH/khk D(2009) 520232 dated 25/05/2009) from the European Commission with a request for a scientific opinion on the Risk of transmission of TSEs via semen and embryos in small ruminants (sheep and goats).

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3. Wrathall AE, Holyoak GR, Parsonson IM and Simmons HA 2008. Risks of transmitting ruminant spongiform encephalopathies (prion diseases) by semen and embryo transfer techniques. Theriogenology 70. 5, 725-45.

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APPENDICES

APPENDIX A

Measures to reduce the risks of transmitting Classical scrapie via semen and embryos.

Depending on the scrapie status and aspirations of importing country, region or flock, measures advocated by international advisory bodies such as the Office International des Epizooties (OIE) and the International Embryo Transfer Society (IETS) could include the following:

- use donors (ewes and/or rams, female and/or male goats)) from low scrapie incidence flocks if these can be identified;
- PrP genotyping of potential donor males and females and, depending on status of the importing country or flock, select genotypes to either a) increase chances that the offspring will be resistant to commonest scrapie strains, or b) increase chances that clinical manifestations of scrapie, if they occur, will do so early while offspring are still under quarantine;
- ensure that the sanitary protocols for preventing transmission of conventional pathogens via semen and embryos, as in the relevant Chapters of the OIE Terrestrial Animal Health Code, and in the Manual of the IETS, are adhered to;
- use only frozen semen and embryos so these can, if required, be stored pending post-collection surveillance or testing of donors;
- take and test biopsies of accessible lymphatic tissues (or test a wider range of tissues, including brain, if the donors are killed at or after collection). Test for PrP^{Sc} by immunological methods, and/or for infectivity by bioassays using genetically susceptible sheep, goats or mice;
- select recipients with appropriate PrP genotypes for either resistance or susceptibility, (depending on circumstances) and transfer the semen or embryos into these;
- use an appropriately isolated quarantine station (e.g. a remote island) which is under official veterinary supervision;
- keep offspring and recipients in quarantine under observation (passive surveillance) for clinical signs of Classical scrapie for at least five years, and do *post mortem* examinations for TSE on all those that die (active surveillance);
- pursue a breeding programme in quarantine to produce cryopreserved semen and/or embryos, and second generation progeny, and keep these progeny under observation also;
- slaughter all surviving first generation offspring, and the recipients, after five years and carry out full *post mortem* examinations, including tests for TSEs (~~s~~ (PrP^{Sc} and for infectivity);
- on satisfactory completion of the quarantine programme, release second and subsequent generations, plus any cryopreserved semen and embryos, to their destined mainland flocks, but ensure these flocks are kept under official supervision for at least a further five years.

Use of all these measures would be appropriate only in the most extreme circumstances, for example importation of sheep/goat semen or embryos from countries with endemic Classical scrapie into countries with large, scrapie-free populations. Otherwise the responsible authorities should select an appropriate package of measures to reduce the risk to an acceptable and cost-effective level.

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APPENDIX B

Details of experimental studies on transmission of Classical scrapie via embryo transfer in sheep.

Early studies

Dickinson, Young and Renwick were probably first to appreciate the potential of embryo transfer to investigate scrapie transmission in sheep, and in 1964 they described a small experiment on this topic (one embryo transferred), but no conclusions were reached (Dickinson et al., 1966). Subsequent studies were started in the 1980s by two separate groups: Foote and his colleagues in the USA, and Foster and his colleagues in Scotland, but results from these two groups were different and need careful interpretation. In essence Foote's group found no evidence for scrapie transmission whereas Foster's group, to quote from their first paper (Foster et al., 1992), reported that *"... under the conditions prevailing in this experiment, scrapie infection* [was] *passed from the dams to their offspring via the preimplantation embryo*". The Scottish group's findings gained international prominence and led to a widespread belief that scrapie is liable to be transmitted via sheep embryos. Details of these studies are as follows.

In 1980, in the USA, Warren Foote and his colleagues commenced their ambitious programme, the objectives of which were to obtain data, by means of reciprocal embryo transfers between scrapie-inoculated and scrapie-free sheep, on a) the occurrence of vertical transmission and b) the efficacy of embryo transfer for obtaining scrapie-free progeny (Foote et al., 1986; Foote et al., 1993). Overall 153 ewes and 26 rams were challenged with scrapie, but not all of these were used in the embryo transfer experiments. Their ages ranged from one to five years and the breeds were Cheviot and Suffolk. The Cheviots were injected subcutaneously with the SSBP-1 scrapie inoculum, whereas the Suffolks were challenged orally, subcutaneously, or by both routes, with a Suffolk-passaged scrapie strain from Texas. All the challenged sheep were kept at the Mission Experimental Station in Texas, and approximately 50% of them eventually developed clinical scrapie after incubation periods averaging 11 months in the Cheviots and 20 months in the Suffolks. The intended five year observation period in the Cheviots (these comprised a third of the challenged animals) had to be curtailed by slaughter at two years, so the percentage with scrapie might otherwise have been higher.

Embryo collections by Foote and colleagues from the scrapie-challenged donor ewes (mated to scrapie-challenged rams) began at less than a month after challenge in some instances and continued for over three years after challenge in others. Intervals to scrapie onset after collection varied from zero (already showing clinical signs) to 32 months in those donors that did eventually succumb. The embryos were not frozen before transfer, and they were washed three, not ten times as later recommended in the 1990 (second edition) Manual of the International Embryo Transfer Society (IETS, 1990). Another IETS Manual recommendation, that embryos without an intact zona pellucida should not be transferred, was not always followed either. The embryos were transferred into a total of 198 scrapie-free recipient ewes, all of which were kept on premises at Utah State University. About a half of these recipients were Suffolks and the rest were either Cheviots or Targhees (a white-faced, western USA range breed) in roughly equal proportions. It appears that embryos from Suffolk donors were mainly transferred into Suffolk recipients, and embryos from Cheviots went mainly into the Cheviots or Targhees. Following the transfers a total of 99 offspring was born, 32 of which died between birth and 23 months, 11 died between 24 and 60 months, and 56 died or were killed at over 60 months. The Suffolk recipients were kept under observation until death or for at least 60 months after transfer, but for most of the Cheviots and all the Targhees the interval was shorter (about two years). No clinical evidence of scrapie was seen in any of the offspring or in the recipients, and histopathological examinations of the brains (done on all but a few of the animals) were uniformly negative.

At first sight Foote's published data encourage optimism that scrapie is not transmitted by embryo transfer, even when IETS Manual protocols (IETS, 1990) were not fully adhered to, but some aspects of the work indicate that a more guarded conclusion is appropriate. Results from his other scrapie transmission experiment which involved transfer of embryos from scrapie-free donors into scrapie-challenged recipients, did not, as might have been expected, yield evidence for transmission of the disease *in utero*. The offspring in this case were removed by caesarean section at term and placed in a clean environment. Counting only those which survived for at least 60 months, and which were from recipients that actually developed scrapie following challenge, there was a total of 19 offspring, but none of these developed scrapie. In only one of Foote's experimental groups, referred to as the "*positive control group*" which consisted of 21 offspring conceived and gestated naturally, reared to weaning (at five months) by their own scrapie-inoculated mothers, and then kept under observation until at least 24, and where possible 60 months old, did any cases of scrapie develop. Two of these offspring, both from the same Cheviot ewe, succumbed to scrapie; one at 31 months and the other at 42 months. The sire, which had been challenged, and the dam (i.e. the Cheviot ewe) succumbed to scrapie also, but of the other 19 offspring, 16 lived to 60 months or more without any evidence of scrapie.

With the benefit of hindsight, other deficiencies can be seen in these embryo transfer studies by Foote and his colleagues. The relatively low incidence of scrapie and fairly long incubation periods in the challenged animals, especially the Suffolks, suggest that variations in genetic susceptibility among the experimental sheep may have contributed to the generally negative results. Analysis of the genotypes (Foote et al., 1993; Maciulis et al., 1992) was done retrospectively and revealed that although about a quarter to a half of the Cheviots, and over half of the Suffolks, carried a PrP gene polymorphism (Q/Q at codon 171) for short incubation, and thus were probably susceptible to the challenge scrapie inocula, many animals in the key groups may not have been susceptible. A further criticism is that most embryos appear to have been collected from the scrapie-inoculated donors early in the preclinical stage, i.e. soon after challenge, so infectivity in the reproductive tract, if it ever existed, would probably have been minimal.

The Scottish team which used embryo transfers to study transmission of scrapie in sheep was led by James Foster and his colleagues at the Neuropathogenesis Unit (NPU), Edinburgh. It is important to note in this case that all the animals came from their long established experimental flock of Cheviot sheep, and information on *Sip* and PrP genotypes obtained by PCR and RFLP was incorporated into their experimental design. In those early studies, the term '*Sip*' referred to 'scrapie incubation period', and sA was the scrapie susceptible haplotype and pA was the resistant haplotype. Foster's team referred to their first experiment (Foster et al., 1992), which started in 1988, as a "*worst case scenario*" because not only were none of the embryos washed prior to transfer but the embryo donors were of susceptible genotypes and had previously been challenged with the highly virulent SSBP-1 inoculum. Six donor ewes, two of sAsA genotype and four of sApA genotype, were used, together with a ram of the sApA genotype. The ewes were inoculated subcutaneously six months prior to embryo collection, and clinical signs of scrapie appeared in all of them between six weeks and six months after collection. The ram was not inoculated until after his semen had been collected for AI but, 11 months after challenge with SSBP-1, he too went down with scrapie and was killed. A total of 37 embryos were transferred into 16 recipient ewes, 15 of the latter being of genotype pApA and one sApA. These recipients were aged three to five years at time of transfer and, while six of them had to be culled fairly early for reasons unrelated to scrapie, nine were still healthy three years after transfer, and one (of sApA genotype) was almost eight years when killed due to old age.

From the 16 recipients a total of 26 embryo transfer offspring were born, but six of them died within a year from causes unrelated to scrapie. Of the remaining 20, three were found to be of pApA genotype, eleven were of sApA genotype, and six were sAsA. Of the latter six, five developed scrapie and were killed at just over two years of age (751-783 days) and the sixth went down about seven months later (979 days). These six cases were confirmed to be scrapie positive by brain histopathology, by electron microscopy for scrapie-associated fibrils (SAF) and/or by immunoblotting for PrP^{Sc}. At the time of

their first paper (Foster et al., 1992) all of the remaining offspring were still healthy, but in their next publication (Foster et al., 1993) Foster and colleagues reported that two of the sApA offspring had subsequently developed scrapie at 1006 and 1270 days. Moreover, a further six of the nine sApA genotype offspring had to be killed due to metabolic illness, and two of these, killed at 988 and 1013 days, although not having clinical signs or histopathological lesions of scrapie, were found positive for PrP^{Sc} by immunoblotting.

At this stage of Foster's studies, with half the surviving embryo transfer offspring being scrapie positive at relatively early ages, many questions were being raised about the work. Some, for example Ridley and Baker (Ridley and Baker, 1995), doubted whether maternal transmission of infection had occurred and instead they favoured a genetic explanation, with the disease having arisen *de novo* in sheep of highly susceptible genotypes. Others suggested that scrapie transmission may have occurred due to lack of washing of the embryos, or postulated that maybe the resistant (*Sip* pApA) recipients were subclinically infected and had infected the offspring *in utero*. Environmental contamination after birth in the recipient flock was another possibility. The authors acknowledged these questions (Foster et al., 1993) and began further transfers from infected and uninfected (control) ewes. This time some embryos were washed according to IETS protocols while others were left unwashed.

Foster and colleagues' further studies were reported in June, 1996 (Foster et al., 1996). As before, all their sheep were from the NPU Cheviot flock, but in addition to *Sip* genotypes (primarily linked with polymorphisms at PrP codon 136; see above) data on PrP codons 154 and 171 were also known (Hunter et al., 1996). Two groups of embryo donors were used; the first consisted of three unchallenged ewes of *Sip* sApA genotype to provide negative control embryos, and the second group consisted of three sApA and three pApA ewes that were inoculated subcutaneously with the SSBP-1 inoculum to provide embryos potentially infected with scrapie. The challenged donors were inoculated about eight months prior to embryo collection and the collections took place 60-100 days before scrapie onset in the sApA donors. As expected, the three challenged pApA donors did not go down with the disease (they were monitored for approx. five years after inoculation). Two sAsA rams were used to provide semen for AI, and, although neither were scrapie-challenged, both developed scrapie naturally when they were about two years old, which was about eight months after semen collection.

All the recipient ewes were of pApA genotype and were over five years old when the embryos were transferred into them, and, although none developed clinical signs or had *post mortem* evidence of scrapie, their observation periods were fairly short. Strict precautions were taken to try to ensure that the embryo transfer media, equipment and operating theatre would not pose scrapie risks to the recipients or the embryos at the time of transfer. Also, at lambing and during rearing, efforts were made by disinfection (with 20 per cent sodium hypochlorite), group segregation and control of husbandry procedures to avoid entry of extraneous infectivity. However, while the groups were kept in separate paddocks on re-seeded pasture that had not previously been used for parturient or experimental sheep, there appear to have been no great distances between them, and this aspect of the experimental protocol might be open to criticism.

A total of 28 embryo transfer offspring were born and ten of these had developed scrapie at the time the paper describing the second experiment by Foster and co-workers was written (Foster et al., 1996). Numbers in the different groups, with the *Sip* genotypes and ages at death with scrapie, are shown in Table 2 which is adapted from their paper.

Rather than providing clear answers to questions arising from their first experiment, these results from the second prompted even more questions. Several (though not all) of the sAsA offspring developed scrapie and most did so at similar ages to those in the earlier work, i.e. between two and three years. The origin of these affected offspring, i.e. from the unchallenged (control) donors or the challenged ones, seems to have had no clear effect on the scrapie rate or age at death. Embryo washing made little difference either. Nevertheless, as Foster and colleagues pointed out in their paper, three embryo

transfer offspring of sAsA genotype survived for well over four years which, they said, is significant because sAsA sheep in the source flock always succumbed to the disease. They suggested that the embryo transfer procedures, together with the stringent precautions to avoid contamination, may have had at least some blocking effect on transmission of scrapie via the embryos.

Table 2: Ages of embryo transfer offspring at scrapie/death in study by Foster et al., (1996)

	sApA Donors: unchallenged		sApA Donors: challenged		pApA Donors: challenged	
	No. born	**Age of death (d.)**	**No. born**	**Age of death (d.)**	**No. born**	**Age of death (d.)**
Offspring type						
sAsA washed	4	860, 1000; two survivors	3	803, 884, 1267	-	-
sAsA unwashed	4	778, 886, 888, 888,	2	769; one survivor	-	-
sApA washed	3	three survivors	1	one survivor	2	two survivors
sApA unwashed	4	four survivors	2	one survivor, one intercurrent death	3	two survivors, one intercurrent death

With regard to the 15 *Sip* sApA offspring, it can be seen in Table 2 that, apart from the two intercurrent deaths, all of these survived for at least four years. However, in their discussion (Foster et al., 1996), and also in that of a parallel paper from the Edinburgh NPU team (Hunter et al., 1996), Foster and colleagues comment that more detailed analyses of the *Sip*/PrP genotypes of these particular animals revealed that "*only one is liable to be susceptible to natural scrapie*". This is interesting since it indicated a suspicion on their part that most if not all of the scrapie cases among the embryo transfer offspring were of natural origin rather than being transmitted from the SSBP-1 inoculated donors. They also stated "*.... it remains possible that the progeny from the embryo transfers were infected at or around the time of lambing, despite the stringent precautions taken to prevent it. Lambs may be particularly susceptible to infection from scrapie in the environment at this time*". This could explain why six of the sAsA genotype offspring derived from the unchallenged donors also developed the disease (see Table 2).

Probably the most compelling evidence pointing to natural (environmental) scrapie rather than the SSBP-1 inoculations of the donors being responsible for the cases in the embryo transfer offspring is that the clinical manifestations and brain histopathology in most if not all of them bore close resemblance to those of the natural type of scrapie endemic in the NPU Cheviot flock rather than to the distinctive changes known to be produced by SSBP-1 inoculum. The development of natural scrapie in the two sAsA rams eight months after their semen was used to sire the embryos also raises the possibility, albeit remote, of paternal infection of the offspring. The experiment appears to shed little new light on whether scrapie infectivity can be passed *in utero* to offspring as a result of the (hypothetical) carrier state in resistant genotypes. Nevertheless the prolonged survival of three of the *Sip* sAsA offspring shows that natural scrapie does not necessarily always arise spontaneously in highly susceptible genotypes (as had been proposed by Ridley and Baker in 1995).

By 1997, despite all the research done by Foote and colleagues in the USA and by Foster and colleagues in Scotland, the question of whether scrapie is likely to be transmitted by sheep embryos remained unanswered and further studies were needed. Priorities in the experimental design of any further studies would have to be: a) the embryos should be obtained from naturally infected sheep and, where possible, from clinical cases; b) the donors, both male and female, and the offspring, should be of known *Sip*/PrP genotype, and c) recipients and their embryo transfer offspring should be properly isolated from all possible sources of extraneous infection.

Recent embryo transfer studies in sheep (published since 2000)

When it became clear that despite all the early work the questions remained unanswered, Foote's team, and another Scottish group (not Foster's), embarked on further studies to try to clarify the situation, at least with respect to transmission of Classical scrapie. The additional work by Foote's team was published by Wang et al. (2002; 2001), and that carried out in Scotland was published in a peer reviewed paper by Low et al. (2009). Low's work had previously been available electronically on the website of United Kingdom's Department for Environment, Food and Rural Affairs (DEFRA) (Low, 2008), but some of the data in the DEFRA account (e.g. numbers of donors and embryo transfer offspring) differ from those given in the peer reviewed paper published in on 2009. Details of these two studies by Wang et al. and Low et al. are as follows.

In the study by Wang et al. (2001), donors were sourced from six flocks of Suffolk sheep with a high incidence of naturally-occurring scrapie. Exact numbers of donors, recipients and offspring are difficult to determine from the paper but appear to be as follows: Fifty two offspring, of which 33 lived to at least five years (60 months) of age, were obtained by embryo transfer from a total of 17 donors which at post mortem examination tested scrapie-positive by histopathology, PrP^{Sc} immunohistochemistry, or by both methods. In addition to the 33 offspring from scrapie-positive donors that survived for at least five years, one offspring survived for four years, two for three years and three for two years, and these too tested negative for scrapie post mortem. Another 35 offspring of which 23 survived for five years were obtained from 18 donors which were found to be scrapie-negative when tested post mortem but, as the authors point out, these donors might have been in the early stages of scrapie incubation. All of the donors were observed until their death or for a minimum of 60 months (five years) before being tested for scrapie, but it is not apparent from the paper whether any of the positive ones had clinical scrapie at the time of embryo collection, or, if not, how long after collection they developed the disease. Prior to transfer, all the embryos were washed ten times (without trypsin) as recommended in the IETS Manual (IETS, 1998). The recipients were of American 'white-face' breeds, and the embryo transfer offspring in the donor-positive and donor-negative groups were obtained from 43 and 14 recipient ewes respectively. As with their embryo transfer offspring, these recipients were, wherever possible, kept alive for five years after transfer, then killed and tested by histopathology or immunohistochemistry. None of the recipients or the offspring derived by embryo transfer from the scrapie-positive or the scrapie-negative donors developed clinical scrapie during the 60 mont observation periods, and all tested negative post mortem by histopathology and immunohistochemistry.

In the second part of their study Wang et al. (2002) determined the genotypes of their donors, recipients and embryo transfer offspring. As they point out, sheep of Suffolk breed with genotype Q/Q at codon 171 are highly susceptible to scrapie. It is difficult to ascertain from the paper the exact numbers with particular genotypes in the different scrapie exposure groups but it seems that 70 % of donors, including all but one of the 17 that were scrapie positive, were of Q/Q genotype (the other being R/Q), and 89 % of the offspring derived by embryo transfer from the scrapie positive donors had the Q/Q genotype. As expected, the 'white-face' recipients had more varied genotypes than the Suffolks, but some did have the high-risk genotypes for those breeds. Based on their results, Wang et al. (2002; 2001) concluded that: "*vertical transmission of scrapie can be circumvented using embryo transfer procedures, even when the offspring have high risk genotypes*".

In the Scottish study by Low et al. which was published initially in 2008 on the DEFRA website (Low, 2008) and later, in 2009, in Theriogenology (Low et al., 2009), embryos were collected from 11 naturally affected ewes in a flock of Suffolk sheep where natural scrapie predominantly occurred in sheep of genotype Q/Q at codon 171, and where incubation periods (from birth) varied from 19 to 64 months, but in most cases were between 23 and 36 months (Hunter et al., 1997). The donor ewes were superovulated and semen from an ARQ/ARQ ram from the same scrapie-infected Suffolk flock was used for laparoscopic insemination of these donors. The experimental embryos, after collection, were passed through five washes of culture medium, two washes of trypsin solution and a further five

washes of medium, as recommended in the IETS Manual (IETS, 1998) and then they were frozen. Repeat embryo collections were made at various intervals from the donors and although the account published on the DEFRA website (Low, 2008) refers to a total of 94 suitable embryos having been collected from the 11 donor ewes, the number of these that came from the eight with pathological lesions (see below) and which were transferred into recipients, is not made clear in the peer reviewed publication by Low et al., (2009). Genotypes of all 11 donors were recorded as Q/Q at the time of embryo collection, but it was discovered later that two of five lambs from one of these donor ewes were of genotype H/Q, not Q/Q, so despite having had clinical signs and having tested PrP^{Sc}-positive post-mortem, this ewe must also have been H/Q. The codon 171 H/Q genotype is usually associated with moderate resistance to scrapie, so those two lambs may not have been fully susceptible, and this may be the reason for not including them in the peer reviewed (Theriogenology) paper. Whereas the report on the DEFRA website includes results for embryos from all 11 donor ewes, the peer reviewed paper (in Theriogenology) refers to embryos from only eight of the 11 donor ewes, and states that results from the other three donors are excluded because, despite their having developed clinical scrapie, post mortem lesions consistent with Classical scrapie were not evident in the brain stems of these three sheep. The remainder of this report refers only to the results given in the peer reviewed, Theriogenology paper (Low et al., 2009).

The experimental embryos were transferred into a total of 50 recipient ewes of New Zealand origin; 31 of genotype R/Q and 19 of genotype Q/Q at codon 171. The transferred embryos resulted in birth of 39 live lambs which were allocated to four categories according to the interval between date of embryo collection and date of onset of clinical signs of scrapie in their donor:

- Category 1 = embryos collected more than 12 months prior to onset of clinical signs
- Category 2 = embryos collected between six and 12 months prior to onset of signs
- Category 3 = embryos collected six months or less prior to onset of signs
- Category 4 = embryos collected from ewes already showing clinical signs

The category 1 embryos yielded five liveborn lambs, four of which survived to at least five years. The category 2 embryos yielded 14 liveborn lambs, 13 of which survived to at least five years. The category 3 embryos yielded three liveborn lambs, two of which survived to at least five years. The category 4 embryos yielded 17 liveborn lambs, 14 of which survived to at least five years. Thus, from the experimental embryos, a total of 33 (85%) of the 39 lambs survived for at least five years (end-point of the study). Of the six that died before five years four did so when less than a month old and the other two died when 7 and 23 months old respectively.

In addition to the experimental embryos that were transferred from infected donors, a further 47 negative control embryos were collected from ten Suffolk donors, genotype Q/Q, that had been inseminated with semen from negative control Suffolk rams of Q/Q genotype. These embryos were transferred into 22 recipients, eight of which were of Q/Q and 14 of R/Q genotypes. Seventeen live control lambs were produced and 12 survived for at least five years. The scrapie-negative donors, rams, and all the recipients in both groups originated from New Zealand (Simmons et al., 2009). Although the sheep in experimental and control groups were kept separate throughout the study, both groups continued to be fed from the same food sources and they grazed pastures within the same premises. All animals were observed for clinical signs of scrapie during life and all that died or were killed (apart from some offspring that died very early) were tested post mortem by histopathology and/or PrP^{Sc} immunohistochemistry.

Despite having in most cases been kept under observation for five years or longer, all of the embryo transfer recipients and offspring remained scrapie-negative clinically and were negative at post mortem examination. The five year observation periods used in the study were significantly longer

than practically all the scrapie incubation periods (assuming exposure to infection occurred at birth) in the flock of Suffolk sheep from which the donors originated. It is also worth emphasising that 14 of the embryo transfer offspring from donor ewes with clinical scrapie at the time of embryo collection survived for five years, indicating that Classical scrapie is unlikely to be transmitted by embryo transfer even when the donors are clinically affected with the disease.

In discussion of their results, Low *et al*. (2009) make some caveats regarding the size of their experiment, which, they state, "*...does not exclude the possibility that the rate of scrapie transmission* [by embryo transfer] *may occur at a low level, and below the limit of detection for the study, or that the transmitted infectivity is so low that the incubation periods are in excess of five years.*" They continue by stating that: "*Formally, the statistical comparison allows us to be 95% confident that if scrapie is transmitted via the transferred preimplantation embryos, then it must occur in fewer than 1 in 11 embryos.*"

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