Risks of transmission of spongiform encephalopathies by reproductive technologies in domesticated ruminants

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Abstract

This paper considers whether transmissible spongiform encephalopathies (TSEs or prion diseases) could be spread by artificial insemination, embryo transfer and other more advanced reproductive technologies which are used for genetic improvement and also for purposes such as production of recombinant drugs for medical use. Although the technologies are most used in cattle, they are increasingly used in sheep, goats and deer as well, all of which can be naturally affected by TSEs. In general, provided appropriate precautions are taken, the risks of TSE carriage specifically by the gametes (spermatozoa and oocytes) or by in-vivo-derived embryos per se appear to be negligible, but further research, some of which is already in progress, will be helpful to give assurance on this point. Greater concerns relate to the many biological products that are used in the technologies, e.g. pituitary hormones used for the superovulation of donors, and various tissues and blood products used in semen and embryo culture/transport media, some of which have the potential to carry TSE infectivity if derived from infected animals. The myriad instruments and items of technical equipment that are used also give cause for concern because if they become contaminated with TSEs they may, due to their construction, be impossible to sterilise properly. Crown Copyright © 2000 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Awareness of the transmissible spongiform encephalopathies (TSEs — also known as prion diseases), particularly scrapie in sheep and bovine spongiform encephalopathy (BSE), has risen dramatically in the past decade. The possibility of a zoonotic link between BSE and new variant Creutzfeldt–Jakob disease (nvCJD) in man, first reported in March 1996 (Will et al., 1996), added ‘fuel to the fire’ and precipitated the so-called ‘beef crisis’ in the UK, Europe and in other parts of the world. In the wake of this there has been a huge increase in research on the TSEs, including studies to elucidate their routes of transmission.

Unconnected with TSEs, but nevertheless momentous, was the revelation early in 1997 that a cloned sheep (‘Dolly’) had been produced in Edinburgh, Scotland, by transfer of the nucleus from an adult cell (Wilmut et al., 1997). This has also stimulated new research and a vigorous debate on the implications of cloning and other reproductive technologies in farm animals, including possible risks of diseases such as TSEs being spread by their use (Evans, 1999). These risks are small but they cannot be dismissed altogether. The possibility, for example,
of a cloned, transgenic animal transmitting TSE infectivity via recombinant products destined for medical use is an alarming but not implausible scenario if sanitary precautions are overlooked. This paper explains how TSE transmission risks might arise in reproductive technologies and how they should be avoided. Topics covered include the following.

- Brief summaries of the reproductive technologies in domesticated ruminants.
- Conventional disease transmission risks via reproductive technologies.
- Differences between conventional infectious diseases and the TSEs.
- Natural TSE transmission, especially during reproduction in ruminants.
- Risks of TSE transmission via reproductive technologies, and their control.

2. The reproductive technologies

2.1. Background

Selective breeding and the science of genetics have been used to good effect over the last 100 years to improve farm livestock, but the reproductive technologies are now enabling even faster progress. Some, such as artificial insemination (AI) and embryo transfer in cattle, sheep and goats, are already well established, and others such as cloning and genetic modification are on the threshold of commercial development. Use of AI and embryo transfer is also starting in other domesticated ruminants such as deer and water buffalo.

Much has been written elsewhere about the reproductive technologies, so the information given here is simply to help understand how TSE transmission risks might arise. Attention is mainly confined to use of the technologies in cattle, sheep, goats, and deer, i.e. the ruminants reported to be susceptible to TSE infection. Essentially the technologies fall into three main categories. Firstly there is AI which enables effective use of the male in breeding programmes. The second category includes superovulation, embryo transfer and in vitro fertilisation; technologies developed mainly in the past 10 to 20 years to enable more oocytes from preferred females to be utilised. Then in the third category is a range of techniques, some relatively new, such as sexing, cloning and genetic modification, which select or modify gametes and embryos to obtain offspring with specific characteristics. Taken together, these technologies not only supplement what can be achieved by conventional breeding but also enable the production of new foods, industrial products, and drugs for use in human medicine.

2.2. Selective breeding

Although not strictly a reproductive technology it is apposite to mention selective breeding because susceptibility to some TSEs is strongly affected by genetic factors. Selective breeding enables promising individuals to be identified and genetic lines to be selected and improved in respect of commercially important characteristics. One of these characteristics is disease resistance, and selection for resistance to conventional diseases, such as mastitis and lameness, has aroused much interest. Discovery of strong correlations between certain genotypes and scrapie susceptibility in sheep and goats (though apparently not in cattle) was followed by development of blood tests to identify those genotypes (Dawson et al., 1998; Goldmann et al., 1998). Combined with selective breeding, these tests are now being used to increase the proportions of scrapie-resistant animals, particularly in sheep populations, and reproductive technologies such as AI and embryo transfer are enabling even faster progress. Conversely if selection is made for other desirable factors without due regard to TSE genetics, the result may be populations of animals that are more susceptible to TSE infections. This could also apply to production of cloned and transgenic animals which, for example in the case of individuals designed to produce recombinant proteins for medical use, should ideally carry TSE-resistant genotypes.

2.3. Semen collection and artificial insemination (AI)

AI was developed in the 1930s as a technique to control venereal disease in cattle, and later to facilitate selective breeding, so it is not a new reproduc-
ative technology. It is used extensively in cattle, especially dairy breeds, but has also been developed for many other species. Partly due to the physical difficulty of insemination, seasonal breeding and their tendency to extensive types of husbandry, AI is not widely used in sheep, goats and deer. Disease agents can often be found in the semen of infected males, and others may contaminate during collection and processing. Nevertheless it is well known that if semen donors are properly selected and managed, and if the semen is properly processed, AI is seldom a cause of disease transmission (Hare, 1985; Philpott, 1993; Eaglesome and Garcia, 1997).

2.3.1. Semen collection and AI in cattle

Semen is routinely collected by stimulating the bull to ejaculate into an artificial vagina, a device consisting of a strong rubber or plastic cylinder with a softer latex liner, and filled with warm water (Parkinson, 1996a). The inner surface of the liner in contact with the penis is lubricated with petroleum jelly or liquid paraffin, and a rubber extension cone leads to a receptacle tube for the semen. Most bulls donate semen voluntarily but occasionally electroejaculation is used, especially with untrained farm bulls. Electroejaculators (the electrodes) are inserted by the operator with gloved hand into the bull’s rectum. Whatever the collection method, the semen is diluted with a liquid extender and stored in plastic straws, usually in a frozen state. Extenders contain animal origin substances such as skimmed milk and/or egg yolk in buffered saline with antibiotics, and cryoprotectants such as glycerol are also used if the semen is to be frozen. It should be noted that although the semen is diluted with extender, the spermatozoa are not separated from the seminal fluids.

Except perhaps in beef cattle where farmers may wish to breed within a narrow time period, it is not normal practice to synchronise oestrus for AI. For insemination the straw is warmed if necessary to thaw the semen, then fitted into a metal catheter-like device (AI ‘gun’) and covered with a disposable plastic sheath (Parkinson, 1996b). With a hand in the animal’s rectum to guide the ‘gun’ through the vagina and cervix, the technician deposits the semen into the uterus. This is normally a quick procedure and no sedation or anaesthetic is required. A promising new technology involves production of sexed semen by flow cytometry (see below) and for this and other situations where available sperm numbers are very low a modified AI technique has been proposed in which the semen is deposited deep within the ipsilateral uterine horn (ovulation side) to increase the chance of a functional sperm reservoir in the active oviduct (Hunter and Greve, 1998). Epidural anaesthesia may be required in this case.

2.3.2. Semen collection and AI in sheep and goats

Rams and bucks, like bulls, can be trained to donate semen into an artificial vagina (small version of the bovine one), and their semen can be frozen. Sometimes, however, semen is obtained by electroejaculation, which entails placing the instrument into the rectum, and sedation or even general anaesthesia is usually required. Insemination presents more problems in sheep and goats than in cattle, partly because of seasonal breeding, and consequently oestrus synchronisation is often used in these species. Further problems arise due to their smaller size and anatomical differences of the cervix. Intravaginal insemination with fresh semen can be successful but pregnancy rates with frozen–thawed semen deposited into the vagina are low. The transcervical route is technically difficult and can cause injury, so AI often involves surgery under general anaesthetic or sedation and a local anaesthetic (McKelvey, 1999). With the animal inverted in a restraining cradle small amounts of frozen–thawed semen are injected directly into the uterus (or oviduct) via laparotomy, or by laparoscopy using a grasping probe and inseminating pipette.

2.3.3. Semen collection and AI in other domesticated ruminants

AI in domesticated deer, especially the red deer (*Cervus elaphus scoticus*), the larger North American sub-species known as wapiti or Rocky Mountain elk (*Cervus e. nelsoni*), the fallow deer (*Dama dama*), and various hybrids, is being developed along similar lines to sheep (Asher and Dixon, 1994). Semen is collected by electroejaculation under sedation, and extenders such as egg yolk/glycerol are used for freezing. In larger deer types, e.g. wapiti/elk, AI is possible by per-rectal manipulation, as in cattle, but in others laparoscopic insemination is the
routine, especially with frozen semen. Semen collection and AI have been practised for many years in domestic buffalo (*Bubalus bubalis*) (Jainudeen, 1996), but are still in the development stages in bison (*Bison bison*) (Dorn, 1995). The techniques resemble those used for cattle.

### 2.4. Oestrus synchronisation and superovulation

Oestrus synchronisation facilitates the supervision of breeding programmes and efficient use of sires by natural mating or AI. It is especially important in species such as sheep and buffalo which show little sign of oestrus in the absence of a male. It is also a pivotal component of embryo transfer programmes. Synchronisation is commonly achieved by the use of intravaginal devices (coils, sponges or other soft polyurethane/plastic appliances) impregnated with slow release synthetic prostegestagens, with or without oestrogen, which inhibit ovarian activity. Sometimes prostegestagen is given as a subcutaneous implant instead of by intravaginal device. Alternatively prostaglandin injections spaced 10 to 12 days apart are used to re-schedule ovarian activity. To overcome limitations caused by seasonal breeding in sheep, goats and deer, photoperiodic conditioning or courses of melatonin treatment may be used.

Withdrawal of progestagen, or injection of prostaglandin, is normally followed by synchronous growth of follicle(s) and ‘rebound’ into oestrus within 2 to 4 days. If, however, instead of natural rebound, a series of injections of follicular stimulating hormone (FSH) is given to coincide with the decline of prostegestagen, this stimulates growth of extra follicles and ‘superovulation’. Small FSH doses may lead to twins or triplets whereas high doses can potentially give many more embryos for collection and transfer. Some programmes incorporate gonadotrophin releasing hormone (GnRH) injections to ensure timely release of endogenous FSH and/or luteinising hormone (LH) from the animal’s own pituitary gland.

In view of their potential for carriage of TSE infectivity (see later) it is pertinent to consider the origin of hormonal preparations used in reproductive technologies. The natural hormones include ovarian steroids (progesterone and oestrogen), hypothalamic peptides (e.g. GnRH), pituitary glycoproteins (e.g. FSH, and LH), and uterine prostaglandins (long chain fatty acid compounds such as PGF2α). Apart from the gonadotrophins these are all available as synthetic analogues, so use of tissue extracts is unnecessary, thus avoiding any associated disease risks. The gonadotrophins, however, are a different matter because they cannot be synthesised, and FSH has a key role, especially for superovulation. Several FSH preparations are commercially available (Gordon, 1994; Christie, 1996) but the most effective and widely used are extracts from the pituitary glands of pigs or sheep. Equine chorionic gonadotrophin (eCG), extracted from pregnant horse blood, is sometimes used, as also is the human menopausal gonadotrophin (hMG) which is extracted from urine, but whilst their origins suggest they should be safer from a disease perspective than the pituitary-derived FSH products, both have disadvantages. Some years ago it was reported (Looney and Bondioli, 1988) that an effective bovine FSH had been produced by recombinant DNA technology but this has not been marketed commercially. Due to their disease risks, cadaver-derived pituitary hormones have now been replaced by recombinant products in human medicine.

### 2.5. Embryo collection and transfer

Embryo transfer began in cattle and sheep in the 1950s and initially involved full-scale surgical intervention. It was not until non-surgical techniques were developed for cattle in the 1970s that the technology began to be widely used commercially. Data from the International Embryo Transfer Society (IETS) (Thibier, 1998) show that almost 400,000 bovine embryo transfers took place world-wide in 1997. Most were in-vivo-derived embryos (i.e. flushed from the uterus after a week after conception) but ~30,000 were produced in vitro. Annual transfers in sheep and goats number at least 6000 and 10,000, respectively (unpublished data), with smaller numbers in buffalo and deer.

#### 2.5.1. Embryo collection and transfer in cattle

Collection is usually preceded by synchronisation and superovulation, and by natural mating or AI at the synchronised oestrus. About 7 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 from the uterine cavity by the non-surgical flushing technique (Christie, 1996). This has similarities to AI, but requires more complex equipment and greater skill. Various types of ‘Foley’ catheter of silicone rubber, plastic, etc. are used, some of which are stiffened with an inner metal stylet during manipulation through the cervix into the uterus, whilst for others a metal tube (introducer) with inner rod (trochar) is passed through first, then the trochar is removed and the catheter is passed through the introducer. Once in position a balloon-cuff on the catheter is inflated to prevent leakage; the collection fluid medium is then injected through into the uterine lumen and the embryos are flushed back via plastic tubing into a collection flask. As with AI, the operator keeps a hand within the cow’s rectum to manipulate the reproductive tract, catheter, etc. by palpation through the rectal wall. At least one assistant is also needed to handle the equipment and to instill the flushing fluid. Donors are almost invariably given an epidural anaesthetic during embryo collection.

Embryos are collected and processed in a fluid medium which essentially consists of buffered saline with low levels of blood protein (e.g. fetal calf serum or bovine serum albumen) to maintain embryo viability and prevent them sticking together. Antibiotics are also added to control bacterial contamination. Using a microscope, the embryos are picked out from the uterine flushings and examined to establish their developmental stage and viability. For purposes of disease control embryos are usually washed 10 times, as specified 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 (0.25 ml), cooled in a freezing apparatus, then stored in a liquid nitrogen refrigeration tank. Eventually, when thawed, the embryo is passed through dilutions of glycerol or sucrose in buffered saline to remove the cryoprotectant; it is then loaded into another straw for transfer into the recipient. Alternative freezing methods such as ‘vitrification’, using ethylene glycol as cryoprotectant, have been developed which give good results and have the advantage that they enable direct transfer of frozen–thawed embryos without cryoprotectant removal (Voelkel and Hu, 1992). Fresh (i.e. unfrozen) embryos can be loaded directly into straws in the original collection medium and transferred into recipients within a few hours of their collection.

Recipients are usually oestrus synchronised to ensure synchrony between the maternal reproductive cycle and the developmental stage of the embryo. The straw containing the fresh or frozen–thawed embryo is loaded into a transfer ‘gun’ of similar construction, although longer, than an AI gun; the embryo is then transferred non-surgically in a manner similar to AI. The need to manipulate the cervix, ovaries and uterus per rectum, and to deposit the embryo well down the uterine horn on the side of the active corpus luteum, means that an epidural anaesthetic is usually required prior to transfer.

2.5.2. Embryo collection and transfer in sheep and goats

As with cattle, oestrus synchronisation and superovulation precede embryo collection from sheep and goats, and the hormonal regimes are broadly similar. Insemination, especially if frozen semen is used, is by laparoscopy, as described above. Embryos are usually collected 5 or 6 days post-insemination when at the unhatched morula or blastocyst stage, and this entails full surgical laparotomy with exteriorization of ovaries and uterus, laparoscopy using similar instruments to those used for AI (McKelvey et al., 1986; McKelvey, 1999), or a combined approach. Small Foley catheters, similar in design to the bovine type, are used, and flushing media tend to be of similar composition to those for bovine embryos. Sanitary processing and freezing of in-vivo-derived sheep and goat embryos are basically the same too. Embryos are transferred via laparotomy under general anaesthesia, or by the laparoscopic method which resembles that used for AI in sheep and goats.

2.5.3. Embryo collection and transfer in other species

Oestrus synchronisation and superovulation can be achieved in deer, buffalo and bison in essentially the
same way as for other ruminants (Asher and Dixon, 1994; Fennessy et al., 1994; Dorn, 1995). Embryos are collected surgically in smaller species of deer, as in sheep and goats, but in larger species (e.g. wapiti/elk), and in buffalo, non-surgical embryo collection and transfer can be done under epidural anaesthesia, as in cattle (Misra et al., 1998). Deer and buffalo embryos are amenable to freezing in the same way as cattle embryos.

2.6. Ultrasound scanning

Ultrasound scanning, especially with the versatile (and expensive) B-mode apparatus, is a key element in some reproductive technologies (Taverne and Willemse, 1989). In large ruminants a probe (transducer) is held and directed by the operator from within the rectum whilst in small species it is applied to the external abdominal wall. The transducer is connected by a rubber enshathed electrical cable to the monitor where tissues of interest are imaged on the screen. Apart from its obvious use for pregnancy diagnosis, ultrasound can provide detailed images of ovarian follicles and corpora lutea. Also, as described later, a specifically designed transducer can be positioned in the vagina of a cow to guide needles for the aspiration of oocytes from ovarian follicles. Sanitary aspects of scanning should not be overlooked, particularly when transducers are placed within the rectum or vagina.

2.7. In vitro fertilisation (IVF) and in vitro production (IVP) of embryos

2.7.1. Background to IVP technologies

Collection of oocytes, IVF, then culture of the resulting zygotes, is principally used to produce embryos in cattle (Gordon, 1994), but the technology is also being developed for sheep and goats (Alvarez et al., 1999; Graff et al., 1999), deer (Pollard et al., 1995) and buffalo (Chauhan et al., 1996; Galli et al., 1998). IVP embryos are less used in routine commercial embryo transfer than the in-vivo-derived type, but are widely used in research. The technology is also the foundation for other more advanced technologies such as cloning and transgenics since it makes available a range of developmental stages, from the oocyte and pronuclear zygote through to the blastocyst, without having to recover them surgically. Reasons why IVP embryos have proved less popular for commercial transfer include their lower survival after cryopreservation, higher disease transmission potential, and a tendency for the resulting offspring to develop a foetal oversize problem with high mortality (Walker et al., 1996; Kruip and den Daas, 1997).

2.7.2. Oocyte collection and IVF/IVP methods in cattle

Essentially oocytes are aspirated from immature ovarian follicles, then matured, fertilised and cultured in vitro to the morula or blastocyst stage. They are aspirated either from excised ovaries or whilst still in situ. In the former case the ovaries may have been removed surgically although more often they are taken from batches of abattoir-slaughtered cattle to a laboratory where follicular aspiration is done with hypodermic needle and syringe. The other method involves aspiration from the ovaries of live cows by trans-vaginal oocyte recovery (TVOR) using a purpose-built ultrasound transducer which houses and guides the aspiration needle (Kruip et al., 1991; Looney et al., 1994). TVOR is a skilled technique, and epidural anaesthesia plus a sedative are required. With the transducer inserted into the vagina and the cow’s pelvic contents visualised on the monitor screen, a long (e.g. 60 cm) single or double lumen needle (with echo-reflective tip) is guided through the anterior vaginal wall and across the peritoneal cavity into the ovary, the latter being held adjacent to the transducer by the operator’s hand within the cow’s rectum. Ovarian follicles are penetrated by the needle and fluids plus oocytes are aspirated via plastic tubing into a receptacle. The transducer unit may be covered with a sanitary latex cover when in use but has to be taken apart for cleaning afterwards, and needles must be sterilised if re-used. TVOR can be used to collect oocytes repeatedly from the same animals, including those with reproductive problems and in early pregnancy, thus enabling an almost unlimited supply of known lineage (Garcia and Salaheddine, 1998). Oocytes from batches of abattoir ovaries, on the other hand, are difficult to trace back to the donors, so disease risks are higher.

Whatever method is used to obtain the oocytes,
their subsequent in vitro maturation, IVF and culture require controlled laboratory conditions, a variety of equipment, sophisticated media, and strict sanitary standards to prevent contamination. Media tend to be similar to those for in-vivo-derived embryos, but with higher levels of serum and antibiotics, and gonadotrophins, steroids, heparin and transferrin may be added at different stages of the culture.

A straw of frozen semen is usually used for the IVF. Prior to its addition to the oocytes the semen is washed to remove cryoprotectant and seminal plasma, then motile spermatozoa are selected by a method such as filtration through glass wool, or separation on discontinuous gradients of bovine serum albumen (BSA) or ‘Percoll’ (silica particles bound with polyvinylpyrrolidone) (Gordon, 1994). In contrast to AI of live cattle, only the sperm fraction is used for IVF, and, as described below, it is now possible to use sexed sperm to produce IVF embryos of known gender. It is also possible to microsurgically inject a single sperm into the oocyte to initiate apparently normal development, a technique known as intracytoplasmic sperm injection (ICSI). ICSI is often used in human IVF to enable infertile men to conceive (Kurinczuk and Bower, 1997) but has had limited success in ruminants (Catt et al., 1996; Chen and Seidel, 1997; Keskin and Brackett, 1999).

The embryos, once fertilised, are usually co-cultured for 7 to 9 days with somatic cells such as granulosa cells (from ovarian follicles), oviductal epithelial cells, or continuous cell lines from other species, e.g. buffalo rat liver (BRL) cells, or monkey kidney (Vero) cells. In the early days of IVF/IVP, newly fertilised bovine embryos were often transferred surgically into ligated oviducts of live sheep and ‘cultured’ there for a week before retrieval and transfer into recipients of the intended species. Temporary culture in sheep oviducts is still used occasionally (e.g. Campbell et al., 1996; Wilmut et al., 1997; Galli et al., 1998) although because there are some potential disease risks this is not a wise practice.

**2.7.3. Oocyte collection and IVF/IVP in other species**

In both large and small ruminants, oocyte aspiration from the ovaries of slaughtered females is straightforward, but aspiration during life by TVOR from smaller species such as sheep and goats is usually impractical, so surgery (laparotomy or laparoscopy) is the method of choice (Smith, 1994; Alvarez et al., 1999; Graff et al., 1999). Production of good quality embryos by IVF/IVP appears to be less efficient than in cattle.

**2.8. Semen and embryo sexing**

Production of sexed offspring is of value in many situations and can be achieved either by semen sexing or by embryo sexing. In the case of bovine semen, separation of male and female spermatozoa is possible because their DNA content differs; those with an X chromosome being about 4% heavier (Cran et al., 1993; Johnson et al., 1994). The technology involves addition of a fluorescent dye to the diluted semen which is then passed through a sophisticated flow cytometer. A laser beam activates the dye-stained DNA which enables male and female sperm to be identified and sorted within an electrical field into separate collection tubes. Although ~95% accurate, the sorting rate (about 1000 live sperm/s) is too slow to provide enough sexed sperm to use for conventional AI in cattle; in fact what many would consider a minimum dose of 2.5 million live sperm (Moller et al., 1972) would take almost an hour, whilst a typical dose for frozen semen (25 million — Parkinson, 1996b) would take up to 10 h. Sorted sperm, however, can be used for IVF where the low numbers are adequate (Cran et al., 1993). Encouraging results have also been reported when small doses of unfrozen, sexed semen (3 × 10^5 sperm), obtained in minutes by a new sorting process, were given by deep intrauterine AI to oestrus-synchronised heifers (Seidel et al., 1998). Semen sexing as used for cattle is possible in other ruminants (e.g. Cran et al., 1997) but has not been developed commercially. Unfortunately the complexity of the flow cytometry equipment means that its effective sterilisation is impractical.

Embryo sexing has been possible for several years in cattle, but its cost and the fact that half the embryos are of the ‘wrong’ sex have restricted its commercial use. One method involves microsurgical removal of a few cells (a ‘biopsy’) from the embryo which are cultured then karyotyped to reveal which
sex chromosome is present (Seidel and Seidel, 1991). Unfortunately this is slow and rather inaccurate, and the biopsy embryos tend not to survive well after freezing. Damage to the zona pellucida also breaches the protective barrier against pathogens, so embryos for export must undergo washing and other necessary treatments before microsurgery. A second sexing method also involves a biopsy but in this case the sex (Y) chromosome is detected by the polymerase chain reaction (PCR) (Herr and Reid, 1991). Although faster and more accurate than karyotyping, the PCR method is quite expensive. The microsurgical equipment required to hold the embryo, cut through the zona pellucida and remove a biopsy is complex, expensive, and difficult to sterilise. Fortunately, only the holding and cutting instruments have contact with the embryo and, being cheaply fashioned from glass tubing or rod using a microforge, these can be discarded after a single use. There appear to be few reports of embryo sexing in ruminant species other than cattle.

2.9. Cloning

Cloning, the production of genetically identical animals, can be achieved in various ways, the simplest being embryo division or disaggregation to produce identical twins, triplets, etc. Triplet calves and quadruplet sheep have been produced by separation and transfer of individual blastomeres from four- and eight-cell stage embryos (Willadsen, 1981; Willadsen and Polge, 1981), but a more practical method for producing identical twins is to split morulae or early blastocysts and to transfer each half into one or two synchronised recipients (Willadsen and Godke, 1984; Seidel and Seidel, 1991). Split embryos, like biopsied ones, have the problem of lowered viability after freezing, but pregnancy rates over 50% can be obtained with singly transferred, fresh demi-embryos, which means over 100% per original embryo.

Identical animals can also be produced by nuclear transfer which involves removing the chromosomes from a mature (usually metaphase I) oocyte and replacing them with the nucleus from a donor cell. The latter may be a blastomere from an early embryo, or a cell from cultured fetal or even adult tissue (Campbell et al., 1996; Bourhis et al., 1998; Kato et al., 1998; Stice et al., 1998). The technique for removing the chromosomes from an oocyte (no distinct nucleus at this stage) and replacing with a donor cell nucleus resembles embryo biopsy, and requires similar microsurgical equipment. Whatever their type, the donor cells are usually co-cultured initially on rodent cell feeder layers in a medium containing a high level (e.g. 10%) of foetal bovine serum. They are then dissociated with trypsin and replication is arrested by further culture in a medium with little serum (e.g. 0.5%). A whole cell is inserted beneath the zona pellucida of the recipient oocyte and, to provoke integration of the donor nucleus into the surrogate cytoplasm, the oocyte is placed between metal wire electrodes on a glass microscope slide and subjected to electrical pulses with ‘electrofuson’ equipment.

Subsequent to nuclear transfer the resultant zygotes are cultured to morula or blastocyst stage before transferring into recipients. Sometimes they are cultured in ligated oviducts of sheep (e.g. Campbell et al., 1996), although this is not ideal due to infection risks. Culture with high levels of serum and co-culture cells can be successful (e.g. Bourhis et al., 1998; Kato et al., 1998) but, as with IVP embryos, fetal oversize, congenital abnormalities and neonatal death have been linked to culture conditions and exposure to serum during these very early stages (Lees et al., 1998).

Probably the best known clone, ‘Dolly’, born in July 1996, was the result of transplanting a nucleus from a culture of mammary gland cells from a 6-year-old sheep (Wilmut et al., 1997). Since then cloned calves have been produced using nuclei from cultured oviductal epithelium and ovarian follicle cumulus cells from an adult cow (Kato et al., 1998). These results are significant because previous attempts to clone mammals by nuclear transfer using any cells other than those from very early embryos, or cell lines derived from them, had failed (Stice et al., 1998), so it had been assumed that nuclear totipotency is lost early in development.

An original aim of nuclear transfer technology was to multiply genetically superior animals for traditional farming, but factors such as the low success rate, poor viability after freezing, and welfare concerns about foetal oversize problems, have tended to dampen enthusiasm. Abnormalities may also arise in
post-natal life, possibly as a consequence of defec-
tive nucleocytoplasmic interactions and genetic dis-
ease (Morris, 1999; Renard et al., 1999). Adult cell
nuclear transfer can, however, be of value for
preserving endangered breeds and species, as shown
by Wells et al. (1999a) who produced a healthy calf
from the last surviving Enderby Island cow by
transplanting granulosa cell nuclei into enucleated
oocytes from a conventional cow. Interest in the
nuclear transfer technology is alsoreviving with
prospects of combining it with genetic modification
technologies to create cloned transgenic animals.

2.10. Genetic modification (transgenesis)
technologies in cattle and sheep

Genetic modification involves taking a gene from
one organism (the donor) and inserting it into the
genome of another. If successful, the modified
('transgenic') individual has a new gene which
functions to produce a protein characteristic of the
donor organism. Traditional techniques for gene
insertion such as pronuclear injection and transfe-
tion (see below) achieve rather random integration
into the genome, but more precise ‘gene targeting’
methods are being developed which will enable them
to be inserted (or deleted) at specific locations on
particular chromosomes (Wilmut, 1998). Gene dele-
tion, for example, can be used to delete the prion
protein (PrP) gene, and individuals modified in this
way should not then succumb to TSE. In ruminants,
however, precision gene targeting is a relatively
futuristic technology.

The primary steps in traditional genetic modi-
fication are to identify and extract the DNA sequence
of the specified gene; this is then used to produce the
desired gene ‘construct’. Next, many copies (usually
hundreds) of the prepared construct are inserted into
the genome of an individual embryo. Typically the
constructs are injected microsurgically into one of
the pronuclei of a single cell embryo (zygote),
whereupon the latter is cultured for some days in
vitro (or, for example, in a sheep’s ligated oviduct).
Embryos surviving this culture period are transferred
into recipient females, hopefully to develop into
transgenic individuals (Simons et al., 1988; Gordon,
1994; Velander et al., 1997). Unfortunately pronu-
clear microinjection is not an efficient technique:
only about 5% of injected zygotes survive to be
transferred and, of these, only a tiny proportion
incorporate the transgene in a balanced way to
become productive transgenic adults (Eyestone et al.,
1998). A higher rate of transgenic offspring has
recently been reported in cattle by using a re-
plication-defective retroviral vector to introduce
genes into metaphase II (arrest phase) oocytes (Chan
et al., 1998).

Another way of producing transgenic animals
utilises nuclear transfer technology plus a process
known as ‘transfection’. This has a major advantage
over microinjection in that potential nuclear donor
cells can be checked to ensure the desired gene is
incorporated before they are used (Cibelli et al.,
1998). Cell lines, usually of embryonic or foetal
origin, are initially propagated in culture, as for
cloning by nuclear transfer. The desired gene con-
struct is linked to a marker gene (e.g. one conferring
resistance to a specified cell toxin) and these genes
are together inserted into the cultured cells by
transfection, a process usually achieved by concur-
rent exposure of the cells and the construct to a
cationic lipid transfection reagent (Schnieke et al.,
1997). After further culture in a medium containing
levels of the toxin lethal to non-transfected cells,
colonies of the resistant cells (i.e. transgenic ones
with marker gene) are selected for further propaga-
tion. These are then used for nuclear transfer in the
sure knowledge that the resulting embryos, if they
survive, will develop into transgenic offspring.
Aliquots of the cells can be frozen down and used
again and again as nuclear transfer donors.

Since only cells expressing the transgene are used,
fewer nuclear transfers and pregnancies are needed
to obtain transgenic individuals. Moreover, because
multiple cloned transgenic individuals are obtained
in the first generation, testing a few individuals for
health and production is predictive for all present and
future clones of that type. Donor cell populations can
also be karyotyped beforehand to ensure that trans-
genic animals will be of the desired sex. Thus, if
transgenic females are needed to produce specific
milk proteins (e.g. for pharmaceutical use) this is
possible in the first generation.

Although a variety of transgenic cattle, sheep and
goats have been produced successfully by zygote
injection and by nuclear transfer, adverse effects
have been reported too, and it is evident that these technologies do carry risks as well as benefits. For example, transgenic goats (and pigs) intended for production of valuable proteins in milk (for human therapy) were found eventually to develop peculiar mammary lesions (Ebert and Schindler, 1993). The foetal oversize problems, already mentioned, which seem to be associated with in vitro culture are also impeding progress with genetic modification technologies. Another more hypothetical risk is that carriage of or susceptibility to infection, including infection with TSE agents, might permeate a narrowly based, genetically modified population and remain in it undetected, only to manifest itself many years later.

3. Overview of the risks of disease transmission by reproductive technologies

3.1. General comments

It must be emphasised at the outset that the risks of transmitting infectious diseases by AI, embryo transfer and other reproductive technologies are extremely small, especially if established sanitary protocols are followed. Broadly it can be said that in-vivo-derived embryos seem to carry the lowest disease risks, with the risks of semen and IVP embryos somewhat higher, but the disease risks of moving live animals are greater than any of these.

Surgical procedures create added disease risks if they are used in reproductive technologies. In small ruminants, for example, it is normal for embryo transfer and sometimes AI to be done surgically, or at least by laparoscopy, so the risks are inevitably higher than for techniques without intentional penetration of the peritoneal cavity. Disease risks of surgery, and also of ultrasound and manual interventions per rectum or per vagina in the larger ruminants, are largely those of mechanical transfer of infection from one animal to another by contaminated instruments, or by the operator’s hands (Divers et al., 1995), and are not necessarily due to carriage of infection via the gametes or embryos per se. The advanced technologies such as semen and embryo sexing, cloning and genetic modification, all tend to carry higher risks simply because they involve prolonged culture and/or complex instrumentation, and often require substantial use of biological materials. Disease risks associated with oestrus synchronisation and superovulation arise mainly because donors and recipients may be treated with potentially contaminated hormonal products.

IVP embryos are the foundation for most advanced reproductive technologies, including cloning and transgenics, but due to the properties of their zonae pellucidae, which seem to make them ‘sticky’, they are less amenable to pathogen removal by washing than in vivo derived embryos (Stringfellow and Wrathall, 1995; Marquant-Le Guienne et al., 1998; Trachte et al., 1998; Booth et al., 1999; Langston et al., 1999) The potential for pathogen exposure during oocyte collection, IVF and culture is further increased by batch production methods, and by the 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 takes up to 9 days, so inevitably there is some overlap, with attendant risks of introducing new infections into ongoing batches.

4. Important characteristics of the TSEs which influence transmission risks

The TSEs are unique diseases, having many characteristics which set them apart from those caused by conventional infectious agents such as bacteria and viruses. For this reason they are often termed ‘unconventional infections’. Notable features of the TSEs include the following.

- The obscure nature of their causal agents which seem to contain no nucleic acid; thus, despite major variations between agent strains, the genetic coding mechanism is a mystery.
- The extreme resistance of the agents to inactivation by standard physical and chemical treatments such as dry heat and radiation, and many chemical disinfectants.
- The epidemiology (natural spread of TSEs between animals) is poorly understood, and there is scant information about threshold infective doses.
- Their incubation periods are very long, i.e. years rather than days, weeks or months, but once clinical disease appears it is invariably fatal.
• The absence of quick and effective tests for presence of infection in living, preclinical (and clinical) cases, and also for infectivity in tissues and fluids.
• The fact that host genetic factors can strongly influence TSE susceptibility and incubation periods, particularly in sheep and goats (and man and mouse).

These and other features of TSEs are now considered in more detail.

4.1. Nature of TSE agents

In all TSEs a characteristic proteinase-resistant insoluble protein, referred to as PrP\textsuperscript{res} or PrP\textsuperscript{Sc}, accumulates in the central nervous system (CNS). Many now believe TSE infections are caused by particles consisting solely of PrP\textsuperscript{Sc}, or ‘prions’, and that infectivity arises when the natural, soluble cell glycoprotein (PrP\textsuperscript{s}) is transformed by abnormal folding at the molecular level into insoluble PrP\textsuperscript{Sc} or prion protein (Prusiner, 1995; Weber and Aguzzi, 1997). However, in recent work, when PrP\textsuperscript{s} was transformed in vitro into protease resistant PrP\textsuperscript{Sc}, the latter was found not to be infectious (Hill et al., 1999a).

TSE strains are subtypes of TSE infectious agents capable of maintaining their distinctive phenotypic characteristics such as disease incubation periods, CNS lesion profiles, and possibly tropisms for specific cell types, when passaged within a host species, or even within other species. A minority view is that nucleic acid might exist as small, hitherto unidentified virus-like particles associated with the PrP\textsuperscript{Sc}, and that the agent strain variations are due to genetic polymorphisms (Farquhar et al., 1998; Hunter, 1999). However, proponents of the protein-only hypothesis (e.g. Collinge et al., 1996; Safar et al., 1998) argue that strain-specific properties of TSE agents are encoded by conformational patterns of the PrP\textsuperscript{Sc} protein.

4.2. Natural transmission (epidemiology) of TSEs in domesticated ruminants

Knowledge of natural disease transmission routes is basic to gauging the risks of transmission by reproductive technologies. For example, if a particular infectious disease is never transmitted by parents to their offspring during natural breeding, then prima facie, it is unlikely that it will ever be carried specifically by semen, oocytes or embryos. Nevertheless, transmission could occur in artificial breeding due to contamination by other tissues, or if contaminated equipment is used. Possible natural disease transmission routes are:

• horizontal (or lateral) transmission: the spread of infection between unrelated animals via direct or indirect contact at any time, or to the offspring after parturition;
• vertical transmission: the spread of infection from parent (male or female) to offspring via germplasm (spermatozoa or oocytes) at fertilisation, or in utero during prenatal life;
• maternal transmission: the spread of infection from the dam to her offspring either vertically (via female germplasm, or across the placenta), or horizontally in the immediate post-parturient period (via milk, saliva, faeces, etc.).

Despite much study, natural routes of TSE transmission are still poorly understood. Long incubation periods make it difficult to link clinical cases to their original sources of infection, and genetic predispositions passed from parents to their offspring, especially in sheep scrapie, make epidemiological interpretations even more complicated. Most TSEs can be transmitted experimentally by injecting or feeding infected material (e.g. brain from affected animals) to others of the same species, and sometimes to different species, but the relevance of this to natural transmission routes is uncertain.

Epidemiological studies have shown that most cases of BSE in the UK arose from dietary exposure to infected meat and bonemeal (Wilesmith et al., 1988; Kimberlin and Wilesmith, 1994) and this, though unintentional, was a man-made transmission route. The mean incubation period for BSE is about 5 years, with a probable range of 2 to 7 years, and it is believed that cows giving birth in the latter stages of incubation, or after clinical onset, are more likely to transmit the disease to their offspring than those calving in early incubation (Donnelly et al., 1997; Wilesmith et al., 1997; Donnelly, 1998). Thus, while no maternal transmission could be detected in offspring born more than 2 years before clinical onset in...
the dam, the risk thereafter was enhanced, rising to ~10% in the last 6 months of the maternal incubation period, and possibly more in those born after clinical onset. Some remain sceptical about these data, arguing that the apparent maternal transmission might in fact be due to genetic variation in susceptibility to BSE. However, there is little evidence that the known PrP gene polymorphisms in cattle affect susceptibility, as they do in sheep. Further, as pointed out by Donnelly et al. (1997), the fact that the maternal transmission risk is positively correlated with incubation stage makes a genetic explanation unlikely. Maternal transmission in cattle is now generally accepted therefore, though how and when the calves are exposed, i.e. transplacentally during gestation, at parturition, or in the early post-natal period via maternal secretions/excretions, is still unknown. Experimental and epidemiological investigations have shown no clear evidence for horizontal transmission of BSE in cattle.

Scrapie, which affects sheep and goats, is the commonest of the natural TSEs, and at least in sheep there is good evidence for both horizontal and maternal transmission (Dickinson et al., 1974; Hoinville, 1996; Wrathall, 1997; Woolhouse et al., 1998). However, because sheep carry a variety of different PrP gene polymorphisms which strongly influence susceptibility, the epidemiology of scrapie in different sheep breeds and populations is very complex. In infected flocks with a high proportion of genetically susceptible animals the disease tends to be common, with clinical signs typically appearing at 2 to 4 years of age, whereas in those with many resistant animals clinical scrapie is rare and mainly occurs in older sheep. In some countries, such as Australia and New Zealand, despite significant numbers of genetically susceptible sheep (Hunter and Cairns, 1998; Bossers et al., 1999), scrapie is not seen, which suggests an absence of endemic infection (and a need to avoid its importation). In countries with endemic scrapie, on the other hand, selective breeding for resistance, based on PrP genotyping blood tests, has good potential for control of the disease (Dawson et al., 1998).

The mechanisms of horizontal transmission of scrapie between animals are still unknown, but ingestion from contaminated pastures leading to infection of the alimentary tract is one possible explanation (van Keulen et al., 1999). Some (e.g. Ridley and Baker, 1998) have proposed that the disease is entirely due to genetic causes; however, the fact that genetically susceptible sheep in scrapie-free countries do not succumb indicates otherwise. As for maternal transmission, this probably occurs transplacentally or soon after birth. Moderate levels of infectivity and PrP$^{Sc}$ have been found in placentae (Pattison et al., 1974; Race et al., 1998) and this is thought to contaminate and persist in pens and pastures, leading also to horizontal transmission. It has been found that scrapie tends to be common in sheep born to infected dams in infected environments (Hourrigan and Klingsporn, 1996). Whether maternal transmission occurs only in late incubation in sheep, as in cattle, is not clear, although Hoinville (1996) mentions that the risk to lambs born the year before onset of the dam’s clinical signs is similar to that in lambs born in the year of disease onset.

Scrapie cases in goats are uncommon and most can be traced to contacts with affected sheep. It is also assumed from tissue infectivity studies that natural transmission routes and pathogenic mechanisms resemble those in sheep (Andrews et al., 1992; Wood et al., 1992). Onset of natural scrapie usually occurs between 2 to 4 years, but after experimental inoculation the incubation can be as little as 20 months. Maternal transmission in goats, although probable, has not been documented. Until recently it was believed that genetic factors had little effect on scrapie incubation in goats, but new studies (Goldmann et al., 1996, 1998) have revealed PrP gene polymorphisms which do have a marked influence.

Chronic wasting disease (CWD), a naturally occurring TSE in deer, was first reported in North America in 1967. Affected species include Rocky Mountain elk or wapiti, mule deer (Odocoileus hemionus), white-tailed deer (Odocoileus virginianus) and certain hybrids. Both captive and free ranging animals have been affected (Williams and Young, 1992; Spraker et al., 1997) and there are mounting concerns about spread into the expanding deer farming industry in North America (Zeman et al., 1998). Horizontal transmission of CWD is thought to occur and maternal transmission is also probable. The minimum incubation period is about 18 months but most cases occur at 3 to 4 years of age. Genetic effects on incubation in deer seem not
to have been studied. The infective agent responsible for CWD differs from those of scrapie and BSE in that it does not readily transmit experimentally to mice or hamsters; transmissions to ferrets, mink and a goat have been reported however (Bartz et al., 1998). Little is known about susceptibility of other domesticated ruminants to TSEs, though one case of BSE in a bison has occurred in a British zoo (MAFF, 1997).

4.3. TSE infectivity in different body tissues

Understanding the risks of TSE transmission by reproductive technologies requires a knowledge of TSE infectivity in different types of tissue, and how it is detected. Presence of PrPSc, the characteristic proteinase-resistant protein of the TSEs, can be demonstrated in the CNS and some other tissues of affected animals by immunohistochemical and immunoblotting tests which, if applied to biopsies of accessible tissues such as lymph node, tonsil or nictitating membrane, can also be useful for ante-mortem diagnosis of TSEs (O’Rourke et al., 1998; Race et al., 1998; Schreuder et al., 1998). However, the presence of PrPSc does not necessarily equate with TSE infectivity, so bioassays, usually in mice, are also required. These are very time consuming and expensive to perform. In peripheral tissues, as distinct from the CNS tissues, neither PrPSc nor TSE infectivity seem to be associated with pathological lesions.

A guide to potential levels of TSE infectivity in various tissues is shown in Table 1 which is based mainly on bioassay results from naturally and experimentally affected sheep, goats and cattle in many different studies, most of which are covered in detail elsewhere (e.g. Hoinville, 1996; Wrathall, 1997; MAFF, 1998). Despite all the studies, allocation of tissues to infectivity categories is fraught with difficulty, and it is unlikely that Table 1 is wholly accurate. Doubts arise for various reasons, not least because some of the published work lacks detail on methodology. Results also vary according to stage of infection, PrP genotype, breed and species. For example, in cattle with BSE (as compared to sheep and goats with scrapie) infectivity has been detected by mouse bioassay in relatively few tissues, i.e. CNS, trigeminal ganglion, retina, distal ileum and

<table>
<thead>
<tr>
<th>Category</th>
<th>Tissue type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No detected infectivity</td>
<td>Skeletal muscle, heart, kidney, urine, thyroid, mammary gland milk, blood (clotted and serum), saliva, connective tissue, skin, cartilage, faeces, testis, epididymis, prostate, seminal vesicle, semen, ovary, uterus, uterine flushings, embryos (in-vivo-derived ones at 7-days post-fertilisation), foetus</td>
</tr>
<tr>
<td>2. Low infectivity</td>
<td>Thymus, liver, lung, kidney, adrenal gland, pancreas, nasal mucosa, salivary gland, bone marrow</td>
</tr>
<tr>
<td>3. Medium infectivity</td>
<td>Lymphoreticular tissues, especially those associated with the pharynx (e.g. tonsil), alimentary tract (e.g. Peyer’s patches) and eye (e.g. nictitating membrane); also spleen, intestines (small and large), placenta, peripheral nerve</td>
</tr>
<tr>
<td>4. High infectivity</td>
<td>Central nervous system (e.g. brain, spinal cord), eye, dorsal root ganglia, pituitary gland, pineal gland, dura mater</td>
</tr>
</tbody>
</table>

*a* Information condensed from many original reports (see text).

*b* Infectivity critically dependent on incubation stage, assay sensitivity and many other factors (see text).

*c* In cattle few non-CNS tissues have been shown to harbour infectivity; i.e. distal ileum, bone marrow.

*d* Inconclusive evidence exists that certain white blood cells might carry infectivity (see text).

*e* Detected by Hourigan (1988, 1990) in sheep by mouse bioassay but not confirmed by other workers.

*f* If killed by stunning/pithing, infected brain emboli may lodge in lung (and possibly other tissues).

*g* Detected by Pattison and Millson (1962) in goat salivary gland by bioassay in goats, but not confirmed.

*h* High risk due to close association with CNS, and evidence of iatrogenic CJD transmission in man.
possibly bone marrow, and other tissues have tested negative. Whereas bioassays, if positive, are valuable for categorising the risk of particular tissues, confidence in negative results obtained when heterologous species (e.g. mice) are used for the tests may be misplaced, particularly if only a few samples are tested, or the tissue dilution factor is high. Testing for BSE infectivity in bovine tissues by inoculating mice could be as much as 1000-fold less sensitive than testing in cattle, the natural host species (Wells et al., 1998). Thus, even if a particular ruminant tissue tests negative in mice, analogies with other species (including man), plus a basic knowledge of TSE pathogenesis, may indicate that it would be prudent to allocate it to a potentially non-negative risk category until proved otherwise. Possible examples include the spleen and placenta in cattle.

In Table 1, in contrast to similar tables in some other publications (e.g. WHO, 1997; Advisory Committee, 1998), potentially high infectivity tissues (e.g. CNS) are placed in the high numerical category, i.e. category 4, whilst those without detectable infectivity are mainly in category 1. This is to facilitate a quantitative approach to risk.

Apart from CNS and some contiguous tissues which can carry high levels of infectivity it is evident from Table 1 that several peripheral tissues can also support at least some TSE replication. Of particular importance in this respect are lymphoreticular tissues, especially those (e.g. tonsil and Peyer’s patches) associated with the alimentary tract. The spleen is another potential source of infectivity in sheep and goats, although none has been found in spleen or lymph nodes of cattle with BSE. Nevertheless, BSE infectivity has been detected to a limited extent in bone marrow of affected cattle (Wells et al., 1998, 1999b), and, surprisingly, it has also been found in the spleen of sheep challenged with BSE (Foster et al., 1996a).

Occurrence of scrapie and BSE infectivity in spleen of sheep, and possible BSE infectivity in the bone marrow (but not spleen) of cattle, might mean that TSE agents infect different types of lymphoreticular cell in these two species. Alternatively their affinity might be for a type of cell residing in different sites. Whatever the case, infectivity levels in peripheral tissues of BSE cattle appear to be proportionately much lower than in sheep and goats with scrapie.

4.4. TSE spread within the body of an infected animal

At this juncture it is important to consider how TSE infection spreads to the CNS from its portal of entry into the body. The traditional view, based on experimental work with scrapie in mice (Kimberlin and Walker, 1988; Fraser et al., 1992) is that after initial replication in the lymphoreticular tissues, especially those of the alimentary tract and spleen, infection spreads along autonomic nerve fibres to the spinal cord, and thereafter to the brain. Replication in the CNS occurs first in those parts of the spinal cord (or brain) from which nerves connect to the sites of primary infection, which implies spread of the agent via the peripheral nervous system. An alternative (or additional) route about which there is currently much speculation is that infectivity might be carried in the blood by mobile cells from the lymphoreticular system.

4.4.1. Possibility of haematogenous carriage of TSE infectivity

Follicular dendritic cells (FDCs) are known to play a key role in TSE replication in the lymphoreticular tissue (McBride et al., 1992; van Keulen et al., 1996; Hill et al., 1997, 1999b) but, being non-mobile cells, they are unlikely to carry infection into the blood stream themselves. Recent work (Blattler et al., 1997; Klein et al., 1997, 1998; Collinge and Hawke, 1998) suggests that B lymphocytes, which are not only mobile and circulate in the blood (part of the ‘buffy coat’ fraction), but also interact closely with FDCs, might act as ‘carriers’ of infectivity. If correct this has important implications for the reproductive technologies.

Direct evidence for TSE infectivity in blood has come mainly from studies with hamsters, mice and humans. For example, Casaccia et al. (1989) demonstrated low levels in concentrated blood from scrapie-infected hamsters (infected intraperitoneally) using other hamsters (challenged intracerebrally) for the bioassays, thereby avoiding the problem of non-homologous testing. Brown et al. (1998) reported
presence of infectivity in samples of buffy coat, plasma, and Cohn plasma fractions I-plus-II-plus III, but not in the red blood cell component or in Cohn plasma fractions IV or V (the albumen fraction) from clinically ill mice that had been inoculated with a human CJD strain. Brown also claims to have detected CJD infectivity in plasma, buffy coat and whole blood of human patients (Brown, 1998). So far as ruminants are concerned, apart from an early report of transmission of a scrapie-like illness to mice by inoculation of serum from an affected ram (Gibbs et al., 1965), infectivity does not appear to have been detected in blood or serum from scrapie-affected sheep and goats, or in blood clots, serum or the buffy coat from BSE-affected cattle that were inoculated into mice. It is important to reiterate, however, that failure to detect infectivity does not necessarily mean it is absent. Samples of concentrated blood or of specific types of blood cells from preclinically and clinically affected ruminants have seldom been tested, and bioassays, particularly in mice, may be incapable of reliably detecting very low and intermittent levels of haematogenous infectivity (Bolton, 1998).

The possibility of haematogenous carriage of TSE infectivity not only raises concerns about the use of blood and blood products but, a priori, would imply that most tissues and some secretions and excretions from TSE-affected animals could also be potentially infected. Concerns about haematogenous infectivity have been particularly acute in the medical field where, because certain individuals who died of CJD had been blood donors during their pre-clinical phase, the risks of transmission via blood or blood components are being taken very seriously (Will and Kimberlin, 1998). Among blood components perceived as a risk is serum albumen, which is used in human IVF, and, to the dismay of those practitioners and their patients who had already used them, some batches of this and other blood products have had to be withdrawn. In that the haematogenous TSE hypothesis has already had an impact on the human reproductive technologies, its potential implications for the ruminant technologies should not be lightly dismissed. Nevertheless, it is important to emphasise that no proven or probable instances of accidental TSE transmission have arisen from blood transfusions or use of blood products in any species, including humans. Thus the risks, if they exist, must be very small, and should be balanced against the established benefits of using blood products.

4.4.2. Iatrogenic transmissions

The term ‘iatrogenic transmission’ means inadvertent and preventable induction of disease by medical/veterinary treatments or procedures (Webster’s Medical Dictionary, 1986) so it includes disease induced by reproductive technologies. Iatrogenic transmissions of CJD in the medical field have had much publicity, with at least 80 known cases arising from transplants of dura mater from cadavers which were subsequently shown or suspected of having had CJD (Brown, 1998). Smaller numbers have resulted from transplantation of eye tissues (cornea and sclera) (Duffy et al., 1974) and also from use of contaminated neurosurgical instruments or intracerebral electrodes. In a recent case-control study of risk factors for sporadic CJD in humans in Australia a range of surgical procedures were found to be significantly associated with development of the disease (Collins et al., 1999). The largest number (over 100) of known iatrogenic CJD cases, however, has arisen from the use of pituitary hormones (growth hormone and gonadotrophins) which were extracted from what were presumably infected human cadavers (Brown, 1998). Gonadotrophins were mainly used for treatment of infertility and in IVF programmes. CJD incubation periods after CNS surgery or ocular exposure were often short (1 or 2 years) whereas incubations following parenteral injection of pituitary hormones tended to be longer (5 to 35 years).

Iatrogenic TSE transmissions have occurred in the veterinary field too, the best known example being an incident in the UK over 60 years ago in which tissues (brain, spinal cord and spleen) from young sheep which must have been incubating scrapie were treated with formalin and used to make a vaccine against the tick-borne disease, loup-ill. Three batches were made and several thousands of sheep were vaccinated. Scrapie appeared 2.5 years later amongst sheep vaccinated with one of the batches, and over 35% were affected on some farms (Gordon, 1946). Another outbreak of what appears to have
been iatrogenic scrapie occurred recently in Italy in sheep and goats vaccinated against the mycoplasmal disease, contagious agalactia, with a vaccine consisting of homogenised, filtered ovine brain, mammary gland and lymph nodes (Capucchio et al., 1998). Of a total of over 1000 goats and 1000 sheep on three farms, 18.5% of the goats and 1.15% of the sheep developed scrapie.

These incidents of iatrogenic TSE are salutary warnings of the hazards of using contaminated instruments or infected biological materials for medical and veterinary purposes, including the reproductive technologies. Vigilance is essential to minimise the risks.

5. Special risks of TSE transmission by reproductive technologies

TSE transmission risks in reproductive technologies include:

- those associated with people, protocols and procedures;
- those associated with instruments and equipment;
- those associated with materials of animal origin;
- those associated with the gametes and/or embryos per se.

5.1. People, protocols and procedures

Avoidance of disease transmission by reproductive technologies depends heavily on the people who carry them out, especially those with a duty to ensure effective sanitary precautions. Team leadership, integrity and training are critical. As the technologies become increasingly complex, national and international regulatory bodies have to devote more and more effort to formulating protocols and regulations. Good progress has been made, including the setting up of AI centres and embryo transfer and production teams supervised by veterinarians which is the basis whereby national regulatory authorities and commercial organisations address the potential disease risks associated with reproductive technologies. Concepts and practical protocols are given in the International Animal Health Code of the Office International des Epizooties (OIE, 1998) and in the Manual of the IETS (Stringfellow and Seidel, 1998). Quality controls should be based on these.

Procedures involving surgery or laparoscopy used for AI and embryo collection and transfer in small ruminants generally carry higher risks than the non-surgical procedures used to collect and transfer embryos in cattle and other large ruminants. The same applies to TVOR from live donors of whatever species, since this also involves invasion of the abdominal cavity.

5.2. Risks of transmission by instruments and equipment

Although the risks of TSE transmission by instruments and equipment in reproductive technologies are small, they are important. Disinfection procedures for use in embryo laboratories are detailed in the IETS Manual (Schiewe, 1998) which emphasises the need to avoid chemical residues toxic to the semen and embryos. TSE agents, however, are extremely resistant to most standard disinfection procedures and, to complicate matters, some instruments used in the reproductive technologies are either too fragile to withstand effective physical or chemical treatments, or so complicated that subsequent removal of toxic residues would be impossible.

Advice on preventing TSE transmission in hospitals has been given by the Advisory Committee (1998) on Dangerous Pathogens, and its recommendations for surgical instruments are also relevant to reproductive technologies. Briefly, instruments are categorised according to whether they are used for human patients without known TSE exposure or symptoms, for symptomless patients having had exposure (e.g. iatrogenically), or for patients with actual or suspected TSE symptoms. The Committee assigned these patient groups to categories (iii), (ii) and (i), respectively, but to facilitate an additive approach the order here is reversed, i.e.:

- category 1 — instruments for animals whose likely exposure to TSEs is zero or minimal;
- category 2 — instruments for animals with medium to high exposure risk (i.e. possibly incubating TSE) but without clinical signs;
- category 3 — instruments for high risk animals
known or suspected to be clinically affected with TSE.

The Advisory Committee (1998) recommends that instruments for high risk patients should be of a single use type and be destroyed by incineration after use, whereas for medium risk patients, provided the surgery does not involve the CNS or eye, the instruments can be reused if they undergo specified TSE decontamination procedures. Instruments for CNS or eye surgery in medium risk patients should be incinerated, as in the high risk category. No special procedures are suggested for instruments in the low risk category apart from conventional cleaning and disinfection/sterilisation. Extrapolation of these recommendations to the instruments and equipment used for reproductive technologies in ruminants seems appropriate.

Specific decontamination procedures recommended for instruments used on medium risk patients (ruminants in our case) are either chemical disinfection with sodium hypochlorite (20,000 ppm for at least 1 h), or autoclaving in a porous load steam steriliser at 134–137°C for a single cycle of at least 18 min (or six successive cycles of 3 min each) (Advisory Committee, 1998). Another recommendation, from the World Health Organisation (WHO, 1997), states ‘if instruments are to be re-used, they should be immersed in 1 N sodium hydroxide solution for 1 h, cleaned, and then autoclaved at 134°C for 1 h’. It must be emphasised, however, that many instruments and pieces of equipment used in reproductive technologies cannot withstand such rigorous procedures. Also listed by the Advisory Committee (1998) are chemical and physical disinfection methods that are not effective against TSE agents, including several that are routinely used in the reproductive technologies, e.g. alcohols, iodophors, phenolics, ethylene oxide, autoclaving in dry heat or moist heat at 121°C for 15 min, and radiation (ionising, ultraviolet or microwave).

Categorisation of the risks of instruments is helpful only if the animals on which they are used are categorised. Instruments used on low risk animals in countries (e.g. Australia and New Zealand) currently believed to be free from specified TSEs could, for example, be in category 1, and should not require specific decontamination, whereas instruments and equipment used on animals with clinical BSE, scrapie or CWD would be in category 3, and ought never to be reused. Category 2 applies to most other situations, i.e. instruments used on clinically normal animals in countries or regions where the relevant TSEs are considered endemic.

Except for salvage of genetic material, or for research, reproductive technologies are unlikely to be used in clinically affected animals. Nevertheless, if required, the best option in large ruminants would be to collect embryos by non-surgical uterine flushing, and with single-use, disposable equipment. In small ruminants non-sterilisable laparoscopy equipment must be eschewed in favour of conventional surgery with fully disposable instruments. In medium risk (category 2) situations in cattle there should be few limitations to AI, embryo collection and transfer (i.e. in-vivo-derived embryos) where disposable instruments and equipment can mainly be used, and the metallic items such as catheter introducers and insemination/embryo transfer ‘guns’ can be effectively sterilised to TSE standards. Manufacturers do claim that certain ‘silicone’ items such as Foley catheters and endotracheal tubes can be sterilised by autoclaving, but it is doubtful whether this is possible to TSE standards, so these should be used once, then incinerated. In the relevant risk situations precautions must be taken not only with operating instruments per se, but also with operators’ clothing (gowns, drapes, gloves etc.) and anaesthetic (laryngoscopes, endotracheal tubes, etc.). The temptation to re-use disposable items to save money must be resisted. Surgical and laboratory premises should be kept clean and disinfected regularly. A further point to emphasise regarding reproductive technologies in larger ruminants (of any risk category) is that disposable arm-length gloves should always be used during rectal manipulations, and should be changed between animals.

Some reproductive technologies, including TVOR in large ruminants, laparoscopic AI and embryo transfer in small ruminants, and semen sexing, could present significant TSE transmission risks due to the difficulty of sterilising the necessary equipment. Items such as ultrasound probes, laparoscopes and flow cytometers tend to be in constant use over long periods and frequent decontamination to TSE standards is seldom an option. Carriage of infectious
agents (bacteria and viruses) by fiberoptic endoscopes in hospitals has been reviewed by Hanson et al. (1991) who emphasise that these agents can lodge in channels and on damaged surfaces of such instruments. They also showed that contamination by blood and other body fluids will exacerbate matters. To prevent transmission of human immunodeficiency virus (HIV) thorough cleaning followed by disinfection with 2% glutaraldehyde was advocated, but these procedures would not be effective against TSEs. Glass micropipettes, needles and other microsurgical instruments used for embryo biopsies, ICSI, cloning and related technologies might also pose risks, so these should never be reused. Likewise materials such as ‘Percoll’, used to select viable sperm for IVF, must be disposed of after use to avoid any residue of contamination. Microscopes, micromanipulators, incubators, electroporators and other ancillary items tend to have little direct contact with the gametes, embryos or tissues, nevertheless it is important to ensure they do not become contaminated.

5.3. Risks associated with materials of animal origin

Guidelines for the international movement of livestock embryos, published by the OIE in its International Animal Health Code (1998), include the following statement: ‘any biological product of animal origin, including co-culture cells and media constituents used in oocyte [and embryo] recovery, maturation, fertilisation, culture, washing and storage should be free of living microorganisms. Media should be sterilised by approved methods according to the IETS Manual (1998) and handled in such a manner as to ensure that sterility is maintained’. Adherence to these guidelines is not easy, particularly with regard to TSEs, so it is no surprise that another international expert group (WHO, 1997) concluded ‘...the ideal situation would be to avoid the use of bovine materials in manufacture of medicinal products, as well as the use of materials from other animal species in which TSEs naturally occur’. Unfortunately animal materials are used to some extent in virtually all reproductive technologies, and often several such materials are used. Almost invariably they are crucial for technological success.

Whilst many materials of animal origin carry little or no TSE risk, the potential risks of others are high. To maximise safety, therefore, they should be selected with care according to their geographical origin, species origin and tissue type. Methods of collection should also be considered, and steps should be taken where possible to remove or reduce any potential infectivity which might be present. These precautions will now be discussed in more detail.

5.3.1. Geographical origin of the material

Movements of biological materials across national borders for medical and veterinary use are strictly controlled due to concerns that they might introduce exotic diseases. This applies not only to the range of materials used in reproductive technologies but also to vaccines, antisera and other biologicals, and to the raw materials used for their production, so choice of disease-free sources is crucial (Owusu, 1995; WHO, 1998).

The geographical origin is especially important with regard to the TSEs. Procurement decisions should take account of veterinary infrastructure, disease surveillance systems, statistics on TSE occurrence, and whether control policies are being effectively applied in the specific exporting countries or regions. The reliability of veterinary certification is also critical, and if the health or traceability of materials and their donors is in any doubt, the risks must be scored accordingly. The OIE in its International Animal Health Code (OIE, 1998) has proposed for BSE (chapter 3.2.13), and will probably also do so for scrapie (chapter 3.3.8), that the status of different countries and zones be formally categorised on the basis of key risk criteria. These OIE Code proposals are complex and still under discussion, but disease-free (category 1) countries or zones would in essence be those able to demonstrate an absence of animal TSEs, whereas category 2 countries would be those claiming absence but not yet having met all the specified criteria. Category 3 and 4 countries/zones would be those with known low or high incidences of the TSE(s), respectively. In the case of BSE, for example, high incidence countries would probably have more than 100 cases per year.

Materials of animal origin for use in reproductive technologies should preferably come from category 1 countries where, if their status is maintained, the TSE risks should be zero. Criteria for category 1 are
extremely stringent, however, and few countries can meet them. Category 4 countries are unlikely to be acceptable, but materials from category 2 or 3 countries may be if the source herds or flocks can conform to strict criteria. They should, for example, have been well recorded and monitored for TSEs for several years (preferably within an accreditation scheme), no potentially infected animals or animal products should have been imported, and feeding of ruminant-derived protein (other than milk) must have been banned. If reliable tests (such as those for BSE recently evaluated by Moynagh and Schimmel (1999), for the European Commission) can be developed to prove that specific animals, flocks or herds are TSE-free, then sourcing animal origin materials will be greatly simplified.

5.3.2. Exclusion of individual donors in high risk categories

Animals known or suspected of having eaten ruminant-derived meat-and-bone meal, and the offspring of parents known to have had TSE, should not be used as donors. Likewise individuals with susceptible genotypes might be considered high risk, although those living well beyond the age when such genotypes would normally show disease might in reality be a low risk. In view of the complexity of risk, selecting animals as tissue donors must be based on excellent recording plus a high degree of owner integrity. In contrast to human medicine where living donors often contribute blood and other tissues periodically, animal donors usually donate once, at death, so the recall of a biological product because a donor is subsequently discovered to have a disease is unlikely.

5.3.3. Tissue type of the material

Many different tissues and tissue derivatives are used in reproductive technologies: cow’s milk and hen’s egg yolk are used in semen diluents; gonadotrophins from pituitary glands of pigs or sheep (occasionally cattle and horses), or from horse serum (eCG) or human urine (hMG) are used for superovulation; blood constituents such as foetal calf serum and bovine serum albumin (BSA) are used in embryo transfer/culture media; bovine and porcine pancreatic trypsin are used to wash and decontaminate embryos, and a variety of cell types are used in embryo co-culture and for nuclear transfer.

Most of these are low rather than high risk materials, but the pituitary, commonly used to produce the gonadotrophin FSH for superovulation, is in category 4 (Table 1). Indeed, soon after BSE was identified in the UK a warning was issued about its possible transmission by ovine and bovine gonadotrophins (Kidd and Gray, 1988). Great care is needed, therefore, to ensure these hormones are sourced from low risk species (e.g. pigs) and also from low risk countries where ruminant protein is never fed to livestock. Cells of neurological origin, including neural stem cells (Bjornson et al., 1999) are also a risk, and should never be used for embryo co-culture or nuclear transfer because they might contain, or be capable of amplifying TSE agents (Chesebro et al., 1993; Windl et al., 1999). Where techniques involve surgery it is possible that surgical catgut, prepared from intestines of sheep and cattle, will be used. Intestine is a category 3 tissue (Table 1) so, because catgut manufacture is unlikely to inactivate any TSE agents that may be present, and because it has prolonged and intimate contact with the sutured tissues, this material can carry an element of risk (McDiarmid, 1996). The practice of culturing IVF or nuclear transfer embryos from the zygote to the blastocyst stage in ligated oviducts of live sheep, though effective, is hard to justify. The oviduct is probably not a high risk tissue but unless the temporary surrogates are in a low risk country or are otherwise guaranteed TSE-free, TSE contamination cannot be ruled out.

5.3.4. Materials from other animal species and non-animal sources

Many of the animal materials used in reproductive technologies are derived from high or medium risk species, so efforts to switch to low risk species, or to synthetic, recombinant or plant materials would be justified. Depending on their PrP gene polymorphisms, tissues from sheep generally pose a higher risk than those from cattle, goats and deer (and perhaps humans), and these in turn probably pose more risk than tissues from pigs, horses and poultry. Even non-animal products might carry some risk, since they could have been exposed to media containing animal tissue extract, or be stabilised with serum albumin. Each product should be assessed individually therefore.

Traditional semen extenders for AI consist of
skimmed milk and/or egg yolk in buffered saline, but recently a new extender based on soya-lecithin has become available which not only maintains semen fertility but also eliminates any infection risks associated with the animal materials (Bousseau et al., 1998). Bovine serum and serum albumin are widely used in embryo collection and culture media, and are difficult to replace, but limited success has been reported with polyvinyl alcohol and certain other chemically-defined substitutes for serum (Palasz et al., 1995; Jones and Westhusin, 1996). Recombinant albumins are also now being produced so their use should be studied. In the case of cells for co-culture with IVP embryos, instead of primary cells from animals of undetermined infectivity status, the option of using authentic, quality controlled cell lines with good viral safety records should be considered (Hay, 1998; ICH Harmonised Tripartite Guideline, 1998).

5.3.5. Conditions under which materials are collected

Whereas practitioners may choose what animal materials they use, they seldom have control over tissue collection, especially if they rely on commercial suppliers. Contamination during collection could occur from instruments or from other tissues of higher risk. Instruments, therefore, must be of the disposable type or be sterilised to TSE standards, and when collecting tissues such as ovaries and oviducts care is needed to prevent contact with intestines, lymphoid tissues, and placentae, all of which are in higher TSE risk categories (Table 1). Likewise, when collecting foetal blood, care is needed to avoid contamination by the placenta and its fluids, and from the maternal tissues.

An important TSE risk arises when tissues are from animals killed by penetrative brain stunning. Embolisation of brain tissue to the lungs and other parts of the body as a result of head trauma has long been recognised in humans (McMillan, 1956; Hauck et al., 1990), and it is probable that haematogenous spread of CNS tissue also occurs in animals which are shot or stunned by some types of captive bolt gun (Garland et al., 1996; Love et al., 1999). Many of the tissues used in reproductive technologies are from abattoir animals killed in this way, so in appropriate circumstances other methods such as barbiturate overdose should be used.

Whilst animal material used in a reproductive technology may be from a single donor, and sometimes even from the same one from which the ova or embryos are collected (e.g. oviductal cells for co-cultures), in most cases it is made up of a pool originating from many different animals. In the latter case the risk tends to be compounded because infection from one donor can contaminate a whole batch, so smaller donor pools are usually considered safer. On the other hand some might argue that dilution by pooling will reduce the risk.

5.3.6. Removal of infectivity from materials of animal origin

It has been reported that exposure to concentrated (6 M) urea plus a series of ultrafiltration steps will effectively decontaminate human pituitary growth hormone (Pocchiari et al., 1991) but whether the hormonal properties remain after such harsh physico-chemical treatments is unclear. In practice it is hard to envisage anything that would totally remove TSE infectivity from animal materials used in reproductive technologies without damaging their biological activity.

5.3.7. Testing materials to detect presence of infectivity

Testing representative samples of source materials, or aliquots of the final product before it is used is a routine method to reduce the risks of conventional pathogens. For TSEs, however, testing by bioassay is expensive, prolonged, and not always reliable. Nevertheless, for high risk materials, such as FSH from pituitary glands of TSE susceptible species, if their safety cannot be guaranteed on the basis of geographical origin, testing, preferably in the homologous species, may be the only alternative.

5.3.8. Route of administration

Experimental work with scrapie in mice has shown that direct injection into the brain is the most efficient transmission route, followed by intravenous, intraperitoneal, intramuscular, subcutaneous and oral routes, in that order. Most drugs used in the reproductive technologies (e.g. for oestrus synchronisation and superovulation) are given intramuscularly, subcutaneously or intravaginally, which may be marginally safer routes.
5.4. Risks associated with gametes and embryos per se

As reported elsewhere (Wrathall, 1997) the general conclusion from all the research done so far is that the risks of carriage of TSE agents by semen and embryos per se from cattle, sheep and goats are extremely small or non-existent. I will briefly consider each of these separately.

5.4.1. The evidence for semen in cattle, sheep and goats

Experiments to assess TSE infectivity in bovine and ovine semen (nothing published on other ruminants) have given negative results. Bioassays in mice, for example, have failed to detect infectivity in testis, seminal vesicle and semen of affected rams, or in testis, epididymis, prostate, seminal vesicle and semen of affected bulls. Testing in mice is not ideal due to the species barrier effect, but in the one homologous bioassay study (Palmer, 1959) in which semen from a scrapie-affected ram was inoculated into 20 one-day-old lambs the result was also negative; however, since it was curtailed after only 30 months, and the lambs' genotype was not known, this experiment was flawed. Studies have not been carried out in which semen from clinically affected males (bulls, rams or bucks) is inseminated into TSE-free and (in the case of sheep and goats) genotyped females, and where the latter plus their offspring are kept isolated in an uncontaminated environment for long periods of time. Thus the negative experimental evidence for the semen of ruminants is not as conclusive as it may initially appear.

Evidence from epidemiological studies also suggests that scrapie and BSE are unlikely to be transmitted via semen but again, as pointed out in my earlier paper (Wrathall, 1997), there are caveats. For example, most studies on the role of the ram in scrapie transmission were carried out before the effects of PrP gene polymorphisms on susceptibility were understood. Thus lambs inheriting susceptibility from either parent would tend to acquire scrapie more readily if born into an infected environment, so it is difficult to differentiate between transmission of TSE infection via semen and transmission of genetic susceptibility. So far as bull semen is concerned, Wilesmith (1994) reported that incidence of BSE in the offspring of two AI bulls which later succumbed to the disease was not statistically different from that in the offspring of two unaffected bulls. Although more conclusive data on this matter is still needed, based on current evidence the OIE has decreed that for purposes of international trade that the risk of BSE transmission by bovine semen is negligible (see OIE, 1998, chapter 3.2.13). Surprisingly, since the evidence for negligible risk seems to be at least as good for embryos as for semen, the OIE has not yet decreed the same about bovine embryos. It is generally accepted, however, at least for conventional diseases, that the risks of transmission via embryos (the in-vivo-derived type) tend to be lower than those of semen.

5.4.2. The evidence for in-vivo-derived embryos

Results of a major study to determine whether embryos from cows clinically affected with BSE can carry infectivity are still in progress in the UK (Wrathall, 1997). Basically these were in-vivo-derived embryos collected by non-surgical uterine flushing 7 days after AI; they were then washed 10 times (without trypsin treatment) as recommended in the IETS Manual (Stringfellow, 1998) and cryopreserved using glycerol. Semen from clinically affected bulls was used for approximately half the inseminations and semen from healthy bulls collected at least 5 years before the first ever recognised case of BSE was used to AI the remainder. This meant that half the embryos had a terminally ill BSE positive sire as well as a terminally ill positive dam. Poor quality embryos and samples of uterine flush fluid (sediment) from these collections were bioassayed in mice with negative results (Wrathall et al., 1997). The good quality embryos were thawed and transferred into recipient heifers which, to ensure their TSE-free status, were imported from New Zealand. The recipients are being kept under observation for 7 years from their transfer date and the embryo transfer offspring likewise for 7 years from their date of birth. At or soon after their 7-year dates recipients and offspring are killed and their brains are removed and examined histopathologically for evidence of BSE. Of the total of 345 recipients approximately a third have now been killed (or died from intercurrent disease) and examined, all with negative results. Almost a third of the 266 liveborn
offspring have also been killed (or have died) and examined, again with negative results. This experiment is not scheduled to finish until the year 2001, nevertheless the absence so far of evidence of BSE in either recipients or offspring, or in the inoculated mice, is encouraging. If the final outcome is the same it will indicate that BSE does not transmit via embryos even if they are collected at a late stage of the disease, i.e. when the risk of maternal transmission is at its highest. It will also support the view that BSE is not transmitted via semen.

Results of studies in Scotland by Foster et al. (1992, 1994, 1996b) have appeared to suggest that scrapie can be transmitted by in-vivo-derived sheep embryos, both washed and unwashed. However, when Foster’s methods and results are critically examined and compared with other observations made by his group (particularly genetic data on his animals), and also the negative scrapie transmission results with embryo transfer reported by Foote et al. (1993) in the USA, the evidence for transmission by embryos is extremely tenuous. The design, results and interpretation of these two sets of experiments are complex, so I will refrain from giving details here; nevertheless, in my earlier review (Wrathall, 1997) I concluded that, taken overall, both Foster’s and Foote’s results provide good evidence that scrapie is not transmitted by sheep embryos. Sanitary precautions must be strict, however, because the procedures, instruments and materials of animal origin used in this transfer technology are probably more likely to carry TSE infectivity than the embryos.

Embryo transfer experiments with goats that had been orally inoculated with scrapie, and also with goats that had been subcutaneously or intracerebrally inoculated with BSE, have been reported (see Wrathall (1997) and also Foster et al. (1999)). During observation periods subsequent to transfer which lasted 5 or more years neither the embryo transfer offspring nor their surrogate mothers developed scrapie or BSE.

5.4.3. TSE risks with gametes and embryos produced by other reproductive technologies

There is no published work specifically on TSE risks in the other technologies so these must be gauged from information about semen and embryos, plus knowledge of the particular technology. A variety of tissues can contain infectivity (Table 1) and some, including blood and its derivatives, should be considered. It is known, for example, that traces of blood are sometimes present in freshly ejaculated semen, and when embryos are collected from the uterus the flushings often contain blood and inflammatory exudates. Likewise when gametes or tissues (e.g. for cloning, nuclear transfer, co-culture) are collected possibilities exist for TSE contamination by instruments or other tissues. Examples include nerves (during epidural anaesthesia), tonsils (during general anaesthesia with intubation), intestines (during rectal manipulation), and lymphoreticular tissues (during laparotomy/laparoscopy and TVOR). Thus, although the gametes and embryos may not of themselves carry TSE risk, risks can arise during their collection and processing. It is often impossible to know at the time, or indeed for long after a technique is performed, whether an animal is a carrier of TSE infection, so it is all the more necessary to take sensible precautions to avoid disease transfer.

6. Measures to prevent TSE transmission by reproductive technologies

Sanitary protocols for international movement of the semen and embryos of various livestock species are given in Appendices in the International Animal Health Code of the OIE (OIE, 1998). Recommendations in the IETS Manual (Stringfellow and Seidel, 1998) form a basis for the embryo Appendices in the OIE Code. Regulatory authorities around the world are generally aware of these protocols which in many respects are as valid for the TSEs as for conventional infectious diseases. However, in view of the unique characteristics of the TSEs, protocols which specifically address the risks of their transmission via reproductive technologies, including new technologies such as cloning and transgenesis, would be desirable.

To guard against risks of transmission of conventional diseases, stud bulls are normally kept in licensed AI centres, and semen is collected, processed and stored under veterinary supervision to ensure sanitary standards are met. Bulls are tested for certain diseases before entry, and undergo regular testing whilst in residence. AI technicians are trained
in sanitary procedures, including cleaning and disinfection of AI ‘guns’ and other equipment. Although recommendations exist for sanitary control of semen collection and AI in sheep and goats (OIE, 1998), the regulations applied in most countries tend to be less strict for these and ruminants other than cattle. Thus, in practice, licensed AI centres for other ruminants are uncommon and AI work tends to be done on farms or semi-residential breeding centres.

Established protocols for in-vivo-derived embryos (OIE, 1998) stipulate they should be collected and processed by an officially approved (licensed) collection team led by a veterinarian to ensure high sanitary standards. However, unlike stud bulls for AI, donor cows (and other ruminants) are seldom confined to licensed collection centres but are usually flushed on their farms of origin, and the flush fluids are either searched there or taken to the team laboratory for searching and embryo processing. Embryos, especially those intended for international trade, should have an intact zona pellucida, be devoid of adherent debris, and be processed according to the internationally agreed sanitary protocols (Stringfellow, 1998) prior to freezing and/or transfer. Processing includes washing at least 10 times and in some cases treating with the enzyme trypsin to ensure that certain viruses (particularly herpes viruses) will be removed if present. Since their eventual destination is not always known at the time of processing, embryos are often washed and trypsin-treated as per the agreed protocols anyway. Protocols for IVP embryos given in the OIE Code (OIE, 1998) and in the IETS Manual (Nibart et al., 1998) recommend that an officially approved embryo production team, supervised by a veterinarian, should have responsibility for the sanitary controls. Although not as effective for removal of pathogens as it is in the case of in-vivo-derived embryos, washing of IVP embryos should be performed as specified above.

6.1. Specific risk reduction measures for the TSEs

Since practically all the evidence indicates that the gametes or embryos per se carry no detectable TSE infectivity, the measures which remain to be considered are primarily those associated with people, instruments, equipment and materials of animal origin involved in their collection or production, and in their processing. If the instruments are contaminated, or suspect materials ever enter the process, it is virtually impossible to prove that potential infectivity has been totally eliminated by any disinfection or inactivation process. The TSE agent in question may be incapable of causing disease in the species for which the technology is used, or insufficient amounts of it may be present to constitute an infective dose, so the risk might be irrelevant. Nevertheless, convincing regulatory authorities (and the general public) with such arguments may be so difficult that it is best to incur the expense of TSE-free instruments and materials at the outset.

As with most risk reduction efforts, measures to prevent TSE transmission by reproductive technologies are cumulative. Thus, whilst a single measure is unlikely to be reliable, several common sense actions will provide a high degree of safety. For example, using a different team for collection/production/processing of the gametes or embryos from that responsible for transfer to, and care of, the recipients is a wise precaution. Embryo washing could also be a risk reducing factor for TSE agents as it is for conventional disease agents, although trypsin treatment is unlikely to be effective because TSE agents are resistant to proteolytic enzymes.

Other measures to ‘dilute’ risk include using less of a particular animal-origin material, or replacing with safer alternatives. Avoiding ruminant pituitary-derived FSH in favour of eCG or hMG for superovulation, for example, is sensible unless the FSH can be sourced from a country free of ruminant TSEs. Likewise if the small TSE risk of blood products is deemed unacceptable, then these too should be sourced from TSE-free countries. So far as instruments are concerned, measures similar to those proposed for human surgical instruments, as outlined above, should be implemented. Other precautions which can be useful when semen or embryos are imported from high-TSE-risk countries into countries where ruminant TSEs are absent are given in my earlier paper (Wrathall, 1997).

Sanitary controls are especially important in IVP laboratories where complex equipment and multiple materials of animal origin (including living cell lines) are used. One-off batch runs should take place specifically for IVP embryos intended for export, or to produce cloned or transgenic offspring, and great care must be taken to ensure sterility of equipment.
before starting. This is crucial when making transgenic animals to produce proteins or tissues for human use. Important for co-culture and cloning by nuclear transfer are the types of cells used; cells that could carry or be excessively susceptible to TSE agents should be avoided.

It is important to remember that in contrast to most of the conventional infectious diseases, a strong element of genetic susceptibility exists with the TSEs. Thus, although the value of reproductive technologies for rapidly introducing desirable genes into livestock populations is widely proclaimed, their potential for equally rapid introduction of harmful genes, such as TSE susceptibility, also deserves some emphasis. As pointed out in a recent report on the pros and cons of cloning (FAWC, 1998), loss of natural genetic diversity can be difficult to reverse, so the risks of this happening with reproductive technologies should be recognised and avoided.

6.2. Quantitative aspects of managing the risks of TSE transmission

Unless a particular technology is banned altogether it is impossible to rule out its risks entirely, hence the need to quantify levels of risk and decide if they are acceptable. Real life measurements are seldom possible, so mathematical modelling with scenario pathway analysis is needed, as described, for example, for the risks of scrapie transmission by ovine semen and embryos (McDiarmid, 1996) and for the risks of transmitting certain viruses by bovine embryos (Sutmoller and Wrathall, 1997). Some risks (e.g. TSE infectivity in blood) are conjectural and hard to quantify, so informed guesses have to be made and probability values assigned to enable the construction of meaningful scenario pathways, and summation of the overall risk.

Quantifying risks in a precise mathematical way requires information on all the technical steps as well as on epidemiological and environmental factors, but it is very illuminating. It reveals the critical points where sanitary controls can best be focused. Having quantified the risks, someone has to decide if they are acceptable, and this will depend on how much is at stake, economic and otherwise. Agreement on what constitutes an acceptable risk can be hard to achieve; expert and lay opinions may differ, but both must be reflected in policy decisions.

7. Conclusions

The risks of transmitting conventional diseases by in-vivo-derived embryos and to some extent semen are extremely low, which is a great advantage when using AI and embryo transfer for livestock breeding. The same applies when these technologies, rather than movement of animals, are used in international trade, and sanitary protocols to regulate and capitalise on the low risk benefits of semen and in-vivo-derived embryos are now widely accepted. A variety of new reproductive technologies are being developed, however, and it is difficult for regulatory bodies to keep pace with these and to devise sanitary controls to ensure that they too are safe. The task deserves priority nevertheless.

The risks of TSE transmission by reproductive technologies also appear to be low, but caution is needed because research data are still limited. In contrast to the risks of most conventional diseases, TSE risks are associated particularly with the instruments and materials of animal origin used in the technologies, and once present these are almost impossible to deal with by traditional decontamination procedures. Instruments, biologicals, vaccines and foodstuffs have all been associated with TSE transmission: for example, use of pituitary-derived hormones has led to transmission of CJD to humans; use of vaccines made with CNS tissues has led to transmission of scrapie to sheep and goats, and use of rendered products from ruminant carcasses as a protein food source has led to an epidemic of BSE in cattle and also indirectly to cases of nvCJD in man. If TSE transmission via a reproductive technology were ever to occur, years would elapse before it was recognised, so awareness of the risks is vital if they are to be properly managed. The legacy of BSE, especially in the UK, is a salutary lesson to anyone inclined to ignore the potential consequences of inadequate precautions.

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