Br. vet. J. (1993) 149, 339

THE DANGERS OF DISEASE TRANSMISSION BY ARTIFICIAL INSEMINATION AND EMBRYO TRANSFER

MARK PHILPOTT

Avoncroft Cattle Breeders Ltd, Sugarbrook, Stoke Prior, Bromsgrove, Worcestershire, B60 3AS

SUMMARY

This review summarizes the major infectious diseases of the three major agricultural species (cattle, sheep and pigs) and horses, and presents the evidence for and against the possibility of infectious agents being transmitted between animals via the venereal route or by the use of semen or early embryos in commercial artificial insemination (AI) or embryo transfer (ET). Cattle feature most prominently in the widespread distribution of frozen semen, and national and international organizations have set out guidelines to work towards disease-free bull studs with semen free from potential pathogens. With the control of major epizootic diseases, attention has been focused on such diseases as IBR, BVD and blue tongue, where clinical signs are rarely evident but the detection of virus in semen is of great importance. New information on the relevance of bacterial disease such as *Mycobacterium paratuberculosis*, campylobacteriosis and leptospirosis is reviewed, along with details of the mycoplasma and ureaplasma species of the bull's genital tract. Bovine spongiform encephalopathy (BSE) has attracted much research and semen is not regarded as a source of infection. New work on the pathogenesis of a number of diseases and the use of new biotechnology in diagnosis is included.

The International Embryo Transfer Society (IETS) has encouraged a great deal of experimental work—much originating in Canada—on the risk of transmission of disease from donors to recipients via a 7-day-old blastocyst. There has been much success in demonstrating that with an approved protocol of handling the embryos, to date there is very little danger in disease transmission with both viruses and bacteria. The mycoplasma group appear more intractable and the role of BSE is still being evaluated.

In sheep, scrapie, *Brucella ovis* infection and blue tongue feature in current work. In the pig there is a surge in international movement of pig semen, and Aujeszky's disease and the new so-called Blue Ear disease feature prominently. Much work is in progress on infectious agents likely to be found in the semen of stallions, with an expanding trade in the inter-

0007/1935/93/040339-32/$08.00/0

national movement of chilled and frozen semen. Equine embryo transfer experiments are hampered by the very limited number of embryos available. Reference is also made to the further risk of disease transmission by *in vitro* manipulated embryos.

INTRODUCTION

Whereas artificial insemination (AI) was set up to distribute advantageous genes and eliminate the problem of venereal disease, great caution must be maintained to ensure that the same vehicle does not spread genetic defects or pathogens. With the ability of bulls to give up to 1000 doses from one ejaculate, and the widespread distribution of these both nationally and internationally, the potential for the spread of disease is considerable. The freezing of semen enables many infectious agents to survive and cryoprotectants may render antibiotics less effective (Bartlett, 1981).

With the aim of producing pathogen-free semen the concept of an enclosed stud of sires with a regular health testing programme under official supervision has become the norm (Bartlett *et al*, 1976). The Office International Des Epizooties (OIE) (1986) has defined guidelines; in the United States there is standardization under the National Association of Animal Breeders (NAAB, 1989) and the recent EEC Directive (EEC, 1988) has encouraged fresh dialogue between member states in Europe and achieved a measure of standardization. With a forever changing spectrum of disease (Anon, 1992b) there must be no room for complacency.

The testing programme will take into consideration the national animal health status as well as the source of the donors. A knowledge of the natural history of the disease will determine the timing of the tests in relation to semen production (Sellers, 1983). Diagnostic tests have limitations as to sensitivity and specificity and there is latency to contend with (Guérin, 1989). New diagnostic techniques will lead to greater precision and confidence (Howard, 1986). The level of risk of using such semen in embryo transfer (ET) operations is still to be clearly defined.

The distribution of embryos is less hazardous for disease transmission. The number of recipients is small, flushing fluids will dilute pathogens present and, most significantly, embryos can be rinsed (Singh, 1988). The zona pellucida surrounding the bovine embryo has a sponge like surface (Chen & Wrathall, 1989), but appears impermeable to pathogens (Wrathall, 1987). Some pathogens do not detach easily but some of these can be removed by a trypsin wash (Singh, 1988).

The protocol for an approved system of treatment post flushing and prior to freezing has been adopted by the IETS (Stringfellow & Seidel, 1990), and these are now accepted by the OIE (1986). The diseases currently accepted as constituting minimal or no risk with the approved protocol are set out in Table I. These guidelines refer to embryos with intact zona pellucidas, and where the zona pellucida is breached in the laboratory, an agreed protocol has yet to be defined (Marchant, 1991).

The OIE has always given a lead in the definition of international health stan-

Table I

Based on a 1990 review by the Research Subcomittee of the International Embryo Transfer Society (IETS) Import/Export Committee of available research and field information on infectious diseases that have been studied apropos of the risk of their transmission through embryo transfer, the IETS has categorized those diseases as follows

Category 1

Diseases or disease agents for which sufficient evidence has accrued to show that the risk of transmission is negligible provided that the embryos are properly handled* between collection and transfer:

Enzootic bovine leukosis
Foot and mouth disease (cattle)
Brucella abortus (cattle)
Infectious bovine rhinotracheitis (trypsin treatment required)
Pseudorabies (swine) (trypsin treatment required)

Category 2

Diseases for which substantial evidence has accrued to show that the risk of transmission is negligible provided that the embryos are properly handled* between collection and transfer, but for which additional transfers are required to verify existing data:

Bluetongue (cattle)
Hog cholera (classical swine fever)

Category 3

Diseased or disease agents for which preliminary evidence indicates that the risk of transmission is negligible provided that the embryos are properly handled* between collection and transfer, but for which additional *in vitro* and *in vivo* experiments are required to substantiate the preliminary findings:

Rinderpest (cattle)
Bovine virus diarrhoea
Bluetongue (sheep)
Campylobacter fetus (sheep)
Foot and mouth disease (swine, sheep and goats)
Swine vesicular disease
African swine fever
Scrapie (sheep)**
Haemophilus somnus.

*Manual of the International Embryo Transfer Society (Recommendations for the Sanitary Handling of Embryos).

**See current review.

dards, and this is reflected in an excellent review by Hare (1985) as a monograph on disease transmission by semen and ET technology. This paper will concentrate on developments since that time. Amongst other reviews FAO (1981), Howard (1986), Wrathall (1987), Singh (1988), Afshar and Eaglesome (1990) and Stringfellow and Seidel (1990) are important. A review of OIE Group A diseases and other viral diseases of particular importance to AI and ET will be followed by a more general update on bacterial disease. Much of the work has been done with the bovine but a review of recent work on the sheep, pig and horse is included.

GROUP A DISEASES

Foot-and-mouth disease

Artificial insemination. There is little new work on foot-and-mouth disease (FMD) in relation to AI, but with the cessation of vaccination in western Europe (Anon, 1992a), and the radical changes adopted by some veterinary authorities to the importation of embryos from countries where FMD is still present (Winegar, 1991), reference can usefully be made to the papers of Donaldson and Sellers (1983), and Afshar and Eaglesome (1990) to gauge the levels of risk.

The virus is readily transmitted in semen (Cottral *et al.*, 1968), and presents a real risk for transmission by AI. Virus excretion can occur several days before clinical signs (Sellers *et al.*, 1968). Semen quality will deteriorate during the height of the fever (Cottral *et al.*, 1968), and there will be little, if any, virus present in semen (Sellers *et al.*, 1968) when the quality recovers. However, convalescent cattle frequently retain virus in the pharynx for many months, which might be a source of infection to naive stock. Equally important is the donor bull with waning immunity, where exposure to the homologous or heterologous type of field virus may lead to superficial lesions and a period of seminal shedding of virus. Furthermore, even when fully protected, bulls exposed to a fresh source of field virus may temporarily shed virus, not only from the pharyngeal region but also from the skin of the prepuce leading to seminal contamination.

Donaldson and Sellers (1983) recommend the following guidelines to eliminate the possibility of importing FMD virus in semen of bulls or boars when the stakes are high and donors are vaccinated.

(1) An adequate period (30 days) following vaccination before semen collection to ensure that the vaccine contains no live virus.
(2) A probang sample from the donor bull at the time of collection, and a confirmation that no clinical signs of the disease have occurred in the stud for the subsequent 30 days.
(3) Samples of ejaculates are cultured for virus and confirmed negative before semen is distributed. A confirmed declaration of freedom from the disease at least 3 months before semen collection, and at least 1 month after collection is necessary under all circumstances.

Where vaccination is not practised, confirmation of freedom from the disease in a specified area and a suitable quarantine period of storage may be satisfactory.

Embryo transfer. Singh *et al.* (1986) and McVicar *et al.* (1988) have described the successful removal of virus from zona pellucida-intact embryos after experimental infection, or from acute and convalescent donors. The same group of workers (Mebus & Singh, 1991) have confirmed this by further *in vitro* studies. Thirty FMD viraemic cattle, some with virus demonstrated in their reproductive tract, were superovulated, and after accepted washing procedures (no trypsin) 149 embryos were transferred. All recipients and all calves remained FMD seronegative. Other embryos in the batch were assayed in cell culture or intradermally in the skin of the tongue. FMD infectivity was not demonstrated. They concluded that if there is proper processing and handling of zona pellucida-intact embryos, and donors are

not in the acute stage of infection, bovine embryos can be harvested safely from an FMD infected area regardless of the serological status of the donor.

Rinderpest

Artificial insemination. Virus is known to be excreted in semen during acute disease, but there is not known to be a carrier state. Tissue culture vaccines are recommended over traditional goat attenuated products (Scott, pers. comm. 1992), but country freedom from the disease is usually mandatory.

Embryo transfer. Singh (1988) exposed 121 bovine embryos to high levels of virus, and after washing assayed them in tissue culture. Mebus (1987) collected 107 embryos from 10 viraemic donors, and after washing assayed them in cell culture and by animal inoculation. Three percent of embryos retained rinderpest virus after *in vitro* exposure, but those from viraemic donors were free from virus. Because little virus is secreted into the reproductive tract of infected animals, these preliminary results indicate that embryos are unlikely to be a vehicle for the transmission of rinderpest virus where approved protocol is observed (Singh, 1988).

Vesicular stomatitis

Artificial insemination. The virus is most likely to be present in semen during the course of the disease but there is no information on excretion in those protected by the attenuated live virus vaccines. Most authorities demand area freedom.

Embryo transfer. Experimental work has been confined to *in vitro* exposure of bovine and porcine embryos to the virus, followed by washing under IETS protocol. Singh and Thomas (1987) found that a trypsin stage was necessary to satisfactorily remove virus from 38 bovine embryos. Yet with embryos exposed to the same titre of virus, Stringfellow *et al.* (1989) found the trypsin technique to be ineffective.

Bluetongue

Artificial insemination. Bluetongue occurs in a broad band around the world between 40° north and 35° south, reflecting the distribution of the vector. Cattle are not the primary hosts for bluetongue, but transmission by *Culicoides* midges leads to a prolonged viraemia of up to 100 days. During this time most cattle are asymptomatic, but virus may be present in semen of bulls during the viraemic phase (Bowen *et al.*, 1983b; Thomas *et al.*, 1985). The presence of virus in semen is not a regular occurrence and is probably due to blood cells carrying virus leaking into the genital tract via damaged capillaries (Bowen *et al.*, 1985). Gard *et al.* (1989) exposed a series of bulls in northern Australia to natural infection of a series of orbiviruses over a 5-year period. They could demonstrate no clear evidence of bluetongue virus-contaminated semen in 18 infection episodes. The longest viraemia recorded was 29 days. This and other Australian field data led them to believe that only laboratory attenuated bluetongue virus is shed in the semen (Hetherington, 1992).

The original experimental observations by Luedke *et al.* (1970) demonstrating

the latent carrier state arising from infection *in utero* have not been clearly substantiated, and many workers question this evidence (Roberts, 1989). Roeder *et al.* (1991) inoculated two groups of 10 pregnant heifers with bluetongue virus at either 40 or 60 days gestation. All became infected, and 17 produced live calves, none of which showed evidence of infection, and all proved susceptible to subsequent experimental infection with bluetongue virus. However, Schultz *et al.* (1985) did describe the isolation of bluetongue virus from the semen of a seronegative bull over a number of years.

There is clear evidence that the disease may be transmitted by bluetongue-contaminated semen (Bowen & Howard, 1984) and therefore sound diagnostic tests are necessary to confirm virus-free frozen semen potentially for international trade. Diagnostic tests are well reviewed by Afshar and Eaglesome (1990), who refer to a group specific ELISA test and a more specific serum neutralization test. Virus isolation tests *in vitro* include calf pulmonary artery, endothelial cell lines (Wechsler & McHolland, 1988), but currently 11-day-old embryonated chick eggs are frequently used (Wechsler & Luedke, 1991). For more stringent confirmation, inoculation of sheep is followed by serological testing, but Squire (1989) describes delayed and variable serological response, and caution is advised. In the experiments of Roeder *et al.* (1991), one experimentally infected cow with a demonstrable viraemia showed a persistent negative reaction to the agar gel test. There are obviously many unanswered questions about the nature of the immune response in bluetongue virus infection (Roeder *et al.*, 1991). The diagnostic sensitivity and specificity of genetic probes for detection of bluetongue virus in semen remain to be studied (Afshar & Eaglesome, 1990; Howard, pers. comm. 1990).

Afshar and Eaglesome (1990) stressed the importance of maintaining stud bulls serologically negative for bluetongue where semen is to be exported. Young sires should be moved out of endemic areas before passive immunity is lost at 6 months of age. In marginal epizootic areas, bulls should enter AI Centres and semen should be collected at the Centre when the flies are not active. The housing of bulls, even in endemic areas, is said to curtail fly challenge considerably (Philips, 1992).

Epizootic haemorrhagic disease virus of deer (EHDV), closely related to bluetongue, is also *Culicoides* transmitted and must be included in screening programmes. Its presence may complicate the interpretation of serological tests for bluetongue (Acree *et al.*, 1991).

Embryo transfer. There is much evidence from *in vitro* and *in vivo* work that embryos from infected donors washed by IETS protocol do not transmit bluetongue virus, despite the presence of virus in uterine washings of donors (Bowen *et al.*, 1983a; Thomas *et al.*, 1983) and even with the use of infected semen (Thomas *et al.*, 1985). In a recent larger field experiment, Acree *et al.* (1991) infected 60 heifers by insect originated virus, and embryos collected were washed under the IETS protocol (no trypsin). A total of 308 embryos were either cultured for virus or implanted into recipients with no evidence of transfer of infection. Caution must arise from two observations. Acree *et al.* (1991), using recovered donors and convalescent donors, did record two recipients as developing antibodies to bluetongue virus 9 months after transfer. However, this was likely due to

subsequent exposure to virus carrying vectors, particularly as the donors were found to have been originally infected with EHDV. Secondly, Gillespie *et al.* (1990), in exposing bovine embryos to seven bovine viruses, found bluetongue virus to persist on the surface of the embryo, but after only five washes.

Roberts (1990) cited the observations of Bowen (1987) on the level of confidence that bluetongue disease transmission will not occur in a large number of embryo transfers. To be 95% confident that transmission will not occur in, say, more than one transfer in 100 between viraemic donor and susceptible recipient, it would be necessary to carry out 300 transfers. This number of transfers has only been carried out for enzootic bovine leukosis virus.

Lumpy skin disease

The virus has been isolated from semen for up to 20 days post-infection, but it has also been isolated from the semen of bulls with subclinical infection (Woods, 1988). Such infection is not easy to detect serologically and a history of herd and country freedom is normally required. However, as it is thought to be principally vector transmitted, there is no published evidence for the venereal route (Carn, pers. comm.). No studies on ET are available.

Rift valley fever

No work has been done in cattle, but transient virus in semen would be expected during the course of the disease (Hare, 1985). It is not expected that infection would occur as a result of insemination.

Enzootic bovine leukosis

Artificial insemination. The virus proliferates in the beta lymphocytes (Bendixen, 1988), and its rare isolation from semen (Lucas *et al.*, 1980) could well be due to the leakage of infected cells to the genital tract (Afshar & Eaglesome, 1990), particularly where electro-ejaculation has been used. The transmission of infection by semen from seropositive bulls appears unlikely (Afshar & Eaglesome, 1990; Straub, 1987), and the authors quote a number of field studies supporting this, notably those of Monke (1986), where semen from serologically positive bulls from several studs in the USA had been used in a closed Jersey herd for 5 years, with no seroconversion in the females.

Nevertheless, the international community understandably demands seronegative sires, and the agar gel test is the most widely used diagnostic tool. An ELISA test is available with slightly higher sensitivity (Afshar & Eaglesome, 1990), and radioimmunoassay gives even greater sensitivity. The EEC Directive prohibits the distribution of semen from young sires under 2 years, where the negative serological status of the dam has not been confirmed because of the phenomenon of late seroconversion (Bendixen, 1988), and associated viral excretion.

Embryo transfer. Enzootic bovine leukeosis virus was one of the first viruses to be shown as controlled by embryo transfer (Eaglesome *et al.*, 1982) and there is sufficient evidence for international acceptance (Hare, 1985; Thibier & Nibart, 1987). It is stressed that multiple washing of zona pellucida-intact embryos must be carried out because of the possibility of infected lymphocytes in the donor's

flushing fluids. In a more recent study (DiGiacomo *et al.*, 1990), 128 calves were transferred as embryos from 18 seropositive cows. Some of these cows had received semen from positive sires. Subsequently, no recipients seroconverted within the first 3 to 6 months post-calving, and equally none of the calves born showed positive serological titres.

Infectious bovine rhinotracheitis

Artificial insemination. Bovine herpes virus type 1 (BHV-1) is the most common herpes virus to be found in bull semen. A good review can be found by Afshar and Eaglesome (1990). The presence of virus in semen may lead to clinical signs in inseminated cows or heifers. More likely it leads to short returns to service in non-immune females, seroconversion and the retention of the virus for life (Parsonson & Snowdon, 1975).

Both modified viral vaccines and inactivated vaccines have been used to promote immunity and decrease viral shedding in carrier bulls (Forschner, 1988). However, some modified vaccine viruses can be shed in semen (Gregersen & Wagner, 1985), and field virus can infect cattle protected with inactivated vaccines (Frerichs *et al.*, 1982). A seronegative stud is the only reliable way to produce virus free semen, but with the development of new subunit vaccines this will need re-evaluating (Ackermann *et al.*, 1990; Howard, pers. comm. 1990; Kit *et al.*, 1990). Virus excretion from asymptomatic carrier bulls in semen is intermittent and can be influenced by stress, transport, etc, and the elimination of such animals from studies depends on reliable diagnostic tests. Eloit *et al.* (1991) have shown considerable variation in ELISA kits used in different laboratories in France. Moreover, where serum neutralization, haemagglutination and ELISA tests were used on 151 sera, 64 of these were positive on all tests, 36 were negative to all tests, but 51 (ie. one-third) gave divergent results between tests. There is evidence for differences between national laboratories.

Guérin (1989) has drawn attention to the late seroconverting bulls that are negative to stud tests at a year old, but later seroconvert and excrete virus. It has been suggested that such an animal may have been born from an infected dam and subsequent infection or vaccination of the calf may have been masked by maternal antibody (Guérin, 1989; Whetstone, pers. comm.). The seroconversion of Swiss cattle after the use of imported semen from a donor sire, seronegative at the time of collection (Kupferschmeid *et al.*, 1986), could have arisen from such a case. Serological testing of donor bulls 2 weeks after collection would be prudent. Danish workers have recommended more sensitive serological tests to identify these latently infected animals and claim the success of their eradication scheme rested on this procedure (Ackermann *et al.*, 1990). The Swiss work demonstrates the greater sensitivity of animal inoculation over *in vitro* cultural tests. Afshar and Eaglesome (1990) reviewed recent developments in new diagnostic techniques, drawing attention to improved tissue culture (Drew *et al.*, 1987) and nucleic acid hybridization (Pacciarini *et al.*, 1988; Giavedoni *et al.*, 1988; Ackermann *et al.*, 1990; Howard, pers. comm.). Much more experimental work needs to be done before these can be routinely used for testing bovine semen.

Two new approaches have been published in connection with the removal of viruses from semen. Bielanski *et al.* (1988) treated frozen thawed bovine semen

experimentally infected with BHV-1 with 0.3% trypsin solution. Virus was not isolated from any trypsin treated samples. Semen was successfully used to inseminate heifers after superovulation, but no major fertility studies on trypsin treated semen have been published. A second approach is that of Schultz *et al.* (1988) using immunoextension. This is the principle whereby hyper-immune serum against the virus in question is added to the extender. It was demonstrated that infectivity could be eliminated without affecting fertility.

Embryo transfer. Extensive work by Singh *et al.* (1983) demonstrated that BHV-1 can be found in the flushing fluid of IBR infected donors, and is not readily eliminated from zona pellucida-intact blastocysts simply by washing. However, when trypsin treatment is incorporated into the washing routine, all virus was inactivated or removed. Stringfellow *et al.* (1990) confirmed the effect of trypsin washing using 139 zona pellucida-intact embryos exposed to virus *in vitro,* and also demonstrated that BHV-4 followed the same principles. Echternkamp *et al.* (1989) demonstrated that exposure of embryos in trypsin for 2–3 min does not decrease embryo survival, and may even have enhanced cryopreservation.

Without the use of trypsin, embryos can be transferred from IBR positive donors to uninfected recipients without disease transmission (Thibier & Nibart, 1987). This is not surprising as *in vivo* experimental work has used viral titres that greatly exceed those found in the reproductive tract, and it is possible that *in vitro* viral adherence to embryos does not take place when exposed *in vivo* (Stringfellow & Seidel, 1990). More field work must be documented (Singh, pers. comm.).

Bielanski *et al.* (1992) have recently described the use of photosensitive agents to disinfect embryo cultures. When added to blastocysts in media and exposed to white light, both IBR and BVD viruses were inactivated. With haematoporphyrin derivatives there was no deleterious effect on the embryos.

Bovine virus diarrhoea

Artificial insemination. BVD is normally a widespread transitory mild infection with the non-cytopathogenic biotype, resulting in a brief viraemia and associated seroconversion (Brownlie, 1991). During viraemia, and certainly up to 14 days after experimental infection, virus can be demonstrated in semen (Paton *et al.*, 1989). Meyling and Jensen (1988) have reported the infection of heifers after insemination with semen taken from acutely infected sires. Virakul *et al.* (1988) recorded early embryonic death and repeat breeding after exposure to infection. In a recently infected herd, conception rates were reduced from 78.6% in immune cows to 22.2% in infected cows.

Great concern arose when the immunotolerant state was seen to occur in apparently normal calves born to dams infected with a non-cytopathogenic strain in the first third of pregnancy (Brownlie *et al.*, 1987). Previous to this, infection early in gestation was thought to give rise to abortion or the birth of abnormal or unthrifty calves (Radostitis & Littlejohns, 1988). On screening European and North American AI centres, a few seronegative immunotolerant bulls were revealed, demonstrating persistent virus excretion in the semen (Meyling & Jensen, 1988; Revell *et al.*, 1988; Howard *et al.*, 1990). It is now regular practice to screen all potential AI

sires for presence of virus in the blood (EEC, 1988). If positive on two consecutive tests they are discarded.

Some workers have described a deterioration in semen quality in association with the infection. Paton *et al.* (1989) demonstrated virus in the semen of one out of four experimentally infected bulls despite all animals seroconverting. The infected bull showed a marked deterioration in semen quality, with a reduced density and motility, and an increase in sperm abnormalities. Improvement was seen by 76 days after infection. Howard *et al.* (1990), in a study of 12 persistently infected bulls, found that such viral contamination was not always accompanied by diminished semen quality. Kirkland *et al.* (1991), in a study of five acutely infected bulls, demonstrated low titre viraemia in four and seroconversion in five. Virus was isolated from semen of three, but not on all occasions. The infection did not appear to affect the quality of the semen. In this work, virus was isolated with equal efficiency from raw or extended semen, and virus excretion in semen continued after the end of viraemia and appeared to reflect a replication of virus in the reproductive tract. It was suggested that prostate glands and seminal vesicles are particularly productive sites for virus replication.

Revell *et al.* (1988) studied two persistently infected bulls and found poor semen quality in both, with low density and motility in one. Also in one of these bulls, gross abnormalities were characterized by so-called collapsed heads in 28% to 45% of the spermatozoa. Electron microscope studies of the defect are described, and it is noted that this particular bull had a corresponding increase of enlarged sperm heads. These workers found that raw semen in one animal failed to yield the virus, but it was recovered after processing. One reason for difficulty in cultivating virus may be its invasion of lymphocytes and presence in semen in this form alone (Brownlie, 1991; Radostitis & Littlejohns, 1988). Isolation of virus in the laboratory often requires several passages. Fluorescent antibody techniques are regularly used (Edwards *et al.*, 1988), and most currently a semi-automated immunoperoxidase staining technique. Details of this and other diagnostic methods are reviewed by Afshar and Eaglesome (1990) who have stressed the importance of laboratory media being checked as free from BVD virus, and also from its neutralizing antibody.

Because of the ubiquitous nature of the non-cytopathogenic virus, the majority of stud bulls will be seropositive (Lucas, 1986), but persistently infected animals are usually seronegative. The value of vaccinating all young bulls with live BVD vaccine, even where the serological status is not relevant, must be viewed with caution (Afshar & Eaglesome, 1990); it has been shown that live vaccines may be immuno-suppressive (Roberts *et al.*, 1988). Killed vaccines are preferable, but recombinant vaccines may yet prove to be more selective (Brownlie, pers. comm.).

Embryo transfer. Earlier work by Singh *et al.* (1982) and Potter *et al.* (1984) indicated that bovine embryos could be washed clean of virus by the accepted ten washing regime at a regular dilution of a hundred-fold. Gillespie *et al.* (1990) demonstrating virus on embryos used only five washes. This observation underlines the necessity to follow stringent protocols in commercial processing (Stringfellow & Seidel, 1990).

In donors persistently infected with the non-cytopathogenic strain, it has yet to

be established whether embryos are infected at the level of the germ cells or subsequent to rupture of the zona pellucida and implantation (Brownlie, 1991). The cytopathogenic strain does not pass through the zona pellucida, but it is not the strain associated with the persistent carrier state. More work needs to be done with the non-cytopathogenic biotype (Brownlie, 1991).

Liess *et al.* quoted by Moenning (1990), set out the following measures for the prevention of the spread of BVD through ET.

(1) All bulls in AI will have tested negative for BVD virus.
(2) Check the embryo donor is not a persistent carrier.
(3) Check recipients are not persistent carriers.
(4) If seronegative, such recipients to be vaccinated at least 8 weeks before embryo transfer, or not mixed with other stock until mid-pregnancy.
5) ET calves born of recipients of unknown BVD status, test before breeding.

It is salutary to note that of 12 young persistently infected bulls on four AI studs, ten were produced by embryo transfer (Howard *et al.*, 1990).

OTHER VIRUSES OF IMPORTANCE

Bovine spongiform encephalopathy

Artificial insemination. Provisional views on BSE transmission stemmed from work with the related encephalopathy, scrapie in sheep. Using bioassay in experimental animals, the scrapie agent has not been detected in semen (Palmer, 1959), testis or seminal vesicle (Hadlow *et al.*, 1982) of sheep affected with scrapie.

In confirmed cases of BSE the only tissue, so far, found to contain the agent is brain tissue, after an incubation period in RIII mice of approximately 320 days (Fraser, McConnell, Wells & Dawson, 1988). Bull semen from three confirmed cases of BSE have been injected parenterally into mice and no clinical signs were evident more than 800 days after inoculation (Fraser, pers. comm.). The endpoint for these studies was set at 700 days. The epidemiological evidence shows that the incidence of BSE in the offspring from infected sires is not at variance with that in the general epidemic for this age group, and that BSE has not occurred in the inseminated dams (Kimberlin, 1992). Taken together, the epidemiological evidence and results of transmission studies undertaken so far, support the conclusion that semen is non-infective.

Embryo transfer. Whereas in the case of scrapie in sheep the agent was demonstrated in placental tissue of affected ewes (Pattison *et al.*, 1972), this is not the case with BSE in cattle where the agent has not been detected in the placentae of confirmed cases (Barlow & Middleton, 1990). Experiments are underway to determine whether embryos derived from BSE-infected cattle are infective for recipient dams and progeny. The first 100 calves have been born in this study (Wrathall & Brown, pers. comm. 1992) but it will take several more years before results will be available.

Ephemeral fever

Ephemeral fever virus has been demonstrated in semen but intra-uterine inoculation failed to affect conception rates, with no seroconversion in cattle (Parsonson & Snowdon, 1974).

Akabani virus

Parsonson *et al.* (1981) were unable to detect Akabani virus in semen of infected bulls by both *in vitro* and *in vivo* tests.

No recent papers have appeared on the risks of parvovirus, enterovirus or PI3 in bovine serum, but they have been implicated in affecting spermatogenesis when isolated from bull semen (Afshar & Eaglesome, 1990).

BACTERIAL DISEASES

Tuberculosis

It is still salutary to recall the paper of Raumy (1966), where a bull in service from 1957 was found, on slaughter 6 years later, to have generalized TB including pelvic lesions. Semen from the bull had been used to inseminate more than 1000 cows, and more than 100 were found on slaughter to have TB of internal organs, including peritoneum or regional lymph nodes. The limitations of the Tuberculin test on a single animal, particularly with the predicted extensive movement of livestock within the European community, must be recognized.

Brucellosis

Artificial insemination. Many authorities still consider the serum agglutination test sensitive enough for routine screening of bulls in *Brucella* free countries (EEC, 1988). Garin *et al.* (1985) introduced a mercapto–ethanol variation to avoid non-specific titres, but the complement fixation test and more recently an ELISA test (VanAert *et al.*, 1984) are of greater sensitivity. There is possible merit in a semen plasma test in areas of high risk (NAAB, 1989).

Embryo transfer. There is plenty of evidence that *Brucella abortus* will not be transmitted by embryo transfer when proper handling of embryos takes place. Stringfellow and Wright (1989) presented accumulated evidence that embryo washing procedures alone ensure freedom from *B. abortus,* even without antibiotics. The use of antibiotics with the proper cryoprotectant provides additional insurance against transmission. Moreover, *Brucella*-infected cows superovulated more than 100 days post-calving do not appear to retain *B. abortus* in the uterus. Stringfellow *et al.* (1988) superovulated artificially infected cows and failed to detect *Brucella* on harvested embryos with ten times washing, and also failed to detect *Brucella* in flushing fluids. Barrios *et al.* (1988) made similar observations with naturally infected cows, some of which had aborted previously. Flushing fluids were negative for *Brucella* after superovulation despite organisms being isolated from the milk of donors. Del Campo *et al.* (1987) in a field trial transferred

embryos from seven *Brucella* serologically positive donors to 39 recipients; the recipients remained serologically negative.

Leptospirosis

Artificial insemination. Natural service sires may be disseminators of *Leptospira* infection (Pritchard *et al.*, 1989), and Ellis *et al.* (1986) have described the isolation of *Leptospira hardjo* from the genital tract of the male. The diagnosis of carriers by serology is unreliable (Ellis *et al.*, 1981), and treatment is not always fully effective (Ellis *et al.*, 1985). The internationally approved treatment level of 25 mg kg^{-1} of streptomycin (OIE, 1986) is a massive dose, two and a half times the approved maximum dose according to the formulary, and it has given rise to concern over the effect on semen quality (Cunningham, 1990; Hahn, 1990). However, Howard (pers. comm.) recorded no effect on semen quality on all bulls entering a major US AI station. Urine concentrations of streptomycin were at antimicrobial levels for 6–12 weeks.

Because of the inefficiency of diagnostic techniques, the addition of antibiotics to semen prior to freezing is necessary (Sleight, 1965). Kiktenko *et al.* (1978) have demonstrated the survival of *Leptospira* spp. in the semen of a number of species with and without penicillin and streptomycin. Antibiotics were not added to the raw semen, and no equilibration at 35°C or room temperature was described. In the work of Shin and Lorton (pers. comm.) it proved very difficult to isolate *Leptospira* from seeded semen samples, and after freezing and thawing no isolates could be made even in the absence of antibiotics.

Embryo transfer. There is no published work on the potential of disease transmission of *Leptospira* in the bovine using ET. The prolonged presence of *Leptospira* in the genital tract of the bovine is well documented (Ellis *et al.*, 1985), and the trabeculae of the zona pellucida might well offer a haven for the spirochaetes. Knowing that negative serology is not sufficient to justify freedom from the disease in the donor (Ellis *et al.*, 1981), there is a clear case to justify the use of antibiotics in washing fluids. More work is needed here.

Campylobacter fetus venerealis

Artificial insemination. *C. fetus* freedom is usually shown by fluorescent antibody or selective cultural tests with transport media where necessary. An efficient sheath lavage is necessary for accurate diagnosis, but Ellis *et al.* (unpublished) have demonstrated the value of examining washings of the artificial vagina after semen collection from a particular sire. Their studies show no significant difference in diagnostic sensitivity compared with regular preputial sampling as determined by fluorescent antibody and culture. Because of the limitations of diagnostic tests, antibiotics are still added to semen. Howard *et al.* (1981) have stressed the advantages of adding antibiotics to raw semen prior to extension, and Shin *et al.* (1988) described the advantages of a combination of gentamycin, lincospectin and tylosin. Subsequently Eaglesome and Garcia (1992) have described the re-isolation of laboratory strains of *C. fetus* after processing and freezing seeded samples in the presence of gentamycin, lincospectin and tylosin; in some cases even after prior incubation of the semen with antibiotics for 40 min at 35°C. Shisong *et al.*

(1990) using penicillin, streptomycin and lincospectin found most efficient reduction of *C. fetus* in semen by allowing equilibration at 35°C for 40 min prior to cooling. Sperm motility was not reduced, but fertility tests have not been done. Streptomycin resistant strains have been reported (Howard, 1986; Bonte, pers. comm.).

There would seem to be a great advantage in *C. fetus* bacterins being given to all stud bulls on entry, where bulls entering the stud have been used for natural service (Bouters *et al.*, 1973). In enclosed studs, where bulls enter at a young age, they are considered unnecessary (Bonte, pers. comm.).

Embryo transfer. No work has been done with this organism. Guérin *et al.* (1988) exposed mouse and sheep blastocysts to *C. fetus fetus in vitro* and failed to recover the organisms after just three rinses without the use of antibiotics. No organisms could be recovered from homogenized embryos.

Trichomonas fetus

It is appropriate to mention this infection at this stage. Bovine trichomoniasis is still widespread in ranch cattle throughout the world, perpetuated by natural service. Although the parasite can survive in diluted semen at 5°C and the freezing process, infection through AI is thought to be rare. However, testing of bulls entering AI has always been mandatory (EEC, 1988).

Recent work on diagnosis, immunology and control is reviewed by Yule *et al.* (1989). Direct examination and culture of preputial washings are still the diagnostic tests of choice with the latter proving 25% more effective. Three successive samplings were shown necessary to confirm freedom.

Development of immunological tests has been confounded by cross reactivity and low levels of antigen present during infection. To date vaccination has only been partially successful.

Mycobacterium paratuberculosis

Artificial insemination. Larsen *et al.* (1970) described the isolation of *M. paratuberculosis* from the genital tract of several infected bulls and the semen of one of these. Subsequent studies by the same team (Larsen *et al.*, 1981) demonstrated a few organisms in the semen of an infected bull by repeated sampling. Such numbers should not be sufficient to establish infection in cattle with raw semen into the uterus, let alone with extended semen through AI (Howard, 1986). Spread within a stud is most unlikely, since experimental infection of adult animals, as opposed to calves, with massive doses does not result in clinical disease (Merkal *et al.*, 1982).

Diagnosis of the pre-clinical condition still presents a problem. Faecal culture is currently the test of choice (Chiodim *et al.*, 1984), but DNA probes are on the market and likely to be in widespread use very soon (Howard, 1986, pers. comm. 1990).

Embryo transfer. The Mycobacteria have not been extensively studied. Rohde *et al.* (1990) isolated *M. paratuberculosis* from washed bovine embryos after *in vitro* exposure, but transient uterine infection requires a massive dose (Merkal *et al.*,

1982). This suggests that there should be caution where ET is practised in herds not confirmed as free from tuberculosis.

Haemophilus somnus

Artificial insemination. No fresh data are available on *Haemophilus somnus* in the semen of bulls (Krogh *et al.*, 1983). The addition of gentamycin, tylosin and lincospectin to raw semen prior to processing has shown to be effective in eliminating the organism (Shin *et al.*, 1988).

Embryo transfer. *H. somnus* still retains much interest. Kaneene *et al.* (1987) inoculated *H. somnus* cultures into the uteri of superovulated heifers at various stages prior to embryo collection. There were minimal pathological changes in the uterus, but *H. somnus* significantly affected ovarian activity as measured by the embryo/corpora lutea ratio. Thomson *et al.* (1988) exposed pre-implantation bovine embryos to cultures of *H. somnus*. After exposure and before washing, some of the embryos were treated with antibiotic media. Washing alone did not remove the organisms, but all those treated with antibiotic were bacteria free.

The Mycoplasma

M. mycoides mycoides is the major bovine pathogen in this group. The presence of contagious bovine pleuropneumonia in Europe (Anon, 1992b) must be a reminder that vigilance is required for stud and day centres, with quarantine and serological screening where necessary (Provost *et al.*, 1987). Although transmission through AI and ET is unlikely, knowing the difficulty of removing this group of organisms *in vitro*, more information is needed.

Artificial insemination

Many species of the mycoplasma are inhabitants of the genital tract (Pilaszek & Truszczynski, 1988). Of these *M. bovis* is regarded as the most pathogenic (Garcia *et al.*, 1986). Heifers inseminated with contaminated frozen semen can become repeat breeders with chronic suppurative salpingitis and endometritis (Nielsen, 1987). Other isolates, in particular *M. bovigenitalium*, are basically considered likely to be saprophytic species in the urogenital flora (Martel *et al.*, 1991). Poumarat *et al.* (1987) describe a passive haemagglutination test for the diagnosis of *M. bovis* infection as widely used in France.

The other organism of importance in this group is *Ureaplasma diversi*, at times associated with acute granular vaginitis. Doig *et al.* (1981) underline the Canadian conviction of the importance of this organism. A number of studies throughout the world have demonstrated a high frequency of ureaplasma in the preputial cavity, and semen of bulls at AI units (Martel *et al.*, 1991).

In field studies Fish *et al.* (1985) showed that 28% of bulls used for AI had semen contaminated with mycoplasma species, but no *M. bovis* was isolated. Garcia *et al.* (1986) cultured 2950 samples from nine Canadian studs, and no *M. bovis* was detected. Ball *et al.* (1987) in culturing 332 samples of fresh semen, found that 46% contained a mycoplasma species and 32% of these also contained ureaplasma. These ureaplasma were not eliminated by treatment with mycoplasmacides, in this case lincospectin.

Martel *et al.* (1991) point out that a principal obstacle to investigation is accurate isolation and identification of strains. Proper collection and transport of samples are very important. On 50 raw semen samples collected from old bulls in France, between 28% and 37% were positive depending upon the isolation techniques. Ball (1990) recommended the addition of hyper-immune serum to sheath washings to suppress the quickly growing *M. bovigenitalium*, and that allowed the less competitive species to appear. To identify isolates Nielsen *et al.* (1987) described an ELISA test detecting levels above 200 parts per ml with excellent specificity. Poumarat *et al.* (1991) describe a dot immunobinding technique.

Because of the uncertainty over the pathogenic role of the mycoplasma group, and the ubiquitous presence of many species in the genital tract of the bull, antibiotics have been added to raw semen or extenders in an attempt to eliminate these organisms. Lincomycin and spectinomycin have been used for many years (Hamdy, 1972), and are efficient against mycoplasma species (Ball *et al.*, 1987), but not against ureaplasma sp. Shin *et al.* (1988) demonstrated the advantage of a combination of gentamycin, tylosin and lincospectin in controlling *M. bovis*, *M. bovigenitalium*, other mycoplasma species and ureaplasmas, as well as *C. fetus* and *H. somnus*. These antibiotics were shown not to be deleterious to semen quality or fertility (Sullivan *et al.*, 1988), and might well be adopted in Europe. The importance of adding antibiotics directly to the raw semen has been stressed. However, where such semen is not frozen, laboratory seeded organisms may survive because of the purely bacteriostatic nature of the antibiotics added (Lorton, 1992).

Embryo transfer. Embryo transfer also presents a risk in the dissemination of mycoplasma species. *In vitro* exposure of bovine embryos to *M. bovis* and *M. bovigenitalium* resulted in isolation of the organism after washing in all cases, demonstrating adherence of these two species to the zona pellucida (Riddell *et al.*, 1989a). Mycoplasmacides and trypsin treatment also surprisingly had no effect. This work is supported by Bielanski *et al.* (1989). Brittain *et al.* (1988) found a similar behaviour with ureaplasma, whereby ten washings had no effect on removing organisms. The recent work of Bielanski *et al.* (1992) on the germicidal effect of photo-sensitive agents when activated by light may have relevance here.

SHEEP

Artificial insemination

General disease conditions Amongst the group A diseases FMD, vesicular stomatitis, rinderpest, peste des petits ruminants, bluetongue, EHDV, sheep pox, Rift Valley fever, Wessalbrons disease and Nairobi sheep disease must all be considered as potential threats through infected semen (Hare, 1985).

Moreover, the following disease agents deserve attention in a similar way to that of the cattle data mentioned above:- Brucellosis (but particularly *B. melitensis* where seminal excretion has been confirmed (FAO, 1981), Johnes disease, leptospirosis, Border disease/BVD, and Akabani virus if relevant.

Scrapie

Although the agent has been detected in a number of tissues other than neural tissue by sheep assay (Pattison *et al.*, 1972) and mouse inoculation (Hadlow *et al.*, 1982; Foote & Pitcher, 1989), it has never been detected in semen, urine or the male genital tract.

Other slow virus infections

There is no data on the detection of Maedi Visna/CAE or pulmonary adenomatosis agents in the semen of rams or bucks.

Brucella ovis. Bulgin (1990) demonstrated the excretion of *Brucella ovis* in the semen of seronegative rams, tested by the complement fixation test and the ELISA test, and he stressed the importance of culturing semen in any flock control programme. Kott *et al.* (1988) in examining 154 rams, both vaccinated and non-vaccinated, found 13% and 25.6% positive on culture respectively, despite an absence of scrotal lesions. Some semen parameters were rated lower in infected rams, but there was no clear-cut delineation.

Mycoplasma

Very little data are available on this group of organisms in the semen of rams and bucks infected with contagious caprine or ovine pleuropneumonia, contagious agalactia or similar conditions. Since balanoposthitis is described as a symptom of agalactia infection in the ram (FAO, 1981), clinical freedom and specific serological tests should be required.

Chlamydia spp.

Chlamydia psittaci has been isolated from the semen of rams (Lozano, 1986), but the relevance to the transmission of enzootic ovine abortion is thought to be slight (Appleyard *et al.*, 1985). Serological tests can be notoriously non-specific (Aitken, 1991).

The variety of organisms isolated in semen from rams with epididymitis, including *Actinobacillus seminis* (Lozano, 1986), underlines the necessity for close clinical appraisal of donor rams.

Embryo transfer

Bluetongue. Gilbert *et al.* (1987) transferred 12 embryos incubated with bluetongue virus, and after four washings transferred to seronegative ewes. Viraemia and seroconversion were observed in nine of the 12. Embryos from eight viraemic ewes were transferred after four washings to 15 serologically negative recipients, and two seroconverted. There was no evidence of bluetongue at the post-mortem of the lambs born. However, Hare *et al.* (1988) transferred 14 embryos from four experimentally infected donors bred by seronegative rams, and 39 embryos from infected donors bred by infected rams into a total of 27 seronegative recipients. None of the recipients or lambs seroconverted, and bluetongue virus was not isolated from pregnant recipient ewes or lambs at slaughter 30 days post partum.

Scrapie. Although Hadlow *et al.* (1982), using mouse assay, did not detect infection in the ovaries, the uterus or placentae of naturally infected sheep, Foote *et al.*

(1989) reported the detection of the agent in ovarian and uterine tissue. Furthermore, Pattison *et al.* (1972) using sheep feeding experiments identified infection in the placentae of experimentally infected ewes.

Foster *et al.* (1992) successfully demonstrated infection associated with unwashed pre-implantation 5 or 6-day-old embryos harvested from experimentally infected sheep. Six of 26 recipient ewe lambs (all homozygous for the short incubation allele of the Sip gene) developed scrapie in 751–959 days. This contrasts with studies of Foote *et al.* (1986), which were not able to take account of the genetic susceptibility of the recipients. Foster *et al.* (1992) have demonstrated a 'worst case' scenario and the work is to be repeated using a full IETS protocol. This will help to determine whether infection occurs at the transovarian level or purely at the surface associated with the zona pellucida.

Pulmonary adenomatosis. The likelihood of eliminating sheep pulmonary adenomatosis has been demonstrated by Parker *et al.* (1991) in a large scale trial. One hundred and twenty-two embryos from 50 donor ewes in endemically infected flocks implanted into 82 recipient ewes have resulted to date in no evidence of disease in recipients or progeny, many of whom are beyond the range of the 1 to 4 year incubation period.

Brucella spp.

Brucella ovis appears to be more closely attached to the zona pellucida than *B. abortus*. Wolfe *et al.* (1988) failed to remove *B. ovis* from blastocysts after exposing them to medium containing *B. ovis* followed by ten washings. The presence of penicillin and streptomycin had little effect. Riddell *et al.* (1990) also exposed ovine embryos to *B. ovis in vitro*, and after ten times washing without the presence of antibiotics, recipient sheep seroconverted and unfertilized embryos yielded the organism on culture. Riddell *et al.* (1989b) exposed ovine embryos to *B. abortus*, and after washing and antibiotic treatment all organisms were removed.

Caprine Arthritis Encephalitis (CAE) in goats

In goats, embryo transfer has been demonstrated as effective in removing contagious arthritis and encephalitis virus infection in goats. Sixteen embryos were transferred from infected sheep into eight recipients, and no seroconversion or clinical signs were observed (Wolfe *et al.*, 1987).

PIGS

Artificial insemination

There is a very active international market in chilled and frozen pig semen, and the EEC directive (ECC, 1990) sets out an agreed protocol of tests to establish disease free boar studs within the European community.

It must be assumed that major epizootic disease such as FMD, African swine fever, classical swine fever, swine vesicular disease and Teschen disease may lead to the presence of virus in semen (Hare, 1985).

Aujeszky's Disease. Pseudorabies virus in swine, a member of the herpes group,

behaves similarly in many ways to BHV-1 infection in the bovine with associated latency (Vannier, 1991). Vannier explains the difficulty of isolating virus from semen as due to viral inhibitors, but there are reports of successful isolation (Hsu *et al.*, 1984; Medveczky & Szabo, 1981). Viral replication is believed to take place in the genital tract of the boar, but the site of this is not well defined (Miry *et al.*, 1987). Vannier (1991) believes that vaccination does not prevent infection or the risk of presence of virus in semen of infected boars. He recommends that all boars in AI Centres should not be vaccinated, should come from herds serologically free of infection and be regularly tested negative.

However, gene-depleted vaccines are now available. It appears they offer some protection and furthermore reduce viral excretion from those infected. Laboratory tests are readily available to differentiate vaccination from field virus infection (Kit *et al.*, 1990; Aumüller pers. comm.).

Parvovirus. Porcine parvovirus is very widespread. The transmission of virus in the semen of boars has been demonstrated (Lucas *et al.*, 1974), but Mengeling (1989) suggests that the vaccination of boars should reduce their involvement in the dissemination of virus.

Porcine respiratory and reproductive syndrome (PRRS—Blue Ear disease). Circumstantial evidence suggests that some boars only, for a brief period following infection, are capable of shedding virus in the semen prior to seroconversion. This has not been confirmed by any isolations (Robertson, 1992). Beyond this initial stage, although a long lasting viraemia has been described, detailed investigations conclude that the risk of semen contamination is very low indeed (Öhlinger, 1992).

Other virus diseases. Madec and Vannier (1992) state that enteroviruses and adenoreoviruses have been isolated from boar semen. Should swine influenza or TGE viruses be present, the major risk is thought to be contamination with faecal material etc, at collection time. This risk can clearly be minimized with pre- and post-stud clinical freedom and sound hygiene at collection.

Brucella suis. *Brucella suis* is a bacteria of major importance in swine, with frequent infection of accessory glands and dissemination of large numbers of organisms in the semen (Vandeplassche *et al.*, 1967).

Special caution is urged in diagnosis by serological means. Of the tests available an ELISA test may be the most efficient, but not all infected individuals appear to produce antibody. Interpretations should be based on a clear herd test (MacMillan, 1992).

Leptospirosis. A variety of serovars of *Leptospira* have been implicated in causing swine abortion. Swine free of clinical symptoms may shed *Leptospira* in the urine in large quantities for up to a year after infection (Schönberg & Brem, 1992). Ellis *et al.* (1986) have shown the likely importance of venereal transmission. In chronic infections, *L. pomona, L. tarassovi, L. grippotyphosa* and *L. bratislava* are particularly prominent. As in cattle, the serum agglutination test may not identify all chronic carriers capable of disseminating the organism in the semen. Serological testing of boars must be used in conjunction with the addition of antibiotics to the diluent (Ellis, 1992).

Gross bacterial contamination of the ejaculates is a major problem in the collec-

tion and processing of boar semen. Madec and Vannier (1992) stress the necessity to reduce bacterial contamination to a minimum. Experimentally they demonstrated reduced fertility of chilled semen seeded with more than 10 000 bacteria ml^{-1}, and a significantly higher level of uterine infection in such groups. They stressed the necessity for regular bacterial screening for extended semen to monitor hygienic practice.

Embryo transfer

Foot and mouth disease. Singh *et al.* (1986) exposed 194 porcine blastocysts to FMD virus *in vitro*, and five of these showed evidence of infection after an approved washing technique.

Other virus infections. Classical swine fever, vesicular stomatitis and pseudorabies respond well to trypsinization, despite the adherence to the zona pellucida of the virus after a ten stage wash. (Singh & Thomas, 1987; Dulac & Singh, 1988; Singh & Thomas, 1990). In an *in vitro* trial, classical swine fever virus was not transferred with 385 embryos (Singh & Dulac, 1990) with the use of trypsin. Over 1000 transfers are recorded from pseudorabies infected donors, with no infection being recorded in the recipients (Veselinovick *et al.*, 1988), but there was evidence of transfer of pseudorabies infection where trypsin was not used (Bolin *et al.*, 1982).

Stringfellow and Seidel (1990) point out the apparent susceptibility of enveloped viruses to trypsin treatment *in vitro*. Non-envelope viruses do not seem susceptible to such treatment. Thus, swine vesicular disease virus and African swine fever virus showed very strong attachment to the porcine zona pellucida after *in vitro* exposure (Singh *et al.*, 1984), with comparatively little effect of trypsinization.

Porcine parvovirus. Bane *et al.* (1990) exposed 4–8 cell stage porcine embryos to strains of porcine parvovirus. They were able to identify PPV–DNA of the avirulent strain, but not a pathogenic strain, by RNA–DNA hybridization within the embryos. They concluded that there was replication of the virus associated with viable embryos. This is the first clear evidence of the possibility of viruses passing through the zona pellucida in any of the domestic species so far referred to.

HORSE

Artificial insemination

Although according to early records horses were inseminated artificially long before other large domestic animals, it is only in recent years that the movement of chilled and frozen semen internationally has been significant (Boyle, 1990). As a result, comparatively little data are available on disease transmission through AI.

Major systemic disease such as African horse sickness and glanders may well lead to the infected organisms' presence in the semen, but there are no data on this aspect of infection. Equine infectious anaemia infection leads to the carrier state in the stallion (Roberts & Lucas, 1987), and the presence of virus in the semen has been demonstrated. There are good serological tests for all these conditions.

More recent attention has been focused on equine viral arteritis (EVA) where

pre-natal infection leads to the persistent carrier state in progeny (Huntingdon *et al.*, 1990). Equine arteritis virus has been isolated repeatedly from semen of infected stallions with short and long term carrier states identified (Timoney *et al.*, 1987). Changes in semen quality were considered to have resulted from the thermal effect of elevated body temperature and scrotal oedema. Repeated culture of semen for the presence of EVA is considered advisable should previously infected stallions be returned to service (Neu *et al.*, 1992).

The equine herpes viruses are currently attracting much attention. Coital exanthema (EHV-3) has long been recognized throughout the world as primarily venereally spread (Pascoe & Bagust, 1975), and infected animals become carriers (Plummer, 1967). There are no data on the presence of virus in semen, but it must be considered likely. Serological tests must differentiate this condition from the ubiquitous EHV-2 infection, and the agents of equine pneumonitis and abortion EHV-1 and 4 which also exhibit latency (Welch *et al.*, 1992). Recent experimental work with EHV-1 infection has demonstrated replication in blood vessels of the testes and epididymes at 8 to 10 days, and lymphocytes may be detected in semen samples from infected animals by day 30 after challenge. Given that a viral vasculitis in the endometrium has been shown to precede placental transfer of virus and abortion in the late pregnant mare (Edington *et al.*, 1991), the potential for EHV-1 shedding in the semen—either as free virus or latently infected cells—is clear (Smith *et al.*, unpublished data).

Diseases such as contagious equine metritis *(Taylorella equigenitalis), Pseudomonas aeroginosa* and some capsule types of *Klebsiella pneumoniae* obviously have a venereal pattern of spread, and isolates have been made from raw semen (Vaissaire *et al.*, 1987; Guérin, pers. comm. 1992). It is concluded that they could contaminate frozen semen. Dourine, the disease caused by *Trypanosoma equiperdum* also has a venereal pattern of spread, but the risk of transmission in the semen has not been properly studied (Wood, pers. comm.).

Embryo transfer

Virtually no work has been done on the transmission of infection by embryo transfer in the equine. Embryos for freezing are flushed at 6 to 7 days to ensure that the zona pellucida is intact (Boyle, pers. comm.). Within a day or so of the development of the blastocysts, the zona pellucida itself is shed leaving the structure termed the capsule to surround the embryo (Betteridge, 1989). The role the latter might play in acting as a barrier to infection has yet to be determined. Eaglesome and Runkhe (1989) record the recovery of *Mycoplasma equigenitalium* from two 6–8 day old equine embryos after exposure to the mycoplasma *in vitro* and after a ten times washing protocol. The risk of flushing embryos from mares carrying *T. equigenitalis* (CEM) demands attention (Guérin, pers. comm. 1992).

TRANSGONADAL INFECTION IN ALL SPECIES

Although the presence of infective agents has been shown in ovarian tissue, for example BHV-1 (Guérin *et al.*, 1989), Border disease (Gardiner, 1980), scrapie (Foote & Pitcher, 1989), and immature spermatozoa e.g. bluetongue (Foster *et al.*,

1980) and Border disease (Gardiner, 1980), there is no field or experimental evidence to clearly demonstrate disease transmission by this route.

In the mouse under *in vitro* conditions, the zona pellucida can be penetrated by mengovirus (Gwatkin, 1967) and Coxsacki B-4 virus (Heggie & Gaddis, 1979), but in domestic animals there is only one report of viral replication inside an embryo after surface exposure—this refers to the porcine parvovirus (Bane *et al.*, 1990). Earlier work by Bolin *et al.* (1982) with pig blastocysts showed Aujeszky's virus to enter sperm tracts in the zona pellucida, and parvo and enteroviruses to enter pores in the zona pellucida. No viral infection of the blastomeres of the embryos was found. Virus biotypes might vary as to their behaviour (Brownlie, 1991).

More *in vitro* work is required on the risk of semen containing virus infecting the oocyte at fertilization, augmenting the *in vivo* work of Thomas *et al.* (1985) with bluetongue virus and DiGiacomo *et al.* (1990) with the EBL virus.

Once the zona pellucida is breached artificially *in vitro* for sexing, splitting, cloning or gene injection, the embryos will clearly be more susceptible to infection by any virus or bacteria contaminating the *in vitro* medium, whether arising from donor ovarian infection or the laboratory media itself.

CONCLUSION

Since the paper of Bartlett *et al.* (1976) on disease free studs, there has been a greater understanding of the pathogenesis of diseases such as IBR, Aujeszky's disease, BVD and bluetongue. This has lead to an improvement in diagnostic techniques, greater precision in confirming the absence of virus and a greater confidence in moving semen internationally.

There is no room for complacency, and particularly where such diseases are still present in the cattle population of the country at large, the health of the herd of origin of all AI sires is of paramount importance. Quarantine may be broken by fly-borne disease, and global warming may change the pattern of vector distribution. Post collection testing of sires is particularly important here. Vaccination may reduce the risk of disease breakdown within the stud, knowing that gene depleted vaccines allow differentiation from field virus.

The far reaching conclusion from current embryo transfer work, particularly in cattle, leads to the real possibility of moving livestock internationally as frozen embryos from any supervised population of donors without the risk of disease spread to the recipient herd, subject to exact protocols being adhered to. Caution must be exercised, but the OIE in conjunction with the IETS proves a helpful watchdog. More data too are required on the risk of viral contaminated semen in embryo transfer.

The development of internationally approved standards of collection of semen and embryos will assist in developing breeds throughout the world wherever they have potential. Integrity must be maintained at a very high level to promote confidence and trust to ensure that the animal health status is not compromised.