Hygienic aspects of storage and use of semen for artificial insemination

M. Thibier a, *, B. Guerin b

Abstract

The artificial insemination (AI) industry has developed over the last 50 years to the extent that it is used in almost every country in the world. One of the main factors contributing to its success is the confidence of the farmers that germplasm is not associated with pathogens, so that AI can be performed without risks. This has been achieved as a result of a considerable amount of research based on sound scientific data that has identified the major risk pathogens. A summary of these studies, given in this section, shows that despite the large number of agents that could be transmitted via the semen, there are cost-effective means to prevent such hazards. One of the basic rules is that the males should be housed in strictly protected semen collection centres (SCCs). Such centres should be approved by the veterinary authorities based upon specific criteria, which include special housing and operating specifications. This also includes specific means of monitoring the health of individual males through regular clinical examinations, assessment of semen and testings for various diseases. Two new challenges can now be identified, one relevant to so-called emerging diseases the impact of which on the status of the semen donors should always be assessed, and the second, relates to endangered genetic resources which may become extinct without active conservation programmes. The experience gained by the AI industry over the last 50 years should help to solve those problems. Currently, the use of semen derived from approved SCCs warrants their disease-free status. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Semen; Pathogen; Antibiotic treatment
1. Introduction

Artificial insemination (AI) is the first generation of reproductive biotechnologies (Thibier, 1990a,b) both in terms of numbers around the world (Chupin, 1992; Chupin and Thibier, 1995) and historically, as being implemented on the field for more than 50 years and its future relies upon health security. Farmers throughout the world wish to use the technology, provided that they take no health risks. Zero risk does not exist in the biological world, but it should be as close to it as possible, and modern risk analysis has provided a quantitative approach. It is generally accepted that a risk lower than $10^{-9}$ may be negligible and hence admissible. Such an analysis requires the clear identification of all factors down the whole line from the male providing the semen to the transfer of the semen into the female genital organs. Further, an exhaustive review should be made of all pathogens that may contaminate the semen. This of course underlines the level of responsibility of the people involved, not only on the production line but also from the regulatory standpoint, to ensure effective control of movement of semen within a region, and between areas around the world. The risk pathogens should not be evaluated individually but from an interacting point of view of such agents with the recipients of semen. When assessing the risk, it should take into account not only the major pathogens [lists A and B of the Office International des Epizooties (OIE) International Animal Health Code, 1999], but also the factors that under special conditions could affect female physiology.

Finally, while most semen is processed for use in AI, semen storage is also valuable for storage of genetic resources of endangered species, as part of an active conservation programme.

The aim of this review is to elaborate on the pathogens which should be considered in semen storage, and to describe the epidemiological considerations related to the housing of males and the premises of a SCC, and how they should be organised to ensure an optimum health environment. The procedure of controlling the critical points of hazards are also reviewed and some of the health constraints involved in the established repositories for ex situ conservation of animal genetic resources are briefly described.

2. Specific and non-specific microorganisms

Microorganisms are present in every ejaculate. The aim of obtaining sterile semen is virtually unachievable. Thus, in the use of AI, it is important to efficiently control the population of microorganisms in the semen and thereby prevent any introduction of diseases into individual animals, herds, areas or countries where they were not previously present. The experience of the last 50 years has demonstrated that a high level of control is achievable. However, to attain this, it has been necessary to clearly identify the agents that pose the main risks, and not only those of intrinsic pathogenicity, but also those likely to contaminate and to lead to a high concentration of microorganisms in the diluted semen. A number of detailed reviews have documented the interactions between sperm and pathogens (e.g. Hare, 1985; Afshar and Eaglesome, 1990; Eaglesome and Garcia, 1992; Eaglesome et al., 1992; Philpott, 1993). This section focuses on the major
points to be learnt from the abundant and yet incomplete literature on microorganisms present in semen. Agents that could inadvertently be introduced during the processing and storage of semen have not been considered.

2.1. Specific agents

For a complete picture of the role of putative pathogenic agents, each candidate agent should be subjected to combined in vivo and in vitro investigations: on the association of pathogens with semen on the one hand, and the insemination with any resulting disease, on the other.

The four main types of investigations are: (i) in vivo collection of semen followed by in vitro agent determination; (ii) in vitro contamination followed by in vivo insemination; (iii) in vitro contamination of semen followed by in vitro determination, and (iv) collection of in vivo contaminated semen followed by in vivo insemination. Such complete experimental designs have seldom been applied, necessitating caution on the part of the regulatory authorities.

Some so-called specific microorganisms gain access to the semen as a result of viraemia or bacteriaemia, whereas others are present due to local infections in parts of the genital tract. In some cases, microorganisms can also be associated with blood cells or with inflammation or trauma of the urinary tract and the preputial cavity. As a result, presence of infection is often an intermittent process, with pulses of high concentration of pathogen in the semen, followed by low concentrations or none at all. This aspect should be taken into account when monitoring semen for pathogens.

2.1.1. The major diseases (OIE List A)

The 15 major diseases listed by the OIE in List A (Table 1) are all of viral origin with the exception of contagious bovine pleuropneumonia which is caused by *Mycoplasma mycoides* subsp. *mycoides* SC. Due to their pathogenesis, one would particularly expect

<table>
<thead>
<tr>
<th>Disease or pathogenic agent</th>
<th>Bovine</th>
<th>Ovine, caprine</th>
<th>Porcine</th>
<th>Equine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foot and Mouth disease</td>
<td>P; Tr</td>
<td>P: (Tr)</td>
<td>P: (Tr)</td>
<td></td>
</tr>
<tr>
<td>Vesicular stomatitis</td>
<td>(P); (Tr)</td>
<td>P; (Tr)</td>
<td>P: (Tr)</td>
<td></td>
</tr>
<tr>
<td>Swine vesicular disease</td>
<td>P; (Tr)</td>
<td>P; (Tr)</td>
<td>P: (Tr)</td>
<td></td>
</tr>
<tr>
<td>Rinderpest</td>
<td>P; (Tr)</td>
<td>P; (Tr)</td>
<td>P: (Tr)</td>
<td></td>
</tr>
<tr>
<td>Contagious bovine pleuropneumonia (Mycoplasma mycoides)</td>
<td>P; (Tr)</td>
<td>P; (Tr)</td>
<td>P: (Tr)</td>
<td></td>
</tr>
<tr>
<td>Lumpy skin disease</td>
<td>P; (Tr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rift Valley fever</td>
<td>(P)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue tongue</td>
<td>P; Tr</td>
<td>P: Tr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep pox and goat pox</td>
<td>P; (Tr)</td>
<td>P: (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>African horse sickness</td>
<td></td>
<td></td>
<td></td>
<td>(P)</td>
</tr>
<tr>
<td>African swine fever</td>
<td>P; Tr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classical swine fever</td>
<td>P; Tr</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P: presence demonstrated; Tr: transmission demonstrated; ( ): highly probable.
these agents to be present in the semen during the viraemic phase. This appears to be the case for all of them, apart from vesicular stomatitis and Rift Valley fever for which there are no conclusive data (Hare, 1985; Philpott, 1993). For most of these diseases, the

Table 2
List B diseases in mammals susceptible to be transmitted through AI (adapted from Hare, 1985)

<table>
<thead>
<tr>
<th>Disease or pathogenic agent</th>
<th>Bovine</th>
<th>Ovine, caprine</th>
<th>Porcine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple species</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aujesky’s disease</td>
<td>P; Tr</td>
<td></td>
<td>(P; Tr)</td>
</tr>
<tr>
<td>Leptospirosis (Leptospira sp.)</td>
<td>P; Tr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q fever</td>
<td>P; (Tr)</td>
<td>P; (Tr)</td>
<td></td>
</tr>
<tr>
<td>Paratuberculosis</td>
<td>P; Tr</td>
<td></td>
<td>(P; Tr)</td>
</tr>
<tr>
<td>Cattle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaplasmosis</td>
<td>(P); (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Babesia sp.</td>
<td>(P)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine brucellosis (Br. abortus)</td>
<td>P; Tr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine genital campylobacter</td>
<td>P; Tr</td>
<td>P; Tr</td>
<td></td>
</tr>
<tr>
<td>Bovine tuberculosis (mycobacterium tuberculosi)</td>
<td>P; (Tr)</td>
<td>(P); (Tr)</td>
<td></td>
</tr>
<tr>
<td>Enzootic bovine leukaemia</td>
<td>(P)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBD-IPV (BHV1)</td>
<td>P; Tr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theileriosis (Theileria spp.)</td>
<td>(P); (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trichomoniasis</td>
<td>P; Tr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypanosomiasis (Trypanos spp.)</td>
<td>(P)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BVD/MD</td>
<td>P; Tr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papilloma virus (*)</td>
<td>P; (Tr)</td>
<td></td>
<td>(P); Tr</td>
</tr>
<tr>
<td>Sheep and goats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brucellosis (Brucella ovis)</td>
<td>P; (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brucellosis (Brucella melitensis)</td>
<td>P; (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Border disease (*)</td>
<td>P; (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAEV</td>
<td>(P)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contagious agalactia (M. agalactiae)</td>
<td>P; (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contagious caprine pleuropneumonia</td>
<td>(P); (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzootic abortion of ewes (Chlamydiae sp.)</td>
<td>P; (Tr)</td>
<td>P; (Tr)</td>
<td></td>
</tr>
<tr>
<td>Salmonellosis (S. abortus ovis)</td>
<td>P; (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brucella suis</td>
<td>P; Tr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papilloma virus (*)</td>
<td>P; (Tr)</td>
<td></td>
<td>(P); Tr</td>
</tr>
<tr>
<td>PRRS</td>
<td>P; Tr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus</td>
<td>P; (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porcine enterovirus*</td>
<td>P; (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porcine parvovirus</td>
<td>P; Tr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influenza virus</td>
<td>P; (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGE</td>
<td>(P); (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlamydia psittaci</td>
<td>P; (Tr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycoplasma sp.</td>
<td>P; Tr</td>
<td>P; Tr</td>
<td>(P); (Tr)</td>
</tr>
<tr>
<td>Listeria</td>
<td>(P)</td>
<td></td>
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</tr>
</tbody>
</table>
pathogenic agent has also been reported to occur in semen during the chronic phases when relevant, which substantiates the need for very strict control of the health status of the donor male. Seminal transmission has been documented for several of these agents, such as Foot and Mouth disease and Blue Tongue in ruminants.

2.1.2. The minor diseases (OIE List B)

About 80 diseases are listed by the OIE in List B, and 9 of them can affect more than one species. There are some other diseases of importance that are not listed by OIE but which should also be considered. Table 2 summarises the present knowledge regarding the diseases to date in terms of their demonstrated presence in semen and their potential for transmission.

2.1.2.1. Bovine diseases. The two bacterial diseases of cattle in List B that have been the most fully investigated are brucellosis and tuberculosis, and this is linked to the fact that major eradication programmes for these diseases have been in progress. It has been known since the late 1940s that the bacterial agents of these diseases may be excreted into semen and that they can be transmitted to inseminated cows. Other bacterial agents of concern found in semen include *Campylobacter fetus*, *Leptospira*, *Mycobacterium paratuberculosis*, *Chlamydia psittaci*. The protozoan parasite *Tritrichomonas foetus* is also important. Although these agents have been detected in semen, their transmission to cows after insemination has not been proven for all. This may be due to insufficient research. *Campylobacter* and *Tritrichomonas* are those that pose the most risk and they have been clearly shown to be transmitted. It is highly recommended, therefore, and in some parts of the world it is a mandatory requirement, that bulls used for AI are free from these pathogens. Other agents may be controlled by a rational administration of antibiotics either to the animal (*e.g.* *Leptospira*) or to the semen during its processing.

The viral diseases in List B that have been the most investigated are Enzootic Bovine Leucosis (EBL), and the Herpes virus disease, Infectious Bovine Rhinotractitis/Infectious Pustular Vulvovaginitis (IBR/IPV). Bovine Viral Diarrhoea (BVD) has also been thoroughly investigated but it is not listed by the OIE (see below). The virus of EBL is exclusively associated with blood cell contamination of semen from infected animals. An intensive eradication programme for EBL has been initiated in the European Union.
IBR/IPV has been the subject of considerable investigation and the virus is known to be shed in the semen and can be transmitted by AI (Kupferschmied et al., 1986; Van Oirschot et al., 1993; Wellemans et al., 1993). There is no worldwide agreement on the best way to monitor bulls for IBR/IPV in AI centres. The main reason is that, as with almost all herpes viruses, the Bovine Herpes virus 1 (BHV-1) responsible for this disease has phases of excretion and phases of latency. Intermittent excretion of BHV-1 in semen has been shown to occur in bulls (Guérin, 1989) and reactivation of latent infection without warning or noticeable signs is a hazard of great concern. This is why France (since 1972) and some other countries in Western Europe have taken the decision to eliminate all seropositive bulls from their AI centres.

BVD virus is usually considered as a specific pathogen but it is not included in List B of OIE. It has been identified in semen and the major risk arises from so-called persistently infected bulls, which continuously shed the virus into the semen and transmit the disease (Kirkland et al., 1997). The usual method of control involves testing of bulls to check that they are not persistently infected.

Other viral diseases (not listed in List B) that could be of potential concern with regard to AI include Akabane, Ephemeral Fever, and Bovine Immunodeficiency (BI). Akabane disease virus has not been detected in semen (Parsonson et al., 1981). Australian studies on Ephemeral fever have shown that this virus may occasionally be present in bull semen, but AI did not transmit the disease (Parsonson and Snowdon, 1974). BI disease is currently under investigation in different parts of the world. It has recently been shown to be shed in semen (Nash et al., 1995), but its capacity to be transmitted via AI is not yet proven.

Finally, with regards to cattle, it should be mentioned that the agent known as “prion”, or non-conventional transmissible agent which causes Bovine Spongiform Encephalopathy (BSE), does not appear to pose a risk of transmission through semen contamination (Wrathall et al., 1991; Wrathall, 1997).

2.1.2.2. Ovine and caprine diseases. Brucellosis is a disease of particular significance in sheep and goats. Both Brucella melitensis and Brucella abortus, which can induce lesions of the genital tract, can be shed in the semen. Brucella ovis is the causal agent of contagious epididymitis and is frequently excreted in the semen (Bulgin, 1990; Homse et al., 1995; Kittelberger et al., 1995). Nevertheless, the donor ram may remain sero-negative, necessitating a bacterial isolation test on semen to detect the disease. At least four other important bacterial agents can be shed in the semen of rams or bucks: Mycoplasma agalactiae, C. psittaci, Haemophilus somnus and Corynebacterium renale, but the degree to which their transmission occurs through AI remains to be determined.

Visna Maedi and Caprine Arthritis Encephalitis Virus (CAEV) are major viral diseases of sheep and goats, respectively. As these viruses are incorporated into the white blood cells, the presence of blood cells in the semen from infected donor animals could be a threat to the inseminated females. However, to our knowledge, the shedding of these viruses in semen has never been conclusively demonstrated, with the notable exception of CAEV in one experimentally infected buck (Travassos et al., 1998), nor has transmission via semen been demonstrated. Sheep and goat pox are important viral diseases on the OIE List A, but there are no conclusive data on whether they are shed in
semen (Bane, 1981). The scrapie agent does not seem to be transmitted through semen (Palmer, 1959; Wrathall, 1997), but there is a need for further research on this topic.

2.1.2.3. Porcine diseases. Transmission of *Brucella* infection and leptospirosis by semen is also relevant in this species. A thorough monitoring for these diseases in pig AI centres is imperative and the donors seropositive for brucellosis must be eliminated. Adequate antibiotic treatment of semen batches must also be applied to prevent transmission of *Leptospira* sp.

The Ausjeszky disease virus is another major threat to the swine industry and appropriate monitoring is essential. The virus can be shed in the semen even of vaccinated animals (Vannier and Gueguen, 1979), and it is particularly difficult to inactivate the virus in processed semen (Duricic, 1993). Although not listed by the OIE, Porcine Reproductive and Respiratory Syndrome (PPRS) is a disease of concern, as the virus is known to be shed in the semen and can be transmitted by insemination (Swenson et al., 1994a,b; Yaeger et al., 1993). Other diseases include Japanese encephalitis and parvovirus disease, the agents of which may be found in the semen and their transmission by insemination has been documented. Viruses such as enterovirus, adenovirus, Transmissible Gastro Enteritis (TEG) virus and influenza virus may occasionally be present in the semen at collection or can contaminate it during processing, as a consequence of their mode of excretion (faecal or aereal). Provided that strict hygienic procedures are taken, the epidemiological risk of transmission of these agents can be reduced to negligible levels (Madec et al., 1994).

2.1.2.4. Equine diseases. A venereal disease of major concern in horses is contagious equine metritis. Apparently healthy stallions may not only shed the agent *Taylorella equigenitalis*, but they can transmit the disease via the semen. This calls for a very strict monitoring and careful bacteriological sampling at the specific location of the agent on the prepuce. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* are other bacteria of significance that can sometimes be found in stallion semen. Monitoring for these diseases is important, although it seems that they are of less risk than *T. equigenitalis*, and they are not included in the OIE List B.

Among the viral diseases of most concern is equine arteritis, the agent of which is shed in the semen of acutely and persistently infected animals. Viral excretion may last for over 5 months without clinical signs of the disease. Moreover, the level of excretion may be so low that only DNA-probe tests can detect the agent. Transmission via semen has been documented and frozen–thawed semen retains its infectivity. Careful monitoring of stallions by serology or virological testing seems to be the best way to avoid risks. The equine rhinopneumonitis agents (EHV 1 and 3) have also been reported to be transmitted venereally. Transmission of Glanders and African Horse Sickness via semen has not been documented, although knowledge of their pathogenesis indicates that there could be some shedding of these agents in semen and transmission by AI. Equine Infectious Anaemia is also listed by the OIE in the B list and has been found in semen; monitoring for this disease can be reliably achieved by serodiagnostic tests. Finally, Dourine is also a venereal disease and, according to Timoney (1996), is transmitted via semen.
2.2. Non-specific agents

2.2.1. Origins and pathogenic properties

The saprophytic flora of the prepuce in healthy semen donors comprises numerous bacterial species which may become associated with the semen at ejaculation and during collection. Under appropriate environmental conditions some of these bacteria may behave as opportunistic pathogens and hence represent a significant risk to inseminated females (Wierzbowski, 1981). Contamination with non-specific agents may also occur during processing and storage of semen, not only from the atmosphere but also through substances (especially of animal origin) that are added to the semen diluents. Other sources of contamination include equipment and materials that have direct or indirect contact with semen, such as the vials in which it is stored. Liquid nitrogen is an effective cryopreservative of pathogens and can be an unsuspected source of contamination. This emphasises the need for well-protected and sealed containers to hold semen straws (Mazurova and Krpatova, 1990).

2.2.2. Problems in livestock species

2.2.2.1. Bovine and buffaloes. Several studies (Fache et al., 1985) have shown that the level of saprophytic flora can be as high as 4 to 5 logs CFU/ml in freshly collected semen. Therefore, it is important that the control is achieved through the use of appropriate antibiotics (Shin et al., 1988). Due to development of bacterial resistance to antibiotics, it is still essential to give careful attention to hygienic procedures.

The best examples of so-called non-specific pathogens affecting semen are the Mycoplasmas occurring in the urethra and in the preputial cavity. They include Mycoplasma bovigenitalium, Acholeplasma laidawii and Ureaplasma diversum. Mycoplasma bovis is also of concern and should regularly checked. Other ubiquitous bacteria frequently found in the preputial cavity are H. somnus and U. diversum. They can become pathogenic under unfavourable conditions. When antibiotics are added to semen, account should be taken of the bacteria most likely to be present and their respective sensitivity. For example, lincomycin, spectinomycin, gentamicin, or tetracyclin are usually used against Mycoplasmas.

Occasionally, Papillomatosis virus can also be found in bull semen and can be transmitted by this route. It is recommended not to collect semen from bulls for 2 to 3 weeks after appearance of visible papillomas.

Studies on semen contamination in buffaloes (see Pal et al., 1989; Aleem et al., 1990) indicate that the bacterial ecology of their genital tract is very similar to cattle.

2.2.2.2. Ovine and caprine. Little is known about the identity of non-specific bacterial agents in these species. Ureaplasmas (Marcus et al., 1994) and Actinobacillus seminis have been found in ram semen (Low et al., 1995), but their transmission to inseminated ewes has not been reported. There is no proof that Listeria monocytogenes could be associated with semen. Little information is available on seminal transmission of the
pestivirus agent of the Border disease, although this might be a possibility by extrapolation from BVD.

2.2.2.3. Porcine. Extensive studies have been conducted on bacterial contamination of boar semen. Madec et al. (1994) have reported a considerable qualitative and quantitative variation in the microflora of semen. The bacterial concentration varied between 0 and 7 logs CFU/ml with Gram-negative bacteria being the most prevalent. Examination of 60 semen samples from 22 boars on four different farms revealed Micrococcii in 30%, Escherichia coli in 25%, Proteus in 10% and Streptococcii in 8% of the samples. It has been reported that a concentration equal to or higher than 5 logs of non-specific bacteria can decrease fertility and reduce litter size (Madec and Vannier, 1992). Use of commercial semen for insemination of Specific Pathogen Free (SPF) sows caused hyperthermia as well as anorexia and there were no pregnancies. Serratia liquefaciens was isolated from the semen in this particular case (Madec et al., 1994).

2.2.2.4. Equine. Equine semen also contains several non-specific bacteria, including Streptococci sp., E. coli, Streptococcus zooepidemicus, Bacillus, Pseudomonas aeruginosa and Micrococcii. The presence of S. zooepidemicus has been associated with a poor semen quality, and concentrations up to 6 logs CFU/ml of this organism have been reported (Clement et al., 1993).

2.2.2.5. Avian. Several species of saprophytic bacteria have been found in semen from cocks and turkeys. They included E. coli, Staphylococci sp., Micrococcii, Enterococci and Salmonella, and an overall concentration of 5 logs (CFU/ml) was reported by Reiber et al. (1995). Grant (1988) noted an association between the presence of Mycoplasma iowae in turkey semen and an increased embryonic mortality.

2.2.3. Prophylactic measures to non-specific contamination

2.2.3.1. The pen and collection room. Bacterial contamination of semen is clearly associated with the hygiene and cleanliness of the donor bull. This directly reflects the hygienic standard of bedding and of the floor in the pen and collection room. The level of bacterial contamination can be significantly reduced by simple sanitary measures, including clean and proper bedding, separate entries before and after semen collection to and from the AV washing room, and clean procedures for semen collection as well as eliminating dust and faecal material (Lechat and Guérin, 1991; Gérard et al., 1991; Manciaux et al., 1994).

2.2.3.2. The processing laboratory. Further precautions should be taken to prevent any contamination after transfer of the collected semen to the processing laboratory. There are many potential sources of contamination, including extender containing egg yolk. The cleanliness of semen straws has to be maintained once the pack is open. A few of the conditions required for maintaining cleanliness are: (i) separate premises for the laboratory work and for the collection of semen; the use of air tubes to move the collected semen from the collection room to the laboratory is to be encouraged; (ii) use
of sterile extenders (additives of animal origin are to be avoided wherever possible); and (iii) adequate addition of efficient and targeted antibiotics to the collected semen (Shin et al., 1988; Visser et al., 1995).

An international inventory of the numerous causes of contamination of processed semen has resulted in the identification of critical steps, and an approach similar to that in use for other products such as food, known as the Hazard Analysis and Control of Critical Points. This analysis is now used in France (Dumont et al., 1994) and The Netherlands (Feitsma, 1996).

3. Housing and premises: epidemiological rule and critical points of hazard in semen production

The discussion below refers to the bovine, but the basic points are also relevant to other species.

3.1. Basic principles on the concept of designated pathogen-free semen

3.1.1. Principles of biosecurity

In establishing principles of biosecurity, the aim is to ensure the production of semen from the male donor which is free of specific pathogens (bacteria, viruses or parasites). One possible approach could be the analysis on each ejaculate to ensure the absence of pathogens. However, this is neither realistic nor feasible and in addition unsafe. In practical terms, the millions of semen doses collected around the world could not all be tested: the volume is too great and the cost would often exceed the value of the semen. This method is also not feasible because the number of pathogens in each batch that

![Diagram](image)

Fig. 1. The epidemiological rule to maintain pathogen-free bull semen for AI (Thibier, 1990b).
could be searched for would exceed normal diagnostic capabilities. In addition, it would be unsafe because the limits of sensitivity and the specificity or accuracy of routine diagnostic methods would not result in the desired level of guarantee. An alternative method used for many decades is illustrated in Fig. 1. The important point is that the bull has never been in contact with a given pathogen. However, there is also the shortcoming that in case of a possible and undetected recent exposure of the male, the pathogen may exist in the genital organs and fluids of the animal. An additional line of defence is the health status of all animals in the AI Centre. If they have never been in contact with the pathogen and are well protected, then it can be claimed that the semen should be pathogen free; the monitoring will be also more cost-effective and efficient.

The basic principal is: the semen may be guaranteed free of a given pathogen if the bull is free from it and housed with other males free from the pathogen. This approach needs a very strict and well-monitored system, including (i) an approved semen collection centre (SCC); (ii) well-trained and experienced staff; and, (iii) a rigorous control program of the health status of the sires.

3.1.2. Specific cases of vaccination

Vaccination can be used in the case of highly contagious diseases such as Foot Mouth Disease (FMD), Rinderpest or rabies. This strategic approach is also necessary if the epidemiological conditions of a disease induce risks that cannot be under control without vaccination (for example an epizootic at the border of a given country). Only inactivated vaccines should be authorised and used and their inocuity should be previously tested on the bulls themselves (and not only on females or castrated animals), so that no secondary and undesirable effects occur on libido, spermatogenesis or on the external genital tract. An accident of this kind was described and reported some 15 years ago (Brastel and Goffaux, 1981).

3.2. The quarantine station

Such a station is the interface between the herd from where originates the male and the SCC. It is a critical element because it influences the entire system. The basic requirements for a station are that:

(i) It should be fenced and protected from environmental threats as prescribed by the veterinary authorities.

(ii) Introduction of animals into the quarantine station should be done in an all in all out system.

(iii) The duration of stay of animals in the station varies according to species and the designated pathogens. For cattle, and as for most domestic farm animals, such a period would be a minimum of 30 days, allowing time for incubation of disease, appearance of clinical symptoms, or for an antibody response after a recent contamination.

(iv) Relevant testings should be performed during the quarantine period of the animal, and for some diseases (see below) two tests may be recommended.

It is good practice to acquire the males from farms identified free of specified diseases. The entry of a diseased animal may require discard and slaughtering of all stock at the station, which can, in some instances, be genetically and financially disastrous.
3.3. Semen collection centre

The first critical point is the maintenance of the health status of the centre which is to be used exclusively for semen collection of a given species. A mix of species in a given premise is not allowed. The condition and function of the centre should be approved by an official veterinary authority. There are many national regulations for AI centres applicable to different species for both domestic and international movements. They are all based on recommendations laid down by OIE (International Animal Health Code): Section 4.2 “Health controls and hygiene” for bovine (Appendices: 4.2.1.1. and 4.2.1.2.), porcine (Appendix 4.2.2.1) and small ruminant semen (Appendix 4.2.2.2.). One of the prime examples of the application of these measures is the EU Council Directives relative to semen such as that referred to as Directive 88/407, amendments 93/60 for bovine species. This directive includes the conditions for the approval of semen collections centres and the conditions relating to the supervision of such centres in its Annex A. Its Annex B also lays down the conditions which apply to the movement of animals into approved SCCs as well as the routine tests and treatments which must be applied to all resident bovine animals.

How should these recommendations be put into practice? First by a logical approach in the design of the centre, and secondly by a rigorous adherence by management to a biosecurity program. As shown in Fig. 2, a given AI Centre includes three major parts with three different levels of health statuses. Part A is for the animals; part B is for semen processing; and part C is for the administration office. Only the latter part may be at the general environmental level, but parts A and B should be in restricted areas and healthwise at the higher level. The animal containment facility should have a level compatible with that of housing ruminants, while the laboratory has that of a biological and microbiological laboratory which is known as level 2 (L2) biocontainment.

3.3.1. The pen and collection room

It is necessary to fence the perimeter of the centre and to control the health status of such premises to prevent the entry of feral animals, vectors or unwarranted visitors. The movements for personnel, animals and supplies/services should be separately specified.

Only authorised personnel should be allowed to enter the animal containment area. Such entry should be via one entrance only and is through a shower that is to be taken on a mandatory basis when entering the animal area and on an optional basis when leaving. These personnel must wear clothes that are exclusively used within the centre. The basic idea is to preclude any herdsman having contact with outside animals that could shed pathogens. Visits by farmers represent a major hazard in terms of potential for contamination of animals housed in the centre. Another hazard is that of the veterinarian. This is why it is mandatory that veterinarians follow the same procedures as those prescribed for the regular personnel (shower and use of special clothing) and to ensure that only new products (veterinary medicines, etc.) are utilised. Furthermore, only equipment owned by the centre may be used for treatments (syringes, needles, etc.).

Only tested animals (see below) should be permitted to enter the centre. The only interface with the exterior should be an adequate loading platform at the edge of the centre (behind fences when not in use), for loading or unloading animals. It should be
designed in such a manner that the transportation truck remains at the outside of the centre with no contact whatsoever with the inside.

The places for food should be located at the edges of the centres in such a way that every delivery vehicle remains on the outside. The food should be distributed to the animals only with equipment belonging to the centre. The same applies for manure stored in a corner of the centre. It should be loaded on trucks for removal from outside the perimeter fence.

Fig. 2. Model of SCC (Thibier, 1994).
The collection room is also a very critical part of the centre. It should have a design, shape, light, etc. compatible with good libido and semen output, while maintaining a high level of hygiene. It must be kept clean before, during and after semen collection. The nature of the floor has long been a matter of debate. Nowadays, strong, resistant and easy to cleanse rubber mats are available. They should be widely used, particularly at the mounting place. There should be no possibility for personnel in the processing laboratory walking directly into the collection arena and vice versa. The place of exchange of artificial vagina (AV) and semen between the collection room and the laboratory should be exclusively of the type of double window sluice, one for AV and one for the semen samples. An interesting alternative is the use of air tubes for sperm dispatch from the collection room to the laboratory. Correct identification of the semen samples is of paramount importance and should be confirmed both at the collection room and in the laboratory.

3.3.2. The processing laboratory

One overall general model of a semen-processing laboratory is shown in Fig. 2. Again, only authorised personnel should be allowed to enter. It should have only one entrance through a cloakroom and the personnel must wear special clothes, including shoes that are exclusively used in the laboratory. The basic idea of organising such a laboratory is the FORWARD rule: once the semen has been collected it should move forward from one room to another with no return or crossing. The collected semen is evaluated, diluted, processed and frozen, after which follows a so-called quarantine storage period of 30 days to ensure that no disease has occurred in the centre. It is safer and easier to treat the batches on a monthly basis. The storage room is at the heart of the centre and should be locked when not in use. The minimum personnel and the minimum time of work in the room should be targeted. Only the introduction of canes from the pre-storage room and exit of canes of semen for dispatch should take place in this room. The preparation room is the place where the semen is handled and prepared for dispatch. The dispatch room is the interface between the laboratory and outdoors. It should have no door and the containers should be transferred from this dispatch room to the external platform through an adequate sluice.

4. Health surveillance and testing

4.1. General aspects

Many of the recommendations regarding animal health are provided in the OIE Animal Health Code and in national regulations. The principles are, first, to control and monitor individual sires before their entry in the SCC, to ensure that the health status of the centre is maintained; and, secondly, to ensure regular examination and testing of the males in the centre. Before entry, health considerations of the area and origin of stock, as well as individual testing are to be undertaken. Once in the centre, three major types of monitoring are required: (i) clinical examination; (ii) spermiogramme; and, (iii) testing patterns to various diseases recognised as the major sources of risks (see above).
4.2. Health surveillance before entry to SCC

Candidates for entry into a quarantine station should come from an area in which diseases on the OIE List A have not been reported in a 10-km radius and for at least the last 4 weeks. This is particularly important regarding Food and Mouth disease, rinderpest and Bovine Contagious pleuropneumonia for ruminants. In many countries, it is now required that the herd of origin be free of tuberculosis, brucellosis and bovine leucosis. It is also recommended, or is compulsory in some countries, that the dam of the young bull candidate to enter in a SCC to be IBR/IPV and Bovine leucosis negative.

The animals arriving to the quarantine stations should undergo a thorough clinical examination as well as being tested for tuberculosis, brucellosis, leucosis, IBR/IPV and BVD (lack of virus). A second series of tests should be performed at least 30 days later which include the above tests, plus a search for Campylobacter and Trichomonas from preputial secretion (sheath washing or scrapping). The clinical examination should be repeated, and if possible, (according to age) a thorough semen examination performed to ensure that no gross abnormalities (often a reflection of chronic diseases) are present. If any of the animals fails to pass the tests, all stock must be tested again within 30 days.

4.3. Health surveillance in an approved SCC

The stock held at the centre and designated as disease free should undergo approved maintenance health examinations and testing at 6 months or at yearly intervals. These include the following.

(i) Thorough clinical examination of all stock.
(ii) Detailed semen examination, regardless of whether the bull is on collection or laid off. Such an examination is often a key point in diagnosing chronic inflammatory processes that may otherwise be undetected. This includes more tests on the semen than routinely done before processing, such as a search for non-germinal cells, detailed sperm abnormalities, resistance to heating etc.
(iii) A complete set of testing, including tuberculosis, Campylobacter, Trichomonas from preputial washings and blood tests for brucellosis, leucosis, IBR/IPV for relevant countries.

A combination of clinical and andrological tests provides the most efficient monitoring of the health status of the stock.

4.4. Specific items

In the case of clinical symptoms, the animal should be transferred immediately to a special location at the edge of the centre for further investigation. If there is a confirmed infectious disease, the animal should be slaughtered, the semen collection in the centre stopped, and the semen doses collected during the previous month discarded. Resumption of semen collection should only occur when all the animals in the centre are again confirmed to be in a satisfactory health status. If only a minor disease was detected, the bull could be relocated for semen collection after treatment and recovery.
In case of semen exports, the international markets may require additional testing for specific diseases. Special areas in the centre may be devoted to the animals tested (for example Leucosis-free in North America for the European market). Frequency of testing (when required two tests a year) or special testings (paratuberculosis, leptospirosis etc.) may also be specified.

In some countries, the semen donor bulls are returned to the herds while they are progeny tested. Such a procedure is not recommended, except under exceptional circumstances, as it could cause a break in the whole security system. In such a situation, the bull leaving the SCC to a herd loses all its health credits and should be considered as a new individual if called back to a SCC for semen collection. The returning animal should enter the quarantine station and undergo complete testing.

5. Conservation of genetic resources at high risk of extinction

Cryopreservation of genetic resources of animals threatened with extinction is a relatively new proposition. The concept is under investigation by several international bodies, and is of particular interest to the Food and Agriculture Organization (FAO) (Thibier, 1994). There are three major problems related to this topic.

The first relates to the health status of the animal which is of genetic interest. It might, for example, be the last survivor of a given breed, but healthwise not be eligible to enter into a regular centre. Whatever methods of collection are used (currently discussed by FAO), the semen will not meet normal international standards. It can be asked: Why the interest to collect and cryopreserve semen if it cannot be used?

The second problem refers to the storage. It would be irresponsible to use a repository containing semen from all kinds of origins. Semen from a single male with unsatisfactory health records could destroy the reported disease-free status of the semen that had fulfilled all health requirements. There is a need for guidelines regarding the repositories receiving and holding semen of low-grade health status.

Finally, there is the problem of use of semen regardless of its health status. The containers and straws holding semen not of the world standard should be very clearly identified and the use of such semen should be determined by special conditions. The problem seems easiest to solve with the use of special outland quarantine stations for accommodation of recipients for the healthwise-uncertified semen.

6. Conclusion

The AI industry has managed to develop to the extent that it is now by far the largest reproductive biotechnology used worldwide with close to 100 million cows inseminated each year. This industry has been able to generate a very large international semen exchange without causing any major disease outbreak. This has been mainly due to the high standard of health surveillance at the semen collecting centres, based upon sound scientific data and a high level of expertise. With the concept of new emerging diseases now recognised worldwide, it is wise to be alert, to continue professionally the same
level of supervision and to be prepared to adjust to any threat to the industry. The problems linked with storage of semen from animals threatened with extinction remains to be solved.

Farmers can be confident about the health status of the semen obtained from officially approved SCCs.

References


