# Chapter 10
# Quality Improvement of Boar Seminal Doses

**Eva Bussalleu and Eva Torner**

**Abstract** The implementation of artificial insemination techniques (AI) has been a turning point in the swine industry. To prepare doses for AI, semen must be collected by following a serial procedure involving high hygiene measures handling in order to minimise microbiological risk. One of these practices is the inclusion of antibiotics in the extenders, the aqueous media used for packing seminal doses that contain elements for assuring the survival of sperm cells for a short or long time period. However, a certain degree of microbial contamination cannot always be prevented, and in this case sperm quality and sanity standards of AI are better preserved if, prior to selling or freezing the doses, microbes are removed by applying methodologies, such as sperm filtration and sperm washing. Additionally, the demand for doses with a high ratio of X- or Y-bearing sperm is increasing due to the particular structure of commercial pig production; hence, it is also necessary to optimise current sex-deviation techniques. All these topics will be fully discussed in the present chapter.

## 10.1 Introduction

### *10.1.1 The Concept of Seminal Dose*

The first time that artificial insemination (AI) was attempted in boars was at the beginning of the twentieth century. Nowadays, the use of AI has become widespread all over the world, and in developed countries it sustains the entire pig production system (see Chap. 12). The biological basis of AI is the high amount of spermatozoa present in an ejaculate, which entails the possibility of fertilising a high number of sows (from 15 to 40), whereas in natural mating there is only one recipient (Domínguez et al. 2006).

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To ensure the success of AI, ejaculates must be diluted in the appropriate medium to prolong their viability, so that a commercial dose can be defined as liquid extended semen. Typically, different ejaculates from a single or multiple boars are collected, and then processed individually or otherwise pooled. Pooling semen takes advantage of the high fertility of some boars to minimise the effects of subfertile boars (or ejaculates). Thus, semen doses can be treated in two different ways:

- Homospermy: Doses are from a single ejaculate and inseminations are carried out with this single ejaculate.
- Heterospermy: Doses that contain sperm from at least two different animals in the same dose.

Heterospermy allows the processing of different ejaculates at the same time (the ones with the best quality are chosen) and it increases prolificity (number of piglets born alive) (Martin and Dziuk 1977; Dziuk 1996; Haugan et al. 2005).

The number of total spermatozoa in seminal doses is an important parameter in fertilisation (see Sect. 12.2.1.3). Normally, the doses destined to AI contain approximately 2 to 4 $\times$ $10^9$ spermatozoa in a volume of 80–100 ml, although the threshold varies with semen quality (Saacke et al. 1991).

### *10.1.2 From Ejaculates to Doses in Commercial Farming: Collection Procedures, Packaging, Refrigeration and Transport*

Semen can be collected from boars mainly in two ways: using an artificial vagina, or by a simpler practice commonly known as the "gloved-hand" method. Although in other species electrical stimulation (electroejaculation) is widely used, it is not predominant in pigs (King and Mcpherson 1973; Basurto-Kuba and Evans 1981; The PigSite 2011). During the collection stage and the entire process, it is mandatory to use hygienic techniques and to control temperature fluctuations. The routinely common procedure for semen collection in the "gloved-hand" technique is the following (adapted from Hancock and Howell 1959; Singleton 2002; Althouse 2007):

1. A filter is placed on a collection flask pre-warmed to 37 °C.
2. The extender is prepared at least 1 h before its use and pre-warmed at the same temperature before being dropped into the flask.
3. The ejaculate is collected. It can be collected from the boar while it mounts a sow or gilt in oestrus. This methodology has two main drawbacks: (1) the boar may be too big to be supported by females; (2) females may begin to walk around the pen, making it difficult to collect semen. Both inconveniences have made breeders decide to train boars to mount a dummy sow. Boars must be trained when they are aged 8–10 months. The trainers must

be patient and the boar should be moved to the collection room two or three times daily to get used to it. If it becomes difficult to make the boar mount the dummy, it can be sprayed with boar scent as this awakens the instinct of the animal. Dummies can be purchased or made in the farm, assuring that they are solid, well-secured on the floor and have no sharp edges, and their height should be adjustable to fit different boar sizes. When boars mount the dummy their sheaths must be visible below. During training, sudden movements and loud noises should be avoided. Semen is collected using polyvinyl, not latex, gloves; once the gloves are on, nothing must be touched except the boar's penis.

4. The penis is grasped and directed inside the collection flask. The first part of the ejaculate (pre-spermatic fraction) is discarded as this watery fluid does not contain sperm but a high bacteria count.
5. The sperm-rich fraction, which is chalky in appearance and contains 80–90 % of all sperm cells in the ejaculate, is collected.
6. The boar is allowed to complete his ejaculation (5–8 min) until the jellified last secretion is observed.
7. The filter is removed and discarded.
8. Within the first 15 min after collection the 37 °C extender must be added to semen. The collection container is placed on a scale to check the volume and the extender should be added slowly.
9. Sperm quality is evaluated (motility, morphology and concentration). The minimum requirements for use of fresh boar semen are the following: the consistency should be milky to creamy; the colour, from grey–white to white; the gross motility of unextended semen should be ≥70 % (if doses are used within 48 h) or ≥80 % (if doses are used later than 72 h after ejaculation); and total abnormalities should not exceed 25 %, including the cytoplasmatic droplets (Althouse 2007).
10. Semen is further diluted and packed into doses. Each standard dose contains about 2 to 4 billion sperms and 80–100 ml of fluid depending on the AI centre. The final concentration should be determined on the basis of the expected duration of storage before use. For example, if the doses are stored for 2 days, a 2 billion sperm/dose is recommended; if the doses are stored for 3 days, a 3 billion sperm/dose, etc. (Althouse 2007). The final number of packed doses depends on the characteristics of the boar, the frequency of collection and semen quality. It also depends on the opaqueness and chalkiness of the ejaculate (Table 10.1). There are different containers for sperm packaging: bottles with screw cap, bottles with clip-clap, plastic blisters, twist-off plastic tubes, and so on. (Fig. 10.1).
11. After packaging, it is advisable to monitor motility for some days as a quality control step for the whole process.
12. Doses are placed into a cooling chamber at a temperature of between 15 and 18 °C and are rotated twice a day to resuspend the sperm cells.
13. Finally, transport must be made using appropriate containers at 15–20 °C to avoid temperature fluctuations.

**Table 10.1** A short guide table for boar sperm dilution based on opaqueness (adapted from Singleton 2002)

| Semen volume (gram/ml) | Opaqueness | Dilution semen/ extender | Ml of semen + extender | Total amount of extended semen | Number of bottles (100 ml each) |
|---|---|---|---|---|---|
| 150 | Watered down milk | 1:3 | 150 + 450 | 600 | 6 |
| 150 | Milky | 1:6 | 150 + 900 | 1050 | 10 |
| 150 | Creamy | 1:10 | 150 + 1500 | 1650 | 16 |

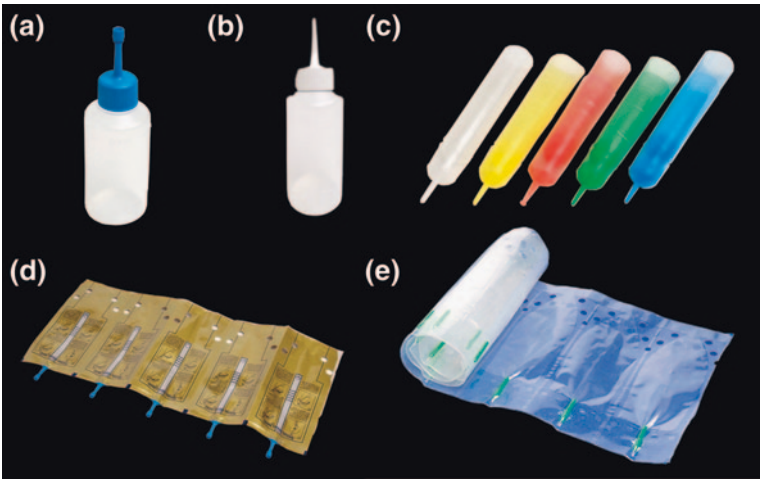


**Fig. 10.1** Some of the products used for seminal dose packaging: **a** bottle with twist off cap; **b** bottle with clip clap; **c** coloured tube flexible 90 cc; **d** twist off bags for semen doses; **e** blister for semen doses (http://www.kubus-sa.com/products.php?id=1

## 10.2 Techniques for Quality Improvement

### *10.2.1 Formulation of Extenders*

#### 10.2.1.1 History and Basic Formulation of Extenders

With the term "extender", we define the aqueous solution used to increase the volume of the ejaculate to that of the required dose (Gadea 2003). The extenders used in AI are a mixture of compounds that are physiologically, biochemically and biophysically equilibrated with the spermatozoa to maintain their integrity and fertilising ability until insemination is performed. The extenders reduce sperm metabolism to prolong its lifespan and also avoid the development of microorganisms. Early diluents for boar semen were proposed by Milovanov in the 1930s and were composed of glucose sulphate and glucose tartrate (Milovanov 1962; Johnson

et al. 2000). In 1965, the Kiev extender, developed by Plisko, introduced an ionic chelator to neutralise ions that could damage the cell membrane structure or trigger a premature acrosomal reaction. In 1975, Pursel and Johnson initially developed the Beltsville Thawing Solution (BTS) for thawing cryopreserved boar sperm but its beneficial properties permitted its development as a conventional extender for refrigerated semen (Johnson et al. 1998). The BTS extender, in comparison with its predecessors, contains a small amount of potassium that allows the maintenance of sodium–potassium pumps in sperm, so it prevents intracellular reduction, and consequently there is no impairment of motility (Alvarez and Storey 1982).

Basically, extenders provide nutrients for the maintenance of sperm metabolism, and substances to control osmotic pressure. It is also necessary to add compounds that reduce the metabolic activity of sperm, whose action is enhanced if the storage temperature is reduced. Due to the characteristic lipid content of boar sperm cell membranes, boar spermatozoa are highly sensitive to temperature changes; so, stabilising the temperature to 15–17 °C also permits keeping a controlled environment. The most common ingredients in extenders are glucose for the sperm metabolic activity, BSA (Bovine Serum Albumin) for cold-shock protection and for the compensation of protein loss, bicarbonate, TRIS (tris(hydroxymethyl) aminomethane) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) for pH control, antibiotics to prevent microbial growth and substances like NaCl or KCl for osmotic pressure control. However, most companies keep the exact composition of their extenders secret for commercial reasons.

#### 10.2.1.2 Long-Term and Short-Term Extenders

Extenders are actually divided into two groups: short-term extenders (those that allow preservation from 1 to 3 days) and long-term extenders (those that allow a preservation period higher than 4 days).

Short-term extenders are mainly used when semen is distributed within a local commercial network (Gadea 2003); in contrast, long-term extenders are used when semen production and insemination sites are far apart. The use of long-term extenders allows the possibility of performing analyses for sanitary controls, and more in-depth quality assessment. As stated before, extenders (both long term and short term) must contain nutrients for the metabolic maintenance of the spermatozoa, substances against cold shock, elements for controlling pH, substances for controlling osmotic pressure and antibiotics for bacterial growth inhibition. Long-term extenders such as DiluPorc™ (Sinus, Germany) have a protein mixture that, apart from helping the maintenance of osmotic pressure and pH, neutralises toxic substances secreted by bacteria and also proteins resulting from dead sperm cells. The pH of all these diluents is set between 6.8 and 7.2 and osmolarity ranges from 240 to 380 mOsm $kg^{-1}$. Sperm metabolism is reduced and viability maintained at temperatures ranging from 15 to 17 °C. However, when doses are warmed sperm activity is stimulated once again (Huo et al. 2002) and the sperm cells begin to consume high amounts of sugars in exchange for releasing free radicals (Parrish

et al. 1999; O'Flaherty et al. 1997). As boar sperm cell membranes have many unsaturated phospholipids, which are vulnerable to lipid oxidation, it is necessary that extenders also include antioxidants (Aitken and Curry 2011; Am-in et al. 2011; Martín-Hidalgo et al. 2011).

Vyt et al. (2004) performed a study comparing three long-term and two short-term extenders for a period of 7 days. They pointed out that motility was dependent upon the time of storage and fell into acceptable values in the two short-term extenders during 3–4 days. After that, motility decreased until being out of the quality range on day 7, although it was still noticeable by this time point. They concluded that the choice of a long-term extender is essential for long-term preservation of extended semen. Likewise, a study by Silva et al. (2011) suggests that the addition of an insulin-like growth factor-I (IGF-I) may be beneficial to semen stored for long periods of time. In their study, they found that the addition of 150 ng/mL IGF-I improved the quality of semen stored for 24 h, and the addition of 78 ng/mL IGF-I improved the quality of semen stored for 72 h.

Antimicrobials are usually added to semen extenders to inhibit bacterial contamination and growth, so that sperm stays viable for days or at maximum for 2 weeks when it is stored at 15–17 °C (Sone 1990). Some studies point out that the presence of antibiotics in the extenders at the appropriate concentration enhances sperm survival, and consequently fertility results (Colenbrander et al. 1993). However, there are certain resistant bacteria that can survive in the extended semen despite the presence of antimicrobials, thereby reducing the fertilising ability of this sample (Sone 1990). Some studies have revealed that the majority of bacteria isolated from extended semen are resistant to the most common antibiotics (Althouse et al. 2000; Althouse and Lu 2005; Bolarín 2011). New studies and strategies are being developed to avoid or reduce the use of antimicrobials. For example, Morrell and Wallgren (2011) tested single layer centrifugation (SLC) as a tool to reduce bacterial contamination, with successful results. There has been great controversy concerning the utilisation of antibiotics in boar semen extenders, so their use is currently legislated.

#### 10.2.1.3 Legislation

In Europe, the addition of antibiotics in boar semen extenders and the sanitary requirements for intra-community trading and importations are regulated by the Council Directive 90/429/EEC. This directive states that "an effective combination of antibiotics, in particular against leptospires and mycoplasmas, must be added to the semen after the final dilution. This combination must produce an effect at least equivalent to the following dilutions: not less than 500 IU per ml streptomycin, 500 IU per ml penicillin, 150 mg per ml lincomycin and 300 mg per ml spectinomycin. Immediately after the addition of the antibiotics, the diluted semen must be kept at a temperature of at least 15 °C for a period of not less than 45 min."

There is another reference body for the legislation of semen extenders and semen collection: the World Organisation for Animal Health (the former Office

International des Epizooties, OIE). This organisation regulates the criteria to be applied to semen extenders, as stated in its International Animal Health Code (OIE 2011). According to these guidelines, diluents must be pathogen-free or sterilised if they contain milk, egg yolk or other animal proteins.

## *10.2.2 Column Filtration*

### 10.2.2.1 Methodology

Sperm morphology anomalies in boar ejaculates account for considerable economic losses in farms and AI centres. Furthermore, the presence of dead or aberrant spermatozoa in ejaculates can have toxic (Shannon and Curson 1972) and lytic (Lindemann et al. 1982) effects on adjacent spermatozoa. In several species of productive interest, different methods of filtration have been used to improve the sperm quality of seminal doses. The use of such techniques is also advisable before freezing-thawing procedures, especially in those ejaculates from asthenozoospermic or teratozoospermic males (Sieme et al. 2003). Filtration serves to increase the number of viable and motile spermatozoa, although its effects vary considerably depending on the nature of the filtration matrix and the species (Johnson et al. 1996; Adiga and Kumar 2001).

Gel-filtration columns allow the separation of molecules by different parameters such as size, hydrophobicity, charge or ligand binding. The matrix used for filtration is poured into a column to form a packed bed and is chosen for its chemical and physical stability and inertness (lack of reactivity and adsorptive properties). The bed is equilibrated with a buffer, the optimal pH varying depending on the type of matrix. The principle of filtration is that non-viable spermatozoa tend to be more entrapped by the matrix than motile sperm and seemingly functional spermatozoa (Jeyendran 2002). In addition, viable spermatozoa are able to cross the filtration barrier without modifying their functional characteristics (Adiga and Kumar 2001). Immotile and dead spermatozoa tend to agglomerate because of changes in their surfaces charges (Ahmad et al. 2003) or in the proteins after capacitation (Januskaukas et al. 2005). It has been reported that filtration methods eliminate leukocytes (sources of reactive oxygen species, ROS) and select motile and morphologically normal sperm (Ibrahim et al. 2001). It is believed that they are also selective for acrosome intact spermatozoa (Anzar et al. 1997).

There are several matrices available for column filtration. The most common ones are the Sephadex™ neuter column matrices (Sephadex™ G-15, Sephadex™ G-25, Sephadex™ G-50Fine, Sephadex™ G-50Medium, Sephadex™ G-75), with different porous sizes ranging in diameter from 60 μm in Sephadex G-15 to 280 μm in Sephadex™ G-75 (in each type of Sephadex there are different particle sizes, the ones mentioned here are the smallest and the biggest in all the range) (GE Healthcare 2012). Apart from neuter Sephadex™, there are ionic Sephadex™: anionic Sephadex™ (DEAE-50), cationic Sephadex™ (CM-50),

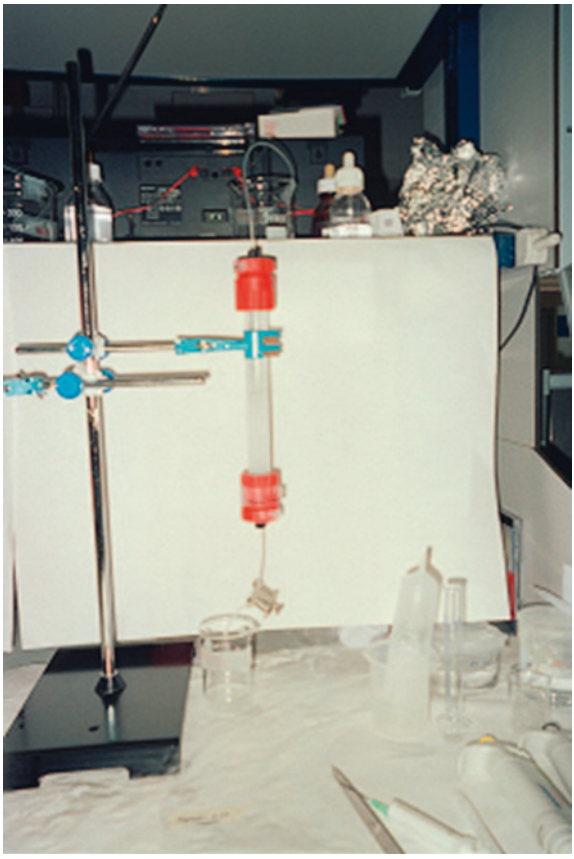

**Fig. 10.2** Sephadex™ column packed and ready for sperm filtration

glass wool, glass beads, Leucoscorb™, and so on. Sometimes it is possible to use mixed columns of different matrices (Fig. 10.2).

#### 10.2.2.2 Column Filtration Versus Other Methods

Apart from filtration alternative methods to improve boar sperm quality exist and are gathered under the name of purification or washing practices. They basically consist of sequential centrifugations to remove poor quality sperm.

In the simplest of these methods, the ejaculate in its seminal plasma and extender is centrifuged once and the pellet is resuspended with an appropriate volume of new fresh extender (Morrell and Rodriguez-Martinez 2010). This method only seeks the separation of the spermatozoa from the seminal plasma (Björndahl et al. 2005), so that no separation of dead, moribund or abnormal cells is achieved (Hallap et al. 2004). Another method consists of colloid or density centrifugation, which comprises some variants: Density Gradient Centrifugation (DGC),

SLC and swim-up (see Sect. 10.2.3). In these colloidal systems only sperm that is motile, viable and with intact chromatin is separated from the seminal plasma and the non-viable sperm (Pertorft 2000).

Filtration is not considered the cleanest technique since some cellular debris remains in the sample, but fewer spermatozoa are lost in comparison with other methods (Januskaukas et al. 2005).

#### 10.2.2.3 Implementation of Column Filtration at AI Centres

Experiments conducted so far have established suitable filtration protocols for different types of pathologies affecting boar sperm quality (Bussalleu et al. 2006, 2008, 2009a, b). Each type of column needs different elution conditions for optimal sperm separation and is recommended for a given pathogeny affecting the male ejaculate (Tables 10.2 and 10.3). Filtration experimental tests showed high efficiency in enhancing boar sperm quality (Bussalleu et al. 2008, 2009a, b). Other authors (Ramió et al. 2009) also pointed out that the use of matrices like Sephadex™ G-15 and C-50 are good tools, not only for the improvement of boar semen quality but also for in vivo fertility. In contrast, the election of an inappropriate matrix could diminish boar sperm quality.

Column filtration could be easily implemented at AI centres since no heavy or expensive equipment is required. However, for large-scale filtration it would be necessary to develop a commercial prototype that suited large volumes, which is why research is ongoing in this respect.

**Table 10.2** Different characteristics and filtration conditions for matrices used in semen filtration at 23 °C

| Type of matrix | Particle diameter (μm) (approximately) | Matrix length (cm) (using a column of 20 cm length and a diameter of 2 cm | Elution velocity |
|---|---|---|---|
| Sephadex™ G-25 medium (neuter) | 85–260 | 10 ± 0.5 | 1 ml/20 s |
| Sephadex™ G-50 fine (neuter) | 40–160 | | |
| Sephadex™ G-50 medium (neuter) | 100–300 | | |
| Sephadex™ G-75 (neuter) | 90–280 | | |
| Sephadex™ DEAE-50 *(anionic) | 182–214 | 2.5 ± 0.5 | 1 ml/40 s |
| Sephadex™ CM-50 *(cationic) | 221–250 | 5 ± 0.5 | |
| Glass beads | 150–212 | 10 ± 0.5 | |
| Glass wool | – | 2 ± 0.5 | |

*The use of peristaltic pump is mandatory

**Table 10.3** Application of different filtration matrices in relation to different sperm pathologies

| Type of males | Best matrix to use | Advantages | Disadvantages |
|---|---|---|---|
| Non-pathological males | Sephadex G-50 fine, Sephadex G-50 medium, Sephadex G-75 | Increase in the frequency of mature spermatozoa | |
| | Sephadex G-25 medium | Increase in the frequency of mature spermatozoa. Decrease in the frequency of spermatozoa with distal droplet | |
| | Sephadex CM-50, glass wool | Decrease in the frequency of spermatozoa with distal droplet | Decrease in the frequency of motile spermatozoa |
| | Glass beads | Increase in the frequency of mature spermatozoa. Decrease in the frequency of spermatozoa with distal droplet | Decrease in the frequency of motile spermatozoa |
| | Sephadex DEAE-50 | Decrease in the frequency of spermatozoa with distal droplet | Decrease in the frequency of motile spermatozoa. Decrease in the frequency of viable spermatozoa. Increase in the frequency of spermatozoa with head abnormalities |
| Pathogenic males | | | |
| Asthenospermia (ejaculates with high frequency of immotile spermatozoa) | Glass beads | Increase in the frequency of viable spermatozoa | – |
| | Glass wool | Decrease in the frequency of spermatozoa with the tail folded in the connection piece | – |
| Astenoteratonecrospermic (ejaculates with high frequency of immotile, morphologically abnormal and death spermatozoa) | Sephadex G-25 medium, Sephadex G-50 fine, Sephadex G-50 medium, Sephadex G-75 | Decrease in the frequency of spermatozoa with proximal droplet, spermatozoa with distal droplet, spermatozoa with tail folded in Jensen annulus and agglutinated spermatozoa. Increase in the frequency of morphologically mature spermatozoa and in the frequency of viable spermatozoa | Decrease in the frequency of motile spermatozoa |
| | Glass beads | Increase in the frequency of viable spermatozoa | |

(continued)

**Table 10.3** (continued)

| Type of males | Best matrix to use | Advantages | Disadvantages |
|---|---|---|---|
| Teratospermia (ejaculates with high frequency of morphologically abnormal spermatozoa) | Sephadex DEAE-50 | Increase in the frequency of mature spermatozoa and decrease in the frequency of spermatozoa with proximal droplet and spermatozoa with broken tails | Decrease in the frequency of motile spermatozoa and in the frequency of viable spermatozoa |
| | Sephadex CM-50 | Increase in the frequency of morphologically mature spermatozoa and decrease in the frequency of spermatozoa with distal droplet | Decrease in the frequency of motile spermatozoa |
| | Glass wool | Increase in the frequency of morphologically mature spermatozoa and decrease in the frequency of spermatozoa with proximal droplet and in the frequency of isolated heads | |
| | Glass beads | Increase in the frequency of morphologically mature spermatozoa and in the frequency of viable spermatozoa | |
| Teratospermia (ejaculates with high frequency of morphologically abnormal spermatozoa) | Glass beads | Decrease in the frequency of spermatozoa with proximal droplet, agglutinated spermatozoa and spermatozoa with abnormal heads. Decrease in the frequency of isolated heads and in the frequency of broken tails | |
| Astenonecrospermia (ejaculates with high frequency of immotile and dead spermatozoa) | Sephadex G-25 medium | Decrease in the frequency of spermatozoa with tails folded in the connection piece | |
| | Sephadex G-75 | Increase in the frequency of viable spermatozoa | |

## *10.2.3 Purification (Washing) Sperm Procedures*

### 10.2.3.1 Applications

As explained in Sect. 10.2.2.2, simple washing and density centrifugation are good techniques to separate motile sperm from other cell types (Agarwal and Ranganathan 2001), but only the use of colloids permits to discard sperm with different pathologies, namely immature, aged and dying sperm (Morrell et al. 2001, 2004), morphologically abnormal sperm (Tomlinson et al. 2001), and sperm with damaged chromatin (Morrell et al. 2001, 2004; Sakkas et al. 2000; Tomlinson et al. 2001). Removal of bacteria and viruses is also possible with density centrifugation (Nicholson et al. 2000; Guibert et al. 2001; Cassuto et al. 2001, 2002; Levy et al. 2001; Bujan et al. 2001, 2002; Englert et al. 2004) as well as the elimination of ROS (Agarwal and Ranganathan 2001).

### 10.2.3.2 Density Gradient Methods

The DGC has been used for the separation of different types of cells, not only spermatozoa. The principle of this technique is that under the centrifugal force applied to a colloidal density gradient the cells move to the isopycnic point; that is, the point at which the gradient matches their density (Mortimer 1994). At this point, they can be easily aspirated and dropped into another tube with buffered medium. As spermatozoa have diff erent density from leucocytes, bacteria, viruses and cell debris, they can be separated using density gradient methods (Morrell 2006). When centrifuged, motile spermatozoa move faster in the direction of the centrifugal force than immotile spermatozoa and are separated. Motile spermatozoa remain at the bottom of the tube, whereas immotile spermatozoa and other cells are retained in the upper layers together with seminal plasma.

Normally, when DGC is applied it uses at least two layers of different density (and up to 12 layers in some protocols). A variant of this methodology is SLC, which only uses one layer. There are several substances used as colloids for gradient centrifugation, most of them commercial, although BSA is applied in some cases. The most common ones are Percoll™ (Sigma, St. Louis, MO), Ficoll™ (GE Healtcare, Salt Lake City, Utah, USA), PureSperm™ (Nidacon, Goteborg, Sweden), Isolate™ (Irvine Scientific, Santa Ana, CA, USA), Ixaprep™ (Medicult, Copenhagen, Denmark), Optiprep™ (Nycomed Pharma, AS, Majorstua, Oslo, Norway), Androcoll™ (SLU, Uppsala, Sweden) and Nycodenz™ (Sigma-Aldrich, St. Louis, MO). However, in 1996 there was great controversy about the use of Percoll™ (colloidal silica coated with polyvinylpyrrolidone (PVP)). The manufacturer withdrew it from human-AI because of possible adverse effects on sperm survival and development of fertilised eggs. Nowadays, this substance is only used for research purposes and not for the preparation of sperm for AI (Mortimer 2000). This product has been replaced by alternative colloidal silica preparations covalently bound to silane molecules (for example, PureSperm™ and Isolate™) or

others (Nycodenz™ is based on the iodinated cyclic hydrocarbon iohexol), which also give great separation yields.

The main inconvenience of density gradients is dealing with the high volume of semen that boars produce, which makes it difficult to use this procedure in routinely AI dose packaging. However, Morrell et al. (2009) proposed that the SLC procedure, with the colloid Androcoll™, is suitable for processing large volumes. Moreover, this technique has also been shown to be highly efficient at removing bacteria from boar ejaculates (Morrell and Wallgren 2011).

#### 10.2.3.3 Swim-Up

Although it can be considered a density gradient method, this technique deserves to be given individual attention owing to its particular features. The swim-up technique has been widely used in andrology laboratories for the selection of motile spermatozoa since at least the 1950s. This migration procedure is considered to be functionally equivalent to the process by which spermatozoa escape from the ejaculate and colonise the cervical mucus (Mortimer 2000). It is based on the fact that fully motile spermatozoa swim from the pellet, after the ejaculate is centrifuged, to the top layer of the swim-up medium, leaving the cellular contents and seminal plasma behind. The technique is quite simple and only requires an incubator. Briefly, the fresh diluted semen is concentrated by centrifugation (600 × *g* for 5 min) and then the sperm pellet is overlaid at the bottom of the tubes containing the swim-up diluent. Afterwards, the tubes are incubated at 37–39 °C for between 40 and 60 min and the medium and top layers, where motile spermatozoa have swum, are removed and processed (Holt et al. 2011). This technique is not highly efficient and the total recovery rate of motile spermatozoa does not exceed 10 or 20 % (Hallap et al. 2004). Moreover, some studies have criticised that this selection method is only based on sperm motility and does not select for general morphology, chromatin or acrosome/membrane integrity (Somfai et al. 2002). For this reason, and despite its simplicity, swim-up is not widely used for preparing spermatozoa for AI (Morrell and Rodriguez-Martínez 2011).

## 10.3 Sanitary Control

### *10.3.1 How can Semen be Microbiologically Contaminated? Economic and Sanitary Implications for the Swine Industry*

The presence of microorganisms in boar sperm is currently one of the problems that the swine industry, and especially artificial insemination (AI) centres, deal with. In the farm industry, AI and the international market of germoplasm require a high sanitary control to avoid the spread of diseases caused by viruses

or bacteria and to avoid the sale of infected doses (also with probably decreased quality due to the presence of microorganisms). Microbial contamination of semen can be due to systemic and/or urogenital tract infections of the boar, or can occur during collection, processing and storage. Sources of bacterial contamination of extended semen can be of animal origin (faeces, preputial cavity fluids, skin/hair, respiratory secretions and personnel), or non-animal origin (water, plant matter, sinks/drains, air and ventilation systems or inanimate objects) (Althouse 2008).

The source of fungi and yeasts is basically individuals and air ventilation systems. In the case of viruses, their presence in semen samples has its origins in the infected blood that goes to the genital tract, in preputial contamination via faeces (this venereal contamination affects semen collection and processing steps) and in aerosol contamination during semen collection and processing (Guérin and Pozzi 2005).

There are several recommendations for avoiding or minimising semen contamination (adapted from Althouse 2008):

Personnel:

- Follow good hand hygiene and use protective gloves.
- Avoid contact of hands with products that will be used in semen processing.
- Avoid sneezing or coughing into material in contact with semen.
- Use caps and hair nets (especially people who perform semen collection).
- Cleanness of overalls and shoes/boots.

Animal housing/handling:

- Regular sanitary maintenance, including the removal of organic material and application of broad-spectrum disinfectants.
- The boar abdomen must be clean and dried prior to semen collection.
- Clean preputial opening and surrounding area with convenient material.
- Preputial fluids can sometimes contain a high number of bacteria, so these must be eliminated prior to the semen collection process.
- When semen collection is done using an artificial vagina or the gloved-hand method, the collector should direct the penis in such a way that gravitational contamination of the semen collection flask with preputial fluids is minimised.
- When collection is done by the gloved-hand method, separation of the pre-sperm fraction helps to reduce the bacterial load.
- At the end of each collection, the working area must be thoroughly cleaned and disinfected.

Laboratory:

- Usage of disposable products whenever possible.
- If recycled laboratory material cannot be sterilised it must be cleaned using a laboratory-grade detergent (residue-free) with water, followed by a distilled water rinse. Allow sufficient time and proper ventilation for complete evaporation of residual alcohol.
- Laboratory purified water should be checked regularly. When there is bacterial growth, the source of contamination must be identified.

- Daily disinfection of the equipment using a residue-free detergent and thorough rinsing.
- The floor should be mopped with disinfectant at the end of the day.
- Break down bulked products into aliquots.
- It is advisable to install ultraviolet lighting in the laboratory surfaces.

The presence of microorganisms in semen can result in poor sperm quality, embryonic or foetal death, endometritis, systemic infection and/or disease in the recipient female (Maes et al. 2008). However, the transmission of pathogens does not always occur. The conditions required for the establishment of infection in the sow are complex, and lack of transmission might be explained by factors such as the sow's innate immunity, or concentrations of the pathogen below the minimum infection dose (Maes et al. 2008). Another example is the results reported by Maroto Martin et al. (2010), in which litter size was significantly reduced when semen was contaminated with *Escherichia coli* above a threshold value of $3.5 \times 10^3$ CFU/ml.

In relation to the regulation of the presence of microorganisms in semen, the Office International des Epizooties (OIE 2011) has defined some guidelines in the annexes of the International Zoosanitary Control (OIE 2011), and different countries all over the world have also established their own regulations.

### *10.3.2 Bacteria (and Fungi) Transmitted Via Semen*

A variety of bacteria can be found in boar semen under pathological conditions; most of them are gram negative and belong to the enterobacteriace family (Althouse and Lu 2005). The most common ones are *E. coli*, *Staphylococcus* spp., *Proteus* spp., *Pseudomonas* spp, *Klebsiella* spp, *Bacillus* spp and *Actinomyces* spp. There are some differences in the genera found depending on the source consulted, but the ones mentioned above are common in all the studies. Other genera of bacteria identified in boar semen are: *Enterobacter* spp, *Pasteurella* spp, *Citrobacter* spp, *Providencia* spp, *Neisseira* spp, *Corynebacterium* spp, *Streptococcus* spp, *Bacteroides* spp, *Lactobacillus* spp, *Acinetobacter* spp, *Actinobacillus spp, Flavobacterium spp, Serratia* spp and *Micrococcus* spp. (Tamuli et al. 1984; Dagnall 1986; Danowski 1989; Sone et al. 1989). Another bacterium prevalent in boar semen used for AI is *Chlamydia* spp (Kauffold et al. 2006). Maroto Martin et al. (2010) have also mentioned the presence of anaerobic bacteria in some seminal samples. Their incidence in semen has also been reported in equine livestock (Corona and Cherchi 2009) but it has not been widely studied in boars. *Clostridium perfringens* can survive in extended boar semen and can also cause lethal damage in sperm quality depending on the bacterial load (Sepúlveda et al. unpublished data).

Bacteria and spermatozoa are unable to regulate their temperature but the latter are much more sensitive to temperature changes than bacteria, so that exposure to a few degrees above body temperature causes sperm death. As environmental temperature decreases, cell plasma membrane fluidity changes and growth rates and metabolism decrease; at a certain point, growth and metabolism stop and cells become

dormant; this phenomenon, used to reduce metabolism and to induce dormancy in extended semen (and, consequently, increase sperm longevity), can be beneficial for contaminant bacteria, which can adapt to low environmental temperatures (Althouse 2008). To avoid their growth, antimicrobials are widely included in the formulation of extenders, but some studies have demonstrated that over 90 % of bacteria isolated from extended semen are resistant to common antibiotics (Bolarín 2011).

One of the most studied bacteria in boar semen is *E. coli* since its presence in semen induces sperm agglutination and motility impairment (Auroux et al. 1991; Diemer et al. 1996), which may be explained by the adhesion of *E.coli* to sperm cell membranes. Moreover, this bacterium produces spermicidal effects without an acidic environment (Althouse et al. 2000). Its presence in doses destined to AI is frequent and compromises sperm quality (Bussalleu et al. 2011a, b). This was demonstrated in an experiment in which different concentrations of enterotoxigenic *E.coli* (ETEC) and verotoxigenic *E.coli* (VTEC) strains ranging from $10^2$ to $10^8$ colony forming units (cfu) per ml, were inoculated in doses destined to AI and left during 11 days at 15ºC (Bussalleu et al. 2011a, b). Both bacteria (ETEC and VTEC) were selected for their high prevalence in farms (Thomson 2001). As shown in Fig. 10.3, the percentage of progressive motile spermatozoa significantly decreased in the tube inoculated with $10^8$ cfu/ml after 24 h of inoculation. This tendency was maintained until the end of storage time, so that the highest inoculation dose dramatically affected the percentage of progressive motile spermatozoa from the very beginning of the experiment. Regarding the percentage of viable spermatozoa (intact nucleus, intact mitochondrial sheath and intact acrosome) (according to Bussalleu et al. 2005), it significantly decreased in the tube inoculated with $10^8$ cfu/ml after 2 days of refrigeration when compared with other treatments

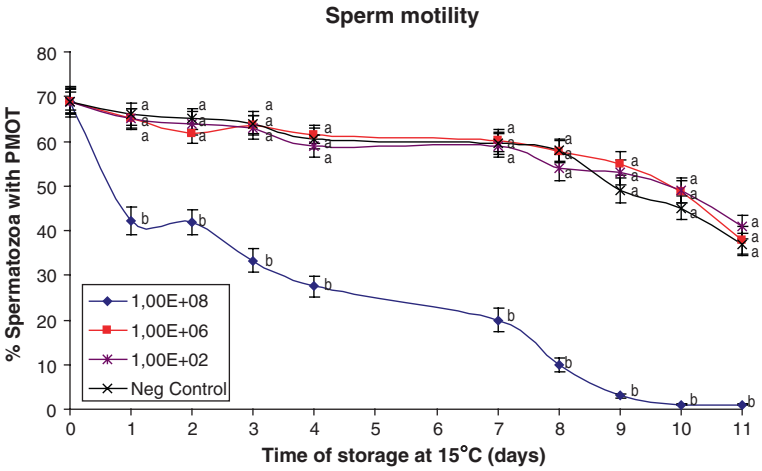


**Fig. 10.3** Percentage of viable spermatozoa (mean ± SEM) after 11 days of storage at 15 °C. Different superscripts (*a*, *b*, *c*) mean significant differences ($P < 0.05$) among treatments at the same given time point

(negative control, inoculation with $10^6$ cfu/ml and inoculation with $10^2$ cfu/ml) (Fig. 10.4). The percentage of morphologically normal spermatozoa, analysed under light microscopy, was not affected by the different concentrations of *E.coli* (Bussalleu et al. 2011a, b). However, previous studies have demonstrated that *E.coli* adhere to the sperm surface via mannose binding sites (Wolff et al. 1993; Monga and Roberts 1994) and produces ultrastructural changes at the level of the midpiece, plasma membrane and acrosome (Diemer et al. 1996), thereby altering sperm function (Villegas et al. 2005). These differences may be explained by the fact that it was not possible to see the ultrastructural damages induced by *E.coli* under light microscopy. On the other hand, Yániz et al. (2010) have reported that deleterious effects in ram semen samples are only evident if the sperm: bacterial ratio is 1:1 or greater. The same authors have pointed out that, during storage at 15 °C, multiplication of bacteria causes the reduction of the ratio, thus increasing the adverse effects on spermatozoa. It would seem that the same process occurs in boar ejaculates. Furthermore, the presence of factors released by bacteria, like α-hemolysin, Shiga-like toxin, lipopolysaccharides and peptidoglycan fragments, may also have important deleterious effects on spermatozoa (Schulz et al. 2010).

Regarding fungi and yeasts, their presence in the reproductive tract and in the semen of boars has not been widely studied. Only an investigation conducted by Cioreni et al. (2008) reveals that different fungi species can also infect boar semen samples. Species such as *Cladiosporum* sp., *Penicillium* sp., *Fusarium* sp., *Aspergillus* sp., *Mucor* sp., *Alternaria* sp and *Geotrichum* sp., apart from different yeasts, have been found in boar semen (Cioreni et al. 2008).

The harmful effects of contamination on sperm quality are not observed immediately, but, as mentioned before, appear after one (in the case of the highest

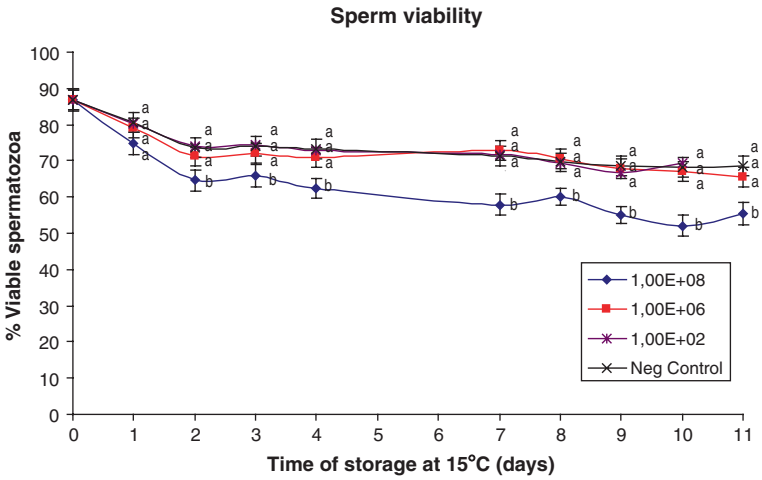


**Fig. 10.4** Percentage of viable spermatozoa with intact acrosome and mitochondrial sheath (mean ± SEM) after 1, 2, 3 and 4 days of storage at 37 °C. Different superscripts (*a, b, c, d*) mean significant differences ($P < 0.05$) among treatments at the same given time point

infective concentration) or more days of storage. This finding may be explained by the fact that once the extended semen is contaminated, bacteria must adapt to the novel environment by recovering from the physical damage or shock of initial transfer (Althouse 2008). Bacteria, fungi and yeasts that can be found in semen samples are susceptible to being transmitted via AI. Thus, it is necessary to control the microbiological sperm quality to avoid the spread of illnesses and the use of semen doses with low quality due to the presence of bacteria, fungi or yeasts.

### *10.3.3 Viruses Transmitted Via Semen*

Regarding viruses, many of them have been recovered from the semen of infected boars (Table 10.4; adapted from Maes et al. 2008). Apart from the strains mentioned in Table 10.4, there are others that have been isolated: adenovirus, reovirus, influenza, transmissible gastroenteritis and swine papilloma virus (Guérin and Pozzi 2005). The presence of these viruses in semen is related to reduced reproductive performance and infertility (Guérin and Pozzi 2005).

Many of the viruses can be transmitted venereally with or without clinical signs of disease. The most likely period to find virus in semen is in the stage of clinical illness of the host (Larson et al. 1980; Wittman 1989). If symptomatology exists, the breeder immediately removes the ejaculate from the AI circuit. The danger comes when the animal does not show any clinical sign, so that its infected semen is used. For this reason, it is advisable to perform analyses to detect the presence of viruses prior to the use of doses. Moreover, the conditions required for the appearance of clinical illness in sows after insemination with infected semen are complex, and the sows do not always become infected or show clinical signs (Guérin and Pozzi 2005).

Guérin and Pozzi (2005) have categorised the viruses that can be transmitted via semen into four types:

- Category I: viruses or viral diseases with proved evidence of transmission via semen, but without any sanitary risks for AI because of the existence of an official eradication policy. In this category there are the following viruses: Foot and Mouth Disease (FMD) virus, Aujezky's disease virus, Classical Swine Fever (CSF) virus, African swine fever virus and swine vesicular virus.
- Category II: viruses or viral diseases with proved evidence of transmission via semen and which can be associated with sanitary risk for AI unless they are accompanied by control measures and/or an official eradication policy. Porcine reproductive and respiratory syndrome (PRRS) virus, Japanese B-encephalitis virus and porcine parvovirus are included in this category.
- Category III: viruses or viral diseases that seem to be associated with sanitary risks for AI. In this case, more research to ensure the risk of transmission via semen is required. In this category viruses Porcine circovirus 2 (PCV-2), rubulavirus, enteroviruses and picornaviruses.
- Category IV: viruses or viral diseases for which there are no proof of their presence in semen or transmission via semen and which are dangerous for AI.

**Table 10.4** Important pathogenic viruses transmitted via semen

| Organism | Boar infection type | Timing of detection (test used) | Potential risk for contamination | Reference |
|---|---|---|---|---|
| Classical swine fever virus (CSFV) | Experimental inoculation | 7 and 11 DPI (virus isolation) | High | de Smit et al. (1999) |
| | Experimental inoculation | 7-63 DPI (RT-PCR); 11, 18, 21 and 53 DPI (virus isolation) | | Choi and Chae (2003) |
| Foot-and-mouth disease virus (FMDV) | Exposure to experimentally inoculated pen mates | Up to 9 d post-exposure (virus isolation) | Low | McVicar et al. (1978) |
| Japanese encephalitis virus | Experimental inoculation | 35 DPI | High | Ogasa et al. (1977) |
| Porcine circovirus type 2 (PCV2) | Natural infection | Detected (multiplex nested PCR) | High | Kim et al. (2001) |
| | Natural infection | Detected (nested PCR) | | Hamel et al. (2000) |
| | Experimental inoculation | Intermittently between 5 and 47 d DPI (nested PCR) | | Larochelle et al. (2000) |
| Porcine enterovirus | Experimental inoculation | 45 DPI (virus isolation) | High | McAdaragh and Anderson (1975) |
| | Natural infection | Detected (virus isolation) | | Phillips et al. (1972) |
| Porcine parvovirus | Natural infection | Detected (virus isolation) | High | McAdaragh and Anderson (1975) |
| | Natural infection | Detected (multiplex seminested PCR) | | Kim et al. (2003) |

(continued)

**Table 10.4** (contniued)

| Organism | Boar infection type | Timing of detection (test used) | Potential risk for contamination | Reference |
|---|---|---|---|---|
| Porcine reproductive and respiratory syndrome virus (PRRSV) | Experimental inoculation | 2-57 DPI (nested PCR) | High | Shin et al. (1997) |
| | | 12-21 DPI (nested RT-PCR) | | Christopher-Hennings et al. (1998) |
| | | Up to 47 DPI (nested RT-PCR) | | Christopher-Hennings et al. (1995a, b) |
| | | Up to 92 DPI (nested RT-PCR) | | Christopher-Hennings et al. (1995a, b) |
| | | 7 and 8 DPI (swine bioassay-sero-conversion) | | Swenson et al. (Swenson et al. 1994a, b) |
| | | 43 DPI (swine bioassay seroconversion) | | Christopher-Hennings et al. (1995a, b) |
| | | Up to 43 DPI (swine bioassay seroconversion and virus isolation) | | Swenson et al. (Swenson et al. 1994a, b) |
| | | 7 DPI (virus isolation) | | Prieto et al. (1996), Shin et al. (1997) |
| | | 11 DPI (virus isolation) | | Christopher-Hennings et al. (1995a, b) |
| Pseudorabies virus (Aujezky) | Natural infection | Detected (virus isolation) | High | Medveczky and Szabó (1981) |
| | Experimental infection | Detected (virus isolation) | | Vannier and Gueguen (1979) |
| Rubula virus | Experimental infection | 2-49 DPI (virus isolation) | High | Solis et al. (2007) |
| Swine vesicular disease virus (SVDV) | Exposed to experimentally inoculated pen mates | Up to 4 DPI (virus isolation) | Low | McVicar et al. (1978) |

*Adapted from Maes et al. (2008)

### *10.3.4 Methods for the Sanitary Control of Semen*

In the farm industry, AI and the international germplasm market require a high sanitary control to avoid the spread of diseases caused by viruses and bacteria. This control must be based on rapid, sensitive and specific diagnostic tests for certifying semen free from pathogenic agents (Afshar and Eaglesome 1990; Eaglesome and Garcia 1992). Currently, the isolation of bacteria from semen is based on culture procedures, which are complex and laborious and can easily be altered by the presence of antibiotics and inhibitors in semen (Gradil et al. 1994). This fact makes it necessary to use sensitive and rapid techniques, such as PCR (Polimerase Chain Reaction), which allows the amplification of a specific DNA sequence of a microorganism present at a low concentration (Mermin et al. 1991; Gradil et al. 1994; Eaglesome et al. 1992). Nowadays, the use of the PCR method is widespread and there are plenty of primers available to match a wide range of known viruses and bacteria. Some laboratories also apply the qPCR (quantitative PCR), which permits, apart from detection, the quantification of the microbial load. Due to its high efficiency and to the fact that results are obtained in a few hours, the use of this rapid technique allows quick detection of microbes, and infected doses can be immobilised on time before they are marketed.

Some PCR techniques are available for the detection of viruses and bacteria in boar semen. For example, the PCR techniques from Zhang et al. (2007) and Yilmaz et al. (2006) were adapted for the detection of ETEC and VTEC in boar semen. Concretely, two multiplex PCR (a type of PCR which allows the simultaneous detection of several genes under the same PCR conditions), and a conventional PCR were used (Bussalleu et al. 2011a, b). These PCRs permit the detection of 14 virulence factors (K88, K99, 987P, F18, F41, AIDA-I, Stx2e, STa, STb, LT, EAE, EAST1, VT1, VT2) present in different pathogenic *E.coli* strains (Fig. 10.5). Furthermore, a multiplex PCR technique developed by Ferrarezi et al. (2008) and adapted to boar semen, combined with a conventional PCR (developed by Bussalleu et al., unpublished), allows the detection of the following virulence genes from *Clostridium perfringens*: *cpa, cpb2, cpb*, *cpe*, *iA* and *etx* (Bussalleu et al., unpublished).

Regarding viruses, the methods used for their isolation are poorly sensitive, time consuming and highly expensive (every virus needs a different cell line to grow; sensitive cells lines for each virus are recommended in the OIE standards (OIE manual 2000) and they generate toxicity to host cells) (van Rijn et al. 2004).

To overcome these problems there are plenty of conventional PCR, nested PCR or quantitative PCR (qPCR) techniques available (more sensitive and less time-consuming than conventional virus isolation), like the two nested PCR techniques for the detection of PRRSV (from a sequence of the ORF7) (Bussalleu et al. 2009a, b) and Aujezky disease (from the gene *gB*) (Figs. 10.6 and 10.7). The nested PCR consists of two sequential PCR, in which the product of the first PCR is the template for the second. With this type of PCR the sensibility of the technique increases in comparison with the conventional one.

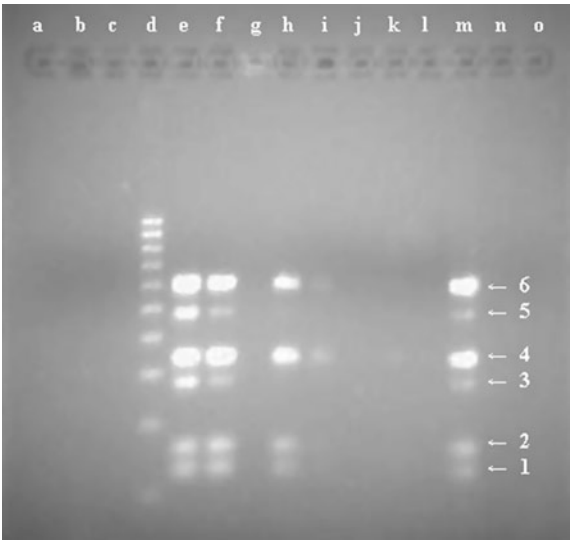


**Fig. 10.5** PCR multiplex assay for the detection of genes *K88*, *F18*, *F41*, *987P*, *K99*, *LT*, *STa*, *STb* and *Stx2e* of different infective loads of *E.coli* in boar semen. Lanes *a*, *b*, *c* and *o*, empty; lane *d*, 100 bp ladder; lane *e*, sample from an infective dose of $\times 10^8$ bacteria $ml^{-1}$; lane *f*, an infective dose of $\times 10^7$ bacteria $ml^{-1}$; lane *g*, reagent control (negative control); lane *h*, an infective dose of $\times 10^6$ bacteria $ml^{-1}$; lane *i*, an infective dose of $\times 10^5$ bacteria $ml^{-1}$; lane *j*, an infective dose of $\times 10^4$ bacteria $ml^{-1}$; lane *k*, an infective dose of $\times 10^3$ bacteria $ml^{-1}$; lane *l*, an infective dose of $\times 10^2$ bacteria $ml^{-1}$; lane *m*, direct cell culture of $\times 10^9$ bacteria $ml^{-1}$ (positive control); lane *n*, negative control. Band 1 corresponds to gene STb (125 bp), band 2 to STa (146 bp), band 3 to LT (280 bp), band 4 to gene F18 (334 bp), band 5 to gene K88 (440 bp) and band 6 corresponds to gene Stx2e (599 pb)

### *10.3.5 How can the Microbiological Contamination of Boar Semen be Eliminated?*

There are different strategies to maintain microbial concentration, of bacteria in particular, below a threshold level to preserve sperm fertility (Althouse et al. 2000). The first and most viable option is to enhance hygienic measures during semen collection and processing. The second option is to establish a shelf-life time limit on the extended semen product in order to limit the amount of time available for bacterial multiplication and to prevent overwhelming the buffering capacity of extenders. The third and most used option is to select antibiotics as preservatives with a broad-spectrum bactericidal or bacteriostatic activity to include them in the semen extender formulation (Althouse et al. 2000; Yániz et al. 2010). The Council Directive 90/429/EEC stipulates the antibiotics and the minimum concentrations that must be added to extended semen doses for their sale within the European Union (Morrell and Wallgren 2011). The most common antibiotics used in porcine extenders are spectinomycin, gentamicin, neomycin, amoxicillin, penicillin,


**Fig. 10.6** Detection of the ORF7 from PRRVS using a nested PCR. Lane *a*, product of first PCR (310 pb); lane *b*, negative control from the first PCR. Lane *c*, ladder of 100 pb. Lane *d*, product of the second PCR (162 pb); lane *e*, negative control of second PCR; lane *f*, negative control of first PCR after second PCR; lane *g*, negative control of reagents used for RNA extraction

lincomycin, tylosin, polymixin and enrofloxacin (Althouse 2008). Antimicrobials must be present at a concentration that provides enough readily-available active product; they must be able to permeate bacteria to a certain degree, and they must also occupy a sufficient number of active sites of the microorganism, and during enough time, to ensure their detrimental effect. If there are interferences in this process, this leads to the apparition of antimicrobial resistance (Althouse and Lu 2005).

Nowadays, some studies show promising results to improve some aspects of sperm quality and to reduce the use of antibiotics, thus minimising the development of antibiotic-resistant strains. Morrell and Wallgren (2011) have reported that it is possible to obtain bacteria-free sperm samples, or sperm samples with reduced bacterial load, without detrimental effects on sperm quality, using the SLC boar-specific colloidal system Androcoll™-P just after collection. Moreover, this technique not only reduces the bacterial load, but also improves the sperm quality of the samples by increasing the proportion of spermatozoa with linear motility and with normal morphology.

Still, more research must be done in the development of antibacterial and antiviral elements, as well as in the packaging systems. Nevertheless, the most important thing is to follow the recommended hygiene rules (see Sect. 10.3.1) during all the collection and processing of AI doses to minimise microbial contamination.

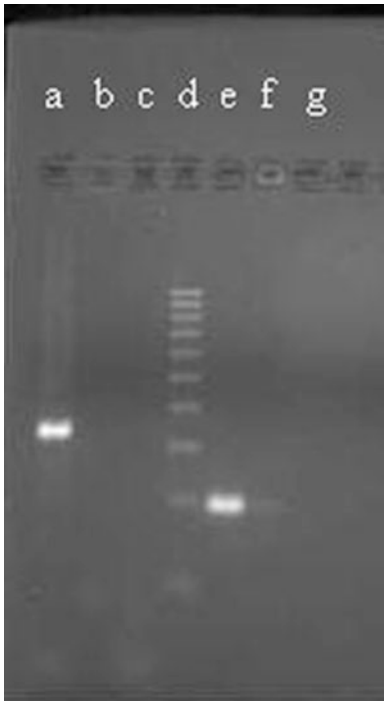


**Fig. 10.7** Detection of Pseudorabies (Aujezky) virus using a nested PCR that allows the amplification of g*B* gene. Lane *a*, product of first PCR (334 pb); lane *b*, negative control from the first PCR. Lane *c*, ladder of 100 pb. Lane *d*, product of the second PCR (195 pb); lane *e*, negative control of second PCR; lane *f*, negative control of first PCR after second PCR; lane *g*, negative control of reagents used for RNA extraction

## 10.4 New Trends: Sex-Ratio Deviation of Doses

### *10.4.1 Why Utilise Sexed Sperm?*

Selection of the sex of the offspring is really important for the improvement of AI management in pigs (see Sect. 12.2.3). In the swine industry, the main purpose of sex sorting is to maintain the elevated sow-to-boar ratio required in commercial breeding. In farms using natural mating, a sow-to-boar ratio of 16:1 is fairly typical. Thus, for a 300-sow unit, 19 boars would be required. In on-farm AI programmes, the sow-to-boar ratio is increased to 100:1 and only 3 boars are required for the same 300-sow unit (Estienne 1999). Another application of sex-sorted semen is the production of male and female crossbred lines for different meat characteristics; depending on the breed and the gender there will be differences in meat quality. Control of the sex ratio also permits faster genetic progress, higher productivity, improves animal welfare (avoids castration) and produces less environmental impact due to the elimination of the unwanted sex before growing to adulthood (Rath and Johnson 2008). The major demand of sex-sorted semen is for the production of females destined to consumption. Sows escape from the effects of androstenone and skatol, the metabolites responsible for the sexual smell in boars that give a particular bad taste to

pork. Moreover, in some countries in Europe the prohibition of castration will be soon implemented, so the production of sex-deviated offspring will be more necessary.

However, application of sex-sorting technology in the livestock industry is dependent on economics, efficiency and easiness of use (Martinez et al. 2005). An alternative to sexed semen is the use of sexed embryos. This technique, which is under development, requires embryos obtained in vitro that can be vitrified and used upon commercial demand.

### *10.4.2 Current Sex-Sorting Methodology*

The most effective and the most used technology for sex-sorting nowadays is flow cytometry, a technique that has been used since the 1980s (Johnson et al. 1987; Garner 2006). Basically, the sperm DNA from a semen sample is quantified with a sorting cytometer that retrieves two populations, one of X- and the other of Y-bearing sperm. Sex-sorting through flow cytometry requires four main steps (Rath and Johnson 2008):

a. Modification of a commercially available flow cytometer sorter into a sperm cell sorter by adding a forward fluorescence detector and a bevelled sample injection needle to accommodate sperm orientation and minimise DNA variability (Johnson and Pinkel 1986).
b. Development of a method to stain sperm cells with intact membranes with a vital fluorescent dye just to ensure the maintenance of sperm viability throughout the sorting process (Johnson et al. 1987).
c. Merging the analytical and sorting capacity of the machine for the production of separated populations of living X and Y sperm based on the differential DNA content (it has been demonstrated that there is a difference of 3.6 % DNA content between X- and Y-chromosomes) (Bathgate 2008).
d. Development of a method for re-analysis of sorted X and Y viable sperm populations to verify their purity in the laboratory (Welch and Johnson 1999). Some studies have used in situ hybridisation to check the purity of sex-sorted sperm (Parrilla et al. 2003).

The efficiency of this technology depends on the number of sexed sperm produced per unit of time, on the fertilising ability of the samples after sorting, on the number of spermatozoa required per insemination and on the percentage of piglets born with the expected gender (Martinez et al. 2005).

Among boars, there is a high variability in the identification and sorting of X- or Y-spermatozoa (Vazquez et al. 2009) There are differences in sperm staining, maybe related to the stability of chromatin (Bathgate 2008), which depends on the number of chromatin disulfide bridges, highly variable between species and within the same species (Rodriguez-Martinez et al. 1985). Other factors are also involved in the variability of sex-sorting results in boars (Vazquez et al. 2009).

Due to the fact that sperm sorting requires handling of the sample, it entails a certain degree of physical damage to the sperm. As will be detailed in Chap. 12, AI requires spermatozoa to be deposited as close as possible to the site of fertilisation and also an accurate control of ovulation time to achieve acceptable fertility (Martinez et al. 2005). The deep intrauterine insemination technique (in which 50–70 million spermatozoa are deposited in the anterior third of the uterine horn), combined with a high accurate hormonal control of ovulation is useful to enhance the success in AI with sex-sorted semen (Rath et al. 2003; Vazquez et al. 2003; Grossfeld et al. 2005). Inseminations in swine require a high number of spermatozoa which still represent a limit if considering the speed of sorting (Johnson et al. 2005; Vazquez et al. 2009). Unfortunately, the speed of sorting and the purity of populations obtained are inversely correlated. Both parameters are defined on a graph by sorting windows, which are regions defined to match the cell populations that will be separated (on the basis of their DNA content, for example). The narrower a sorting window is, the more pure the population obtained, and the fewer cells sorted per unit of time. Increasing the size of the sorting window by 15 % (in terms of sperm DNA content) increases the speed of sorting to 20 %, but in contrast, decreases the purity of X-/Y- sperm populations by 10 % (Johnson 1997). Moreover, inseminations with sex-sorted sperm run the risk of inducing alterations on the expression patterns of mRNA blastocysts, as observed in bovine livestock (Morton et al. 2007), and on the distribution of some heat-shock proteins in boar sperm (Spinaci et al. 2006).

Another technique tested for the separation of X- and Y-chromosomes is the use of density gradients. The separation of X- and Y- bearing sperm with this method was first reported in human sperm in 1977 (Shastry et al. 1977) and is based on the theory that: (a) a small fraction of X-sperm is the fastest of all spermatozoa, (b) the next fastest spermatozoa are the Y-sperm, and (c) the slowest are the majority of X-spermatozoa. Despite this, this technique is not completely suitable for gender separation (Upreti et al. 1998).

Other techniques like Sephadex™ gel filtration have been revealed to be unsuccessful in the separation of X- and Y-bearing sperm (Schilling et al. 1978). Nor do washing procedures enrich the population of Y-bearing spermatozoa, as observed in humans (Flaherty and Matthews 1996). There are also some products available on the market that are said to alter the sex-ratio both in fresh and frozen/thawed semen, like PigPlus™ (Emlab Genetics, Arcola, IL). The formulation of this product is under patent and its principle is to make Y-bearing spermatozoa become dormant. Six or eight hours after the addition of PigPlus™ in a dose, sperm bearing the Y-chromosome recover their motility. Up to the present, there are no available data about the effectiveness of this product and only flow cytometry produces a significant enrichment of X- or Y- bearing spermatozoa.

New research is ongoing to improve the efficiency of the flow cytometry procedure and to experiment with new techniques. For example, it has been suggested that it may eventually be possible to use other genetic markers for mammalian sex selection besides the sex chromosomes (Holt et al. 2007).

## 10.5 Conclusion

The development of AI has represented a great improvement in the swine industry, since it has allowed the multiplication of litter production with a sole ejaculate. Another important point to take into account in the development of the AI industry is the formulation of extenders, which increase the longevity of doses and assure the survival of spermatozoa. Currently, there are two major types of semen extenders: short and long term, and their utilisation will depend on the preference of the centres where AI doses are prepared and their final destination. Nowadays, the majority of developed countries are running AI programmes (more than 90 % of pig inseminations in some countries). Collection, manipulation and packaging of semen require high hygienic measures to prevent microbial contamination due to intrinsic or extrinsic factors. Unfortunately, this contamination is not always avoidable, so that it is necessary to take some measures to eliminate or minimise it. As a common principle, antibiotics are added to the extenders under different legislations (depending on the country). However, a large list of bacteria is becoming resistant to them, so it is necessary to continue research based on the development of alternative or complementary methods. The presence of bacteria and viruses in doses destined to AI is a double-edged sword, since not only does semen become a vector of transmission, but sperm quality diminishes. Several methods are available to improve a poor quality sample, namely, column filtration, density gradients and swim-up. Each technique has its advantages and disadvantages and depending on the farmer or on the AI centre, one or other methodology is used. The improvement of seminal doses is a suitable practice before AI and also before sperm cryopreservation. Finally, there is an increasing requirement for sex-sorted doses in the swine industry. To date, the most suitable methodology for sex-sorting in boars is flow cytometry but research is ongoing in order to improve its efficiency and for the development of new methods better adapted to AI requisites.

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