Preselection of sex of offspring in swine for production: current status of the process and its application

Lawrence A. Johnson a,*, Detlef Rath b, Juan M. Vazquez c, William M.C. Maxwell d, John R. Dobrinsky a

aBiotechnology and Germplasm Laboratory, ARS, USDA, Bldg. 200, Barc-East, Beltsville, MD 20705, USA
bDepartment Biotechnology, Institute of Animal Science, Mariensee (FAL), Neustadt, Germany
cDepartment of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Spain
dFaculty of Veterinary Science, University of Sydney, NSW, Australia

Abstract

It is estimated that as many as 30,000 offspring, mostly cattle, have been produced in the past 5 years using AI or some other means of transport with spermatozoa sexed by flow cytometric sperm sorting and DNA as the marker of differentiation. It is well documented that the only marker in sperm that can be effectively used for the separation of X- and Y-chromosome bearing spermatozoa is DNA. The method, as it is currently used worldwide, is commonly known as the Beltsville Sperm Sexing Technology. The method is based on the separation of sperm using flow cytometric sorting to sort fluorescently (Hoechst 33342) labeled sperm based on their relative content of DNA within each population of X- and Y-spermatozoa. Currently, sperm can be produced routinely at a rate of 15 million X- and an equal number of Y-sperm per hour. The technology is being applied in livestock, laboratory animals, and zoo animals; and in humans with a success rate of 90–95% in shifting the sex ratio of offspring. Delivery of sexed sperm to the site of fertilization varies with species. Conventional AI, intrauterine insemination, intra-tubal insemination, IVF with embryo transfer and deep intrauterine insemination are effectively used to obtain pregnancies dependent on species. Although sperm of all species can be sorted with high purity, achieving pregnancies with the low numbers of sperm needed for commercial application remains particularly elusive in swine. Deep intrauterine insemination with 50–100 million sexed boar sperm per AI has given encouragement to the view that insemination with one-fiftieth of the standard insemination number will be sufficient to achieve pregnancies with sexed sperm when specialized catheters are used. Catheter design, volume of
inseminate, number of sexed sperm are areas where further development is needed before routine inseminations with sexed sperm can be conducted in swine. Cryopreservation of sex-sorted sperm has been routinely applied in cattle. Although piglets have been born from frozen sex-sorted boar sperm, freezing and processing protocols in combination with sex-sorted sperm are not yet optimal for routine use. This review will discuss the most recent results and advances in sex-sorting swine sperm with emphasis on what developments must take place for the sexing technology to be applied in commercial practice.

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1. Introduction

The most sought after reproductive technology of modern times is pre-conception sex preselection. The only accurate and potentially cost-effective approach for achieving sex preselection involves separating the X- from the Y-chromosome bearing sperm using flow cytometry and sperm sorting, followed by the use of the sperm for AI or IVF with subsequent embryo transfer (ET). Management schemes for livestock production benefit from sex preselection because of the ability to plan matings for a specific sex. In addition to faster genetic progress from the use of sexed sperm or embryos, there are additional advantages for the management and efficiency for livestock production. Swine production would benefit from sex preselection if it was economically available by allowing for the production of male and female crossbred lines. In some countries, the use of castration will be outlawed and hence alternatives for producing higher numbers of females will need to be found. Female production through sex preselection must be considered as one alternative. As with the utilization of most assisted reproductive technologies (ART), which include IVF and ET, sexed semen for production of livestock with preselected sex is dependent on economics, efficiency and ease of use. These factors are the key to the widespread use of sexed sperm, whether by means of ART or using AI with low numbers of sperm. At the present time, economics and ease of use remain goals to be attained since low-dose AI has not been perfected. The effectiveness and efficiency of the sexing technology are not in question as results have been published worldwide that demonstrate the effectiveness of the sexing process based on sorting sperm with differential DNA content as the X- and Y-sperm marker [1–3].

Since the first report utilizing DNA as a marker for separating X- from Y-chromosome bearing living sperm [4], in which more than 50 rabbits were born of the predicted sex, an estimated 30,000 animals have been born worldwide using this method. Most of the births are from cattle since the technology is used commercially in that species (Cogent Ltd., Chester, UK). Application of sex preselection through separation of sperm by DNA and flow cytometry is not practiced in the swine industry because of the large numbers of sperm that are required to achieve pregnancies. The following references describe the primary application of the Beltsville Sperm Sexing Technology for sex preselection [4,5,7–12]. This paper will be devoted to describing the current application of the Beltsville Sperm Sexing Technology with particular emphasis on swine.
2. Basis for separating X- from Y-chromosome bearing sperm

Early in the 20th century, it was established that the DNA content of X- and Y-chromosome bearing sperm is different in virtually all mammals. The Y-chromosome is smaller and carries less DNA than the larger X-chromosome, while the autosomes carried by X- or Y-bearing sperm are identical in DNA content. A review of the early efforts to differentiate X- and Y-sperm using DNA and flow cytometric analysis and the necessity for orienting aspherical cells during the flow cytometric process has been published earlier [1,6] and will not be repeated here. The basis for routine X- and Y-sperm DNA analysis using commercial instrumentation modified to orient the sperm to the excitation source was demonstrated by Johnson and Pinkel [13]. The routine determination of sperm sex ratios based on the relative DNA content of the X- and Y-sperm populations by analysis and sort reanalysis was a critical advance in ensuring that X- and Y-sperm proportions could be determined in the laboratory rather than waiting for fertility results [14,15].

3. Sorting living sperm for use to produce sexed offspring

The sorting of sperm into separate populations of X- and Y-bearing sperm based on DNA requires a cell sorter. When sperm are to be sorted, however, the cell sorter, which incorporates the analytical aspects of the flow cytometer with sorting capability in normal circumstances, must also incorporate the modification of a forward detector and a means of orienting the living sperm [13]. Cell sorting instrumentation was originally developed in the 1970s. Numerous generational changes in cell sorting instrumentation have taken place over the past 30 years consistent with computer technology advancement. The most advanced systems currently being used for sorting of cells as well as spermatozoa are based on higher pressures and higher event rates and thus higher throughput. A detailed description of standard speed sorters and their use for sperm sorting has been presented earlier [1,6]. The further advance of using high-speed sorters for sperm sorting was described in detail in a previous publication [11]. Although standard speed sorting can be effectively used in research settings, the use of high-speed sorting systems is currently universal in commercial and research applications.

3.1. Modification of cell sorter to make it a sperm sorter

The modification of a cell sorter for sperm sorting [13] consists of a forward fluorescence detector in place of the light scatter detector that is standard in orthogonal configured flow systems and a means of orienting the sperm to the laser beam. This is necessary in order to collect the fluorescent light from both the edge of the sperm (90°) as well as from the flat side (0°) of the sperm. By means of electronic gating, one can collect edge fluorescence and eliminate much of the variability associated with differential fluorescence, thereby resolving the two populations. The second modification as originally designed incorporates a beveled sample injection needle to replace the usual cylindrical sample injection needle common to cell sorters. This bevel at the exit tip of the needle is designed to produce a flat ribbon type sample-sheath stream, which orients the paddle-
shaped heads of the sperm cells to align them within the stream, so that as the spermatozoa pass the laser beam, a high proportion will be facing the beam in the proper plane.

A significant improvement in the needle modification aspect was reported in 1998 by Rens et al. [16]: the tip of the flow cell was remolded to provide 2–3 times the orientation capability of the needle system. An elliptical shape was molded into the interior nozzle tip just above the exit orifice. The orienting tip of the nozzle brings the orienting action much closer to the laser beam detection system, thus increasing the orienting efficiency. All the sperm sorters in use worldwide at the present time have incorporated the orienting tip design [11]. The modified flow cytometer and cell sorter is essential for attaining separate populations of X- and Y-chromosome bearing living sperm [4] on a repeatable basis. The modifications are also essential for reanalyzing sorted sperm for DNA to determine the proportions of X- or Y-sperm in a given sorted sample [15,17]. Differentiation of the amount of DNA present in the X- and Y-chromosome bearing sperm for sorting can be done on virtually all commercial cell sorters that have been manufactured in the past 30 years if they have been modified as described. The only aspect (in addition to improved optics) that is variable is the rate of sperm being sorted and the subsequent sexed sperm throughput.

3.2. High-speed sperm sorting

High-speed cell sorters appropriately modified for sperm sorting as described above have the ability to sort cells at much faster rates and at much higher pressures [22]. Instead of the standard rate of 5000–10,000 events per second that was common in older cell sorting instrumentation, the high-speed system has the capability of more than 30,000 events per second. The desired drop formation frequency at this event rate is 66,000 kHz (at 40 psi). Currently, there are three manufacturers offering high-speed sorters that can be adapted for sperm sorting if modified as described above [11]. They are the MoFlo from Dako-Cytomation Inc., Fort Collins, CO, the Altra from Beckman-Coulter, Miami, FL and the Facs Vantage SE from Becton-Dickinson, Palo Alto, CA. Experience at Beltsville with boar sperm since 1996 has been with the MoFlo. The MoFlo is capable of sorting approximately 15–20 million sperm per hour [11]. Similar results have been obtained with the MoFlo in Mariensee, Germany since 2000 and at the University of Sydney, Australia since 2000. The Altra has been effectively used for boar sperm sorting since 2000 at the University of Murcia, Spain.

3.3. Sperm preparation for sorting

The guiding principle for preparing and sorting sperm, which has been adhered to with living sperm, is to minimize in every way possible the effects of staining, incubation and handling so as to attain the highest viability possible in the collected sorted sperm [4]. The current protocol for swine semen in particular has been refined from the original protocol [5]. Using high-speed sorting requires one to prepare an aliquot containing 100–150 million sperm or 10–15 times the amount needed for the standard sorter. Proportionally the same amount of stain (Hoechst 33342) is added to the sperm. The suspension is then incubated at 32–39 °C (most frequently 35 °C is used) for approximately 1 h. Following
the incubation, the spermatozoa are uniformly stained so as to have minimal variation in the staining intensity within the X- or Y-bearing sperm population. The most critical part of the sorting/separation process depends on uniform staining within and across the sperm population while maintaining resolution of the X- and Y-sperm populations. Detailed protocols for sorting sperm at high speed have been published earlier [11].

3.4. Recovering sorted sperm

As mentioned above, the least insult that the sperm faces while in preparation is critical. This carries through to the collection of the sorted sperm. An environment that will minimize the dilution that accompanies cell sorting is essential. Once the fluorescently stained sperm pass into the flow cell, the stream is surrounded by a sheath fluid of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), which contributes to decreasing the dilution effect. Different sheath fluids that are physiological are also being used with good results. The primary function of the sheath is to provide physiological conditions while giving the optimum salt concentration for proper sorting. Minimizing dilution is accomplished by sorting sperm into a tube containing an egg yolk extender at the outset. Test-yolk (2–20%) has been the most successful for this purpose [4,5,9,11]. The fluid volume in the tube increases as sorting progresses. The sperm fall or are projected into the tube and the motile sperm continue their downward movement into the Test-yolk buffer at the bottom of the tube and remain there in a fairly concentrated environment. Once sorting is terminated, the sperm are centrifuged (300 × g) to concentrate them for insemination in vivo or in vitro, depending on the species. In our experience, sperm sorting is most effective and efficient when done at room temperature.

The use of seminal plasma as a portion of the collection fluid is also a possibility for minimizing the dilution effect. In several experiments, Maxwell et al. [18], and Maxwell and Johnson [19] showed that 10% heterologous seminal plasma as part of the Test-yolk (2%) reduced the percentage of sperm showing the acrosome reaction after sorting. However, additional results using the seminal plasma protocol in combination with IVF in the pig showed that fertilization by sorted sperm was inhibited when the sperm were collected into a Test-yolk (2%) medium containing 10% seminal plasma [19]. Premature capacitation is clearly a problem with sex-sorted sperm. This phenomenon appears to be similar to the capacitation effect that seems to take place during the cryopreservation and thawing of boar sperm, shortening their viable lifespan but improving their in vitro fertilizing capacity.

3.5. Sort reanalysis to determine the sperm sex ratio

At some point during a sort or immediately after the completion of a sort to be used for insemination or IVF, an aliquot of about 50,000 sperm is sorted into a tube without egg yolk extender. Additional Hoechst 33342 is added to maintain staining uniformity and after brief incubation, the sperm are sonicated to remove the tails. The stained spermatozoa are then reanalyzed by flow cytometry but not re-sorted [15]. The proportions of X- and Y-bearing sperm are determined based on the DNA difference and histograms are analyzed by computer fitting to double gaussian peaks [14].
3.6. Fluorescent in situ hybridization (FISH) to determine the sperm sex ratio

The sperm sex ratio can also be assessed with FISH. The fluorescent signal is counted to determine the proportion of Y-chromosome bearing sperm carrying the Y microsatellite DNA probe. In a comparison of FISH and sort reanalysis on sexed boar semen, there was no significant difference between the accuracy of the testing sort purity by FISH or by sort reanalysis [20]. More recently, paints have been produced and used with bull sperm to distinguish the X from the Y population [21]. Two-color direct FISH with porcine chromosome-specific DNA probes prepared by Nick Translation has recently been reported. The ease of the labeling procedure and the quality of the fluorescent signal demonstrate that Nick Translation is an effective method for preparing porcine DNA probes that are then used for monitoring the sorting process for purity [23]. FISH has the advantage that sort purity can be assessed by a process other than flow cytometry. However, several hours are required to conduct a FISH sort purity analysis while sort reanalysis can be done immediately following the sort. Both FISH and sort reanalysis are an accurate reflection of the proportion of X- and Y-sorted spermatozoa.

4. Cryopreservation of sexed boar sperm

Cryopreserved boar sperm has been available on the commercial market since 1975 [24]. However, even though it is used in some commercial production situations [25] and in more specialized genetic transfer opportunities, frozen boar semen cannot be used as efficiently in production situations as is liquid-preserved semen. The magnitude of overcoming sperm membrane damage due to freezing and thawing is increased in sorted sperm due to the dilute concentrations of sperm and the need to cool and freeze dilute samples. The first litters of pigs from sorted boar sperm that had been frozen and thawed were produced at Beltsville in 1999 after intra-tubal insemination. The process of maintaining small numbers of sperm in 0.25 ml straws and avoiding warming of the straw during transfer was one of the major difficulties encountered [26,27]. A total of four litters were born with an average litter size of 6.8. A control sow farrowed 12 pigs (Table 1).

A recent report by Centurión et al. [28] demonstrates the benefit of spermadhesins for stabilizing membranes and improving the viability of sorted sperm. These findings may be useful in preserving sorted sperm by freezing. Cryopreservation of sorted sperm along with low-dose insemination techniques are the two most critical areas that will benefit from increased research emphasis seeking to achieve greater utilization of sexed semen.

<table>
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<th>Gilt no.</th>
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<th>Born alive</th>
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<td>Control-frozen</td>
<td>12</td>
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Table 1
Farrowing results from gilts surgically inseminated with frozen sorted boar spermatozoa [27]
5. Insemination of sex-sorted sperm and the advent of special insemination catheters

At present, it is clear that intrauterine insemination offers an opportunity for applying the current sexing technology to swine. Conventional AI in the pig requires about two to three billion spermatozoa per AI dose of semen, which is far above the capability of sorted sperm production. Intra-tubal insemination is effective in the pig for producing sexed offspring [5]. However, it is not likely to be used in general production systems as they are currently designed. Cattle, on the other hand, lend themselves to deep intrauterine insemination [2,10] where pregnancies can be obtained using as little as 200,000 sperm per insemination with deep intrauterine AI to 2 million sperm for insemination into the body of the uterus. Insemination into the body of the uterus is being used for most sexed sperm inseminations undertaken in commercial cattle production at the present time.

Krueger et al. [29], and Krueger and Rath [30] demonstrated that 50 million sperm were sufficient to obtain fertilization if the sperm were placed near to the utero-tubal junction surgically. In 1999, the University of Murcia, Spain group led by E. Martinez presented their extensive work on developing a catheter that would reach deep into the uterus of the pig. They produced the specialized fiberoptic endoscope that could penetrate the cervix and come within about 25 cm of the utero-tubal junction in non-sedated sows [31,32]. This was followed by a development of a simplified technology that could be used without an endoscope and was quite successful in producing pregnancies [33]. During 2002, the technology was refined to increase its usefulness on a practical scale with the development of a disposable deep intrauterine catheter (Firflex™ catheter; Magapor, Spain). Results have been shown to be applicable to frozen semen [34]. Bathgate et al. [35] reported pregnancy rates of 63% in 45 and 43% in 42 sows after deep intrauterine insemination with 250 million fresh and frozen-thawed spermatozoa, respectively. The catheter has also been used for sorted but unsexed sperm [36,37]. Controlling the time of AI in relation to ovulation, as well as the depth of insemination are critical to success. In this regard, Martinez et al. [33] have shown that in sows hormonally treated with eCG and hCG, pregnancy and farrowing rates did not differ from that in controls (3 billion spermatozoa inseminated in the cervix) when 150 or 50 million of spermatozoa were deeply inseminated. However, sows inseminated with 50 million spermatozoa showed a tendency toward lower pregnancy and farrowing rates. When sorted but unsexed spermatozoa were inseminated instead of fresh spermatozoa, a significant decrease in farrowing rate was observed (39% versus 79% with 70 million spermatozoa inseminated and 46% versus 88% with 140 million spermatozoa inseminated). Litter size showed a tendency to be lower after inseminations with sperm that had passed the sorter but were unsexed.

More recently, Rath et al. [38] reported the birth of the first piglets produced from flow cytometrically sexed semen, employing 50 million sperm for deep intrauterine AI. Further results that will be presented at this conference show an average pregnancy rate of 33% (4/12) and an average litter size of 7.2 piglets. Unsorted controls inseminated with 50 million sperm from the same ejaculates had 21% higher pregnancy rates.

Several suppliers of swine AI equipment market catheters that are designed to penetrate through the cervix and deposit semen about 10 cm into one horn or the other. A variation of this type of catheter was made and used by Johnson et al. [26,27] when they reported
pregnancies with 50–400 million unsorted sperm. However, attempts to use this deep insemination catheter, which incorporates the Melrose catheter as the sheath for locking into the cervix, for sexed sperm were unsuccessful. It is clear that more work on this particular area of semen transport by deep insemination would be beneficial to the commercial application of sexed semen and to standard AI also.

6. Sexed offspring production using both standard and high-speed sperm sorters

Studies to validate the efficacy of the Beltsville Sperm Sexing Technology were initially conducted in rabbits and swine with standard speed sperm sorters. The sex ratios of the litters produced from females surgically inseminated with sorted X- or Y-sperm confirmed the sperm sex ratio results determined by reanalyzing aliquots of sorted X- and Y-sperm populations for DNA [4,5]. A summary of the results obtained in these studies was presented at the IVth International Conference on Boar Semen Preservation [27].

New and improved nozzle technology [11] that increases the percentage of oriented sperm and thus increases the number of sexed sperm that can be produced per unit of time has significantly advanced our ability to provide sexed semen to the producer. High-speed sperm sorting using this special orienting nozzle [16] in place of, or in combination with a beveled needle is used to routinely sort bull or boar semen with production rates of 15 million X- and 15 million Y-sperm in an hours time [11].

The effectiveness of high-speed sorting in combination with IVF was demonstrated in collaborative studies between USDA, ARS and University of Missouri. These studies involved sorting the sperm at Beltsville and shipping to Missouri to be used in IVF while another portion was used for fertilizing ova at Beltsville [27]. These studies showed that healthy offspring in significant numbers (17 litters) could be produced with sort purities of 97% and phenotypic sex of 97% females [39,40]. These two studies clearly demonstrate the efficacy of high-speed sperm sorting in conjunction with a special orienting nozzle to orient a greater percentage of sperm from each sample than could be done with the standard nozzle and standard speed sorting.

In addition to IVF, the use of ICSI has also been successful in pigs. In a study reported by Probst and Rath [41], 256 injected (sorted Y-sperm) oocytes were transferred to four previously synchronized gilts. All four produced litters, averaging 3.3 pigs per litter. Utilizing assisted reproduction techniques in combination with sorted sperm is another way of effectively producing sexed offspring in specialized situations.

7. Concluding remarks

At the present time, the only means of farrowing pre-sexed offspring in the pig is by using the Beltsville method of sperm sorting, which is based on using a DNA marker to differentiate X- from Y-sperm. Through the use of intra-tubal insemination or IVF with sexed sperm one can obtain litters that are 90–100% of one sex or the other. Utilization of sexed sperm offers the swine industry a wide variety of options for producing swine more efficiently. Future advances incorporating some form of AI with deep intrauterine catheters
and using sexed sperm will make sex preselection available to a wide spectrum of the swine industry. The full potential of sexed boar sperm for improving the reproductive efficiency of swine will never be realized without progress in the low-dose AI area and the ability to improve the sorting process to produce sexed spermatozoa at a much faster rate than is currently available. However, sex-sorted boar sperm can be used effectively even the current rates of sexed sperm production in specialized production–management situations.

References


