PRECISION AND ACCURACY OF VARIOUS COUNTING METHODS

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INTRODUCTION

In the swine industry, there is an increasing demand for extended semen samples containing a defined number of sperm cells with a given quality. Accurate and precise concentration determination of boar semen samples is of major importance to increase the amount of straws per ejaculate. Various methods are used to determine the number of sperm cells: manual or computer-assisted counting, opacity measurement, spectrophotometric technique, coulter counting, and flow cytometry. Each method has its own advantages and disadvantages regarding precision, accuracy, rapidity, costs, and ease of conduct.
The Institute for Pig Genetics (Beuningen, The Netherlands) wanted to compare various methods for the determination of the concentration of a semen sample. Routinely, opacity measurements using a colorimeter or a spectrophotometer are used. These measurements are compared to computer-assisted counting with the Hamilton Thorne IVOS, and manual counting using a Leja 4-chamber slide and a haemocytometer (BürkerTürk). Flow cytometry (FACS) measurements were included as an independent concentration reference.
The experimental setup of this experiment allows comparing the average values of the various methods (accuracy). Furthermore, the variation per method is given by comparing the standard deviations and ranges (precision).

EXPERIMENTAL SETUP

The concentration of one semen sample of excellent quality was determined 10 times using the colorimeter, according to standard procedures (Varkens K.I. Noord Brabant).
The concentration of the sample appeared to be 146.4 M/ml.

This sample was diluted to: Concentration A (2.44x, supposed to be ± 60 M/ml)
Concentration B (from A, supposed to be ± 40 M/ml)
Concentration C (from B, supposed to be ± 20 M/ml)

The concentration of dilution A was determined with the colorimeter again, and using the ACCUCEL (spectrophotometer) method. A concentration of ± 60 M/ml is out of range, and therefore 2.44x the normal amount was added to the colorimeter assay (730 µl semen in 6770 µl Na-citrate, instead of the normal amounts: 300 µl semen in 7200 µl Na-citrate), and 5x the normal

amount to the ACCUCEL assay (500 µl semen in 2000 µl Na-citrate, instead of the normal amounts: 100 µl semen in 2400 µl Na-citrate). Both assays were done 10 times.

The concentration of dilutions A, B, and C was then determined using:

1. Leja 4-chamber slide (batch 020422, average height 19.8 µm) in combination with the IVOS (standard setting as installed by Hamilton Thorne, with the exception that besides 45 frames acquired also 10 frames were used). 10 independent measurements were done.
2. Leja 4-chamber slide in combination with a grid (10x10=100). The samples were frozen for about 15 minutes to immobilize the sperm. For concentration A: 50 squares were counted, for concentration B: 100, and for concentration C: 200. The 3 samples were each counted 10 times.
3. Haemocytometer (BürkerTürk). First, the samples were diluted in saturated $NaHCO_3$ to be able to count a similar number of cells in a 1 mm^2 square. Sample A was diluted 40x, B: 25x and C:12.5x. The dilutions were done 10 separate times and part of the dilutions were subsequently pooled to eliminate dilution inaccuracies. 10 independent countings of 1 mm^2 squares (0.1 µl) were performed on the pooled dilutions.
4. The same pooled dilutions as used for the haemocytometer countings were used for the FACS. For details on the FACS experiment see: Accurate boar sperm counting using a disposable non-toxic counting chamber, by Alike van der Velden. Measurements were done 5 times.

RESULTS AND DISCUSSION

Initial measurements on Concentration A

The concentration of the starting material was 146.4 M/ml on average, as was determined by the colorimeter. The minimum and maximum values of the 10 measurements were 137 and 151 M/ml, and the SD was 5.3 M/ml.

After diluting this sample to concentration A (supposed to be 60 M/ml), it was tried to use the colorimeter assay and the spectrophotometer to determine the exact concentration of dilution A. As 60 M/ml is not in the linear range of both measurements, more sperm was added to the assays. The colorimeter gave an average concentration of 63 M/ml (SD = 1.3 M/ml) and the ACCUCEL assay resulted in 60.7 M/ml (SD = 0.8 M/ml).

Comparison of the concentrations of A, B, and C, as determined by the IVOS, Leja slide, haemocytometer, and the FACS

Boar sperm was diluted to concentrations A, B, and C, and the precise concentrations were determined as described in the Experimental Setup. The results are depicted in Table 1 and Figure 1, showing the average concentrations, minimum/maximum values, the ranges, and standard deviations (SD).

To date, measurements using the FACS were not successful as the derived concentrations are much lower as compared to the other three methods. This issue is still under investigation.

The results show that the remaining three methods of concentration determination yield very similar results, especially when the SDs are taken into account. The results of these methods are however much lower than was predicted by the colorimeter and spectrophotometer; the results of these two are at least 14% higher than of the counting methods. This result has been described before and was attributed to the interference of fat droplets, somatic cells, debris and bacteria.

In this experiment, the average concentrations as determined by the IVOS are about 10% lower than counting by eye using the Leja slide and the haemocytometer (BürkerTürk). It has yet to be determined whether this difference is significant. According to the WHO, the Improved Neubauer haemocytometer is the standard for counting human sperm. In our previous studies, with human as well as with boar sperm, we used the Improved Neubauer instead of the BürkerTürk, and found smaller differences between CEROS and haemocytometer. At higher concentrations, the IVOS values are different for 10 and 45 acquired frames. Changing the frames setting to 10 reduces the negative influence of collisions. Indeed, with 10 frames instead of 45 the result is closer to the other values.

The SDs and ranges belonging to the IVOS outcome are smaller than belonging to the manual counting methods. At least part of this difference can be explained by the larger number of sperm cells analyzed by the computer (Table 1). Counting more sperm cells improves the reliability of the result by reducing Poisson distribution-related errors. The large SDs of the haemocytometer counting can be explained by the inclusion of two extra potential inaccuracies, the dilution and assembly steps. In conclusion, the IVOS system with the Leja slide provide a higher precision than the other counting methods.

It appeared that the FACS method is also prone to errors, and therefore we cannot answer the 'accuracy question': which method gives the real sperm concentration? Answering this question is difficult as the various methods have inherent errors, and are also differently influenced by external factors like the quality of the semen sample, dilution/sampling inaccuracies, experience of the lab technician, haemocytomer brand etc. To fully answer the question more data are needed from various laboratories. Once the absolute standard is defined, the IVOS system can be calibrated by adjusting the settings or algorithm.

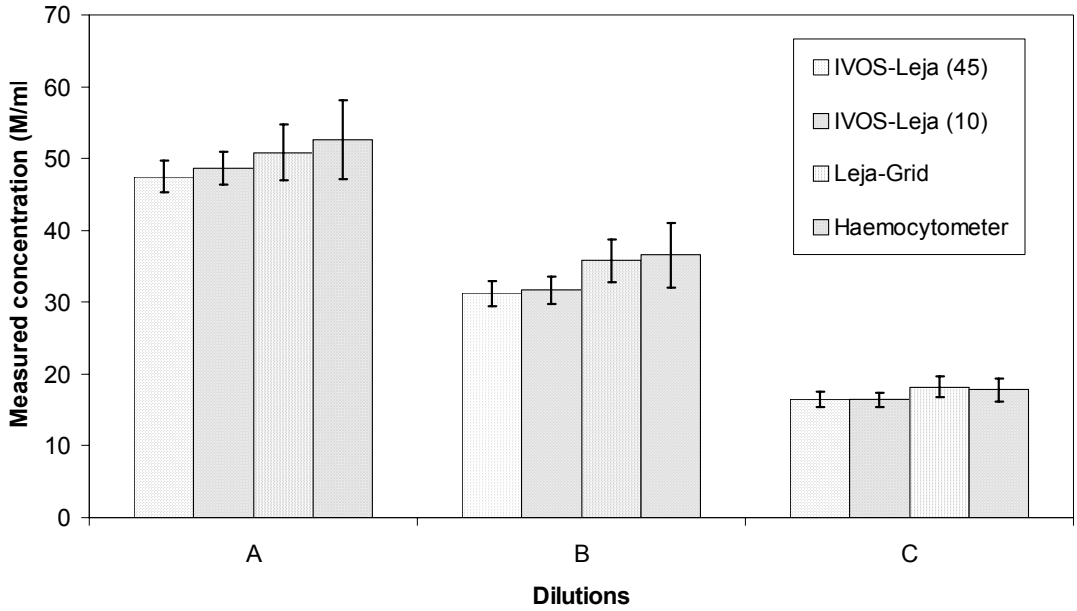

Figure 1: Boar sperm concentration measurements using the IVOS, Leja slide, haemocytometer and the FACS.

The precise concentrations of three dilutions of boar sperm were determined as described in the experimental setup. The mean values and standard deviations are shown. Data are given in Table 1.

Table 1: Concentration determination of 3 dilutions of a boar sperm sample.
The average concentrations, minimum, maximum values, ranges, and standard deviations are in M/ml. The average number of counted cells per measurement is given in the first column.

Concentration A:	*counted cells*	*average con*	*min-max (range)*	*SD*
IVOS + Leja chamber (45)	769	47.5	44-50 (6)	2.2
(10)	784	48.7	44-50 (6)	2.3
Leja-chamber + grid	126	50.8	46.1-56.6 (10.5)	3.9
Haemocytometer	132	52.6	45.2-63.6 (18.4)	5.5
FACS	15.000 events	42.1		1.5
Concentration B:	*counted cells*	*average con*	*min-max (range)*	*SD*
IVOS + Leja chamber (45)	505	31.2	29-34 (5)	1.8
(10)	512	31.7	28-34 (6)	1.9
Leja-chamber + grid	177	35.8	30.1-40.6 (10.5)	3.0
Haemocytometer	150	36.6	30.0-41.7 (11.7)	4.5
FACS	15.000 events	28.8		1.4
Concentration C:	*counted cells*	*average con*	*min-max (range)*	*SD*
IVOS + Leja chamber (45)	269	16.5	15-18 (3)	1.1
(10)	262	16.4	14-17 (3)	1.0
Leja-chamber + grid	180	18.2	16.4-20.7 (4.3)	1.5
Haemocytometer	142	17.8	16.6-21.8 (5.2)	1.6
FACS	15.000 events	13.4		0.2