

## Comparison of five different methods to assess the concentration of boar semen

### *Vergelijking van vijf verschillende methoden voor de bepaling van de concentratie van varkenssperma*

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#### ABSTRACT

Both for research and practical purposes, accurate and repeatable methods are required to assess the concentration of boar semen samples. Since the method which is used may influence the results considerably, the aim of the present study was to compare 5 frequently used techniques to determine boar semen concentration. Fifty ejaculates were collected from 37 different boars at an artificial insemination centre. Subsequently, each ejaculate was analyzed for sperm concentration by means of 2 different types of colorimeters (Colorimeter 1: Model 252, Sherwood Scientific Ltd, Cambridge, UK ; Colorimeter 2: Ciba-Corning, Schippers, Bladel, The Netherlands), the Bürker counting chamber (golden standard), and the Hamilton Thorne Analyzer (Ceros 12.1) using 2 types of Leja chambers (the ‘former’ and the ‘recently developed’). Each ejaculate was assessed 5 times with each of the 5 methods, and the repeatability, expressed by coefficient of variation (CV), was determined for each method. The different methods were compared using Pearson’s correlations and limits of agreement. The colorimeters yielded the lowest CV’s (both 3.7%), while the former Leja chamber resulted in the highest CV (12.4%). Moreover, significant ( $P < 0.01$ ) and high correlations ( $r > 0.71$ ) were found between the results obtained by the different methods. The limits of agreement plots showed that none of the methods consistently over- or underestimated the sperm concentrations when compared to the Bürker chamber, although there was a tendency toward higher over- or underestimation in highly concentrated sperm samples. Based on our results, there were no major differences in the assessment of sperm concentration between the evaluated methods. The choice of method used in a laboratory could therefore be based on factors such as cost, number of samples to be assessed and practical use, without thereby negatively affecting the validity of the results thus obtained.

#### SAMENVATTING

Accurate en herhaalbare methoden voor de concentratiebepaling van varkenssperma zijn belangrijk zowel voor onderzoek- als praktijkdoeleinden. Omdat de resultaten afhankelijk kunnen zijn van de methode die wordt gebruikt, was de doelstelling van dit onderzoek 5 frequent gebruikte methoden voor de concentratiebepaling van varkenssperma te vergelijken. Hiertoe werden 50 ejaculaten van 37 verschillende beren in een KI-station onderzocht. Van elk ejaculaat werd de concentratie bepaald door middel van 2 verschillende types colorimeter (colorimeter 1: Model 252, Sherwood Scientific Ltd, Cambridge, UK ; Colorimeter 2: Ciba-Corning, Schippers, Bladel, The Netherlands), de Bürker telkamer (gouden standaard), en de Hamilton Thorne Analyzer (HTR Ceros 12.1; Hamilton-Thorne Research, Beverly, CA, USA) waarbij 2 types Lejakamers werden gebruikt (de ‘oude’ en de ‘recentelijk ontwikkelde’ Lejakamer). Elk ejaculaat werd 5 keer onderzocht met elk van de 5 methoden en de herhaalbaarheid, uitgedrukt door middel van een variatiecoëfficiënt (VC), werd bepaald voor elke methode. De overeenstemming tussen de verschillende methoden werd onderzocht door middel van de Pearsons correlatie en “limits of agreement”. De colorimeters hadden de laagste VC (beide 3,7%) terwijl de oude Lejakamer de hoogste VC had (12,4%). Significante ( $P < 0,01$ ) en hoge correlaties ( $r > 0,71$ ) werden gevonden tussen de resultaten van de verschillende methoden. De “limits of agreement” grafieken toonden aan dat geen enkele methode de concentratie steeds over- of onderschatte in vergelijking met de Bürkertelkamer alhoewel er een tendens was naar meer over- of onderschatting bij hogere spermaconcentraties.

Als conclusie kan gesteld worden dat er tussen de verschillende onderzochte methoden geen grote verschillen zijn in de concentratiebepaling. De keuze van een bepaalde methode wordt daarom vooral bepaald door de kostprijs, het aantal te onderzoeken monsters en de praktische uitvoering.

## INTRODUCTION

Most andrology centers and laboratories routinely evaluate the main sperm parameters (i.e. concentration, motility and morphology) using light microscopic techniques (Rijsselaere *et al.*, 2005). The determination of the sperm concentration is one of the most important parameters for the assessment of a sperm sample, especially in pigs. Accurate assessment of the sperm concentration and consequently of the total number of spermatozoa in an ejaculate has major economic implications in porcine artificial insemination (AI) centers, since this factor determines the number of doses which can be obtained from the ejaculate of a single boar. Moreover, AI centers are inclined to dilute the ejaculates as much as possible to maximize the semen dose production (Vyt *et al.*, 2007).

In most laboratories, the sperm concentration is routinely performed by the use of hemocytometry (such as the Bürker, Thoma and Makler counting chambers), which is often considered to be the 'gold standard' (Rijsselaere *et al.*, 2003; Prathalingam *et al.*, 2006). However, this method is rather time consuming, since it requires the loading of a sperm sample onto a grid and the counting of a relatively high number of immobilized spermatozoa in order to achieve an acceptable level of precision (World Health Organization 1999; Christensen *et al.*, 2005). Moreover, the different types of chambers and the evaluation of the same sample by different observers may lead to variable results (Christensen *et al.*, 2005). Therefore, the results obtained with this method largely depend on the level of training and skills of the investigator (Knuth *et al.*, 1989), which frequently leads to a lack in agreement between different laboratories examining the same specimens (Davis and Katz, 1993). Moreover, with this conventional microscopic evaluation, the subjectivity of the analysis makes comparison of results difficult (Vyt *et al.*, 2004).

To overcome subjectivity and variability in sperm concentration assessment, several novel techniques have been proposed (Rijsselaere *et al.*, 2005; Prathalingam *et al.*, 2006). During the last decade, commercial AI centers have integrated automated systems such as colorimeters or computer assisted sperm analysis (CASA) to determine the sperm concentration (Vyt *et al.*, 2004). In Belgium, a recent study showed that colorimetry is currently the most frequently used method to determine the sperm concentration in commercial pig AI centers (Vyt *et al.*, 2007).

CASA systems were first proposed by Dott and Foster (1979) approximately 30 years ago. Nowadays, these systems are commonly used in andrology, in university research laboratories and in AI centers. These devices have been introduced in the laboratory routine mainly to improve the accuracy of data collection, to avoid errors due to subjective evaluation of different technicians and to save time in the evaluation procedure (Johnson *et al.*, 1996). In the laboratory of the first author, the Hamilton-Thorne computer-aided semen analyzer (HTR Ceros 12.1; Hamilton-Thorne

Research, Beverly, CA, USA) is routinely used for the assessment of canine (Rijsselaere *et al.*, 2003; Rijsselaere *et al.*, 2007), feline (Filliers *et al.*, 2008), bovine (Hoflack *et al.*, 2007) and porcine (Vyt *et al.*, 2004) semen. When this system was used with dogs, however, the measurements for sperm concentration were consistently lower (on average 14.8 %) in comparison with the values obtained by the Bürker chamber (Rijsselaere *et al.*, 2003). In pigs, as well, sperm concentrations with the HTR Ceros 12.1 were lower (-32%) compared to the values obtained with the Bürker chamber (Vyt *et al.*, 2004).

The aim of the present study was to compare different techniques that can be used in a commercial AI center for the assessment of the sperm concentration in pigs: a conventional technique based on the use of a Bürker counting chamber as the gold standard, two types of colorimeters and a computerized method: the HTR Ceros 12.1. The computerized measurements were performed using two types of Leja counting chambers: the 'former' and the 'recently developed' one. The results obtained from HTR Ceros 12.1 were subsequently compared with those obtained with the Bürker counting chamber and from the colorimeters.

## MATERIALS AND METHODS

### Semen samples

A total of 50 ejaculates were collected from 37 different Piétrain boars using the gloved hand technique (Shiple 1999). The animals varied in age from 15 months to 3 years and they were housed in individual pens (sawdust as bedding material) at the same AI center (Hypor NV, Olsesne, Belgium). All ejaculates were collected in the same period of the year (i.e. between March and April) using standard procedures. Semen from approximately five boars per day was tested. A disposable gauze (Bastos Viegas, Penofiel Portugal) was used at collection to eliminate the gel fraction in the semen. Prior to processing in the laboratory, the semen was filtered using a milk filter sleeve (Eurofarm, Halle Germany) to eliminate possible remnants of sawdust particles. No fertility problems were recorded for the boars included in the study.

### Assessment of semen concentration

#### Colorimeter

Immediately after collection and filtration, a 1:1 dilution was made and the sperm concentration of the ejaculate was assessed five times using two types of colorimeters (Colorimeter 1: Model 252, Sherwood Scientific Ltd, Cambridge, UK ; Colorimeter 2: Ciba-Corning, Schippers, Bladel, The Netherlands) at the AI center. Both colorimeters were calibrated approximately twice per year at the AI center using the Bürker counting chamber.

Subsequently, 10 mL of each semen sample was transported at 17°C in isotherm boxes to the sperm la-

boratory at the Faculty of Veterinary Medicine (Ghent University, Belgium). There, approximately 1.5 hours after collection, the concentration was determined by means of the Bürker counting chamber and the Hamilton Thorne Analyzer (Ceros 12.1) using two types of Leja chambers. This short time interval between collection of semen and evaluation did not influence the results for concentration since spermatozoa are immobilized with water for the assessment with the Bürker Counting Chamber and the Hamilton Thorne Analyzer (Ceros 12.1) takes both motile and immotile spermatozoa into account for the determination of the sperm concentration (Rijsselaere *et al.*, 2003).

#### *Bürker Counting Chamber*

The sperm concentration of the semen was determined using a Bürker counting chamber (Merck, Leuven, Belgium) after a 40 times dilution with tap water (10 µl semen + 390 µl water). The measurements were repeated 5 times and were conducted by the same person. The numbers of spermatozoa in 40 different fields within the Bürker counting chamber were counted.

#### *Use of the Hamilton-Thorne analyzer*

The Hamilton-Thorne computer-aided semen analyzer (version 12.1 Ceros; Hamilton-Thorne Research, Beverly, USA), a previously validated CASA system, was used to evaluate the sperm concentration (Vyt *et al.*, 2004). The software settings for the HTR Ceros 12.1 were those recommended by the manufacturer for

analysis of boar sperm, namely: frames per second (Hz) 60, number of frames 45, minimum contrast 18, minimum cell size (pix) 7, cell size (pix) 9, cell intensity 125, slow-static cells with average path velocity (VAP) cut-off (µm/s) 20 and straight-line velocity (VSL) cut-off (µm/s) 5, minimum static intensity gates 0.5, maximum static intensity gates 2.5, minimum static size gates 0.65, maximum static size gates 2.6, minimum elongation gates 20 and maximum elongation gates 85.

Based on the mean sperm concentration obtained by the Bürker Counting Chamber, each ejaculate was diluted with physiological saline solution to  $50 \times 10^6$  spermatozoa/mL. Subsequently, 7.5 mL of diluted semen was mounted on each of 2 disposable Leja counting chambers (depth 20 µm) (Orange Medical, Brussels, Belgium, Art n° SC-20-01-C): a 'former' and an 'improved, recently developed' chamber which was claimed to correct for the Segre-Silberg effect.

To assess sperm concentration ( $\times 10^6$ /mL), five randomly selected microscopic fields were investigated 5 times each by the same person. Using this procedure, at least 1000 spermatozoa were analyzed individually. The mean of the 5 scans for each microscopic field was used for the statistical analysis. The sample/diluent ratio was computed to recalculate the original sperm concentration.

#### **Statistical analysis**

Throughout the study, the results were presented as means and the variation was expressed as standard de-

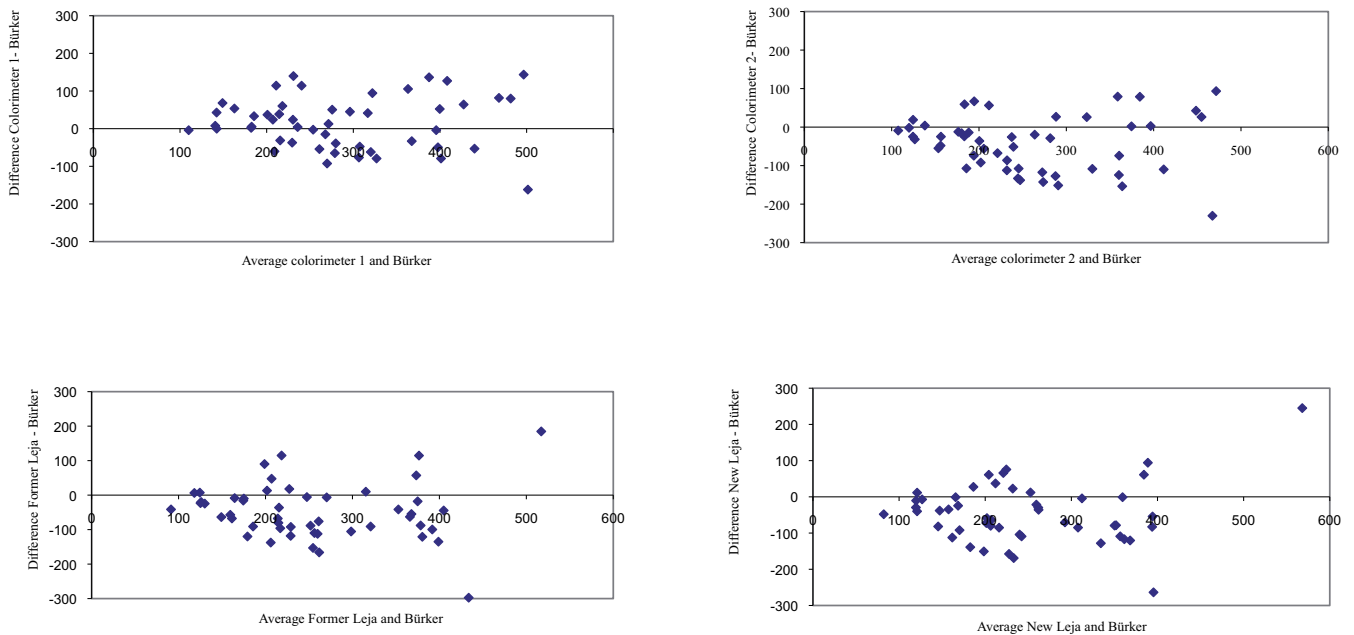
**Table 1. Mean, minimum and maximum sperm concentrations, coefficient of variation and mean over- or underestimation (compared to the Bürker chamber) of the 2 colorimeters, the Bürker counting chamber and the 2 types of Leja chambers (n =50 pig semen samples; each sample was analyzed 5 times).**

	Colorimeter 1	Colorimeter 2	Bürker Chamber	Leja chamber (former)	Leja chamber (recently developed)
Mean sperm concentration ( $\times 10^6$ /ml)	294.1	236.0	279.0	231.3	233.5
Minimum sperm concentration ( $\times 10^6$ /ml)	108.0	103.0	112.0	70.6	64.0
Maximum sperm concentration ( $\times 10^6$ /ml)	568.4	518.3	582.4	609.8	669.9
Coefficient of variation (%)	3.7	3.7	11.1	12.4	11.0
Mean % over- or underestimation in comparison with Bürker	9.8	-13.4	0 <sup>a</sup>	-14.7	-14.3

<sup>a</sup> Bürker was considered to be the golden standard.

**Table 2. Pearson correlations between the five different methods for sperm concentration evaluated in this study (n=50 pig semen samples; each sample was analyzed 5 times). All correlations were statistically significant (P<0.01).**

	Colorimeter 1	Colorimeter 2	Bürker Chamber	Leja chamber (former)	Leja chamber (recently developed)
Colorimeter 1	1				
Colorimeter 2	0.992	1			
Bürker	0.796	0.771	1		
Leja (former)	0.900	0.896	0.711	1	
Leja (recently developed)	0.875	0.872	0.712	0.966	1



**Figure 1.** Plots of agreement between the Bürker chamber and colorimeter 1 (Figure 1A), colorimeter 2 (Figure 1B), the former Leja chamber (Figure 1C) and the recently developed Leja chamber (Figure 1D). The difference in concentration measured by the Bürker chamber and each of the 4 other methods is plotted against their average ( $\times 10^6/\text{ml}$ ) (with --- : 2 times the SD of the difference between the methods).

viation (SD) or minimum and maximum. Coefficients of variation were calculated by dividing the SD by the mean. The different methods for the assessment of boar sperm concentration were compared using Pearson's correlations and limits of agreement (Bland and Altman 1986). Statistical analyses were performed with procedures available in SPSS 15.0 (SPSS Inc. Headquarters, Chicago, Illinois, US). Values were considered statistically significant when  $P < 0.05$  (two-sided test).

## RESULTS

The mean, the minimum and maximum sperm concentrations, and the coefficient of variation of the 2 colorimeters, the Bürker counting chamber and the 2 types of Leja chambers are summarized in Table 1. The mean over- or underestimation of the 2 colorimeters and the 2 types of Leja chambers compared to the Bürker chamber are also presented in Table 1. The mean sperm concentration obtained by the Bürker chamber ranged from 112 to 584  $\times 10^6$  spermatozoa/ml (Table 1).

The 2 different types of colorimeters yielded the lowest mean of CV (3.7%), while the Bürker chamber and the 'former' and the 'recently developed' Leja chambers showed a mean CV of 11.1%, 12.4% and 11.0%, respectively. Compared to the Bürker chamber, the 'former' and the 'recently developed' Leja chambers underestimated the sperm concentration on average by 14.7 and 14.2%, respectively. Colorimeter 1 overestimated the sperm concentration on average by 9.8%, while colorimeter 2 underestimated the sperm concentration by 13.4% compared to the Bürker chamber.

In all cases, significant results ( $P < 0.01$ ) and high correlations ( $r > 0.71$ ) were found between the results obtained by the different methods (Table 2). In Figure 1, the averages of each method and the Bürker chamber were plotted against their difference by means of scatter diagrams. For the range of sperm concentrations, none of the methods consistently over- or underestimated the sperm concentration when compared to the Bürker chamber. However, there was a tendency for a higher over- or underestimation in highly concentrated sperm samples since several measurements were not in the interval of 2 x SD (Figure 1).

## DISCUSSION

The present study compared several commonly used techniques for the assessment of porcine semen, namely the Bürker counting chamber (gold standard) and the Hamilton-Thorne Semen Analyser (HTR Ceros 12.1) using two different types of Leja chambers and two types of colorimeters. Our study showed that (1) significant and high correlations were found between the different methods, (2) the HTR 12.1 Ceros and colorimeter 2 underestimated the sperm concentration in comparison with the Bürker Chamber, and (3) no differences in sperm concentration could be elucidated between the two types of Leja chamber.

As the mean sperm concentration obtained by the Bürker chamber ranged from 112.0 to 584.4  $\times 10^6$  spermatozoa/mL, a sufficiently wide range of sperm concentrations was covered in this study. Significant and positive correlations were found between the different methods (Table 2), indicating a good agreement between the evaluated techniques. However, the HTR Ceros 12.1 measurements for sperm concentration

were on average 14.7 % (former Leja Chamber) and 14.3% (recently developed Leja Chamber) lower in comparison with the values obtained by the Bürker chamber. The underestimation of the sperm concentration in the present study was in agreement with findings in previous studies in dogs (Rijsselaere *et al.*, 2003) and pigs (Vyt *et al.*, 2004) using the HTR Ceros 12.1 with the former Leja Chamber. The discrepancy may be due to a number of factors. Firstly, any method that is used to determine the sperm concentration, taking an aliquot from the original sample, is only an estimation of the true sperm concentration (Coetzee and Menkveld 2001). Even the hemocytometer method recommended by the World Health Organization (WHO) has been criticized because several factors may interfere with sperm counting such as type of pipette, dilution, calculation errors and semen viscosity (Johnson *et al.*, 1996; Mahmoud *et al.*, 1997). Another possible factor is the presence of clumped spermatozoa, which may be observed in clinical material. These spermatozoa are either digitized as a single image or, being too large for a sperm head according to the pixel size range set in the system parameters, rejected from the analysis (Mortimer *et al.*, 1988; Chan *et al.*, 1989), consequently causing a reduction in the sperm concentration. The larger underestimation at higher sperm concentrations is probably due to the increased likelihood of clumping in the more concentrated sperm samples (Mortimer *et al.*, 1988). Other reported explanations for the underestimation of sperm concentration by the CASA include the small variance (18-20 mm) in chamber height (Coetzee and Menkveld 2001) and the overestimation of sperm concentration mentioned for the Bürker chamber when compared with other counting chambers such as the Cellvision and Makler Counting Chamber (Mahmoud *et al.*, 1997). Moreover, an additional dilution of 40x with subsequent change of medium (i.e. water) and the rather low number of cells counted in the Bürker chamber presumably makes hemocytometry more sensitive to occasional variations compared to the HTR Ceros 12.1, which determines the concentration directly on the diluted semen and which evaluates several thousands of sperm cells (Vyt *et al.*, 2004). The underestimation of the sperm concentration in the CASA system corroborated with the estimations of previous authors (Knuth and Nieschlag 1988; Mortimer *et al.*, 1988; Rijsselaere *et al.*, 2003; Vyt *et al.*, 2004), but was in contrast with others who found markedly higher sperm concentrations using a CASA system (Vantman *et al.*, 1988; Chan *et al.*, 1989; Neuwinger *et al.*, 1990).

Douglas-Hamilton *et al.*, (2005a) explained the lower sperm concentration in terms of the physical properties of the Leja counting chamber used by CASA, stating that particles in solution tend to move at a higher velocity and have the tendency to accumulate at the meniscus when the fluid enters a low depth chamber by capillary force. This phenomenon, referred to as the Segre-Silberberg effect, is due to the decreasing velocity of the fluid near the wall, which results in a high transverse velocity gradient (Douglas-

Hamilton *et al.*, 2005a). Consequently, a relatively lower concentration of cells is counted when evaluating the center of the microscopic field, which results in an underestimation of sperm concentration by CASA. Since multi-usable chambers such as the Bürker counting chamber have a depth of 100 µm, they are less likely to be susceptible to the Segre-Silberberg effect in laminar flow due to their lower transverse velocity gradient (Douglas-Hamilton *et al.*, 2005b). However, the recently developed Leja chamber used in this study was claimed to correct for the Segre-Silberberg effect but, based on our findings, no major differences were found in the assessment of the sperm concentrations between the former and the more recently developed Leja counting chambers, at least not for porcine semen.

Our study additionally showed that the colorimeters yielded the lowest CV's (both 3.7%), while the former Leja chamber had the highest CV (12.4%), which means that the two colorimeters yield the most repeatable results of the evaluated methods. The low CV for the colorimeters is in agreement with a recent study in bulls examining the sperm concentrations determined by 6 different methods (Prathalingam *et al.*, 2006); no significant differences were found between spectrophotometry, hemocytometry, flow cytometry and image analysis (Prathalingam *et al.*, 2006). These authors showed that flow cytometry has the lowest coefficient of variation, while the colorimeter was considered the second most precise method. However, many other factors may influence the variability of a measurement, such as operator expertise, the calibration of the system and the use of different systems in various animal species with variable sperm concentrations.

In conclusion, our study of porcine semen showed that the colorimeters yielded the most repeatable results, and that high correlations were found between the different methods. The choice of method used in a laboratory could therefore be based on factors such as cost, number of samples to be assessed and practical use, without thereby negatively affecting the validity of the results thus obtained.

The HTR Ceros 12.1 is the most expensive method, but it makes it possible to examine a large number of samples very easily and in a short period of time. The Bürker counting chamber is the cheapest method, but also the most time-consuming and the least practical for examining a large number of samples.

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