Acta Veterinaria (Beograd), Vol. 57, No. 5-6, 563-572, 2007.

DOI: 10.2298/AVB0706563M UDK: 619:612.616.2

METHOD AGREEMENT BETWEEN MEASURING OF BOAR SPERM CONCENTRATION USING MAKLER CHAMBER AND PHOTOMETER

MRKUN J, KOSEC M, ZAKOŠEK MAJA and ZRIMŠEK PETRA

Clinic for Reproduction and Horses, Veterinary Faculty, University of Ljubljana, Ljubljana, Slovenia

(Received 11. January 2007)

Determination of boar sperm concentration using a photometer is used routinely by many artificial insemination (AI) laboratories. The agreement between determining sperm concentration using Makler chamber and a photometer has been assessed. Method agreement was evaluated on the basis of scatter plots with Deming regression line, absolute bias plots with limits of agreement, and relative bias plots. Coefficients of variance for the Makler chamber and a photometer were calculated as 6.575±3.461% and 1.635±0.632%, respectively, showing acceptable precision for both methods. The estimated Deming regression equation indicates that the points are close to the line of equality, and the SE of the slope (0.0600) indicates that there is almost no pivoting of the line about the central point through the means of x and y. The estimated intercept for the regression line (4.7069 M/mL) does not differ greatly. Average absolute bias was close to zero at −1.092±15.237 M/mL. Sperm counts obtained with the Makler chamber and photometer agree; 90% of the differences lie within the limits of agreement. The simplicity of sperm counting with a photometer greatly enhances the usefulness of sperm count determination in on-farm AI laboratories.

Key words: *semen concentration, porcine, method agreement*

INTRODUCTION

The increasing use of artificial insemination (SI) in swine emphasises the need for the distribution of good quality sperm by AI centres (Vyt *et al*., 2004). Boar sperm quality is routinely assessed by measuring the concentration, morphology and motility of spermatozoa (Shipley, 1999). Determination of sperm concentration is essential in evaluating fertility, whether *in vivo* or *in vitro*. However, there is no agreed method for use as a standard. Knuth et al. (1989) showed that the introduction of an unevaluated laboratory method, without an appropriate quality control, can cause a bias in semen analysis. However, the methodology of semen evaluation is complex, and standardisation is difficult (Brazil *et al.*, 2004). For example, the first large scale, nation-wide proficiency testing program for clinical andrology laboratories in the United States reported that the inter-

Acta Veterinaria (Beograd), Vol. 57. No. 5-6, 563-572, 2007.
Mrkun J *et al.*: Method agreement between measuring of boar sperm concentration using Makler chamber and photometer

laboratory coefficient of variation for manual sperm concentration determination was 80%, with a range for a single semen specimen of 3 – 492 x 10^6/mL (Kell *et al*., 2000). Variation in the results from different laboratories could be due to the lack of standardisation of methods between laboratories (Maatson, 1995).

The reason for comparing methods is often that a quicker, more convenient and more economical adaptation has been made to an existing method. The counting chamber technique for estimating sperm count appears to be adequate because of its simplicity, low cost and reproducibility. However, photometers are widely used routinely for determining sperm concentrations by many artificial insemination (AI) organisations, for bulls and boars as well as other species (Woelders, 1991). For this purpose a correct assessment of sperm concentration is essential to ensure that the number of sperm per insemination dose meets requirements and that the maximal number of doses can be produced per ejaculate.

In the present study we compared two clinical laboratory methods for determining boar sperm concentration: the Makler chamber and the photometer. Prior to method comparison, the precision of each method was assessed. Scatter plots with fitted regression lines, and absolute and relative bias plots were used to get the best overview of comparative data (Twormey, 2004; Twormey, 2005). Deming regression was applied to describe the relationship between variables both measured with error by proposing that the sum of the squares of the deviations from a line should be minimised in both the x and the y directions at the same time, thus taking account of the analytical imprecision of each method (Jones and Payne, 1997). The purpose of this study was to compare the two methods and to assess method agreement together with the appropriate regression analysis used in data interpretation.

MATERIALS AND METHODS

Semen samples

Twenty-three semen samples were obtained from eight 12 to 24 month old boars of various breeds. Each semen sample was collected with a gloved hand using a clean semen collecting flask that filters out gel, dust and bristles, while the boar mounted a dummy sow. Semen samples were diluted 1:2 with BTS semen extender (Beltsville Thawing Solution, Truadeco, Netherlands) and delivered to the laboratory.

Counting with the Makler chamber

Immediately before each semen aliquot was analysed, the entire semen specimen was vortexed. To render the spermatozoa immotile and to prepare the semen samples for the Makler chamber (Sefi Medical Instruments, Israel), semen samples were diluted 1:1 with distilled water. Parallel dilutions (n=6) of each semen sample were prepared and the average of the measurements of each used as the representative value.

Following dilutions, sperm suspensions were again vortexed, and an aliquot of 5 μL was loaded into the Makler chamber. The next step was to assess whether spermatozoa were evenly distributed or whether there were movements in the fluid in the counting chamber. If either of these problems was observed; the chamber was cleaned and refilled. The fields were chosen according to a prescribed pattern: 10 fields spaced left to right and 10 fields spaced top to bottom. Chosen fields formed a plus sign centred in the middle of the chamber, excluding the areas 2-3 mm from the chamber edges. Only recognizable spermatozoa, including lost heads, were counted, while other cells and lost tails were ignored. The concentration in the original semen sample was calculated from the total number of spermatozoa in the counting area.

Counting with a photometer

Sperm concentration was determined by measuring the sample opacity, as the percentage transmittance of light through a sample, using a photometer (Photometer SDM5, MiniTüb, Germany). Boar ejaculates are normally opaque, so a small semen sample was diluted with an isotonic solution before measuring. A blank tube was loaded with 3.5 mL 0.9% NaCl and a sample tube with 70 μL semen sample added to 0.9% NaCl. Sperm concentration was determined from a previous calibration of the spectrophotometer, performed by the manufacturer (Photometer SDM5, MiniTüb, Germany). Six measurements were made for each semen dilution.

Precision of each method for sperm counting

The precision of each method was determined by making 6 measurements of each of 23 semen samples. Coefficients of variation (CV) were calculated for each method and scatter graphs of CV versus average sperm count for each semen sample were constructed.

Method agreement

Bias determination

Differences between pairs of measurements of sperm counts – by Makler chamber and photometer – were calculated for each semen sample. In absolute bias plots, the biases were plotted against their average value for each sample. In order to assess how well the paired measurements agreed with each other, we determined the limits of agreement. The upper and lower limits of agreement were calculated as $\bar{d} \pm 2s_{diff}$, where $\bar{d}$ is the mean of differences for all the samples (average bias) and s_{diff} is the standard deviation of the differences; $2s_{diff}$ is also referred to as the British Standard Institution repeatability (or, reproducibility, as appropriate) coefficient and indicates the maximum difference likely to occur between two measurements. This coefficient is the value below which the bias between paired results may be expected to lie (Petrie and Watson, 1999).

Deming regression

While comparing the measurements obtained with the Makler chamber and the photometer, we developed scatter graphs to which we fitted a Deming regression line. Deming regression was used to solve the problem of describing the relationship between sperm counting with the methods, both measured with error. Deming's method gives only a single regression line, whether x or y is used as the "independent variable".

The intercept is calculated, as in conventional least squares regression, as the mean of y minus the product of the slope and the mean of x. The standard error (SE) of the intercept defines how much the line might vary in the y direction, and SE of the slope defines how much the line might pivot about the central point through the means of x and y. Thus, SEs allow calculation of the confidence intervals of the slope and the intercept (Jones and Payne, 1997).

RESULTS

Precision of sperm counting using the Makler chamber and the photometer

Coefficients of variance (CV) for Makler chamber and photometer were 6.575±3.461% and 1.635±0.632% respectively. From the plots of CV against mean sperm counts calculated as the average of six parallel counts for each sample, it is evident that the scatter of the points is random for the photometer (Fig.1), whereas higher CVs are observed for lower sperm counts using the Makler chamber (Fig. 2).

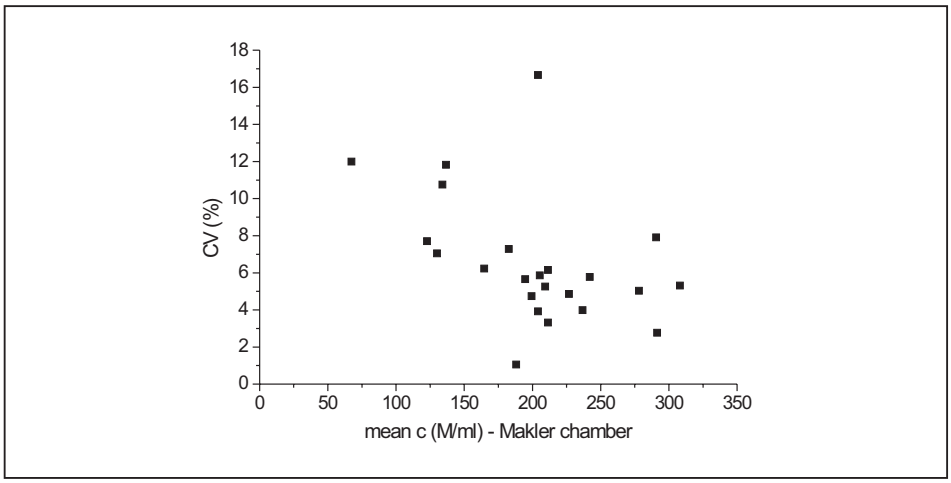

Figure 1. Coefficient of variation (CV) versus mean sperm count for the Makler chamber method

Mean counts were calculated as the average of six parallel counts for each sample

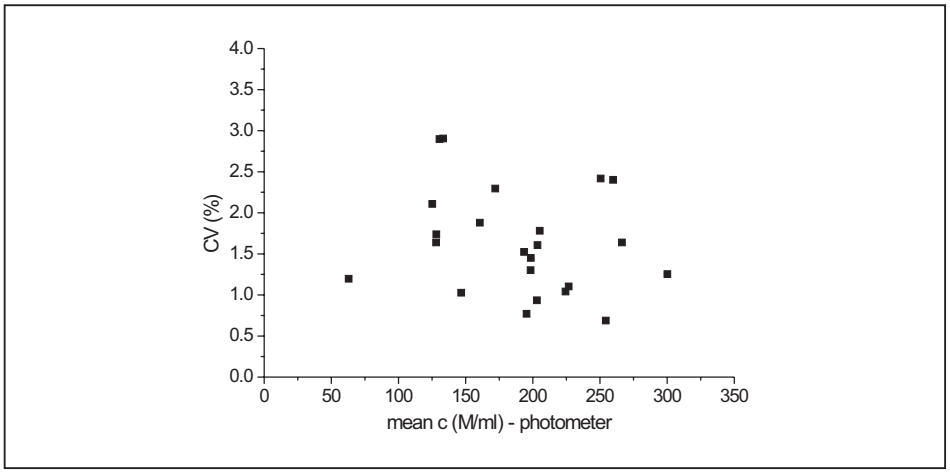

Figure 2. Coefficient of variation (CV) versus mean sperm count determined by the photometer method

Mean counts were calculated as the average of six parallel counts for each sample.

Method agreement between the Makler chamber and the photometer

Using Deming regression, we described the relationship between sperm counting using the Makler chamber and the photometer, by determining the straight line that most closely approximates the data points on a scatter diagram (Fig. 3). The estimated intercept for the regression line, 4.7069 M/mL, does not differ much from zero. The estimated regression equation indicates that the points are close to the line of equality, i.e. the 45° line and SE of the slope (0.0600) indicate that there is almost no pivoting of the line about central point through the means of x and y (Table 1).

Table 1. Results of Deming method comparison for photometer versus Makler chamber

	Coefficient	SE	95% Confidence interval
Intercept	4.7069	12.3609	-21.2623 to 30.6762
Slope	0.9706	0.0600	0.8446 to 1.0967

Equation of Deming regression line: c (photometer) = 0.9706 x c (Makler chamber) + 4.7069

The mean percentage bias between methods was – 0.6±6.9%. The scatter of the points lies in the interval –15 to 15 % (Fig. 5), which is in the range of satisfactory between-run reproducibility of the assay. From the absolute bias plot (Fig. 4) it is also evident that the scatter is random, indicating that the size of the

difference between sperm counts obtained by two methods is not related to the size of the counts. Thus, no proportional bias has been detected.

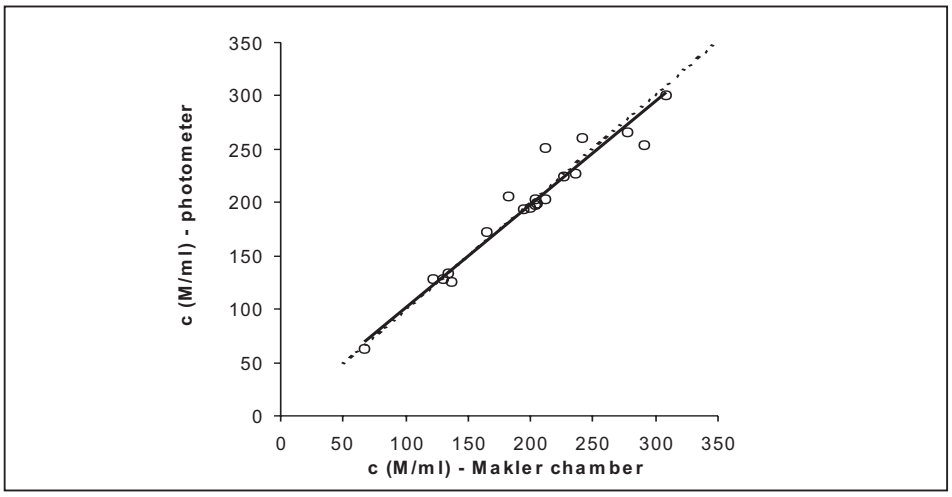

Figure 3. Scatter diagram of sperm concentration obtained by photometer versus sperm concentration obtained with Makler chamber, with Deming regression line fitted
- - - - - : line of equality (Y=X)
_____ : Deming regression line:
c (photometer) = 4.7069 + 0.9706 x c (Makler chamber)

Average absolute bias was close to zero (–1.092±15.237 M/mL). Sperm counts obtained with Makler chamber and photometer agree; 90% of the differences lie within the limits of agreement (Fig. 4).

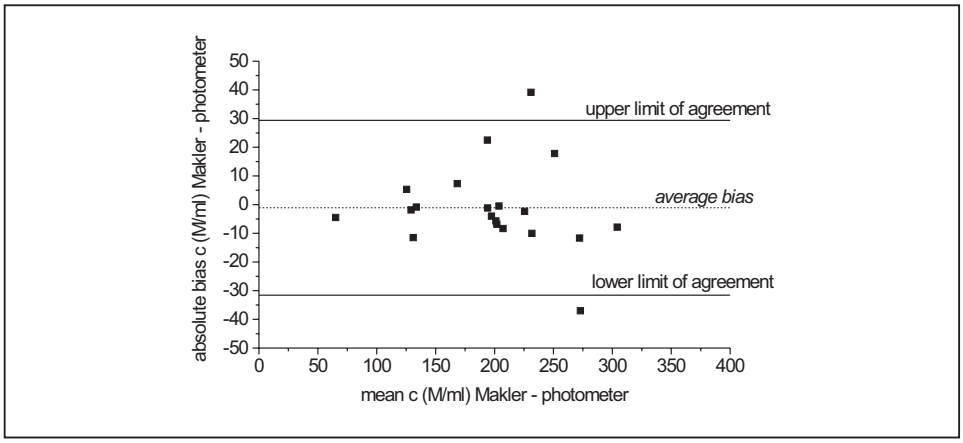

Figure 4. Absolute bias plot of sperm concentrations obtained by the Makler chamber versus concentrations obtained by photometer showing average bias and limits of agreement

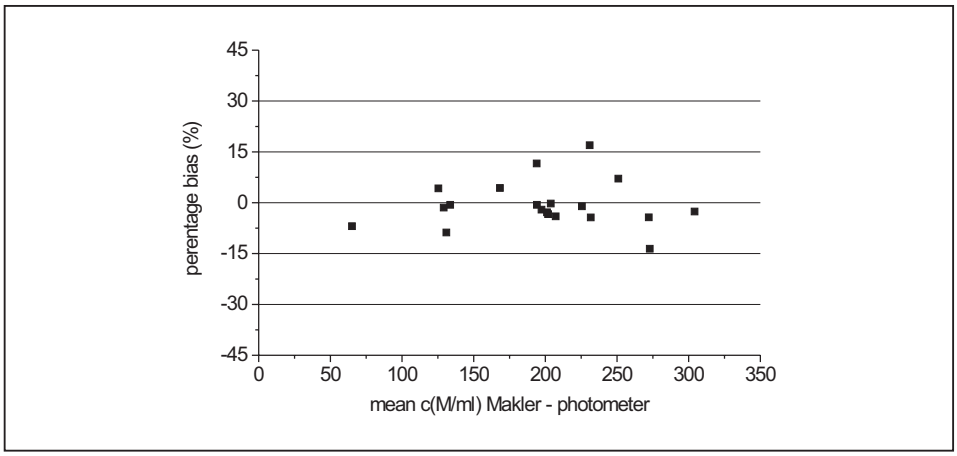

Figure 5. Relative bias plot of sperm concentration obtained by the Makler chamber versus concentration obtained by the photometer

DISCUSSION

Semen samples, which often contain a variety of cells (immature germ cells, blood cells, epithelial cells, and cellular debris) in addition to spermatozoa, differ markedly from blood samples because of their heterogeneity. There is also no specific standard available for sperm cells of each species. This is probably the reason why no established method for counting sperm has been fully validated until now. It is therefore important to compare a new, more appropriate or additional method to a conventional one (Eutache *et al*., 2001).

It is necessary to establish that a method is repeatable before comparing two measurements for reproducibility (Petrie and Watson, 1999). In our study CVs were calculated to be 6.575±3.461% and 1.635±0.632% for the Makler chamber and the photometer, respectively. Both methods yielded acceptable precision (Porstman and Kiesig, 1992; Christensen *et al*., 2005), although the precision of the Makler chamber was significantly poorer. Imade *et al*. (1993) reported a similar overall precision (5.9%) for the Makler chamber, whereas CV for sperm counts in sperm suspensions can be higher, for example 18.6% (Christensen, 2005) or even 26.3% (Mahmoud *et al*, 1997). It is generally admitted that intra-observer CVs are often greater than 10%. Although guidelines for standardising the procedure have been proposed, relatively important degrees of intra- and inter- technician or inter-laboratory variability have been reported. In the external quality assessment (EQA) reported by Neuwinger *et al*. (1990), which involved 10 experienced German laboratories in the evaluation of 8 sperm samples, the mean CV was 37.5%. From the data of the external quality control obtained under the British Fertility Society and reported by Matson (1995), the calculated inter-individual CV for sperm concentration was 64.7% for 24 semen samples collected by technicians from 20 laboratories.

In a diagram of the CV plotted against the average for each sperm concentration, the scatter of the points is random for the photometer (Fig.1). In contrast, for the Makler chamber, the size of CV is related to the size of sperm concentration, shown by the higher CVs for lower sperm counts (Fig. 2).

According to literature, a very common way of investigating method agreement is to perform a paired t-test or to calculate a correlation coefficient to provide a measure of agreement. However, in this instance, neither method is appropriate for the following reasons (Petrie and Watson, 1999). The paired t-test tests the null hypothesis that the difference is zero. If the differences between pairs are large – indicating that the methods do not agree – but are evenly scattered around zero, then the result is non-significant. We can only conclude that there is no bias, not that the methods agree. Correlation is a statistical method used to quantify any association between two continuous variables (Ma and Smith, 2003). The correlation coefficient provides a measure of the linear association between the measurements obtained by the two methods. It provides an indication of how close the observations in the scatter diagram are to a straight line. R measures the strength of a relation between two variables, not the agreement between them (Bland and Altman, 1986). For example, the Pearson correlation coefficient gives no information of value in method comparison studies, because R can be highly significant even when there is an obvious bias between the two methods. It measures the strength of association, rather than agreement, although in literature it has been used in many studies, such as comparison between different methods to determine sperm concentration (Prathalingam *et al*., 2006). R was also used to evaluate the agreement between assessments within lab technician in sperm analysis (Christensen *et al*., 2005). In order to assess the agreement, it is necessary to know how close the points are to the line of equality, i.e. the 45^{o} line (Petrie and Watson, 1999). Therefore, in the study of Sokol *et al*. (2000), comparison of two methods for measuring sperm concentration using only Wilcoxon signed rank test and F-test, appears to be insufficient.

We performed method agreement using the statistical programme Analyse-it, General + Clinical Laboratory statistics, version 1.71, where linear regression, Deming regression and Passing Bablok regression can be applied in the evaluation. We chose Deming regression, because it is appropriate for describing the relationship between two variables, both measured with error. In the case of observed increasing imprecision, i.e. where a proportional bias between methods is detected, the Passing Bablock regression procedure is more accurate than Deming's method. When the assumption that the independent variable is determined without error is satisfied, linear regression should be used to describe the agreement between two methods (Jones and Payne, 1997).

Scatter plots and absolute and relative bias plots give the best overview of comparisons of data (Twormey, 2004; Twormey, 2005). Absolute bias plots are also called Bland and Altman plots, usually used for method comparison (Bland and Altman, 1986). Using scatter diagrams with regression lines fitted, we established that the paired measurements, sperm counts obtained with Makler chamber and with photometer, were close to the line of equality.

We were interested in assessing the similarity between sperm counts measured with the Makler chamber and a photometer, so we compared pairs of measurements. For this purpose, we calculated the differences between sperm counts obtained by each method for each sperm sample. The mean of these differences ($\overline{d}$) is an estimate of the average bias of one method relative to that of the other. If this value is zero, then the two measurements agree on average. However, this does not imply that they agree for each individual measurement. In order to assess how well the measurements agree on an individual basis, we determined the limits of agreement (Petrie and Watson, 1999). 90% of the absolute differences were less than the reproducibility coefficient, confirming that the level of agreement between the methods was satisfactory. Therefore, measurements of sperm concentration with photometer and counting chamber techniques are equally appropriate for estimating sperm counts.

The usefulness of sperm counting is greatly enhanced by the simplicity of determination by photometer in on-farm AI laboratories. The use of a photometer for determining sperm concentration would, therefore, be of benefit also to livestock producers in evaluating the quality of boar semen.

ACKNOWLEDGEMENTS

This work was supported by the Slovenian Ministry of Higher Education, Science and Technology, programme group "Endocrine, immune, nervous and enzyme responses in healthy and sick animals" (P4-0053).

Address for correspondence:
Janko Mrkun
Clinic for Reproduction and Horses,
Veterinary Faculty, University of Ljubljana,
Gerbičeva 60,
1000 Ljubljana, Slovenia
E-mail: petra.zrimsek@vf.uni-lj.si

REFERENCES

1. *Bland JM*, *Altman DG*, 1986, Statistical methods for assessing agreement between two methods of clinical measurement. *The Lancet*, 1 (8476), 307-10.
2. *Brazil C*, *Swan S, Tollner C, Treece C, Drobnis E, Redmon JB et al.*, 2004, Quality control of laboratory methods for semen evaluation in a multicenter research study. *J. Androl.*, 25, 645-56.
3. *Christensen P*, *Stryhn H, Hansen C,* 2005, Discrepancies in the determination of sperm concentration using Bürker-Türk, Thoma and Makler counting chambers. *Theriogenology*, 63, 4, 992-1003.
4. *Eustache F, Jouannet P, Auger J*, 2001, Evaluation of Flow Cytometric Methods to Measure Human Sperm Concentration. *Journal of Andrology*, 22, 4, 558-67.
5. *Imade GE, Towobola OA, Sagay AS, Otubu JAM*, 1993, Discrepancies in sperm count using improved Neubauer, Makler, and Horwells counting chamber. *Arch Androl*, 31, 17-22.
6. *Jones RG, Payne RB*, 1997, Analytical methods: control and comparison. In: Jones RG, Payne RB, editors, *Clinical Investigation and Statistics in Laboratory Medicine*. London: ACB Venture Publications, 27-65.
7. *Keel B, Quinn P, Schmidt C, Serafy N Jr, Serafy N Sr, Schalue T*, 2000, Results of the American association of Bioanalysts national proficiency testing programme in andrology. *Hum Reprod*, 15, 680-6.
8. *Knuth UA, Neuwinger J, Nieschlag E*, 1989, Bias to routine semen analysis by uncontrolled changes in laboratory environment – detection by long-term sampling of monthly means for quality control. *Int J Androl*, 12, 375-83.

9. *Maatson P*, 1995, External quality assessment for semen analysis and sperm antibody detection: results of a pilot scheme. *Hum Reprod*, 10, 620-5.
10. *Ma D, Smith FG*, 2003, Correlation and regression, In: Smith *FG*, Smith *JR*, editors, *Key topics in Clinical Research*, Oxford: BIOS Scientific Publishers Limited, 147-51.
11. *Mahmoud AMA, Depoorter B, Piens N, Comhaire FH*, 1997, The performance of 10 different methods for the estimation of sperm concentration. *Fertil Steril*, 68, 2, 340-5.
12. *Neuwinger J, Behre HM, Nieschlag E*, 1990, External quality control in the andrology laboratory: an experimental multicenter trial. *Fertil Steril*, 54, 308-14.
13. *Petrie A, Watson P*, 1999, Additional Topics. In: Petrie *A*, Watson *P*, editors, *Statistics for veterinary and animal science*, Oxford: Blackwell Science, 168-81.
14. *Prathalingam NS, Holt WW, Revell SG, Jones S, Watson PF*, 2006, The precision and accuracy of six different methods to determine sperm concentration. *J Androl*, 27, 2, 257-62.
15. *Sokol RZ, Shulman P, Paulson RJ*, 2000, Comparison of two methods for the measurement of sperm concentration. *Fertil and Steril* , 73, 2, 591-4.
16. *Vyt P, Maes D, Rijsselaere T, Dejonckheere E, Castryck F, Van Soom A*, 2004, Motility Assessment of Porcine Spermatozoa: a Comparison of Methods. *Reprod Dom Anim*, 30, 447-53.
17. *Twormey P*, Plasma glucose measurement with the Yellow Springs Glucose 2300 STAT and the Olympus AU640. *J Clin Pathol* 2004, 57, 752-4.
18. *Twormey P*, How do we really compare methods in the clinical laboratory? In: Statistics Workshop & Clinics, *EuroMedLab* 2005, Glasgow May 8-12th.
19. *Woelders H*, 1991, Overview of in vitro methods for evaluation of semen quality. *Reprod Domest Anim Suppl*, 145-64.

PODUDARNOST METODA ZA ODREĐIVANJE KONCENTRACIJE SPERMATOZOIDA NERASTA MEKLEROVOM KOMOROM I FOTOMETROM

MRKUN J, KOSEC M, ZAKOŠEK MAJA i ZRIMŠEK PETRA

SADRŽAJ

Određivanje koncentracije sperme nerasta uz pomoc fotometra se upotrebljava rutinski u mnogim laboratorijima za veštačko osemenjivanje. U našem radu smo upoređivali metode brojanja spermija fotometrom ili u Maklerovoj komorici na osnovu različitih statističkih postupaka kao što su: "scatter plots" sa Demingovim pravcem regresije, apsolutnih graf razlika sa granicama podudarnosti i relativnih graf razlika. Izračunati koeficienti variance za Maklerovu komoricu i fotometar bili su 6,575±3,461 % i 1,635±0,632 %, što ukazuje na dobru preciznost obe metode. Jednačina Demingovog pravca regresije ukazuje, da su tačke u blizini pravca podudarnosti, a SE krivulje (0,0600) ukazuje, da nema praktično nikakvog nagiba u pravca regresije. Secište pravca regresije nije puno udaljeno od nule (4,7069 M/ml). Prosečna vrednost apsolutnih razlika bila je takođe u blizini nule (-1,092 ± 15,237). Za broj spermija koji je bio utvrđen sa Maklerovom komoricom i fotometrom može se reći, da je podudaran; 90% nađenih razlika su u granicama podudarnosti. Jednostavna a dovoljno precizna metoda određivanja broja spermija uz pomoć fotometra omogučava širu upotrebu ove metode u terenskim uslovima.