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Original article

Method agreement between three different chambers for comparative bull semen computer assisted sperm motility analysis

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Abstract

The aim of this study was to compare computer assisted sperm analysis (CASA) results of frozen thawed bull semen using three different chambers. Sixty bull frozen semen samples were thawed (37°C; 30 sec), extended in PBS (30×10^6 spermatozoa/mL; 37°C) and incubated (37°C; 2 min). Each semen sample was analyzed by CASA [total motility, progressive (pro)/ non-progressive/rapid/medium/slow movement spermatozoa, VCL, VSL, VAP, ALH, BCF, LIN, STR, WOB and hyperactive spermatozoa] using three different chambers: a Makler® chamber (MC; 10 μm); a Leja 4 chamber slide (LC; 20 μm); and a Glass slide covered with a coverslip (GSC; 10.3 μm). The Makler chamber gave higher values compared to both the LC and GSC for almost all examined parameters. No systematic effect was evident between LC and GSC for VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF. Method agreement between MC and LC was generally moderate, between MC and GSC poor and between LC and GSC moderate to good. In general, narrower limits of agreement were found in samples with lower values. In conclusion, the CASA outcomes could be influenced by the analysis chambers. This finding should be taken into consideration when comparing results from different laboratories.

Key words: analysis chamber, bull semen, computer assisted sperm analysis, motility

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Introduction

In bovine reproduction, the quality of frozen thawed (FT) semen is of paramount importance due to the widespread use of artificial insemination (AI) with semen derived from a relatively small number of donor bulls. As a result, semen quality evaluation is pivotal. Sperm motility, although it is not a fertility marker per se (Holt et al. 1997), is one of the most important sperm quality parameters for evaluating the fertilizing capacity of an ejaculate. This parameter provides information on the ability of spermatozoa to move toward the fertilizing site and reflects the integrity of plasma membranes and the metabolic status of sperm (Johnson et al. 2000). According to World Health Organization (WHO) guidelines regarding human semen analysis (WHO 2010), only four categories of spermatozoa movement can be defined by conventional microscopic visualization. However, this inexpensive and simple technique is subjective and associated with large discrepancies between laboratories (Jørgensen et al. 1997) or technicians, even when the same semen sample is evaluated (Contri et al. 2010).

The computer assisted sperm analysis (CASA) system has been commercially available since the 1980s. Total motility and numerous kinetic parameters are evaluated objectively and simultaneously for a high number of spermatozoa, allowing the whole sperm population to be divided into subpopulations with specific track characteristics (Contri et al. 2010, Amann and Waberski 2014). Hence, it has largely replaced the conventional light microscopy technique in research and clinical andrology laboratories, and in AI centers which produce marketed semen doses from farm animals (Amann and Waberski 2014). According to WHO (WHO 2010), CASA systems provide a detailed and reliable analysis of human sperm motility. However, the accuracy and sensitivity of CASA measurements can be influenced by a variety of factors, such as the software settings, the frame rate acquisition, the semen sample temperature etc (Del Gallego et al. 2017, Bompart et al. 2019). According to the literature, the type of chamber used for analysis could also affect the CASA motility results (Contri et al. 2010, Hoogewijs et al. 2012, Palacín et al. 2013). A variety of analysis chambers with different technical characteristics in terms of shape, size, depth, and charging method of the semen sample are commercially available

Recent research is focusing on the further standardization of CASA processes in order for data from different laboratories to be comparable (Palacín et al. 2013, Gaczarzewicz 2015). In this respect, the aim of this study was to evaluate the agreement between three chambers (Makler, Leja 20 μm, Glass slide coverslip) regarding FT bull semen CASA motility assessment.

Materials and Methods

The semen samples used in the present study were commercially available. No approval by the Ethics Committee on Animal Use (School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece) was necessary as no operations on research animals were carried out.

Reagents and media preparation

All chemicals used in the study were of analytical grade and purchased from Sigma-Aldrich Chemical Co (St Louis, MO, USA). EDTA-free phosphate-buffered saline solution (PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4; pH 6.8 6.9; 280-300 mosmol/kg) supplemented with 0.058 gr/L penicillin G and 0.05 gr/L streptomycin sulfate was used for the extension of thawed semen samples prior to CASA analysis.

Semen sample processing and evaluation

Commercial FT semen samples from 10 Holstein bulls were included in the study. Sixty straws of 0.5 mL (6 straws/bull) were examined. The thawing process was performed in a water bath at 37°C for 30 seconds. Immediately after thawing, the semen samples were extended in PBS (37°C) at a concentration of 30 x 10^6 spermatozoa/mL and incubated (37°C) in the dark for 2 min prior to analysis.

Each incubated (37°C) semen sample was evaluated with each one of the examined analysis chambers (preheated to 37°C) as follows: a) a volume of 10 μL semen sample was loaded into the Makler® chamber (MC; Makler® counting chamber, 10 μm deep, Sefi Medical Instruments, Israel) and covered with a glass coverslip, b) a volume of 2.3 μL semen sample was loaded into the Leja 4 chamber slide (LC; Leja, 20 μm deep, Leja Products B.V., Nieuw Vennep, Netherlands), and c) a volume of 5 μL semen sample was placed on a glass slide (GSC) and covered with a coverslip (22 mm × 22 mm). All the filled chambers were maintained at 37°C for 1 min prior to evaluation in order to avoid passive flow of liquids (Contri et al. 2010). The preparation process was repeated in case air bubbles were detected in the chambers. The chambers were used in a random rotation to exclude the effect of time.

Sperm motility was assessed using the Sperm Class Analyser® CASA system (SCA®; Microptic S.L., Automatic Diagnostic Systems, Spain) and a microscope (AXIO Scope A1, Zeiss, Germany) equipped with a heating stage (37°C) and a camera (Basler scA780 54fc, Basler vision technology, Germany). The analysis was performed using SCA® software

Table 1. Mean or median differences, limits of agreement (mean difference ± 2SD or 5th and 95th quantile) and Intraclass Correlation Coefficient (ICC, 95% Confidence Intervals) between Makler chamber (MC), Leja chamber (LC) and glass slide chamber (GSC).

Variable	MC-LC	limits of agreement MC-LC	ICC (95% CI) MC LC	MC-GSC	limits of agreement MC-GSC	ICC (95% CI) MC GSC	LC-GSC	limits of agreement LC-GSC	ICC (95% CI) LC GSC
Totmot (%)	17.43**	33.47, 1.39	.57 (0-.85)	25.83**	51.13, 0.53	.47 (0-.79)	8.4**	32.10, -15.30	.79 (.44-.90)
Nonpro (%)	5.55**	24.23, -13.13	.84 (.62-.92)	10.76**	26.18, -14.70	.73 (.25-.88)	5.54**	14.18, -11.18	.88 (.72-.94)
Pro (%)	8.08**	27.43, 1.49	.65 (0-.87)	9.70**	40.70, 2.39	.35 (0-.65)	2.26**	18.14, -9.24	.65 (.35-.81)
Rapid (%)	16.63**	33.21, 0.05	.56 (0-.85)	18.76**	38.58, 8.47	.45 (0-.79)	4.37**	18.45, -9.71	.83 (.60-.92)
Medium (%)	2.17**	6.45, -3.05	.34 (0-.60)	5.75**	10.54, -8.68	.13 (0-.41)	2.79**	7.09, -6.99	.55 (.24-.73)
Slow (%)	-0.98	4.54, -10.30	.86 (.75-.92)	3.00	7.76, -12.72	.78 (.64-.87)	2.8**	8.16, -6.78	.83 (.65-.91)
VCL (µm/s)	20.41**	46.45, 6.96	.56 (0-.85)	22.87**	56.94, 8.60	.28 (0-.62)	1.87	30.52, -10.29	.56 (.26-.74)
VSL (µm/s)	8.90**	25.40, 2.22	.68 (0-.88)	8.31**	36.49, 1.25	.31 (0-.60)	0.00	19.55, -4.93	.62 (.36-.78)
VAP (µm/s)	11.96**	27.90, 3.44	.57 (0-.84)	12.20**	41.23, 4.13	.22 (0-.52)	0.37	20.91, -5.61	.46 (.12-.68)
LIN (%)	1.75**	17.61, -2.03	.72 (.35-.86)	0.72	22.09, -5.27	.53 (.20-.73)	-0.91	11.59, -8.40	.71 (.52-.83)
STR (%)	3.33**	14.20, -2.20	.76 (.44-.88)	2.54**	20.63, -5.67	.59 (.25-.77)	-0.64	11.02, -8.55	.79 (.65-.88)
WOB (%)	0.23*	11.82, -2.55	0 (0-.30)	0.18	22.37, -3.45	0 (0-.04)	0.57	9.75, -4.59	.56 (.27-.74)
ALH (µm)	0.51**	1.19, -0.17	.34 (0-.66)	0.55**	1.67, -0.06	0 (0-.20)	0.00	1.23, -0.64	0 (0-0)
BCF (Hz)	2.64**	5.60, -0.32	.88 (0-.97)	2.37**	8.11, -3.37	.78 (.31-.90)	-0.27	5.95, -6.49	.82 (.69-.89)
Hyper (%)	2.27**	33.00, -1.50	.79 (.54-.90)	2.84**	64.50, -0.60	0 (0-.28)	0.58**	40.50, -1.30	0 (0-.29)

Totmot: total motility (%), Nonpro: non-progressive spermatozoa (%), Pro: progressive spermatozoa (%), RapidMediumSlow: rapid, medium, slow spermatozoa (%; 10<slow<25<medium<45<rapid µm/sec), VCL: curvilinear velocity (µm/sec), VSL: straight line velocity (µm/sec), VAP: average path velocity (µm/sec), LIN: linearity (VSL/VCL x 100), STR: straightness (VSL/VAP x 100), WOB: wobble (VAP/VCL x 100), ALH: amplitude of lateral head displacement (µm), BCF: beat/cross-frequency (Hz), Hyper: hyperactive (%).
Symbols * and ** denote significant differences of the mean or median from 0 at $p<0.003$ and $p<0.0001$ level, respectively.

(v.6.3.) and the CASA configurations were: 1) 8 fields (MC and LC) or 5 fields (GSC; a central and 4 peripherical fields 5mm from the edges; WHO 2010) were recorded (× 100) for each semen sample, 2) >500 spermatozoa, 3) 25 frames/sec, 4) region of particle control 10-18 microns, 5) progressive movement of >70% of the parameter STR, 6) depth of field 10, 20, 10.3 microns (MC, LC, GSC, respectively), and 7) temperature of the microscope plate at 37°C. Objects incorrectly identified as spermatozoa were manually deleted prior to final analysis.

The following CASA motility parameters and kinetics were estimated for each semen sample analyzed: 1) total motility (totmot); %, 2) progressive (pro; straight line velocity>70) and non-progressive (nonpro) movement spermatozoa; %, 3) rapid, medium and slow movement spermatozoa (10<slow<25<medium<50<<rapid µm/sec); %, 4) curvilinear velocity (VCL); µm/sec, 5) straight line velocity (VSL); µm/sec, 6) average path velocity (VAP); µm/sec, 7) amplitude of lateral head displacement (ALH); µm, 8) beat/cross-frequency (BCF); Hz, 9) linearity (LIN); VSL/VCLx100, 10) straightness (STR); VSL/VAPx100, 11) wobble (WOB); VAP/VCLx100 and 12) hyperactive spermatozoa (hyper); %.

Statistical analysis

Statistical analysis was performed using SAS® version 9.3 (SAS Institute Inc., 1996, Cary, N.C., U.S.A.) and SPSS® version 25 (IBM-SPSS, Armonck, NY, USA) software. The guidelines of Watson and

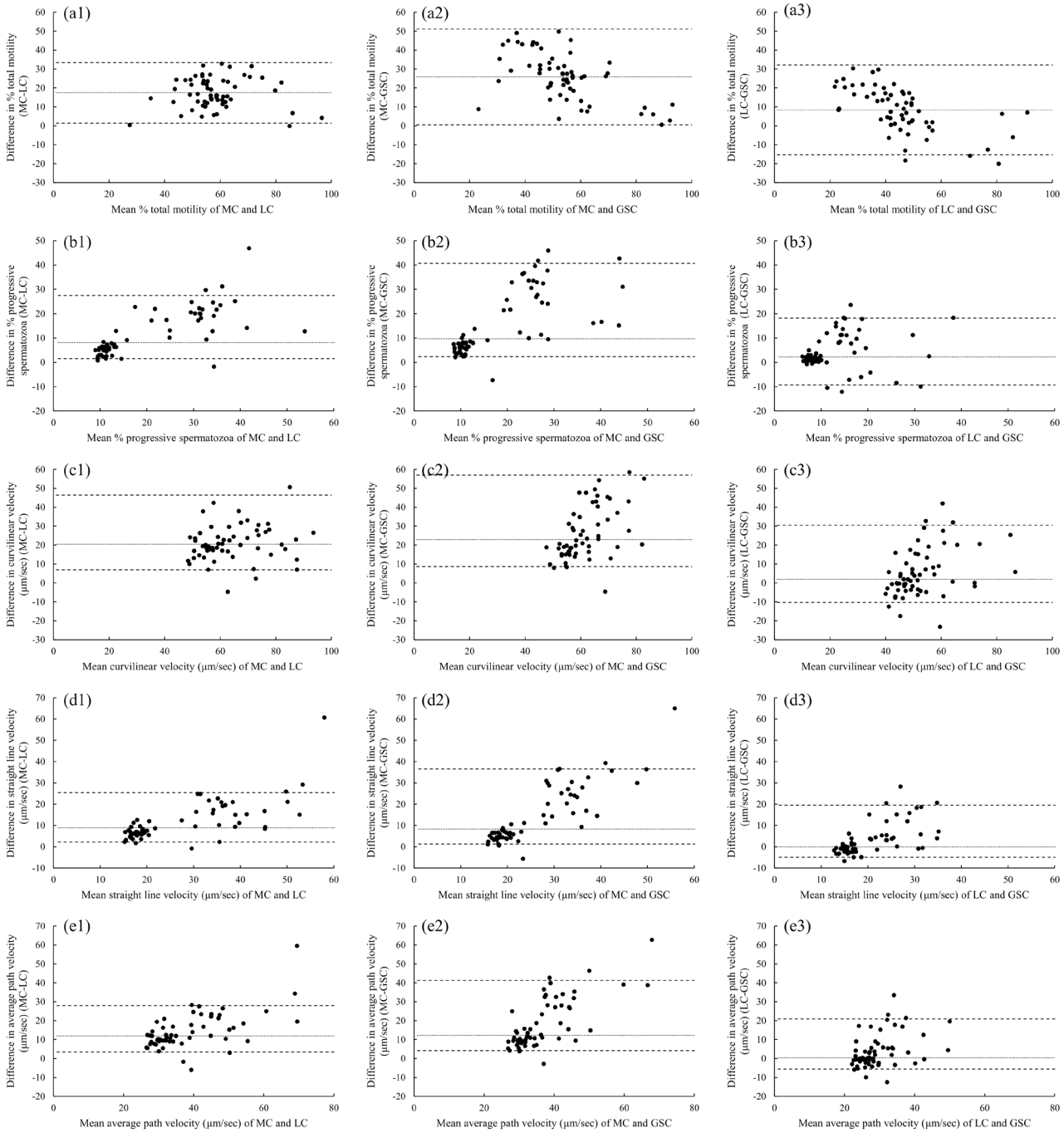

Fig. 1. Bland and Altman plots showing mean or median differences (dotted lines) and limits of agreement (mean difference ± 2SD or 5th and 95th quantile, dashed lines) between Makler chamber (MC), Leja chamber (LC) and glass slide chamber (GSC) for main CASA motility parameters and kinetics.

Petrie were followed to perform sample size and method agreement analysis (Watson and Petrie 2010). The anticipated Intraclass Correlation Coefficient (ICC) was set at 0.60 and the acceptable confidence interval at 0.30. Based on these values sample size was set at 60. Paired differences between MC, LC and GSC were calculated for every ejaculate and parameter. The normality of the differences was tested using the Shapiro-Wilk Test. A paired t-test for cases of normal distribution and a Wilcoxon signed rank test for cases of not normal distribution were respectively applied to examine the null hypothesis that the true mean or median difference was zero. Results significantly different from zero are indicative of a systematic effect. To reduce the chance of type I error, a Bonferroni correction was applied separately for every pair of comparisons (i.e. MC-LC, MC-GSC or LC-GSC), including the 15 variables under consideration. Thus, the statistically significant difference was defined as $p<0.003$. Additionally, upper and lower limits of

agreement were estimated as the mean ± 2 standards deviation or as the 5th and 95th quantiles for normal and not normal distribution of the differences, respectively. Agreement between the methods was subjectively evaluated with the use of Bland and Altman plots (Bland and Altman 2010). The differences between pairs of methods were plotted against their mean. Finally, method agreement was objectively estimated with the use of ICC and the 95% Confidence Intervals. Estimates were calculated based on a mean rating, absolute-agreement, 2-way mixed-effects model. Agreement between the methods based on ICC was considered poor if ICC was lower than 0.5, moderate if ICC was between 0.5 and 0.75, good if ICC was between 0.75 and 0.9 and excellent if ICC was above 0.9 (Koo and Li 2016).

Results

A systematic effect was evident in all parameters except slow for the comparison between MC and LC and except slow, LIN and WOB for the comparison between MC and GSC (Table 1). This effect was almost entirely positive, as MC derived, on average, higher values compared to both LC and GSC. Intraclass Correlation Coefficients revealed moderate to good average agreement between MC and LC for most of the assessed parameters (Table 1). However, in all parameters except from nonpro and slow, the 95 % Confidence Intervals were wide, especially regarding the lower limit. The limits of agreement between MC and LC were generally moderate (Fig. 1, a1-e1). Additionally, in numerous parameters (pro, VSL, VAP), Bland and Altman diagrams revealed narrow limits of agreement in lower average values (Fig. 1, b1-d1-e1). The agreement between MC and GSC was moderate to poor for all parameters, except for slow and BCF which showed good agreement. The 95 % Confidence Intervals and the limits of agreement were generally wide (Table 1, Fig. 1, a2-e2). Narrower limits of agreement were again evident regarding samples showing lower values.

The comparison between LC and GSC revealed a positive systematic effect for some CASA assessed parameters (totmot, nonpro, pro, rapid, medium, slow, hyper) (Table 1). Limits of agreement were generally modest, close to the values obtained from the comparison between MC and LC. Moreover, the effect of narrower limits in lower values was again evident (Table 1 and Fig. 1). Based on ICC values, method agreement was moderate to good for most of the evaluated parameters (totmot, nonpro, slow, LIN, STR, BCF) with the upper limit of the Confidence Interval being close to or above 0.90 in many cases (totmot, nonpro, rapid, slow, BCF).

Discussion

The development of CASA is an invaluable tool for recording human and animal sperm kinematic characteristics. However, the different settings and configurations among the available CASA systems and the lack of standardized procedures for sample handling prior to analysis complicate the comparison of results obtained from different laboratories (Lannou et al. 1992, Rijsselaere et al. 2003, Contri et al. 2010, Hoogewijs et al. 2012). In the present study, three different chambers were compared for CASA motility analysis of FT bull semen. The assessment of sperm number/ml was excluded from our analysis, since we did not perform any gold standard techniques (such as Neubauer haemocytometer, Burker counting chamber or NucleoCounter) for sperm concentration.

According to the literature, the effect of type of chamber on CASA outcomes is mainly attributed to the different properties of each chamber. The depth of the chamber could facilitate the natural movement of spermatozoa (Soler et al. 2018) in many species such as goat (Del Gallego et al. 2017), bull (Gloria et al. 2013), stallion (Hoogewijs et al. 2012), boar (Basioura et al. 2019), ram (Palacín et al. 2013), and human (Lannou et al. 1992). The rotational movement of spermatozoa could be affected by the available space provided under *in vitro* analysis conditions and interestingly enough, sperm motility could be prevented when the analysis chamber is of 2 μm depth or lower (Makler 1978). Additionally, the charging method can influence CASA outcomes. It has been proposed that the capillary loading of chambers results in different sperm distribution (Contri et al. 2010, Palacín et al. 2013) and to passive hydrodynamic movement of fluid, known as the Segre and Silberberg effect (Segré and Silberberg 1961), when compared to droplet loaded chambers. Moreover, it is hypothesized that capillary loaded chambers could damage the sperms' tail movement due to capillary forces and therefore reduce sperm motility (Lenz et al. 2011, Hoogewijs et al. 2012). In the present study, the reported discrepancies between MC and LC as well as between LC and GSC could be explained by the different depth (MC; 10 μm, LC; 20 μm, and GSC; 10.3 μm) and/or the different charging method of the chamber. The Makler chamber gave higher values compared to LC, which is in accordance with previous studies on bovine and equine semen (Hoogewijs et al. 2009, Contri et al. 2010, Gloria et al. 2013). However, the results obtained with LC were higher compared to GSC for some CASA examined parameters, which is in contrast to some published studies. Specifically, equine (Hoogewijs et al. 2012) and bovine (Lenz et al. 2011) semen CASA motility analysis with

GSC gave higher values compared to LC; however, in these two studies the depth of GSC was different (approx. 20 μm) compared to the present study (10.3 μm). Additionally, the different CASA system used in the above mentioned studies, with different settings and configurations, could be another factor contributing to these discrepancies. Based on a recent study of our laboratory, in which the same chambers were compared for CASA motility analysis of boar semen, LC gave higher values compared to GSC (Basioura et al. 2019). Thus, similar findings were obtained when the experimental design and the CASA system were the same (excluding the species-related configurations), although the species and the type of semen were different between the two studies.

In the present study, differences between the two droplet loaded chambers were observed and MC overestimated CASA parameters compared to GSC. According to Hoogewijs et al. (2012), MC gave higher values for equine sperm progressive motility compared to the slide chamber using WHO guidelines for human semen examination. The same finding was made for ram (Palacín et al. 2013) and boar (Basioura et al. 2019) semen CASA parameters. It is interesting that, although the depth of the GSC was different in the above-mentioned studies, the results were similar. However, Lenz et al. (2011) did not find any difference between these two chambers for bovine CASA semen analysis. Under the experimental conditions of the present study, the differences between MC and GSC are unlikely to be related to the filling method or the depth of chamber, since they were almost identical (10 and 10.3 μm for MC and GSC, respectively). A possible explanation could be the different pressure on sperm suspension. In MC, a stable depth of 10 μm is created by the glass coverslip which is held in position by 4 supports. However, in conventional coverslip of GSC, the depth could be unevenly distributed and this could result in the compression of the sperm population due to surface tension (Lenz et al. 2011).

Method agreement between MC and LC was found generally moderate, between MC and GSC poor, and between LC and GSC moderate to good, while narrow limits of agreement were noticed in samples showing lower values. Results of another study at our laboratory indicated that the comparative CASA motility analysis of boar semen using the same three chambers revealed a similar phenomenon for total and progressive motility, although in this case it was noticed in higher values (Basioura et al. 2019). Based on these findings, we make the hypothesis that when the sperm population is largely of similar quality (either high or low), the discrepancies between the chambers under investigation tend to be low.

In conclusion, the motivation of this study was not so much to favor the accuracy of one chamber over another, as to enhance the need for standardized procedures for CASA semen motility analysis. Under the experimental conditions of this study, it is reported that although semen analysis was conducted by the same CASA system, the outcomes were influenced by the analysis chamber. This is an important finding that should be considered when results obtained by different laboratories are compared. However, the narrow limits of agreement observed in lower values indicate that the differences between the compared chambers tend to be limited when the sperm population provides the same kinetic characteristics.

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