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Assessment of motility of ejaculated, liquid-stored boar spermatozoa using computerized instruments

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Abstract

Visual-motility assessment is a tool used to determine the quality of boar ejaculates. This method is subjective by nature, and consequently, computer-assisted sperm analysis (CASA), with different software designs, has been developed for more objective assessment using conventional image analysis or particle counting. In the present study, we compared the results of sperm analysis using a conventional CASA system (Cell Motion Analyzer, SM-CMA^{TM}), with those using a novel software (QualiSperm^{TM}) and those of visual assessment performed by two operators. Ejaculates were collected weekly from four Swedish Landrace boars for 4 weeks. Each ejaculate was divided into three aliquots of different sperm concentration (300, 125, and 40 million spermatozoa/mL) and stored at ~17 °C for 96 h. Only samples at 40 million spermatozoa/mL concentration were analyzed using both automated systems; for the remaining concentrations, the SM-CMA^{TM} was not used due to its inability to examine higher sperm concentrations. The number of spermatozoa analyzed was highest for the QualiSperm^{TM} (~300–5000 spermatozoa), followed by the SM-CMA^{TM} (~200 spermatozoa), and lastly, by subjective motility evaluation (~100 spermatozoa). There was a time-course decrease in motility of the liquid-stored semen, detectable by either computerized method. Although the percentage of motile spermatozoa measured by the two automated systems correlated well ($r \geq 0.75$), there was disagreement between operators. In conclusion, because of the lower degree of variation, the numbers of spermatozoa analyzed, and the speed of analysis (~1 min per sample), QualiSperm^{TM} appears to be a suitable instrument for routine work, provided it maintains stability and is available at an affordable price.

Keywords: Computer-assisted sperm analysis (CASA); QualiSperm; Boar; Motility; Sperm storage

1. Introduction

Many methods are used to estimate the viability of a semen sample and thus evaluate potential fertility of the male from whom the semen has been collected, in order to eliminate male animals with substandard fertility and also, to avoid the use of substandard samples that may

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result in lower fertility after artificial insemination (AI) [1]. Most methods of semen assessment *in vitro* measure general characteristics of the spermatozoa (morphology, motility patterns, membrane and organelle integrity, etc.), all essential to fertility, provided these attributes are maintained until the spermatozoa are confronted with the oocyte. More complicated methods (which are therefore mostly used for research purposes, rather than being intended for routine use by the AI industry) attempt mimicking *in vitro* the interactions between the spermatozoa and the female genital tract, the oocyte vestments, and the fertilization process *in vivo*. The outcomes of these explorations relate differently to fertility, depending on the method used, as well as on the number of spermatozoa evaluated at one time. For instance, maintenance of membrane integrity appears to be more closely related to semen fertility than does sperm motility, but only when a large number of spermatozoa are examined (using a fluorescent-activated cell sorter (FACS) or fluorometry [2,3]).

The use of AI in the swine industry has increased exponentially, particularly in Europe, where some countries such as Spain artificially inseminate more than 80% of sows [4]. Most of the semen is still used as liquid, extended and stored at temperatures slightly below room temperature (17–20 °C), although a certain percentage is also used deep-frozen for gene-banking purposes or for export of genetics. Consequently, there is an increasing interest in the diagnostic methods used for semen analysis in AI. However, the battery of diagnostic methods used by the industry is as yet restricted. Under routine conditions, only sperm concentration and sperm motility are assessed, as indicators of sperm production and viability. Sperm morphology is rarely checked, when boars enter the production line, or where pathologies are suspected.

Assessment of sperm motility is usually done by visual evaluation of sperm movement under phase contrast microscopy. This visual evaluation is rapid and cheap, but its accuracy depends on the experience of the operator, which explains the large intra- and inter-assay variation documented in the literature [5–8]. To overcome this problem, different evaluation techniques, such as turbidimetry, laser Doppler spectroscopy, photometric systems, and computer-aided instrumentation, have been developed [9]. Among them, the most successful systems have been grouped into what has been termed ‘‘computer-assisted sperm analysis (CASA)’’ instrumentation. A CASA instrument records, by means of a video camera, the path followed by spermatozoa placed on a wet smear over a certain time interval. The signal picked up by the camera is digitized and the information processed by a computer which reconstructs, for a certain number of frames, each individual spermatozoon’s fixed (most CASA instruments) or summary (Hobson instrument) path trajectory. The different systems locate a certain point in each spermatozoon (often the head) for the signal, and also check for presence of a tail, so that spermatozoa are separated from the neighboring debris, based on size, presence of tail, and speed of translation. The computer is, thereafter, able to use a series of variables considered absolute kinematic parameters, such as sperm velocities and the lateral displacement of the sperm head (LDH). Using these, it recalculates other derived parameters, such as proportions of spermatozoa with various patterns of movement (e.g., linear, nonlinear, circular, or even local, non-translational motility), their degree of linearity, dance, etc. [10]. The CASA examination is considered fast and more ‘‘objective’’, yielding a large amount of data, but analyzing a restricted number of spermatozoa (often of the order of hundreds per sample), which in most instruments are followed for a restricted time. Computer-assisted sperm analysis instruments are, therefore, useful for research purposes, since they provide data the human eye cannot register. Moreover, CASA analysis make it possible to determine the presence of sperm subpopulations coexisting in an ejaculate [11,12], the effect of cryopreservation [13,14], and the appearance of sperm changes such as hyperactivation [15,16].

On the other hand, CASA instruments are not widely used in commercial practice. There are several reasons for this. Firstly, CASA requires a certain degree of calibration and validation [17], and proper programming of species-specific settings [18]. Also, it is costly [9]. It does not always show a relationship between motility and fertility [1,19]. Furthermore, CASA instruments can present errors of measurement, caused by too many spermatozoa in a sample, the crossing of trajectories, or collisions of spermatozoa, all of which produce variation in the results [20]. Moreover, under certain conditions (such as during handling and storage, and particularly, post-cryopreservation), the data gathered show some seemingly paradox results when compared with the initial readings, due to the fact that the system only records the surviving spermatozoa, whose number and motility patterns may differ from those of the original sperm population [10].

Alternative systems have been tried, and newer computerized sperm analysis systems (such as QualiSperm™; Biophos, Pfäffikon, Switzerland; http://www.biophos.com) work on a different principle.

QualiSperm™ determines the number of particles (spermatozoa) crossing fields of view, yielding a regression fluctuation algorithm of sperm numbers and translation classes. The regression fluctuation algorithm is a method for measuring dynamic parameters and the concentration of particles in a solution, such as spermatozoa. It runs analyzing the movement of the particles across the boundaries between of the several detections areas in which the digital picture are divided. The fluctuation of the number of particles with respect to the detection areas is analyzed by the correlation function of the fluctuation signal, which is generated by light emission of the particles. It further analyzes thousands of spermatozoa per sample. This restricts the output of information to numerical sperm classes, based on their velocity of translation in a large field, making it potentially suitable for routine use.

Considering all the above, it is clear that the industry needs standard methods of sperm motility analysis, which will allow a more objective quality control of production, for comparison of performance within and among enterprises, and comparison of handling methods, sperm storage variations prior to AI, and the implementation of new technologies.

Therefore, the objective of the present study was to compare the QualiSperm™ instrument with a proven, conventional CASA instrument (SM-CMA™; MTM Medical Technologies, Montreaux, Switzerland) [21,22] and with the subjective assessment of sperm motility by two independent operators. Mimicking routine management of semen for preparation, storage, and analysis of AI doses, the analyzes were done on semen split-extended to three different sperm concentrations (considered optimal for analysis) and stored at ~17 °C for 96 h.

2. Material and methods

2.1. Semen samples

Four young boars of the Swedish Landrace breed were used, ranging from 8 to 14 months of age and selected according to normal semen quality (i.e., $\geq 50 \times 10^9$ total number of sperm per ejaculate, initial motility ≥70%, and containing >85% morphologically normal spermatozoa). All boars were kept on straw beds in individual pens at the Division of Reproduction, Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, fed according to Swedish standards [23], and provided with water *ad libitum*. The experimental protocol had previously been reviewed and approved by the Ethical Committee for Experimentation with Animals, Uppsala, Sweden.

Semen was manually collected from each boar once a week for four consecutive weeks using the gloved-hand technique [24]. Immediately after collection, each ejaculate was extended with an equal volume (1:1, v/v) of isothermic (~35 °C) Beltsville Thawing Solution (BTS) extender (IVM, L'Aigle, France) and, following counting of the sperm concentration using a Bürker counting chamber (VWR International, Stockholm Sweden), re-extended to provide three split samples, as follows:

(1) sample C300, with a sperm concentration of $\sim 300 \times 10^6$ spermatozoa/mL;
(2) sample C125, with a sperm concentration of $\sim 125 \times 10^6$ spermatozoa/mL;
(3) sample C40, with a sperm concentration of ~40 $\times 10^6$ spermatozoa/mL.

The mean sperm concentrations (± standard deviation (S.D.)) for the total of 16 ejaculates considered were, after Bürker countings, $284.0 \pm 21.81 \times 10^6$ spermatozoa/mL for sample C300; $133.9 \pm 10.96 \times 10^6$ spermatozoa/mL for sample C125; $43.9 \pm 5.79 \times 10^6$ spermatozoa/mL for sample C40. All samples (C300, C125, and C40) were kept at ~17 °C in closed recipients for 96 h. Once daily within this period, flasks were periodically shaken and the temperature registered, and the sperm motility was registered immediately thereafter.

2.2. Assessment of sperm motility

2.2.1. Subjective evaluation

After gentle mixing, a portion of each sample, C300, C125, and C40, was re-warmed to 38 °C for 5 min. Thereafter, a 5-μL drop of the sperm suspension was placed on a pre-warmed glass slide and covered with a cover slip. The evaluation was independently performed by the two operators, three times each, using a microscope (Nikon Optiphot 2; Nikon, Tokyo, Japan) equipped with phase contrast optics and a thermal plate, at 100× magnification. All the material used in this process was warmed to 38 °C on a thermal plate.

2.2.2. Computer-assisted sperm analysis evaluation

After gentle mixing, a portion of each sample, C300, C125, and C40, was re-warmed to 38 °C for 5 min. A 5-μL drop of the sperm suspension was placed in a 10-μm deep Makler counting chamber (Sefi-Medical, Haifa, Israel) warmed to 38 °C, and examined under phase contrast microscopy using the same microscope as for the subjective examination (Nikon Optiphot 2; Nikon,

Tokyo, Japan), linked to the SM-CMA™ (MTM Medical Technologies, Montreaux, Switzerland). For each sample, eight predetermined optical fields around the central reticulum of the chamber were used to count a minimum of 200 spermatozoa per sample. In conjunction with the percentage of motile spermatozoa, the following motility patterns were recorded: proportion of linearly motile spermatozoa (LinMS, %), of nonlinearly motile spermatozoa (NLinMS, %), of circularly motile spermatozoa (CMS, %), and of locally motile spermatozoa (LMS, %). As well, the degree of sperm lateral head displacement (LHD, μm) was registered, as were sperm speeds, namely, straight linear velocity (VSL, μm/s), average path velocity (VAP, μm/s), and curvilinear velocity (VCL, μm/s). The parameter settings for the SM-CMA™ software were 32 frames with a spermatozoon present in at least 16 in order to be counted, time resolution 20 ms (50 Hz). An object with VAP $\leq$10 μm/s was considered immotile and objects with a velocity $\geq$25 μm/s were deemed as motile; velocities between 10 and 25 μm/s represented spermatozoa that were locally motile. Those spermatozoa with a radius <25 μm were classified as circularly motile. Spermatozoa deviating $\leq$10% from a straight line were designated as linearly motile, while those with a deviation >10% were considered nonlinearly motile. This assessment was performed by operator 1 (O1).

2.2.3. QualiSperm™ evaluation

At the same time, operator 2 (O2) carried out the analysis of the same extended semen with QualiSperm™, version 1.3 (Biophos, Pfäffikon, Switzerland). Following gentle mixing, a 5-μL drop of each sample, C300, C125, and C40, re-warmed to 38 °C for 5 min, was loaded onto a Makler chamber at 38 °C and the sperm movement observed using a Nikon E200 microscope (Nikon, Tokyo, Japan) equipped with phase contrast optics and a thermal plate, at 100× magnification. The spermatozoa were recorded in one field at a rate of 50 frames/s (200 frames in total) using a MV-D640-48-U2-10 Photon Focus camera (Photon Focus, AG, Lachen, Switzerland). The data recorded were the proportions of motile and immotile spermatozoa and their mean speed.

2.3. Statistical analysis

The data were analyzed using the General Linear Model (GLM) procedure of the Statistical Analysis Systems software, version 9.1 (SAS Institute Inc., Cary, NC, USA). The mean and coefficient of variation were calculated for the percentages of sperm motility and CASA parameters. The normality of the data was checked using the Shapiro–Wilk test, and since the data did not follow a normal distribution, they were subjected to a Kruskal–Wallis test for differences between methods, days, and sperm concentration of the extended semen. Correlations between different methods and different days, as well as between the parameters measured by the computer-assisted systems, were calculated using Spearman's rank correlation coefficient. For all analyzes, a significance level of $P < 0.05$ was set up.

3. Results

The evaluations of sperm motility were done using the concentrations given. As the SM-CMA™ has been used in this laboratory for many years, the sperm concentration used was 40 million/mL, which is close to the optimal determined for the instrument. The number of spermatozoa being retrieved and analyzed per assessment time therefore differed between instruments, being approximately 200 spermatozoa for the SM-CMA™, while the QualiSperm™ retrieved a mean of 315 spermatozoa per sample analyzed in C40, 1580 in C125, and 5165 in C300, i.e., a significantly higher proportion than assessed by the SM-CMA™. QualiSperm™ was used to analyze sperm motility in all three sperm concentration classes (C40, C125, and C300), the output showing significant differences between classes. The most uniform measurements (i.e., with the least variability) were obtained when C300 was analyzed, suggesting that the system is able to analyze high sperm numbers. The values for the percentage of motility measured by the QualiSperm™ system and SM-CMA™ in C40 showed a good correlation between instruments for consecutive analyzes ($r \geq 0.75$, $P < 0.006$). The correlation between consecutive analyzes was, however, poorer for the subjective measurements performed by either operator for any class of extended semen ($r = 0.1$–0.5).

Sperm motility decreased steadily along the 96 h of experimental storage at ~17 °C, for either sperm concentration considered or sperm evaluation procedure used (see Fig. 1). For QualiSperm™, the decrease was highly significant ($P < 0.001$) at 24 h at all sperm concentrations; it was significant ($P < 0.05$) at 72 h in C40 and C125 and at 96 h in C40 and C300. In the remaining analysis periods, the decrease was not statistically significant ($P > 0.05$). For the SM-CMA™ system (where the only extension analyzed was C40), the decrease in the proportion of motile spermatozoa

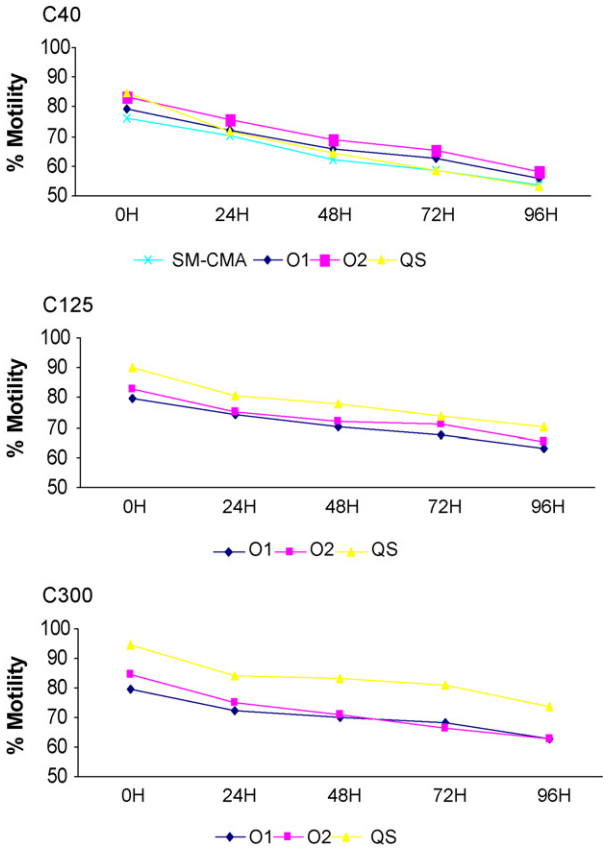

Fig. 1. Changes in the mean proportions of motile spermatozoa (% motility) in boar semen extended to three different concentrations (C40: ~40 × 10^6 spermatozoa/mL; C125: ~125 × 10^6 spermatozoa/mL; C300: ~300 × 10^6 spermatozoa/mL) and stored at ~17 °C for 96 h (H, $n = 16$ ejaculates). Split samples were analyzed subjectively (by two operators, O1 and O2) and by the computer-assisted sperm analysis (CASA) instruments SM-CMA^{TM} (SM-CMA) and QualiSperm^{TM} (QS).

was registered as significant by 24 and 48 h ($P < 0.05$), but not by 72 or 96 h ($P > 0.05$). Regarding the subjective evaluations, for O1, sperm motility decreased significantly ($P < 0.01$) in the three concentration groups by 24 h, and significantly ($P < 0.05$) at 48 and 96 h in C40, at 72 h in C125, and at 96 h in C300. In the rest of the intervals, sperm motility was maintained ($P > 0.05$). For O2, sperm motility also decreased significantly ($P < 0.01$) at 24 h in all concentration classes (C40, C125, and C300) and by 96 h in C125, but was significantly lower by 48 and 96 h in C40 ($P < 0.05$). In the rest of the evaluation times and concentrations, the decrement was not statistically significant ($P > 0.05$).

Table 1 summarizes the proportions of motile spermatozoa and their coefficient of variation (CV, intra-assay) in each measurement interval, for the four methods of evaluation and the three groups of concentration assayed. It also includes the maximum–minimum CV for the five consecutive measurements. In general, the CVs increased with storage time. For C40, with the exception of the 96-h interval and results using the SM-CMA^{TM} system, the mean of CVs of the measurements performed by the two operators was lower than the same mean given by the two CASA instruments. For O2, the mean of CV never exceeded 10% (ranging from 4.78% at 0 h to 9.72% at 96 h), while the results of O1 and QualiSperm^{TM} exceeded 10% at 96 h. The SM-CMA^{TM} showed erratic behavior (see Table 1). In the other two concentration classes (C125 and C300), the means of CVs were, for most measurements, higher when measured using the QualiSperm^{TM} system compared with evaluations by O1 or O2, increasing with storage time. Maximal values of the mean of CVs for QualiSperm^{TM} were 11.02% in C300 and 11.01% in C125.

The inter-assay variation for C40 (checking repeatability of motility measurements in the same boar between weeks) showed that CVs were always highest with examinations done using SM-CMA^{TM}, and for all systems, the mean of CVs increased with the time of storage, being, for SM-CMA^{TM}, 13.35–33.64%; for O1, 6.20–26.33%; for O2, 3.48–30.35%; finally, for QualiSperm^{TM}, 5.61–30.98% (data not shown). In the other two concentration classes (C125 and C300), the CVs were lower, especially with QualiSperm^{TM}.

The measurements of percentages of motility, obtained using the SM-CMA^{TM} and QualiSperm^{TM} systems, were highly correlated ($r \geq 0.75$, $P < 0.0006$). The correlation between O1 and QualiSperm^{TM} or SM-CMA^{TM}, when examining C40, increased along the experimental period, from $r = 0.16$ at 0 h to $r = 0.75$ by 96 h ($P < 0.1$–0.05). For O2, on the other hand, the correlations between subjective motility assessment and QualiSperm^{TM} or SM-CMA^{TM} were consistently around $r = 0.5$ ($P > 0.05$). The correlation between the two operators increased as well, from $r = 0.24$ at 0 h to $r = 0.71$ by 96 h ($P < 0.36$–0.002). On the other hand, when analyzing C125 or C300, the methods correlated ($P < 0.82$–0.004) but the correlation coefficient never exceeded 0.6.

Table 2 summarizes absolute kinematic values, measured using the QualiSperm^{TM} (average speed, AS) and the SM-CMA^{TM} instrument (VSL, VCL, and VAP). Particularly interesting is the high correlation between the AS and the VAP, i.e., average velocity, measured by the instruments, indicating that both instruments are able to accurately measure this relevant absolute kinematic value.

Table 1
Summary of the mean percentages of motile spermatozoa (mean), coefficient of variation (CV), and maximum and minimum coefficient of variation (CV max–min), for the four methods of evaluation (SM-CMA™, QualiSperm™, and evaluation by operators O1 and O2) and the three groups of sperm concentration assayed (C40, C125, and C300[a])

Sample	Method used		Storage time (h)				
			0	24	48	72	96
C40	SM-CMA™	Mean (%)	76.21 a	70.16 b	62.09 c	58.47 c	54.34 c
		CV (%)	12.41	8.28	8.59	14.52	7.91
		CV max–min (%)	1.45–56.31	1.17–24.37	1.42–22.71	1.32–33.43	1.16–20.84
	QualiSperm™	Mean (%)	84.50 a	71.44 b	64.31 bc	58.77 c	55.96 d
		CV (%)	5.96	8.52	9.29	9.36	17.72
		CV max–min (%)	2.22–22.58	2.71–22.53	1.91–32.99	1.47–18.23	2.97–52.92
	Subjective (O1)	Mean (%)	79.17 a	71.88 b	65.83 c	62.81 c	53.31 d
		CV (%)	5.12	5.56	7.12	6.67	13.62
		CV max–min (%)	0–16.88	0–13.32	0–19.52	0–12.39	4.22–75.18
	Subjective (O2)	Mean (%)	83.54 a	75.52 b	69.06 c	65.42 c	58.18 d
		CV (%)	4.78	5.00	7.57	8.33	9.72
		CV max–min (%)	3.27–9.75	3.46–10.19	3.53–34.64	0–34.64	4.03–34.64
C125	QualiSperm™	Mean (%)	89.60 a	80.48 b	77.23 b	71.98 c	69.94 c
		CV (%)	4.11	5.74	6.28	8.61	11.01
		CV max–min (%)	1.22–9.36	0.76–12.52	0.72–12.57	0.77–15.39	5.52–24.69
	Subjective (O1)	Mean (%)	79.79 a	74.79 b	70.10 c	67.71 d	63.13 d
		CV (%)	4.57	5.08	5.52	6.65	5.59
		CV max–min (%)	0–16.88	0–8.66	0–12.06	0–12.39	0–15.06
	Subjective (O2)	Mean (%)	82.50 a	75.00 b	71.98 bc	71.25 c	65.94 d
		CV (%)	4.77	5.35	5.07	5.00	7.35
		CV max–min (%)	0–11.55	3.46–12.06	0–8.66	0–10.66	0–21.65
C300	QualiSperm™	Mean (%)	94.65 a	84.21 b	83.19 b	80.73 b	73.54 c
		CV (%)	2.02	4.74	6.47	7.61	11.02
		CV max–min (%)	0–3.72	0.63–9.71	1.09–13.60	1.37–15.61	2.74–21.17
	Subjective (O1)	Mean (%)	79.58 a	72.19 b	70.10 b	68.33 b	62.92 c
		CV (%)	4.59	5.16	6.17	6.22	6.18
		CV max–min (%)	0–16.88	3.69–10.66	0–11.18	0–12.39	0–15.06
	Subjective (O2)	Mean (%)	84.38 a	74.90 b	70.73 bc	66.46 cd	62.81 d
		CV (%)	3.94	5.30	5.98	6.86	7.83
		CV max–min (%)	3.15–6.30	3.46–9.75	3.77–14.52	0–16.88	4.03–15.75

a–d: different letters denote significant difference between times of storage.
[a] C300 = sample with a sperm concentration of $\sim 300 \times 10^6$ spermatozoa/mL; C125 = sample with a sperm concentration of $\sim 125 \times 10^6$ spermatozoa/mL; C40 = sample with a sperm concentration of $\sim 40 \times 10^6$ spermatozoa/mL.

4. Discussion

Sperm motility is the parameter that is most frequently used to measure boar sperm viability in the ejaculate and during/after the process of storage or cryopreservation. Indirectly, analyzes of sperm motility are expected to provide cues on the potential fertility of the spermatozoa. Since subjective motility assessment is considered less objective than computer-assisted motility analyzes, there has been an interest in including these instruments in practice. However, several elements constrain the application of these instruments in commercial practice, from costs to the plethora of data they provide, which makes the use of this equipment still a matter for research rather than for commercial interest. Many of the parameters provided by most conventional CASA instruments are questionable, since they are data recalculated from absolute values. Moreover, their value for decision making, e.g., whether an ejaculate or a dose is substandard, is as yet not higher than the decision taken by an experienced operator while observing a drop of the semen in question, without incurring in purchase of sophisticated equipment.

The subjective assessment is therefore practical and inexpensive, and results in the elimination of ejaculates or processed semen depicting poor motility, as a good clinical indicator of potential sub-fertility or infertility.

Table 2
Summary of sperm velocities (μm/s) in sample C40, determined using QualiSperm™ (average speed, AS) and SM-CMA™ (straight line velocity, VSL; curvilinear velocity, VCL; average path velocity, VAP)

Storage time	Sperm velocities (μm/s)			
	AS	VSL	VAP	VCL
0 h				
Mean	84.50	44.80*	64.71*	126.61*
CV	3.28	7.54	8.77	8.84
CV max–min	1.45–7.18	1.47–19.34	2.12–23.86	0.73–22.59
24 h				
Mean	71.44	43.00*	75.18	159.27*
CV	3.51	6.89	7.91	8.46
CV max–min	0–10.19	2.02–19.87	1.64–19.82	1.01–21.95
48 h				
Mean	64.31	42.53*	74.02	159.97*
CV	4.20	9.84	9.33	9.20
CV max–min	0.82–14.79	3.97–30.49	2.03–18.50	1.20–19.76
72 h				
Mean	68.10	43.22*	71.78	155.54*
CV	3.46	9.31	7.51	7.67
CV max–min	0.79–10.46	3.96–37.24	2.54–14.04	1.32–15.15
96 h				
Mean	65.20	42.42*	71.52	155.79*
CV	9.12	10.60	7.32	7.44
CV max–min	0.9–54.15	1.20–28.53	1.17–24.81	0.57–33.95

C40 = sample with a sperm concentration of ~40×10^6 spermatozoa/mL. Mean = average of the measurement of the parameters in each analysis; CV = coefficient of variation of this measurement; CV max–min = maximum and minimum coefficient of variation in each time interval.
* Significant difference from average speed (AS).

However, under commercial conditions and particularly in enterprises providing semen for AI from selected boars of high-genetic value, the semen of these boars is highly motile and often shows acceptable motility during processing, even post-thaw. It is here that CASA instruments appear more valuable since they provide much more data with a degree of objectivity which would hopefully reveal subtle differences in motility not perceivable to the human eye. Most of these instruments digitize microscope images of sperm trajectories, providing information on proportions of motile spermatozoa, motility patterns, and other kinematic variables. However, the results are often based on comparatively few (hundreds) spermatozoa analyzed per sample which, in most cases, are followed for a mere few seconds of their trajectory.

In light of the above, the present study was undertaken to examine the value of a novel computerized sperm analyzer (the QualiSperm™ instrument) which is designed to determine the number of particles (spermatozoa) crossing fields of view, yielding a regression fluctuation algorithm of sperm numbers and translation classes. Using a high resolution camera [25], the instrument captures a large area of the wet smear under analysis, and allows a longer capture time for the particles (4 s), all of which is supposed to increase the number of spermatozoa captured, potentially improving the precision of the measurement [30]. Such performance makes the analysis time much shorter, a matter that was clearly determined in the present study. The QualiSperm™ instrument provided results within seconds, much faster than the conventional CASA (SM-CMA™) instrument. Obviously, such a gain in time is of utmost importance, since it is similar to the time needed for an experienced operator to analyze a sample. Two more interesting features of the QualiSperm™ instrument are appealing, namely, the fact that it analyzes thousands of spermatozoa per sample, and the fact that it restricts the output of information to two motility parameters: percentage of

motility and mean speed of the spermatozoa, the two most relevant variables for an AI enterprise. All these reasons make the instrument more suitable for routine use compared with a conventional CASA instrument. However, the conventional CASA instrument, which screens more kinematic variables, renders more information than does QualiSperm™, which is useful in comparisons with other studies.

In the present study, the QualiSperm™ system showed a high correlation with the SM-CMA™ system regarding the minimal output provided, both as an absolute parameter (average sperm speed) and as a recalculated parameter (total percentage of motile spermatozoa). However, it is important to note that the comparison was only done at a concentration of 40 million spermatozoa/mL, which has previously been found to be optimal for the SM-CMA™ instrument under previous studies in our laboratory [14,21,22,28]. Use of a higher concentration makes analyzes with the SM-CMA™ instrument difficult, while it proved not to be a problem with the QualiSperm™ instrument, which analyzed concentrations of about 300 million spermatozoa/mL without difficulty. Interestingly, the subjective measurements correlated with both CASA systems when sperm concentrations were low, but overestimated the output when examining higher sperm concentrations.

Because of their peculiar plasma membrane composition, boar spermatozoa are extremely sensitive to cooling (chilling), freezing, and thawing [26]. This sensitivity to low temperatures requires storage at moderately reduced temperatures (i.e., 16–20 °C), which restricts the sperm's storage capacity because the cell metabolism cannot be slowed down and because microbiological conditions may not be as effectively controlled as at temperatures around 5 °C. Injuries to the sperm membrane and changes in cell function appear already at temperatures around 20 °C if storage is not proper, resulting in alterations in the permeability of the membrane and a decrease in energy production [27]; both reducing sperm motility as well as impairing membrane integrity or the levels of tyrosine phosphorylation [28–30]. Besides, sperm kinematics is also affected by temperature decrease [12], a phenomenon mostly noticeable when CASA instruments are used.

At all time intervals, our motility data measured by the SM-CMA™ instrument agreed with a previous study performed with the same extender and CASA system [28]. In that study, however, the first significant decrease in motility was reported to have taken place by 72 h of storage, while in the present trial, such decrease ($P < 0.05$) was observed already at 24 h by both the computerized analyzers and both operators. Most likely, individual boar differences were responsible for these discordant results.

The number of motile spermatozoa, measured using QualiSperm™, was higher in C125 and C300 along all the liquid storage periods. This contrasts with the widely held belief that boar sperm viability is best preserved at 15–20 °C at concentrations of ~30 million spermatozoa/mL. However, we believe that our results do not contradict this general theory and the overestimation is, in our opinion, the result of increasing sperm collisions, with subsequent multiple evaluations of the same individual spermatozoa [31] and the resulting artificial increase in motility [32]. In other studies using higher sperm concentrations, a larger part of the faster moving cells was excluded from the analyzes [9], leading to lower motility values. Nevertheless, most present systems cannot perform the analysis if the concentration is as high as in our trial. With the new QualiSperm™ instrument, we could determine the decrease in sperm motility in all concentration classes. The fact that we were able to detect the first significant decrease in motility at 24 h, using either the conventional CASA (C40) system or QualiSperm™ (all concentration classes), points to another advantage with QualiSperm™, namely, that the number of cells analyzed is high and there is a decrease in variability of the results [32]. We can, therefore, suggest that the results of the highly concentrated classes, analyzed by QualiSperm™, were more precise compared with SM-CMA™-generated results. The results of kinematic parameters (VCL, VAP, and VSL) measured by the SM-CMA™ system at time 0 were very close to previously published data using the same equipment [28,33].

The subjectivity of the visual assessment of sperm motility is a well-known disadvantage of this method [9], leading to disagreement between results of different technicians/laboratories in the analysis of the same sample. In our trial, the results of visual analysis by two technicians did not agree, showing that O1 and O2 used different criteria when scoring sperm motility. The results were, however, closer with decreased motility, probably because the human eye is better able to record lower numbers of spermatozoa per microscopic field.

The time spent on the analysis is an important factor for AI enterprises. The visual scoring of motility of about 100 spermatozoa takes ~1 min, while a conventional CASA system (such as the SM-CMA™ system) takes 3–4 min to capture ~200 spermatozoa. The QualiSperm™ instrument, on the other hand, took ~2 min to capture and analyze several hundreds to

thousands of spermatozoa (depending on the sperm concentration of the original sample), thus making it a suitable system for genetic enterprises.

In conclusion, the QualiSperm™ system showed a high correlation with the SM-CMA™ CASA system with regard to the minimal output provided, both as an absolute parameter (average sperm speed) and as a recalculated parameter (total percentage of motile spermatozoa). However, it is important to consider that the comparison was done only at a concentration of 40 million spermatozoa/mL, which has already been tested as optimal for the CASA equipment. Use of a higher concentration makes analyzes difficult with the SM-CMA™ CASA system, while it proved not to be a problem with the QualiSperm™ instrument, which is easily able to analyze concentrations of about 300 million sperm/mL. Interestingly, the subjective measurements correlated with both computer-assisted systems at low sperm concentrations, but overestimated the output when examining higher sperm concentrations. In sum, QualiSperm™ appears to be a suitable instrument for routine work, provided it maintains stability and is available an affordable price. The ultimate test would be to compare its output *in vitro* with AI results on the farms.

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