OPEN ACCESS

GAVIN PUBLISHERS

Advances in Biochemistry and Biotechnology

Research Article

Carvalho PHA, et al. Adv Biochem Biotechnol 7: 1087.
DOI: 10.29011/2574-7258.001087

Bovine Frozen Semen Motility Analysis Using Two Different Laser Wavelengths

PHA Carvalho[1], RODS Rossi[2], E Lopes[1], APC Santos[1], JNS Sales[3], GF Rabelo[4], RA Braga Jr[4], JB Barreto Filho[1*]

[1]Department of Veterinary, Animal Reproduction Laboratory, Federal University of Lavras, Lavras, Minas Gerais, Brazil

[2]Federal Institute of Triângulo Mineiro, Campus Uberlândia. Sobradinho Farm S/N Zona Rural, Uberlândia, Brasil

[3]Veterinary Medicine, Federal University of Juiz de Fora, Minas Gerais, Brazil

[4]Department of Automatica, Centre for Development of Applied Instrumentation, Federal University of Lavras, Lavras, Minas Gerais, Brazil

[*]**Corresponding author:** Joao Bosco Barreto Filho, Department of Veterinary, Animal Reproduction Laboratory, Federal University of Lavras, Lavras, CEP 37200-000 CP3037 Minas Gerais, Brazil. Tel: +55-3538291718; Email: barreto@ufla.br

Citation: Carvalho PHA, Rossi RODS, Lopes E, Santos APC, Sales JNS, et al. (2019) Bovine Frozen Semen Motility Analysis Using Two Different Laser Wavelengths. Adv Biochem Biotechnol 7: 1087. DOI: 10.29011/2574-7258.001087

Received Date: 16 April, 2019; **Accepted Date:** 09 May, 2019; **Published Date:** 17 May, 2019

Abstract

Laser of 632 and 532 nanometers were evaluated for bovine semen kinetic analysis. Numerical index of dynamic laser speckle (DLSI) was compared with motility assessment by light microscopy. Straws (n = 123) were thawed and evaluated as to velocity and cell motility. In assay one, each sample was lit by either kind of laser (red, n = 56; green, n = 20). In the second assay 46 samples were lit alternately by both laser sources. An index grouping velocity and motility was used to compare the evaluations. In a third assay a single sample was illuminated with both laser sources to assess the motility decreasing, and regression analysis of dynamic laser speckle index, velocity and motility index have been done. Correlations between dynamic laser speckle index, velocity and cell motility were r = 0.594; r = 0.734 and r = 0.665; r = 0.684 for samples illuminated by red and green laser, respectively. Correlation coefficients for red and green laser found between DLSI index and velocity (r = 0.801; r = 0.590), cell motility (r = 0.826; r = 0.613) and velocity and motility index (r = 0.840; r = 0.618), were significant ($p < 0.01$). No difference was observed between the laser sources that were sensitive in detecting semen activity, thus both of them can be used to generate the biospeckle phenomena and assess motility in the bull frozen semen.

Introduction

Semen analysis intends to determine the fertility potential of the ejaculate by conventional methods. Sperm motility is considered to be a good fertilization predictor, and high positive correlations were observed between spermatozoa motility and fertility in the human species [1], in the stallion [2], ovine [3] and cattle [4]. On the contrary, poor correlations were mentioned in some species [5].

In sperm motility analysis the semen kinetics evaluation by routine methods usually implies a subjective component [6,7] and an uncertainty degree [8]. Thus, methodology and the precision of the measurement are important to semen evaluation, as well as the choice of the right attribute, such as progressive motility [9]. In the last few years, the use of computer-assisted technologies improved the accuracy of semen analysis, however, these are high cost techniques and not suitable in many situations [10,11]. In addition, many studies observed positive and even high correlations between motility evaluation by conventional methods and computer-aided systems [12,13].

Many efforts have been done to increase the objectivity of the ancillary methodologies used to analyze semen samples [14,15]. Optical metrology is one such approach and was pioneering presented by Ross, et al. using laser illumination, the Doppler effect evaluation and correlation with semen motility [16].

The earliest works on spermatozoa (fish and rabbit sperm) showed that laser light scattering was strongly influenced by mobile cells, indicating its value as an assay of per cent motility [17]. First models (point scatterers) proposed by Harvey & Woolford assumed that spermatozoa behave as point particles moving with constant velocity [18]. However, these models proved to be inadequate, because sperm cells, particularly from rams and bulls, are large and far from being spherical in form. The interpretation of correlation

functions on light scattered from bovine motile sperm requires a model which considers the size, shape, and unusual swimming trajectories of these cells.

In addition, it was found that the spermatozoa exhibited a strong orientation effect, and the light scattered intensity was dominated by those cells swimming in a narrow angle which were almost perpendicular to the scattering vector. Further studies have pointed to the importance of immotile cells present in the ejaculate, interactions or collisions between motile and immotile cells, the distribution of spermatozoa within the scattering cell, spatial orientation effects and the shape of the bull spermatozoon in order to understand the spectrum or autocorrelation function of the scattered light by semen [19].

Other methodologies using the optical approach are the dynamic laser speckle or biospeckle laser techniques that was initially adapted in blood flow [20,21] and after applied to many phenomena related or not to a flow movement [22,23]. Previous report of our laboratory [24] showed the reliable use of the biospeckle laser as an approach to evaluate semen kinetic parameters. Nevertheless, different laser wavelengths can influence the activity of spermatozoa [25-27], compromising the DLSI outcomes.

The objective of this study was to evaluate the biospeckle technique using two distinct wavelengths as a tool to measure the quality of bovine frozen semen samples under the view of motility parameters.

Materials and Methods

Semen Samples

Semen of the bulls were diluted in citrate-yolk extender and frozen according to routine procedures. Briefly, ejaculates were diluted in yolk extender (lactose 7,4 nmol x L^{-1}; egg-yolk 20% - v/v; added with 10^5 UI of penicillin K and 10^5 µg of diidroestreptomyicin), cooled at 5 ˚C for approximately 90 minutes, filled into 0.5 ml straws (IMV technologies, France) and after 4 hours at 5 ˚C for equilibration they were frozen. Samples were kept in liquid nitrogen (-196 ˚C) until the time of evaluation. Each sample was thawed in a water-bath at 37 ˚C, for 30 seconds, and then analyzed. The average sperm cell concentration used in all experiments was 10 million viable cells, and total concentration was 30 to 35 x 10^6 sperm cells per straw.

Biospeckle Setup

Experimental equipment consisted of low-power Helium-Neonium (He-Ne) and Solid State laser (power and wavelengths were 10 and 3 mW and 632 and 532 nm, respectively), a beam splitter, a mirror, a color digital Charge-Coupled Detector Device (CCD) and a computer with an image processor (Figure 1). A density filter was also used to ensure the same light intensity in the sample submitted to both laser sets in experiments II and III. The time history speckle patterns (512 images) were constructed using a CCD camera of 640x480 pixels. The speckle grains were always larger than the pixels; the backscattering configuration was the adopted to illuminate and to get the images in a frame rate of 0.08 seconds, which were processed forming the Time History Speckle Pattern of 512 x 512 pixels. That was the database adopted in the biospeckle image analysis and to obtain the Inertial Moment (IM), (numerical biospeckle index) based on a second order statistical result, with the use of an intensity dispersion matrix as a secondary image derived from the Time History Speckle Pattern (THSP), in accordance with Arizaga, et al. [28].

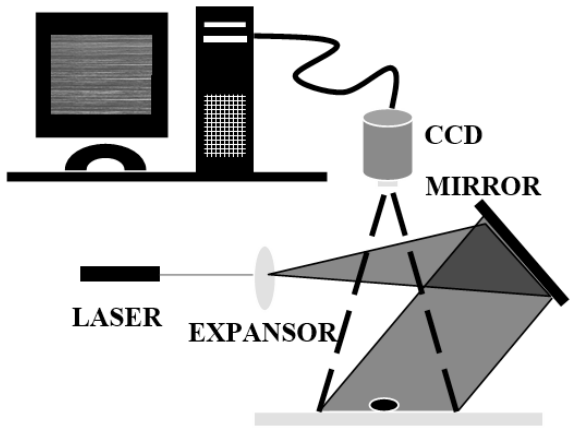

Figure 1: Equipment set-up.

Experimental Design

In experiment I, the straws (n=76) were thawed at 37 ºC for 30 seconds and 10 µL were evaluated microscopically to measure sperm velocity (V) in a relative scale (1 - slow to 5 - fast movements) and Percent Sperm Cell Motility (PSCM) in slides with cover slips (15 x 15 mm), warmed in a hot platinum device [29]. In this study, 76 samples were illuminated by the red (n=56) and green (n=20) laser without a sequence, which means that specimens were lit by red laser and in other set of illuminations they were lit by the green one. Each sample was lit by either kind of laser (red and green), after a 30 seconds period of stabilization in the slide before biospeckle readings. The two laser sets in the visible range were analyzed by the biospeckle routine analysis. Inertial moment values were compared to routine semen evaluation using two evaluators, both trained in the same laboratory, who analyzed V and PSCM by light microscopy.

In experiment II, 46 samples were illuminated by red and green lasers alternately, using the same set up and without moving the sample. Samples were submitted to the same period of stabilization described in the first experiment. The results

accounted by IM were compared to V, PSCM and the Velocity and Motility Index (VMI) proposed in this work. Velocity and motility index was proposed as a form of direct comparison of a single measure evaluated by light microscopy and the IM generated by BSL. According to VMI, the same value, obtained in the same metrics, was given to V (measured in a 0 to 5 scale; folded by 20) and PSCM (measured in percentage; 0-100%) because up to this experiment the influence of each parameter in BSL analysis was unknown. This index was composed in accordance with the equation:

$$VMI = \frac{1}{2}(20V + PSCM) \quad \text{(Eq. 1)}$$

In experiment III, one single sample was illuminated alternately, throughout time, with red and green laser in the same intensity, and the evaluations provided by IM and VMI were compared. Eight illuminations were performed at room temperature allowing two-minute intervals (0, 2, 4, 6, 8, 10, 12 and 14 minutes after thawing). To each BSL results obtained, another light microscopy evaluation was done. The choice of light microscopy was due to the short time elapsed among the evaluations.

Statistical Analysis

In the first and second experiments, IM values - for the combinations found among the classes of V and PSCM investigated with the two sources of laser (red and green) - were evaluated by means of an exploratory analysis, involving the calculation of the mean and standard deviation. In the second experiment, the analyses included VMI data. In these experiments, three classes of PSCM (C1 ≤ 30%; C2 – 30≤50%; C3>50%) and V (C1≤ 2; C2 – 2≤3 and C3>3) were established to functional analysis. The correlation coefficient between IM and the variables V, PSCM and VMI, as ascribed by two raters, was obtained and tested by the Spearman correlation test, with a nominal significance level of 5%. The adjustment of a simple linear regression equation performed for the IM as related to the VMI for the sources of red and green laser. Three classes of VMI were also established (C1≤ 50; C2 – 50≤ 70; C3>70) as mentioned before. In order to assess decreasing motility throughout the time a temporal regression analysis of IM and VMI was done in the third assay. For all statistic analysis, the statistical software Sisvar® version 4.0 [30] and R® version 3.4.3 [31] were utilized.

Results

Experiment I

The results from red laser illumination of the 56 samples showed positive correlations among V, PSCM and the IM. Apparently, correlations between PSCM and IM were greater than V and IM (Table 1). The green laser illumination of 20 samples also exhibited similar behavior (Table 2). Spearman coefficients relating inertial moment to percent sperm cell motility and velocity for two evaluators are presented. Tables 3 and 4 classified the features analyzed in groups of inertial moment ranges considering red and green laser illuminations. The inertial moment was influenced by both parameters.

	V A1	V A2	PSCM A1	PSCM A2	IM
V A1	1.0000				0.5906
					(p<0.0001)
V A2	0.8362	1.0000			0.5436
	(p<0.0001)				(p<0.0001)
V	----	----	----	----	0.5945
Me					(p=0.0002)
PSCM	0.8767	----	1.0000		0.6747
A1	(p<0.0001)				(p<0.0001)
PSCM	-----	0.8631	0.9228	1.0000	0.6469
A2		(p<0.0001)	(p<0.0001)		(p<0.0001)
PSCM	----	----	----	----	0.6651
Me					(p<0.0001)

Table 1: Spearman correlation coefficients estimate with their respective significances for all the variables velocity (V) and Percent Sperm Cell Motility (PSCM) ascribed by two raters (A1 and A2) and for their means (Me), and Inertial Moment (IM) on the basis of 56 samples of bovine frozen semen analyzed under red laser.

	V A1	V A2	PSCM A1	PSCM A2	IM
V A1	1.0000				0.6811
					(p=0.0009)
V A2	0.8709	1.0000			0.7999
	(p<0.0001)				(p<0.0001)
V	----	----	----	----	0.7342
Me					(p=0.0002)
PSCM	0.7679	----	1.0000		0.6563
A1	(p<0.0001)				(p=0.0017)
PSCM	-----	0.8461	0.9378	1.0000	0.7579
A2		(p<0.0001)	(p<0.0001)		(p=0.0001)
PSCM	----	----	----	----	0.6840
Me					(p=0.0009)

Table 2: Spearman correlation coefficients estimate with their respective significances for all the variables velocity (V) and percent sperm motility (PSCM) ascribed by two raters (A1 and A2) and for their means (Me), and Inertial Moment (IM) on the basis of 20 samples of bovine frozen semen analyzed under green laser.

		Percent sperm cell motility classes*			Mean
		1	2	3	
Velocity classes*	1	101.79	145.16	----	106.13
		(13.06)			(18.43)
	2	100.19	145.75	172.75	135.40
		(15.77)	(37.40)		(38.51)
	3	----	158.96	180.21	176.79
			(41.44)	(48.49)	(47.45)
Mean		101.29	149.84	179.94	
		(13.28)	(36.57)	(47.57)	
*Classes of percent sperm motility: 1 (≤30); 2 (30 a 50); 3 (>50). Classes of velocity: 1 (≤2); 2 (2 a 3); 3 (>3).					

Table 3: Mean values (standard deviation) of the Inertial Moment (IM) related to the classes of velocity and percent sperm motility for bovine frozen semen obtained with the red laser.

		Percent sperm cell motility classes*			Mean
		1	2	3	
Velocity classes*	1	64.96	65.82	----	65.13
		(6.04)			(5.25)
	2	75.58	66.37	70.50	68.59
			(3.95)		(4.88)
	3	----	80.85	87.74	85.44
			(5.62)	(16.02)	(13.43)

Mean		67.08	71.73	85.28	
		(7.07)	(8.53)	(16.02)	
*Classes of percent sperm motility: 1 (≤30); 2 (30 a 50); 3 (>50). Classes of velocity: 1 (≤2); 2 (2 a 3); 3 (>3).					

Table 4: Mean values (standard deviation) of the Inertial Moment (IM) related to the classes of velocity and percent sperm motility for bovine frozen semen obtained with the green laser.

Experiment II

Table 5 shows that the index can represent both features, once its value is in the same metrics of V and PSCM, and positive correlations were verified. Similar values were observed even to V and PSCM separately or grouped in the VMI index. The classification in classes allowed a relationship of IM values with some defined ranges of VMI (Table 6). Figure 2 presents curves fitted to IM versus VMI values under red and green lasers, although fitted curves expressed a linear pattern.

	V	PSCM	VMI	IM Red.	IM Green
V	1.0000				0.5906
					(p<0.0001)
PSCM	0.9506	1.0000			0.6132
	(p<0.0001)				(p<0.0001)
VMI	----	----	1.0000		0.6186
					(p<0.0001)
IM Red	0.8019	0.8267	0.8403	1.0000	----
	(p<0.0001)	(p<0.0001)	(p<0.0001)		
IM Green	0.5906	0.6132	0.6186	----	1.0000
	(p<0.0001)	(p<0.0001)	(p<0.0001)		

Table 5: Spearman correlation coefficients estimate with their respective significances for all the variables velocity (V), Percent Sperm Motility (PSCM), Inertial Moment (IM) and Velocity and Motility Index (VMI) on the basis of 46 samples of bovine frozen semen analyzed under red and green lasers light.

Laser light	*VMI classes		
	1	2	3
Red	130.19 (31.26)	205.94 (69.36)	402.61 (261.25)
Green	187.75 (58.24)	248.29 (130.9)	545.69 (362.15)
*Classes of VMI: 1 (≥50); 2 (50 a 70); 3 (>70).			

Table 6: Mean values (standard deviation) of the Inertial Moment (IM) obtained with the green and red lasers related to the Velocity and Motility Index (VMI) for bovine semen.

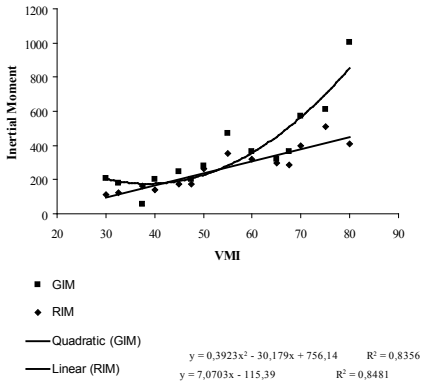

Figure 2: Mean values of Inertial Moment (IM) obtained by the types of red (RIM) and green (GIM) laser related to Velocity and Motility Index (VMI).

Experiment III

The behavior of semen motility in time under the two wavelengths is presented in Figure 3 with the evolution of the IM in time fitted by a second order curve with R2 about 0.98 to green and 0.96 to red laser. The VMI behavior is observed in Figure 4. In both evaluations it was observed a decrease in semen activity.

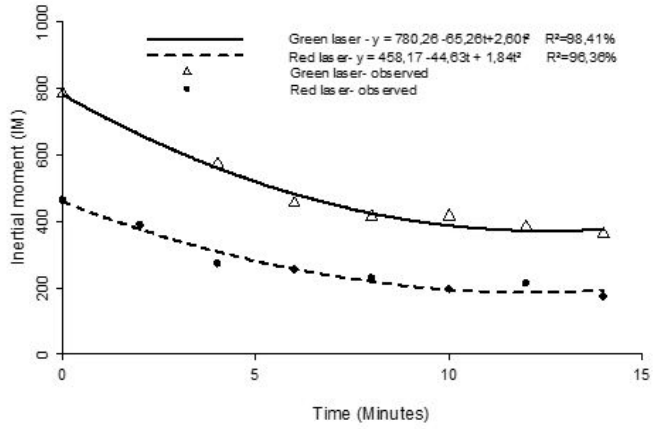

Figure 3: Inertial Moment (IM) values obtained by red and green lasers light related to the time.

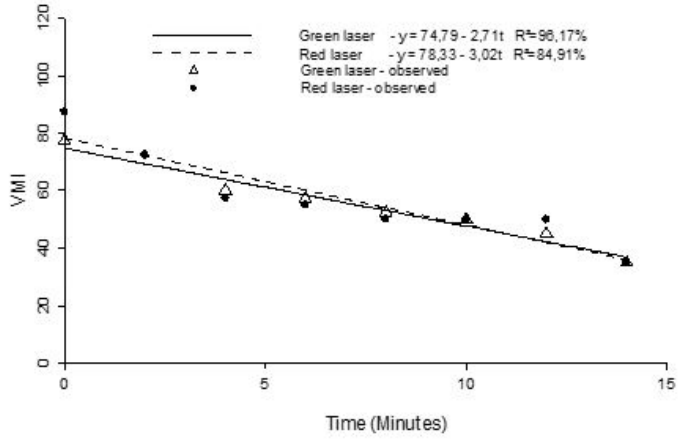

Figure 4: Velocity and Motility Index (VMI) values obtained by microscopy evaluation for samples by red and green lasers light related to the time.

Discussion

Experiment I

Variations between estimates made by the two evaluators were kept within 10% limits and so average values were used to subsequent analysis. Several studies uphold the reliability of light microscopy data to examine sperm motility, used as control of IM results in our work.

Coefficient of Variation (CV) of 21% for sperm motility evaluation by manual methods was verified [32], close to that observed (24%) in Computer Assisted Sperm Analysis (CASA) [33]. Nevertheless, a CV of 44.3% was observed among 26 technicians who estimated sperm cell concentration in a single semen sample using light microscopy [34]; similar results were obtained for motility and morphology evaluation. Interestingly, CVs of 39.5% and 45.0% were obtained in an inter laboratory evaluation when using CASA and subjective (light microscopy) analyses, respectively [13]. The small variation observed between the two technicians in this study was due to the standard training received in our laboratory and was considered precise enough to support our results.

Inertial moment values ranged from 78 to 311 and 60 to 111 in red and green laser analyses, respectively. For both laser sources, increasing velocity and percent sperm cell motility values corresponded to progressively higher average IM values in the BSL analysis. Previous work of our laboratory (data not published), using undiluted raw semen of the ram, showed IM values ranging approximately from 1000 to 3000 in a different setup. IM was strongly influenced by velocity and percent sperm motility in this species. It was not possible to affirm that other features of the ejaculate, like seminal plasma differences, were responsible for interfering with the IM behavior.

Whereas mean values presented a clear tendency, individual relations expressed some stable states mainly in the velocity feature. Otherwise, the percent sperm motility values were reliable in all configurations, which suggest a strong relation between PSCM and IM, allowing the conclusion that the laser technique is better to evaluate activity related to the movement. Further studies comparing computer-assisted sperm analysis and the BSL technique, using bovine frozen semen, indicated that IM values were related to motile and progressive percentual count, progressive concentration as well as curvilinear velocity (unpublished data).

The results of the present study allowed the conclusion that the inertial moment presented coherent values to V and PSCM to both evaluators under the two light sources.

Experiment II

Apparently no effect of stimulation on the spermatozoa was observed caused by the incidence of either red or green wavelength, as we can see by the decreasing of the VMI and IM values throughout the time of analysis (Figures 2 and 3), which indicates that short time incidence of laser on the specimen was not sufficient to alter their biological properties. Some authors [25] observed significant ($p<0.05$) velocity increase from five minutes of irradiation with the green laser (532nm; 0.38 J/cm^2), while others [27], using red laser (660 nm; 4.0 Joules; power from

30-100 mW; 133 seconds), also observed an increment ($p<0.05$) of sperm progressive motility in the bull. In our study these kind of improvement was not detectable, probably because of the short time of exposition.

The regression curve between inertial moment and the VMI index showed that 83% of IM value variations could be explained by variations that occur in the VMI index. The off-set between the laser sources can be attributed to the back-scattering configuration, with the back ground and the liquid absorbing more the real wavelength without interfering with the dynamics of the phenomenon monitored, thus presenting the same tendency to both cases (laser sources).

Results showed that the index proposed in this work is a reliable tool to simultaneously compare V and PSCM with IM. Its parametric characteristic can be pointed out as a positive factor.

Experiment III

The off-set between the evolution of red and green laser curve over a sample in the third experiment reinforces the hypothesis about the absorptive property of the light by the background-sperm. When light microscopy evaluation was compared to IM data over the time it has been observed that spermatozoa motility decreasing judged by the BSL was less intense in relation to the human estimates. This fact can be interpreted as the human tendency to force a continuous decrease in the result of VMI, whereas the objective results of IM curves point to an expected or reliable tendency of physical phenomena. Besides, the BSL analysis is not subjective and inertial moment values are more precise to assess the behavior of a biological phenomenon.

In conclusion, evaluation of semen motility by the IM could be a reliable and objective approach to estimate fertility in the frozen semen.

Acknowledgment

The authors wish to express their gratitude to Dr Fernando Vilela (ABS-Pecplan, Uberaba, Brazil), for the technical assistance. Reported research was supported financially by FAPEMIG (Grant EDT – CVZ 94/07) and CNPq.

References

1. Marnet B, Vieitez G, Milhet P, Richoilley G, Lesourd F, et al. (2000) Computer-assisted assessment of sperm morphology: Comparison with conventional techniques. Int J Androl 23: 22-28.
2. Love CC (2011) Relationship between sperm motility, morphology and the fertility of stallions. Theriogenology. 76: 547-557.
3. Del Olmo E, Bisbal A, Maroto-Morales A, et al. (2013) Fertility of cryopreserved ovine semen is determined by sperm velocity. Anim Reprod Sci 138: 102-109.
4. Gliozzi TM, Turri F, Manes S, Cassinelli C, Pizzi F (2017) The combination of kinetic and flow cytometric semen parameters as a tool to predict fertility in cryopreserved bull semen. Animal 11: 1-8.
5. Nie GJ, Wenzel JGW, Johnson KE (2002) Comparison of pregnancy outcome in mares among methods used to evaluate and select spermatozoa for insemination. Anim Reprod Sci 69: 211-222.
6. Iguer-Ouada M, Verstegen JP (2001) Evaluation of the Hamilton Thorn computer-based automated system for dog semen analysis. Theriogenology 55: 733-749.
7. Vyt P, Maes D, Rijsselaere T, Dejonckheere E, Castryck F, et al. (2004) Motility assessment of porcine spermatozoa: A comparison of methods. Reprod Domest Anim 39: 447-453.
8. Van Der Steeg JW, Steures P, Eijkemans MJ, F Habbema JD, Hompes PG, et al. (2010) Role of semen analysis in subfertile couples. Fertil Steril 95: 1013-1019.
9. Seidel GE (2012) Several insights on evaluation of semen. Anim Reprod 9: 329-332.
10. Amann RP, Waberski D (2014) Computer-assisted sperm analysis (CASA): Capabilities and potential developments. Theriogenology 81: 5-17.
11. Mortimer D, Mortimer S, Horst G der (2015) The future of computer-aided sperm analysis. Asian J Androl 17: 545.
12. Komori K, Tsujimura A, Ishijima S, Tanjapatkul P, Fujita K, et al. (2006) Comparative study of Sperm Motility Analysis System and conventional microscopic semen analysis. Reprod Med Biol 5: 195-200.
13. Brito LF (2016) A multilaboratory study on the variability of bovine semen analysis. Theriogenology 85: 254-266.
14. Graham JK (2001) Assessment of sperm quality: a flow cytometric approach. Anim Reprod Sci 68: 239-247.
15. Moscatelli N, Spagnolo B, Pisanello M, Lemma ED, De Vittorio M, et al. (2017) Single-cell-based evaluation of sperm progressive motility via fluorescent assessment of mitochondria membrane potential. Sci Rep 7: 17931.
16. Ross DA, Dhadwal S, Foulkes JA (1983) Laser measurement of the motility of bull spermatozoa in an egg-yolk diluent. J Reprod Fertil 67: 263-268.
17. Bergé P, Volochine B, Billard R, Hameli A (1967) Mise en evidence du mouvement propre de microorganisms vivants grace a l'etude de la diffusion inelastique de lumière. C. R. Acad. Sci. (Paris) Ser. D.265:889-892. Comptes Rendus l'Académie des Sci 265: 889-892.
18. Harvey JD, Woolford MW (1980) Laser light-scattering studies of bull spermatozoa. I. Orientational effects. Biophys J 31: 147-156.
19. Woolford MW, Harvey JD (1982) Light-scattering studies of bull spermatozoa. II. Interaction and concentration effects. Biophys J 40: 7-16.
20. Briers JD (1975) Wavelength Dependence of Intensity Fluctuations in Laser Speckle Patterns From Biological Specimens. Opt Commun 13: 3-5.
21. Asakura T, Takai N (1981) Dynamic laser speckles and their application to velocity measurements of the diffuse object. Appl Phys 25: 179-194.

22. Zdunek A, Adamiak A, Pieczywek PM, Kurenda A (2014) The biospeckle method for the investigation of agricultural crops: A review. Opt Lasers Eng 52: 276-285.

23. Braga Júnior RA (2017) When noise became information: State-of-the-art in biospeckle laser. Ciência e Agrotecnologia 41: 359-366.

24. Carvalho PH, Barreto JB, Braga R, Rabelo GF (2009) Motility parameters assessment of bovine frozen semen by biospeckle laser (BSL) system. Biosyst Eng 102: 31-35.

25. Abdel-Salam Z, Dessouki SHM, Abdel-Salam SAM, Ibrahim MAM, Harith MA (2011) Green laser irradiation effects on buffalo semen. Theriogenology 75: 988-994.

26. Iaffaldano N, Paventi G, Pizzuto R, et al. (2013) The post-thaw irradiation of avian spermatozoa with He-Ne laser differently affects chicken, pheasant and turkey sperm quality. Anim Reprod Sci 142: 168-172.

27. Fernandes GHC, de Carvalho PDTC, Serra AJ, et al. (2015) The Effect of Low-Level Laser Irradiation on Sperm Motility, and Integrity of the Plasma Membrane and Acrosome in Cryopreserved Bovine Sperm. PLoS One 10: e0121487.

28. Arizaga R, Trivi M, Rabal H (1999) Speckle time evolution characterization by the co-occurrence matrix analysis. Opt Laser Technol 31: 163-169.

29. Henry M, Neves JP, Jobim MIM (2013) Manual Para Exame Andrológico e Avaliação de Sêmen Animal. Terceira. (CBRA, ed.). Belo Horizonte, MG, Brasil.

30. Ferreira DF (2011) Sisvar: a computer statistical analysis system. Cienc e Agrotecnologia 35: 1039-1042.

31. The R Core Team (2017) R: A Language and Environment for Statistical Computing 3:3585.

32. Neuwinger J, Behre HM, Nieschlag E (1990) External quality control in the andrology laboratory: an experimental multicenter trial. The investigators and centers involved in this study were Prof. Dr. Julian Frick, Urologische Abteilung, Landeskrankenhaus Salzburg, Austria; Dr. Werner G. Gehring an. Fertil Steril 54: 308-314.

33. Holt W, Watson P, Curry M, Holt C (1994) Reproducibility of computer-aided semen analysis: comparison of five different systems used in a practical workshop. Fertil Steril 62: 1277-1282.

34. Jequier AM, Ukombe EB (1983) Errors Inherent in the Performance of a Routine Semen Analysis. Br J Urol 55: 434-436.