Archives of Veterinary Science **ISSN 1517-784X**
v.25, n.3, p.33-45, 2020 www.ser.ufpr.br/veterinary

USE OF A SMARTPHONE CAMERA ATTACHED TO A LIGHT MICROSCOPE TO DETERMINE EQUINE SPERM CONCENTRATION IN IMAGEJ SOFTWARE

(Uso de câmera de smartphone em microscópio óptico para determinar concentração espermática equina no software ImageJ)

[1]Giovanna Polo Freitag[https://orcid.org/0000-0002-4771-1243], [1]Luis Gustavo Freitag de Lima[https://orcid.org/0000-0003-2186-9461], [1]Luiz Ernandes Kozicki[https://orcid.org/0000-0002-3700-1811], [1]Liedge Camila Simioni Felicio[https://orcid.org/0000-0001-5009-1148], [2]Romildo Romualdo Weiss[https://orcid.org/0000-0002-7218-1184]

[1]Pontifícia Universidade Católica do Paraná, Curitiba, Paraná; [2]Universidade Federal do Paraná, Curitiba, Paraná

E-mail: giovanna_w7@hotmail.com

ABSTRACT: This study aimed to determine stallion sperm concentration by ImageJ analysis of images captured using a cell phone camera attached to a LED light microscope and compare the obtained results to those of a standard method (Neubauer chamber). A total of 108 gel-free semen samples (fresh, unextended semen = 89; cooled diluted semen = 19) were analyzed by both methods. For ImageJ analyses, photographs were taken from fields outside of the grid zone of Neubauer chambers, after visual counting with the standard method. Diluted samples were also analyzed using a commercial computer-assisted sperm analysis (CCASA) system. ImageJ overestimated sperm concentration by 2.5% compared with the standard method, whereas CCASA resulted in an overestimation of 165.7%. Given that Neubauer chamber counts have a 10% tolerance related to chamber variations and human errors, differences between the standard method and ImageJ were deemed acceptable, being under 10%. ImageJ and standard values correlated positively (r = 0.99, P < 0.0001). The Bland–Altman test showed excellent agreement between ImageJ and standard results, with a bias close to zero and a range of 30 × 10^6 cells/mL. On the other hand, there was a high estimated bias between the standard method and CCASA, with a range of −52.4 to 335.7 × 10^6 cells/mL, indicating a significant overestimation of results by CCASA. Analysis time was significantly lower by ImageJ than by Neubauer chamber counting. Equine semen concentration can be accurately determined using ImageJ, but photographs taken with a cell phone camera and light microscope may need to be enhanced before analysis. ImageJ analysis is a simple, fast, and low-cost method for estimation of equine semen concentration.

Keywords: semen; sperm counting; equine; software; cell phone.

RESUMO: O objetivo desse estudo foi determinar a concentração espermática de garanhões com o software ImageJ por meio da análise de imagens capturadas usando uma câmera de telefone celular acoplada a um microscópio óptico e comparar os resultados obtidos com os do método padrão (câmara de Neubauer). Um total de 108 amostras de sêmen sem gel (sêmen fresco, não diluído = 89; sêmen refrigerado, diluído = 19) foram analisadas pelos dois métodos. Para

Recebido em 20/02/2020
Aprovado em 23/06/2020

análises com ImageJ, foram tiradas fotografias de campos fora da zona de grade da câmara de Neubauer, após contagem visual com o método padrão. As amostras diluídas também foram analisadas usando um sistema comercial de análise de espermatozoides assistida por computador (CCASA). ImageJ superestimou a concentração de espermatozoides em 2,5% em comparação com o método padrão, enquanto o CCASA resultou em uma superestimação de 165,7%. Devido à contagem na câmara de Neubauer apresentar uma tolerância de 10% relacionada a variações de câmara e erros humanos, as diferenças entre o método padrão e o ImageJ foram consideradas aceitáveis, estando abaixo de 10%. ImageJ e valores padrão correlacionaram-se positivamente ($r = 0,99$, $P < 0,0001$). O teste de Bland–Altman mostrou excelente concordância entre o ImageJ e o método padrão, com um viés próximo de zero e um intervalo de 30×10^6 células/mL. Por outro lado, houve um alto viés estimado entre o método padrão e o CCASA, com um intervalo de -52,4 a $335,7 \times 10^6$ células/mL, indicando uma superestimação significativa dos resultados pelo CCASA. O tempo de análise da concentração espermática foi significativamente menor pelo ImageJ do que na câmara de Neubauer. A concentração de sêmen equino pode ser determinada com precisão usando o ImageJ, mas as fotografias tiradas com uma câmera de telefone celular em um microscópio óptico devem ser aprimoradas antes da análise. A análise ImageJ é um método simples, rápido e de baixo custo para estimar a concentração de sêmen equino.

Palavras chave: sêmen; contagem espermática; equino; software; celular.

INTRODUCTION

Sperm concentration is a fundamental component of breeding soundness examination and provides important information to determine the number of semen doses and the required semen extender for preservation. These parameters are particularly important when the semen is intended to be used for insemination of many mares (McKinnon et al., 2011; Turner, 2005).

Sperm concentration can be assessed by a variety of methods. The Brazilian College of Animal Reproduction defines hemocytometer counting as the most accurate method (CBRA, 2013). More recent and rapid methods exist, such as NucleoCounter (Morrell et al., 2010) and flow cytometry; however, a previous study (Rigby et al., 2001) showed that care must be taken when using methods other than hemocytometer counting to ensure that the proper dose is defined, especially when a large number of mares are to be inseminated. Nevertheless, all methods are susceptible to errors, as they depend on pipetting, dilution, and chamber filling, which are performed manually (Amann & Waberski, 2014; Zinaman et al., 1996).

A quality control test revealed that differences in hemocytometer counts between laboratories reached 37.5%; variations were higher for samples with low sperm concentrations and lower for samples with high concentrations (Neuwinger, 1990). In another study (Brazil et al., 2004), differences in hemocytometer counts obtained by a single technician, multiple technicians, and in relation to an established value ranged from 12.5 to 16.6%. Although hemocytometer counting is more time-consuming than other methods, it is considered the standard for sperm counting (Estrada & Samper, 2007; World Health Organization, 2010; CBRA, 2013).

Computer-assisted sperm analysis (CASA) is an objective method that uses hardware and software to detect, characterize, track, and count cells using a phase-

contrast microscope coupled to a camera (Amann & Waberski, 2014; Amann & Hammerstedt, 1980). The method is efficient in evaluating motility parameters but can be unreliable for counting cells of different species. The presence of cells that are similar in shape and size to sperm cells can lead to overestimation of sperm concentration by CASA, greatly affecting the count of low-concentration samples. In highly concentrated samples, some sperm cells are not counted, leading to underestimation (Verstegen et al., 2002; Iguer-Ouada & Verstegen, 2001; Rijsselaere et al., 2004). Zinaman et al. (1996) found that CASA and hemocytometer counts differed by 12.1%.

Motility testing is also influenced by inadequate sperm concentration (Verstegen et al., 2002). CASA cannot provide accurate results for samples with more than 50×10^6 or less than 20×10^6 sperm cells/mL (Verstegen et al., 2002; Rijsselaere et al., 2004). Iguer-Ouada and Verstegen (2001) counted dead sperm cells and obtained accurate results by CASA, indicating that limitations in sperm measurement are due to cell collisions. Errors can be minimized by staining sperm cells or improving the software with anti-collision systems (Zinaman et al., 1996; Klimowicz et al., 2008). Verstegen et al. (2002) suggested confirming sample concentrations manually or using dead sperm cells as well as adjusting sample concentration prior to motility evaluation. CASA results can be seriously affected by slight changes in settings, necessitating standardization (Verstegen et al., 2002; Davis & Katz, 1993; Schleh & Leoni, 2003).

Amann and Waberski (2014) emphasized the importance of developing a simplified, field-suitable CASA system that can measure three or four seminal parameters for field theriogenologists. In a previous study (Nafisi et al., 2005), a camera was coupled to a light microscope for seminal evaluation as a means to reduce costs, as CASA systems tend to be costly. The authors concluded that the contrast and sharpness of images must be enhanced for better identification of sperm cells. Wilson-Leedy and Ingermann (2007) developed a CASA plugin (available free of charge) for ImageJ, a free open source image processing program provided by the United States National Institutes of Health (https://imagej.nih.gov/ij/). The authors accurately assessed Zebrafish sperm motility-related parameters using a phase-contrast microscope, with the size of zebrafish sperm cells defined as 0–40 pixels.

This study aimed to test the ability of ImageJ to accurately determine stallion sperm concentrations using images acquired by a cell phone camera coupled to a LED light microscope and compare the results to those of the standard method (Neubauer chamber counting). A secondary objective was to compare the accuracy of ImageJ and other computerized systems (CCASA) regarding the standard method.

MATERIALS AND METHODS

The experiment was approved by the Animal Research Ethics Committee of the Pontifical Catholic University of Paraná, Brazil (protocol no. 1021).

Animals and semen collection

Semen collection was performed using a dummy mare and artificial vagina (Conboy, 2011). Thirty 3 to 20year-old healthy stallions without a history of reproductive diseases, of different breeds (Crioulo, Quarter-Horse, and Appaloosa) and from different private farms, were used in this study. Semen was collected 3 to 4 times per horse, according to stallion availability, resulting in a total of 108 samples.

Not all collections were made on the same day or farm. Farms were located in or on the outskirts of Curitiba (25°25′40″S 49°16′23″W), Paraná, Brazil. Samples were collected between March 2015 and March 2016, prior to and during the breeding season.

Evaluation of sperm concentration

Sperm concentration was evaluated by three methods. Hemocytometer (improved Neubauer chamber) counting was considered the standard method. ImageJ and CCASA (Hamilton-Thorne Sperm Analyzer, Hamilton Thorne Research, Beverly, MA, USA) results were compared to standard values.

A total of 108 samples were analyzed by Neubauer chamber and ImageJ, including 89 fresh, raw samples and 19 cooled, diluted samples. The 19 diluted samples were also evaluated by CCASA (Figure 1).

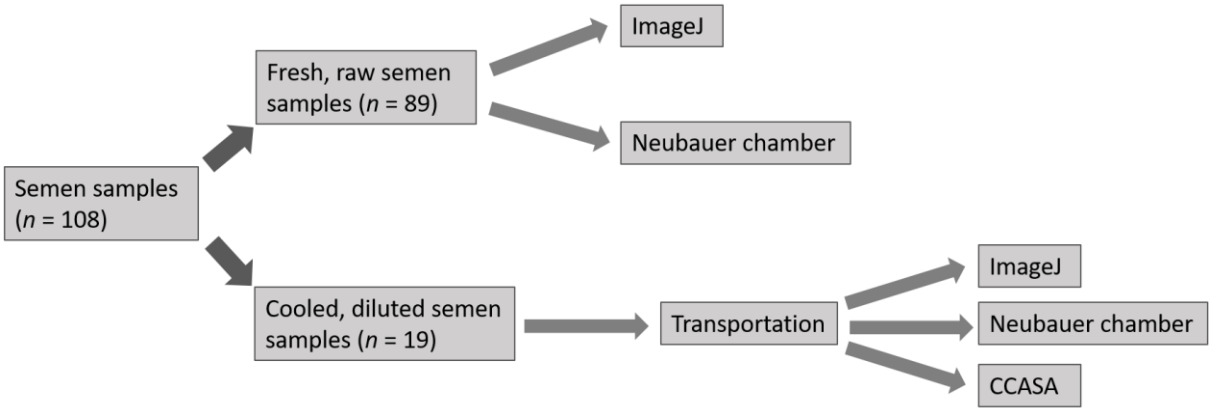

Figure 1. Diagram of the study design for comparison of stallion sperm concentrations determined by Neubauer chamber counting (standard method), commercial computer-assisted sperm analysis (CCASA), and ImageJ analysis. Cooled samples were used for comparisons involving CCASA; comparisons between ImageJ and Neubauer chamber included 108 samples (cooled and fresh samples).

Microscope and camera

Images were captured using a smartphone (Samsung Galaxy S5, 16 MP) attached, by a universal smartphone adapter (adapted from: http://www.thingiverse.com/thing:78071), to the eyepiece of a light microscope (Bioptika B20) with light-emitting diode (LED) illumination, as depicted in Figure 2.

Neubauer chamber counting

Fresh, gel-free, raw samples were diluted 1:20 in formaldehyde buffered saline before analysis. Both sides of the chamber were filled using a pipette, and, after 1 min, sperm cells located in 5 central squares of each side of the chamber were counted (400× magnification), from a total of 25 squares (Figure 3) (CBRA, 2013). Results were considered acceptable when the difference in sperm counts between the two sides of the chamber was below 10% (CBRA, 2013). Calculations were performed considering chamber size, number of counted squares, and dilution factor, as follows: Sperm concentration (10^6 cells/mL) = $n \div 1/10 \times 5/25 \times 1/20$, where n is the mean number of sperm cells in the two sides of the chamber (CBRA, 2013). The

mean time taken to perform the analysis, from the start of the counting procedure to the calculation of the results, was monitored in seconds.

Figure 2. Cell phone attached to a LED light microscope by a universal smartphone adapter for determination of stallion sperm concentration using image analyses with ImageJ.

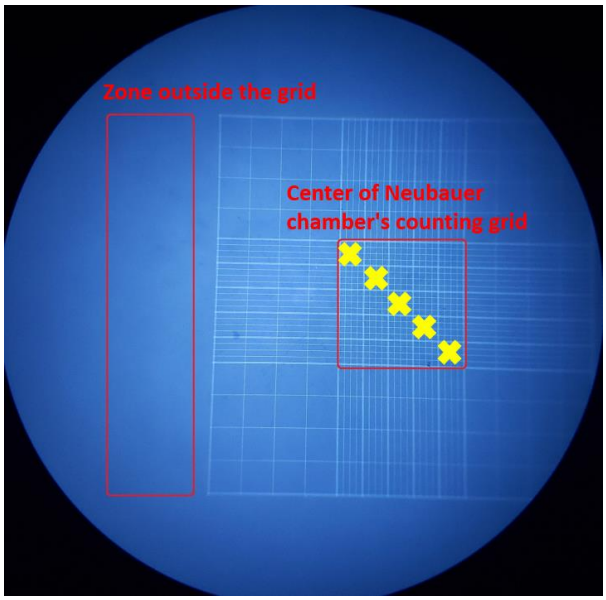

Figure 3. Microscopy image of a Neubauer chamber showing the central region of the grid where sperm cells were counted (squares marked with an X) and the outer zone photographed for ImageJ analysis.

ImageJ analysis

Immediately after the standard analysis was performed, a smartphone was attached to the microscope and the same chamber was photographed for ImageJ analysis. Photos were taken at 400× magnification from fields outside the grid zone (Figure 3). The chamber counting grid may interfere with ImageJ results because the software estimates the number of sperm cell heads based on pixel data; therefore, images need to have a single-colored background.

Before image processing, a photograph of the central grid of the hemocytometer was used to obtain the area equivalent to 1 grid square in ImageJ (Figure 4).

Aiming to determine whether ImageJ can provide accurate and representative results in a short analysis time, we analyzed only one photograph per sample. Images were imported to ImageJ in their native resolution (2988 × 5312 pixels), and the analyze tool was used to place a new grid with an area per point equivalent to that of one square of the Neubauer chamber. A selection was made to include two squares in the new grid, corresponding to two central squares of the Neubauer chamber. Then, the crop tool was used to analyze only sperm cells within the selected region (Figure 5).

Background signals were removed, and the image was enhanced using the default black and white threshold (Figure 6A and B).

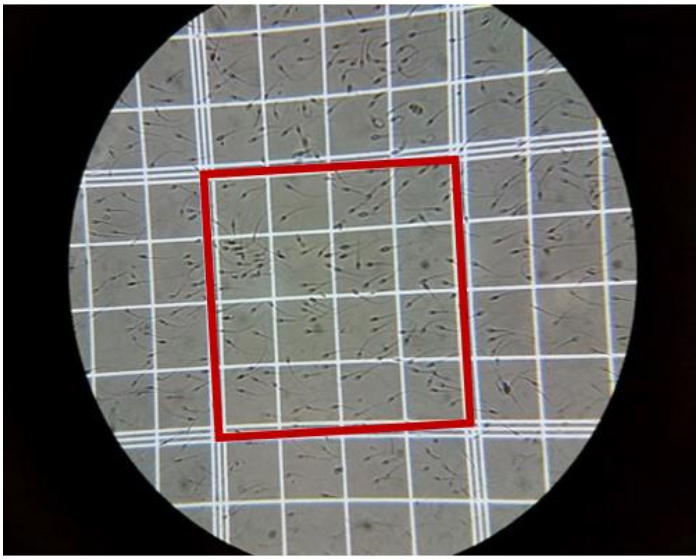

Figure 4. Micrograph of the central area of a Neubauer chamber, considered equivalent to one square for calibration prior to sperm cell counting using ImageJ.

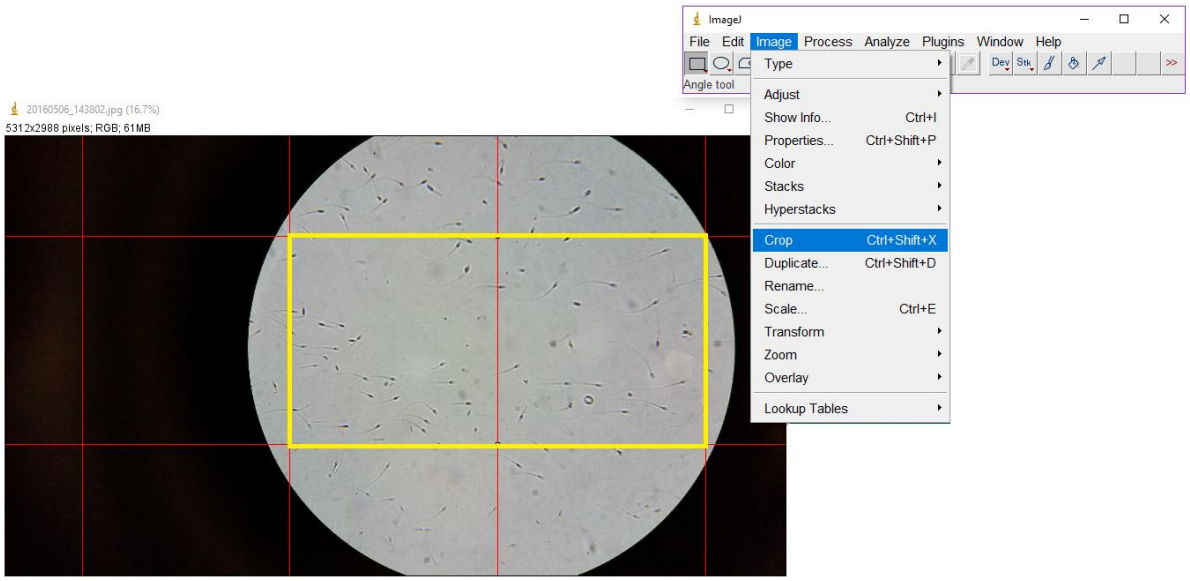

Figure 5. Use of ImageJ crop tool to select an area corresponding to two Neubauer chamber squares for stallion sperm cell counting.

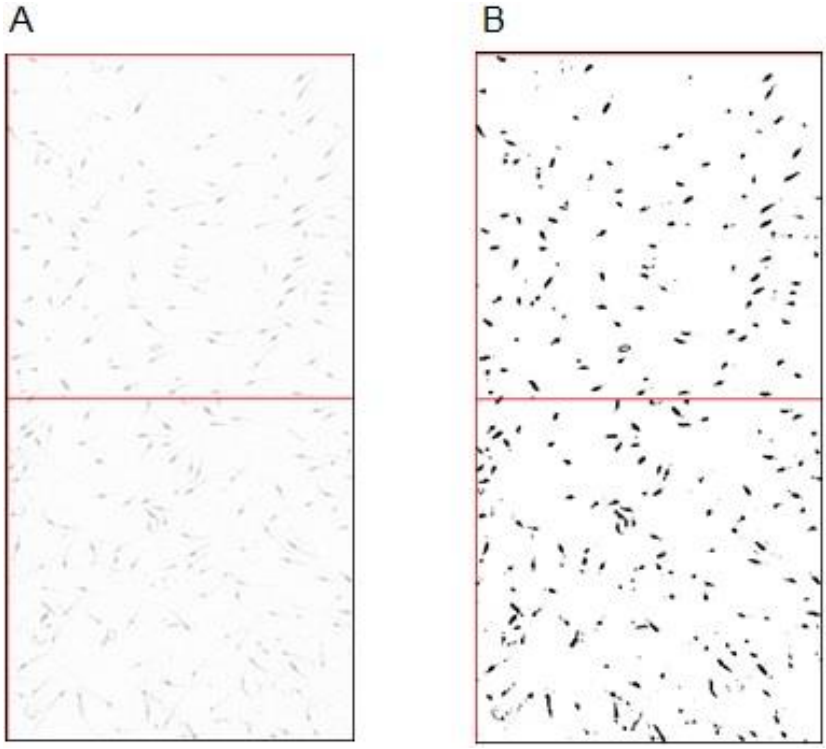

Figure 6. (A) Selected image area after background removal. (B) Sperm cells ready for counting in ImageJ after image enhancement with the threshold tool.

Finally, the analyze tool was selected, followed by analyze particles using a size of 170-infinity and 0.0–1.00 circularity. These parameters were defined prior to the experiment on the basis of trial and error and visual inspection of the obtained results. The total cell number provided by the program represents the total number of sperm cells in two grid squares; therefore, the value was divided by 2 to obtain the number of cells in one square and multiplied by 5 to obtain the sperm concentration in 10^6 cells/mL (CBRA, 2013).

The mean time taken to perform the analysis (from opening the image in the software to calculating the results) was monitored in seconds.

CCASA

CCASA was carried out in ISASD4C20L disposable counting chambers. Manufacturer-defined settings were used. The options equine species and count cells were selected. Results given represented sample concentration, as 10^6 sperm cells/mL. Because of the distance between the farms and the CASA equipment, samples had to be diluted with the extender in a proportion of 1:3 and cooled in proper boxes to support transportation, so they could be evaluated using the CCASA method. The diluted samples were then also evaluated with the Neubauer chamber and ImageJ. A limited number of samples were evaluated since we had to use cooled samples and not all the collections were made on the same day/ farm.

Statistical analysis

Pairwise comparisons between standard, ImageJ, and CCASA results were performed using Friedman's test. Pearson's correlation and linear regression analyses were performed for comparison of the standard method with computerized methods (ImageJ and CCASA). The Bland–Altman test was used to investigate the

agreement of ImageJ and CCASA estimates with standard results. Analysis times of ImageJ and Neubauer chamber analysis were compared by *t*-tests. Comparisons were always made between methods, not between samples.

The percentage variation of ImageJ and CCASA results in relation to standard results was calculated by subtracting the standard value from the computerized estimate and dividing the resulting value by the standard value. Mean percentage variations were used to assess differences between methods.

All statistical analyses were performed using GraphPad Prism version 6.0 (2012). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The objective of the current study was to determine whether the free software ImageJ can be used to perform stallion sperm counting with comparable accuracy to those of standard (Neubauer chamber) and computerized (CCASA) methods as a means to reduce costs in relation to CCASA.

The main challenge was to acquire images with good focus for the program to perform the analysis. Accurate results were obtained using images that focused on the sperm head rather than on the whole sperm cell (Figure 7A and B), as different tonalities of the same cell, when evident, can lead to double counts in ImageJ. We observed that “well-focused” images (Figure 7B), according to standards for human viewers, are not adequate for analysis by ImageJ. In Figure 7B, it is possible to observe different tonalities within a single sperm cell. However, ImageJ understands that differences in tonalities indicate different cells, leading to inadequate sperm counting. Homogeneous sperm cells are counted only once by the program. For this, the focus had to be modified so that the entire cell had the same gray tonality (Figure 7A). These findings showed that focus quality standards for human viewers are not the same as those for ImageJ analysis. It is also important to choose a field with homogeneous cell distribution. Images were taken from the same chamber in which visual counting was performed.

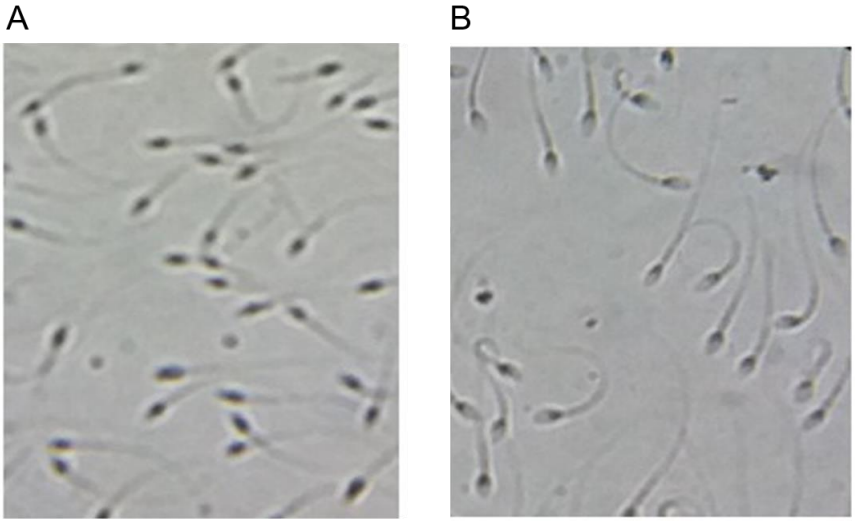

Figure 7. (A) Ideal focus quality for sperm counting using ImageJ. Note how sperm cells have a uniform tonality. (B) Ideal focus quality for sperm identification by the human eye, with differences in tonality between sperm head and body.

The Friedman test showed that results obtained using Neubauer chamber, ImageJ, and CCASA differed significantly (P < 0.0001), with CCASA results differing from those of Neubauer chamber and ImageJ. Means, standard deviations, and mean percentage variations of ImageJ and CCASA results compared to standard results are shown in Table 1.

Table 1. Mean ± standard deviation (10^6 sperm cells/mL) of stallion sperm concentrations determined by ImageJ and a commercial computer-assisted sperm analysis (CCASA) system compared to results obtained by the standard technique (Neubauer chamber counting), using cooled semen samples.

Samples (*n* = 19)	**Sperm concentration (10^6 sperm cells/mL)**	**Mean percentage variation from Neubauer chamber results (%)**
CCASA	381.1 ± 212.2[b]	165.7%
Neubauer	239.4 ± 180.3[a]	-
ImageJ	239.7 ± 179.2[a]	2.5%

Means in a column followed by different letters differ significantly at *P* < 0.05.

ImageJ overestimated sperm concentrations by only 2.5% in relation to the standard method. Given that the standard method has an error tolerance of 10% (CBRA, 2013) or higher (Neuwinger et al., 1990; Brazil et al., 2004), a variation of 2.5% from the value obtained with the Neubauer chamber is acceptable. The source of this error can be attributed to manual counting by humans on the hemocytometer or to the program itself. CCASA overestimated the values by 165.7% in relation to the standard method, corroborating previous reports of high overestimation (Iguer-Ouada & Verstegen, 2001). The authors obtained CASA results 70.0% higher than those obtained by hemocytometer counting.

Linear regression lines of ImageJ and CCASA results versus standard results are presented in scatter diagrams (Figure 8).

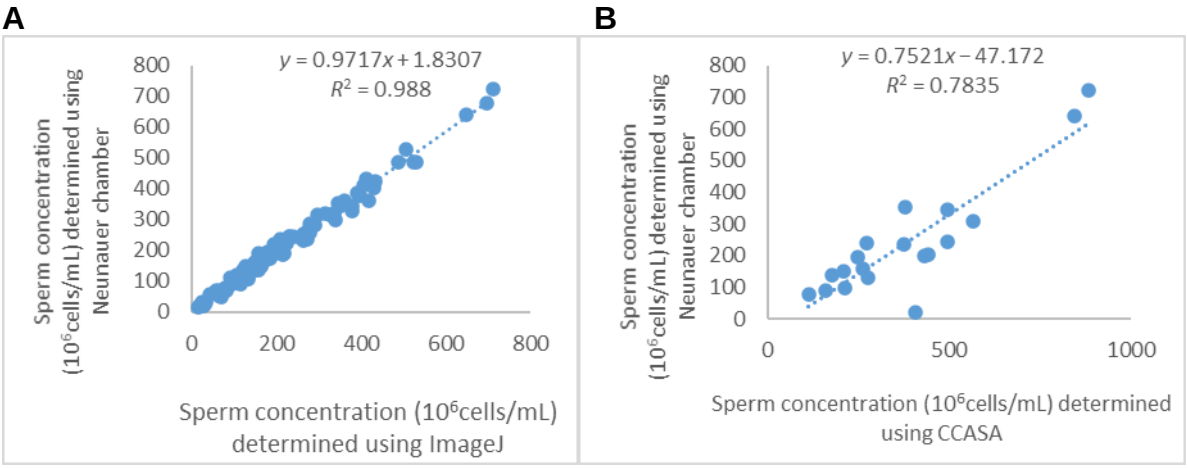

Figure 8. Scatter diagram with linear regression of stallion sperm concentration determined by ImageJ (A) and a commercial computer-assisted sperm analysis (CCASA) system (B) in relation to the results obtained by Neubauer chamber counting. R^2, coefficient of determination.

The correlation between ImageJ and Neubauer chamber results (*n* = 108 samples) was strong and significant (*r* = 0.99, *P* < 0.0001), as was the correlation

between CCASA and Neubauer chamber results (n = 19 samples) (r = 0.88, P < 0.001). The coefficient of determination of the relationship between standard results and ImageJ results was close to 1, indicating a good fit. Of the 19 samples analyzed by CCASA, 2 had extremely high concentrations because of individual variations among stallions. The correlation between methods was greatly influenced by these extreme values. Excluding the two samples, the correlation decreased to 0.63 (P = 0.005).

Bland–Altman plots are presented in Figure 9.

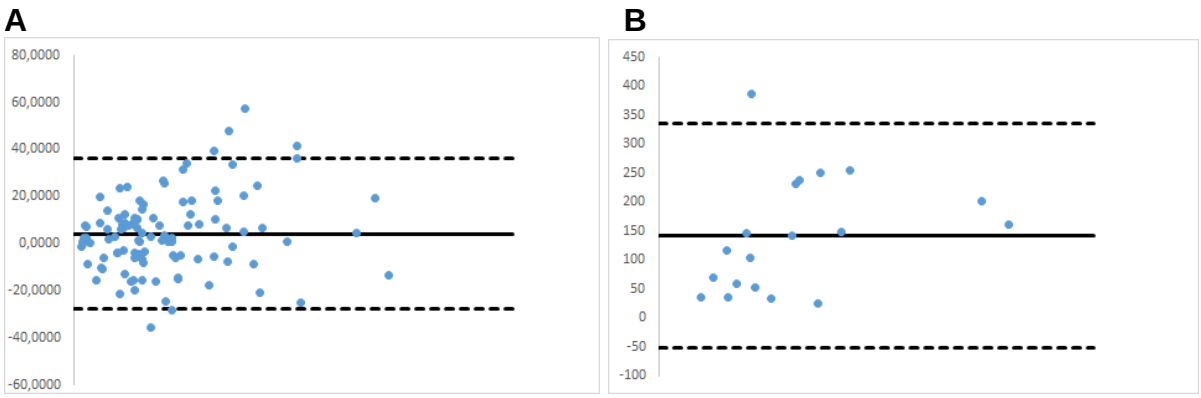

Figure 9. Bland–Altman plots showing differences in stallion sperm concentration determined using ImageJ (A) and a commercial computer-assisted sperm analysis (CCASA) system (B) in relation to that determined by Neubauer chamber counting. The central line represents the mean difference (bias), and dashed lines represent the limits of agreement.

Bland–Altman analysis was carried out to determine the agreement of results obtained with ImageJ and CCASA in relation to standard results. The discrepancy between ImageJ and standard results was small. Most values were plotted near the bias line, which was about 4.12. The results indicate that there is a 95% chance that ImageJ results are equal to Neubauer chamber counts, as only 4.63% of the samples were outside the limits of agreement. The upper and lower limits shown in Figure 9A indicate that the range of variation is about 30 × 10^6 sperm cells/mL, increasing as sperm concentration increases. This is a good result because a difference of 30 in a high-concentration sample (>300) is not as problematic as the same difference in a low-concentration sample. An error of up to 10% is acceptable for the standard method (CBRA, 2013). The mean difference line between CCASA and standard results was 142, a very high value when considering differences between concentrations. The results show that CCASA tends to overestimate sperm concentration. Lower and upper limits ranged from −52 to 336 × 10^6 sperm cells/mL. The bias indicates a clinically significant discrepancy between methods and, combined with the large range of variation, demonstrates the lack of agreement between methods.

Computerized methods count sperm cells based on the size of the sperm, in pixels. Therefore, particles with the same size as sperm cells may be counted as sperm, as frequently occurs in CASA (Verstegen et al., 2002). Because ImageJ was calibrated visually before analysis, differentiation between cells and debris was likely more accurate than that in CCASA, which was used with predefined settings. Semen extender may contain particles that interfere with cell counting (Verstegen et al.,

2002; Amann & Waberski, 2014), leading to overestimation by CCASA. Underestimation of highly concentrated samples can occur because of cell collisions or other unidentified factors (Zinaman et al., 1996; Verstegen et al., 2002; Iguer-Ouada & Verstegen, 2001). In the present study, CCASA results were overestimated in relation to standard results, possibly because the software considered debris and nonsperm cells during counting. Such overestimation can lead to the use of semen doses with insufficient sperm cells, hampering fertilization (Rigby et al., 2001; McKinnon et al., 2011; Verstegen et al., 2002).

The mean time required to perform ImageJ (58.7 ± 5.0 s) and Neubauer chamber counting (286.1 ± 97.3 s) differed significantly. A shorter analysis time in relation to the standard method is advantageous, as visual counting of cells can be very time-consuming, depending on the sample (Ax et al., 2004).

In summary, ImageJ afforded better results than CCASA. CCASA had very high bias, range, and percentage variation compared to the standard method. ImageJ afforded similar results to those of the standard method, with low bias, range, and percentage variation (2.5%). ImageJ results were strongly correlated with standard results and were obtained in a significantly shorter analysis time.

CONCLUSION

ImageJ can be used to determine stallion sperm concentration under field conditions from images acquired by a cell phone camera coupled to a LED microscope after enhancement (using grid, crop, background removal, and threshold tools). ImageJ results were consistent and agreed with standard results. The use of ImageJ obviates the need for visual sperm counting, reducing analysis time. ImageJ is a simple, fast, and low-cost method to determine stallion sperm concentration.

ACKNOWLEDGMENTS

The authors thank Professors Marco Flávio Mattana, Alceu de Souza Britto Junior, Edson José Rodrigues Justino, Jacques Facon, and Luis Fernando Fonseca Kasprzak from different departments of the Pontifical Catholic University of Paraná, Brazil, for their assistance with ImageJ analysis; Dr. Marcilio Nichi from the São Paulo State University for permitting the use of the CCASA system; Dr. Saulo Henrique Weber for the assistance with statistical analysis; and the Pontifical Catholic University of Paraná for the scholarship provided to one of the authors. This research did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit-sector.

ETHICAL STATEMENT

All experimental procedures were approved by the Animal Research Ethics Committee of the Pontifical Catholic University of Paraná, Brazil (protocol no. 1021).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research.

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