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Review Article

The Use of Computer Assisted Sperm Analysis (CASA) in Domestic Animal Reproduction: A Review

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Abstract

Computer Assisted Sperm Analysis (CASA) was developed in the late 1980s to study the sperm movement characteristics or kinematics. Since then, CASA is becoming among the most important tools in reproductive biotechnologies laboratories and research centers related to animal breeding although the doubt about its effectiveness. This review aimed to investigate the different studies and reports performed by the scientific community recently regarding CASA system, as well as to suggest new areas of use and improvement for this automated device to better interpret the complexity surrounding the sperm sample. The main problem is related to the standardization and optimization of the equipment and procedures. CASA system has evolved dramatically over the past two decades to become powerful tool for the rapid and objective assessment of sperm concentration, motility and kinematics, as well as morphology, in almost all mammals, including humans. Despite the lack of full universal standardization, the various CASA instruments have all currently demonstrated high levels of accuracy and reliability.

Keywords: Computer assisted sperm analysis, CASA, Domestic animal, Sperm, Clinical applications, Standardization

Introduction

Sperm analysis is of great importance when investigating male infertility [1]. A variety of cells enters into sperm composition, in particular, spermatozoa (spz). The latter are composed of several membrane-bound sections, consisting of the plasma membrane, acrosome membrane, and mitochondrial membrane, that must be intact to ensure the viability of the spz to fertilize the oocyte [2].

Andrology laboratories around the world assess semen quantity (volume, sperm concentration, color, density, and viscosity) and quality parameters (total and progressive motility, morphological abnormalities, oxidative status, mitochondrial Activity and DNA fragmentation) [3-12]. Different studies have been performed in pigs [3], rams [4-6], dogs [7], goats [8,9], roosters [10], horses [11], and bulls [12] to examine relationship between such *in vitro* laboratory standards of fertile semen and their relationship with *in vivo* field fertility. The most important objective is to ensure the successful packaging of a straw with fertile spz that meets semen laboratory standards.

Computer Assisted Sperm Analysis (CASA) was developed to study sperm movement characteristics or kinematics and has proven to be very effective in research [1]. CASA has also been used with great success to record sperm characteristics such as concentration and proportions of total and progressive motility in many animal species, including wide application in domestic animal breeding laboratories and research centers [13]. The results obtained allow a great advance in the field of reproduction of domestic animals, especially providing solutions to the threat of animal disappearance by extinction of the breed [14].

This review aimed to document the recent contribution of the scientific community who used and benefited from CASA system in different experiments. Furthermore, the authors tend to suggest new areas of use and improvements

for CASA to better interpret the complexity of the sperm sample. Specifically in this literature, the authors focused on the use, the advantages, and the limits of CASA system.

Presentation and History

CASA is an automated system consisting of hardware and software that is used to view and digitize a sequence of sperm images. It enables clear, precise results to be obtained, which inform about different qualities of sperm parameters, including concentration, motility, morphology, and morphometry [15]. After spz scanning and placing the coordinates (x, y) on the observation field, the same procedure is repeated during the following successive captures. Then an algorithm is used to analyze all the data and reconstruct the individual trajectories of the sperm [16].

The development of computer-assisted sperm analysis (CASA) during the 1980s aroused great excitement, although the systems were only black boxes capable of tracking sperm movement, without being able to generate verifiable data, with a frame rate limited to 30 frames per second [17,18]. In the 1990s and early 2000s, advances in computer science led to several improvements to CASA systems. During this time, CASA has gained in value, especially in research area.

In the early stages of development of the CASA system, various problems arose; initially, CASA could not differentiate sperm from particles of the same size. To solve this problem, several approaches have been tried through modifications and improvements of the software, for example, the presence of the flagellum as a prerequisite [19] or the use of dyes for sperm DNA staining [20]. Currently, more than twelve different CASA systems brands are available for semen quality evaluation in laboratories and AI centres [17]. We cite IVOS and CEROS by Hamliton-Thorn and Motion Analysis (USA) *(Fig. 1)*, the Sperm Class Analyzer by Microptics SL (Spain), Hobson Tracker (UK), Sperm Motility Quantifier (South Africa), Sperm Vision (Germany) and Proiser/ISAS (Spain) and many others. Therefore, it is necessary to update the standardization of the methods for the objective evaluation of sperm quality [21].

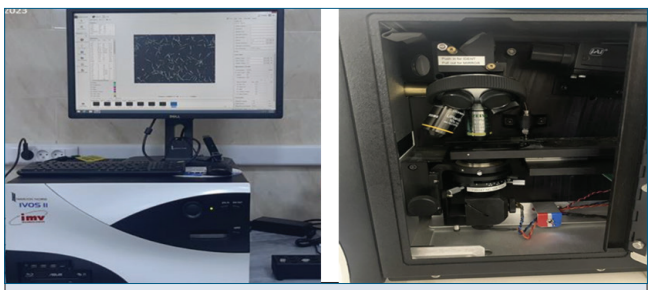


Fig 1. CASA system (Hamilton Thorne, HT-IVOS II)

Parameters Assessed Using Casa System

CASA allows to assess various Sperm parameters.

Sperm Concentration

Concentration is certainly one of the most important parameters when evaluating sperm, as infertility has been associated with low sperm count in many species [22]. Accurate assessment of sperm concentration using CASA systems remains a problem for all species in which this parameter has been studied [23]. If the CASA system proves to be reliable for motility analysis compared to the conventional technique, there is not yet a consensus on its reliability in measuring concentration in the different species [24]. Indeed, the values obtained were often overestimated or underestimated compared to the reference method [25]. The spz detected, because of analytical errors [26], are no longer representative of the microscopic fields in which they were analyzed "the principle of spz homogeneous distribution in a cell suspension is no longer respected". This results in concentration and motility evaluation errors. If the system detects non-spz particles (with the size of a spz head), it will probably overestimate the population of static spz [27]. Agarwal et al.[28] reported in Humans that CASA systems can have difficulties in distinguishing between immotile sperm, non-sperm cells, and debris. This lack of distinction causes inaccurate evaluation of sperm motility as well as counting of spermatozoa, which also affects the evaluation of sperm concentration.

Sperm Motility

Sperm motility evaluation provides essential information on its functional competence and fertilizing potential [12]. CASA analyzer is considered the Gold Standard in Motility assessment [26]. CASA-derived motility parameters include total motility, progressive motility, non-progressive, static, progressive fast [type a], slow progressive [type b], non-progressive [type c] and immobile [type d] [24]. Additionally, this system provides verifiable data, as previously analyzed video images can be re-examined for periodic internal and external quality control evaluations.

Current CASA-Mot systems focus almost exclusively on scanning and tracking the sperm head, with some newer systems starting to look for the presence of the flagellum to help exclude debris and other foreign objects from scans. Mechanically, however, it is the flagellum that propels the sperm and, as such, the behavior of the flagellum is the fundamental characteristic governing sperm motility. Taking this into consideration, it is believed that the most effective way to develop CASA-Mot, and thus enlarge the circle of use of computer-assisted analyzes in clinical

diagnosis is the introduction of flagellar monitoring, as has been done the pioneers Hiramoto and Baba [29].

Sperm Kinematic

In addition to being powerful analytical tools for assessing sperm motility, CASA systems provide additional details on sperm motion via determining their kinematic characteristics. Each individual sperm in the field of view is identified and a series of digital images of the spz head movement is captured. This allows for the reconstruction of their individual trajectories [18]. CASA-based kinematics include the following measurements: straight line velocity (VSL, µ/ms), corresponding to the straight line from the beginning to the end of the track; curvilinear velocity (VCL, µm/s), measured over the actual point-to-point track followed by the cell; average path velocity (VAP, µm/s), the average velocity over the smoothed cell path; amplitude of lateral head displacement (ALH, µm), defined as the maximum of the measured width of the head oscillation as the sperm cells swim; beat cross-frequency (BCF, Hz), defined as the frequency with which the actual track crossed the smoothed track in either direction; motility (%), the percentage of the total motile cells; and progressive motility (%), corresponding to spermatozoa swimming forward quickly in a straight line, (STR ≥45%; VAP ≥25 µm/s). Three progression ratios, expressed as percentages, were calculated from the velocity measurements described above: linearity of forward progression

(LIN = VSL/VCL·100), straightness (STR = VSL/VAP·100) and wobble (WOB = VAP/VCL·100) [21]. These kinematic parameters provide valuable information on sperm quality, which cannot be obtained by the subjective evaluation using manual microscopic examination, and could become important components of male fertility evaluation, thereby enhancing the routine semen analysis [5]. Indeed, using CASA, it was revealed that sperm cells with the highest velocity and progressive motion were positively correlated with their resilience post-cryopreservation [2].

The 3 individual sperm velocity assessed by CASA are presented in *Fig. 2*.

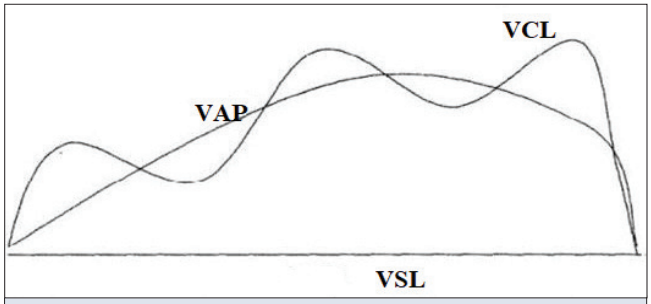
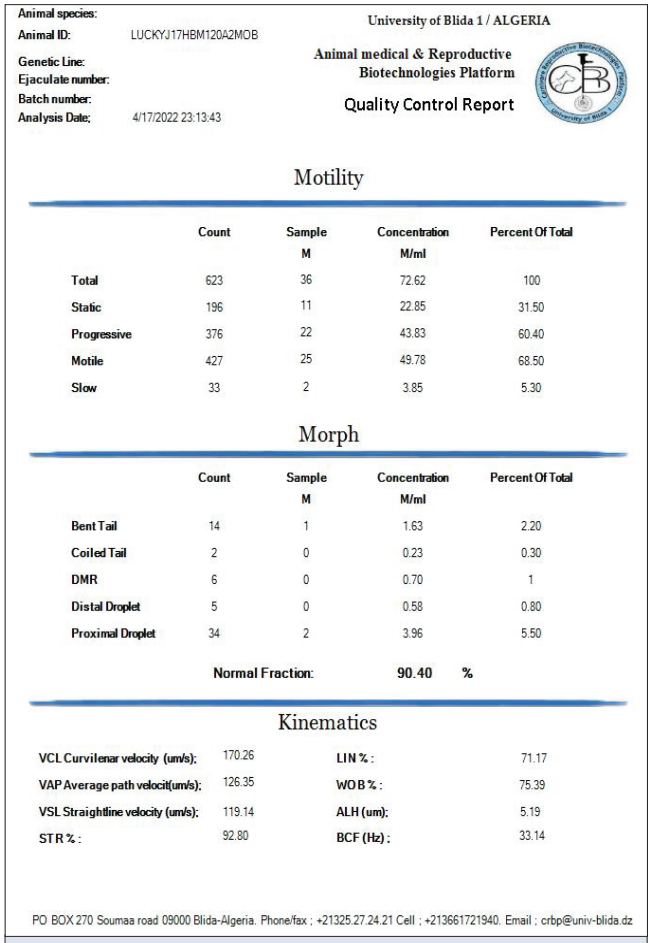


Fig 2. The main kinematic measurements involved in sperm tracking (Velocity)

Sperm Morphology and Morphometrics

Spermatozoa morphology constitutes an important parameter in the exploration of sperm quality and its effectiveness in fertilizing the egg [30]. The competitiveness of spz in the female reproductive organs and their ability to penetrate an ovum may result from their dimensions and shape which they often show a relationship with ejaculate traits [31]. In fact, fertilization process is affected more by morphological defects of spz than motility [10]. However, the predictive value of sperm morphology in male fertility has always been a controversial issue mainly due to the great variability of manual assessment of morphology within and between laboratories, which then limited its practical application [14]. Investigators reported that the changes of classification and standards in successive editions of WHO classification participated in keeping this parameter in a conflictual cercle. The standard values have evolved considerably in 30 years and the doubts about this parameter have been more raised [13,14].

Antoni van Leewenhoek reported microscopic sperm morphology in 1678 for human and dog sperm using an early single-lens microscope [32]. This time-consuming


Animal species:
Animal ID: LUCKYJ17HBM120A2MOB
Genetic Line:
Ejaculate number:
Batch number:
Analysis Date: 4/17/2022 23:13:43

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Quality Control Report

Motility

	Count	Sample M	Concentration M/ml	Percent Of Total
Total	623	36	72.62	100
Static	196	11	22.85	31.50
Progressive	376	22	43.83	60.40
Motile	427	25	49.78	68.50
Slow	33	2	3.85	5.30

Morph

	Count	Sample M	Concentration M/ml	Percent Of Total
Bent Tail	14	1	1.63	2.20
Coiled Tail	2	0	0.23	0.30
DMR	6	0	0.70	1
Distal Droplet	5	0	0.58	0.80
Proximal Droplet	34	2	3.96	5.50

Normal Fraction: 90.40 %

Kinematics

VCL Curvilenar velocity (um/s):	170.26	LIN % :	71.17
VAP Average path velocit(um/s):	126.35	WOB % :	75.39
VSL Straightline velocity (um/s):	119.14	ALH (um):	5.19
STR % :	92.80	BCF (Hz) :	33.14

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Fig 3. Sperm parameters assessed by HT-IVOS II analyzer (Report of canine semen evaluation)

technique is quite precise depending on the experience of the operator, but requires specialized microscopes or stains and can induce visual fatigue which is a probable source of error. Besides, it can also suffer from subjectivity when it is not carried out by highly experienced investigators [33].

To avoid the limits of conventional methods, computer-assisted sperm morphometry analysis systems (CASMA, generically CASA) have been developed and have first appeared on the market since 1990s. CASMA has been widely evaluated and validated for several systems and in different animal species [14]. Compared to morphometry on stained slides, automatic morphometry could thus offer the advantage of speed, lower analysis cost and the large number of spz analyzed [14,34]. It also helps avoid fixation and staining already identified as error factors in morphometry on stained slides [13].

Fig. 3 showed the different parameters assessed by a CASA analyzer.

Casa System Benefits

CASA plays an important and growing role in ensuring the quality of seed products for use in the AI industry at large, accelerating a trend that started a decade ago in many species [35].

Currently, CASA is a very useful laboratory tool, the simpler and more affordable option for the objective evaluation of semen in farm animals. It allows quick and repeatable sperm motility and morphometry assessment [1]. Examining large numbers of samples and acquiring reliable results in a short period of time can support the control of reproductive problems in male animals and lead to maintaining high rates of embryo production within laboratories [36]. The precondition is the existence of specialized and well-trained operators with appropriate functionality of the system.

Casa and Manual Analysis

Unlike manual counting, CASA uses hardware and software to visualize and to evaluate consecutive images of viable sperm in order to obtain accurate and valid information on the kinematics of individual sperm. Both methods (manual and CASA) have their advantages and disadvantages but the most promising technique is CASA. One of the drawbacks of manual method is that efficient use of the hemocytometer is highly dependent on precise pipetting, dilution and careful calculation, all of which are common sources of error [37]. CASA allows to obtain fast results with detailed objective analysis combined to high reproducibility [35].

The sperm parameters obtained in accordance with WHO recommendations remain a reference for clinical examinations. However, they do not always reflect the male fertilizing potential. Manual semen analysis is a very cost-effective and straightforward procedure, but it requires analysis by well-trained laboratory investigator. To date, no procedure has been validated by the WHO as a gold standard for semen analysis [38].

When using frozen sperm, it is interesting to notice that CASA compared to manual methods often overestimates sperm counts before freezing and underestimates this parameter after thawing [25]. It could be due to the variable agglutination rate to which immotile cells after thawing are subject. Fresh sperm settings of the analyzer parameters may not be optimal for counting cryopreserved sperm, where the freezing medium often contains egg yolk and/or a cryoprotectant. In humans, washed sperm samples require different CASA parameters than seminal sperm for semen analysis; similarly, samples after thawing with freezing medium may require different settings when the viscosity of the freezing medium is different from that of the ejaculate. Manual microscopic evaluation of sperm motility is subjective and strongly associated with inter- and intra-laboratory variations. Within the same semen sample, variations of 30-60% have been reported in manually assessed human and animal ejaculates [39].

Unfortunately, although there are general and important guidelines for the measurement of sperm motility analysis and the use of CASA, detailed and generally accepted guidelines for specific internal parameters of CASA and important laboratory species adjustments are lacking [27]. Obviously, due to specific software algorithms for specific CASA devices (which are able to modify the results obtained from particular parameters such as the amplitude of lateral head displacement (ALH)), it is difficult but possible to generate a general directive with specific CASA parameters. Using CASA, significant inter-laboratory variations were observed, multi-centric studies on male topics demonstrated that one of the least subjective parameters is the sperm concentration with a coefficient of variation of 21% to 80% [27]. Therefore, the standardization of assessment methods is becoming more and more necessary with the increasing worldwide export of semen straws and communication between animal breeding centers. Such standardization requires a large amount of organization and involves several laboratories working on the same samples [40].

CASA is very sensitive to small changes in internal parameters that can lead to a considerable modification in the results. For example, a small tick in the box "Slow sperm are counted as motile/static" can lead to completely different results, e.g. an observation of 49% motile cells instead of 80%. Although valid arguments exist for both possibilities - 1: slow cells are motile; or 2: slow cells will never reach the oocyte. There is a high risk of misinterpretation of the results for each of the choices;

a possibility of overinterpretation of the respective information due to missing background knowledge. To which groups do the hyperestimated slow cells belong?

Another example of an important piece of information is the frame rate; e.g. 50 Hz and 60 Hz. Different rates can have a significant impact on the results obtained [41]. Those parameters should be considered before purchasing a specific CASA device.

Lammers et al.[42] performed a prospective double-blind study comparing two automated semen analyzers with manual sperm assessment. Statistical analysis revealed no significant differences for most of the measured sperm parameters. Komori et al.[43] found high agreement between automated and microscopic methods in assessing sperm motility. In the same context, Fuse et al.[44] showed that the measurement of total sperm concentration and percentage of progressive motility by IIB sperm quality analyzer revealed high correlations with those of the conventional manual method recommended by WHO [24] "Enhanced Neubauer cell type". On the contrary, Vested et al.[45] reported significant differences when comparing the results obtained by using CRISMAS CASA and those performed by conventional method regarding sperm concentration and motility analysis.

Both CASA and manual method presented acceptable agreement. However, CASA is a better tool to avoid subjective variations and for better standardization. Moreover, the latter is undoubtedly the best regarding the evaluation of kinematic parameters [38] as well as spz concentration [25].

For many years, the CASA analysis has been one standard in the laboratory for motility and kinetic parameters [5], with no clear description of a species-specific setup [26]. Besides, although the recent advances in automation technology, CASA systems still require manual intervention to rectify errors and provide reliable results [28]. According to Jorge-Neto et al.[26], the correct use of the CASA system, coupled with a detailed description of the setup and procedure employed, facilitates the replication of methodologies and comparisons of studies. Recently, O'meara et al.[40] worked to adjust the kinematic and morphometric setup of the HT-IVOS II analyzer used for frozen bovine spz. These authors confirmed concerns and issues on the variability of the results obtained using different CASA settings and would recommend to the Research Centers to validate systems and ideally engage in a standard-setting for the IVOS II CASA system.

Casa Applications in Clinical Diagnosis

Due to the considerable efforts made to improve the technical performance and efficiency of current CASA systems, it is necessary and has become crucial to assess the biological relevance of parameters derived from CASA in the prediction of male fertility potential. CASA-derived measurements have been shown to be very useful in monitoring subtle changes in sperm distribution among different subpopulations of motility and speed in response to various physiological conditions and environmental exposures, which cannot be observed manually by light microscopic analysis [46,47].

Definition of Risk Factors Affecting Motility

Many factors can affect the quality of sperm movements. These include measurement temperature, sperm processing (freezing/thawing), sperm concentration and other technical factors [48].

Some CASA systems (IVOS II) are equipped with heated specimen stage, which allows precise control of temperature during the analysis, constant to within 0.5°C, whereas manual semen analysis is usually conducted under a phase-contrast microscope at room temperature. This represents an unquestionable advantage, as changes in temperature may significantly affect the analysis of sperm parameters, particularly motility assessment [47].

In humans, measurement of sperm concentration and movement characteristics requires different parameter settings for fresh and washed (after removal of seminal plasma) sperm samples. Similarly, in cryopreserved samples, post-thawed sperm samples may require different parameter settings due to the presence of the freezing medium added to the ejaculate.

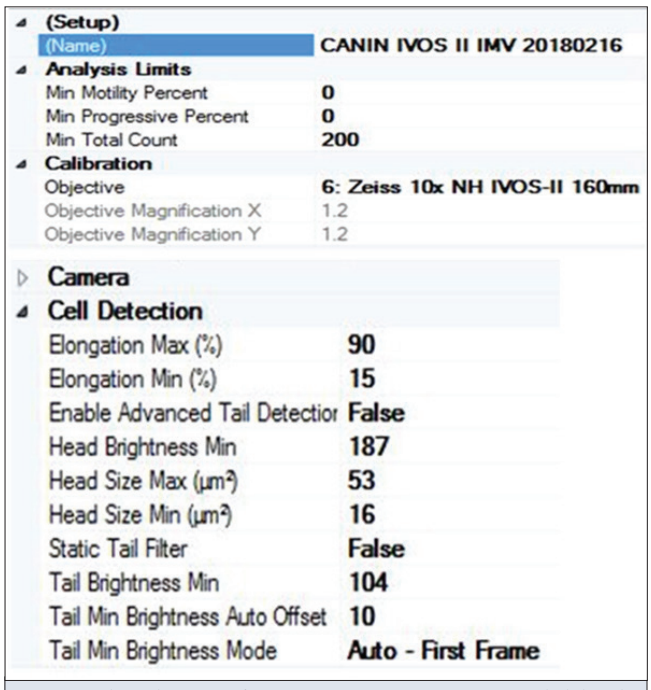

Fig 4. Technical setup of HT-IVOS II system recommended by the manufacturer (IMV Technologies) for canine spz analysis

Accordingly, sperm movement characteristics should not be considered absolute values, but should rather be interpreted in light of the parameter settings. Currently, the potential effects of environmental and occupational hazards on sperm function have been widely studied. External factors like sample temperature, sample dilution factor, mixing, pipetting, time to analysis, technician and the use of counting chambers (Type and depth) caused a large degree of variation or loss of cell motility [14,23,40,49]. Besides, the factors related to the technical configuration *(Fig. 4)* of the analyzer (pre-established setting of the image analysis software) do lead to different final analysis results [40]. These observations indicated that some risk factors may lead to specific changes in sperm movement characteristics.

Prediction of Motility Parameters Increasing the Chances of In-vitro Fertilization

Motility is accepted as the most important parameter in evaluating sperm fertilization capacity [50]. Several scientific laboratories deal with the topic of determining a particular parameter, evaluated by the CASA system, and its close correlation with *in vivo* fertility. However, this parameter or laboratory test, which could accurately report information on the fertilization capacity of the insemination dose, has not yet been established. This cannot be predicted because the reproduction process is very complex [17] and can be affected by different conditions [1,23].

Analysis of Hyperactive Spermatozoa Due to Capacitation Phenomenon

Hyperactivation is a form of non-progressive motility observed in the oocyte at the time of fertilization and is characterized by extremely asymmetrical flagellar undulations with higher amplitudes and lower frequencies leading to highly curved swimming trajectories, like whiplashes [51]. Sperm hyperactivation was initially assessed manually, describing flagellar movement patterns subjectively based on purely visual observations. However, since it is a flagellar phenomenon associated with changes in the amplitudes and frequencies of flagellar waves, manual analysis of the latter remains very laborious, time-consuming and uncontrolled. It has been strongly recommended that CASA systems are a more practical option for the identification of hyperactivated spz [18].

Under normal circumstances, spz that pass through the fallopian tube undergo the capacitation reaction and enter a hyperactive state, which is conducive to pass through the twisted fallopian tube, and fertilize the egg. Although the correlation between the percentage of hyperactive spz and the *in vitro* fertilization rate is not conclusive, but the detection of the percentage of hyperactive spz could predict the capacitation ability of spz *in vivo* and *in vitro,* and guide the treatment of infertility, so it is necessary to evaluate the dynamic characteristics of hyperactive spz by using the CASA system [52].

Dynamic characteristics of hyperactive spz are very different from those of ejaculated and non-capacitated spz. There are two types of sperm hyperactivity: progressive and non-progressive [18].

Factors Affecting Results Provided by Casa

A very important factor for standardizing CASA system data is the pre-analytical part, which includes the sample preparation for analysis [17]. Thus, the frame rate of the CASA system, sample concentration and volume, type of counting chamber, temperature and type of extender are essential for correct interpretation of CASA results.

Frame Rate

The Frame rate (FR) is defined as the number of images acquired per second while obtaining the trajectory of the sperm in the CASA. It depends mainly on the quality of the camera and the imaging standard applied [52]. The FR has a direct effect on the sperm movement trajectories, which can further affect the results of sperm movement parameters. The trajectory of sperm movement varies with the FR. When the path is built with a FR of 30 Hz, it will be relatively simple, while with a 60 Hz FR, it will be relatively complex.

For analysis of sperm movement, images typically represent 0.02 sec exposure at a rate of 50 frames per second (fps). Image exposure can be controlled via a camera shutter or the pulse duration of the strobe light.

The speed at which images are captured and the duration of a "scene" (for example, 60 frames per second for 0.5 sec) affect the distance a sperm can move between successive frames or during an entire scene (i.e. a curvilinear path). These have a direct effect on the shape of the calculated "mean path" for each sperm, deviations from the recorded path of a sperm's center of gravity on successive images, and other output values for motion of sperm. For the analysis of sperm morphology, one or more images are usually evaluated although some systems apparently calculate morphology statistics based on all the images in a scene [16].

As the trajectory of sperm movement changes, the dynamic parameters of the sperm will therefore be altered. The CASA-Mot frame rate used was limited by hardware restrictions from 16 to 60 Hz. Nevertheless, as has been previously indicated, the rate at which images are captured and the length of video recording both affect the distance that spz might move between successive frames [21].

This has a direct effect on the estimated trajectory for each sperm cell, deviations from the recorded path of a spermatozoon's centroid over successive frames, and other output values for sperm motion [17].

It is also stated that grade a and b sperm can be assessed correctly by the commercial CASA system with a FR of 30-60 Hz, while accurate assessment of grade c sperm requires increased FR. Now, it is generally accepted that the CASA system with a FR of 60 Hz can essentially meet the requirements of analyzing the sperm dynamics in mammals [16]. Recently, in boars, using the ISAS® v1 CASA-Mot system, with a video camera working up to 200 Hz while six FRs (25, 50, 75, 100, 150 and 200 fps) were compared, authors concluded that FR affected all the kinematic parameters, with curvilinear velocity (VCL) and BCF the most sensitive ones [21]. These latter reported that high fps values bring significant changes in the value of some sperm kinetic parameters and this must be considered. They mentioned also that the correct determination of sperm tracks results in a fundamental shift in the determination of motility and morphology subpopulation structure (the fact that the ejaculate in many species is constituted of different subpopulations of spermatozoa).

Type of Counting Cell Associated with CASA System

According to the literature, the type of chamber used for analysis could affect the CASA motility results. A variety of analysis chambers with different technical characteristics in terms of shape, size, depth, and charging method of the semen sample are commercially available *(Fig. 5)*. Recent research is focusing on the further standardization of CASA processes in order for data from different laboratories to be comparable [53,54]. Even though the CASA system has very high FR, very good video processing system, and advanced analysis software, if the depth of the sperm counting chamber has a large error, the results of sperm concentration and motility will be directly affected.

For motion analysis, disposable chambers (several manufacturers), capillary charged, with a carefully controlled depth (Z axis; certified depth) of 20 or 10 µm are often recommended. These depths make it less likely that a sperm can move up and/or down outside the useful depth of field of the microscope. However, this does mean that the sperm of some species cannot swim in their normal way; for bull spermatozoa, unlimited helical tail excursions require 12 µm in each direction from the plane of the head when a given sperm reposition itself [55].

Fig 5. Leja® slides with 4 chambers of 20 µm used with HT-IVOS II analyzer

In equine semen, Hoogewijs et al.[56] found that concentration and motility parameters were significantly influenced by the chamber type used. These authors after using the NucleoCounter as the gold standard for determining concentration, revealed that the correlation coefficients were low for all of the various chambers evaluated (Leja chambers of different depths were compared with disposable and reusable ISAS chambers, a Makler chamber and a World Health Organization (WHO) motility slide), with the exception of the 12 µm deep Leja chamber.

For morphology analysis, most systems use a colored dry preparation (on a conventional slide) although some now provide a complete analysis of head and/or tail morphology using a wet preparation [17].

CASA System Algorithms

An irregular path of sperm movement will produce quite a different shape and complexity than the path of sperm movement when FR changes. Therefore, it is necessary to process these tracks using certain principles and algorithms. In general, the values of the kinematic parameters of sperm, such as VAP, ALH, and others, are calculated by the algorithms after smoothing the sperm trajectory [57]. Thus, if the algorithms are different between CASA systems, the results will lack comparability.

Nafisi et al.[58] presented a kind of algorithm for calculating the sperm trace, which was not sensitive to the imaging acquisition conditions, but background and other particles can be successfully removed by an arithmetic method improved in two stages. This arithmetic has been proven to work very well under different imaging acquisition conditions. In addition, the calculation of the percentage of motile sperm is linked to the setting of the threshold between motile and immobile sperm. Threshold settings can be arbitrary, which currently lacks a uniform standard.

Sperm Concentration

Sample concentration effect on sperm quality parameters was very obvious when it was evaluated with CASA system. A high sperm concentration can affect the results of sperm count, motility and kinematics [31].

WHO [24] recommended that the concentration of sperm in semen samples detected with CASA should be less than 50 x 10^6 mL^{-1}, and Mortimer [52] recommended even less values than 40 x 10^6 mL^{-1}.

In fact, this is well understandable. When the kinematic parameters of the sperm are analyzed using CASA, the spz

trajectories in the detectable fields must be reconstructed. If there are too many spz in a field, the risk of collisions between sperm increases. Two or more spz trajectories that appear to be too close cannot be accurately reconstructed by CASA. As a result, the dynamic parameters of the sperm will be altered, and the motility categories of the sperm may be affected.

Therefore, when the semen samples were analyzed with the CASA system, samples with high concentrations of spz must be diluted with their corresponding seminal plasma [16]. The diluent used should not contain particles similar in size to the sperm heads (for example, unclarified egg yolk), as they will not be differentiated from non-motile spz [23].

Standardization and Quality Control Measures for CASA

The human factor can also affect the results of the analysis in different ways, so specific requirements must be set. The laboratory technician plays a crucial role in these complex assessment conditions and can influence almost all of the above-mentioned factors, in order to keep the results obtained reliable and reproducible [17,36]. Therefore, CASA, as a sophisticated system, places high demands on qualified operators. In addition, as there is no defined gold standard for animal sperm motility analysis methods, it is therefore recommended to establish a standard operating procedure for a specific laboratory. This should be accompanied by the determination of the repeatability of the assessment, when repeatability greater than 95% is possible. In other words, one of the difficulties in using CASA is the human factor. The purchase and establishment of CASA not only involves the maintenance of the facilities, but the operators must also know the principles of CASA and must be trained periodically [36].

CASA System Limits

While CASA is a reliable tool that has the ability to provide detailed measurements of the dimensions of the sperm head and midpiece, most commercially available CASA systems are not capable of analyzing characteristics of the sperm tail and therefore limit the application of this technology in clinical settings [18]. Moreover, CASA systems are not ready-to-use robots and can be influenced, like any other automated technique, by several artifacts related to inappropriate configurations and technical errors [59].

With recent advancements in CASA software, many of the limitations affecting CASA measurement performance have been partially or fully reversed. For example, when assessed manually, sperm motility is defined and categorized based on its flagellar movement and beat pattern, while CASA is primarily dependent on tracking the movement of the sperm head. It has been argued that the assessment of the percentage of sperm motility using CASA might be unreliable due to the potential misidentification of particulate debris such as immobile sperm [24].

However, the new CASA models are incorporated with smart filters removing some particles similar in size to sperm but mostly using positive phase contrast where most of the background images are now viewed in dark and therefore not represented as part of the white reflective semen. In addition, a function called "drift" can be defined to eliminate not only Brownian motion but also minor flow and even help counter the detection of immotile spz slightly displaced as motile due to a collision with motile spz. These characteristics allow a more accurate and objective assessment of sperm count and motility [18].

Potential Future of CASA

The conditions for studying the movement of sperm are a compromise. The sperm are suspended in an environment different from anything they will encounter *in vivo*. The suspension is seen in a chamber (or droplet) in which the spz accumulate at the interfaces between the suspension and the air, or in the wall of the chamber, where they swim differently than if they were far from the interface [60]. The laws of optics dictate a shallow depth of field in which an object sperm can be detected by a typical matrix chip and even in a 20 µm deep chamber, some sperm may not be detected. Approaches to acquiring images of three-dimensional swimming sperm are emerging. Researchers studying the biology of sperm should push to increase their availability, as the shallow depth of field hinders the free movement of sperm.

The successful collaboration and fruitful cooperation between science and industry has resulted in establishing CASA as a reliable tool that has the capacity to quantitatively assess sperm motility, kinematics as well as morphological and morphometric features, in a rapid and precise manner. The combination of these basic characteristics with advanced functional parameters will enhance the diagnostic value of semen analysis and provide a more accurate as well as quantitative approach for the assessment of idiopathic male infertility. Therefore, for the future development of CASA technology, advanced markers of sperm functionality (i.e. mitochondrial function, DNA status, hyperactivation, cervical mucous penetration) need to be integrated. This will enable a precise objective description of numerous aspects of sperm quality based on automated assessment. It is important also for clinicians in the field (e.g., examining the quality of breeding bulls under field or wildlife conditions) or in a small fixed-site practice to provide a market for a robust, simple, functional CASA system which does not require a specific location to use.

Conclusions

CASA systems have evolved dramatically over the past two decades to become powerful tools for the rapid and objective assessment of sperm concentration, motility and kinematics, as well as morphology, in almost all mammals, including humans. Within seconds, hundreds to thousands of sperm can be analyzed with great precision. In this regard, CASA is far superior to subjective manual assessments; it quantitatively measures different aspects of sperm speed, hyperactivation and morphometry, which cannot be done manually. New generation CASA devices have recently been developed to automatically quantify various aspects of sperm functionality, such as sperm DNA fragmentation, sperm vitality, and acrosome integrity.

This investigation mainly focused on studies relating to the motility and morphology of sperm in domestic animals, as well as in various wildlife; providing parameter settings, in addition to new basic data for predominantly normospermia samples; showing that even the most difficult samples, such as rat and mouse sperm, can now be routinely analyzed for motility and morphology, thus presenting a powerful tool for toxicology studies. As the current literature showed, there are many applications for the routine use of CASA in the research laboratory, wildlife management and clinical fertility assessment (human and veterinary).

Despite the lack of full universal standardization, the various CASA instruments have all currently demonstrated high levels of accuracy and reliability. Although they can sometimes be disappointing, the availability of these tools and the adherence to a strict partial standardization will give the opportunity to objectively compare the movement and the morphology of the sperm. Therefore, CASA provides an efficient, precise and reliable tool to objectively assess fertility, to improve artificial reproduction technologies and to develop physiological or toxicological studies.

Declarations

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