Indian Journal of Animal Sciences **91** (6): 419–429, June 2021/Review Article
https://doi.org/10.56093/ijans.v91i6.115435

Computer-assisted sperm analysis (CASA) in veterinary science: A review

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Received: 20 July 2021; Accepted: 23 July 2021

ABSTRACT

Computer-assisted sperm analysis (CASA) allows an assessment of sperm motion and morphology more accurately and objectively than by subjective evaluation. Although, CASA instruments have improved significantly during last 40 years especially in terms of software, image capture and computer settings, little has changed regarding processes for analyzing sperm motion attributes. The main problem is related to validation, consistency and optimization of equipment and procedures. Differences among CASA systems denote problems of objective analysis of results between different semen processing units. If validated, CASA systems can provide a great tool to objectively compare sperm motility and morphology. Sperm motility is one of the indicators most evaluated before and after cryopreservation vis-à-vis quality and fertilizing ability. Researchers have determined a possible relationship of CASA outputs with bull fertility in vivo; however, a clear association has not yet been confirmed. Most CASA measures depend upon concentration, sample volume, type of extender, duration of analysis and thawing temperature. For each attribute, CASA software should provide outputs based on a range rather than means or medians for transformed data. The current review describes development, validation requirements, limitations and future expansions associated with CASA technology.

Keywords: CASA, Fertility, Semen analysis

During semen evaluation, one of the most important element is precise and quality assessment of sperm motility (Gil *et al.* 2009). Examination of semen basically dates back to 1678 when Anton Von Leeuwenhoek first looked at sperm movements using a light microscope subjectively. Even today, methods routinely used for the assessment of spermatozoa are laden by subjectivity and variability and can be affected by bias and inaccuracy (Rijsselaere *et al.* 2012). Therefore, more objective methods for assessment of sperm motion are needed (Rijsselaere *et al.* 2003). One such method of enhancing accuracy of semen motility evaluation is through the use of computer-assisted sperm analysis (CASA) systems (Gil *et al*. 2009). CASA represents, interchangeably, the terms 'computer-aided sperm analysis' and 'computer-assisted sperm analysis'. CASA was developed first in early 1980s (Amann and Waberski 2014) that aimed at providing a more objective and detailed analysis of sperm motility/kinematics for quality assessment and fertility estimation, morphological features and characteristics (concentration, morphology and viability) using optical microscopy and two dimensional videomicrography. CASA analyzes more number of sperm cells in a shorter time and plays an important role in assurance of quality semen for artificial insemination (AI; Verstegen *et al.* 2002, Kathiravan *et al.* 2011). The output values obtained from CASA system lead to reduced bias which could provide a reliable basis to reject or use a given semen sample and assure that quality of product meets specifcations. Periodic training of CASA operators/ technicians might further improve accuracy (Broekhuijse *et al.* 2011b).

Currently, CASA systems use special software to produce desired output and information, detect objects based on intensity of pixels in a frame or light scatter and project successive images of a sperm-suspension on to a detector array (Amann and Katz 2004). These electronic systems provide measures for motion or morphology of each sperm and allow accurate prediction of fertility since sperm motility is accepted to be closely related to fertility (Puglisi *et al.* 2012). Accuracy and sensitivity of each output measure depends upon instrument settings, sperm-suspending medium, sample chamber depth and software system. Motility is the most validated parameter measured by CASA. Kathiravan *et al.* (2011) reported that analysis of sperm motility using CASA system is more effective and precise for fertility assessment than subjective evaluation among different breeds and age categories of breeding males. Studies on the use of CASA measurements exhibiting significant correlations between motility and fertility have been conducted in bovine (Gillan *et al.* 2008, Kumar *et al.* 2015), equine (Køí•ková *et al.* 2017), human being (Hirano *et al.* 2001), rabbit (Lavara *et al.* 2005), ovine (Palacin *et al.* 2013) and swine (Broekhuijse *et al.* 2011a).

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The current review reports the use of CASA systems with animal sperm and also reflects a possible connection of sperm motility to bull fertility *in vivo*, followed by possible future developments.

Functioning of different components of CASA system

To elucidate the proper functioning of CASA operations for accurate measurements of motion parameters, working of various components of CASA system have been described here.

Sperm-suspension: Neat semen is diluted in an extender or dilutor to examine the sperm. As such, there is no *in vitro* measure of sperm motion that refects its competence within highly variable environment of a female reproductive tract (Hunter *et al.* 2011, Olson *et al.* 2011). As an alternative, CASA provides information only on sperm motility in defned conditions and allows comparison with other samples evaluated similarly.

Optical principles: A spermatozoa comprises of many object points each with greater or lesser intensity in the specimen plane of a microscope. Each object point has a three dimensional diffraction sphere which combine to form an image of sperm head. The microscope objective forms an 'intermediate image' of the object. The distance from nearest to farthest points in the specimen plane is called as 'depth of field' which decreases with higher numerical aperture, refractive index of sperm-containing medium and wavelength of illumination (Amann and Waberski 2014). An array (eye) chip can detect the intermediate image only if majority of center points fall within its focal plane (in focus). Objects which are out of focus can be better detected by human eye through 'accommodation'. Newer technologies can increase depth of field and allow visualization of larger areas.

Automated mechanical stage: This stage allows movement of a slide to programmed locations which is followed by autofocusing that provides faster processing of semen.

Specimen chamber: For analysis of motion traits, disposable chambers with fixed depth of 10 or 20 µm are used and are loaded using capillary action. Capillary filling of chamber depends on viscosity of suspension and size of sperm (Segre Silberberg effect) which affects the measure of sperm concentration (Douglas-Hamilton *et al.* 2005a). After filling, the Segre-Silberberg effect causes excessive sperm movement towards the walls of the chamber. This effect can be reduced by analyzing the sample along the central long axis of chamber and also by the use of correction factor (Douglas-Hamilton *et al.* 2005b, Tomlinson *et al.* 2010). This phenomenon also helps in relocation of motile sperm from the walls to the centre of the chamber and prevents redistribution of immotile sperm.

Image capture: An image exposure of <0.02 sec and at a frequency of >50 frames per second is desirable for optimum sperm motion analysis. The shape of average path, curvilinear path and other output values calculated for each sperm in a 'scene' are influenced by the frame rate and the duration of a scene (60 frames per sec for 0.5 sec; Amann and Waberski 2014).

Illumination and optical systems: Commercially promoted CASA systems use ultraviolet (343 nm) illumination to excite a fluorochrome (Hoechst 33342 dye intercalated with sperm DNA). To reduce the exposure of sperm to harmful ultraviolet illumination, certain systems use pulsed illumination. Some systems provide broad-band illumination within the visible spectrum (390–700 nm; maximum visual sensitivity near 550 nm). In few systems, fluorescent epi-illumination and optics are available to detect lack of normal acrosome, sperm head and sperm membrane damage. Some systems use negative or positive phase-contrast objectives (10× or 20×) for motion analysis. Evaluation of sperm morphology includes differential interference-contrast optics (unstained sperm, wet preparation) and bright-field (stained sperm) objectives (20×–100×, Farrell *et al.* 1996).

Defining output measures of CASA system

CASA systems use software to calculate output measures describing and defining motion traits in successive frames, viz. total motility (TMOT, %): [percentage of total motile spermatozoa]; progressive motility (PMOT, %): [percentage of spermatozoa with a progressive motility]; velocity average path (VAP, µm/s): [average velocity of the smoothed cell path]; velocity straight line (VSL, µm/s): [average velocity measured in a straight line from the beginning to the end of the track]; velocity curvilinear (VCL, µm/s): [average velocity measured over the actual point-to-point track followed by the cell]; amplitude of lateral head displacement (ALH, µm): [mean width of the head oscillation as the sperm cells swim]; beat cross frequency (BCF, Hz): [frequency of sperm head crossing the average path in either direction]; straightness (STR, %): [estimates the proximity of the cell's pathway to a straight line and is a measure of forward progression, also expressed as the ratio of VSL/VAP]; linearity (LIN, %): [estimates the proximity of the cell's track to a straight line, also expressed as the ratio of VSL/VCL]; sperm size (SS, µ): [elongation ratio of minor to major axis of each sperm]; and sperm nucleus (SN, µ): [size of each sperm nucleus] (Verstegen *et al.* 2002; Fig. 1). These CASA parameters have been modeled and refined mathematically to best describe the motion parameters of each spermatozoon as it travels through a microscopic field (Boyers *et al.* 1989). The overall sperm population is subdivided into four categories based on low VAP cut-off (LVV) and medium VAP cut-off (MVV) values, viz. rapid (VAP>MVV), medium (LVV<VAP< MVV), slow (VAP<LVV) and static (fraction of all cells that are not moving during the analysis). The percentage of progressive spermatozoa includes cells moving with VAP>MVV and STR>So (So=straightness threshold cut-off to determine the progressive spermatozoa). Minimum 15 points at 30 Hz or 30 points at 60 Hz are required to identify trajectories (Mortimer *et al.* 2015). For motion measures, the values for each sperm examined

Table 1. CASA-based mean (±SD) sperm motion characteristics in different species

Parameter	Pre-freeze					Post-thaw				
	Buffalo bull	Cattle bull	Stallion	Ram	Boar	Buffalo bull	Cattle bull	Stallion	Ram	Boar
TM (%)	91.18± 5.25	82.9± 12.0	92.60± 5.94	77.0± 1.2	87.4± 6.4	56.51± 11.48	48.2± 3.8	41.84± 0.54	65.2± 4.5	20.31± 10.82
PM (%)	45.59± 9.71	68.9± 11.7	46.20± 10.03	50.1± 1.2	78.2± 8.6	31.12± 5.41	24.3± 2.3	26.0± 1.41	39.7± 3.5	13.45± 9.02
VAP (µm/s)	159.03± 25.77	120.9± 14.4	98.36± 12.10	104.6± 2.8	95.1± 20.5	102.36± 17.20	86.9± 2.5	77.90± 6.93	–	39.02± 13.18
VSL (µm/s)	104.08± 9.77	109.4± 14.2	73.34± 7.93	88.6± 2.7	68.5± 18.4	81.29± 12.81	68.1± 1.9	58.79± 2.61	–	31.28± 11.35
VCL (µm/s)	284.09± 53.09	–	177.94± 19.89	127.3± 2.1	175.2± 37.3	185.09± 14.17	172.2± 5.4	113.33± 2.97	120.3± 4.0	56.82± 15.18
ALH (µm)	9.42± 1.04	4.5± 0.8	6.78± 0.38	3.3± 0.1	7.3± 1.3	7.24± 0.76	9.5± 0.3	5.73± 0.18	3.3± 0.1	2.60± 0.46
BCF (Hz)	34.26± 1.96	39.0± 3.0	35.44± 4.04	8.9± 0.2	39.3± 2.8	35.01± 2.81	28.1± 0.4	10.32± 1.03	–	9.35± 1.21
STR (%)	67.97± 7.02	89.2± 3.1	71.80± 5.76	81.0± 0.7	–	80.28± 3.67	76.4± 0.8	68.89± 2.06	–	81.26± 5.46
LIN (%)	40.14± 4.74	–	41.40± 4.16	67.2± 1.4	–	47.09± 3.70	40.3± 0.7	51.93± 2.12	63.5± 1.6	54.00± 8.78
Reference	Kumar *et al.* (2015)	Hoflack *et al.* (2007)	Rezagholizadeh *et al.* (2015)	Palacín *et al.* (2013)	Broekhuijse *et al.* (2011a)	Kumar *et al.* (2015)	Kathiravan *et al.* (2005)	Seifi-Jamadi *et al.* (2016)	Alvarez *et al.* (2012)	Ravagnani *et al.* (2018)

TM, total motility; PM, progressive motility; VAP, velocity average path; VSL, velocity straight line; VCL, velocity curvilinear; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity.

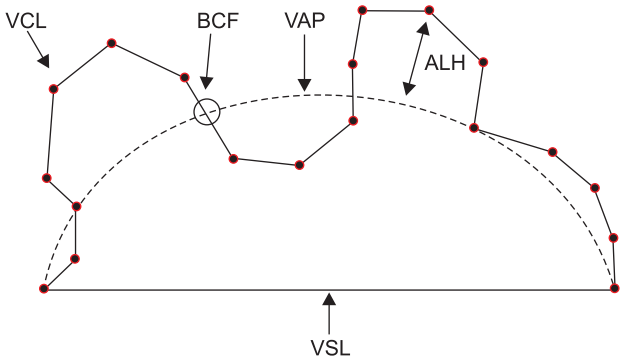

Fig. 1. Assessment of CASA-based sperm kinetic parameters showing different motion tracks (VSL, velocity straight line; VCL, velocity curvilinear; BCF, beat cross frequency; VAP, velocity average path; ALH, amplitude of lateral head displacement).

across all fields are evaluated for >500 sperm/sample and ideally >1000 sperm/sample. Variations in sperm motion characteristics across different species on the basis of CASA measurements have been reported (Hoflack *et al.* 2003). Table 1 depicts comparison of sperm motility and kinematic parameters in different species based on CASA systems with different settings to obtain a rough estimation of motility differences among species.

Different CASA settings for semen analysis

There are various setting components of CASA systems like frame rate (Hz) or frames/second, number of frames, minimum contrast, minimum cell size, cell size (pixels), cell intensity (pixels), minimum cells, minimum tracking time, low gate size, high gate size, low intensity gate, high intensity gate, slow gate velocity, VAP (µm/s), VSL (µm/s), STR (%), chamber type, chamber depth (µm), stage set temperature, field selection, critical linear index and illumination (Table 2). These settings vary amongst species. Presently, there are different CASA systems available in the market with updated parameter settings and adjustments for multi-species use like Hamilton Thorne (IVOS), Minitube (Androvision), PROiSER R+D (ISAS), Microptic (SCA), DITECT (SMAS), Medical Electronic Vision (SQA-vision) and Biophos (Qualisperm) (Boe-Hansen and Satake 2019). Solar *et al.* (2016) advocated that for better application to research, CASA could be named on a particular attribute actually being assessed, viz. CASA-Mot (motility), CASA-Morph (morphology) and CASA-Conc (concentration). However, this may lead to additional burden on the cost since for each attribute separate CASA system would be required. All operating procedures of CASA systems should be clearly defined, especially chamber type and depth, software, sampling time, concentration and composition of sample, temperature, sample preparation and processing and magnification.

CASA and sperm biology

CASA instruments are used to assess and capture changes in sperm motion or morphology. The sperm-suspension is placed in 1–3 chambers of CASA system. For each chamber, about 3–8 fields are evaluated and for

Table 2. Variations in CASA settings for semen analysis in different species

Parameter	Species					
	Buffalo bull	Cattle bull	Stallion	Dog	Ram	Boar
Equipment	IVOS 12.2 Hamilton-Thorne	HTR IVOS	Model HT M2030, Hamilton-Thorne	HTR IVOS V10	ISAS®, Version 1.0, PROISER	UltiMate Hamilton Thorne
Frame rate (Hz) (Frames/second)	60	30	30	60	50	60
Frames acquired	30	30	30	30	–	45
Minimum contrast	60	8	6	75	–	4
Minimum cell size	5	8	5	3	–	7
Cell size (Pixels)	9	–	–	–	–	7
Cell intensity (Pixels)	110	21	85	–	–	50
Minimum cells	–	>200	v	>100	–	–
Minimum tracking time(s)	–	–	100	–	0.6	–
Low gate size	–	0.4	0.3	0.37	–	–
High gate size	–	1.6	2.0	2.48	–	–
Low intensity gate	–	0.4	0.4	0.31	–	–
High intensity gate	–	1.6	2.0	1.54	–	–
Slow gate velocity	–	–	20	–	–	–
Medium cut-off VAP (μm/s)	50	80	35	50	50–300	25
Low cut-off VAP (μm/s)	30	–	–	–	–	20
Threshold VSL (μm/s)	15	–	–	–	25–100	5
Threshold STR (%)	70	60	–	75	40–100	30
Chamber type	Leja-8	Microcell	–	Makler	Microcell	Leja-4
Chamber depth (μm)	19.7	12	–	10	20	10
Stage set temperature (°C)	37	37	37	38	37.5	20
Critical linear index	–	–	25	–	–	–
Illumination	–	–	Dark field	–	–	–
Reference	Kumar *et al.* (2015)	Farrel *et al.* (1998)	Love (2011)	Iguer-ouada and Verstegen (2001)	Robayo *et al.* (2008)	Broekhuijse *et al.* (2011a)

each field, CASA captures images containing >100 spermatozoa (observational unit). The data for all fields could be used to describe a particular attribute of motion using all values for that experimental unit. With regard to morphology, all values represent one experimental unit (Amann and Katz 2004). The number of observational units represents one experimental unit when subpopulations of cells are recognized. The effect of treatments on ejaculated semen, if any, depends on sample size. For each attribute, data should be based on a range rather than means or medians for transformed data. The data should be repeatable both at male and ejaculate levels. Thus, CASA plays an important role in sperm selection for inclusion in preservation media.

Importance of CASA analysis of sperm motility in predicting bull fertility

Sperm motility is considered as the most important attribute vis-à-vis fertilizing potential of spermatozoa. Most research andrology laboratories aim in determining the association of sperm motility assessed by CASA system with *in vivo* fertility. However, this parameter has not yet been validated to provide substantial information on the fertilizing ability of spermatozoa owing to complexity of reproductive process (Vincent *et al.* 2012, Ferraz *et al.* 2014). Nonetheless, numerous studies (Farrell *et al.* 1998, Gillan *et al.* 2008, Oliveira *et al.* 2013) in bulls have demonstrated relationship among motion parameters of sperm evaluated by CASA and field fertility. Farrell *et al.* (1998) described a positive correlation (r=0.34) for total motility measured by CASA to predict bull fertility. Likewise, total motility and progressive motility of AI doses exhibited high correlation (r=0.97) with fertility *in vivo* and could be used as predictors of field fertility (Oliveira *et al.* 2013). Similar observations in frozen-thawed semen of buffalo bulls also presented a strong positive correlation between CASA outputs of total motility (r=0.694, P<0.01) and progressive motility (r=0.387, P<0.05) with fertility (Singh *et al.* 2014). Correspondingly, a significant (P<0.05) positive correlation of CASA-based post-thaw total motility (r=0.369) and progressive motility (r=0.629) with buffalo bull field fertility has been reported (Singh *et al.* 2016). In another study, CASA measures of progressive motility were linked to buffalo bull fertility as revealed by high values of progressive motility (33.2±1.9%) and high first service conception rate (FSCR, 57.0±2.6%) in good fertility bulls and vice versa (23.4±1.9% progressive motility and 16.7±1.7% FSCR in poor fertility buffalo bulls; Singh *et al.* 2017). Similarly, in boars, Broekhuijse *et al.* (2011a) demonstrated that CASA evaluation of sperm motility is an indicator of fertility. On the contrary, no correlation of parameters evaluated by CASA and *in vivo* fertility was

observed, rather subjectively assessed motility (r=0.672) showed strong correlation to fertility (Gillan *et al.* 2008).

Evaluation of sperm kinematics presented that VAP and VCL are predictors of fertilization (Holt *et al.* 2007). A higher VAP and VCL enhanced high proportion of sperm showing hyperactive movement which increased *in vitro* fertilization (IVF) rates (Rashid *et al.* 1998). Farrell *et al.* (1998) noticed a higher correlation (r=0.98) for combination of parameters (BCF, LIN, VSL, VCL, STR) measured by CASA for prediction of bull fertility. Likewise, in cross bred bull, a significant positive correlation of VAP (r=0.612) and VSL (r=0.625) analyzed by CASA with fertilization rate has been reported (Kathiravan *et al.* 2008). In buffalo bulls, CASA analysis of VSL (r=0.759, P=0.018) and VCL (r=0.693, P=0.039) were highly correlated to field fertility (Kumar *et al.* 2016). Similarly, assessment of VAP, VSL and VCL using CASA depicted a positive correlation with *in vivo* buffalo fertility (Singh *et al.* 2020). Another CASA-based parameter affecting the outcome of IVF is ALH which has been associated with the ability of spermatozoa to penetrate cervical mucus and fuse with oocytes (Verstegen *et al.* 2002). In another study, Robayo *et al.* (2008) observed a positive correlation of VCL (r=0.51) and VSL (r=0.25) with cervical mucus penetration in rams. On the contrary, BCF and LIN were negatively correlated with IVF (Herrara *et al.* 2005, Arman *et al.* 2006). Similar disparities were also noticed when bulls with higher field fertility displayed lower motility parameters (VSL, STR and LIN) assessed by CASA (Oliveira *et al.* 2013).

Importance of CASA measurement of sperm concentration

Concentration is an important parameter during semen evaluation since infertility has been related to low sperm count. However, CASA evaluation of semen concentration still remains an unmet challenge in majority of the species. Iguer-ouada and Verstegen (2001) opined that the most common observation noticed during sperm count by CASA is overestimation. They further reported that overestimation of sperm concentration at low counts is due to debris and at higher counts due to collisions. Optimum sperm concentration required for evaluation by CASA ranges between 20×10^6/ml to 50×10^6/ml depending upon species (Contri *et al.* 2010). When compared manually, CASA measures of sperm concentration result in overestimation of pre-freeze count and underestimation of post-thaw count. Variable clumping and agglutination seemed to be a predisposing factor of frozen-thawed immotile sperm cells resulting in underestimation of sperm concentration by CASA (Douglas and Hamilton 2005b). Evaluation of sperm concentration by CASA than using conventional microscope revealed an overestimation of 1.7 times in dogs which could due to collisions (Iguer-ouada and Verstegen 2001). Inorder to minimize these collisions, dilution of semen was advocated, however, semen dilution did not always lead to correctness of overestimation (Verstegen *et al.* 2002). Moreover, extensive dilution may also cause changes in motility parameters. In this context, Iguer-ouada and Verstegen (2001) suggested to estimate the sperm concentration using 9% hypertonic NaCl solution and evaluate motion attributes in samples having concentrations in the range recommended by the producer. Presently, evaluation of sperm concentration and motility parameters by most CASA systems is performed in samples ranging from 20–50 million/ml to 300 million/ml, however, these estimates need to be validated and corroborated with manual methods as well. Accuracy of CASA measurements should also be compared for semen from different sires against reference values determined with a sperm counter (NucleoCounter) or hemacytometer (Hansen *et al.* 2006). The use of DNA staining (Hoechst 33342) which can distinguish sperm cells from seminal debris or other contaminants has improved the working efficiency of CASA analysis (Farrell *et al.* 1996).

Importance of CASA-based sperm morphology in fertility

Assessment of morphology and sperm head is considered important by clinical theriogenologists during semen examination which is still performed with manual methods subjectively. However, large variations exist between technicians and laboratories in the subjective evaluation of semen characteristics which revealed a clear cut need for improvement in repeatability (Broekhuijse *et al.* 2011b, Lu *et al.* 2013). Objective methods measure head length, width, area (total number of pixels), perimeter, shape ellipticity, elongation, rugosity and regularity of each sperm head (Marti *et al.* 2012). Over the years, the methods for assessment of morphology developed progressively. Initially, sperm morphology was assessed manually using digitalizing tablets, followed by video imaging processors to capture sperm images and subsequently, CASA systems were introduced to exhibit morphological differences objectively in comparison with previous subjective evaluations (Amann and Waberski 2014). There are three steps involved in CASA measurement of sperm morphology, viz. (1) sample preparation (specimen washing, fixing, staining); (2) image capture by microscope under bright field illumination; and (3) image processing and assessment. The CASA systems categorize the sperm cells into normal (oval shape head), amorphous (irregular head), tapered head, large and small head and immature (Yaniz *et al.* 2015). Eventually, time requirements for completion of an analysis also appeared to cause hindrances in morphological assessments (Hidalgo *et al.* 2005). Finally, Holt and Satake (2018) noticed that CASA system actually derives a sample value from sperm motion rather than the measurement of normal sperm percentage. Cluster analysis of data would allow selection of thresholds in order to group sperm based on a particular attribute. These thresholds should be validated by each user to calculate the percentage of motile sperm and circumvent the problem of normality. Combination of motion and morphology measures for each spermatozoa could help in providing a group containing 'satisfactorily rapid non-abnormal' cells.

Abnormal morphology is an important indicator of poor

fertility in bulls (Ghirardosi *et al.* 2018), buffaloes (Bisla *et al.* 2021), stallions (Køí•ková *et al.* 2017) and rams (Maroto-Morales *et al.* 2009). Previous studies (Gravance *et al.* 2009, Sellem *et al.* 2015) in bulls have shown the relationship between CASA-based morphology and fertility. CASA determination of abnormally shaped sperm cells is less likely to progress through the female reproductive tract and fertilize the oocytes (Sundararaman *et al.* 2007). A high correlation (r=0.762, P=0.010) was observed between sperm morphology analyzed by CASA and fertility in bulls (Gillan *et al.* 2008). Likewise, morphologically normal sperm assessed by CASA were highly correlated (r=0.42, P<0.05) with fertility in stallions (Love 2011). A similar relationship of CASA-based sperm morphology with fertility has been depicted in rams (Robayo *et al.* 2008, Maroto-Morales *et al.* 2009) and buffaloes (Rishipal *et al.* 2017). Failure of fertilization was associated with high percentage of abnormal sperm measured by CASA in rabbits (Lavara *et al.* 2008). However, morphology measures alone may not be adequate in determining the fertilization and should be associated with motility parameters in clinical applications. Combination of motion and morphology measures for each spermatozoa could help in providing a group containing 'satisfactorily rapid non-abnormal' cells. Although, CASA systems are available for more than four decades in the field of veterinary medicine, morphological evaluations of CASA have not yet fully explored in eliminating the biases of subjectivity.

Significance of CASA in quality assurance

Quality assurance is defined as the ability to certify that a product/system is devoid of any defects, works properly and the quality control program is effective. The main aim of quality control is to test, inspect and ensure that the product/system is complete and meets all required criteria. Both quality assurance and quality control go hand-in-hand. To ensure proper working of a CASA system validation is vital and must be repeatable and reliable. In a semen production facility, validation is referred as system, settings and species specific for any measurement of motion or morphology (Amann and Waberski 2014). For each instrument or system, optimum validation of each output measure is must, failing which one cannot be sure that the data available is true or not. Consistency and validation of a CASA system starts with the manufacturer and should fulfill all the specifications with respect to use (Boyers *et al.* 1989). Each CASA system should provide data of each output parameter with precision and accuracy, capable of reproducing primary data and facilitate internal and external validations using stored digitized images (Verstegen *et al.* 2002, Lu *et al.* 2013). The end user should validate the system before each use and after any update of software. Precision and accuracy of each sperm measurement using CASA can be affected by chamber depth and filling, sample type, sample mixing pipetting technique, magnification and focus, illumination and optics (Broekhuijse *et al.* 2012). Apparently, high-quality certified chambers with set chamber depth must be used in the software (Douglas-Hamilton *et al.* 2005a, 2005b). For each sample chamber used, each software setting must be optimized. These issues can affect the predictive value for fertilizing potential of sperm. The goal of each validation procedure should include working with a reference laboratory wherein advice from skilled personnel can be accessed. In this context, all technicians must be trained at regular intervals as defined in the quality assurance program (Broekhuijse *et al.* 2011b). Validation across laboratories is necessary for commercial semen processing. In any validation process, the system should correctly detect and identify each sperm in a field and exclude all particles which do not represent complete spermatozoa. Ideally, correctness of these decisions should be >95%. Sperm motion is the most important CASA output. Precision of curvilinear path is essential since it forms the basis for other motion traits (Boyers *et al.* 1989). Therefore, minimum guidelines of quality assurance for total system validation are desirable.

Fertility prediction of semen following artificial insemination

The actual fertility of particular semen can be achieved after its insemination. Therefore, it is not wise to consider that only CASA measures of a given sample or a group of samples which may otherwise be normal can accurately predict fertility. This could be due to the fact that each female inseminated with a particular semen sample changes the appearance of each sperm attribute and subsequently modifies the combined effect of all attributes for each cell. Nevertheless, this may not stand true in all the females inseminated with that particular semen sample (Amann and Hammerstedt 2002). Moreover, environment, management and genetic lineage of male and female also affect the success of inseminated sperm (Broekhuijse *et al.* 2011b, Broekhuijse *et al.* 2012). Ultimately, commercial semen is processed to reduce the differences among samples, if any, thereby resulting in lower variation in pregnancy rates (Amann and DeJarnette 2012). Although CASA analysis of motion assessment is notable, association with other sperm characters is still deficient, which are equally important for fertility but remain non-validated.

Role of CASA in a commercial production setting

In a production center, the primary aim of CASA systems is to (1) correctly identify ejaculates having more subpopulation of sperm which is abnormal in order to discard bad samples before processing; (2) provide desired fertility via motion and/or morphology analysis; and (3) optimize the number of sperm placed in each dose through measurement of concentration (Hunter *et al.* 2011). Under certain conditions where management of herds is less ideal, the production centers struggle to achieve optimum outcomes (pregnancy rate and litter size) for their users. Therefore, operators/technicians should be able to correctly identify ejaculates with poor potential taking into consideration that such ejaculates may not provide desirable

fertility in managed herds also (Amann and Hammerstedt 2002). Some CASA systems detect sperm having impaired plasma membrane, cytoplasmic droplet or bent tail and provide relevant information for its discard/use. All semen marketing organizations discard samples having morphologically abnormal sperm in excess (Broekhuijse *et al.* 2011a). Primarily, CASA systems rely on VCL and STR for each spermatozoon to process the samples meeting a sufficient percentage of satisfactory sperm and discard unsatisfactory ones. The actual demarcations for VCL and STR or any other attribute should be validated because these can help the computer in providing a decision to discard or process the sample and in calculating the percentage of sperm in each group. Moreover, CASA analysis of motion traits is advantageous because the data can be easily downloaded and shared. All semen processing units pack doses of each ejaculate or pool of ejaculates having optimum sperm concentration (3×10^9 chilled boar sperm or 10×10^6 cryopreserved bull sperm) to minimize differences within batches and achieve maximum outcome after insemination (Holt 2009, Amann and DeJarnette 2012). Therefore, identification of inferior samples based on outcome of AI is imprecise for use in commercial herds. In most bull artificial insemination centres, determination of sperm concentration by CASA has been considered inaccurate and imprecise in comparison with manual methods. On the other hand, use of CASA tool as a measure of sperm concentration is more common in centres where stallion or boar semen is processed (Amann and Waberski 2014).

Factors affecting the accuracy of output data of CASA systems

Accuracy and precision of CASA outputs has been attributed to various factors given below.

Sperm concentration: Prior to CASA analysis, the samples need to be diluted since the concentration of neat ejaculate is too high for the successful analysis of individual spermatozoa tracks (Verstegen *et al.* 2002). Contri *et al.* (2010) reported a lower sperm concentration (20×10^6/ml) for evaluation by CASA since at higher concentration inferior sperm were detected.

Sample volume: Following dilution, the volume of semen used for CASA analysis varies between 4–10 µl and depends upon the counting chamber. Due to small volume, the samples need to be homogenized and processed gently. Different producers market chambers of different volumes, viz. 4 µl for Makler and Leja chambers, and 7 µl for Microcell chambers. Sperm motility can be influenced by different volumes of chambers (Gloria *et al.* 2013, Amann and Waberski 2014).

Type of extender: The diluting media used also has an impact on CASA analysis. In egg yolk extenders, the yolk granules are similar in size to sperm cells which can adversely affect the assessment of motile sperm percentage and ultimately the accuracy of results (Vincent *et al.* 2012). Higher viscosity in egg yolk type media resulted in reduction of kinematic parameters like VAP, VCL and VSL (Simonik *et al.* 2013).

Duration of analysis: Contri *et al.* (2010) reported that the duration of analysis should not exceed five minutes and could affect sperm motion measures.

Number of fields and cells to analyze: The precision of results increases as the number of fields and cell analyses increase. In bulls, the measure of 30 fields and approximately 300 cells is recommended whereas in stallion analysis of about 300–500 cells appear to be optimal (Verstegen *et al.* 2002). A minimum over 100 cells should be counted.

Sperm-suspending medium: The type of sperm-suspension solution also has an impact on CASA outputs. Using TALP or HEPES compared to saline solution for dilution of sample increased the percentage of motile and rapidly moving spermatozoa (Rijsselaere *et al.* 2003).

Frame rate: This factor is important since several kinetic parameters are dependent on frame rate. However, the choice of frame rate is not only equipment-dependent, but also species-specific. In most mammalian semen analysis, 60 frame/sec is widely accepted for most CASA applications (Kathiravan *et al.* 2011)

Thawing temperature: A possible effect of thawing temperature on some motion parameters has been observed during the analysis of post-thaw AI doses (Iguer-ouada and Verstegen 2001, Contri *et al.* 2010).

Thresholds: It is vital to use suitable optics and illumination to augment the contrast of the spermatozoa head which in turn facilitates the manual control of the detection threshold

System settings: Different CASA softwares having different settings that affect the reliability of results (Amann and Waberski 2014).

Individual sperm differences: Another indicator to predict the fertilizing capability of insemination dose could be distinct changes in each ejaculate (Muino *et al.* 2008).

Hyperactivation: This process is shown by sperm as they progress through the female genital tract to the point of fertilization. During hyperactivation, capacitated sperm exhibit vigorous, non-progressive and non-linear motion and could be a prognostic indicator of sperm characteristics associated with fertility *in vivo* (Shojaei *et al.* 2012). The shift from progressive to hyperactivated motion is a progressive phenomenon involving intermediary or transitional pattern. The distinguishing characteristics of hyperactivated spermatozoa are VCL >70 µm/s, ALH >7 µm, LIN < 30%, and VSL <30 µm/s (Marquez and Suarez 2007).

Human factor: This factor can influence almost all of the aforementioned factors. Therefore, high skilled operators and/or technicians are required to operate latest CASA systems that need to be trained periodically (Broekhuijse *et al.* 2011b, Michos *et al.* 2013).

Limitations of CASA

In addition to motility assessment, CASA systems can also be used for evaluation of sperm concentration, morphology and viability. However, an accurate assessment

of sperm concentration using CASA is a limitation in most species (Verstegen *et al.* 2002). The main problems are overestimation of the sperm numbers and subsequent re-counting of already analyzed objects. The current CASA instruments are not able to analyze sperm morphology for routine use due to lack of ability to determine the central part of sperm flagellum (Björndahl *et al.* 2010). Morphology analysis using CASA is influenced by magnification, type of staining, output quality and software settings (Verstegen *et al.* 2002). Differences among CASA systems also represent obstacles of objective comparison of results across laboratories and in investigating prediction values of CASA outputs to bull field fertility. Additionally, high cost of CASA systems has resulted in limited marketing in veterinary medicine. This has led to the development and validation of open-access and adaptable plugins for different species which are of low cost with better user background knowledge and understanding (Elsayed *et al.* 2015). Above all, do CASA-based parameters make possible to forecast bull fertility, is still a question of debate? Although CASA provides many parameters for each sperm cell, along with data for semen evaluation and prediction of bull fertility, accuracy of motility parameters to predict field fertility has still not been fully explored.

Future developments in CASA systems

Although CASA systems have greatly improved during last 40 years, still there is continuous need for further up-gradation of CASA systems to acquire accurate, precise and validated results. Accordingly, a simple, portable and cost-efficient CASA system capable of measuring sperm motion and morphology accurately is the need of hour for andrologists working in the field. This can be accomplished by improving the computer software to provide sufficient and reliable data, reprogramming detector arrays and providing thermal control adapter (Sanz *et al.* 2017). Using laminar-flow instruments with low-pressure based on fluorescence microscopy with imaging can compute fluorochromelabeled probes (upto 5 colors and 5000 cells per second) and provide a high-resolution image of each sperm morphology under bright-field and/or dark-field (Zuba-Surma *et al.* 2007). However, such instruments cannot measure sperm motion. Another approach is through use of lens-free holographic microscopy to acquire three dimensional sperm motility patterns and trajectories with quality image capture. This system involves usage of enhanced depth of field in a deep chamber (150 µm depth), and capture images at <143 frames per sec for 10–20 sec in <50 fields of view to provide high-resolution digital image of cells (Su *et al.* 2012).

Conclusion

Today CASA is widely accepted by the veterinary fraternity for conducting research on sperm biology as well as for use in production units. CASA represents a tool for more objective and accurate analysis of sperm motion than subjective evaluation. Sperm motility is one of the most traditional parameter evaluated by CASA. The accuracy and precision of CASA output in comparison of results across laboratories purely depends upon algorithms, software, object recognition, threshold values for subpopulations and training of technician/operator. Validation of above-mentioned factors can provide quality assurance of CASA analysis. Future improvements in CASA might help in visualization of 3-dimentional image of sperm motion as well as production of a portable CASA system for use in veterinary practice.

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