Andrology laboratory review: evaluation of sperm motility

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Abstract

This review is an effort by the andrology taskforce of the Association of Applied Animal Andrology, American College of Theriogenologists, European College of Animal Reproduction, and Society for Theriogenology. It is intended to serve as a reference on methods to evaluate sperm motility in domestic animals and to contribute to adoption of best practices in veterinary andrology laboratories and semen processing centers. Sperm motility evaluation topics covered include sample preparation, subjective evaluation, computer-assisted semen analysis, and use of sperm quality analyzers. Emphasis is given to principles of the methods, equipment, performing evaluation, and common mistakes and/or pitfalls. In addition, precision and accuracy of the various methods are discussed.

Introduction

Semen analysis constitutes the most important clinical laboratory test currently available to evaluate male fertility. In domestic animals, this analysis is commonly used to: attest breeding soundness, characterize semen samples for trade, diagnose suberfertility/infertility, and guide clinical and management decisions.

There are 2 major quantifiable traits involved in semen analysis, i.e. sperm number and semen quality. The first of these was addressed by this task force in a previous publication[1] which represented the first of a series. Semen quality includes sperm vitality, motility, morphology, and seminal fluid composition, aspects of which will be addressed in subsequent publications, with the current work focusing on sperm motility assesment. Although sperm motility evaluation is generally considered an essential routine component of semen analysis, it is sometimes viewed dismissively, with results taken for granted without proper validation.[2] Nonetheless, studies have reported interlaboratory coefficients of variation (refer precision and accurancy of methods) > 20% for sperm motility.[2,3,4] This illustrates the difficulties in comparing results among laboratories and in generalizing the findings of scientific studies.

Recognition of relative subjectivity, and variability, in sperm motility assessment, particularly as traditionally perfomed, has led to a number of attempts to develop more objective and consistent methods, some of which are described herein. This review is intended to address the lack of formal training materials for semen analyis laboratory personnel and to serve as a reference on methods to evaluate sperm motility.

Sample preparation

Species-specific considerations

Differences in reproductive biology, including testicular size, sperm production capacity, epididymal sperm storage capacity, and ejaculate volume contribute to physiological differences in sperm motility among species. Methods of semen collection, sexual stimulation, and the environment can also affect ejaculate parameters. In addition, ejaculates of some species consist of distinct fractions that differ in number of sperm, thereby affecting sperm motility evaluation. A critical objective is to obtain a representative sample that can be used for evaluation.

Sperm motility reflects an essential functional sperm trait that allows sperm to reach the site of fertilization and fertilize oocytes. Sperm motility is a complex phenomenon that is finely regulated by its environment. Ions, and metabolic and enzymatic factors influence sperm kinematics to various extents, depending on the responsiveness of sperm subpopulations in the semen sample.[5,6] Additionally, the type of extender and dilution may affect sperm motility traits, at least in the first hours after dilution before sperm can adapt to a changed micromilieu.[7]

As suggested for dogs,[8] sperm motility, in general, should be performed as soon as possible after collection in all species or at least within 30 minutes. If possible, sperm motility should be first assessed in raw semen, because addition of extenders may change the natural environment and compromise the assessment of male intrinsic sperm motility values. Since temperature dependence of sperm motility is broadly acknowledged, assessment of motility in a heated environment at 38°C, with a possible range of 37 - 39°C, is standard in andrology laboratories.

Differences in ejaculate composition and sperm metabolism mandate differing techniques of sample preparation and evaluation. For example, the possibility to estimate mass motility is restricted to raw semen of species with high sperm density ejaculates as in ruminants.[9]

Boar sperm are unique in that preserved semen is incubated at 38°C prior to motility assessment to have optimal results. Dog semen is usually collected in fractions and sperm motility is assessed without prostatic fluid, as that may induce sperm hypermotility.[10] In addition, sperm motility estimation in liquid or frozen semen might require different sample preparations, depending on the preservation method. Resuspension and incubation in specific postthaw extender might be required, according to semen processing center instructions.

In most species, 30 minutes incubation is generally recommended to display stable sperm motility from extended and cooled samples. Some extender manufacturers stipulate extender-specific incubation times, ranging from 2 to 30 minutes.

Subjective assessment of sperm motility

Principles

Sperm, unlike other cells, are designed to function principally outside the body which produces them. The functional environment of sperm is essentially a foreign object adrift in an external aqueous fluid or engulfed within a new biological niche. Although capable of intense activity, sperm generally remain quiescent until ejaculation.[11] Assessment of motility of ejaculated sperm is considered an important measure of function. Hence, in veterinary practice, subjective evaluation of sperm motility has been a fundamental component of assessing the potential fertility of males. Besides, determination of sperm motility score, to some extent, enables practitioners to decide the number of sperm per dose.[12]

The concept of gross or wave motion and individual sperm motility was adopted by the Society for Theriogenology for evaluation of bull semen[13] and later adopted to other ruminant species. Traditionally, gross and individual sperm motility has been evaluated subjectively using light microscopy at magnifications ranging from 100 - 400 x, a method that is rapid, simple, and inexpensive.[14]

Gross motility is estimated under lower (100 x) magnification without a coverslip and is scored in a scale (e.g. 0 - 4; 0 - 5; or 'poor,' 'fair,' 'good,' and 'very good'). Gross motility results from the interaction of individual sperm motility with sperm concentration and refers to the swirl pattern observed. Gross motility ranges from no swirl (score 0 or poor) to a fast distinct swirls referred as 'waves' and 'eddies' (score 4 - 5 or very good).[9,15] It shoulde be noted that gross motility is not a

measure of individual sperm motility patterns since immoitle sperm are also carried along with motile sperm.

Determination of individual progressive motility involves the use of higher (200 - 400 x) magnification and mostly in a diluted sample with a coverslip. The scoring system is based on percent progressive motile sperm. When every sperm in a field has rapid, progressive, forward motion, then the score is ~ close to 100%.[15] Progressively motile sperm are those in which the sperm exhibit forward motion in a 'relatively' straight line. For most species, the concept of individual progressive motility is intended to describe the population of sperm that are moving in a forward manner.

In stallions, sperm motility is often represented as both total or progressive. Gross sperm motility is not analyzed because the sperm concentration of stallion ejaculates, in general, is lower than ruminant ejaculates. Total sperm motility is the percent of sperm that display any type of movement, including those that lack forward motility but exhibit tail movement and includes those that are actively moving forward (i.e. progressively motile). Definition of progressive sperm motility in stallions varies considerably amongst examiners and laboratories. Stallion sperm do not typically have straight trajectories, and this may be due to the abaxial attachment of midpiece to the head of sperm in a relatively high percentage of the sperm population. This is considered to be a normal occurrence. For example, the stallion manual published in 1983 by the Society for Theriogenology described progressive motility as follows: 'the most important and critical aspect of motility is progressive motility, i.e. sperm are actively moving forward. Large, circular motion of some normal sperm is mostly due to high incidence of normal, abaxial connections between sperm head and neck'. Later, it was suggested[16] that 'the percent of sperm that appear to be morphologically normal and moving relatively straight across the microscopic field. Since stallion sperm have abaxial midpieces, they sometimes move in a circle. Movement of sperm in small, very tight circles are considered undesirable' and it was further described[17]as follows: 'for a spermatozoon to be called progressively motile, it must move across the microscopic field reasonable rapidly and with each back and forth lash of the tail, the head must rotate 360°'.

Suggested standards prescribed for progressive motility are not consistent across species. Furthermore, depending on the qualifier (threshold) adopted, an animal can be classified as a 'satisfactory breeder' or an 'exceptional breeder.' For example, in small ruminants, an animal with 30% progressive sperm motility in raw semen is classified as a satisfactory breeder and one with 90% progressive motility is classified as an exceptional breeder, provided they meet the requirements of other parameters of a breeding soundness evaluation. It is also important to recognize that the acceptable percent progressively motile sperm is not similar across organizations and countries and its description is beyond the scope of this review. For example, Society for Theriogenology uses a score of 30% for bulls,[18] whereas the Western Canadian Association of Bovine Practitioners uses a score of 60%.[15] In addition, for dogs, it is 70% (sperm must be moving in a straight line)[19] and for stallions it is 60%.[20] For boar semen, estimation of gross or total motility (instead of progressive motility) is recommended, as boar sperm tend to display circular movement, which in some cases was explained by the abaxial tail attachment to the head.[21]

Although the evaluation of gross motility is subjective, determination of individual progressive motility is even more so. The degree of inaccuracy in the evaluation of gross and especially individual progressive motility has profound clinical consequences (i.e. 'pass' or 'fail'), especially when sperm motility is performed as the only sperm quality test. Since the introduction of individual progressive motility as a method of determining sperm quality, knowledge of sperm kinetics and factors that alter sperm motility has expanded. We understand that some changes in sperm trajectory may not influence sperm viability or fertility. For example, immotile sperm can be viable; temperature reversibly (an artifact) alters the type of sperm motility (progressive to nonprogressive and vice versa) as well as the velocity; and removal of most of seminal plasma will improve the longevity of motility.

Equipment

Microscope type and magnification can affect the estimation of sperm motility. Brightfield type microscopes provide a flat image with relatively poor detail discrimination, resulting in poor identification of immotile sperm. Properly aligned phase contrast or differential interference contrast microscopes are recommended and considered essential for accurate motility estimation.[22] The

contrast in the image field provides a more detailed and clearer image of both motile and immotile sperm in the field of view.

Microscopes must be equipped with appropriate objectives and a heated stage at 37 - 39°C. A second associated heated stage and or warming cabinet should also be available to warm everything (e.g. pipette tips, slides, and cover slips) that contacts semen. For preserved boar semen evaluation, a water bath or dry block heater is required. All material must be intact, clean and stored protected from dust.

Slide and coverslip type (plastic versus glass) can affect the quality of sperm motion. Slides should be glass and clean. Depending on the manufacturer, glass slides can vary considerably in their clarity and cleanliness. If a slide is cloudy or dirty, it should be cleaned with alcohol, rinsed thoroughly with distilled water, and dried to assure no toxic residue is present. Coverslips should also be glass, as that assures a more even distribution of semen compared to a plastic coverslip. Avoid leaving finger marks while handling slides and cover slips.

Evaluation

Any circumstances that may have compromised sperm motility (e.g. failures or fluctuations in storage temperature, storage duration, and semen packaging) must be recorded. Semen samples should be evaluated immediately after collection or at the latest within 30 minutes after collection for all species. If it has to be transported to a laboratory, the evaluation should be performed immediately on arrival. Variation can be expected, if there is a delay in evaluation. If samples are not evaluated immediately following collection and poor sperm motility is recorded, the evaluator must take this into account and reexamine the male, ensuring optimal handling conditions. If evaluation of the sample is delayed, raw sperm should be diluted in a suitable extender immediately following collection.

At extended intervals following semen collection, deleterious effects of dehydration, pH and temperature changes, and metabolic waste products might become apparent. Semen is a nonhomogeneous suspension and sperm tend to sediment. Therefore, immediately before taking an aliquot, careful mixing of the semen container without creating air bubbles or sample foaming is required.

Type of wet preparation as determined by droplet size and dimensions of the glass slide cover slip systems or the measurement chamber has a major influence on motility estimates.[23] It is considered that currently applied subjective motility measurement is limited to analysis of 2 dimensional movements of sperm. Hence, in vitro conditions need to be thoroughly defined and standardized. Based on these aspects, it is important to recognize the relative value of subjective motility estimates and resultant limits for inter-laboratory comparisons.

Placement of semen on the microscope slide depends on both the coverslip dimensions (i.e. 22 x 22 mm) and 'drop size' used. A prewarned pipette tip is used to deposit 5 - 10 µl semen on a warm microscope slide and overlaid with a prewarmed coverslip. Volume of semen and dimensions of coverslip must be standardized, so that ideally the analyses are done on a preparation of fixed depth of ~ 20 µm. Lower sample depth may impair rotational motion, whereas higher sample depth hinder assessment by creating a multilayer of sperm. For example, a 6.5 µl sample covered with an 18 x 18 mm coverslip (area 324 square mm) provides a depth of 20.1 µm, whereas 10 µl of semen delivered covered with a 22 x 22 mm coverslip (area 484 square mm) provides a depth of 20.7 µm.[23] Clarity can be improved with the use of # 11/2 cover slips compared to # 1 cover slips. Sample volume and size of cover slips must be standardized for each species, and for raw and diluted semen.

Assessment of raw semen may require dilution to minimize sperm overlay. Dilution however, may alter sperm kinematics as outlined above. Alternatively, the droplet size can be reduced, but a sample depth should not be < 10 µm. Sperm from some samples are more susceptible to adhere to a coverslip and thereby give the impression that the sperm are immotile. Upon closer inspection, it will become apparent that sperm heads are immotile, although midpieces and tails are vigorously moving. This is usually an artefactual change that is alleviated once semen is extended.

Dilution of raw semen to a specific sperm concentration (e.g. 20 - 40 x 10^6 sperm/ml) allows the evaluation of individual sperm trajectories and provides fewer sperm for the examiner to evaluate in a field. This is critical in evaluating total and progressive sperm motility. For example, for bull semen samples, an acceptable dilution is to have 10 µl of semen diluted in 790 µl of physiological saline.[24] Raw semen should be diluted in a medium/extender that does not reduce sperm motility. If motility estimates are lower in extended semen than raw semen, then alternative extender batches or

formulations should be used. Reduced motility in the extended sample can only be diagnosed if the motility of the raw sample has been evaluated. If sample concentration is standardized, then the number of sperm visualized per field will be uniform across samples. Sperm concentration of the semen sample evaluated will also create artifacts. More concentrated samples tend to look better than less concentrated samples of similar motility; this tends to be a perception rather than real difference, for several reasons. In more concentrated samples, more motile sperm occupy the microscope image, and thus collide with immotile sperm, giving the perception that immotile sperm are also motile. Human eyes tend to follow motile rather than immotile objects, and thus immotile sperm may be ignored.

The wet preparation is examined under lower magnification to verify a clear layer of sperm that is in sample homogeneity without air bubbles. The limitation of poor image quality is not only the inability to clearly visualize relevant features, but in the case of evaluating sperm motility, may result in an artificially high motility values, since the only sperm evaluated may be those that are motile. The quality of the microscope can influence the degree of subjectivity and variability, resulting in erroneous scores.

Freshly prepared wet preparations are assessed as soon as sperm are no longer drifting. Total motility and progressive motility are estimated at 200 - 400 x magnification by assessing the amount of sperm activity to the nearest 5%. Multiple fields (≥ 5) should be evaluated, avoiding the periphery of the coverslip where motility is often lower. Fields near sample edges and those with lower motility should be excluded from assessment, due to potential artifacts.[25] If motility estimations are below threshold values, a second sample should be prepared to confirm the findings. Sperm are only considered motile if a flagellar beating can be observed. Progressively moving sperm have either a linear movement or move in large circles. Nonprogressive sperm move in small circles with a diameter < sperm's length. Sperm are classified as immotile if they are static and do not have flagellar beating. Percent total motile sperm is estimated or calculated from the total percent sum of progressive and nonprogressive sperm. Sperm velocity of movement (scale of 0 -5) determination[26] provides supplemental information.

Mistakes and pitfalls

Regardless of the method of semen collection (e.g. artificial vagina, gloved hand, electroejaculation), all components that come in contact with the semen and environmental temperature have the potential to negatively impact sperm motility. Therefore, materials that contact semen should be scrutinized for potential spermicidal influence and should be maintained at 37 - 39°C to prevent cold shock. These materials include the artificial vagina, collection cone, disposable gloves, collection receptacle, glass microscope slides, coverslips, and pipettes. As previously mentioned, the microscope stage should also be temperature controlled to assure that sperm motility is maintained. Sperm motility can be easily affected by heating, chilling, and contamination with urine or other fluids, including soap.[15]

The size of the semen drop should be consistent; therefore, use of a pipette to measure droplet size is essential. Variation in droplet size can create artefactual changes in sperm motility. A small drop that does not cover the whole coverslip can result in low motility due to the thinness of the drop causing resistance and impeding the ability of the sperm to move freely. Conversely, large drops result in significant drifting of sperm, making discernment between the drift of an immotile sperm and a motile sperm challenging. The semen drop used should represent the sample of sperm, as an error may be caused by assessment of a nonrepresentative sample of sperm.

Computer-assisted semen analysis

Principles

Computer-assisted semen analysis (CASA) refers to the automated assessment of motility of individual sperm. CASA involves hardware (video camera, video-frame grabber, and computer) and software designed to acquire and digitize successive images of sperm, process and analyze the sequence of images, and finally provide information on the kinematics of individual sperm.[27,28] Results of sperm motility analysis by CASA are considered more accurate, objective, and precise than other analyses.[29] Various CASA systems for analysis of sperm motility are available.

Sperm motility is completely dependent on functions of the sperm midpiece and tail, whereas the sperm head does not influence the movement, but moves in response to the flagellum. Nevertheless, CASA will assess movement of the sperm head because technically this is easier to follow as a focal point for analysis[27] since it can be more readily captured owing to its size and lumniosity. Furthermore, as a direct relationship between head movement and flagellar beats can be assumed, analysis of sperm head movements provides significant information on differences in sperm kinematics under varying conditions. With most systems, CASA depends on sperm images via dark field, negative or positive phase-contrast microscopy. The image of the sperm head is digitized and the number of pixels covered by the sperm head is determined. The computer recognizes any object that falls within an designated size range of a sperm head. This range has to be defined and depends on the species. After identification of the sperm head, its coordinate position on the microscope field is calculated based either on the center of the sperm head (also termed centroid) or on the brightest spot of the sperm head.[30,31] After all sperm heads in a single field or frame have been identified and located, the next frame is analyzed. The head of an individual sperm is recognized on the consecutive frame if it appears within a zone of probability (i.e. a circle of a particular radius around the sperm head). This radius depends on the maximum distance a sperm would be expected to move within the time period. It is usually set by the user and is influenced by the medium used to dilute the semen. Finally, the trajectory of motion is reconstructed for each sperm based on its centroid trajectory.[27]

Dynamic values (sperm motion parameters) are then calculated (Table 1). These usually include sperm motion velocity (VCL: curvilinear velocity; VSL: straight-line velocity; and VAP: average path velocity), velocity ratios (LIN: linearity; STR: straightness; and WOB: wobble) and sperm wobble characteristics (ALH: amplitude of lateral head displacement; and BCF: beat-cross frequency). Some sperm motion parameters may not be comparable among CASA systems, due to differences in the algorithms used to compute them (e.g. VAP, ALH).[31] Further analysis of datasets by cluster analysis allows for grouping of sperm into subpopulations; however, the biological meaning of these subgroups still has to be established. [27,28]

Future developments of CASA technique aim for capturing clearer images, such that captured sperm motion trajectory would more accurately reflect the actual sperm motion characteristics.[32] One key feature of CASA is the frame rate (number of images per second) of the video camera. Frame rates were low (~ 4 - 5 Hz) in early systems, but today 30 - 60 Hz are routinely used and CASA systems with 80 - 100 Hz are available. For reliable analysis of mammalian sperm motion characteristics, > 50 Hz is recommended[27] and is commonly used. Frame rate influences the shape of the trajectory, with low frame rates providing tracks that appear much simpler than in reality.[30,32] Minimal sampling time is 0.5 seconds to aquire reliable kinematic values of sperm trajectory in human sperm.[33]

If disposable specimen chambers are used, chamber depth must allow for unconstrained sperm movement and will therefore depend on the species-specific movement pattern of sperm. Furthermore, chamber depth must match optics to achieve an appropriate depth of focus.[33] For a 10 x objective, the chamber depth cannot exceed 20 µm to provide reliable tracking of sperm.[34] A depth of 10 or 20 µm is recommended, but will not always provide optimal space for physiologic swimming of sperm in certain species.[30] A 30 µm chamber (eflow) is currently used. It is clear that CASA has already evolved substantially and is currently superior to other techniques in the research and clinical setting.[35] Future CASA systems will probably be able to integrate several sperm quality parameters with motility[36] and also use 3 dimensional tracking of sperm movement and flagellar analysis through flagellar and sperm tracking.[37,38]

Equipment

The CASA system consists of a phase contrast microscope, video camera and a computer (hardware) with specific software. A list of commercial manufacturers of CASA is provided elsewhere.[39]

Evaluation

Common steps in evaluation include instumentation settings, sample preparation, and assesement of motility. Protocols may differ, depending on the device developed and species of interest.

Table 1. Explanations for some common CASA kinematic parameters, their abbreviations and dimensions [27]

Abbreviation	Meaning	Explanation	Dimension
VCL	Curvilinear velocity	Total distance that the sperm head covers in the sampling period	µm/s
VSL	Straight-line velocity	Straight-line distance between the first and last points of the trajectory	µm/s
VAP	Average-path velocity	Distance the spermatozoon has travelled in the average direction of the movement in the observation period	µm/s
LIN	Linearity	(VSL/VCL) x 100 Linearity of the curvilinear path	%
STR	Straightness	(VSL/VAP) x 100 Linearity of the average path	%
WOB	Wobble	(VAP/VCL) x 100 Oscillation of the actual path about the average path	%
ALH	Amplitude of lateral head displacement	Width of the lateral movement of the sperm head, it is calculated as the total width of the head trajectory	µm/s
BCF	Beat-cross frequency	The number of times the sperm head crosses the direction movement, calculated by counting the number of times the curvilinear path crosses the average path per second	Hz

Mistakes and pitfalls

Despite the application of many automation principles to CASA, manual intervention is still necessary for correction.[31] Technical errors are mainly based on failure to discriminate between sperm and nonsperm objects, and between immotile and motile objects.[34] A major problem of CASA is related to the standardization and optimization of the equipment and procedures. Internal image settings such as minimum contrast, frame rate or analysis time, which are important for identification and reconstruction of the trajectory of sperm, clearly influence the results.[40]

It is unwise to assume that outcome values from 2 different types of CASA system, analyzing the same sample, would be almost identical even if end points of study were the same. Hardware configurations and software algorithms could differ. Second, conditions to study sperm motion are a compromise. Sperm are suspended in a medium unlike any they will encounter in vivo. The suspension is viewed in a chamber (or droplet) in which sperm accumulate at interfaces between the suspension and air, or chamber wall, where they swim differently from the interface.[30] A summary of factors that may affect CASA measurements of sperm motility and recommedations to avoid variations and misestimations are reported.[39] They can be summarized under 4 major categories (sample and slide, microscope, hardware and software, and user).

Other factors that can influence the outcome are sperm concentration, settings, medium (seminal plasma and extender used for anlysis alter sperm kinematics), sampling time, chamber size (alter velocity and motion pattern of sperm since sperm tend to associate with surfaces because of surface tension). Analysis should be postponed until specimen drift is gone, i.e. all visible flow of medium has ceased.

Perhaps the greatest misunderstanding surrounding CASA is the implicit acceptance that it provides a 'gold standard' for sperm motion. CASA cannot and should not serve as a 'gold standard' in respect to type or nature of motion, because we do not know how sperm, regardless of species, should swim at any stage of their sojourn through the female reproductive tract.[41] Direct comparisons among laboratories might not be possible, because the estimation of end points is affected by the settings, type of slide/chamber used during testing, preparation of sperm for analysis, and operator regulated selection of microscopic fields for examination. Role of training staff is very crucial in quality control and periodical evaluation is essential. There are no perfect systems, but a crucial point is that standarization and validation of the system are performed and optimization of protocols are assured.[39]

Sperm quality analyzer

Principles

The term sperm quality analyzer (SQA), represents a proprietary technology in which fluctuations in optical density (OD) are recorded using a modified light beam as it passes through a

sperm population contained in a thin glass capillary. A photometric cell registers OD variations as electrical pulses that are digitally transformed to provide a sperm motility index (SMI).[42] In turn, the SMI is claimed to provide an indication of overall sperm sample quality, including sperm concentration, progressive motility and normal morphology. [42] Today, several hardware models are available with specific software for various species.

Studies using SQA/SMI methodologies have been conducted in various species[43] including humans, cattle, pigs, dogs, horses[44] (SQA-Ve [Vequine]) and birds. Claimed advantages over traditional systems include relative speed of operation, reduced cost, and less human error, whilst providing automated data recording,[45] all of which represent potential advantages for industry application. Good statistical relationships have been reported for motile sperm concentrations[46] within prescribed sperm dilution windows, although similar relationships with sperm morphology or fertility are not as well established.[47] Utility of the SQA (i.e. an SQA-Vb) as an industry screening tool was demonstrated in an Israeli study, in which progressively motile sperm numbers were strongly associated with pregnancy rates following 8000 bovine inseminations.[48] Some of the units provide information on percent progressive motility and total motility, as well as values for velocity, concentration of raw sample, and percentage of morphologically normal sperm.[49]

Equipment

Equipment has to be selected based on the species. For example, SQA 11-C was used for bulls,[47] dogs,[50] and boars,[46] SQA-Vp was used for boars[51] and SQA-Ve was used for stallions.[49]

Evaluation

Each version of SQA is species specific and it is currently not possible to provide detailed information on protocol for each unit. The reader is encouraged to follow manufacturers' insructions carefully to avoid errors. Certain versions are meant for raw semen, whereas some are intended for extended semen and others for postthaw motility evaluation. To provide an example, a study used block heaters that were preheated for at least 15 minutes at 40ºC for the sample and empty capillaries (to be loaded in the system) to attain a temperature of 37ºC. The raw samples were incubated in the block heater for 5 minutes prior to testing.

Mistakes and pitfalls

Depending on the comparisons to other methods (e.g. light microscopy and CASA) and species, results may or may not validate all aspects of a SQA device that is evaluated.[51] Version and methodolgy differences have to be recognized when an equipment is chosen. Some units may be good for parameters other than motility; however, may be insufficiently accurate to determine motility for both raw and extended semen. Although auomated motility analyzers could lead to an increased standardization in and between laboratories, automated devices need to be validated for repeatability.

Precision and accuracy of methods used to determine sperm motility

Precision (or repeatability) of sperm motility results is usually reported as the coefficient of variation (CV) for results obtained across technicians or laboratories (inter-assay CV) or from multiple evaluations from the same sample (intra-assay CV). Several factors affect the CV observed in a study, such as the number of samples, the range of motility of the samples, the number of persons performing the evaluations, and the number of replicates per semen sample.

Studies investigating inter-technician or inter-laboratory CV of subjective sperm motility evaluation may use fresh semen samples when technicians work in the same laboratory (or during training workshops), whereas videos or frozen semen samples are used in studies where samples are distributed across multiple laboratories. Reported inter-laboratory or inter-technician CVs for sperm motility subjective estimates are summarized in Table 2. Considerable variation is observed among studies, but most report CVs > 15%. The only multilaboratory study involving animal samples (bovine frozen semen) reported CVs ranging from 20 to 55% in 3 separate experiments. Reported intra-technician CVs in human studies are mostly < 15% (Table 3), whereas intra-laboratory CVs ranged widely in veterinary laboratories evaluating frozen bovine samples (Table 4).

Several studies have demonstrated that variation is much greater when different motile sperm populations (e.g. rapid progressive, progressive, and nonprogressive) are categorized subjectively when compared to simply determining total sperm motility,[4,52] suggesting that laboratories should

refrain from adopting the former evaluation approach. Experience does not necessarily improve subjective sperm motility evaluation precision, as demonstrated by the relatively unchanged variation observed over time in studies with laboratories participating in external quality control programs.[4,53] However, targeted training can significantly reduce intra- and inter-technician variation of sperm motility evaluations.[52,54]

Reported intra-technician CVs for CASA sperm motility are mostly < 10% (Table 5). However, in a multilaboratory study with frozen-thawed bovine semen, CASA evaluation of sperm motility from frozen and thawed bovine samples resulted in very good precision in some laboratories (CVs < 12%) but not in others (Table 4), demonstrating that the adoption of CASA does not necessarily result in improved precision.[1] Proper training and use of standard protocols are essential to achieve adequate precision when using CASA for semen evaluation.[55]

Table 2. Inter-technician or inter-laboratory coefficients of variation for sperm motility evaluated subjectively.

Sample	Species	n	Technicians or laboratories	Parameter	CV (%)	Reference
Fresh	Human	20	4	Total motility	8.2	Dunphy et al[56]
Frozen	Human	5	10	Total motility	21.0	Neuwinger et al[3]
				Progressive motility	22.0	
Fresh	Human	17	12	Total motility	21.8	Auger et al[54]
Video	Human	128	7	Total motility	5.5	Brazil et al a[52]
				Progressive motility	25.1	
Video	Human	-	26 - 40*	Total motility	13.9 -19.2*	Alvarez et al[4]
				Progressive motility	17.3 - 27.0*	
Video	Human	59	121	Total motility	13.8	Punjabi et al[53]
				Progressive motility	15.1	
Frozen	Bovine	10	5	Motility	54.7	Brito[2]**
			2	Motility	45.0	
			4	Motility	20.2	

*Range for 9 separate trials (test periods).**Data reanalyzed according to method of evaluation.

Table 3. Intra-technician coefficients of variation for total sperm motility evaluated subjectively

Species	n	Technicians	CV (%)	Reference
Human	20	4	15.6	Dunphy et al[56]
Human	-	5	8.4	Cooper et al[57]
Human	5	12	26.2	Auger et al[54]
Human	509	4	3.5 - 10.1	Brazil et alb[58]

Studies in veterinary andrology laboratories using the SQA have reported conflicting results related to the method's precision (Table 6). A crucial detail of experimental design must be considered and is likely behind various reports, and that is whether replicate results are obtained simply by repeating the reading on the same preparation or replicates involve the preparation of a completely different subsample. Obviously, the variation with the former design is expected to be much lower than the variation with the latter.

Low CV's have been reported in SQA studies with canine, porcine, and human semen, but studies in other species have had much greater variation. With extended bovine semen samples, CVs for SMI between 2 separate capillaries ranged from 2.1 to 58.8%, although CVs could be reduced to acceptable levels (< 10%) when sample concentration ranged from 35 to 705 x 10^6 sperm/ml.[47] The CVs for SQA sperm motility from the analysis of 5 straws from 10 frozen-thawed bovine semen samples ranged from 13 to 78% (mean 47.3%), a variation considerably greater than that observed when using subjective evaluation or CASA.[1] Repeatability for SQA total and progressive motility was considered only just acceptable for extended equine semen and CVs were at least twice the CVs of CASA.[49]

Table 4. Intra-laboratory coefficients of variation (%) for sperm motility according to evaluation method from 10 frozen-thawed bovine semen batches evaluated in five replicates. Laboratories included semen processing centers and veterinary university laboratories in the United States and Brazil (Adapted from Brito[2]).

	Mean	Range
Subjective		
Laboratory 2	12.7	5 - 25
Laboratory 3	13.0	0 - 25
Laboratory 4	14.6	0 - 25
Laboratory 5	15.1	7 - 35
Laboratory 6	16.3	9 - 22
Laboratory 7	17.7	7 - 31
Laboratory 8	18.9	9 - 29
Laboratory 9	22.7	7 - 56
Laboratory 10	26.5	0 - 55
Laboratory 11	32.4	17 - 56
Laboratory 12	54.0	16 - 111
CASA		
Laboratory 13	8.5	5 - 18
Laboratory 14	8.6	4 - 12
Laboratory 15	11.9	7 - 17
Laboratory 16	15.2	5 - 44
Laboratory 17	16.9	9 - 36
Laboratory 18	19.6	7 - 42

Table 5. Intra-technician coefficients of variation for sperm motility evaluated using CASA

Species	n	Parameter	CV (%)	Reference
Bovine	4	Motility	6.0	Budworth et al[59]
Canine	8	Total motility	0.06	Iguer-ouada and Verstegen[60]
		Progressive motility	0.15	
Porcine	30	Total motility	3.1	Vyt et al[46]
		Progressive motility	6.5	
Bovine	10	Total motility	3.1 - 5.8*	Ehlers et al[55]

*Range before and after training

Table 6. Intra-technician coefficients of variation for sperm motility evaluated using the semen quality analyzer (SQA)

Species	n	Parameter	CV (%)	Reference
Canine	14	Sperm motility index	0.025 - 0.46*	Rijsselaere et al[61]
Porcine	30	Sperm motility index	6.6	Vyt et al[46]
Bovine	5	Sperm motility index	2.1 - 58.8*	Hoflack et al[47]
Porcine	50	Motility	3.2	Rodriguez et al[51]
Bovine	10	Motility	47.3	Brito[2]

*Range for different samples

Determining the accuracy of sperm motility estimates is more complex, as it requires a method to be defined as the 'gold standard' against which estimates obtained using other methods are compared. Defining a 'gold standard' for sperm motility is a controversial subject, as no 'gold standard' exists. However, any method should probably be compared to subjective evaluation from trained technicians. Accuracy is determined using a variety of statistical methods, including simple correlation, regression, comparison of means, estimation of percentage differences, and Bland-Altman plots.

The difference between subjective and CASA total sperm motility was 2.0% (95% CI = 0.8 - 3.2%) for extended canine semen and -8.1% (95% CI = -13.3 - -2.9%) for extended equine semen.[49] Inter-laboratory CVs ranging from 18 to 45% were observed when CASA was used for evaluation of frozen bovine semen.[2] These observations highlighted the several differences among various CASA systems in hardware and in algorithms used for sperm detection and tracking. Moreover, classification of sperm as motile is based on user-defined thresholds of velocity and

straightness; therefore, caution must be exercised with the notion that sperm motility estimates from various laboratories are comparable simply because CASA systems were used for analysis.

The SMI with SQA system ranged from 275 to 305 and CASA sperm motility ranged from 82 to 84% in extended porcine semen stored for 3 days.[46] Extended bovine semen samples with percent of total and progressive sperm motility determined subjectively ranged from 60 to 85% and from 10 to 85%, respectively and SMI ranged from 9 to 527.[47] The correlation of SMI with motility and progressive motility evaluated subjectively was low (-0.17 - 0.04) for extended dog semen and low to moderate (0.49 to 0.71) for extended porcine semen.[46] The obvious issue with SMI, as is the case for any type of index, is the inability to properly discern deficiencies in sperm concentration, motility, and morphology, as 1 variable may compensate for the other. Conversely, semen samples with identical SMI values may have very different individual semen characteristics. This greatly limits the ability to offer a proper interpretation of the analysis.

Motility ranged from 9 to 57% in frozen-thawed bovine samples evaluated using the SQA and from 11 to 48% in samples evaluated subjectively or using CASA. The difference between SQA and all other methods was ~ ± 20%.[2] The difference between sperm motility assessed with SQA (66%) and CASA (72%) was -6.4% (95% CI = -32.8 - 20%), whereas the difference from with subjective evaluation (64%) was 1.7% (95% CI = -27.5 - 30.9%) in extended porcine semen samples.[40] The agreement of SQA sperm motility with subjective evaluation was nonexistent for extended equine samples (95% CI = -34.3 - 35.9%).[49]

Review of the available literature indicates that caution must be used when comparing sperm motility results from various laboratories or even from the same laboratory when strict standardization and internal quality control procedures are not followed. Precision and accuracy favor the use of well-validated CASA systems for evaluation of sperm motility over subjective evaluation. Additional studies are required before the SQA can be recommended for evaluation of animal semen.

Conclusion

Semen analysis constitutes the most important clinical laboratory test currently available to evaluate male fertility. Sperm motility estimations should not be regarded as absolute measurements but, rather, they have a relative value that needs to be interpreted in the context of specific systems and settings. As suggested,[62] it is important to optimize quality of semen evalution in veterinary practice by creating standardized protocols for evaluation of all semen parameters and updating those protocols as needed, thereby creating some form of quality control for the clinic laboratory.

Conflict of interest

None to declare.

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