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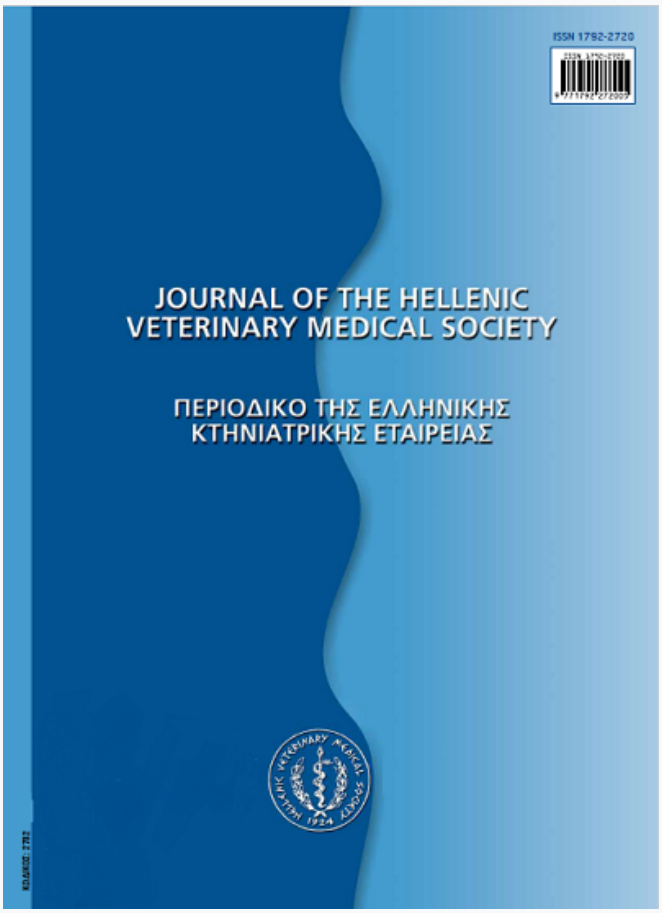

Ορθή χρήση και συνεισφορά του 'Αναλυτή Σπέρματος Υποβοηθούμενου από Ηλεκτρονικό Υπολογιστή' στην εκτίμηση του σπέρματος των παραγωγικών ζώων

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Review article
Ανασκόπηση

Proper use and impact of 'Computer Assisted Semen Analysis' technique on semen evaluation of farm animals

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Ορθή χρήση και συνεισφορά του 'Αναλυτή Σπέρματος Υποβοηθούμενου από Ηλεκτρονικό Υπολογιστή' στην εκτίμηση του σπέρματος των παραγωγικών ζώων

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ABSTRACT. Objective of this review is to present the use of 'Computer Assisted Semen Analysis' technique application in farm animal health management, in particular the steps for accurate semen evaluation and the impact of the method in predicting male animal fertility under field conditions. Requirements for proper use of the equipment, factors affecting the evaluation process and the role of the estimated parametres for fertility under field conditions are described. Special reference is made in sperm motility evaluation. It is concluded that the method is an effective and efficient tool for semen evaluation, provided good practices are strictly applied and adhered to, by means of which valid results may be obtained.

Keywords: CASA, farm animals, motility, semen.

ΠΕΡΙΛΗΨΗ. Σκοπός της παρούσας ανασκόπησης είναι η παρουσίαση της λειτουργίας του αυτόματου αναλυτή σπέρματος υποβοηθούμενου από ηλεκτρονικό υπολογιστή (CASA) και των εφαρμογών του στον τομέα της αναπαραγωγής των παραγωγικών ζώων, ώστε να γίνει ευρέως αντιληπτή η συμβολή του ως εργαλείο πρόγνωσης της γονιμότητας των αρσενικών ζώων Παρατίθενται οι προϋποθέσεις ορθής λειτουργίας του αναλυτή, οι παράγοντες που επηρεάζουν αυτήν και η αξιολόγηση των εκτιμούμενων παραμέτρων, με ιδιαίτερη έμφαση στην κινητικότητα των σπερματοζωαρίων. Ο αναλυτής CASA αποτελεί σημαντικό εφόδιο για τους ασχολούμενους με τη διαχείριση υγείας των παραγωγικών ζώων, ωστόσο απαιτείται τήρηση των κανόνων για ορθή εκτίμηση των δειγμάτων και για αξιοπιστία και δυνατότητα σύγκρισης των αποτελεσμάτων.

Λέξεις ευρετηρίασης: CASA, κινητικότητα, παραγωγικά ζώα, σπέρμα.

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INTRODUCTION

Genetic improvement of farm animals is a factor directly affecting animal production and farm profitability. Artificial insemination has been used to achieve this, which highlights the importance of male animals. In all cases, semen to be used in artificial insemination should be extensively evaluated before used.

Semen evaluation may be still performed by conventional techniques: direct microscopy, conventional dyes and Neubauer chamber (Verstegen at al., 2002). Nevertheless, sperm motility is an important parametre affecting of male fertility, hence its subjective evaluation can be influenced by the operator and result in varying measurements in the very same sample (JØrgensen et al., 1997). An additional disadvantage of subjective assessment of sperm motility is the difficulty in a detailed description, as only three levels of classification are available: (a) progressive motility, (b) non progressive motility and (c) immobility (World Health Organization, 2010).

The continuous effort for improved assessment of semen parametres has led to development of various technological systems, e.g., the laser Doppler spectroscopy (Budworth et al., 1987), the turbidimetry (Donelly et al., 1998), as well as and various photometric methods (Burkman et al., 1991). The above mentioned techniques had limited possibilities, as they lacked capacity to analyse movement of individual spermatozoa. Dott and Foster (1979) indicated the requirement for a system that might take successive digital images of spermatozoa as units and analyze their individual movement, which might lead to improved motility estimation (Gravance and Davis, 1995; Holt and Palomo, 1996). In 1985, the first 'Computer Assisted Sperm Analyser' system (CASA) was developed and became commercially available under the name of Cellsoft™ (CRYO Resources Ltd, New York, NY, USA). CASA was originally used for human semen evaluation and led to great expectations (Mortimer and Mortimer, 1988). Later, researchers started using it for farm animal semen samples evaluation; nowadays, it has become the most popular method for objective assessment of qualitative and quantitative sperm traits (Verstegen et al., 2002; Gil et al., 2009).

Among semen characteristics, sperm motility is particularly important for successful ovum fertilisation. Only the most motile and viable spermatozoa will be able to move through the female's cervix, although sperm transportation is assisted by the negative vaginal pressure and the increased vaginal and uterine contractions. Movement in the oviduct is also an important procedure, as, after sperm leaves isthmus, it becomes hyperactive (Schillo, 2009). Immotile and damaged spermatozoa are not able to pass through the uterotubal junction. After capacitation, motile spermatozoa are the only ones that can detach from the oviduct epithelium and find their way to the fertilization point (Burkitt et al., 2011).

CASA is a valuable method for objective evaluation of male fertility and becomes particularly powerful especially in combination with other diagnostic tests. Objective of this review is to present the development of CASA, as well as to point out the crucial steps in semen evaluation of farm animals by means of the CASA equipment. Within this context, the main components and the function of a CASA system are presented. Moreover, assessment of sperm kinematic values, parametres that may affect proper evaluation of samples, reliability of the results and, finally, input and predictive value of CASA parametres are discussed.

DESCRIPTION AND FUNCTIONING OF THE CASA SYSTEM

The CASA system consists of a video camera adjusted on a negative-phase contrast objective, a videograbber card, a computer and the image processing software (Fig. 1). Initially, CASA is taking pictures of the microscope field with the video camera and converts them into digital images. In order to make this possible, proper settings are required. By using a negative-phase contrast objective lens, there is the possibility for the spermatozoa to appear as white in a dark background with no alteration of the brightness during their movement. The image of sperm is then digitized and the software determines the total number of pixels that each sperm head occupies.

In early stages of development of the CASA system, various problems had arisen, e.g., initially CASA could not differentiate spermatozoa from particles of the same size. As part of the solving process, various approaches have been tried through modifications and improvements of the software, e.g., the presence of the sperm's tail as prerequisite (Neuwinger at al., 1990; Wijchman at al., 1995) or the use of dyes for staining sperm DNA (Zinaman et al., 1996).

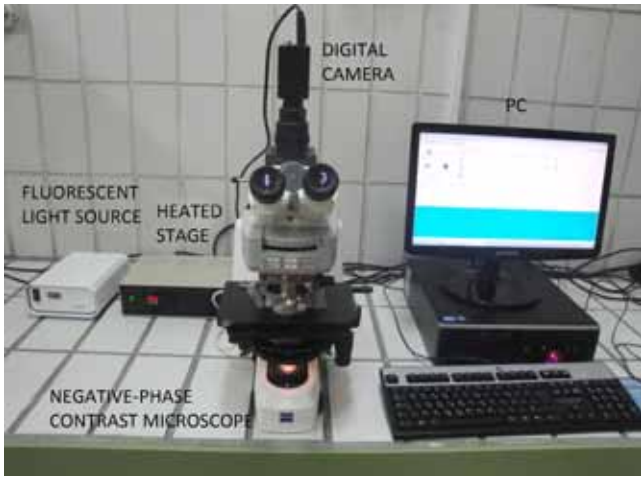

Fig. 1. Equipment for Computer Assisted Sperm Analysis.

After digitization of spermatozoa and setting of coordinates (x, y) in the field, the same procedure is repeated at subsequent successive captures. Then, an algorithm is employed, in order to analyse all data and to reconstruct trajectories of individual spermatozoa. In this process, the first step is to identify all sperm heads and record them. Some CASA systems recognize the sperm head as a series of pixels, calculating the centre of the formation which is termed 'centroid'; others digitize the circumferential pixels of an object, using them for calculating the centroid. Thus, the brightest point on the sperm head or the center of the head is used and is considered as reference point. Although, the flagellum actually is responsible for movement of spermatozoa, assessment of sperm head movement (i.e., centroid-based analysis) is more important in sperm kinematic evaluation. The method used for identification of spermatozoa must be taken into account when results from different laboratories are compared (Mortimer, 1997). The second step involves identification and re-recording of the position of spermatozoa on the next capture. The computer looks for the sperm at each successive take, within a circular boundary around the place of the previous capture. The threshold radius depends on the speed and distance that is expected to go into the interval between two successive shots. The third step involves the combination of coordinates from each sperm individually, trajecting the course at the computer and calculating parametres of motility for each sperm individually and also all of them as a population (Mortimer, 2000; Kathiravan et al., 2011).

Results obtained from the analysis depend both upon the image quality, as well as the frequency. Most CASA systems operate at 50 Hz (phase altering line standard) or 60 Hz (National Television Standards Committee standard) depending on the country (Mortimer, 2000). CASA systems that record images with a higher frequency have an improved accuracy in charting the path of spermatozoa, in particular of those that have not been moving in a slow and linear way. Instead, CASA systems operating at low frequencies (< 50 Hz) may extrapolate a trajectory likely different than the real one and may conceal data on sperm motility (Castellini et al., 2011).

ASSESSMENT OF SPERM KINETIC VALUES BY MEANS OF CASA

CASA systems have now the ability to assess objectively various quality parametres of semen, i.e., motility, individual movement styles, density, vitality, morphology, dimensions of sperm head (morphometry) and DNA fragmentation by using fluorescent dyes.

Results of semen motility are based on the proportion of sperm cells with strong progressive movement, non-progressive movement or no movement. The first class includes spermatozoa that move into straight or circular path and can be separated into rapidly and slowly moving sperms; for that, a speed of 25 μm s^{-1} (bull or boar semen) or 45 μm s^{-1} (ram or buck semen) is used as the threshold. The second class includes all individual forms of sperm motility, whilst the third class includes motionless sperms (World Health Organization, 2010).

The various individual movement styles that can be evaluated by the CASA system are described below and presented graphically in Figure 2 (World Health Organization, 2010).

- Curvilinear velocity (VCL), expressed in μm s^{-1} and defined as time-averaged velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope; this can be used as a measure of cell vigor.
- Straight-line (rectilinear) velocity (VSL), expressed in μm s^{-1} and defined as the time-averaged velocity of a sperm head along a straight line between its first and last detected positions.
- Average path velocity (VAP), expressed in μm s^{-1} and defined as the time-averaged velocity of a sperm head along its average path, which is com-

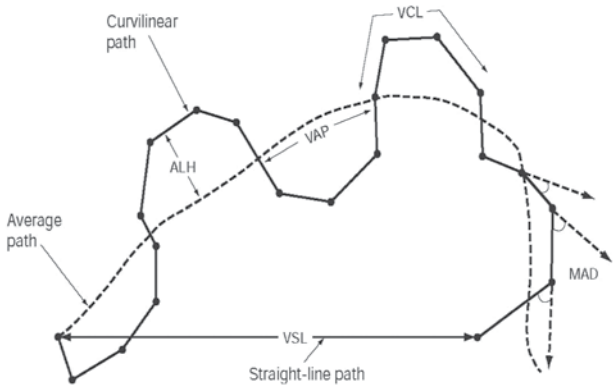

Fig. 2. Standard terminology for variables measured by CASA systems (World Health Organization, 2010).

puted by smoothing the curvilinear trajectory according to algorithms in the CASA instrument; as such algorithms vary between equipment, values of this parametre may not be comparable among various systems and laboratories.

- Amplitude of lateral head displacement (ALH), expressed in μm, defined as the magnitude of lateral displacement of a sperm head in relation to its average path and expressed as a maximum or an average of such displacements; as algorithms used in computation of this parametre vary between equipment, its values may not be comparable among various systems and laboratories.
- Linearity of a curvilinear path (LIN), defined as the ratio VSL:VCL.
- Wobble (WOB), defined as the ratio VAP:VCL, which is a measure of oscillation of the actual path in relation to the average path.
- Straightness (STR), defined as the ratio VSL:VAP, which expresses the linearity of the average path.
- Beat-cross frequency (BCF), expressed in Hz and providing the average rate at which the curvilinear path crosses the average path.
- Mean angular displacement (MAD), expressed in ^{o} (degrees) and providing the time-averaged absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory.

Sperm velocity is determined by parametres VCL, VSL and VAP; for any sperm, VCL is always the highest, while VSL is always the lowest of the three. When a spermatozoon has a regular and linear path with little lateral movement, VSL is almost equal to VAP, whilst when it has an irregular path, VSL is smaller than VAP (Mortimer, 2000).

There is a special type of sperm movement termed 'hyperactivated motility', which takes place at the isthmus and is responsible for prevention of sperm entrapment into the folds and crypts of the oviductal epithelium, as well for the penetration into the zona pellucida (Mortimer, 2000). Hyperactivated motility is characterized by vigorous, non-linear and non-progressive movement and is occurring just before the acrosomal reaction (Kathiravan et al., 2011). Moreover, it is considered to be important, due to the hypothesis that it could be used for prediction of the *in vivo or in vitro* fertilisation success (Mortimer, 2000). The proportion of spermatozoa that present hyperactivated motility is automatically measured by CASA.

The particular characteristics of hyperactive motility are increased VCL and ALH and decreased LIN, STR, WOB and BCF (Schmidt and Kamp, 2004). For hyperactivated sperms of men, kinematic definition motility is as follows: VCL ~150 μm s^{-1}, ALH ~7 μm and LIN ≤0.5 (Mortimer, 1997). In hyperactivated sperms of bulls, kinematic definition motility is VCL ≥70 μm s^{-1} and ALH ≥7μm (Kathiravan et al., 2011) and in those of boars VCL >97μm s^{-1}, ALH >3.5 μm, LIN <0.32 and WOB <0.71 (Schmidt and Kamp, 2004).

The evolution of CASA systems coupled with current requirements for more reliable assessment of reproductive capacity of male animals have resulted in further integration options and capacities in semen evaluation. By using fluorescent dyes and fluorescent optics microscopes, it has become possible to automatically and objectively assess various other parametres, such as sperm morphology, vitality and DNA fragmentation of spermatozoa.

PARAMETRES AFFECTING PROPER EVALUATION OF SAMPLES AND RELIABILITY OF RESULTS

During CASA analysis performance, particular attention is required by the operator, in order to correctly assess the samples and extract reliable and repeatable results. The precision of the results depends on many parametres, e.g. expertise of users or rational setup of the process. Various factors, which include but are not limited to errors during semen processing, inadequate mixing or homogenisation of sample, errors in filling the counting chamber with the wrong

semen volume or incorrect software settings, may adversely affect the results (Feitsma et al., 2011).

Increased concentration of spermatozoa in semen can lead to misperception of actual motility, due to limiting spermatozoa into a confined space, leading to multiple conflicts between them and reduction of motility (Rijsselaere et al., 2002). Therefore, semen must often be diluted before analysis, so that the system can properly detect sperm heads and not reconstruct false trajectories (Contri et al., 2010). Ideal semen sperm concentration for CASA evaluation varies between 12×10^6 and $40\text{-}60\times10^6$ sperms mL^{-1}; a concentration around 25×10^6 to 30×10^6 spermatozoa mL^{-1} is considered to be optimal (Kathivaran et al., 2011). Moreover, diluents may affect results, because some of their components can improve semen characteristics. Commercial extenders of classic or specific composition have been used as diluents of farm animals semen (Ehlers et al., 2011). Rijsselaere et al. (2003) reported that when using Hepes or TALP as diluent media, improved velocity parametres (VAP, VSL, VCL) and more motile and rapidly moving spermatozoa were recorded than when using Tris or physiological saline. Seminal plasma, that had been also used to dilute sperm samples, can affect measurements, while it is not available when stored frozen semen has to be evaluated (Farell et al., 1996). Finally, the diluent solution must not contain particles and debris with size similar to that of sperm head, because that might lead to false results.

Volume of semen sample should range from 4 to 10 μL (Januskauskas et al., 2000). Correct volume of sample to be used is indicated by the equipment's manufacturer and volumes other than the recommended may influence the results (Kathiravan et al., 2011). The structure and quality of the counting chamber (type, age, possible damage etc.) may affect sperm motility. Increased sperm motility has been observed in the middle of the chamber, than at its edges, due to passive movement of dead and immotile spermatozoa to the outer area of the coverslip (Lenz et al., 2011). Variations may also occur when different chambers are used. Lenz et al. (2011) evaluated sex-sorted frozen thawed bull semen and reported that approx. 25% more total and progressive motility in Makler chamber or common slides with coverslips compared to Leja slides; however, they recorded no differences between Makler and common slides with coverslips. In addition, as many kinematic parameters are depending on frame rate, it is assumed that it correlates with the rightness of the results (Castellini, 2011). According to Davis and Katz (1992) and World Health Organization (2010), in order to obtain reliable results, examination of six fields per sample and estimation of at least 200 spermatozoa are required. Finally, ambient conditions should not be extreme and temperature of materials that come into direct contact with the sample must be similar to that of the sample; as in classical semen evaluation procedures, a 37 ^{o} C heated stage must be used, so that temperature of the chamber is stable and consistent.

INPUT AND PREDICTIVE VALUE OF CASA

Use of CASA in farm animal health management has improved productivity of farm animals through correct evaluation and management of male animals (Verstegen et al., 2002).

Bull semen

There are studies providing conflicting results about the relationship of subjectively estimated sperm motility and bovine fertility (Andersson et al., 1992; Januskauskas et al., 1999). CASA provided the possibility to study motility in an objective way. Kathiravan et al. (2011) reported that only the parameters VCL, VSL, VAP can be used to predict *in vivo* fertility. Other researchers reached the same conclusion, moreover adding that the parameters STR, LIN, ALH did not affiliate with *in vivo* fertility of bulls (Sukcharoen et al., 1995, 1998). Finally, several sperm motion characteristics, such as total motility, progressive motility or BCF, were found to be predictors of bovine *in vivo* fertility (Oliveira et al., 2013). Multiple combinations of sperm parametres seem to correlate in a stronger way than single parametres with field fertility. Among CASA semen evaluated parameters, assessment in combination of progressive motility, ALH, BCF and VSL is much more correlated with *in vivo* fertility than the estimation of single total motility (Farell et al., 1998).

Ram semen

The literature does not provide adequate information about the relationship between the estimated by CASA motility parameters and *in vivo* fertility. Nevertheless, Smith et al. (1998) observed positive correlation between them and fertility. Moreover, Robayo

et al. (2008) limited this correlation to VCL and VAP. Specifically, both parametres were found to be related to the ability of spermatozoa to migrate through the female genital tract, increasing the chance of fertilisation. Generally, most literature references indicate that CASA is an objective semen evaluation technique for rams and it provides useful results (Tsakmakidis, 2010).

Buck semen

In general, there is limited information on how characteristics of buck semen are correlated to fertility. Cox et al. (2006) found that ejaculates with improved VCL, VAP and VSL had better spermatozoan migration efficiency in homologous cervical mucus. However, Furstoss et al. (2010) used a combination of parametres to predict fertility, but, surprisingly, predicting *in vivo* fertility with using CASA parametres was less precise than results of traditional methods of semen evaluation.

Boar semen

Didion (2008) did not detect any significant correlation of semen motility with *in vivo* fertility in boars. On the other hand, two more recent studies by Broekhuijse et al. (2011; 2012) showed that progressive motility, VCL and BCF were positively related with farrowing rate, while total motility, VAP, VSL and ALH were positively related to the total number of born piglets. Holt et al. (1997) also reported that the VSL was positively related to the litter size.

CASA provided the opportunity to assess association of semen motility parametres with *in vivo* and *in vitro* fertility. Velocity parametres, such as VAP, VSL and progressive motility, have been found to correlate with in vitro fertility, while STR, LIN and ALH have been found to correlate negatively (Kathiravan et al., 2008). Other studies have reported a weakly relation of motility and its parametres to *in vitro* fertility, highlighting the fact that motility is correlated better to *in vivo* fertility (Blondin et al., 2009). Although Gillan et al. (2008) found no correlation between motility and in vitro fertility, they reported that a combination of diagnostic tests, such as assessment of sperm morphology, motility and chromatin integrity, can contribute in prediction of in vitro fertility. Suzuki and Nagai (2003) did not observe any characteristic tendency between semen evaluation parametres and in vitro fertility in boars.

CONCLUDING REMARKS

CASA is currently a useful laboratory equipment to be employed for objective evaluation of farm animal semen. Examination of large number of samples and acquirement of reliable results within a short period can support control of reproductive problems in male animals and can sustain high rates of laboratory embryo production. Prerequisite for these is the existence of specialized and well trained personnel and reassurance of appropriate system functionality.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest to declare.

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