RELIABILITY AND ACCURACY OF A COMPUTERISED SEMEN IMAGE ANALYSES TO EVALUATE VARIOUS BIOLOGICAL PARAMETERS IN RABBIT SEMEN

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Abstract - In order to study the reliability and accuracy of a computerised semen analyser (HTMA-IVOS Hamilton-Thorn), 47 ejaculates were analysed. PH and visual parameters were measured immediately after collection and a 1:5 dilution. 400 µl were put in 3 tubes containing diluent (final dilution 1 : 40) ; in each tube 3 drops were taken ; each drop was put on a Makler chamber where 4 fields were analysed ; so 36 analyses were made by ejaculate. The analysis of variance indicated that the stability of the HTMA parameters was correct when only 2 tubes, 2 drops and 2 fields were analysed. This work demonstrates that 2 tubes are necessary to detect a possible technical problem, as well as two drops per tube to detect sampling errors and finally, several fields as soon as this analysis does not exceed 2 minutes. The authors conclude that the accuracy and reliability of HTMA system is confirmed but point out the importance of analysis conditions on the results of Computer Assisted Sperm motility Analyser.

INTRODUCTION

In artificial insemination programs, the optimal use of genetically superior bucks primarily depends upon the fertilising ability of ejaculated spermatozoa. Among parameters commonly used to depict this trait are the motility and the percentage of live spermatozoa. Meanwhile, visual estimations, even simple and rapid, are highly subjective. Quantitative aspects can be estimated with a counting chamber, but this method is tedious. Furthermore, for rabbits, spectrophotometry is generally not used, because the seminal plasma contains a varied number of prostate secreted particles often very refringent.

Computer Assisted Semen Analyses (CASA) have been of practical use in human and animal reproduction. The performances of computer assisted semen analyses greatly depend on the correct setting of the system. These settings may differ greatly between species. The right set up must first be determined before practical use of the equipment.

The aim of this work is to study the reliability and accuracy of a commercially available computerised semen analyser (HTMA-IVOS Hamilton-Thorn Research, Beverly, MA/U.S.A.).

MATERIAL AND METHODS

Semen from 47 A1029 and A1077 bucks was analysed. Rabbits were individually housed in cages drawn up in flat deck, photoperiodism was 16L : 8D photoperiod (THEAU-CLEMENT *et al.*, 1995), they were fed *ad libitum* with a commercial diet containing 17.5% of protein and of 14.5% fibres. They were watered *ad libitum*. Semen was collected twice a day at 15 mn interval. Only the second ejaculate was kept for further analyses (BENCHEIKH, 1995). Immediately after, standard visual parameters were determined by an expert technician. Then, the semen sample was diluted (1:5) in a commercial extender (Dilap 2000, IMV France). The culture tube was gently swirled seven times, and 400 µl of the sperm suspension were placed in a first tube containing 2.8 ml of Dilap 2000 (dilution 1:40). This was repeated twice to analyse HTMA-IVOS repeatability. The diluted semen was placed at ambient temperature until the moment of analysis. Ten minutes before the beginning of the analysis with the Hamilton Thorn motility analyser (HTMA), the tube was placed at 37 °C. For each sample, a 10 µl aliquot was thoroughly mixed and placed in a pre-warmed (37°C) Makler chamber (depth =10µm) and covered with a coverslip without grid. The chamber was then placed on the stage of the HTMA internal microscope, four fields were manually selected and individually analysed. This procedure was

repeated with three aliquots per sampling. Therefore, for each ejaculate, a total of 3 tubes, were assessed with 3 drops per tube and four fields per drop, i.e. 36 analyses. Within each tube, concentrations were estimated using both a Thoma-Zeiss cell counter (final dilution 1:200) and a spectrophotometer (wave-length : 520 nm). Visual measurement methods are described in BOUSSIT (1989).

The version 10 of HTMA-IVOS operates as a cell motion analyser, a cell morphometry analyser, and a computer. Integrated hardware components include : an internal phase-contrast optics system with stroboscopic illumination, an internal automated heated specimen stage, an image digitizer, and an 80486 computer. The algorithm for recognition of sperm used by the HTMA-IVOS depends on mean size and mean optical density of all moving objects within a semen sample. The settings were determined for minimum size and minimum contrast by examining various samples with the "play back" option, ensuring that only sperm cells were included in the analysis. Other options were adjusted as proposed by the manufacturer. A list of parameters involved during semen analyses has been reported (Table 1).

Table 1 : Set-up parameters

Frames Acquired :	20	
Frame rate :		50 Hz
Minimum contrast :	40 (arbitrary units of brightness)	
Minimum cell size :	3 pixels	
Threshold Straightness :	80.0 %	
Low VAP Cut-off :	20 µ/sec	
Medium VAP Cut-off :	40 µ/sec	
Non-motile head size :	11	
Non-motile intensity :	115	
	Minimum	Maximum
Static head size :	0.96	2.54
Static head intensity :	0.61	1.34
Static elongation :	20	70

PH and visual parameters were recorded : general motility, individual motility, percentage of live spermatozoa for each ejaculate, concentration (number of spermatozoa/ml) and optical density for each tube. HTMA parameters available for each field were : analysis temperature (°C), total number of cells per field, concentration, percentage of motile cells, percentage of progressive cells (if VAP>40µ/sec and straightness> 80%), path velocity (VAP : average velocity of the smoothed cell path in µ/sec), the amplitude of lateral head displacement (ALH, which corresponds to the average sperm track width) and linearity (LIN, measures the departure of the cell track from a straight line). Following each analysis, the play back option was used to count manually all cells incorrectly identified (uncounted or counted erroneously) by the HTMA, which allowed further quantification of the cell populations present in each field (exact total number of cells).

To study reliability and accuracy of the HTMA, the tube, drop and field fixed effects as well as the first order interactions were estimated by analysis of variance, from data corrected by the effect of the ejaculate. As far as necessary, percentages were substituted by deviations to the regression line. Results presented in Table 2 are least square means.

RESULTS

Analysis of 36 measurements per ejaculate (3 tubes, 3 drops, 4 fields)

The mean delay between collection and beginning of the HTMA analysis was 1h 30 mn (minimum = 30 mn, maximum = 4 h). For a 1 : 40 dilution, the HTMA analyses average 49.9 cells per field (minimum = 4, maximum = 318). A preliminary analysis including only ejaculate effect showed that about 50% of the variability is explained by the model. So the discrimination of the HTMA-IVOS seems to be important but, its relationship with biological phenomena has to be proved.
The analysis of variance results are given on Table 1.

Table 2 : Variability of HTMA parameters with the tube, the drop and the field. Means and standard deviations. Means (standard deviation)

	Analysis température (°C)	Total cells	Concentration 10^6/ml	Motile cells (%)	Progressive cells (%)	VAP (µ/sec)	ALH	LIN	Uncounted cells	Cells counted by error
36 analysis (3 tubes, 3 drops, 4 fields)										
TUBE	40.0^a *(0.02)*	50.2 *(1.03)*	1342 *(26.8)*	75.7 *(0.20)*	30.0^a *(0.24)*	70.3^a *(0.42)*	6.4^a *(0.05)*	34.7^a *(0.20)*	2.5 *(0.08)*	5.5 *(0.12)*
	40.2^b *(0.02)*	51.3 *(1.03)*	1383 *(26.8)*	74.6 *(0.20)*	32.8^b *(0.24)*	66.2^b *(0.42)*	6.4^a *(0.05)*	35.3^b *(0.20)*	2.5 *(0.08)*	5.5 *(0.12)*
	40.3^c *(0.02)*	48.1 *(1.03)*	1297 *(26.8)*	79.6 *(0.20)*	36.1^b *(0.24)*	63.4^c *(0.42)*	6.1^b *(0.05)*	35.3^b *(0.20)*	2.5 *(0.08)*	5.2 *(0.12)*
DROP	40.3^a *(0.02)*	51.9^a *(1.03)*	1389 *(26.8)*	73.4 *(0.20)*	31.5 *(0.24)*	67.8^a *(0.42)*	6.3 *(0.05)*	35.3 *(0.20)*	2.6 *(0.08)*	5.6 *(0.12)*
	40.1^b *(0.02)*	49.6^{ab} *(1.03)*	1337 *(26.8)*	77.5 *(0.20)*	33.2 *(0.24)*	67.6^a *(0.42)*	6 4 *(0.05)*	35.3 *(0.20)*	2.5 *(0.08)*	5.3 *(0.12)*
	40.1^b *(0.02)*	48.1^b *(1.03)*	1296 *(26.8)*	79.0 *(0.20)*	34.3 *(0.24)*	64.4^b *(0.42)*	6.2 *(0.05)*	34.9 *(0.20)*	2.5 *(0.08)*	5.3 *(0.12)*
FIELD	40.2 *(0.02)*	51.0 *(1.18)*	1359 *(30.9)*	76.0^a *(0.23)*	27.9^a *(0.28)*	67.9^a *(0.49)*	6.6^a *(0.06)*	33.9^a *(0.23)*	2.4 *(0.09)*	5.7^a *(0.13)*
	40.2 *(0.02)*	50.3 *(1.18)*	1356 *(30.9)*	75.5^b *(0.23)*	33.2^b *(0.29)*	68.2^a *(0.49)*	6.4^b *(0.06)*	35.5^b *(0.23)*	2.5 *(0.09)*	5.4^{ab} *(0.13)*
	40.1 *(0.02)*	48.9 *(1.18)*	1345 *(30.9)*	77.4^b *(0.23)*	35.3^{bc} *(0.28)*	65.5^b *(0.49)*	6.1^c *(0.06)*	35.7^b *(0.23)*	2.5 *(0.09)*	5.4^{ab} *(0.13)*
	40.1 *(0.02)*	48.3 *(1.18)*	1302 *(30.9)*	79.1^{ab} *(0.23)*	36.3^c *(0.28)*	64.8^b *(0.49)*	6.1^c *(0.06)*	35.5^b *(0.23)*	2.6 *(0.09)*	5.1^b *(0.13)*
R^2	0.095	0.016	0.017	0.012	0.082	0.127	0.068	0.041	0.014	0.018
8 analysis (2 tubes, 2 drops, fields 2 and 3)										
TUBE	40.1^a *(0.03)*	49.2 *(1.67)*	1326 *(44.7)*	76.9 *(0.35)*	31.9^a *(0.42)*	72.3^a *(0.80)*	6.5 *(0.08)*	35.4 *(0.36)*	2.5 *(0.12)*	5.4 *(0.19)*
	40.2^b *(0.03)*	53.5 *(1.67)*	1441 *(44.7)*	71.0 *(0.35)*	32.1^b *(0.42)*	67.1^b *(0.80)*	6.3 *(0.08)*	35.8 *(0.36)*	2.4 *(0.12)*	5.7 *(0.19)*
DROP	40.2^a *(0.03)*	51.7 *(1.67)*	1395 *(44.7)*	73.2 *(0.35)*	32.6 *(0.42)*	70.2 *(0.80)*	6.4 *(0.08)*	35.9 *(0.36)*	2.5 *(0.12)*	5.8 *(0.19)*
	40.1^b *(0.03)*	50.9 *(1.67)*	1373 *(44.7)*	74.5 *(0.35)*	31.5 *(0.42)*	69.4 *(0.80)*	6.4 *(0.08)*	35.2 *(0.36)*	2.3 *(0.12)*	5.2 *(0.19)*
FIELD	40.2 *(0.03)*	51.5 *(1.67)*	1389 *(44.7)*	73.2 *(0.35)*	31.0 *(0.42)*	70.6 *(0.80)*	6.5 *(0.08)*	35.4 *(0.36)*	2.3 *(0.12)*	5.7 *(0.19)*
	40.1 *(0.03)*	51.1 *(1.67)*	1378 *(44.7)*	74.5 *(0.35)*	33.1 *(0.42)*	69.0 *(0.80)*	6.3 *(0.08)*	35.8 *(0.36)*	2.5 *(0.12)*	5.3 *(0.19)*
R^2	0.062	0.015	0.014	0.003	0.029	0.062	0.025	0.016	0.016	0.022

Within columns, means with different letters (a,b,c) are significantly different P<0.05.

Tube effect - Temperature significantly increased when the tube number increased, but tube and drop effects interact. The percentage of progressive cells increased too with the tube number. On the contrary, VAP and ALH decreased with the tube number. The linearity increased between tube 1 and 2, then appeared stable thereafter. Meanwhile, we could notice an interaction between the tube and the drop effect. No tube effect was observed for concentration, and percentage of motile cells. Uncounted or cells counted erroneously were independent from the tube number.
Drop effect - The analysis temperature of the first drop was significantly higher. Even if the semen has been carefully homogenized just before each drop taken, the number of analyzed cells tended to decrease. The spermatozoa velocity was significantly reduced between the second and the third drop. No drop effect was observed for other variables.
Field effect - The field effect was analysed from the same drop within four minutes. During this interval, no variations in this parameter were observed. Meanwhile, all parameters in relation to cell movements varied between fields, therefore, there is a detrimental effect of "chamber time" on spermatozoal motility. With an exception for VAP and ALH, other parameters observed in fields 2 and 3 were maintained quite constant. ALH and cell velocity of the first 2 fields were significantly greater than the last ones, but there is an interaction drop x field. In field 4, we observed lower numbers of errors among the cells counted.

Therefore another approach to the statistical analyses was carried out with the first 2 tubes, the first 2 drops and fields 1 and 2 or fields 2 and 3. The residual standard deviation of this analysis was generally lower for fields 2 and 3. Consequently, we chose to present the results of 8 analyses per ejaculate, taking into account the second and third field.

Analysis of 8 measurements per ejaculate (2 tubes, 2 drops, fields 2 ant 3)

The HTMA parameters were slightly different between tubes. We noticed a temperature difference of only 1/10°C between the two tubes, as well as a slight, even significant, increase of the percentage of progressive cells (31.9 *vs* 32.1 %, for tubes 1 and 2 respectively) and a slight diminution of spermatozoa velocity (72.3 *vs* 67.3 µ/sec). With an exception for the temperature, there were not significant differences between drops or fields whatever the HTMA parameters. No significant interactions appeared in this model of analysis of variance.

DISCUSSION

When temperature increases the percentage of progressive cells increases; we can hypothesize a relationship with analysis temperature rise. Because of the decrease of VAP and ALH with the tube number, the multiplication of the tubes to increase HTMA accuracy, is limited by the diminution of the sperm velocity according to time. It is likely that the semen is waiting at ambient temperature (37 and 75 minutes respectively for tubes 2 and 3) has a slight but significant detrimental effect on cell velocity and amplitude lateral head displacement (tube 3). It seems that the analysis of 2 tubes is a good compromise. Furthermore, it seems that the analysis of 2 drops is sufficient (analysis time =12 mn), more can lead to lower cell velocity. As observed by VARNER *et al.* (1991), all movement characteristics change according to the fields, so, there is a detrimental effect of "chamber time" on spermatozoal motility. Results in the first field on progressive cells and linearity indicate that the first field analysis is too early. As indicated by TULI *et al.* (1992), the HTMA analysis has to start after allowing one minute for sperm to settle.

Frequent changes of Makler chamber was responsible for HTMA difficulties with rapid regulation. For a routine use of HTMA, the time taken to carry on all the analyses appeared too long (nearly two hours). Cell velocity decreased when the duration of the analyses increased. Because of this delay, there was a confusion between tube, drop and time effects.

In general, the optimum conditions for computerised semen image analyses should be a compromise between the repetition of measurements (allowing a better accuracy) and the duration of the analysis which itself integrates the progressive decline in *in vitro* sperm survival. A homogeneous temperature is essential ; as when too high it may generate artefacts and punctually accelerate sperm metabolism. The second analysis of variance indicates the stability of HTMA parameters is right and supports an easier experimental design, adaptable in routine work. But, two tubes are necessary to detect a possible technical problem (ex : observation too early although the temperature equilibration has not been reached), as well as two drops per tube to detect sampling

errors and finally, two fields per drop after one minute of equilibration in the Makler chamber on the microscope stage.

Meanwhile, in our study, field analyses were carried out on individually and therefore lasted longer for routine work. For this reason, it appears that global analyses issued from several fields should be preferred under field conditions, as soon as this analysis does not exceed two minutes (or 3 mn after placing the Makler chamber on the microscope stage).

The low number of uncounted or cells counted erroneously demonstrates the validity of the set up used and proves HTMA accuracy.

CONCLUSION

A method for estimating biological parameters of semen needs to be accurate, repeatable and free of bias. We could observe acceptable reliability of the HTMA system for motility parameters and accuracy, but we also noted the importance of the analysis conditions, which must be strictly controlled (ex : duration of the analyses). In conclusion, we have to confirm the suitability of the conditions of the analyses proposed.

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Fiabilité et efficacité de l'analyse d'image pour évaluer différents paramètres biologiques de la semence de lapine - Afin d'étudier la fiabilité et la précision d'un analyseur de semence assisté par ordinateur (HTMA-IVOS Hamilton-Thorn), 47 éjaculats ont été analysés. Le pH et les paramètres classiquement utilisés pour l'observation de la semence ont été estimés immédiatement après récolte sur la semence diluée 5 fois. 40 µl sont prélevés et déposés dans 3 tubes contenant 2,8 ml de dilueur, 3 gouttes de chaque tube sont analysées et chacune de ces gouttes est placée sur une chambre de Makler, 4 champs sont étudiés, 36 analyses sont donc réalisées pour chaque éjaculat. Les résultats d'analyse de variance démontrent la bonne stabilité de l'HTMA quand seulement 2 tubes, 2 gouttes et 2 champs sont analysés. Plusieurs champs peuvent être observés dès l'instant que la durée de leur analyse n'excède pas 2 minutes. Les auteurs concluent que l'analyse d'image utilisée pour évaluer les caractéristiques biologiques de la semence est un outil précis. Ils mettent cependant en évidence l'importance des conditions d'analyse sur les résultats.