



CORRELATIONS BETWEEN CASA ANALYZED SPERMATOLOGICAL PARAMETERS AND FERTILITY OF BULLS

Abstract of: BALTISSEN, J., 2007, Dr. med. vet. Thesis

1. Summary

The objective of this study was to investigate the relationship between spermatological parameters and the fertility of AI-bulls, identified by the Non-Return-Rate (NRR). Additionally, the identification of subfertile bulls by a thermoresistance test and a storage test after thawing of semen was tested. Thirty-four young dairy sires were included in a widely set-up field trial for which only investigated sperm was used. After dilution, thawing and at 3 time points during each resistency test the parameters progressive motility, motility, integrity of plasma membrane (via SYBR 14 and Propidium-Iodid), VSL, VAP, VCL, DSL, DAP, DCL, BCF, Linearity, Wobble, Straightness and ALH were measured using the CASA-System Sperm Vision™. Sperm morphology was investigated microscopically using fixed samples after dilution and after thawing. In total, 28,219 inseminations were included in the analysis, on average 830 per bull.

After dilution, progressive motility, changes in middle piece, tail and acrosome defects showed the highest correlation with the NRR. After thawing, these were progressive motility, changes at the neck, changes in middle piece and tail as well as acrosome defects. Regression analyses explained 67% of the variance of the NRR after dilution. After thawing, it decreased to 42% with progressive motility and acrosome defects respectively changes at the neck and BCF contributing most to the explained variance. Integrity of plasma membrane did not increase the prediction of fertility. Differences between the fertile (quartile highest NRR) and the sub fertile group (quartile lowest NRR) were not found in the MANOVA. Using descriptive analyses of the single parameters however, linearity and wobble analyzed during the storage test proved to be especially useful for the identification of sub fertile bulls.

Confirming previous results in the literature, alongside the progressive motility, morphological parameters contributed significantly more to the prediction of the NRR than the kinetic parameters. The prediction rate of 67% was rather high, but still not sufficient for use in practice. Since the CASA-System was fully integrated into the routine work flow of the AI-station, routine analyses of linearity and wobble during the storage test in future trials can ensure the successful identification of fertile bulls. It seems useful to develop further parameters which describe the quality of the single sperm to improve the prediction of the fertility rate. By the identification of subpopulations of sperm through the analysis of physiological and functional parameters, additional information can be retrieved to predict fertility more efficiently.





2. Material and methods

Bulls	The semen was taken from 34 test bulls (Holstein-Friesian, 15 Red & White and 19 Black & White) in the period from 26.03.2004 to 02.9.2004 at the Rinder-Union West (RUW) in Borken / Westphalia.																										
Semen collection	Three times in two weeks, semen was collected with the help of an artificial vagina (manufactured by Minitüb) and pre-diluted. The second collection was pre-diluted as well, and both ejaculates were admixed in a plastic cone and final diluted down to 15 million sperms per dose (Minitüb SmartDispenser), under computer control through the laboratory software (Minitüb IDA Lab.).																										
CASA analysis	<p>After the final dilution of the double ejaculate collection, the semen was examined with Sperm Vision™ (manufacturer Minitüb, including a fluorescent microscopy unit and viability module). The following data were collected:</p> <table border="0"> <tr> <td>Total motility</td> <td>(tMOT)</td> </tr> <tr> <td>Progressive motility</td> <td>(pMOT)</td> </tr> <tr> <td>Viability</td> <td>(VIA)</td> </tr> <tr> <td>Distance curve line</td> <td>(DCL)</td> </tr> <tr> <td>Distance average path</td> <td>(DAP)</td> </tr> <tr> <td>Distance straight line</td> <td>(DSL)</td> </tr> <tr> <td>Velocity curve line</td> <td>(VCL)</td> </tr> <tr> <td>Velocity average path</td> <td>(VAP)</td> </tr> <tr> <td>Velocity straight line</td> <td>(VSL)</td> </tr> <tr> <td>Linearity</td> <td>(LIN) [VSL/VCL]</td> </tr> <tr> <td>Straightness</td> <td>(STR) [VAP/VCL]</td> </tr> <tr> <td>Beat cross frequency</td> <td>(BCF)</td> </tr> <tr> <td>Amplitude of lateral head displacement</td> <td>(ALH)</td> </tr> </table> <p>The evaluation in the disposable chamber was designed according to the technical specifications of Minitüb. The number of examined sperms was 400 per examination. The CASA analysis was repeated after thawing, and at 3 points during a storage resistance test.</p>	Total motility	(tMOT)	Progressive motility	(pMOT)	Viability	(VIA)	Distance curve line	(DCL)	Distance average path	(DAP)	Distance straight line	(DSL)	Velocity curve line	(VCL)	Velocity average path	(VAP)	Velocity straight line	(VSL)	Linearity	(LIN) [VSL/VCL]	Straightness	(STR) [VAP/VCL]	Beat cross frequency	(BCF)	Amplitude of lateral head displacement	(ALH)
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Artificial insemination	A bull had to produce a given number of 850, respectively 1000 servings that featured a least motility of 45% after thawing. Subsequently the semen doses were delivered to AI technicians, veterinarians and on-farm inseminators of the Rinder-Union West who used the semen within 3 months for artificial insemination.																										
Determination of NRR (60)	The resulting 60 days non return rate from these inseminations is not subdivided by semen collection days, due to organisational reasons, but is calculated from the ratio of successful inseminations to the total of executed inseminations. A second service with the same bull on the same day or the subsequent day was not counted as a new insemination. Hereby it was of no importance how often the respective animal was inseminated.																										
Statistical methods	The statistical evaluation of the data was performed with the help of the statistic programs SAS® (SAS® Institute Inc., Cary/North Carolina, 1998), SPSS® 14.0 (SPSS GmbH Software Munich/ Deutschland) and Microsoft® Excel®.																										



3. Results

3.1. Spermatologic examinations (CASA and morphology)

After thawing, the motility and plasma-membrane integrity data lied significantly below the data after dilution. No significant difference between data of mean lateral head displacement (LHD) and the velocity quotient (linearity, straightness, wobble) could be detected. The variation coefficient was, besides the morphologic parameter, with few exceptions below 10%, i.e. the dispersion expressed in units of the mean was very low, being less than 10% of the mean.

3.2. Insemination results (NRR)

34 test bulls produced 31,750 doses in the time from 26.03.2004 to 02.09.2008 that were distributed to technicians, veterinarians and on-farm inseminators. From these servings, 28,219 inseminations, a mean of 830 (659 to 966) per bull, were evaluated. In the mean it took every animal about 9.09 mounts (3 to 23) to produce the necessary amount of straws for the test. This corresponds to the standard spermatologic criteria of 57% usable ejaculates of all collected ejaculates. The number of produced doses per usable ejaculate was about 227 (112 to 459). As fertility parameter, the non-return rate was applied. The data varied between 51.0% and 70.2%. A majority of bulls had values of 60% and higher. Only two bulls had a NRR of less than 55%, the benchmark of 70% was also achieved by only two bulls. The mean of the NRR was 62.1%.

3.3. Correlation between NRR and spermatologic parameters

The correlation between the non-return rate and the spermatologic parameters after dilution and after thawing are presented in table 1. After dilution, both motility parameters (total and progressive motility), and the BCF correlated significantly positive, the morphology parameters acrosome defects (A), neck deformations (N) and mid and end piece defects (ME) significantly negative with the NRR. The alterations on mid and end piece, the acrosome defects and the progressive motility hereby showed the highest correlation with the NRR.

After thawing only the progressive motility, the total motility, the BCF and the neck deformations correlated with the NRR. The mid and the end piece defects, as well as acrosome changes showed correlations of $r > 0.30$ with the NRR, but missed the significance level by little. Between all other parameters and the NRR there was no significant correlation. This also applies to the correlation between viability and NRR.

It was shown that the velocity and distance indicators, due to the high inter-correlation collected similar data. From these measures the DSL was selected for the highest correlation to NRR. Out of the motility parameters that practically measure identical data ($r = 0.98$), the progressive motility was included as a predictor into the regression analysis for the point of time after dilution due to its marginal higher correlation with NRR. Finally, 6 parameters were selected as possible fertility predictors:

- Progressive motility
- DSL
- BCF
- Mid piece defects
- Head and tail defect
- Acrosome defects

The stepwise regression analysis rendered a forecast efficiency of the NRR of 62.5% (rectified R^2 ; $F(3/30) = 19.34$; $p < 0.00$). As a significant predictor, the acrosome deformation (A), progressive motility (pMOT) and mid and end piece deformations (ME) had influence on this analysis. Based on the regression analysis an approximation formula can be created to determine the NRR. Hereby the factor in front of the single predictors emphasizes their respective quantifier.

$$\text{NRR (estimate)} = 38 - 0.529 \times A + 0.40 \times \text{pMOT} - 0.228 \times \text{ME}$$



Table 1: Correlations (r) of spermatologic parameters (CASA, morphology) to Non-Return-Rate (n = 34 bulls), *p < 0.05; **p < 0.01

	Parameter	r after dilution	r after thawing
CASA	tMOT	0.47**	0.39*
	pMOT	0.53**	0.40*
	DCL	0.27	0.06
	DAP	0.29	0.13
	DSL	0.30	0.21
	VCL	0.24	0.05
	VAP	0.23	0.10
	VSL	0.26	0.19
	LIN	-0.02	0.11
	STR	0.08	0.20
	WOB	-0.10	0.06
	BCF	0.36*	0.43*
	ALH	0.01	-0.13
	VIA	0.15	0.01
Morphology	A	-0.59**	-0.32
	H	-0.08	0.06
	N	-0.39*	-0.51**
		0.08	0.18
	ME	-0.59**	-0.32
		0.15	-0.09
		-0.29	-0.30

For creating a maximal forecast with a minimal predictor number a model could be specified in which only two predictors could still bring a forecast merit of 60%. This model consisted of the two parameters acrosome deformation (A) and progressive motility (pMOT) with a forecast merit of 60.4% (rectified R; F (2/31 = 26.14; p < 0.00)). The decrease of the forecast merit by 2.1% was not significant. Insofar this model brings ideal forecast of the NRR after dilution. The approximation formula for this regression equation is:

$$\text{NRR (estimate)} = 26.77 - 0.583 \times A + 0.533 \times \text{pMOT}$$

Including all features into the regression analysis and allowing, via a forward selection, the exclusion of variables to receive a maximal forecast by the statistically ideal selection of the predictors, you would achieve a total forecast merit of 67% (rectified R²; F(4/29 = 17.73; p < 0.00)). In this model the features mid- and end piece deformation (ME), acrosome deformation (A), progressive motility (pMOT) and head deformation (H) proved to be significant predictors. The regression equation for these four predictors is:

$$\text{NRR (estimate)} = 31.355 - 0.521 \times A + 0.468 \times \text{pMOT} - 0.298 \times \text{ME} + 0.256 \times H$$

For the regression analysis after thawing, the features progressive motility, BCF, neck deformation, mid- and end piece deformation and acrosome defects were selected. In the stepwise regression analysis the neck deformations and BCF proved to be significant predictors with a forecast merit of 38.5% (rectified R²; F(2/31 = 11.31; p < 0.00)). The regression equation is:

$$\text{NRR (estimate)} = 33.16 - 0.493 \times N + 0.408 \times \text{BCF}$$

Here also all features of forward selection were included in the regression analysis. In case of stepwise regression the features mid- and end piece deformation, BCF, progressive motility, acrosome deformation and neck deformation proved to be significant predictors with a forecast merit of 42% (rectified R²; F(5/28 = 5.78; p < 0.00)). For five predictors the forecast merit was by 3.5% higher than when using 3 features. The additional explanation merit was not significant.



$$\text{NRR (estimate)} = 43.85 - 0.386 \times N - 0.267 \times A + 0.255 \times \text{BCF} - 0.206 \times \text{ME} + 0.140 \times \text{pMOT}$$

Regarding the question which features proved to be significant predictors to both sides, the indicators progressive motility, acrosome deformation and mid- and end piece deformation could be identified. After dilution they had a forecast merit of 62.5% (rectified R²; F(3/30 = 19.34; p < 0.00)), after thawing the merit was only at 30% (rectified R²; F(3/30 = 5.70; p < 0.00)) though.

$$\text{NRR (estimate)} = 54.614 - 0.379 \times \text{ME} - 0.353 \times A + 0.328 \times \text{pMOT}$$

Thus, the features progressive motility, acrosome deformation and mid- and end piece deformation were the predictors that at both times had a significant forecast of the insemination merit. The single analysis showed, though, that the significance of the features, meaning their quantifier, was dependent on the time of measure. After dilution acrosome defects and progressive motility were the most significant indicators for the insemination competency, and after thawing the neck deformation and BCF were the most significant.

3.4. Correlations between the NRR and the results of the storage resistance test

Coherence between the motility results from the storage resistance tests and the non-return-rate could be also identified. The progressive (r = 0.37; p < 0.05) and the total motility (r = 0.42; p < 0.05) correlated after 24h at 5°C significantly with the NRR. At all three points of time of measure, there was also a significant negative correlation between NRR and the derived features linearity and wobble (see table 2).

3.5. Differences between groups of bulls with low and high NRR

A further question of this work refers to the identification of sub fertile bulls by means of spermatologic features. To answer this question the bulls were classified into quartiles according to their NRR. The group of the 25% bulls with the highest NRR were compared with the group of the 25% bulls with the lowest NRR. The comparison of the means was executed via multivariable variance analysis including all collected features.

The data were compared

1. after dilution and after thawing,
2. at the three points of time of measurement of the thermo resistance test and
3. at the three points of time of measurement of the storage resistance tests.

The parameters that could be used for identification of sub fertile bulls were, besides a few exceptions, the same that had already significant correlations with NRR. These were the parameters progressive motility, deformations of the acrosome and defects on mid- and end piece. Furthermore, differences between the fertile and sub fertile bulls in the distance (DCL, DAP, DSL) and the velocity parameters (VAP, VSL) as well as the BCF could be determined.

In the thermo resistance test, none of the features showed significant differences between fertile and sub fertile bulls in the single analysis.

The parameters Linearity and Wobble showed differences between the 25% bulls with high NRR and the 25% bulls with low NRR at all three points of measurement of the storage test. Straightness and DCL were different after 24h at 5°C, and progressive motility after 72h at 5°C.

Table 2: Correlations (r) of spermatologic parameters (CASA) with the non-return-rate during the storage resistance test (n=34 bulls), *p < 0.05; **p < 0.01

Parameter	24 h, 5°C	48 h, 5°C	72 h, 5°C
tMOT	0.22	0.33	0.42*
VIA	0.14	0.09	0.21
pMOT	0.20	0.30	0.37*
DCL	0.25	0.27	0.26
DAP	0.12	0.21	0.23
DSL	0.01	0.26	0.33
VCL	0.20	0.24	0.24
VAP	0.08	0.19	0.20
VSL	-0.03	0.21	0.28
LIN	-0.51**	-0.36*	-0.38*
STR	-0.31	-0.13	-0.12
WOB	-0.48**	-0.45**	-0.45**
BCF	-0.32	-0.02	0.28
ALH	0.17	0.15	0.14



4. Discussion

Focus of this study was the question of correlation between computer based, spermatologic parameters and the fertility of bulls. Special features of the present study are the high number of animals and analysed ejaculates, as well as the direct correlation between analyzed semen and the by insemination identified fertility. By including 34 AI bulls with a total of 28,219 analyzed inseminations the analysis was put on a broad, statistically safe basis.

4.1. Significance of parameters

If the significance of parameters is ranked according to the correlation to NRR, then the number is reduced down to 6 parameters according to the results of the present study. The parameters progressive motility and BCF showed relevant correlations to NRR. Next to kinetic parameters it was especially the morphology parameters acrosome deformation, deformation of mid- and end piece and neck deformation that showed a correlation to NRR. The correlation between NRR and the relevant parameters was in a range of $r = 0.36$ to $r = -0.59$ and are thus to be assessed as relatively high for spermatologic examinations. The here identified correlations between spermatologic features and the NRR accord to former results from BUDWORTH et al. (1988), FARELL (1998) as well as GSCHWEND (1986).

In all studies the correlation between progressive motility and the NRR was higher than between total motility and NRR, also according to our present results: this can be traced back to the total motility being composed by progressive motile and local motile sperms. It can be assumed that the bigger part of the local motile semen cells will not reach the ovarian tube and thus an insemination won't take place and therefore the analysis of progressive motile sperms has a higher significance for the insemination merit. From this follows that not the total motility but the progressive motility measured with the CASA-device is the more relevant parameter for semen selection.

To evaluate the multitude of in this study measured parameters, also the analysis for inter correlation between the parameters is important; especially regarding their selection in future studies. Regarding the correlation of velocity and distance parameters that was quite high with

an average of $r = 0.83$, it appears clearly that these features measure comparable characteristics.

In comparison to kinetic parameters the morphologic parameters only showed little correlation in-between each other. Also the correlation to other spermatologic features was small. This result indicates a relative independency and autonomy of the morphologic parameters, also in term of prediction of the insemination merit. The only (negative) correlation between morphologic and other features was between neck, respectively mid- and end piece deformations and the motility indicators and derived data from the velocity parameters as well as the BCF. This can be explained through defects of the tail that can influence the velocity, respectively the motility and therefore also the BCF.

The control of the correlation between fertility and plasma membrane integrity did not reveal any positive results despite the expanded sample amount, is to say a correlation could not be proven. Also the high correlation of $r = 0.97$ between the motility rate of frozen semen and viability, which was described by GARNER in 1997, could not be reproduced in this study. The observed lower and not significant waste of plasma membrane faultless sperms during the storage period in a ratio to the motility as well as the missing correlation between both parameters in the resistance tests, allows to infer that damages of the membrane, resulting from stressful conditions during the resistance test, are only explained to a small amount. It is to be assumed that e.g. the use of ATP as energy base or functional losses of the membrane integrity, which cannot be displayed with fluorescence colouring, play a more important role for the decreased motional activity of the sperm cells.



4.2. Prediction of the insemination competence

The forecast for the non-return-rate, determined by the regression analysis, could be significantly improved by the combination of several parameters. For spermatologic parameters after dilution it was at 67%. The quantifier of the predictors in the estimation formula showed that the features of the acrosome deformations and progressive motility had the highest part in clarification. Taking into account only these two features in the analysis, the explained variance is 60.4%.

After thawing the semen the variance clarification was 42% when including all spermatologic features into the regression analysis. Just like after dilution it were deformations of the mid- and end pieces, progressive motility and acrosome deformations that accounted, next to neck deformations and BCF for variance clarification. Also at this point of analysis the main part of the forecast was due to two features; the neck deformations and the BCF. These two features alone explain 38.5% of the variance of NRR so that neither at this point a significant increase of the forecast of the variance of NRR could be achieved by including other features.

The fact that after freezing other features account for prediction of the insemination success than after dilution can be explained by the damages due to the freezing process. However, it seems difficult to reason at this point in time, why these two features (neck deformation and BCF) received their quantifier. For the insemination, neck dislocations (54% after dilution, 70 after thawing) seem to be the bigger exclusion criterion, as without, respectively with dislocated flagellum the sperm cannot autonomously move forward.

The measure of additional parameters, been made possible only in the last few years through the development of CASA, accounts for improvement of the forecast merit of fertility. The beat cross frequency (BCF) is a parameter after thawing that accounts for a big part in explaining NRR. Same counts for progressive motility.

4.3. Identification of sub fertile bulls

No differences could be detected between the quartile of the sub fertile bulls and the 25% bulls with the highest NRR in the multi variable variance analysis including all parameters. It is notable that both features, linearity and wobble, successfully separated the groups in the storage tests and thus showed differences, just like at the inter correlation with the morphologic parameters, not in velocity parameters (VCL, VSL, VAP) itself but from derived values. It is to be assumed that the differences between the groups under stressful conditions of the storage test manifests in changes of velocity of the sperms, but this effect only became clear after creating ratios. While a recommendation was derived on basis of the correlation results that a velocity or distance parameter should be determined, the results of the analysis of differences between fertile and sub fertile bulls was cause to determine all velocity indicators (after dilution). In the storage test then the different quotients wobble and linearity can be calculated, to use further significant parameters for the selection of semen.

4.4. Practicability of the CASA device Sperm Vision™

A further objective of this work was the integration of the CASA system SpermVision™ into the daily routine of a laboratory of an insemination center. This target could successfully be achieved. It is possible to do the analysis that have to be made in a short period of time during production, fast and efficient with the help of the device. Thereby the objective assessment, the increase of number of analysis parameters and last but not least the easy filing of the data resulted to be advantageous compared to the usual analysis process.

Successful use of the device Sperm Vision™ requires applying a consistent and exact technique, according to the manual of the manufacturer, including charging of the measuring chamber with semen. A certain routine herefore is premise for a good repeatability of the results.

5. Complete thesis and literature

BALTISSSEN, J., 2007: Zusammenhänge zwischen computergestützt ermittelten spermatologischen Merkmalen und der Fertilität von Bullen