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Evaluating Bovine Spermatozoa Using the Hamilton-Thorne Integrated Visual Optical System (IVOS)

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Abstract: Traditionally, spermatozoa from yearling bulls have been evaluated manually as a part of a breeding soundness evaluation (BSE). Recent advances in computer technologies may allow for a more objective evaluation of spermatozoa. Our objective was to determine the relationship of bull spermatozoal characteristics generated by traditional methods under criteria established by the Society for Theriogenology (SFT) in 1993 to those generated by an automated semen analyzer. Semen specimens from 173 yearling beef bulls from five discrete bull test stations were evaluated by the traditional method (SFT 1993) and by an automated semen analyzer [Hamilton-Thorne Integrated Visual Optical System (IVOS) v.10, Beverly, MA]. The IVOS and manual methods were moderately correlated for motility ($r = 0.64$, $P < 0.001$). The minimum threshold of 30% motile spermatozoa, used with the manual method (SFT 1993), is applicable to the IVOS method. Correlations for normal morphology between the two methods were variable; the overall correlation was $r = 0.26$ ($P < 0.001$). The minimum threshold of 70% normal morphology, used with the manual method (SFT 1993), is not applicable to the IVOS method.

Key words: Spermatozoa, IVOS, semen analysis, CASA, breeding soundness evaluation

Introduction

Semen analysis is an integral component of the breeding soundness evaluation (BSE). Bulls should be subjected to a BSE before each mating season to determine their breeding potential. Unfortunately, most bulls used for breeding in the United States are never subjected to a BSE (Chenoweth *et al*, 1992).

During the routine BSE, a technician manually conducts a semen analysis to determine percent motile spermatozoa and percent spermatozoa exhibiting normal morphology. This manual method is not only subjective, but the results can also be quite varied between technicians (Davis Katz, 1993, Dunphy *et al.*, 1989 and Jequier *et al.*, 1983). The field of semen evaluation was updated over fifteen years ago, with the advent of computerized equipment and software capable of analyzing spermatozoa (Dott *et al.*, 1975; Jecht *et al.*, 1973 and Katz and DoH, 1975). Computerized semen evaluation can be more advantageous than manual methods. This technology offers the possibility of objectivity, and of increased sensitivity and reliability (ESHRE, 1996,Gravance *et al.*, 1999 and Holt *et al.*, 1994).

The Hamilton-Thorne Integrated Visual Optical System (IVOS) (version 10.0; Hamilton-Thorne Research, Beverly, MA) has been used by animal practitioners and human infertility clinics to evaluate spermatozoal characteristics (Farrell *et al.*, 1998). Computer assisted semen analysis (CASA) has been the focal point of many research efforts over the past years. This technology is not only capable of determining population averages such as motility, morphology, and kinematic measurements, but it also has the power to provide incisive measurements on individual cells (Davis *et al.*, 1993). A great deal of research conducted thus far through the use of CASA has been with human spermatozoa. Studies have shown that computer assisted semen analyzers can accurately determine percent motility and percent normal morphology of human sperm (Coetzee *et al.*, 1999 and Kruger *et al.*, 1995). Furthermore, human infertility clinics do not need to change minimum acceptance values (designed for manual evaluation) when they use computer methods (Coetzee *et al.*, 1999).

Thresholds for normal morphology of human sperm are quite low (5% to14%) compared to the threshold for bovine spermatozoa (70%). When they undergo semen analysis, human and bovine populations are generally complete opposites in regard to breeding ability. Humans solicit the assistance of infertility clinics when there is a problem with reproduction. Therefore, their seminal characteristics are often low or poor. A typical bovine that undergoes a BSE is a yearling bull (Chenoweth *et al.*, 1992) that has been selected to become a herd sire based on his genetics, physical appearance, and/or performance. Hence, he is expected to be an exceptional breeder and to have above-average seminal quality.

The goal of this study was to evaluate the ability of the Hamilton-Thorne IVOS to analyze bovine spermatozoal

motility and morphology by comparing computer results to manual results, and to determine if the thresholds used with the manual method are applicable to those of the computer method.

Materials and Methods

Semen was collected from 173 yearling beef bulls as part of a routine BSE from five discrete bull tests in South Carolina: Clemson University Bull Test, Jan. 1999 (CU), Black Bull Test, Feb. 1999 (SP), Edisto Bull Test, Oct. 2000 (ED), Clemson University Bull Test, Jan. 2000 (CA), Black Bull Test, Feb. 2000 (TA).

Breeding Soundness Evaluation: The same trained technician conducted all of the BSEs. Bulls were subjected to a physical examination, a thorough examination of the reproductive system, a scrotal circumference measurement, and a semen evaluation (Chenoweth *et al.*, 1992; Chenoweth *et al.*, 1993, Elmore, 1985a; Elmore, 1994 and ESHRE, 1996). A scrotal circumference was measured to the nearest 0.5 cm. Bulls with a scrotal circumference < 32 cm were removed from the bull test before the BSE (because of test requirements).

A semen sample was collected via electroejaculation into a pre-warmed insulated tube through a cone and evaluated manually according to the standards set by the Society for Theriogenology in 1993 (Table 1) (Chenoweth, 1992). After completing the entire BSE, the technician classified the bull as a satisfactory potential breeder, unsatisfactory potential breeder, or classification deferred (the bull will be retested at a date deemed appropriate by the technician).

Table 1: BSE Minimum Requirements set by the Society of Theriogenology in 1993a

Category	Threshold
Scrotal Circumference	30 cm at ≤ 15 mo
	31 cm at > 15 ≤ 18 mo
	32 cm at > 18 ≤ 21 mo
	33 cm at > 21 ≤ 24 mo
	34 cm at > 24 mo
Seprm Morphology	≥ 70% normal sperm
Sperm Motility	≤ 30% individual motility

[a]Modified from Chenoweth *et al.*, 1992

Manual Evaluation: The semen sample was placed in a heating block and maintained at 37°C. A drop of semen was placed on a warmed slide (37° C), diluted with sodium citrate or sterile saline (if necessary), and covered with a cover slip. An estimation of motility (rounded to the closest multiple of ten) was obtained by using a light microscope at X400. Another slide was prepared by placing a drop on a warmed slide (37° C), mixing with eosin-nigrosin stain, and smearing. An estimate of spermatozoa morphology (rounded to the nearest whole number) was determined under oil immersion by using a light microscope at X1000. Exactly 100 individual spermatozoa were counted and classified as being either normal cells or cells with primary or secondary abnormalities.

Table 2: IVOS Analysis Setup for Motility Evaluation

Items	Settings
Frames Acquired	25
Frame Rate	60 Hz
Minimum Contrast	30
Minimum Static Contrast	30
Straightness (STR) Threshold	80.0%
Low VAP Cutoff	25.0 $\mu m\ s^{-1}$
Medium VAP Cutoff	75.0 $\mu m\ s^{-1}$
Low VSL Cutoff	0.0 $\mu m\ s^{-1}$
Head Size, Non-Motile	5 Pixels
Head intensity, Non motile	55
Static Head Intensity	0.64 to 2.40
Static Elongation	0.63 to 1.57
Static Elongation	15 to 93
Slow Cells Motile	NO
Magnification	1.95
Temperature Set	37.0°C
User Defined Cell Depth	20 μm

Computer Evaluation of Motility: Once manual evaluation was completed for an individual sample, the semen specimen was subjected to computer evaluation. The IVOS was available at the site of collection for motility determination. Dilutions were made by using 1% BSA: PBS (37.0° C) so that the concentration of a given sample would be between 20-50 million spermatozoa / mL (Mortimer, 1995; Spirpoulos, 2001; Spitzer *et al.*, 1988; Van *et al.*, 1971). A 10-μl aliquot of the diluted specimen was placed in a MicroCell 20-micron chamber slide (Conception Technologies, San Diego, CA), allowed to fill, and blotted to remove excess fluid (Johnson *et al.*, 1996; Morrow and Gage, 2001 and Spitzer *et al.*, 1988). The slide was loaded into the IVOS stage (37.0° C) and retracted into the machine for motility assessment (Farrell *et al.*, 1996). Three slides for each semen specimen were analyzed and for each slide a minimum of six fields were evaluated between IVOS stage positions of 0.0 to 7.9 (Katz *et al.*, 1975). The parameter settings used for motility evaluation are presented in Table 2 (Farrell *et al.*, 1998; Holt *et al.*, 1994).

Computer Evaluation of Morphology: A 400-μL aliquot of the original semen specimen was placed on ice and transported back to the laboratory. Calculations to determine the amount of sample needed to produce 3

million spermatozoa per mL were performed by using the concentration information generated by the IVOS during motility assessment: 300 (target amount) / sample concentration (M/mL) = Z (amount to mix with dilutent - 20 μL) (Fig. 1).

Z = [300/initial concentration (M/ml) x 20μL
Example: initial concentration = 100M/mL
Z = [300/100] x 20 = 60 μL

Fig. 1: Example of Dilution

Sperm preparations were conducted in the following manner: 2 mL of 1% BSA: PBS were placed in a 15-mL centrifuge tube, the semen sample was vortexed, the calculated amount (Z) of the sample was pipetted into the centrifuge tube, vortexed, centrifuged for 3 min at 800 X G, and the supernate discarded. The pellet was resuspended with 20 μL of 1% BSA: PBS (vortexed), a 10-μL aliquot was placed on a glass slide (two slides were made per sample), smeared [pulling across the slide to avoid detaching heads (Le Lannou *et al.*, 1992, pp.1417-1421)], and allowed to dry. The slides were stained with Diff-Quik (Dade Behring Ag, CH-3186 Düdingen, Switzerland): fixed (Diff-Quik fixative) for 30 sec at room temperature, stained (Diff-Quik solution 1) for 2 min. at 40.0° C, and counterstained (Diff-Quik solution 2) at 40.0° C. The slides were gently washed with distilled water, allowed to dry, and cover slipped. Before analysis, the back of each slide was wiped clean by using 95% alcohol to remove excess stain.
After loading a slide into the IVOS, the slide was positioned at 20 mm and evaluated. The "field" was viewed by the internal microscope with a X60 objective. A field typically contained 0-6 spermatozoa. Approximately 200 individual sperm were captured, analyzed, and classified as normal, abnormal, or rejected (Coetzee *et al.*, 1999; Davis *et al.*, 1993 and Gravance *et al.*, 1999. After an entire slide was analyzed, the computer technician reviewed the sperm classifications and made appropriate changes. For example, if the IVOS marked a tail on a tail-less sperm, the technician changed the classification from normal to abnormal. The technician did not change any of the parameter measurements, only the gross classification. The evaluation of morphology was conducted by using the acceptance ranges presented in Table 3 (Boersma *et al.*, 1999; Cummins and Woodall, 1985; Gravance *et al.*, 1998; Mortimer *et al.*, 2000 and Van Duijn, 1971).

Statistical Analysis: Correlations between the two methods were determined by using the PROC CORR (correlation) procedure from SAS (Statistical Analysis Systems, Cary, NC). Scatterplots were constructed by using the PROC PLOT command in SAS. Chi-square analysis was used to determine whether the thresholds currently used with the BSE were applicable to the computer evaluation. Not all of the assumptions for chi-square analysis were met for threshold determination with the motility analysis.

Table 3: Gate Settings for the Hamilton - Thorne IVOPS v. 10[a]

Item	Abnormal	Normal	Normal	Abnormal
Major Axis (μm)	7.0	8.0	12.0	15.0
Minor Axis (μm)	3.0	4.0	5.4	7.0
Elongation (%)	35.0	40.0	64.0	70.0
Are (μm^2)	15.0	26.0	40.0	50.0
Perimeter (μm)	18.0	20.0	28.0	34.0

Hamilton-Thorne IVOS v. 10: Bovine Setup

Results

During manual evaluation, the technician estimated percent motile spermatozoa. The IVOS calculated numerous kinematic measurements. Three parameters were initially compared to the manual evaluation: the percentage of motile spermatozoa (moving > 25 μ s^{-1}) (MOT), the percentage of progressive spermatozoa (moving > 25 μ s^{-1} and possessing a straightness > 80%) (PROG), and the percentage of rapid spermatozoa (moving > 75 μ s^{-1}) (RAPID). Correlations between manual estimates and the three computer parameters for motility are presented in Table 4.
According to 1993 standards from the Society for Theriogenology (SFT) a percentage of motile sperm 30% is satisfactory. This standard was used to convert the numeric value assigned to each bull to pass (P) or fail (F). Table 5 shows the number of bulls that would have passed or failed the BSE after the numeric values were categorized.

Table 4: Correlations for Percentage Motile Spermatozoa between Manual and Computer Methods

Bull Test	MOT	PROG	RAPID
ED	0.58[b]	0.50[c]	0.67[b]
CA	0.70[a]	0.67[a]	0.66[a]
CU	0.60[a]	0.63[a]	0.59[a]
SP	0.54[a]	0.47[a]	0.51[a]
TA	0.45[b]	0.56[a]	0.55[a]
OVERALL	0.64[a]	0.60[a]	0.63[a]

[a]P<0.01, [b]P<0.01, [c]P<0.015

The MOT parameter was more similar to the manual results than were the other two parameters. Fig. 2 shows the relationship between manual motility and computer motility (MOT). Values on and to the right of

Table 5: Pass/Fail Classification by the 30% Acceptance Threshold for Motility

Items	Pass (Satisfactory)	Fail (Unsatisfactory)
Manual	171	2
IVOS MOT	166	7
IVOS PROG	147	26
IVOS RAPID	156	17

Table 6: Correlations for Morphologically normal spermatozoa between manual and computer methods

Bull Test	Correlations
ED	0.42 ($P<0.05$)
CA	0.61 ($P<0.001$)
CU	0.13 ($P>0.4$)
SP	0.06 ($P>0.6$)
TA	-0.02 ($P>0.9$)
OVERALL	0.26 ($P<0.001$)

Table 7: Chi-square results at 70% threshold for Morphology

Items	Pass (Satisfactory)	Fail (Satisfactory)
Manual	156	17
IVOS	93	80

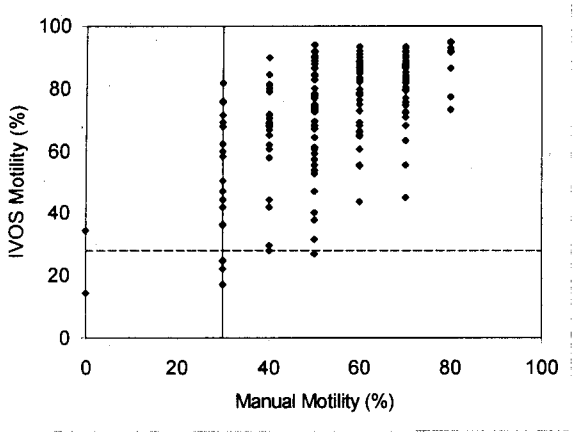

Fig. 2: Scatterplot of Mortility Estimates Generated Via Manual and Computer Methods

the solid line passed the manual evaluation, and values to the left of the solid line failed the manual evaluation. Values above the dashed line passed the computer evaluation of the MOT parameter, and values below the dashed line failed the BSE when the computer analysis was performed.

For the evaluation of spermatozoa morphology, both the manual and computer methods determine the percentage of spermatozoa exhibiting normal morphology. Correlations between the manual and computer method for the percentage of morphologically normal spermatozoa are presented in Table 6.

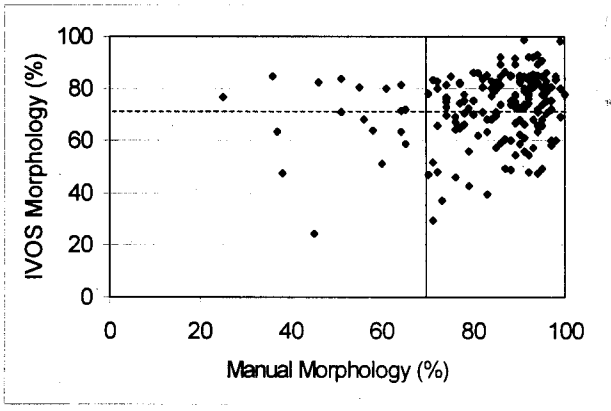

Fig. 3: Scatterplot of Morphology Estimates Generated Via Manual and Computer Methods

Table 8: Chi-square Results at 60% Threshold for morphology

Items	Pass (Satisfactory)	Fail (Satisfactory)
Manual	156	17
IVOS	123	50

Table 9: Chi-square Results at 50% Threshold for Morphology

Items	Pass (Satisfactory)	Fail (Satisfactory)
Manual	156	17
IVOS	151	22

The guidelines for bovine BSE set by the Society for Theriogenology (SFT) in 1993, suggest a semen sample contain at least 70% morphologically normal spermatozoa. This standard was used to convert the numeric value assigned to each bull to pass (P) or fail (F). Chi-square analysis was used to compare pass/fail classification between the computer and manual methods to determine whether the results were equal when the 70% threshold was used. Table 7 shows the number of bulls that would have passed or failed the BSE after the numeric values were categorized. The Chi-square analysis was not significant [$\chi^2 = 0.34$ ($P = 0.6$)]; therefore, the classification was not the same between the two methods when the 70% threshold was used. Fig. 3 is a scatterplot showing the relationship between the two methods. Values on and to the right of the solid line passed the manual evaluation, and values to the left of the solid line failed the manual evaluation. Values above the dashed line passed the computer evaluation, and values below the dashed line failed the BSE when the computer analysis was done.

Because the 70% threshold, which had been designed for manual evaluation, appeared to be too stringent for the IVOS method, we reclassified numeric values for IVOS evaluation to use a 60% threshold. Chi-square analysis was performed to determine if the classification between the two methods was the same if a 60% threshold were applied to the IVOS method. The Chi-square analysis was not significant [$\chi^2 = 1.38$ ($P = 0.2$)]; therefore, the classification is not the same between the two methods when the 60% threshold is used. The Chi-square results are presented in Table 8. More bulls failed at the 60% threshold according to the computer method, therefore, numeric values for IVOS evaluation were re-classified to use a 50% threshold. Chi-square analysis was performed to determine whether the classification between the two methods was the same if a 50% threshold were applied to the IVOS method. The Chi-square analysis was again not significant [$\chi^2 = 0.41$ ($P = 0.5$)]; therefore, the classification was not the same between the two methods when the 50% threshold was used. The Chi-square results are presented in Table 9.

Discussion

The use of automated semen analysis systems in the field of human reproduction has been investigated extensively. The Hamilton-Thorne IVOS has proven to be an acceptable alternative to subjective manual assessment of human spermatozoa. The question now presented is whether or not this system is applicable to non-human andrology. The IVOS has been used to investigate various species from the mouse to the elephant. However, no one has presented research to describe how to use the system in a practical manner with the bovine.

The data presented in Tables 4 and 5 show the Hamilton-Thorne IVOS v.10 is capable of accurately analyzing bovine motility. The parameter that seems most appropriate to use as a diagnostic tool during the BSE is overall motility (MOT); of the tested parameters, this one produces results most similar to those gathered by the traditional method (Table 5). Of the two bulls that had < 30% motility (fail) when subjective methods were used, one had < 30% (fail) with the IVOS method and one had > 30% (pass) with the IVOS. So the computer only classified one bull as pass that the technician classified as fail.

In previous studies, motility analysis by the computer was found to be more discriminating than were subjective methods (Davis and Katz, 1992). The authors attribute this finding to the fact that during manual evaluation (of human sperm), a technician considers spermatozoa motile if the flagellum is moving, even if it is non-progressive. During the breeding BSE, the technician considers only progressively moving sperm to be motile. So we would expect the computer and the breeding soundness technician to agree more than the computer and the technician evaluating human spermatozoa. Davis and Katz (1992) believes the computer's lower estimates of motility are caused by the fact that to be considered motile spermatozoa must obtain a threshold average progressive velocity for a minimum duration (Davis and Katz, 1992. This fact could account for the six bulls that passed the manual evaluation but did not pass the IVOS evaluation.

Morphological analysis is slightly more complex than is the motility analysis because of slide preparation. Slide preparation (i.e., staining) can add great variability to the computer analysis (Boersma *et al*, 1999 and Van *et al.*, 1971. Slides for each of the five bull tests were prepared separately (because of the differing times of test / BSE). Perhaps slide preparation contributed to the inconclusive results presented in Table 6.

The data in Table 6 indicate that a possibility for consistency exists with correlations of $r = 0.61$ ($P < 0.001$) and $r = 0.42$ ($P < 0.05$). However, the inconsistency among the later three bull tests [(CU, SP, TA) ($P > 0.4$)] indicates that future studies need to be conducted before the IVOS can accurately be utilized during the BSE to evaluate morphology.

Studies of human infertility show that the IVOS is capable of correctly evaluating human spermatozoa without technicians' having to change minimum thresholds. However, with human semen analyses, the minimum threshold, when the strict criteria is used, is 5% normal morphology. This percentage differs greatly from the 70% threshold set forth by the Society for Theriogenology in 1993 and currently used during BSE. In fact, the lowest reading by the IVOS for the 173 bulls was 24%.

This difference raises the question whether changing the threshold would allow for more consensus between the two methods in terms of pass and fail. When the 70% threshold was used, 80 bulls failed the computer evaluation. Of those 80, 71 had passed the manual evaluation. After the threshold for the computer evaluation was lowered to 60%, 50 bulls failed the computer evaluation. Of those 50, 43 had passed the manual evaluation. And if the threshold for the computer evaluation was lowered to 50%, 22 bulls failed the computer evaluation. Of those 22, 19 had passed the manual evaluation.

Even though the correlations between the computer and manual methods for sperm morphology are not high enough or consistent enough to suggest replacing the manual semen evaluation with the computer evaluation, these data do suggest that the threshold will need to be lowered when the computer method is used during a BSE. The computer is by nature more

critical than the technician when it evaluates sperm morphology. The computer is able to take exact measurement of the sperm whereas the technician is evaluating the gross shape. One would expect the computer to be more discriminating than the technician is.

Another factor to consider is the range of acceptance values used to classify the sperm. In this lab, we are currently working on a study to determine how a group of highly qualified technicians classify image printouts of individual spermatozoa in order to see whether the technicians and the computer are in agreement on the acceptance ranges. Results from this exercise may provide insights into the differences between individual technicians and differences between technicians and the computer.

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Conclusion

Results of this study indicate that the Hamilton-Thorne Integrated Visual Optical System (IVOS) v. 10 can accurately determine bovine spermatozoal motility. The threshold of 30% set by the Society for Theriogenology in 1993 is applicable to the IVOS analysis. However, correlations for morphology results between manual and computer methods were not consist over the five bull tests. The moderate correlations seen with two bull tests suggest this study should be repeated or continued to determine the true relationship between the two methods. However, much care should be taken to ensure all samples are handled uniformly. Although these data are inconclusive, it does appear that the threshold currently used with manual evaluation is not applicable to the computer method. The threshold of 70%, set by the Society for Theriogenology in 1993, appears to be too stringent; a lower, more appropriate threshold should be determined in future studies.

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