

# Comparison of bull sperm morphology evaluation methods under field conditions

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## Abstract

Sperm morphological assessment is a critical component in bull breeding soundness evaluations. Although sperm morphology is an important parameter to identify subfertile from fertile bulls, the evaluation can be biased because sperm staining methods used under field conditions may not be exact due to various factors, including artifacts. Objective was to compare 2 sperm morphological evaluation methods. Prebreeding season ejaculates of 1,216 Angus cross bulls collected via electroejaculation were evaluated. For each bull, an unstained (UNS) and an eosin-nigrosin stained (ENS) semen smear were viewed under a phase contrast microscope and a brightfield microscope with an oil immersion lens, both at 1,000 × magnification. Normal and percentage of abnormal sperm were identified by counting 200 sperm. Inter-rater agreements between 2 clinicians for the percentage of abnormal sperm determination and its categories were very good (ENS method,  $r = 0.84 - 0.96$ ; UNS method,  $r = 0.76 - 0.96$ ;  $p < 0.01$ ). No differences ( $p > 0.1$ ) were observed for abnormal sperm percentage determination and its categories between 2 methods. Correlation was very good between 2 methods for total abnormal sperm percentage determination ( $r = 0.91$ ;  $p < 0.01$ ) and its categories ( $r = 0.84 - 0.96$ ;  $p < 0.05$ ). Additionally, 60 ejaculates were evaluated by triple stain (TS), ENS, and UNS methods. Agreements between TS (percentage of sperm with damaged membrane) and ENS (percentage of abnormal sperm) and between TS and UNS methods were moderate ( $r = 0.58$ ;  $p < 0.05$ ) and fair ( $r = 0.43$ ;  $p < 0.05$ ), respectively. Based on our findings, either technique can be used for bull sperm morphological evaluation under field conditions. Considering the ease of semen smear preparation, the UNS method can be a viable alternative to the ENS method.

**Keywords:** Bull, sperm morphology, eosin-nigrosin, triple stain, microscope, phase contrast

## Introduction

Spermiogenesis includes reorganization of sperm nucleus, development and positioning of acrosome from the Golgi apparatus, organization of tail structures, and restructuring of cytoplasm.<sup>1</sup> Transformation of spermatids during spermiogenesis is a key postmeiotic event contributing to major sperm morphological reorganizations.<sup>2</sup>

An exact evaluation of sperm parameters is necessary to predict bull fertility for natural breeding and for assisted reproductive technology (ART).<sup>3,4</sup> Evaluation of bull's breeding potential includes analysis of basic sperm characteristics (motility and morphology).<sup>5,6</sup> Sperm use in ART procedures is expanding, especially for semen cryopreservation, semen sexing, and in vivo and in vitro embryo production, validating the need for diagnosing subfertile and infertile bulls.<sup>7-10</sup> Sperm morphological characteristics are the most distinguishing

sperm parameter of subfertile and fertile bulls.<sup>11-13</sup> Additionally, sperm morphology has a high prognostic potential for evaluating bull fertility, and thus morphological assessment is critical for selecting bulls for breeding purposes.<sup>6,11-13</sup>

Sperm morphological evaluation determines the percentage of normal and abnormal sperm.<sup>5,6</sup> Microscopic examination of ejaculates indicated that sperm morphological assessment has discrepancies, even within the same ejaculate, and these discrepancies create difficulties in determining bull fertility potential.<sup>6,14</sup> Various stains and methods were used for sperm morphological analysis, resulting in ambiguous outcomes.<sup>2,15</sup> In this regard, eosin-nigrosin (ENS) staining has remained the most commonly used technique for detecting sperm morphological abnormalities.<sup>5,6</sup> However, it should be noted that sperm morphological evaluation methods can critically affect sperm morphology outcomes.<sup>16-18</sup> Veterinary practitioners also perform sperm morphological evaluation using unstained (UNS)

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semen smears under a phase contrast microscope under an oil immersion lens. Easy-to-use inexpensive standard sperm morphological evaluation technique is important for exact, unambiguous analysis of sperm.<sup>19</sup> Therefore, we compared 2 methods (ENS versus UNS) for evaluating sperm morphological characteristics of bulls. Additionally, we also evaluated ejaculates from 60 bulls by the triple stain (TS), ENS, and UNS methods to determine the agreement among these 3 methods to determine their field application. We hypothesized that bull sperm morphological evaluation using these methods is similar.

## Materials and methods

### Animals

Our prospective study used ejaculates (n = 1,216; Angus cross; yearling to 5 years) collected from bulls (2017 to 2020) presented for routine bull breeding soundness evaluation.<sup>5</sup> Bulls were reared by private commercial beef cattle producers and were chosen without considering their fertility potential. Ejaculates were collected from bulls via electroejaculation (Pulsator IV, Lane Manufacturing, Inc., Denver, CO, USA) applying minimal electrostimulation to obtain ejaculates.

Since it is a routine theriogenology procedure (with no extra manipulation of bulls involved), institutional animal care and use committee approval is not required, and the study is exempt.

### Sperm morphological examination

Once sperm motility was assessed, sperm morphology slides were prepared using both methods for each bull. For the UNS method, 10  $\mu$ l of semen was placed on one end of a preheated (36°C) microscopic slide, and a thin smear was prepared by feathering technique. For the ENS method, a drop of semen (5  $\mu$ l) was placed on a preheated (36°C) microscopic slide, mixed with an equal volume of eosin-nigrosin dye, and a thin smear was prepared using feathering technique. Slides were air-dried, and 1 clinician prepared slides for sperm morphology. For the ENS method, sperm morphology slides were viewed under a brightfield microscope at 1,000  $\times$  magnification under an oil immersion lens. For the UNS method, sperm morphology slides were evaluated at 1,000  $\times$  under a phase contrast microscope under an oil immersion lens. Sperm morphology slides were evaluated by 2 clinicians, on the same or on the following day, at the laboratory. For both techniques, slides were scored after counting a minimum of 200 sperm per sample and categorized based on morphological abnormalities.

### Sperm abnormality classification

Morphological abnormalities<sup>5,6</sup> were calculated as a percentage of the total number of counted sperm. Morphological categories used were abnormal heads (pear-shaped, narrow at the base, abnormal contour, acrosome defects, vacuoles and craters, diadem, and undeveloped, detached, narrow, big, little-normal, and short-broad heads), abnormal midpieces (bent, short, and rough edge), proximal cytoplasmic droplets, Dag defect, bent tail, and coiled tail. Comparison of sperm plasma, acrosomal, and mitochondrial membrane assessment was by the triple stain (TS) method with sperm abnormality assessment by ENS and UNS methods.

Semen samples from 60 bulls were used. Immediately after semen collection, semen smears were prepared for ENS and

UNS methods. Additionally, an aliquot of the ejaculate was extended with a semen extender and was transported to the laboratory. Semen samples were washed in PBS, diluted in TALP sperm medium to reach the final concentration of  $25 \times 10^6$  sperm per ml, and then stained using the TS staining method.

Triple staining procedure was performed according to the method described.<sup>20-22</sup> For each bull, 12  $\mu$ l of propidium iodide (PI) (0.5 mg/ml), 2  $\mu$ l of mitochondrial (JC-1) (153  $\mu$ M), and 50  $\mu$ l of *Pisum sativum* agglutinin (FITC-PSA) (100  $\mu$ g/mL) fluorescent probes were added to 150  $\mu$ l of the diluted semen sample. The mixture was incubated at 37°C for 8 minutes, then placed on a warmed slide, covered by a coverslip, and evaluated using fluorescence microscopy (Leitz, Laborlux S) at 1,000  $\times$  magnification. Two hundred sperm were examined per slide and were classified into 6 classes (Table 1) according to the fluorescence emission from each dye. Plasma (PI), acrosomal (FITC/PSA), and mitochondrial (JC-1) membranes were simultaneously assessed.<sup>20-22</sup> Percentage of sperm with low membrane integrity (sperm exhibiting damaged plasma membrane), damaged acrosome, and low mitochondrial potential on triple stain was considered for comparison with the percentage of sperm abnormalities by ENS and UNS methods. Furthermore, comparisons were made for sperm organelles among staining methods, and parameters compared are provided (Table 2).

### Data analyses

Data were analyzed using a statistical program (SAS, Version 9.4; SAS Institute, Cary, NC, USA), and p value was set at  $\leq 0.05$  for significance. Concordance correlation coefficient of Lin was used to analyze inter-rater agreement for determining the percentage of abnormal sperm.<sup>23,24</sup> The Shapiro-Wilk test was used to verify the normality of the data and Levene's test to verify the homoscedasticity of the variance. When necessary, data were transformed with log10 or arcsine for analysis, and nontransformed values were reported. Mean abnormal sperm percentage by the 2 techniques was compared using the Student's t-test. Correlation of abnormal sperm percentage by ENS and UNS methods was determined using the Lin correlation coefficient method. Correlation between the percentage of low sperm membrane integrity by the TS method and the percentage of abnormal sperm by the ENS method, and the percentage of abnormal sperm by UNS method were determined using the Lin correlation coefficient method. Furthermore, the percentage of plasma membrane integrity by TS versus the percentage of live sperm by ENS or head defect by UNS; intact acrosome membrane by TS versus normal acrosome by ENS or UNS methods; and high mitochondrial potential by TS versus normal midpiece by ENS or UNS methods were also determined using the Lin correlation coefficient method.

### Results

Images of morphologically abnormal sperm by ENS (Figure 1) and UNS (Figure 2) methods are provided. Images of 6 classes (Figure 3) by simultaneous assessment of plasma, acrosomal, and mitochondrial membranes are given. Agreement (concordance correlation of Lin, r) between 2 clinicians for determining various types of abnormal sperm percentage (Table 3) by the ENS method was very strong (r = 0.85 – 0.96; p < 0.01), whereas by the UNS method, the agreement was moderate to very strong (r = 0.76 – 0.96; p < 0.01).

**Table 1.** Sperm classification for simultaneous assessment of plasma (propidium iodide – PI), acrosomal (*Pisum sativum* agglutinin – FITC/PSA), and mitochondrial (JC-1) membranes by triple stain fluorescent probes

Sperm classification	PI	FITC-PSA	JC-1
A. Intact plasma membrane, damaged acrosome, and high mitochondrial potential (Class III)	-	+	Red
B. Intact plasma membrane, intact acrosome, and high mitochondrial potential (IPIAH – Class I)	-	-	Red
C. Damaged plasma membrane, damaged acrosome, and high mitochondrial potential (Class VII)	+	+	Red
D. Damaged plasma membrane, damaged acrosome, and low mitochondrial potential (Class VIII)	+	+	Green
E. Damaged plasma membrane, intact acrosome, and low mitochondrial potential (Class VI)	+	-	Green
F. Damaged plasma membrane, intact acrosome, and high mitochondrial potential (Class V)	+	-	Red

PI positive (+) = red stained nucleus; FITC-PSA positive (+) = green acrosome region; PI negative (-) = unstained nucleus; FITC-PSA negative (-) = unstained acrosome.

**Table 2.** Categories for comparison of sperm organelles

Categories	Triple stained	Eosin-nigrosin stain	Unstained
Plasma membrane integrity	Intact plasma membrane (PI, %)	% Live sperm <sup>‡</sup>	% Normal heads <sup>†</sup>
Intact acrosome	% Intact acrosome membrane (FITC)	% Normal acrosome*	% Normal acrosome*
Mitochondrial potential	% High mitochondrial potential (JC-1)	% Normal midpiece <sup>§</sup>	% Normal midpiece <sup>§</sup>

<sup>‡</sup>Sperm stained in pink/red color (sperm with structurally intact cell membranes, live sperm, are not stained, while dead sperm, with disintegrating cell membranes, are stained) were excluded; <sup>†</sup>Abnormal heads (including pear-shaped, narrow at the base, abnormal contour, vacuoles and craters, diadem, and undeveloped, detached, narrow, big, little-normal, and short-broad heads) were excluded; \*Acrosomal defects (knobbed acrosome and acrosome reacted) were excluded; <sup>§</sup>Abnormal midpieces (bent, short, rough edge, and Dag defect) were excluded.

Percentage abnormal sperm did not differ ( $p > 0.1$ ) between ENS and UNS methods (Table 4). The percentage of various sperm abnormalities by 2 methods was positively correlated ( $r = 0.84 - 0.96$ ;  $p < 0.01$ ). Both methods detected acrosome defects and cytoplasmic droplets; however, nuclear vacuoles were detected most readily by the UNS method. The percentage of total sperm defect did not differ between TS and ENS but differed between TS and UNS (Table 5). The percentages of head, mid-piece, and tail sperm defects did not differ among methods (Table 5).

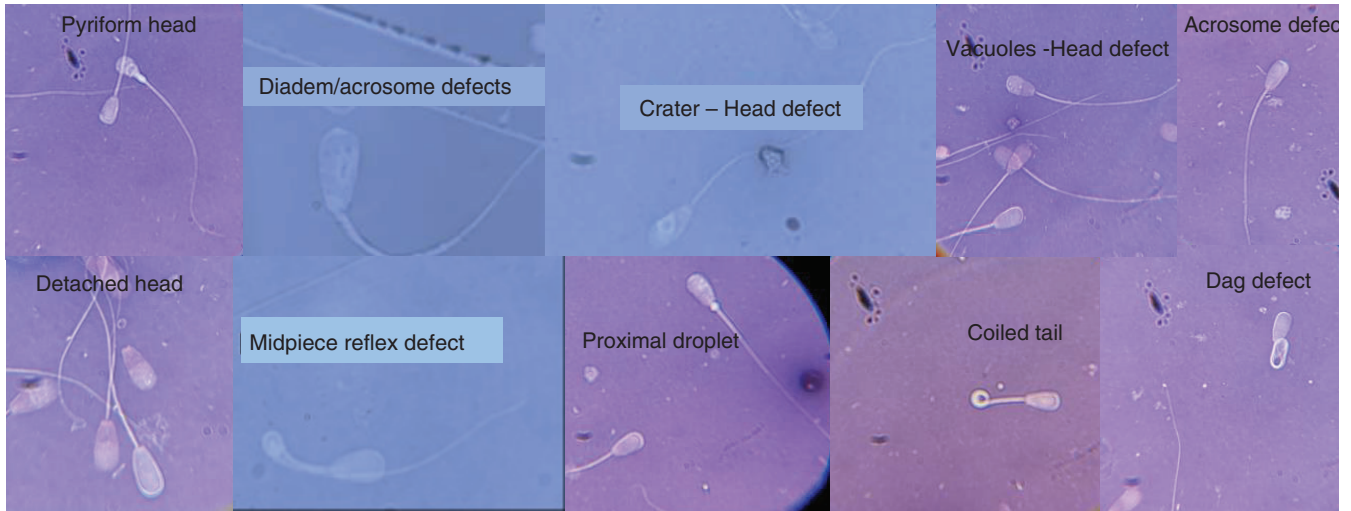
Correlation between the percentage of abnormal sperm with damaged membranes by the TS method and the percentage of abnormal sperm by the ENS method was moderate ( $r = 0.58$ ;  $p < 0.05$ ). Similarly, the correlation between the percentage of sperm with damaged membranes by the TS method and the percentage of abnormal sperm by the UNS method was fair ( $r = 0.43$ ;  $p < 0.05$ ) (Table 5). Correlations between TS and ENS methods for sperm head, mid-piece, and tail defects were fair to very good, 0.73, 0.49, and 0.64, respectively ( $p > 0.05$ ); furthermore, correlations between TS and UNS methods were

also fair to very good, 0.80, 0.40, and 0.61, respectively ( $p > 0.05$ ) (Table 5).

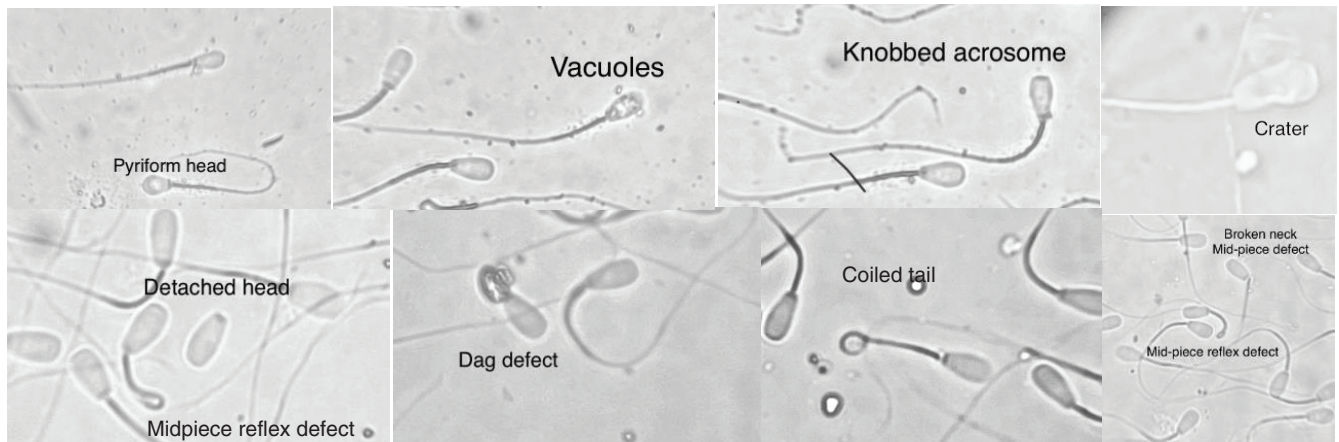
Plasma membrane integrity by TS versus live sperm percentage by ENS or head defect by UNS had a very good correlation ( $p < 0.01$ ; Table 6). Correlation between intact acrosome membrane by TS versus normal acrosome by ENS was moderate ( $p < 0.05$ ; Table 6) and by TS versus UNS was good ( $p < 0.01$ ; Table 5). Correlation between high mitochondrial potential by TS versus normal midpiece by ENS was moderate ( $p < 0.05$ ; Table 5) or by TS versus UNS was poor ( $p > 0.1$ ; Table 6).

## Discussion

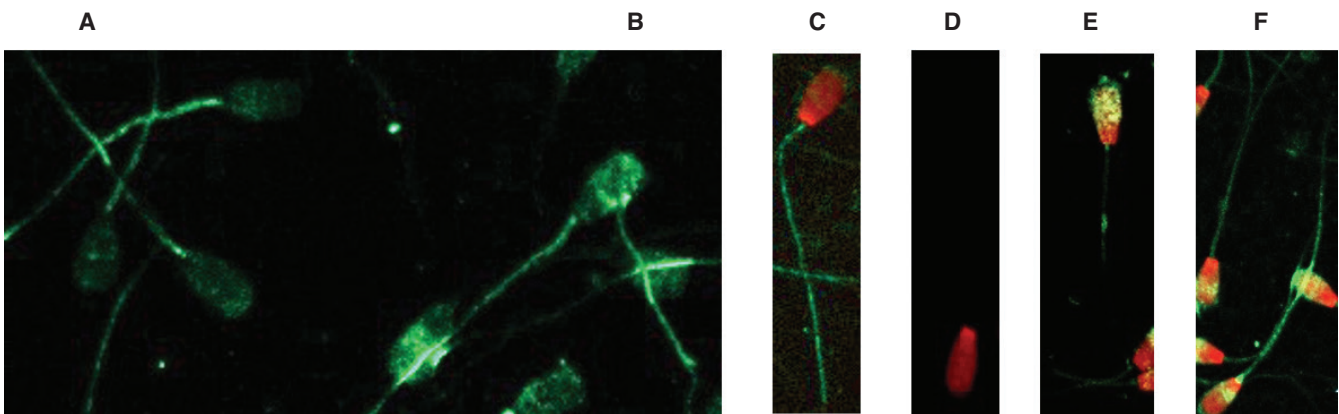
Sperm morphology in bulls was compared using the UNS method and the commonly used ENS method. Both methods evaluated sperm morphology similarly. Although similar studies<sup>16,17</sup> have been conducted, the current study investigated sperm samples from a larger population of bulls to determine the repeatability of earlier studies' outcomes.



**Figure 1.** Sperm with abnormal morphology (eosin-nigrosin stain); images were captured by iPhone 12 under brightfield microscopy.



**Figure 2.** Sperm with abnormal morphology (unstained); images were captured by iPhone 12 under phase contrast microscopy.



**Figure 3.** Images for simultaneous assessment of sperm plasma (propidium iodide – PI), acrosome (*Pisum sativum* agglutinin – FITC/PSA), and mitochondrial (JC-1) membranes by triple stain fluorescent probes in bulls. (A) Intact plasma membrane, damaged acrosome membrane, and high mitochondrial potential; (B) intact plasma membrane, intact acrosome membrane, and high mitochondrial potential; (C) damaged plasma membrane, damaged acrosome, and high mitochondrial potential; (D) damaged plasma membrane, damaged acrosome membrane, and low mitochondrial potential; (E) damaged plasma membrane, intact acrosome membrane, and low mitochondrial potential; (F) damaged plasma membrane, intact acrosome, and high mitochondrial potential.

**Table 3.** Degree of inter-rater agreement among independent observers of sperm abnormalities

Sperm abnormalities (%)	Eosin-nigrosin stain		Unstained	
	r	p value	r	p value
Head abnormalities	0.85	0.01	0.79	0.01
Detached head	0.94	0.01	0.94	0.01
Mid piece abnormalities	0.84	0.01	0.76	0.01
Proximal droplet	0.91	0.01	0.92	0.01
Distal droplet*	0.94	0.01	0.93	0.01
Bent tail	0.94	0.01	0.96	0.01
Coiled tail	0.96	0.01	0.96	0.01
Total abnormalities	0.91	0.01	0.92	0.01

r: Lin's concordance correlation coefficient, the degree of agreement among independent observers who assessed sperm abnormalities; significant difference if  $p \leq 0.05$ ; \*not classified as sperm abnormalities and not included to calculate percentages of morphologically normal sperm.

**Table 4.** Differences in sperm morphological abnormalities (means  $\pm$  SD) relative to techniques

Sperm abnormalities (%)	Eosin-nigrosin stain	Unstained	p value	r	p value
Head abnormalities	4.54 $\pm$ 3.19	5.42 $\pm$ 3.52	0.58	0.85	0.01
Detached head	3.17 $\pm$ 2.35	4.11 $\pm$ 3.19	0.43	0.91	0.01
Mid piece abnormalities	5.13 $\pm$ 3.31	6.12 $\pm$ 3.07	0.55	0.84	0.01
Proximal droplet	6.36 $\pm$ 6.10	7.20 $\pm$ 8.54	0.49	0.91	0.01
Distal droplet*	4.47 $\pm$ 4.81	4.99 $\pm$ 5.12	0.73	0.94	0.01
Bent tail	5.41 $\pm$ 5.08	5.03 $\pm$ 5.73	0.67	0.94	0.01
Coiled tail	2.90 $\pm$ 3.10	3.21 $\pm$ 4.12	0.84	0.96	0.01
Total abnormalities	25.61 $\pm$ 13.31	28.42 $\pm$ 15.52	0.31	0.91	0.01

SD: standard deviation; r: Lin's concordance correlation coefficient; significant difference if  $p \leq 0.05$ ; \*Not classified as sperm abnormalities.

**Table 5.** Mean  $\pm$  SD % abnormal sperm by triple staining (TS), eosin-nigrosin stained (ENS) and unstained (UNS) methods, and association among 3 staining methods

Sperm defect (%)	TS	ENS		UNS		TS versus ENS		TS versus UNS	
		(%)	p value TS versus ENS	(%)	p value TS versus UNS	r	p value	r	p value
Total	22.61 $\pm$ 16.31 <sup>†</sup>	25.61 $\pm$ 13.31	> 0.1	28.42 $\pm$ 15.52	0.05	0.58	0.05	0.43	0.05
Head <sup>§</sup>	9.10 $\pm$ 8.23	8.44 $\pm$ 6.91	> 0.1	9.55 $\pm$ 6.67	>0.1	0.73	0.01	0.80	0.01
Midpiece*	12.16 $\pm$ 9.89	11.08 $\pm$ 9.34	> 0.1	13.12 $\pm$ 11.02	>0.1	0.49	0.05	0.40	0.05
Tail	9.22 $\pm$ 9.01	7.96 $\pm$ 8.11	> 0.1	9.01 $\pm$ 8.98	>0.1	0.64	>0.01	0.61	> 0.01

<sup>†</sup>Damaged plasma membrane, damaged acrosome, and low mitochondrial potential (TS) versus total abnormal sperm defect (ENS or UNS); <sup>§</sup>damaged plasma membrane and damaged acrosome (TS) versus head defect (ENS or UNS); \*low mitochondrial potential (TS) versus midpiece defect (ENS or UNS); r: Lin's concordance correlation coefficient; Significant difference if  $p \leq 0.05$ .

**Table 6.** Correlation among normal sperm morphology parameters identified by triple staining (TS), eosin-nigrosin stained, and unstained methods.

Triple stain sperm parameter	ENS		UNS	
	R	p	r	P
IPIAH (triple stain)	0.85	0.01	0.82	0.01
Plasma membrane integrity	0.88	0.01	0.43	0.05
Acrosome integrity	0.52	0.05	0.67	0.01
High mitochondrial potential	0.49	0.05	0.34	0.22

ENS (eosin-nigrosin stain); UNS (unstained); IPIAH (intact sperm plasma membrane, intact acrosome, and high mitochondrial potential).

Additionally, sperm evaluation by the triple staining method used in this study can be considered as an unbiased quantitative assessment. Using fluorescence microscopy to assess organelles functions with simultaneous acquisition of brightfield microscopic images of sperm morphology can identify subtle sperm features to predict negative impacts on fertility.

Sperm morphology is widely regarded as the most accurate and exact assessment of fertility under field conditions. Moreover, sperm morphological characteristics can be used in diagnosing male reproductive disorders and studying the effect of exposure to reproductive toxins and hazards on male fertility.<sup>25-27</sup> Hence, standardized techniques for evaluating sperm morphology including morphometry are required. Sperm morphological characteristics have historically been evaluated using a variety of staining methods – Papanicolaou, eosin-nigrosin, trypan blue, Giemsa, and Diff-Quick. Nevertheless, a specific method of sperm staining for various species of livestock animals is lacking. Stains used for sperm morphological analysis might induce morphologic or morphometric alterations to bull sperm.<sup>17</sup> Furthermore, dyes used have differing pH and osmolarity, and lengthy procedures may affect sperm shape and size, and thus the outcome of the sperm morphological evaluation.<sup>28-30</sup>

A study<sup>17</sup> compared bull sperm morphology evaluated by brightfield microscopy of eosin-nigrosin stained dry-mount semen smears and differential interference phase contrast microscopy of wet-mounted semen, both at 1,000× magnification. Differential interference phase contrast method was more effective in visualizing major defects. In contrast, brightfield microscopy was considered to cause more minor defects. Differences ( $p < 0.05$ ) were generally noted for defects in acrosome, midpiece, tail, and cytoplasmic droplets but not for sperm head defects. Though some studies<sup>17,18</sup> observed differences in identifying percentage of head, mid-piece, and tail sperm defects, those defects did not differ between the 2 methods in the current study.

In the current study, bulls that failed to meet the normal sperm morphology criteria set forth by the Society for Theriogenology (70% normal sperm) were 12.4% (151/1,216) and 12.9% (157/1,216) by the ENS and UNS methods, respectively. Both methods identified 151 bulls producing abnormal sperm, and the UNS method identified an additional 6 bulls producing abnormal sperm. Correlation between the 2 methods in identifying the total percentage of abnormal sperm was

very good. Further correlations for identifying percentage abnormal sperm categories (percentage of head, midpiece, and tail sperm) defects were also very good.

The presence of a sperm defect may not always depict an artifact; main sources of variation in sperm morphometry are not only related to the staining technique but also due to heterogeneity in the sample preparation, the classification systems used, and evaluator competency for the assessment that is necessarily subjective.<sup>16,18</sup> Therefore, it is important to homogenize semen smear preparations, reading techniques, and classifications, and stress the importance of conducting quality controls and training programs to maintain operator competency.<sup>30-34</sup> Current study observed very good agreement between interpreters. In the current study, the 2 clinicians who evaluated the sperm morphology were experienced, and 1 clinician prepared sperm morphology slides for all bulls evaluated in the study. This substantiates the importance of qualified and experienced evaluators interpreting sperm morphology.

In the present study, the percentage of head, midpiece, and tail defects among the 3 staining methods (TS, ENS, and UNS) was the same; however, the percentage of total abnormal sperm defects was different between TS and UNS methods. The detection of the percentage of acrosome defects and nuclear vacuoles by the UNS and TS methods in the current study was comparable and consistent with earlier studies.<sup>17,18</sup> Comparisons between the efficiency of these methods (TS versus ENS and TS versus UNS methods) identified the correlations between these methods were fair to very good. An explanation for the result observed could have been due to fresh semen used for the preparation. Earlier studies demonstrated the effect of cooling/freezing and the duration of storage on membrane permeability as the procedure itself could have altered sperm lipid fraction, increased membrane permeability, reduced enzyme activities, changed membrane proteins, or reduced sperm cell membrane stability.<sup>35-37</sup> Therefore, sperm morphology evaluation after cooling and postthaw processes may require a different technique. Both ENS and UNS techniques can be used for different purposes. The stain used in the ENS method is a negative differential dye that recognizes intact live and dead sperm. The UNS method is simple since it includes single-step preparation, it is fast, and it is easy to learn and perform consistently by everyone, which is a primary criterion for robustness and repeatability in the results. Furthermore, the changes in pH and osmolarity during storage may affect sperm's shape and size.

The triple stain method is effective because it simultaneously detects damage to plasma membrane, acrosome status, and inner mitochondrial membrane potential, but it is not possible to perform under field conditions.<sup>12</sup> This method was used in the current study as this offered a different perspective in terms of membrane integrity comparison with the ENS and UNS methods. A positive correlation was observed between the sperm viability test by PI and ENS methods for identifying sperm plasma membrane integrity in dogs ( $r = 0.88$ ),<sup>38</sup> boars ( $r = 0.71$ ),<sup>39</sup> and bulls ( $r = 0.78$ <sup>40</sup> and  $r = 0.83$ <sup>39</sup>), which is consistent with the current study.

Percentages of sperm with normal and abnormal acrosome membranes that were identified by TS and ENS methods had a positive correlation. However, the correlation between TS and UNS methods was better than the correlation between TS and ENS methods for identifying acrosome-reacted sperm. This supports the findings of studies<sup>16,17</sup> in which the sperm acrosome was visualized by phase contrast.

The acrosome reaction is a time-dependent phenomenon that should not take place prematurely or too late.<sup>41</sup> Premature acrosome reaction and the inability of the sperm to release the acrosomal contents in response to proper stimuli (acrosome reaction insufficiency) were associated with male infertility.<sup>42</sup> Although the cause of premature acrosome reaction is unknown, the stimulus of independent premature initiation of acrosomal exocytosis appears to be related to a perturbation of the plasma membrane integrity. In this situation, the acrosome reaction may not involve a premature activation of the receptor-mediated process but reflect an inherent fragility of the sperm membrane, leading to a receptor-independent acrosomal loss.<sup>43</sup> In the current study, the sperm characteristics were evaluated in fresh semen, and all necessary precautions were taken to avoid premature acrosome reaction. A study<sup>44</sup> conducted on marmoset sperm observed that ENS had a marked differentiation between sperm with intact and reacted acrosomes, with and without ionophore treatment, and closely correlated to the results obtained with FITC-PSA.

A study<sup>45</sup> observed no significant correlations between the ENS and JC-1 methods for the determination of total sperm defects and high mitochondrial membrane potential (MMP) ( $r = -0.22$ ) and between total sperm defects and low MMP ( $r = 0.11$ ). Similarly, correlations between head defect and high MMP ( $r = -0.24$ ), head defect and low MMP ( $r = 0.17$ ), tail defect and high MMP ( $r = -0.35$ ), and tail defect and low MMP ( $r = 0.28$ ) were not significant between the ENS and JC-1 methods. It should be noted that a structurally deficient mitochondrial sheath (swollen; gaps) may structurally damage the axoneme, resulting in a wide variety of midpiece defects.<sup>46</sup> Furthermore, sperm with low MMP or midpiece defects with mitochondrial aberrations may exhibit mitochondrial dysfunction, leading to high ROS production since mitochondria are the main source of sperm-produced ROS via the electron transport chain.<sup>47,48</sup> In the current study, positive correlations between the TS and ENS methods for the identification of percentage of normal and abnormal sperm mitochondrial membrane defects were identified; however, no correlation was observed between the TS and UNS methods. Though the fluorochrome staining methods have varying levels of correlation with conventional methods used to evaluate sperm morphology, the benefits of the detailed assessment of sperm structure and functions by fluorochromes should be taken into consideration when advanced methods are warranted to diagnose male infertility.

Results support the conclusion that either ENS or UNS technique can be used for bull sperm morphological evaluation under field conditions. Considering the ease of semen smear preparation, the UNS method can be a suitable alternative to the staining method.

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## Conflict of interest

Authors declare that they have no conflict of interest.

## Authors' contribution

Rachel Hanson: Investigation, Methodology, Data Collection, Writing – original draft; Final approval.

Sadie Reddick: Investigation, Data Collection; Image acquisition; Final approval.

Sabrina Thuerauf: Investigation, Data Collection; Image acquisition; Final approval.

Katie Webb: Investigation, Data Collection; Image acquisition; Final approval.

Vanmathy Kasimanickam: Conceptualization, Investigation and supervision, Methodology, Writing – original draft, Final approval.

Ramanathan Kasimanickam: Conceptualization, Methodology, Formal analysis, Writing – review & editing, Final approval.

## References

1. Moreno RD, Ramalho-Santos J, Chan EK, et al: The Golgi apparatus segregates from the lysosomal/acrosomal vesicle during rhesus spermiogenesis: structural alterations. *Dev Biol* 2000;219:334–349. doi: 10.1006/dbio.2000.9606
2. Gatimel N, Moreau J, Parinaud J, et al: Sperm morphology: assessment, pathophysiology, clinical relevance, and state of the art in 2017. *Andrology* 2017;5:845–862. doi: 10.1111/andr.12389
3. Kasimanickam R, Tibary A, Kastelic J: Fundamentals of bull selection. *Clin Theriogenol* 2013;5:97–107.
4. Butler ML, Bormann JM, Weaver RL, et al: Selection for bull fertility: a review. *Transl Anim Sci* 2019;4:423–441. doi: 10.1093/tas/txz174
5. Koziol JH, Armstrong CL: Manual for breeding soundness examination of bulls. 2<sup>nd</sup> edition, Montgomery, AL, USA; Society for Theriogenology: 2018.
6. Barth AD, Oko RJ: Abnormal morphology of bovine spermatozoa. 1<sup>st</sup> edition, Ames, Iowa, USA; Iowa State University Press: 1989:285.
7. Vishwanath R, Moreno JF: Review: semen sexing – current state of the art with emphasis on bovine species. *Animal* 2018;12:s85–s96. doi: 10.1017/S1751731118000496.

8. Morgentaler A, Fung MY, Harris DH, et al: Sperm morphology and in vitro fertilization outcome: a direct comparison of World Health Organization and strict criteria methodologies. *Fertil Steril* 1995;64:1177–1182. doi: 10.1016/s0015-0282(16)57981-3
9. Kandel ME, Rubessa M, He YR: Reproductive outcomes predicted by phase imaging with computational specificity of spermatozoon ultrastructure. *Proc Natl Acad Sci USA* 2020;117:18302–18309. doi: 10.1073/pnas.2001754117
10. Eliasson R: Semen analysis with regard to sperm number, sperm morphology and functional aspects. *Asian J Androl* 2010;12:26–32. doi: 10.1038/aja.2008.58
11. Al-Makhzoomi A, Lundeheim N, Håård M, et al: Sperm morphology and fertility of progeny-tested AI dairy bulls in Sweden. *Theriogenology* 2008;70:682–691. doi: 10.1016/j.theriogenology.2008.04.049
12. Nagy S, Johannisson A, Wahlsten T, et al: Sperm chromatin structure and sperm morphology: their association with fertility in AI-dairy Ayrshire sires. *Theriogenology* 2013;79:1153–1161. doi: 10.1016/j.theriogenology.2013.02.011
13. Holroyd RG, Doogan W, De Faveri J, et al: Bull selection and use in northern Australia. 4. Calf output and predictors of fertility of bulls in multiple-sire herds. *Anim Reprod Sci* 2002;71:67–79. doi: 10.1016/S0378-4320(02)00026-X
14. Auger J: Assessing human sperm morphology: top models, underdogs or biometrics? *Asian J Androl* 2010;12:36–46. doi: 10.1038/aja.2009.8
15. Gatimel N, Mansoux L, Moreau J, et al: Continued existence of significant disparities in the technical practices of sperm morphology assessment and the clinical implications: results of a French questionnaire. *Fertil Steril* 2017;107:365.e3–372.e3. doi: 10.1016/j.fertnstert.2016.10.038
16. Brito LFC: A multilaboratory study on the variability of bovine semen analysis. *Theriogenology* 2016;85:254–266. doi: 10.1016/j.theriogenology.2015.05.027
17. Freneau GE, Chenoweth PJ, Ellis R, et al: Sperm morphology of beef bulls evaluated by two different methods. *Anim Reprod Sci* 2010;118:176–181. doi: 10.1016/j.anireprosci.2009.08.015
18. Brito LF, Greene LM, Kelleman A, et al: Effect of method and clinician on stallion sperm morphology evaluation. *Theriogenology* 2011;76:745–750. doi: 10.1016/j.theriogenology.2011.04.007
19. Hook KA, Fisher HS: Methodological considerations for examining the relationship between sperm morphology and motility. *Mol Reprod Dev* 2020;87:633–649. doi: 10.1002/mrd.23346
20. Celeghini ECC, Arruda RP, Albuquerque R, et al: Utilization of fluorescent probe association for simultaneous assessment of plasmatic, acrosomal, and mitochondrial membranes of rooster spermatozoa. *Braz J Poultry Sci* 2007;9:143–149. doi: 10.1590/S1516-635X2007000300001
21. Angrimani DSR, Bicudo LC, Luceno NL, et al: A triple stain method in conjunction with an in-depth screening of cryopreservation effects on post-thaw sperm in dogs. *Cryobiology* 2022;105:56–62. doi: 10.1016/j.cryobiol.2021.12.001
22. Oliveira BM, Arruda RP, Thomé HE, et al: Fertility and uterine hemodynamic in cows after artificial insemination with semen assessed by fluorescent probes. *Theriogenology* 2014;82:767–772. doi: 10.1016/j.theriogenology.2014.06.007
23. Lin LI: A concordance correlation coefficient to evaluate reproducibility. *Biometrics* 1989;45:255–268.
24. Altman DG, Altman E: *Practical statistics for medical research*. 1st ed. Boca Raton, FL, USA: Chapman & Hall/CRC; 1999.
25. Hatamoto-Zervoudakis LK, Duarte Júnior MF, Zervoudakis JT, et al: Free gossypol supplementation frequency and reproductive toxicity in young bulls. *Theriogenology* 2018;110:153–157. doi: 10.1016/j.theriogenology.2018.01.003
26. Komsky-Elbaz A, Kalo D, Roth Z: Effect of aflatoxin B1 on bovine spermatozoa's proteome and embryo's transcriptome. *Reproduction* 2020;160:709–723. doi: 10.1530/REP-20-0286
27. Silva EFSJD, Missio D, Martinez CS, et al: Mercury at environmental relevant levels affects spermatozoa function and fertility capacity in bovine sperm. *J Toxicol Environ Health A* 2019;82:268–278. doi: 10.1080/15287394.2019.1589608
28. van den Hoven L, Hendriks JC, Verbeet JG, et al: Status of sperm morphology assessment: an evaluation of methodology and clinical value. *Fertil Steril* 2015;103:53–58. doi: 10.1016/j.fertnstert.2014.09.036.
29. Natali I, Muratori M, Sarli V, et al: Scoring human sperm morphology using Testsimplets and Diff-Quik slides. *Fertil Steril* 2013;99:1227.e2–1232.e2. doi: 10.1016/j.fertnstert.2012.11.047.
30. Henkel R, Schreiber G, Sturmhoefel A, et al: Comparison of three staining methods for the morphological evaluation of human spermatozoa. *Fertil Steril* 2008;89:449–455. doi: 10.1016/j.fertnstert.2007.03.027
31. Davis RO, Gravance CG: Standardization of specimen preparation, staining, and sampling methods improves automated sperm-head morphometry analysis. *Fertil Steril* 1993;59:412–417. doi: 10.1016/s0015-0282(16)55686-6.
32. Mallidis C, Cooper TG, Hellenkemper B, et al: Ten years' experience with an external quality control program for semen analysis. *Fertil Steril* 2012;98:611.e4–616.e4. doi: 10.1016/j.fertnstert.2012.05.006
33. Menkveld R: Sperm morphology assessment using strict (tygerberg) criteria. *Methods Mol Biol* 2013;927:39–50. doi: 10.1007/978-1-62703-038-0\_5
34. Eustache F, Auger J: Inter-individual variability in the morphological assessment of human sperm: effect of the level of experience and the use of standard methods. *Hum Reprod* 2003;18:1018–1022. doi: 10.1093/humrep/deg197
35. Barthelemy C, Royere D, Hammah S, et al: Ultrastructural changes in membranes and acrosome of human sperm during cryopreservation. *Arch Androl* 1990;25:29–40. doi: 10.3109/01485019008987591
36. Aalseth EP, Saacke RG: Morphological change of the acrosome on motile bovine spermatozoa due to storage at 4 degrees C. *J Reprod Fertil* 1985;74:473–478. doi: 10.1530/jrf.0.0740473
37. Kasimanickam R, Kasimanickam V, Pelzer KD, et al: Effect of breed and sperm concentration on the changes in structural, functional and motility parameters of ram-lamb spermatozoa during storage at 4 degrees C. *Anim Reprod Sci* 2007;101:60–73. doi: 10.1016/j.anireprosci.2006.09.001
38. Graham JK, Kunze E, Hammerstedt RH: Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. *Biol Reprod* 1990;43:55–64. doi: 10.1095/biolreprod43.1.55.



39. Peña AI, Quintela LA, Herradon PG: Flow cytometric assessment of acrosomal status and viability of dog spermatozoa. *Reprod Dom Anim* 1999;34:495–502.
40. Pintado B, de la Fuente J, Roldan ER: Permeability of boar and bull spermatozoa to the nucleic acid stains propidium iodide or Hoechst 33258, or to eosin: accuracy in the assessment of cell viability. *J Reprod Fertil* 2000;118:145–152.
41. Cummins JM, Fleming AD, Crozet N, et al: Labelling of living mammalian spermatozoa with the fluorescent thiol alkylating agent, monobromobimane (MB): immobilization upon exposure to ultraviolet light and analysis of acrosomal status. *J Exp Zool* 1986;237:375–382. doi: 10.1002/jez.1402370310
42. Tesarik J, Mendoza C: Alleviation of acrosome reaction prematurity by sperm treatment with egg yolk. *Fertil Steril* 1995;63:153–157. doi: 10.1016/s0015-0282(16)57311-7
43. Esteves SC, Sharma RK, Thomas AJ Jr, et al: Effect of in vitro incubation on spontaneous acrosome reaction in fresh and cryopreserved human spermatozoa. *Int J Fertil Womens Med* 1998;43:235–242.
44. Valle RR, Valle CM, Nichi M, et al: Validation of non-fluorescent methods to reliably detect acrosomal and plasma membrane integrity of common marmoset (*Callithrix jacchus*) sperm. *Theriogenology* 2008;70:115–120. doi: 10.1016/j.theriogenology.2008.03.011
45. Bruinjé TC, Ponce-Barajas P, A. Dourey A, et al: Morphology, membrane integrity, and mitochondrial function in sperm of crossbred beef bulls selected for residual feed intake. *Can J Anim Sci* 2019;99:456–464. doi: 10.1139/cjas-2018-0103
46. Veeramachaneni R: Spermatozoal morphology. In: Mckinnon AO, Squires EL, Vaala WE, et al: editors. *Equine reproduction*. 2<sup>nd</sup> edition, Oxford; Wiley: 2011:1297–1307.
47. Koppers AJ, De Iuliis GN, Finnie JM, et al: Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J Clin Endocrinol Metab* 2008; 93:3199–3207. doi: 10.1210/jc.2007-2616
48. Amaral A, Lourenço B, Marques M, et al: Mitochondria functionality and sperm quality. *Reproduction* 2013;146:R163–R174. doi: 10.1530/REP-13-0178