Technique Report

Interaction of semen smear preparation method and slide region evaluated affects the probability of detecting morphologically normal sperm

Shelby Noel, Shaun Huser, Todd Gunderson
Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA

Abstract

Bull breeding soundness examination (BSE) is important in identifying subfertile bulls, with sperm morphology being a key determinant. It is recommended that eosin-nigrosin stained semen smears are used for sperm morphology assessments. These smears are often made on glass slides with either another slide or a wood applicator. However, evaluation within only 1 smear region may not account for variations across regions that may be influenced by smear method. This may affect sperm evaluation and BSE classification, depending on smear region evaluated. Objective of our study was to determine if normal sperm morphology percentage varies across semen smear and whether smearing method modifies such variation. Coverslipped, stained semen smears were divided into 3 equal zones. Each zone was evaluated independently by experienced evaluators, with nontarget sections masked and each zone assigned a unique identifier to ensure blinding. Of 124 slides assessed, 33 (27%) contained at least 1 zone having a different classification category using a cutoff of 70% morphologically normal sperm. A linear mixed model with percentage of normal sperm as the outcome identified that sperm normality probability decreased from smear initiation site to smear end, with more pronounced effect ($p < 0.001$) for smears made with wood applicators. Decompositional models were applied to determine the probability of independent defect categories contributing to decreases in normal sperm morphology; most was from midpiece defects. These findings highlighted potential sources of variation in sperm morphology assessment and a need for standardized smear preparation methods.

Keywords: Bulls, breeding soundness examination, variation, sperm morphology

Introduction

Bull breeding soundness examination (BSE) is a widely used tool for identifying subfertile bulls, with sperm morphology serving as a key determinant of reproductive potential.[1–6] Identifying subfertile bulls is important for beef cattle operations to maximize production and profits.[7,8] Society for Theriogenology recommends that eosin-nigrosin stained semen smeared onto slides are used for sperm morphological assessments and evaluated at 1000X magnification.[9] Percentage of morphologically normal sperm is typically determined by assessing 100-200 sperm.[9,10] However, if sperm are assessed only within 1 smear region, variations across regions in proportion of morphologically normal sperm may not be accounted. Furthermore, studies have identified the possibility that staining and smearing methods create tertiary (i.e. iatrogenic) sperm defects. Prevalence of detached sperm heads increased significantly in stained dry smears compared to wet preparations; differences in acrosomal defects and proximal droplets relative to preparation methods were also observed.[11] A more recent study observed differences in major and minor defects (including acrosomes, midpiece, tail and droplet defects) among semen smears observed with bright-field microscopy versus wet mounts observed with differential interference contrast microscopy.[12]

Heterogenous variations in sperm percent that are abnormal across various regions of a stained semen smear could result in misclassification and compromise BSE's predictive value. This type of variation could also account for some discrepancies in interevaluator agreement. Cohen's Kappa coefficients among 3 board certified theriogenologists evaluating same stained bull semen smear slides ranged from 0.32-0.64.[10] Although differences in how each evaluator subjectively classified individual sperm may have accounted for some observed disagreement, variations in morphology among various slide regions may have affected interevaluator agreement.

CONTACT Todd Gunderson **tggundy@ksu.edu**
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Our objective was to determine whether sperm morphology percentages vary significantly across various regions of stained semen smears made by 2 methods, and whether slide preparation method affects variation across regions. Additionally, we evaluated how often this variation is large enough to impact a bull's BSE classification (i.e. satisfactory versus unsatisfactory). We hypothesized that sperm morphology percentages vary depending on slide region examined and by slide preparation method.

Materials and methods

Slides with stained bull semen smears evaluated in this study were collected for a separate study at the US Meat Animal Research Center in Clay Center, Nebraska according to USMARC Institutional Animal Care outline number 154.1.[13] Slides were selected for analysis if they were coverslipped, contained ≥ 4 cm of legible smear and were legible in all 3 predefined zones. To determine these zones, total length of each smear was measured on each individual slide (from where stained sample began to end, whether smear ended before or at slide end). This length was then divided into 3 equal portions and designated as Zones 1, 2, or 3 (Zone 1: near smear initiation site; Zone 2: mid region; Zone 3: distal region). This individual approach ensured that stained area of each zone was similarly proportionate to total stained area. Two slide smear preparation techniques were evaluated: stick method, where semen was transferred to a glass microscope slide using a clean wooden applicator stick, mixed with eosin nigrosin stain (JorVet J0326S Dead/Live Semen Stain, Jorgensen Laboratories, Loveland, CO, USA) on the slide, then spread to create a smear; pipette method, where a drop of semen was placed on the slide via pipette, mixed with stain and smeared across the slide with another glass slide. After stained smears were dry, they were coverslipped using a xylene based mountant (CoverSeal™-X, Cancer Diagnostics, Durham, NC, USA). Stick method smears were coverslipped immediately after drying whereas pipette method smears were coverslipped 2-8 hours later. Attempts were made to identify semen samples with slides that were made using both methods, though this was not a necessary criterion for inclusion.

Two veterinarians with at least 10 years of BSE experience evaluated semen morphology; they evaluated different sets of slides and therefore interevaluator agreement was not assessed. Prior to allocation, slides were stratified by preparation method to ensure that each evaluator examined roughly equivalent numbers of slides made with each method. Slides were then randomly allocated to each veterinarian to be evaluated in 3 rounds (1 for each zone). Prior to evaluation, masking tape was used to cover original slide identification and the 2 zones not scheduled for evaluation during the corresponding round. For each zone, counts of morphologically normal and abnormal sperm out of 200 were recorded. Both evaluators classified defects as follows:

1. Head defects (excluding detached heads and acrosomal defects)
2. Midpiece defects (excluding distal midpiece defects and proximal droplets)
3. Tail defects
4. Detached heads
5. Acrosomal defects
6. Distal midpiece defects (DMR)
7. Proximal droplets
8. Other (defects that did not fit well in above categories)

All statistical models were run in R using glmer() function (v4.4.1, R Foundation for Statistical Computing, Vienna, Austria). For the primary objective, a generalized linear mixed-effects model was fit, modeling the probability of sperm normality with fixed effects for slide preparation method and zone, interaction of these effects, and a random intercept for slide to account for within-slide variation. Random effect of evaluator did not contribute significantly to model variance and was not significant as a fixed effect; hence, it was excluded from final model. P-values for pairwise comparisons of modeled results were adjusted using Sidak adjustment. An α of 0.05 was set a priori and used for all statistical tests.

Because statistical model included random intercepts, 2 fixed effects and an interaction term, sample size calculations using a deterministic function were not feasible. To get an estimate for the approximate number of slides needed to detect meaningful differences in sperm morphology assessment, a simulated dataset was created that assumed a 3% difference in normal sperm percentage between at least 2 zones on a slide. Simulated morphology counts were randomly selected from normal distributions centered on means from previously recorded assessments. Normal distributions standard deviation ranged 1-6% depending on slide preparation method and evaluator. Analysis of simulated data identified that counting 200 sperm per zone on 100 slides would yield statistically significant results if an α of 0.05 was set a priori and used for all statistical tests.

Separate generalized linear mixed-effects decomposition models were fit for each category of abnormal sperm to model the probability of a specific abnormality category within abnormal count, with slide preparation method and zone as fixed effects. These models also evaluated the interaction of these effects and had a random intercept for slide. Linear trends for each defect across zones and by preparation method were estimated. With an exception, analyses that evaluated percent contribution of each category of sperm abnormality to overall changes in probability of abnormal sperm, only evaluated mutually exclusive categories. An exception was a decompositional model evaluating the probability of observing any midpiece defect by combining midpiece category with DMR category.

Results

Of 124 slides evaluated, 91 (73%) were prepared by pipette method and 33 (27%) were prepared by stick method. Smear lengths ranged 4.0-5.5 cm with an average length of 4.8 cm; therefore, average zone length evaluated was 1.6 cm. Evaluator A evaluated 45 slides prepared by pipette method and 19 prepared by stick method. Evaluator B evaluated 46 slides prepared by pipette method and 14 prepared by stick method. There were 12 bulls with semen samples that were smeared by both methods (24 slides total).

Twenty-seven percent (33 of 124) of slides contained at least 1 zone that would have resulted in a different BSE classification than other zones of the same slide using a cutoff of 70% morphologically normal sperm to be classified as satisfactory. Largest discrepancy in crude normal morphology percentage observed was 43% (Figure 1).

Statistical modelling of sperm normality revealed an interaction effect ($p < 0.001$) between slide preparation method and zone (Figure 2). There was also a main effect ($p < 0.001$)

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Slide	Z1/Z2	Z1/Z3	Z2/Z3	Slide	Z1/Z2	Z1/Z3	Z2/Z3
1	11	31	20	63	11.5	3	8.5
2	6.5	13.5	7.0	64	7.5	16	23.5
3	24	8	16	**65**	3.5	16.5	13
4	0.5	32	32.5	66	3	4	1
5	0.5	12.5	12	**67**	23.5	34.5	11
6	14.5	12.5	2	68	12	24	12
7	5	16	11	69	7.5	14.0	6.5
8	9.5	24.5	15	**70**	19	8.5	10.5
9	4	10	6	71	11.5	6	17.5
10	10	10.5	20.5	**72**	14.5	6	20.5
11	6	7.5	13.5	**73**	3.5	43	39.5
12	15.5	24	8.5	74	0.5	0.5	1
13	10.5	4.5	6	75	5	0.5	4.5
14	12.5	11	1.5	76	0.5	1	1.5
15	4	11.5	7.5	77	2	3.5	1.5
16	3	0.5	3.5	78	19.5	26	6.5
17	2.5	2	0.5	**79**	4	5	1
18	5	13	18	80	10.5	4	6.5
19	6.5	12	5.5	**81**	3	24	27
20	16.5	17	0.5	**82**	17	3.5	13.5
21	2	8.5	10.5	83	3.5	6.5	3
22	5	2.5	2.5	**84**	5.5	24	18.5
23	7.5	0.5	8	85	6	9.5	3.5
24	12.5	11	1.5	86	6	9	3
25	10	4.5	5.5	87	4.5	7.5	12
26	5	16	21	88	3.5	11.5	8
27	5	3	8	**89**	22.5	16.5	6
28	12.4	9.4	3	90	12.5	2	14.5
29	4.5	9	4.5	91	4.5	4	0.5
30	5.5	2	7.5	**92**	2.5	1	3.5
31	3.5	1.5	5	**93**	11.5	3	14.5
32	2	1	1	94	5	4.5	0.5
33	5.5	13	7.5	95	2	2	0
34	2.5	0	2.5	**96**	4.5	6	10.5
35	5.5	1	4.5	**97**	26	9.5	16.5
36	1	0.5	1.5	98	3.5	10	6.5
37	3	21	24	99	8	3	11
38	5.5	7.0	1.5	100	8	8.5	16.5
39	14.5	25.5	11	101	2.5	2	4.5
40	11	4.6	6.4	102	1	2	3
41	1.5	0.5	1	103	0	3.5	3.5
42	12	18	6	**104**	7.0	6.5	13.5
43	9	7.5	1.5	105	5	7.0	2
44	1	3.4	2.4	106	4	4.5	8.5
45	8	5.5	2.5	107	6.5	8	1.5
46	1	1.5	2.5	108	0.5	2.5	3
47	2.5	15.5	13	109	5	7.5	12.5
48	1	0	1	110	1	4	3
49	1	1.5	0.5	111	1	0.5	0.5
50	20	13.5	6.5	112	5.5	2.5	8
51	0.5	2.5	3	113	11	6.5	4.5
52	0.5	6	5.5	114	1.5	0	1.5
53	1	23	22	**115**	14.0	4.5	9.5
54	2	6.5	8.5	**116**	19	14.5	4.5
55	8	19.5	11.5	117	13	7.0	6
56	1.5	0.7	2.2	**118**	0	9.5	9.5
57	5	8	3	119	19.5	24	4.5
58	1.5	25.5	24	120	7.5	0.5	8
59	5	9	4	121	2.5	3	0.5
60	5	8.3	3.3	122	2.5	7.0	4.5
61	9.5	10	0.5	**123**	5	3.5	1.5
62	5	14.5	9.5	124	1.1	8.4	9.5

Difference (%)
30
20
10
5
0

Figure 1. A heat map of differences in percentage of normal sperm across 3 zones of each slide. Darker colors represent larger differences. Bold slide numbers indicate the classification category discrepancy of pass or fail among zones within same slide, based on a cutoff of 70% normal sperm

observed for zone, though no effect (p = 0.07) was detected for slide preparation method. Probability of sperm normality decreased as zones progressed from smear initiation site to distal zones (i.e. in the direction the smear was made) and was more pronounced for stick method.

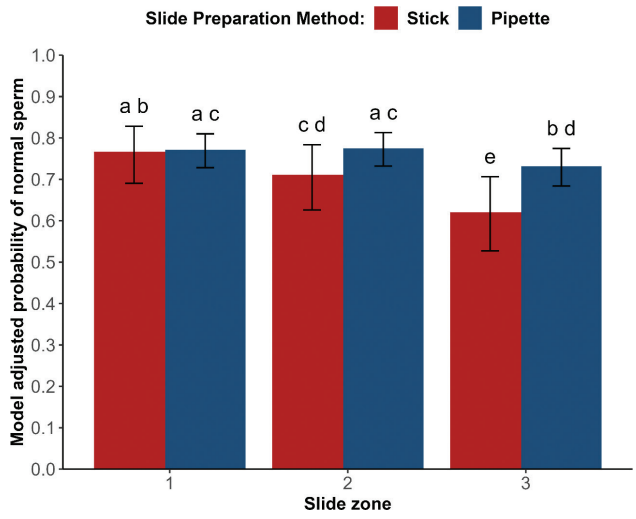


Figure 2. Predicted probability of morphologically normal sperm by slide smear zone and preparation method. Bar heights represent model-adjusted probabilities (error bars represent 95% confidence intervals) of observing normal sperm in Zones 1 (near inoculation site), 2 (mid region) and 3 (distal region). Bars are grouped by slide preparation method. Bars with different superscripts represent significantly different probabilities after a Sidak correction for multiple comparisons

Decomposition modelling of the probability of abnormal sperm category within total number of abnormal sperm identified midpiece defects (including DMRs) accounting for most (~ 50%) of the differences in abnormal sperm from Zones 1-3 between stick and pipette methods (Table 1). Defects for which method modified the probability of detection across zones were all midpiece defects ($p = 0.03$), tail defects ($p < 0.001$) and others ($p = 0.01$). Although there was a main effect of zone on head defects ($p < 0.001$), detached heads ($p < 0.001$) and proximal droplets ($p = 0.04$), interactions of zone and method was not significant for these defects (Table 2).

Discussion

Our findings demonstrated that sperm morphology assessments can be influenced by smear region, and that preparation method can modify this effect. It is unclear why these effects were particularly pronounced for some defects. It is possible that sperm midpiece abnormalities may make them more prone to attach to the smearing device, resulting in these defective sperm being more likely to be dragged to the distal smear end. This effect might be particularly pronounced when a wooden applicator is used, because wood fibers may be more likely to gain purchase on defective midpieces. This rationale may also explain why detached heads were more likely to be observed in proximal zones. If smearing device is more likely to gain purchase on midpiece, and connection between midpiece and head is weak, then detached head would be left behind as midpiece is dragged toward the distal end. Regardless, there are insufficient data in this study to test these hypotheses further; authors are unaware of literature that confirms whether defective midpieces are more likely to attach to smearing devices. Further research is needed to elucidate mechanisms behind observed effects.

It is less clear why head defects on fully intact sperm would be more likely to be observed distally. One possible

Table 1. Overall contribution of each defect to the difference in probability of abnormal sperm across zones slide between stick and pipette methods; each reported value indicates the percentage of each abnormality's contribution to the total difference between methods

Abnormality	Percent of contribution to all variation in abnormal sperm
Head (Detached & acrosome excluded)	8.9
Detached Head	24.1
Acrosome	1.1
Midpiece (DMR excluded)	31.2
DMR	21.4
Proximal droplet	5.6
Tail	6.9
Other	0.9

explanation would be sperm with defective heads being more buoyant than normal sperm and therefore riding higher in the wave of stained semen as it is smeared across the slide. Although this could theoretically be true, density of bull sperm may vary based on several factors including stain osmolarity and sperm viability.[14] Therefore, these rationale remain largely speculative.

Another factor that may have contributed to variation in morphology assessment across the slide was the number of sperm per high power field. In semen samples that were highly concentrated, excessive numbers of sperm in the viewing field may have resulted in some abnormalities being obscured or difficult to observe. If concentration of semen decreased as the smear was drawn out, this may have led to more defects observed in distal zones. Notwithstanding, including the effect of individual slide as a random variable in the statistical models should have accounted for the variation in morphology across zones that was dependent on individual slide characteristics.

These findings highlighted the importance of standardized techniques in semen analysis to ensure accurate and

Table 2. Comparison of linear trends across zones of a stained bull semen smear in the odds of normal sperm. The odds ratios (OR) represent the linear relative change in the odds of identifying a given defect among abnormal sperm across zones of the slide

Defect	Effect	LogOR	Standard Error	OR	p-value
Head	stick method	0.608	0.091	1.84	< 0.001
	pipette method	0.469	0.054	1.6	< 0.001
	zone x method interaction				0.3
Detached Head	stick method	–0.906	0.087	0.4	< 0.001
	pipette method	–0.805	0.053	0.45	< 0.001
	zone x method interaction				0.3
Acrosome	stick method	–0.191	0.311	0.83	0.5
	pipette method	0.168	0.221	1.18	0.5
	zone x method interaction				0.3
Midpiece (excluding DMR)	stick method	1.04	0.087	2.83	< 0.001
	pipette method	0.379	0.052	1.46	< 0.001
	zone x method interaction				< 0.001
DMR	stick method	–0.789	0.118	0.45	< 0.001
	pipette method	0.083	0.074	1.09	0.3
	zone x method interaction				< 0.001
Proximal droplet	stick method	–0.245	0.117	0.78	0.04
	pipette method	–0.222	0.077	0.8	0.004
	zone x method interaction				0.9
All Midpiece	stick method	0.554	0.078	1.74	< 0.001
	pipette method	0.359	0.048	1.43	< 0.001
	zone x method interaction				0.03
Tail	stick method	–0.805	0.258	0.45	0.002
	pipette method	0.227	0.123	1.26	0.06
	zone x method interaction				< 0.001
Other	stick method	–1.44	0.326	0.24	< 0.001
	pipette method	–0.447	0.221	0.64	0.04
	zone x method interaction				0.01

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reliable results. Survey data from practitioners in southeastern US had gross inconsistencies in the methods used to evaluate sperm morphology.[15] Even when methods are relatively uniform, differences in interevaluator agreement may also produce discrepancies in results.[10,16] A possible solution would be increased use of automated semen analysis and flow cytometry. Computer assisted semen analysis platforms have been commercially available for several years.[17] However, their use in BSE has traditionally been limited by cost and laboratory/technician requirements.[18] Newer, less-expensive models designed for chute-side have been produced, though they currently only provide detailed analysis on density and motility, but not accurate assessments of morphology.[19–22] Notwithstanding, advances in machine-learning and computer vision may result in algorithms capable of accurately assessing sperm morphology in a high throughput manner, thereby having standardized and objective spermiogram analysis with higher statistical power for quantifying sperm normality.[23,24] In scenarios where only manual methods are available, guidelines on which/how many regions of the smear to evaluate may also decrease test variability. Wet fixation of semen with neutral buffered formalin and evaluation with phase microscopy may also bypass potential issues associated with evaluating stained semen smears.

Although this study provided a detailed examination of various zones' impact and preparation methods on sperm morphology percentages, it is not without limitations. Bulls evaluated may not fully represent bull populations' diversity and semen smears may not be representative of smears made by other practitioners. Additionally, samples were not collected specifically with this study's objectives in mind and therefore data should be considered observational rather than experimental.

Future research should include a broader range of samples and consider other factors (e.g. sperm concentration, stain quality) that may influence sperm morphology assessments and explore underlying mechanisms that contribute to observed variations.

Conclusion

Differences in sperm morphology assessment among regions of a stained semen smear slide may affect a bull's morphology classification; specific region of slide evaluated affected morphology percentage, with distal region having a lower normal morphology percentage. This effect was modified by the staining/smearing method, with stick method resulting in a more marked decrease in normal morphology percentage moving proximally to distally. Furthermore, midpiece defects appeared to have contributed most to the decreasing normal morphology associated with interaction of slide region and smearing method.

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Conflict of interest

Authors have no conflicts of interest to disclose.

Authors' contributions and declaration

SN, **SH**, **TG** contributed to the experiment design and methods, implemented experiments and collected data, interpreted analyzed data; **SN** drafted the initial manuscript; and **TG** edited the draft. All authors have read and approved the final version of the manuscript.

Statement on the use of generative large language models and other artificial intelligence

Generative large language models (e.g. Chat GPT, OpenAI, San Francisco, California; Notebook LM, Alphabet, Mountain View, California) were used for the development of code for data analyses, to evaluate and summarize reference material and to assist with manuscript preparation (e.g. editing text for conciseness and clarity). Notwithstanding, the investigators take full responsibility and ownership for study design, analysis and interpretation of results.

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