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Effect of method and clinician on stallion sperm morphology evaluation

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Abstract

The objective of this study was to determine the effects of method and clinician on stallion sperm morphology evaluation. Five clinicians evaluated 60 semen samples using wet-mount preparations with phase-contrast, eosin/nigrosin-stained semen smears, and Papanicolaou-stained semen smears. There were significant differences among methods for all sperm morphology categories and most intra-class correlation coefficients were only fair to moderate. The use of wet-mount preparations facilitated detection of acrosome defects, nuclear vacuoles, and cytoplasmic droplets when compared to stained smears. Smearing stallion semen samples onto slides increased the proportion of detached sperm heads. In addition, acrosome defects, nuclear vacuoles, rough/swollen midpieces, and cytoplasmic droplets were difficult to observe with Papanicolaou stain; this method resulted in overestimation of normal sperm when compared to other methods. There were significant differences among clinicians for all sperm morphology classification categories. In conclusion, this study demonstrated that sperm morphology evaluation results varied, depending on the evaluation method and clinician. Wet-mount preparation with phase-contrast microscopy appeared to be more sensitive for identification of abnormal stallion sperm when compared to stained smears. Veterinary andrology laboratories should invest in training, continuing education, proficiency testing, and other quality control measures to minimize the variation of sperm morphology evaluation results among clinicians.
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1. Introduction

Sperm morphology evaluation is an essential component of semen analysis and provides the clinician invaluable information for assessing the breeding soundness of a stallion and the potential fertility of individual semen samples. Although the Society for Theriogenology (SFT) recommends the use of wet-mount semen preparations and phase-contrast or differential interference contrast microscopy for evaluation of sperm morphology in stallions [1], comparisons among different methods have apparently not been reported. Eosin/nigrosin is a stain recommended by the SFT for evaluation of bull sperm morphology [2] and is widely used, mainly because of its ease of use. In contrast, the World Health Organization (WHO) recommends the use of Papanicolaou stain for evaluation of human sperm morphology [3].

Several studies from laboratories evaluating human sperm described variations in sperm morphology results that could be attributed to technician differences [4–7]. One of the main reasons for this variation was likely the

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lack of quality control measures, including appropriate training, continuing education, and proficiency testing. Although no similar studies have been reported, the same issues are expected to affect sperm morphology assessment in veterinary andrology laboratories.

The objectives of the present study were to determine the effect of method and clinician on stallion sperm morphology evaluation.

2. Materials and methods

2.1. Preparation of semen samples

Sixty semen samples from 34 stallions (1 to 4 samples per stallion) were evaluated in this study. Stallions represented 12 breeds, ranged from 3 to 23 y of age, and were part of the population presented to the New Bolton Center of the University of Pennsylvania School of Veterinary Medicine for breeding soundness evaluation, fertility problems, breeding management, or semen processing for shipment or freezing. As such, this diverse population represented a wide range of fertility, from normal and fertile to severely sub-fertile stallions. Most samples consisted of gel-free semen processed immediately after collection with an artificial vagina, but single samples from four stallions were evaluated after extension with skim milk-based extenders and cooling for approximately 24 h.

Sperm morphology was evaluated using: 1) wet-mount preparations of semen samples fixed in buffered-formol saline (BFS); 2) semen smears stained with eosin/nigrosin stain; and 3) semen smears stained with Papanicolaou stain. The same clinician prepared all the BFS-semen dilutions; each individual clinician was responsible only for preparing his or her own wet-mount for evaluation. Eosin/nigrosin and Papanicolaou smears were also all prepared and stained by the same clinician.

For wet-mount, semen samples were fixed in BFS (1:4 ratio; [8]) and approximately 5 μL of the sample was placed on a slide and covered with a coverslip. Eosin/nigrosin (sperm morphology stain; Lane Manufacturing Inc., Denver, CO, USA) semen smears were prepared by mixing a droplet of semen and a droplet of stain (approximately 15 μL of each) on a warm slide and spreading the sample with a wood applicator after quickly mixing it. The slide was immediately placed on a slide warmer at 37 °C and gently blown over in order to quickly dry the sample. Papanicolaou samples were prepared by staining semen smears (approximately 20 μL smeared on a clean slide with a wood applicator) as recommended by the WHO [3] (reagents from Sigma Chemical Co., St. Louis, MO, USA).

2.2. Evaluation of sperm morphology

Semen samples were coded to eliminate any identification of the stallion and date of the collection and were examined by five clinicians. Participating clinicians all had specialty training in theriogenology and had experience with sperm morphology evaluation in stallions; years of experience ranged from 1 to 19. Prior to the beginning of the study, the clinicians performed a review of sperm morphology classification criteria and elaborated a classification chart. The objective of this initiative was to minimize the variation of classification criteria among clinicians. Sperm morphology was evaluated under 1000X magnification and phase-contrast (wet-mounts) or bright field microscopy (stained semen smears). For wet-mount evaluation, sperm that were not properly oriented, i.e., that were not lying flat on the slide, were not examined. For each sample, 100 sperm were examined and classified.

Sperm were classified into nine main categories according to morphology: normal, acrosome defect, head defect, midpiece defect, principal piece defect, detached head, proximal cytoplasmic droplet, distal cytoplasmic droplet, and others. In addition, sperm head abnormalities were classified into abnormalities of shape and/or size (including, but not limited to pyriform, tapered, microcephalic, and macrocephalic) and nuclear vacuoles. Midpiece abnormalities were classified into bent and/or coiled, fractured (either at the neck, at the middle, or at the annulus), roughed and/or swollen, and others (segmental aplasia of the mitochondrial sheet and duplication). Other sperm abnormalities included duplication of the head and tail and teratoids. These abnormalities have been documented elsewhere [9]. All abnormalities on a single spermatozoon were recorded. The only exception was the presence of distal cytoplasmic droplets in sperm with distal midpiece reflex; in these cases, only the midpiece defect was recorded.

2.3. Statistical analyses

Statistical analyses were performed using the Statistix and SPSS software packages. Sperm morphology results were evaluated by ANOVA, using a Tukey test for post-hoc comparisons. The model included the main effects of sample, clinician, and method; sample-by-clinician-by-method was used as error term. Two-way random intra-class correlation coefficients (ICCs) of

Table 1
Mean (± SEM) stallion sperm morphology classification according to method. Each method included 60 samples each evaluated by five clinicians.

	Wet-mount	Eosin/nigrosin	Papanicolaou
Normal	36.7 ± 1.2[a]	39.3 ± 1.2[b]	50.3 ± 1.1[c]
Acrosome defects	5.5 ± 0.3[a]	5.5 ± 0.3[a]	3.1 ± 0.2[b]
Head defects	19.8 ± 0.9[a]	20.3 ± 0.8[a]	16.8 ± 0.8[b]
Abnormal head shape/size	14.0 ± 0.7[a]	15.9 ± 0.7[b]	15.6 ± 0.8[b]
Nuclear vacuoles	6.8 ± 0.6[a]	6.1 ± 0.4[a]	1.6 ± 0.2[b]
Midpiece defects	25.4 ± 1.0[a]	26.0 ± 0.9[a]	19.8 ± 0.8[b]
Bent/coiled midpiece	15.5 ± 0.7[a]	11.5 ± 0.5[b]	11.6 ± 0.6[b]
Rough/swollen midpiece	8.3 ± 0.5[a]	12.3 ± 0.6[b]	6.9 ± 0.4[c]
Other midpiece defects	1.3 ± 0.1[a]	2.3 ± 0.2[b]	1.3 ± 0.1[a]
Principal piece defects	2.2 ± 0.1[a]	2.7 ± 0.2[b]	1.7 ± 0.1[c]
Detached head	5.2 ± 0.5[a]	9.2 ± 0.7[b]	8.3 ± 0.5[b]
Proximal droplets	9.3 ± 0.5[a]	5.4 ± 0.3[b]	1.9 ± 0.1[c]
Distal droplets	8.6 ± 0.6[a]	5.4 ± 0.3[b]	4.0 ± 0.3[c]

[a–c] Within a row, means without a common superscript differed ($P < 0.05$).

agreement were calculated between results obtained with various methods.

3. Results

The proportion of sperm with "other sperm defects" (duplicate heads and tails, and teratoids) was very low (< 0.5%) and was excluded from the analysis. There were method effects ($P < 0.05$) on the proportions of all sperm morphology categories (Table 1). The proportion of normal sperm was greater ($P < 0.05$) and the proportions of acrosome, head, midpiece, and principal piece defects, and cytoplasmic droplets were less ($P < 0.05$) in Papanicolaou-stained samples when compared to wet-mount preparations and eosin/nigrosin-stained samples. The proportions of normal sperm, abnormal head shape/size, other midpiece defects, and principal piece defects were less ($P < 0.05$) and the proportions of rough/swollen midpiece and cytoplasmic droplets were greater ($P < 0.05$) in wet-mount preparations when compared to eosin/nigrosin-stained samples. The proportion of detached sperm heads was greater ($P < 0.05$) in stained samples (both eosin/nigrosin and Papanicolaou) than in wet-mount preparations.

Most intra-class correlation coefficients between sperm morphology categories classified by different methods were fair to moderate (Table 2); in general, correlation coefficients between Papanicolaou and wet-mount preparation or eosin/nigrosin were less than that between wet-mount preparation and eosin/nigrosin. There were good to very good correlations among all methods only for midpiece defects, bent/coiled midpieces, and detached heads. Furthermore, there were good to very good correlations between wet-mount preparation and eosin/nigrosin for normal sperm and

Table 2
Two-way random intra-class correlation coefficients of agreement for sperm morphology classification between evaluation methods.

	Eosin/nigrosin	Papanicolaou
Normal		
Wet-mount	0.70	0.54
Eosin/nigrosin	—	0.58
Acrosome defects		
Wet-mount	0.46	0.25
Eosin/nigrosin	—	0.27
Head defects		
Wet-mount	0.60	0.49
Eosin/nigrosin	—	0.58
Abnormal head shape/size		
Wet-mount	0.55	0.54
Eosin/nigrosin	—	0.64
Nuclear vacuoles		
Wet-mount	0.57	0.13
Eosin/nigrosin	—	0.21
Midpiece defects		
Wet-mount	0.76	0.62
Eosin/nigrosin	—	0.59
Bent/coiled midpiece		
Wet-mount	0.69	0.69
Eosin/nigrosin	—	0.77
Rough/swollen midpiece		
Wet-mount	0.55	0.35
Eosin/nigrosin	—	0.31
Other midpiece defects		
Wet-mount	0.21	0.24
Eosin/nigrosin	—	0.20
Principal piece defects		
Wet-mount	0.49	0.28
Eosin/nigrosin	—	0.25
Detached head		
Wet-mount	0.61	0.67
Eosin/nigrosin	—	0.78
Proximal cytoplasmic droplets		
Wet-mount	0.32	NS
Eosin/nigrosin	—	0.16
Distal cytoplasmic droplets		
Wet-mount	0.45	0.49
Eosin/nigrosin	—	0.50

NS, non-significant; all other coefficients were significant ($P < 0.005$).

Table 3
Mean (± SEM) stallion sperm morphology classification (%) according to clinician. Each clinician evaluated 60 samples using three methods (wet-mount with phase-contrast, eosin/nigrosin stain, and Papanicolaou stain).

	Clinician A	Clinician B	Clinician C	Clinician D	Clinician E
Normal	44.7 ± 1.6^{a,b}	37.7 ± 1.4^{c}	47.4 ± 1.5^{a}	37.5 ± 1.6^{c}	43.4 ± 1.4^{b}
Acrosome defects	6.0 ± 0.5^{a}	6.7 ± 0.4^{a}	1.4 ± 0.1^{c}	5.6 ± 0.4^{a}	3.8 ± 0.3^{b}
Head defects	20.7 ± 1.1^{b}	20.6 ± 1.0^{b}	19.4 ± 0.9^{b}	25.8 ± 1.3^{a}	8.1 ± 0.5^{c}
Abnormal head shape/size	12.8 ± 0.7^{c}	15.6 ± 0.9^{b}	18.0 ± 0.9^{b}	22.1 ± 1.2^{a}	7.0 ± 0.5^{d}
Nuclear vacuoles	10.9 ± 0.8^{a}	7.0 ± 0.5^{b}	1.4 ± 0.2^{d}	3.6 ± 0.4^{c}	1.1 ± 0.1^{d}
Midpiece defects	22.1 ± 1.1^{b}	30.6 ± 1.3^{a}	19.0 ± 1.0^{c}	22.4 ± 1.1^{b}	24.4 ± 1.1^{b}
Bent/coiled midpiece	12.4 ± 0.8^{b}	17.2 ± 0.9^{a}	11.0 ± 0.7^{b}	11.4 ± 0.7^{b}	12.1 ± 0.8^{b}
Rough/swollen midpiece	8.0 ± 0.7^{c}	13.0 ± 0.9^{a}	6.7 ± 0.6^{c}	8.2 ± 0.5^{c}	10.1 ± 0.6^{b}
Other midpiece defects	1.8 ± 0.2^{a,b}	1.2 ± 0.1^{c}	1.3 ± 0.1^{b,c}	1.5 ± 0.1^{b,c}	2.2 ± 0.2^{a}
Principal piece defects	3.3 ± 0.2^{a}	2.5 ± 0.2^{b}	1.7 ± 0.1^{cd}	1.3 ± 0.1^{d}	2.1 ± 0.2^{b,c}
Detached head	7.5 ± 0.8^{a,b}	8.3 ± 0.9^{a}	7.9 ± 0.7^{a}	6.4 ± 0.6^{c}	7.8 ± 0.8^{a,b}
Proximal droplets	6.2 ± 0.4^{a}	4.5 ± 0.4^{c}	5.8 ± 0.6^{a,b}	6.2 ± 0.5^{a}	5.0 ± 0.4^{b,c}
Distal droplets	5.6 ± 0.6	5.8 ± 0.5	6.5 ± 0.6	6.3 ± 0.6	5.7 ± 0.5

$^{a-d}$ Within a row, means without a common superscript differed ($P < 0.05$).

head defects, and between Papanicolaou and eosin/nigrosin for abnormal head shape/size.

There were clinician effects ($P < 0.05$) on the proportion of all sperm morphology categories except distal cytoplasmic droplets (Table 3). There were substantial differences in the proportions of normal sperm (as much as 10%), and sperm head (as much as 17%) and midpiece defects (as much as 11%). Smaller differences were observed for other morphology categories.

4. Discussion

Differences in sperm morphology results among different evaluation methods can only be attributed to introduction of artifacts, poor resolution/definition of sperm structures, and unfamiliarity of the evaluator with the distinctive appearance of sperm processed by a particular method. Studies comparing methods of sperm morphology evaluation have either used only the proportion of normal sperm, which by itself is not a reliable endpoint from which to draw conclusions, or have made little effort to explain the possible causes of differences in the proportions of different sperm defects [10–13]. It is not logical to conclude that two methods are similar just because the proportion of sperm classified as normal are similar, when concurrently, the proportions of different defects are not; it is clear that the factors described above play a role in these cases. Moreover, correct classification and quantification of specific sperm defects can provide valuable information regarding potential fertility of a stallion or ejaculate, and help to formulate a diagnosis and prognosis for reproductive problems [9,14].

In the present study, specific sperm defects were enumerated and all defects in a single spermatozoon were recorded in an attempt to paint a comprehensive picture of differences among evaluation methods. The use of wet-mount preparations and phase-contrast facilitated the observation of acrosome defects, nuclear vacuoles, and cytoplasmic droplets, as demonstrated by the increased proportions of these defects when compared to stained smears; these results were similar to that of sperm morphology studies in bulls using phase-contrast or DIC [13,15]. Although some authors have suggested that increased proportion of sperm defects in wet-mount preparations might be the result of artifacts [11], it is very unlikely that the specific defects mentioned above would be artifactual. The use of wet-mount preparations seemed to reduce the introduction of some artifacts (detached sperm heads), but increased others (bent/coiled midpieces). Alternatively, although less likely, sperm with bent/coiled midpieces were more susceptible to head-tail detachment during smearing onto slides. This could have resulted in a decrease of bent/coiled midpieces concomitantly with the increase in detached heads in stained samples. Wet-mount preparation with phase-contrast microscopy appeared to be more sensitive for identification of abnormal stallion sperm when compared to stained smears; these results supported the SFT recommendations for stallion sperm morphology evaluation [1].

Some authors discouraged the use of the method because of the necessity of high-quality phase-contrast microscopes, lack of experience in using the method, and difficulty in storing samples [12]. However, equipping a microscope with phase-contrast is a straightfor-

ward and relatively inexpensive upgrade, experience with wet-mount preparations should be no more difficult to obtain than with other methods, and storing digital images could be a more efficient way to maintain records for re-evaluation and quality control than storing slides. In addition to the likely benefit of increased sensitivity for identification of abnormal sperm, processing wet-mount preparations is also simpler and quicker than preparing stained smears. One potential disadvantage of wet preparations is the observation of improperly oriented sperm, i.e., sperm that do not lie flat on the slide and therefore cannot be properly classified. This problem can be minimized by adjusting the volume of the semen droplet on the slide and the size of the coverslip, gently pressing down the coverslip and blotting dry any excess semen from the borders, and letting the sample settle for a few minutes before the examination. Adjusting the osmolarity of the fixing solution to approximately 300 mOsm is also recommended to avoid wet-mount artifacts associated with bent/coiled tails.

In the present study, smears were prepared with a wood applicator as described for bull semen [16]; this method was similar to the "pipette method" described for preparation of human smears [3]. The results obtained with stallions indicated that smearing semen samples on the slide increased the proportion of detached sperm heads when compared to wet-mounts. Studies in bulls also report an increase in the proportion of detached sperm heads and broken midpieces when eosin/nigrosin-stained smears where compared to wet-mount preparations [13,15], but the same was not observed in men [11] or dogs [17]. These discrepancies might represent species differences in the resilience of sperm, or might be related to different methods of preparing the smears; perhaps the "feathering method" [3] might eliminate or at least minimize the separation of sperm heads from the tails. The relatively low coefficients of agreement between wet-mount preparations and stained smears for most categories of sperm defects were also of concern and seemed to indicate that the appearance of specific defects in unstained and stained sperm varied considerably.

A common concern with eosin/nigrosin is the hypotonicity of the stain and the possibility of introduction of artifactual tail defects, e.g., bending and coiling. These types of artifacts have been observed in other studies [13,17], but the use of warm slides and stain combined with quick drying of the smear by blowing to minimize the time of contact of sperm with the hypotonic stain prevented any increase in bent and coiled sperm tails in the present study. Although the use of Papanicolaou-stained smears is the method recommended by the WHO for evaluation of human sperm [3], the results obtained with stallion sperm were extremely poor. Abnormalities of sperm head and size were readily identified, but more subtle defects, including acrosome defects and nuclear vacuoles were difficult to observe with this stain. Moreover, staining of the tail was generally light and contributed to the difficulty of observing rough/swollen midpieces, principal piece defects, and cytoplasmic droplets. Correlations of agreement of Papanicolaou-stained smears with the other two methods were generally the lowest. Finally, comments from the clinicians indicated that this stain was the most difficult to interpret, due to its pale color and the resulting difficulty in visualizing stained sperm clearly.

Reports from human andrology laboratories have demonstrated that sperm morphology results varied significantly not only among laboratories, but also within the same laboratory, with coefficients of variation among laboratories for the proportion of normal sperm in a semen sample ranging from 20 to 100% [4–7]. In the present study, there were significant differences among clinicians for all sperm morphology classification categories. This variation makes it difficult for clinicians to compare results and to diagnose problems that might be related to specific sperm defects. In addition, it might also hamper efforts to establish reference values and guidelines for satisfactory semen quality.

Evaluation of sperm morphology is considered the biggest technical challenge for the andrology laboratory [18]. Sperm morphology evaluation is subjective and results are largely dependent on the proficiency and experience of the evaluator. Unfortunately, more often than not, evaluators are trained incidentally, since well-defined training methods have not been developed. Although reference materials with detailed descriptions and comprehensive documentation of sperm morphology are available for some species [3,19], similar materials have not been available for stallions. Most of the older, classical literature contains only drawings and sperm morphology pictures can only be found scattered in book chapters and journal articles, but a recent publication has attempted to document stallion sperm morphology in samples stained with eosin-nigrosin [9]. Initiatives from human reproduction laboratories have demonstrated that differences among laboratories and technicians can be minimized with training, continuing education, and quality control measures including pro-

ficiency testing [20–22]; similar initiatives might also benefit veterinary andrology laboratories.

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