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Theriogenology 61 (2004) 691–703

# Determining sample size for the morphological assessment of sperm

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Received 12 March 2003; accepted 13 June 2003

**Abstract**

Morphologic assessment of spermatozoa is an integral component in the analysis of semen. Whether a technician rapidly screening semen quality at a commercial stud, a veterinarian performing breeding soundness examinations, a clinician at a reference andrology laboratory providing auditing or diagnostic services, or a researcher evaluating morphology as a part of a fertility study, it is important to make an informed decision regarding the number of spermatozoa to include in the morphology assessment. Application of basic statistical principles such as the nature of proportions, level of confidence in an observed value, and the interaction of sample size with precision, can and should be used in the decision process. This paper outlines in detail the application of these statistical principles in relation to the morphologic assessment of spermatozoa. Guidelines on how these principles can be utilized in practical situations are discussed. Additionally, methodologies for comparison of results within and between laboratories (an area easily prone to misinterpretation) are reviewed. It is hoped that through the use of these fundamental statistical principles, this paper will bring clarity and delineation to the science of quantifying the morphology of spermatozoa.
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*Keywords:* Spermatozoa; Morphology; Sample size; Statistics; Comparisons

## 1. Introduction

Given the complex demands placed on spermatozoa in order to achieve fertilization and sustain early embryonic development, it has been difficult to identify a single in vitro test capable of accurately predicting the fertility potential of an individual ejaculate [1].

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0093-691X/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0093-691X(03)00240-1

Therefore, the emphasis remains on screening out overtly poor quality ejaculates, with standard morphology estimation often the method of choice to achieve this goal [2,3]. These analyses are conducted by a broad range of individuals, including commercial boar/bull stud personnel, practicing veterinarians, clinicians at reference andrology laboratories, and research scientists. Thorough explanations concerning how many spermatozoa should be counted and the statistical basis for this decision are not as readily available as one might expect, although the question has been posed in various ways for quite some time [4,5]. Five different Breeding Soundness Examination (BSE) forms and two Frozen Semen Evaluation/Transport forms are available to member veterinarians through the Society for Theriogenology (SFT). Only one of these, the Bovine Frozen Semen Evaluation form, provides guidelines concerning the number of spermatozoa to be assessed for the morphology evaluation [6]. The Bull BSE Evaluation form refers the clinician to another resource where general suggestions concerning morphology counts can be found, albeit without presenting the statistical foundation for their recommendations [7]. Although a formal boar BSE form is not available from SFT, a manual has been prepared which briefly outlines differential counting methods for characterizing sperm abnormalities [8].

Finding answers in the scientific literature is no less frustrating, as a wide range of cell numbers have been suggested, often with little explanation regarding the appropriateness of the stated sample size, while others may not report the number counted [9–13]. Those studies that do discuss morphology counting strategy generally report correlation or repeatability within sample or justify the numbers they counted based on preliminary results in which the time-consuming effort of counting more cells failed to convince the authors of an obvious benefit over lower counts that were quicker and more efficient to perform [4,14]. While the assessment of spermatozoa with flow cytometry offers certain inherent advantages over routine microscopy, such as objectivity, the ability to assess additional parameters and the rapid analysis of large numbers of spermatozoa [15,16], the expense of this instrumentation often limits its availability in clinical settings.

Clinical laboratories involved with human assisted reproductive technologies lack standardization as well, as a recent investigation revealed that most laboratories (83%) evaluated $\leq$100 spermatozoa per slide when assessing sperm morphology, while 16% evaluated $\geq$200 or more [17]. It is the purpose of this paper to serve as a single source, providing the background necessary for the reader to make an informed decision on the number of spermatozoa to include in sperm morphology assessments and to offer guidelines on how this information can be put into practice. Appendix A is provided which includes definitions of statistical terms and the mathematical formulas discussed in the text.

## 2. Sperm morphology threshold values

Defining scientifically relevant threshold values for normal sperm morphology in animal breeding programs, or debating whether these threshold values are applicable at all, is beyond the scope and intent of this work. However, in the interest of providing practical examples, it should be noted that there is currently a substantial amount of support for the recommendation that the minimum percentage of morphologically normal spermatozoa in an ejaculate should lie in the range of 70–80% in order to achieve optimum fertility [18–20].

For this reason, a threshold value of 75% normal spermatozoa will be used in the examples discussed below.

## 3. The nature of proportions

The estimated percentage of morphologically normal spermatozoa in an ejaculate is calculated as a proportion, consisting of the number of normal cells divided by the total number of spermatozoa counted, with the quotient multiplied by 100. For instance, a sample reported to be 75% normal could be the result of 75 normal cells out of 100 counted or 375 normal cells out of 500 counted. In this case, each sperm cell is categorized into one of two mutually exclusive categories, normal or abnormal. This type of situation is known as a Bernoulli trial. An everyday example of a Bernoulli trial is the coin toss, where each toss results in one of two mutually exclusive outcomes, either heads or tails. For each Bernoulli trial, there exists a certain probability of success; in the case of the coin toss, there is a 50% probability of obtaining heads on each individual flip of the coin.

A collection of independent Bernoulli trials in which the probability of success remains constant is known as a binomial experiment, where the probability of observing a specified number of successes can be calculated. In the example of the coin toss, the probability of a head on each toss is 50%, and thus we would expect to observe five heads in 10 tosses. This expected outcome is known almost instinctively; however, there is a formula that defines this relationship. The mean (or expected) value for the number of successes in a binomial distribution is calculated as $n \times P$, where $n$ is the number of independent Bernoulli trials and $P$ is the probability of success on each trial. If the true proportion of normal cells in an ejaculate is 80% and we count 100 cells, we would expect to observe 80 normal cells in the sample that is counted ($100 \times 0.8$).

It is critical to recognize that the above calculations are used to generate the expected values for a given binomial distribution. Although the probability of a head on a coin toss is always 50%, we do not always observe exactly 50% heads and 50% tails with an even number of tosses. For example, we can calculate the probability of getting eight heads on 10 coin tosses due to chance as approximately 4.39%. As with the coin toss, just because an ejaculate contains a certain proportion of normal spermatozoa does not mean that the observed proportion will match this parameter. In the case of sperm morphology counts, we could have a situation in which the true proportion of normal cells in the ejaculate is 80%. By chance alone, we would expect to observe 70 normal cells out of 100 counted (and thus reject the ejaculate) 0.51% of the time. This leaves us with the following questions: (1) How confident are we that the observed proportion reflects the true proportion? (2) How many spermatozoa should be evaluated so that the observed proportion accurately reflects the true proportion?

## 4. Confidence

For screening purposes, such as a BSE performed in the field by practicing veterinarians or routine semen analysis by technicians at commercial studs, the goal is to ensure that the ejaculate in question surpasses the designated cut-off value for normal sperm morphology.

Before making this decision, we must first ask ourselves if the observed proportion accurately reflects the true value for a given ejaculate. Because we cannot reasonably count every sperm in an ejaculate that may contain billions of cells, we can never know the exact proportion of normal cells it contains. However, we can classify a sample of cells and calculate a bound of statistical uncertainty around the estimate, forming a range known as the confidence interval (CI). A working knowledge of the CI allows the investigator to determine if the estimate is within the clinically 'normal' or 'abnormal' range. A 'borderline' result from an ejaculate can indicate that additional cells must be counted to classify the result more precisely. For the purpose of this discussion, borderline ejaculates will be described as those in which the limits of the CI for the observed percentage of normal cells overlap the designated threshold value.

As previously discussed, a single count of 100 spermatozoa will provide a point estimate of the proportion of normal cells in the ejaculate. Each time this count of 100 cells is performed, a different point estimate will be obtained. After repeatedly counting 100 cells, the most common point estimates should be a more accurate reflection of the true proportion of normal cells in the ejaculate; this is an over-generalization of the Central Limit Theorem. The CI is based on this idea of repeated sampling. Specifically, if 100 counts of 100 cells were performed, we can generate statistical CIs around each of these 100-point estimates, such that 95% of the intervals would contain the true parameter. This can be loosely interpreted to mean that there is an approximate 95% probability that the CI will contain the true proportion of normal cells in the ejaculate [21].

How then do we calculate and use a CI from a single count of spermatozoa? In order to calculate the CI for a proportion, we must have three pieces of information: (1) the observed proportion ($p$); (2) the total number of cells counted ($n$); and (3) the desired confidence level ($1 - \alpha$), where $\alpha$ represents the Type I error rate (see Formula I). For example, the 95% CI for the observation of 80 normal cells out of 100 cells classified would be 71.1–86.7%, while the 95% CI for the observation of 800 normal cells out of 1000 classified would be 77.4–82.4%. The calculation of the CI generally starts with the assumption of a 'large' ($n > 30$) sample size. When smaller sample sizes are used, specialized equations are required [21].

The width of the CI that can be expected based on variable sample sizes is shown in Fig. 1. As anticipated, we would not have much confidence in an estimate that falls within an extremely wide interval (i.e. $n = 50$), but we can be extremely precise by including more cells in the count (i.e. $n = 10{,}000$). This example highlights the relationship between CI width and sample size. Intuitively, we can be more ''confident'' of our point estimate if we count more cells. However, the CI is calculated after the data are collected. How then do we determine in advance (a priori) the appropriate number to count?

## 5. Sample size and precision

The width of the CI depends on the random selection of spermatozoa from within the ejaculate (variability) and the level of statistical confidence desired. Variability of the observed sample is partially a function of sample size. If you decide a priori the level of precision you desire (i.e. how wide you are willing to allow the CI to be), the appropriate

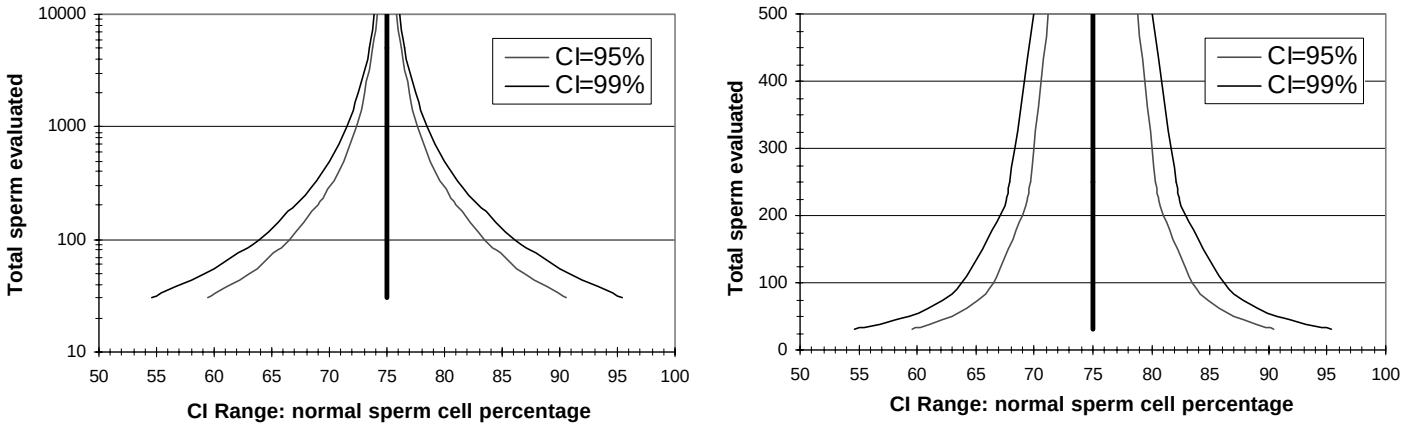


Fig. 1. Sample size (total number of sperm evaluated) required to obtain specific confidence interval (CI) around an expected percentage of 75% normal (adapted from [29]).

sample size can be calculated. For example, if we think that the ejaculate contains 80% normal spermatozoa and we wish to be 95% confident that the true mean proportion of normal cells is greater than 75% from a sample of this ejaculate (i.e. the lower bound of the CI being equal to the minimum acceptable level), then we need to generate a sample size using a desired precision of 5%. Note that a one-tailed sample size should be calculated because we are only interested in whether the proportion of normal cells is greater than 75%. In this example, the total number of spermatozoa that must be counted is 174 (see Formula II). It is also important to recognize that the precision described above is known as absolute precision ($80 \pm 5\%$). In contrast, a relative precision (Formula III) of 5% implies a precision that is 5% of the 80%, or $80 \pm 4\%$.

We cannot reasonably predict the observed proportion of normal cells before evaluating an ejaculate, but we can prepare a reference table containing common sample sizes and the corresponding decision values. The decision values are derived from a pre-selected cut-off value for accepting or rejecting an ejaculate based on normal morphology and desired precision. Table 1 is an example of such a reference to determine when the minimum numbers of spermatozoa have been counted in order to accept or reject an ejaculate based on a cut-off value of 75% normal morphology with 95% confidence. To use this table, start by classifying 50 spermatozoa as normal or abnormal. If the number of normal cells is $\geq$43 (85%), the ejaculate can be accepted. If the number of normal cells is $\leq$32 (65%), the ejaculate should be rejected for processing. If the number of normal cells falls within the borderline range of 33–42, then an additional 25 cells (total of 75) should be counted and the number of normal cells compared to the table in the same manner. This process continues until a value is obtained that allows a decision to be made. It is apparent from this table that the numbers needed to confirm classification can become unrealistically high, and some provision for practicality must over-ride statistical precision.

Historically it seems that sample sizes were chosen more out of tradition or habit, rather than by analytical methods as described above. Probably the most common number of spermatozoa counted in morphology analysis is 100. Fig. 2 illustrates the error range to be

Table 1
Decision table for a threshold value of 75% morphologically normal sperm

| Total sperm counted | Decision | | | | |
|---|---|---|---|---|---|
| | Pass ($\geq$) | | Fail ($\leq$) | | Borderline |
| | Normal | % | Normal | % | Normal |
| 50 | 43 | 85.1 | 32 | 64.9 | 33–42 |
| 75 | 62 | 83.3 | 50 | 66.8 | 51–61 |
| 100 | 82 | 82.1 | 68 | 67.9 | 69–81 |
| 200 | 160 | 80.1 | 140 | 69.9 | 141–159 |
| 250 | 199 | 79.5 | 176 | 70.5 | 177–198 |
| 500 | 391 | 78.2 | 359 | 71.8 | 360–390 |
| 1000 | 773 | 77.3 | 727 | 72.7 | 728–772 |
| 5000 | 3801 | 76.0 | 3699 | 74.0 | 3700–3800 |
| 10000 | 7571 | 75.7 | 7429 | 74.3 | 7430–7570 |

As morphology is assessed, this table can be used to decide if the ejaculate meets minimum requirements (pass), fails, or if counting should continue (borderline). One-tailed; $\alpha = 0.05$; $Z = 1.65$.

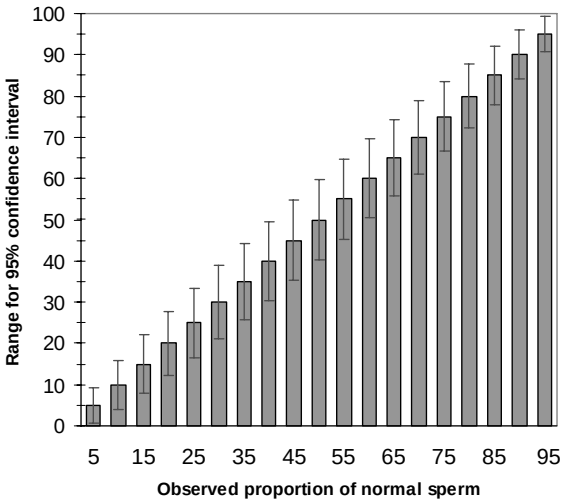


Fig. 2. Observed proportion of normal sperm with error bars representing the range of values included in a 95% CI with a sample size of 100.

expected when estimates are made with this sample size. It has been suggested that there is little precision gained by counting more than 100 spermatozoa in most cases, but that ‘‘500 or more’’ spermatozoa may need to be counted when the proportion of morphologically abnormal spermatozoa is high and many types of abnormalities are present [7]. Normal sperm morphology in ejaculates from fertile, healthy domestic animals will often exceed 85%, in which case a count of 100 cells may be more than sufficient (see Table 1). Likewise, if the proportion of abnormalities is particularly high, and the decision is simply pass/fail, it may not be practical to count more than 100 cells. However, estimates near the designated cut-off value may warrant greater sample sizes.

## 6. Comparisons within and between laboratories

Making comparisons between laboratories can be problematic, even when samples from the same source are analyzed simultaneously. In addition to considering whether or not the results fall within an acceptable range in the absence of systematic error, it is necessary to consider the sources of variation that exist independent of chance alone. Method of sub-sampling the original ejaculate, shipping conditions, sample preparation, and technician experience can all introduce systematic errors. The difference in results obtained by using a staining method for contrast versus wet-mount fixation can contribute substantially to the variation in certain classes of abnormalities, which in turn impacts the estimate of percentage of normal spermatozoa [22,23]. Additionally, classification systems may differ between laboratories, affecting sub-categories of morphology independent of the overall proportion of normal and abnormal (Fig. 3).

Comparing morphologic analyses between laboratories is not as straightforward as it sounds. Applying common statistical methods, such as the Chi-square test [24], or making

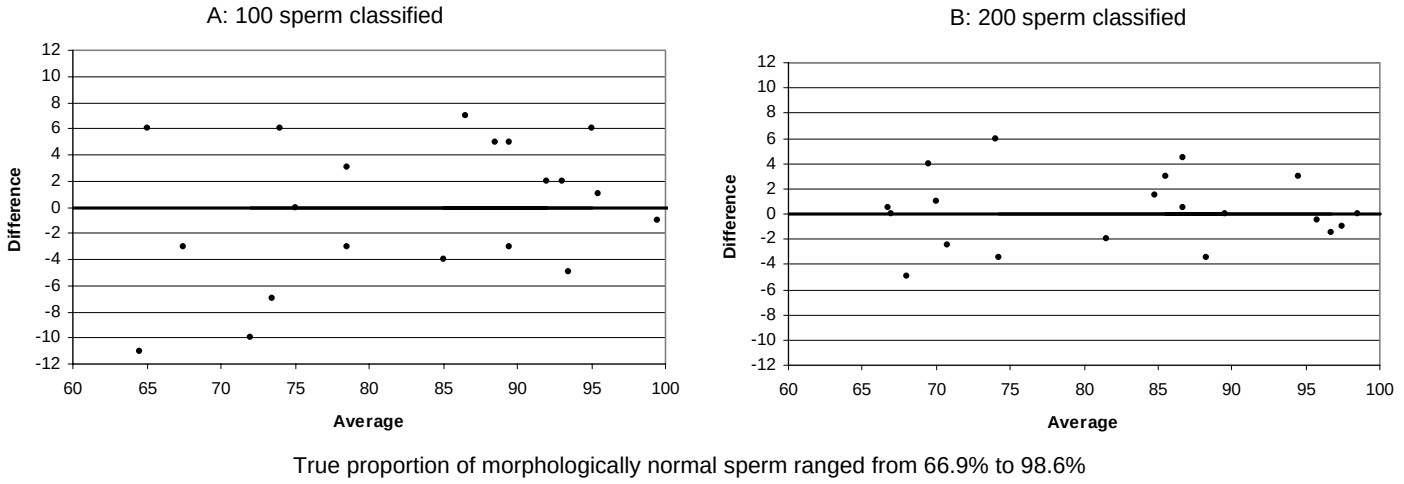


Fig. 3. Distribution expected for the difference between proportions for two laboratories assuming no systematic error or bias.

inferences based on a CI for the difference between the two proportions [25] are problematic for a variety of reasons. Kappa statistics are familiar to many as a method of assessing agreement between diagnostic tests, and could be used to analyze inter-laboratory agreement in pass/fail ratings for the morphology of sperm cells. However, the application of kappa in this instance may not be particularly useful. Kappa is an index of agreement, and therefore lacks the ability to make distinctions between various types and sources of disagreement. When comparing results across laboratories the goal is not only to assess agreement, but also to understand the factors that are associated with disagreement and to improve consistency and accuracy. For example, it is helpful to know if one laboratory is biased, such that the values it reports are consistently higher or lower than other laboratories, and which samples are the subject of the most disagreement. Kappa is also influenced by prevalence, where the value of the kappa statistic is negatively biased as prevalence becomes very high or very low. This bias can make it difficult to compare across studies and/or populations. For example, if two laboratories analyze high quality samples that tend to fall into the 'pass' range, the prevalence of 'pass' will be high, and the kappa statistic would underestimate the actual level of agreement. The kappa statistic also provides no information about the accuracy of the tests being evaluated; a high kappa statistic can imply that the two tests are equally good or equally bad. For these reasons, alternative approaches for assessing inter-laboratory agreement are warranted.

An alternative approach is to have the two laboratories perform morphology assessments on multiple ejaculates of variable quality. Each laboratory will generate a proportion of normal cells for each sample, and the difference between the proportions generated from each laboratory can be calculated for each sample. One way to visualize these data is to plot the proportion differences for each sample against the mean of the proportions from that sample. If the two laboratories are in agreement (absence of a systematic error or bias), the mean difference between their estimates should approximate 0, and the points on the graph should be randomly distributed around the null value of 0. Fig. 4 was created by using the binomial distribution to randomly generate point estimates for a hypothetical set of semen samples with either 100 (IIIa) or 200 (IIIb) spermatozoa counted per ejaculate. It is apparent from this figure that no systematic bias exists between these 'laboratories'. As expected, the variability decreases as the mean values approach the limit (100%), and larger sample sizes reduce the likelihood of observing extreme values.

Although laboratory-to-laboratory comparisons should always be based on multiple samples, circumstances occasionally arise where a single sample needs to be compared among the laboratories. In this case a CI can be constructed around the difference between the observed proportions (Formula V). The null value of this CI is 0, and thus, if the CI overlaps 0 it is assumed that no significant difference exists between the two estimates. However, the difference between proportions is directly influenced by sample size, so that if both laboratories include a large number of cells in their analysis, they are more likely to report a statistically significant difference that is not biologically relevant, and conversely, if the sample size is small, it is more difficult to statistically identify a true biological difference. Additionally, since the proper application of the CI is in the context of multiple sampling (see Section 4), drawing conclusions based on a single interval calculated for one point estimate may be misleading. For these reasons, this method of comparison is highly undesirable and therefore should be interpreted with caution.

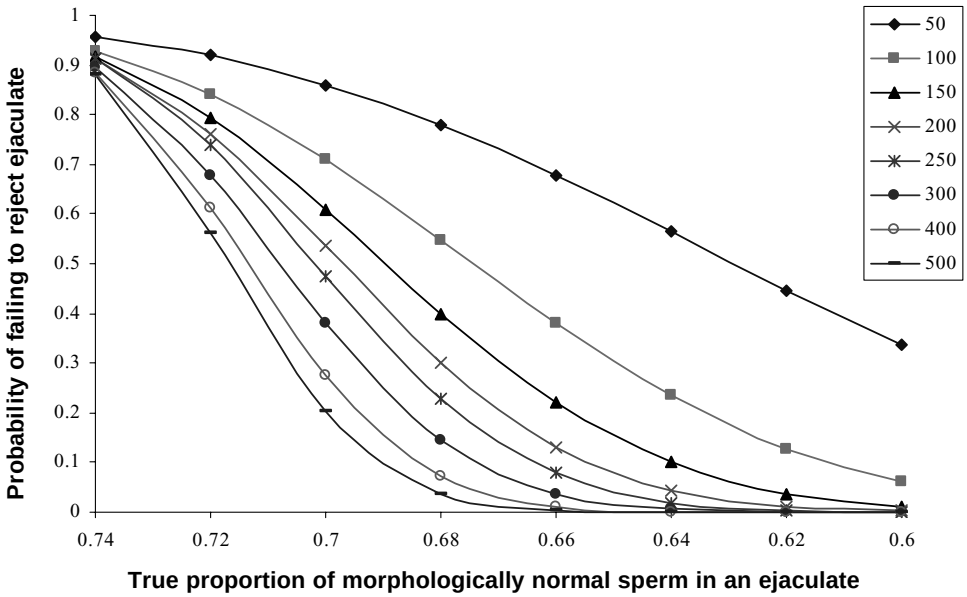


Fig. 4. Probability of failing to reject an ejaculate for various samples (threshold value: 75%).

For within-laboratory comparisons of duplicate estimates of sperm morphology, the difference between the two proportions can be evaluated using Formula VI, provided the same numbers of spermatozoa were counted each time [26]. Comparing morphology estimates between slides starts with the assumptions that the distribution and prevalence of sperm abnormalities are uniform for each slide prepared from the original sample. If these assumption are not met, each slide would represent an experimental unit, with many slides required to estimate a mean value for the population of spermatozoa represented in the ejaculate. High slide-to-slide repeatability can be expected when morphology estimates are made from the area distal to where the smearing process was initiated on stained slides and from the center of aldehyde-fixed wet mounts [7]. Techniques have been described to further standardize this procedure [27].

## 7. Implications

It is not the intention of the authors to reveal a magical number of spermatozoa that must be counted in order for a morphology analysis to be considered ‘correct’ in every situation; however, some general guidelines can be suggested. For relatively high-volume field observations, such as routine screening exams at boar studs, a practical goal would be to assess a minimum of 50 cells on each sample, then refer to Table 1 to determine if an immediate pass/fail judgment can be made, or continue counting for ‘borderline’ ejaculates. Counting more than 200 cells is often considered impractical due to time constraints, and the benefits of increased precision are unlikely to be worth the opportunity cost.

Practicing veterinarians conducting a BSE would be advised to count 100 cells initially and then refer to a resource that is built on the applicable threshold value, similar to Table 1, to determine if greater precision is required. If the purpose of the analysis is to monitor the progression of semen quality over time (i.e. pubertal development in maturing males or determine prognosis for recovery in previously proven sires), higher counts will be necessary to more precisely define the subcategories considered 'abnormal' as previously described. The relationship between the number of cells classified and the probability of failing to reject an ejaculate with a true proportion of spermatozoa below the specified threshold value is presented in Fig. 4.

For reference andrology laboratories, the expectations are higher, and this should be reflected in the accuracy and precision of the results that are ultimately reported. Once again, 100 cells represent an acceptable starting point, but at least 200 cells will need to be assessed in most cases, with even higher numbers included if sub-categories containing relatively small percentages are important for interpretation of the final results. In addition, it may be prudent to prepare and review multiple slides of the same sample for cases that fall into the 'borderline' category, especially if one or more stakeholders may contest the results. Reference laboratories should be held accountable to document not only the point estimate, but also the calculated error range for their results.

Researchers concerned with sperm morphology will need to consider their specific aims. Is the purpose of the morphology analysis simply to report routine semen parameters, or is morphology a critical component, as in IVF or breeding trials studying the link between sperm morphology and subsequent fertility? The number of cells assessed in each unique instance should reflect the answers to these questions while taking into account the time that is available for allocation to this task, repeatability between workers and the risk of unreliable results due to fatigue if large numbers of cells are counted for multiple samples within a short time frame.

So, when is enough really enough? By understanding how that decision is made and documenting it for customers, clients, and reviewers, ultimately you decide.

## Acknowledgements

The authors wish to thank Drs. Ian Gardner and Jim Lowe for their input during the draft stages of this manuscript.

## Appendix A. Definitions and formulas

I. *Confidence interval (proportion)*: A range of values that have a specified probability of containing the parameter of interest. An individual CI either does or does not contain the parameter of interest. The specified probability of 95% confidence means that 95 out of 100 intervals created from repeated samplings from the same population would contain the parameter of interest [21].

$$p = Z_{(1-\alpha)/2}\sqrt{\frac{p(1-p)}{n}}$$

II. *Absolute precision*: A measure of how close an estimate is to the true value of a population parameter, expressed in absolute terms [28].

$$n = \frac{Z^2_{(1-\alpha)/2} \times p(1-p)}{d^2}$$

III. *Relative precision*: A measure of how close an estimate is to the true value of a population parameter, expressed in relative terms [28].

$$n = \frac{Z^2_{(1-\alpha)/2} \times p(1-p)}{\varepsilon^2 \times p}$$

IV. *Standard error*: A measure of dispersion that provides a way to estimate the expected error for a particular sample size [24].

$$\text{S.E. of } p = \sqrt{\frac{p(1-p)}{n}}$$

V. *Confidence interval*: Difference between two proportions [25].

$$(p_1 - p_2) \pm Z_{1-\alpha/2}\sqrt{\frac{p_1(1-p_1)}{n_1} + \frac{p_2(1-p_2)}{n_2}}$$

VI. *Difference between two proportions*: Indicates whether an absolute difference between two estimates from the same sample in the same laboratory are due to random variation or systematic error [26].

$$|p_1 - p_2| = Z_{(1-\alpha)/2} \times \sqrt{2 \times \frac{\overline{p}(1-\overline{p})}{n}}$$

where $n$ is the same number of sperm must be counted for each estimate.

Abbreviations: $n$, sample size (assumes infinite population); $p$, anticipated population proportion or prevalence; $d$, maximum absolute difference between observed and true value (expressed as a decimal); $\varepsilon$, maximum relative difference between observed and true value; $Z$, value obtained from Student's $t$-distribution (represents confidence) (common values: 90% CI = 1.645, 95% CI = 1.960, 99% CI = 2.576).

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