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SpermBlue®: A new universal stain for human and animal sperm which is also amenable to automated sperm morphology analysis

G van der Horst, L Maree

Department of Medical Biosciences, University of the Western Cape, Private Bag X17, Bellville 7530, South Africa

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Abstract

Our study was aimed at exploring a simple procedure to stain differentially the acrosome, head, midpiece, and flagellum of human and animal sperm. A further prerequisite was that sperm morphology of the stained samples could be analyzed using automated sperm morphology analysis (ASMA). We developed a new staining process using SpermBlue® fixative and SpermBlue® stain, which are iso-osmotic in relation to semen. The entire fixation and staining processes requires only 25 min. Three main steps are required. First, a routine sperm smear is made by either using semen or sperm in a diluting medium. The smear is allowed to air dry at room temperature. Second, the smear is fixed for 10 min by either placing the slide with the dried smear in a staining tray containing SpermBlue® fixative or by adding 1 ml SpermBlue® fixative to the slide. Third, the fixed smear is stained for 15 min by either immersing the slide in a staining tray containing SpermBlue® stain or adding four drops of SpermBlue® stain to the fixed smear. The stained slide is dipped gently in distilled water followed by air drying and mounting in DPX® or an equivalent medium. The method is simple and suitable for field conditions. Sperm of human, three monkey species, horse, boar, bull, ram, mouse, rat, domestic chicken, fish, and invertebrate species were stained successfully using the SpermBlue® staining process. SpermBlue® stains human and animal sperm different hues or intensities of blue. It is possible to distinguish clearly the acrosome, sperm head, midpiece, principal piece of the tail, and even the short end piece. The Sperm Class Analyzer® ASMA system was used successfully to quantify sperm head and midpiece measurements automatically at either 600 × or 1000 × magnification for most of the species studied.

Key words: acrosome, ASMA, head, human, invertebrates, mammals, midpiece, morphology, primates, sperm, staining, tail, vertebrates

Sperm morphology assessment is one of the most important criteria for determining the quality of a semen sample for humans and animals (Gravance et al. 1998, WHO 1999, Henkel et al. 2007, Van der Horst et al. 2009). Moreover, many claims have been made about the relation between sperm morphology assessment and fertility (Enginsu et al. 1991, Menkveld et al. 2003). Accordingly, many stains and staining combinations have been employed to determine the percentage of sperm with normal morphology.

The accuracy of sperm morphology assessment depends on careful preparation, fixation, and staining of sperm (García-Herreros et al. 2006), because these procedures can affect sperm dimensions significantly (Meschede et al. 1993, Gago et al. 1998, Hidalgo et al. 2006, Lukaszewicz et al. 2008). Thus, one of the criteria for a staining method should be that the processes involved cause as little change to sperm morphology as possible.

Address for correspondence: Gerhard van der Horst, Department of Medical Biosciences, University of the Western Cape, Private Bag X17, Bellville 7530, South Africa. E-mail: gvdhorst@uwc.ac.za

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Papanicolaou® stain (Papanicolaou 1942), and to a lesser extent Shorr® stain (Shorr 1941, Henkel et al. 2007), have become the standards for human sperm morphology assessment for both the World Health Organization criteria and the Tygerberg Strict Criteria (Menkveld et al. 1990, WHO 1999). Despite the extensive application of Papanicolaou® stain for staining human sperm and its suitability for automated sperm morphology analysis (ASMA; Coetzee et al. 1999, 2001), the procedure involves processing through more than 20 steps and more than 12 different chemical solutions (WHO 1999). It is not only time consuming, but alcohol fixation and alcohol or xylene dehydration may cause cell shrinkage (Ross 1953, Katz et al. 1986). It is not surprising, therefore, that researchers in the field of sperm morphology have searched for simpler and faster staining methods for the routine and clinical laboratory.

The Diff-Quik®, Hemacolor®, and Giemsa® type staining procedures are rapid methods for evaluating sperm morphology and sperm chromatin status (Sousa et al. 2009) and involve fixation followed by staining with two staining solutions. Despite the findings that Diff-Quik® causes sperm swelling (Kruger et al. 1988) and background staining (WHO 1999), it is recommended by WHO (1999) for human sperm morphology assessment. Spermac® is another rapid sperm staining procedure for determining morphology (one fixative and two staining solutions) that is particularly effective for staining the human acrosome (Oettle 1986, Chan et al. 1999). It has the disadvantage of background staining, however, and is not easily used for ASMA (personal observation).

Eosin–nigrosin staining developed for ''live-dead'' staining of sperm (Bjorndahl et al. 2003) also has been used to assess sperm morphology for many animal species (Van der Horst et al. 2009). This stain does not clearly differentiate the various components of the sperm, however.

Many other staining combinations (Jager et al. 1984, Gravance et al. 1998, Belletti and Melo 2004) have been used for assessing sperm morphology for humans and several animal species, but have not been shown to have wider application in the routine laboratory.

Several studies have shown the need for ASMA in the routine clinical laboratory to improve accuracy and avoid bias when determining the percentage of normal sperm (Gago et al. 1998, Coetzee et al. 1999, 2001). These studies were disappointing, however, in terms of differences encountered among stains and different ASMA equipment.

There currently is not a sperm morphology stain available which represents a simple methodology (one staining solution), is a rapid procedure, is osmotically and/or physiologically adapted for semen, stains the various components of sperm differentially, does not stain background material, can be applied to almost any species (invertebrates to humans), and also is useful for ASMA. Our aim was to develop a staining procedure that fulfills the criteria above by using the newly developed sperm stain, SpermBlue®.

Materials and methods

Semen samples of human and animal species

Ethical clearance was obtained for all of the following methods of sperm collection: masturbation (humans), artificial vagina (horse, boar, bull, and chicken), electrostimulation (three monkey species and merino ram), epididymal puncture (rat and mouse), ''milt stripping'' (trout), chemical stimulation (abalone), and direct isolation of sperm from the testis (black mussel). All procedures were in accordance with ethical guidelines of the University of the Western Cape and/or the various research or animal units where animals were maintained and semen was collected. Table 1 lists the general and species names of all the animals used in this study, their location, as well as the number of individuals of each species used. All animals had access to a balanced diet and water on a daily basis and all animal husbandry requirements as prescribed by the various ethics committees were followed (Table 1). The Helsinki Declaration governing research on humans also was followed (Christie 2000). A consent form was signed by each human donor and these donors were assured of their anonymity, that their semen sample was used for research purposes only, that it would not be used for cloning research, and that the semen sample was destroyed after experimentation. Human sperm donor selection was based on sample size (≥ 2 ml), sperm concentration ($\geq 20 \times 10^6$ ml^{-1}) and percentage of sperm motility ($\geq 50\%$ forward progression) (WHO 1999).

The osmotic concentration of semen samples of humans and two monkey species were measured within 1–3 h after masturbation or sampling using a vapor pressure osmometer (Wescor 5100C Vapor Pressure Osmometer, Logan, UT) that was calibrated with 290 and 1000 mM kg^{-1} standard solutions. This analysis was performed to adjust the osmotic concentration of the SpermBlue® fixative and stain with a standard phosphate buffer to an average value

Table 1. Sperm of human and 13 South African species stained using SpermBlue®

General names	Species names	Number	Unit/place where collection took place
Human	*Homo sapiens*	10	Stellenbosch Medical School, Bellville
Vervet monkey	*Chlorocebus aethiops*	10	Primate Unit, Medical Research Council, Parow
Chacma baboon	*Papio ursinus*	5	Delft Animal Centre, Cape Town
Rhesus maqacue	*Macaca mulatta*	5	Delft Animal Centre, Cape Town
Merino sheep	*Ovis ovis*	10	Elsenburg Experimental Farm, Stellenbosch
Arabian horse	*Equus equus*	3	Veterinary practice, Malmesbury, Western Cape
Bull (Nguni)	*Bovis bovis*	4	Agricultural Research Council, Irene, Pretoria
Boar (Kolbroek)	*Sus scrofa*	3	Agricultural Research Council, Irene, Pretoria
Wistar rat	*Rattus norvegicus albinus*	3	MBS, University of the Western Cape, Cape Town
Mouse	*Mus musculus*	10	MBS, University of the Western Cape, Cape Town
Chicken (Venda)	*Gallus domesticus*	3	Agricultural Research Council, Irene, Pretoria
Rainbow Trout (Fish)	*Salmo gairdneri*	10	Jonkershoek Trout Hatchery, Stellenbosch
Abalone	*Haliotis midae*	10	HIK Abalone Unit, Hermanus, Western Cape
Black mussel	*Choromytilus meridionalis*	3	Blouberg Strand, Cape Town

that was almost iso-osmotic to the semen (sometimes slightly hypo-osmotic and sometimes slightly hyper-osmotic in relation to the various semen samples) as shown in Table 2.

SpermBlue® fixative and stain and other chemicals

SpermBlue® fixative and stain were supplied by Microptic SL (Barcelona, Spain). All other chemicals were purchased from Sigma-Aldrich (Cape Town, South Africa) and only analytical grade molecular grade chemicals were used.

Table 2. Osmotic concentration of human and primate semen and SpermBlue® fixative and stain; n = number of samples of each species or medium

Species/medium	Osmotic concentration mOsm/kg or mM ± SD
Human (*Homo sapiens*) ($n=10$)	358 ± 27.6
Vervet monkey (*Chlorocebus aethiops*) ($n=13$)	335 ± 49.8
Chacma baboon (*Papio ursinus*) ($n=4$)	358 ± 16.9
SpermBlue® fixative ($n=3$)	375 ± 2.5
SpermBlue® stain ($n=3$)	317 ± 1.8

Smears of semen and/or sperm in diluting media and staining procedure

All semen samples were processed for staining within 2 h after collection and after liquefaction in humans. Before a semen sample was stained, the sperm concentration was assessed by pipetting 5 µl of semen into a 20 µm deep chamber of a Leja slide (Leja Products B.V., Nieuw-Vennep, The Netherlands) using a positive displacement pipette. The motility module of the Sperm Class Analyzer® (SCA), version 4.0.0.5 (Microptic S.L.) was used to determine sperm concentration. When the sperm concentration was greater than 100×10^6 ml^{-1}, only 3–5 µl of semen/sperm suspension was used to make the smear. For sperm concentrations between 20 and 90×10^6 ml^{-1}, approximately 7−10 µl was used. This procedure resulted in two to 10 sperm in the field of view using the 100 × oil immersion objective.

Clinicians and researchers use undiluted semen for morphological analysis and often need to study sperm morphology in culture medium and/or after treatment in a diluting medium. Sperm smears of human and all animal species listed in Table 1 also were prepared by taking an aliquot of sperm from the top fraction of a swim-up preparations. For this purpose, approximately two volumes of diluting medium (e.g., Ham's F10 medium) was placed in an Eppendorf centrifuge tube. One volume of semen then was released slowly with a positive displacement pipette into the Eppendorf tube at the bottom of the diluting medium. Sperm "swim-up" into the surrounding diluting fluid and the motile sperm then were harvested from the top of the fluid in the Eppendorf tube after 10 min to 1 h

incubation (appropriate temperature and 5% CO_2). Sperm concentration was determined as described above.

For animals such as abalone that live and release gametes in the sea, sea water was used as a diluting medium. The sea water was filtered using a 0.2 μm Millipore filter before use. The SpermBlue® fixative and stain also were prepared using filtered sea water.

Once the correct volume of semen or swim-up sperm (sperm concentration adjusted) was placed on a glass slide, a routine sperm smear was made in duplicate and allowed to air dry. The slides with sperm smears then were placed in a staining tray and fixed with SpermBlue® fixative (pH 7.6 and osmotic concentration 375 mOsm kg^{-1}) for 10 min. The osmotic concentrations of semen of various species ranged from about 320–420 mOsm kg^{-1} (Table 2 indicates averages) and are hyper-osmotic in relation to blood plasma (about 290 mOsm kg^{-1}). Alternatively, the slides were placed horizontally on filter paper, smear side up, and a plastic disposable pipette was used to put 1 ml of SpermBlue® fixative on the fixed sperm smear, covering the whole area of the smear. After the 10 min fixation step, the slides were held at an angle of about 60–80° to drain the excess fixative.

During the third step of the staining procedure, the slides were placed in a staining tray containing SpermBlue® stain and stained for 15 min (immersion technique). Alternatively, the slides were placed horizontally on filter paper, smear side up, and a plastic disposable pipette was used to put 0.45–0.5 ml of SpermBlue® stain on the fixed sperm smear (''drop-on'' technique). During the 15 min staining procedure for the latter technique, the slides were rolled regularly from side to side to ensure proper staining across the entire sperm smear surface. After staining, the excess stain was drained off as for the fixative. Thereafter, the slides were gently dipped in distilled water with each dip lasting about 1 sec. When the immersion technique was used, only one dip was necessary to wash off the excess stain, but for the ''drop-on'' technique, a second dip was necessary to wash off excess stain. The main purpose for the washing step was to remove most of the background blue color. Slides must be dipped very gently in distilled water to prevent too many sperm from being lost during the washing step. The slides then were placed at an angle of about 60–80° to remove excess fluid and left to air dry. After drying, one slide could be mounted using DPX or an equivalent mounting medium. The quality of staining was evaluated immediately using a light microscope at 400 × magnification. If nuclear staining is not sufficiently intense, the duplicate smear can be re-stained for another 3–5 min and even longer if required.

Photomicrography

A Basler A312fc digital camera (Microptic S.L.) was mounted (C-mount) on either a Zeiss Photomicroscope III (Zeiss, Cape Town, South Africa) or a Nikon Eclipse 50i (IMP, Cape Town, South Africa), both with bright field optics. The Basler camera was connected by a six pin firewire cable (IEEE1394) to a Belkin firewire card of a desktop computer. Sperm were photographed and images captured digitally using the counter module of the SCA system (Microptic S.L.). Images were saved as bitmap files using the 40, 60, or 100 × objectives of the relevant microscopes. Calibrations were carried out for the different microscopes and objectives using a Nikon micrometer scale marked in 10 μm units.

Automated sperm morphology analysis (ASMA)

The camera and microscope systems described above and the Morphology module (version 4.0.0.5) of the SCA system (Microptic S.L., Barcelona, Spain) was used for ASMA. This system automatically detects the acrosome, head and midpiece of sperm. The SCA system furthermore makes rapid and accurate measurements of the sperm head length, head width, head perimeter, head surface area, head ellipticity, head elongation, head regularity and the percentage acrosome coverage over the head. Several midpiece dimensions are also measured (width, area and angle of insertion). ASMA analysis requires that the sperm are sufficiently and differentially stained with a clear background.

For the purpose of this investigation, not all species were analyzed using the SCA morphology module; representative examples were measured here. Human, monkey, and horse sperm were analyzed using the 100 × objective. Because bull, ram, and boar sperm are almost twice the length of human sperm, they were analyzed using the 60 × objective. At least 100 sperm were analyzed per semen or swim-up sample and the number of animals used for each species is indicated in Table 1. In some species, only three or four animals were used; however, this should not affect the results, because the aim of this study was to show whether sperm were stained properly and differentially and whether sperm could be

analyzed accurately using ASMA when SpermBlue® was used as stain.

Results

SpermBlue® staining patterns in different species

Two distinct sperm staining patterns have been observed among the species studied using SpermBlue® stain. Staining pattern 1 is found in human, vervet monkey, horse, and chicken. In these species, the acrosome stains pale blue and the post-acrosomal area stains intensely dark blue. The midpiece stains slightly less dark blue than the head, the principal piece of the tail is slightly less blue than the midpiece and the end piece of the tail stains pale blue (Fig. 1a–d). There are some variations in the staining pattern observed within and between species. With chicken sperm, the principle piece of the tail stains blue–pink (Fig. 1d). All the features of staining pattern 1 in all of the species above are equally clear for semen and swim-up samples.

Staining pattern 2 is nearly the reverse of pattern 1. Ram, boar, bull, mouse, and abalone are representative of pattern 2 (Fig. 1e–i). Here the acrosome stains dark blue and the post-acrosomal area stains pale blue. The very distinct dark blue staining of the acrosome in ram, boar, and bull also shows additional acrosomal detail. The acrosomal lip is clearly evident and the species specific posterior edge or border area of the acrosome can be distinguished. The equatorial segment just behind the acrosome is well differentiated as a very pale staining zone. The midpiece is clearly stained darker blue than the post-acrosomal area and the principal piece of the tail is lighter blue

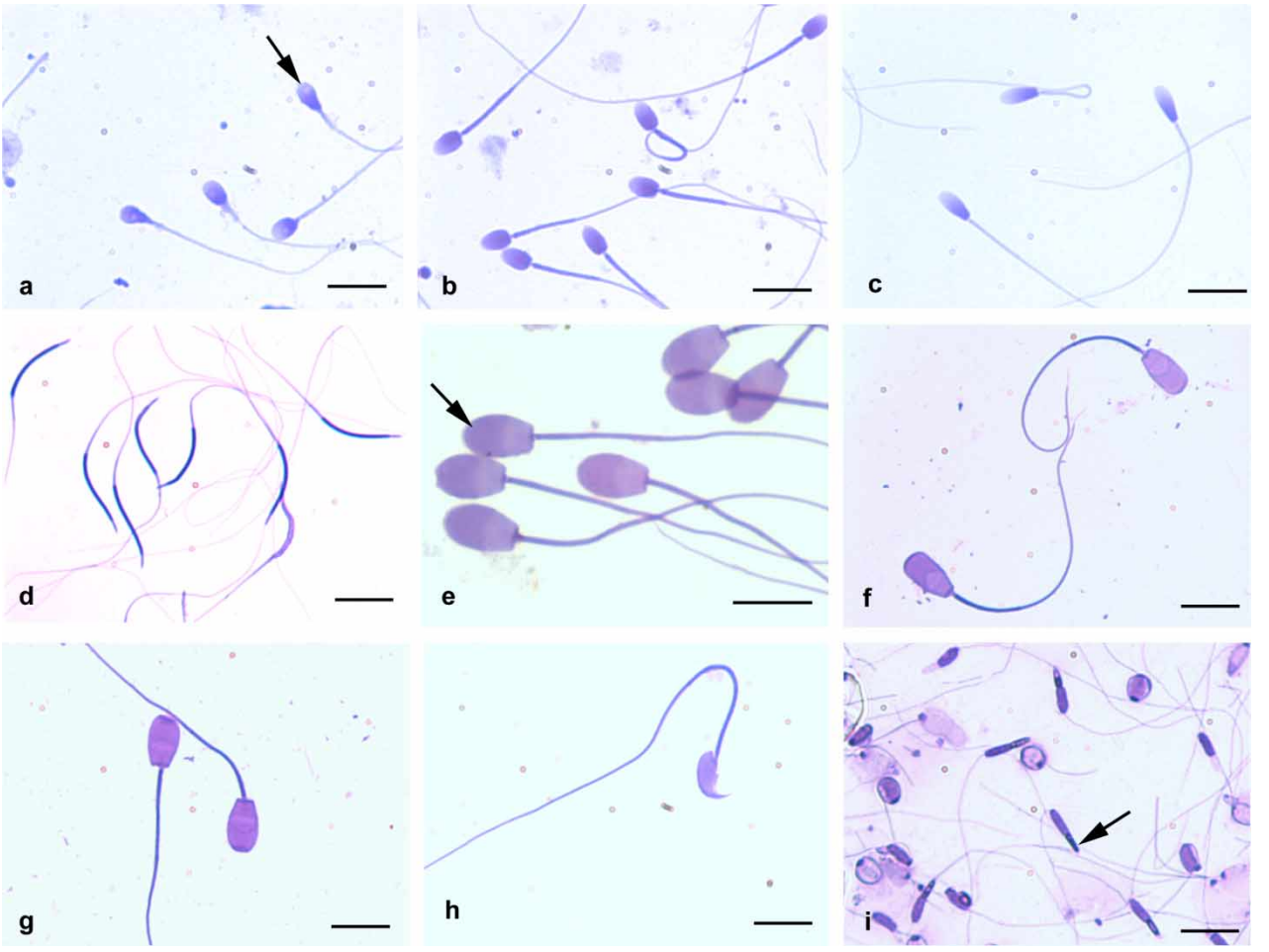

Fig. 1. a–i Sperm morphology of human and eight representative species stained with SpermBlue®. a) Human b) Vervet monkey c) Horse d) Chicken e) Ram f) Boar g) Bull h) Mouse i) Abalone. Each scale bar represents 10 μm and the large arrows show the acrosome in three representative examples.

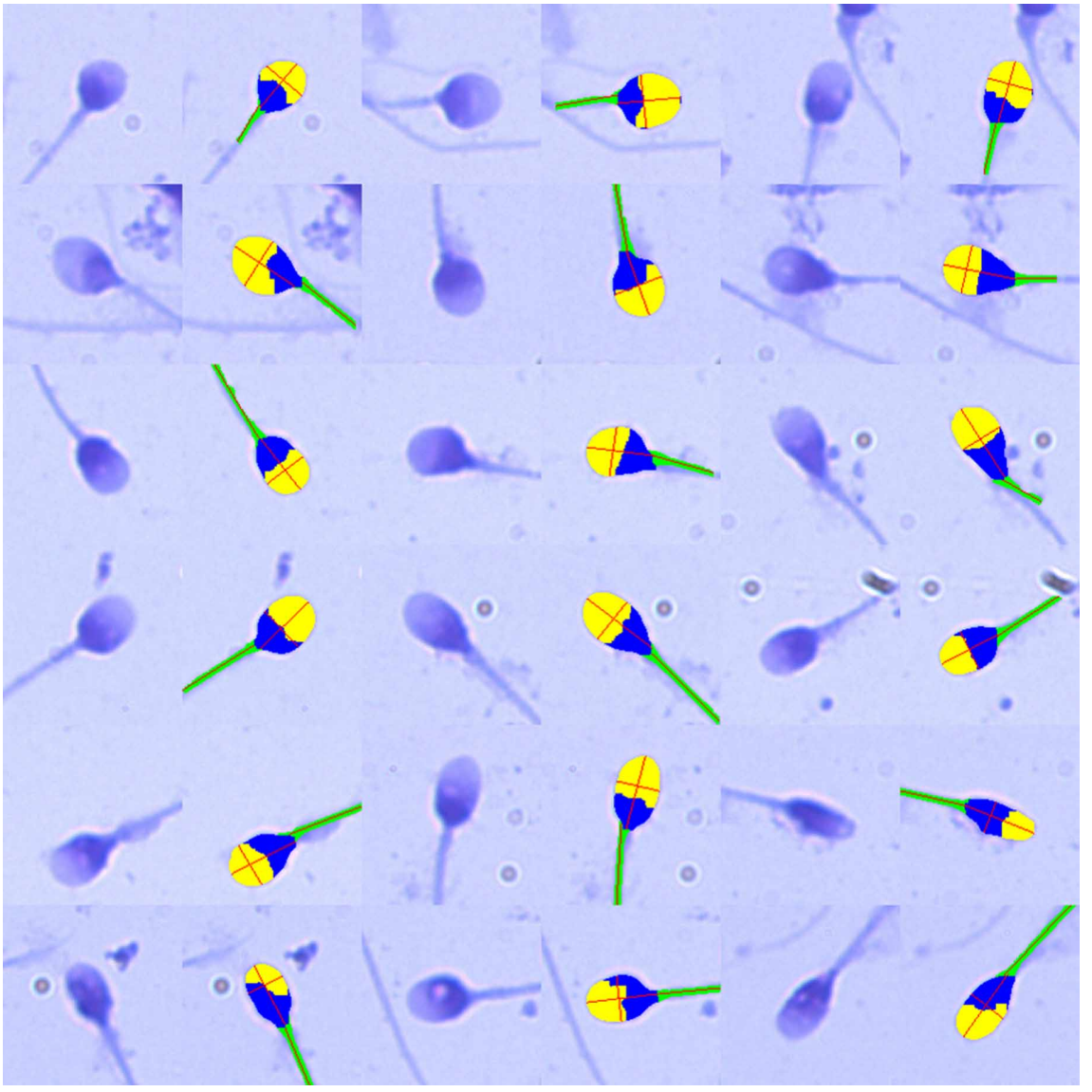

Fig. 2. SCA analysis of human sperm stained with SpermBlue®. The SCA system recognizes the acrosome (yellow), head (blue), and midpiece (green). Each sperm stained with SpermBlue® is shown on the left and to its immediate right the SCA analysis of that particular sperm is shown. Actual measurements of this SCA analysis are given in Table 3.

than the midpiece. Rat and mouse sperm acrosomes are clearly observed, but the acrosome has almost the same blue color as the head (Fig. 1h). Staining pattern 2 features are equally clear for semen and swim-up preparations.

Automated sperm morphology analysis (ASMA)

Figure 2 shows an example of human sperm stained with SpermBlue® and analyzed using the SCA morphology module. For each sperm analyzed, the actual stained sperm is shown on the left side, and on the right side, the analysis of the same sperm (acrosome yellow, head blue, and midpiece green) is superimposed on it (Fig. 2).

Table 3 shows the SCA morphometry analysis of 10 human donors (100 sperm analyzed per donor semen) as well as the human morphometry results reported by other investigators using different stains.

Figure 3 shows an example of boar sperm stained with SpermBlue® and analyzed using the SCA system. The SCA morphology module (version 4.0.0.5) has the option of selecting "acrosome inversion" for type 2 staining patterns. This refers to the fact that the acrosome stains dark (e.g., boar) in contrast to human sperm, where the

Table 3. SCA morphometric analysis of human sperm and comparisons with published data

	This investigation	Schrader et al. (1990)	Leubane-Janse van Rensburg (1998)	Garrett and Baker (1995)	Pérez-Sánchez et al. (1994)
Stains	SpermBlue®	PAP®	PAP®	Shorr®	Hemacolor®
Number of donors	10	45	30	23	186
Length (μm)	4.71 ± 0.25	4.53	4.49	4.35	5.31
Width (μm)	2.78 ± 0.23	2.85	2.57	2.89	3.92
Area (μm^2)	10.50 ± 1.11	8.72	8.61	9.6	14.93
Perimeter (μm)	12.98 ± 0.77	12.7	12.03	12.4	–
Ellipticity	1.72 ± 0.12	–	–	–	–
Elongation	0.26 ± 0.03	–	–	–	–
Roughness	0.78 ± 0.02	–	–	–	–
Regularity	0.98 ± 0.20	–	–	–	–
Acrosome coverage (%)	50.67 ± 6.71	–	–	–	–

acrosome stains pale blue. ''Acrosome inversion'' is used for species showing type 2 staining patterns. If acrosome analysis fails when this option is used, SCA still recognizes and measures all head and midpiece dimensions (Fig. 3).

Table 4 presents the SCA morphometry measurements for boar sperm in this investigation and boar sperm measurements reported by other investigators using different stains. The results of our investigation are preliminary, because a relatively small number of individuals was used. At least 100 sperm, however, were measured for each semen sample from each animal.

Microscopic observations of human and animal sperm studied in our investigation did not show overt signs of either swelling or shrinkage.

Discussion

Papanicolaou® stain, Shorr® stain, Diff-Quik® stain and a group of closely related rapid stains, as well as Spermac® and eosin–nigrosin are the most widely used stains for sperm of humans and animals. The Papanicolaou® and Shorr® staining procedures are complex, involve many chemicals, and are time consuming compared to some of the rapid methods. Some of the rapid staining procedures, however, have the disadvantage that they swell sperm (Kruger et al. 1987) and rapid stains such as Testsimplets® produce inaccurate information compared to Papanicolaou® and Shorr® (Henkel et al. 2007) for evaluating normal sperm morphology. Henkel et al. (2007) also pointed out that values obtained with each staining method differ and this should be taken into account when defining ''normal values'' for a particular laboratory.

SpermBlue® is a rapid staining method (25 min) that involves a simple fixing and staining procedure. Furthermore, no overt signs of swelling have been observed. This is not surprising, because our fixation and staining media were largely iso-osmotic in relation to semen. Sperm readily swell when placed in hypo-osmotic solutions and shrink in hyper-osmotic solutions (Liu and Foote 1998, Rutlland et al. 2003).

SpermBlue® also is suitable for clearly defining the main components of sperm. Sperm morphology assessment requires that sperm be differentially stained, clearly indicating the boundaries of the acrosome, head, midpiece, and tail to evaluate each part. Several studies have reported an association between an increase in morphologically abnormal sperm and reduced fertility in humans (Kruger et al. 1988) and animals (Chandler et al. 1988, Jasko et al. 1990). For example, a positive correlation was found between the fertilization rate in humans and the proportion of sperm with normal head shape and normal acrosome size (40–70% of total head area) (El-Ghobashy and West 2003). Sperm midpiece and tail abnormalities also can be indicators of possible infertility.

Many ASMA systems have been developed during the past two decades. The advantages of these systems are that they reduce the subjectivity of manual sperm morphology assessment and increase the precision and accuracy of these analyses (El-Ghobashy and West 2003). Thus optimization of ASMA is a prerequisite for standardization and inter-laboratory comparisons of sperm morphology. The accuracy of an ASMA system depends on the staining properties of the sperm and the degree of contrast between the sperm and the background (Gago et al. 1998). The problem

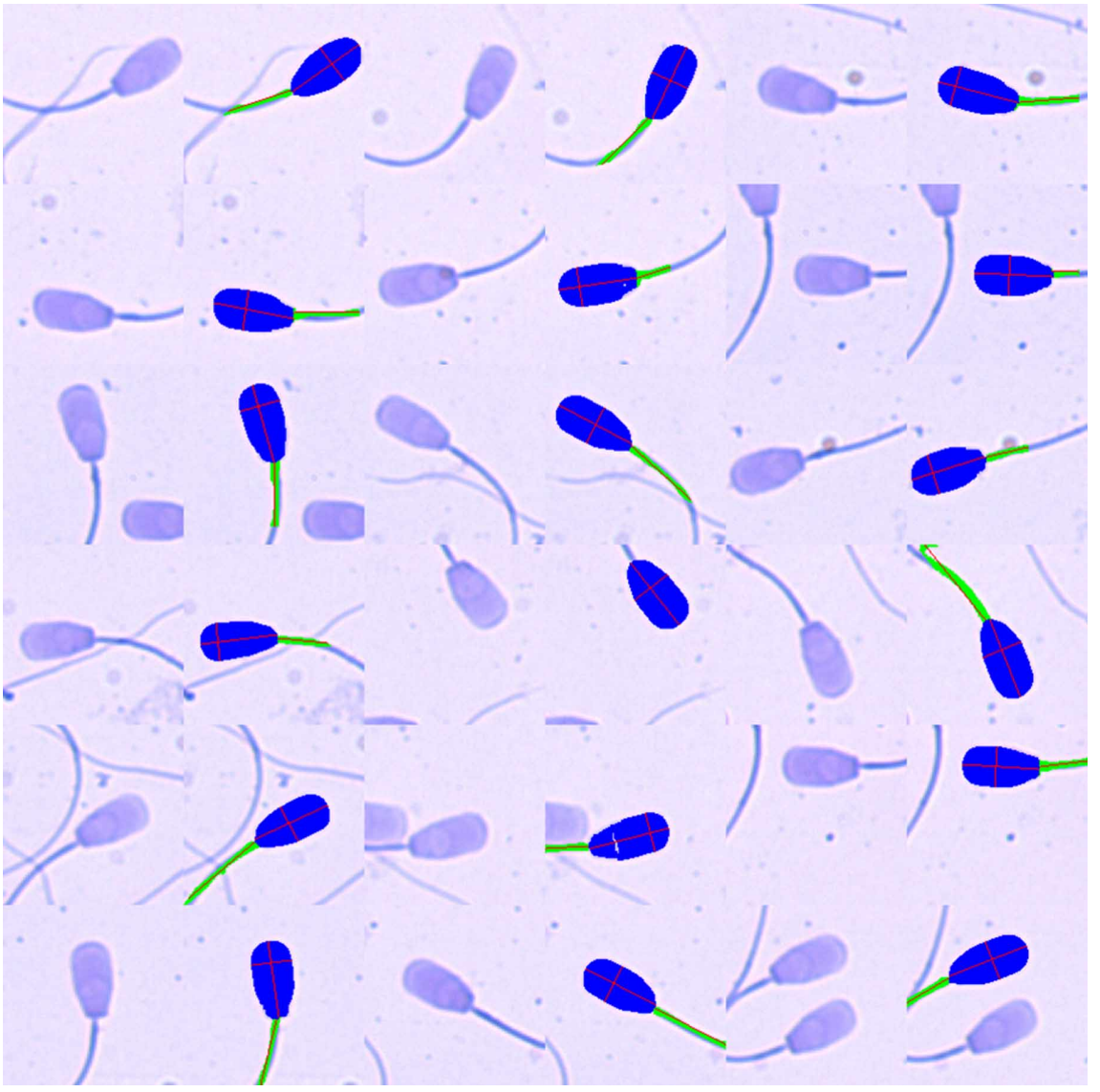

Fig. 3. SCA analysis of boar sperm stained with SpermBlue®. In this analysis, the acrosome was not analyzed (acrosome function switched off). The SCA system recognizes the head (blue), and midpiece (green). Each sperm stained with SpermBlue® is shown on the left and to its immediate right the SCA analysis of that particular sperm is shown. Actual measurements of this SCA analysis are given in Table 4.

with background staining is that if these particles are in contact with any part of the sperm, it masks the true boundaries of the sperm and could result in incorrect analysis or rejection by the ASMA system. Apart from the fact that SpermBlue® stains sperm differentially, another advantage is that no background staining has been found in any of the semen or swim-up smears. Therefore, SpermBlue® largely meets the minimum criteria for ASMA as shown here.

Human sperm morphology and standardization of its assessment have received considerable attention (Mortimer and Menkveld 2001). For domestic animals, however, there are either very limited studies of sperm morphology assessment (Tuset et al. 2008) or the procedures for sperm evaluation have not been standardized. Several studies of sperm staining techniques for domestic animals have reported that the same fixatives and stains have different reactions with the sperm of individual species (Gago et al. 1998, García-Herreros et al. 2006, Hidalgo et al. 2006, Lukaszewicz et al. 2008). It is important, therefore, to find the most suitable staining technique for each species (García-Herreros et al. 2006), but such investigations are time consuming. In the study reported here, the sperm of several mammalian, avian, and invertebrate species were stained with the same method and all

Table 4. SCA sperm morphometry analysis of boar sperm and comparisons with published data

	This investigation	Hirai et al. (2001)	García-Herreros et al. (2006)	Saravia et al. (2007)
Stains	SpermBlue®	Farelly®	Hemacolor®	Eosin and methylene blue
Number of animals	3	12	4	35
Length (μm)	8.96 ± 0.24	9.27	8.22	9.1
Width (μm²)	4.52 ± 0.03	4.66	4.21	4.6
Area (μm²)	34.45 ± 1.05	35.7	29.8	36.2
Perimeter (μm)	23.78 ± 0.55	–	22.62	26.6
Ellipticity	1.98 ± 0.04	–	1.96	2
Elongation	0.33 ± 0.01	–	0.32	0.3
Roughness	0.76 ± 0.02	–	0.73	0.6
Regularity	0.92 ± 0.01	–	0.91	0.9

results were favorable. No adjustment was necessary for either the fixative or stain for the different species, except for the sea water species where SpermBlue® was prepared using filtered sea water. Furthermore, the results of the measurements of the various human sperm and boar sperm head parameters after staining with SpermBlue® fall within the range reported in the literature (Tables 3 and 4).

SpermBlue® can be regarded a universal sperm stain that can be applied to almost any species and can be used also for staining smears made from semen and/or sperm in diluting media. Care should be taken, however, when interpreting the results depending on whether a particular sperm stains according to staining pattern 1 or 2.

It is important for future investigations that SpermBlue® be compared to the well recognized staining methods such as Papanicolaou® and Diff-Quik® for ASMA. This will provide useful information concerning sperm dimensions and differences due to potential swelling or shrinkage artefacts.

SpermBlue® should also be evaluated for use for both normal human sperm morphology assessment in the clinical setting and animal sperm. This also will assist standardization and will simplify inter-laboratory comparisons.

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