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The performance of 10 different methods for the estimation of sperm concentration

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Objective: To evaluate the performance of different methods of sperm counting using latex beads and sperm suspension.
Design: A quality-control study.
Setting: University-based andrology laboratory.
Intervention(s): None.
Main Outcome Measure(s): Counting latex beads six times in two standard suspensions using 10 methods and counting spermatozoa with 4 methods.
Result(s): When air-displacement pipettes were used, the disposable chambers Standard Count (Leja, Amsterdam, Holland), Cellvision (Anthos-ec, Heerhugowaard, The Netherlands), and Cell Vu (Fertility Technologies, Inc. Natick, MA) showed small variation and correct estimation of bead concentration. All the reusable chambers gave relatively large variability, with tendency to underestimate (improved Neubauer; Hawksley, Lancing, United Kingdom) or to overestimate the bead concentration. The use of plunger-displacement pipettes resulted in an overestimation of bead concentration in medium but not in seminal plasma. Counting the sperm suspension using plunger-displacement pipettes indicated that the Bürker hemocytometer overestimated concentration relative to that obtained by Cellvision and Makler Counting Chambers (Sefi Medical Instruments, Haifa, Israel) and that the improved Neubauer presented the lowest variability (7.1%).
Conclusion(s): The improved Neubauer hemocytometer is the standard for sperm counting, though disposable chambers give reliable results as well. If beads are used to evaluate the accuracy of counting chambers, it is recommended to dilute them with seminal plasma. (Fertil Steril® 1997;68:340–5. © 1997 by American Society for Reproductive Medicine.)

Key Words: Sperm counting, quality control, latex beads, male infertility

Counting of sperm concentration is an essential step for the evaluation of male fertility, whether in vivo or in vitro (1). However, no method is agreed on as the standard for the estimation of sperm concentration, and the hemocytometer method recommended by the World Health Organization (WHO) (2, 3) has been criticized (4). The factors that may interfere with sperm counting are numerous and include the type of pipette used (5), dilution and calculation errors, semen viscosity and inhomogeneity, errors in identifying spermatozoa in some computerized systems (6), variations in the depths of the counting chambers of the same brand (Douglas-Hamilton DH, personal communications) (5), differences between the two sides of the same hemocytometer (4), and variations between technicians. Therefore, standardization of the techniques of semen analysis and quality control studies were recommended by WHO (2, 3) and are conducted in many laboratories (7–13). It has been suggested that counting latex beads of a known concentration (14)

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or mixing these beads with spermatozoa and adjusting the sperm concentration according to the sperm/bead ratio (15) may help to standardize sperm counting.

In our study, we compared 10 different methods and/or devices for the microscopic evaluation of sperm concentration using latex beads (14). To investigate whether these results are applicable to the counting of spermatozoa, the performance of some of these methods was evaluated using a homogenized sperm suspension (10).

MATERIALS AND METHODS

Table 1 summarizes the devices, materials, pipette type, and counting methods. All the determinations described below were repeated six times.

Materials Tested

Latex Beads

Bead suspensions. Using all the devices described below, we counted two standard quality-control suspensions of latex beads in liquid medium developed to validate the accuracy of methods of sperm counting (Accu Beads; Hamilton-Thorne Research, Danvers, MA), with known concentrations of 18×10^6/mL (range, 15.5 to 20.5 $\times 10^6$/mL) and 35×10^6/mL (range, 30 to 40 $\times 10^6$/mL), using negative-displacement (air-displacement) pipettes (Eppendorf, Hamburg, Germany). In addition, the DROP method also was performed using plunger-displacement pipettes (DROP1, Finnpipette P.C.R.; Labsystems, Helsinki, Finland).

Beads Diluted With Seminal Plasma. Latex beads diluted with seminal plasma were prepared by mixing equal volumes of bead suspension (35×10^6/mL) and seminal plasma, and the beads were counted in the improved Neubauer hemocytometer (Hawksley, Lancing, United Kingdom) as described below, using plunger displacement pipettes.

Beads in the Pipette Tips. The concentration of latex beads in the used pipette tips was determined after pipetting and discarding 11.5 μL of bead suspension (35×10^6/mL) into each of six plunger- and six air-displacement pipette tips. The outer surface of the pipette tips was wiped gently. Then distilled water (11.5 μL) was drawn into each of the used tips and the concentration of latex beads was determined in the resultant fluid using the DROP method (described below).

Sperm Suspension

A homogenized sperm suspension (10), prepared by mixing equal volumes of a semen pool and 20% Hayem's reagent for erythrocyte counting (Merck, Darmstadt, Germany), was counted in the Bürker and improved Neubauer hemocytometers, the Makler Counting Chamber (Sefi Medical Instruments, Haifa, Israel) and the Cellvision counting chamber (Anthos-ec, Heerhugowaard, The Netherlands) as described below, using plunger-displacement pipettes.

Table 1 Summary Materials and Methods

Device	Material Tested	Bead Concentration	Pipette Displacement	Counting Technique	Diluting for Counting
DROP	Beads	18, 35	Air	Autosperm	No
	Beads from pipette tips	35	Air and plunger	Autosperm	No
DROPl	Beads	18, 35	Plunger	Autosperm	No
Disposable counting chambers					
Standard count	Beads	18, 35	Air	Autosperm	No
Cell Vu	Beads	18, 35	Air	Manual	No
Cellvision	Beads	18, 35	Air	Autosperm	No
	Sperm suspension	—	Plunger	Autosperm	
MicroCell	Beads	18, 35	Air	Autosperm	No
Multiple-use chambers for semen					
2X-CEL	Beads	18, 35	Air	Autosperm	No
JCD	Beads	18, 35	Air	Manual	1:20
Makler	Beads	18, 35	Air	Manual	1:20
	Sperm suspension	—	Plunger	Manual	1:20
Multiple-use hemocytometers					
Bürker	Beads	18, 35	Air	Manual	1:20
	Sperm suspension	—	Plunger	Manual	1:20
Improved Neubauer	Beads	18, 35	Air	Manual	1:20
	Beads with spermatozoa	18	Plunger	Manual	1:20
	Beads in seminal plasma	35	Plunger	Manual	1:20
	Sperm suspension	—	Plunger	Manual	1:20

Bead / Sperm Mixture

A mixture of the sperm suspension and latex beads (18×10^6/mL) was counted in the improved Neubauer hemocytometer as described below, using plunger-displacement pipettes. In addition, the adjusted sperm concentration was calculated using Peters' formula (15): adjusted sperm concentration = (counted sperm concentration/counted bead concentration) × known bead concentration

Methods of Counting

The Autosperm Method

This computer-assisted method for sperm counting (Autosperm; FertiPro, Lotenhulle, Belgium) (16) uses a square drawn on a digitizing tablet equipped with a cursor connected to a microcomputer. A zoom drawing tube is introduced between the objective and the eyepiece of a phase-contrast microscope. The drawing tube permits simultaneous observation of the microscopic field and the digitizing tablet, which is placed beside the microscope. The zoom of the drawing tube is adjusted so that the superimposition of the tablet's square on the microscopic slide corresponds exactly to 100 × 100 μm. The technician screens several squares to identify a minimum of 50 beads or spermatozoa and records them by pressing a button on the cursor. The computer then calculates the concentration according to the number of squares scanned by the technician (16).

Manual Methods

The concentration was assessed manually by counting the number of latex beads and/or spermatozoa in the counting chamber as described elsewhere (17).

Devices for Counting

Disposable Devices

These included the DROP method, in which a fixed volume of 11.5 μL of latex beads was brought on a microscope slide and covered with a 24 × 24-mm coverglass to obtain a preparation with a standard depth of 20 μm (16, 18). The concentration of the preparations was assessed by the Autosperm method.

The Standard Count (Leja bv, Amsterdam, The Netherlands), Cellvision, and MicroCell Counting Chambers (Conception Technologies, Inc., La Jolla, CA) are counting chambers with fixed preparation depth of 20 μm. The counting chamber is filled with 5 μL (Standard Count, Cellvision) or 3 μL (MicroCell) of the fluid to be tested. The fluid flows into the counting chamber by capillary force. The concentration of the preparations was assessed by the Autosperm equipment (see above).

In the Cell Vu counting chamber (Fertility Technologies, Inc., Natick, MA), the glass cover is equipped with a grid, and the glass slide has a rim, allowing a fixed volume of fluid to be trapped between it and the glass cover. The concentration of beads was assessed manually by counting their number inside the grid.

Reusable Devices

The 2X-CEL counting chamber (Hamilton-Thorne Research) has a reusable frame into which fits a disposable glass slide; a jointed magnetic lock compresses the coverglass against a rim on the slide, forming two compartments with a fixed depth of 20 μm. The concentration of the preparations was assessed by the Autosperm equipment (see above).

For the Bürker, improved Neubauer, and JCD (JC Diffusion International, Gauville, France) counting chambers, a 1:20 dilution was made of a thoroughly mixed test fluid by adding 50 μL of that fluid to 950 μL of Hayem's reagent. After a thorough mixing, a drop of 20 μL in volume was transferred to the counting chamber.

The Makler Counting Chamber (19) was loaded with 5 μL of undiluted test fluid. The concentration was assessed manually.

The same hemocytometer/counting chamber was used for all determinations in each method.

Statistics

Statistical analysis made use of coefficient of variation (CV), Wilcoxon test, and Box and Whisker plots (Medcalc; Medcalc Software, Mariakerke, Belgium) (20). The Bland and Altman plot (21) was used to test the agreement between the different methods of counting. In this method, the difference between the results of two methods is plotted against their average. The two methods are considered to be in good agreement if the mean difference ± 1.96, its SD, is not clinically important.

RESULTS

Counting the Latex Beads

Counting the Bead Suspensions

The Box and Whisker plots with dots (Figs. 1 and 2) show the bead concentration determined by the 10 methods used, whereas Table 2 shows the CVs for these methods.

The use of the air-displacement pipette in the DROP method produces little variation but under-

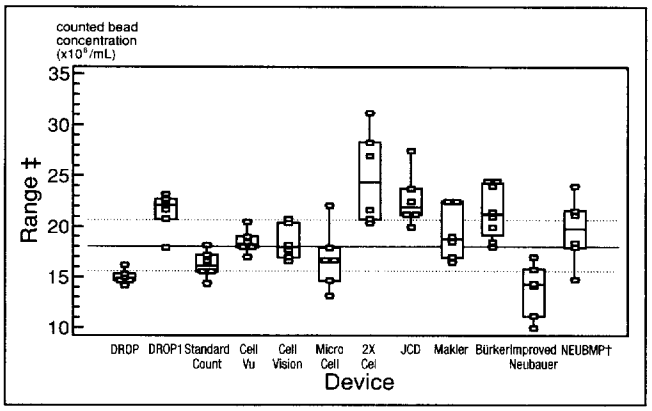

Figure 1 Box and whisker plot with dots (indicating individual values) showing results of counting latex beads in a concentration of 18 × 10^6/mL. † Beads in mixture (with spermatozoa), using a plunger-displacement pipette and an improved Neubauer hemocytometer. ‡ Range of bead concentration specified by manufacturer.

estimates the concentration of beads. The plunger-displacement pipette DROP1 clearly overestimates the concentration, both in the 18 and the 35 × 10^6/mL reference bead suspensions.

Standard Count, Cellvision, and Cell Vu performed equally well, with small variation and correct estimation of bead concentration. MicroCell yielded a good median value but large variation.

All the reusable chambers yield relatively large variability, with four of them tending to overestimate concentration (2X-CEL, JCD, Bürker, and Makler) and one to underestimate (improved Neubauer). For the 2X-CEL counting chamber, the bead concentration in the compartment close to the joint of the chamber was higher than that in the other compartment (data not shown). An angle could be observed visually between the frame of the chamber and the magnetic lock when the chamber was closed.

Counting the Beads Diluted With Seminal Plasma

Counting bead suspension mixed with seminal plasma shows a correct estimation of beads' concentration (Fig. 3) and a CV of 14.3%.

Counting the Beads in the Pipette Tips

The concentration of latex beads at the inside of the used tips was significantly higher in the air-displacement pipettes (median, 5.25 × 10^6/mL; range, 3 to 7 × 10^6/mL) compared with that in the plunger-displacement ones (median, 1.0 × 10^6/mL; range, 0.5 to 1.5 × 10^6/mL; $P < 0.01$, unpaired Wilcoxon test).

Counting the Sperm Suspension

Bland and Altman graphs (21) (not shown) and Box and Whisker plots with dots (Fig. 3) indicate that the Bürker hemocytometer overestimated sperm concentration when compared with the other three methods, which showed good agreement between their results. Table 2 shows the CVs for these methods, the highest being that of the Makler chamber.

Counting the Bead/Sperm Mixture

When the mixture of bead suspension and sperm suspension was counted (plunger-displacement pipettes were used), a slight tendency toward overcounting the latex beads was noted (Fig. 1), the median sperm concentration did not differ significantly when compared with sperm counting in improved Neubauer without beads (Fig. 3), and a high CV was noted for both the bead and sperm concentrations (11.5% and 16.5%, respectively). Adjusting the sperm concentration according to Peters' formula (15) resulted in the underestimation of sperm concentration and a CV of 10.1%.

DISCUSSION

In this study, we compared the performance of 10 commonly used methods of sperm counting to estimate the concentration of latex beads of a known concentration (14). Our data indicate that, for counting latex beads in medium and using air-displacement pipettes, the reusable devices show unacceptably high CVs and/or tended to overestimate or underestimate the bead concentration. On the other hand, most of the disposable chambers evaluated, namely Cell Vu, Cellvision, and Standard Count, were acceptably reproducible and yielded a correct estimation of the concentration of beads. The large

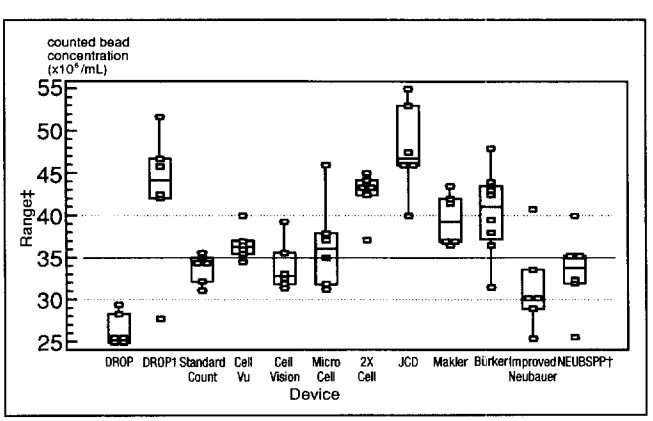

Figure 2 Box and whisker plot with dots (indicating individual values) showing results of counting latex beads in a concentration of 35 × 10^6/mL. † Beads diluted in seminal plasma, using a plunger-displacement pipette and an improved Neubauer hemocytometer. ‡ Range of bead concentration specified by manufacturer.

Table 2 Counting Devices: Coefficients of Variation*

	DROP	DROPl	Standard Count	Cell Vu	Cellvision	MicroCell	2X CEL	JCD	Makler	Bürker	Improved Neubauer
Bead suspension (18×10^6/mL)	4.6†	9.0‡	8.3†	6.4†	9.5†	18.0†	18.2†	11.8†	13.6†	12.2†	19.5†
Bead suspension (35×10^6/mL)	7.2†	19.0‡	5.2†	5.1†	8.8†	14.7†	6.6†	11.3†	7.8†	12.7†	16.6†
Sperm suspension	—	—	—	—	11.7‡	—	—	—	26.3‡	4.3‡	7.1‡
Counting technique	Autosperm	Autosperm	Autosperm	Autosperm	Autosperm	Manual	Autosperm	Manual	Manual	Manual	Manual

* Values are percentages.
† Air-displacement pipettes were used.
‡ Plunger-displacement pipettes were used.

variability noticed with MicroCell might be explained by the small volume of fluid used, which may not be representative of the entire sample. Both the overcounting and the high variability observed with 2X-CEL may be, at least in part, due to differences between the two compartments of the slide. This might result from unequal pressure of the magnetic lock on the cover slide, since an angle was observed between the former and the chamber frame.

An interesting finding is the overestimation of the concentration using plunger-displacement pipettes DROP1 versus underestimation using air-displacement pipettes in the DROP method in bead suspension. These differences cannot be explained by differences in the volume of the pipetted fluid as assessed by weighing (6). Our finding that the number of latex beads in the used air-displacement pipette tips was high might explain the underestimation using the former method, but not the overestimation in the latter. The recommendation of using plunger-displacement pipettes for sampling semen was based on their low variability in sperm counting compared with the air-displacement ones (5). Because there is no air dead space between the sample meniscus and the plunger in the former, the aliquot volume is fixed and is not affected by sample viscosity. In the air-displacement pipettes, increased viscosity may induce a disequilibrium between the air pressure in the large dead space inside the pipette body and that outside the pipette, as well as a coating of the inside of the sampling tip (5).

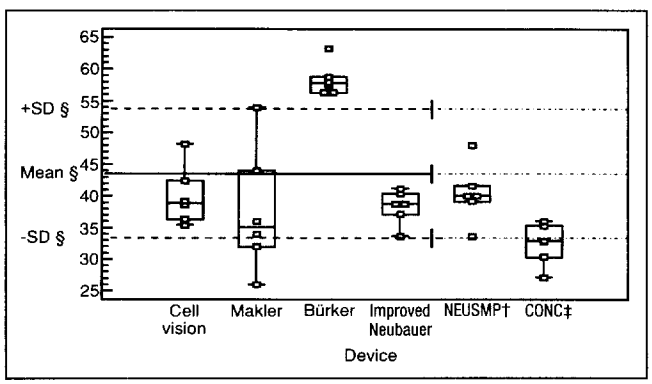

Figure 3 Box and whisker plot with dots (indicating individual values) showing sperm concentration (plunger-displacement pipettes used). † Spermatozoa in mixture (with beads), using an improved Neubauer hemocytometer. ‡ Adjusted sperm concentration according to Peters' formula (15). § Mean ± SD of sperm concentration for four methods together (Cellvision, Makler, Bürker, and improved Neubauer).

On the other hand, our data on sperm counting indicate that the improved Neubauer hemocytometer, Makler, and Cellvision counting chambers show good agreement but that the Bürker chamber overestimates sperm concentration. However, the improved Neubauer hemocytometer should be considered the standard for sperm counting because it showed the lowest CV, in agreement with previous studies (7) and the recommendations of WHO (2, 3).

Counting beads or counting spermatozoa can lead to different conclusions regarding the accuracy of counting chambers, as is evidenced by the results using the improved Neubauer hemocytometer (plunger-displacement pipettes). This might be due to the effect of seminal plasma. Our data indicate that mixing the bead suspension with the sperm suspension ($\frac{1}{4}$-strength seminal plasma) leads to a slight overestimation of the bead concentration, whereas mixing the bead suspension with seminal plasma (with resulting $\frac{1}{2}$-strength seminal plasma) leads to a correct estimation of bead concentration. Therefore, if latex beads are to be used to evaluate the accuracy of counting chambers for sperm counting (14), the latex beads should be diluted with seminal plasma and plunger-displacement pipettes should be used. The data on mixing the beads with spermatozoa indicated that adjusting the sperm concentration according to sperm/bead ratio (15) yields acceptable variability but tends to underestimate the sperm concentration. A better alternative may be counting a standardized suspension of spermatozoa.

In conclusion, the Cell Vu, Cellvision, and Standard Count methods give the best results in terms of accuracy and reproducibility for counting the standard beads. When reusable chambers are used, the improved Neubauer chambers seem to be the most

reliable for sperm suspension and for latex beads, provided the latter are mixed with seminal fluid. The results of the Cellvision counting chamber with sperm suspension are comparable to those of the improved Neubauer hemocytometer.

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