

Automation of Semen Analysis Using Flow Cytometer in Comparison with Manual Methods

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In order to standardize techniques and limit the effect of human factors on the results of analyses of biological fluids, automation seems to be mandatory. In an attempt to automate semen analysis, computer assisted sperm analysis (CASA) system has been developed, however its use is still limited and its practical applications have many criticisms. In a trial to automate semen analysis, this study aimed to evaluate the usefulness of flow cytometer in the detection of some seminal parameters in comparison with the traditional manual methods. Isolated spermatogenic cells and isolated sperms from semen and EDTA blood of volunteers were analyzed by flow cytometer in order to define their respective regions. Ejaculates of 28 male patients were subjected to routine semen analyses, leucocytes detection by peroxidase test and monoclonal antibody CD53 using flow cytometer after preparation of the patients' semen samples for flow cytometric analysis. A highly significant correlation ($r=0.96$, $p=0.001$) of absolute neutrophils (pus cells) detected by peroxidase versus flow cytometer using CD53 monoclonal antibody. A poor correlation ($r=0.39$, $p=0.035$) of sperm counts assessed by manual technique and flow cytometer and a spurious sperm counts of 1.08 million/ml detected by flow cytometry in azoospermic patients. Flow cytometer could be used for the assessment of pus cells in semen but seems to be non reliable for the assessment of sperm count if gating depend on sperm size and granularity alone.

Infertility is an emerging medical and social problem. Semen analysis is necessary for studying male infertility. Semen analysis, including sperm count, motility, viability and morphology, immature sperm cells and leucocytes (WBCs) count, is a commonly requested analysis in clinical laboratory, as the first step of evaluating the male factor. The average sperm in normal fertile males is $70-80 \times 10^6$ sperms/ml, but this finding is not indicative of the minimal requirements for fertilization (Macheod and Wang 1979). Oligospermia corresponds to a sperm concentration of less than 20×10^6 sperms/ml. Immature sperm cells (also called round cells) don't exceed 5×10^6 cells/ml, and leucocytes count don't exceed 1×10^6 leucocytes/ml (WHO, 1992) in normal ejaculate.

High leucocytes count in semen has been associated with genital tract infection, poor semen quality and in vitro fertilization and embryo transfer failure (Cohen et al., 1985;

Talbert et al., 1987; Wolf, 1995). WBCs are usually counted manually and microscopically, but these methods are inappropriate for counting low numbers of WBCs in semen. Moreover, direct counting of round cells is highly inaccurate because WBCs can't be distinguished from immature germ cells. WHO recommends the peroxidase test but it detects only granulocytes and not other WBCs types (Endtz, 1974). Immunohistological staining employing monoclonal antibodies against all specific WBCs subpopulations is now considered by many to be the gold standard of semen WBCs detection but it is expensive, time consuming and not standardized (Harrison et al., 1991; and Kiessling et al., 1993).

In an attempt to standardize the manual semen analysis, computer assisted sperm analysis (CASA) system has been developed. In CASA system, a series of videotaped microscopic images are digitalized, and computer software programs are used to

evaluate sperm count, motility and morphology. Current CASA system is not suitable for evaluating semen samples for many patients, spurious counts of 3.6×10^6 sperms/ml were reported in azoospermic donors, sperm concentration reportedly was over estimated by 30% (Bar-Chama and Lamb, 1994) and CASA presented the poorest performance of sperm concentration in the range around 5×10^6 sperms/ml (Knuth et al., 1987), a critical limit for a pregnancy (WHO 1992). Moreover, CASA system is dedicated only to sperm analysis, very expensive and not suitable for general laboratory having small batches of semen analysis. The purpose of this study was a trial to automate some semen parameters by flow cytometer and to compare it with the manual methods.

Subjects and Methods

This study included twenty-eight randomly selected adult males who were attending the Andrology clinic, Suez Canal University Hospital for evaluation of their fertility potential. It also included five volunteers, three of them were normal fertile males and two infertile males with non-obstructive azoospermia, who were known through previous testicular biopsies to have maturation arrest with absence of mature sperm cell and presence of reasonable count of sperm precursors in their semen samples. Patients' ages ranged from 22 to 55 years (mean 33 ± 2.3 years). After taking their consent, patients were subjected to history taking, clinical examination and were asked to obtain their semen samples.

Semen samples were collected in the hospital by masturbation into a sterile container after 3-4 days of sexual abstinence. Routine manual semen analysis including seminal volume, pH, count using Mackler counting chamber, and progressive motility were performed according to WHO recommendation (WHO, 1992).

For the detection of leukocyte count, peroxidase test was performed by the Endtz protocol (1974). A stock solution was prepared by mixing 50 ml distilled water with 50 ml 96% ethanol and adding 125 mg Benzidine (Sigma, Milan, Italy). The working solution was obtained by adding 5 μ l 30% H_2O_2 to 4 ml of stock solution. Twenty μ l of working solution were mixed with 20 μ l of liquefied semen in a small test tube. After incubation for 5 minutes at room temperature, 20 μ l of working solution was mixed with 20 μ l of phosphate

buffered saline (PBS), then 10 μ l were placed in a haemocytometer, and peroxidase-positive cells i.e. dark brown round cells, were counted.

For preparation of isolated sperms and sperm precursors, an aliquot of 1.5 ml semen samples was treated with standard percoll (Sigma, Saint Louis, Missouri, USA) gradient separation procedure (Diemer et al., 1994), each of these aliquots was added with 1 ml of percoll with concentration 90% (900 μ l percoll + 100 μ l F10), 70% and 40% (total volume: 3 ml) and centrifuged at 500x g for 20 minutes. After centrifugation the sample was washed twice with 3 ml of PBS, pH 7.4 and further centrifuged at 300x g for 10 minutes. The pellet was resuspended in 1 ml of PBS, pH 7.4. The same technique was done for semen samples with total absence of mature sperm cells and large amount of spermatogenic cells (at least 1×10^6 cells/ml.).

In order to detect the leucocytes region in the dot plot, an EDTA blood sample was used. Aliquots of 100 μ l whole blood was incubated with 20 μ l of CD53 fluorescein isothiocyanate (FITC)-conjugated (Sector, Oxford, UK), for 15 minutes in dark at room temperature. Lysing the RBCs was done by adding 2 ml of the lyse solution (Becton Dickinson, USA) for 10 minutes at room temperature. Lysis was followed by three times wash with 3 ml PBS buffer, the mixture was centrifuge at 300 xg for 5 minutes. The final pellets were resuspended in 300 μ l PBS buffer and were analysed by flow cytometer. This procedure is adopted from Beckton Dickinson Immunofluorescence Staining of Human Cells by Lysed Whole Blood Method online protocols.

For flowcytometric analysis, aliquots of semen samples (500 μ l) were added to 500 μ l PBS, pH 7.4 and centrifuged at 300 xg for 10 minutes, the supernatant was discarded. The remaining pellet was resuspended in 100 μ l of PBS and 20 μ l of CD53 (FITC)-conjugated. As negative control, samples were stained with isotype monoclonal antibodies FITC conjugated (Becton Dickinson) and unstained samples were also used to identify the autofluorescence background of the samples. The samples were incubated for 30 minutes in room temperature then washed three times with 3 ml PBS buffer, the mixture was centrifuge at 300 xg for 5 minutes, the supernatant was discarded. The pellet was resuspended in 300 μ l according to the method described by Ferrara et al 1997.

Samples acquisition was then done using FACScalibur (Becton Dickinson) equipped with 15mW air-cooled argon-ion laser. FL 1(FITC) signals were detected through 515/30 nm band pass filter. The Cell Quest software was used for the samples acquisition. 20,000 events were acquired from each sample. The samples containing isolated sperms, isolated

spermatogenic cells and isolated leucocytes were run first to identify the regions of sperms, spermatogenic cells and leucocytes, using the forward and side scatters. Both neutrophils and spermatogenesis cells shared the same light scattering properties and differentiation was made by using CD53. Neutrophils are CD53 positive while spermatogenic cells are CD53 negative. The same maneuver was used to differentiate between the sperms and lymphocytes that both shared the same light scattering properties. Sperms are CD53 negative while the lymphocytes are CD53 positive. Having the four regions set and further dot plot for the full identification of the four targeted populations (sperms, spermatogenic cells, neutrophils and lymphocytes), the studied samples were run, analyzed and the relative counts of sperms, neutrophils, lymphocytes and spermatogenic cells were estimated. The events accumulated in the lower left corner, corresponding to debris, were excluded from analysis.

For calculation of the absolute sperm counts in semen samples, the following formula was applied:

$\% \text{ of relative count of sperms} \times \text{absolute count of neutrophils by peroxidase test} / 100.$

While, the absolute count of neutrophils was calculated by applying the following formula:

$\% \text{ of relative count of neutrophils} \times \text{absolute count of sperms per ml} / 100.$

Statistical analysis

Statistical analysis was carried out using SPSS computer software. Data were expressed as mean \pm SD. Parametric data were analyzed by t test of unpaired samples. Spearman correlation analysis was used when required. Results were considered significant at 5% level of probability.

Results

Seminal parameters for patients were summarized in table (1).

Table 1. Summary of seminal parameters in the studied semen samples.

Variable	No of samples	%	
Semen volume	Less than 1 ml	2	7.2
	1-2.5 ml	16	57.1
	3-4.5 ml	6	21.4
	More than 4.5 ml	4	14.3
Sperm motility	Normal motility	19	67.9
	Asthenospermia	7	25
	<u>Azoospermia nonmotile</u>	<u>2</u>	<u>7.1</u>
Sperm count	Normal count	23	82.1
	Oligospermic	3	10.7
	Azoospermic	<u>2</u>	<u>7.1</u>

Flowcytometric analysis, acquiring 20,000 events allowed a reliable enumeration of the leucocytes even at low concentrations. On the sole basis of the forward versus side scatter it was impossible to distinguish sperms from leucocytes. The use CD53 was a must to identify the leucocytes populations together with the side scatter. Together with the help of the isolated leucocytes sample, the regions of the leucocytes was identified. Using the light scatters properties and CD53 positivity, the neutrophils and spermatogenic cells were separated, the same was applied on

distinguishing between the sperms and lymphocytes (figure 1, 2, 3). The results of seminal parameters as detected by flowcytometry are summarized in table (2).

Table 2: Relative counts of seminal parameter as detected by flow cytometer.

Variable (%)	Mean \pm SD	Range
Sperm	79.19 \pm 19.89	13-93 %
Neutrophils	0.78 \pm 1.1	0.01-3.9 %
Lymphocytes	0.80 \pm 1.9	0.03-7.4 %
Spermatogenic cells	1.75 \pm 1.81	0.28-6.3 %

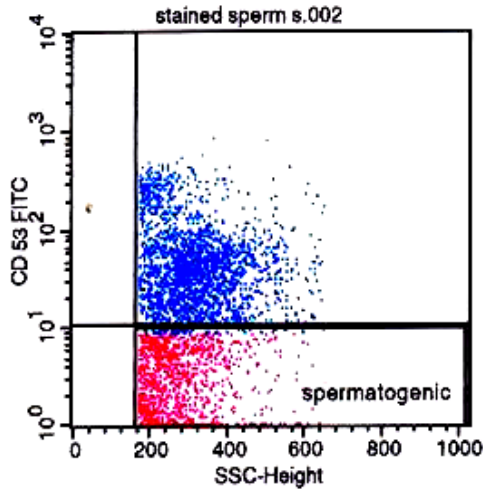


Figure 1. Flow cytometric analysis of semen sample shows high neutrophilic and high spermatogenic cell counts.

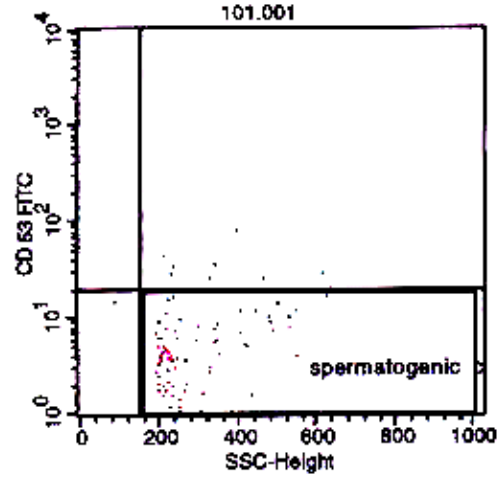


Figure 3. Flow cytometric analysis of semen sample shows low neutrophilic count.

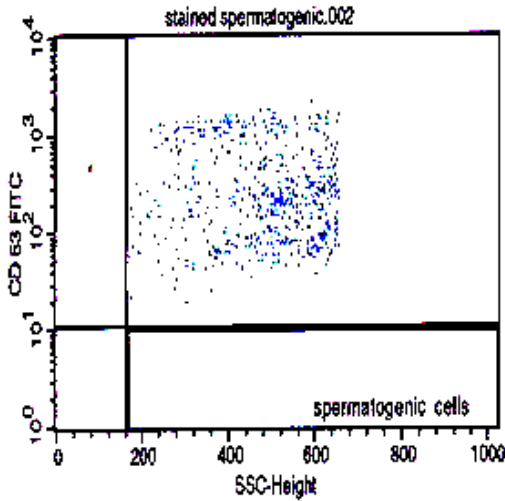


Figure 2. Flow cytometric analysis of semen sample shows low spermatogenic count.

The results that compare flowcytometric method and manual semen analysis with regards to sperm count and pus cells are shown in table (3). A positive correlation was found between concentrations of pus cell as detected by the peroxidase test and that detected by the flow cytometer ($r= 0.966, p= 0.001$), while a poor correlation was found between sperm counts as detected by the flowcytometer and by using Makler chamber ($r = 0.399, p < 0.035$).

Table 3: Comparison between manual methods and flow cytometry for detection of sperm count and pus cells of semen samples.

Variable	Sperm count (million/ml)		Pus cells (cell/ml)	
	Makler chamber	Flow cytometer	Peroxidase test	Flow cytometer
Mean	37.3	93.35	206923	142585
Std Deviation	21.3	49.13	369365	184574
Range	0-67	1.08-263.1	30,000-1,440000	57,000-740,000

Discussion

Although automation of semen analysis, based on image analysis system, is pushing its way into the laboratory world, yet its high costs confined it to the specialized centers (Amamm et al, 1993). On the other hand the manual technique is tedious, objective and not standardized; it is hindered by its imprecision, related to high variability of microscopic evaluation (Ferrara et al, 1997). This urged the search for a new technique allowing flow cytometer to enter the andrology laboratory. It was first used to detect anti-sperm antibodies (Ke et al, 1995) and to study sperm chromatin structure (Pasteur et al, 1994). The advantage of flow cytometer is that many millions of cells can be analyzed in a few seconds and on cell to cell basis.

In this study a poor correlation was observed in sperm count analysis between manual versus flowcytometric techniques that may be attributed to the aspherical shape of human sperm which increases the variability in light scatter and fluorescence signals as a result of inconsistency of particle orientation with respect to the excitation beam and sensing device leading to the presence of multiple signals related to the different orientation of one sperm through the light beam (Steen, 1985). Another reason for the poor correlation between flow cytometer and manual method is the low intensity light scatter signals observed in the sperm region during evaluation of semen samples from azoospermic patients. In this study there was a spurious sperm count noticed in azoospermic patients (1.08 million/ml), these signals may be due to the presence of some protein aggregates as azoospermia is associated with altered pH values and other biochemical abnormalities in seminal fluid, easily inducing protein aggregation (Evenson et al, 1983). In addition aggregates of inorganic elements can be observed in semen samples of azoospermic

patients affected by some prostatic diseases (Wilson, 1980).

On the other hand Ferrara et al (1997) reported a better correlation between flow cytometric analysis and optical microscopy evaluation. However, this could be contributed to the fact that they used closed flow chamber Xenon lamp equipped flow cytometer in which the flow velocity of the sample jet can be reduced below 3m/sec, increasing the sensitivity of the machine and prevent the multiple signal related to the different orientation of sperm through the light beam. All flow cytometers employ some type of nozzle with hydrodynamic focusing in order to induce a laminar flow; the cell follows the narrowest possible path through the measuring region. The water flowing through the nozzle is usually supplied from a pressurized container, while the sample is introduced through a thin tube along the center of the stream in the nozzle. In order to maintain a laminar flow in air, the flow velocity of the jet in air open flow chamber used in this study cannot be reduce below a critical limit, which is about 3m/sec.

Meanwhile Eustache et al (2001) and Christensen et al (2004) demonstrated that human sperm concentration can be accurately assessed by the flow cytometer. However they used new method of sperm detection, combining gating on cell size, granularity, DNA staining by propidium iodide, and direct counting of sperms by using fluorescent microspheres (beads).

A good correlation was found between pus cells detected by the peroxidase and CD 53. This is consistent with other reports (Ferrara et al 1997, Ricci et al, 2000). However, controversial result reported by Barralt et al, (1991); and Diemer et al (1994) could be attributed to the use of different monoclonal antibody like; CD45 and different immunocytochemistry technique.

In conclusion, this study demonstrates that Flow cytometer is a rapid and accurate technology for the detection of leucocytes by the use of only one monoclonal antibody; anti-CD53. Yet it seems to be non reliable for the assessment of sperm count if gating depend on sperm size and granularity alone.

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