Review Article

Semen Analysis: An Update

Tummidi Santosh*

Department of Pathology, AIIMS, Kalyani, W.B.

DOI: 10.21276/APALM.3229

***Corresponding Author**:
Dr Tummidi Santosh
born_vss@yahoo.co.in

Submitted: 02-Nov-2022
Final Revision: 22-Nov-2022
Acceptance: 03-Jul-2023
Publication: 02-Aug-2023

Abstract

Male factor for infertility is responsible in 50% of cases. In recent times there has been a significant deterioration in semen quality due to various lifestyle factors. In the year 2021 WHO has released the 6th ed. of semen analysis manual. The main highlight of the edition is to provide a step-by-step guide for the evaluation of the semen sample for calculation and interpretation of results.

Keywords:

Semen, Infertility, The Male, Basic examination, Index.

Introduction

Semen analysis has been used for centuries for the evaluation of the male infertility. The basic parameters includes the physical, biochemical and morphological parameters. WHO had five editions for reporting and interpretation of semen. The recent 6th edition has been the most upgraded version including various extended examinations for incorporation of CASA and research parameters. [1-3] Semen examination not only helps for the evaluation of the male genital tract but also helps in monitoring the trend of semen quality over the years, treatment response and sub-fertility cases. It has also been used for the detection of efficacy of male contraception. [4]

The article highlights the various test parameters used in reporting of a semen and techniques for doing the procedure.

Purpose of semen analysis:

- To describe the procedure for semen analysis to investigate male reproductive organ function,
- Follow-up of andrological treatment,
- Assessment of male contraceptive,
- Choice of assisted reproductive treatment modality
- Epidemiological and environmental studies.

Responsibility: Technician and Pathologist

Method: [1, 3]

- Patient is informed regarding the required period of abstinence (min 2 days – max 7 days) and the method to collect the semen (by masturbation) into the sterile container provided after cleaning the penile area with soap and proper washing. Not recommended: Coitus interruptus, special condoms/latex condoms, lubricants.[5]
- Patient is advised to pass urine before sample collection.
- If the specimen is collected at home, inform the patient ensuring that the sample has been collected properly, completely and immediately noting the time of collection maintain a temp between 20-37°C while transport.
- In case the sample could not be collected or is incorrectly collected, the test is postponed for the abstinence period and the bill is not raised.

Test procedure:

- Time of receiving the sample, the examination time are to be noted.

Macroscopic examination

- Measure the volume of ejaculate, color: Weight an empty container with lid, post collection of sample weight the container with lid again. The difference in weight is the volume of sample collected considering the fact that the density of a semen is 1g/ml. Freshly ejaculated semen is a highly viscid, opaque, white/gray white (slight yellow-long abstinence, reddish-RBCs present, thick yellow-jaundice/drugs) coagulum will spontaneously liquefy to form a translucent, turbid, viscous fluid which is mildly alkaline. [Table 1] High volume: infection, Low volume: retrograde ejac/androgen def.

Table 1: Flow time for processing of semen sample.

Flow time	Procedure
Within 5 mins	Placing the specimen container on the bench or in an incubator (37°C) or orbital mixer for liquefaction. Measure the volume.
Between 30 - 60 mins	Assessing liquefaction, appearance of the semen, volume, pH, assessing wet mount microscopic appearance, motility and the dilution required for assessing sperm conc., sperm vitality, smears for morphology, MAR, anti-sperm abs.
Within 3 hrs	Microbiological analysis (If any)
After 4 hrs	Stain and assess smears for sperm morphology. Biochemical analysis of accessory glands.

Note:

- If the sample on collection is viscous, totally clear and colorless it could be pre-ejaculate from Cowper glands only, repeat sample is advised with abstinence.
- Toxicity testing of container: Collect high sperm conc. with good sperm motility into known safe containers (control). Then transfer some amount in to new containers (test). Examine the results from both containers (C&T) after 1hrs and 4hrs.

Liquefaction:

Liquefaction should be completed within 30 min. (no longer than 60 minutes). It is important to distinguish persistent viscosity from delayed liquefaction. Place the specimen container in an incubator (37°C) or orbital mixer/ 15-30 sec swirling for liquefaction. [3, 6]

Note:

- Induce liquefication by Dulbecco's PBS or passing through 18Gz needle with the syringe, bromelain digestion. These may alter biochemical, motility and culture results. [3]
- The time taken to liquefy to be mentioned in the form (if liquefaction is prolonged >60 mins, include in the final report). [6]

Viscosity

Aspirate into a wide bore disposable pipette (1.5mm dia.) and allowed to drop by gravity or by inserting a glass rod into the sample, if it falls as discrete drops (Viscosity is normal). If the length of the thread formed is > 2 cm then the sample is abnormal. Observe and enter in the form as normal/increased. [7]

- Odour: Strong odour can be of clinical significance.
- pH of semen is checked with wide range of pH papers (6.0-10.0) within 30-60 mins of collection. Report as alkaline/acidic.

Note:

- A low pH is of significance- indicating low alkaline seminal vesicular fluid or contamination from urine. (Alk-seminal vesicle, Acidic Prostrate) [3]

Chemical analysis for fructose by semi-quantitative/ seliwanoff's (50 mg resorcinol + 33 ml conc. Hydrochloric acid + make volume up to 100 ml with D.W). Take 3 ml of seliwanoff's reagent and add to it 3 drops of semen, heat it and look for color change at bottom of a tube in 30 sec. A reddish-orange color indicated the presence of fructose > 2mg%.

Motility

After liquefaction, and within one hour of collection examine in eyepiece reticle for motility (rapidly progressive ≥25µm/s, slowly progressive 5-24µm/s, non-progressive <5µm/s, non-motile/no active tail movements). The velocity of spermatozoa is temperature-dependent. For assessing motility, mix the sample properly without any air bubbles formation, 10 µl semen is taken on to a pre-warmed slide (37°C) and 22 x 22 mm coverslip for a depth of 20 µm. Examine the wet preparation as soon as the

contents are no longer drifting and at least 5mm from the edges of the coverslip. Count at least 200 spermatozoa, the whole depth of the fluid should be screened and the percentage of spermatozoa showing actual progressive motion should be recorded. The lower reference limit for total motility (progressive + non-progressive) is ≥40%. [3]

Aggregation

The adherence either of immotile spermatozoa to each other or of motile spermatozoa to mucus strands, non-sperm cells or debris is considered to be non-specific aggregation. [Fig 1a-b]

Agglutination

It specifically refers to motile spermatozoa sticking to each other, head-to-head, tail-to-tail or in a mixed way noted under 10x. The presence indicates anti-sperm antibodies testing. [Fig 1c-d] [3,8]

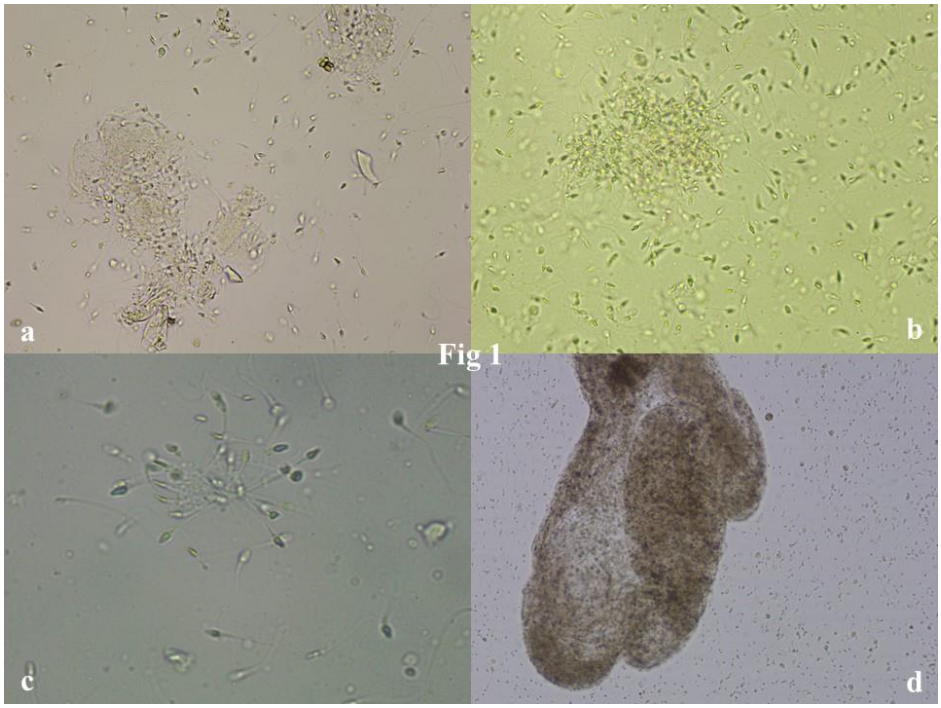

Fig 1: a, b: Wet mount showing the spermatozoa aggregated with squamous epithelial cells and with spermatozoa itself. [Wet Mount, x10] Fig1c, d: Wet mount showing tail to tail agglutination and large agglutination of spermatozoa seen. [Wet Mount, x40, x10

Sperm vitality

Assessing membrane integrity, but not necessary when the motile count is >40%. Important in discrimination of immotile live (flagellum) vs immotile dead (epididymal/ immunologic) causes. To be done within 30 - 60 mins of collection. Mix 1:1 of eosin-nigrosin and semen in the test tube and smear it, allow it to dry and examine under a microscope. Proportion of Dead (stained pink or red head/faint stain) and Live (White unstained head or with stain in neck/leaky neck membrane) is counted in the laboratory counter for 200 spermatozoa. Not mandatory if motility is good. [Fig 2] [3, 9]

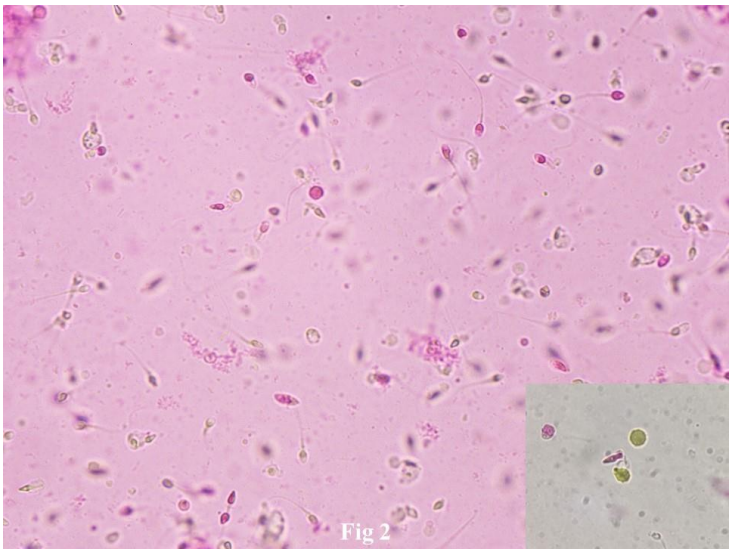

Fig 2: Wet mount prepration showing the pale looking viable spermatozoa without eosin uptake and the pinkish looking non-viable spermatozoa with eosin uptake. Oval shaped golden structures are the RBCs (Inset) [WM Eosin, x40]

Note:

- Eosin alone can be used too. Use 0.5gm Eosin Y in 100ml of 0.9% NaCl.
- Proportion of live & immotile spermatozoa are determined. i.e., Live proportion minus motile (Rapid Progressive + Slow Progressive + Non progressive). The normal range is 25-30%. If it's more than normal possible genetic ciliary problem.

Sperm count

Check the sperm count after liquefaction, by diluting (1:20) [Table 2] 50 µl of semen in 950 µl of semen diluting fluid. Charge the Neubauer chamber, and let stand for 2 minutes to allow the sperms to settle down. Count only spermatozoa with head and tail. If any headless/pinhead spermatozoa or head with >1 tail in present in 1 in 5 spermatozoa report it.

Table 2: Dilution of semen sample for various concentrations of spermatozoa in wet mount examination.

Spermatozoa per x 400 field	Spermatozoa per x 200 felid	Dilution	Ejaculate (µl)	Fixative (µl)
> 200	> 800	1:50 (1 + 49)	**50**	2450
40 - 200	160 - 800	1:20 (1 + 19)	**50**	950
16 - 40	64 - 160	1:10 (1 + 9)	**50**	450
2 - 15	8 - 64	1:5 (1 + 4)	**50**	200
< 2	< 8	1:2 (1+1)	100	100

The RBC chambers are counted for spermatozoa (4corner & 1central), Sperm Conc. = Number of spermatozoa * 5 * Dilution factor * 10,000 /ml. If the numbers of spermatozoa counted is less than 200 in central 25 squares, then spermatozoa in 4 sq.mm (WBC 4 large squares) are counted, multiply this number by 50,000 to get the number of sperms per ml of semen. [Fig 3 & Table 3]

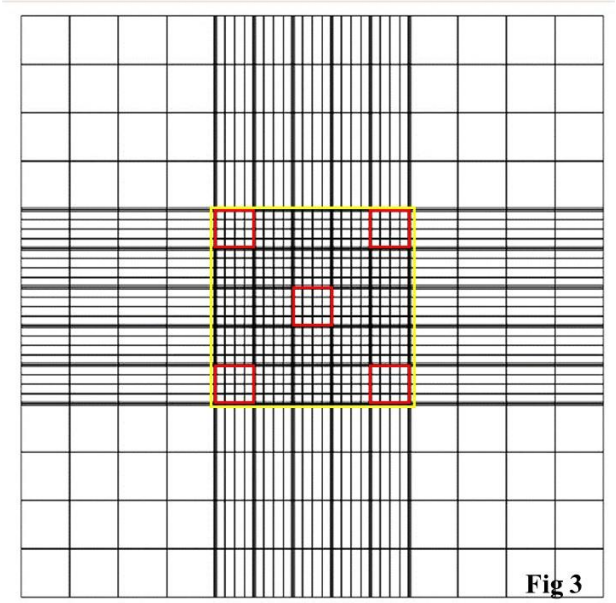

Fig 3: Modified Neubauer chamber with red chambers to be counted on both sides and average is taken in case of sample with >200 spermatozoa on wet mount. The yellow squares i.e., 25 central boxes are counted on both sides with average is taken in cases with sample < 200 spermatozoa on wet mount.

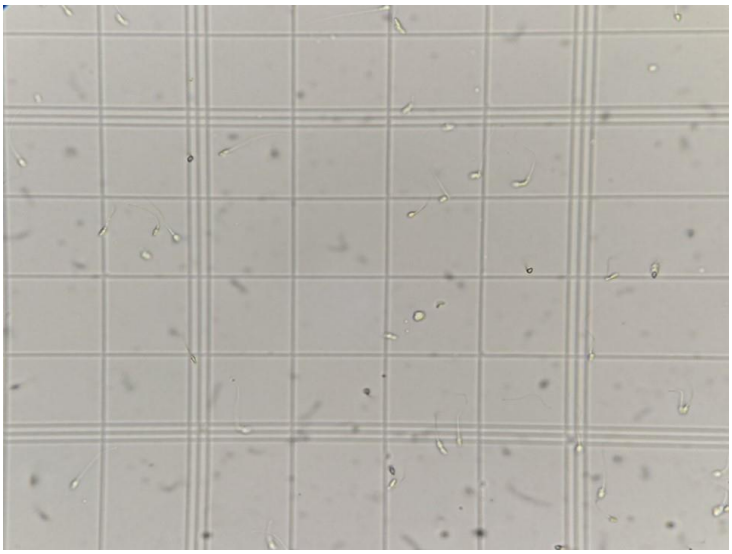

Fig 4: Modified Neubauer chamber with central box focused showing may spermatozoa. [Wet mount, x100]

Table 3: Neubauer chambers squares counted for the number of spermatozoa per field.

Number of spermatozoa	Central squares
< 10 spermatozoa	25 central squares
10 - 40 spermatozoa	10 central squares
> 40 spermatozoa	05 central squares *(4corner & 1central),*

Note:

- Semen diluting fluid= 0.595M / 50gm $NaHCO_3$ + 0.14M / 10ml of 40% HCHO in 1 lt aq. water solution. To it add 0.25gm of trypan blue or 5ml saturated solution of gentian violet for highlighting sperm heads. Store at 2-8°C use it for 1 year. If crystals are formed filter, it before use.
- Azoospermia: When no sperm are found in the centrifuged sample. Don't use the term for a wet mount with no spermatozoa. Counts on the such sample should carry remarks.
- Total sperm counts should be reported in integer number (no decimal places) of millions of spermatozoa. The exception is when counts are less than 10 million, one decimal place is acceptable.
- Total spermatozoa per ejaculate is more important indicator of testicular sperm production.

Morphology (Tygerberg strict criteria)

Smear is prepared after the semen has liquefied. Take 5-10µl of semen in a clean slide and smear it. Air dried and wet fixed slides fixed in isopropyl alcohol and stained by Papanicolaou stain. At least 200 spermatozoa should be examined under oil immersion and differential counts (%) of morphologically normal and abnormal spermatozoa to be given (abnormal %: head, midpiece/neck, Tail/principal piece, cytoplasm). Abnormal morphology and its percentage is recorded. Normally semen has ≥ 4% normal forms (head with tail). [Fig 5a-l, 6a-l & Table 4, 5] [10]

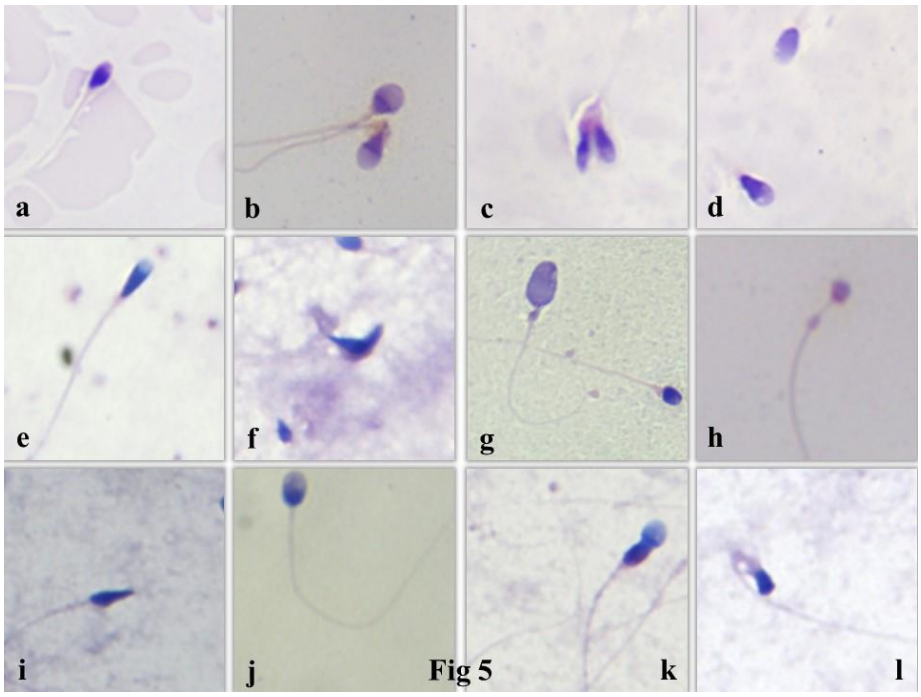

Fig 5: Various normal and defective forms of head shown. 5a Normal spermatozoa; 5b Bulb shaped head; 5c Bifid head; 5d Pyriform head; 5e Tapered head; 5f Bow shaped head; 5g Large head along with a normal spermatozoon; 5h Pinpoint head; 5i Pointed head; 5j Vacuolated head; 5k Fusiform head; 5l No acrosome in spermatozoa. [Pap, x100]

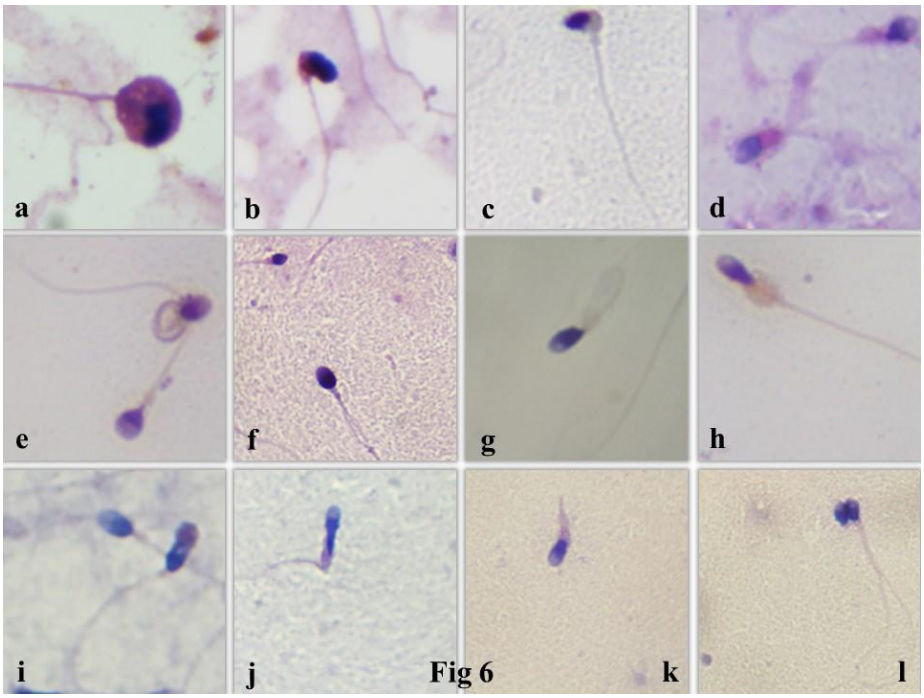

Fig 6: Various defective forms of midpiece, tail & cytoplasm are shown. 6a Abundant cytoplasm; 6b Side insertion/bent neck; 6c Broad side insertion of neck; 6d Broad/thick insertion; 6e Coiled tail with absent midpiece; 6f Bifid tail; 6g Ring tail; 6h Excess residual cytoplasm; 6i Bent tail; 6j Bent tail; 6k No tail; 6l Fused tail & head. [PAP, x100]

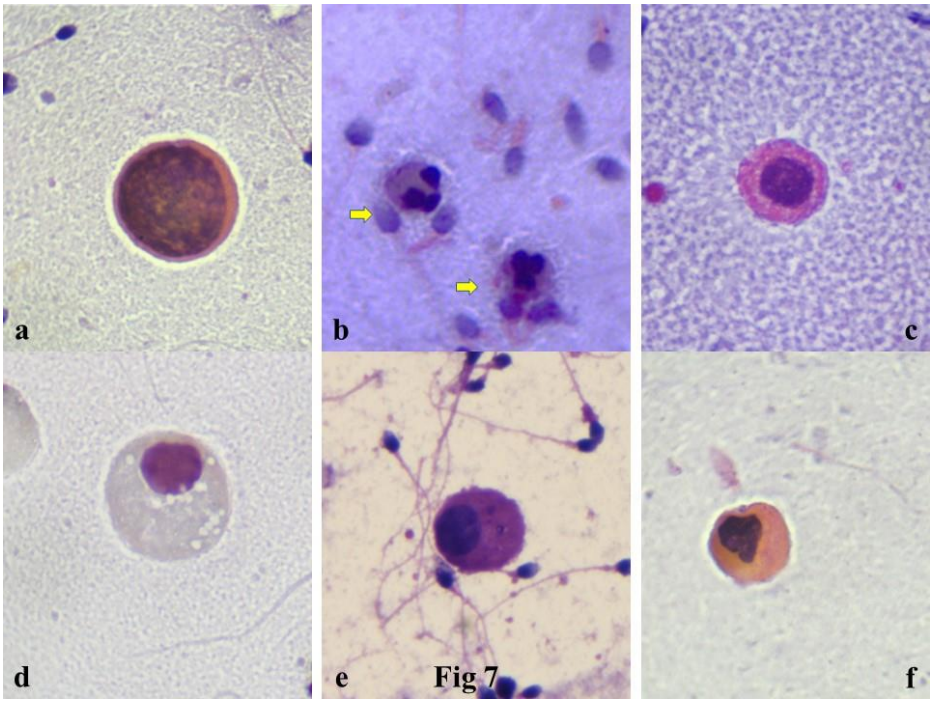

Fig 7: Macrophage along with mature spermatozoa; Fig 7b: Neutrophils along with spermatozoa; Fig 7c: Spermatocyte along with mature spermatozoa; Fig 7d & e: Spermatid; Fig 7f: Degenerated spermatid along with few scattered tails. [Pap, x100]

Table 4: Normal and abnormal morphological changes in spertmatozoa

Location	Normal	Abnormal
Head	Smooth, regular, oval, with 40-70% acrosomal area	• Acrosome < 40% or > 70% • Length: Width <1.5(round) or >2(elongated) • Shape pyriform, amorphous, asymmetrical, non-oval apical portion • Vacuoles • Double head • Any combination
Midpiece/ neck	Slender, regular, major axis in aligned to head	• Irregular, thin, thick, asymmetrical, angled insertion, sharp bent, any combinations.
Tail / Principle piece	Uniform caliber, 45µm long (10 times head length), looped	• Sharp angulation, smooth hair pin bends, coiled, short, irregular width, multiple tails, any combination.
Cytoplasmic residue	Cytoplasmic droplets of <1/3rd normal sperm head size	• >1/3rd normal sperm head

Table 5: Comparison of the WHO reference manual for semen analysis as per the 5th & 6th edition

Parameter 6th ed WHO Lower reference limit	6th ed 5th centile data (95%CI)	5th ed 5th centile data (95%CI)
Semen volume	> 1.4 ml	>1.5 ml
Semen conc x 10^6/ml	≥ 16 million	≥15 million
Total sperm no. x 10^6 /ejac	≥ 39 million	≥ 39 million
TM (PR+NP) %	≥ 42 %	≥ 40%
PR%	≥ 30 %	≥ 32%
NP%	1 %	1 %
IM%	20 %	22 %
Vitality %	≥ 54 %	≥ 58%
Normal forms %	≥ 4.0%	≥ 4%
Leucocytes	1 - 2 x 10^6/ml	1 x 10^6/ml

- Slides of PAP & Leishman are to be stored for 1 week from the issue of report.
- Also Count Pus cell/RBC/Epithelial cells/ Germinal cells per high power field.
- Normal Acrosomes. The same criteria for a normal sperm head, regarding the acrosome, namely clearly visible and well defined with a smooth oval configuration and comprising 40% to 70% of the sperm head was used to classify the acrosome as normal. Normal acrosomal staining had to be a homogeneous light blue. [Fig 5]
- Staining Defects. Irregular staining, the presence of three or more vacuoles or large cysts was considered as a staining defect. Furthermore, the equatorial junction had to form a regular clear line across the sperm head. [Fig 5]
- Too Small. In cases where the acrosome covered <40% of the anterior part of a normal head it was considered as too small. Acrosomes of spermatozoa classified as too small or from spermatozoa with severe elongation, usually

associated with small acrosomes, also was considered as too small. [Fig 5]

- Amorphous. All other abnormalities, including acrosomes that were too large, were classified under amorphous. [Fig 5]

Morphological indices: [3, 11,12,13]

- Sperm deformity index (SDI)- in vitro (No. of defects/No. of sperms counted), normal values ≤1.6
- Teratozoospermic index (TZI) (1.00-4.00) = (c + p + q) / x, c = head defects, p = midpiece defects, q = tail defects, x = total number of abnormal sperm. TZI two decimal places. In vivo
- Multiple anomalies defect (MAI): (≤1.75) Mean number of defects per sperm (the multiple anomalies index, defined as the average number of abnormalities per abnormal sperm, also was calculated for each semen sample by continuing the morphological evaluation using the stricter criteria for normal sperm morphology until 100 abnormal sperms had been assessed). In vivo
- Acrosomal index (%) = (Spermatozoa with normal acrosomes/ Total number of spermatozoa evaluated) x 100

What is kept in 6th ed 2021? [3]

Basic semen examination (Concentration; motility-Rapidly progressive, slowly progressive, non-progressive, Immotile; vitality only if the motility is <40%; morphology with PAP-head, midpiece, tail, cytoplasm; no replicate assessment required. [Table 5, 6]

Extended analysis

- Sperm DNA damage/fragmentation (sDF) using {TUNEL assay, sperm chromatin dispersion test (SCD), Comet assay using single gel electrophoresis, Acridine orange flow cytometry},
- Markers for genital tract inflammation- Leucocytes Cellular peroxidase using ortho-toluidine, CD45 IHC, Range is 0.5-1 x 106/ml PMNs or 1-2 x 106/ml leucocytes, IL levels.
- Sequence of ejaculation- Fraction 1/2/3/4 and levels of SC, zinc, fructose, volume, and PR in each fraction. Affected in stenosis, ejaculatory duct obs, etc.
- Semen Biochemistry- Prostrate (Citric acid, Zn, Acid phosphatase, GGT), Seminal vesicle (fructose, PGs), epididymis (neutral α-glucosidase, L-carnitine, glycerophosphocholine / GPC)
- Anti-sperm antibodies (ASAB)- IgA=IgG >> IgM, Mixed antiglobulin test (MAR), Immunobead test (IB),
- Indices of multiple sperm defects: MDI, TZI, SDI.

Research procedures

Oxidative stress/ROS, acrosome reaction, sperm chromatin structure and stability, CatSper channels, CASA, sperm preparation techniques,

- Cryopreservation of spermatozoa: donor sperm, fertility preservation (vasectomy, CT agents, dangerous occupation, transgender conversion, testicular trauma), infertility treatment (ICI, IVF, ICSI
- QC (Internal QC, External QA)

Table 6: Methods for evaluation of test parameters in semen analysis.

Received semen in proper labelled container (e.g. Pt's name, age, id no, date) from requisition form

⬇

Check pH of semen

⬇

Check liquification time after 30mins if not liquified, then check after every 5 min interval at 37℃,

⬇

After liquification check these parameters given below at 37℃.

⬇

Sperm Motility	**Sperm Vitality**	**Sperm Count (By hemocytometer)**	**Chemical Examination**	**Sperm Morphological Analysis**
Making a wet preparation Mix the semen sample properly Place a standard volume of semen e.g. 10µl onto a clean glass slide Also, a mix. of NS to Semen 1:1 Cover it with cover slip Check under microscope	Mix the semen sample properly Prepare a dilution of Eosin Y (0.5%) Ratio of 1:1 Semen: Eosin Y Onto a clean glass slide Cover it with cover slip and leave 30 seconds Check under microscope	Mix the sample well Prepare a dilution of semen & semen diluting fluid in the ratio of 1:20 e.g. Take 50µl of semen and 950µl of semen diluting fluid Mix well Place the Neubauer chamber on plane surface, Cover it with standard size cover slip Charge the Neubauer chamber with the help of micro pipette e.g take 10µl of prepared dilution for each side charging Count under microscope	**Fructose test (Seliwanoff's test)** Take 3 ml of seliwanoff's reagent, add 5 drops of semen in the test tube Heat the tube gently Check color of solution at 30sec Cherry red color + for ketose sugar/fructose	(Giemsa & PAP Stain) Prepare 2 semen smears each for Geimsa & PAP stain Do staining as per routine Giemsa and PAP staining Examine head, midpiece, principal piece and cytoplasm defects Calculate the MAI, TZI, SDI

Removed:

Examples of calculations, Hamster egg penetration test, sperm cervical penetration test

Stains used for semen morphology: [14]

- The conventional staining techniques used were- [Table 7]
- Leishman's stain
- Giemsa Stain
- DiffQuik stain
- Papanicolaou stain

Table 7: Methods of staining used for semen analysis

PAP Stain	Time	Leishman's	Time
50% ethanol	If spray fixative has been used	Air dry	--
95% ethanol	Fix slide for 15 mins	Fix in methanol	15 mins
Tap water	10 dips	Pour Leishman's stain	2 mins
Harris hematoxylin	3 mins	Buffer solution	8 mins
Running tap water	2 mins	Wash with tap water	--
1% acid alcohol	2 dips	Air dry	--
Tap water	2 mins	**Giemsa**	**Time**
Ethanol 50%	10 dips	95% methanol	10 min
Abs. ethanol	10 dips	1:10 :: Geimsa: Distilled water	30mins
Orange G6	2 mins	Tap water	Rinse
Ethanol 95%	10 dips	**DiffQuik**	**Time**
EA 50	3 mins	95% methanol	1 min
Abs. ethanol	10 dips	Solution B Eosin	6 dips
Abs. ethanol	10 dips	Solution A Methylene blue + Azure A	10 dips
DPX	Mount	Tap water	10-15 dips

Terms used in reporting. Aspermia, azoospermia, Haemospermia etc. are used for the interpretation of samples.

Table 8 Terms for interpretation

Terms	Semen	Spermatozoa	Total Conc.	Normal Morph.	PR	IM	RBCs	Leukocytes
Aspermia	--							
Azoospermia		--						
Asthenozoospermia					↓			
Asthenoteratozoospermia				↓	↓			
Crytozoospermia		-- (+ centrifuged sample)						
Haemospermia							↑	
Leucospermia								↑
Necrozoospermia					↓	↑		
Normozoospermia	↔	↔	↔	↔	↔	↔		
Oligoasthenozoospermia			↓		↓			
Oligoasthenoteratozoospermia			↓	↓	↓			
Oligoteratozoospermia			↓	↓				
Oligozoospermia			↓					
Teratozoospermia				↓				

- Preference should be given to total number over concentration.
- ↓- Below the lower reference limits.
- ↔- Normal or above the lower reference limits.
- ↑- Increased % of spermatozoa.
- Spermia- ejaculate; Zoospermia- spermatozoa.
- Leucospermia aka *Leucocytospermia/ Pyospermia*

Conclusion

The semen analysis forms an important investigation for the evaluation of couples with infertility. The semen analysis parameters can take into a reference for screening of various other risk factors and the prevalence of infertility in the community. The concise workup mentioned in the article would help the reporting under a standardized format to be used for patient care.

Acknowledgements: None
Funding: None
Availability of data and materials: Not applicable.
Ethics approval and consent to participate: Not applicable.
Consent for publication: Not applicable.
Competing interests: The authors declare that they have no competing interests.

References

1. Cooper TG, Noonan E, Eckardstein S, Auger J, Gordon Baker HW, Behre HM. World Health Organization reference values for human semen characteristics. Human Reproduction Update 2010;16(3):231–245.
2. Jeelani R, Bluth MH. Reproductive Function and Pregnancy. In Mc Pherson RA, Pinicus MR, ed. Henry's Clinical diagnosis and management by laboratory methods, 23rd ed. China: Elsevier; 2017.
3. World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen 6th ed. Geneva: World Health Organization, 2021
4. Mishra P, Negi MPS, Srivastava M, Singh K, Rajender S. Decline in seminal quality in Indian men over the last 37 years. Reproductive Biology and Endocrinology 2018; 16:103-11.
5. Jones DM, Kovacs GT, Harrison L, Jennings MG, Baker HW. Immobilization of sperm by condoms and their components. Clin Reprod Fertil. 1986;4(6):367-72.
6. Anamthathmakula P, Winuthayanon W. Mechanism of semen liquefaction and its potential for a novel non-hormonal contraception†. Biol Reprod. 2020;103(2):411-426.
7. Vasan SS. Semen analysis and sperm function tests: How much to test? Indian J Urol. 2011;27(1):41-8.
8. Berger GK, Smith-Harrison LI, Sandlow JI. Sperm agglutination: Prevalence and contributory factors. Andrologia. 2019;51(5):e13254.
9. Baskaran S, Finelli R, Agarwal A, Henkel R. Diagnostic value of routine semen analysis in clinical andrology. Andrologia. 2021;53(2):e13614
10. Menkveld R. Sperm morphology assessment using strict (tygerberg) criteria. Methods Mol Biol. 2013;927:39-50
11. Gatimel N, Moreau J, Parinaud J, Léandri RD. Sperm morphology: assessment, pathophysiology, clinical relevance, and state of the art in 2017. Andrology. 2017;5(5):845-862.
12. Talwar P, Hayatnagarkar S. Sperm function test. J Hum Reprod Sci. 2015;8(2):61-9.
13. J. Auger, F. Eustache, A.G. Andersen, D.S. Irvine, N. Jørgensen, N.E. Skakkebæk, J. Suominen, J. Toppari, M. Vierula, P. Jouannet, Sperm morphological defects related to environment, lifestyle and medical history of 1001 male partners of pregnant women from four European cities. Human Reproduction 2001; 16(12):2710–2717.