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**Graduation Memory in view of obtaining the Master's degree**
**Option: Biology and Physiology of Reproduction**

***Theme***

# Assessment of The Techniques of The Sperm Preparation of Bovine

**Graduate on 01 /10/2020**

**Presented by: Miss Boubekeur Rym**

***In front of the Jury:***

| | | | |
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| **Mr. Bessaad M.A.** | **MC B** | **U. Blida 1** | **President** |
| **Mr. Kaidi R.** | **Professor** | **ISVB** | **Examinateur** |
| **Mr. LARBI DOUKARA K.** | **MC B** | **U. Blida 1** | **Promoteur** |

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Mr. **Bessaad M.L.,** MC B at Blida University 1.

Mr. **Kaidi**, National Institute Of Veterinary Medicine, Blida.

At the end, I would like to thank all the people who participated in one way or another in the realization of this memoir.

# Dedication

To my mother's soul.

To my grandmother who gave me joy "god protects her".

To my dear sister "**Selma**" who is always standing by me.

To my grandfather, my aunts and uncles who have always supported me.

To "**Abd-elbari** "who encourage me.

**Abstract:**

The evaluation of the quality of the sperm is indispensablefor its usefulness in artificial insemination. For this reason, there are several tests and analyses of the sample using different methods in order to guarantee a sperm of good quality.

In this study, we have studied the semen of bulls using different techniques, the light microscope, CASA and the flow cytometry, which determines parameters that allow us to evaluate the quality of bovine sperm.

For this purpose, volume, concentration, motility, activity, pH and morphology were evaluated using the "light microscope" technique. On the other hand, viability was assessed by CASA and flow cytometry, which is one of the most elaborate techniques.

We then conducted a comparative study between these previous methods in order to determine the reliability and efficiency of each one.

Keywords: Bovin sperm, viability, light microscope , CASA, flow cytométrie,

**Résumé :**

L'évaluation de la qualité du sperme est indispensable pour son utilité en insémination artificielle. Pour cette raison, il existe plusieurs tests et analyses de l'échantillon selon différentes méthodes afin de garantir un sperme de bonne qualité.

Dans cette étude, nous avons étudié la semence de taureaux en utilisant différentes techniques, le microscope optique, la CASA et la cytométrie de flux, qui détermine les paramètres permettant d'évaluer la qualité de la semence bovine.

À cette fin, le volume, la concentration, la motilité, la viabilité, ont été évalués à l'aide de la technique du (microscope optique). D'autre part, la viabilité a également été évaluée par la CASA et la cytométrie en flux, qui est l'une des techniques les plus élaborées.

Dans l'ensemble, nous avons mené une étude comparative entre ces méthodes précédentes afin de déterminer la fiabilité et l'efficacité de chacune d'entre elles.

Mots-clés : Sperme bovin, viabilité, microscope optique , CASA, cytométrie en flux.

**ملخص:**

تقييم جودة الحيوانات المنوية ضروري لفائدتها في التلقيح الاصطناعي. لهذا السبب ، هناك العديد من الاختبارات والتحليلات للعينة بطرق مختلفة من أجل ضمان جودة السائل المنوي.
في هذه الدراسة قمنا بدراسة السائل المنوي للثيران باستخدام تقنيات مختلفة مثل المجهر الضوئي و CASA

وقياس التدفق الخلوي التي تحدد معايير تقييم جودة السائل المنوي البقري.
ولهذه الغاية تم تقييم الحجم والتركيز والحركة والحيوية باستخدام تقنية "المجهر الضوئي". من ناحية أخرى ، تم تقييم الجدوى بواسطة CASA و قياس الدفق الخلوي احد التقنيات الاكثر تعقيد .
بشكل عام ، أجرينا دراسة مقارنة بين هذه الطرق السابقة لتحديد موثوقية وفعالية كل منها.
الكلمات المفتاحية: السائل المنوي البقري ، الجدوى ، الطريقة التقليدية ، قياس الدفق الخلوي CASA

## Summary

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**List of abbreviations:**

CASA: Computer Assisted Semen Analysis.

CNIAAG: National Center for Artificial Insemination of Genetic Improvement.

FC: Flow cytometry.

DNA: Deoxyribonucleic acid.

PI : propidium iodide.

LIN: l'index de linéarité

LIN: Linearity index.

Spz : Sperm.

STR: Percentage of Correctness or Linearity of Average Path.

UV: Ultra violet

VAP: Average Path Velocity.

VCL: Curvilinear Velocity.

VSL: Straight-Line Velocity.

W.H.O: World Health Organization.

# Introduction

## Introduction:

The improvement of livestock is a major concern of breeders who seek to select the best breeders in order to obtain more efficient and better adapted descendants to the breeding conditions (Colombani, 2012).

In Algeria, beef is an important source in the production of meat and milk. To this end, efforts are being made in this direction through genetic enhancement testing of our local breeds, the importation of high-productivity foreign breeds, the introduction of animal biotechnologies, including artificial insemination and embryonic transfer. (Laghrour, 2012).

Sperm production begins with semen collection (Kabera, 2008).Then semiological parameters are evaluated mainly by macroscopic semen analysis and then by microscopic analysis. Once the quality of the semen has been checked, it can be used, in fresh form, directly for artificial insemination, or in refrigerated form, if its use is short-term or it can be diluted and then cryoconserved. A cryopreserved seed can be maintained for several years (Rigal, 2008).

As a result, the success of artificial insemination depends largely on the quality of the sperm used. For this reason, it is important to have reliable analytical techniques to evaluate it (Cabannes, 2008).

In addition, the success of artificial insemination is strongly linked to the quality of the sperm freezing. During the freezing-thawing process, various biochemical and anatomical compartments of the sperm may be adversely affected affecting the reproductive capacity of the sperm (Briand-Amirat et *al*., 2006).

The original contributions of this study relate the comparison of the methods of assessment of the sperm quality, and discuss the limits of conventional methods and evocates the technical improvements that could be developed in the future.

This work will be presented in two parts:

The first part is a bibliographic synthesis in which of the recalls anatomo-physiological of the bull's reproductive system and the conservation of the seed.

The second represents the synthesis of experimental part understanding the methodology of work, the presentation and discussion of the results as well as the perspectives.

# Bibliographic synthesis

## I. Reproduction in cattle:

### I.1 Anatomy of the bull's reproductive system:

#### I.1.1 Anatomy:

The major structures of the bull reproductive tract are the penis, testicles, and the accessory organs and glands responsible for sperm maturation and transport, including epididymis, seminal vesicles, and prostate (Reinaldo, 2011).

Some of their features and functions are:

##### I.1.1.1 Testicles :

Bulls have a pair of testicles (figure 1) that has two main functions (Reinaldo, 2011):

**a.** Production of sperm cells (spermatozoa).

**b.** Synthesis of testosterone (the male hormone).

The testicles (figure 1) are located outside the body cavity of bulls and housed in the scrotum (a sac of skin containing sweat glands, muscles, and tissues to protect the testicles from impact and extreme temperatures).

Sperm cells are constantly synthesized within the testicles by the seminiferous tubes, a collection of hundreds of winding tubules surrounded by testosterone-producing cells, which fuse into few dozen larger tubules and culminate in the epididymis (Reinaldo, 2011).

##### I.1.1.2 Secondary sex organs :

**A. Epididymis :**

Attached to the testicle, the epididymis (figure 1) is divided into three regions: the head, body, tail.

The head and body of the epididymis are responsible for receiving the sperm cells from the testicles. Sperm cells stay in these regions for a few days, while they mature to become fertile and move toward the tail of the epididymis.

In the tail of the epididymis, fertile sperm cells are stored up to 8 days until they are either reabsorbed or shipped out of the epididymis if ejaculation occurs (Reinaldo, 2011).

**B. Ductus deferens:**

Also known as vas deferens, these tubes are responsible for transporting the sperm cells from the tail of the epididymis into the penis during ejaculation (figure 1). During transportation, sperm cells are mixed with fluids, known as seminal plasma, produced by accessory sex glands (seminal vesicles, prostate, and Cowper’s glands) attached to the ductus deferens with the purpose of diluting and enhancing fertility of sperm cells (Reinaldo,2011).

**C. The urethra:**

Is an odd canal running from the bladder to the urinary meatus and serving as a passage for semen during ejaculation and urine during urination (figure 1). The length of the urethra can reach 100 to 120 cm in an adult bull. It has two parts: the pelvic urethra located in the pelvic cavity and the penile urethra located in the penis. In the bull, the urethra has the peculiarity of having an S-shaped path: it is the penis. It contributes to the lengthening of the penis during erection (Konfe 2014).

D. **Penis:**

Organ responsible for copulation and deposition of semen into the cow's reproductive tract. The penis (figure 1) is maintained inside the body of the bull by the sigmoid flexure, an anatomical structure that prevents exposure of the penis to prevent injuries. During copulation, the musculature responsible for the sigmoid flexure (retractor muscles) relaxes and the penis extends and becomes exposed (Reinaldo, 2011).

### II.1.1.3 The three accessory sex glands include :

- **The seminal vesicles :**

The seminal vesicles (figure 1) are symmetrical, bumpy, paired organs located behind the bladder neck above the prostate (Kohler 2011).

- **The prostate gland :**

The prostate is a gland of the male reproductive system that is the shape and size of a chestnut and weighs 15 to 25 g in adulthood. Located in the front part of the rectum, the prostate surrounds the neck of the bladder and the initial part of the urethra, the canal that allows the evacuation of urine and semen (figure 1). It has three main areas: the central zone, the transition zone and the peripheral zone (Dayon 2008).

- **The bulbo-urethral gland (Cowper's gland) :**

The bulbo-urethral glands, also called Cowper's glands, are paired and located on either side of the dorsal surface of the pelvic part of the urethra, close to the bulb of the penis. They are absent in dogs, very small in cats and relatively small in bulls (Marc 2015).

### II.1.1.4 Protective, supporting, and other structures :

- **Scrotum :**

The bull's scrotum is an appendage dependent on the ventral abdominal skin that supports and protects the testicles and helps regulate testicular temperature (Wolfe, 2017).

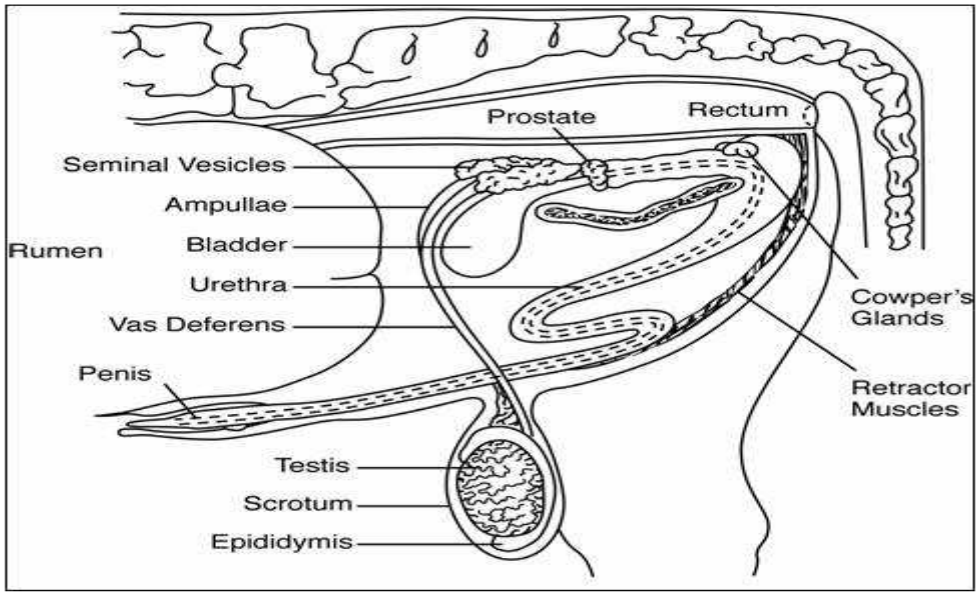



**Figure 1:** The reproductive tract of the bull (Hamilton,2006).

## I.2 Physiology of the bull's reproductive system :

### I.2.1 The puberty:

Sexual maturity, or puberty, 1s the time in the animal's life when it normally becomes capable of reproduction (bull able to produce an ejaculate containing 50 million sperm with a minimum of 10% motility (Hamilton, 2006).

Bulls usually reach puberty from 8 to 12 months of age, depending on breed and nutritional level (Wilson et *al*., 1967).

Bulls exhibit first sexual interest about 3 weeks prior to puberty and attain mating ability about 6 weeks after puberty. Although bulls that have reached puberty can breed, reproductive capacity increases as the bull continues to mature (Hamilton, 2006).

### I.2.2 Sperm production:

#### a. Definition of sperm :

In the bull approximately 5 billion sperm are ejaculated at each service. Mature bulls may produce upwards of 70 billion sperm each week (Wilson et *al*., 1967).

A sperm consists of two parts: the head and the tail. The bull's genetic contribution (genes) is carried in the head. Although the tail may help to propel the sperm it is question able if the tail is necessary for fertilization of the egg (Wilson et *al*., 1967).

Although 5 billion sperm may be ejaculated at one service, normally only one sperm unites with an egg to form the new individual. The rest of the sperm are

wasted. One advantage of artificia1 insemination is that 300 to 1000 cows may be inseminated artificially with the sperm contained in one ejaculation (Wilson et *al*., 1967).

**b. The anatomy (morphology) of the sperm:**

The morphology (anatomy) of the sperm has been shown to be one of the most important indicators of fertility in the bull and a very useful selection tool for improving herd fertility.

While the sperm head contains the materials necessary for fertilization and paternal DNA, the tail comprises the apparatus necessary for sperm energy production and motility (Kaya et *al*., 2014).

**1. The nucleus:**

The largest component of the sperm head is the nucleus, which contains highly condensed DNA, the result of the involvement of sperm-specific proteins, "the protamines". (Ward et coffey, 1991;Hazzouri et *al*.,2000 *in* Kaya et *al*.,*2014*).

This condensed DNA state characterizes the cell as being non-dividing and transcriptionally inactive.Further, the compacted sperm DNA occupies a very small volume compared with the DNA in the mitotic chromosome. The nucleus is surrounded by a special cytoskeletal complex of perinuclear theca called the post-acrosomal sheet (Kaya et *al*., 2014).

**2. The tail**

The sperm tail consists of four segments:

A. the neck (connecting piece),
B. the midpiece,
C. the principal piece
D. the endpiece

Each of these four segments is surrounded by a common cell membrane.

The primary structural parts of the mammalian sperm tail are the axoneme, the mitochondrial sheath, the outer dense fibers and the fibrous sheath.

The centrally located axoneme is composed of nine evenly spaced microtubule doublets and a central pair of singlet microtubules. These extend throughout the length of the tail.

The outer dense fibers have a very remarkable cytoskeletal structure, consisting of nine fibers that surround the axoneme and extend through the neck, the midpiece and principal piece of the mammalian sperm tail (Kaya et *al*., 2014).

**A. The neck :**

The neck is a short connecting segment between the head and the tail of the spermatozoon. The sperm head and tail are connected via the basal plate at the caudal end of the nucleus, and the capitulum, which adheres to the basal plate of the implantation fossa of the nucleus (Kaya et *al*., 2014).

The neck and the axoneme are formed by a pair of centrioles that are composed of nine circularly arranged microtubular triplets (Kaya et *al*., 2014).

These two centrioles are present in the spermatid at the time of sperm tail formation. The distal centriole forms the axoneme, whereas the proximal centriole is associated with the formation of capitulum (Kaya et *al*., 2014).

**B. The midpiece**

The midpiece is the region of the tail between the neck and the annulus. The annulus is a structural element of the mammalian sperm tail, which connects the midpiece and the principal piece. In the midpiece segment, the outer dense fibre–axoneme complex is surrounded by a helically-wrapped mitochondrial sheath, which extends from the neck to the annulus (Kaya et *al*., 2014).

**C. The principal piece**

The principal piece is the longest part of the sperm flagellum and extends from the annulus to the terminal piece. Due to the termination of mitochondria in the mid-piece, the diameter of the tail in the principal piece is reduced. The principal piece is characterized by the presence of a fibrous sheath, which provides stability for the contractile elements of the tail (Kaya et *al*., 2014).

**D. The endpiece**

The endpiece is the region beyond the distal end of the fibrous sheath. This region contains only the terminal segment of the axoneme, surrounded only by the cell membrane of the sperm tail (Kaya et al., 2014).

### I.2.3 The spermatogenesis :

Spermatogenesis is a finely regulated process of germ cell multiplication and differentiation leading to the production of spermatozoa in the seminiferous tubules (Staub et Johnson, 2018).

Spermatogenesis can be divided into three parts: Spermatocytogenesis; Meiosis; Spermiogenesis.

### I.2.3.1 Spermatocytogenesis :

During spermatocytogenesis stem germ cells engage in the spermatogenetic process by performing a first mitotic division that generates spermatogonia (Staub et Johnson, 2018).

The latter proliferate in turn by performing several successive mitotic divisions which result in the production of preleptotene spermatocytes. The preleptotene spermatocytes cross the blood-testis barrier and engage in meiotic prophase (Staub et Johnson, 2018).

### I.2.3.2 Meiosis:

During the prophase of the first division of meiosis, the germinal cells differentiate successively into different stages (leptotene, zygotene, pachytene, diplotene) before undergoing the two meiotic divisions.

The first meiotic division is the reductional division (reduction of chromosome number, separation of homologous chromosomes), while the second meiotic division is the equational division (separation of the daughter chromatids). Meiosis therefore allows the production of round haploid spermatids (Staub et Johnson, 2018)

### I.2.3.3 Spermiogenesis :

Consists of the differentiation of round spermatids into spermatids at various degrees of elongation and finally into spermatozoa. Spermatozoa are released into the lumen of seminiferous tubules during a final stage called spermiation (Staub et Johnson, 2018).

### I.1.4 Endocrinology : bull's hormones

From birth to puberty the bull's reproductive tract goes through a period of growth and development. This growth is brought about chiefly by hormones, which are chemical substances manufactured by glands located in different parts of the body.

These hormones are usually secreted into the bloodstream and carried with the blood to other glands and tissue (Kaya et *al*., 2014).

Once the hormones reach a particular gland or tissue, they change the activity or growth of that gland or tissue. In the immature bull the hormonal action is less complex than in the heifer. The pituitary gland, located at the base of the brain, produces hormones which stimulate the onset of sexual maturity (Kaya et *al*., 2014).

The pituitary consists of two parts:

a) **The posterior lobe**: is believed to play an important role in the development of the bull's reproductive system.

b) **the anterior lobe** : produces two substances wiche are follicle

stimulating hormone (FSH) and luteinizing hormone (LH), which are very important.

**FSH:** is found in high concentrations in the developing bull fetus and stimulates growth of the testes and spermatogenesis (sperm production).

As puberty approaches the LH concentration increases, further stimulating sperm production and release (Kaya et *al*., 2014).

**LH:** also stimulates certain ce11s in the testes to produce a third hormone, testosterone (Kaya et *al*., 2014).

Testosterone passes directly into the blood stream and is carried throughout the body, these three substances (FSH, LH, and testosterone) continue to be produced in the mature bull (Kaya et *al*., 2014).

### I.1.5 Ejaculation of semen :

In the bull the penis is rigid even when non erect, the erection is due partly to the temporary inability of blood to drain freely from the penis. However, most of the actual length of the erect penis is due to a straightening of the sigmoid flexure.

During the courtship or teasing period, the sperm are transported from the tail of the epididymis (Kaya et *al*., 2014).

At the same time, contractions of the secondary glands (vesicular, prostate, and bulbo - urethral) force their fluids into the urethral cavity where the fluids are mixed with the sperm. The resulting mixture of sperm and fluids (semen) is then propelled to the outside through the penis by muscular contractions of the reproductive tract (Kaya et *al*., 2014).

#### a. Seminal Plasma:

Seminal plasma is the main component of semen; it consists of the secretions of the adjoining glands of the epididymis and Sertoli cells. It contains inorganic constituents and various organic constituents (Konfe, 2014).

The major role of seminal fluid is the supply of energy substrates to sperm that were largely absent when stored in the epididymis.

In addition, the dilution of gametes in seminal plasma allows the activation of their progressive motility and a facilitated transfer of sperm into the female genital tract during ejaculation. Secretions of the adjoining glands constitute the majority of the liquid fraction of the ejaculate, from 50 to 95% of the total volume of seminal fluid following the species. Some elements of seminal fluid come from blood plasma filtration, while others are produced by sexual glands according to (Dacheux et *al*., 2001*in* Marc, 2015).

## II. Cattle seed study :

### II.1 Harvesting (Collect) of sperm :

#### II.1.1 Sperm collection:

Sperm collection is the set of processes by which sperm is collected in a live animal. Sperm is taken from healthy animals, which are found to be free of legally recognized contagious diseases (MLRC) and all zoonoses (brucellosis, tuberculosis).In addition, several harvesting techniques have been used over time with advances science and technology; some are inspired by natural mating conditions while the others are the result of investigations, experimental in the light of physiology (Boly, 1986)

#### II.1.2 Semen collection via artificial vagina :

The most common technique for collecting sperm is using the artificial vagina (figure 2) used in the world at the level of the bovine species. It is a harvesting processes that closest to natural ejaculation conditions (Boly, 1986). It allows you to simulate the natural conditions offered by the cow's vagina.

The principle of the artificial vagina is to reproduce all the sensations presented by the female genital tract during coitus (heat, pressure, lubrication), and collect rapidly a total and unstained ejaculate (Dumont, 1997).

**Figure 2:** Artificial vagina (Rigal,2008)

##### II.1.2.1 Description:

The artificial vagina (figure 3): has a shape and dimensions related to the species, for which it is designed taking into account the conformation of the penis and the size of the animal. It is a simple and practical device; the artificial vagina has two parts (Meskini,2017) :

a) **An exterior cylinder**: Made of rigid material most often made of hard and thick rubber or plastic with an opening closed by a stopper (Meskini,2017) .

b) **The latex or artificial rubber inner shirt**: Is inserted into the outer cylinder and its ends folded and maintained by an elastic band ends of that one. The cavity (figure 3), thus,

formed by the outer cylinder and the shirt is filled with water in sufficient quantity to obtain pressure equivalent to the female vagina (Meskini,2017).

The end of the artificial vagina (figure 3) is lubricated to introduce the penis; on the other side, a rubber cone is attached, and a glass tube or a plastic tube is adapted to collect the semen. Some artificial vaginas are equipped with a thermometer (Hanzen, 2015). Sometimes the rubber cone carries an opening allowing the air to start so as to avoid excess pressure at this level. In many cases, the artificial vagina is protected by a coating that ensures that the sample of the thermal shock is preserved and that the device is protected from possible damage (Shoenian, 2005).

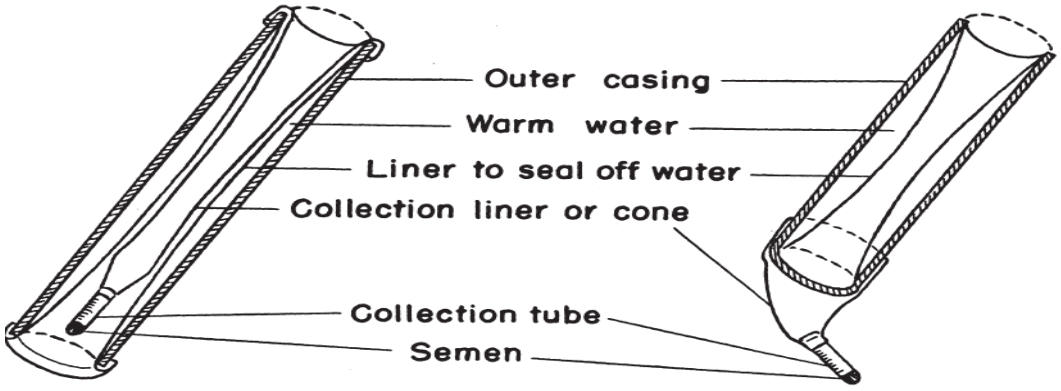



**Figure 3:** The artificial vagina (AV) designed to protect the semen from coldshock during cold weather (Salisbury and Willett, 1940) is shown on the left. The AV shown on the right is typical of those used commercially, with an insulated jacket (not shown) slipped over the AV for protection (Foote, 2005).

#### II.2.3.1 <u>The way we applied the artificial vagina:</u>

In the morning, before the collection of semen, the bull does not receive food because a full animal has lower sex drive, the collection of semen is performed in a specially prepared place called "manege", the room must meet appropriate sanitary and health conditions because it is considered a laboratory room, the floor has to be flexible and anti-slip (Barszcz et *al*., 2011).

In the first stage of the preparation the bull makes a few rounds in the “manege”, is allowed to sniff around, to jump other bulls and to perform empty jumps on a dummy cow.

A well aroused male with distinctive sexual impulses is brought to the dummy cow or the teaser in order to produce semen The semen is collected in an artificial vagina composed of (Barszcz et *al*., 2011):

1. A glass container for semen with a water jacket and a volume scale;
2. A thin, flexible latex sleeve;

3. A latex cone joining the end of the vagina with the collection tube;
4. A rubber cylindrical casing with a valve for pouring water and blowing air;
5. A bag – a thermal protector and a mechanical container.

When the artificial vagina is installed attention has to be paid that (Barszcz et *al*., 2011):

a. Every ejaculate is collected in a separate vagina;
b. The latex sleeve has to be installed in such a way that there are no fold;
c. Inside the casing; the sleeve should be moderately stretched in order to pour water and blow air;
d. The water temperature in vagina should be 40-42˚C;
e. The temperature of the container should vary between 35-37˚C;
f. The inlet to the vagina should be lubricate such as vaseline.

When collecting the sperm, attention must be paid that the bull does not touch the rump of the teaser with its penis. The person collecting the semen should take the bull's penis by the prepuce, and direct it to the inlet of the vagina.

The artificial vagina should never be put over the penis. Characteristically for its species, the bull performs a single copulation push. Next, the collected semen is evaluated (Barszcz et *al*., 2011).

#### III.1.2.3 The interests of collecting with artificial vagina:

The quality of sperm: Artificial vagina collection gives a natural ejaculate, induced by necess a and sufficient libido, and produced by physiologically close behavior to coitus. For this reas on it provides the best possible sperm at any given time (Fabrice et *al*., 2008).

It also allows the obtaining of the whole ejaculate, and the exact measurement of the ejaculat a better viability of sperm compared to other methods and the absence of external secretions.

### II.2.3 Semen collection via electro-ejaculator :

#### II.1.3.1 Electro-ejaculation :

Electro-ejaculation is achieved by electrical stimulation of the smooth muscles of the bulb and the deferensing canal (figure 4) using an intrarectal probe and an electrical source with tension control.

It allows sperm to be taken from the bull without the intervention of normal sensory, and psychic mechanisms of ejaculation (Haskouri, 2001).

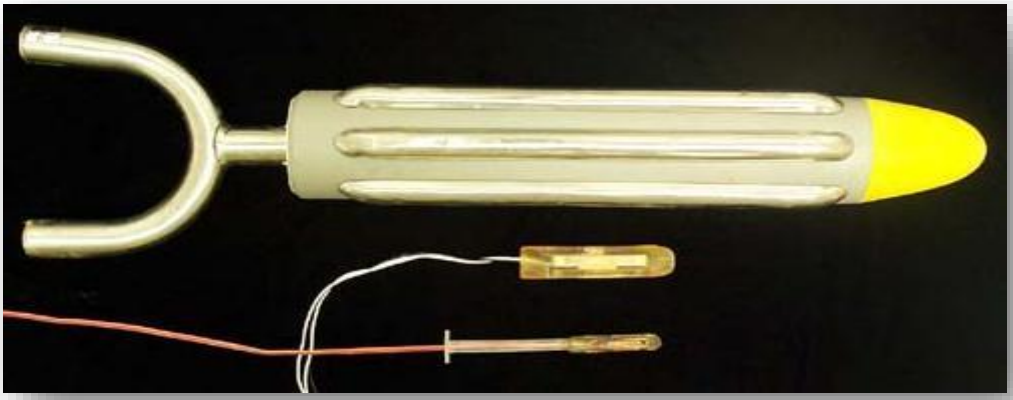


**Figure 4:** Electro-ejaculation (Rouge et Bowen, 2002).

In comparison with the harvest using artificial vagina (figure 2), it is generally accepted that the ejaculate volume is greater and sperm concentration is lower in the overstimulation of the adjoining glands, but without decreased motility of these (Akusu et *al*., 1984).

This method is not widely used for sperm collection, it is reserved for males having lost their libido or who cannot serve the artificial vagina.

### III.2 Analysis of the quality of the sperm harvested:

**III.2.1 Physical examination:** The bull needs to be able to see, eat, smell, and move properly to be physically able to breed cows. Therefore, eyes, mouth, feet, legs, and nutritional status of bulls should be evaluated and be in proper condition prior to breeding (Reinaldo, 2011).

**III.2.2 Seman examination :**

**III.2.2.1 Macroscopic examination :**

After semen collection, several parameters need to be evaluated to perform initial macroscopic investigation. The overall appearance of the sample in terms of color, pH, volume, viscosity and liquefaction is estimated (Dovere et *al*., 2017).

**a) Volume :**

The ejaculate volume tested on the graduated collection tube. This volume varies from 0.5 to 14 ml, depending on age, larce, food, health status, harvest conditions and harvest frequency (Gauthier et Varo, 1985).

**b) Color** :

The normal bull sperm is white according to (Parez et Duplan,1987).

However, this color can be changed for physiological and especially pathological reasons.

The yellow color of the sperm is due to high carotene content from the seminal vesicles. It can also be caused by the presence of pus or urine in the sperm. A pink or reddish coloration reflects the presence of blood in the sperm. The brownish color reflects the presence of alterd blood or degenerated blood in the sperm or an infliction. The blue color of the ejaculate is du e to low sperm concentration or methylene blue administration (Konfe, 2014).

**c) The Viscosity:**

The viscosity of the sperm is highly dependent on sperm concentration. Ejaculate is all the m-ore viscous as the number of sperm is high. Compared to distilled water, the bull's normal sperm has a viscosity of 3.7 according to(Parez et Dupln, 1987). Good viscosity is probably synonymous with good sperm concentration.

**d) The pH of sperm:**

The pH of the sperm is measured by a pH meter or by means of indicator paper. This is done immediately after the harvest. In fact, the semen rapidly acidifies by lactic acid formation.The pH of the normal sperm is between 6.5 and 6.8 in the bull according (Hanzen, 2009 *in* Konfe, 2014).

**e) Specific weight :**

The specific weight of the sperm depends on the ratio of the concentration tosperm and volum e of seminal plasma. It is 1,035 in the bull (Konfe, 2014).

### III.2.2.2 Microscopic examination:

They include the assessment of motility, sperm concentration, percentage of live sperm, and morphology of sperm.

**a) Motility:**

It is an element of appreciation of the life or death of sperm and their level of vivacity. It can be applied to the total sperm after harvest (massage motility and individual motility) or to the diluted sperm in interested in individualized sperm (individual motility) (WHO, 2010).

**1. Massage motility:**

The examination of the mass motility is carried out as quickly as possible after the sperm is taken, by keeping it rigorously at a temperature close to 38°C, as it decreases very rapidly. Sperm usually moves straight, massage motility depends on three main factors: concentration, percentage of mobile sperm and rate of movement of sperm. In bulls, mass motility is assigned a score ranging from 0 to 5 (Table 1), depending on (Parez and Duplan, 1987).

**Table 1:** Rating of mass mobility in the bovine species (Parez et Duplan, 1987)

| Note Approximate | percentage | Nature of the transaction |
|---|---|---|
| 0 | 0% | No surface movement |
| 1 | 20% | Slight movement on the surface |
| 2 | 40% | Net movement but not forming waves |
| 3 | 60% | Wave Start |
| 4 | 80% | Very sharp waves |
| 5 | 100% | Turbines clearly visible |

**2. Individual motility**

Is measured by optical microscope at a 200x enlargement between blade and lamella, it correspods to the proportion of sperm with a straight movement that crosses the microscope field. Sperm mo ving on the spot, turning in small circles or moving backward because of afolded tail are not cons-dered mobile according to (Gérard and Khirredine, 2002) (Table 2).

**Table 02:** Determination of the individual motility score of spermatozoids (Baril et *al*., 1993).

| Note | Individual motility |
|---|---|
| 0 | No movement of sperm. |
| 1 | Very slow or no movement, tremors of the sperm, tail oscillations. |
| 2 | Slow movement, tremors, unorganized movements, some sperm move faster. |
| 3 | SpermZoids perform curvilinear movements without tremors. |
| 4 | Fast move, some cells with a straight path, others with a curved path. |
| 5 | Sperm movement fast and straight. |

**b) Morphology : ( Take a look at Annexe 01)**

Sperm morphology: Morphological examination differentiates normal sperm from abnormal and live sperm from dead. This method is based on selective staining of certain organs, whether normal or abnormal, or whether cells are dead or alive.

Morphology is appreciated on colored sperm smears (Chinese ink, Giemsa, Eosineaniline or bromophenol blue). To be eligible, sperm must contain less than 25% of abnormal sperm and more than 60% of live sperm (Gacem, 2016).

**c) Concentration of the ejaculate:**

The purpose of this measure is to determine the number of sperm per milliliter of pure semen using the minimum of sperm possible. The sperm concentration generally varies from 2 to

10 x $10^9$ sperm per milliliter of ejaculate seed. Several possibilities exist to measure this concentration:

a. Direct visual appreciation of the consistency of the ejaculate.
b. Exact count with a hematimeter.
c. Measurement of optical density in a spectrophotometer (Baril et al., 1993).

- **Classic hematimeter method:**

The concentration is measured by counting sperm in the naked eye using a hematimeter (Thomas or Malassez cell), this method is considered the reference method (W.H.O, 1999).Indeed, this is the oldest and has been used for a long time both in production centers and in the diagnosis of fertility disorders in men (Kumar et *al*., 2010).

- **Spectrophotometry:**

The use of a spectrophotometer is the most effective technique because it combines speed and precision. The general principle is to measure the optical density (at a wavelength of 550 manometers) of the previous formulated saline solution, containing the spermatozoids, and to compare it to a white (not containing of the spermatozoids). Before using this technique in routine conditions, it is necessary to obtain a standard curve using 20 to 50 samples of different and known sperm concentrations, determined beforehand using the hematimeter. The correlation and slope of the linear regression are calculated between the optical density of the sample (X) and its sperm concentration (Y). The correlation coefficient must be greater than 0.9 and the slope close to 1, then a diagram, or the same calculation formula, can be used in routine conditions. Each year an audit (comparing the counts to the hematimeter and the optical density) must be carried out to prevent any drift of the measuring instrument (Baril et *al*., 1993).

**d) Vitality**:

The determination is made using special dyes (Eosine-negrosin, bleu-demethyl-eneou-bleu-debromophenol)that can cross the membrane of dead sperm and thus differentiate them from living sperm.

- **Vitality analysis**:

- **By the Eosin-nigrosine test:**

Vitality assessment is based on the principle of permeability of the dead sperm membrane.The head of the dead sperm becomes pink or red when colored with eosinenigrosin,while the living sperm does not take the dye and remains white (neckolor).

The average of the results obtained with both samples is expressed as a percentage of live sperm in the report.

- **By hypo-osmotic test:**

The hypoosmotic test (figure 5) is used to evaluate the membrane integrity of the cells. Cells with an intact plasma membrane have the ability to deform and discharge water.When the cell is exposed to hypoosmotic conditions, the water will enter the intracellular medium until osmotic equilibrium is reached on both sides of the membrane. So the cell is going to inflate. This phenomenon is particularly visible at the sperm level, which will show aincurvation of their flagella or swelling of it (Pena Martinez ,2004 *in* Marc,2015).

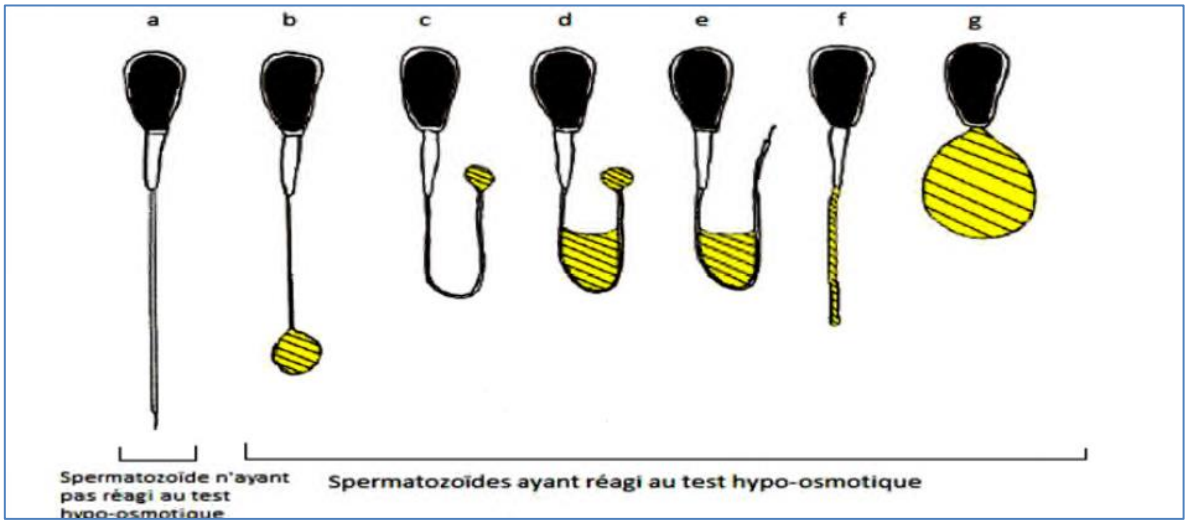



**Figure5:** Morphological Changes in Sperm Subjected to Hypoosmotic Testing (Marc 2015).

### III.2.4 **Computer-Assisted Sperm Analysis (CASA) : ( Take a look at Annexe 02)**

The Computer Assisted Semen Analysis (CASA) system is an image analyses that allows automated assessment of the concentration, vitality, morphology and, above all the characteristics of the mobility of sperm in a sample (Hakima et *al*., 2012).

It therefore provides a simple and rapid quantitative and qualitative assessment of bovine semen and can even predict its ability to fertilize an egg. The main interest of CASA systems is their ability to measure motion parameters based on the kinematic of sperm heads (Auger, 1997).

The images captured by the online video camera are recorded on video tape for further analysis or directly processed by the system consisting of a high-capacity micro-computer with software and hardware dedicated to the analysis of sequential images.

Parameters commonly used for motion assessment are curvilinear velocity (VCL), linear velocity (VSL), linearity index (LIN), lateral head flow amplitude (ALH), and head rotation frequency (BCF) (figure 6).

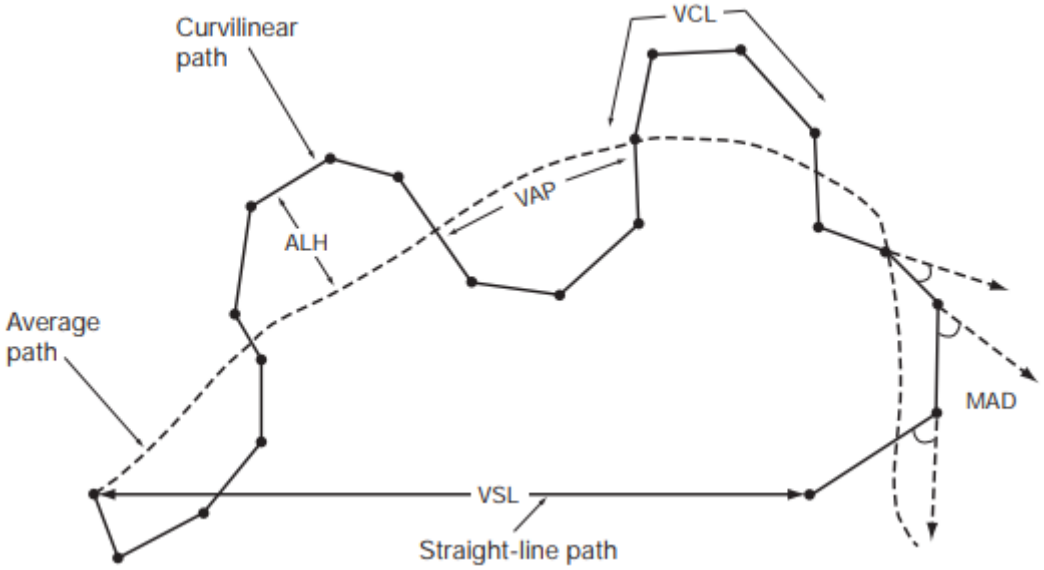



**Figure 6:** Standard terminology for variables measured by CASA systems (WHO, 2010).

VCL, curvilinear velocity (m/s): Time-averaged velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope (WHO, 2010);

VSL, straight-line (rectilinear) velocity (m/s): Time-averaged velocity of a sperm head along the straight line between its fi rst detected position and its last (WHO, 2010);

VAP, average path velocity (m/s):Time-averaged velocity of a sperm head along its average path. This path is computed by smoothing the curvilinear trajectory according to algorithms in the CASA instrument; these algorithms vary between instruments, so values may not be comparable among systems (WHO, 2010);

ALH, amplitude of lateral head displacement (m): Magnitude of lateral displacement of a sperm head about its average path. It can be expressed as a maximum or an average of such displacements. Different CASA instruments compute ALH using different algorithms, so values may not be comparable among systems (WHO, 2010);

BCF, beat-cross frequency (Hz): The average rate at which the curvilinear path crosses the average path. (WHO, 2010);

MAD, mean angular displacement (degrees): The time-averaged absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory (WHO, 2010).

### III.2.5 Analysis of sperm function by flow cytometry (FC):

#### III.2.5.1 Flow cytometry: ( Take a look at Annexe 02)

The application of FC minimizes, in part, several analytical difficulties encountered. FC is an automated approach that can measure the amount of one or more fluorescent markers associated with the cell in an unbiased way.

This device offers incomparable properties of precision, sensitivity, speed and allows multiparametric analysis on a statistically appropriate number of cells (Cordelli et *al*., 2005). In addition, the discovery of a variety of fluorochromes and fluorescent probe-conjugated components makes possible a wide range of analyzes for the evaluation of the sperm at biochemical, ultra-structural and functional levels (Gillan et *al*., 2005).

### II.2.5.2 Cell Viability and Concentration :

A viable sperm is defined as a cell that has an intact plasma membrane; this attribute is evaluated by staining a sperm sample with PI, a fluorescent probe that binds to DNA. PI binds selectively and quantitatively to the double helix nucleotide poly structure by intercaling between base pairs of nucleic acids (Le Pecq and Paoletti, 1967).

An intact plasma membrane prevents the penetration of PI within the cell, unlike a damaged membrane. As soon as it enters the cell, the PI binds to the DNA, causing a red fuorescence in cells with damaged plasma membrane.

However, no fluorescence occurs in cells with intact plasma membrane (Graham et *al*., 1990; Wilhelm et *al*., 1996). The use of PI also allows the determination of sperm concentration at the same time as the assessment of viability (Eustache et *al*., 2001).

### III.2.5.3 Integrity of the acrosome:

The acrosome assessment can be useful by using isothyocyanate (FITC) related plant lectins, for example, Peanut Agglutinin (FITC-PNA) or Pisum Sativum Agglutinin (FITC-PSA). These lectins are bound by method to glycosylated compounds of the outer acrosomal membrane. If the acrosome is intact, this binding causes a very high fluorescence of the acrosomal region, which it fluorescence in mature in case the acrosome is involved (Cheng et *al*., 1996).

### III.2.5.4 Mitochondrial function :

The mitochondrial function is evaluated by the use of a JC-1 dye (Mitochondrial Membrane Potential Probe), which monitors green fluorescence. This dye is used to check the sperm of the misused mitochondria sperm of the direct mitochondria sperm. In 2001, JC-1 is transported within the effects mitochondria, its increased concentration, and forms an aggregate that has orange fluorescence. Y emission of green fluorescence only the specific

sperm of poorly defined mitochondria, whereas orange fluorescence only the sperm of mitochondria (Thomas et *al*., 1998).

### III.2.5.5 Sort sperm during enhancement by sexing

During meiosis, the number of chromosomes increases from 2n to n. The sperm therefore have one chromosome of each original pair. In the case of sex chromosomes, they have either the X chromosome or the Y chromosome. Because the X chromosome is larger than the Y chromosome, its DNA content is greater than that of the Y sperm. The X sperm of cattle contain about 3,8% more DNA than the Y sperm (Peters and Bail, 2004).

As a result, sorting the seed of the main domestic species is technically feasible by separating sperm based on their DNA content by flow cytometry (Dmart and Bento, 2004). This is the method currently used for the production of sexed sequins (Chevalier, 2011).

The principle of this technique is based on the precise coloring of the DNA of the sperm with Hoechst 33342 (nucleic acid-specific fluorophore), to differentiate subpopulations of sperm X and Y (Duane et *al*., 2013). Indeed, when Hoechst is bound to DNA and is excited by a ultra-violet light, it emits blue fluorescence. The intensity of fluorescence emitted by a Hoechst-labeled sperm excited by a UV beam is directly proportional to its DNA content. The amount of fluorescence emitted by a bovine X sperm will therefore be 3,8% greater than that emitted by a Y sperm. Sensors then accurately measure the amount of fluorescence emitted and assign positive or negative electrical charges to each microdroplet containing a sperm each. Then, electrically charged metal plates (magnetic field) sort the sample into three streams. Positively charged particles corresponding to one sex are diverted in one direction, negatively charged particles corresponding to the opposite sex are diverted in another direction, while uncharged particles, possibly containing more than one sperm per microdroplet, unidentified sperm or dead or deficient sperm (Hamano, 2007 *in* Gacem,2016 ).

# Experimental part

## I. The objective:

The objective of this study is to evaluate the different techniques of the sperm and to study the main parameters, that allow evaluating the quality of the bovine sperm, trough flow cytometry, we have the following parameter: viability of spermatozoids, CASA's motality and by microscopic, and macroscopic examination the parameters are defined as follows: volume, pH, motility, activity of sperm, concentration.

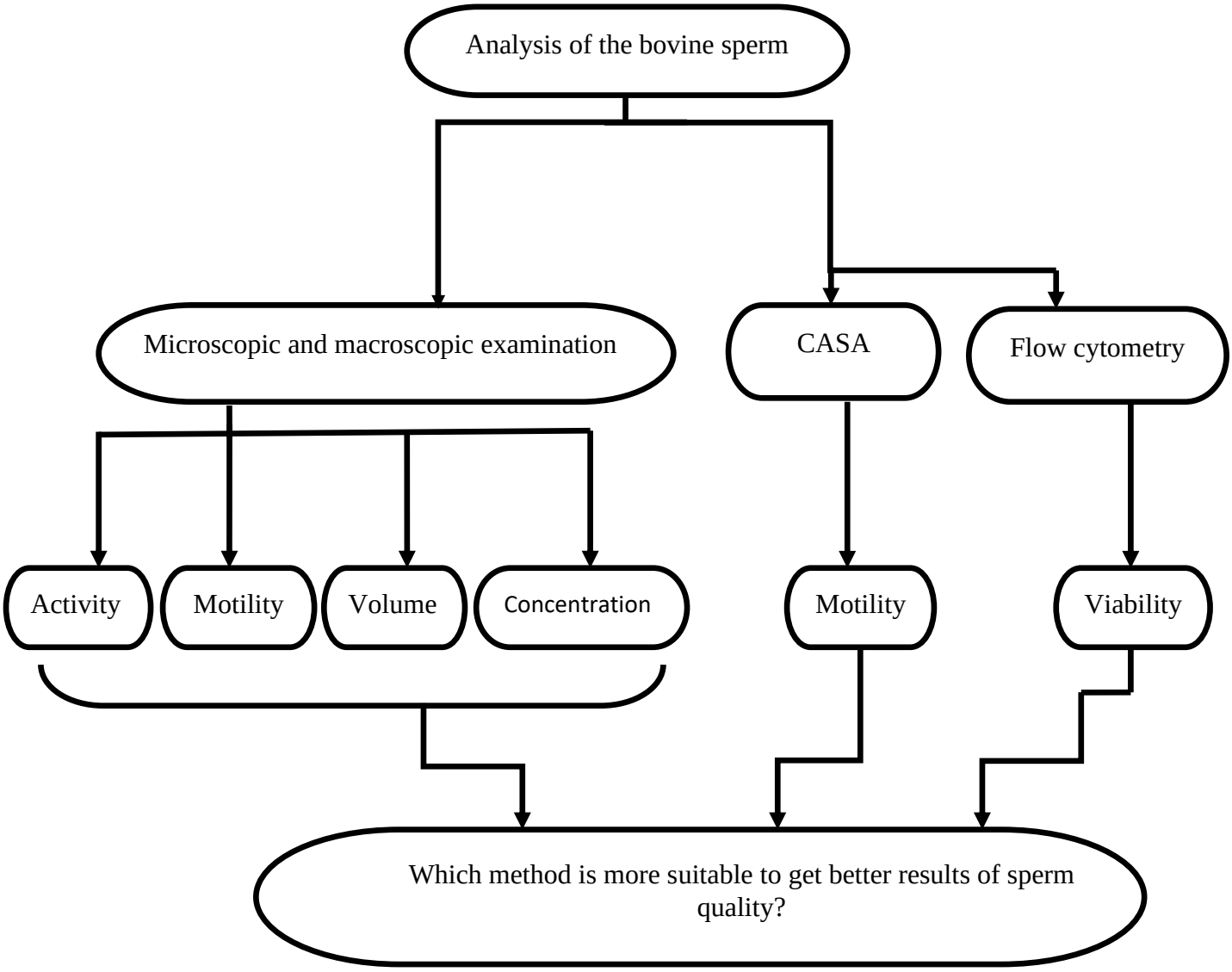



**Figure 7:** Deferent's analysis of bovine sperm.

"This work was expected to be done before Coronavirus - COVID-19."

## II. Study Environment:

National Center for Artificial Insemination and Genetic Improvement (CNIAAG) located in Baba Ali Birouta.

## III. Material: (Take a look at Annexe 03)

### 1. Biological material:

The study examined bulls of different breeds in the National Center for Artificial Insemination of Genetic Improvement (CNIAAG), Baba Ali-Alger.

### 2. Semen collection equipment:

We need to, an artificial vagina, protective sleeves (against ultraviolet rays) for tube and cone and the bull's boom.

### 3. Semen evaluation equipment:

It consists of, a photonic microscope, a hot plate, blades and lamellae, a digital spectrophotometer ACCUCEL-IMV for bovine sperm and a water bath set at 37°.

### 4. Semen dilution equipment (seed preparation):

In the dilution stage we used the diluter MICROLAB 500 as well as the diluter OPTIXCELL which is composed of : a distilled water, fructose, glycerol and plant phospholipids (acting as cryoprotectors), citric acid (acting as a buffer), antibiotics (tylosine), gentamicin, spectinomycin, lincomycin), vitamins and antioxidants.

### 5. Cooling and balancing equipment:

It includes a refrigerated showcase, and a thermometer.

### 6. Sequins printing equipment:

We use Fine sequins with a diameter of 2 mm and a volume of 0.25 ml, and for filling, printing (name of bull, identification number, date of collection and identification of the insemination center) and welding of sequins we use a filling and welding machine (MRS3) and freezer ramps.

### 7. Pre-freezing equipment:

It includes a Digitcool programmable freezer coupled with a tank of liquid nitrogen (vertical freezing), a computer coupled with a control box and ramps to hold the packaged sequins.

### 8. Semen freezing and conservation equipment:

Freezing is done in a tank (digit cool) and then in liquid nitrogen at -196°C.

### 9. Motility test :

The sperme Class Analyzer® (SCA®) is a CASA (Computer Assisted Semen Analysis) system that allows the management and analysis of human and animal semen samples according to World Health Organization (WHO) criteria.

The basic components of the system are:

- Phase contrast microscope.
- Camera.
- A computer (consult minimum requirements) with the SCA® analysis software installed.

**10. Viability test equipment:**

The FC method using the Easy Cyte5 HT cytometry which requires the use of pilot software called guava Soft IMV and a kit called Easy Kit, for the calculation of the viability rate. In addition, we also use bleach, distilled water, eppendorf tubes, micropipettes, a cytometry rinsing kit, a kit for the evaluation of the viability rate, tips for the micropipettes and a rack.

## IV. Method: (Take a look at Annexe 03)

### 1. Semen collection:

According to a protocol described by (Gacem, 2016):

Semen collection was carried out using an artificial vagina heated to a temperature between 35 and 38°C to avoid any thermal shock to the sperm. Before collection, the bulls were prepared. To do this, the bull trainer walks the bull and then brings it to the contact of the tips on the train.

As the animal climbs the mud by train, the bull grabs the penis's sheath with one hand and introduces it into the artificial vagina held with the other hand.As soon as the penis comes into contact with the internal surface of the vagina, the animal ejaculates.

The bull trainer returns the artificial vagina and the sperm flows into the graduated collector t ube then is transported to the laboratory to reduce the risk of damage due to the action of light or temperature.

At the end the resulting ejaculate must be identified in a special form called the spermiogram, which contains the name of the bull, the date of collection, the breed of the donor, the volume harvested, the quality of the semen (massal motility and concentration) and the volume necess ary to carry out the dilution.

### 2.1 First semen quality control:Macroscopic examination:

According to a protocol described by (Gacem, 2016):

The first control allows a general assessment of the quality of the ejaculate. by a visual examination that is carried out after collection, in order to evaluate the following parameters like the volume (measured by direct reading on the collection tube), the color, viscosity and odor of the ejaculate.

### 2.2 Microscopic examination:

According to a protocol described by (Gacem, 2016):

First, we begin with the evaluation of the massale motility as a first microscopic control. It is made from pure sperm, within minutes of collection. A drop of pure sperm is deposited on the previously heated to 37°C on a hot plate and then observed under a photonic microscope at XI0 magnification. The intensity of waves caused by sperm movement is then evaluated.

### 2.3 Concentration calculation:

According to a protocol described by (Gacem, 2016) :

The concentration of semen from bulls of different breeds was evaluated by spectrophotometry, in which pure semen is deposited in a bowl and diluted 1/100 with a physiological solution (this step is performed by an automatic dilution device).

The bowl is then collected and placed in an ACCUCEL-IMV spectrophotometer, which is used to measure the concentration of semen on the one hand and to determine the volume of diluents to be added to complete the dilution on the other. Finally, a printer attached to the spectrophotometer is used to print out all calculated data.

### 2.4 Dilution:

According to a protocol described by (Gacem, 2016):

The purpose of dilution is to obtain a sufficient quantity of sperm for use in artificial insemination and to maintain the viability of sperm in artificial insemination.

Dilution is carried out in sterile, graduated glass bottles. For this, the volume of diluents indicated by the spectrophotometer is added to the ejaculate which is always held at 37°C in the water bath.

### 2.5 Cooling and sequencing:

According to a protocol described by (Gacem, 2016):

After dilution, the bottles are placed in a refrigerator at +4°C for 3 hours. Once cooled, the seed will be packaged in fine sequins, with a diameter of 2 mm and a volume of 0.25 ml and for filling, printing (name of bull, identification number, date of collection and identification of the insemination center) and welding of sequins we use a filling and welding machine

(MRS3), latter is located in a refrigerated window at +4°C; the sequins are then placed on freezer ramps

**2.6 Freezing:**

According to a protocol described by (Gacem,2016):

The first stage of freezing is carried out in a tank (Digit Cool), when this stage the temperature is lowered gradually, thanks to nitrogen vapors, from +4°C to -140°C. After 7 minutes, the glitter is recovered from the Digit Cool and then plunged into liquid nitrogen at -196°C.

**2.7 Second sperm quality control and storage :**

According to a protocol described by (Gacem,2016):

This control is used to check the effect of freezing on the quality of the sperm produced. For this, 24 hours after freezing, a few sequins are chosen at random. They are thawed in warm water for 30 seconds.

They are then dried and their contents are examined under a photonic microscope to verify that the sperm have kept their motility. If freezing has not had a negative effect on the quality of the sperm, the sequins are placed in cups, which are placed in the seed canisters.

The latter are immersed in biostats of liquid nitrogen. A regular nitrogen level check is performed. After 24 hours of collection, frozen sperm is transferred to the sperm bank, which will be stored indefinitely.

**2.8 Motility with CASA:**

According to a protocol described by Purday and Graham (2004):

The bull sperm was heated as quickly as possible. The straw was first shaken to remove the remaining liquid nitrogen and then immersed and agitated in water at 34-37°C. The sequins was then shaken to remove the remaining liquid nitrogen. Thawing was observed after about 30 seconds . The content of the thawed straw was diluted in an eppendorf tube to the order of one V: V (250µl of the seed +250µl of the Nacl at 0.9%). This step was carried out, still, in thermal conditions close to 37°C (in a water bath). Deposit of a fairly rich and homogeneous sample of 10 microliters on a glass slide which was covered, by horizontal deposit, with a 22 x 22 mm glass slide. In these conditions, the space obtained between slide and slide was about 10 to 20 micrometers. This space is sufficient to observe the undulatory movement of the spermatozoa that will evolve in this liquid thickness. The kinematic analysis was performed using a 10x and Ph- magnification objective above an integrated heating plate (37°C) in the microscope. The microscope has a light source with a green filter. The analysis was performed using SCA®CASA software, under the nail of SCA Mobility®, by digitizing the

images obtained by the camera. These images are then processed by an integrated computer, which allows us to define objective and repeatable parameters such as the proportion of motile spermatozoa, their speed, the linearity of their trajectories and the lateral mobility of the head. After analyzing the mobility, we exported the data obtained (the different kinematic parameters) in the form of Excel.

### 2.9 Viability test with the Flow Cytometry:

According to a protocol described by (Gacem, 2016):

Washing of the device is the common step that precedes all the tests performed by the cytometry, the latter is done using distilled water, bleach and a washing solution called Easy Clean; the purpose of this is to avoid that:

The capillary of the cytometry from clogging, and to recover all the cells that remain inside the system after the previous analysis and to recover all the molecules that can alter the results of the next analysis.

- A volume of each ejaculate is mixed with 199ul of distilled water and deposited in wells placed on a special plate called the well holder. The number of wells is determined according to the number of samples to be analyzed.
- The well plate was incubated for 10 minutes in an incubator at 37°C protected from light.
- On the first window that appears on the Cytosoft IMV software interface, the bovine species is selected and the type of analysis (in this case: viability) is chosen.
- The plate is then retrieved from the incubator and placed in the Cytometry.
- After assigning the names of the bulls to the corresponding wells and saving the generated files in a chosen location on the microcomputer, the analysis are started.
- Once the analysis is completed, the results are retrieved in the form of a data table (Excel file) and corresponding graphs (scatter plots).

# Result and discussion

**Due to covid-19 (corona virus) situation, I was unfortunately unable to do my internship; therefore, I counted on synthesis in my study.**

Among the articles that we relied on and studied are:

• Advanced Techniques of Bovine Semen Analysis, (Cenariu et al., 2018).

• Assessment of the sexual function of the breeding bull (Dumont, 1997).

• Computer assisted sperm analysis of Brahman crossbred breeding bull semen, (MR et al., 2017).

• State of play on artificial animal insemination in West African countries, (Dotche et al., 2019).

• Evaluation of Sperm Viability and Acrosomal Integrity by Flow Cytometry Analysis in Jersey Crossbred Bulls, (Rajaram et al., 2017).

• Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art, (Hossain et al., 2011).

• Bovine artificial insemination, (Parez and Duplan, 1987).

• What place for the automated spermogram in 2016 (Lammers et al., 2016).

• SCSA and Sperm – Bos – Halomax to analyze bull sperm DNA fragmentation, (Garcıa-Macıas et al., 2007).

**The main goal of our work has been to evaluate the quality of bovine semen using different methods and to identify the difference by indicating the limits and advantages of each one.**

## I. The classic method:

The analysis by macroscopic and microscopy allows evaluating the semen characteristics of the bull's.

The table 3 below represents the rate (volume, concentrastion, motality, etc.) of bovine sperm for the Borgou, N'Dama, Lagoon, Baule, Zebu Peulh and Azawak breeds in West Africa.

| Espèces | Pays | Volume (ml) | Concentration ($x10^6$/ml) | Mobilité (%) | Motilité | Vivant (%) | Auteur |
|---|---|---|---|---|---|---|---|
| Bovin | | | | | | | |
| Borgou | Bénin | 3,6 | 1340 | 80,2 | 4,5 | | Akpo *et al.* (2018b) |
| Borgou | Bénin | 3,1 | 775,7 | - | 4,3 | 90,2 | Gbangboche *et al.* (2011) |
| Borgou | Bénin | 3,4 | 720 | - | 2,5 | 86 | Konfe (2014) |
| Lagunaire | Bénin | 2,2 | 919 | - | 3,7 | 73,8 | Konfe (2014) |
| N'Dama | Mali | 2,2 | 743 | - | 2,7 | 71,5 | Konfe (2014) |
| Baoulé | Burkina-Faso | 2,4 | 1050 | - | 3,8 | 80 | Cloe *et al.* (1989) |
| Zébu | Burkina-Faso | 1,6 | 1550 | - | 2,8 | 75,3 | Konfe (2014) |
| Azawak | Bénin | 5,2 | 740 | 73,4 | 4,7 | | Akpo *et al.* (2018b) |
| Azawak | Niger | 3,6-5,5 | 413-766 | 72-82,5 | 4-4,2 | 72,33-86 | Cristofori *et al.* (2005) |

**Table 3**: Characteristics of the semen of domestic animals (bull's) in West Africa (Dotche et *al.*, 2019).

### 1. Macroscopic analysis:

- **The volume:**

The volume of sperm produced by the Borgou bull ranges from 3.1 to 3.6 ml and for Azawak bulls from 3.6 to 5.5 ml (Table 1) .Whereas for the N'Dama and Lagunaire breed is 2.2 ml, 1.64 ml for the Zebu Peulh , 2.7 ml for the Baoule.

As reported by (Dotche et *al.*, 2019), the volume of ejaculate recorded in Borgou by (Akpo et *al.*, 2018b) is close to that reported by (GBANGBOCHE et *al.*, 2011) and (Konfe,2014).

On the other hand, the ejaculate volume of the N'Dama and Lagunaire breed is close to that reported by (Cloe et *al.*, 1989).

And also for the Azawak breed volume which is determined by (Akpo et *al.*, 2018b) is close to (Cristofori et *al.*, 2005).

Furthermore, the volume of the ejaculate varies is according to the breed and the average volume of ejaculate recorded in all breeds was more than 1 ml, one of the requirements for freezing sperm (Parez et Duplan, 1987).

## 2. Microscopic analysis:

- **Concentration:**

The sperm concentration of Borgou bull semen ranges from 720 to 1340x$10^{6}$spz/ml also for the Azawak bull from 413 to 766x $10^{6}$spz/ml (Table 1), on the other hand for the Boule bull the sperm concentration is 1050 x $10^{6}$ spz/ml, 919 x $10^{6}$spz/ml for the Lagunaire breed and 743 x$10^{6}$spz/ml for the N'Dama breed (Table 1). Otherwise,the Zebu Peulh, produces a more concentrated sperm (1550 x $10^{6}$spz/ml ) than the bulls (Borgou, Lagoon, N'Dama).

As said by (Dotche et *al.,* 2019), the sperm concentration recorded in Borgou by (Akpo et *al.,* 2018b) is higher than that determined by (Gbangboche et *al.,* 2011) and (Konfe , 2014) and represents a value close to the concentration between them.

For the Azawak breed the sperm concentration noted by (Akpo et *al.,* 2018b) is consistent with (Cristofori et *al.,* 2005).Also the sperm concentration of the Lagunaire breed is close to the one registered by (Cloe et *al.,* 1989).

The Zebu breed (Konfe, 2014) has a higher sperm concentration compared to other breeds of bulls.

In addition, the concentration of sperm in the ejaculate is used to determine the correct dilution rate for the production of frozen sperm straws used for artificial insemination (Cabannes,2008)

There is a general rule, only sperm with a minimum concentration of 0.6 X $10^{9}$spz/ml is diluted in the insemination centers (AdamouN'Diaye et *al.,* 1990).

- **Motality:**

Borgou bull sperm has a motility score from 2.5 to 4.3 and 3.8 for the Baoule bull, 3.7 for the Lagunaire breed and 2.7 for the N'Dama breed (Table 1) and for Zebus sperm motility varies from 72 to 83% .

Dependent on (Dotche et al., 2019), the sperm motility recorded in Borgou by (Konfe, 2014) is lower than that reported by (Akpo et *al.,* 2018b) and (Gbangboche et *al.,* 2011).

The sperm motility recorded in N'Dama bulls corroborates those reported by (Cloe et *al.,* 1989) in the Baule bull.

The sperm motility of the Azawak breed by (Akpo et *al.,* 2018b) agrees with that recorded by (Cristofori et *al.,* 2005).

The sperm motility of Zebus falls with time and is between 8 and 20% 48 hours after collection (Cristofori et *al.,* 2005).

Moreover, for bulls used in artificial insemination, motility must be greater than 60 %, a score of more than 3 in mass motility(Dumont, 1997).

- **Motile**:

According to (Dotche et al., 2019) Borgou bull sperm has 80.2 to 89.7% motile of spermatozoa (Akpo et al., 2018b), while the motile of the Azawak spermatozoa varies from 72 to 83% (Cristofori et al., 2005). This motility drops over time and is between 8 and 20% 48 hours after collection (Cristofori et al., 2005).

The combination of macroscopic and microscopic parameters such as sample volume, sperm concentration and sperm motility allows the calculation of the number of motile spermatozoa in the ejaculate according to (Lammers et *al.*,2016).

- **Live sperm:**

According to (Dotche et *al.*, 2019), the percentage of live sperm found in Borgou by (konfe,2014) is consistent with that recorded (Gbangboche et *al.*, 2011) for the same breed in Benin.

The percentage of live sperm recorded in the Lagunaire, N'Dama and Zebu peulh by (knofe,2014) is close to that reported by (Cloe et *al.*, 1989) in the Baule bull,

The concentration of spermatozoa in zebus sperm is higher in the rainy season than in the dry season (Rekwot et *al.*, 1987).

Freezable semen must contain at least 60% live spermatozoa (Parez et duplan, 1987).

All breeds meet this condition (knofe, 2014). But , If the live sperm count is less than 60%, the sperm is not stored. Moreover, this examination is not performed routinely, as the most relevant quality criterion for Bovine AI use is the percentage of live sperm after thawing. (Cabannes,2008).

As specified by (Dotche et *al.*, 2019), The rate of sperm abnormalities in bulls ranges from 3% to 6% (Cloe et *al.*, 1989; Sekoni et al., 2004; Cristofori et *al.*, 2005) and the rate of dead sperm from 14% to 26% (Cristofori et *al.*, 2005).Furthermore, the season and health status of the bull influence the rate of abnormalities. Thus, under the influence of high temperature and lack of feed resources, the rates of sperm abnormalities and dead sperm are higher in the dry season than in the wet season (Rekwot et *al.*, 1987).

- **Limitations and advantages of classical sperm examination techniques:**

Among the limitations of the classical method used to determine semen quality is:

Subjective and their reliability depends on the experience of the observer (risk of maximizing errors (IFIP, 2013)) according to (Cabannes, 2008; Dotche et *al.*, 2019), also this method is time-consuming as reported by (Cabannes,2008; Garner et *al.*,1986 ).

Moreover, the analyses can suffer from a lack of precision (Lammers et *al.*, 2016).And the conventional semen examination is only allows for the analysis of a small number of spermatozoa (Cabannes,2008).

On the other hand, the advantage of this method is: Inexpensive according to (Cenariu et *al.*,2018) and Allows to perform the same analyses (the examination of the seed) as the advanced methods according to (Boukari et *al.*, 2018).

## II. Computer-Assisted Sperm Analyses (CASA):

The CASA system is one of the most advanced techniques for determining the different types of sperm to assess quality,and allow an objective measure of many measurements including: motility, and CASA measurements.

The result of sixty (60) ejaculates were collected from eight (8) matured Brahman crossbred breeding bulls with a view to assess the motility and velocity parameters of ejaculates sperm using Computer-Assisted Sperm Analyser (CASA) ,the table 4 below represents the square mean with standard error of the CASA parameters.

| Parameter | Number of records | Brahman crossbred bull (50%) |
|---|---|---|
| Volume (ml) | 60 | 6.24±0.15 |
| TM (%) | 41 | 72.85±1.46 |
| FPM (%) | 41 | 64.80±1.51 |
| FM (%) | 41 | 45.91±2.09 |
| CM (%) | 41 | 1.92±0.48 |
| VCL (µm/s) | 52 | 166.05±13.00 |
| VSL (µm/s) | 52 | 80.93±7.03 |
| VAP (µm/s) | 52 | 112.01±7.51 |
| LIN (VSL/VCL) (%) | 52 | 48.73±0.86 |
| STR (VSL/VAP) %) | 52 | 72.25±0.03 |
| RD (%) | 52 | 5.75±2.55 |
| ALH (µm) | 52 | 7.36±3.54 |
| ROT (%) | 52 | 53.01±0.05 |
| BCF (Hz) | 52 | 28.58±0.97 |
| WOB (%) | 52 | 55.13±0.02 |

TM, Total Motility; FPM, Forward Progressive Motility, FM=fast motility, CM=Circular Motility, VCL=Curvilinear Velocity, VSL=Straight line Velocity, VAP=Average Path Velocity, LIN=linearity, STR=Straightness, WOB=Wobble, ALH=Amplitude of Lateral Head Displacement, RD=Radius, ROT=Rotation and BCF=Beat/Cross-Frequency

**Table 4**: Least square mean with standard error of motility and velocity parameters of bulls (MR et *al.*,2017) .

The least square mean value of total motility, forward progressive motility, fast motility and circular motility were 72.85±1.46, 64.80±1.51, 45.91±2.09 and 1.92±0.48%, respectively but for the mean value of velocity parameters such as curvilinear velocity (VCL), straight line velocity (VSL) and average pathway velocity (VAP) were 110.05±13.00, 52.93±7.03 and 64.01±7.51 µm/s, respectively. For linearity (LIN, VSL/VCL) and straightness (STR, VSL/VAP) were 48.73 and 72.25% (Table4).

According to (MR et *al.*,2017), this observation strongly agrees with the studies of (Vincent *et al.*,2012 ; Contria *et al*., 2010) who obtained the mean value of total motility of sperm .In addition, the result of forward progressive motility strongly supports with local and Friesian bulls who obtained by (Ahmed *et al.*,2014) .

However, this result are differs from that of (Vincent *et al.*,2012) who reported higher progressive motility of bovine sperm, also for (Contria *et al.*,2010) who obtained the mean value of progressive motility slightly lower.

On the other hand, As reported by (MR et al.,2017) ,the observation of VCL, VSL and VAP is strongly supports with the result of (Perumal et *al.*,2014) and (Amanda,2011) for Mithun semen and Holstein bulls. However, the observation differs from that of (Sundararaman et *al.*, 2012) who reported lower velocity in bull sperm.

A spermatozoon has significantly higher VCL and ALH, indicating that there is major bending of the mid piece and large amplitude of lateral head displacement. This signifies the hyperactivation of the spermatozoa.

Hyperactivation in turm implies high energy state of the spermatozoa, which is essential for sperm penetration through cervical mucus, zona pellucida, fuse with the oocytes, and successful fertilization (Aitken et *al.*, 1985).

In addition, linearity (LIN, VSL/VCL) and straightness (STR, VSL/VAP) are agreeable to the average of results found by (Perumal *et al.*, 2014 ; Amanda, 2011) and comparable to (Farrell *et al.*, 1998, Budworth *et al*., 1988). The linearity or linear motility is higher indicating that spermatozoa have higher rate of fertilization potential in comparison to the total motility percentage (Cremades *et al*., 2005) and sperm samples containing such spermatozoa have higher fertility rates and pregnancy rates after artificial insemination (Farrell *et al*., 1998).

The results of amplitude of lateral head displacement (ALH) (μm) and beat/cross-frequency (BCF) (Hz) were approximately equal to the results get by (Perumal *et al.*,2014 ; Amanda, 2011).Also for the percent value of wobble is similar to the results of (Perumal *et al.*,2014) in Mithun semen.

Moreover, the parameter LIN is a measure of linearity and the BCF motion parameter indicates the number of times the sperm track crosses the smoothed path, both of which indicate linear progression.

In other result of 4 ejaculates, obtained from 4 Pinzgauer bulls (1 ejaculate from each male).when motility and concentration were assessed using the SCA Motility module of the CASA system, the following results were obtained (Table 5):

| | Bull 1 | Bull 2 | Bull 3 | Bull 4 |
|---|---|---|---|---|
| **CASA total motility** | 100% | 98.75% | 90.41% | 98.31% |
| **CASA progressive motility** | 90.27% | 58.11% | 52.84% | 59.64% |
| **CASA rapid velocity** | 88.55% | 51.34% | 47.86% | 54.40% |
| **CASA Concentration** | 2.176 | 2.089 | 2.311 | 1.879 |

**Table 5**: CASA motility and concentration (billion spz./ml) results in the four bulls (Cenariu et *al.*, 2018).

CASA total motility are 100%, 98.75%, 90.41%,98.31% in Bull 1 , 2 , 3 and 4 , respectively. In addition, the concentration in bull's is between 2.17 and 1.87 billion spz. /ml

According to (Cenariu et *al.*,2018) , CASA was able to differentiate between progressively and non-progressively motile spermatozoa, which is extremely important, taking into consideration the fertilization capacity of those cells. As the results show very clearly, only one of the bulls (Bull 1) had great parameters regarding progressive motility, correlated with a proper velocity, thus providing an adequate level of confidence regarding its fertility.

In the other three bulls, although gross motility was very good, the CASA analysis pointed out a large number of non-progressively motile spermatozoa, which have a questionable ability to fertilize an ovum.

Moreover, if those spermatozoa were submitted to the process of cryopreservation, motility would decrease and their fertilization capacity would be even more unreliable. It is often relatively easy to identify completely infertile bulls because their sperm usually are considerably less motile or have abnormal shapes.

The results of concentration were very reliable, as shown by CASA. For that matter is one of the most important sperm parameters, which provides important information about a male's fertility.

- **Advantages and limitations of computer assisted sperm analysis (CASA) techniques:**

First of all, we have several advanced techniques such as CASA, which is a rapid and objective technique according to (Cenariu et *al.,* 2018) that allows a more accurate estimation of sperm motility in a minimum amount of time as reported by (Salson, 2008) and (Cabannes,2008) and to assess viability (Allimbant, 2010). On the contrary, among the limitations of this technique is the need for expensive equipment and specialized personnel as reported by (Cenariu et *al.*, 2018).

In addition several models of CASA instruments are now available for assessing sperm quality and motility. Each system operates on similar principles, but they differ in their setup and use different algorithms to determine speed and trajectories. These systems are designed to be used in a variety of ways. Many factors are known to affect CASA results, such as the temperature at which semen is analyzed, as said by (Vincent et *al.*, 2012).

### III. Flow Cytometry:

FC it is one of technique who allows counting viability in a different way either by Statistic or by probes or dyes.

The evaluation of sperm viability in the collected sperm sample was determined by which was displayed in (table 6):

| Bull No | Total sperm | Viable sperm | Non-viable sperm |
|---|---|---|---|
| 1 | 61338 | 50408 (82.18) | 10930 (17.82) |
| 2 | 61542 | 48885 (79.43) | 12657 (20.57) |
| 3 | 61078 | 49736 (82.89) | 11342(17.11) |
| 4 | 61235 | 43312 (70.73) | 17923 (29.27) |
| 5 | 61589 | 49436 (80.26) | 12153 (19.74) |
| 6 | 60977 | 45854 (75.20) | 15123 (24.80) |
| **Chi square test ($\chi^2$)** | | 348.82** | |

Note: Figures in parenthesis indicate the percentage value.

**Table 6:** Evaluation of sperm for viability (Rajaram et *al.*, 2017).

There is a high significant difference (P<0.01) in the sperm viability between the bulls. Among the six bulls, Bull number 3 has higher percentage of viable spermatozoa (82.89%), followed by 82.18%, 80.26%, 79.43%, 75.20% and 70.73% for Bull number : 1, 5, 2, 6 and 4 respectively. The percentage of dead sperm was 29.27%, 24.80%, 20.57%, 19.74%, 17.82% and 17.11% for Bull: 4, 6, 2, 5, 1 and 3 respectively.

The percentage of viable and non-viable sperm by flowcytometry was ranged from 70.73 to 82.89 and 17.11 to 24.80 respectively, as claimed by (Rajaram et *al.*, 2017). These results were in accordance with (Graham *et al.*,1990) who studied sperm cell viability, acrosomal integrity and mitochondrial function using flow cytometry and found that propidium iodide stained cells (red- non viable) ranging 7% to 41%

In another results obtained in the form of graphs and in the form of a cloud of points by the FC, Figure below (figure 7) explain how the sperm distribute and deviated to population after determination of viability.

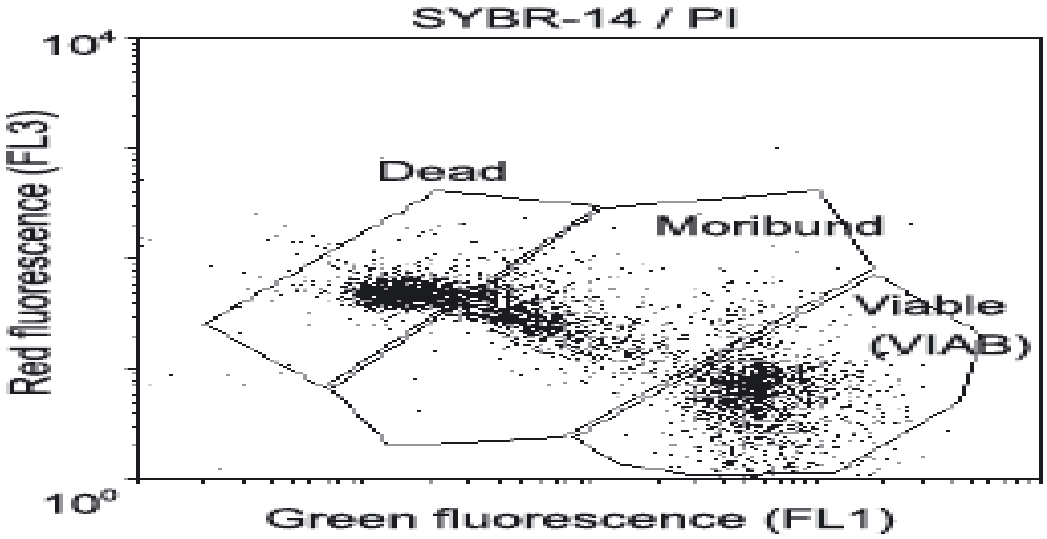



**Figure07**: General flow cytometric plots of double dye SYBR-14/PI.(Garcıa-Macıas et *al.*, 2007).

Moreover,figure 8 show that viable spermatozoa are indicated by green color, dead ones are indicated by red color and dying spermatozoa are indicated by blue color. Fluorescent microbeads are indicated by magenta color.

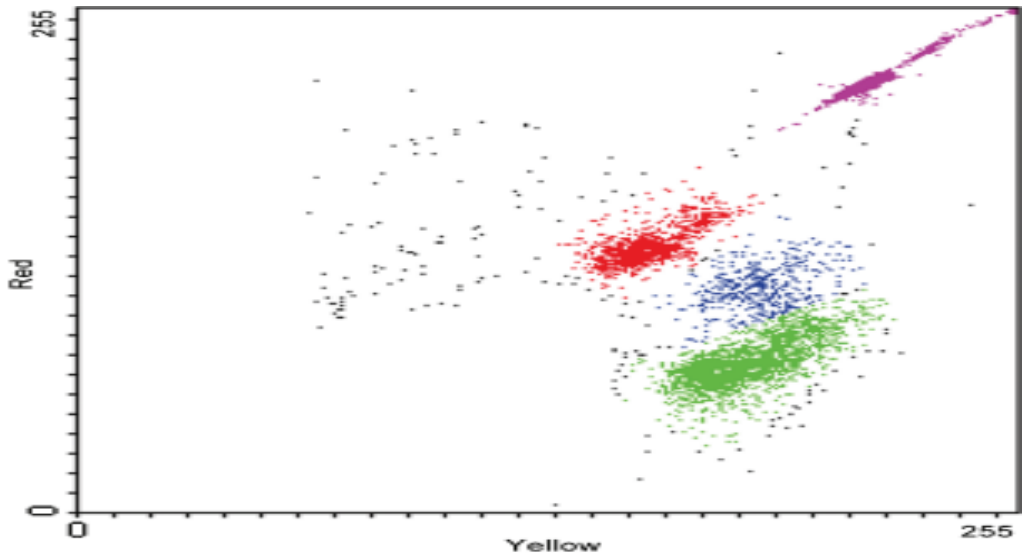



**Figure 8:** Color dot plot representing the simultaneous evaluation of viability (SYBR 141/PI staining) and cell concentration (Hossain et *al.*, 2011).

In addition, another result by FC in form the three-dimensional contour plot shows distinct peaks for viable and dead spermatozoa; moreover, dying spermatozoa and cell debris can be easily recognized as well.

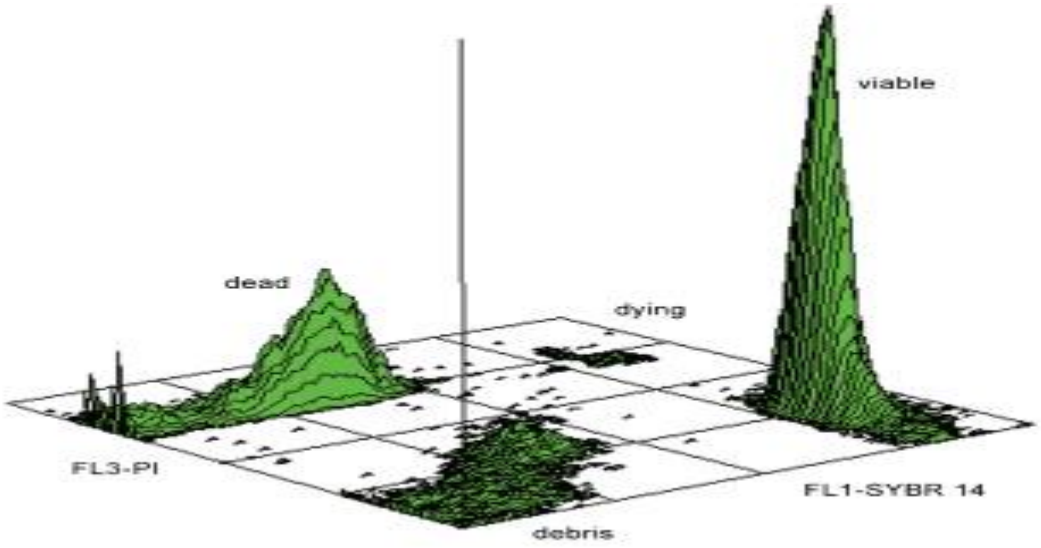



**Figure 9:** Viability evaluation by SYBR 14/PI labeling (Hossain et *al.,* 2011).

According to (Hossain et *al.,* 2011), The most commonly used combination of dyes used for membrane integrity checks (to detected sperm viability) is SYBR-14/ PI.

For that matter the SYBAR-14 is a membrane-permeable nuclear dye that penetrates to the head, forms a complex with DNA and causes green fluorescence in the nuclei of living cells. The advantage of this dye is that it is not based on an enzyme system and is therefore not time-dependent. Also the PI penetrates at the sperm head into cells with membrane damage to bind to the DNA, as claimed by (Allimant,2010), In this combined stain, the nuclei of viable spermatozoa fluoresce green, while those with eroded plasmalemma are counterstained red, including a moribund cell subpopulation (green–red), as claimed by (Hossain et al., 2011).

Furthermore, PI is normally used as the viability probe of choice in flow cytometre , as this supravital stain rapidly penetrates non-viable spermatozoa when their plasma membrane is disrupted , as reported by (Riedy et al.,1991*in* Hossain et *al.,* 2011).

According to (Hossain et *al.,* 2011), the main advantage of this dye combination is that both fluorochromes have the sperm intracellular target (DNA).

While the determination of sperm concentration by flow cytometry can be used to maximize production of straws, simultaneous determination of sperm viability can be used for the rejectionthe poorest ejaculate and thus increase fertility after insemination ,as reported by (Christensen et *al*,2004b *in* Christensen et *al.*,2005).

- **Advantages and Limitations of flow cytometry :**

The flow cytometry based sperm evaluation is a fast, accurate, highly sensitive and highly repeatable technique, as reported by (Rajaram et *al.,* 2017) and offers incomparable precision properties (Cordelli et *al.,* 2005 *in* Fortier,2010) , with the possibility of objectively measuring thousands of cells for multiple characteristics in a short time with minimum preparation

(Graham et *al.*, 1990 *in* Rajaram et *al.*, 2017) Also, it will help in the better understanding of the sperm physiology and functionality according to (Hossain et *al.*, 2011).

The application of flow cytometry minimizes, in part, several analytical difficulties encountered. Moreover, it's an automated approach capable of measuring the quantity of one or more fluorescent markers associated with the cell in an unbiased way. In addition, the discovery of a variety of fluorochromes and components conjugated to a fluorescent probe makes possible a wide range of assays for sperm evaluation at biochemical, ultrastructural, and functional levels (Gillan et *al.*, 2005 *in* Fortier, 2010).

On the other hand, several factors influence the choice of the cytometry to use for the analysis of sperm cells. Multiparametric analysis is usually obtained with instruments containing more than one laser and many photomultiplier tubes. However, some software requires certain knowledge of flow cytometry concepts, making the instrument more difficult to operate. Moreover, some programs have gaps in export and data compilation, making it more difficult to analyze the data and these shortcomings are time-consuming for the user as stated by (Vincent et *al.*,2012).

Consequently, the use of flow cytometry remains limited to research for certain reasons, notably the cost of the equipment, the operating difficulties requiring a qualified operator, and the fragility of the device in terms of the impact associated with movement as reported by (Gillan et *al.*, 2005 *in* Fortier, 2010).

# Conclusion and Perspectives

**Conclusion:**

This work discusses the importance of having reliable analytical techniques to evaluate bovine sperm by study the main parameters that allow evaluating the quality,in general we discussed are volume, motility , viability , and to identify the difference by indicating the limits and advantages of each techniques.

Firstly, the analysis by automaton has shown accuracy and precision and allows performing simultaneously several sperm parameters on thousands of individual cells in a short period of time.

Therefore, the examination by the classic method can be a fairly reliable alternative method for viewing and measuring sperm parameters when the number of samples is not high, but this method is time consuming.

In brief, sperm freezing can affect the quality, which may lead to different results obtained through the techniques.

Overall, the artificial insemination industry will benefit from the implementation of flow cytometry, CASA, light microscopic to evaluate sperm quality.

## *Perspectives:*

The time allocated to this study was insufficient to determine the comparison of the methods of assessment of the seed quality and defined the most reliable and appropriate technique.

In our modest work, we touched upon some techniques, among them the classic method, CASA and flow cytometry which allowed us to obtain a statistical analysis of semen quality for artificial insemination .

The evaluation can be enhanced by other techniques. For instance, The Eosin-Nigrosine one to establish the percentage of live sperms. As well as, the use of a Chromatin Structure Assay System (CSAS) or the use of Flow cytometry to assess the viability in terms of mitochondria, acrosome integrity and chromatin integrity, and by the concentration . Furthermore, it is possible to use the oxidative stress to detect the functionality of the sperm.

Finally, the use of these techniques may contributes in knowing the presence or absence of fertility.

# Bibliographic Reference

1. Akusu, MO., Agiang, E.A., Egbunike, G.N., 1984. Ejaculate and plasma characteristics of west African Dwarf (WAD) buck. 10th Intl Congr. Anim. Reprod. A.I,2.50.

2. Allimant,M.,2010,Actualites sur les methodes d'evaluation de la qualite de la semence de l'etalon.These de Doctorat Vétérinaire ,Universite Claude Bernard Lyon1,France.138.

3. Auger,I., 1997.Perspectives de l'analyse de la mobilité des spermatozoïdes. Andrologie, 7,433-442.

4. Briand-amirat , L., Anton, M., Gerard, O., Tainturier, D.,2006. Revue médecine vétérinaire. 4,205-212.

5. Baril, G., Chemine, P., Cognie, Y., Guerin, Y., Leboeuf, B., Orgeur, P., Vallet, J.C., 1993. Manuel de formation pour l'insémination artificielle chez les ovins et les caprins.Rome, FAO.2,3-1.

6. Barszcz,K.,Wiesetek,D.,Wąsowicz,M., Kupczyńska,M., 2011. Bull Semen Collection and Analysis for Artificial Insemination,Journal of Agricultural Science.10,3.

7. Boly, H., 1993. Effet pathogène de Trypanosoma congolense sur la fonction de reproduction mâle des taurins « Baoulé ». Thèse de doctorat d'université. Université PIERRE et MARIE CURIE (PARIS VI), Physiologie de la reproduction.France. 100.

8. Boly, H., 1986. La récolte du sperme chez le babouin (Papio papio) Parla technique de l'électro-éjaculation. Thèse de Dr Vétérinaire, Université Cheick A. Diop de Dakar, EISMV, 76.

9. Cabannes, C.R., 2008. Comparaison des méthodes d'évaluation de la qualité de la semence dans les espèces bovine, canine et humaine. Thèse de doctorat vétérinaire. Université Paul-Sabatier,Toulouse. 107.

10. Cenariu, M.,Pall,E.,Borzan,M.,Bogdan,L.,Groza,I.,2018. Advanced Techniques of Bovine Semen Analysis. Bulletin UASVM Veterinary Medicine.75,59-64.

11. Cheng, F.P., Fazeli A., Voorhout, W.F.,1996. Use of Peanut Agglutinin to assess theacrosomal status and the zona-pellucida induced reaction in stallion spermatozoa. Journal of Andrology.17, 674-682.

12. Chevalier, M.E., 2011. Gestion du sexe du produit en élevage bovin. Thèse de doctorat vétérinaire. Université claude-bernard - lyon I.

13. Christensen, P., Hansen,C., Liboriussen, T., Lehn-Jensen,H., 2005.Implementation of flow cytometry for quality control in four Danish bull studs. Animal reproduction science.85, 201–208.

14. Cordelli, E., Eleuteri, P., Leter, G., Rescia, M., Spano, M., 2005. Flow cytometry applications in the evaluation of sperm quality: semen analysis, sperm function and DNA integrity. Contraception. 72,273-9.

15. Dayon A. 2008. Role de la sphingosine kinas-1 dans la survie et la progresion des cellule tumorales prostatiques LNCaP vers l'androgén-indépendance , l'université de Toulouse.
16. Dotche, IG., Kiki, P., Govoeyi , B., Dahouda, M., Antoine-moussiaux,N., Dehoux, JP., Mensah,GA., Farougou, S., Thilmant, P., Youssao Abdou Karim, I., Koutinhouin, B.,2019. Etat des lieux sur l'insémination artificielle animale dans les pays de l'Afrique de l'Ouest. Journal of Applied Biosciences.143, 14712 - 14730.

17. Dovere,L.,Maggiulli,R.,Stoppa, M., Sanges,F., Romano,S.,Albricci, L., Tacconi,L., Soscia,D., Ubaldi,F.M., Rienzi,L.,2017. Semen collection, analysis and preparation:a mini review ,GENERA Centre for Reproductive Medicine,6,2.

18. Dumont, P., 1997.Appréciation de la fonction sexuelle du taureau reproducteur. Le Point Vétérinaire.28, 19-32.

19. Eustache, F., Jouannet, P., Auger, J., 2003. Evaluation of flow cytométrie methods to measure human sperm concentration.Journal of andrology. 22,558-567.

20. Foote, RH., 2005. Highlights in Dairy Cattle Reproduction in the Last 100 Years, The Internet-First University Press.Ithaca, New York.

21. Fortier, M., 2010.La cytometrie en flux comme outil pour caractériser et évaluer le potentiel de fertilité des spermatozoides bovins.Université Laval.Québec.115.

22. Gacem, A., 2016. Etude des paramètres spermatiques: Comparaison entre la viabilité des spermatozoïdes réfrigérés et spermatozoïdes décongelés.Mémoire de master, Université de Mostaganem, Alger.59.

23. Garcıa-Macıas,V., Paz, P., Martinez-Pastor, F., Alvarez, M., Gomes-Alves,S., Bernardo,J., Anel,E., Anel,L., 2007.SCSA and Sperm–Bos–Halomax to analyse bull sperm DNA fragmentation.international journal of andrology.30,88–98.

24. Garner, D.L., Johnson, L.A., Yue, S.T., Roth, B.L., Haugland, R.P., 1994.Évaluation de la double coloration de l'ADN de la viabilité des spermatozoïdes bovins à l'aide de SYBR-14 et d'iodure de propidium. Journal of Andrology.15, 620-629.

25. Gauthier D., Varo H., 1985.Caracteristiques spermatiques des taureaux en Guadeloupe. Variations avec la race et la saison. Ann de zootech 34, 463-470.

26. Graham, J.K.,Kunze, E., Hammerstedt, R.H., 1990. Analysis of sperm cell viability,acrosomal integrity, and mitochondrial function using flow cytometry.Biol. Reprod. 43, 55-64.

27. Gayrard ,2005, mémento des critères numériques de reproduction des mammifères domestiques.

28. Gillan, L., Evans, G., Maxwell, WM., 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology.63, 445-57.

29. Hakima, N., Sermondade, N., Sifer, C., 2012. Causes spermatiques et échecs de fécondance : quelles explorations autres que le spermogramme ? Can specialized sperm analysis predict fertilization ability? GynécologieObstétrique&Fertilité.40, 543–548.

30. Hanzen Ch., 2015. Cours doctorat, chapitre 12. L'anoestrus saisonnier des petits ruminants.Hanzen L'insémination artificielle chez les ruminants 2016 Université de Liège Faculté de Médecine Vétérinaire service de Thériogenologie des animaux de production.

31. Hamilton,T.,2006,January. Beef Bull Fertility.ontario. Consulté le 5 mars 2020 sur http://www.omafra.gov.on.ca/english/livestock/beef/facts/06-015.htm

32. Haskouri, H., 2001.Insémination artificielle et détection des chaleurs.-In : Gestion de la reproduction chez la vache. page consultée le 20 juin 2020 sur http://www.iav.ac.ma/veto/filveto/guides/repro/students/haskouri.pdf

33. Hossain, MS.,Johannisson,A., Wallgren, M.,Nagy,S., Siqueira, AP.,Rodriguez-Martinez,H., 2011.Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. Asian Journal of Andrology .13, 406–419.

34. kabera , F.,2008. Appreciation de la qualité de la semence bovine produite au centre national d'amélioration génétique (CNAG) de Dahra au Sénégal. Mémoire de diplôme d'études approfondies de productions animales. Ecole inter-états des science et medecine vétérinaires de Dakar.

35. Kaya,A., Birler,S., Enwall,L.,Memili,E.,2014.Determinants of Sperm morphology,Chenoweth P.J., Lorton S., Animal Andrology Theories and Applications,CAB International,USA.34-55.

36. Kohler, C., 2011. L ' appareil génital masculin. Support Cours (Version PDF) –Université Médicale Virtuelle Francoph.15.

37. Konfe, H., 2014. Etude spermiologique des bovins de races locales de l'Afrique de l'Ouest: cas du Borgou , du taurin Lagunaire , du taurin N'Dama et du Zébu Peulh.,Université polytechnique de Bobo-Dioulasso,87.

38. Kumar, D., Naqvi, A-J. S. M. K., 2010. Objective assessment of sperm motion characteristics of Malpura ram lambs raised under intensive management system in semiarid tropical environment. Trop Anim Health Prod. 42,653–658.

39. Laghrour, W., 2012. Comparaison de deux méthodes de traitement de Maitrise des cycles associant la progesterone oeustrogénes et la prostaglandine f2atpha chez la vache laitiére. Thése de magister. Université el-hadj lakhdar Batna.

40. Lammers, J., Splingart, C.,Reignier, A., Catteau, A.,Barrière, P., Fréour, T.,2016.Quelle place pour le spermogramme automatisé en 2016 ?. Journal de Biologie Médicale.5,28-30.

41. LePecq, JB., Paoletti, C.,1967. A fluorescente complex between ethidium bromide and

nucleic acids.Physical-chemicalcharachterization.J.Mol Biol. 27, 87-106.

42. Marc, S., 2015. Actualités en cryoconservation des semences des principales espèces d'intérêt vétérinaire.Lyon.163.

43. Meskini, Z., 2017.L'insemination artificielle chez les caprins de la race arbia dans la region de Tiaret.Mémoire de master, Université de Mostaganem,Alger.94.

44. MR, I., Husain, SS., Hoque1, MA., Talukder, MK., Rahman, MS., Alil, MY.,2017.Computer assisted sperm analysis of Brahman crossbred breeding bull semen. Bang. J.Anim.Sci. 46, 1-9.

45. Parez, M., Duplan, J.M., 1987. L'insémination artificielle bovine, ROGER MARION Edition. reproduction et amélioration génétique, Paris, France. 256.

46. Peters, A.R., Bail P.J.H., 2004. Reproduction in cattle.Third Edition Oxford.Blackwell Publishing.13,193-195.

47. Purdy, PH., Graham, JK., 2004. Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction, and fertility. biolReprod.71, 522-527.

48. Rajaram, V., Palanisammi, A., Maroudam, A., Sivaselvam,S.N.,Kumanan, K.,2017. Evaluation of Sperm Viability and Acrosomal Integrity by Flow Cytometry Analysis in Jersey Crossbred Bulls. Int.J.Curr.Microbiol.App.Sci. 6, 87-93.

49. Reinaldo, F. C., 2011. Basics of Reproductive Function in Bulls. Beef Cattle Library.4,1-2.

50. Rigal,GBF.,2008. Comparaison de la qualité de la semence de taureaux collectes àl'électroéjaculateur ou au vagin artificiel. Thése du doctorat. Université Paul-sabatier. Toulouse.

51. Ronot, X., Grunwald, D., Mayol, J.,F., Boutonnant, J., 2006. La cytométrie en Flux. Editions Tec et Do.456.

52. Rouge, M., Bowen, R., 2002.Collecte et évaluation du sperme: introduction et index. Consulted on le 16/09/2020 sur http://www.vivo.colostate.edu/hbooks/pathphys/reprod/semeneval/collection.html

53. Salson, OGL., 2008. Recolte et conservation du sperme chez les felins:etude bibliographique. Thèse de doctorat vétérinaire. La faculte de medecine de creteil.201, 98-100.

54. Shoenian,S., 2005. Reproduction in the ram.,Prolongation de coopérative du Maryland,Université de maryland.

55. Staub,C., Johnson,L., 2018.Spermatogenesis in the bull. The Animal Consortium.9,1.

56. Thomas, C.A., Gamer, D.L.,DeJarnette JM., Et Marshall CE.,1998. Effect of cryopreservation of bovine sperm organelle function and viability as determined by flow cytometry.Biol.Reprod.58, 786-93.

57. Vincent, P., Underwood, S.L., Dolbec, C., Bouchard, N., Kroetsch, T., Blondin, P., 2012.Bovine semen quality control in artificial insemination centers. Anim Reprod.9, 153-165.

58. WHO,2010.WHO laboratory manual for the examination and processing of human semen 5émé edition.WHO press,Switzeland.286.

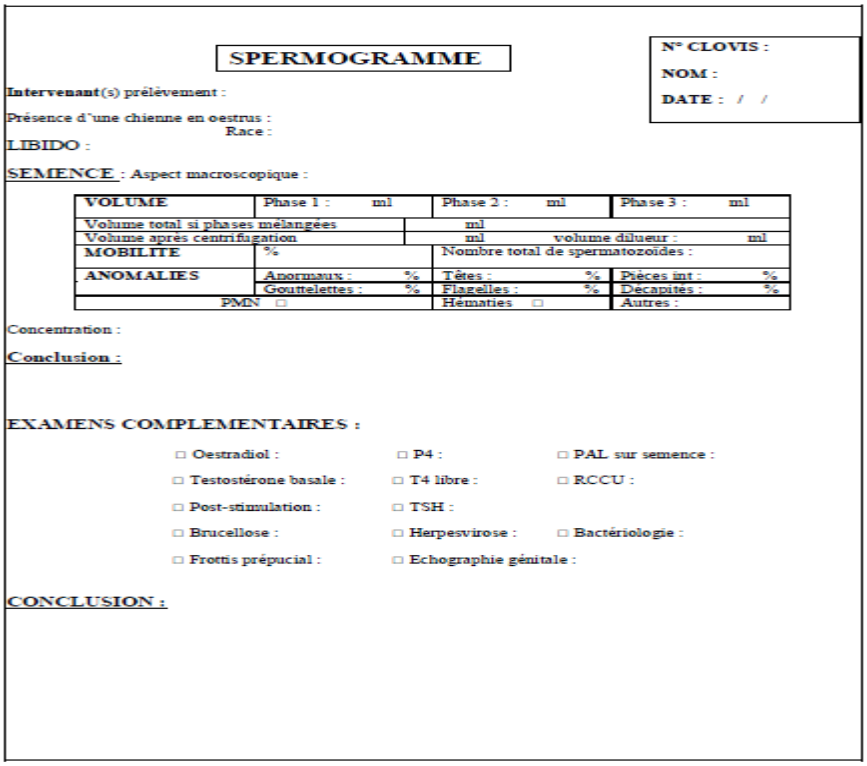
59. Wilson,L.L.,Foley,C.W.,Animal Sciences Department,1967.Reproductive Organs and Hormones of the Bull. Historical Documents of the Purdue Cooperative Extension Service.6, 3-5.

60. Wolfe, DF., 2017.Chirurgie du scrotum et de son contenu. Clé de vétéran. Consulté le 27/09/2020 sur https://veteriankey.com/surgery-of-the-scrotum-and-its-contents/

# Annexe

## Annexe 01

- The form established during the examination of the sperm at CERCA, which summarizes the identity of the animal, the anamnesis, the seminological parameters as well as any additional examinations carried out.



**SPERMOGRAMME**

**N° CLOVIS :**
**NOM :**
**DATE : / /**

**Intervenant(s) prélèvement :**

**Présence d'une chienne en oestrus :**
**Race :**

**LIBIDO :**

**SEMENCE** : Aspect macroscopique :

| | | | |
|---|---|---|---|
| **VOLUME** | Phase 1 : ml | Phase 2 : ml | Phase 3 : ml |
| Volume total si phases mélangées | | ml | |
| Volume après centrifugation | | ml | volume dilueur : ml |
| **MOBILITE** | % | Nombre total de spermatozoïdes : | |
| **ANOMALIES** | Anormaux : % | Têtes : % | Pièces int : % |
| | Gouttelettes : % | Flagelles : % | Décapités : % |
| | PMN □ | Hématies □ | Autres : |

**Concentration :**

**Conclusion :**

**EXAMENS COMPLEMENTAIRES :**

□ Oestradiol : □ P4 : □ PAL sur semence :
□ Testostérone basale : □ T4 libre : □ RCCU :
□ Post-stimulation : □ TSH :
□ Brucellose : □ Herpesvirose : □ Bactériologie :
□ Frottis prépucial : □ Echographie génitale :

**CONCLUSION :**

Figure 1: Sheet used at CERCA during the examination of the sperm (Cabannes, 2008).

- Classifications of sperm morphology by sperm anomalities (figure 2):

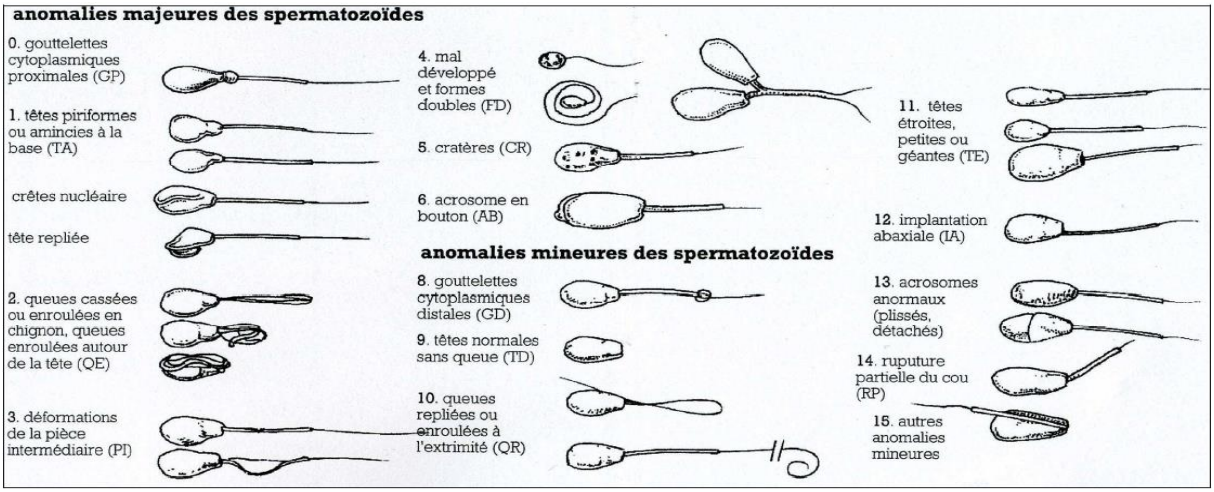
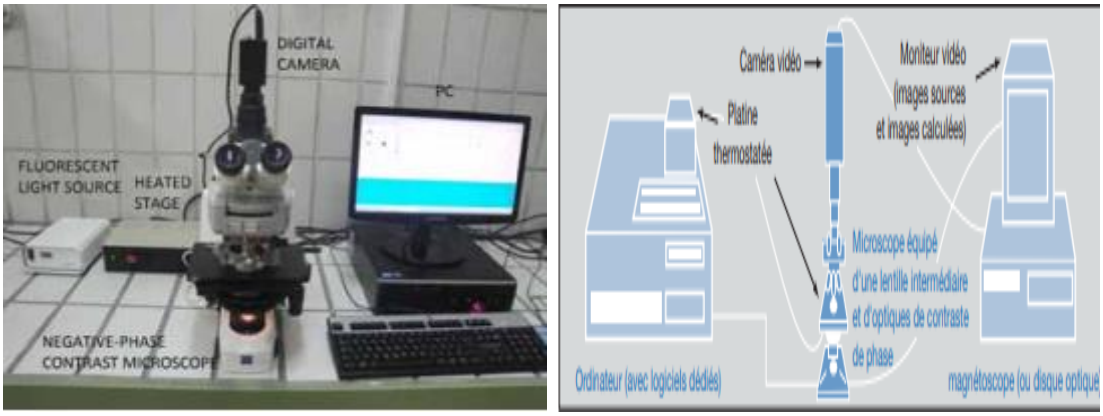



Figure 2: Major and minor sperm abnormalities in bovine species (Dumont, 1997)

## Annexe 02

- **Computer-Assisted Sperm Analysis (CASA):**




(A) (B)

**Figure 3:** Equipment for Computer Assisted Sperm Analysis , (A): real picture(Michos et *al.*, 2017) ; (B): Schematic of CASA.

- **Flow cytometry:**

**Principe de la cytométrie en flux :**

In flow cytometry, the cells are sucked in by a pump system, certain cellular parameters. Signals related to the intrinsic optical properties of the analyzed particle or to its fluorescence are separated by optical filters (filters dichroics) and collected by photo-multipliers (PMT). The diffracted light, measured at 90° (SSC parameter) gives a measure of the granulosity of the cell, i.e. the internal complexity of the cell under consideration. The use of fluorochromes (fluorescent dyes) allows to detect, in a specific way, the presence of certain molecules, the fluorochrome is chosen according to the marker to be highlighted.(Cabannes, 2008) (Figure 5,6).

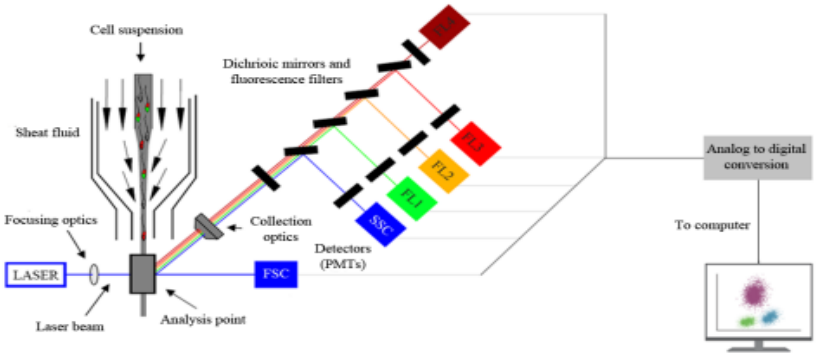



**Figure 5:** The functional components of a flow cytometer showing the fluidic, optical, and electronic systems. Thehydrodynamic forces of the sheath fluid create a stream of single cells in the cell suspension. The cells then pass through the analysis point (laser focal point) leading to scattering of light, and the excitation of bound fluorescent dyes and emission of

fluorescent light (the fluidic system). As the fluorescent light reaches the dichroic mirrors and fluorescence filters, it is separated and led to specific detectors called photomultiplier tubes (PMTs; FL1, FL2, FL3, FL4) that enhance the signal. In addition to these tubes, detectors of forward scatter (FSC) and side scatter (SSC) comprise the optical system. Analog signals gathered by the optical system are then translated to digital data for computer analysis by the electronic system. Modified from Brown and (Wittwer ,2000b; Waterhouse ,2007 *in* Berg,2020) .

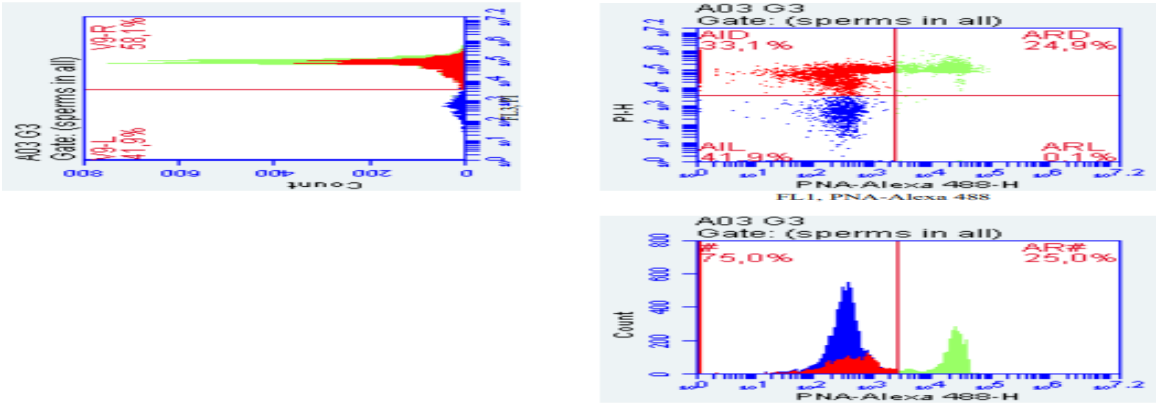



**Figure 6:** Two-parameter (cytogram) and one-parameter (histogram) distributions of acrosome intact live (AIL) (Berg,2020).

## Annexe 03

1. **Semen collection equipment:**

The artificial vagina is a method that uses for the collection of sperm at the CNIAAG center.

The principle of the artificial vagina is to reproduce all the sensations presented by the female genitalia during mating (heat, pressure, lubrication), and to quickly collect a total and unsullied ejaculate. After each use, the whole vagina is completely dismantled to be washed, dried and disinfected.

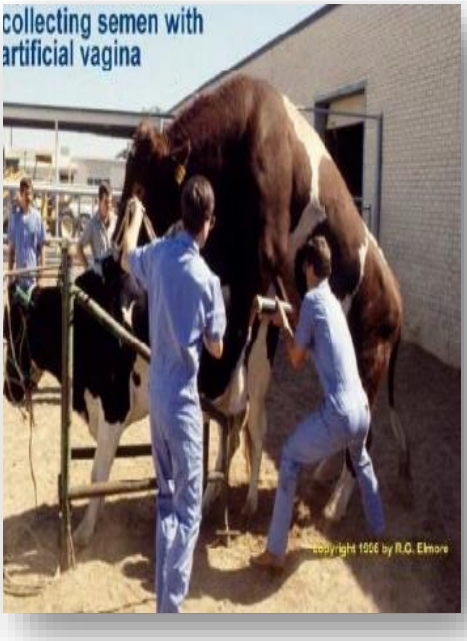



**Figure 7:** Semen collection

**Figure 8:** Artificial vagina

2. **Examination of sperm:**

- **A macroscopic examination:**

The volume (in ml) is evaluated by direct reading on a graduated collection tube.

Aspects of the ejaculate such as color, viscosity, etc. are analyzed.

by simple observation of the ejaculate in the collection tube; normal semen is whitish to yellowish-white in color and of milky to creamy consistency. This observation usually allows the detection of abnormalities such as the presence of blood or pus.

- **Amicroscopic examination:**

**Evaluation of mass motility:**

Observation under a binocular magnifying glass with a heating plate the intensity of the waves caused by the movement of the spermatozoa is evaluated.

note 0: absence of sperm movement

note 1: slight noticeable movement, no waves

note 2: few waves

note 3: numerous waves

note 4: fast and intense waves

note 5: very fast swirls

Semen with a mass motility of less than or equal to 3 is usually eliminated.

**Sperm concentration:**

The sperm concentration is measured in billions of sperm per milliliter using a calibrated spectrophotometer.

- to measure the sperm concentration of the ejaculate
- the quantity of diluent to bring to the ejacula
- the number of doses that can be produced are then calculated

3. **Individual motility evaluation :**

Measured under the microscope at magnification ×40 between slide and lamina, it corresponds to the proportion of spermatozoa with a rectilinear movement crossing the microscope field.

Spermatozoa moving in place, turning in small circles or moving backwards due to a folded tail are not considered to be motile

4. **Dilution of the seed:**

The diluent must be brought to a temperature of 35°C before being added to the seed.

It provides the spermatozoa with the nutritive and protective elements necessary for their survival after freezing.

The diluter/sperm is kept at 4°C for one hour after mixing to refrigerate the semen. An additional 3 hours of equilibration are then necessary to allow exchanges between the diluter and the cells.

5. **Putting in sequins:**

After dilution, the bottles are placed in a refrigerator at +4°C for 3 hours.

Once cooled, the semen will be conditioned into fine sequins by using an filling and welding machine.

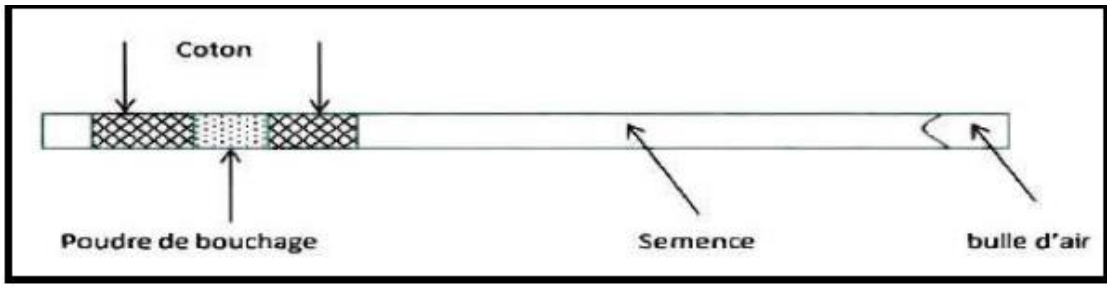



**Figure 9:** Schematic drawing of a CASSOU-type straw (Mbaindingatoloum, 1982).

6. **Freezing :**

1. Slow cooling to 4°C (1 h30min)
2. Fast cooling down to - 196°C

7. **Conservation:**

The sequins are then immersed in liquid nitrogen and stored in containers.

**Figure 10:** The sequins in liquid nitrogen.

8. **Viability test with the flow Cytometry:**

The Cytometer used is the EasyCyte 5HT, IMV which requires the use of a pilot software called guavaSoft IMV and a Kit called EasyKit.

Flow cytometry was used to analyze sperm viability.

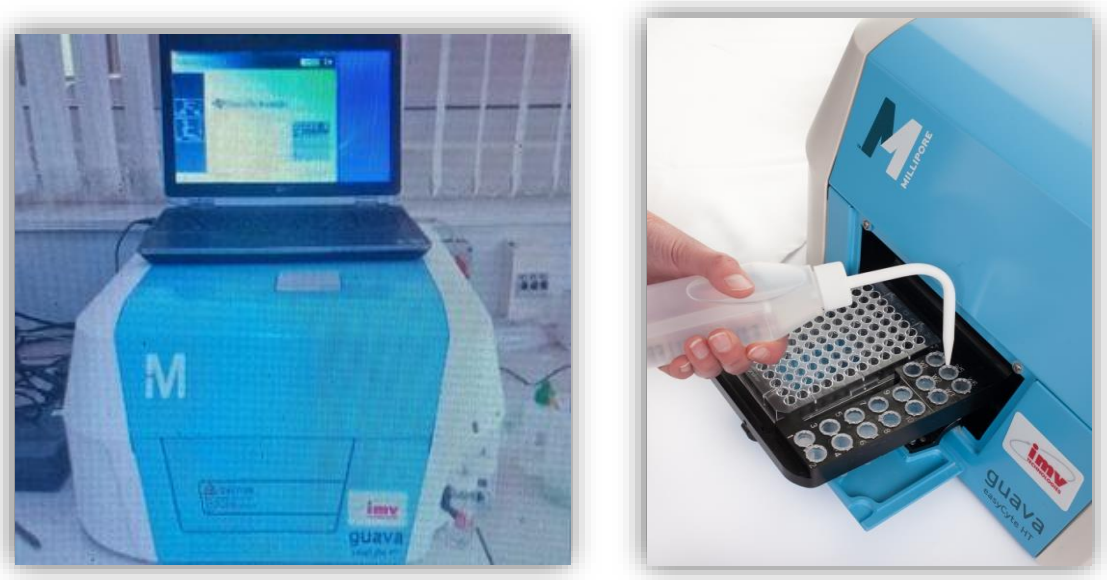
Before and after each manipulation, a wash is performed using distilled water, bleach and a washing solution called EasyClean.

The following is added :

- Distilled water in positions 1 and 5 or 9 (the position you have to mention is the one you have used) and w1, w2, w3, w4, w5, w6.
- Bleach in position 2.
- ICF in positions 3 and 4.

After washing, take 1 µl of each seed and mix with 199ml of EasyBuffer.

The mixture is left in the incubator for 10 minutes to start the viability analysis of the refrigerated and thawed sperm.




A B

**Figure 11:** (A): flow Cytometry type Easy Cyte 5HT IMV ; (B): The way we flood cytometry (Easy Cyte 5HT, IMV);