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Original Scientific Paper

ANALYSIS OF THE QUALITY OF SEXED BULL SEMEN BY COMPUTER-ASSISTED SEMEN ANALYSIS-CASA

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Summary

The quality of the ejaculate is determined by examination of the macroscopic (volume, density, color, smell) and microscopic properties of the sperm (concentration of spermatozoa in 1 ml and the total number of spermatozoa in the ejaculate, morphology, vitality, mobility of spermatozoa and integrity of the acrosomal membrane). Computer assisted semen analysis (CASA) is an automated system that measures sperm motility, kinetics and concentration. Some systems have modifications for evaluating the morphological characteristics of spermatozoa. The assessment of morphological characteristics of spermatozoa can be performed by microscopic examination (presence of normal and pathological spermatozoa, integrity of sperm cell membrane and acrosome abnormalities). The Blom method, as the method of the staining of the sperm smear with eosin and nigrosine, determines the vitality of the semen. In modern cattle breeding, sexed bull semen (semen from which desired gender of calf will be delivered) is used more and more. The analysis of sexed semen, and especially the comparison of its quality with conventional semen, is an increasingly common requirement on the cattle production market. The aim of the work was to examine the quality of 15 samples of sexed bull semen, originating from imports. The tests included determination of concentration and kinetics using the CASA system, as well as assessment of vitality and morphological characteristics by Blom staining. The average sperm concentration in 15 samples was 19.61×10^6/ml of ejaculate. The total motility was 39.42%, while the number of immobile spermatozoa in the samples was 60.58%. The number of morphologically normal spermatozoa varied from 2.91×10^6 to 6.11×10^6, and the percentage of pathological spermatozoa from 8% to 28%. Taking into account the results obtained and the absence of standards

related to sexed semen in the currently valid Regulation on the manner of marking of sperm, the manner of keeping records on sperm production, as well as on the conditions that must be met by animal sperm in terms of quality (Official Gazette of the Republic of Serbia, 38/14), it is necessary to carry out more frequent analyzes of sexed semen using the CASA method in order to obtain values that would become an integral part of the Regulation and thereby facilitate the manipulation of sexed semen on the market.

Keywords: sexed bull semen, CASA, sperm quality.

INTRODUCTION

One of the key goals of cattle production is to improve the reproductive parameters of the herd and achieve the maximum exploitation of reproductive capacities. The way to achieve these goals is the implementation of modern biotechnological procedures, as well as methods for controlling and ensuring semen quality.

Computer assisted semen analysis (CASA) is a method of semen analysis that is constantly being developed and improved. It enables an insight into the characteristics of ejaculate quality, important for their fertility, both conventional and sexed semen. CASA is a method that is considered objective and reliable, but relatively expensive, so it is most often used in large centers for artificial insemination, i.e. when producing insemination doses from genetically superior bulls (Maes et al., 2011). CASA uses image analysis, on the basis of which it determines the number and concentration of spermatozoa. The accuracy of this system depends on the optical properties and settings of the instruments and software, but it excludes errors caused by the analyst's subjectivity (Amann and Waberski, 2014; Finelli et al., 2021).

Morphologically intact spermatozoa in the ejaculates of bulls used for artificial insemination should be at least 70% (Menon et al., 2011). Sperm with poor morphology of spermatozoa provide poorer results of artificial insemination, as a result of reduced fertilizing ability of sperm with an increased number of abnormal spermatozoa. Different anomalies of the morphology of spermatozoa, cell membrane and acrosome can be established by histological staining methods. The Blom staining method (eosin-nigrosin staining) is most often used, on the basis of which live and dead, or morphologically changed spermatozoa can be determined (WHO, 2010). The integrity of the acrosomal membrane, which covers 2/3 of the head of the spermatozoa and contains the enzymes necessary for penetration through the oocyte during the fertilization process, is a significant indicator of the fertilizing ability of the spermatozoa. Therefore, good ejaculates

must have more than 51% spermatozoa with a normal acrosome (Vincent et al., 2012). Abnormalities of the acrosome structure can be examined by applying different staining methods and by examining with phase-contrast microscopy (Maes et al., 2011) or flow cytometry (Vincent et al., 2012). Deep freezing can significantly increase the number of live spermatozoa with damaged acrosome (Ugur et al., 2019).

The average speed of bull sperm is about 100 μm per second, or 4-7 mm in 1 minute. At this speed, the sperm reaches the cow's oviducts in two hours. The movement takes place by rotation around the extended axis from left to right, with a frequency of 3 to 15 rotations per second and with 9 tail strikes in the form of waves. The rotation of the head and tail takes place in three dimensions with the simultaneous movement of spermatozoa forward - progressive movement (Miljković and Veselinović, 2005).

Sexed semen means ejaculate that has been separated in a way that it contains spermatozoa that will most likely produce the desired sex of the offspring (Seidel, 2014; Dragin et al., 2016). Limiting factors for the use of sexed semen are the smaller number of spermatozoa in the dose of which there are on average 2 million in sexed semen compared to 20 million in conventional semen. Some authors established a reduction in the conception rate using sexed semen, as a consequence of the low number of spermatozoa in the dose, but also potential physical damage, as well as mistakes during sorting (Frijters et al., 2009).

In addition to the above, the disadvantages of using sexed semen are the high costs of acquiring and maintaining equipment and the necessary qualified workforce. Also, the process that provide a small number of spermatozoa in a dose and a smaller number of doses per unit of time (7-10 doses / hour) is slow, there is a high proportion of sperm with undetermined gender (only 30% of sperm are properly sexed) and, finally, sexed semen has the higher price (Frijters et al., 2009; Seidel, 2014).

The advantages of using sexed semen are achieving the desired sex of the animal in a high percentage. It is stated that the gender ratio in dairy breeds of cattle is 85-90% female versus 10-15% male calves. There are significant potentials for improving the genetic trend in the herd, by selecting parents with the best production potential and obtaining female calves from the best cows in the herd. Also, a reduction in the occurrence of difficult calving and associated reproductive problems was recorded (Seidel, 2014).

Steele et al. (2020) described the analysis of sexed semen using the CASA method ($n = 5$ bulls) which showed significantly reduced percentages of progressive motility of fast and slow spermatozoa compared to conventional semen (25.6% and 4.3% versus 60.8% and 13.3%, respectively). The same author states that the

percentage of immobile spermatozoa is significantly increased in sexed semen as it is 64.3% compared to 18.4% of the same class of spermatozoa in conventional semen.

The determined number of spermatozoa per dose of sexed semen is $2.1x10^6$. This number is significantly lower than the number of spermatozoa in a conventional dose of bull semen, which is on average about $20x10^6$. Holstein heifers, as well as cows, have the same percentage of conception when inseminated with sexed semen of $2x10^6$ or conventional semen of $3.5x10^6$ spermatozoa per dose. However, there is an increased percentage of conception when heifers, as well as cows, are inseminated with conventional semen in a dose of $15x10^6$ spermatozoa (Macedo et al., 2013).

The percentage of immotile spermatozoa in the sexed semen of three Holstein and one Angus bulls immediately after thawing was 33%, 30%, 47% and 50% tested by the CASA method. Three hours later, the percentage of immotile spermatozoa was 53%, 71%, 77% in Holstein bulls and 82% in Angus bull (Brogliatti et al., 2003). The same author states that the percentage of progressively motile spermatozoa immediately after thawing was 23.1±4.9%, and after three hours the percentage decreased to 3.7±3.2%.

Article 5 of the Regulation on the manner of marking of sperm, the manner of keeping records on sperm production, as well as on the conditions that must be met by animal sperm in terms of quality (Propis, 2014) defines the minimum conditions regarding the quality of bull semen. The mentioned Regulation does not regulate the sexed semen at all, and consequently does not prescribe the conditions for obtaining sexed semen, as well as the quality requirements that must be met by sexed semen at market. The requirements of the modern market, where sexed semen is commercially available, impose the need for regulations that will define the production, trade, as well as the quality and safety conditions of this product.

The aim of this work is to examine the quality of sexed semen of imported bulls using CASA.

MATERIALS AND METHODS

Analysis of sexed semen samples using the CASA and staining of the samples by Blom method

Fifteen samples of sexed semen, originating from imports, were tested. The tests were performed at the Department of Obstetrics, Sterility and Artificial Insemination, Faculty of Veterinary Medicine, University of Belgrade.

Sperm concentration, motility and speed were determined by Computer assisted semen analysis (CASA). For the purposes of CASA, a sub-sample of 5 µl was used, which was applied to chamber beds (Proiser D4C20, Valencia, Spain), 20 µm deep, placed on the heating plate of the microscope. After the cessation of passive movement of spermatozoa, imaging was performed on all 7 defined fields of the chamber. The number of analyzed spermatozoa per sample was 1500-5000, or 150-250 spermatozoa per one video. The program is set to analyze 25 images per second, with a 2-second exposure (50 images in total). After image processing, the following parameters were obtained:

- concentration of spermatozoa ($x10^6$ in ml and in dose);
- the percentage of individual classes of spermatozoa according to mobility (progressively mobile, non-progressively mobile and immobile), as well as according to speed (fast, medium speed, slow and static spermatozoa).

Morphological analyzes of semen were carried out in order to determine the relationship between live and dead spermatozoa, findings of intact and damaged acrosomes, protoplasmic droplets, as well as primary, secondary and overall pathological forms of spermatozoa by specific supravital Blom staining method.

Determination of the total number of living aerobic microorganisms in deep-frozen bull semen

The total number of living aerobic microorganisms was determined by the standard method ISO/TR 8607 (ISO, 1991).

Statistical data processing

The normal distribution of the data was tested using the D'Agostino & Pearson normality test. Since the data were normally distributed ($p>0.05$), the t-test for independent samples was used to compare statistically significant differences between the two groups. Statistical processing of experimental data was performed using GraphPad Prism version 6 software (GraphPad, San Diego, CA, USA).

RESULTS

The results of testing the concentration and mobility of the tested semen samples determined by the CASA method are shown in Table 1.

Table 1 Parameters of semen concentration and mobility determined by the CASA method

Sample No	Con ($\times 10^6$/ml)	Con D ($x10^6$/dozi)	TM (%)	PM (%)	FS (%)	SS (%)	LM (%)	IM (%)	C (%)
1	15.43	3.85	34.47	28.14	18.50	9.51	6.34	65.53	0.13
2	16.72	4.18	36.14	30.41	10.76	19.65	5.73	63.86	0.00
3	17.81	4.45	40.40	34.80	24.70	10.10	5.60	59.60	0.00
4	21.72	5.43	31.41	22.59	13.14	9.45	8.82	68.59	0.00
5	12.90	3.22	34.85	31.82	24.24	7.58	3.03	65.15	0.00
6	19.75	4.93	45.54	42.57	21.78	20.79	2.97	54.46	0.00
7	15.40	3.85	44.44	38.10	23.17	14.92	6.35	55.56	0.00
8	19.65	4.91	45.51	42.47	21.58	20.59	2.91	54.36	0.00
9	17.30	4.32	41.13	36.27	28.81	7.46	4.86	58.87	0.00
10	20.61	5.15	56.52	52.73	39.29	13.36	3.79	43.48	0.08
11	24.28	6.08	42.42	35.03	18.39	16.51	7.38	57.58	0.13
12	23.46	5.86	35.00	27.02	13.33	13.57	7.98	65.00	0.12
13	21.06	5.25	33.70	25.53	15.97	9.29	8.17	66.30	0.28
14	28.44	7.11	44.23	37.66	22.31	15.13	6.57	55.77	0.23
15	19.59	4.89	25.55	19.36	12.28	7.09	6.19	74.45	0.00

Legend: Con – semen concentration in mL; Con D – semen concentration in dose; TM – total mobility; PM – progressive mobility; FS – fast spermatozoa; SS – slow spermatozoa; LM – locally motile spermatozoa; IM – immobile; C – circular or spermatozoa that move in a circle;

After the statistical analysis of the sexed semen samples, the following results were obtained: by examining the concentration of spermatozoa in 15 samples of sexed semen, an average concentration of 19.61 ± 3.958 x 10^6/ml of ejaculate was determined (Table 2). The lowest determined concentration was 12.9 x 10^6/ml, and the highest was 28.44 x10^6/ml. The lowest concentration of spermatozoa in a dose was 3.22x10^6/dose, and the highest was 6.08 x 10^6/dose.

Table 2 Descriptive statistical values of the analysis of parameters of sexed semen - concentration of spermatozoa in mL [10^6/ml]

Semen concentration	**n**	$\bar{x}$	**SD**	**SE**	**X min**	**X max**	**CV (%)**
	15	19.61	3.958	1.022	12.9	28.44	20.18

By examining sperm motility in 15 samples of sexed semen, a total motility of 39.42±7.528% was determined, and the percentage of spermatozoa that were immobile in the samples was 60.58±7.528% (Table 3).

Table 3 Descriptive statistical values of the analysis of sexed semen parameters [%]

Parameter	n	$\bar{x}$	SD	SE	X min	X max	CV (%)
Total motility	15	39.42	7.528	1.944	25.55	56.52	19.10
Progressive motility	15	33.64	8.659	2.236	19.36	52.73	25.74
Fast spermatozoa	15	20.56	7.389	1.908	16.47	24.66	35.93
Slow spermatozoa	15	13.01	4.844	1.251	7.09	20.79	37.23
Local motility	15	5.783	1.927	0.4975	2.97	8.82	33.32
Immobile	15	60.58	7.528	1.944	43.48	74.45	12.43
Circular mobility	15	0.065	0.094	0.0242	0.00	0.28	145.05

Progressive motility varied from 19.36% to 52.73%, and the average values were 33.64±8.659%, the percentage of fast spermatozoa in the sample was on average 20.56±7.389% (from 10.75% to 39.29%), and the average percentage of slow spermatozoa was 13.01±4.844% (from 7.09% to 20.79%). The average percentage of spermatozoa that move in the circle was 0.065±0.094%, and the locally moving spermatozoa, i.e., oscillating in place, was on average 5.783±1.927%.

The percentage of live spermatozoa ranged from 34% to 60% in the examined samples of sexed semen. The percentage of changes in the head and tail of the spermatozoa ranged from 2% to 12%, and the changes in the middle part of the spermatozoa were less and amounted to 2% to 6%. The number of morphologically normal spermatozoa in sexed semen samples varied from $2.91x10^6$ to $6.11x10^6$, and the percentage of pathological spermatozoa varied from 8% to 28% (Table 4).

Table 4 Values of cytomorphological parameters of semen, by Blom's method

Sample No	Live/dead %	Changes on head %	Changes in the middle part %	Changes on tail %	Total number of pathological forms %	Number of morphologically normal spermatozoa in dose ($x10^6$)
1	44 / 56	4	/	4	8	3.55
2	40/60	4	4	8	16	3.51
3	48/52	4	2	8	14	3.83
4	60/40	8	/	4	12	4.78
5	52/48	2	2	6	10	2.91
6	58/42	/	/	12	12	4.35
7	52/48	2	/	6	8	3.39
8	58/42	/	/	12	12	4.32
9	56/44	4	2	8	14	3.73
10	60/40	8	/	12	20	3.50
11	44/56	8	6	8	22	4.61
12	48/52	8	2	6	16	3.55
13	40/60	6	2	2	10	4.73
14	52/48	6	2	6	14	6.11
15	34//66	12	4	12	28	3.53

DISCUSSION

The determined average number of spermatozoa per dose of sexed semen in our study is $4.9x10^6$, which is 57% more than described in study done by Macedo et al. (2013) where it was $2.1x10^6$. Brogliatti et al. (2003) observed that the percentage of progressively motile spermatozoa immediately after thawing was 23.1±4.9%, and three hours later the percentage was reduced to 3.7±3.2%, while in our study progressive motility was 33.64±8.659% immediately after sample dissolution. While working on field with sexed semen, Seidel et al. (1997) established that doses for insemination of heifers ranging from 1×10^6 to 2.5×10^6 spermatozoa gave a satisfactory conception rate, that was between 35% and 48%.

Steele et al. (2020) determined that the percentage of progressively moving fast and slow spermatozoa were 25.6% and 4.3%, respectively, while in our study the percentage of fast spermatozoa was 20.56±7.389% and slow spermatozoa was 13.01%±4.844%. So, in our study, there was a much higher percentage of slow spermatozoa than the above-mentioned author determined. The same author states that the percentage of immobile spermatozoa in sexed semen was 64.3%, which is similar to our results of this parameter of semen quality (60.58±7.528). Contrary to our results, they obtained a much lower percentage of immobile spermatozoa in the sexed semen and it was 33%, 30% and 47% in three Holstein bulls and 50% in an Angus bull.

Based on the Regulation on the manner of marking of sperm, the manner of keeping records on sperm production, as well as on the conditions that must be met by animal sperm in terms of quality (Propis, 2014), which prescribes the fertilizing ability of bull semen after thawing, the progressive motility of spermatozoa should be at least 50%, which is not fulfilled by the fourteen sexed semen samples in our study, so that the percentage of progressive motility in the samples was 33.64±8.659. Only one sample had progressive mobility greater than 50% (52.73%). Paragraph 2 of the Regulation prescribes that the percentage of morphologically changed spermatozoa should be up to 30%, which all the samples in our study fulfilled and it was amounted less than 28%. Paragraph 4 of the Regulation prescribes that the number of progressively motile and morphologically normal spermatozoa in a dose after thawing should be at least 10 million, which cannot be fulfilled with sexed semen, because their concentration in a dose is from 3.22 to 7.11 $x10^6$, while the number of morphologically normal spermatozoa in sexed semen samples varied from $2.91x10^6$ to $6.11x10^6$. All semen samples were bacteriologically and mycologically negative, which is in

accordance with paragraph 5 of the same Regulation, which prescribes that the total number of bacteria in the sample must be up to 500 CFU/mL.

CONCLUSION

The valid Regulation on the manner of marking of sperm, the manner of keeping records on sperm production, as well as on the conditions that must be met by animal sperm in terms of quality (Official Gazette of the Republic of Serbia, 38/14) does not prescribe quality parameters for sexed semen. Taking into account the trend of increased use of sexed semen by farmers, it is necessary to carry out more frequent analyzes of sexed semen using the CASA method in order to obtain norms that would become an integral part of the Regulation. This would facilitate the manipulation of sexed semen on the market and establish standards for assessing the quality of sexed semen.

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