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EVALUATION OF SEMEN SEX RATIO IN COOLED AND FROZEN SEMEN STRAWS BY REAL-TIME PCR

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Key word: Sex ratio, ZFX, SRY.

ABSTRACT

The study was demonstrated on quantitative evaluation of SRY and ZFX gene of (120) semen straws from 10 Holliston bull (60) straws in cooling state 5C° and 60 straws after deep freezing in liquid nitrogen) . The samples collected from the Artificial Insemination Center of Abo-Ghreeb / Baghdad . All samples were sent to the laboratory for DNA extraction using (Qiamp DNA extraction Kit) and primer design then testing in real time PCR. The results showed there were highly significant variation in the sex ratio of cooled and frozen semen straws which varied between (35%-59% in ZFX , 37%-58% in SRY and 40%-69% in ZFX , 30%-53% in SRY for frozen and cooled semen respectively) . The study conclude that it was easy and possible to detect the quantity of sex ratio for each bull through using real time PCR . The freezing process of semen could cause decrease in the percentages of SRY (minimum 30%) and increase in the percentage of ZFX (maximum 69%) .

INTRIDUCTION

Many of developed countries are trying to pay attention to livestook, through that and for good dairy industry female are more desirable at . It would be more profitable for dairy farmers to use sexed sorting semen to produce daughters from genetically goal [(1)]. Sex sorting of bull spermatozoa has applications for genetic improvment in dairy industry with great economic advantages and preselection for females will reduce the losses in both income and genetic progress get by the producer [(2)].

In artificial insemination industry, it is possible to collect, extend, package and ship a billions of sperms cells from one male that will be used to breed thousands of females [3] . This allows for the dispersal of superior genetic material through the these country [4] . Since the sperm cell determining the sex of the offspring, semen sorted by its ability to produce males or females would be very marketable[5] . Many techniques in past focused on the physical separation of X- and Y- bearing spermatozoa, these methods were developed based on motility, DNA content ,surface charge, sperm surface antigenic determinants and the majority of these methods had non disirable effects on semen quality [6]. Other methods of separation had also been reported [7], which were density gradient, electorophoresis, albumin centrifugation and sedimintation. All these methods were not fully successful except flow cytometric cell sorting, in other hand a new technique of quantification real time-Polymerase chain reaction (qRT-PCR) [8] has used to determine the sex ratio of individual ejaculates of bull semen with an accuracy of 99% [9] depend on the sex determination region of Y-chromosome gene(SRY) and ZFX gene (Zinc like finger of X-chromosome gene)[10]. The study has designed to investigate the effect of cooling and freezing of semen on the bull's semen sex ratio depending on the percentages of sex determination region of (SRY gene)and (ZFX gene).

MATERIAL AND METHODS

Ten (10) Holstein bulls located in Artificial Insemination Center, Baghdad-Abo Ghreeb, were used for semen collection. Ages of these bulls ranging between (2-5year), and these were kept under healthy environment and good management along the period of collection .Duration of the study were (5 month) from 25-1-2016 to 25-6-2016 . The semen samples had collected by artificial vagina then sent to the laboratory for routine semen tests(semen characteristics). Bad semen (less than 49%) was discard, while the good semen (more than 50%) was acceptable for the study and diluted by using egg yolk seminal (11)

The first group of samples collected at the cooling steps and the other group after 2 days post deep freezing with liquid nitrogen (-196ºC) (Diagram :1) from the same ejaculate, each bull alone .

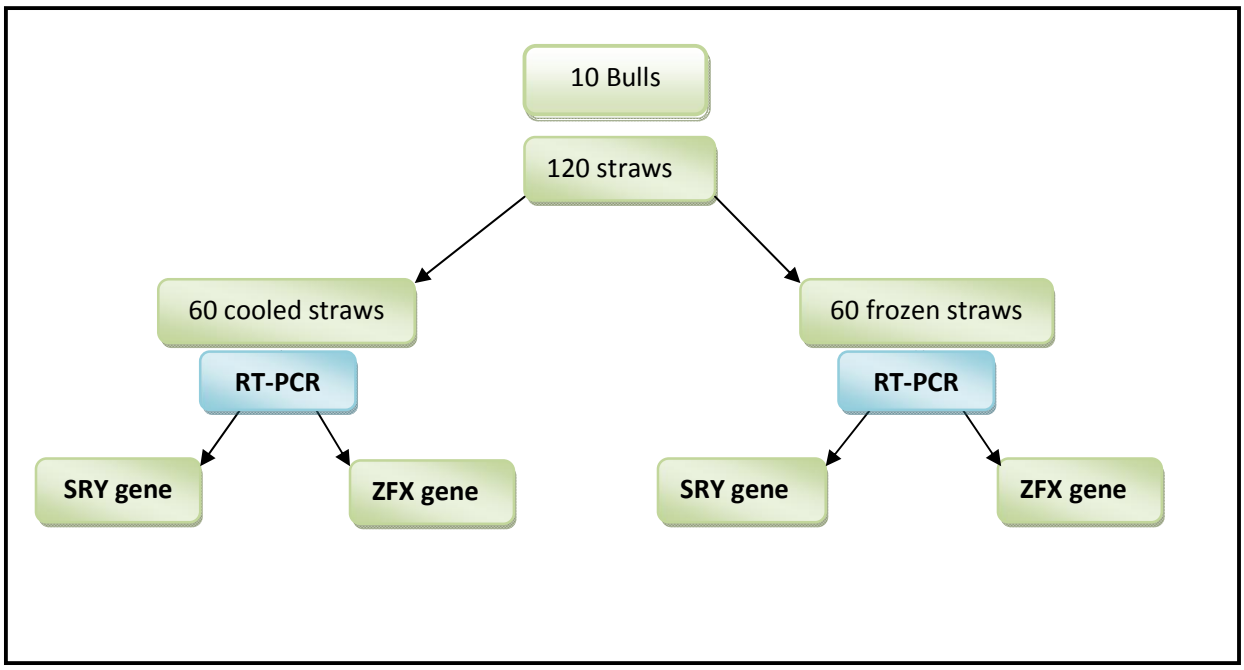

Diagram (1): The semen samples and experimental design.

PRIMER DESIGN: The present study was used primers that reported by [9] to determine the sperm sex ratio by using of qRT-PCR which are forward (5-CCA CGT CAA GCG ACC CAT-3) and reverse (5-AGA GCC ACC TTT CGT CG-3), which were used to amplify a 66 bp fragment of SRY gene-y chromosome linking [12]**.** The primershad designed for ZFX gene (X-chromosome) (5-GTT GTG TTA GTT TCT GCT GTA CAA TAA AGT G-3) and the reverse (5-GAT GGC AGG TGA GGG TAG GA-3) for amplify 96 bp of DNA fragments [13] .

DNA EXTRACTION: Each one of the (120) samples placed in Eppendorf tubes at 37ºC water path. Twentymicroliter of proteinase K had added, then 20 µl of ADTA and 10µl lyses buffer solution also were added to each semen samples after mixed together and incubated for more than 15 hours (overnight). The purification procedure of DNA used in this study based on spin columns procedure which centrifuged at (500 cycle/minute) for 10 minute to collect the sperm

pellets [14]. The samples were washed 4 times with 2.9% sodium citrate solution and incubated at 56 C for (30) minutes [15] then removing of cell depress by centrifugation with 1000 cycle/minute for 5 minute. After that DNAs extract had purified using (QIAamp, DNA Mini Kit, Cat. No. 51304, QIAGE, GmbH, D. 40724 Hilden)

Real time PCR: After DNA extraction and purification, all samples had tested in real time PCR (Sacycler% real time PCR system, 5x RUO, Italy)as the program below:

	Step	Temperature°C	Time min
1	Pre-denaturation	95:0	14:35
2	Denaturation and Annealing	95:0	00:25
3	Extension	95:0	00:15
4	Melting curve	60:0 - 90:0	01:00 - 00:15

Table (1): Real time PCR program.

STATICAL ANALYSIS: The Statistical Analysis System [18] program was used to effect of difference factors in study parameters. Chi-square test also had used for significant comparing between percentages and Least Significant Difference (LSD) test.Through that the study was estimating the correlation coefficient between characters of current study [9]

RESULTS AND DESCUSION

The results showed there were highly significant variation in the mean ratio of cooled and frozen semen straws (Table:2). According to table-2the minimum mean percentage of SRY after six weeks collection of semen and after cooling of semen straws were37% in bull number (41). So that mean it was tend to produce females more than males because the mean percentage of ZFX in the semen of this bull was more than 59%(percentage of Y-chromosome is less than percentage of X-chromosome). While the maximum mean percentages of SRY gene in cooled

semen straws was 59% in bull number (17769) ,which mean it was tend to produce males more than females because of this mean percentage of ZFX gene in same bull semen not exceed 35% (mean percentage of Y-chromosome is more than X-chromosome) the result agreed with [16] .

The result after freezing show highly significant increase the ratio of ZFX (mean 56.5 %) and decrease of SRY (mean 42.5%), the result was differenced with [16] when he found the ratio of ZFX was (mean 50.3%) while the SRY was (49.6%) and even differenced with [9] who found the semen sex ratio of ZFX (mean 54.7%) this variation in sex ratio may result from either using of diluents or affected by semen processing and packaging in straws (the packaging process of semen straws didn't depend on the semen motility or activity but it is works according to injection by special syringe with special system designed to kept in cooled condition) [17].

Bulls	**Mean ± SE**			
	After cooling		**After freezing**	
	ZFX (X%)	SRY (Y%)	ZFX (X%)	SRY (Y%)
4	48.83 ±9.51	51.17 ±9.51	47.67 ±1.20	52.33 ±1.20
57	55.67 ±8.48	39.67 ±7.91	56.17 ±7.61	43.83 ±7.61
145	58.50 ±8.15	41.17 ±7.98	66.17 ±5.04	34.00 ±5.09
41	59.40 ±4.95	37.00 ±1.67	69.20 ±2.95	30.80 ±2.96
109	47.50 ±2.78	54.67 ±1.90	62.50 ±3.58	37.50 ±3.58
528	37.83 ±2.79	56.00 ±4.08	59.17 ±2.66	40.83 ±2.66
409	47.33 ±1.45	52.67 ±1.45	55.00 ±1.52	45.00 ±1.52
17769	35.17 ±3.10	59.17 ±4.32	51.50 ±2.39	45.83 ±1.64
17768	58.75 ±2.65	44.00 ±5.36	58.25 ±3.31	41.75 ±3.30
451	42.00 ±4.51	58.00 ±4.50	40.33 ±1.20	53.67 ±6.43
LSD value	18.736 *	18.596 *	12.386N *	12.663 *
* (P<0.05).				

Table (2): Effects of bulls in % X and Y after cooling and freezing.

At cooling steps , the results showed there were significant increase in ratio of ZFX in the first two weeks (55.9% , 56.25% respectively) (table : 3) and increasing of SRY in the third week (57.28%) (figure :1) , this results were apposite with [16] when he found an increase in the ratio of ZFX in the third and sixth weeks (53.8% , 58.8% respectively) , and increase in the ratio of SRY in the first , 4th ,5th , weeks (53.4% , 54.2% , 54.4% respectively) . This variation in the semen ratio may skewed from individual ejaculates of semen [9]

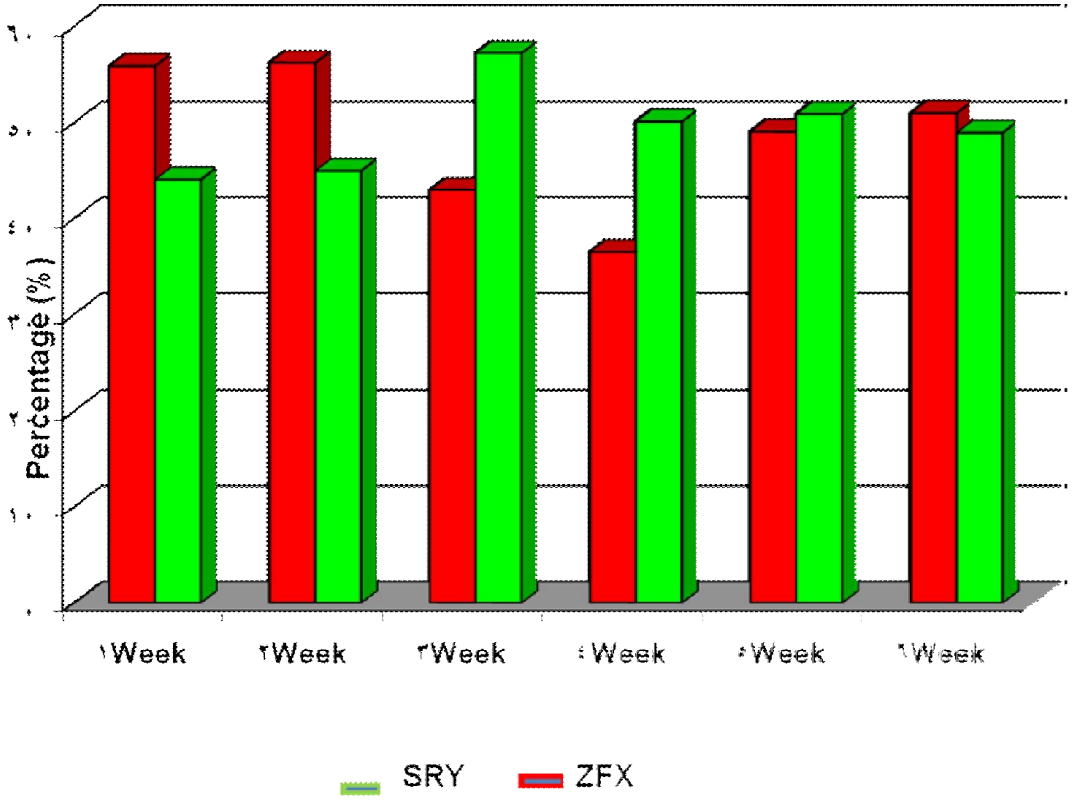

Figure 1: Effect of week in % X and Y after cooling

The frozen semen straws (figure:2) showed maximum increase in the ratio of ZFX in the second week (62.12%) while the third week showed maximum increasing of SRY ratio (46.14%) (table :3)

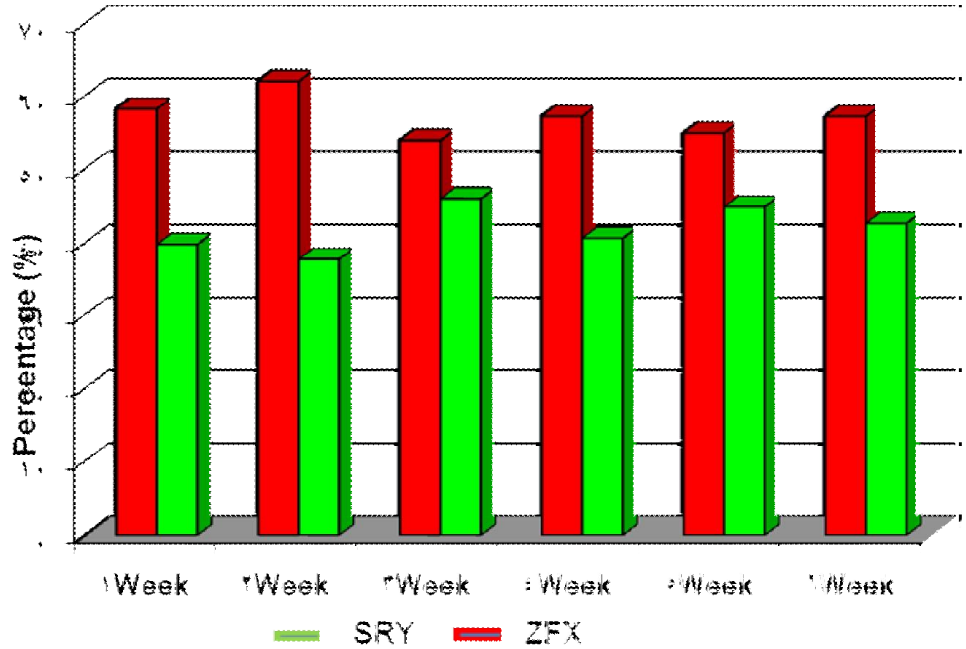

Figure 2: Effect of week in % X and Y after freezing

Weeks	Mean ± SE			
	After cooling		After freezing	
	ZFX (X%)	SRY (Y%)	ZFX (X%)	SRY (Y%)
Week 1	55.90 ±6.17	44.10 ±6.17	58.30 ±4.28	39.90 ±3.83
Week 2	56.25 ±7.20	45.00 ±7.22	62.12 ±6.81	37.87 ±6.81
Week 3	43.00 ±4.96	57.28 ±4.71	54.00 ±2.91	46.14 ±2.87
Week 4	36.50 ±3.00	50.12 ±4.88	57.25 ±3.52	40.75 ±2.78
Week 5	49.10 ±3.35	50.90 ±3.35	55.00 ±2.21	45.00 ±2.21
Week 6	51.00 ±3.80	49.00 ±3.80	57.25 ±3.22	42.75 ±3.23
LSD value	14.465 *	12.055 *	6.294 *	11.685 NS
* (P<0.05), NS: Non-significant.				

Table 3. Effect of week in % X and Y after cooling and after freezing

CONCLUSION

The study had concluded that it was easy and possible to detect the quantity of sex ratio of each bull alone by revealing the percentage of SRY and ZFX gene, which had referred to the percentage of Y-bearing spermatozoa and X-bearing spermatozoa respectively by using real time PCR. There was significant variation between cooling and freezing mean percentages of semen in SRY and ZFX gene quantity, which mean the freezing process of semen could cause decrease in the percentages of SRY and increase in the percentage of ZFX gene (increase probability of female newborns).

الكشف الكمي للنسبه الجنسيه لقصبات السائل المنوي المبرده والمجمده باستخدام تفاعل الدنا المتسلسل الكمي

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الخلاصة

صممت الدراسة لمعرفة التقدير الكمي لكل من SRY جين و ZFX جين لمائة وعشرون (120) قصبة سائل منوي من ثيران نوع هولشتاين على شكل مجموعتين . تضمنت المجموعة الاولى (60) قصبة مبردة بدرجة حرارة (5) خمسة مئوية، في حين كانت المجموعة الثانية تتضمن (60) قصبة اخرى اخذت بعد التجميد بالنايتروجين السائل.

جمعت العينات من مركز التلقيح الاصطناعي / ابوغريب / بغداد. ثم ارسلت الى المختبر لغرض استخلاص ال DNA (باستخدام عدة استخلاص الدنا Qiamp) وتم تصميم البادئات ثم اجري تفاعل الدنا المتسلسل الكمي (Real time PCR) لمعرفة النتائج.

بينت النتائج بان هناك اختلاف ملحوض في النسبة المئوية الجنسية للقصبات اثناء التبريد وبعد التجميد (تراوحت مابين 35%-59% بالنسبة ل ZFX ، 37%-58% ل SRY للقصبات المبردة و 40-69 ل ZFX ، 30-53% ل SRY للقصبات

المجمدة غلى التوالي) . الدراسة تمكنت من الاستنتاج انه من السهل والممكن الكشف الكمي للنسبة الجنسية لكل ثور على حدة باستخدام تفاعل الدنا المتسلسل الكمي (real time PCR)، كما استنتجت بان عملية التجميد ممكن ان تؤدي الى تقليل في النسبة المئوية لجين SRY (اقل قيمة 30%) وتزيد من نسبة جين ZFX (اعلى قيمة 69%)

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