Fragility in X-chromosomes in Sahiwal (Zebu) cattle

N.S. Magar[1], G.S. Sonawane[2*], B.R. Yadav[3] and V.B. Dongre[2]

[1]Department of Animal Husbandry, Satara, Govt. of Maharashtra. [2]Maharashtra Animal and Fishery Sciences University, Nagpur, Maharashtra, [3]ICAR-National Dairy Research Institute, Karnal, India
*Corresponding Author e-mail: gokulsonwane@mafsu.ac.in

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Abstract

Fragile site (FS) is a region in mitotic chromosome and at metaphase lacks the normal compaction of chromatin, which can be visible as constriction, break or gap. Cytogenetic examination followed by DNA primers amplification were used to study FS in 27 Sahiwal females inflicted with fertility problems (repeat breeding (17) and abortion (8)) both (2) along with 10 control normal fertile females. Chromosomes were examined with three different culture media conditions: RPMI-1640 with Aphidicolin (APH), TC-199 without folic acid and Ham's F-10 as a control medium. FSs were detected on different locations in X- chromosomes. In problematic animals percentage of metaphases showing FSs ranged from 17.14 to 28.79 while in control group the range was 7.89 to 10.76. Three media used revealed different frequencies of FSs on X- chromosomes. Significant revelation of FS was shown by RPMI-1640 with APH and TC-199 without folic acid as compared to Ham's F-10. Confirmation of FSs was done with specific seven DNA primers. FSs were found significantly associated with reproductive problems viz. repeat breeding, abortion and incidence of both in the same animals.

Key words: Fragile site (FS); X-chromosome; Sahiwal, Reproductive problems, DNA primers

Introduction

Cytogenetics in the last 60 years has progressed tremendously and achieved that status of disciple and in recent past has emerged as molecular Cytogenetics (Iannuzzi et al., 2023). Since, the first chromosome anomaly of Robersonian Translocation reported by Gustavsson and Rockborn (1964), a large number of structural and numerical aberrations and their effects have been reported in various species of animals including man. There are several reports and reviews on such anomalies in the animals (Iannuzzi et al., 2021), buffaloes (Albarella et al., 2017), pigs (Donaldson et al., 2021) and sheep (Danielak-Czech et al., 2010; Berry et al., 2018). In the recent past molecular cytogenetics is exploring the finer details in the structure and function of chromosomes, and the present report deals with fragility of X-chromosomes of Sahiwal breed of zebu cattle. In India and other Asian countries, the demand to multiply the genetic material of valuable *Bos indicus* females has increased (Kumar et al, 2025).). Sahiwal cow is one of them which are more suitable to our climatic conditions as compared to *Bos Taurus.* Sahiwal cattle is renowned for its higher milk production, remarkable power of endurance for hot climate of sub–tropics, relative resistance to diseases and low maintenance cost (Dey 2017).

Fragile sites (FS) are non-random lesions in mitotic chromosomes, where the normal appearance of chromatin remains feeble and subsequently this result into gap or break or rearrangement in the carrier during cell divisions. Rare fragile sites constitute to a class of heritable DNA regions with specific molecular constitution, with or without any clinical or biological significance (Danielak-Czech et al., 2013). Chromosomal fragile sites have been shown susceptible to breakage under specific experimental conditions (Pauciullo *et al.*, 2011; Iannuzzi *et al.*, 2016). Several investigations have been carried out on FS in humans correlating with infertility, abortions, malformations, cancer and mental retardation (D'Hulst & Kooy, 2009); however, domestic animals have received a little less attention. Furthermore, some researchers attempted work on chromosome rearrangements that affect fertility in cattle (Jennings et al., 2020). In animal breeding programmes infertility and sub fertility are the major causes of economic losses. So it is of paramount importance to work out strategies for early detection of problems for breeding animals. There are various cytogenetical means to identify genetic impairment; however, each has some limitations. One of the potent tools with modest resources is to untangle fragile sites using tools of molecular cytogenetics and to find their association with reproductive problems such as infertility and sub fertility. In the event of revelation of association of fragile sites with the reproductive problems, it can be a tool in culling of problematic breeding animals early in the age and the financial burden of rearing of these animals in future can be prevented. Hence, the present study was carried out in reproductively problematic females of Sahiwal cattle.

Materials and Methods

The present investigation was carried out on 37 animals including reproductively problematic and normal group belonging to Sahiwal breed of indigenous cattle. These all animals were selected from an animal herd maintained at National Dairy Research Institute, Karnal. The problematic animals were the females with history of reproductive problems such as repeat breeder (17), abortion (8) and abortion-repeater (2). Normal animals were females selected as control (10) with record of three successive normal calving and completion of lactations each of 305 days or more.

Cytogenetic Preparations

Metaphase chromosomes preparations were made using short term whole blood lymphocyte standard culture technique with some modification. Sample of blood from each animal was collected in vacutainer tube (Becton-Dickinson) containing 143 USP units of sodium heparin. An aliquot (0.5 mL) of blood was used for setting up of culture. Lymphocytes were grown in three different media viz. RPMI-1640, TC-199 without folic acid and Hams F-10. After 72 hours of growth, Aphidicolin (Sigma) was added to the cultures grown in RPMI-1640 at 0.15 µM (final concentration) for further incubation of 24 hours to reveal common fragile sites. Subsequent to a total of 94 hours of incubation, 2 drops (0.5 µg/mL) of colcemid solution (Sigma) were added to each culture bottle, and then after 45 minutes cells were harvested. During harvesting, cells were treated with hypotonic (0.75M KCl) at 37^{o}C and fixed with chilled Corney's solution. The slides were stained for 30 minutes with 4% Giemsa solution at pH 6.8. Metaphases were examined at X100 magnification under Leica microscope. The number of metaphases critically evaluated varied from 40 to 100 in different animals. Selected metaphase plates were photographed using Leica digital camera fitted on microscope. The images were stored in PC and evaluated for number and structural details.

Molecular Approach

Blood sample of about 20 mL of was collected from each animal in Falcon's tubes containing ACD solution @ 1 mL / 6mL blood. DNA was isolated from 10 mL of blood by phenol chloroform extraction method with some routine modifications. FSs specific primers were selected from reported literature, their sequences were recorded and got synthesized from a commercial firm (M/s. Sigma-Aldrich Pvt. Ltd., Bangalore, India). These primers in suitable conditions and procedure were used to confirm the presence of fragile sites. The primers used are listed in Table 1.

Subsequent to PCR amplification, the product was checked on 1.5% agarose gel containing ethidium bromide (@ 7µL /100 mL in horizontal mini electrophoresis unit (Stratagene) using 0.5X TBE as running buffer. In this process 5 µL of PCR product and 1 µL of Xylene Cyanol dye (6X) were loaded in agarose gel along with 50 bp ladder as a marker and was run at 80 V for 1 hour 30 minutes. In the end of run, gel was viewed under UV light and photographs were taken with Nikon DX camera for analysis and interpretation.

Statistical analyses

The initial data of the experiment was recorded in percentage. In order to normalize the distribution, it was transformed into arcsine scale. The transformed data were subjected to ANOVA to study the effect of different media along with specific chemical agent to check the level of significance of fragile sites in reproductively problematic animals. The least squares mean along with standard error were calculated. Fisher's LSD was used for pair wise comparison of the means of the effect of three different media.

Results and Discussion

Cytogenetic Evaluations

All the animals studied were females and had 60, XX chromosomes complement. There was no any numerical anomaly, however, in evaluation of structural details, FSs were observed in one or both the chromatids of X-chromosomes in significant number of metaphase plates of reproductively problematic animals. These were present on the p and q arms at different locations: towards telomeric end, close to telomere, middle of the arm, close to centromere. Though a few gaps and breaks were also seen in some autosomes, however, were not included in analysis for association.

The cumulative percentage of FSs in all the problem cases ranged from 17.14 to 28.79, while in control group the range was 7.89 to 10.7. The variation in percentages within each anomaly and other details are presented in Table 2. Some of the prominent fragile sites at different location on X- chromosome are shown in Figure 1. The observations showed that repeat breeding and abortion cases had fragile sites in metaphase plates, which were the highest in the medium RPMI 1640 containing aphidicolin (APH) followed by medium TC 199 (without folic acid) and the Ham's F-10. The animals inflicted with both repeat breeding and abortion revealed fragile sites the highest in the medium TC 199 (without folic acid) followed by medium RPMI 1640 containing aphidicolin (APH) and the Ham's F-10. In control animal's fragile sites were much less than experimental; however, the trend was reverse in all the three media.

Molecular evaluations of fragile sites

Fragile sites observed in cytogenetic examinations were confirmed in genomic DNA with specific primers. The specific sequences of genomic DNA of all the animals were amplified in PCR and examined after electrophoretic separation in agarose gel. The observations on UV Transilluminator showed the primer specific product length in the animals inflicted with fragile sites on X-chromosomes. Among the 27 reproductively problematic animals examined 18.5%, 51.9%, 48.2%, 40.7%, 0.0%, 22.2% and 40.7% showed the PCR amplification with primers IDVGA82, F9, XIST, DMD, IL1RAPL1, FMR1 and HPRT1, respectively. However, control animals did not show band with any of these primers. The pattern of optimized PCR products obtained from IDVGA82 and F9 genes along with 50 bp DNA ladder are shown in Figures 2.

Amplification of a band of the expected size with the specific primers demonstrated the specificity of the fragile sites on X-chromosomes in problematic breeding animals. On the other hand, these primers were not amplified in control animals suggestive of absence of fragile sites in these animals. The details of animals confirmed with PCR amplification using seven fragile site-specific primers are given in Table 3.

Association of fragile sites with reproductive problems

The effect of different culture media conditions on revelation of fragile sites on X chromosomes in reproductive problem cases was studied using ANOVA. The p-value indicated that effect of media was significant ($p < 0.1$) on fragile sites on X chromosome of reproductively problematic animals. Pair wise comparison of effect of different media showed that RPMI 1640 was significantly different from Ham's F 10. Rest two combinations of media were not different from each other. Least squares mean ± SEM for media RPMI 1640 (with APH), TC 199 (without folic acid) and Ham's F-10 were 26.9983 ± 0.176582, 22.5977 ± 0.176582 and 15.39181 ± 0.176582, respectively. Further the effect of fragile sites on reproductive problematic conditions was studied using ANOVA. P-value obtained indicated that fragile sites on X chromosome were significantly shown by APH ($p < 0.01$) and TC 199 without folic acid ($p < 0.1$) in all the three types of reproductively problematic animals. However, revelation was not significant in Ham's F-10 medium ($p > 0.1$).

Fragile sites are specific points on chromosomes, which are shown as non- randomly distributed gaps and breaks when exposed to specific agents or culture conditions (Berger et al., 1985). Chromosomal fragility is considered to play a role in karyotype evolution, chromosomal rearrangements and disease etiology related to productive and reproductive efficiency of farm animals (Danielak-Czech et al., 2010). In the present study, three types of media were used in culture of lymphocytes of Sahiwal cattle for preparation of metaphase chromosomes. Fragile sites on X-chromosomes revealed as non- staining gaps and breaks with higher percentage when exposed to specific agents or culture conditions.

Table 1 List of primers used in the study

S. N.	Name	Sequence (5'-3')	T_m	Primer Length (bp)	Product Length (bp)
1.	IDVGA82	F-ACAATGATGAGGGGCTCTG	58°C	19	191
		R-GGCAAACCATTCCAGTATTC		20	
2.	F9	F-GGCCAAAGAGGTATAATTCAGG	59°C	22	347
		R-CAACATACTGCTTCCAAAATTCAG		24	
3.	XIST	F-AATTGTGGTATCATGAGGTGGG	62°C	22	350
		R-GTCAGCCATATTGTCCCTGCAGC		23	
4.	DMD	F-TGAGAGCTTTATTGCTGCATTT	56°C	22	125
		R-CATGCCATGTGATGTTTATGC		21	
5.	IL1RAPL1	F-ACGGTCATTAAATGGCATGG	55°C	20	125
		R-GCCCTTGCTCACTGACATCT		20	
6.	FMR1	F-AGCACTTCAGGGCAGATTTTAG	58°C	22	268
		R-ACACACATTTCAGGGTCCAC		20	
7.	HPRT1	F-AGCTTGCTGATGAAAAGGAC	60°C	20	275
		R-TTATAGTCAAGGGCATATCC		20	

IDVGA82, microsatellite loci mapped to region Xq31-34; *F9*, coagulation factor IX gene; *XIST*, X- inactive specific transcript gene; *DMD*, Duchenne Muscular Dystrophy gene; *IL1RAPL1*, interleukin 1 receptor accessory protein – like 1; *FMR1*, Fragile mental retardation 1 gene; *HPRT1*, Hypoxanthine phosphoribosyl transferase-1 gene.

Table 2 The percentage of metaphase plates showing fragile-X in three culture media in different categories of reproductive problems

S.N.	Culture media	Reproductive problems				Control
		Repeat breeding	Abortion	Both repeat breeding & abortion	Total	
1.	RPMI- 1640	30.73 (362/1178)	26.92 (140/520)	17.89 (22/123)	28.79 (524/1821)	7.89 (58/735)
2.	TC-199	28.19 (323/1146)	17.43 (99/568)	22.30 (31/139)	24.45 (453/1853)	10.18 (64/629)
3.	Ham's F-10	19.50 (218/1118)	12.61 (73/579)	17.01 (25/147)	17.14 (316/1844)	10.76 (81/753)

Table 3 PCR amplification result with seven fragile site specific primers in Sahiwal cattle.

Parameter	**Primers/markers**						
	IDVGA82	**F9**	**XIST**	**DMD**	**IL1RAPL1**	**FMR1**	**HPRT1**
Abnormal (27)	05	14	13	11	00	06	11
Percentages (%)	18.5	51.9	48.2	40.7	0.0	22.2	40.7
Control (10)	00	00	00	00	00	00	00
Product length (bp)	191	347	350	125	125	268	275

Among the media used, RPMI 1640 along with aphidicolin (APH) for revelation of common fragile sites, TC-199 (without folic acid) for rare fragile sites and routinely laboratory medium Ham's F-10 as a control media. The observations showed that reproductively problematic animals revealed fragile sites in metaphase plate, which were highest in the medium containing aphidicolin (APH) followed by medium TC 199 (without folic acid) and the Ham's F-10.

In different age groups of Holstein cattle, chromosomal fragility has been identified in autosomes and X-chromosome associated with reproductive abnormalities, baldy calf syndrome, and dwarfism (Danielak-Czech *et al.,* 2013). In bovines a fragile site was discovered in the region FRAXq3.1, associated with fertility problems such as repeat breeders, long calving interval, and abortions (Szczerbal *et al.,* 2021). In the present study it was also found that fragile sites were present significantly in the reproductively problematic animals. Among the media used for fragile sites revelation RPMI (with APH) was found to be best followed by TC 199 (without folic acid) and Ham's F-10 as interpreted from least squares means. The gross gaps and breaks or fragile sites can be easily observed in conventionally Giemsa stained metaphases. In routine usually this approach is in practice, however, with the advent of molecular markers; primers and probes have been developed for most of the regions of genome and particularly related with different traits. In this process primers

have also been developed for revelation of fragile sites. Homologous sequences of the gene FMR1 associated to human fragile X chromosome have been identified using specific primers in cattle DNA. The amplification of a band of the expected size (191bp) with the primers IDVGA82 (mapped to the Xq34) on the fragile region (region Xq31-34) has been reported (Llambí and Arruga, 2008). Goldammer *et al.* (2003) physically assigned coagulation factor IX gene (F9), the hypoxanthine phosphoribosyl transferase-1 gene (HPRT1), and the X-inactive specific transcript gene (XIST) in cattle to evaluate chromosomal breakpoints on BTAX. In the present study also, the fragile sites were found significantly high in the reproductively problematic animals. McAvoya et al. (2007) showed that DMD (Duchenne Muscular Dystrophy) gene and its immediately distal neighbour the IL1RAPL1 (interleukin 1 receptor accessory protein – like 1) gene are CFS genes contained within the FRAXC CFS region (Xp21.2-p21.1). In the present study seven primers were used to confirm the presence of fragile sites on X-chromosomes of Sahiwal cattle. The product length of specific size was obtained on the some of the reproductively problematic animals. The studies so far carried out suggest the importance of knowing the distribution and morphological characteristics of animal fragile sites, as a first step to finding the possible relationships between any defined pathology or syndrome and the presence of chromosomal fragility.

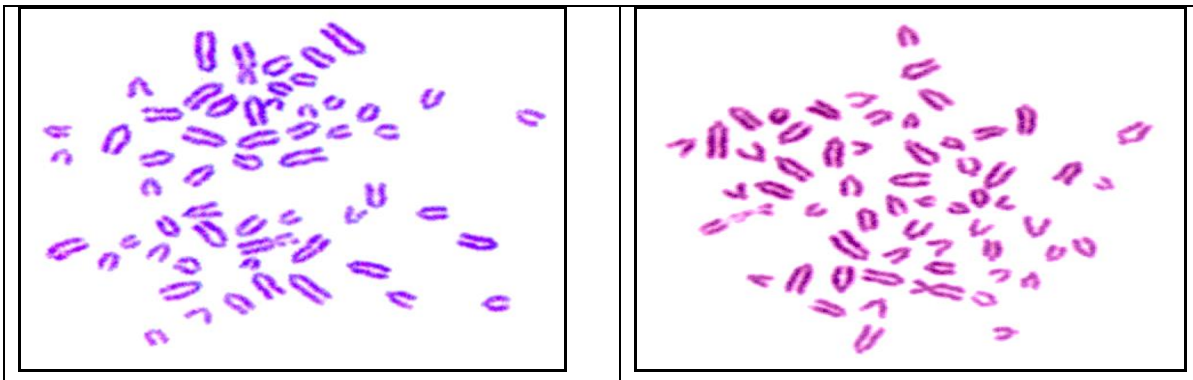

Fig. 1. Fragile sites (a) in the middle of one chromatid on the short arm and (b) in the middle of both the chromatids of the long arm of X- chromosome

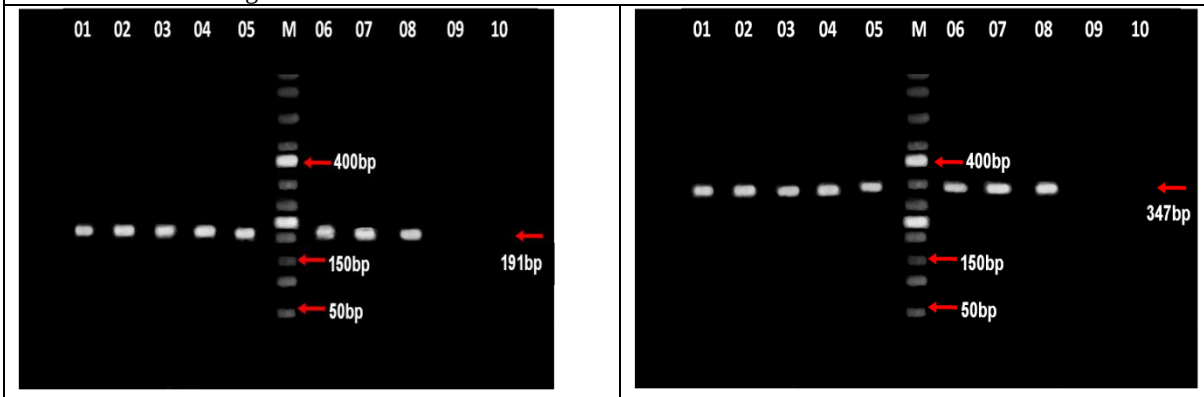

Fig. 2. PCR product of (a) IDVGA82 and (b) F9 genes showing 191 bp and 347bp along with 50 bp DNA ladder on 1.5% agarose gel. Lanes represent as M: DNA ladder, 01-04: Repeat breeders, 05-06: Abortion cases, 07-08: Repeat breeders - Abortion cases and 09-10: Control groups.

Conclusion

Three types of tissue culture media namely RPMI-1640 with aphidicolin (APH), TC-199 (without folic acid) and Ham's F-10 revealed different frequencies of fragile sites on X- chromosome, which were interpreted with least squares means. Among these media RPMI (with APH) was found to reveal highest number followed by TC 199 (without folic acid) and Ham's F-10, which are considered as suitable for exposure of common, rare and routine types of gaps and breaks, respectively. The existence of fragile sites was confirmed with specific DNA primers. FSs were found significantly associated with reproductive problems such as repeat breeding and abortion. It is further stated that those cases where cytogenetical observations showed gaps but could not be confirmed by DNA primers could be mechanical gaps and breaks. Secondly, the primers used were limited and might not be covering whole genome. The association found can be useful as a tool in identification and culling of carrier animals early in the age and stage. Further for this cause, these chromosome defects, without cytogenetic control, escape selection, with subsequent detrimental effects on fertility (and production),

especially in female carriers. Chromosome anomalies can also be easily spread by offspring, especially when using artificial insemination, with adverse financial effects on animal breeding. Certainly, clinical cytogenetics remains a vital step in the genetic improvement of livestock. The findings can be applied in cytomolecular comparative studies to evidence relationships between chromosome instability and reproductive performance in the other breeding animals.

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Disclaimers/Conflict of interest The authors hereby declare that there is no conflict of interest regarding the experimental data and manuscript.

References

1) Albarella S., Ciotola F., D'Anza E., Coletta A., Zicarelli L., Peretti V. (2017). Congenital Malformations in River Buffalo (Bubalus bubalis). Animals. **7(2):** 9
2) Berger R., Bloomfield C.D., Sutherland G.R.(1985). Report of the committee on chromosome rearrangements in neoplasia and on fragile site. 8th International Workshop on Human Gene Mapping. Cytogenetics and Cell Genetics.**40**:490-535.
3) Berry D.P., O'Brien A., O'Donovan J., McHugh N., Wall E., Randles S., McDermott K., O'Connor R.E., Patil M.A., Ho J., Kennedy A., Byrne N., Purfield D. C. (2018). Aneuploidy in dizygotic twin sheep detected using genome-wide single nucleotide polymorphism data from two commonly used commercial vendors. Animal. **12:** 2462–2469.
4) Danielak-Czech B., Babicz M., Rejduch B., Kozubska-Sobocińska A. (2013). Cytogenetic and molecular analysis of chromosome instability in cattle with reproductive problems. Annales UMCS Zootechnica **30(4):**18-25
5) Danielak-Czech B., Kaczor U., Sharan M. (2010). Cytomolecular analysis of chromosome X fragility in subfertile sheep. Ukrainian Academy of Agrarian Sciences. The Animal Biology. **12**: 234–239.
6) D'Hulst C., Kooy R.F. (2009). Fragile X syndrome: from molecular genetics to therapy. Journal of Medical Genetics.**46**: 577-584.
7) Donaldson B., Villagomez D., King W.A. (2021). Classical, Molecular, and Genomic Cytogenetics of the Pig, a Clinical Perspective. Animals. **11**:1257.
8) Dey D. 2017. Sahiwal – a policy pointer in Indian context. Journal of Livestock Science 8: 88-91
9) Goldammer T., Amaral M.E.J., Brunner R.M., Owens E., Kata S.R., Schwerina M., Womack J.E. (2003). Clarifications on breakpoints in HSAX and BTAX by comparative mapping of F9, HPRT, and XIST in cattle. Cytogenetic and Genome Research **101**:39-42.
10) Gustavsson I., Rockborn G. (1964). Chromosome abnormalities in three cases of lymphatic leukaemia in cattle. Nature. **203**:990
11) Iannuzzi A., Iannuzzi L., Parma P. (2023). Molecular Cytogenetics in Domestic Bovids: A Review. Animals. **13**: 944.
12) Iannuzzi A., Parma P., Iannuzzi L. (2021). Chromosome Abnormalities and Fertility in Domestic Bovids: A Review. Animals. **11**: 802.
13) Iannuzzi A., Perucatti A., Genualdo V., Pauciullo A., Melis R., Porqueddu C., Marchetti M., Usai M., Iannuzzi L. (2016). Sister chromatid exchange test in river buffalo lymphocytes treated in vitro with furocoumarin extracts. Mutagenesis.**31**:547–551.
14) Jennings R.L., Griffin D.K., O'Connor R.E. (2020). A new approach for accurate detection of chromosome rearrangements that affect fertility in cattle. Animals. **10**:114.
15) Kumar A., Kumar A., Sheetal S.K., Kumar A., Azad C.S., Yadav D. 2025. Effect of CIDR on superovulatory response of FSH administration in Sahiwal cows. Journal of Livestock Science 16: 247-252 doi. 10.33259/JLivestSci.2025.247-252
16) Llambí S., Arruga M.V. (2008). Molecular approach of the fragile chromosomal region Xq31-34 in cattle (*Bos taurus*) by microdissection and DOP-PCR. Brazilian Journal of Veterinary and Animal Sciences**. 60(4):** 926-931.
17) McAvoy S., Ganapathiraju S., Perez D.S., James C.D., Smith D.I. (2007). DMD and IL1RAPL1: two large adjacent genes localized within a common fragile site (FRAXC) have reduced expression in cultured brain tumors. Cytogenetic and Genome Research. **119**:196-203.
18) Pauciullo A., Cosenza G., Peretti V., Iannuzzi A., Di Meo G.P., Ramunno L., Iannuzzi L., Rubes J., Di Berardino D. (2011). Incidence of X-Y aneuploidy in sperm of two indigenous cattle breeds by using dual color fluorescent in situ hybridization (FISH) Theriogenology. **76**:328–333.
19) Szczerbal I., Komosa M., Nowacka-Woszuk J., Uzar T., Houszka M., Semrau J., Musial M., Barczykowski M., Lukomska A., Switonski M. (2021). A Disorder of Sex Development in a Holstein–Friesian Heifer with a Rare Mosaicism (60,XX/90,XXY): A Genetic, Anatomical, and Histological Study. Animals. **11**:285.