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Cytogenetic Screening of Karan Fries and Tharparkar Males for Selection of Elites at an Early Age

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Abstract

The importance of sire selection in the herd is reflected in the total genetic gain through sire-to-sire path. The present investigation was envisaged with the objective of screening chromosomes with conventional cytogenetic techniques in males of Tharparkar and Karan-Fries cattle and to find association between chromosomal anomalies and performance of males. 25 and 15 males of KF (Karan Fries) and TP (Tharparkar) respectively, were utilized for cytogenetic and semen evaluation work at ICAR-National Dairy Research Institute, Karnal. Cytogenetic screening of animals could not show gross numerical and chromosomal aberrations, while fragile sites were revealed by induction with aphidicolin. The average percentage of fragile sites in autosomes and sex chromosomes were found 14.66 and 6.16 respectively in KF males, while in TP males average percentages were 7.66 and 4.9, respectively. Association study revealed that fragility in autosomal chromosomes had significant effect on the birth weights in KF males and no association was found between any libido and reproductive trait with the percent fragile sites either in autosomes or sex chromosomes. Cytogenetic screening is recommended at the initial stage for the propagation of only elite bull semen used for A.I. Association between autosomal fragility and birth weights although found highly significant but still need to be validated with large sample size.

Keywords: Association Studies, Cattle, Chromosomal Fragility

Introduction

The sustained economics of dairy enterprise depends on the overall profitability of animals maintained in herd, which can be enhanced by selecting the animals as early as possible. Furthermore, the fact that maintenance of reproductive efficiency in the herd is of particular concern cannot be disputed, however, increasing growth is important to increase output from production systems (Eler *et al.,* 1995).

For success of genetic improvement programme, it is also necessary to have a nucleus herd of high genetic merit required for production and dissemination of quality germplasm to meet the requirement of pure as well as cross bred bulls (Singh *et al.,* 2019). The importance of sire selection in the herd is reflected in the total genetic gain through sire-to-sire path and sire-to-dam path being more than 50 percent. Males reserved initially on the basis of pedigree performance and growth traits, are raised for semen production. Bulls are presently selected at the age of 2-3 years for further reproduction and even a bull with high production index is culled due to some reproductive anomalies. However, all the males reserved for breeding do not reach the semen freezing stage. Prior to the inclusion of breeding bulls in test matings, a number of high genetic merit bulls are disposed due to various sub fertility problems viz., poor libido in zebu cattle, poor semen quality and freezability in crossbred males (Mukhopadhyay *et al.,* 2010). The importance of male animals in selection emphasizes a set of existing criteria for selection of young male animals under the breeding program.

Alterations in chromosome number and structure have been found to be the best-known genetic variations having direct effects on fertility and reproductive outcome in cattle. Number of autosomal and sex chromosomal genes have been reported to affect the reproductive traits of males (Di Meo *et al.,* 2011). Fragile sites are intriguing cytogenetic phenomena that have been extensively investigated in human and laboratory animal chromosomes over the past 40 years, but domestic animal species have been studied sporadically. A chromosomal fragile site is a specific heritable point on a chromosome that tends to form gaps and breaks on metaphase chromosomes following partial inhibition of DNA synthesis (Durkin *et al.,* 2007). These are chromosomal sites showing susceptibility to breakages and discontinuities in specific conditions of cell culture and also following induction with chemical substances (Wojcik *et al.,* 2012). The association between abnormal chromosome constitutions and disorders of sexual development (DSDs) in domestic animals has been recorded since the beginnings of conventional cytogenetic analysis. Deviated karyotypes consisting of abnormal chromosome sets seem to be the main causes of reproductive anomalies (Favetta *et al.,* 2012). The fragile site regions in chromosomes are associated with the sub-fertility problems and abnormal semen quality in breeding bulls (Anil, 2011). Hence it is imperative to have cytogenetic evaluation of males for culling of undesirable males, which are prone to sub-fertility problems, at an early age. The definition of the species-specific 'fragile site map' in domestic animals might be an important step towards a more precise characterization of the karyotypes and the clues of the degree of chromosome stability and instability of chromosomes. More effort is needed to clarify economic and clinical significance that may be associated with chromosome fragility. Keeping this in view, the present investigation was envisaged to screen chromosomes with conventional cytogenetic techniques in males of Karan-Fries cattle and to find association between chromosomal anomalies and reproductive performance of males (if any).

Materials and Methods

Sample Collection

The present investigation was carried out on males of Tharparkar (TP) and Karan Fries (KF) cattle breed maintained at National Dairy Research Institute (NDRI), Karnal. 25 and 15 males of KF and TP respectively were utilized for cytogenetic and semen evaluation work, investigation was conducted on reserved and semen donor bulls of Tharparkar and Karan Fries cattle maintained at Artificial Breeding Research Centre (ABRC) herd of National Dairy Research Institute, Karnal. These studies were carried out in Livestock Genome Analysis Laboratory, Dairy Cattle Division and ABRC, NDRI, Karnal.

Chromosome Preparations

Short term whole blood lymphocyte culture technique as described by Yadav (1981) was followed for the preparations of chromosomes necessary for subsequent staining and banding for the present studies. Blood was collected from the jugular vein of the animals in standard vacutainer tubes (Becton-Dickinson & Co., NJ 07070)

containing 143 USP units of sodium heparin per tube. An aliquot (0.5 ml) of blood was used for setting up of cultures and the remaining blood was stored at 5°C until harvesting for reuse should a culture fail. The serum from healthy mature cattle was utilized to culture medium to support metabolism of the cells in the cultures. All glassware used was properly sterilized and siliconized for the experiment.

RPMI-1640 was prepared as the culture medium. To 100 ml glass distilled water in a conical flask, 1.04 gm of RPMI media was added in flask and mixed by slight shaking. Antibiotics (streptomycin & penicillin) & Pokeweed mitogen 1 ml each were added in it. The flask was swirled gently to dissolve all the chemicals completely. The pH of the medium was adjusted to 7.2 by adding drop by drop sterile $NaHCO_3$ solution (4.4%). The prepared medium was sterilized by filtering through millipore filter assembly (0.22 µm). After filtration, the sterile medium was transferred to laminar hood and 20 ml serum (from healthy mature cattle) was added to it. One drop of sterile sodium heparin was added to prevent the coagulation. Gentle swirl was given for proper mixing. This complete culture medium (CCM) was distributed in screw cap culture bottles (30 ml) in aliquotes of 6 ml each.

Whole blood cultures were established either in freshly prepared medium or stored medium (-20°C). With the help of sterile glass syringes, 0.5 ml of blood was transferred to the culture bottles containing completed culture medium. The contents were given a gentle swirl for quick mixing. The culture bottles were transferred to an incubator at 37.5°C (±0.5°C) for a period of 96 hours. After 72 hours of growth, Aphidicolin (Sigma) was added to the cultures grown in RPMI-1640 at 0.15 µM (final concentration) for further 24 hours to induce fragile sites. 7 hours before the harvesting, BrdU (20 µg/ml, f.c.) was added to the cultures for labeling late replication regions. After incubating for 94 hours, 2 drops (0.5 µ g/ml) of colcemid solution (Sigma) were added to each culture bottle, 45 minutes prior to actual harvesting procedure and again kept in the incubator. Culture materials were transferred to centrifuge tubes (15 ml) after 45 minutes of incubation with colcemid treatment. These tubes containing cultured cells in medium were centrifuged for 15 minutes at 1400-1500 rpm. Cell suspension in the bottom of the centrifuge tubes in the form of pellets was used. The pellets in each tube were treated with about 10 ml warm hypotonic solution (0.075 M KCl). The pellets were mixed gently with pasteur pipette and the tubes were again transferred to the incubation for another 10 minutes. 1 ml of chilled fixative was added to each centrifuge tubes and mixed gently. Subsequently, centrifugation was done for 10 minutes at 1200 rpm, after which the supernatant was discarded and pellets were resuspended in 5 ml chilled fixative. This procedure was repeated thrice. After third centrifugation, supernatant was removed with the help of pasteur pipette judging the button size. The button was broken and mixed by gentle pipetting to obtain a slightly milky cell suspension.

The slides [deluxe microslides (Blue Star) of 0.8 mm thickness] were dipped overnight in chromic acid solution, thoroughly rinsed in running tap water and then with GDW. These were stored in a plastic bottle containing 5 percent HCI and absolute methanol Slides were prepared both by flame drying for Conventional Giemsa Staining and by air-drying methods for banding study.

Staining of Slides

About 50 ml of Giemsa working solution was taken in a glass coplin jar. The required slides were kept into the coplin jar. The slides were allowed to stay in the staining solution for 30 minutes. Subsequently, the slides were rinsed thoroughly with GDW and dried in folds of filter paper. The slides were then transferred to the incubator and kept for a few hours before starting of screening.

Screening and Photomicrography of Slides

Slides were screened by using LEICA microscope. Normally 50 well spread metaphase plates were screened. If any chromosomal abnormality was suspected, then at least 100 plates were recorded. Plates with excellent spread and chromosome morphology were selected for photography. Selected plates were photographed by using LEICA camera fitted on microscope.

Assessment of Seminal Attributes

Semen was collected using bovine artificial vagina (IMV model-005417) with smooth neoprene liner over a male dummy bull once a week. On each collection, two ejaculates were taken with 20 to 30 min gap between two successive ejaculates. The volume of semen was measured directly from the collecting tube (15 ml) having 0.1 ml

calibration. Mean of the successive two ejaculates were taken as volume. Mass activity was evaluated as per method described by Tomar *et al.* (1966). The progressive motility and percentage motile spermatozoa were determined by mixing 100 µl of undiluted semen into pre-warmed tubes containing 900 µl of Tris buffer. A thin drop of diluted semen was placed on a warmed glass slide (37°C) and a cover slip was placed on it. The slide was observed under phase contrast microscope equipped with a 37°C heated stage in 20X objective lens and in a phase combination (D2). The percentage of progressively motile (0-100%) spermatozoa was estimated by observing five representative areas of the slide. Usually, ejaculates having more than 60 per cent motility were used for the study. Sperm concentration was estimated by haemocytometric slide (Neubar's chamber) method. Non-eosinophilic (live) Sperm Count was determined by Eosin-Nigrosine stain which was-prepared by the method described by Blom (1950) and Hancock (1951). The procedure used in the present study for the Hypo-osmotic swelling Test **(**HOST) was similar to the one developed by Jeyendran *et al.* (1984) with some modifications. Assessment of acrosome integrity was carried out by following the staining procedure as described by Hancock (1952). The sexual behaviour scoring was adopted as described by Anzar *et al.* (1993) with some modification. Sexual behaviour score is divided into two parts; libido score and mating ability score. Libido was scored on the basis of reaction time (in seconds), sexual aggressiveness and tactile stimulation.

Classification of Percentage of Fragility

The data on percentage of fragility was classified into different age groups based on Sturges rule (Sturges, 1926) using the following formula:

$$\frac{Range}{1 + 3.322\ log_{10}N}$$

Where, N = No. of observations

Results and Discussion

Cytological Investigations of KF and TP Males for Fragile Sites

In 1250 metaphase plates studied for KF males, no gross numerical and structural chromosomal aberrations were detected by cytogenetic screening (Figure 1). However, addition of aphidicolin (APH) to media revealed the secondary structural chromosomal aberrations as fragile sites. The induced fragile sites were identified as chromatid gaps or breaks.

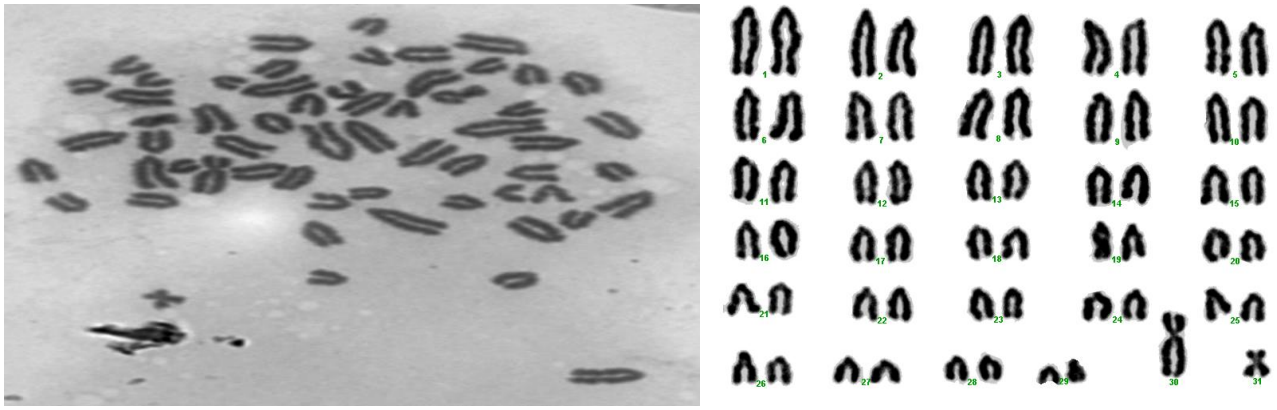

Figure 1: Normal Karyogram of KF without aphidicolin treatment

Fragile sites on X-chromosomes and autosomes of KF are shown in Figures 2, 3 and 4. The observations revealed that percentage of fragile sites in autosomes varied from 5 % to 22 % with mean 14.66 % and for sex chromosomes it ranged from 0 to 14 % with an average of 6.16 %, indicating relatively moderate fragility in autosomes and low in sex chromosomes of KF males. The fragile sites were consistently present on the centromeric regions and the terminal end of the q-arm of different chromosomes.

Figure 2: Fragile sites in the terminal portion of long arm (q) of both the chromatids of X chromosome in KF bulls

Figure 3: Fragile sites in the short arm (p) of both the chromatids of the X-chromosome in KF bulls

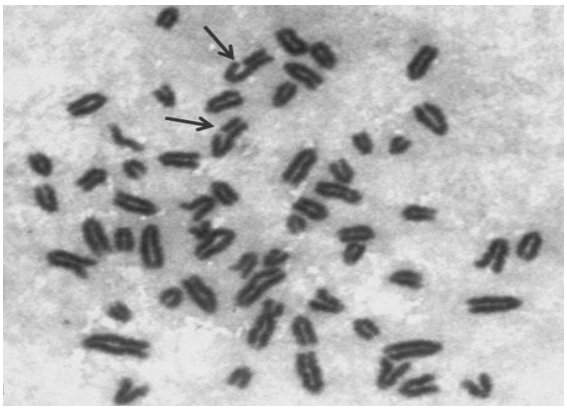

Figure 4: Giemsa stained metaphase plates of KF showing fragile sites in autosomes

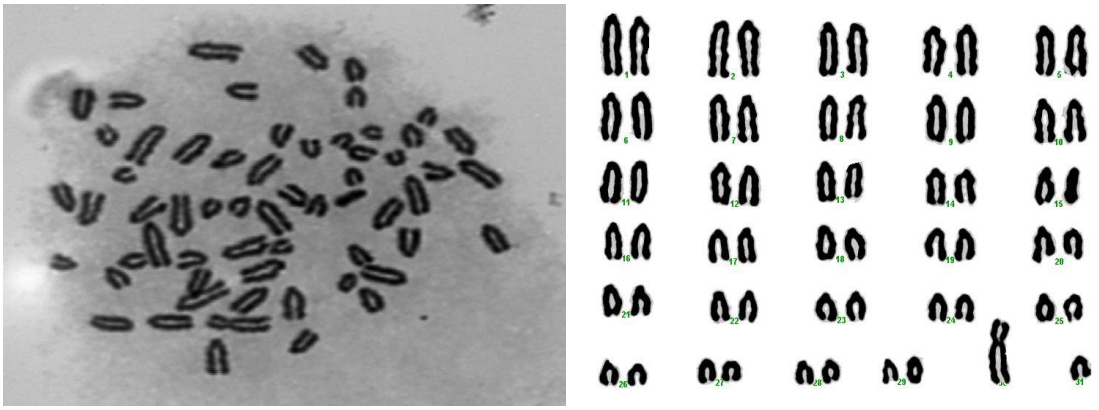

Figure 5: Normal Karyogram of TP without aphidicolin treatment

In TP well spread complete metaphase plates were screened from the prepared slides. The chromosome complement in male is (60, XY). All the 58 autosomes and Y-chromosomes are acrocentric; X-chromosomes are the sub-metacentric (Figure 5). In 500 metaphase plates studied, no gross numerical and structural chromosomal aberrations were detected by cytogenetic screening. However, addition of aphidicolin (APH) to media revealed the secondary structural chromosomal aberrations as fragile sites. The induced fragile sites were identified as chromatid gaps or breaks. Fragile sites on X-chromosomes and autosomes of TP are shown in Figures 6, 7 and 8. The observations revealed that percentage of fragile sites in autosomes varied from 3 % to 11 % with mean 7.66 % and for sex chromosomes it ranged from 0 to 8 % with an average of 7.99 %, indicating relatively low fragility in autosomes sex chromosomes of TP males. The fragile sites were consistently present on the centromeric regions and the terminal end of the q-arm of different chromosomes.

Figure 6: Fragile sites in the terminal portion of long arm (q) of both the chromatids of the X chromosome in TP bull

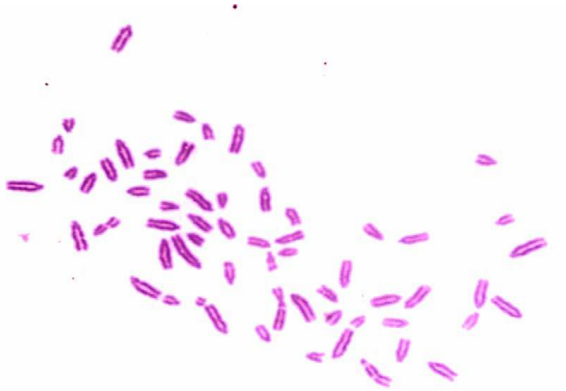

Figure 7: Fragile sites near to centromere on the long arm (q) of both the chromatids of X chromosome in TP bull

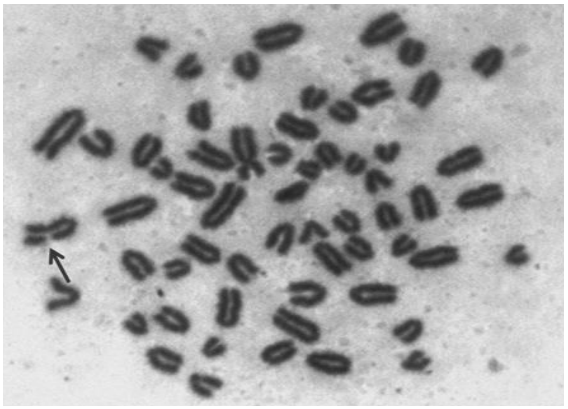

Figure 8: Giemsa stained metaphase plates of TP showing fragile sites in autosomes

Saroj (2012) reported that in Sahiwal calves fragile sites range from 4.48% to 36.07% of total metaphase plates screened with mean of 19.27 ± 1.63. Further, out of metaphase plates revealing fragile sites, in 0 to 100% of cases with mean of 59.39 ± 5.19 fragile sites were associated with X-chromosomes. In Tharparkar, fragile sites ranged from 7.69% to 39.39% of total screened metaphase plates with mean of 18.89 ± 2.48. Further, in 0 to 87.5% of plates showing fragile chromosomes, with mean of 66.28 ± 5.88, fragility was associated with X-chromosomes. And in KF, same author reported fragile sites ranging from 3.95% to 41.89% of total metaphase plates screened were found with mean of 21.28 ± 2.21. Out of these metaphase plates revealing fragile sites, in 0 to 86.96% of cases with mean of 66.96 ± 4.16, fragile sites were associated with X-chromosomes. Revelation of fragile sites in metaphase plates was highest (20.95%) in Karan-fries calves followed by Murrah (20.67%) and Sahiwal calves (19.36%), respectively. Further, fragile X-chromosomes were highest (78.21%) in Murrah buffalo calves followed by Karan-fries (72.28%) and Tharparkar (70.63%) calves. Anil (2011) reported that the overall mean of percent total fragile site was observed as 15.61± 0.69 in different breeds and categories of bulls. The least squares means of total fragile sites for Sahiwal, Karan Fries and Murrah were found to be 15.89 ± 0.03, 13.21± 0.03 and 17.89 ± 0.04, respectively. The overall average of total fragile sites for poor libido, poor semen freezability, poor semen quality and control groups were observed to be 19.93 ± 0.04, 21.43 ± 0.04, 18.40 ± 0.04, and 5.53 ± 0.04, respectively. And also, revealed that there were significant differences in total fragile sites (%) between categories of bulls. However, the differences between breeds were not found to be significant. Thus, our findings were lower than Saroj (2012) and were higher than the Anil (2011) for Karan Fries. Our findings were lower than Saroj (2012) and Anil (2011) for Tharparkar. In the present study it can be concluded that, fragile sites were revealed as non-staining gaps and breaks were present in Tharparkar and Karan Fries as in other breeds and species reported by different authors around the world.

Guven *et al.* (1999) reported Aphidicolin (58%) as more potent fragile site inducer than FUdR (53%) and caffeine (33%), based on the frequency of breakages at the different sites in healthy Turkish individuals. Aphidicolin (APH) inducible fragile sites have been detected in the chromosomes of cattle (Rodriguez *et al.,* 2002); buffalo (Nicodemo *et al.,* 2008); pigs (Riggs *et al.,* 1993); dog (Wurster-Hill *et al.,* 1988; Stone *et al.,* 1991); 1993); mice (Elder &

Robinson, 1989; McAllister & Greenbaum, 1997; Rozier *et al.* 2004) and rabbits (Poulsen & Ronne, 1991). Pires *et al.* (1998) reported fragile X-chromosomes in 2.86 to 41.03% of metaphase plates in different breeds of river buffalo. Nicodemo *et al.* (2007) reported significant difference in fragile sites of male and female river buffalo mainly due to inactive X- showing twice as many breaks compared to the active counterpart in females. Fuster *et al.* (1990) reported telomere association of chromosomes of a phenotypically and mentally normal individual in 11.7% of metaphase plates cultured in RPMI-1640 with aphidicolin. Fry and Loeb (1986) described aphidicolin as a selective inhibitor of DNA polymerase-A, the major eukaryotic polymerase which adds deoxynucleotide monophosphates to the 3'-end of a DNA primer, during both replicative and excision repair DNA synthesis. Riggs *et al.* (1993) reported association of APH-induced breakage and reciprocal translocation breakpoints in domestic pig chromosomes.

Association between Autosomal Chromosome Fragility and Reproductive Performance in KF

Association of different levels of autosome chromosome fragile percentage and semen parameters has been presented in Table 1. Overview of the results revealed that upon conducting Analysis of Variance, no significant association could be established between autosomal chromosome fragility and the reproductive performance of KF bulls.

Table 1: Association between reproductive performance and birth weight with autosomal chromosome fragility in KF bulls

Traits	Chromosome fragile percent group		
	< 12 % (N=8)	**12 – 17 % (N=8)**	**> 17 % (N=9)**
Volume	4.19	4.28	4.73
Concentration (millions/ml)	1142.25	943.75	1077.89
Mass activity (0 to 5)	3.12	3.17	3.17
Progressive motility	72.25	74	72.67
Live (%)	69.25	70.25	68.11
HOST (%)	65	64.25	63.33
Acrosomal activity (%)	77.88	82.13	81.89
Libido (%)	82.63	82.13	80.33
Birth Weight	36.38	29.88	25.89

N = Number of animals

Association Between Autosomal Chromosome Fragility and Birth Weight in KF

With the increasing autosomal chromosome fragility there was decrease in birth weight (Table 2) and the analysis of variance revealed that there was highly significant effect of between different autosomal chromosome fragility on birth weight of KF bulls. This is an important factor for consideration as sire is half the herd therefore selection of bulls taking chromosomal fragility into account may significantly increase the herd performance.

Table 2: Classification of sex chromosome fragile percentage

Bin	Frequency	Cumulative %
< 5	9	36.00%
05-Jul	8	64.00%
> 7	8	100.00%
Total	25	

Association Between Sex Chromosome Fragility and Reproductive Performance in KF

To assess the association, autosome chromosome fragility percentage was classified according to Sturges formula (Sturges 1926) (Table 2). Association between reproductive performance and sex chromosome fragility in KF bulls is give in Table 3. The result of the analysis of variance revealed that no significant association could be established between sex chromosome fragility and the reproductive performance of KF bulls.

Table 3: Association between reproductive performance and sex chromosome fragility in KF bulls

Traits	Chromosome fragile percent group		
	Below 5	**5 to 7**	**Above 7**
Volume	3.92	4.16	5.21
Concentration (millions/ml)	1073.89	1039	1051.5
Mass activity (0 to 5)	3.25	3.14	3.06
Progressive motility (%)	74.33	71.38	73
Live spermatozoa (%)	69.67	71	66.75
HOST (%)	65.22	63.25	63.88
Acrosomal activity (%)	81.11	79.38	81.5
Libido (%)	83.44	80.25	81

Association Between Sex Chromosome Fragility and Birth Weight in KF

The KF bulls with sex chromosome fragility percentage as below 5, 5 to 7 and above 7 % were having mean birth weight of 31.22, 28.86 and 31.11 kg respectively. Analysis of variance revealed no significant effect between different sex chromosome fragility on birth weight of KF bulls.

Conclusion

Comparative study revealed that KF chromosomes are more prone to fragility than TP. Autosomal chromosome fragility was higher than sex chromosomes in both breeds under study. Association study revealed significant effect of autosomal fragility on birth weights in KF males. No association could be established between chromosomal fragility with libido and semen quality parameters. There may still need to be validated with large sample size.

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Conflict of Interests

There is no conflict of interest.

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