QUANTITATIVE AND QUALITATIVE ASSESSMENT OF SEX SORTED SEMEN OF *BOS INDICUS* AND *BOS TAURUS* BREEDS

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THESIS SUBMITTED TO

P. V. NARSIMHA RAO TELANGANA VETERINARY UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE AWARD OF THE DEGREE OF

MASTER OF VETERINARY SCIENCE

(ANIMAL GENETICS AND BREEDING)

IN THE FACULTY OF VETERINARY SCIENCE

DEPARTMENT OF ANIMAL GENETICS AND BREEDING

COLLEGE OF VETERINARY SCIENCE

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CERTIFICATE

This is to certify that **Mr. NARESH GADDAM, RVM/2018-39** has satisfactorily prosecuted the course of research and that the thesis entitled **"QUANTITATIVE AND QUALITATIVE ASSESSMENT OF SEX SORTED SEMEN OF *BOS INDICUS* AND *BOS TAURUS* BREEDS"** submitted is the result of original research work done and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any University.

(Dr. S.T. VIROJI RAO)
Major Advisor

Date: 11-03-2022
Place: Hyderabad

CERTIFICATE

This is to certify that the thesis entitled **"QUANTITATIVE AND QUALITATIVE ASSESSMENT OF SEX SORTED SEMEN OF *BOS INDICUS* AND *BOS TAURUS* BREEDS"** submitted in partial fulfillment of the requirements for the degree of **MASTER OF VETERINARY SCIENCE** of **P.V. NARSIMHA RAO TELANGANA VETERINARY UNIVERSITY, HYDERABAD,** is a record of the bonafide research work carried out by **NARESH GADDAM (RVM/2018-39)** under our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of the investigations have been duly acknowledged be the author.

The final *Viva Voce* examination was held on 11-03-2022 and the Thesis is approved by the Student Advisory Committee.

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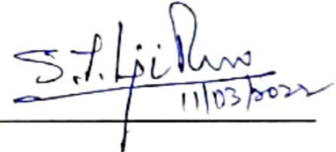

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LIST OF ABBREVIATIONS

Abbreviation	**Full form**
%	Percentage
μ	Micron
μg	Microgram
μl	Microliter
°C	Degree Celsius
°F	Degree Fahrenheit
bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic acid
SDS	Sodium dodecyl sulphate
F	Forward
Fig.	Figure
R	Reverse
PLP gene	Proteo Lipid Protein gene
SRY Gene	Sex determining region Y
hrs.	Hours
g	Grams
Kbp	Kilo base pair
l	Litre
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	milli molar
Min.	Minutes
Mol.wt	Molecular weight
OD	Optical Density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
rpm	Revolutions per minute
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
TAE	Tris acetate EDTA
w/v	Weight/volume

DECLARATION

I, **NARESH GADDAM (RVM/2018-39)** hereby declare that the thesis entitled **"QUANTITATIVE AND QUALITATIVE ASSESSMENT OF SEX SORTED SEMEN OF *BOS INDICUS* AND *BOS TAURUS* BREEDS"** submitted to **P.V. NARSIMHA RAO TELANGANA VETERINARY UNIVERSITY** for the Degree of **MASTER OF VETERINARY SCIENCE** is a result of original research work done by me. It is further declared that the thesis or any part thereof has not been submitted for any other degree or diploma.

Date: 11-03-2022

Place: Hyderabad

(NARESH GADDAM)

ACKNOWLEDGEMENTS

First and foremost, I would like to thank ***God*** *Almighty for giving me the strength, knowledge, ability and opportunity to undertake this research study and to persevere and complete it satisfactorily. Without his blessings, this achievement would not have been possible. I have to thank my beloved parents* ***Sri G. Narsingh & Smt. G. Laxmi for*** *their endless love, support and encouragement throughout my life, which making me capable of reaching this peak of success and upbringing me with precious value.*

I wish to avail this golden opportunity in my life to express my heartfelt and sincere gratitude to the Chairman of my advisory committee ***Dr.S.T. VIROJI RAO,*** *Registrar, PVNRTVU, Rajendranagar, Hyderabad. His kind support, careful planning and inspiring suggestions have allowed this research work to be successful.*

Fervently and modestly, I extol the genuine co-operation and affection offered to me by my member of the Advisory Committee ***Dr. D. Sakaram****, Professor & Head, Department of Animal Genetics and Breeding, College of Veterinary Science, PVNR TVU, Rajendranagar, Hyderabad, from the initiation of the work to the ship-shaping of the manuscript. It was indeed a rare privilege for me to work under him unending inspiration and indomitable spirit.*

I deem it my privilege in expressing my heartfelt gratitude to the member of the Advisory Committee ***Dr. P. Kalyani,*** *Assistant Professor and Head, Department of Veterinary Biotechnology for her excellent guidance, unreserved enthusiastic moral support, diligence and valuable time in guiding me throughout this investigation.*

I would like to thank ***Dr. P. Jayalakshmi, Professor,*** *Department of Animal Genetics and Breeding, College of Veterinary Science, Rajendranagar, for her valuable suggestions & encouragement during this research work.*

I would like to thank ***Dr. B. Sridevi****, Assistant Professor, Department of Animal Genetics and Breeding, College of Veterinary Science, Rajendranagar, for her valuable suggestions during this research work.*

I would like to thank ***Dr. G. Visveshwar****, Assistant Professor, Department of Biotechnology, College of Veterinary Science, Rajendranagar, for his valuable suggestions during this research work.*

I respectfully acknowledge and express my profound thanks to ***Bhagyalakshmi****, SRF at Department of Veterinary Biotechnology for her cooperation and help throughout my investigation.*

I feel short of space to express my sincere gratitude to my colleagues ***Dr. Mounika, Dr. Anusha, Dr. Swati, Dr. Jyosthsna, Dr. Rachana, Dr. Spandana, Dr. Sri Teja and Dr. Avinash*** *for their help, transcendent suggestions and endless cooperation during the prosecution and presentation of this research work. I would like to thank my seniors* ***Dr. Rajith Reddy, Dr. Ravali, Dr. Narmadha, Dr. Tejasri*** *and juniors* ***Dr. Nagasri, Dr. Vasanti, Dr. Suvarna***

I would like to extend my thanks to ***Sudarshan, Mohan Krishna, Savitri, Mahender, Narasimha, Madhu, and Kumar the*** *non-teaching staff of Departments of Veterinary Microbiology and Veterinary Biotechnology and Animal Genetics and Breeding. I apologize for the faux pass of the persons who have extended the help in a way or other and deserved such thanks.*

It gives me immense pleasure to express my profound sense of gratitude and sincere thanks to ***Dr. L. Atchamamba,*** *Assistant Professor, and* ***Staff*** *of College Library for their help in providing the necessary facilities to carryout research work.*

I thank my friends Mahesh Gaddam, shekar Velma, Navin, Baapu Reddy, Suresh Reddy and **Rajender Reddy** *for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.*

I also thank my well-wishers **Dr. Venkat Reddy, Dr. Abhinav, Dr. Vinay, Dr. Srinivas Thota, Dr. Nijanand, Dr. Giri, Dr. Srinivas Tummala and Dr. Ajay Veer Reddy** *for their help in this research. My vocabulary utterly fails in expressing my accolade to my revered parents Sri* **G. Narsingh** *and* **Smt. Laxmi** *who brought me to this stage. I am grateful for their kind blessings, love, patience, overwhelming support and inspiration. I fall short of words for the moral and unrelenting support extended by my family members,* **Manasa Reddy** *(wife),* **Sahasra Reddy** *(daughter)* **Suresh Reddy** *(brother),* **Mounika** *(Sister) and* **Manasa** *(sister) who were beside me in all my ups and downs.*

(NARESH GADDAM)

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Title of the thesis : **QUALITATIVE AND QUANTITATIVE ASSESEMENT OF SEX SORTED SEMEN OF *BOS INDICUS* AND *BOS TAURUS* BREEDS**

Degree to which it is submitted : **MASTER OF VETERINARY SCIENCE**

Faculty : **VETERINARY SCIENCE**

Department : **ANIMAL GENETICS AND BREEDING**

Major Advisor : **Dr.S.T. VIROJI RAO**

University : **P.V. NARSIMHARAO TELANGANA VETERINARY UNIVERSITY**

Year : **2022**

ABSTRACT

The current study was carried out with the aim of quantitative assessment of X and Y chromosome bearing spermatozoa in sexed/sorted semen produced through various semen sorting techniques in *Bos indicus* and *Bos taurus* breeds by quantitative real time PCR. The qualitative assessment of the sorted semen was also done using standard protocols.

It was found that the percentage of X chromosome bearing spermatozoa ranged from 62.88% to 71.21% and Y chromosome bearing spermatozoa ranged from 28.79% to 37.12% in unsexed semen samples of four cattle breeds namely Holstein Friesian (HF), Jersey, Gir and Sahiwal. The corresponding values in semen sorted through flow cytometry technique ranged from 99.02 % to 99.99% (X) and 0.07% to 0.98% (Y) in the above four cattle breeds. Whereas the sexed semen obtained through the decapitation of Y chromosome technique showed slightly lower concentrations of both X and Y bearing spermatozoa; the X chromosome percentage ranged from 95.92 % to 97.99% and Y chromosome ranged from 2.1 % to 4.08 % across the four breeds.

A 518bp product of *PLP* gene specific to X chromosome was amplified through PCR in four cattle breeds from sexed as well as unsexed semen samples for comparison of nucleotide changes and amino acid changes if any. Similarly, a 928 bp Y chromosome specific *SRY* gene fragment was also amplified and compared for any nucleotide and amino acid changes.

For *PLP* gene, it was observed that at nucleotide position 10610, a **T/C** base substitution (intron region) was observed between reference sequence (Gen bank accession number AJ009913) and gene sequences of all (sorted plus un sorted) semen samples of the breeds tested in the current study. Interestingly, there was a double insertion (intron region) of **A** nucleotide at positions 10679 and 10680 in all the sorted and unsorted semen samples. When compared with reference sequence only *Bos taurus* breeds (both sorted and unsorted samples) showed a base substitution (intron region) **G/C** at position 10721. Entire amino acid sequence of *PLP* gene of all cattle breeds under study showed 100% similarity with the reference sequence whereas the nucleotide sequence showed 99% homology.

A 928 bp product of *SRY* gene from all the samples (sexed and un sexed) was compared for nucleotide and amino acid homology with a reference sequence (Gen bank accession number ABO39748) which showed 100% homology in sorted as well as in unsexed samples.

Sexed and unsexed semen samples were compared for their quality parameters such as concentration, motility, livability, acrosomal integrity and structural abnormalities. Unsexed semen samples were found significantly ($P<0.01$) superior with high semen concentration (19.93 ± 0.01millions/straw), semen motility (65.98% $\pm$ 0.23), acrosomal integrity (75.28% $\pm$ 0.05), live spermatozoa (67.83% $\pm$ 0.42) and less sperm abnormalities (13.82% $\pm$ 0.02) than sexed semen samples. Sexed semen samples were having low semen concentration (1.98 ± 0.00 millions/straw), semen motility (60.88% $\pm$ 0.18), acrosomal integrity (70.79% $\pm$ 0.26), live spermatozoa (63.58% $\pm$ 0.25) and more sperm abnormalities (18.12% $\pm$ 0.18). Ours is the first study to compare the quantitative and qualitative parameters of sexed and unsexed semen samples of major cattle breeds in India.

INTRODUCTION

CHAPTER I

INTRODUCTION

Semen bearing solely X chromosome or Y chromosome bearing sperm to get progenies of a desired sex i.e., either female or male, respectively with about 80-90% accuracy is known as sexed semen. Predetermining the sex has valuable impact on livestock industry because of its economic gains. In Indian dairy sector, farmers can use sexed semen for the production of replacement daughters from genetically superior cows and for the production of bulls for progeny testing from the population of elite dams. Sexed semen is now gaining popularity amongst the dairy and beef industries and has reached a point of commercialisation (Seidel *et al.,* 2009).

Sperm is sorted by identifying differences between the X and Y bearing sperms. The X-chromosome contains about 3.8% more DNA than the Y chromosome in cattle. This difference in DNA content is used to sort the X from the Y bearing sperm (Welch *et al.,* 1995). The different semen sexing techniques include flow-cytometry, identification of H-Y antigen, decapitation of Y chromosome, detection of sex specific proteins, free-flow electrophoresis, centrifugal counter current distribution, albumin. gradient (or) gradient swim down procedure, percoll density gradient method, volumetric differences and quinacrine mustard staining (Joerg *et al.,* 2004). Among several methods of semen sexing, flow cytometry-based sorting has emerged as most efficient. The technology was refined through the decades and finally sex sorting was possible at the purity of more than 90% (Carvalho *et al.,* 2009). In India, various private companies viz., BAIF, Select Sires, Genex, Accelerated Genetics, CRV, ABS Global WWS, Sandors Animal Biogenics, and Prime Genetics are producing and marketing sexed semen of various *Bos taurus* and *Bos indicus* breeds by employing different semen sorting techniques.

Most of the research in the field of semen sorting was confined to *Bos taurus* breeds. The studies in this area in *Bos indicus* were grossly neglected and need to be compared with the breeds of *Bos taurus* in terms of quality parameters, nucleotide and amino acid sequences of genes associated with X and Y chromosomes to elucidate any variation at nucleotide level. During the process of semen sexing the spermatozoa undergoes a series of processes and hence it is essential to understand whether such

process affects the quality and concentration of semen which in turn affects the conception rates.

Hence, the present study was undertaken with the following objectives.

1. Quantitative assessment of X and Y chromosome bearing spermatozoa in sexed semen produced through the different semen sorting techniques in *Bos indicus* and *Bos taurus* breeds by quantitative real time PCR.
2. Comparative sequence analysis of sex chromosome bearing genes (*PLP* and *SRY* genes) in *Bos taurus* and *Bos indicus* breeds.
3. Comparative analysis of quality parameters i.e., motility, livability, concentration, acrosomal integrity, and structural abnormalities between sexed and unsexed semen.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Semen having X or Y chromosome bearing sperm to produce progenies of a desired sex i.e., either female or male with about 80-90% accuracy is known as sexed semen.

2.1 HISTORY OF SEXED SEMEN

Gledhill (1976) first attempted to separate X and Y sperm by analytical flow cytometry. First successful separation of sperm was made in mammalian sperm (Pinkel *et al*., 1982).

Sex sorting technology was developed by the USDA (United States Department of Agriculture) researchers in Livermore, California, and Beltsville, Maryland. The technology was patented as “Beltsville Sperm sexing technology”. The commercialization of sexed semen started in United States in 2001 with a license granted to Sexing Technologies (ST), Texas. At present, ST commercially produces sex sorted semen in many countries of Europe, USA, Canada, Mexico, Brazil, China, Japan etc.

2.2 IMPORTANCE OF SEXED SEMEN

Pre determining the sex has valuable impact on livestock industry because of its economic gains. In Indian dairy sector farmers can use sexed semen for the production of replacement daughters from genetically superior cows and for the production of bulls for progeny testing. The latest breakthrough in animal reproduction is sexing of semen. Pre determination of sex in livestock offspring is in great demand and is of great importance for profitable dairy industry. Semen from many mammalian species can be sexed by flow cytometry or cell sorting machine at about 90% accuracy without damaging them (Seidel *et al*. 2009).

As per Boro *et al.* (2016) the below advantages can be seen by semen sorting.

1) To produce calves of desired sex in both dairy and beef cattle.
2) Herd replacement and herd extension can be done very quickly.
3) To ensure birth of heifers when progeny testing of young bulls is desired.

4) Combination of super-ovulation and insemination with sexed semen further increases the desired calf crop.
5) In in-vitro fertilization programmes, one dose of sexed sperms can be used to produce many embryos of desired sex.

Several technological limitations such as high cost of sex sorting machine, low sorting efficiency and speed, need of highly skilled person to operate sex sorting machines, damage to the sperm due to shear force, electrostatic charge, droplet formation, waste of approximately 50% of sperm and reduced freezing potential of the sorted sperm limit the usage of sorted semen (Mohteshamuddin 2017).

2.3 PRINCIPLE OF SEMEN SEXING TECHNOLOGY

Sperm is sorted by identifying differences between the X and Y bearing sperms. The X-chromosome contains about 3.8% more DNA than the Y chromosome in cattle. This difference in DNA content is used to sort the X from the Y bearing sperm (Welch *et al.,* 1995). The observed average X-Y sperm DNA difference in *Bos indicus* cattle was 3.73%. Whereas in Murrah and Nili- Ravi buffaloes it was 3.59% and 3.55% respectively (Lu *et al.,*2007).

Prasad *et al*. (2010) observed the following differences between the X and Y sperms (Table 2.1). The X spermatozoa has more DNA content than the Y spermatozoa. The size of the X spermatozoa is larger and motility is slower than Y spermatozoa. The H-Y antigen is present in Y spermatozoa whereas absent in X spermatozoa.

Table 2.1 Potential differences between X and Y spermatozoa.

Parameter	X spermatozoa	Y spermatozoa	Method
DNA content	More DNA	Less DNA	Flow cytometry
Size	X sperm is larger	Relatively smaller	Percoll method
Motility	X sperm is slower	Y sperm is faster	Swim up
Surface charge	Migrate to cathode fast	Migrate to cathode slow	Free flow electrophoresis
Cell surface antigen	Absence of H-Y antigen	Presence of H-Y antigen	Immunological sexing

2.3.1 Differences in the DNA Content Between X- and Y- Bearing Spermatozoa Among Different Breeds of Cattle and Buffaloes.

Prasad *et al*. (2010) observed the following differences in the DNA content between X and Y sperms among different breeds of cattle and buffaloes as shown below (Table 2.2).The DNA content in different breeds of cattle ranged from 3.55% to 4.24%. The Jersey breed has highest DNA content in X spermatozoa whereas Nili Ravi breed has lowest DNA content in X spermatozoa.

Table 2.2 Differences in the DNA content between X- and Y- bearing spermatozoa among different breeds of cattle and buffaloes.

Breed	Difference in DNA content	References
Holstein Friesian	3.98%	Garner *et al.,* 1983; Garner, 2001; 2006
Jersey	4.24%	Garner *et al.,* 1983; Garner, 2001; 2006
Angus	4.05%	Garner *et al.,* 1983; Garner, 2001; 2006
Hereford	4.03%	Garner *et al.,* 1983; Garner, 2001; 2006
Brahman	3.73%	Garner *et al.,* 1983; Garner, 2001; 2006
Murrah	3.59%	Lu *et al.,* 2007
Nili Ravi	3.55%	Lu *et al.,* 2007

2.4 METHODS OF SPERM SEXING

The different semen sexing techniques were flow- cytometry, identification of H-Y antigen, detection of sex specific proteins, free-flow electrophoresis, centrifugal counter current distribution, albumin gradient (or) gradient swim down procedure, percoll density gradient method, volumetric differences and quinacrine mustard staining (Joerg *et al.,* 2004 ; Singh *et al*., 2019).

2.4.1 Principle of Flow Cytometry

Flow cytometry was thought to be the best technique which works based on difference in DNA content between X and Y spermatozoa. X spermatozoa contains more DNA. In most of the mammals the differences in DNA content between X and Y were in the range of 3-4.2% (Johnson *et al*., 2000).

Garner (2006) reviewed the parameters of optimizing flow cytometric sexing of mammalian sperms and revealed that the DNA content of all cells for each mammalian species was highly conserved, slight but measurable DNA content

differences of sperm occur within species even among cattle breeds due to different sizes of Y-chromosomes.

Among several methods of semen sexing, flow cytometry-based sorting has emerged as most efficient. The technology is refined through the decades and finally sex sorting is possible at the purity of more than 90% (Carvalho *et al.,* 2009).

Sharpe and Evans (2009) studied on advances in flow cytometry for sperm sexing and reported that routine high-purity sorting of X- or Y-chromosome-bearing sperm can be achieved at rates up to 8000/sec for an input rate of 40,000 X- and Y-sperm/sec. Recent protocols of sexed semen straws intended for use in artificial insemination contained approximately $2x10^6$ sperm.

Bhalakiya *et al.* (2018) in their sexed semen studies observed that the flowcytometry was the effective among all other techniques which resulted in 90% desired sex.

As per Boro *et al.* (2016) the below factors influence the efficiency of flow cytometry.

1) Orientation of sperm head.
2) Angle of sperm presentation towards the excitation source, 450 is adequate.
3) Orientation of fluid in the nozzle.
4) Even with the correct orientation of cell and fluid 20-40% of live sperms are not measurable, passes directly to the waste tube.
5) Optical techniques
6) Speed of computer processor

2.4.2 Other Methods of Semen Sexing

Hoppe *et al.* (1984) reported that. reacting mouse sperm with monoclonal HY antibodies does not influence sex ratio of eggs fertilized in vitro and reported that H-Y antigen appears on the sperm surface during association with testicular constituents and is removed during epididymal transport and capacitation.

Upreti *et al.* (1988) studied on sexing of bovine spermatozoa by fractionation on a Percoll density gradient and reported that bovine spermatozoa were fractionated on Percoll density gradients into two major subpopulations of motile spermatozoa and a minor fraction containing mostly nonmotile spermatozoa with abnormal morphology, and when the subpopulations were evaluated by flow cytometry, their Y:X ratios were similar to that of an unfractionated control.

Blecher *et al.* (1999) developed a non-invasive, immunological method for sexing mammalian sperm based on the hypothesis that sex-specific proteins (SSPs) are evolutionarily more highly conserved than non-SSPs and reported that ex-specific antibodies (SSAbs) raised against the sex specific proteins (SSPs) appear to bind to sex-chromosome-specific proteins (SCSPs) on the sperm membrane and made possible a sperm-sexing procedure.

Hendriksen (1999) reviewed on possible differences between X- and Y-chromosome-bearing spermatozoa (X and Y sperm) in relation to immunological method of separation of X and Y spermatozoa and reported that X and Y sperm may differ in proteins, but it has not been confirmed by comparative studies between flow-cytometrically sorted X and Y sperm for H-Y antigen or other membrane proteins.

Ollero *et al*. (2000) studied on separation of ram spermatozoa bearing X and Y chromosome by centrifugal counter current distribution in an aqueous two-phase system and reported that by using centrifugal counter current distribution in a sensitive-charge aqueous two-phase system, they achieved the separation of a sperm population enriched in Y chromosome-bearing ram spermatozoa (75%) with a high viability rate (57%).

Van munster *et al.* (2002) studied on interferometry in flow to sort unstained X-and Y-chromosome-bearing bull spermatozoa and found earlier that the difference in volume between unstained X- and Y-chromosome-bearing sperm heads could be detected using interference microscopy in visible light and this novel technique was introduced combining interferometry with flow cytometry.

Yan *et al*. (2006) studied on influence of swim-up time on the ratio of X-and Y-bearing spermatozoa and concluded that there was no significant effect of swim-up time on the ratios of X- and Y-bearing spermatozoa using a modified swim-up procedure.

Resende *et al*. (2009) studied on separation of X-bearing bovine sperm by centrifugation in continuous percoll and optiprep density gradient effect in sperm viability and in vitro embryo production and found that sexual deviation in the Percoll density gradient was achieved without reduction of sperm viability and in vitro embryo production rates.

Sang *et al.* (2011) studied on immunological method to screen sex-specific proteins of bovine sperm and concluded that 30-kDa protein might be a sex-specific

protein in bovine X-sperm, which has the potential to be used in immunological procedures for sexing sperm.

Lucio *et al.* (2012) studied on association of the modified swim-up method with centrifugation in density gradient for the separation of X-bearing spermatozoa. Sperm viability and integrity were evaluated and quality control of centrifuged spermatozoa was performed in in vitro produced embryos. They reported that X-bearing spermatozoa can be separated by discontinuous density gradient centrifugation, modified swim-up and modified swim-up associated with discontinuous density gradient centrifugation with about 60% accuracy. Furthermore, they suggested that use of X-bearing spermatozoa was possible in IVP of embryo systems with the purpose of reducing the deviation towards males without compromising embryo development.

Hadi (2013) Studied on separation of Y-chromosome bearing Rams sperms using an albumin gradient technique and identification of embryos by PCR and reported that bovine serum albumin sexed sperms resulted in more percentage of male embryos by using one layer of BSA (8%) at 200 Ã— g (M1a) and 300Ã— g (M1b) which were 72.7% and 54.5% respectively, showing a deviation ($p<0.05$) from the 50% expected percentage for male and female embryos.

Susilawati *et al.* (2014) studied on effect of different centrifugation duration on Simmental bull sperm quality and membrane status after sexing, cooling and freezing processes and found that sexing using Percoll density-gradient centrifugation method at 850 x g for 5 min could afford 85% of X and Y sperm separation. Whereas the treatment for 7 min centrifugation had a high-level separation power as sperms were able to reach the tube base.

Wolf *et al.* (2018) determined the percentage of male and female bovine embryos produced after oocyte fertilization with Percoll density gradient centrifugation or with self-migration (swim-up) selected semen. Swim-up and continuous Percoll gradient centrifugation for 10 min showed a deviation toward males ($P = 0.044$) and females ($P = 0.015$), respectively, when compared to the 50% expected percentage of each gender. They concluded that semen selected by swim-up increased the percentage of in vitro produced male embryos, while 67.5% continuous Percoll density gradient centrifugation for 10 min increased the percentage of in vitro produced female bovine embryos.

Bhat *et al.* (2020) studied on X-sperm enrichment of bovine semen by percoll density gradient method and its effect on semen quality, sex ratio and conception rate and concluded that the volume, progressive motility, live sperms, concentration and host reactive sperms significantly decreased at stage II and stage III compared to stage I, no significant change was observed in pH and mass motility of semen at all three stages. There was non-significant effect on per cent spermatozoa with intact acrosomal membrane and fully damaged acrosomal membrane at all three stages. However, partially damaged acrosomal membrane sperms were significantly decreased at Stage II and Stage III compared to Stage I.

2.5 ECONOMICS AND GENETIC ASPECTS OF SEXED SEMEN

Abdel – Azeem *et al.* (2007) studied on genetic impact of using female sorted semen in commercial and nucleus herds and reported that the sexed semen could be used extensively both inside and outside the nucleus breeding schemes, which will have greater effects on genetic progress in dairy cattle populations.

Olynk and Wolf (2007) reported that sexed semen has been a long-anticipated tool for dairy farmers to obtain more heifer calves, but challenges exist for integrating sexed semen into commercial dairy farm reproduction programs. It was recommended that the virgin heifers were better suited to inseminate with sexed semen than lactating dairy cows. The percentage of calves expected to be born with conventional and sexed semen used was 49.2 and 90%, respectively.

Butler *et al.* (2014) studied application and cost benefit of sexed semen in pasture-based dairy production systems and reported that conception rates (CR) to 1st service with frozen-thawed sexed semen are 75% to 80% of those achieved with conventional frozen-thawed semen.

2.6 QUANTIFICATION OF X AND Y BEARING SPERMATOZOA BY RT-PCR

Welch *et al.* (1995) conducted studies on sperm sorting through flow cytometry and quantification of X and Y chromosome bearing sperms in bovines by RT PCR and reported that the quantitative flow cytometric reanalysis was preferred for routine determination of X- and Y-chromosome sperm percentage with 90% purity of *PLP* and *SRY* in given semen samples.

Checa *et al.* (2002) conducted prediction studies on X and Y chromosome content in bovine sperm DNA through capillary electrophoresis. Bull ejaculates were used for PCR amplification of a segment of the X-Y homologues amelogenin gene in order to estimate the X and Y chromosome frequencies by capillary electrophoresis. They found that an average of 50.02±2.79% X chromosome content was found with normal distribution ranging from 38.7 to 58.2%.

Deprez *et al.* (2002) studied on sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green and reported that SYBR® Green detection was used to determine DNA contamination in plasmid preparations and provides a reliable alternative to both the traditional blotting methods and expensive TaqMan protocols.

Whelan *et al.* (2003) deduced a method for absolute quantification of cDNA by real-time PCR and reported that the absolute quantification of cDNA is more accurate by a combination of extremely accurate double-stranded DNA quantification and a plasmid reference curve.

Joerg *et al.* (2004) validated the bovine sexed semen samples though real-time polymerase chain reaction (RT PCR) assay. They calculated male cell proportion by amplifying a *SRY* fragment and an autosomal gene (MSHR) fragment. It was found that the stable amplification of *SRY* for 100–1 ng of genomic DNA, which allowed detection of 1% of male cells if 100 ng of target DNA is used.

Taylor *et al.* (2005) in their studies of comparing calf sex ratio and semen sex ratio through conventional PCR in HF cattle reported that the predicted percentage of Y spermatozoa and percentage male calves showed significant correlation to each other. Also, it was stated that the PCR technology used for determining the percentage of Y spermatozoa in ejaculates was shown to be an adequate method to determine semen sex ratio.

Parati *e .al.* (2006) applied a new method of determining X and Y chromosome bearing spermatozoa in bovine semen samples using Taq Man chemistry in real time PCR. They found that there were no significant ($P > 0.05$) differences in the frequency of X- and Y-chromosome sperms between real time PCR technique and flow cytometry. The average X-chromosome bearing sperm content in unsorted sample was 51.11 ± 0.56% for ejaculate and 50.17 ± 0.58% for the commercial semen.

Lu *et al.* (2006) in their studies of determining DNA content of X and Y chromosome bearing sperms in Murrah and Nili Ravi buffaloes used flow cytometry analysis of the DNA content after staining the chromosomes with Hoechst 33342 stain. They found that the difference in fluorescence intensity, which related to the DNA content, between the X- and Y-sperm was 3.59 ± 0.11% for Murrah buffalo and 3.55 ± 0.14% for Nili-Ravi buffalo, respectively.

Leong *et al.* (2007) conducted studies on absolute quantification of gene expression in biomaterials research using real-time PCR and reported that standards were linear double-stranded DNA molecules instead of the typical gene-in-plasmid format. A qPCR could also be used to give relative quantification comparisons between samples simply by dividing the copy numbers readings of the gene of interest with that of the normalization gene.

Lu *et al.* (2007) developed a fast and reliable method for bovine sexing through amplification of the bovine high motility group (HMG) box of the sex-determining region of the Y chromosome gene (*SRY*). First time this method was applied in bovine breeding programs to facilitate manipulation of the sex ratio of offspring and also allows a quick diagnosis for the XY-bovine offspring by amplification of the HMG box of the bovine *SRY* gene.

Rath and Johnson (2008) in their studies of application and commercialisation of flow cytometrically sex sorted semen and reported that this technology was capable of producing sexed sperm at greater than 90% purity.

Resende *et al.* (2011) used density centrifugation with percoll and optiprep to concentrate the X and Y bearing sperms and produced embryos. It was reported that the percoll and optiprep centrifuged sperms resulted in 62% and 47.1% female embryos respectively.

Wang *et al.* (2011) applied both PCR and flow cytometry to determine the purity of X or Y enriched semen. Through this combined technology in determining the X, Y bearing sperms it was found that the unsorted semen contained 48% of PLP and 52% of *SRY* gene and the corresponding values in sexed semen were 92% and 8%.

Maleki *et al.* (2013) used a SYBR Green chemistry in Real-Time PCR to determine the sex ratio in bovine semen by amplifying *PLP* and *SRY* genes in bovine and reported no significant difference in unsorted semen X/Y ratio (54.7 ± 0.52% X

and 47.6 ± 060% Y), whereas, significant difference was observed in sorted semen (93.3 ± 0.08% X-Sperm and 91.4 ± 0.06% Y-Sperm).

Mukherjee *et al.* (2013) studied the absolute copy number differences of Y chromosomal genes in healthy crossbred (*Bos taurus* × *Bos indicus*) and Indicine bulls. It was found that the crossbred bulls showed higher copy number of both (*SRY*, *PLP*) the genes than in Indicine bulls.

Khamlor *et al.* (2014) in their study, amplified both the *PLP* and *SRY* genes in a single tube through multiplex real time polymerase chain reaction using X and Y chromosome-specific primers and reported that the multiplex PCR showed high amplification efficiency (97-99%) than simplex PCR even though both assays did not differ ($p>0.05$) significantly.

Shende *et al.* (2014) in their studies of molecular cloning and expression of buffalo *SRY* gene reported that the recombinant *SRY* could be used to raise monoclonal antibodies for labelling the Y chromosome bearing spermatozoa, which can be used for assessing the enrichment of Y chromosome bearing spermatozoa in sex sorted semen.

Tan *et al.* (2015) cloned and quantified the bovine spermatozoal *PLP* and *SRY* genes using SYBR® Green RT PCR for sex determination. The percentages of unsexed X- and Y- chromosome-bearing spermatozoa (1:1) did not differ much from the hypothetical value.

Awan *et al.* (2017) used modified swim up technique to separate X and Y bearing chromosome sperms and confirmed the separation by RT PCR based quantification, in Nili Ravi buffaloes. They reported that the technique yielded higher percentage of X chromosome bearing sperm fractions compared to Y chromosome bearing spermatozoa which can have future prospects for its implication in the dairy industry.

Kumari *et al.* (2019) carried out studies on SYBR green chemistry based real time PCR for validation of sperm sex ratio in buffalo bull semen and reported that the difference in X and Y chromosome bearing sperm ratio (sex ratio) in unsorted semen was not significant (51.7±0.52 % X and 49.6±0.60% Y).

2.7 COMPARATIVE SEQUENCE ANALYSIS OF *PLP* AND *SRY* GENES BETWEEN *BOS TAURUS* AND *BOS INDICUS* BREEDS

Gou *et al.* (2010) in their sequence analysis studies of mitochondrial DNA and a 1305 bp *SRY* gene of Y chromosome identified two mutations at sites 748 and 1100 bp in *Bos taurus* and *Bos indicus*, respectively in *SRY* gene.

Shende *et al.* (2014) in their study on molecular cloning and expression of buffalo *SRY* gene, concluded that the HMG-box of SRY protein had shown 6 amino acid substitutions as compared to the *Bos taurus* and these would probably make buffalo SRY functionally more active compared to its closely related species.

Hartaik *et al.* (2014) analysed the PCR RFLP Polymorphism in *SRY* Gene of Madura cattle and reported different type of Y chromosome in crossbred cattle indicating the contribution of *Bos taurus* gene through the paternal line.

Hartaik *et al.* (2018) studied SNP polymorphism in promoter region of SRY gene in native and crossbreed cattle and reported that five SNPs were found in the promoter region of the *SRY* gene. Bali cattle showed three SNPs (-966 C/G, -907 T/deletion, -402 C/T), while two SNPs were found in Nellore cattle (-140 G/A dan -117 G/A). However, Wagyu-BX, BX, Simmental, Limousin, PO, Madura, and Hereford cattle showed complete homology with respect to promoter region of the *SRY* gene.

Prihatin *et al.* (2018) studied the genetic variation in the *SRY* gene of Madhura cattle through PCR and sequencing and reported that Madura cattle *SRY* showed close resemblance to *Bos indicus*. The variations observed in the *SRY* gene were due to mutations and deletions of nucleotides.

2.8 QUALITY PARAMETERS OF SEXED AND UNSEXED SEMEN

During the process of semen sexing, the spermatozoa undergoes a series of processes and hence it is essential to understand whether such process effects the quality of semen. As sperm concentration in sexed semen straw is far less than the conventional semen straw and the sorting procedure itself damages the sexed sperm, it was reported that the conception rate was 10 to 15 % less with sexed semen as compared to conventional semen. When sperm concentration in sexed sperm was 2×10^6 per insemination, the conception rate was 56% and when the sperm

concentration in unsexed sperm was 10-20x10^6 per insemination, the conception rate was 61% (Garner and seidel, 2003).

Brito *et al*. (2002) studied effect of environmental factors, age and genotype on sperm production and semen quality in *Bos indicus* and *Bos taurus* AI bulls in Brazil and reported that neither ambient temperature and humidity nor month (season) significantly affected sperm production or semen quality. However, *Bos indicus* bulls had significantly higher sperm concentration compared to *Bos taurus* bulls with fewer defective spermatozoa.

Beletti *et al.* (2005) investigated the sperm morphometric characteristics in terms of head area, perimeter, width, length, width-length ratio, ellipticity, shape factor, width of sperm base, symmetry and hydrodynamics of fertile *Bos taurus* and *Bos indicus* bulls in Brazil by using several traditional and advanced computer vision concepts and methodologies. It was observed that the sperm cells of Zebu bulls tend to be smaller and less elliptic. These differences clearly indicate that the geometrical characterization of bull sperm cells should take into account morphological peculiarities that are specific to each subspecies.

Hoflack *et al.* (2006) studied on comparison of sperm quality of Belgian Blue (BB) and Holstein Friesian bulls and found that the BB semen differs from HF semen in terms of lower percentage of live and normal spermatozoa. This lower percentage of live spermatozoa influence the percentage of totally and progressively motile spermatozoa.

Nichi *et al.* (2006) conducted studies on seasonal variation in semen quality in *Bos indicus* and *Bos taurus* bulls raised under tropical conditions and revealed that *Bos taurus* bulls showed greater reactive oxygen species (ROS) and lower activity of antioxidant enzymes in their semen than *Bos indicus* bulls. It was also observed that the summer season had significantly higher (20.3±3.1%) percentages of major sperm defects than winter (12.2 ± 2.4%).

Barros *et al.* (2007) carried out the experiments to evaluate membrane integrity, vitality, and mitochondrial cytochemical activity, in frozen semen samples of buffalo bulls and compared it with the field fertility. It was observed that there was no correlation between pregnancy rate and motility or vigour.

Blondin *et al.* (2009) characterized the bovine semen parameters through CASA and flow cytometry of four types (fresh and frozen non-sexed semen, fresh and frozen sexed semen to determine the best IVF conditions to produce a maximal

percentage of blastocysts. It was found that the sexed semen resulted in production of fewer blastocysts than non-sexed semen ($P < 0.05$). Freezing and not sexing had a significant negative effect on semen quality.

Carvadho *et al*. (2009) compared the kinetics of cryopreserved non sexed and sexed for X, Y sperm cells before and after selection using Percoll gradient. The samples were analysed for motility by CASA subjected to Percoll (45:60%) gradient selection. It was observed that the sperm motility was higher in the non-sexed group than in the sexed groups.

Fiaz *et al.* (2010) examined the effect of seasons on quality and quantity of semen of Holstein-Friesian and Jersey bulls. It is found that the number of ejaculates per bull recorded during three seasons were not significantly different ($P>0.05$) in both breeds. However Friesian bulls produced lower ejaculatory volume during dry summer season, whereas Jersey bulls produced higher ($P<0.05$) volume during wet summer when compared to other seasons. Seasonal pattern of mass motility and individual motility of semen was differed between two breeds.

Koivisto *et al.* (2009) carried out studies to evaluate the effects of season on semen characteristics in terms of sperm concentration, gross- motility, progressive motility and sperm morphology, freezability and peripheral plasma concentrations of testosterone. Vigour and morphological sperm defects were significantly influenced by season and genotype ($p < 0.05$). Heat tolerance was better in *Bos indicus* bulls than in *Bos taurus* bulls characterized by lower values of sperm abnormalities. The highest values were recorded for abnormal heads followed by cytoplasmatic droplets in *B. taurus* bulls.

Carvadho *et al.* (2010) assessed the quality of bovine cryopreserved sexed semen for use in in *vitro* embryo production. It was found that the unsexed sperm had better ($P<0.05$) quality in terms of motility and greater percentages of cells with an intact membrane and acrosome (58.0 ±3.0, 58.2 ± 3.0, and 60.9 ± 3.3) than sexed X (29.6± 1.3, 36.0±2.9, and 37.1 ±3.3), and sexed Y (26.2 ±2.1, 36.4±2.9, and 37.5±3.3).

Bhakath *et al.* (2011) studied the effects of age and season on semen quality in Sahiwal bulls and reported that the bulls produced best semen during rainy season followed by summer and winter, probably due to variation in quality of feeding, ambient temperature, and humidity. Also reported that semen quality of first ejaculates was superior as compared to second ejaculate.

Sohail *et al*. (2013) in their studies on effect of sperm motility parameter (%; motile, progressive, rapid and medium) and motion characteristics (path velocity = VAP, µm/sec; progressive velocity = VSL, µm/sec; curvilinear velocity = VCL, µm/sec; amplitude of lateral head = ALH, µm and beat cross frequency = BCF, Hz) on invitro fertilization in five buffalo bulls semen and concluded that at post thaw , bull 2 showed the highest sperm progressive and rapid motility parameters, and motion characteristics (VAP, VSL and VCL) than other four bulls, but the conception rate was similar.

Joydip *et al.* (2013) reported in Sahiwal sex sorted semen that some modification of cell sorter may increase the purity of sorted sperm at high speed in flow cytometry sexing by reducing the damage of the sperm and improve post thaw motility, hypo-osmotic swelling response and acrosome integrity of sorted sperm.

Goncharenko *et al.* (2016) compared the sexed and unsexed semen of Holstein bulls and observed that the motility and survivability of the sexed semen were reduced by 15-20%.

Kumar *et al.* (2016) in their review on sexed semen technology in cattle reported that during flow cytometry sorting, sperm under goes several physical stresses such as passing through nozzle with pressure, dyeing of DNA, passing through ultraviolet laser beam, electrostatic separation and centrifugation which leads to alteration of membrane and other changes like pre-capacitation in the sorted sperm leading to decrease in fertility.

Sonar *et al.* (2016) compared the characteristics of unfrozen and frozen semen in Gir bulls and reported that the freezing significantly lowered (69.10 ± 0.75 vs 53.81 ± 0.61) the progressive sperm motility, intact acrosome (84.42 ± 0.77 vs 75.69 ± 1.10), HOST (60.12 ± 1.19 vs 55.71 ±1.33) and CMPT (31.32 ± 0.70 vs 27.97 ±0.72) in comparison to fresh un frozen semen.

Prihatin *et al.* (2017) studied the effects of genetic and environmental factors such as age, seasons, interval of semen collections and frequency of ejaculations on semen production and its quality in local Madura cattle breeds (*Bos indicus*) and reported that the age and ejaculation frequency affected all variables, interval of semen collections affected to both semen volume and sperm concentrations; while seasons only affected to sperm concentrations. Repeatability estimation of semen

volume, sperm concentration and sperm individual motility were 0.376, 0.445 and 0.567 respectively.

Gopinathan *et al.* (2018a) studied the effects of non-genetic factors on semen quality traits of crossbred bulls (*Bos taurus* X *Bos indicus*) in organized farm conditions and reported that the semen volume per ejaculate increased with age of the bull and exhibited negative correlation with sperm concentration over the ages. It was found that the first ejaculates showed better sperm concentration, mass activity and number of doses per ejaculate whereas the second ejaculate had higher initial semen motility and post thaw motility. No seasonal influence was observed on semen quality parameters except semen concentration and initial sperm motility.

Gopinathan *et al.* (2018b) in their studies of influence of non-genetic factors on semen quality parameters in crossbred Jersey bulls observed that the effects of farm, ejaculate, period and age of the bulls were significant ($P<0.05$) on all semen quality parameters. Season had significant ($P<0.05$) effect on sperm concentration, mass activity and initial sperm motility.

Saffari *et al.* (2018) investigated how individual differences and season affect the proportion of sperms containing Y-chromosomes to sperms containing X-chromosomes in bull semen. The results showed that the sex ratio of sperms varied among different bulls. Moreover, season affected primary sex ratio that Y-bearing sperm increase in summer.

Isnaini *et al.* (2019) studied effects of different seasons on semen quality of Ongole crossbred and Simmental bulls and reported that the Simmental bull was superior in terms of semen concentration and the number of approved fresh semen than Ongole crossbred bulls.

Bhave *et al.* (2020) studied on genetic and non-genetic factors affecting semen production and quality characteristics of Gir cattle breed under semi-arid climate and reported that the seasonal variations in semen parameters were due to the influence of climatic condition during the sensitive stages of spermatogenesis.

MATERIALS AND METHODS

CHAPTER-III

MATERIALS AND METHODS

3.1 GENERAL LABORATORY MATERIALS

Molecular grade reagents were used for the preparation of all solutions and buffers. All the reagents used in the study were availed from Bangalore GeNei, Himedia (Mumbai), Merck (Mumbai), Sigma Aldrich (Hyderabad) and Invitrogen (USA). All aqueous solutions were prepared using double glass distilled water. Where necessary, solutions were sterilized by autoclaving at 121ºC, 15 lbs. pressure for 15 min. or by membrane filtration using 0.22 μm membrane filter. (Millipore Corporation, Bedford, MA, USA).

3.1.1 Labware

Neutral glassware of Borosil and Schott Duran were used in the study. Petri plates for bacterial cultures were procured from Galaxy. Centrifuge tubes (15 ml), microfuge tubes (2 ml, 1.5 ml), PCR tubes (0.2 ml) and micropipette tips (1 ml, 200 μl, 10 μl) procured from Axygen and Tarsons were used in the study. MicroAmp® Fast 96-Well Reaction Plate (0.1 ml) (Part No.4346907) from Applied Biosystems and thermal seal RTTM (TSRT2100) from Excel Scientific were used in molecular study.

3.2 STERILIZATION OF LAB WARE

Labware were scrubbed with neutral detergent (Labolene) and washed thoroughly under running tap water till all traces of soap were removed. Later, they were soaked and rinsed thrice with distilled water. Finally, they were rinsed with double distilled water and kept inverted for air drying. Dried glassware was packed and sterilized in hot air oven at 160°C for one and a half hour. Dried plastic ware like bottle screw caps, filter assembly along with filter membrane were packed and sterilized by autoclaving at 121°C/15 psi/15 min.

Micropipette tips, microfuge tubes and PCR tubes were sterilized by autoclaving at 121°C/ 15 psi/ 15 min. Decontaminated thoroughly using 70% ethyl alcohol and UV light, wherever necessary.

3.3 CHEMICALS AND REAGENTS

Molecular grade reagents were used for the preparation of all solutions and buffers. The reagents and labware were availed from suppliers including Merck (Mumbai), Qualigens, Sigma Aldrich (Hyderabad) and Fischer scientific Chemicals used for molecular work in the present study were of molecular grade from TaKaRa, HiMedia, New England BioLabs, Sigma (USA) and Life Technologies.

All aqueous solutions were prepared using double distilled water. Where necessary, solutions were sterilized by autoclaving at 121 ºC, 15 psi for 15 min. or by membrane filtration using 0.22 μm membrane filter (Catalogue No. GVWP04700; Millipore).

3.4 DNA ISOLATION PROTOCOL

DNA was extracted from the unsexed and sexed frozen semen straws of *Bos indicus* breeds (Gir, Sahiwal) and *Bos taurus* breeds (HF, Jersey) according to the protocol described by Anvar *et al.* (2015) with slight modifications.

Steps in DNA isolation

1) 0.5ml of semen was taken into a 1.5 ml eppendorf.
2) It was centrifuged @ 8000rpm for 5 minutes and the supernatant was discarded.
3) 500 μl of lysis buffer, 2.5 μl of triton X -100, 21 μl of DTT and 10 μl of proteinase-K (20mg/ml) was added to the pellet and mixed well and kept overnight at 50ºC in water bath.
4) Centrifuged @ 12000rpm for 3 minutes.
5) The supernatant was collected into a separate 1.5 ml eppendorf and equal amount of phenol: chloroform: isoamyl alcohol (25:24:1, pH.8.0) was added and incubated on ice for 15-20 minutes.
6) Centrifuged @ 12000rpm for 10 minutes and the upper aqueous phase was collected into a separate eppendorf.
7) Equal volume of isopropanol, was added and mixed well and incubated at -20ºC for overnight.
8) Centrifuged @ 12000rpm for 10 minutes and the supernatant was discarded.
9) 1ml of 70% ethanol was added to the pellet and mixed well.
10) Centrifuged @ 12000rpm for 10 minutes and supernatant was discarded.

11) 20 µl of NFW or TE buffer was added to the pellet and stored at - 20ºC for future use.

3.5 QUANTIFICATION AND QUALITY ASSESSMENT OF DNA

DNA was quantified by Nano Drop TM Lite (Thermo Fisher Scientific). For nucleic acid quantification, the Beer- Lambert equation is modified to use a conversion factor with units of ng-cm/µl. the modified equation for nucleic acid calculations is the following:

$C = (A*CF)/l$

Where,

C = the nucleic acid concentration in ng/µl

A = the sample absorbance

CF = the conversion factor in ng-cm/µl

l = the path length in cm

The U. V absorbance was analyzed at 260 and 280 nm wavelength for determination of sample concentration and purity. Quality of DNA was judged on the basis of O.D. ratio at 260:280. The DNA having ratio in a range of 1.8 to 2.0 was considered to be of good quality.

3.6 CLONING OF *PLP* AND *SRY* GENES

A 150 bp *PLP* and 66bp *SRY* gene were amplified through PCR and cloned into T-vector and transformed into the DH5α E. coli competent cells. PCR was carried out using Emerald Amp® GT PCR master mix (TaKaRa) with *PLP* and *SRY* primer set (Table 3.1), in Prima-Duo PCR system (instrument) from Himedia. Primers were reconstituted with nuclease free water to obtain 100 pmol/µl stocks. Stocks were made into 10 pmol/µl working solution and used for PCR.

3.6.1 PCR Amplification of *PLP* Gene (150bp)

A set of primers mentioned in Table.3.1 were used for amplification of *PLP* (150 bp) gene. The PCR reaction mix for *PLP* gene (150 bp) is shown below.

S. No	Name of reagent	Quantity in (µl)
1	Nuclease free water	11.6
2	Master mix	15.0
3	Forward primer (10µM)	1.20
4	Reverse primer (10µM)	1.20
5	Template	1.00
Total		**30 µl**

The PCR cycling conditions for *PLP* gene (150bp) are shown below.

S. No	Condition	Temperature (°C)	Time	Cycles
1	Initial Denaturation	95	10 min.	1
2	Denaturation	95	15 Sec	35
3	Annealing	58	15 Sec	
4	Extension	72	30 Sec	
5	Final extension	72	10 min.	1
6	Idle temp after completion of reaction	4	∞	1

Table 3.1 Primers used for Real-Time PCR (Parati *et al.* 2006, Maleki *et al.* 2013).

S.No	Primer name	Primer sequence '5 – 3'	Nucleotide position	Fragment amplified
1	PLP - FP	TAAAGGTGGGTAGGGTCAAGG	1120 to 1140	150bp
	PLP - RP	GATGTGGCAGCATAAGTGTCG	1249 to 1269	
2	SRY – FP	CCACGTCAAGCGACCCAT	1196 to 1213	66bp
	SRY - RP	AGAGCCACCTTTCGTCTTCG	1242 to 1261	

3.6.2 PCR Amplification of *SRY* Gene (66bp)

A set of primers mentioned in Table.3.1 were used for amplification of *SRY* (66bp) gene. The PCR reaction mix for *SRY* gene (66bp) was as shown below.

S. No	Name of reagent	Quantity in (µl)
1	Nuclease free water	11.6
2	Master mix	15.0
3	Forward primer (10µM)	1.20
4	Reverse primer (10µM)	1.20
5	Template	1.00
Total		**30 µl**

The PCR cycling conditions for *SRY* gene (66bp) were as shown below.

S. No	Condition	Temperature (°C)	Time	Cycles
1	Initial denaturation	95	10 min.	1
2	Denaturation	95	30 Sec	35
3	Annealing	60	30 Sec	
4	Extension	72	30 Sec	
5	Final extension	72	5 min.	1
6	Idle temp after completion of Reaction	4	∞	1

1) The reagents were allowed to thaw completely. Then were mixed gently and spun briefly. The PCR master mix was prepared by scaling up based on the volumes listed above to the desired number of PCR reactions.
2) Using the template DNA, PCR mixture was prepared.
3) The above-mentioned contents were transferred to a 200µl tube on ice.
4) The PCR mixture was mixed thoroughly by using pipette and was spinned briefly.
5) The tube was kept in a thermal cycler for amplification at above specified Cycling conditions.
6) The amplified PCR product of *PLP* and *SRY* gene were confirmed through 2% agarose gel electrophoresis with DNA ladder.

3.6.3 DNA Ligation

3.6.3.1 DNA ligation reaction mix for *PLP* gene (150bp): Shown below.

S. No	Name of reagent	Quantity in (µl)
1	Nucleus free water	6.0
2	5 X buffer	4.0
3	PCR product of *PLP* gene (150bp)	8.0
4	T-vector	1.0
5	T_4 Enzyme	1.0
Total		**20 µl**

The above mixture was incubated at 16° C in thermal cycler overnight.

3.6.3.2 DNA ligation reaction mix for *SRY* gene (66bp): Shown below.

S. No	Name of reagent	Quantity in (µl)
1	Nuclease free water	6.0
2	5 X buffer	4.0
3	PCR product of *SRY* gene (66bp)	8.0
4	T-vector	1.0
5	T_4 Enzyme	1.0
Total		**20 µl**

The above mixture was be incubated at 16°C in thermal cycler overnight.

3.6.4 Preparation of DH5α Competent Cells

1. From the stock culture plate of *E. coli* (DH5α strain), a single colony was picked and streaked on LB agar plate and incubated overnight at 37°C.
2. At the end of next day, a single colony was picked, added to a 5 ml LB broth and was kept in horizontal shaker incubator for overnight at 37°C, 250 rpm.
3. One ml of overnight culture was added to 250 ml flask containing 99 ml of LB

media and allowed to grow at 250 rpm and 37°C till the OD600 reached 0.35-0.4.

4. Once it reached the required OD value, it was immediately chilled on ice for 5-10 minutes and an intermittent swirling was done in order to ensure even cooling.
5. This was further transferred into a sterile disposable ice-cold 50 ml tube and was kept at 4°C for 10 minutes
6. Cells were then harvested by process of centrifugation at 5000 rpm/ 4°C/15 min.
7. The supernatant was discarded leaving the cell pellet. The pellet was resuspended in 0.1 M $CaCl_2$ by gentle pipetting in ice-cold sterile 50 ml tube and incubated on ice for 1 h 30 minutes
8. Following incubation, the cell suspension was spinned at 5000 rpm, 4°C for 15 minutes and the supernatant was discarded.
9. The remaining cells were then resuspended in 0.1 M $CaCl_2$ in a tube containing 10% glycerol by pipetting gently.
10. Finally, aliquots of 200 µl of above mixture were poured into each pre-chilled sterile 1.5 ml tubes and stored at -80°C until further use.

3.6.5 Transformation into Competent Cells

The procedure of transformation of ligated product (*PLP* and *SRY*) into the competent cells was as follows:

1. Using pre-chilled pipette tips, the overnight ligated product (5 µl) was added to a vial having suspension of freshly thawed 200 µl of competent cells.
2. The vial was then mixed gently by swirling and incubated on ice for 30 minutes
3. Following incubation they were subjected to heat-shock at 42˚C for 1 min. 30 seconds and snap cooled immediately on ice.
4. To each vial, about 200 µl of LB broth medium was added at room temperature under laminar air flow.
5. Vials were tightly capped and incubated at 37˚C for 1 h @ 200 rpm in horizontal shaker.
6. This resulted mixture (200 µl) was spread on LB agar plates containing antibiotic ampicillin (100 mg/ml) 20 µl, IPTG (100Mm) 100 µl, X-gal (2%) 100 µl and was incubated overnight at 37˚C.
7. Next day, about 5-10 ampicillin-resistant white recombinant colonies were picked with the help of sterile pipette tip and dipped into tube containing LB broth with 50 µg/µl of ampicillin and cultured overnight (37˚C/150 rpm).

3.6.6 Screening for Positive Clones

The broth containing grown culture was checked for the presence of desired product by colony PCR and plasmid PCR using gene specific primers and was also confirmed by the sequencing. The positive clones were sub cultured for plasmid isolation and stored @ -70˚C with 15% glycerol for further use.

3.6.7 Colony PCR

Gene specific PCR was kept for *PLP* and *SRY* gene fragment using 2 µl broth culture used as template to amplification of genes and after conformation of product the culture were allowed to grow over night at 37˚C in LB broth containing ampicillin (50µg/ml) for plasmid isolation.

E. coli containing *PLP* and *SRY* were kept as stock for multiplication in Luria-Bertani (LB) media added with 15% of glycerol.

3.6.8 Plasmid Isolation

QIAprep® Spin Mini prep kit (50) (With Cat.No.27104, Lot No.148030228) was used to isolate plasmid DNA from the positive culture.

1. The bacterial culture was harvested by centrifugation (13000 g/2 min./ 37˚C) in 2 ml of micro centrifuge tube and the supernatant was discarded leaving the cell pellet undisturbed.
2. To this pelleted cell, about 250 µl of buffer P_1 was added, vortexed till no cell clumps were observed and incubated for five minutes at room temperature.
3. 250 µl buffer P_2 was added to the tube, mixed thoroughly till they become slightly clear viscous and incubated on ice for 5 minutes
4. Subsequently, 350 µl of buffer N_3 was added and mixed immediately by inverting the tube 4-6 times till the bacterial lysate became cloudy. This tube was then incubated for 5-10 min. on ice and was centrifuged (13000 g/ 15 minutes) in order to pellet the cell debris and chromosomal DNA.
5. 800 µl of top supernatant was transferred carefully to a fresh QIA prep 2.0 spin column by pipetting without disturbing the white precipitate and centrifuged @ (13000g/2 min.) and washout the flowthrough.
6. Washed the QIA prep 2.0 spin column by adding 750 µl of buffer PE and centrifuge @13000g/2 min. and washed out the flow through.

7. Transferred the QIA Prep 2.0 spin column to the collection tube and centrifuged @13000g/ 2 min. to remove wash buffer.
8. Placed the QIA Prep 2.0 spin column in a 1.5ml of fresh Eppendorf and added 50 µl of buffer EB (Pre warmed for 56 °C) at the center of the spin column and incubated for 1 min. Centrifuge @13000g/ 2 min. and collected the plasmid DNA in Eppendorf tube.
9. The resulted plasmid DNA was loaded, checked on 1% agarose gel and stored further in -20°C till use.

PCR amplification was carried out using gene (*PLP* and *SRY*) specific primers for the presence of recombinant clone by taking plasmid DNA as template along with a negative control (no template) and a positive control (genomic DNA). The amplified PCR product (5µl) was checked on 1% agarose gel and the fragment size was determined accordingly.

3.6.9 Generation of Plasmid Dilutions for Use in Real Time PCR

***PLP* gene:**

Mol wt: 892405.54Da

Mass: 256ng/µl

Calculation: Required Plasmid Concentration= $\frac{10^{10} \text{ x } (MW*2) \text{ x}10^{9}}{6.023 \text{ x } 10^{23}}$ = 28 ng/µl

***SRY* gene:**

Mol wt: 867352.32Da

Mass: 150.2ng/µl

Calculation: Required Plasmid Concentration= $\frac{10^{10} \text{ x } (MW*2) \text{ x } 10^{9}}{6.023\text{x}10^{23}}$ = 28 ng/µl

Therefore, a) 28ng/µl of SRY plasmid=10^{10} copies/µl

b) 28ng/µl of PLP plasmid= 10^{10} copies/µl

3.7 REAL-TIME PCR

Real-time PCR was carried out using SYBR® Premix Ex Taq (Catalogue No. RR420; TaKaRa) with *PLP* and *SRY* primer set (Table 3.1), in Step One Plus Real-time PCR system (instrument) from Applied Biosystems. Primers were reconstituted with nuclease free water to obtain 100 pmol/µl stocks. Stocks were made into 10

pmol/µl working solution and used for PCR. Real-time PCR was standardized for the primer set of *PLP* gene (150 bp) and *SRY* gene (66bp) as reported by Maleki *et al.* (2013) and Parati *et al.* (2006), respectively.

SYBR® Premix Ex Taq (2X concentration) contains SYBR® Green I, TaKaRa Ex Taq HS, Mg2+, Tli RNase H, dNTPs and Optimized buffer components, ROX reference dye. The SYBR Green dye binds to DNA, thus providing a fluorescent signal that reflects the amount of ds DNA product generated during PCR.

Real time PCR reaction mix was prepared as below:

1) The SYBR® Premix Ex Taq and the working solution of primer set were allowed to thaw completely. Then were mixed gently and spun briefly.
2) PCR master mix was prepared by scaling the volumes listed below to the desired number of PCR reactions.

Real Time PCR Reaction Mix for *PLP* Gene (150bp) was as shown below.

S. No	Name of reagent	Quantity in (µl)
1	Nuclease free water	6.8
2	Syber green master mix	10
3	Rox	0.4
3	Forward primer (10µM)	0.4
4	Reverse primer (10µM)	0.4
5	DNA template	2.0
Total		**20 µl**

Real Time PCR Reaction Mix for *SRY* Gene (66bp) was as shown below.

S. No	Name of reagent	Quantity in (µl)
1	Nuclease free water	6.8
2	Syber green master mix	10
3	Rox	0.4
3	Forward primer (10µM)	0.4
4	Reverse primer (10µM)	0.4
5	DNA template	2.0
Total		**20 µl**

3) The PCR mixture was mixed gently and spin briefly.

4) Then PCR reaction plate was prepared by distributing 18 µl of PCR mixture into each well of MicroAmp® Fast 96-well reaction plate, to that 2 µl of template DNA was added, from this a duplicate reaction made in below well.
5) The loaded reaction plate was sealed with thermal seal RTTM sealing foil and was centrifuged for two min. at 1500×g by placing the multi well plate in standard swing bucket centrifuge containing a rotor with a suitable adaptor for multi well plate and balancing it with a suitable counter weight (e.g., another multi well plate).
6) The multi well plate was transferred into the plate holder of the Step One Plus Real-time PCR system thermal cycling block. Then the programmed plate-document was run according to following cycling conditions for amplification and melt curve generation.

Cycling Conditions of Real-Time PCR were as shown below.

<table>
<tr><th>Enzyme activation</th><th colspan="3">PCR</th><th colspan="3" rowspan="3">Melt curve stage</th></tr>
<tr><td rowspan="2">Hold</td><td colspan="3">Cycles (40 cycles)</td></tr>
<tr><td>Denaturation</td><td>Annealing</td><td>Extension</td></tr>
<tr><td>10 min.</td><td>10 sec</td><td>15 sec</td><td>40 sec</td><td>15 sec</td><td>1 min.</td><td>15 sec</td></tr>
<tr><td>95ºC</td><td>95ºC</td><td>58ºC</td><td>72ºC</td><td>95ºC</td><td>50ºC</td><td>95ºC</td></tr>
</table>

3.7.2 Assay Optimisation and Syber® Green Melting Curve Analysis

Suitability of primers for their uniqueness to amplify a single PCR product in conventional thermal cycler was assured. qPCR was carried out on standards using a real time thermal cycler (Applied Bio System 2.3). Melting curve analysis was carried out after the final amplification. A confirmation step using 2% agarose gel electrophoresis in TAE buffer confirmed the appropriate fragment sizes of all real time assay products.

3.7.3 Standard Curves

The concentration of plasmids carrying the *PLP* and *SRY* genes was measured using Nanodrop ND-1000 spectrophotometer. Using the average molecular weight of the product and mathematical calculations in accordance to the method by Parati *et.*

al. (2006) and Applied Biosystems, the number of copies per unit volume was calculated. The volume of the purified linear double stranded *PLP* and *SRY* plasmid DNA standards was adjusted to 1.023 x 10^{10} copies. This stock solution was serially diluted by 10 folds to obtain a standard series from 1.023 x10^{4} to 1.023 x 10^{10} copies. A corresponding standard was prepared under the same conditions using different copy numbers in triplicates.

The optimum real-time program was shown above. The C_T values were plotted against the logarithm of their initial template copy numbers under the optimum threshold based on the highest regression coefficient, r^2. Each standard curve was generated by a linear regression of the plotted points.

The spermatozoa DNA with various concentrations of plasmid copy number were used as a DNA template. The qPCR step was carried out together with triplicate of standard dilution to create standard curve. PCR conditions were described above. The absolute copy of X- and Y- chromosome bearing spermatozoa was obtained by comparing the C_T value in the standard curves. The percentages of X- and Y-chromosome bearing spermatozoa were obtained.

3.7.4 Data Analysis

A standard curve of 1.023 x 10^{4} to 1.023 x 10^{10} serial dilutions of plasmid molecules was obtained and compared to the samples in order to get absolute copy numbers of X- and Y- chromosome bearing bovine spermatozoa.

3.8 POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction was carried out with Emerald Amp® GT PCR master mix and primers (IDT-DNA) specific *(*Table 3.2*)* for *PLP* and *SRY* Genes. All the primers were obtained in lyophilized form. The primer sets were obtained from Bioserve Biotechnologies India Pvt Ltd. Primers were reconstituted with nuclease free water (Himedia) to get 100pmol/µl stock solution. Stock solutions were further diluted to 10pmol/µl working solution and used for PCR. PCR was standardized for the primer set of *PLP* and *SRY.*

PCR amplification of *PLP* and *SRY* genes was carried out using genomic DNA *PLP* and *SRY* specific primers on Himedia thermal cycler. The reaction mixture contained 10 ng template DNA, in a final volume of 30 µl. The amplification reactions were performed using the conditions listed above. The PCR products were

resolved by 1% agarose gel electrophoresis and visualized under UV illumination. The size of the products was determined by comparison with 100 bp molecular weight marker as a DNA ladder. PCR was standardized for the primer set of *PLP* gene (518bp) as reported by Parati *et al*. (2006) and *SRY* gene (928bp) as reported by Hartatik *et al.* (2014).

Table 3.2. **Primers used for conventional PCR** (Parati *et al*. 2006, Hartatik *et al.* 2014).

S.No	Primer name	Primer sequence '5 – 3'	Nucleotide position	Fragment amplified
1	PLP - FP	GGGCTGGCAGAGAGAGATGAG	10289 to 10309	518bp
	PLP - RP	GATGGCAGGTGAGGGTAGGA	10787 to 10806	
2	SRY – FP	GTT GAT GGG TTT GGG CTGACT	0871 to 0891	928bp
	SRY - RP	AAA TTG AGA TAA AGA GCG CCT	1778 to 1798	

3.8.1 PCR Reaction Setup

1) The reagents were allowed to thaw completely. Then were mixed gently and spun briefly. The PCR reaction mix was prepared by scaling up based on the volumes listed below to the desired number of PCR reactions.
2) Using the template DNA, PCR mixture was prepared as following.

PCR reaction mix for *SRY* gene (928 bp) was as shown below.

S. No	Name of reagent	Quantity in (µl)
1	Nuclease free water	11.6
2	Master mix	15.0
3	Forward primer (10 µM)	1.20
4	Reverse primer (10 µM)	1.20
5	Template	1.00
Total		**30 µl**

PCR reaction mix for *PLP* gene (518 bp) was as shown below.

S. No	Name of reagent	Quantity in (µl)
1	Nuclease free water	11.6
2	Master mix	15.0
3	Forward primer (10 uM)	1.20
4	Reverse primer (10 uM)	1.20
5	Template	1.00
Total		**30 µl**

3) The above-mentioned contents were transferred to a 200µl tube on ice. The PCR mixture was mixed thoroughly by using pipette and was spinned briefly.

4) The tube was placed in a thermal cycler and the following cycling conditions for *PLP* and *SRY* genes.

PCR cycling conditions for *SRY* gene (928bp) were as shown below.

S. No	Condition	Temperature (°C)	Time (Min.)
1	Initial denaturation	94	5 min.
2	Denaturation	92	1 min.
3	Annealing	60	1 min.
4	Extension	72	1 min. 10 sec
5	Cycles	35	
6	Final extension	72	7 min.
7	Idle temp after completion of reaction	4	∞

PCR cycling conditions for *PLP* gene (518bp) were as shown below.

S. No	Condition	Temperature (°C)	Time
1	Initial denaturation	94	5 min.
2	Denaturation	94	1 min.
3	Annealing	61	45 Sec
4	Extension	72	45 Sec
5	Cycles	35	
6	Final extension	72	10 min.
7	Idle temp after completion of reaction	4	∞

5) Agarose gel electrophoresis was carried out for PCR products of *PLP* and *SRY* genes, and was confirmed by comparison of migration distances with 100 bp DNA ladder (Cat.No.3422A; TaKaRa) for their sizes using gel documentation system.

3.9 AGAROSE GEL ELECTROPHORESIS

1) 1 g of Agarose (MB002-500G; HiMedia) powder was dissolved in 100 ml TAE buffer and heated in a microwave oven till melted completely.
2) After letting it cool down to 50ºC, ethidium bromide (E8751-10G; Sigma-Aldrich) was added to a final concentration of 0.5 µg/ml. The content was swirled gently to mix the reagents.
3) The molten agarose was poured into a casting tray and after positioning the required comb, it was allowed to solidify for at least 20 min.
4) The comb was removed gently and the gel tray was positioned in the electrophoresis tank and the buffer was added so that the gel was just submerged within the buffer.
5) The 5 µl of PCR product was mixed with 1 µl of gel loading dye, Purple (6X) (Cat. No. B7025S; New England BioLabs) and 100 bp DNA ladder (Cat.No.3422A; TaKaRa) were loaded in wells.
6) Electrophoresis was carried out at 80V for 1 hr.
7) The gel was transferred to gel documentation system (Gene flash, Syngene bio imaging) having UV trans-illuminator and observed under medium wavelength for the desired bands.

3.10 PARTIAL GENE SEQUENCING OF *SRY* AND *PLP* GENES

3.10.1 PCR for Sequencing

PCR was carried out using Emerald Amp® GT PCR master mix (Catalogue. No. RR310; TaKaRa) with a set of forward and reverse primers of *PLP* and *SRY* that amplifies 518 bp and 928 bp respectively as mentioned in section 3.8.

3.10.2 Purification of PCR Product for Sequencing

The PCR amplified *PLP* and *SRY* gene products was purified using XcelGen® PCR purification mini kit (Catalogue No. XG3511-01/3514; XcelGen) as per the manufacturer's instructions with minimum modifications.

1) To 1 volume of the PCR product, 3 volumes of GC buffer was added in micro-centrifuge tube and mixed by inverting the tubes several times.
2) 1 volume of isopropanol was added to the sample and mixed thoroughly.
3) Sample was then transferred to the XcelGen® spin column with collection tube, and centrifuged for 1 min. at 13,000g at room temperature. Flow through was discarded and the column was kept back to the collection tube.
4) 650 µl of DNA wash buffer was added to the spin column and centrifuged at 13,000g for 1 min. at room temperature. Flow through was discarded.
5) XcelGen® spin column was placed in fresh 1.5 ml micro centrifuge tube.
6) PCR product was eluted by adding 30 µl of prewarmed (60ºC) elusion buffer to the center of the column; columns were incubated at room temperature for 2 min. and then centrifuged for 2 min. at 13,000g.
7) Eluted product was quantified by Nano Drop TM Lite (Thermo Fisher Scientific).
8) The bands of expected sizes 518 bp and 928 bp for *PLP* and *SRY* respectively, were purified from the gel using XcelGen® PCR Purification Mini Kit and were sent for sequencing.

3.10.3 Sequencing of *PLP* and *SRY* Genes

The *PLP* and *SRY* gene sequencing was done at sequencing facility, Bioserve Biotechnologies India Pvt. Ltd, Hyderabad using ABI 3730 (48 capillary) electrophoresis instrument by sangers method, using *PLP* and *SRY* gene specific primers as mentioned in Table.3.2.

Using ClustalW, multiple sequence alignment was generated using MEGA X and the nucleotide sequences were assembled and edited using the EditSeq in the Laser gene software program (DNASTAR). Sequence alignment generated by comparing the nucleotide sequence of *PLP* and *SRY* genes between unsexed and sexed semen samples of *Bos indicus* and *Bos taurus* breeds.

The sequences obtained after sequencing of *PLP* and *SRY* genes were further confirmed by Basic Local Alignment and Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/).

3.11 SOURCE OF SEMEN STRAWS

The frozen semen straws of HF, Jersey, Gir, Sahiwal breeds were collected from teaching veterinary clinical complex (TVCC), which is a constituent department under P.V. Narsimha Rao Telangana Veterinary University of Telangana State.

The frozen sexed semen straws of HF, Jersey, Gir, Sahiwal breeds were collected from Sandors animal Biogenics limited, Hyderabad, BAIF, ABS company Hyderabad.

3.12 CHEMICALS AND REAGENTS FOR SEMEN QUALITY EVALUATION

The following chemicals and reagents were prepared for semen analysis as per the standard protocols.

1. Semen diluting fluid was prepared as per standard method (Saxena 2000)
2. Eosin and Nigrosine stain was prepared as per standard method (Saxena 2000)
3. Giemsa working solution
4. Giemsa stock solution
5. Sorenson's phosphate buffer
6. Rose Bengal stain
7. Sperm swim media (Catalogue number SP/MR/001, Sperm processor private Ltd)

3.13 SPERM QUALITY PARAMETERS

3.13.1 Mass Activity

Soon after thawing, a drop of semen was placed on a dry, clean glass slide without coverslip, kept on biotherm stage maintained at 37°C, and examined under low power (10X) microscope. The scoring of mass activity of the semen samples was done as per the following score (Saxena, 2000).

Table 3.3. Scoring of mass activity of the semen samples as per the wave pattern of spermatozoa.

S. No	Mass activity of semen sample	Score
1.	Immotile sperms	0
2.	Stationary bunting or weak rotary movements, 10-20% scattered progressive sperms	1
3.	Oscillatory or rotary movement, no waves and eddies, 30-40% progressively motile sperms	2
4.	Progressive rapid movement of sperm, slow moving waves and eddies, 50-60% sperms are motile	3
5.	Vigorous, progressive movement with rapid and abruptly forming waves and eddies	4
6.	Very vigorous forward motion, extremely rapid waves and eddies	5

3.13.2 Sperm Concentration

Sperm concentration was expressed as the total number of spermatozoa in millions (10^6) per ml of frozen semen and was estimated by using improved Neubauer counting chamber after 1:200 dilution of semen with a diluting fluid.

The sperm concentration was calculated using the standard formula (Saxena, 2000).

Number of sperm/ml =N X D X 4/n million sperms/ml

Where N = Number of spermatozoa counted;

D = Dilution rate;

n = number of tertiary squares counted.

3.13.3 Individual Motility

The individual motility of sperm was observed under microscope as per the method described by Verberckmoes *et al.* (2004) and assessed by placing a cover slip on a drop of diluted semen (10µl semen mixed with 200µl normal saline was used for fresh semen evaluation) on a clean glass slide under the microscope (10X) with biotherm stage attached. The motility was observed under high power at 40X magnification and expressed in terms of percentage of progressively (0-100) motile sperms.

3.13.4 Live and Dead Spermatozoa Percentage

To ascertain the percentage of live spermatozoa, the mixture containing 1 drop semen and 2 drops of Eosin-nigrosine (Eosin 1.67 gm, nigrosine 10 gm, distilled water 100 ml) was examined under oil immersion lens (100X). Staining solutions, petri dishes were kept at 37ºC before diluting semen with stain to avoid the manual damage to spermatozoa. Smears were prepared within 30 seconds of mixing. All stained (pink color) and partially stained spermatozoa were considered dead and the unstained (colorless) spermatozoa as live. The percentage of live spermatozoa was determined by counting at least 200 spermatozoa.

3.13.5 Structural Abnormalities

Rose Bengal stain (3%) was used for counting the percentage of normal, abnormal sperm (Pervage *et al.,* 2009). Two drops of sodium-citrate buffer were placed on a clean dry glass slide; one drop of mixed semen was added and spreaded by covering with another slide. The slide was air dried and stained with rose bengal stain for 15-20 minutes, rinsed of excess stain by dipping the slide in distilled water. The slide with smear was air dried and observed under oil immersion lens (100X).

3.13.6 Acrosomal Integrity

The intact acrosome of spermatozoa was essential until they bind to zona pellucida. The spermatozoa may be highly motile but not fertile due to acrosomal damage.

The acrosomal damage (percent damaged acrosomes in unsexed and sexed semen) was studied with Giemsa-stained smears according to the method suggested by Barth and Oko (1989). The methodology was as below.

Procedure:

1. Semen sample was diluted with 2.9% freshly prepared sodium citrate buffer to 1:4 in a test tube.
2. A smear was drawn on clean grease free slide and air dried. The slides were put into 5% formaldehyde solution for fixing at 37ºC for 30 min. (Campbell *et al.,*1960).
3. The slides were removed from the solution, washed in running tap water and air dried for further processing.
4. The working solution of Giemsa was prepared by mixing Giemsa's stock-3 ml, SPS-2 ml and distilled water- 45 ml in a coupling jar and warmed at 37ºC for 30 min.
5. The smeared slides of spermatozoa were put into the working solution and kept at 37ºC for 3 hrs.
6. The slides were removed from the stain and washed in running tap water and finally air dried.
7. The counting of intact, partially damaged and fully damaged acrosome was carried under oil immersion lens of microscope at 1000X (10X100) magnification.

A total of 200 spermatozoa were counted for acrosomal status after staining. The acrosome was considered to be normal when the stain was clearly and evenly distributed over the spermatozoa anterior to the equatorial segment.

$$\text{Acrosomal Integrity (\%)} = \frac{\text{Number of Acrosome-stained spermatozoa}}{\text{Total number of spermatozoa counted}} \times 100$$

3.13.7 Swim Up Technique

1. Equal quantity of sexed semen (decapitation of Y chromosome technique) and sperm swim media (Catalogue number SP/MR/001, Sperm processor private Ltd) were taken into a centrifuge tube.
2. Centrifuged @ 2000rpm for 15 minutes and the supernatant was discarded.

3. To the above pellet 2 ml of sperm swim media (Catalogue number SP/MR/001, Sperm processor private Ltd) was added and the tube was kept inclined at 30 ^{0} angle in incubator at 37ºC for 45 minutes
4. After 45 minutes, upper and middle part was aspirated in another centrifuge tube and centrifuged @ 2000 rpm for 1 min.
5. The supernatant was discarded.
6. 0.5 ml of sperm swim media was added to the pellet and mixed well
7. The above sample is used for extraction of DNA from sexed semen sample (sorted via decapitation of Y-chromosome technique).

3.14 STATISTICAL ANALYSIS

The accumulated data was analysed statistically by using one-way analysis of variance test (ANOVA) with the help of statistical software SPSS version 25. The post hoc analysis was performed using Duncan's multiple range tests. The level of significance was set at $P<0.01$.

RESULTS

CHAPTER - IV

RESULTS

The present study was under taken with an aim of quantification of X and Y chromosome bearing spermatozoa in unsexed and sexed (sexed based on flow cytometry, and decapitation of Y chromosome techniques) frozen semen samples of *Bos taurus* (HF, Jersey) and *Bos indicus* (Gir, Sahiwal) breeds of cattle. Also, the qualitative parameters like motility, acrosomal integrity, liveability, concentration, sperm abnormalities of sexed and unsexed frozen semen among the breeds were compared. The results are presented here.

4.1 QUANTIFICATION OF X AND Y CHROMOSOME BEARING SPERMATOZOA IN SEMEN

4.1.1 Cloning of *PLP* and *SRY* Partial Genes into TA Cloning Vector for Generation of Standards

Two individual sets of primers were used specifically to amplify *PLP* and *SRY* genes from X and Y chromosome, respectively. The *PLP* and *SRY* partial genes were amplified from semen DNA with amplicon sizes of 150 bp and 66 bp for *PLP* (Fig. 4.1) and *SRY* genes (Fig. 4.2), respectively. The *PLP* and *SRY* fragments were purified using PCR purification kit. These amplicons were cloned into TA cloning vector as per manufacturer's protocol. Fig. 4.3 shows the annotation map of pMD20 T vector whereas the Fig. 4.4 and 4.5 show the annotated map of vectors pMD20 T-PLP and pMD20 T-SRY respectively post-ligation with the *PLP* and *SRY* genes, respectively. The ligated products were transformed into DH5α cells; the transformants were screened on LB-Amp-IPTG-X gal plate. Fig. 4.6 shows the transformant colonies

carrying either *PLP* or *SRY* genes were white coloured. The resulting clones were reconfirmed by PCR and sequencing.

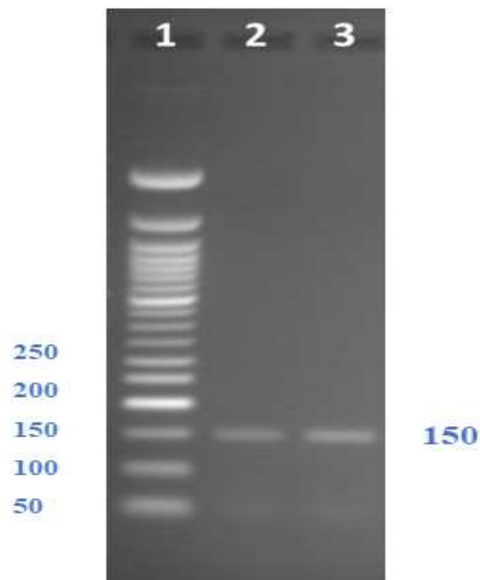


Fig. 4.1 PCR amplified partial *PLP* gene on 1.5% agarose gel. The 150 bp PCR product is amplified from genomic DNA extracted from semen sample. Lane 1 is a 50 bp Ladder, Lane 2 and 3 show amplified PCR product of *PLP* gene from semen genomic DNA.

Fig. 4.2 PCR amplified partial *SRY* gene on 1.5% agarose gel. The 66 bp PCR product is amplified from genomic DNA extracted from semen sample. Lane 3 is a 50 bp Ladder, Lane 1, 2 and 4 show amplified PCR product of *SRY* gene from semen genomic DNA.

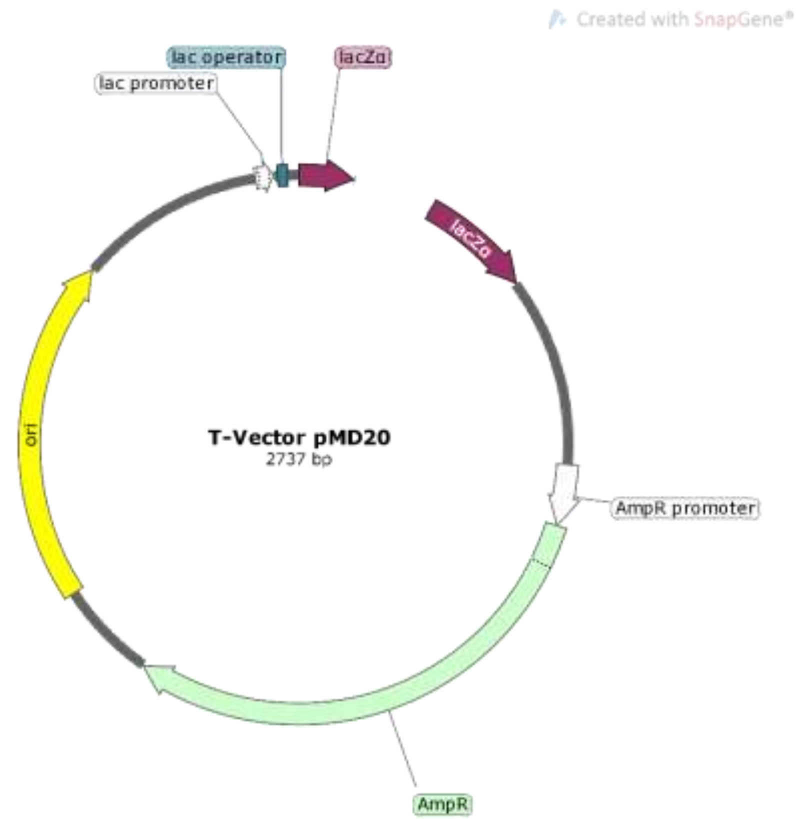


Fig. 4.3 Annotation map of pMD20 T-Vector.

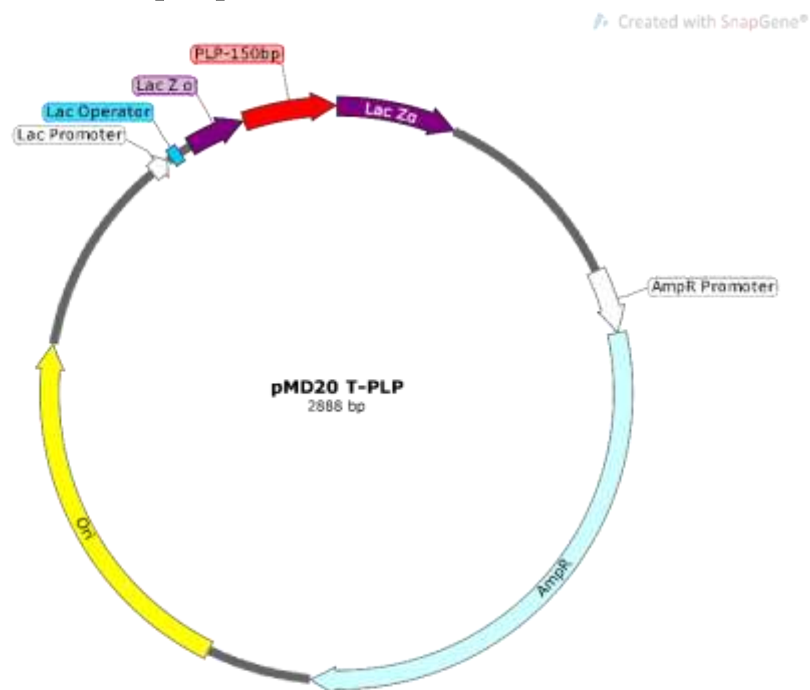


Fig. 4.4 Annotation map of pMD20 T-PLP. The plasmid is generated by cloning the partial *PLP* gene into pMD20 T-Vector.

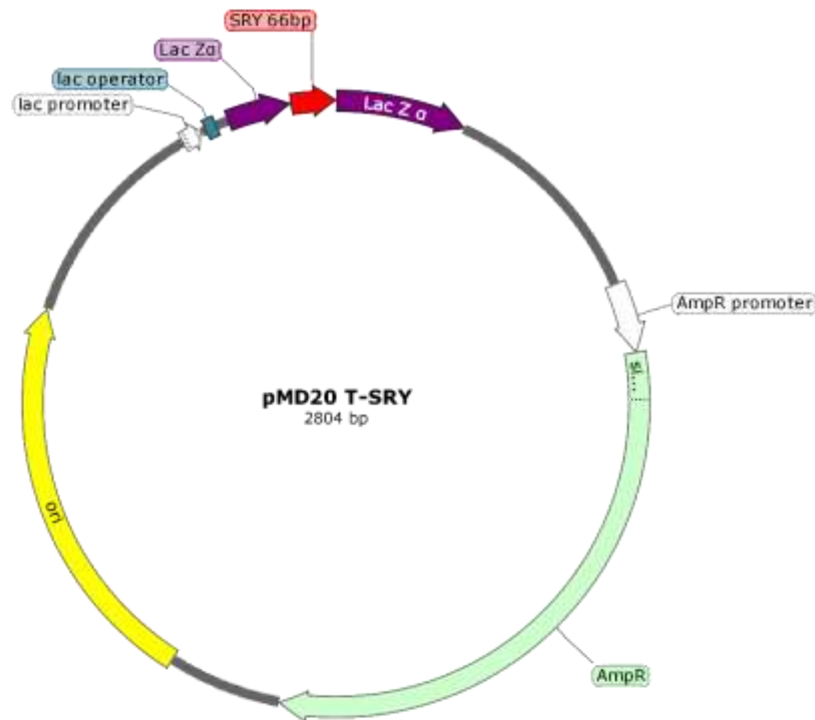


Fig. 4.5 Annotation map of pMD20 T-SRY. The plasmid is generated by cloning the partial *SRY* gene into pMD20 T-Vector.

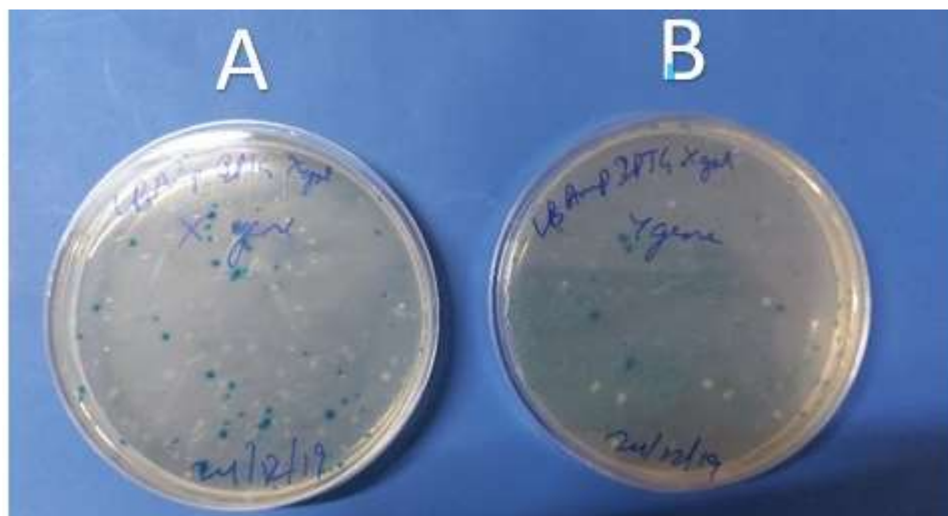


Fig. 4.6 Blue-white screening for selection of transformants with partial gene insert. After transformation of ligated plasmids into DH5α cells the culture is spread on LB Amp IPTG X-gal plate. The transformants show blue colour if the insert is absent and white colour if insert is present for both pMD20 T-PLP (A) and pMD20 T-SRY (B) plasmids.

4.1.2 Quantification of X and Y Chromosome Bearing Spermatozoa in Unsexed Semen Samples.

Quantification of X (*PLP*-gene) and Y (*SRY*-gene) chromosome bearing spermatozoa in unsexed semen samples was done by using SYBR® Green based quantitative real-time PCR (qPCR) technique.

The pMD20 T-PLP and pMD20 T-SRY were used as standards to construct standard curves. The plasmid DNA was used at concentrations ranging from 1.0213 x 10^{10} to 1.0213 x 10^4 copies/µl with 10-fold dilution for generating standard curve. The standard curve was fit by linear regression having $r^2 = 0.9889$ for *PLP* and $r^2 = 0.9998$ for *SRY*. The melt curve analysis showed a clear single peak for *PLP* and *SRY* genes (Fig. 4.7).

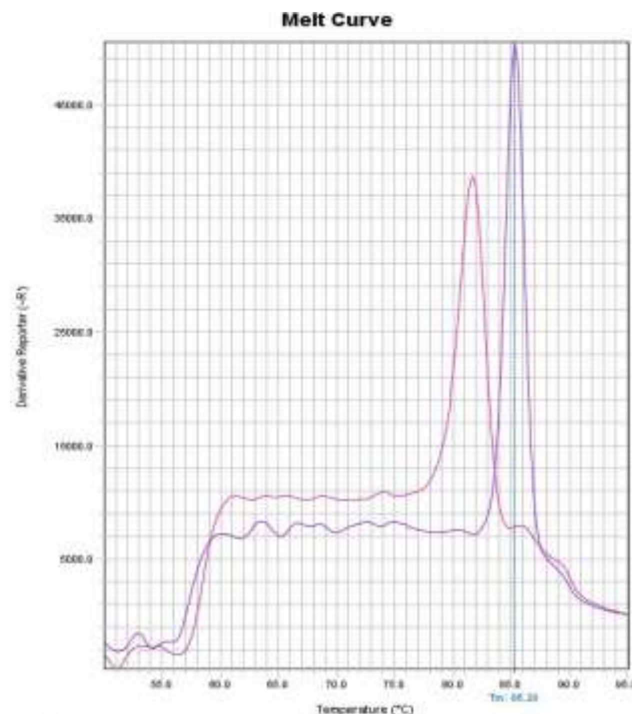


Fig. 4.7 Representative image showing the melt curve of *PLP* and *SRY* genes. The curve in blue colour represents amplicon of *PLP* gene and the curve in pink colour represents amplicon of *SRY* gene.

DNA extracted from the unsexed semen of different cattle breeds was used for quantification of X and Y chromosome bearing spermatozoa along with the standards (Fig. 4.8).

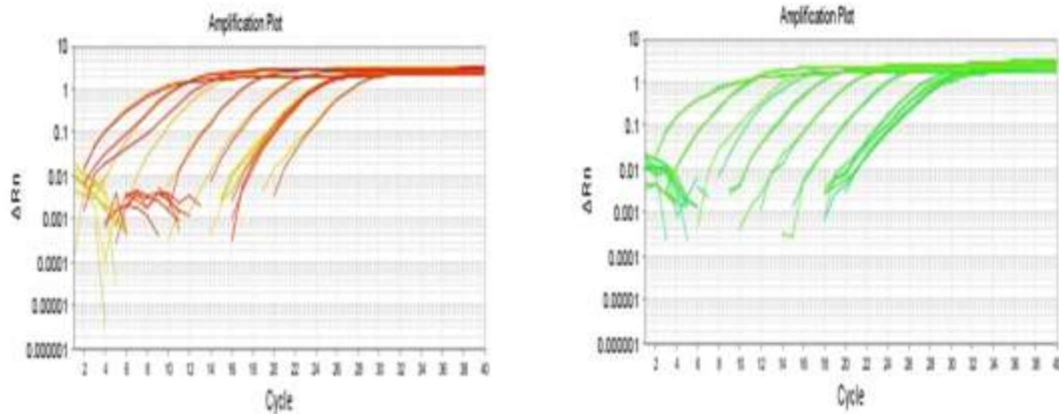


Fig. 4.8 Amplification Plot of *PLP* (left) and *SRY* (right) genes from respective plasmid standards and unsexed semen DNA samples. The plasmids are serially diluted by 10-fold from 1.023×10^{10} to 1.023×10^{4} and the qPCR was performed. The q-PCR was performed without any dilution for unsexed semen DNA samples. The amplification chart with red colour curves are for *PLP* genes and the amplification chart with green colour curves are for *SRY* genes.

The construction of standards curves of *PLP* and *SRY* generated the linear relationship between the input plasmid DNA and the C_T values. The C_T values of the standards are shown in Table 4.1.

Table 4.1 The C_T values of the standards in qPCR assay.

Copy number of plasmids	C_T Mean value	
	PLP	SRY
1.0213×10^{10}	10.41	4.09
1.0213×10^{9}	12.65	7.23
1.0213×10^{8}	15.22	10.96
1.0213×10^{7}	19.22	14.15
1.0213×10^{6}	22.55	17.23
1.0213×10^{5}	26.10	20.61
1.0213×10^{4}	29.31	23.94

A linear relationship between the input plasmid DNA and the C_T values with regression coefficient (r^2) more than 0.98 was obtained for both markers. The coefficient of determinations of standard curves for *PLP* and *SRY* are $r^2 = 0.9889$;

$y = 8E+12e^{-0.705x}$ and $r^2 = 0.9998$; $y = 2E+11e^{-0696x}$ respectively. (Fig. 4.9 and 4.10). C_T values were the parameter by which quantified values are assigned in qPCR assay.

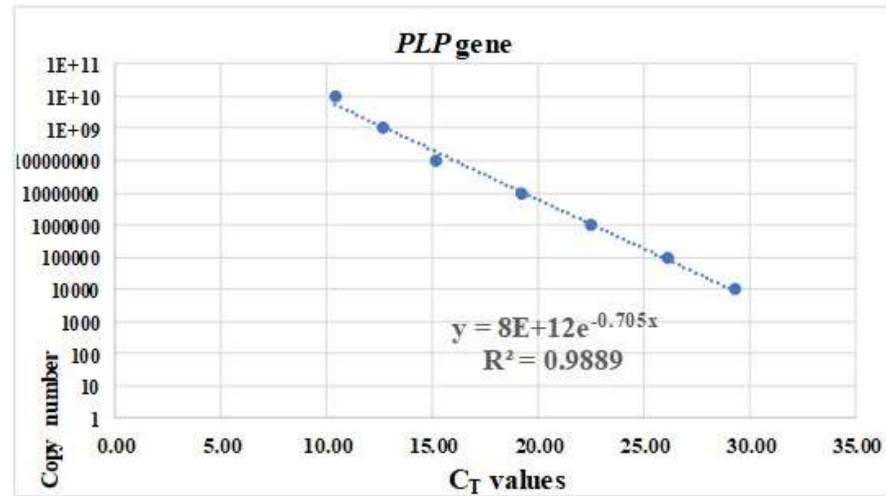


Fig. 4.9 Standard curve (graph) of *PLP* gene standards for unsexed semen performed by Real-time PCR.

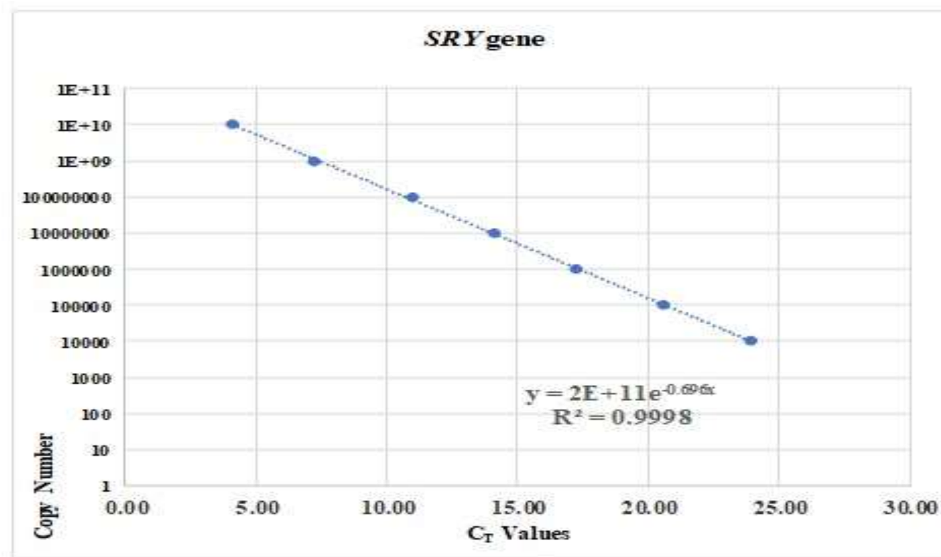


Fig. 4.10 Standard curve (graph) of *SRY* gene standards for unsexed semen performed by Real-time PCR.

Absolute quantification was successfully performed using the standard curves constructed. It was found that the percentage of X chromosome bearing spermatozoa ranged from 62.88% to 71.21%, while Y chromosome bearing spermatozoa ranged

from 28.79% to 37.12% in unsexed semen samples of the four cattle breeds namely Holstein Friesian (HF), Jersey, Gir and Sahiwal (Fig 4.11). The result from the absolute quantification of the DNA samples from spermatozoa percentages is shown in Table 4.2.

Table 4.2 The percentage of the X and Y chromosome bearing spermatozoa in unsexed semen determined by using quantitative Real - time PCR.

Breed	C_T values		Percentage	
	PLP	***SRY***	***PLP* (X%)**	***SRY* (Y%)**
HF	23.6±0.13	22.9±0.18	65.35±2.02	34.65±2.02
Jersey	23.82±0.36	22.88±0.20	62.88±3.39	37.12±3.39
Gir	23.53±0.22	22.79±0.05	64.38±3.07	35.62±3.07
Sahiwal	23.52±0.30	23.14±0.25	71.21±1.96	28.79±1.96

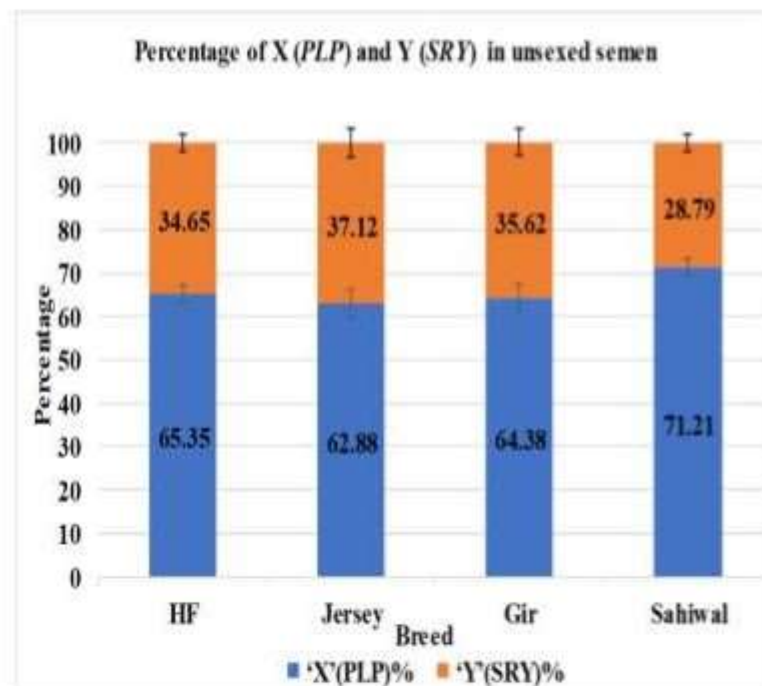


Fig. 4.11 Percentage bar diagram showing the X (*PLP*) and Y (*SRY*) spermatozoa in unsexed semen.

4.1.3 Quantification of X and Y Chromosome Bearing Spermatozoa in Sexed Semen Samples Produced by Flow Cytometry, and Decapitation of Y Chromosome Techniques

X (*PLP* gene) and Y (*SRY* gene) chromosome bearing spermatozoa that were present in sexed semen obtained through flowcytometry were quantified as above (section 4.1.2) by SYBR® Green chemistry of real-time quantitative PCR to compare copy number of *PLP* and *SRY* genes in *Bos indicus* (Gir, Sahiwal) and *Bos taurus* (HF, Jersey) breeds.

The standard curve was fit by linear regression having $r^2 = 0.9889$ for *PLP* and $r^2 = 0.9998$ for *SRY*. The construction of standards curves of *PLP* and *SRY* generated the linear relationship between the input plasmid DNA and the C_T values. The C_T values of the standards are shown in Table. 4.3.

Table 4.3 The C_T values of standards in qPCR assay.

Copy number of plasmids	C_T Mean value	
	PLP	SRY
1.0213×10^{10}	9.61	6.4
1.0213×10^{9}	11.48	7.3
1.0213×10^{8}	15.81	13.0
1.0213×10^{7}	20.61	13.4
1.0213×10^{6}	22.85	16.3
1.0213×10^{5}	25.66	16.0
1.0213×10^{4}	27.96	21.8

A linear relationship between the input plasmid DNA and the C_T values with regression coefficient (r^2) more than 0.98 was obtained for both markers. The coefficient of determinations of standard curves for *PLP* and *SRY* are $r^2 = 0.9889$; $y = 8E+12e^{-0.705x}$ and $r^2 = 0.9998$; $y = 2E+11e^{-0696x}$ respectively.

Absolute quantification was successfully performed using the standard curves constructed. The results from the absolute quantification of the DNA samples from spermatozoa of sexed semen (flow cytometry technique) samples revealed that the

percentage of X chromosome bearing spermatozoa ranged from 99.02% to 99.93% and Y chromosome bearing spermatozoa ranged from 0.07% to 0.98% in the sexed semen samples of the four cattle breeds tested (Fig. 4.12). The result from the absolute quantification of the DNA samples from spermatozoa Percentages is shown in below Table No. 4.4.

Table 4.4. The percentage of the X and Y chromosome bearing spermatozoa in semen sexed through flow cytometry technique determined by Real - time PCR.

Breed	C_T values		Percentage	
	PLP	*SRY*	*PLP* (X%)	*SRY* (Y%)
HF	24.8	23.2	99.02	0.98
Jersey	24.6	25.6	99.90	0.10
Gir	24.3	25.4	99.90	0.10
Sahiwal	24.2	25.6	99.93	0.07

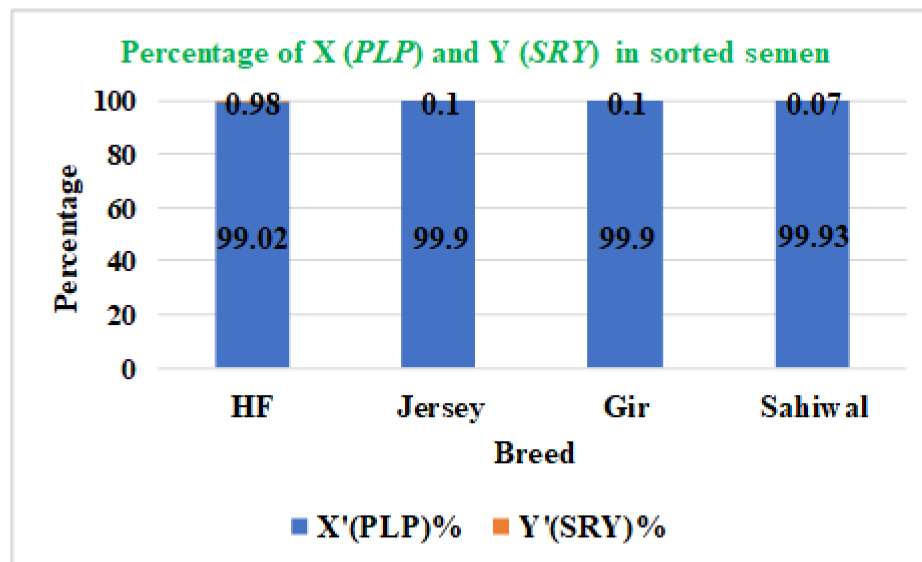


Fig. 4.12 Percentage bar diagram showing the *PLP* gene (X) and *SRY* gene (Y) in sexed semen (flow cytometry technique).

The results from the absolute quantification of the DNA samples from spermatozoa of sexed semen (decapitation of Y sperm technique) samples revealed that the percentage of X chromosome bearing spermatozoa ranged from 95.92% to 97.90% and Y chromosome bearing spermatozoa ranged from 2.10% to 4.08% in sexed semen samples of three cattle breeds namely Holstein Friesian (HF), Jersey, and

Gir (Fig. 4.13). Semen sorted by decapitation of Y sperm technique could not be obtained for Sahiwal breed of cattle for this study. The result from the absolute quantification of the DNA samples from spermatozoa Percentages is shown in below Table 4.5.

Table 4.5 The percentage of the X and Y chromosome bearing spermatozoa in semen sexed through decapitation of Y chromosome technique determined by quantitative real - time PCR

Breed	C_T values		Percentage	
	PLP	*SRY*	*PLP* (X%)	*SRY* (Y%)
HF	21.64	25.45	97.90	2.10
Jersey	21.87	25.27	97.22	2.78
Gir	21.88	24.71	95.92	4.08

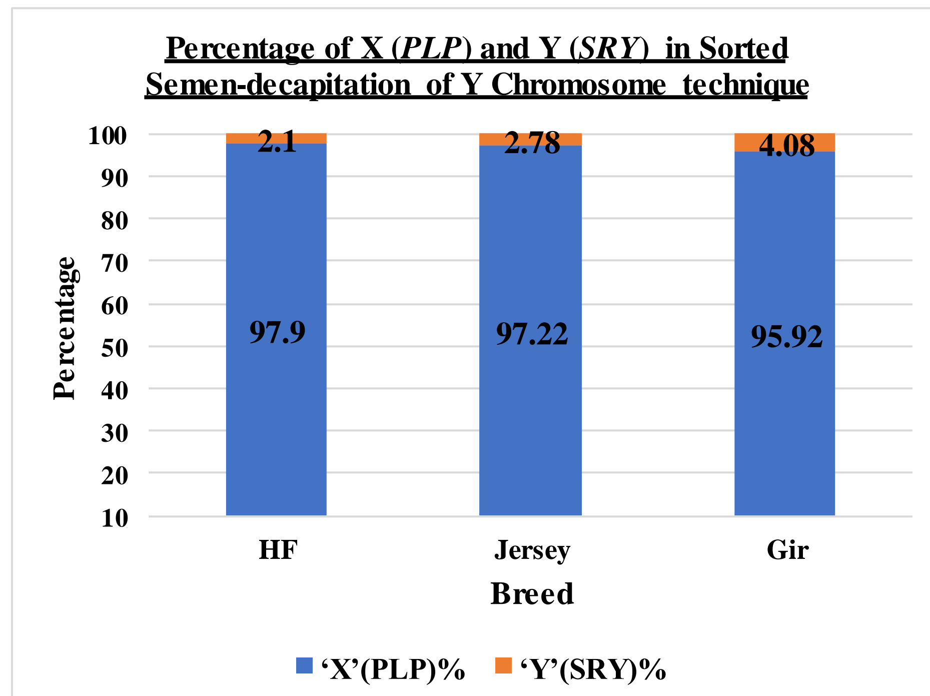


Fig. 4.13 Percentage bar diagram showing the *PLP* and *SRY* genes in semen sexed by (decapitation of Y chromosome bearings technique).

4.2 COMPARATIVE SEQUENCE ANALYSIS OF *PLP* AND *SRY* GENES

4.2.1 PCR Amplification and Sequencing of Partial *PLP* and *SRY* genes

The PCR amplification with specific primers yielded a 518bp product (Fig.4.14) *PLP* gene and a 928bp product (Fig.4.15) *SRY* gene.The PCR products of partial *PLP* gene (518bp) and *SRY* gene (928bp) (8 samples i.e., unsexed: 4 and sexed: 4) (Fig 4.16) were subjected to sequencing followed by comparing for nucleotide changes among breeds. The generated sequence chromatograms were initially edited with Chromas V 2.0 software and low-quality sequences were trimmed from raw sequence data file. A representative chromatogram image showing nucleotide peaks is represented in (Fig. 4.17).

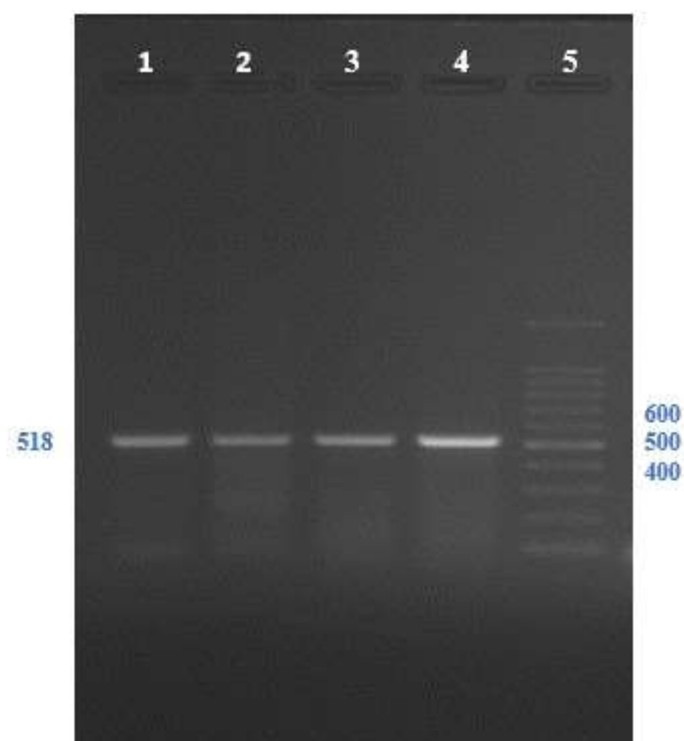


Fig. 4.14 Agarose gel electrophoresis on 1% confirmed a 518bp PCR product of *PLP* gene from unsexed semen samples of *Bos indicus* and *Bos taurus* breeds. Lane -1- HF, Lane -2- Jersey, Lane -3- Gir, Lane -4- Sahiwal, Lane -5- 100bp Ladder.

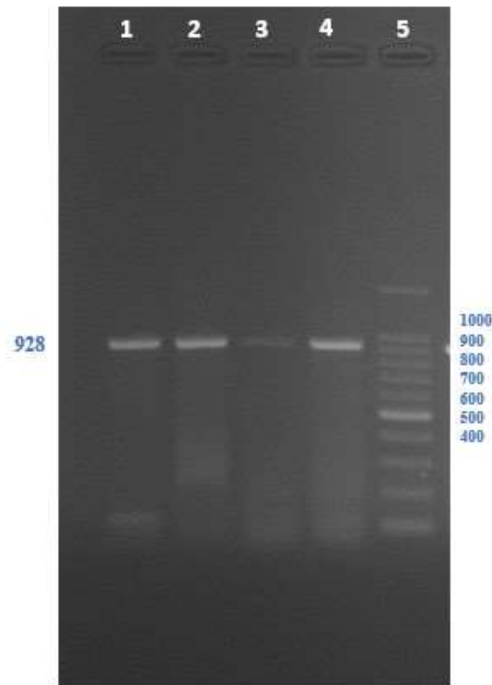


Fig. 4.15 Agarose gel electrophoresis on 1% confirmed a 928bp PCR amplified product of *SRY* gene from unsexed semen samples of *Bos indicus* and *Bos taurus* breeds. Lane -1- HF, Lane -2- Jersey, Lane -3- Gir, Lane -4 Sahiwal, Lane -5- 100bp ladder.

Fig. 4.16 Agarose gel electrophoresis on 1% confirmed the PCR amplified products of *PLP* (518bp) & *SRY* gene (928bp) from sexed semen (flow cytometry) samples of *Bos indicus* and *Bos taurus* breeds. (Lane 1 to 4 *PLP* 518bp) Lane-1- HF, Lane -2- Jersey, Lane -3- Gir, Lane -4-Sahiwal, Lane -5- 100bp Ladder, (Lane 6 to 9 *SRY* 928bp) Lane-6- HF, Lane -7- Jersey, Lane -8 - Gir, Lane -9-Sahiwal.

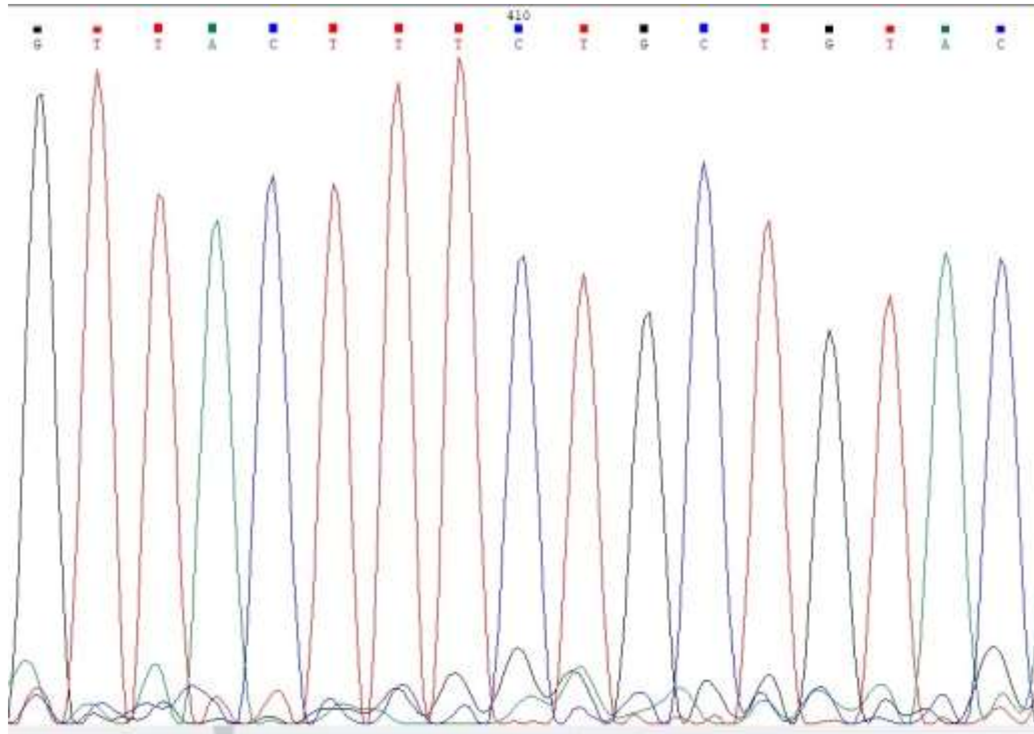


Fig. 4.17 Chromatogram as visualized in chromas V 2.0.

The good quality sequences were taken, which were distinguishable clearly with high peaks to study the evolutionary relationship of isolates with other reference sequences of *Bos indicus and Bos taurus* breeds. The sequences obtained in this study were submitted to the GenBank and GenBank accession numbers are given in Table 4.6.

Table 4.6 GenBank accession numbers from NCBI for *PLP* and *SRY* genes of unsexed and sexed semen samples of *Bos indicus* and *Bos taurus* breeds from this study

S. No	Gene	Designation	Submission ID	GenBank accession number
1	PLP	HF-Unsexed/PVNRTVU/01/2020	2443473	MW815576
2	PLP	Jersey-Unsexed/PVNRTVU/01/2020	2443480	MW815577
3	PLP	Gir-Unsexed/PVNRTVU/01/2020	2443485	MW815578
4	PLP	Sahiwal-Unsexed/PVNRTVU/01/2020	2443493	MW815579
5	PLP	HF-Sexed/PVNRTVU/02/2020	2443498	MW815580
6	PLP	Jersey-Sexed/PVNRTVU/02/2020	2443499	MW815581
7	PLP	Gir-Sexed/PVNRTVU/02/2020	2443501	MW815582
8	PLP	Sahiwal-Sexed/PVNRTVU/02/2020	2443505	MW815583
9	SRY	HF-Unsexed/PVNRTVU/01/2020	2443514	MW815584
10	SRY	Jersey-Unsexed/PVNRTVU/01/2020	2443516	MW815585
11	SRY	Gir-Unsexed/PVNRTVU/01/2020	2443519	MW815586
12	SRY	Sahiwal-Unsexed/PVNRTVU/01/2020	2443520	MW815587
13	SRY	HF-Sexed/PVNRTVU/02/2020	2443521	MW815588
14	SRY	Jersey-Sexed/PVNRTVU/02/2020	2443525	MW815589
15	SRY	Gir-Sexed/PVNRTVU/02/2020	2443528	MW815590
16	SRY	Sahiwal-Sexed/PVNRTVU/02/2020	2443529	MW815591

4.2.2 Nucleotide Sequence Comparison of *PLP* and *SRY* Genes between Sexed and Unsexed Semen

Both the sexed and unsexed semen samples of *Bos taurus* (HF, Jersey) breeds in their nucleotide sequence of *PLP* gene showed a base substitution (transversion) G/C at nucleotide position 10721 (Fig.4.18, Fig.4.19). Both the sexed and unsexed semen samples of *Bos taurus* (HF, Jersey) and *Bos indicus* (Gir, Sahiwal) breeds showed a base substitution (transversion) T/C at 10610 position (Fig. 4.20, 4.21), insertion of A at 10679 and 10680 position (Fig.4.18, Fig.4.22). All these changes were in non-coding (intron) region of the genes. Both the sexed and unsexed semen samples of *Bos taurus* (HF, Jersey) and *Bos indicus* (Gir, Sahiwal) breeds showed no changes in nucleotide sequence (Fig.4.23).

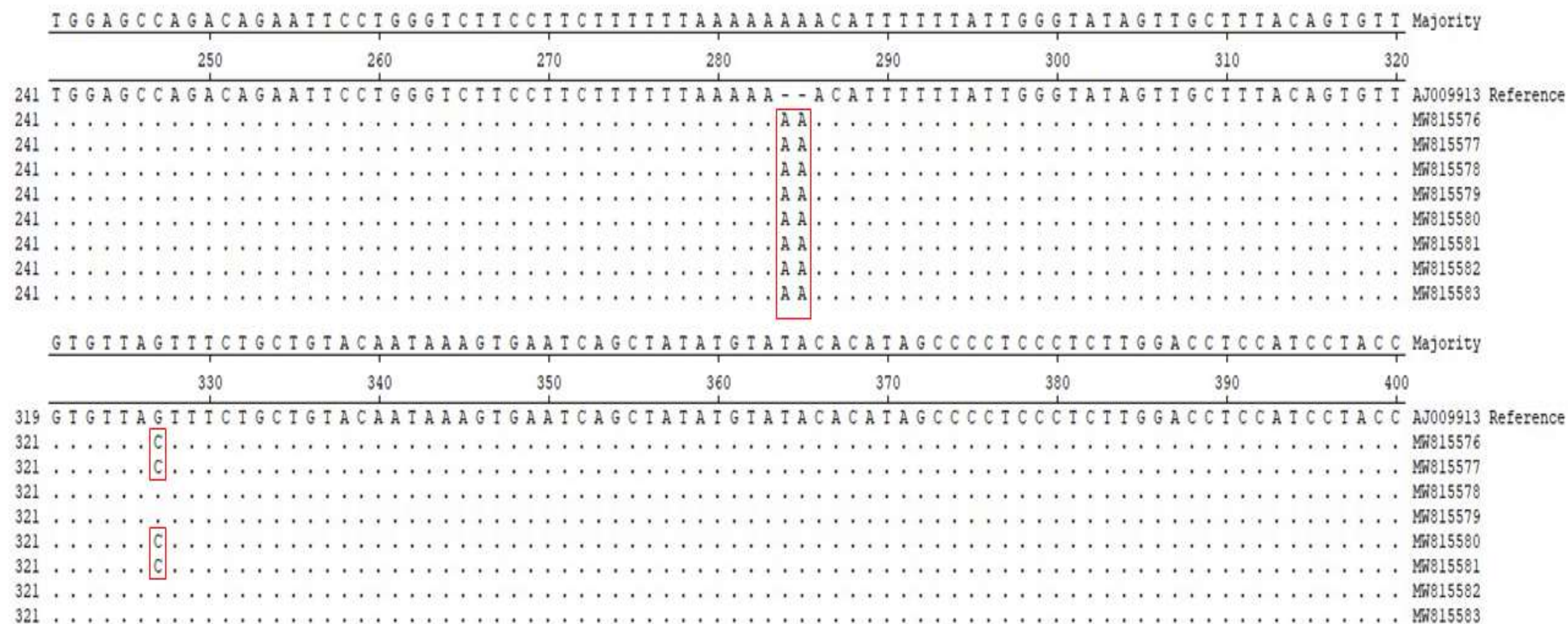
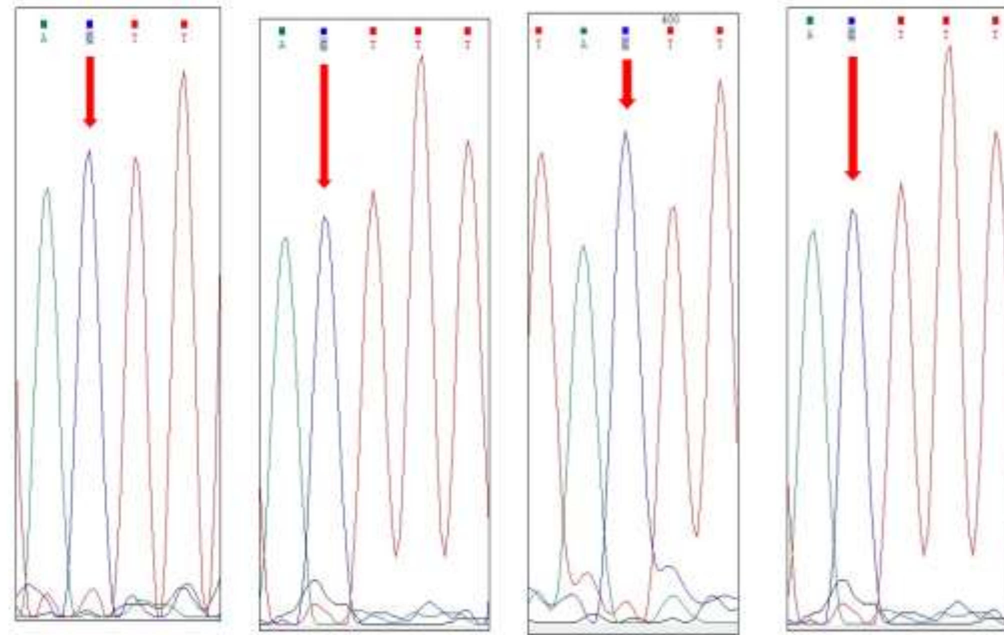


Fig. 4.18 Nucleotide comparison of *PLP* gene with reference sequence showing that there is substitution of G to C at 10721 position in *PLP* of HF, Jersey breeds of sexes and unsexed semen samples and insertion of A at 10679 & 10680 position in *PLP* gene of all breeds of unsexed and sexed semen and all these insertions fall in non-coding (intron) region only.



HF Unsexed **HF sexed** **Jersey Unsexed** **Jersey sexed**

Fig. 4.19 Chromatogram showing G to C transversion at position 10721 of *PLP* gene in HF, Jersey breeds of unsexed and sexed semen samples.

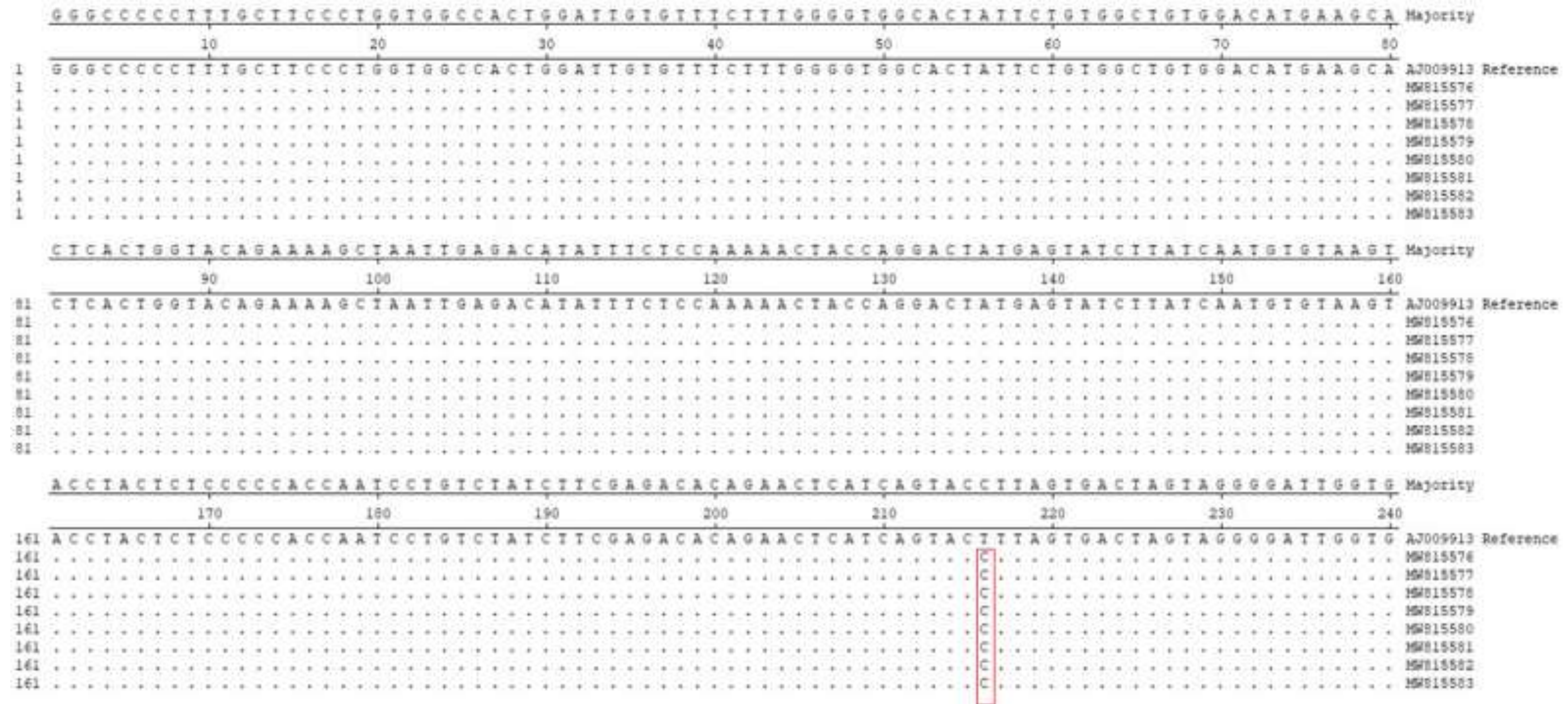


Fig. 4.20 Nucleotide comparison of *PLP* gene with reference sequence showing that there is substitution of T to C at 10610 position and all these insertions fall in non- coding (intron) region only.

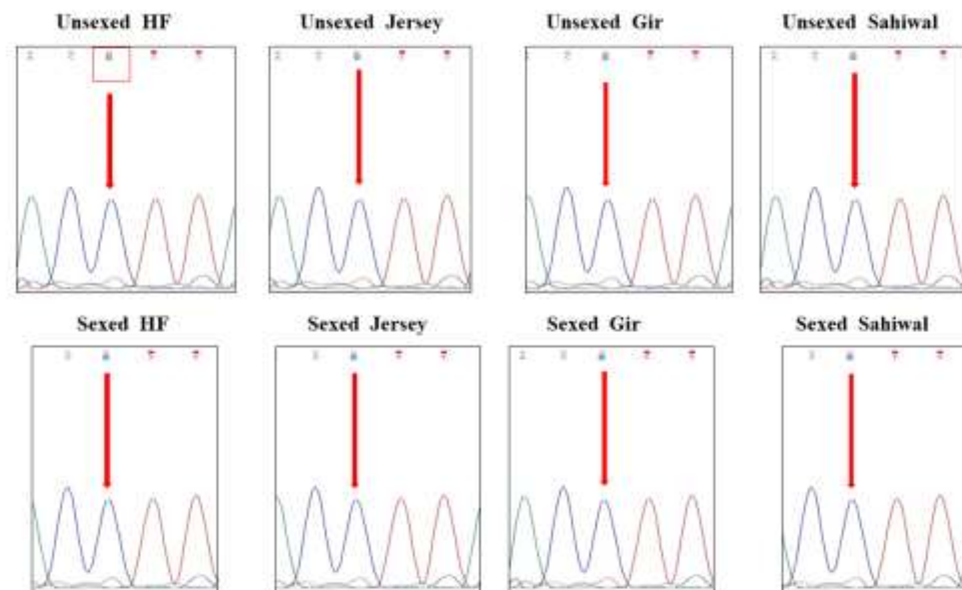


Fig. 4.21 Chromatogram showing a transversion of T to C at position 10610 in all breeds of *PLP* in unsexed and sexed semen.

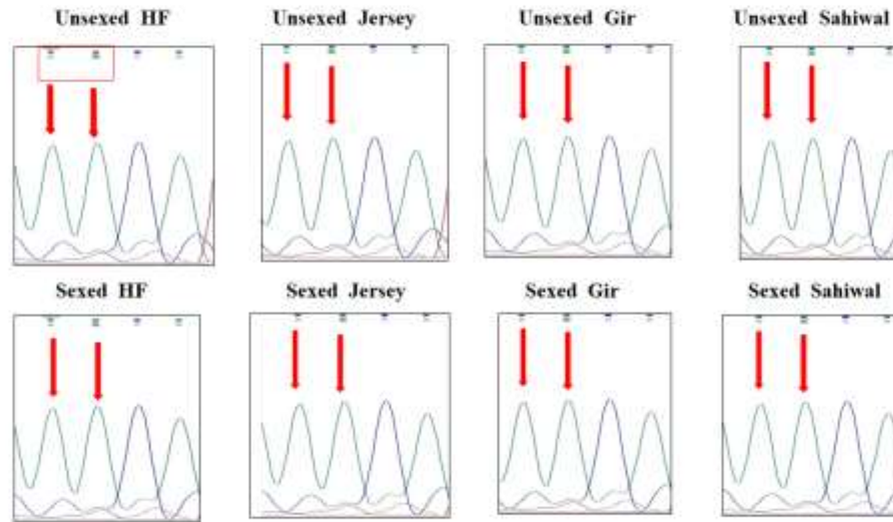


Fig. 4.22 Chromatogram showing insertion of A at 10679 & 10680 position in *PLP* gene in of all breeds in both unsexed and sexed semen.

```
    TTAAACAGTGCAGTCGTATGCTTCTGCTATGTTCAGAGTATTGAACGACGATGTTTACAGTCCAGCTGTGGTACAGCAAC Majority
             10        20        30        40        50        60        70        80
1   TTAAACAGTGCAGTCGTATGCTTCTGCTATGTTCAGAGTATTGAACGACGATGTTTACAGTCCAGCTGTGGTACAGCAAC AB039748.1 Reference
1   ................................................................................ MW815584
1   ................................................................................ MW815585
1   ................................................................................ MW815586
1   ................................................................................ MW815587
1   ................................................................................ MW815588
1   ................................................................................ MW815589
1   ................................................................................ MW815590
1   ................................................................................ MW815591

    AAACTACTCTCGCTTTTAGGAAAGACTCTTCCTTGTGCACAGACAGTCATAGCGCAAATGATCAGTGTGAAAGGGGAGAA Majority
             90       100       110       120       130       140       150       160
81  AAACTACTCTCGCTTTTAGGAAAGACTCTTCCTTGTGCACAGACAGTCATAGCGCAAATGATCAGTGTGAAAGGGGAGAA AB039748.1 Reference
81  ................................................................................ MW815584
81  ................................................................................ MW815585
81  ................................................................................ MW815586
81  ................................................................................ MW815587
81  ................................................................................ MW815588
81  ................................................................................ MW815589
81  ................................................................................ MW815590
81  ................................................................................ MW815591

    CATGTTAGGGAGAGCAGCCAGGACCACGTCAAGCGACCCATGAACGCCTTCATTGTGTGGTCTCGTGAACGAAGACGAAA Majority
            170       180       190       200       210       220       230       240
161 CATGTTAGGGAGAGCAGCCAGGACCACGTCAAGCGACCCATGAACGCCTTCATTGTGTGGTCTCGTGAACGAAGACGAAA AB039748.1 Reference
161 ................................................................................ MW815584
161 ................................................................................ MW815585
161 ................................................................................ MW815586
161 ................................................................................ MW815587
161 ................................................................................ MW815588
161 ................................................................................ MW815589
161 ................................................................................ MW815590
161 ................................................................................ MW815591
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Fig. 4.23 Nucleotide sequence comparison of *SRY* gene with reference sequence.

```
    GGTGGCTCTAGAGAATCCCAAAATGAAAAACTCAGACATCAGCAAGCAGCTGGGATATGAGTGGAAAAGGCTTACAGATG Majority
             250       260       270       280       290       300       310       320
241 GGTGGCTCTAGAGAATCCCAAAATGAAAAACTCAGACATCAGCAAGCAGCTGGGATATGAGTGGAAAAGGCTTACAGATG AB039748.1 Reference
241 ................................................................................ MW815584
241 ................................................................................ MW815585
241 ................................................................................ MW815586
241 ................................................................................ MW815587
241 ................................................................................ MW815588
241 ................................................................................ MW815589
241 ................................................................................ MW815590
241 ................................................................................ MW815591

    CTGAAAAGCGCCCATTCTTTGAGGAGGCACAGAGACTACTAGCCATACACCGAGACAAATACCCGGGCTATAAATATCGA Majority
             330       340       350       360       370       380       390       400
321 CTGAAAAGCGCCCATTCTTTGAGGAGGCACAGAGACTACTAGCCATACACCGAGACAAATACCCGGGCTATAAATATCGA AB039748.1 Reference
321 ................................................................................ MW815584
321 ................................................................................ MW815585
321 ................................................................................ MW815586
321 ................................................................................ MW815587
321 ................................................................................ MW815588
321 ................................................................................ MW815589
321 ................................................................................ MW815590
321 ................................................................................ MW815591

    CCTCGTCGGAGAGCCAAGAGGCCACAGAAATCGCTTCCTGCAGACTCTTCAAT Majority
             410       420       430       440       450
401 CCTCGTCGGAGAGCCAAGAGGCCACAGAAATCGCTTCCTGCAGACTCTTCAAT AB039748.1 Reference
401 ..................................................... MW815584
401 ..................................................... MW815585
401 ..................................................... MW815586
    .....................................................
```


Fig. 4.23 (cont.) Nucleotide sequence comparison of *SRY* gene with reference sequence.

4.3 SEMEN QUALITY EVALUATION OF UNSEXED AND SEXED SEMEN

Comparative analysis of quality parameters i.e., motility, livability, concentration, acrosomal integrity, and structural abnormalities between sexed and unsexed semen was further performed in this study.

4.3.1 Sperm Concentration

The sperm concentration in unsexed semen samples was 19.93 ± 0.01 (million per french mini frozen semen straw) and sexed semen sample was 1.98 ± 0.00 (million per french mini frozen semen straw). There was a significant difference (P<0.01) between unsexed semen samples and sexed semen samples pertaining to sperm concentration. (Table 4.7) (Fig. 4.24).

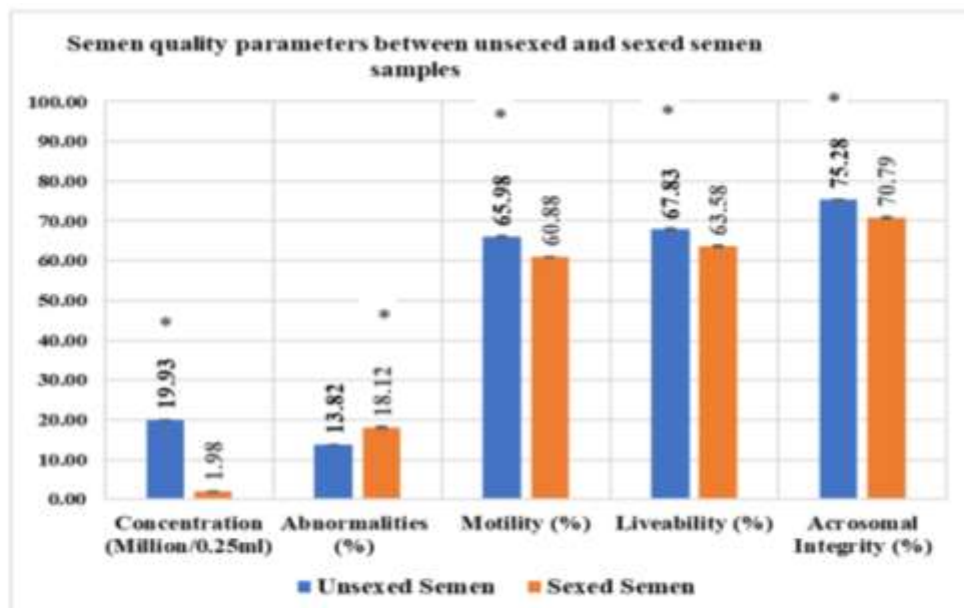

Fig. 4.24 Comparative analysis (P<0.01) of quality parameters between unsexed and sexed semen samples.

Table 4.7 Comparative analysis (P<0.01) of quality parameters between unsexed and sexed semen samples by ANOVA

Semen	Concentration (Million/0.25ml)	Abnormalities (%)	Motility (%)	Liveability (%)	Acrosomal Integrity (%)
Unsexed (n=4)	19.93 ± 0.01	13.82 ± 0.02	65.98 ± 0.23	67.83 ± 0.42	75.28 ± 0.05
Sexed (n=4)	1.98 ± 0.00	22.12 ± 0.18	60.88 ± 0.18	63.58 ± 0.25	70.79 ± 0.26

4.3.2 Sperm Motility

The sperm motility percentage in unsexed semen samples was 65.98 ± 0.23% and in sexed semen was 60.88 ± 0.18%. There is significant difference (P<0.01) between unsexed semen samples (65.98 ± 0.23%) and sexed semen samples (60.88 ± 0.18%) with respect to sperm motility (Table.4.7) (Fig.4.24).

4.3.3 Live Spermatozoa Percentage

The live spermatozoa percentage in unsexed semen samples was 67.83 ± 0.42% and sexed semen sample was 63.58 ± 0.25%. There was significant difference (P<0.01) between unsexed semen samples (67.83 ± 0.42%) and sexed semen samples (63.58 ± 0.25%) with respect to live spermatozoa percentage (Table 4.7) (Fig. 4.24, 4.25).

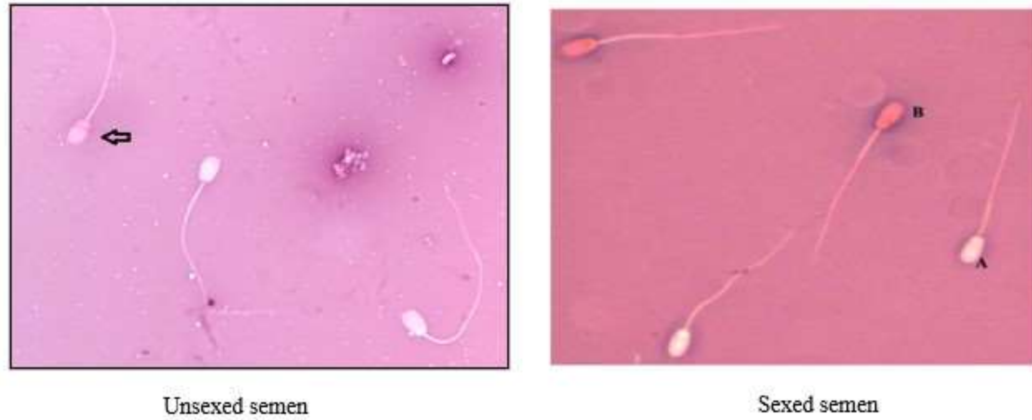

Fig. 4.25 Eosin nigrosine stain – unstained spermatozoa (live) & partially stained (dead) sperm indicated by (↑) (at 1000X) in unsexed and sexed semen samples.

4.3.4 Sperm Abnormalities

The percentage of sperm abnormalities in unsexed semen samples was 13.82 ± 0.02% and sexed semen sample is 22.12 ± 0.18%. There was significant difference (P<0.01) between unsexed semen samples (13.82 ± 0.02%) and sexed semen samples (22.12 ± 0.18%) with respect to sperm abnormalities percentage (Table 4.7) (Fig.4.24).

4.3.5 Sperm Acrosomal Integrity

The sperm acrosomal integrity percentage in unsexed semen samples was 75.28 ± 0.05% and sexed semen sample was 70.79 ± 0.26%. There was significant difference (P<0.01) between unsexed semen samples (75.28 ± 0.05%) and sexed semen samples (70.79 ± 0.26%) pertaining to sperm abnormalities percentage (Table 4.7) (Fig. 4.24, 4.26, 4.27).

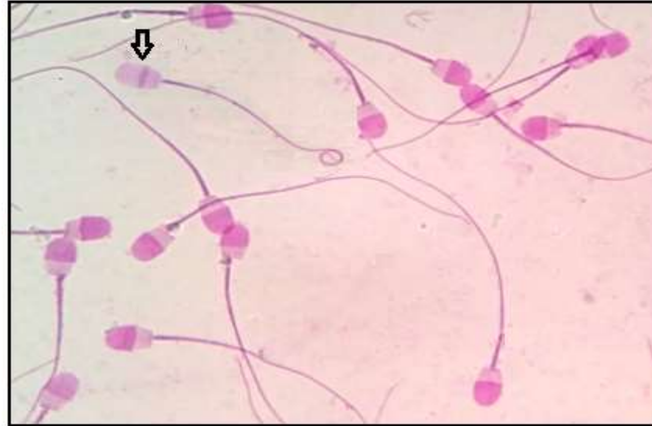

Fig. 4.26 Giemsa staining showing acrosome integrity of sperm heads & (↑) indicates lost acrosomal cap in unsexed semen samples.

Fig. 4.27 Giemsa staining showing acrosome integrity of sperm heads in sexed semen samples.

DISCUSSION

CHAPTER V

DISCUSSION

Cattle are reared mainly for their milk production and hence selection of female progeny is highly desirable. Semen sexing enables to sort the X- and Y-chromosomes, and helps specifically to sort spermatozoa with X- chromosome in the semen samples (Welch *et al.,* 1995; Johnson *et al.*, 2000). This X-sorted semen will account for around 99% of X-spermatozoa (Carvalho *et al.,* 2009), thus making the ova to fertilize and produce female progeny only (Seidel *et al.,* 2009). However, having the semen sorted for X-spermatozoa, during the process they undergo a series of chemical and physical processes. Hence it is essential to understand whether such process affects the quality of semen which in turn affect the conception rates. Most of the research in the field of semen sorting was confined to *Bos taurus* breeds, grossly neglecting *Bos indicus*. There is a need to evaluate the sexed semen in terms of quality parameters, and nucleotide and amino acid sequences of genes associated with X and Y chromosomes to elucidate any variation at nucleotide level. Hence, the present study has been carried to address the qualitative and quantitative parameters of sexed semen.

5.1 QUANTIFICATION OF X- AND Y- CHROMOSOME BEARING SPERMATOZOA

5.1.1 Construction of Reference Plasmids for Copy Number Analysis

The *PLP* and *SRY* genes were identified on the X- and Y- chromosomes (Tan *et al.,* 2015) and were used as gender markers in unsexed and sexed spermatozoa in this study. The *PLP* fragment was specific for female that is located on the X- chromosome and was not amplified in Y- chromosome bearing spermatozoa and vice-versa for the *SRY* amplification.

Both *PLP* and *SRY* genes exist as single copy on X- and Y- chromosomes, respectively in the bovine genome (Tan *et al.,* 2015). Therefore, every single copy of *PLP* and *SRY* sequence detected in qPCR indicated the presence of respective quantity of X- and Y- chromosome bearing spermatozoa.

Plasmid containing clone of a target sequence was commonly used as standard in qPCR (Whelan *et al*., 2003) and *E. coli* is the most popular host cell for bacterial transformation (Lee *et al*., 2006). The reference plasmids were constructed by cloning *PLP* and *SRY* genes separately in pMD20 T-vector and were named as T-PLP and T-SRY,

respectively. The known copies of plasmid were used by 10-fold serial dilution to construct a standard curve and the unknown sample DNA copy numbers were estimated (Sambrook *et al*., 2001).

5.1.2 Quantification of X and Y Chromosome Bearing Spermatozoa in Unsexed Semen Samples

The percentage of *PLP*- and *SRY*- genes bearing spermatozoa was determined using quantitative real-time PCR in unsexed and sexed semen (flow cytometry and decapitation of Y chromosome technique) samples. In qPCR analysis, SYBR® Green was chosen as a DNA interchelating fluorescent dye due to several advantages over sequence-specific probes when single plex PCR are performed. The dye is the simplest and least expensive compared to the other known dyes (Leong *et al.,* 2007). However, SYBR® Green dye tends to bind to all double-stranded nucleic acid molecules, hence the accumulation of primer dimers and the amplification of non-specific PCR products can also be detected in SYBR® Green (Deprez *et al*., 2002). To overcome this, melt curve was run after each cycle to ensure presence of a single melt peak indicating absence of nonspecific PCR products. It had been reported that SYBR® Green detection was used to determine DNA contamination in plasmid preparations and provides a reliable alternative to both the traditional blotting methods and expensive TaqMan protocols (Lee *et al.*2006).

The copy numbers below 1.0213 x 10^4 in qPCR analysis indicated inconsistent C_T values as mentioned earlier (Workenhe *et al.* 2008). Therefore, the copy numbers of the plasmid standards in this study were ranged from 1.0213 x 10^4-1.0213 x 10^{10}. The PLP and SRY markers exist as single copies in X- and Y- chromosome bearing spermatozoa (Tan *et al.,* 2015), indicating the copy number directly relates to number of spermatozoa.

In this study the percentage of X chromosome bearing spermatozoa ranged from 62.88% to 71.21% and Y ranged from 28.79% to 37.12% in unsexed semen samples. However, the studies of Rosenfeld and Roberts (2004) and Whyte *et al*., (2007) revealed 1:1 ratio for X and Y sperms. However, a series of studies conducted on percentage of X and Y bearing chromosome bearing sperms previously suggest that the ratio might differ (Parati *et al*., 2006, Tan *et al.,* 2015 and Kumari *et al*., 2019). The deviation from 1:1 ratio might be due to individual variation in sires, no. of ejaculations, quantity of X and Y chromosomes in an ejaculation, environmental factors, genetic factors, feeding and nutrition, etc (Brito *et al*., 2002).

5.1.3 Quantification of X- and Y- Chromosome Bearing Spermatozoa in Sexed Semen Samples

The sex-sorted semen straws sorted by flowcytometry or decapitation of Y chromosome methods were collected from respective manufacturers. The manufacturer reported more than 90% X-chromosome bearing sperms and less than 10% Y- chromosome bearing sperms for sex-sorted semen sorted by both flow cytometry and decapitation of Y chromosome methods.

The quantification has been carried out similar to unsexed semen to quantitatively verify the reports of manufacturer. The X-chromosome bearing sperms ranged from 99.02% to 99.99% and Y- chromosome bearing sperms ranged from 0.07% to 0.98% for sex-sorted semen sorted by flow cytometry. Wang *et al*., (2011) reported the average purity of X sperm in the X enriched samples by flow cytometry was 92% based on qPCR. Welch *et al.,* 1995 reported that the average purity of X sperm in the X enriched samples by flow cytometry was 90% based on qPCR. Maleki *et al.,* 2013 reported that there is no significant difference in semen sex ratio in unsorted semen (54.7±0.52% X and 47.6±0.60% Y) and there was significant difference was observed in sorted semen by flow cytometry (93.3±0.08% for X sperms) based on qPCR.

The main challenge we encountered in quantifying X- and Y- chromosome bearing spermatozoa from sex sorted semen by decapitation of Y chromosome technique is to eliminate the dead Y- chromosome bearing sperms. It was reported that the swim-up technique helps to segregate the live and dead sperms (Magdanz *et al*., 2019). Briefly, the live sperms swim-up by consuming the nutrients in the media which can be collected from top layer (Garcia-Herreros and Leal 2014). This method was successfully employed in the current study. The X-chromosome bearing sperms ranged from 95.92 % to 97.99% and Y-chromosome bearing sperms ranged from 2.1% to 4.08% for sex-sorted semen sorted by decapitation of Y chromosome technique. However, we found no data on quantitative analysis of sex-sorted semen sorted by decapitation of Y chromosome technique to compare the findings of the current study. Ours is the first study to report quantitative evaluation of semen sorted by decapitation of Y chromosome technique.

In our study, we noticed that the semen sorted by flow cytometry technique edged the superiority with above 99% of X chromosome bearing sperms as compared to 95-98% of X chromosome bearing sperms for semen sorted by decapitation of Y chromosome technique.

5.2 COMPARATIVE NUCLEOTIDE SEQUENCE ANALYSIS OF *PLP* AND *SRY* GENES

In order to understand if there are any variations at the nucleotide level due to the sorting process, partial *PLP* and *SRY* genes of sexed and unsexed semen samples were amplified and sequenced for each of four major cattle breeds reared in India: HF, Jersey, Gir and Sahiwal. It was found that the *PLP* gene sequences of four major cattle breeds in India from this study showed 100% homology among themselves, and 99% homology when compared with exotic HF breed from Germany (GenBank accession number: AJ009913). Similarly, the *SRY* gene sequences of four major cattle breeds in India from this study along with exotic HF breed from Japan (GenBank accession number: AB039748) exhibited 100% homology.

It was noticed that both the sexed and unsexed semen samples of *Bos taurus* (HF, Jersey), *Bos indicus* (Gir, Sahiwal) breeds in their nucleotide sequence of *PLP* gene showed base substitutions G10721C and insertions with A at 10679 and 10680 position where as base substitution T10610C was noticed only in *Bos taurus* (HF, Jersey) breeds and all these were falling in the non-coding (intron) region with no effect on protein coding sequence. It was further noticed that both the sexed and unsexed semen samples of *Bos taurus* (HF, Jersey), *Bos indicus* (Gir, Sahiwal) breeds in their nucleotide sequence of *SRY* gene showed no base substitutions.

5.3 QUALITY PARAMETERS OF UNSEXED AND SEXED SEMEN SAMPLES

It was earlier reported that the concentration in sexed semen was lower than the unsexed semen due to damage to sperms in processing which led to the reduction of conception rate by 10-15%. (Goncharenko *et al.,* 2016). Hence, it was essential to understand whether such processes effect the quality of semen.

It was reported that the standard sperm concentration in sexed semen should be 2×10^6 per insemination and conception rate to be 56% whereas the sperm concentration in unsexed sperm to be $10\text{-}20\text{x}10^6$ per insemination and conception rate to be 61% (Garner and Seidel, 2003). Sperm concentration for the sexed and unsexed semen used in this study was as per the standards mentioned.

In the current study, the quality parameters such as sperm concentration, sperm motility, acrosomal integrity, live spermatozoa and less sperm abnormalities for unsexed semen show superiority over sexed semen. Similar findings were reported previously stating

the superiority of unsexed semen over sexed semen in semen quality parameters (Carvalho *et al*., 2009, 2010 and Goncharenko *et al.,* 2016).

It was reported that the semen quality parameters can be influenced by genetic and non-genetic factors (Gopinathan *et al*., 2018a and Bhave *et al.,* 2020). The non-genetic factors include seasonal effects (Nichi *et al.,* 2006, Fiaz *et al.,* 2010 and Koivisto *et al.,* 2009), number of ejaculations, temperature, humidity, feeding and nutrition (Isnaini *et al.,*2019). The genetic factors include to individual variation in sires, quantity of X and Y chromosomes in an ejaculation (Brito *et al*. 2002). It is also possible that during sorting of sperm by flow cytometry, sperm passes through nozzle with pressure, dyeing of DNA, passing through ultraviolet laser beam, electrostatic separation and centrifugation. All of these might lead to alteration of membrane and other changes such as pre capacitation in the sorted sperm leading to decreased semen quality which might ultimately affect in fertility and conception rates (Kumar *et al*. 2016).

From the current study, we draw the following conclusions

1. The *PLP* and *SRY* gene recombinant plasmids were constructed that can be used for reference standards.
2. The qPCR technique was optimized for quantification of X- and Y- chromosome bearing spermatozoa.
3. We report that the sex sorting can effectively concentrate the X-chromosome bearing spermatozoa in the semen.
4. Comparative sequence analysis (of *PLP* and *SRY* genes) between sexed and unsexed semen samples of *Bos indicus* and *Bos taurus* breeds showed there was no change in nucleotide and protein sequence in *SRY* gene region. Whereas, for *PLP* gene region it was noticed that there was a nucleotide substitution at T10610C, insertion of A at 10679 and 10680 position of all (*Bos taurus* and *Bos indicus)* breeds of unsexed and sexed semen samples. Base substitution G10721C was noticed only in *Bos taurus* and all these insertions fall in non-coding (intron) region only without affecting the protein coding sequence.
5. However, we acknowledge the damage of spermatozoa during sorting process, ultimately leading to deterioration of semen quality, which in turn may affect the conception rate in cattle.

SUMMARY

CHAPTER VI

SUMMARY

In India, the main purpose farmers rear the cattle is for milk production. It is for this reason; female progeny are selectively preferred over male. Sex sorting of semen is one of the recent techniques being popularized for their ability to specifically select the sperms bearing X- chromosome and thus result increasing the probability of giving birth to female progeny. It was reported that the sexed semen consists of about 90% of X-chromosome bearing spermatozoa. The X-chromosome contains about 3.8% more DNA than the Y chromosome in cattle. This difference in DNA content is used to sort the X sperm from the Y bearing sperm (Welch *et al.,* 1995). The different semen sexing techniques are flow cytometry, decapitation of Y-chromosomes, identification of H-Y antigen, detection of sex specific proteins, free flow electrophoresis, centrifugal counter current distribution, albumin. gradient (or) gradient swim down procedure, percoll density gradient method, volumetric differences and quinacrine mustard staining (Joerg *et al.,* 2004). Among several methods for semen sexing, flow cytometry-based sorting has emerged as most efficient. The technology is refined through the decades and finally sex sorting is possible at the purity of more than 90% (Carvalho *et. al.,* 2009).

Although there are several techniques for sorting of spermatozoa, definitive methods to quantify them by molecular techniques are rather limited. The present study has been taken up to develop a cheaper and definitive molecular based quantification technique, and to also understand the effect of sorting on semen quality.

A quantitative analysis of sex bearing chromosomes of bovine spermatozoa by real-time PCR was successfully performed using double strand DNA-binding dye SYBR® Green. *PLP* and *SRY* primers successfully amplified fragments of 150bp and 66 bp from the genome of HF (*Bos taurus)* respectively. The amplified fragments of *PLP* and *SRY* were successfully cloned into vectors and transformed into *E. coli* competent (DH5α) cells. The recombinant T-PLP and T-SRY plasmids were used as reference to generate standard curves that ranged from 1.0213 x 10^{10} to1.0213 x 10^{4} copies.

The unsexed semen samples show X chromosome bearing spermatozoa in the range of 62.88% to 71.21% and Y chromosome bearing spermatozoa in the range of 28.79% to 37.12% for *Bos indicus* and *Bos taurus* breeds. The sex-sorted semen samples by flow cytometry technique show the X chromosome bearing spermatozoa in

the range of 99.02 % to 99.99% and Y chromosome bearing spermatozoa in the range of 0.07% to 0.98% for *Bos indicus* and *Bos taurus* breeds. The sex-sorted semen samples by decapitation of Y chromosome technique show the X chromosome bearing spermatozoa in the range of 95.92 % to 97.99% and Y chromosome bearing spermatozoa in the range of 2.1 % to 4.08 % for *Bos indicus* and *Bos taurus* breeds. The X bearing spermatozoa in sex-sorted semen samples were more than 95% indicating very high probability for birth of female progeny.

The qPCR technique described in the present study is a rapid and reliable technique to quantifying the sex ratio of X- and Y- chromosome bearing spermatozoa in sexed and unsexed semen sample. This method is a suitable tool for routine verification of bovine X- and Y- chromosome bearing spermatozoa in sorted semen samples or for validation and calibration of other related techniques, not only in chromosome studies, but in any analysis of quantitative detection of various genes for other applications.

Comparative sequence analysis between sexed and unsexed semen samples of *Bos indicus* and *Bos taurus* breeds showed there is no change in nucleotide and protein sequence in *SRY* gene region whereas for *PLP* gene region it is noticed that there are nucleotide substitution T10610C and insertion of A at 10679 and 10680 position in *PLP* gene of all (*Bos taurus* and *Bos indicus)* breeds of unsexed and sexed semen samples whereas the base substitution G10721C was noticed only in *Bos taurus* and all these insertions fall in non-coding (intron) region only without affecting the protein coding sequence.

Unsexed semen samples in each dose/straw are having high semen concentration (19.93 ± 0.01 millions/straw), semen motility (65.98 ± 0.23%), acrosomal integrity (75.28 ± 0.05%), live spermatozoa (67.83 ± 0.42%) and less sperm abnormalities (13.82 ± 0.02%) when compared to the sexed semen sample doses/straw which have low semen concentration (1.98 ± 0.00 millions/straw), sperm motility (60.88 ± 0.18%), acrosomal integrity (70.79 ± 0.26%), live spermatozoa (63.58 ± 0.25%) and more sperm abnormalities (22.12 ± 0.18%). The inferior quality of the sex-sorted semen over unsexed semen may be due to sorting procedure.

In conclusion, the reference plasmids for *PLP* and *SRY* genes were constructed, described a definitive molecular method for quantification of sex-sorted spermatozoa, which is cheaper, rapid and more reliable. Further, there is no evidence of genetic variations found in *SRY* and *PLP* genes upon sex sorting. However, the quality

parameters of sex-sorted semen are likely to have been affected due to processing steps involved in it. An elaborate study may be performed to protect the spermatozoa during sex sorting.

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APPENDICES

APPENDIX – A

1X Phosphate-buffered saline

8 g of NaCl, 0.2 g of KCl, 2.44 g of Na2HPO4, 0.24 g of KH2PO4 were added in a glass bottle. The volume was made up to 1 L with Millipore water. The pH of the solution was adjusted to 7.2 and then sterilized by autoclave.

APPENDIX – B

10X TAE buffer

Tris base-24.2 g, Glacial acetic acid-5.7 ml, 0.5 M EDTA-10 ml was added in glass bottle. The volume was made up to 500 ml with distilled water.

APPENDIX – C

DNA lysis buffer (50 ml)

1. 1M tris base = 0.5ml (500µl)
2. 0.5M EDTA = 0.1ml (100µl)
3. 10% SDS = 0.5ml (100µl)

Volume makes up to 50ml by adding Double Distilled Water.

1M Tris

Tris Base = 0.6055 grams

Distilled Water = 5ml

0.5M EDTA (PH 7.5)

EDTA = 0.9305 grams

Distilled Water = 5ml

Adjust the PH by Adding one NAOH Pellets and for dissolve of EDTA powder also

10% SDS

SDS powder = 0.5 gram

Distilled Water = 5ml

APPENDIX – D

<u>1M DDT (Di thriothreitol)</u>

Dissolve 0.15 grams of DDT powder in 1ml of NFW in a 2ml of Eppendorf tube and wrap with an alumin.um foil and store at -20°C.

APPENDIX – E

Preparation of semen diluting fluid

Water soluble Eosin – Y	- 0.05 gm
Sodium chloride	- 1.00 gm
Formalin (Qualigens chem, Mumbai, India)	- 1 ml
Distilled water made up to	- 100 ml

APPENDIX – F

Preparation of Eosin and Nigrosine stain

Eosin solution (Nice chemicals private limited, Cochin, India) and Nigrosine solution (Hi-media laboratories, Mumbai, India) were used for this study.

APPENDIX – G

Preparation of Giemsa working solution

A. Stock Giemsa solution	- 3 ml
B. Sorensons phosphate buffer	- 2 ml
C. Double distilled water	- 35 ml

A) Giemsa stock solution

Giemsa's stain solution (Qualigens fine chemicals, Mumbai, India) was used as stock solution for staining the semen smear for acrosomal evaluation.

B) Preparation of Sorenson's phosphate buffer

Solution A:

Disodium hydrogen phosphate	- 21.682 gm

Distilled water - 500 ml

Solution B:

Potassium dihydrogen phosphate - 22.254 gm

Distilled water - 500 ml

200 ml solution of A mixed with 80 ml of solution B. Then from the resultant 280 ml, 100 ml is taken as stock buffer solution.

APPENDIX - H

Preparation of Hypo-osmotic Solution (150 milliosmole/kg):

Composition:

Sodium citrate dihydrate - 0.735 gm

Fructose - 1.351 gm

Dist. Water - 100 ml

Taken 100 ml of distilled water in conical flask and added sodium citrate and fructose one after another after dissolving.

APPENDIX - I

Preparation of Rose Bengal stain:

Composition:

Rose Bengal stain (powder) - 3 gm

Commercial formalin - 1 ml

Distilled water - 100 ml

To the distilled water, formalin was added and mixed well. Then, rose Bengal powder was added and mixed well. The mixture was filtered using whatmann filter paper No. 1.

APPENDIX - J

Preparation of semen extender

Composition of Egg yolk citrate (EYC) extender:

Part A:	Sodium Citrate	-	2.9 gm
	Streptomycin	-	1000 µg/ml
	Penicillin	-	1000 IU/ml
	Distilled water made up to	-	100 ml
Part B:	Egg yolk		

20%-part B was mixed with Part A i.e., 20 ml of egg yolk was mixed with 80 ml of part A.

Preparation of Egg yolk citrate extender:

a. After buffer preparation, it was autoclaved at 5 psi for about 10 minutes and cooled.

b. Then unfertilized fresh egg (one day old) was washed with warm water and wiped with 70% Alcohol.

c. Shell was cracked and broken at the narrow end with the help of sterilized knife.

d. Albumin. and yolk were separated and yolk was transferred on to a sterile filter paper and rolled gently on it for removing the traces of albumin. without damaging the yolk membrane.

e. The yolk membrane was punctured with a sterile needle and yolk was collected in sterilized measuring cylinder.

f. Egg yolk was added to buffer solution and then it was mixed properly by using magnetic stirrer for 30 min.

Finally, Streptomycin and penicillin antibiotic were added to the dilutor just one hour prior to semen collection and the dilutor volume was made up to 100 ml by adding Egg yolk citrate buffer solution.