OPEN ACCESS
Advances in Animal and Veterinary Sciences

Review Article

Molecular Approaches for Gender Identification and Sperm Sex Ratio Determination in Farm Animals

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Abstract | Mammalian males are heterogametic (XY), while females are homogametic (XX). The accurate identification of the X- and Y-chromosome bearing sperm population and sort them precisely before fertilization event holds promising applications in gender pre-selection and for effectively managing the herds of farm animals. Further, gender identification of embryos during early pregnancy has also been a subject of great interest for animal breeders. Interestingly, the recent reports demonstrated the significant deviation of X- and Y- sperm population from normal (1:1) sperm sex ratio within ejaculates from the same bull or among different bulls. Several techniques, based on the different hypotheses proposed by different research groups, have been used for semen sexing. But none of these, except for flow cytometry based semen sexing, gained wide acceptability due to their low reproducibility and accuracy. Moreover, the reanalysis of flow sorted sperm populations is still required to further validate the purity of the sexed semen. The most commonly used molecular techniques for purity validation of sexed semen include fluorescence in situ hybridization and polymerase chain reaction based approaches. The present review discusses the use of these molecular techniques for gender identification, sperm sex ratio determination and purity validation of semen sexing techniques in farm animals.

Keywords | Fluorescence in situ hybridization, Polymerase chain reaction, Sperm, Semen sexing, Sex ratio

Editor | Kuldeep Dhama, Indian Veterinary Research Institute, Uttar Pradesh, India.
Received | March 04, 2017; **Accepted** | April 17, 2017; **Published** | September 06, 2017
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Citation | Singh J, Yadav SK, Gangwar DK, Singla SK (2017). Molecular approaches for gender identification and sperm sex ratio determination in farm animals. Adv. Anim. Vet. Sci. 5(9): 377-387.
DOI | http://dx.doi.org/10.17582/journal.aavs/2017/5.9.377.387
ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331

INTRODUCTION

Production of the calves of desired sex has attracted attention long back in dairy as well as beef industry. The gender of a mammalian offspring is determined by the type of sperm (X- or Y- sperm) fertilizing the ovum. The techniques to identify the X- and Y- sperm individually and sort them precisely offer great advantage in preselecting the gender of the offspring of agriculturally important animals. Over the last few decades, several techniques such as X- and Y- chromosome detection by semi-quantitative polymerase chain reaction (PCR) (Lobel et al., 1993; Chandler et al., 1998; Chandler et al., 2002, Chandler et al., 2007), capillary electrophoresis (Checa et al., 2002), Quincarine mustard staining for the identification of Y-chromosome (Ogawa et al., 1988), Southern blotting (Beckett et al., 1989), single sperm PCR analysis (Szyda et al., 2000) have been attempted for sperm sex determination in semen samples from different mammalian species. The most reliable and robust tool for separating X- and Y-chromosome bearing spermatozoa (CBS), till date, is fluorescence activated cell sorter (FACS) which utilizes the DNA content differences found in X- and Y- sperm of different mammalian species. Thousands of offspring of desired sex via artificial insemination using flow sorted sexed spermatozoa have been produced (Siedel and Garner 2002). Determination

of the proportion of X- to Y- sperm population (i.e. sperm sex ratio) in semen samples has been a subject of great interest for animal breeders. Several studies have focussed on sperm sex ratio determination in individual ejaculates and possible variation among ejaculates from same bull or different bulls. Significant variation from expected ratio (1:1) in the semen ejaculates and their correlation studies with progeny data (Clutton Brock and Iason, 1986; Chandler et al., 1998; Chandler et al., 2007; Rorie et al., 2014) sparked further interests for sex ratio determination in semen samples. In addition to semen sexing, the molecular approaches used for sex ratio determination could also be used for purity validation of different sorting techniques (Maleki et al., 2013). The purity of FACS sorted semen which is generally determined by reanalysis of the sorted semen using the same instrument makes the analysis approach not truly unequivocal and thus emphasizes the need for reliable alternative approaches for true validation (Parati et al., 2006; Oi et al., 2013). Furthermore, a large number of spermatozoa are required for sort reanalysis by FACS (Colley et al., 2008; Prakash et al., 2014). To address these issues, the two major alternative approaches reported for sort reanalysis include whole semen sex ratio determination and single sperm sex identification (Maleki et al., 2013). Fluorescence in situ hybridization (FISH) technique has been used by various groups for single sperm sex identification (Schwerin et al., 1991; Kawarasaki et al., 1998; Piumi et al., 2001; Rens et al., 2001; Di Berardino et al., 2004; Habermann et al., 2005; Yan et al., 2006). This approach appears quite promising and is particularly helpful to determine sperm sex ratio for valuable low sperm count population (Maleki et al., 2013). PCR based approaches, due to their inherent simplicity and high sensitivity, have recently been used as promising techniques for whole semen sex ratio determination. Gender identification of early stage embryos using molecular approaches has been helpful for avoiding sex linked inheritance and to manage herds by making early decision on pregnancy. The present review focuses on the FISH and PCR based molecular approaches used for the gender identification of sperm and early stage embryos; and sex ratio determination in semen samples from different farm animal species. The review also discusses the important role of these molecular techniques for purity validation of sperm enrichment or sorting techniques used for semen sexing.

Fish Approach for Sexing and Validation of Sperm Enrichment Techniques

FISH is a sensitive technique for molecular diagnosis of chromosomes on single cell level and has been applied successfully for embryo sexing, sex ratio determination in semen samples and for the validation of different sperm enrichment methods as discussed below.

Embryo and Sperm Sexing by Fish

Sex pre-selection before conception is of growing interest for animal breeders especially in bovines. Bovine embryo sexing with >90% accuracy has been reported from biopsied blastomeres using Y-chromosome specific DNA probes (Lee et al., 2004; Cenariu et al., 2008). Hassanane et al. (1999) developed double colour FISH using sex chromosome specific probes for simultaneous detection of X- and Y- sperm in the semen samples of Swedish Holstein Friesian bulls. The observed ratio of X- and Y- chromosome bearing sperm cells was close as expected (1:1) with no inter-individual variation. Revay et al. (2002) reported the method for simultaneous evaluation of viability and sex of the bovine spermatozoa. In this approach, only live sperm were decondensed using modified decondensation protocol thereby making the distinct identification of live sperm heads from that of dead ones very easily. FISH provides the advantage of giving clear picture of individual sperm rather than the whole population of spermatozoa. Further, the chromosomal aneuploidy in the individual sperm (XX, YY, XY) can also be detected when multiple probes hybridize simultaneously in the same sperm cell (Flaherty and Matthews, 1996). Thus, the technique bears important application to help avoid inheritance of sex linked genetic defects in offspring due to their early detection during pre-implantation embryonic stages.

Validation of Sperm Enrichment Techniques by Fish

FISH has been used as an important tool for efficiency validation of different sperm enrichment techniques emerged during the history of semen sexing. Chromosome Y and chromosome 1 specific DNA probes were used in FISH for purity validation of flow sorted porcine sperm (Kawarasaki et al., 1998; Parilla et al., 2003). Piumi et al. (2001) performed cytogenetic labelling of bovine X- or Y-CBS using X-chromosome specific probe and Y- chromosome specific probe. Positive signals were obtained from approximately 45% of the sperm for the each probe used. Rens et al. (2001) used XY paint set developed from sorted yak chromosome for sexing cattle spermatozoa. The proportion of X- and Y-spermatozoa in unsorted samples did not differ statistically from expected normal ratio (1:1). Kobayashi et al. (2004) used FISH technique to study the efficacy of discontinuous Percoll density gradient sperm enrichment method. After Percoll separation, the percentage of Y-sperm in top fraction (52.9%) exceeded slightly than the bottom fraction (44.3%) showing deviation from theoretical ratio (1:1) of X- and Y- sperm. Further, the sperm washing with Bracket and Oliphant (BO) medium also made significant difference in sex population of sperm compared to unwashed sperm. To validate the accuracy of sperm sexing in *Bos taurus*, Habermann et al. (2005) developed a simple, fast and reliable dual colour FISH approach. Using this approach, Y-CBS were identified by a DNA

fragment hybridizing to a large pericentromeric repetitive DNA region on the bovine Y-chromosome. Bovine autosome 6 specific DNA probe was used as a positive control to avoid underestimation of the Y signals. The FISH protocol evaluated on unsorted sperm as well as on FACS sorted sperm samples worked reliably with hybridization efficiency close to 100%. Oi et al. (2013) demonstrated direct visualization of sex chromosomes in individual canine sperm. The purities of flow sorted spermatozoa of the three dogs as verified by dual color FISH ranged from 88% to 93% for the X-chromosome fraction and 86% to 93% for the Y-chromosome fraction with the hybridization efficiency ranging between 97–99%.

The FISH method of embryo sexing is time consuming, labour intensive and more difficult to apply than the PCR sexing, but it can represent an alternative especially for research purposes (Cenariu et al., 2008). Further, the sperm being highly condensed structure requires appropriate nuclear de-condensation which is crucial for obtaining successful hybridization results (Parrilla et al., 2003).

Gender Identification by Pcr Based Approaches

Sex determination in farm animals is of utmost importance for animal breeders to manage their breeding stocks effectively (Tavares et al., 2016). Sex pre-selection of the offspring before implantation requires embryo biopsy (Johnson et al., 2005). The sexing of embryos prior to transfer bears commercial application in dairy and beef industries. For wide application, the sex determination techniques need to be simple, repeatable, robust, cost effective and easy to perform. PCR based approaches hold promise for sensitive and precise sex determination in animals at early embryonic stages as well as after birth. Several studies reported the use of PCR based approaches for gender identification in farm animals (Table 1). Several sex chromosome specific genes such as *SRY*, *TSPY*, *FBNY*, *AMELY*, *ZFY* (Y chromosome specific), *ZFX*, *AMELX*, *F9*, *PLP*(X chromosome specific) have been used as markers for sex identification. Initial efforts were made for sex determination of biopsied bovine embryos at different pre-implantation stages by conventional PCR using sex specific primers (Machaty et al., 1993; Ennis and Gallagher, 1994). Another study demonstrated simultaneous amplification of autosomal (*FBN17*) and male specific DNA sequence (*FBNY*) from bovine DNA samples and embryo biopsies by conventional PCR performed using only one primer pair (Weikard et al., 2001). The bovine male specific target could be detected even in very low concentrations of genomic DNA. Subsequently, unique non-electrophoretic PCR was developed for bovine embryo sexing. Accuracy of sex determination by this method was 98.7% for male embryos and 94.4% for female embryos as confirmed by fetal ultrasound sexing and calving (Hasler et al., 2002). Manual biopsies from the bovine embryos have been utilized for sex determination based on the Y-chromosome amplification detected directly from the tube (Bredbacka et al., 1995), thus avoiding the need for micromanipulators and gel electrophoresis. Chen et al. (2007) developed a simple and accurate (97.4%) conventional PCR for sex determination from goat embryos by amelogenin locus amplification. Lopatarova et al. (2008) performed sex determination from bisected bovine embryos (day 7) and evaluated conception rate of sexed demi embryos after transfer for production of sex desired calves (heifers). The method appears useful for producing sex-desired calves in embryo transfer programs. Pre-implantation genetic diagnosis to determine gender of equine embryos before transfer has recently been successfully performed (Herrera et al., 2014). Heat treatment of equine embryo biopsies (10 min at 95°C) before PCR amplification resulted significant increase in the gender determination rate. Multiplex PCR for the sexing of 8- to 16-cell stage bovine embryos from single blastomere without causing trauma to biopsied embryos was successful for sexing groups of 8, 4, 2 and 1 blastomere(s) with the sexing efficiency of 100.0, 96.3, 94.3 and 92.1%, respectively (Park et al., 2001). Recently, a fast and highly sensitive PCR based method has been reported for livestock embryo sexing (Tavares et al., 2016). The sexing efficiency of whole embryos or embryo biopsies (sheep and cattle) was 100% for embryo biopsies, 98% for sheep embryos, and 90.2% for cattle embryos. The authors claimed that the protocol takes very less time (approximately 2 h) and can be applied to field conditions.Lemos et al. (2005) used Y-chromosome specific gene, *TSPY* for sex identification in cattle. The assay was found highly specific and sensitive enough to work at very low DNA concentrations (1 pg/µl). Another study reported for the first time, the applicability of *TSPY* as a Y-specific marker for sexing of pre-implantation bovine (*Bos indicus*) embryos from single blastomere (Carneiro et al., 2011). In mammals, the amelogenin genes are present on both X- and Y- chromosomes (Pajares et al., 2007). Several studies have used conventional PCR based amplification of amelogenin from genomic DNA for gender identification in various species including porcine (Fontanesi et al., 2008), bovine (Khaledi et al., 2009), sheep and red dear (Pfeiffer et al., 2005). The assay was found highly sensitive for sex identification from very small amount of genomic DNA (20 pg) and showed high specificity without any cross amplification with other species (Fontanesi et al., 2008). However, very small sample size (3 male and 3 female) was used for the bovine study and might need further validation with large sample size and other breeds of *Bos indicus* for differentiating *Bos taurus* and *Bos indicus* males (Khaledi et al., 2009) Pomp et al. (1995) tested *SRY/ZFY-ZFX* duplex PCR system for sex determination in several mammalian species including cattle, sheep, goats, llamas, horses, humans, baboons, dogs, cats, rats and mice. Highly specific and accurate duplex PCR assays using SRY gene specific

Table 1: Different PCR based approaches used for gender identification

Reference	Species	Technique(s) used	Gene(s) studied	Salient findings
Machaty et al., 1993	Bovine	Conventional PCR	Y chromosome and bovine DNA specific	Determined sex of bovine embryos (16-32 cell stage) taking single blastomere biopsied from each embryo.
Bredbacka et al., 1995	Bovine	Conventional PCR	*ZFX* and *ZFY*	Developed simplified protocol for detection of Y chromosome amplification from bovine embryos in the tubes directly.
Hasler et al., 2002	Bovine	Modified conventional PCR	Ampli-Y ™	Non-electrophoretic PCR based embryo sexing method developed for use in commercial embryo transfer program.
Weikard et al., 2001	Bovine	Conventional PCR	*FBNY* and *FBN 17*	Developed PCR based method for sex determination of bovine DNA samples and embryo biopsies. New male specific DNA sequence (*FBNY*) was amplified.
Ennis and Gallagher, 1994	Bovine	Conventional PCR	*AMEL*	Established a method for sexing bovine embryos (6-7 days old) using amelogenin locus specific primers.
Kageyama et al., 2004	Bovine	Conventional PCR	Repeat sequence *S4*	Bovine embryo sexing based on novel repeat sequence (male specific) PCR from genomic DNA with high sensitivity (0.5pg).
Khaledi et al., 2009	Bovine	Conventional PCR	*AMEL*	Molecular sexing of *Bos indicus* (Brakmas Brahman × Kedah Kelantan) cattle using amelogenin sex specific primers.
Lopatarova et al., 2008	Bovine	Commercial PCR kit	Y-chromosome determinant	Performed sex determination after micro-surgical splitting of bovine embryos and evaluated the conception rates of sexed embryos after transfer.
Zeleny et al., 2002	Bovine	Conventional PCR	*AMEL*	Developed a robust and accurate amelogenein PCR based method for beef sexing.
Gokulkrishnan et al., 2012	Bovine	Conventional PCR	*AMEL*	Amelogenin specific locus was amplified for cattle meat sex determination.
Curi et al., 2002	Bovine	Conventional PCR	*BRY1*	Demonstrated cheap and simple method for sexing of bovine carcass using male specific primer.
Lemos et al., 2005	Bovine	Conventional PCR	*TSPY*	Developed PCR based sex identification in cattle using *TSPY* gene specific primers.
Fontanesi et al., 2008	Porcine	Conventional PCR	*AMELX* and *AMELY*	Developed highly sensitive and species specific sex determination assay based on the sequence variability between porcine *AMELX* and *AMELY* genes.

Chen et al., 2007	Caprine	Conventional PCR	*AMEL*	Developed method for sex determination from goat biopsied embryos.
Tavares et al., 2016	Bovine and Ovine	Conventional PCR	*AMEL*	A fast and highly sensitive PCR based method reported for livestock embryo sexing.
Pfeiffer et al., 2005	Ovine and Red deer	Conventional PCR	*AMEL*	Developed sex determination method from genomic DNA isolated from tissue samples of sheep and red deer using X- and Y- chromosome specific variants of the amelogenin gene.
Torner et al., 2013	Porcine	Duplex PCR	*X12696, SUS12S, X515551*	Developed highly sensitive and reliable novel duplex PCR method for sexing in porcine based on the amplification of porcine repetitive sequences.
Phua et al., 2003	Caprine	Duplex PCR	*SRY* and *AMELX*	A highly reliable and accurate method for sex identification in caprine using simultaneous amplification of *SRY* and *AMELX*.
Choi et al., 2009	Porcine	Duplex PCR	*SRY*	Sexing of porcine offspring was done by amplification of the HMG box of *SRY* gene.
Herrera et al., 2014	Equine	Duplex PCR	Equine *SRY* and *AMEL*	Developed method for equine embryo sexing before embryo transfer and confirmation by ultrasonography.
Pomp et al., 1995	Bovine, Ovine, Caprine, Equine, Canine, Feline and Llamma	Duplex PCR	*SRY, ZFX* and *ZFY*	Developed duplex PCR method for sex determination in several mammalian species and performed porcine early stage embryo sexing to study effect of sex on embryonic diameter.
Prashant et al., 2008	*B. frontalis*, *B. grunniens*, *B. indicus*, *Bubalus bubalis*, *Capra hircus*, *Ovis aries*.	Duplex PCR	*SRY* and *GAPDH*	Developed highly specific and accurate method for sex determination of 6 major domesticated species of the family Bovidae.
Carneiro et al., 2011	Bovine	Nested PCR	*TSPY*	First report demonstrating applicability of *TSPY* as a Y-specific marker for sexing of pre-implantation bovine (*Bos indicus*) embryos from single blastomere.
Ballin and Madsen, 2007	Bovine	qPCR	*AMEL*	Developed melt curve analysis based highly reliable and fast method for beef sex determination.
Park et al., 2001	Bovine	Consecutive and multiplex PCR	*BOV97M* and bovine 1.715 satellite DNA sequences	Developed a rapid (within 2 hours) and effective method for the sexing of 8- to 16-cell stage bovine embryos using a single blastomere without compromising the developmental potential of demi-embryos.

Bai et al., 2010	Bos gruniens	Multiplex PCR	*SRY*	Demonstrated method for Yak meat sexing using *SRY* gene specific primer.
Herrero et al., 2013	Bovine	Multiplex qPCR	Bovine specific and Y chromosome specific	Developed a highly specific, sensitive and rapid method for beef sex determination using multiplex real time PCR.
Zhang et al., 2014	Bovine, Ovine and Caprine	Multiplex PCR	Microsatellite markers and *SRY*	Developed method for sex determination in ruminants using microsatellite markers and *SRY* gene specific primers.

primer in combination with housekeeping gene or other chromosome Y specific primer have also been reported for gender identification in 6 major domesticated species of the bovidae family (Prashant et al., 2008) and for porcine sexing (Choi et al., 2009) respectively. The porcine sexing primers were also successfully used further for validation of sperm sorting method. Phua et al. (2003) reported a highly reliable and accurate method for sex identification in caprine using simultaneous amplification of *SRY* and a portion of amelogenin gene on X-chromosome (*AMELX*). The method was sensitive enough to detect sex specific amplification up to 1 microgram of DNA. Torner et al. (2013) reported a novel duplex PCR for porcine sexing based on the amplification of porcine repetitive sequences for sexing porcine tissues, embryos and single cells. The *SUSYb*/*SUS12S* primer-based procedure were found successful for sexing porcine single cells, in vitro produced embryos (100% efficiency) as well as blastocysts (96.6% accuracy; 96.7% efficiency). Kageyama et al. (2004) identified a novel repeat sequence S4 (male specific) from bovine genomic DNA isolated from liver cells. The S4 specific primer amplified male specific product (178 bp) in addition to a product common (145 bp) for both sexes. PCR amplification was accurate and highly sensitive (requires only 0.5 pg of template DNA) due to high copy number of the sequence. Zhang et al. (2014) developed multiplex PCR for sex determination in ruminants viz. sheep, cattle and goat, using four microsatellite markers and *SRY* gene specific primers. In addition to sex identification, individual differences between different species as well as within the same species could be detected based on the expression pattern of microsatellite markers.

Several studies emphasized on precise sexing of beef meat to minimize frauds in beef trade. Beef meat sexing by conventional PCR has been reported using male specific BRY1 primer (Curi et al., 2002) and allele specific primer for amelogenin gene (Zeleny et al., 2002; Gokulkrishnan et al., 2012). Ballin and Madsen, (2007) reported highly reliable and robust qPCR for beef meat sexing based on melt curve analysis of sex specific PCR amplicons of amelogenin loci. Multiplex real time PCR was also found highly sensitive and rapid method for beef sexing. This method worked well with all kind of meat products including intensively processed meat samples (Herrero et al., 2013). Another study reported yak meat sexing using multiplex PCR via amplification of male specific *SRY* gene (Bai et al., 2010).

Sex Ratio Determination and Purity Validation by PCR Based Approaches

Various groups employed different PCR based approaches for sex ratio determination in farm animals and for the purity validation of sex sorted semen samples as discussed in this section. These PCR based approaches mainly include conventional PCR and other variants of PCR viz. nested PCR, duplex PCR, qPCR (Table 2) Chandler et al. (1998) studied the percentage of Y chromosome bearing sperm (%Y-CBS) within sires and in different ejaculates from the same bull using PCR for Y-chromosome specific gene. Results indicated that %Y-CBS varied from 24 to 84% and these variations contributed to variation in the percentage of male calves (16.1 to 72.3%) as evidenced from calving data. Subsequently, the same group studied the effect of collection frequency on semen sex ratio using conventional PCR (Chandler et al., 2002). In the first collection, the percentage of Y-sperm was highly variable among all bulls and these variations decreased in subsequent collections with the least variation in last 2 collections. However, another study found no significant difference in % Y-CBS between bulls or ejaculates (Madrid Bury et al., 2003). Taylor, (2005) studied semen sex ratio and the ratio of the calves produced based on the expression of *SRY* and *PLP* genes using conventional PCR. A significant correlation was found between predicted % Y-sperm and % male calves but significant variance was found between ejaculates within the bull for both. Jorge and co-workers, (2004) were the first to validate the flow sorted bovine spermatozoa using *SRY* and *MSHR* (autosomal) gene specific primers by qPCR. The ratio of male to female sperm cells in unsexed semen was determined. However, the authors opined the need for further optimization of the method for different samples. Accurate determination of the proportion of X- and Y-CBS in bovine semen samples using qPCR was reported by Parati et al. (2006). The results of X- and Y- sperm sorted semen samples analysed by qPCR

Table 2: Different PCR based approaches used for sperm sex ratio determination and purity validation.

Reference	Species	Technique used	Gene(s) studied	Salient findings
Chandler et al., 1998	Bovine	Conventional PCR	*BRY1a* and *BRY1b*	Studied sex ratio variation between ejaculates within sires.
Chandler et al., 2002	Bovine	Conventional PCR	Y-chromosome specific	Effect of semen collection frequency on sperm sex ratio was studied.
Madrid-Bury et al., 2003	Bovine	Conventional PCR and semi-quantitative PCR	*AMEL*	Studied feasibility of double swim up procedure to alter sperm sex ratio.
Taylor et al.,2005	Bovine	Conventional PCR	*SRY* and *PLP*	Compared calf sex ratio and semen sex ratio.
Wang et al., 2011	Bovine	Rapid single sperm PCR	*SRY*	Single sperm sexing of sperm isolated from unsorted semen (Holstein cattle) was done by using rapid single sperm PCR.
Colley et al., 2008	Bovine	Nested PCR	*AMEL*	Developed an adaptable, accurate, and reliable tool for single sperm sex typing.
Malik et al., 2011	Bovine	Nested PCR	*AMEL*	Verification of X and Y chromosome carrying spermatozoa was performed after separation with swimming speed using oestrus cows vagina mucus, Percoll discontinuous gradient (45 to 90%) and swim-up using TALP medium.
Chandler et al., 2007	Bovine	Duplex PCR	*SRY* and *F9*	Studied the correlation between predicted semen sex ratio with calving sex data.
Joerge et al., 2004	Bovine	qPCR	*SRY* and *MSHR*	Validated sperm sorting efficiency by calculating Y sperm proportion in sexed semen samples based on *SRY* amplification relative to autosomal (*MSHR*) gene fragment.
Parati et al., 2006	Bovine	qPCR	*SRY* and *PLP*	Novel real time PCR based method for determination of the proportion of X- and Y- sperm in bovine semen sample was developed.
Puglisi et al., 2006	Bovine	qPCR	*SRY* and *PLP*	Developed efficient protocol for in vitro production of predefined sexed embryos using sexed frozen-thawed bull semen.
Resende et al., 2011	Bovine	qPCR	*SRY* and *PLP*	Enrichment of X-chromosome bearing spermatozoa was performed after one centrifugation in a Percoll or OptiPrep continuous density gradient, using qPCR.
Maleki et al., 2013	Bovine	qPCR	*SRY* and *PLP*	Study provides reliable and inexpensive way to test sexual chromosome content in bovine semen samples.
Korchunjit et al., 2014	Porcine	qPCR	Y-chromosome specific	Rapid single sperm typing protocol was described using SYBR green real time PCR
Somarny et al., 2014	Caprine	qPCR	*ZFX* and *SRY*	Real time PCR based validation of free flow electrophoresis sex sorted caprine spermatozoa using sex specific primers was performed.
Rorie et al., 2014	Bovine	qPCR	*SRY* and *F9*	Studied variation in the ratio of X- to Y-sperm of individual ejaculates to determine association between skewed sex ratio and routine morphological evaluation or CASA.
Tan et al., 2015	Bovine	qPCR	*ZFX* and *SRY*	qPCR based quantification of bovine X and Y sperm from frozen thawed unsexed semen samples was performed.
Khamlor et al., 2014	Bovine	Multiplex qPCR	*SRY* and *PLP*	Developed multiplex qPCR based method for evaluating purity of sexed semen.

and by flow cytometric reanalysis showed no significant difference (P > 0.05). Another study used frozen thawed bovine semen for sperm sorting by flow cytometry and then performed IVF to produce embryos of predefined sex (Puglisi et al., 2006). Chandler et al. (2007) found significant correlation(r = 0.82, P < 0.0002) between predicted semen sex ratio and calving sex ratio. No significant variance between sires was found in predicted semen sex ratio and calving sex ratio, but lots within sires differed significantly for both. Single sperm sexing based on amelogenin expression by nested PCR was reported for sex ratio prediction in bovine (Colley et al., 2008). However, the technique might be impractical for routine semen evaluation for X/Y sperm ratio. Duplex PCR based amplification of the HMG box of *SRY* gene was used as a fast and reliable method for precise sexing and evaluation of sorting accuracy in porcine (Choi et al., 2009). Malik et al. (2011) performed verification of bovine X- and Y- CBS after separation with swimming speed using oestrus cows vagina mucus, Percoll discontinuous gradient (45 to 90%) and swim-up in Tyrode's albumin lactate pyruvate medium. Another study reported enrichment of X-CBS in bovine semen after Percoll or continuous density gradient by qPCR (Resende et al., 2011). Rapid single sperm PCR for the first time was done for identification of X- and Y-CBS in Holstein cattle (Wang et al., 2011). Around 90% sperm could be successfully typed, out of which 48% of this population constituted X-chromosome specific sperm fraction. The authors concluded that this technique could be successfully used for sperm typing from unsorted samples. A highly reproducible and inexpensive qPCR based method was developed by Maleki et al. (2013) to test sex chromosomes content in semen samples using Taqman probes for *SRY* and *PLP* genes. Significant differences were observed for sorted semen (93.3±0.08% X- and 91.4±0.06% Y-sperm). The method was highly accurate (98.2%) with good repeatability and reproducibility. Rapid single sperm typing protocol was reported for boar semen sexing by SYBR green real time PCR using Y chromosome specific primers (Korchunjit et al., 2014). Validation of this protocol for whole semen revealed 52% Y-sperm population and 48% X-sperm population comparable to that of theoretical (1:1) ratio of X- and Y-sperm. A rapid, cost effective and reliable method for determining the sex ratio was developed using a multiplex qPCR for purity validation of bovine sexed semen (Khamlor et al., 2014). Both X- and Y- sperm were simultaneously quantified in a single tube with high amplification efficiency (97-99%) comparable to that of separate tubes simplex real time assay. Rorie et al. (2014) studied the sex ratio of individual ejaculates of semen using the relative standard curve of the qPCR based on amplification of X- and Y- chromosome specific fragments. The percentages of X-CBS were found similar (P > 0.5) across all collections. However, the mean %X-CBS between bulls was different (P < 0.05). No significant correlation was observed between CASA (computer assisted sperm analysis) parameters and %X-CBS across bulls. The results confirmed that the ratio of X- to Y- CBS may be skewed in some ejaculates of bull semen. Different combinations of CASA and morphological parameters were found to correlate with %X-CBS within 3 of 6 bulls. Validation of caprine sex sorted spermatozoa through free flow electrophoresis using sex specific primers (*ZFX* and *SRY*) has been reported (Somarny et al., 2014). Tan et al. (2015) used *ZFX* and *SRY* primers for quantitation of X- and Y- sperm in bovine semen samples. The percentages of unsexed X- and Y-CBS did not differ much from the expected (1:1) ratio, as reported in unsexed spermatozoa population. Further advancements in the PCR based techniques might help sex ratio determination at large scale for commercial applications.

CONCLUSION

Gender pre-selection methods may provide a huge leap in the genetic improvement programme and for the production of the offspring of desired sex in farm animals. Commercial sex pre-selection in cattle, an important animal of economic importance, is of utmost importance for dairy as well as beef industry. Gender pre-selection may also be helpful for production of elite bulls for breeding programs. Additionally, gender pre-selection holds promising potential for controlling rising stray cattle (male) population in coming future for the developing country like India where cattle slaughter is legally banned. For these applications to become reality, the very first step is to identify the X and Y sperm population accurately and thereafter sort them effectively. FISH approach bears important advantage of sperm sex identification at individual sperm level and screening for genetic abnormalities in pre-implantation embryos. The use of novel PCR based techniques like single sperm PCR based sexing, quantitative real time PCR, digital droplet PCR appear very promising for accurate identification of X- and Y- sperm population in unsorted semen as well as for reanalysis of semen sorted by FACS or by any other approach. Further, the gender pre-selection may help avoid the several sex linked inheritable diseases. PCR based methods for sex identification and validation of sex sorting are simple, accurate, easy to perform, cost effective and faster than previously employed *in situ* hybridization technique like FISH. PCR based methods can also be used for the validation of recently evolving techniques of sperm separation. In addition, the cattle meat sexing might have important impact on beef industry to avoid frauds in beef sale. Further advancements in FISH and PCR based molecular approaches may help achieve the sex identification and validation with higher accuracy in a highly robust and ultrasensitive manner and could be applied on large scale for commercial application.

ACKNOWLEDEGEMENTS

This work was financially supported by Indian Council of Agricultural Research through Incentivizing Research Scheme (grant number CS.11/7/2014-IA-IV).The authors are thankful to Sunny Dholpuria (Senior Research Fellow) and Sudha Saini (Research Associate), Embryo Biotechnology Laboratory for going through and making critical comments on the manuscript.

AUTHOR'S CONTRIBUTION

JS conceptualized, designed and wrote the manuscript. SKY and DKG contributed in literature collection, manuscript design and writing. SKS conceptualized, designed, made critical comments on manuscript draft and edited the manuscript for final submission.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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