Molecular identification of infertile bulls by using newly developed DDX3Y based on human STS markers

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Abstract To determine the role of DDX3Y gene in spermatogenesis and infertility in bulls, blood samples were collected from five infertile bulls (azoospermic; no sperm in the semen) at the Animal Breeding Center in Karaj, Iran. The recommended human primers by EAA/EQMN were investigated using the BLASTn database for STS marker detection. Alignment of STS marker genes with bovine genome was performed. Primer Premier 5 and CLC Main Workbench 5.5 softwares were used in designing common primer for the bovine and human DDX3Y gene. Genomic DNA screening of peripheral blood was conducted for detection of DDX3Y gene deletion in the Y chromosome by the PCR method. The bioinformatics analysis of human binding-primers of STS markers indicated that there was no chance connection and target fragment production for bovine samples. Investigation of DDX3Y gene on fertile bovine samples showed that the designed primer could detect the target gene as well. The results showed that three bulls had partial deletion in DDX3Y gene as a cause of their infertility. In conclusion, the DDX3Y gene can be a valuable marker to detect infertility in young male calves.

Keywords: bioinformatics, bull, DDX3Y gene, infertility, STS marker

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Introduction

Genomic selection has been applied for production traits in livestock breeding programs (VanRaden et al., 2009); however, molecular studies on male fertility and genomic selection for semen quality and male infertility are very limited. The Y chromosome carries several testis-specific genes that play fundamental roles in spermatogenesis and male fertility. Recent studies on human and bull Y chromosome have identified at least 3 types of sequence variations including SNP, insertion, deletion, and copy number variation (CNV) which provide genetic markers for male reproductive functions (Balaresque et al., 2008; Ginja et al., 2009; Bonfiglio et al., 2012; Chang et al., 2013). Of particular interest is the deletion (CNV) of the Y-linked multi-copy gene families that are all expressed predominantly or solely in testis (Skaletsky et al., 2003; Chang et al., 2013).

Tiepolo and Zuffardi (1976) reported six azoospermic patients with deletion (or microdeletions) on the long arm of the human Y chromosome and assumed that genes or gene families were located in the distal region of Yq11, defined as the AZF region. It has been reported that AZF region is divided into at least four non-overlapping regions (AZFa, AZFb, AZFc and AZFd) associated with spermatogenesis. Microdeletions of these subregions lead to spermatogenic failure, and are related to azoospermia and oligozoospermia at different stages of spermatogenesis (Raicu et al., 2003). Microdeletion of AZFa region is related to azoospermia and complete Sertoli cell-only syndrome (SCOS) (Nakashima et al., 2002). The most frequent genetic cause of severe oligozoospermia and azoospermia occurs in the AZFb and AZFc regions, while microdeletion of the AZFa region is relatively rare. Biochemical characterization of some gene products and functional studies of different animal orthologues led to the assignment of at least one strong candidate gene for each microdeletion interval, namely DDX3Y for AZFa (Brown et al., 1998), RBMY for AZFb (Ma et al., 1993, Elliott et al.,1997), and DAZ for AZFc (Reijo et al., 1995), all of which may cause azoospermia if deleted.

The European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) recommend using six STS (sequence-tagged sit) markers to screen the AZF microdeletions. The STS markers are short sequences of genomic DNA that can be uniquely amplified by the polymerase chain reaction

(PCR) using a pair of primers. Because each STS is unique, STSs are often used in linkage and radiation hybrid mapping techniques. The STSs serve as landmarks on the physical map of the human genome (Ishii et al., 2008; Elhawary et al., 2010). The absence of STS marker sequence is indicative of the site deletion (Kraucz et al., 2014). Three STS markers including of sY86, sY127 and sY254 have the highest sensitivity in detection of microdeletions (Iishi et al., 2008). The AZFa region is located in the proximal Y long arm, near the centromere and in the Yq11.21 position. The AZFa region contains the DDX3Y (former DBY) and USP9Y genes which are important in spermatogenesis (Vogt, 2004). This region is not found in domestic animals but the DDX3Y and USP9Y genes are located on the short arm of the Y chromosome in the bull (Liu et al., 2009). It has been suggested that DDX3Y plays an important role in fertility, and lack of this gene leads to remarkable reduction in the number of germ cells (Vogt, 2004). The DDX3Y is a member of the DEAD box protein family with 2 functional DDX3 genes on the sex chromosome (DDX3X and DDX3Y), one pseudogene on the X chromosome, and another pseudogene on the autosome in the human genome (Kim et al., 2001). The Y chromosome homolog DDX3Y is located in the AZFa interval of the male-specific region (MSY). Deletion of the AZFa region has been revealed to disrupt spermatogenesis, causing subfertility and infertility in healthy male (Foresta et al., 2008). Partial deletion in DDX3Y gene sequence in mice can lead to failure in spermatogenesis. The human and bovine DDX3Y have 3.16 and 8.10 Kb in length, respectively, and both encode one ATP-dependent RNA helicase which plays an important role in the final stages of spermatogenesis (Sun et al., 2000). The bovine and human DDX3Y are very similar and both have 17 exons and 16 introns (Liu et al., 2009).

The human DDX3Y gene has been considered as a strong candidate for AZFa, because the DDX3Y protein was found only in male germ lines (Ditton et al., 2004), and patients with the deletion of DDX3Y on their Y chromosomes exhibited either Sertoli cell-only syndrome or severe hypospermatogenesis (Foresta et al., 2000). The bovine DDX3Y gene has two transcript variants named DDX3Y short transcript variant (DDX3Y –S) and DDX3Y long transcript variant (DDX3Y –L) which are similar to long and short transcripts in humans and mice (Liu et al., 2009). These two transcripts are identical except for a 3-bp (AGT) insertion at the position of nt 2025. Both bovine DDX3Y isoforms have most likely overlapping with its homolog on X chromosome. Unlike the mouse counterpart, the DDX3Y and its homologs may play a different and indirect role in spermatogenesis and fertility such as a role in regulation of testosterone secretion (Liu et al., 2009).

To the best of our knowledge, the molecular identification of infertile bulls has not been reported, therefore, the aim of this study was to investigate the role of DDX3Y gene in bull infertility. Because no STS marker for diagnosis of infertility bulls is available, human known STS markers were used in the present study.

Materials and methods

Blood sampling

Blood samples were prepared from five infertile bulls (5 year of age) and one fertile bull kept at the Animal Breeding Center, in Karaj, Iran. Blood samples (about 5 mL) were collected from the jugular vein into tubes containing 12 mg of EDTA, and then stored at -20 °C until DNA extraction. Blood sample, with the consent of a fertile man with children and no history of assisted reproductive technology, was used as the control.

Bioinformatics analysis

The recommended primers by EEA/EMQN were analy-

Table 1. The primers sequence information used in this study

Primer name	Primer sequence	Amplicon size (nt)	Source
Forward- DDX	5´-CCTTGAGATTTCACGGGTTAGA-3´	338	Designed in this study
Reverse- DDX	5´-ATAGCACCGTAACAAATGACCT-3´	338	Designed in this study
sY86- Forward	5´-GTGACACACAGACTATGCTTC-3´	320	Simoni et al., (2004)
sY86- Reverse	5´-ACACACAGAGGGACAACCCT-3´	320	Simoni et al., (2004)
sY127- Forward	5´-GGCTCACAAACGAAAAGAAA-3´	274	Simoni et al., (2004)
sY127- Reverse	5´-CTGCAGGCAGTAATAAGGGA-3´	274	Simoni et al., (2004)
sY254- Forward	5´-GGGTGTTACCAGAAGGCAAA-3´	380	Simoni et al., (2004)
sY254- Reverse	5´-GAACCGTATCTACCAAAGCAGC-3´	380	Simoni et al., (2004)
SRY- Forward	5´-GAATATTCCCGCTCTCCGGA-3´	470	Simoni et al., (2004)
SRY- Reverse	5´-GCTGGTGCTCCATTCTTGAG-3´	470	Simoni et al., (2004)
AZF- Forward	5´-CTGTACTGACTGTGATTACAC-3´	495	Simoni et al., (2004)
AZF- Reverse	5´-TCTTTGGTATCCGAGAAAGT-3´	495	Simoni et al., (2004)

zed using the NCBI's BLASTn database for identification of STS markers. The sequences of STS markers were also analyzed using the online Multalin interface software (http://multalin.toulouse.inra.fr/multalin) and NCBI's BLASTn software for the multiple sequence alignment with the bovine genome. Primer Premier 5 and CLC Main Workbench 5.5 softwares were used in order to design common primer for bovine and human DDX3Y gene. Primer sequences and the size of related PCR products are shown in Table 1.

DNA extraction

The DNA extraction was performed with slight modifications on K0721 # GeneJET PCR Purification Kit (Thermo Scientific, USA). Briefly, 20 µL of Proteinase K Solution (50 µg/mL) and 200 µL of Lysis Solution (Tris pH 8.0+ NaCl+ EDTA pH 8.0+ SDS) were added to 200 µL of whole blood. The solution was mixed by vortexing until a uniform suspension was obtained. After incubation at 56°C for 20 minutes, the samples were transferred to the spin column and DNA was purified using Washing Buffer. The DNA was extracted by addition of Elution Buffer. The quality of extracted DNA was determined by using a NanoDrop® Spectrophotometer (Thermo ND-2000, USA) and agarose gel electrophoresis.

Polymerase chain reaction

The identified human primers of STS marker sequence were tested for human positive sample (fertile male) and negative control sample (fertile female) using multiplex-PCR for 5 pairs of primers (SRY, AZF, sY86, sY127, and sY254). Then, the PCR reaction was performed for the bovine samples under the same conditions as for the human samples. To amplify the DDX3Y gene on human and bovine Y-chromosome, a routine PCR was performed using the DDX primer, and the human and bovine genomic DNA were used as templates by Thermocycler (Biometra T-personal model) according to the following procedures.

In total, 25-µL reaction contained 18 µL of distilled water, 1 µL of each primer (5 pmol/µL), 1 µL $MgCl_2$ (50 mM), 2.5 µL PCR Buffer (10 ×), 0.5 µL dNTPs (10 mM), 1 unit Taq DNA polymerase, and 1 µL of genomic DNA (10 ng/µL).

Initial denaturation at 95 °C for 3 min and a subsequent series of 35 cycles of 94 °C for 30 sec (denaturation), 58 °C for 20 sec (annealing), and 72°C for 30 sec (extension) was performed. Final extension was carried out at 72°C for 10 min. The PCR products were subjected to electrophoresis on a 2 percent agarose gel prepared in 0.5 × TBE, stained with GelRed and visualized by exposure to ultraviolet light.

Results

The bioinformatic analysis of human binding-primers of STS markers used in this study (SRY, AZF, sY254, sY127, and sY86) indicated that there is no chance for connection and target fragment production for bovine samples. The DDX primer that was produced for the current study could amplify the target fragments in the man and bull. The Electrophoretic and spectrophotometry results revealed that the extracted DNA had good quality for PCR (Figs. 1 and 2). Electrophoresis of multi-PCR products detected five different bounds for positive human sample (fertile male) and one bound for the negative control sample (fertile female), but did not observe any bound on multi-PCR for bovine samples (Fig. 3). The investigation of PCR results for DDX3Y gene on fertile human and bovine samples indicated that the designed primer could amplify target gene as well (Figs. 4 and 5). The results of infertile samples also sho-

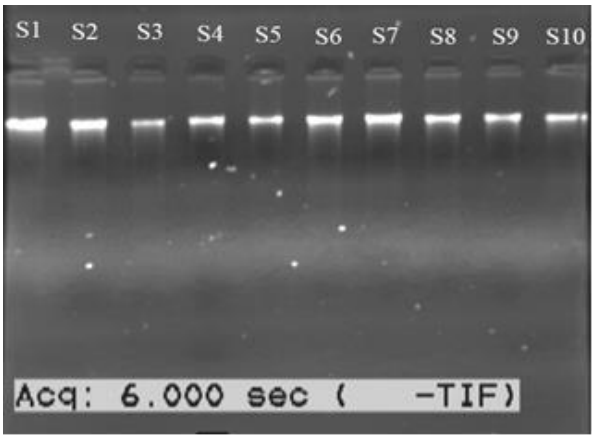

Figure 1. DNA extraction products on a 1% agarose gel (S1 to S10).

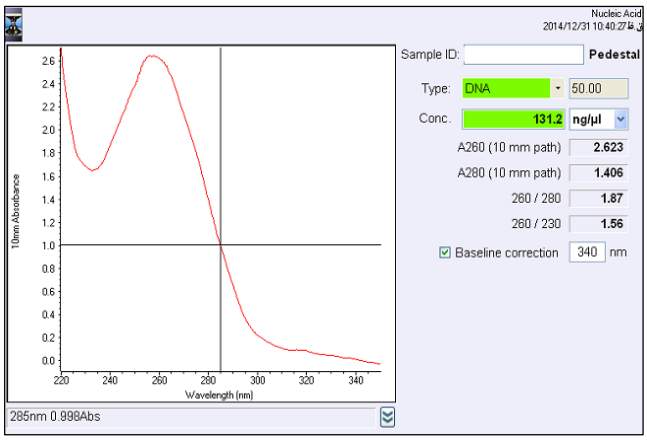

Figure 2. Measurement of DNA concentration using Nanodrop® spectrophotometer.

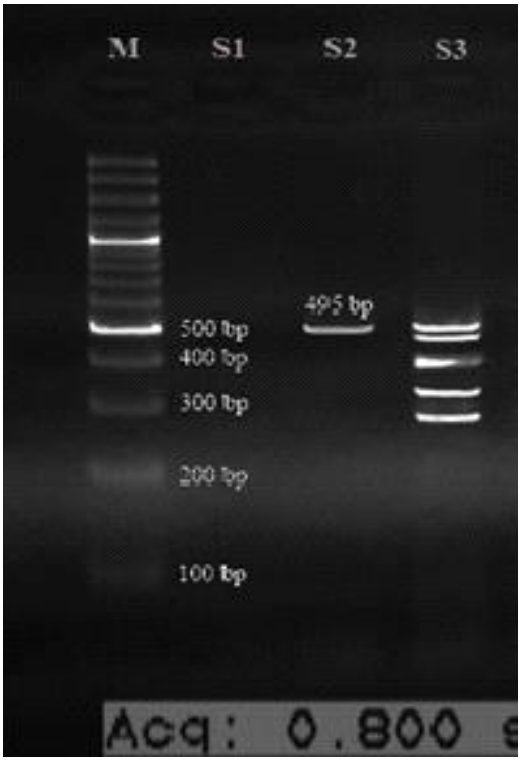

Figure 3. Multi-PCR products for fertile human samples (positive). S1: negative control (without DNA), S2: negative control (DNA of fertile female), S3: DNA of fertile male, M: size marker 100 bp.

wed that three bulls had partial deletion in DDX3Y gene and their infertility was due to genetic deletion of DDX3Y gene.

Discussion

Despite the high similarity between human and bovine genome, STS marker sequences that have been designed for the diagnosis of microdeletion and infertility in humans, could not be found in the bovine genome. Because of the short sequence of STS markers and minor differences between human and bovine genomes, these sequences were not found in bovine genome (Moore et al., 2001). According to the European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) protocol, diagnosis of microdeletions with STS markers does not need DNA sequencing. Therefore, only standard PCR and a subsequent agarose electrophoresis are enough to detect microdeletion occurrence. In contrast to previous reports, the present study indicated that the DDX3Y gene was directly associated with infertility. In addition, the deletion of sequence associated with DDX3Y primer indicative deletion of this gene causes infertility in human and bulls. The extensive similarity between human and bovine genomes confirms the hypothesis of occurrence of microdeletions, and consequently azoospermia and

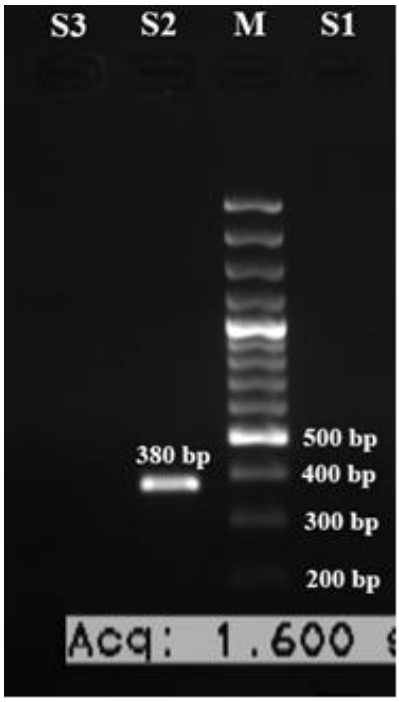

Figure 4. Presence or absence of DDX3Y gene sequence on DNA (fertile bull). S1: negative control (without DNA), S2: PCR product of fertile bull, S3: PCR product of infertile bull, M: size marker 100 bp.

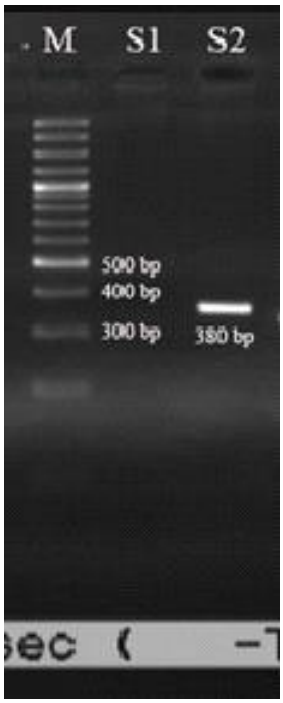

Figure 5. DDX3Y marker in the genome of fertile male and without microdeletion. S1: negative control, S2: amplified fragment by DDX3Y primer, M: size marker 100 bp.

oligospermia in bulls (Chang et al., 2013). Study on AZF regions in terms of structure and gene content, and compliance of these regions between human and bovine can be helpful in determining the genetic causes of unexplained infertility in bulls.

In contrast to previous reports (Ditton et al., 2004; Liu et al., 2009), the present study indicated that the DDX3Y gene was directly associated with infertility and its deletion causes infertility in human and bulls. The extensive similarity between the genomes of human and animals confirms the hypothesis of occurrence of microdeletions, and consequently azoospermia and oligospermia in bovines.

Conclusions

The results of this study indicated that the specific human primers recommended by EAA/EMQN were not applicable for molecular identification of infertility in the bull. However, investigation of genes associated with human male infertility led to a more accurate identification of genetic infertility in bulls. The current research for the first time reports the role of DDX3Y gene in bull infertility by using bioinformatic analysis and the polymerase chain reaction. This can be a valuable marker for infertile bull detection at an early age.

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شناسایی مولکولی گاوهای نر نابارور با استفاده از مارکر تازه توسعه یافته DDX3Y بر پایه STS مارکرهای انسانی

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چکیده به منظور بررسی نقش ژن DDX3Y در اسپرماتوژنز و ناباروری گاوهای نر، نمونه خون ۵ راس گاو نر نابارور (فاقد اسپرم) شناسایی شده در مرکز اصلاح نژاد کرج جمع آوری شد. پرایمرهای توصیه شده توسط اتحادیه آکادمی اندرولوژی و شبکه کیفیت مولکولی اروپا (EAA/EQMN) برای شناسایی STS مارکرها با استفاده از نرم افزار BLASTn مورد بررسی قرار گرفت. همچنین همردیف سازی ژن‌های STS مارکرها با ژنوم گاو انجام شد و به منظور طراحی پرایمر مشترک برای ژن DDX3Y گاو و انسان از نرم افزار Primer Primer 5 و نرم افزار CLCWorkbenche 5.5 استفاده شد. پس از انجام مطالعات بیوانفورماتیک، غربالگری مولکولی بر روی DNA ژنومی خون محیطی با هدف شناسایی حذف ژن DDX3Y در کروموزوم Y با استفاده از تکنیک PCR صورت گرفت. آنالیز بیوانفورماتیکی اتصال پرایمرهای انسانی STS مارکرها نشان داد که هیچ شانس اتصال و تولید قطعه هدف برای نمونه‌های گاوی وجود ندارد. بررسی ژن DDX3Y بر روی نمونه‌های گاو بارور نشان داد که آغازگر طراحی شده به خوبی می‌تواند ژن هدف را تکثیر سازد. نتایج نشان داد که ۳ گاو حذف جزئی را در ژن DDX3Y داشتند که علت ناباروری آن‌ها بود. ژن DDX3Y می‌تواند مارکری ارزشمند در تشخیص ناباروری گوساله‌های نر جوان باشد.