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# Towards the understanding of bull fertility: phenotypic traits description and candidate gene approach

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**ABSTRACT -** We presented the preliminary results of the analysis of semen phenotypic and quality data provided by ANARB along with the result of SNPs discovery on seven candidate genes for male fertility and the ongoing study on mtDNA. The phenotypic data recorded by Computer Assisted Sperm Analyzer of fresh semen for 1,014 bulls were evaluated. The least squared means of volumes were lower than data in literature generally referred to mature bulls, whereas the spermatozoa concentrations, total and progressive motility were satisfactory. As expected in young bulls, the percentage of spermatozoa morphological abnormalities was high. The SNPs discovery in seven candidate genes revealed 30 new SNPs and confirmed the polymorphism in STAT5A gene. The complete sequencing of mtDNA of 48 extreme bulls for semen phenotypic traits revealed 274 mutations. Association between SNPs and semen phenotypes is on going. Our results represent a preliminary step towards the sounding of genetic mechanisms of bull fertility and highlight the paramount importance of reliable phenotypes for association studies.

*Key words:* Male fertility, Dairy cattle, SNP, Candidate genes.

**Introduction -** In dairy cattle, the impressive genetic progress in milk yield and quality is accompanied by the deterioration of functional performance and fertility (Lucy, 2001). Until now, this problem has been faced mainly from the cow side because of either the low number of sires respect to dames or the direct implication of female in all phases of calving. Nonetheless, sire also plays a key role in fertility, not yet adequately investigated. In the last 15 years, knowledge on mammalian physiology and genetics of reproduction increased considerably producing a growing list of sperm structural defects considered of genetic origin. More than 200 nuclear candidate genes in key steps of male reproduction process have been already identified, providing potential candidates for male fertility traits association studies (Leeb, 2007). Increasing evidence suggests also the involvement of mitochondria in male infertility. Sperms mtDNA is particularly prone to accumulate deletions (O'Connell *et al.*, 2002) and some studies have demonstrated that, in human, such deletions are associated with a decline of sperm motility and fertility (Kao *et al.*, 1998). Optimized male fertility will help the selection of bulls with a high semen quality, increase the rate of successful fertilization, facilitate the physiological early embryonic development and great benefit dairy production. Objectives of this paper were: i) the description of the phenotypic variation of several sperm traits recorded by the Italian Brown Breeders' Association (ANARB); ii) the identification of SNPs in candidate nuclear genes and in mtDNA for future association studies.

**Material and methods -** *Semen traits* - The dataset produced by the genetic centre of ANARB comprised: i) volume and concentration of fresh semen in the first and second jump sessions for 1,014 bulls;

ii) progressive motility, average motility, and percentage of primary and secondary defects of spermatozoa head, intermediate trait and tails recorded in 358 bulls; iii) sperm parameters of 277 young bulls recorded by CASA computer analysis (SpermVision™). *Statistical analyses* - The dataset was accurately edited and descriptive statistics were estimated. Last squared means (LSM) of sperm phenotypic traits were obtained by an ANCOVA model that included each recorded trait as independent variable, the effect of bulls and the covariate "age of bulls" at jumping session (Jump software ver. 3.1, SaS Inst. Inc.). *SNPs discovery in candidate genes* - Seven genes candidate to affect male fertility were selected from literature: Transition protein 1 (TNP1), Cystein-rich secretory protein 2 (CRISP2), Prion-like protein doppel precursor (PRND), Tumor necrosis factor precursor alpha (TNFa), Cation channel sperm-associated protein 2 (CATSPER2), Cyclic nucleotide-gated cation channel alpha 3 (CNGA3), and Signal transducer and activator of transcription 5A (STAT5A). Target regions of these genes were amplified in panel of eight animals using customized primers. PCR products were sequenced in outsourcing and aligned using the program BioEdit. *SNPs discovery in mtDNA* - Complete mitochondrial DNA sequencing of extreme animals for phenotypic traits related to sperm motility is planned. Protocols are set up and amplicons assemblage is ongoing. SNPs will be detected by alignment and direct comparison to the GenBank *Bos taurus* reference sequence (NC_006853).

Table 1. LSM and SD of semen traits recorded on young bulls of the ANARB Genetic Center.

| Semen traits | Bulls no. | LSM | SD |
|---|---|---|---|
| Volume: 1st jump (ml) | 1014 | 3.22 | 1.09 |
| Volume: 2nd jump (ml) | 1013 | 3.18 | 0.91 |
| Concentration1st jump (no. Spermatozoa x $10^6$ ) | 1014 | 627.5 | 278:9 |
| Concentration 2nd jump (no. Spermatozoa x $10^6$ ) | 1013 | 657.4 | 258.7 |
| Progressive motility (%) | 358 | 75.24 | 17.19 |
| Total motility (%) | 358 | 81.87 | 14.68 |
| Primary sperm defects: heads (%) | 350 | 27.34 | 9.31 |
| Secondary sperm defects: intermediate tract (%) | 350 | 18.05 | 7.50 |
| Primary sperm defects: tails (%) | 350 | 1.21 | 0.95 |
| Primary sperm defects: total (%) | 350 | 46.53 | 12.09 |
| Secondary sperm defects: heads (%) | 350 | 7.33 | 6.08 |
| Secondary sperm defects: proximal droplets (%) | 350 | 6.07 | 6.26 |
| Secondary sperm defects: tails (%) | 350 | 1.07 | 1.02 |
| Secondary sperm defects: total (%) | 350 | 14.48 | 8.97 |
| Normal (%) | 350 | 41.01 | 15.93 |
| Total abnormalities (%) | 350 | 57.82 | 15.50 |
| Distance average path (micron) DAP | 277 | 38.54 | 6.08 |
| Distance curved line (micron) DCL | 277 | 68.16 | 10.57 |
| Distance straight line (micron) DSL | 277 | 32.68 | 5.39 |
| Velocity average path (micron/sec) VAP | 277 | 86.10 | 13.62 |
| Curvilinear velocity (micron/sec) VCL | 277 | 153.12 | 23.50 |
| Linear velocity (micron/sec) VSL | 277 | 73.79 | 11.77 |
| Straightness (VSL/VAP) | 277 | 0.84 | 0.07 |
| Linearity (VSL/VCL) | 277 | 0.47 | 0.05 |
| Wobbing (VAP/VCL) | 277 | 0.56 | 0.05 |

**Results and conclusions** - Semen phenotypic and quality data were provided by ANARB where a lab facility with specialized technician is operative. The semen traits were recorded weekly in sessions with two consecutive jumps at the end period of the performance test. The 60% of young bulls in the dataset had six sessions recorded whereas 24% had information on 8 sessions.

Sperm parameters of 277 young bulls in performance test were measured through a computer sperm analyzer and collected in a more accurate and complete database available in Italian dairy breeds. In Table 1, the LSM obtained with the ANCOVA model are shown. The LSM of the ejaculated volumes were

lower than data in literature generally referred to mature bulls, whereas the spermatozoa concentrations were satisfactory.

As expected, the distribution of total and progressive motility presented a negative skewness as our samples missed bulls with clinical disease (quickly removed from performances tests). The percentage of spermatozoa morphological abnormalities was particularly high with only 2.5% of young bulls below 30%, considered the limits for high quality semen (Parkinson, 2004). However, morphological abnormalities are negative correlated with the age of the bulls thus expected to decrease with sexual maturity.

In Table 2 the SNPs discovered in seven candidate genes selected from literature are reported. Sequence comparison revealed 30 new SNPs and confirmed the polymorphism of two SNPs in exon 8 of the gene STAT5A already associated with low embryonic survival in Holstein (Khatib *et al.*, 2008). In CRISP2 gene, we observed about 1 mutation every 40 bp sequenced. We tested the primer specificity to exclude the amplification of unspecific fragments. The meaning of this mutation hot spot as well as the possibility to have amplified a pseudogene required further investigation. mtDNA of 48 extreme bulls for semen phenotypic traits were completely sequenced and 274 mutations were scored. Among these, 104 were within coding regions, 97 in ribosomal RNA genes, and 12 within tRNA sequences. Association between individual SNPs or haplotypes and the male fertility traits is ongoing.

In conclusion, our results represent a preliminary step towards the understanding of the complex genetics mechanism of bull fertility. In the high throughput genotyping era, where it is possible to inquire ten-thousands of SNPs in a single essay, our results highlight also the importance of reliable phenotypes for a cost-effective association studies.

Table 2. Genes investigated, sequencing effort, number of SNPs identified, and their localization.

| Gene | GeneID | Gene length (bp)[A] | Number of bp sequenced | SNP | Region |
|---|---|---|---|---|---|
| TNP1 | 281537 | 618 | all | 1 | Ex. 2 |
| CRISP2 | 512443 | 25452 | 830 | 20 | Int. 8 and 9; Ex. 9 |
| PRND | 281426 | 5039 | 640 | 1 | Int. 1 |
| TNFa | 280943 | 2766 | 2504 + 600 of 5'UTR | 2 | Int. 1, Ex 2 |
| CATSPER2 | 517699 | 18112 | 3206 | 4 | Ex. 6 and 12; Int. 8 and 13 |
| CNGA3 | 281701 | 21047 | 4000 + 250 of 5'UTR | 2 | 5'UTR, Ex. 3 |
| STAT5A | 282375 | 20306 | 440 | 2 | Ex. 8 |

[A] *Gene length excluding 5'- and 3'-UTR.*

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