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PROMISING NEW TECHNOLOGIES FOR SEMEN EVALUATION

George R. Dawson, J.N. Oyarzo, M.E. Bellin, H.M. Zhang, T.C. McCauley and R.L. Ax
Department of Animal Sciences, University of Arizona, Tucson, AZ

Introduction

Assessment of seminal plasma and sperm-membrane associated constituents has led to the isolation of four proteins that provide utility for predicting fertility potential of bulls. All four of these proteins have been isolated and characterized biochemically. They have also proved to be very valuable as a diagnostic indicator of fertility beyond the traditional breeding soundness exam (BSE) identified by Chenoweth et al. (1992), since bulls with identical physical semen characteristics still may vary greatly in fertility when used for natural mating or artificial insemination.

The specific proteins found in bovine semen that reflect fertility potential include osteopontin (OPN), a lipocalin-type prostaglandin D (PGD) synthase, fertility-associated antigen (FAA) and type-2 tissue inhibitor of metalloproteinases (TIMP-2). Since a specific gene encodes for particular protein, the potential exists to easily screen for these predictors of fertility. Single nucleotide polymorphisms (SNPs) in genes are becoming valuable tools in both proteomics and genomics. Subtle mutations in the gene from one individual to another can be detected, leading to the prediction of fertility at a day of age rather than puberty to collect semen.

Within the FAA gene, SNPs have been isolated that cause conformational changes in the carboxyl terminus of the protein. When a monoclonal antibody (M1) is used to screen for the FAA protein, the aberrant FAA form is not detected and the bull is subsequently labeled FAA-negative. The use of polymerase chain reactions (PCR) techniques will allow researchers to amplify and easily diagnose those individuals containing mutations or SNPs.

The four proteins as fertility markers in bulls and the promising new technologies for assessing semen/donors will be discussed as evaluators of fertility potential.

Semen Proteins as Predictors of Bull Fertility.

Utility of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) led to the recovery of four proteins in seminal plasma that were linked to fertility (Killian et al., 1993). This original work led to the development of a regression equation (R=.89) to predict fertility based on the density of these proteins in Holstein bull seminal plasma (for formula see Killian et al., 1993). The proteins discovered consisted of a 55 kDa (pI 4.1) protein and a 26 kDa (pI 6.2) protein, which were correlated with above, or high fertility bulls, and two 16 kDa proteins with pI's of 6.7 and 4.1, respectively. The latter two proteins were correlated with below average fertility bulls and are currently uncharacterized.

The 55 kDa protein was later characterized and found to be osteopontin (OPN; Cancel et al., 1997). Based on protein density, OPN was positively correlated (.48) with fertility (Cancel et al., 1997). An OPN localization study illustrated the primary sources in seminal plasma were contributed from the ampulla and seminal vesicle secretions (Cancel et al., 1999). The functional role of OPN with respect to regulating bull fertility remain to be elucidated, however OPN has been shown to play an integral part in a number of signal transduction pathways (for review see Denhardt et al., 2001) including defense mechanisms and inflammatory conditions. The potential also exists for it to serve as a functional cell attachment protein that may assist in stabilization of the plasma membrane.

When bulls are categorized as above or below average fertility (average = 0), lipocalin type PGD synthase is 3.5-fold more prevalent in high fertility Holstein bulls (Killian et al., 1993; Gerena et al., 1998; 2000). Fluorescent microscopy revealed lipocalin-type PGD synthase to be associated with the apical ridge of the acrosome on ejaculated sperm, as well as being localized in a number of other areas (Gerena et al., 2000). Its localization in numerous tissues including the epididymis lends support to having a role in maturation of sperm and may reflect why individuals with a 3.5-fold higher concentration are more fertile.

In the early 1990's a series of proteins were isolated using liquid chromatography, which possessed a high affinity for binding heparin. Of these heparin-binding proteins (HBPs), a 31 kDa protein was later isolated and coined fertility-associated antigen (FAA; Bellin et al., 1996). Biochemical studies indicate FAA is basic and non-glycosylated yielding a N-terminal 26 amino acid sequence which is 73 % identical to human deoxyribonuclease (DNase) I-like protein (McCauley et al., 1999). Tissue extracts indicate the presence of FAA primarily to be in the seminal vesicles and prostate glands (McCauley et al., 1999), however previous work illustrated a similar molecular weight protein in all three accessory sex glands (Nass et al., 1990). Heparin-binding proteins are present on ejaculated sperm, but not on epididymal sperm (Miller et al., 1990). They augment the effects of heparin, a glycosaminoglycan, commonly used to capacitate bovine sperm in vitro (Miller et al., 1990).

Another 24 kDa HBP was also isolated and characterized as being a predictor of bull fertility. The 24 kDa protein was identified as tissue-inhibitor of metalloproteinases-type 2 (TIMP-2) as it was shown to be 90 % identical to the N-terminus of a bovine aortic TIMP-2 (McCauley et al., 2001). TIMP-2 RNA has been isolated from all three bovine accessory sex glands (McCauley et al., 2001). This was the first report of TIMP-2 in bovine semen and potential roles and mechanisms are currently under investigation.

Using SDS-PAGE gels and Western blotting techniques with the monoclonal antibody designated as M1, bovine sperm extracts can be assayed and characterized as FAA or TIMP-2 positive or FAA or TIMP-2 negative. Tables 1 and 2 represent field trials that have occurred and the impact of segregating beef bulls as positive or negative with respect to either FAA or TIMP-2 content.

Table 1. Fertility of range beef bulls following a 60 d exposure period and AI beef bulls after three projected services (equivalent to 60 d exposure) when categorized as FAA-positive or FAA-negative.

FAA Status	Number Of Bulls	Number Of Cows	Pregnant at 60 days or 3rd Service	Percent of Cows Pregnant
FAA-negative	199	4,267	2,764	**64.8 %**
FAA-positive	260	6,081	4,998	**82.1 %**
Total	459	10,348	7,762	75 %

(Data adapted from Bellin et al., 1994; 1996; 1998; and Sprott et al., 2000)

Table 2 summarizes results from field trials recently completed to compare pregnancy outcomes of beef cows exposed to bulls after their spermatozoa had been tested for presence or absence of TIMP-2. Bulls were pastured for 60 days at a ratio of 25 cows per bull in multiple-sire pastures. Fertility was 13% higher for bulls whose sperm was qualified as TIMP-2 positive compared to their herdmates categorized as TIMP-2 negative.

Table 2. Fertility of range beef bulls after a 60 d exposure period following characterization of sperm as either TIMP-2 positive or TIMP-2 negative.

TIMP-2 Status	Number Of Bulls	Number Of Cows	Number Of Cows Pregnant	Percent Of Cows Pregnant
TIMP-2 Positive	180	3,985	3,431	**86.1 %**
TIMP-2 Negative	67	1,225	894	**72.9 %**
Total	247	5,210	4,325	83 %

(Data adapted from Dawson et al., 2002)

Bulls which are categorized at positive for FAA or TIMP-2 represent a 17 % or 13 % higher fertility rate (based on pregnancy) than their counterparts. This represents a

potential large increase in economic gain versus the cost of assaying for these proteins which is estimated to be approximately $1.00 per cow in a 100-head herd.

As mentioned earlier, proteins are unique and they are encoded for by a specific gene, so what other technologies may we have access to in the near future?

Gene Frequencies and Genotypic Frequencies:

Since a gene is unique, we can calculate its frequency if we have a measure of its distribution in a population. Using FAA as an example, a genotypic frequency is distributed as $p^2 + 2pq + q^2$. To date, approximately 6,000 bulls have been screened and 16 % of those are FAA-negative, that means they are homozygous recessive, providing q^2. The square root of .16 = .4. One minus .4 = .6, the frequency of p. From this information, we deduce that $.6^2 = .36$, the frequency of animals homozygous FAA-positive, 2pq = 2(.6)(.4) = .48, so 48 % of the population is presumed heterozygous, carrying one chromosome with FAA positive form of the gene, and one chromosome carrying the FAA-negative form of the gene. They would be classified overall as FAA-positive if FAA were assayed in a semen sample. If different breeds had varying genotypic distributions of FAA, or if we culled homozygous recessive FAA-negative animals to decrease their frequency, the following table illustrates what is happening mathematically. Keep in mind, we are referring to animals. The gene frequencies should be similar in males and females because no coordinated industry-wide culling has yet occurred.

Table 3. Hypothetical gene and genotypic frequencies based on the proportion of homozygous recessive (FAA-negative) individuals in a population.

Proportion of Homozygous Recessive (FAA-negative)	**Gene Frequency**		**Genotypic Frequencies**		
	p	q	Homozygous Dominant (FAA+)	Heterozygous (FAA+)	Homozygous Recessive (FAA -)
25%	.5	.5	.25	.50	.25
16%	.6	.4	.36	. 48	.16
5%	.78	.22	.61	.34	.05

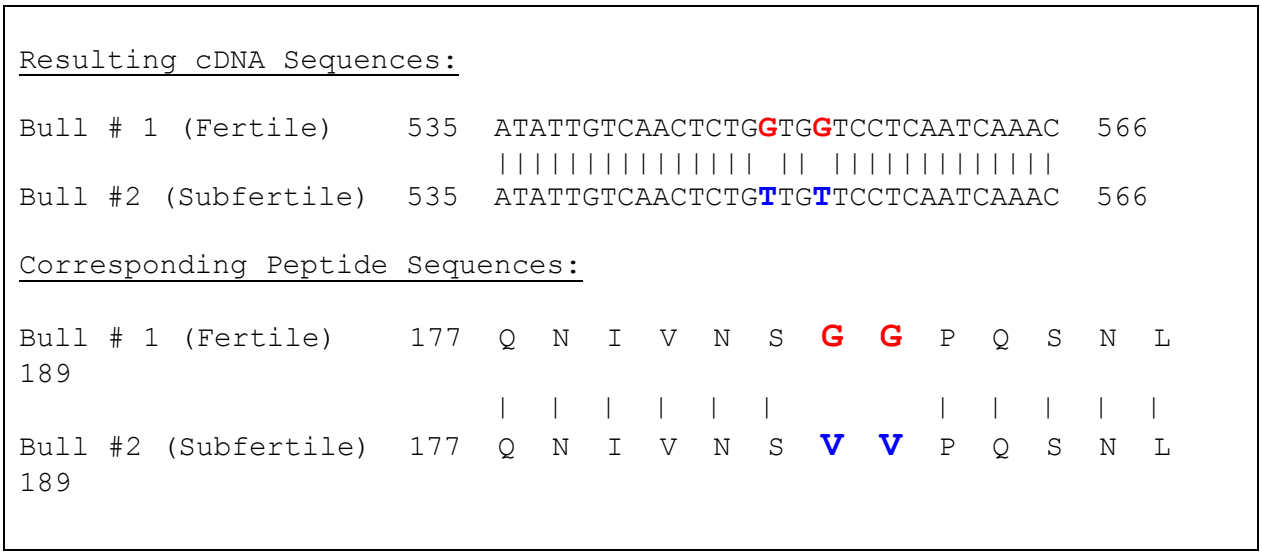
From the Table above, it is clear that if only 5 % of bulls sampled were classified as FAA-negative from a semen test, there would still be 34 % of cattle that would be heterozygotes carrying one copy of the undesirable form of the FAA gene. How then, do we develop a method to find animals lacking two copies of the good form of FAA (or any other protein of choice)?

Mutations

Bulls with sperm categorized as FAA-negative actually produce a protein which is almost identical to FAA. That aberrant form represents a subtle mutation within the FAA protein that causes it to fold slightly different from normal. Obviously, that fold occurs at or near the portion of the FAA which is recognized by the monoclonal antibody (M1) that was originally produced against FAA in 1992 (Bellin et al., 1996). A specific domain on a protein recognized by an antibody is referred to as an epitope. Bulls with semen classified as FAA-negative produce the protein lacking the epitope, leading to them labeled as FAA-negative.

Molecular biology techniques can now be applied to semen for defects within the FAA gene that lead to an altered form of the protein lacking the epitope. First, the FAA genes from a normal fertile bull and subfertile bull were sequenced. The portion of the gene where the mutation was detected, along with the corresponding change in amino acids encoded at that portion of the FAA protein are illustrated below.

Figure 1. Two DNA SNPs found between the cDNA sequences of bovine FAA gene isolated from sex glands of a normal bull and the sex glands of a subfertile bull. Both of the 550 and 553 nucleotides of the normal bull FAA gene were deoxyguanilic acids (**G**), whereas two deoxythymidylic acids (**T**)were found instead at the same positions in the subfertile bull FAA gene. The two SNPs led to two (183 and 184) amino acid substitutions of Glycine (**G**) in the normal bull by two Valine (**V**) in the subfertile bull FAA.



The illustration above is an example of SNP genotyping and clarifies what is happening genetically (for more intensive reviews and/or methods see Kwok, 2001; Mir and Southern, 2000). A bull designated as FAA-negative would have the gene and amino acid sequences shown above for a subfertile bull on both of his chromosomes. A heterozygote would have each form shown above, and a homozygous FAA positive bull would have the normal forms on each chromosome inherited from each of his parents.

DNA-Based Diagnostics

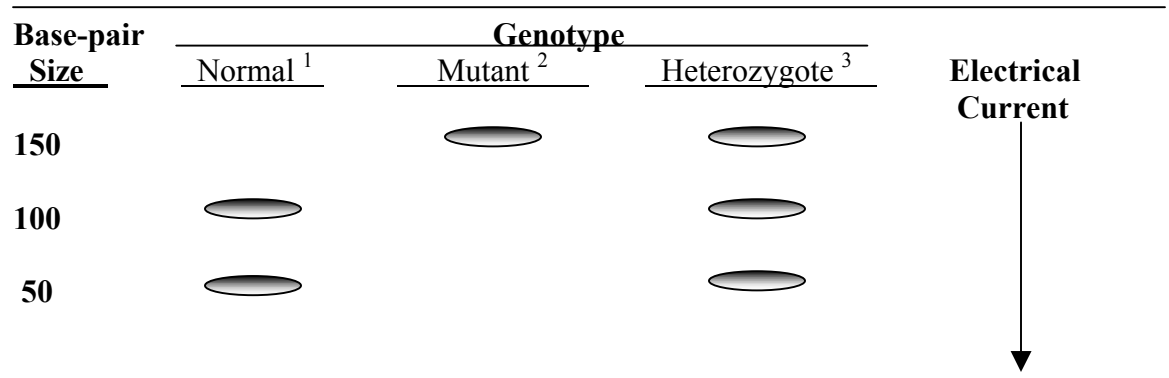
Enzymes referred to as restriction endonucleases will cut DNA at specific sequences. Thus, DNA can be fragmented chemically using these enzymes.

When a mutation occurs within a particular gene, such as the FAA mutation shown in an earlier section of this paper, that mutation may lead to appearance or disappearance of a site recognized by a restriction endonuclease enzyme. Fortunately, that is the case with FAA. In the normal genotype, there is a site between nucleotides 532 and 534 recognized by a restriction enzyme, so DNA is subsequently snipped at that specific location. The mutated form of FAA lacks that recognition site, so cleavage of DNA cannot occur. For example, if PCR is used to amplify 150 nucleotides containing the normal restriction site, and the enzyme is added, two pieces of DNA will result that can be visualized after separation in an electric current. An animal with two copies of the aberrant FAA will yield a single 150 nucleotide piece, making it simple to identify diagnostically.

A heterozygous FAA bull will have the normal and mutated DNA strands amplified. Therefore, three total bands will be visible, leading to the distinction that segregates those animals form the homozygous ones carrying the desirable or mutant genotypes.

An example is portrayed schematically below:

Figure 3. Example of DNA-based identification of FAA genotypes assuming a 150 nucleotide piece of DNA was amplified with the restriction site approximately two-thirds into the DNA sequence.



[1] The restriction site is present on both chromosomes. Therefore, the entire amplified DNA is susceptible to restriction enzyme cleavage.

[2] Mutant lacks enzyme restriction site, no DNA digestion occurs.

[3] Heterozygote has one normal/one mutant copy. Therefore, one-half of the DNA in the sample is digested, with one-half stable. Thus, three bands of DNA are visible.

Sexing Semen

Like many new biotechnologies, the first attempts to sort X and Y-bearing sperm was unsuccessful (Gledhill et al., 1976). In the late 1980's and early 1990's, rapid progress was made in an attempt to provide individuals with the ability to pre-select for gender. Met at first by controversy and concern ranging from ethical issues, to monetary costs and normality of the resulting offspring the refinement of this technology weathered the storm. Predictions have been made that sex-sorted semen will be under artificial insemination use by the year 2005 (Amann, 1999). Sexed semen has already reached the marketing phase in the United Kingdom (Garner, 2001). So, where are we in terms of efficiency, accuracy and marketing of sorted sperm?

Since the DNA content of the Y chromosome is less than the larger X chromosome, this allows for sperm to be segregated easily following the application of a DNA dye using a high intensity light beam. Johnson (2000) reported their original flow cytometer would sort approximately 350,000 sperm per hour, whereas today's high-output sorters now reach near six million per hour when sorting both X and Y sperm. If only the X-bearing sperm are desired, output has reached nearly 18 million per hour (Johnson, 2000). Pressures exerted for segregating at this speed range from .84 kg/cm^2 for standard units to 4.22 kg/cm^2 for high-speed cell sorters. Many hours are needed to sort multiple AI doses. As a result, low-dose insemination numbers of spermatozoa have been under investigation in multiple species. Successful attempts in cattle have been reported with sexed semen using 2×10^5 sperm per insemination of nonfrozen sperm (Seidel et al., 1997). This is significantly less than the 20 million normally used in a commercially available frozen insemination dose, even if one considers 50 % of those are rendered incapable of fertilization following the rigors of freezing/thawing. Continued instrument refinement will enhance this process, but currently, multiple machines, people-power and hours will be required to produce large lots of commercially available sexed semen.

The accuracy of sorting X and Y-bearing is not 100 %, but most laboratories report reaching 90 % or greater (Rath et al., 1999; Seidel, 1999; and Johnson 1992). When sorting sperm, the accuracy is dependent on the amount of DNA difference between X and Y sperm. Johnson (2000) reported the highest accuracies are obtained when the differences in DNA content are greater than 3.5 %. For the all livestock species, the differences have been reported to be greater than 3.5 % while the human resides at approximately 2.8 % (Johnson, 2000). Other factors that may affect the accuracy of the sorting process include dead sperm and any morphological variation in sperm. Post-thaw motilities of sorted frozen bull sperm have been reported around 30-35 % (Schnek et al., 1999; and Johnson et al., 1996) and percentage of intact acrosomes (PIA) of 40% (Johnson et al., 1996).

The United States beef industry is greater than 95% natural mating in contrast to the dairy industry where it has been reported to be approximately 65 % AI (Amann, 1999). For the dairy industry, sexed semen has tremendous advantages where bull calves are undesirable and the cost of replacement heifers often a limiting factor in increasing cow numbers. The beef industry will be slower to benefit from this technology, except for purebred breeders who will more likely accept added costs to sorted sperm to capture those genetically superior male and female lines.

Cost of operating a flow cytometer for one year is estimated at $183,200, this excludes cost of raw semen, storage and distribution (Amann, 1999). Using dairy semen that has been sex sorted may have a 2-fold increase in sale price, and supply may be limited due to current sorting efficiency. Large-scale use in the dairy and beef industry may be limited to those genetically superior individual previously mentioned. However, if the current cost of equipment decreases significantly, sorted X and Y-bearing semen will be met with less hesitation.

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