Fertility-Associated Proteins in Holstein Bull Seminal Plasma

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ABSTRACT

This study was undertaken to determine whether bovine seminal plasma contained protein markers associated with bull fertility, and whether these markers were of value in predicting bull fertility. Seminal plasma was obtained from 35 Holstein bulls of known fertility. Two-dimensional PAGE of seminal plasma samples indicated that two proteins (26 kDa, pl 6.2; 55 kDa, pl 4.5) predominated in higher-fertility bulls, and two proteins (16 kDa, pl 4.1; 16 kDa, pl 6.7) predominated in lower-fertility bulls. Densitometry data for these proteins in individual samples were combined for bulls grouped by fertility level. Average density of the 26-kDa protein was significantly greater in seminal plasma of high-fertility bulls, and high-fertility seminal plasma also contained more of the 55-kDa protein than that of average- or below-average-fertility bulls. Below average- and low-fertility bull seminal plasma had significantly more of both 16-kDa proteins than that of average- and high-fertility bulls. A regression model was developed to predict bull fertility using the four fertility-associated protein densities. A plot of actual bull fertility versus that calculated by this model was linear and positively correlated \( r = 0.89 \). These findings indicate that bull seminal plasma contains fertility-associated proteins that are predictive of bull fertility.

INTRODUCTION

Reports for several species suggest that seminal plasma contains factors that may influence male fertility. These studies are generally based on comparisons of seminal plasma composition between males of differing fertility [1–6] or the isolation of factors from seminal plasma that facilitate or inhibit sperm capacitation, fertilization, or related events [7–14].

We developed an interest in this topic after surveying the fertility of reproducibly normal dairy bulls primarily housed at artificial breeding cooperatives in Pennsylvania and New York. In vivo fertility data summarized for 131 bulls with normal semen parameters, as assessed by laboratory evaluation, indicated that the fertility of the bull population followed a distribution ranging from 8.5 percentage points below to 5.4 points above average fertility, designated as zero (Fig. 1). This relatively small range of fertility differences among bulls in the normal population was distinguishable because fertility data were available for individual bulls based on more than 1000 breedings by artificial insemination. We are not aware of any other species for which such data are available on individual sires. These circumstances provide a unique animal model for the study of factors contributing to the relative fertility of the normal male.

The objective of the present study was to determine whether seminal plasma contained protein markers associated with relative fertility in a population of reproducibly normal bulls, and whether these markers were of value in predicting bull fertility. In addition, we compared the molecular masses and isoelectric focusing points for the fertility-associated proteins we detected to bovine seminal plasma proteins characterized by other investigators.

MATERIALS AND METHODS

Samples of seminal plasma were obtained for protein analyses from 35 mature Holstein bulls of known reproductive history. These bulls were a subset of those represented in Figure 1 and were maintained at Atlantic Breeders Cooperative (14 bulls), Eastern Artificial Insemination Cooperative (16 bulls), and Sire Power Inc. (5 bulls).

Fertility data on each bull were provided by the respective artificial insemination (AI) centers that housed the bulls and were based on breeding records for more than 1000 inseminations using frozen semen. Because the method of preparing fertility data varied slightly among the AI centers, data for each bull were expressed as a percentage point deviation from the average fertility for all production bulls at that AI center sampled during the same period. To maximize the possibility of detecting differences among bulls associated with fertility, the bulls on which seminal plasma analyses were conducted were selected to include adequate numbers of bulls in each fertility level separated by vertical lines in Figure 1. This selection process also was influenced by the availability of samples from the cooperating AI centers. For the 35 bulls supplying seminal plasma for analysis, the percentage point deviations ranged from 6.6 to +4.5, with average fertility designated as 0.0.

Bulls providing seminal plasma were subjected to a regular semen collection schedule, dictated by the AI center, which typically amounted to one to three ejaculates per week. A single ejaculate was analyzed from each bull without regard to month, season, or order in a sequence of ejaculates.
The date a seminal plasma sample was taken fell within the same time interval used to calculate the fertility of the bull.

Within 15 min of ejaculate collection, semen was centrifuged (1000 × g, 15 min), and the supernatant seminal plasma was transferred to cryovials (GL72,694,007 Sardstedt, Princeton, NJ) for storage in liquid nitrogen. Samples were shipped in liquid nitrogen from the AI center to our laboratory, where they remained in liquid nitrogen storage until analyzed. After being thawed at ambient temperature, seminal plasma samples were recentrifuged (10,000 × g, 60 min at 5°C), assayed for protein concentration [15], divided into aliquots, and refrozen in liquid nitrogen. Immediately before electrophoresis was performed, samples were thawed at ambient temperature.

One-dimensional PAGE of seminal plasma was performed under denaturing conditions by methods previously described [16–18]. Seminal plasma samples were subjected to two-dimensional PAGE according to the method of O'Farrell [19] as modified for use in our laboratory [20]. The isoelectric focusing tube gels contained a mixture of amphiolytes consisting of 0.4 ml of pH 3–7 and 0.1 ml of pH 3–10 (Serva, Heidelberg, Germany) to establish a pH gradient. Tube gels were prefocused at a constant voltage of 200 V for 15 min, 300 V for 30 min, and 400 V for 30 min. Samples of seminal plasma consisting of 400–500 μg protein in less than 50 μl were solubilized in urea and beta- mercaptoethanol, giving a final volume of 100 μl to be loaded onto the gel. Tube gels then were electrofocused for 20 h at 375 V, followed by 1 h at 800 V. After electrofocused gels were extruded from the tubes, they were placed on 4% stacking gels covering the top of 10–17.5% acrylamide gradient slab gels, with 1% molten agarose used to seal them in position. Molecular mass standards (Sigma Chemical Co., St. Louis, MO) were electrophoresed in the second dimension along with the proteins originating from the isoelectric focusing gel. The standards were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

Gels were stained overnight in 0.125% Coomassie Brilliant Blue R-250 dye (Sigma), 50% methanol, and 10% acetic acid in distilled water. Gels were destained in 25% methanol and 10% acetic acid, and photographed with LPD-4 positive film (Kodak, Rochester, NY). One positive film of each gel for each bull was scanned with a Bio-Rad model 620 densitometer (BioRad, Rockville Centre, NY), interfaced with a Dell system 310 computer. These data were used to prepare a map of the proteins present in each gel and to objectively estimate the amount of each protein.

The fertility-associated protein densities were adjusted by subtracting the average background for each gel image from the protein densities of interest. Densities for each fertility-associated protein were then compiled, and average (SE) protein densities were calculated for bulls grouped by fertility level.

Densities of the four fertility-associated proteins of each bull also were used as a database to develop a model to predict bull fertility. Several regression models were evaluated to determine which provided the best empirical prediction of fertility. Evaluations were made on log and square root transformations of protein densities. The criteria for comparing models were the calculated R-squared, adjusted R-squared, C-P, and multicollinearity values [21].

**RESULTS**

Preliminary studies with two different ejaculates from each of three bulls indicated similar protein profiles on one- and two-dimensional electrophoresis gels between ejaculates from the same bull and virtually identical profiles between replicate gels of the same ejaculate. Therefore, only one ejaculate was evaluated from each bull in subsequent studies. Densitometry data of one-dimensional SDS-PAGE of seminal plasma from bulls of differing fertility did not provide consistent evidence for differences in protein banding patterns associated with fertility. Although visual inspection of two-dimensional gels of seminal plasma from the 35 bulls indicated some variation in protein maps among individuals, four different polypeptides appeared to occur in association with bull fertility level. Two proteins (26 kDa, pI 6.2; 55 kDa, pI 4.5) occurred with greater frequency and density in bulls of higher fertility (Fig. 2, top), and two pro-
FIG. 2. a and b) Representative two-dimensional polyacrylamide gels of seminal plasma proteins from bulls of higher (top) and lower (bottom) fertility. Locations of fertility-associated proteins are indicated with arrows. (a) 55 kDa, 4.1 pI; (b) 26 kDa, 6.2 pI; (c) 16 kDa, 6.7 pI and (d) 16 kDa, 4.1 pI.

Proteins (16 kDa, pI 4.1; 16 kDa, pI 6.7) were more prominent in bulls of lower fertility (Fig. 2, bottom). On the basis of these preliminary observations, we subjected all two-dimensional gels to analysis by video densitometry to obtain quantitative data on the polypeptides of interest. The density and area of the individual polypeptides on a gel were determined for each bull sample, and these quantitative data were summarized for bulls combined by fertility level into...
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FIG. 3. Average densities (SE) of the high-fertility proteins determined in two-dimensional gels of seminal plasma for bulls grouped by fertility. LF, −5.5, n = 6; BA, −2.8, n = 7; AF, +0.5, n = 11; HF, +2.7, n = 11.

Four groups. Seminal plasma from bulls in the high-fertility group (HF), ranging from 2.0–4.5 percentage points above average fertility, had significantly more (p < 0.05) of the 26-kDa protein than did seminal plasma from bulls in the average-fertility (AF; +1.4 to −0.2 percentage points), below-average-fertility (BA; −2.3 to −3.8), and low-fertility (LF; −4.3 to −6.6) groups (Fig. 3). HF bull seminal plasma also had more of the 55-kDa protein than seminal plasma of AF and BA bulls.

Plasma from BA and LF bulls had significantly more (p < 0.05) of the 16-kDa, pI 4.1 protein than did AF and HF bull seminal plasma (Fig. 4); HF seminal plasma had significantly more (p < 0.05) of the protein than did AF seminal plasma. LF bull seminal plasma had significantly more of the 16-kDa, pI 6.7 protein than did AF, BA, and HF plasma, and BA bull seminal plasma had significantly more (p < 0.05) of the 16-kDa, pI 6.7 protein than did plasma of AF and HF bulls.

The regression model that provided the best empirical prediction of fertility based on the four fertility-associated proteins in seminal plasma was:

\[ \text{Fertility} = -2.12 + 3.58\sqrt{D_1} - 0.90\sqrt{D_2} + 1.61\sqrt{D_3} + 0.35\sqrt{D_4} - 1.69\sqrt{D_4} - \sqrt{D_4} \]

where fertility represents the predicted percentage point deviation from the average fertility of bulls at an AI center, and \( D_1 \) through \( D_4 \) represent the densities of the fertility-associated proteins: 26 kDa, pI 6.2; 16 kDa, pI 4.1; 55 kDa, pI 4.5; 16 kDa, pI 6.7, respectively. The model used square root transformations of the protein densities. Although the main effect of the square root of \( D_4 \) was not significant, it was retained in the model because of a significant interaction between the square roots of \( D_1 \) and \( D_4 \).

Estimated fertility values for each bull were generated by comparison to a data set that included seminal plasma

FIG. 4. Average densities (SE) of the low-fertility proteins determined in two-dimensional gels of seminal plasma for bulls grouped by fertility as described in Figure 3 legend.

FIG. 5. Plot of the actual bull fertility (percentage point deviation from the mean) versus the calculated fertility values for the 35 bulls undergoing analysis of seminal plasma by two-dimensional PAGE.

\[ N = 35 \]
\[ R = 0.89 \]
fertility protein data from the 34 other bulls in the study. A plot of actual in vivo fertility versus calculated fertility (Fig. 5) was linear, and there was a positive correlation (r = 0.89) indicating that the predictive model was valid over the range of bull fertilities studied.

Calculated and actual fertility values differed by an average of 1.19 ± 0.15 percentage points over all bulls. Average percentage point differences (mean SE) between the calculated and actual fertility values among fertility groups were minimal (HF, 1.30 ± 0.34; AF, 1.07 ± 0.29; BA, 0.83 ± 0.18; LF, 1.59 ± 0.28).

DISCUSSION

The present study clearly suggests the existence of four fertility-associated proteins in bovine seminal plasma that appear to be of value in predicting small differences in relative fertility among bulls. The bulls studied represented a population of reproductively normal males with normal semen parameters. Because similar numbers of sperm from each bull were used to inseminate the females, the effect of sperm numbers on fertility was similar among bulls. Although studies with other species have reported the presence of factors in seminal plasma related to in vivo fertility [1, 3-5], the males being compared were typically categorized as fertile or infertile, with the infertile males often oligo- or azoospermic. Because the absence of or reduction in sperm numbers in the infertile male could contribute to infertility, the biological significance of differences in seminal plasma composition associated with fertility was unclear.

Antifertility factors from seminal plasma have been described for several species and include decapacitation factors purified to various degrees [22], human antifertility factor 1 (AF1) [10, 13, 23], rabbit acrosome stabilizing factor (ASF) [9], and bull seminal plasmin (SPLN) [12]. Generally, these factors are believed to inhibit sperm capacitation, the acrosome reaction, or acrosomal enzymes and ultimately to interfere with fertilization.

Recent studies [14, 24] have suggested that heparin-binding proteins in bull seminal plasma are taken up by cauda epididymal sperm membranes. Because other studies have indicated that the ability of sperm to bind heparin and other glycosaminoglycans is correlated with semen quality and fertility [6, 25-27], heparin-binding proteins in seminal plasma may positively influence fertility.

Although the results of the present study demonstrate a high correlation between the amount of certain seminal plasma proteins and bull fertility, we do not know whether this relationship is causal or coincidental. Furthermore, it is important to note that the differences in amount of fertility-associated proteins we observed in a gel based on staining intensity may reflect post-translational modifications of proteins rather than more or less of a protein, or different proteins, per se.

The fertility-associated proteins we detected in bull seminal plasma have biochemical similarities to bull seminal plasma proteins characterized by other investigators. Miller et al. [14] described a group of 15-17-kDa seminal plasma heparin-binding proteins that had pls of 4.1-6.0 and were associated with the sperm membrane. These proteins are similar to the two 16-kDa proteins we observed with pls of 4.1 and 6.7. Likewise, the 26-kDa, 6.2 pl fertility-associated protein we observed in seminal plasma is within the range of basic heparin-binding proteins Miller et al. [14] described for seminal plasma at 24 kDa.

The biochemical characteristics of a group of acidic bovine seminal plasma proteins at 16 kDa have been studied in detail [28-31], Esch et al. [28] named three 16-kDa seminal plasma proteins BSPI, BSPII and BSPIII. These proteins have amino acid sequences [32] identical to those of seminal plasma proteins BSPA1, BSPA2, and BSPA1, respectively, studied by Manjunath et al. [31]. Furthermore, BSPII and BSPIII, collectively referred to as PDC-109 [28], have considerable amino acid sequence homology [32] with the major protein (MP) synthesized by the seminal vesicle [29]. Interestingly, BSPA1, BSPA2, and BSPA4 apparently bind heparin [33], and PDC-109 (BSPA/2) is exclusively bound by the sperm midpiece [34], where it inhibits calcium uptake by bovine sperm [35]. The fact that the 16-kDa acidic BSP proteins bind heparin and influence calcium uptake by sperm raises the possibility that they are the 16-kDa heparin-binding proteins described by Miller et al. [14, 36] and associated with sperm fertility.

In addition to the proteins already mentioned, numerous enzymes and protease inhibitors also have been detected in bovine seminal plasma [37]. However, with the exception of phospholipase A2, these do not have molecular masses and isoelectric points consistent with the fertility-associated proteins we detected in seminal plasma. Purified phospholipase A2 from bovine seminal plasma and seminal vesicle secretions exists in two forms [38]. One form has 15- and 16-kDa subunits with a pl of 4.7. The other form has subunits of 16 kDa and 60 kDa with multiple pl values from 3.75-5.0. The molecular masses and pls of the subunits of the purified phospholipase [38] and the 16-kDa, pl 4.1 and 55-kDa, pl 4.5 fertility-associated proteins we identified in seminal plasma are sufficiently similar to suggest that the fertility-associated proteins may be related to phospholipase A2. Because phospholipase has been shown immunohistochemically to be present on the surface of ejaculated bovine sperm acrosome, post-acrosome, and middle piece, but not on epididymal sperm [39], it is likely that some phospholipase is adsorbed from seminal plasma to the sperm surface.

Given the role that has been proposed for phospholipase in sperm capacitation and the acrosome reaction [40], it is possible that the phospholipase in seminal plasma that...
is adsorbed by sperm could account for the relatively small differences in bull fertility we observed in conjunction with the fertility-associated proteins.

It was beyond the scope of the present study to biochemically and functionally characterize the fertility-associated proteins we detected by electrophoresis in two-dimensional polyacrylamide gels of bovine seminal plasma. Nevertheless, the similarities between the fertility-associated proteins we observed and those biochemically characterized by other investigators provide impetus for further studies to link biochemistry with the biological function of proteins in seminal plasma.

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