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Porcine sperm-zona binding ability as an indicator of fertility

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Abstract

The escalated use of artificial insemination in swine has increased the importance of determining fertility of a semen sample before it is used. Multiple laboratory assays have been developed to assess fertilizing potential but they have yielded inconsistent results. This experiment sought to determine the relationship between *in vitro* competitive zona binding ability and *in vivo* fertility based on heterospermic inseminations and paternity testing. The zona pellucida binding ability and fertility of sperm from 15 boars was assessed by comparing sperm from one boar with sperm from other individual boars in a pair-wise fashion using four ejaculates. The relationship of zona binding ability to the mean number of piglets sired per litter for each boar as well as historic fertility data (litter size and farrowing rate) was assessed. The *in vitro* competition assay consisted of labeling sperm from each boar of the pair with a different fluorophore and incubating an equal number of sperm from each boar in the same droplet with porcine oocytes. The competitive assay was highly effective in ranking boars by zona binding ability ($R^2 = 0.94$). Paternity testing using microsatellite markers was used to determine the mean number of piglets sired per litter for each boar during heterospermic inseminations. The pairwise heterospermic insemination assay was effective in ranking boar fertility ($R^2 = 0.59$). Using historical data from these boars, average litter size and farrowing rate were correlated ($r = 0.81$, $p < 0.001$). However, zona binding ability was not significantly correlated with historic farrowing rate data or historic average litter size. Boar sperm zona binding ability was also not correlated significantly with the mean number of piglets sired per litter following heterospermic insemination. But the number of piglets sired by each boar was related to a combination of zona binding ability, sperm motility, normal morphology, acrosomal integrity, and the presence of distal droplets ($R^2 = 0.70$). These results suggest that zona binding ability is not an accurate predictor of fertilizing ability when used alone; however, when coupled with other sperm assessments, fertility may be predicted successfully.

Keywords

Pig - Fertility; Fertilization; Heterospermic Insemination; Sperm; Zona Pellucida

1. Introduction

The expanded use of artificial insemination (AI) in swine has increased the importance of determining fertility of a semen sample before it is used for AI. Motility, morphology, and sperm concentration are characteristics frequently used to assess fertility of a semen sample.

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Measurement of those characteristics usually identifies very poor samples but does not always identify sub-fertile samples (Ruiz-Sanchez et al., 2006). Because successful fertilization requires that sperm complete an entire series of events, the use of additional tests that assess fertilization characteristics of sperm could yield more accurate results. Examples are assays that measure the ability to maintain the acrosome under osmotic stress (Jeyendran et al., 1984), penetrate zona-free hamster eggs (Yanagimachi, 1984), or fertilize homologous eggs *in vitro* (Gadea et al., 1998). Unfortunately, these assays have also shown inconsistency in fertility prediction (Braundmeier and Miller, 2001).

Previous results using an *in vitro* competitive zona pellucida binding assay coupled with homospermic insemination indicated a relationship between boar zona binding ability and litter size, but not farrowing rate (Braundmeier et al., 2004). Curiously, one boar in that study showed lesser zona binding ability and a small average litter size, but greater farrowing rate (80%). Sows in that study were inseminated with semen from an individual boar (homospermic insemination). Heterospermic insemination (HI) provides a more accurate assessment of fertility when compared with homospermic insemination, particularly when large numbers of females are unavailable (Robl and Dziuk, 1988). Heterospermic insemination of rabbits showed a difference between male fertility for males with similar routine semen quality evaluations (Vicente et al., 2004). The reduction in female variability by using HI increased the ability to detect fertility differences between males in sheep (Berger and Dally, 2001), pigs (Stahlberg et al., 2000), and bulls (Beatty et al., 1969). To further determine the accuracy of competitive zona binding ability for predicting fertility rates, we coupled an *in vitro* competition binding assay with heterospermic inseminations. The *in vitro* assay controlled for variability in oocytes as well as treatment of samples, whereas the *in vivo* assay controlled for sow variability and insemination technique/conditions, allowing for more accurate results. The aim of these experiments was to determine if *in vitro* sperm zona binding ability (HI) of sperm was related to *in vivo* fertility rates after insemination.

2. Materials and methods

2.1. Animals used

Fifteen crossbred boars (Landrace $\times$ Large White $\times$ Duroc $\times$ Hampshire) that were 2.4 ± 0.3 years of age and 201 ± 7 kg (mean $\pm$ SEM) were used in the study. Historical data indicated that these boars differed significantly in their *in vivo* estimates of fertility (Table 1). Boars were housed in individual pens (3.6×2.7 m) in an environmentally controlled building. They were given *ad libitum* access to water and were fed a corn-soybean meal diet (14% crude protein) that exceeded all nutritional requirements for adult boars (Council, 1998). Crossbred sows (Landrace $\times$ Large White $\times$ Yorkshire; $n = 270$) with a mean parity of 3.8 ± 0.2 (SEM) were bred with semen collected from the 15 boars during the experimental period. Sows were maintained in individual crates (1.6×2.8 m) during breeding, gestation and farrowing. Sows were provided *ad libitum* access to water and fed corn-soybean meal diets formulated to meet or exceed their specific nutritional needs (Council, 1998) during breeding, gestation, and lactation.

2.2. Semen collection, preparation and insemination

The entire ejaculate from each boar was collected by the gloved-hand technique (Almond et al., 1998) every other week during January 2005 to December 2005. Four ejaculates per boar were collected over the duration of this study. The percentage of sperm exhibiting normal head and tail morphology, and normal acrosome morphology was evaluated as described (Popwell and Flowers, 2004). In addition, ejaculates were weighed after collection to estimate the total volume of semen collected and analyzed for motility estimates with SpermVision® (Minitube of America, Verona, WI), a computer-assisted semen analysis system. A 1-ml sample of neat

semen was diluted with 19 ml of BTS semen extender (Pursel et al., 1972) and warmed to 35° C. Five, 5- μ l samples were loaded into Leja® chambers (IMV International, Minneapolis, MN) maintained at 35° C. Ten different fields on each chamber were analyzed for the following semen motility estimates: the percentage of motile spermatozoa; the percentage of progressively motile spermatozoa; average path velocity of motile spermatozoa; straight-line velocity of motile spermatozoa; and the curvilinear velocity of motile spermatozoa. The total number of spermatozoa analyzed for each boar each week in this manner varied between 1500 and 3000 depending on the concentration of spermatozoa in the ejaculate. Only ejaculates with 70% or greater normal sperm in each category were processed for inseminations. The number of sperm per ml of each ejaculate was determined using a hemocytometer as described (Almond et al., 1998). The number of sperm per ml was multiplied by the percentage of sperm exhibiting progressive forward motility to estimate the number of motile sperm per ml per ejaculate. The extension process consisted of adding 100 ml of Beltsville Thawing Solution (Pursel and Johnson, 1975) to each ejaculate. After a 30 min equilibration period, ejaculates from two boars were combined such that the mixture contained equal numbers of motile sperm from each boar. After a second 30 min equilibration period, additional Beltsville Thawing Solution was added to produce insemination doses of 6×10^9 motile sperm in 80 ml of extended semen (3×10^9 motile sperm per boar). Sows were inseminated once each day of estrus beginning on the first day of detected estrus. Sows were inseminated a minimum of two times during estrus. All inseminations were performed by the same three experienced breeding technicians during the experimental period and semen was used within 60 h of collection. The disposition of boars and sows is summarized in Table 2. The number of sows bred to each combination of boars ranged from 5 to 10 (Table 3). All procedures involving sows and boars were approved by the North Carolina State University Institutional Animal Care and Use Committee.

2.3. DNA isolation

DNA was extracted from boar sperm obtained at semen collection using the Puregene® Purification kit for 60 to 100 million sperm according to the manufacturer's instructions (Gentra Systems, Minneapolis, MN). DNA was extracted from skin (tail samples) of sows and piglets shortly after farrowing using the Puregene® Purification kit following the manufacturer's procedure for 5 to 10 mg of mouse tail tissue (Gentra Systems).

2.4. Oligonucleotide primers

Oligonucleotide primers for paternity identification were selected based on normal variation inherent to the population of boars for DNA microsatellite markers from the X-Y chromosome map published by U.S.D.A. (www.marc.usda.gov/genome/swine/ChromosomeX-Y/) (Table 4). Primers were purchased from Qiagen (Valencia, CA).

2.5. PCR amplification

For each of the microsatellite markers, reactions were performed in volumes of 20 μ l consisting of 0.2 μ M of each primer (forward and reverse), 0.5 U of Taq DNA Polymerase, 100 ng DNA, 200 μ M dNTPs and PCR buffers supplied by the manufacturer. Mineral oil was placed on top of the reaction mixtures and the 0.5 ml reaction tubes were placed in a thermocycler. All reagents were purchased from Qiagen (Valencia, CA) unless otherwise indicated. Amplification reactions were conducted according to the following program: 2 min at 94° C; 40 cycles of denaturing (10 sec at 93° C), annealing (20 sec at 58° C), and extension (20 sec at 72° C) followed by a final extension phase for 5 min at 72° C. For each reaction, a control was performed in which distilled water was added to the mixture instead of DNA before processing via the amplification cycle. Single and double-stranded DNA fragments from PCR amplification reactions were purified using QIAquick spin columns according to the instructions supplied by the manufacturer (Qiagen). Alleles were identified via gel

electrophoresis (40 V/cm for 2 h) with 8% denaturing acrylamide gels using a sequencing gel apparatus.

2.6. Collection of porcine oocytes

Porcine ovaries were obtained from Excel Inc. (Beardstown, IL) and Meadowbrook Farms (Rantoul, IL) for oocyte collection. Follicles greater than 2 mm in diameter were aspirated with a 10 ml syringe using an 18 gauge needle until 100 ml of follicular fluid was acquired. Collected follicular fluid was centrifuged at $600 \times g$ for 10 min. Follicular fluid was aspirated and follicular debris was suspended in 10 ml dmTALP (100 mM NaCl, 3.1 mM KCl, 1.5 mM $MgCl_2$, 2.1 mM $CaCl_2$, 0.29 mM potassium phosphate, 10 mM sodium bicarbonate, 25 mM HEPES, 1 mM sodium pyruvate, 21.6 mM lactate, 6 mg/ml fatty-acid free bovine serum albumin [BSA $\geq 96\%$ essentially fatty-acid free, fraction V; Sigma], 100 units/ml penicillin, and 100 $\mu g/ml$ streptomycin, pH 7.4). Oocytes were collected from the follicular debris under a dissecting microscope. Collected oocytes were added to a 1.0 ml microcentrifuge tube and vortexed at maximum speed for 5 min to remove cumulus cells. Oocytes were removed from the cumulus debris under a dissecting microscope. The oocytes were washed thrice in Medium B (127 mM NaCl, 5.3 mM KCl, 18.2 mM HEPES, pH 7.2) and, in 20-oocyte batches, fixed in Medium B containing 1.5 % formaldehyde for 1 min. Fixed oocytes were washed through three droplets of Medium B. All oocytes were transferred to one storage droplet of dmTALP that was covered with embryo-tested mineral oil (Sigma Chemical Company, St. Louis, Missouri). Oocytes were stored at $4^\circ C$ for up to 2 months.

2.7. Preparation of porcine sperm

Extended semen from the same collections used for fertility assessment was shipped overnight in a Styrofoam box with temperature stabilizer packs and processed within 24 h of collection. Extended semen was washed through a Percoll cushion containing 5.4 ml of Percoll (Sigma, St. Louis, MO), 0.6 ml of 10X HB saline (1.3 M NaCl, 40 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 140 mM fructose, and 50 mg/ml BSA, and 4.0 ml of dmTALP in a 15 ml conical tube. Sperm was centrifuged at $800 \times g$ for 10 min, the supernatant was removed and pellet was suspended in 15 ml of dmTALP. After centrifuging for 5 min at $600 \times g$, the supernatant was removed and pellet was suspended in 1 ml of dmTALP. Sperm concentration was assessed using a hemocytometer and 2.0×10^7 cells/ml were added to a 1.5 ml microcentrifuge tube, and the volume was adjusted to 300 μl with dmTALP. Sperm were capacitated for 4 h at $37^\circ C$ and 5% CO_2 .

2.8. Staining of sperm with lipophilic dyes

The dyes used were DiIC₁₈ (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; an orange-red fluorophore) and DiOC₁₆ (3,3'-dihexadecyloxycarbocyanine perchlorate; a yellow-green fluorophore) (Molecular Probes, Eugene, Oregon). Each fluorescent lipophilic dye was prepared in 10 mM stock solutions. DiIC₁₈ was dissolved in dimethyl sulfoxide and DiOC₁₆ in dimethylformamide. Five-microliter aliquots of stock solutions were stored at $-20^\circ C$ in 1.5 ml microcentrifuge tubes covered with aluminum foil. Prior to use, an aliquot was thawed, sonicated, and centrifuged. After capacitation, sperm were incubated with 83 μM (2.5 μl of 10 mM stock) of each dye for 60 min at $37^\circ C$ in dmTALP. The sperm suspensions were centrifuged for 30 seconds at $10,000 \times g$, the supernatant was removed to eliminate excess dye from the solution, and sperm were suspended in 300 μl of dmTALP.

2.9. Competitive sperm-oocyte binding assay

Following capacitation and fluorophore labeling, sperm were used in a competitive zona binding assay. Competitions were arranged (Table 5) to obtain the best estimates without

performing all comparisons between boars. In this design, each boar was assayed against six other boars. Each individual assay consisted of two competitions with reversed dye assignment to minimize any effect of the dye on zona pellucida binding ability. Sperm from two boars, each labeled with opposing dyes, was combined (volume of 12.5 μ l from each boar) and triplicate 25 μ l droplets were made with a final concentration of 5.0×10^5 sperm/ml. Ten oocytes were added to each droplet for a total of 30 oocytes per competition. Gametes were co-incubated for 15 min at 37° C to allow for maximal binding without inducing the acrosome reaction. After incubation, sperm-bound oocytes were gently transferred through three 50 μ l wash droplets of dmTALP to remove loosely bound sperm. Sperm-bound oocytes were fixed in a 25 μ l droplet of 4% paraformaldehyde in phosphate-buffered saline. Fixed oocytes were transferred to a 25 μ l droplet of dmTALP covered with embryo-tested mineral oil and viewed with fluorescence using an inverted Zeiss Axiovert 25 microscope (Zeiss, Thornwood, New York). To detect DiOC₁₆ fluorescence, the Zeiss 09 filter set that, for excitation, has a band-pass cutoff of 450 to 490 nm, a 510 beam splitter, and a long-pass 515-nm emission filter was used. For detecting DiIC₁₈ fluorescence, the Zeiss 15 filter set that has a band pass of 534 to 558 nm, a 580-nm beam splitter, and a 590-nm long-pass emission filter was used.

2.10. Statistical analysis

Analysis of variance using GLM procedures of SAS (SAS Inst. Inc., Cary, NC) was used to rank the boars on the number of offspring in a litter sired by either boar. The statistical model for the *in vivo* data was:

$$nborn = week + boar + tborn + random\ error$$

where nborn is the number of piglets/litter/boar, week is the effect of the collection week, boar is the male effect, and tborn is the regression on the total number of piglets/litter. Boars were ranked by the calculated least squares means of the number of piglets born/litter/boar.

Analysis of variance was used to rank the boars on their ability to bind to the zona pellucida of the oocyte. The statistical model for the *in vitro* data was:

$$nbound = assay + comp(assay) + boar + tbound + dye + dye * boar + random\ error$$

where nbound is the number of sperm bound/oocyte/boar, assay is the effect of the assay, comp (assay) is the effect of each competition droplet within each assay, boar is the male effect, tbound is the regression on the total number of sperm bound/oocyte, dye is the effect of each dye, and dye*boar is the interaction term. Boars were ranked by the calculated least squares means of the number of sperm bound/oocyte for each boar. *In vivo* fertility rankings and *in vitro* zona binding ability rankings were compared by correlation analysis to assess their relationship.

Fertility data obtained in November 2003 to December 2004, approximately the year prior to collecting competitive insemination data, were calculated (historic fertility data). Farrowing rates were calculated for each boar as the number of sows that had successfully farrowed after artificial insemination. Litter size of each boar was calculated as the total number of piglets born alive from each farrowing and then averaged for all farrowings. The variance terms are reported as standard errors of the means using data for each ejaculate as an observation. Historic fertility data were compared to both heterospermic binding data and heterospermic insemination data by correlation analysis. All zona binding assays were performed with no knowledge of historic fertility data and before any offspring were born to paternity test.

To elucidate the importance of zona binding ability as a variable in fertility prediction, analysis of variance was used to analyze its importance with that of other sperm characteristics. Least squares means were calculated for all semen characteristics assessed in the four ejaculates from each boar and used to formulate a model for fertility prediction.

The statistical model for fertility assessment was:

$$\text{lsm born} = \text{motil} + \text{morph} + \text{acro} + \text{distal} + \text{lsm spermbound} + \text{random error}$$

where lsm born is the least squares means of the number of piglets sired per litter for each boar, motil is the regression on the percentage of motile sperm, morph is the regression on the percentage of sperm with normal head and tail morphology, acro is the regression on the percentage of sperm with normal acrosome morphology, distal is the regression on the percentage of sperm with a distal cytoplasmic droplet, and lsm spermbound is the least squares means of the number of sperm bound to the zona pellucida for each boar.

3. Results

Semen was collected from 15 boars, extended and used for competitive zona binding assays within 24 h or AI within 60 h. Boar H died while the experiment was in progress. The limited data from that boar were included in the analysis. Boars used in this study had historic farrowing rates ranging from 64% to 94% and an average litter size ranging from 9.1 to 11.4 pigs/litter, based on semen that was collected prior to this study from November 2003 to December 2004 (Table 1). A comparison between historic litter size and farrowing rate revealed a positive correlation ($r = 0.81$, $p < 0.001$, Supplementary Figure 1, Table 6).

Least squares means were calculated for the number of sperm bound per oocyte for each boar, allowing the boars to be ranked by zona binding ability. The model for the *in vitro* competition data was highly effective at ranking boars by zona binding ability ($R^2 = 0.94$). The 15 boars ranged from 16.4 to 20.9 mean sperm bound per oocyte. As a first assessment of whether zona binding ability was related to fertility, zona binding ability was compared to historic values for farrowing rate and litter size. The number of sperm bound per oocyte revealed no significant correlation to historical average litter size ($r = 0.04$, $p = 0.88$, Figure 1, Table 6) or historical farrowing rates ($r = 0.002$, $p = 0.99$, Figure 1, Table 6).

In vitro zona binding ability was compared to the number of piglets sired by each boar in heterospermic inseminations. Least squares means were calculated for number of piglets sired per litter for each boar. The model included effects due to collection week, male variability, and the regressions on total number of piglets in each litter. The number of days from weaning to estrus of sows was also tested as an independent variable and deleted from this model because its coefficient was not significant. The model designed for the heterospermic insemination data was effective in ranking boars by mean number of piglets sired per litter ($R^2 = 0.59$). The mean number of piglets sired per litter ranged from 0.05 to 7.03 for this group of 15 boars. A negative but statistically insignificant correlation was found between zona binding ability and competitive fertility ($r = -0.34$, $p = 0.22$, Figure 2, Table 6).

One explanation for a reduced relationship between zona binding ability and fertility might be inconsistent semen quality. However, historic litter size was positively correlated with heterospermic fertility ($r = 0.85$, $p < 0.001$, Figure 3, Table 6). Furthermore, historic farrowing rate was also correlated with heterospermic fertility ($r = 0.83$, $p < 0.001$, Figure 3, Table 6). These correlations suggest that sperm fertility was consistent during the time of these experiments.

Although competitive zona binding ability alone was not simply related to fertility, inclusion of this parameter in an overall semen quality analysis may be useful. Sperm motility, morphology, acrosomal status and the presence of distal cytoplasmic droplets were assessed immediately after semen collection (Table 7). A model was designed that included sperm motility, morphology, acrosomal status, presence of a distal cytoplasmic droplet, and average number of sperm bound to the zona as independent variables to assess their relationship to heterospermic fertility. Motility and morphology had the greatest effect on fertility, followed

by zona binding ability, acrosome status, and presence of a distal cytoplasmic droplet. The model showed that these traits were related to fertility although the presence of a distal droplet had a marginal relationship to fertility ($R^2 = 0.70$, Table 8).

4. Discussion

Fertilization is a complex process comprising several steps that gametes must complete successfully. To accurately identify sub-fertility, it is likely that a number of tests must be performed to assess as many steps in fertilization as is reasonable to identify those steps that may be defective (Amann, 1989). These experiments use zona pellucida binding to investigate its relationship with the fertility of that semen sample. An *in vitro* competitive zona binding assay was coupled with heterospermic inseminations to determine that association.

Competitive types of assays control the highest number of fertility variables (Foote, 2003). The competitive binding assay and heterospermic insemination assays ranked effectively the 15 boars by zona binding ability and piglets sired per litter. Despite using competition assays, results from the present study suggest no simple relationship between zona binding ability and fertility. This poor association may be because zona binding does not measure a sufficient number of traits. Alternately, it may be due to the small difference in the number of sperm bound per oocyte or the small difference in the average number of piglets sired among this group of 15 boars. Because a competitive zona binding assay does not measure sperm transport or defects in post zona-binding events, it may be best when combined with other sperm traits, such as motility, morphology, and acrosomal integrity in a laboratory fertility assessment. When these characteristics were included in a model to estimate heterospermic fertility, the model provided a good prediction of fertility ($R^2 = 0.70$).

The R^2 value for the heterospermic insemination data ranking boar fertility was less than reports from a previous study ($R^2 = 0.83$) (Saacke et al., 1980). A possible explanation is that boars do not rank as a group the same that they do when compared in pairs. The cause of this variability is not clear, but could be related to ejaculate variation making the results of the comparisons inconsistent. Using multiple ejaculates provides a larger sample size to estimate overall male fertility but includes potential ejaculate inconsistency. In the current study, four ejaculates from each boar were used and only one ejaculate was used in a previous study (Saacke et al., 1980). However, fertility in the current study seemed fairly consistent because during the 2 years of experimental procedures, heterospermic fertility was similar to historic fertility (Figure 3). It is also possible that the ranking of boars could improve by decreasing the number of sperm inseminated. In the current study samples were used with 70% or greater motility to create a total insemination dose of 6 billion motile sperm. Perhaps by decreasing the number of sperm inseminated, males that produce sperm with a shorter lifespan in the oviduct may be identified when inseminated in sows that have a long interval between insemination and ovulation. However, weaning to estrus interval was not a significant factor in the analysis. A long weaning to estrus interval often indicates late ovulating sows (Knox and Zas, 2001, Knox et al., 2002). It is possible that short-lived sperm may be at a greater competitive disadvantage in late ovulating sows when fewer sperm are inseminated. However, because weaning to estrus interval was not a significant variable, it suggests that reducing sperm number may not affect the ranking in the current study. Furthermore, other evidence indicates that changing the number of total sperm in the insemination dose does not affect the fertility ranking of males (Martin et al., 1974).

In addition to that of males, variability due to females is also possible even though heterospermic insemination controls for female variability. Maternal factors that affect sow fertility include technician variability, heat stress, sow parity, or number of inseminations during estrus (Clark et al., 1989). Technician variation in estrous detection or insemination can

impact success rates. Sows in this study were bred by experienced breeding technicians during all seasons. Though technician variation is minimized, heat stress during inseminations performed in the warmer months could be a cause of variation. Heat stress in sows is frequently presented by delay of return to estrus after mating (Love, 1978); however, including the independent variable of weaning to estrus interval did not reduce variation in our experiment. To minimize the effects of heat stress, the data from sows that did not farrow were not included in the present experiment. Last, using sows with average parity from two to five coupled with two or more inseminations per estrus can reduce variability in farrowing rate (Clark et al., 1989). Sows used in this study had an average parity of 3.8 ± 0.2 and were inseminated two or more times per estrus, thus decreasing variability in farrowing rate. When sow variability was included in the present study in the analysis, based on residuals, it did not improve the model R^2 value significantly, consistent with the notion that heterospermic insemination controls for female variability. Therefore it seems unlikely that sow variation affected this study and reduced the R^2 of the heterospermic fertility statistical model.

Successful fertilization requires that sperm complete a number of steps. Other variables not assessed may have affects on sperm fertility, such as sperm transport, acrosome reaction, zona pellucida penetration and the ability to fuse with the oocyte. A more complete assessment of fertility potential may require a large battery of tests. It will be important to determine which tests are most efficacious as predictors of sperm fertility.

5. Conclusion

A pairwise competitive assay was able to accurately rank boars on zona binding ability. Results revealed that zona binding ability was not correlated with other fertility measures, including the proportion of piglets sired following heterospermic insemination. However, zona binding ability was a useful addition to routine laboratory assessments used to estimate sperm fertility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. (Upper Panel). Relationship of zona binding ability to historic litter size. No significant correlation ($r = 0.04$, $p = 0.88$) was detected between average number of sperm bound per oocyte and litter size using homospermic insemination data from the group of 15 boars. (Lower Panel). Relationship of zona binding ability to historic farrowing rate. No significant correlation ($r = 0.002$, $p = 0.99$) was detected between average number of sperm bound per oocyte and farrowing rate using homospermic insemination data from the group of 15 boars.

Fig. 2. Relationship of zona binding ability to average number of piglets sired per litter in heterospermic insemination. The average number of sperm bound per oocyte and the average number of piglets sired per litter for each boar were not correlated significantly ($r = -0.34$, $p = 0.22$).

Fig. 3.

(Upper Panel). Relationship of piglets sired per litter to historic litter size. A positive correlation ($r = 0.85$, $p < 0.001$) was detected between number of piglets sired per litter (heterospermic insemination data) and average litter size (historic homospermic insemination data) from 15 boars. (Lower Panel) Relationship of piglets sired per litter to historic farrowing rate. A positive correlation ($r = 0.83$, $p < 0.001$) was detected between number of piglets sired per litter (heterospermic insemination data) and mean farrowing rate (historic homospermic insemination data) from the group of 15 boars.

Table 1
Boar fertility records prior to start of heterospermic inseminations

Boar I.D.	No. of Sows	Farrowing Rate (%) [*]	Number Born Alive per litter [*]
A	100	87.3 ± 5.3	10.2 ± 0.3
B	111	80.4 ± 4.7	10.4 ± 0.4
C	152	78.3 ± 5.2	10.2 ± 0.3
D	161	65.3 ± 4.6	9.1 ± 0.4
E	187	74.3 ± 4.7	9.8 ± 0.3
F	142	88.3 ± 5.1	10.6 ± 0.4
G	100	82.4 ± 4.8	10.5 ± 0.3
H	98	80.8 ± 4.7	10.6 ± 0.3
I	145	83.8 ± 4.3	10.5 ± 0.3
J	251	94.3 ± 5.1	11.4 ± 0.3
K	234	64.3 ± 4.3	9.4 ± 0.4
L	221	84.4 ± 5.8	11.0 ± 0.4
M	254	85.3 ± 6.1	9.5 ± 0.4
N	245	68.2 ± 5.1	9.3 ± 0.4
O	221	88.3 ± 4.2	10.4 ± 0.3

^{*} Data included outcomes of inseminations of semen collected from November 2003 to December 2004. Three billion sperm were deposited at insemination. Data are means ± SEM based on each ejaculate.

Table 2

Disposition of boars and sows

Variable	
Total Number of Sows Bred	270
Number of Sows Farrowed	206
Number of Litters with ≤ 3 piglets	16
Number of Litters Analyzed	190
Boar H	Died on 4/10/2005

Table 3

Number of sows bred to each combination of boars

Boar Combination	Number of Sows Bred	Boar Combination	Number of Sows Bred
AB	5	GJ	6
AC	5	IJ	9
AD	6	IK	9
BC	8	IL	10
BD	8	JK	5
BE	8	JL	5
CD	8	JM	6
CE	10	KL	8
CF	8	KM	9
DE	6	KN	8
DF	7	LM	6
DG	6	LN	5
EF	6	LO	5
EG	5	MN	7
EH	6	MO	7
FG	7	MA	6
FH	8	NO	7
FI	8	NA	8
GH	6	NB	8
GI	5		

Table 4

Oligonucleotide primers used for paternity analyses

Primer	Sequence 5' to 3'	Primer Length (base pairs)	Molecular Weight (Daltons)
Sw1325F	CATTCATTTTGTCTTCTGCC	22	6608.44
Sw1325R	CACCACTTCGTCTTCACTTCC	21	6228.11
S0022F	CTGGGCAGCTCTATAATATC	20	6092.05
S0022R	TTACCCTTTCTACTCTGTGC	20	5984.99
SW259F	CCTTTCATGCTGTATTTAACCC	22	6611.41
SW259R	CAGAGAACAGAAGTTGGGGG	20	6280.14
SW290F	CTTCAGTGTAGTCCAAGTGGC	21	6437.26
SW290R	GATGTTTGTCTGATAGGAAGGG	22	6885.89
SW2456F	GAGCAACCTTGAGCTGGAAC	20	6151.04
SW2456R	AATGTGATTGATGCTGTGAAGC	22	6829.56
SW2137F	CATTGACACAGTCCCTGG	19	5748.78
SW2137R	GGTAAATACAAAACCTCCACG	22	6681.41
S0117F	CAGGAAGCTGAAGCTGAGATTTGA	24	7465.95
S0117R	CTGTCAGGGAGCTAGTTTCACTCA	24	7343.86

Table 5

Diagram of competition schedule*

Boars	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
A															
B		X		X											
C			X	X	X										
D				X		X									
E					X		X								
F						X									
G							X								
H								X							
I									X						
J										X					
K										N/A					
L											N/A				
M												X			
N	X												X		X
O	X	X	X											X	X

* X indicates a direct comparison was made. N/A indicates data were not available because Boar H died.

Table 6

Correlation coefficients between variables*

Variable	Litter Size	Farrowing Rate	Number of Sperm Bound per Oocyte	Number of Piglets Sired per Boar
Litter Size	1.00	$r = 0.81, p < 0.001$	$r = 0.04, p = 0.88$	$r = 0.85, p < 0.001$
Farrowing Rate		1.00	$r = 0.002, p = 0.99$	$r = 0.83, p < 0.001$
Number of Sperm Bound per Oocyte			1.00	$r = -0.34, p = 0.22$
Number of Piglets Sired per Boar				1.00

* The correlation coefficients and p-values for historic litter size and farrowing rate and for the number of sperm bound to oocytes in vitro and the number of piglets sired in heterospermic insemination are listed.

Table 7
Semen quality parameters from each of the 15 boars*

Boar I.D.	Motility (%)	Normal Morphology (%)	Acrosome Integrity (%)	Sperm with a Distal Droplet (%)
A	74.2 ± 0.7	84.5 ± 0.6	91.2 ± 0.7	8.7 ± 0.4
B	85.2 ± 0.6	87.1 ± 0.6	90.1 ± 0.6	7.1 ± 0.4
C	81.0 ± 0.7	89.2 ± 0.6	88.4 ± 0.7	9.9 ± 0.4
D	72.8 ± 0.6	86.9 ± 0.5	90.1 ± 0.6	10.5 ± 0.4
E	84.5 ± 0.6	89.6 ± 0.5	90.0 ± 0.6	6.3 ± 0.4
F	80.2 ± 0.6	83.0 ± 0.5	88.1 ± 0.6	6.5 ± 0.4
G	93.8 ± 0.7	92.3 ± 0.6	94.2 ± 0.7	2.4 ± 0.4
H	74.1 ± 0.8	78.7 ± 0.7	85.1 ± 0.8	14.6 ± 0.5
I	78.9 ± 0.6	86.6 ± 0.6	90.2 ± 0.6	11.2 ± 0.4
J	82.4 ± 0.7	80.9 ± 0.7	87.2 ± 0.8	6.0 ± 0.5
K	83.1 ± 0.6	85.6 ± 0.5	82.3 ± 0.6	5.1 ± 0.4
L	82.0 ± 0.6	79.0 ± 0.5	81.9 ± 0.6	11.6 ± 0.4
M	75.2 ± 0.6	80.6 ± 0.6	84.1 ± 0.6	7.6 ± 0.4
N	76.2 ± 0.6	81.2 ± 0.5	88.0 ± 0.6	6.1 ± 0.4
O	80.3 ± 0.9	82.7 ± 0.8	87.9 ± 0.9	6.1 ± 0.5

* The average motility (%), percentage of sperm with normal morphology, the percentage of sperm with intact acrosomes and the percentage of sperm with distal cytoplasmic droplets from the four semen collections obtained during the heterospermic insemination study are included. Data are means ± SEM, based on each ejaculate.

Table 8

Multiple linear regression analysis to assess the relationship of sperm characteristics to heterospermic fertility*

Variable	Estimate	Standard Error	t value	p value
Intercept	0.24	10.28	0.02	0.9821
Motility (%)	0.29	0.09	3.41	0.0078
Morphology (%)	-0.45	0.12	-3.59	0.0058
Acrosomal Integrity (%)	0.29	0.13	2.18	0.0576
Sperm with a Distal Droplet (%)	0.21	0.13	1.58	0.1474
Average Sperm Bound to Zona	-0.52	0.24	-2.21	0.0545

* Estimate represents the regression of heterospermic fertilization on each variable. The R^2 for this model was 0.70.