# RELATIONSHIP OF FIBRONECTIN 1 (FN1) TO THE EXTENDED SEMEN SHELF LIFE OF DUROC AND PHILIPPINE NATIVE BOAR-QUEZON AT DIFFERENT STORAGE TEMPERATURES

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## ABSTRACT

**An investigation to assess the seminal plasma protein level of Fibronectin 1 (FN1) as indicator of semen self-life was done using fresh semen from Quezon and Duroc boars diluted with commercially available medium-term extender and stored at either room (22-25°C) or refrigerated (4-8°C) temperature. The seminal plasma FN1 protein levels were quantified using western blot followed by a chemiluminescent screening. Meanwhile, sperm motility parameters and morphology were assessed using a computer-assisted sperm analyzer (CASA) every 4 hours until acceptable values for good extended semen quality were observed. The contrast between the shelf-lives of extended semen between breeds was not significant at room or refrigerated temperature. Moreover, the comparison of extended semen shelf life between the two storage temperatures yielded a non-significant difference for both Quezon and Duroc boars. Association between FN1 levels and extended semen shelf life revealed a trend ($P < 0.10$), where a strong relationship in room storage was observed. This study demonstrated comparable characteristics of extended semen from Quezon and Duroc boars, and the potential use of FN1 as a marker for boar semen shelf life analysis.**

Key words: Duroc, Fibronectin, Philippine native boar, semen, shelf life

## INTRODUCTION

The Philippine native pig is known for being small, prolific, and with innate resistance to local diseases. The preference for raising native breeds has been brought by their distinctive taste and provision of less management inputs like the use of organic kitchen wastes as feeds. However, the production and reproductive performance of Philippine native pigs are different from that of the commercial pig breeds. The proliferation of commercial swine breeds has since then threatened the diversity of pigs taking a hit on the native breeds (FAO, 2007). Based on the speed of commercialization, conservation efforts should also be at a pace to prevent genetic diversity loss while promoting the use of native breeds as an alternative food source for future human generations. Such efforts include the identification

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of useful biological markers that could help increase the efficiency of *in vitro* conservation schemes. One of these markers is Fibronectin 1 (FN1), which is a glycoprotein normally present in the extracellular matrix or seminal plasma (Rungruangsak *et al*., 2017). It is identified as a multifunctional molecule that is involved in cell adhesion and reported to have a role in cell survival by specifically preventing cellular apoptosis (Gonzalez-Cadavid *et al*., 2014). In addition to its studied functions, its abundance in the seminal plasma allows it to be tagged as a freezability marker indicating good freezing properties among boar ejaculates (Vilagran *et al*., 2015).

Semen extension is the process of diluting fresh semen using a mixture of chemicals that may be of natural or synthetic source containing nutrients, buffers, salts and antimicrobial agents. These components are essential for the sperm metabolic needs, pH and osmotic pressure regulation and prevention of microbial growth (Carillo, 2016; Holtgrew-Bohling, 2016). These components will also ensure sperm motility and viability for a certain amount of time in different storage temperatures. Depending on the desired period of extension, different extender components are varied to satisfy the environmental requirements of the spermatozoa. As a reproductive biotechnology, the process of semen extension has allowed the distribution of good genetic lines by acquiring the minimum sperm concentration for fertilization. Thus, fresh semen is diluted into semen extender according to the required sperm concentration. As a result, there is an increased boar to sow ratio compared to the natural mating system.

However, there are limited reports available on the fresh and extended semen characteristics of Philippine native boars; moreover, on biomarkers that can predict their semen storage capacity. This information, if made available, will be very useful in the conservation and propagation programs of Philippine native pigs.

Thus, this study analyzed and compared the extended semen characteristics of Philippine native Quezon and Duroc boars. This study has also demonstrated the identification and quantification of seminal plasma FN1 protein in the semen of Philippine native Quezon and Duroc boars using western blot and chemiluminescent screening techniques. Lastly, the study also assessed the potential use of FN1 as a biomarker for semen shelf life during extension.

## MATERIALS AND METHODS

A total of 6 sexually mature boars (i.e. 3 Quezon native and 3 Duroc boars) were used as semen donors. These animals were housed in different farms namely, University Animal Farm (UAF) of the University of the Philippines Los Baños, Agricultural Training Institute - International Training Center for Pig Husbandry (ATI-ITCPH), and Bureau of Animal Industry - National Swine and Poultry Research and Development Center (BAI-NSPRDC). The biosecurity and herd health program instituted in the said respective farms were strictly observed during the conduct of the experiment. The boars were trained for semen collection and the collected semen samples were processed and analyzed at the Animal Physiology Laboratory, Institute of Animal Science (IAS), College of Agriculture and Food Science (CAFS), University of the Philippines Los Baños (UPLB). The experimental period lasted from March 2019 to April 2019.

All semen samples were collected from experimental boars using the gloved hand method wearing vinyl gloves. The gel fractions were removed using sterile gauze and stored

in artificial insemination (AI) squeeze bottles at 35-37°C and maintained in a thermal insulator.

Semen samples were evaluated for initial sperm concentration via manual counting with the aid of a conventional hemocytometer. The fresh ejaculates were diluted in a medium-term commercial boar semen extender with antibiotic - MIII™ Minitube (Teifenbach, Germany) based on the initial concentration with a final sperm/dose concentration of 3 billion sperm cells/dose. Two aliquots of extended semen were placed in 50 mL conical tubes and stored separately at room temperature (22-25°C) and refrigerated temperature (4-8°C). Computer-assisted sperm analyzer (CASA) CEROS II (Hamilton Thorne Inc., Beverly, USA) was used to assess the sperm motility (i.e. percent static, percent motile, percent progressive, and percent slow), and percent normal sperm morphology by loading 10 uL of the extended semen in a preheated slide at 37°C, which was then topped with a coverslip. This was done in a 4-hour interval until the extended semen parameters failed to reach the threshold expected for artificial insemination.

Meanwhile, about 15 mL of the filtered ejaculate per boar was centrifuged at 640 rpm at 17°C for 15 mins. The supernatant (seminal plasma) was pipetted into 1.5 mL polyvinyl Eppendorf Tubes and flash-frozen. The flash-frozen samples were kept in 50 mL conical tubes, which were stored inside a liquid nitrogen tank until use.

To obtain the total protein concentration, the flash-frozen seminal plasma was thawed at 70°C for 5 mins. Total seminal plasma protein concentrations were obtained using EPOCH 2 Nanodrop (Winnooski, VT, USA) using 1 uL of the thawed sample into the microplate and read under the protein analysis preset.

To detect and assess the level of FN1 in the seminal plasma, western blot analysis was conducted. The samples were prepared by diluting 10 uL seminal plasma sample with 10 uL 4X Bolt™ LDS Sample Buffer, 4 uL 10X Bolt Reducing Reagent and, 16 uL deionized water. The resulting solution was heated to 70°C for 10 min. The samples were loaded to 1-mm Bolt™ 4-12% Bis-Tris Plus precast gels (Thermo Fisher Scientific, Carlsbad, CA, USA) in a 1x SDS running buffer (i.e. 20 mL 20X Bolt™ MOPS Running Buffer and 380 mL deionized water) in Mini Gel Tank™ (Thermo Fisher Scientific, Thermo Fisher Israel Ltd., Israel) at 200V for 35 min. The separated proteins in the gel were transferred to a nitrocellulose membrane using iBlot2™ Dry Transfer System Device (Thermo Fisher Scientific, Thermo Fisher Israel Ltd, Israel) using the P3 preset. One Pierce ECL Fast Blotting Kit (Thermo Fisher Scientific, Rockford, IL, USA) was used for the western blotting technique. The nitrocellulose membrane was washed with Fast Western 1X Wash Buffer (i.e. 10 mL Fast Western 10X Wash Buffer and 90mL deionized water) at room temperature for 10 min with agitation. It was followed by incubation in a solution containing the anti-FN1 polyclonal rabbit antibody (PA5-29578; Rockford, Illinois, USA) diluted in 1:3000 (v:v) Fast Western Antibody Diluent for 30 min at room temperature. Next, the membrane was incubated in Fast Western Optimized HRP Reagent Working Dilution (i.e. 1 mL Fast Western Optimized HRP Reagent and 10 mL deionized water) for 1 hour at room temperature with shaking. The membrane was washed by suspending it in 20 mL Fast Western 1X Washing Buffer with agitation for 10 mins. This step was repeated 4 times. After washing, the membrane was incubated in the Detection Reagent Working Solution (i.e. 5mL of Detection Reagent 1 and 5 mL of Detection Reagent 2) for 5 min at room temperature. The resulting membrane was placed in a loading tray. Using iBright™FL1000 (Thermo Fisher Scientific, Life Technologies Holdings Pte Ltd., Singapore), the blot was scanned and analyzed for

protein band density. The generated band densities were corrected by multiplying the densities with the quotient of the volume of seminal plasma loaded and the respective volume of fresh ejaculate in every boar.

All data were analyzed using STATA 12.0 statistical program (STATACORP LLC, USA). Mann-Whitney U test was used in comparing the fresh extended semen shelf life in both room and refrigerated temperature between Duroc and Philippine native boar-Quezon. Meanwhile, the Wilcoxon Signed-Rank test was used to determine the difference in the fresh extended shelf life between room and refrigerated temperature. Furthermore, Pearson's Correlation was used to determine the strength of relationships between FN1 level and quantitative sperm parameters (i.e. percent static, percent slow, percent motile, percent progressive, and percent normal morphology).

## RESULTS

Sperm morphology and motility are key parameters in evaluating semen prior to processing and artificial insemination. Using computer-assisted sperm analysis, initial percent total sperm motility (which includes percent progressive sperm, percent slow sperm and percent static sperm) and percent total morphologically normal sperm of diluted semen were observed. As shown in Table 1, the mean % total sperm motility between the two breeds were similar, while a higher mean % progressive sperm was detected in Duroc semen. Further, a higher mean % morphologically normal sperm was detected in Quezon semen.

The shelf life of the extended semen was based on time lapsed in hours before the observed total sperm motility drops below 70%. Table 1 also shows that there is longer mean shelf life in Duroc diluted semen maintained in both room (52.00 ± 8.64) and refrigerated (53.33 ± 4.99) temperatures as compared to Quezon extended semen. Meanwhile, Quezon extended semen has a longer shelf life at room (46.67 ± 1.47) than in refrigerated (40.00 ± 0.00) temperature. However, these differences were not significant.

Additionally, the quantification in FN1 bands was done using iBrightFL1000. The mean band density in Duroc (23,143.33 ± 4,225.75) was greater than that of Quezon (20,256

Table 1. Diluted semen characteristics and seminal plasma FN1 levels in Quezon native and Duroc boars (Mean ± SD).

| Parameters | Duroc | Quezon |
|---|---|---|
| Shelf life at room temperature (h)* | 52.00 ± 8.64 | 46.67 ± 1.47 |
| Shelf life at refrigerated temperature (h)* | 53.33 ± 4.99 | 40.00 ± 0.00 |
| Total sperm motility (%) | 90.73 ± 4.41 | 90.73 ± 6.63 |
| Progressive sperm (%) | 37.20 ± 9.05 | 29.83 ± 9.91 |
| Slow sperm (%) | 4.00 ± 1.72 | 7.90 ± 4.65 |
| Static sperm (%) | 9.27 ± 4.41 | 9.27 ± 6.63 |
| Total normal sperm morphology (%) | 78.93 ± 2.06 | 83.70 ± 1.49 |
| Fibronectin1 (band density) | 23,143.33 ± 4,225.75 | 20,256.00 ± 1,334.79 |

*Shelf life based on time lapsed in hours before the total sperm motility drops below 70%

± 1,334.79). It could be seen in Figure 1 that the bands were 200-250kDA with 2 bands among all except for Boar Duroc1 (BD1). The two bands that reacted to the detection reagent were both FN1 since it is a dimer with an approximately 250 kDa molecular weight as previously reported (Druart *et al*., 2013; Gonzalez-Cadavid *et al*., 2014).

The correlation results, as presented in Table 2, showed a trend (*P*=0.0591) where FN1 protein density is strongly (r=0.7943) related to fresh extended semen shelf life at room temperature.

## DISCUSSION

The present study was able to show that FN1 level in seminal plasma tend to correlate positively with shelf life of extended semen at room temperature. In the study of Johnson *et al*. (1988), the viability of semen is dependent on the initial quality and the interaction present in the extender used. Fibronectin 1 is an abundant soluble protein in the seminal plasma (Rungruangsak *et al*., 2017). The functional analysis of FN1 suggests its role in the binding of extracellular products to the plasma membrane (Gonzalez-Cadavid *et al*., 2014). Cryopreservation efforts of Vilagran *et al*. (2015) tagged FN1 as protein freezability marker, which indicates that semen containing high levels of FN1 could better resist cold shock. However, the association made between FN1 level and fresh extended semen shelf life at refrigerated temperature was found to be insignificant (*P*=0.3956).

The shelf life in both room (*P*=1) and refrigerated (*P*=0.0722) temperatures for the two breeds are not significantly different indicating that the shelf life of the extended semen of Quezon is neither superior nor inferior to that of the Duroc. During the collection of ejaculates, it was noted that most of the samples from Quezon were creamy which indicates higher sperm concentration while those in Duroc ranges from watery to creamy. According to Kommisrud *et al*. (2002), sperm concentration varies among porcine species. Sperm concentration plays a vital role in the success of fertilization (Saacke *et al*., 1994). Little is known on the semen characteristics of Philippine native boars. However, in this study, it

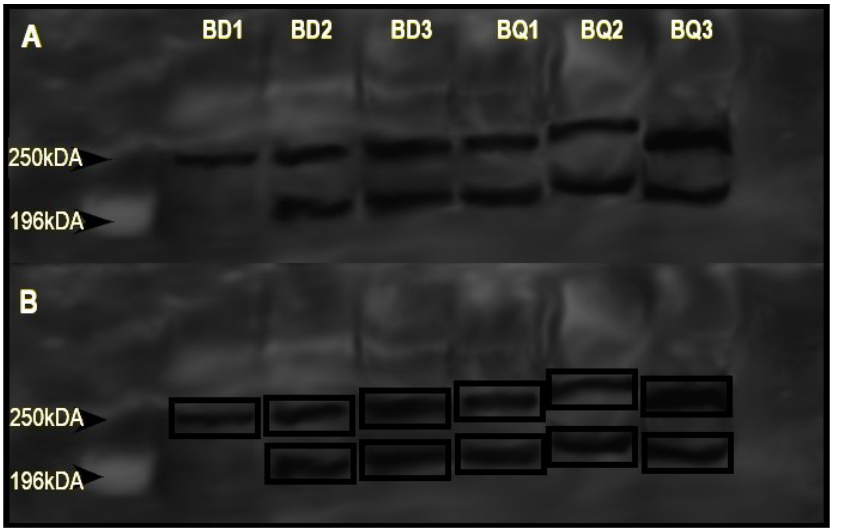


Figure 1. Image of seminal plasma Fibronectin 1 blot under chemiluminescent band detection and quantification using iBright FL1000.

Table 2. Relationship among protein density, number of hours at room temperature, number of hours refrigerated, and percent morphologically normal sperm.

| Pair of Variables | | Pearson's Correlation Coefficient | *P*-Value |
|---|---|---|---|
| FN1 protein density | Shelf life at room temperature (h) | 0.7943 | 0.0591 |
| FN1 protein density | Shelf life at refrigerated temperature (h) | 0.4293 | 0.3956 |
| FN1 protein density | Percent morphologically normal sperm (%) | 0.2423 | 0.6437 |
| Shelf life at room temperature (h) | Shelf life at refrigerated temperature (h) | 0.4602 | 0.3584 |
| Shelf life at room temperature (h) | Percent morphologically normal sperm (%) | 0.6708 | 0.1448 |
| Shelf life at refrigerated temperature (h) | Percent morphologically normal sperm (%) | 0.1386 | 0.7934 |

was found that the semen quality and extended semen shelf life is comparable to that of the Duroc breed.

Analysis performed to check if there is a significant difference in the shelf life between the two temperature profiles revealed that the shelf life reported from the room temperature profile was not significantly different compared to the shelf life in refrigerated temperature for both the diluted semen samples from Duroc ($P$=0.1655) and Quezon ($P$=0.4142) boars. This means that either of the two temperature profiles would yield the same shelf life to the extended semen. However, the data suggest that longer shelf life can be observed at room temperature. The result for the room temperature is similar to the reported optimal temperature of Paulenz *et al*. (2000), which noted that 20°C is the optimal temperature for liquid semen storage using the Beltsville Thawing solution. Arguably, the result for the refrigerated temperature yielded a similar result in terms of shelf life when theoretically it exposes the sperm cells to cold shock. However, the use of efficient extenders could provide protection against cold shock (Althouse *et al*., 1998).

In conclusion, it was found that FN1 density in seminal plasma tend to strongly correlate with extended semen shelf life at room temperature, which confirms that FN1 can be a potential protein marker for the extended semen shelf life at room temperature. The comparison between the two breeds showed that there is no significant difference in terms of semen shelf life at room and a trend on refrigerated temperature. This implies that the semen quality of Philippine native boar-Quezon is comparable to that of Duroc.

For further studies, it is recommended to increase the number of boars used and the number of semen ejaculate per boar. All boars should be housed in the same housing facility to avoid variation due to the environment and other factors related to the transport of specimens. Further, sample loading for western blot analyses should be based on total protein concentration determined through Bradford assay. In the presence of low total protein

concentration, protein precipitation can be done. The use of the housekeeping gene is also advisable for the normalization of band densities.

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