

Project Summary

The presence of co-evolution of sexual partners in animals is evidenced in the ability of the animals to protect against the invasion of foreign objects into the female reproductive system. Females of many mammalian species, including cows, pigs, dogs and horses, produce a quantity of neutrophils, a type of white blood cells, in the uterus directly after intercourse. These neutrophils extend extracellular traps, or NETs, to attract bacteria to prevent them infecting the uterus and causing abortion of the fetus. The consequential effect of these NETs is the binding of the sperm as well. The sperm that are caught in these NETs are broken down and therefore cannot fertilize the egg, or eggs, that are present in the oviduct.

Males of each of these species have developed a protein to prevent against this detrimental linkage. The protein, called Fertility-associated antigen or FAA, is found in the seminal plasma of these males and associates itself with the sperm plasma membrane. The important functionality of this protein is the DNase activity in specific regions of the amino acid sequence which allows for the sperm to be able to break down the NETs and migrate to the appropriate site.

The antibody that was created against the FAA for use in detection in bulls does not react with the same protein in stallions. Therefore, it has been hypothesized that the DNase activity created by the protein in the stallion is higher than in the other mammals, as a result of co-evolution toward greater activity. The DNase activity level would correspond to that of the level of neutrophil concentration within the recipient female animal, as these neutrophils in the uterus exude single stranded DNA that is used to trap foreign material including sperm. This will be tested using the samples of multiple stallions, bulls, dogs, and boars that have been previously been documented to be positive for FAA.

Background

In many mammals, fertility has been compared to the molecular composition of the semen to determine if any molecules present aid in the fertility of the specimen and thus can be used as a testable marker for fertility. As was first discovered in cattle, Fertility-associated antigen (FAA; McCauley et al. 1999) and Type-2 tissue inhibitor of metalloproteinases (TIMP-2; McCauley et al. 2001) were only present in bulls with higher fertility as compared to other animals of the same species. These markers were found to bind to the sperm head by associating themselves with the plasma membrane, as shown by immunofluorescence of sperm with a monoclonal antibody (McCauley et al., 2006).

The FAA in cattle was then characterized as a heparin-binding protein with a 73% identity to a deoxyribonuclease (DNase) I-like protein (McCauley 1999). In following experiments, the effect of heparin-binding allowed for increased ability for sperm to reach capacitation, or the ability of sperm to bind the zona pellucida and undergo the acrosome reaction which leads to fertilization of the egg (Ax 1985). Also, there is a proposed DNase site that interacts with neutrophil extracellular traps (NETs) that are composed of single stranded DNA molecules that are typically used in the prevention of bacteria but also trap sperm as a side effect (Cropp 2006). In digestion of the protein, two internal amino acid sequences had 80 and 85 percent identity with the DNase 1-L3 active sites (Fig. 1). This DNase activity, the breaking down of



Figure 1: Fertility-associated antigen in cattle as compared to the DNase1-L3 type protein by amino-acid structure. (McCauley et al. 1999)

DNA into smaller fragments, is important for the sperm to be able to escape the neutrophil NETs and make their way to the oviduct to fertilize the egg. Thus, the presence of FAA in the seminal plasma increases fertility in bulls by 13-19% (Bellin et al. 1994; 1996; 1998). As a result, a chute-side test was created to determine if bulls were either positive or negative for FAA by using a rabbit antibody reaction that was colorimetrically enhanced to allow for visualization of the positive tests (McCauley et al. 2004).

FAA was then discovered in pigs and dogs by using the antisera for cattle to determine its presence. When the antibody that was used for the cattle was used against the stallion seminal plasma, the test was negative. However, when the seminal plasma was characterized, FAA was discovered as it is a 31-kD protein that has the aforementioned qualities (McCauley 1999). This indicated that the equine FAA has a different sequence than the rest of the species that were studied, and that the other species has sequences that were similar enough to react with the same antibodies.

Hypothesis

H₁: Since the stallion FAA has already been shown to have a different structure from the bull, boar, and dog samples, the DNase activity in the stallion sample should be significantly greater ($P < 0.05$) from the other samples due to the co-evolution of the sexual partners to greater activity. This could either be greater or less activity as the difference would reflect the difference in structure. Therefore, there would also be a difference in structure shown on the amino acid sequence. Also, the three other samples should be similar in activity due to the ability for the antibody to bind them.

H₀: After purification of seminal plasma and characterization of FAA, there will be no differences in DNase activity or nucleotide sequencing among the species tested.

H_A: The FAA among the different species will all have different DNase activities or nucleotide sequences that are significantly different ($P < 0.05$), showing that the co-evolution of sexual partners has no effect.

The differences between species could have developed from the necessity for greater DNase activity as a result of more effective invasion of the neutrophils into the reproductive system of the female. This would account for all of the animals having FAA as a result of the mammalian female reproductive reaction to the invasion of foreign objects and male co-evolution to that influence to allow for the sperm to move through the tract to be able to fertilize the egg. The stallion, being proven different in its structure from the rest of the mammals, will have a different DNase activity as a result of a greater need for the invasion of neutrophils into the tract.

As seen in the bulls, the presence of FAA greatly increases the fertility of the male that produces it as compared to males without the protein. If the DNase activities are different among the species, the most effective FAA protein against the invading neutrophil DNA NETs could convey the greatest fertility differences between FAA-positive and FAA-negative members of the species. This would allow for future experimentation among the species that would see if the greatest DNase activity FAA could bind to other species sperm.

Proposed Study

The Fertility-associated antigen from four different species (cattle, pigs, dogs, and stallions) will be characterized to determine DNase activity and nucleotide sequence. This allows for the DNase activity to be compared to the structure on the nucleotide level to determine if the structural differences have anything to do with the active sites of the DNase 1-L3 protein. The four species selected have already been determined to have the appropriate 31-kD protein of interest. These species also have easily accessible and well-documented methods of sampling the semen. The number of different species will allow for a wide variety of mammals such that similarities can be easily attributed to the species that are along the same evolutionary pathway, whereby divergences in structure can be attributed to either the necessity for the animal to change the functionality of the protein or the distance away from each other in an evolutionary standpoint. Seminal plasma will be collected from four specimens from the same species to allow for differences in genetic mutation among the species. Specimens will be assigned numbers that will be used instead of being identified by their species to prevent prejudice.

Sample Collection and Storage

Samples will be obtained by either self-ejaculation or assisted ejaculation into an artificial vagina or sterile apparatus. The sample will be transported to the Arizona Andrology Laboratory and Cryobank at which time it will be frozen. The sample does not need to contain live sperm, and the sample can be used if from specimens that have had vasectomies. The sample will be stored in a liquid nitrogen tank using egg-yolk base freezing media and techniques outlined by the AALC until a time when each can be purified and characterized. Each sample will be separated into different, smaller vials upon freezing that will be combined when the samples are

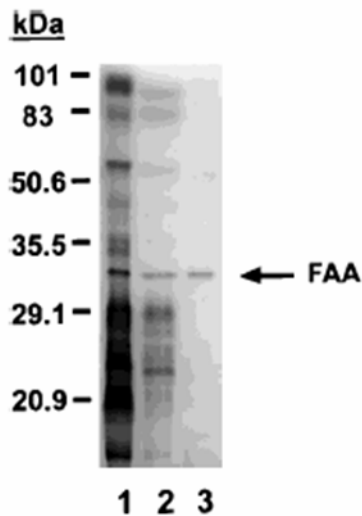


Figure 2: SDS-PAGE showing the progression of heparin-binding protein column elutions to show presence of FAA (McCauley et al. 1999).

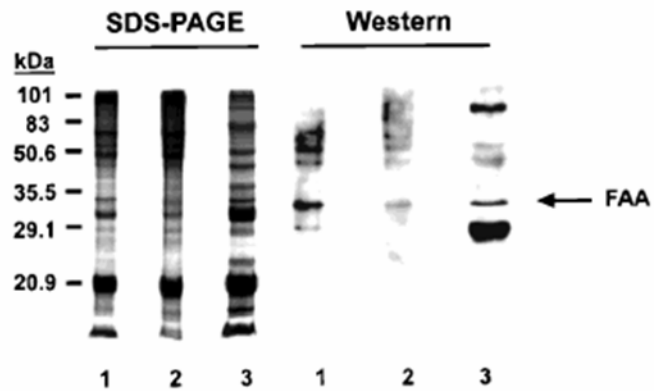


Figure 3: SDS-PAGE and Western blot using rabbit anti bovine antibodies (McCauley et al. 1999).

being characterized. The vials will be thawed in a water bath at 37 degrees Celsius to allow for greatest recovery of the proteins on the plasma membranes. All of the samples will be collected for evaluation within a 3 month period of time.

Sample Characterization

The extraction of FAA from sperm will be followed using the method outlined in McCauley et al., 1999, whereby the sperm will be pelleted and the washed in a saline solution that allows for the disassociation of FAA from the sperm plasma membranes. The isolation of FAA will use a heparin-affinity column that will bind the FAA to the column will separate it from the other proteins in the solution, and will be performed as outlined in McCauley et al., 2001. Sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS-PAGE gels will be run to determine that the protein expressed is actually the 31-kD target protein, as the proteins are

separated by molecular weight with the smallest molecules running through the gel the fastest with the weight determined by a SDS-PAGE ladder. Also, N-Terminal Amino Acid sequencing will be used to sequence the protein, and will be performed at the University of Arizona Macromolecular Structure Facility as outlined by McCauley et al., 1999. Finally, DNase activity will be assayed by the addition of specific concentrations of FAA to DNA, followed by timed extractions of the solution and separation by agarose gel. This allows for the determination of activity by the amount of smearing that appears in the gel. These characterizations will take approximately 9 months, for a total length of the experiment being 12 months.

When the DNase activity assay is done, the average of the assays over the different animals in the same species will be taken. However, if there is a large difference between the DNase activity in one animal within a species, then the amino acid sequence will be determined. If the amino acid sequence differs significantly from the other specimens, then the FAA of the different specimen will be considered a mutant version and therefore will not be used in the experiment. The specimen will be replaced as to keep the number of specimens among all of the groups equal with a random selection from the available pool of specimen candidates. If sufficient candidate cannot be found, then the remaining specimens will be the only ones used.

The stallions' samples will have a difference in the amino acid structure as compared to the other four species, as the antisera test has shown that there is a difference that prevents the reaction from occurring (McCauley unpublished). The other four samples are also assumed to be different with respect to amino acid sequence, as they are from different species. However, these are assumed to be closer in homology to each other than to stallion FAA. If they do not have homology over the entire protein, whatever regions do have homology are potential sites for the antibody reaction to have occurred. This would show structurally where the sites are for binding,

and can also be compared to the stallion to view for non-homology whereby the antibody would not bind. In the situation that the horse has a greater homology with some animals than others, sites of homology can be determined to be regions where the antibody would not bind.

In the DNase activity, the stallion should differ as well, as the different species that require greater DNase activity would require a different structure to uphold said activity. The stallion should be different as the structure has already been proven different by the aforementioned antisera test. The other animals should be more similar in DNase activity, as they are similar enough in sequence to be able to bind the same antibodies. If DNase activity is similar among all species, then the differences in the structure of the protein, such that the antisera does not bind and therefore react, is not in the region of DNase activity. It is also possible that the antisera binds to the portion of the protein responsible for the heparin-binding active site, in which case the DNase activity would be unaffected but the ability of the sperm to bind the heparin-like proteins and capacitate would be affected. At that point, more tests would be needed to further the experiment.

Resources

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Budget

• Sample Collection and Storage	Costs:
○ Sample containers (50 mL, quantity 100) are used for the collection of sterile samples on site for transport to the Arizona Andrology clinic.	24.05
○ Money for transportation to sites around Tucson, Arizona to where the animals are housed (750 miles).	100.00
○ Payment for workers who collect samples from all of the animals on the experiment.	3000.00
○ Freezing	
▪ Freezing tubes (1.5 mL, 500/bag) that are specialized to be able to freeze samples in nitrogen for further analyses at a later date.	99.00
• Sample Characterization	
○ Extraction of FAA	
▪ Dialysis tubing (35 feet) to desalt the solution once the proteins have been salted for disassociation from the sperm.	123.00
▪ Phosphate-buffered saline (PBS) is used to suspend the proteins as a solvent.	313.11
▪ Heparin-affinity column (microbeads) to attract the protein for purification.	350.00
▪ Centrifuge tubes (1.5 mL, quantity 500) for the beads in the affinity column.	34.95
▪ Centriprep tubes to concentrate the protein for future analysis.	74.95
▪ Bovine serum albumin, bSA, (10 g) for standardization of the protein assay for determining the concentration of FAA in the sample.	376.80
▪ Salts (KCl and NaCl) to release the proteins from the tubing.	(included in PBS)
○ Characterization of FAA	
▪ SDS-PAGE chemicals (SDS, acrylamide, Tris buffer, Temed, APS) to make gels to separate out the proteins by size.	334.21
▪ Rabbit anti-bovine (used from previous experiments) and goat anti-rabbit antibodies to be able to react with the FAA to confirm that the protein is present.	140.00
▪ Western blot materials (polyvinylidene difluoride and sponges) to perform immunoblotting to confirm the presence of the protein.	580.69
▪ N-terminal protein sequencing (multiple samples) performed at the University of Arizona Macromolecular Structure facility to determine the amino acid sequence.	1200.00
○ DNase activity	
▪ Premeasured DNA for the determination of DNA breakdown.	270.00
▪ Small aliquot tubes (1.5 mL) to separate out different concentrations of FAA to DNA.	(covered under centrifuge tubes)

TOTAL COSTS:

\$6909.76

