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**14. ABSTRACT** Chronic wasting disease (CWD) of deer and elk is unique among the transmissible spongiform encephalopathies. Our long-term goal is to better understand the epidemiology of CWD and thus develop strategies for management and control. The specific goals of these studies are to develop sensitive assays for PrPres as a marker for infectivity, and use these techniques to monitor the dynamics and modes of shedding of PrPres from orally infected mule and white-tailed deer and elk. Finally these techniques will be applied to investigating the nature of environmental contamination that may be associated with CWD transmission. Protease resistant prion protein from brains of CWD affected deer and elk (PrPres) and cellular PrPc were purified and used in a variety of detection assays. PrPres was detected using antibody-based techniques, which although substantially more sensitive than any current assay still need improvement. Deer and elk have been and infected orally to determine CWD shedding in vivo. We have not identified several protein biomarkers as indicators of prion infection in urine from deer and elk. As the grant ends we have established a very large bank of various deer and elk tissues and fluids starting prior to infection and periodically throughout the infection.

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## EXECUTIVE SUMMARY

Obviously the most important goal is to develop an extremely sensitive assay for the infective prion protein. We have made substantial progress since the start of the grant period but are still short of the goal. We now have a graduate student at Scripps Clinic where he can easily get the antibody chips made and where we have had access to the PrPres "specific" antibodies. Unfortunately, as we described last year antibodies that show certain characteristics in one venue don't hold those characteristics when a different venue is used. Those antibodies do seem specific in tissue assays but in both the bead based and chip assays they were no more specific than any of the other sets of antibodies we are using. The paper describing this is now in press in Analytical Biochemistry.

In a continuation of the proteomics work initiated last year we identified several candidate proteins that are found in urine of infected deer but not in uninfected controls. For some of these we were able to find antibodies and have confirmed the presence of three of these proteins over time using the samples that have been collected for deer over the infection period. Very preliminary results indicate that the levels of the proteins during the infection period. We have not started optimizing the assays until we are certain which protein(s) to follow.

Both the deer and elk infection studies are near completion with the deaths of all but a couple animals. The findings with the deer substantially reinforce the previous data that the genotype of the PrP protein is a major determinate in susceptibility to infection. As noted below all 96SS deer died of the disease but only 1 0f 10 of the 96GG has died. Based on our analysis of the elk genotypes we found no resistant ones. It does appear that the M/L genotype takes longer to progress to disease but the data is weak due to low numbers of any other than the M/M genotype. In a side note, the cattle that were infected orally with the same material used in these studies several years ago by Dr. Elizabeth Williams (the original PI on this grant) were euthanized and examined for disease. None of the animals has exhibited clinical symptoms nor was infected brain tissue found.

## INTRODUCTION

Chronic wasting disease (CWD) of deer (*Odocoileus* spp.) and elk (*Cervus elaphus*) is unique among the transmissible spongiform encephalopathies (TSEs) in that it occurs in free-ranging as well as captive wild ruminants and environmental contamination appears to play a significant role in maintenance of the disease. The precise modes of transmission of CWD are not known although we have shown that horizontal transmission and environmental contamination associated with excreta and carcasses may occur (Miller et al., 2004). But maternal transmission does not appear to play a significant role (Miller and Williams, 2003) in maintenance of CWD in cervid populations. Our long-term goal is to better understand the epidemiology of CWD and apply that information to development of strategies for management and control. To that end we are investigating the dynamics and modes of CWD agent shedding from infected mule deer, white-tailed deer, and elk. The approach includes experimentally infecting cervids, serial collections of a variety of biological samples, and assay of these materials by various means to attempt to detect protease resistant prion protein ($PrP^{res}$). In addition, because of the concern about environmental contamination associated with excreta, we will be collecting and assaying a variety of environmental specimens collected from areas of presumed high, moderate, and low contamination in CWD endemic facilities.

## BODY

**Aim 1**: Develop analytical tools to detect $PrP^{CWD}$ in excreta, blood, and environmental samples.

### Background

Last year's report indicated promising results with the detection of prion protein utilizing the bio-bar code assay (BBCA) and/or magnetic bead ELISAs (MB ELISA). However, while conducting studies with prion protein with these assay formats several important mitigating factors soon became apparent. First, the bio-bar code assay (BBCA) requires extensive expertise in silver development. Similarly, signal development in the magnetic bead ELISA (MB ELISA) requires baseline knowledge to master; however, the cues are simple visual cues to stop development and are easily acquired. BBCA has minimal cues, visual or otherwise, which affects the robustness of the assay. Second, in order to accurately quantitate protein amount in the sample requires running the BBCA six to eight times per sample. Not only does robustness suffer because of this requirement, but the cost of the assay becomes almost prohibitive. Lastly, both the MB ELISA and BBCA assays are physically demanding, time intensive, and mentally taxing, which affects both the simplicity and robustness of these assays.

Based on initial requirements for an assay and the experience over the past years in attempting to develop an assay, an acceptable assay should meet the following characteristics:

1. Specific- The assay needs to have minimal false positives. This is problematic as $PrP_{sen}$ is so ubiquitous in most tissues and fluids associate with CWD.
2. Sensitive-The assay needs to have minimal false negatives. In order to accomplish this, two factors are critical including have (1) low background from both the system and from antibody interactions and (2) high and linear signal amplification.
3. Quantitative-The assay needs to be quantitative and maintain a reasonable range of values for a given amount of target protein (10% coefficient of variation).

4. Rapid-The assay needs to be reasonably rapid in one of three ways. First, the assay could have the ability to allow a single technician to run significant numbers of samples simultaneously. Second, the assay could have the ability to be robotically automates so that large numbers of samples can be run in an automated fashion. Third, the assay time could have a short run time allowing for large numbers of samples to be run sequentially.
5. Robust-The assay needs to be robust in two senses. First, the assay needs to be reproducible across multiple tests across multiple days. Second, the assay needs to be able to easily run by a normal technician without steps which are physically exhaustive or without steps that are easily fallible.
6. Ante Mortem-The assay needs to utilize tissues or fluids that can be easily obtained ante mortem. In addition the tissues or fluids need to be able to dilute and still maintain the ability to detect PrPres.
7. Peer Accepted-The assay needs to readily peer accepted in two ways. First, the assay format should be reported and used in other protein detection applications. Second the antibodies should be introduced and accepted in peer review papers.

After an extensive number of studies with the BBCA, it became apparent that while assay may hold future merit, presently, the BBCA assay fails to meet several important assay requirements as noted above. An alternative solution based on the expertise gained while doing BBCA was to utilize an Antibody Array technology (AA) based on the BBCA system. In an AA, the capture antibody of choice is printed on glass slides with a functionalized surface such as aldehyde, amino, or lysine chemistries (Figure #1). From there, the target protein and biotinylated target antibody are incubated in the slide well. After washing, gold nanoparticles coated with strepavidin are incubated in the wells. The slide is then developed in a silver solution and scanned for quantitation. A main advantage of this AA system is that the system utilizes the same technologies as the BBCA assay for silver development without the variability associated with the DNA slide format. Ab arrays with other target protein are sensitive to the femptograms per mL level and are available for use with Ab specific to the PrPres and Ab Arrays also exhibit internal robustness when appropriate Ab pairs are selected. Thus, the AA format exceeds the requirements for an acceptable assay format. As with the BBCA, screening antibody pairs is critical to achieving assay requirements. In order to do this, different buffers were selected in order to assess potential pair functionality. These buffers intially included 10% urine and AA buffer.

**Results**

After initial pilot experiments and consultations with collaborators, ten antibodies which exhibit high capture potential were selected (136–158, 98–110,19-33,F99, 12B2, D13, 11G5, 94B4, 6H4, and BG4) and printed on GE Amersham's Codelink slides. Two screen were conducted, one with purified prion protein in AA buffer and the other in 10% urine. Both screens revealed several potentially successful pairs as shown in Table #1 and Figure #1. In addition, these screen resulted in important discoveries in understanding printing conditions.

**Discussion**

The data collected indicates that several potential pairs exist which are highly specific and sensitive. The data in the chart suggests that 6H4 capture with 11G5 or 94B4 as the target will

provide an excellent candidate for further characterization. Several other pairs also show strong results.

Characterization of highly specific Ab pairs is undesirable at this time for two reasons: first, pairs that are exhibit high sensitivity but low specificity possess significantly higher signal than the pairs that exhibit high sensitivity and high specificity and second, dogma suggests that current assays for the detection of $PrP_{res}$ utilize protease digestion. Proving a highly sensitive and highly specific assay may have trouble with peer acceptance. It is thus prudent to characterize pairs with the highest signal but lower specificity. Experimentation proving specificity can then follow after proving the sensitivity of the assay.

The data in summary Table 1 indicates that certain capture antibody work well including 6H4, 94B4, and 11G5. The critical nature of the kinetics of capture antibody was suggested by Nanosphere and shown in internal pilot studies, however, Table 1 clearly shows how critical the capture antibody is. Target Ab also showed some level of consistency, especially 94B4. The target Ab appears more normally distributed suggesting exposure to the epitope as more critical. As in previous assays utilized, including the magnetic bead assays, this data suggests that the capture Ab is critical for separation of the protein from the other contaminants whereas the target Ab is critical for amplification of the signal.

**Future Directions**

Two experimental paths are logical based on the primary screens. First, further characterization of the Ab pair interactions while titrating the amount of $PrP_{sen}$ and $PrP_{res}$ from 1ng/ml to 1pg/ml or lower. Characterization would entail determining (1) dose response of the pair (slope greater than .5 signal to target ratio or for every unit of target the signal increases by .5 signal units) (2) sensitivity of pair, (3) robustness of the pair, (4) specificity of the pair to CWD+ PrP versus $PrP_{sen}$ or no protein, and (5) quantification of the protein amount with an error factor less than 10%. The second approach that is evident is a screen to analyze the pair's performance in other buffers including plasma, feces, and saliva. The first experimental path is to establish a standardized buffer for sample preparation. Extension of an assay developed in a standardized buffer would allow for a broad spectrum of biological samples to be utilized. Problems establishing standardized buffer for each biological sample has necessitated the use of dilution of each sample to 10% in a PBS buffer. For the reason discussed above, characterization of Ab pairs with high sensitivity and low specificity should be the next experimental path.

PROBLEMS WITH DEVELOPING AN ULTRASENSITIVE ASSAY FOR THE ANTE MORTEM DETECTION OF THE INFECTIOUS ISOFORM OF THE CWD-ASSOCIATED PRION PROTEIN.

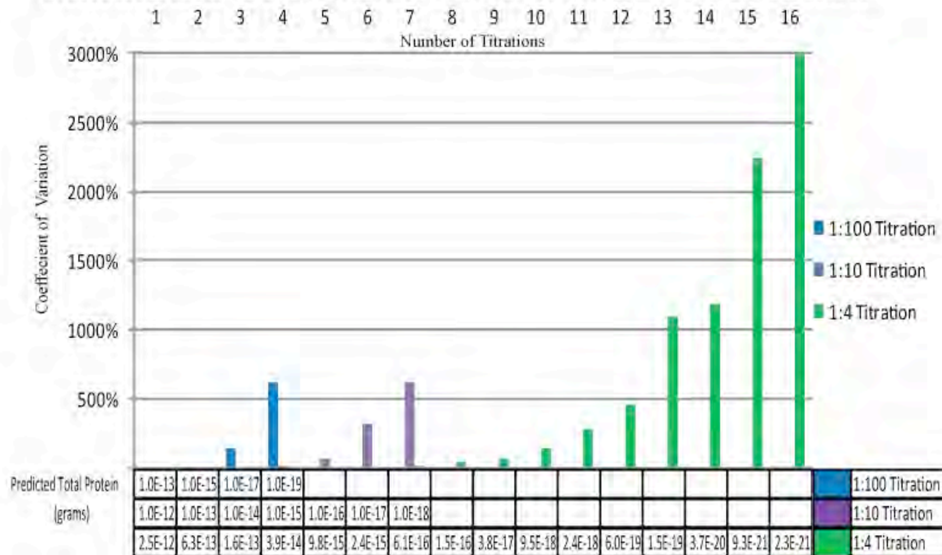
Here we report on problems associated with developing an ultrasensitive immunoassay for CWD including: 1) the lack of specific and sufficiently sensitive and specific antibodies for the infectious isoform(s) of $PrP^{CWD}$, 2) problems associated with serial titration of $PrP^{res}$, and 3) the distribution of $PrP^{res}$ particle sizes. Each of these problems individually or in combination creates a barrier to establishing a consistent signal in a prion immunoassay. Overcoming these

problems require more sophisticated antibody design and a creative engineering of an ultrasensitive protein assay systems for $PrP^{res}$.

The driving force in $PrP^{res}$ assay development has been specificity. Although specificity is critical to the development of an assay, research in sensitivity is also critical as it will open understanding of disease pathology and transmission. We have been dramatically less successful in developing a sensitive (< ng/mL), much less an ultrasensitive assay(<fg/mL). There are two main reasons for this lack of success. First, the antibodies developed to detect prion protein have poor affinities; moreover, they lose even more sensitivity in order to achieve specificity as samples require protease digestion, protein precipitation, or extensive processing in order to distinguish $PrP^{res}$ from the normal conformation of prion protein. Based on bioinformatics simulations we conducted, current antibodies have affinities around 100 nM with little to no specificity in either ultrasensitive format and/or in body tissues and fluids. While certain antibodies exhibit great specificity and sensitivity (Solforosi 2006) in non-sensitive assay formats like immunoblots, these same antibodies do not perform outside of these formats or without highly specific buffer conditions. It may be possible to engineer these antibodies to overcome these limitations both from an assay format perspective as well as a buffer perspective. Undoubtedly, antibodies are continually being generated for prion protein; however, antibodies need to be engineered for specific assay formats, for $PrP^{res}$ to minimize sample processing, and for detecting multiple epitopes per particle possibly even for prion protein's posttranslational modifications. Lastly, prion protein's particle distribution also affects the sensitivity of these assays, in particular the LOD of the assay; however, the variation of the assay, as expressed in the CV, is the greatest casualty of the particle distribution.

Infectious prion protein is found in a distribution of particle sizes ranging from pentamers to high order oligomers (Silviera 2006). Based on bioinforamtics studies, the distribution of particle size causes several unique properties related to variation in detection as shown in simulations described here. First, as the antibody reaches the limit of its $K_D$, the capture antibody sampling fails to follow the particle distribution due to the minimal captured sample size. The consequence is a dramatic increase in the variation in the amount of protein captured and thus a dramatic increase in the variation in assay signal (shown in figure below). Second, another larger variation is due to the capture sampling effect which is seen when the amount of the total protein approaches attogram levels. The particle distribution in this situation fails leading to high variance in total signal. Third, the greater the number of exposed epitopes per particle leads to not only greater variance but also a corresponding loss in sensitivity. At some point, depending on the number of epitopes exposed per particle, an antibody engineered with high affinity will have a smaller and smaller increase in sensitivity due to a greater variability caused by particle distribution and the number of exposed epitopes. Lastly, if the number of titrations is large or if a small percentage volume titration is used, the variation increases almost logarithmically as the number of titrations is performed. This effect is likely to be seen in very dilute samples as well; however, this effect may not hold in other tissues and fluids as the particle distribution may be dramatically different than found in the brain. This differential distribution is especially likely in fluids like urine when the kidney's filter is likely to filter larger particles more selectively. Nevertheless, several of these variations affect the amount of signal and will affect immunoassays as well as replicated assays such as QuIC and PMCA. These effects can combine resulting in huge variations of over than 1000% leading to the errorneous

identification of samples and false negatives; both situations are unacceptable. Solutions to these problems need to be addressed in order to develop an ultrasensitive assay.




Generation of good antibodies is an absolute necessity for the successful development of an immunoassay of this type. An antibody used in an immunoassay will be highly specific, highly sensitive, buffer adaptable, and multiformat compatible. In other words, an antibody that performs well in an immunoassay will have high affinity for its target protein in many different buffers and in many different assay formats. Dozens if not hundreds of antibodies have been developed for prion protein, and while some antibodies are better than others under different conditions, none of these antibodies meets all of these requirements. Amongst all of the requirements, however, the requirement with the greatest impact on the success of the array is specificity.

A major concern relating to antibodies is their ability to effectively function within the selected assay format. The selected highly sensitive immunoassays utilize attachment chemistry, usually an amino chemistry, to attach the antibody to either magnetic beads or functionalized glass slides. The ability of the antibody to attach is poorly characterized, and thus a shotgun approach was taken in that many antibodies were tested for their effectiveness. Three problems arise from this approach in relation to antibody kinetics. First, an engineered antibody attachment may result in changes to the affinity of the antibody, i.e. the affinity could increase or decrease (Lefebvre 2005). Second, the antibody's kinetics will change due to the attachment of a one to ten nanometer magnetic bead, which is logs larger in size than the antibody. Third, the antibody's complementarity determining regions (CDR) may be the region of attachment on the functionalized surface. For instance the 135-55 CDR has a region of five to six lysine residues,

which may be the result in the antibody attaching to the surface of the bead or glass with the CDR face down (Solforosi, Criado et al. 2004; Solforosi, Bellon et al. 2007; Solforosi 2008).

There are many antibodies available for assay development and while there are many protein systems where functional assays have been developed, antibody engineering is an area that must improve if a quantitative immunoassay for $PrP^{res}$ is to be developed (Safar 2008). In order to engineer an effective antibody, it needs to be highly specific for $PrP^{res}$, sensitive in multiple buffers, especially those buffers with high levels of $PrP^{C}$, and engineered for the selected assay format and surface chemistry.

While antibodies pose a problem for the development of an assay, a larger problem is prion protein itself. Prion protein was hypothesized to be the infectious agent in TSEs over 25 years ago, and yet, the protein itself remains largely uncharacterized beyond sequencing and simple biochemical characterization. Important characteristics such as protein function, domains, and glycolsylation function have eluded researchers. This is due to several dynamic properties of PrP including altered conformational state, glycosolation state, and physiological state. Because of infectious concerns, $PrP^{res}$ can be difficult to work with; however, even the normal conformation of the protein is difficult to purify, to keep soluble, and to prevent attachment to surfaces and self-aggregation. The most challenging problems, however, remain separating $PrP^{res}$ from the ubiquitous $PrP^{C}$, a comprehensive understanding of how the particle size distribution affects prion protein properties, and determining the structure of many conformations.

$PrP^{C}$ is ubiquitous in every tissue and fluid in the body. $PrP^{C}$ is even found in large quantities in unlikely fluids as urine (Andrievskaia, O. 2008). In physiological conditions, $PrP^{C}$ is a monomer, however, even a small change in physiological conditions like pH or protein concentration can cause PrP to aggregate. A central obstacle in immunoassays is that epitopes that normally capture $PrP^{res}$ also capture ubiquitous $PrP^{C}$. The processing of samples to eliminate $PrP^{C}$ consequently reduces the signal for $PrP^{res}$, which often is only in minimal quantities in most tissues and fluids. Even antibodies raised against $PrP^{SC}$ fibrils, such as the SAF series from Cayman Chemical, effectively capture $PrP^{C}$. Since $PrP^{C}$ and $PrP^{res}$ have the same primary amino acid sequence, exposed epitopes, especially uniquely exposed conformational epitopes, become very important in trying to specifically capture $PrP^{res}$, and even though the differences between the conformations are dramatic, these differences are not favorable toward better capture of $PrP^{res}$.

Unlike $PrP^{C}$ which is usually found as an individual protein, $PrP^{res}$ is found in aggregates of different sizes (Silveira, Hughson et al. 2006). Computer simulations performed clearly show that the distribution of particles affects both the amount of captured protein and signal variation, probably due to the differential kinetics associated with the position of the exposed epitope within the aggregate. Particle distribution may be tissue or fluid specific so that the data may not hold; nevertheless, it is also likely that any particle distribution will impact immunoassays. This particle distribution makes structural characterization important. Unfortunately, the tertiary and quaternary structure of the aggregates is unknown, thus making the use of molecular dynamics of any kind ineffective. Further, characterizing the posttranslational modifications of prion protein, especially glycolsylation, may be critical to determining the structure, epitope exposure, and

biochemical properties of $PrP^{res}$. With a structure and molecular dynamics one could begin to predict the epitope/paratope interactions. The bottom line is that particle size distribution and particle structure affects the effectiveness of an immunoassay. Two logs of variation of protein capture and total signal renders the immunoassay incapable of producing consistent results.

The last important factor affecting exposed epitopes associated with the protein is the sequestration of the protein. Prion protein is sequestered in several well known ways in the body including as a GPI-anchor in lipid rafts and as a complex with LDL (Safar JG 2006; Taylor DR 2007). This sequestration may provide an important purification or isolation mechanism in the future; however, with present technologies, the sequestration of $PrP^{res}$ effectively minimizes the sensitivity of immunoassay by masking exposed epitopes.

When prion protein aggregates into $PrP^{res}$, many epitopes are believed to become obscured. This effect is caused by the ubiquitous normal conformation of the protein binding antibodies , by the obscuring of epitopes through the aggregation of the protein into fibrils, or the obscuring of epitopes by sequestering by LDL and other complexes. Since the protein is difficult to work with, selecting the appropriate assay format becomes critical.

While the successful assay formats such as IHC, Western Blots, Immunoprecipitations, and ELISA have focused on specificity, extensive research continues for the development of ultrasensitive detection of $PrP^{res}$. While some formats such as mass spectrometry were thought to be viable, formats utilized with at least limited success to date include cell free conversion assays including PMCA and QuIC, immunoassays such as CDI, and hybrids of the two formats such as AS-FACTT. All of these formats provide unique advantages and disadvantages; moreover, none of the assay formats in their present state have met the requirements outlined in the introduction.

Initially, BBCA immunoassay format was selected because of 1) its ability for ultrasensitive detection of proteins, 2) its ability to minimize the dissociation constant of antibodies, and 3) the available expertise. Evidence suggested BBCA would be a successful assay format due to the hypothesized ability to effectively capture a protein with the limited affinity antibodies that have been created for prion protein. The ability to concentrate the antibodies on a magnetic bead leads to not only a lower disassociation constant, but also better kinetics than traditional ELISA formats. While the BBCA has been successfully utilized in limited research protein system settings, the assay failed to meet minimal levels of success in prion systems although it did produce minimal but inconsistent successful results (Reference chapter). The first problem was BBCA robustness. In order to develop this assay further, more basic research is necessary to address 1) the elution of the DNA from the antibody/protein sandwich for more consistent DNA recovery, 2) the development of silver chemistries for a more consistent signal, and 3) most importantly the ability to visually (by the operator) or optically (by a optical scanner) stop development at the optimal time in order for tighter control of signal development. The largest problem, however, was the robustness of the assay. The robustness of BBCA is seriously impacted by the physical demands of the assay. The assay required extensive pipetting steps and extreme organization which leads to three problems: the assay's physical demands made errors by the operator more likely, the assay's physical demands made the possibility of high throughput logistically challenging if not impossible, and the assay intricacy of pipetting made

training difficult. This format needs to address these problems with more basic research before wide scale adoption can be made.

In order to capitalize on the expertise developed while using the BBCA, Antibody Arrays (AA) were explored because of their similarity to BBCA while reducing the problems associated with BBCA. The AA format still has silver development problems, although they are dramatically less than the BBCA as the development is more consistent; however, the other issues associated with the BBCA robustness are overcome as the assay's pipetting requirements are drastically reduced. Overcoming the assay format problems associated with BBCA, it became increasingly apparent that while the AA had problems, these problems were minimal in comparison to the problems associated with the protein and with the affinity of the antibodies as determined by utilizing other protein pairs with AA. Studies developing appropriate controls for the assay revealed that the source of the obstacles did not lie in the assay technique itself but instead was integrally related with the prion protein. This format thus will work for protein systems; nevertheless, the baseline requirements for successful assay development include an antibody pair with high enough affinity when attached to a functionalized surface as well as a monomeric protein or consistent protein behavior when polymerized. Neither one of these requirements is presently attainable for prion protein.

In order to overcome these problems, others have utilized prion replication techniques. While advances are being made on a regular basis, these assays including PMCA and QuIC still require extensive advances in basic research. Solutions to current problems with PMCA and QuIC, which include robustness, scalability, and the ability to quantify protein, continue to advance and may yet lead to the development of an assay; nevertheless, the assay still needs years more research to overcome these problems.
Based on recent advancements in assay formats, especially $PrP^{res}$ replication assays, it is probable that in the coming years an innovative assay will emerge; nevertheless, a fundamental understanding of $PrP^{res}$ particle distribution and the development of higher affinity $PrP^{res}$ specific antibodies are necessary. Further, the ability to develop antibodies compatible for these formats is necessary. Advances in assay formats are also hampered by decontamination issues; however, solutions to many of these problems have been discovered.

Even in the past two years, significant advances have been made toward the development of an immunoassay. The discovery of particle size distribution, the engineering advances in PMCA, and the engineering of partially specific antibodies have moved the field forward. In the future it will be necessary to advance several key areas of research. The further advancement in the understanding of the mechanisms of PMCA may allow PMCA to quantitate $PrP^{res}$ amounts in different buffers, to structurally characterize $PrP^{res}$, and to further characterize strains and species barriers. In the same vein, additional characterization of QuIC may also lead to a quick and ultrasensitive assay. The biochemical characterization of these assays will take research analogous to the development and characterization of the polymerase chain reaction (PCR).

Antibody engineering is also critical. Two areas of research outside of prion therapeutics are important. The engineering of an antibody with high affinity for $PrP^{res}$ is the first. The existing $PrP^{res}$ antibodies, which have limited applications, should be optimized through such technologies as phage display to increase their affinity and specificity. Second, the engineering

of existing antibodies to fit emerging assay formats such as antibody array and magnetic bead immunoassays is important. Engineering antibodies to fit these platforms may provide enough additional affinity to enhance sensitivity for a quick and effective yes/no assay.

**Biomarker Discovery for Chronic Wasting Disease**

We have used the relatively new technology of PF2D to identify potential biomarkers of Chronic Wasting Disease (CWD) that could potentially be used as animal side indicators of the diseases. The PF2D is a liquid means by which to separate proteomes into useful and manageable fractions that are readily submitted for trypsin digests and mass spectrometry analysis for peptide mapping and identification. A proteome is initially separated via cation exchange then further separated using reversed phase chromatography thus providing 2 dimensional separation. Figure 2 demonstrates how the data is analyzed from such a 2 dimensional run, and it in fact shows the actual data used to select our biomarkers. The software, represented in Figure 1, allows us to compare a known positive CWD sample to a known negative CWD sample and it only shows us what is different between the two samples thus making the task of identifying up regulated or new proteins relatively easy.

**Figure 2**

Display of a difference spectrum from the Beckman PF2D of positive vs. negative deer urine. The green lines, contained within the imposed circle, indicate proteins that are present in the positive animal that are not present in the negative animal. Potential biomarkers were selected based on their intensity, or abundance, and were submitted for peptide mapping for identification based on homology to known mammalian proteins.

To date, we have accomplished an extensive analysis of urine from CWD positive animals. The analysis has identified 8 potential biomarkers represented in Table 1. Urine is not an ideal source of biomarkers but we feel strongly that markers found in the urine will also be present in the serum and other fluids of infected animals and our results are bearing this out. The proteins were identified based on their homology to known proteins from other mammal. The deer genome is not known making homology the only means by which to identify potential biomarkers in this instance. Urine from male deer was the only urine deemed suitable for this experiment. Very early in our experimentation there was some difficulty with the reproductive hormones/proteins present in female urine which tended to be significantly more abundant than

any of the since identified biomarkers making it difficult at best to identify them. Urine was pooled from several positive animals as the positive sample, and pooled also from several negative animals to negate the impact that one animals health or its particular environmental stress had on our ability to identify global markers of the disease. Most of the identified biomarkers are neurological in origin, which is a promising indicator that they are in fact linked to the diseased state and not proteins found in the urine due to environmental conditions or stresses.

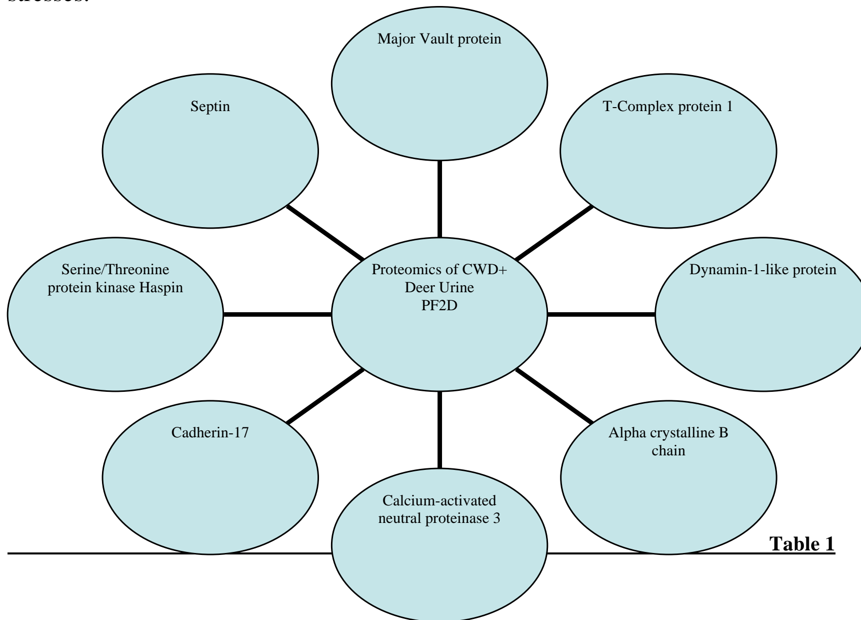



**Table 1**

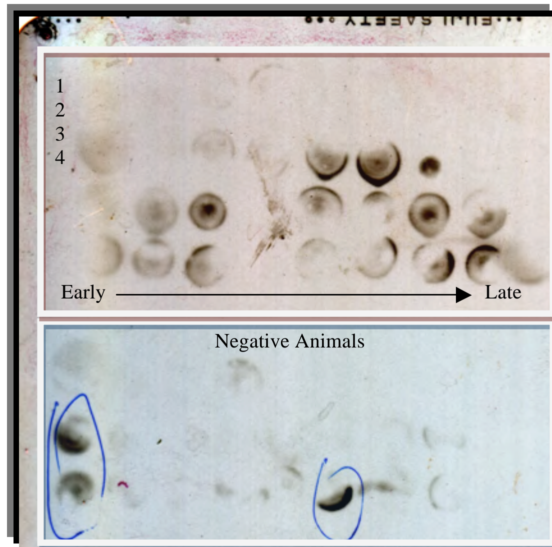
The identified potential biomarkers of Chronic Wasting Disease in no particular order.

To identify whether or not the identified proteins hold any merit as biomarkers for CWD we purchased commercially available antibodies against the proteins. The antibodies tested are, of course, not against the deer form of the proteins rather they are against human, rat and mouse forms of the protein, in general. This predisposes the results to be overly cautious. However, the fact that a couple of the proteins of interest are showing tendencies to be up regulated, or overly expressed, in response to the diseased state strongly suggest we are on the right track.

Initially we challenged infected deer serum, along with a series of negative animal sera's, using ELISA methods. The problem quickly became that trying to develop ELISA's for antibodies that are not specific to the species posed more challenges than we were willing to accept. So, to get a yes/no answer to our question of whether or not they had potential for being decent biomarkers we challenged infected deer serum to our antibodies in a dot-blot western assay. This indicated that at least two of the identified biomarkers hold strong potential to indicate that an animal is or perhaps more importantly, is not infected very early in the disease. The results are certainly not perfect and are meant to represent a jumping off point for further

study.  Figure 3 is a dot blot of one of the proteins that clearly demonstrates an upregulation of the protein in response to CWD .

**Figure 3**




Initial dot blot results for one of the identified biomarkers.  The samples shaded in red represent 4 different blood series taken from deer deliberately infected with CWD.  Samples to the left are early in the infection and progress to later in the infection as you move to the right.  Samples shaded in blue are all known negative samples from a captive deer herd closely monitored and protected from CWD.

The results of the dot blot are promising.  The initial blots were prepared using antibodies that are likely less than specific to the cervid form of the protein thus opening the door for a more specific antibody to more adequately detect the protein.  We are basing our premise that these will be good biomarkers for the disease on the fact that even with the imperfect antibodies we are still seeing much more positive signal in the positive animals than we are in the uninfected animals.  In addition, all of the proteins are likely to be present at some background level at all times in the animals, and with these imperfect antibodies, we can clearly see the difference between supposed normal levels and elevated levels associated with the disease. Given the imperfection of the available antibodies and the already promising results it is now our intention to clone the proteins from deer and generate DNA sequence from them.  This information allows us to design and produce antibodies that will allow us to very accurately determine the presence or absence of the biomarkers that indicate they are suitable for the purpose.

**Aim 2.** Evaluate multiple biological samples collected from experimentally infected mule deer, white-tailed deer, and elk throughout the CWD incubation period.

Colorado: We continued sampling experimentally infected deer (*Odocoileus* spp.) at the Colorado Division of Wildlife's Foothills Wildlife Research Facility (FWRF, Fort Collins, Colorado) in support of Aim 2. During Sep 2007−Aug 2008, we collected urine, feces, saliva, and blood about every 42 days from surviving white-tailed deer (*O. virginianus*) that were inoculated in Dec 2004 according to established study protocols. Samples have been sorted and stored at −20 C pending successful development and availability of assays under investigation (see Aim 1).

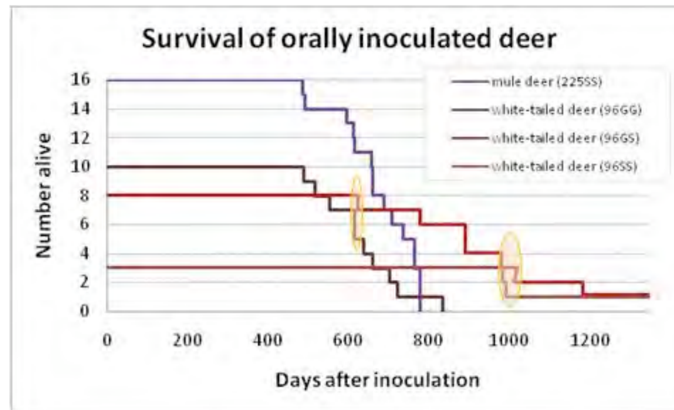



*Figure 2.1.* ***Survival of mule deer (*****Odocoileus hemionus*****) and white-tailed deer (*****O. virginianus*****) orally inoculated with cospecific pooled brain tissue containing about 3 µg $PrP^{CWD}$. Mule deer (all PrP genotype 225 SS) and PrP genotype 96GG white-tailed deer had similar survival patterns after inoculation, whereas genotype 96GS and 96SS white-tailed deer showed prolonged survival after inoculation. Shaded ovals indicate periods where epizootic hemorrhagic disease (EHD) was diagnosed in inoculated white-tailed deer; seasonal EHD epidemics killed six otherwise healthy white-tailed deer during the course of this study, thereby diminishing differences in overall patterns of survival after inoculation among white-tailed deer of different PrP genotypes.***

Although both deer species included in this study were inoculated with conspecific brain pool containing about 3 µg $PrP^{CWD}$, delivered *per os* on the same day (21 Dec 2004), genetic differences appeared to influence the course of prion infection in inoculated white-tailed deer. All 20 of the inoculated mule deer (*O. hemionus*) that survived beyond 252 days post inoculation (dpi) were infected with CWD and died or were euthanized by 778 dpi (Fig. 2.1). Of these, at least 15 died or were euthanized with evidence of clinical CWD (clinical score ≥3) 491−778 dpi. The remaining five mule deer also were infected with CWD, but died or were euthanized 344−487 dpi because of other health problems; whether CWD infection also contributed to the demise of these five deer was unclear. All inoculated mule deer were PrP genotype 225SS. Survival of the 21 inoculated white-tailed deer that lived beyond 252 dpi appeared to be influenced by the PrP codon 96 G/S polymorphism: The 10 96GG individuals survived 488−834 dpi (Fig. 2.1), and at least 7 died or were euthanized with evidence of clinical CWD 517−721 dpi; in contrast, 4 of 8 96GS individuals died or were euthanized with evidence of clinical CWD 778−1182 dpi (Fig. 2.1), and none of the 3 96SS individuals showed evidence of clinical CWD through 1346 dpi despite showing evidence of CWD infection via tonsil biopsy at 477 dpi (Wolfe et al. 2007). Unfortunately, seasonal epizootic hemorrhagic disease (EHD) epidemics in Aug−Sep 2006 and 2007 killed six otherwise apparently healthy white-tailed deer (1 96GG, 3 96GS, and 2 96GG individuals) during the course of our study, thereby diminishing differences in overall patterns of survival after inoculation among white-tailed deer of different PrP genotypes (Fig. 2.1). Unlike mule deer, some affected white-tailed deer did not show

obvious signs of CWD until shortly before their death or termination. These survival patterns in inoculated white-tailed deer are similar to patterns of $PrP^{CWD}$ accumulation across PrP genotypes previously observed in tonsil and rectal mucosa biopsies from these same individuals (Wolfe et al. 2007), and also resemble influences of the 225 S/F polymorphism on survival of CWD-inoculated mule deer reported previously (Fox et al. 2006). The genetic variation in infected white-tailed deer under study here should afford an opportunity to evaluate the influence of genotype on agent shedding once tools have been developed to make such assessments.

**KEY RESEARCH ACCOMPLISHMENTS**

- CWD infections established, confirmed, and monitored to terminus in mule deer and white-tailed deer.
- Serial samples of excreta collected from throughout the disease course from both mule deer and white-tailed deer and available for analysis of prion shedding patterns.
- Genetic influences on disease course in infected white-tailed deer demonstrated, affording opportunities to evaluate the influence of genotype on agent shedding.
- Archived materials shared with other laboratories to advance overall progress on developing sensitive assays for prion detection in blood and excreta, investigating potential routes of prion shedding in deer, and exploring patterns of prion shedding during the disease course.

<u>Wyoming</u>

DOD CWD Annual Report – 9/1/08

Progress during the last year, August 2007-August 2008, for the Chronic Wasting Disease (CWD) study funded by the Department of Defense.

Five positive elk kept at the Sybille Research Center over the last year were sampled as per SOP and euthanized once CWD clinical signs were observed. Biological samples (urine, feces, saliva, and blood) were cataloged and stored at the Wyoming State Veterinary Laboratory (WSVL). Tissues samples after extensive necropsy were also recorded and stored WSVL.

No abortions were performed on the remaining animals nor was there a need for neonate euthanizations from positive control animals. Brain perfusions were performed on two of the neonates shortly after being euthanized using phosphate buffered saline (PBS) as part of the assay development portion of the study. Orphaned neonate deer and pronghorn from the Laramie region were also given brain perfusions as part of the development of an assay.

Eight negative control elk kept at the Red Buttes Research facility over the last year were subjected to routine sampling of blood, saliva, feces, and urine. Samples were archived with the WSVL. Upon fruition of the study all the elk were euthanized, necropsied using standard CWD necropsy protocols, tissues documented, and stored at the WSVL.

Five neonates from five negative control female elk were euthanized, archived, and are stored at the WSVL.

Negative mule and white-tailed deer, which are kept in Pullman, WA. in pens at Washington State University, were sampled in metabolic chambers for urine, feces, blood, and saliva monthly. These samples were frozen (except the blood) and shipped to the WSVL and Colorado Division of Wildlife Foothills Research facility (CDOW). Through the course of the year, one animal died resulting from an accident. The carcass was necropsied and tissues shipped to the WSVL. All other animals were euthanized upon conclusion of the study using our standard protocols for CWD necropsies. Tissues were again frozen and shipped back to the WSVL. A brain perfusion was performed and the brain harvested on one animal using the same protocols as before.

Three of the females were aborted in the spring after being diagnosed pregnant by ultrasound two months earlier. Fetuses were frozen and shipped next day air to the WSVL.

All samples were archived and stored at -20°C until they were utilized by the Molecular biology researchers, at which time requested samples were delivered directly to them.

**PUBLICATIONS TO DATE ARISING FROM GRANT WORK**

M. W. Miller CO Divisi…, 8/28/08 1:19 PM
Comment: These are from last year – not sure whether they should be listed again. Other papers arising from collaborations will be listed in final report.


Chang, B., X. Cheng, S. Yin, T. Pan, H. Zhang, P. Wong, S.-C. Kang, F. Xiao, H. Yan, C. Li, L. L. Wolfe, M. W. Miller, T. Wisniewski, M. I. Greene, and M.-S. Sy. 2007. Test for detection of disease-associated prion aggregate in the blood of infected but asymptomatic animals. *Clinical and Vaccine Immunology* 14:36–43.

Wolfe, L. L., T. R. Spraker, L. González, M. P. Dagleish, T. M. Sirochman, J. C. Brown, M. Jeffrey, & M. W. Miller. 2007. $PrP^{CWD}$ in rectal lymphoid tissue of deer (*Odocoileus* spp.). *Journal of General Virology* 88: 2078–2082.

Brooks, B. and Lewis, R.V. Efficient Screening of High Signal and Low Background Antibody Pairs in the Bio-Bar Code Assay using Prion Protein as the Target (in press, Analytical Biochemistry)


**OTHER COLLABORATIONS ARISING FROM GRANT WORK**

Surplus samples collected in the course of investigations supported by this grant have been shared with at least four other collaborating institutions (Rocky Mountain Laboratories, NIH-NIAID; Case Western Reserve University; Institute for Neurodegenerative Diseases, University of California, San Francisco; Laboratory of Neurodegenerative and Emerging Infectious Diseases, State University of New York Downstate Medical Center) in the hopes of advancing scientific understanding of CWD in particular and prion diseases in general. Other similar collaborative endeavors will be supported as feasible using materials arising from our work.

**REFERENCES**

Fox, K. A, J. E. Jewell, E. S. Williams and M. W. Miller. 2006. Patterns of $PrP^{CWD}$ accumulation during the course of chronic wasting disease infection in orally inoculated mule deer (*Odocoileus hemionus*). Journal of General Virology. 87:3451–3461.

Wolfe, L. L., T. R. Spraker, L. González, M. P. Dagleish, T. M. Sirochman, J. C. Brown, M. Jeffrey, & M. W. Miller. 2007. $PrP^{CWD}$ in rectal lymphoid tissue of deer (*Odocoileus* spp.). *Journal of General Virology* 88: 2078–2082.

Wyoming:

**SAMPLING**

At the start of this period, samples were collected from 31 CWD-inoculated elk. Elk were sampled a total of 8 times at approximately 6 week intervals during this period. Samples collected included blood serum, saliva, urine, and feces. Samples were transported to the Wyoming State Veterinary Laboratory, Laramie, and banked.

MORTALITIES

During this period, 18 elk died and their carcasses were submitted to the Wyoming State Veterinary laboratory for necropsy. All exhibited clinical signs of CWD prior to their deaths.

| DEATH | ELK ID# |
|---|---|
| 29-Aug-06 | 518 |
| 25-Oct-06 | 536 |
| 6-Nov-06 | 507 |
| 20-Nov-06 | 513 |
| 8-Dec-06 | 512 |
| 12-Dec-06 | 514 |
| 1-Jan-07 | 545 |
| 2-Feb-07 | 525 |
| 9-Feb-07 | 522 |
| 16-Feb-07 | 510 |
| 22-Feb-07 | 532 |
| 2-Mar-07 | 520 |
| 6-Mar-07 | 540 |
| 9-Mar-07 | 539 |
| 21-Mar-07 | 506 |
| 2-Apr-07 | 519 |
| 3-May-07 | 548 |
| 14-May-07 | 517 |

PREGNANCY

Six cows calved during this period. Calves were euthanized within 24 hours of birth and sent to the Wyoming State Veterinary laboratory for necropsy. No placentas were recovered from any cow.

| BIRTH | ELK ID# |
|---|---|
| 29-May-07 | 524 |
| 9-Jun-07 | Unk |
| 21-Jun-07 | 544 |
| 28-Jun-07 | 530 |
| 2-Jul-07 | 509 |
| 30-Jul-07 | 516 |

OTHER

In November several elk in a nearby corral died from <u>*Pasteurella multocida*</u> infections. Several elk in this study group appeared depressed and possibly sick during this event. As a precautionary measure, all of the elk in this study group were treated with Nuflor (Schering-Plough Animal Health, Union, New Jersey). The sampling events that were scheduled in December were postponed until January to reduced stress and allow for recovery. No deaths were attributed to this event.

**Aim 3.** The goal of this Aim is to determine if $PrP^{res}$ can be detected in samples collected from facilities contaminated with the CWD agent.

Both CWD endemic research facilities, FWRF and Sybille, have been qualitatively evaluated based on history and pen and pasture usage by CWD-affected animals and categorized into areas regarded as having low, moderate, or high probability of surface contamination with CWD agent (see previous annual reports for details).

<u>Colorado</u>: At FWRF, we previously designed a replicated transect system and collected soil, vegetation, and invertebrate samples from areas regarded as having low, moderate, or high probability of surface contamination with CWD agent (Fig. 3.1) to support the goals of this Aim. Samples were sorted (Table 3.1) and have been stored at −20 C pending successful development and availability of assays under investigation (see Aim 1). In addition, small mammals were been sampled opportunistically in conjunction with other research studies and facility operations and tissues have been archived pending analysis.

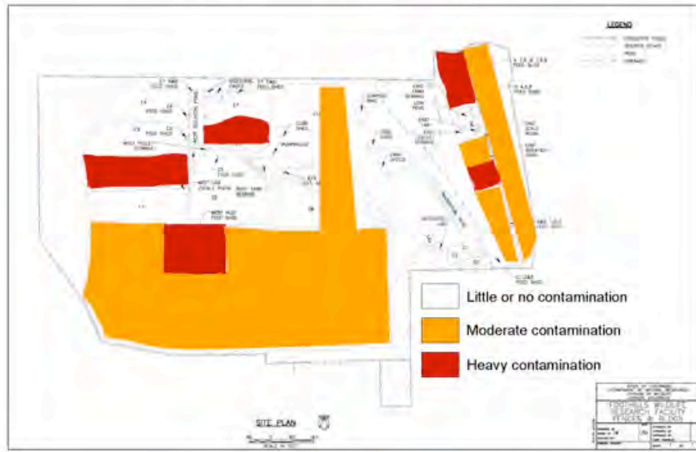



Figure 3.1. Qualitative assessment of areas of CWD-associated surface contamination at the Colorado Division of Wildlife's Foothills Wildlife Research Facility, Fort Collins, Colorado.

Table 3.1. Invertebrates collected from Colorado Division of Wildlife's Foothills Wildlife Research Facility, Fort Collins, Colorado. (Yellow highlights are estimated numbers.)

| Sample Area | Miscellaneous | Orthoptera | Collembola | Hymenoptera | Coleoptera | Araneae | Isopoda | Julida | Scolopendromorpha | Opiliones | Annelida | Gastropoda | Hemiptera | Lepidoptera | Dermaptera | Diptera | Non-insects | Acari | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Low | 61 | 3 | 6 | 223 | 207 | 39 | 175 | 44 | 3 | 3 | 3 | 2 | 1 | 2 | 5 | 0 | 60 | 36 | 873 |
| Moderate | 27 | 3 | 15 | 215 | 363 | 54 | 222 | 0 | 0 | 4 | 3 | 0 | 36 | 17 | 12 | 2 | 103 | 21 | 1,097 |
| High | 78 | 6 | 30 | 157 | 240 | 61 | 207 | 1 | 4 | 6 | 3 | 0 | 0 | 11 | 37 | 5 | 55 | 2 | 903 |
| Incidental | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 4 |
| Compost | 2 | 1 | 7 | 45 | 128 | 75 | 106 | 0 | 1 | 0 | 3 | 0 | 4 | 2 | 5 | 1 | 0 | 0 | 380 |
| | 169 | 13 | 58 | 640 | 938 | 231 | 710 | 45 | 8 | 13 | 12 | 2 | 41 | 33 | 59 | 8 | 218 | 59 | 3,257 |

## Future Work

In order to finalize analysis of the prion protein in urine, antibody pairs in the bead based ELISA will be screened in diluted urine against the infectious isoform of PrP in similar experiments as were conducted using the noninfectious form of PrP. For additional sensitivity, bio-bar code assay experiments will likely be necessary to achieve the desired sensitivity. In addition antibodies from Anthony Williamson's group at Scripps that are specific for the infectious form or PrP have been acquired and will be tested in order to see if digestion can be eliminated.

Other transmission mechanism fluids such as feces and saliva will also be tested using bead based ELISA, bio-bar code assays and the PF2D proteomic system. Currently, experiments are being done in feces to determine optimal resuspension of the prion protein from feces. Saliva experiments will commence after completion of urine and feces studies.

## KEY RESEARCH ACCOMPLISHMENTS

- CWD infections established and confirmed in mule deer and white-tailed deer.
- $PrP^{CWD}$ demonstrated in tonsil and rectal mucosa biopsies from infected mule deer and white-tailed deer.
- Clinical CWD demonstrated in experimentally infected mule deer and white-tailed deer.
- Archived materials shared with other laboratories to advance overall progress on developing sensitive assays for prion detection in blood.

## REPORTABLE OUTCOMES

- Demonstrating $PrP^{CWD}$ in plasma of mule deer (Chang et al. 2007) has applications to both antemortem CWD diagnosis and to other prion diseases that may improve the efficacy and efficiency of ongoing surveillance and health management programs worldwide.

## PUBLICATIONS ARISING FROM GRANT WORK

Chang, B., X. Cheng, S. Yin, T. Pan, H. Zhang, P. Wong, S.-C. Kang, F. Xiao, H. Yan, C. Li, L. L. Wolfe, M. W. Miller, T. Wisniewski, M. I. Greene, and M.-S. Sy. 2007. Test for detection of disease-associated prion aggregate in the blood of infected but asymptomatic animals. *Clinical and Vaccine Immunology* 14:36–43.

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## OTHER COLLABORATIONS ARISING FROM GRANT WORK

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