Test for Detection of Disease-Associated Prion Aggregate in the Blood of Infected but Asymptomatic Animals

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Received 19 September 2006/Returned for modification 23 October 2006/Accepted 25 October 2006

We have developed a sensitive in vitro assay for detecting disease-associated prion aggregates by combining an aggregation-specific enzyme-linked immunosorbent assay (AS-ELISA) with the fluorescent amplification catalyzed by T7 RNA polymerase technique (FACTT). The new assay, named aggregation-specific FACTT (AS-FACTT), is much more sensitive than AS-ELISA and could detect prion aggregates in the brain of mice as early as 7 days after an intraperitoneal inoculation of PrPSc. However, AS-FACTT was still unable to detect prion aggregates in blood of infected mice. To further improve the detection limit of AS-FACTT, we added an additional prion amplification step (Am) and developed a third-generation assay, termed Am-A-FACTT. Am-A-FACTT has 100% sensitivity and specificity in detecting disease-associated prion aggregates in blood of infected mice at late but still asymptomatic stages of disease. At a very early stage, Am-A-FACTT had a sensitivity of 50% and a specificity of 100%. Most importantly, Am-A-FACTT also detects prion aggregates in blood of mule deer infected with the agent causing a naturally occurring prion disease, chronic wasting disease. Application of this assay to cattle, sheep, and humans could safeguard food supplies and prevent human contagion.

Transmissible spongiform encephalopathy (TSE), or prion disease, is a group of fatal neurodegenerative diseases (15, 16). For decades, the only diagnostic test for TSEs was the demonstration of the presence of spongiform lesions in the brains of infected animals or humans by histopathology (15, 16). No reliable in vitro diagnostic test was available until the discovery that TSEs are caused by the conversion of a normal cellular prion protein, PrPC, into the pathogenic scrapie PrP isoform, PrPSc (4, 28). An important effect of this conformational change is that while the entire PrPSc is protease sensitive, the C-terminal domain of PrPSc becomes relatively protease resistant (12, 25). Consequently, the presence of protease-resistant PrPSc in central nervous system (CNS) tissue has provided the basis for the in vitro diagnosis of all prion diseases (19, 39). However, available tests are mostly postmortem, invasive, not very sensitive, and nonquantitative. These deficiencies have also hampered our ability to follow the migration, replication, and accumulation of PrPSc in infected animals.

The exact chemical composition of an infectious PrPSc particle is not known. In vivo, PrPSc exists as aggregates referred to as scrapie amyloid fibrils (22, 29). PrPSc infectivity in hamster brain has a sedimentation coefficient of 40S (22). In another study, it was estimated that the smallest PrPSc has a molecular mass of about 600 kDa (44). However, ionizing radiation inactivation experiments found that the minimum size of a PrPSc molecule has a molecular mass of 50 kDa, which corresponds to a PrP dimer (3). A recent study found that the smallest infectious PrPSc particle has a molecular mass of between 300 and 600 kDa, which corresponds to between 14 and 28 PrPSc molecules (38). The relationships between these smaller PrPSc aggregates and scrapie amyloid fibrils are not known. Whether PrPSc is present in the circulation of infected animals has also been controversial for decades (1, 5, 6, 9, 31, 45). This uncertainty is most likely due to the lack of a more sensitive assay that can detect PrPSc in the circulation. However, a recent study reported that two patients who had received blood from donors who had died from variant Creutzfeldt-Jakob disease (vCJD) had also developed vCJD (21, 27). Therefore, the presence of infectious prion in blood is no longer a theoretical possibility but rather is a tangible threat to public health. An in vitro test that can detect PrPSc in blood would greatly enhance our ability to monitor the occurrence and spread of prion diseases.

To increase the detection limit of currently available immunoblotting assays, Soto and colleagues developed a “protein misfolding cyclic amplification” (PMCA) technique. PMCA allows an undetectable amount of PrPSc to convert exogenously provided PrPSc to PrPSc after multiple cycles of sonication (11, 33, 40). Another study suggested that simple incubation without sonication is sufficient (41). PMCA has been used to amplify and detect PrPSc in the blood of terminally sick

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† Published ahead of print on 1 November 2006.
hamsters with 89% sensitivity and terminally sick mice with 80% sensitivity and specificity (11, 32). Using “seeded polymerization” of PrP, a concept similar to PMCA, it was reported that PrP fibrils could be detected in the plasma of cattle naturally infected with bovine spongiform encephalopathy (43).

Recently, we developed a novel aggregation-specific (AS) enzyme-linked immunosorbent assay (ELISA), termed “AS-ELISA,” for PrPSc aggregates present in the brains of mice infected with the ME7, 139A, or 22L strain of PrPSc (26). The assay can detect disease-associated PrPSc aggregates in the brains of mice 70 days postinfection, a time when prion-resistant PrPSc is undetectable. The fluorescent amplification catalyzed by T7 RNA polymerase technique (FACTT), another newly developed amplification platform, can detect target proteins at fetomolar levels (51). In this paper, we describe the development of an ultrasensitive prion assay. The assay combines an in vitro amplification (Am) step similar to PMCA with AS-ELISA and FACTT. We named the assay Am-A-FACTT. We used Am-A-FACTT to determine whether PrPSc aggregates are present in the brains or blood of infected but asymptomatic mice as well as in deer and elk infected either naturally or experimentally with the agent causing chronic wasting disease (CWD).

MATERIALS AND METHODS

Recombinant PrP protein and monoclonal antibodies. The generation of recombinant PrPc proteins and anti-PrP monoclonal antibodies (Mabs) was described in detail previously (20, 49, 50). All Mabs were affinity purified. MAb, were biotinylated with the EZ-Link Sulfo-NHS-Biotin kit (Pierce Endogen, Rockford, IL).

Mice. Mouse-adapted scrapie strain ME7 was injected (0.1 ml of a 10% brain homogenate) intraperitoneally (i.p.) into 7-week-old CD-1 mice as described previously (37). The ME7 infectant has a titer of about 105 50% infectious doses/ml (26, 37). Sham-infected, age- and sex-matched CD1 mice, normal CD1 mice, and PrP−/− mice were used as controls. Blood was collected from individual mice by cardiac puncture with a 30-gauge needle. Plasma samples were collected after centrifugation and stored at aliquots at −80°C. All animal experiments were carried out according to institutional regulations and standards. All experiments have been repeated at least twice with comparable results.

Naturally infected mule deer and elk. Blood (plasma) and brain tissue (5-mm slices of medulla oblongata cut immediately caudal to the obex) were collected from CWD-infected cervids and presumed to be free-ranging mule deer (Odocoileus hemionus) as described previously (23, 46–48). Five paired sets of blood and brain tissue samples were collected at euthanasia from naturally infected, free-ranging deer previously diagnosed as being CWD agent infected by tonsil biopsy (47, 48). Blood from five unexposed live deer and brain tissue from five different hunter-killed deer were also collected. Three additional CWD brain samples (one mule deer and two elk [Cervus elaphus nelsoni]) and five non-CWD samples (two mule deer and three elk) were kindly provided by the late Elizabeth S. Williams of the Wyoming State Veterinary Laboratory. Plasma and brain tissue samples were stored at −80°C until assayed. All animal handling and sampling were carried out according to institutional policies and standards.

Experimentally infected mule deer. As part of an ongoing study (M. W. Miller, L. L. Wolfe, and R. V. Lewis, unpublished results), in December 2004, mule deer fawns were inoculated with 1 g of homogenized deer brain tissue by using a small syringe inserted into the diastema of the oral cavity as described previously (36). This deer brain tissue homogenate was prepared from the brains of 26 captive mule deer naturally infected with the CWD agent and was previously demonstrated to contain the agent of infectious CWD (36). All fawns were shown to be negative for CWD infection by tonsil biopsy immunohistochemistry (47, 48) prior to inoculation.

Preparation of mouse brain homogenate. To prepare 20% (wt/vol) total mouse brain homogenate, individual mouse brains were homogenized in ice-cold lysis buffer (phosphate-buffered saline [PBS] with 1% Nonidet P-40, 0.5% sodium deoxycholate, 5 mM EDTA, pH 8.0) in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, MO). The homogenate was to be treated with PMSF, omitted. After centrifugation at 1,000 × g for 10 min, the supernatant was stored in aliquots at −80°C.

AS-ELISA assay. In a conventional sandwich or capture ELISA, two Mabs with distinct binding epitopes are required: one MAb is immobilized on a solid phase to capture the antigen, and a second MAb that reacts with a distinct epitope is then used to detect the bound antigen. We reasoned that when PrP proteins dimerize or further aggregate, some epitopes would be buried, while others would be present more than once. We further postulated that if the epitope is present more than once, we might be able to use the same MAb as the capture MAb as well as the detecting MAb. Therefore, in our aggregation-specific ELISA, the same MAb is used as the capture MAb and the detecting MAb.

Ninety-six-well plates (Costar, NY) were coated with affinity-purified capture Mab (ME7, 139A, or 22L strain of PrPSc) and diluted 1:20 in PBS at 4°C overnight. The plates were washed three times with PBS and 100 μl of ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] (Roche Diagnostic, IN) was dispensed into each well. After 20 min, the absorbance was read at 405 nm on a Kinetic Micro-Plate reader (Molecular Devices, CA). The results presented are the averages of the duplicates, and all experiments were carried out blind and repeated at least twice.

AS-FACTT assay. For the AS-FACTT assay, a 384-well plate was coated with a capture antibody in carbonate-bicarbonate buffer (pH 9.6) at 5 μg/ml and 20 μl/well overnight at 4°C. The plate was washed three times with PBS (0.1% Tween 20 in PBS) and blocked with 1x casein buffer (PBS-casein, 1x concentrated; BioFX Laboratory) for 1 h. After three washes with PBS, the tested samples (from a 20% total brain homogenate) were diluted in PBS and added to the coated plate, in the amount of 20 μl per well, for a 60-min incubation at room temperature. The plates were washed three times with PBS, and 20 μl of a diluted biotinylated detection antibody (1 μg/ml) was added to each well. Plates were incubated at room temperature for 60 min. Streptavidin (Chemicon) and the biotin-DNA template (the amplification module [AM]) (51) was added sequentially at 5 μg/ml and 250 ng/ml, respectively, with a 30-min incubation at room temperature for each step, followed by three washes with PBS between each incubation. After excess AM and proteins were removed by washing, 20 μl of a reaction mixture containing 60 units of T7 RNA Polymerase Plus (Ambion), 1.25 mM nucleoside triphosphate, and 120 g/ml of proteinase K (Sigma, MO) at 37°C for 1 h. The RNA-intercalating dye RiboGreen (Molecular Probes) was added to the reaction mixture (20 μl, diluted 1:200 in the Tris-EDTA buffer supplied by the manufacturer), and the plate was read at an excitation wavelength of 485 nm and an emission wavelength of 520 nm in a TECAN Safire reader (Mdrak, MA).

In vitro amplification. For amplification, 10 μl of plasma was mixed with 500 μl of PBS (pH 7.0) and 30 μl of a 20% (wt/vol) brain homogenate from normal mice or mule deer in PBS with 5 μl of a protease inhibitor cocktail, PMSF, in a 15-ml conical tube. Some plasma was also mixed with the same volume of 20% brain homogenate from PrP−/− mice as controls. The presence of PrPc in the brain homogenate is required for amplification because we did not detect any signal in samples containing infected plasma and brain homogenate from PrP−/− mice (results not shown). The mixture was incubated in a 37°C shaking (240 rpm) water bath for 1 h. Another 10 μl of a 20% normal brain homogenate with PMSF was then added, and the mixture was again incubated in a 37°C shaking water bath for 1 h. This procedure was repeated four more times. The sample was then placed in a 4°C rotating platform overnight. Tubes were centrifuged, and the supernatants were discarded. The pellet was then dissolved in 120 μl PBS, and 20 μl of the mixture was removed and used for AS-FACTT.

Enzymatic treatment of brain homogenates. Each brain homogenate was treated with 50 μg/ml of proteinase K (Sigma, MO) at 37°C for 1 h as described previously (26). The protease was inactivated by the addition of PMSF to a final concentration of 3 μM.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. To detect PrP species in brain homogenates, brain samples containing 60 μg of total brain protein were dissolved in 2x sample buffer and incubated at 95°C for 5 min before being separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (26). The 12% polyacrylamide gel was then transferred onto a nitrocellulose membrane and probed by Mab 8H4. After incubation with horseradish peroxidase-conjugated goat anti-mouse immuno-
gated the temporal appearance of PrP Sc in the brains of mice at 70 days but not at 35 days postinfection (23). To improve the sensitivity of AS-ELISA, we combined AS-ELISA with FACTT and developed a new assay, AS-FACTT. Approximately 5% of recombinant PrP (rPrP) occurs as dimers (26). We first demonstrated that AS-FACTT is more sensitive than AS-ELISA for detecting rPrP dimers. With anti-PrP MAb 11G5, which was previously identified as being able to react with the rPrP dimer (26), the lowest detection limit of AS-ELISA is about 20 ng/ml or 2 ng/well of rPrP. On the other hand, AS-FACTT can detect 10 pg/ml or 0.2 pg/well of rPrP (results not shown). We estimate that while AS-ELISA has a detection limit of about 100 pg of rPrP dimers, AS-FACTT has a detection limit of about 10 fg. Thus, AS-FACTT is about 1,000-fold more sensitive than AS-ELISA, an estimation that is in good agreement with recent studies using FACTT (51).

We then compared the sensitivities of AS-FACTT and AS-ELISA for the detection of PrPSc aggregates in the brains of PrPSc-infected mice. Brain homogenates from noninfected or from infected, terminally sick mice were serially diluted and assayed for the presence of PrPSc aggregates by AS-ELISA (Fig. 1A) or AS-FACTT (Fig. 1B). AS-FACTT was more sensitive than AS-ELISA for the detection of PrPSc aggregates. AS-FACTT detected significant reactivity in samples from infected brains, even when diluted to 0.04 μg/ml of total brain protein, which corresponds to ~0.8 ng/well (P < 0.01). In hamster brains, it was estimated that there were between 100 and 1,000 50% lethal dose units of infectivity per ng of brain protein (13). We estimate that the titer of infectivity in hamster brain is about 20-fold higher than the titer in ME7-infected mouse brains. Hence, AS-FACTT potentially detected between 4 and 40 50% lethal dose units of infectivity.

Detection of PrPSc aggregates during early stages of disease. Previously, we found that AS-ELISA can first detect PrPSc aggregates in brain tissues at approximately 70 days postinfection (26). We therefore determined whether AS-FACTT could detect PrPSc aggregates earlier. We inoculated mice intraperitoneally rather than intracerebrally to avoid the possibility of detecting the injected PrPSc in the brain tissues. At 35 days after inoculation, brains from some mice individually were assayed for PrPSc aggregates by AS-ELISA and AS-FACTT. Brains from a group of mice at terminal stages of disease were used as positive controls. Both AS-ELISA and AS-FACTT detected aggregates in every animal at terminal stages (Table 1). However, in brains from animals inoculated 35 days earlier, only AS-FACTT was able to detect PrPSc aggregates (P < 0.0001) with 100% sensitivity and specificity.

Temporal appearance of PrPSc in brain. We next investigated the temporal appearance of PrPSc in the brains of mice inoculated intraperitoneally. At 1, 7, 21, or 35 days after inoculation, brain homogenates from individual mice were prepared. A control group of mice was inoculated with identical amounts of normal brain homogenate, and their brains were harvested 1 day later. Brain homogenates from PrPSc−/− mice, which did not express PrPSc, were also included as controls. Each brain homogenate was assayed for aggregates by AS-FACTT (Fig. 2A). The immunoreactivities detected in normal mice, mice injected with normal homogenates, or mice injected with PrPSc−/− mouse brains were used to establish backgrounds. Backgrounds were subtracted from all readings from controls as well as from infected samples. AS-FACTT was able to detect small but significant levels of immunoreactivity in samples containing 0.04 μg/ml of total brain protein (P < 0.01).

**RESULTS**

**AS-FACTT is more sensitive than AS-ELISA.** AS-ELISA can detect disease-associated PrPSc aggregates in the brains of mice at 70 days but not at 35 days postinfection (23). To improve the sensitivity of AS-ELISA, we combined AS-ELISA with FACTT and developed a new assay, AS-FACTT. Approximately 5% of recombinant PrP (rPrP) occurs as dimers (26). AS-ELISA is able to detect signals at 120 μg/ml (P < 0.01) but not at 60 μg/ml of total brain proteins. Results presented are the averages of the duplicates ± standard errors for individual mice. (A) Detection of PrPSc aggregates by AS-ELISA. Individual brain homogenates from noninfected, normal, or infected mice were serially diluted and assayed by AS-ELISA (26). AS-ELISA is more time points are summarized in Fig. 2B. The levels of immunoreactivity in samples containing 0.04 μg/ml of total brain protein (P < 0.01).

![Image](http://cvi.asm.org/)
protease-resistant PrPSc species in the amplified materials by immunoblotting (not shown). Therefore, the amount of amplified PrPSc aggregates must be below the detection limit of immunoblotting but is detectable by the more sensitive Am-A-FACTT. To validate the utility of Am-A-FACTT, we determined its intra-assay coefficient of variation (CV) and inter-assay CV. The assay is stable, and its discriminating power is robust. The intra-assay CV was less than 10% and the inter-assay CV was less than 14% in all groups (Table 2).

We next investigated if PrPSc aggregates were detectable in the plasma of mice at very early stage of disease, at 35 days postinoculation. Am-A-FACTT detected higher immunoreactivity (3 standard deviations above the mean of controls) in 11 of the 22 infected mice (Fig. 3B). Hence, even at this very early stage of disease, Am-A-FACTT has a sensitivity of 50% and a specificity of 100%.

Detection of PrPSc aggregates in deer and elk infected with the agent causing chronic wasting disease. It was important to determine whether the assays can detect PrPSc aggregates in naturally infected animals, such as deer and elk infected with the CWD agent. We first demonstrated that the CWD agent-infected deer have proteinase K (PK)-resistant PrP species. Data from representative experiment using five infected deer and four noninfected, control deer are shown in Fig. 4. It is clear that all CWD agent-infected deer have PK-resistant PrP species. We then determined whether AS-FACTT could detect PrPSc aggregates in CWD agent-infected deer. We found that AS-FACTT detected PrPSc aggregates in the brains of all CWD agent-infected mule deer and elk (Fig. 5A). We next investigated whether Am-A-FACTT could detect PrPSc aggregates in blood. All blood samples from presymptomatic, infected mule deer were positive (Fig. 5B).

Detection of PrPSc aggregates in experimentally infected mule deer during disease progression. Plasma samples were collected from a group of deer \((n = 18)\) in October or early December 2004, prior to inoculation with PrPSc orally on 21 December 2004. These plasma samples were used as controls. At 22, 84, 127, 167, 209, and 252 days after infection, plasma samples were again collected from individual deer and assayed.
for PrPSc aggregates by Am-A-FACTT. The results of such an experiment are shown in Fig. 6. Eighty-four days after inoculation, one of the infected deer had detectable PrP Sc aggregates (3 standard deviations above the mean of normal controls); by 127 days, eight of the deer had significant PrPSc aggregates; and by 167 days, all inoculated deer had detectable circulating PrPSc aggregates. All inoculated deer had CWD as confirmed by tonsil biopsy immunohistochemistry at 252 days postinoculation. As of August 2006, more than 600 days after inoculation, most of these experimentally infected deer were still alive. Therefore, Am-A-FACTT was able to detect PrPSc aggregates in the plasma of mule deer at very early stages of infection.

**DISCUSSION**

A test that can detect asymptomatic carriers infected with PrPSc is needed to prevent accidental transmission of prion diseases from animals to humans and from humans to humans; such a test would also be valuable as a tool in the surveillance and control of animal prion diseases. Preferably, the test should be minimally invasive and able to detect PrPSc in body fluids such as blood. We have taken the first step toward this goal. We improved the detection limits of our original AS-ELISA by incorporating a signal amplification step, FACTT. We then further improved the sensitivity of AS-FACTT by incorporating a PrPC-to-PrPSc amplification step and developed an ultrasensitive assay, termed Am-A-FACTT. Am-A-FACTT detects PrPSc aggregates in the blood of infected but asymptomatic mice with a high degree of sensitivity and spec-
ificity. Moreover, Am-A-FACTT was also able to detect disease-associated prion aggregates in mule deer naturally or experimentally infected with the CWD agent. It is believed that in naturally occurring prion diseases, such as scrapie in sheep and goats, CWD in deer and elk, bovine spongiform encephalopathy in cattle, and vCJD in humans, the infectious agent enters the host via the oral route. However, the mechanisms by which PrPSc enters the CNS are not completely understood (2, 7, 24, 42). It is believed that PrPSc is imported into the CNS either by follicular dendritic cells (10, 18) or by neurons (17, 30). Irrespective of the pathway, disease-associated PrPSc aggregates are detectable in the brains of some mice at 1 week after an intraperitoneal inoculation by AS-FACTT. Therefore, some PrPSc aggregates are able to migrate from the periphery to the CNS within 10 days (14). PrPSc could be detected as early as 2 weeks after intracerebral inoculation by PMCA (40). We found that by 35 days after intraperitoneal inoculation, PrPSc aggregates were detected in the brains of all mice by AS-FACTT.

The AS-ELISA is specific for PrP aggregates composed of at least a dimeric complex, because it does not react with either PrP monomer or brain homogenates from PrPC "knockout" mice (26). AS-ELISA can also detect PrPSc aggregates in the brains of mice infected with any one of the three strains of PrPSc, ME7, 139A, or 22L (26). Previous studies revealed that the immunoreactivity detected by AS-ELISA included PK-resistant PrPSc that had a molecular mass of about 2,000 kDa (26). By ultracentrifugation in a sucrose gradient, most of the immunoreactivity detected by AS-ELISA is present in fractions 3, 4, and 5. These fractions are mostly devoid of PrPC, as it partitions into the top two fractions. On the other hand, AS-ELISA reacts poorly with the largest PrPSc aggregates, which tend to partition into the bottom fractions. Recent studies by flow field-flow fractionation found that the most infectious hamster PrPScs have a molecular mass of 300 to 600 kDa, which corresponds to 14 to 28 molecules of PrP C (38). Using flow field-flow fractionation, which is much more refined and precise, we found that the AS-ELISA preferentially reacts with hamster PrPSc aggregates with a molecular mass of between 200 and 420 kDa (B. Chang, J. R. Silveira, B. Caughey, and M.-S. Sy, unpublished results). Therefore, it is likely that the AS-ELISA detects infectious PrPSc.

The level of PrPSc in the blood is much lower than that in the CNS (1, 6, 8). Therefore, an additional in vitro amplification step similar to PMCA is required for the detection of PrPSc in blood. The new assay, Am-A-FACTT, was able to detect PrPSc aggregates in the blood of all mice at terminal stages of disease (total, $n > 40$), as well as mice at late but presymptomatic stages of disease (total, $n > 20$). Furthermore, Am-A-FACTT was able to detect higher immunoreactivities in 11 of the 22
mice inoculated 35 days earlier, at very early stages of disease. It should be noted that by 35 days postinoculation, PrPSc aggregates were detected in the brains of all infected animals. On the other hand, at this time, only 50% of mice had detectable PrPSc aggregates in their blood by Am-A-FACTT. This difference most likely reflects the lower levels of PrPSc in the circulation. By using immunoblotting, we did not detect any protease-resistant PrPSc species after the amplification procedures (results not shown). Therefore, the amount of amplified PrPSc aggregates must be below the detection limit of immunoblotting but is detectable by the more sensitive Am-A-FACTT. Am-A-FACTT did not detect any reactivity in blood from animals infected either 1 day or 7 days earlier (results not shown). Hence, the PrPSc aggregates that are detected are most likely host derived rather than the injected PrPSc, and it takes time for PrPSc to appear in the blood. Accordingly, we also failed to detect PrPSc in the blood of PrPSc mice inoculated with PrPSc (results not shown).

Perhaps more significantly, Am-A-FACTT was also able to detect disease-associated PrPSc aggregates in mule deer naturally infected with CWD with a high degree of specificity and sensitivity. This is important because experimentally infected animals tend to accumulate much higher levels of PrPSc. In 18 deer orally inoculated with a low dose of CWD PrPSc (1 gram of CWD agent-infected brain), one deer had disease-associated PrPSc aggregates at 84 days postinoculation. By 120 days, all inoculated deer had detectable PrPSc aggregates in their circulation. In a previous CWD pathogenesis study (36), PrPSc could be detected by immunohistochemical staining in lymph nodes at between 42 and 90 days postinoculation; however, these animals were inoculated with concentrations of PrPSc that were 10 times higher than concentrations used to inoculate the deer that we studied here. Therefore, it appears that Am-A-FACTT may be at least as sensitive as immunohistochemical staining in identifying preclinical infected deer.

Currently, all in vitro diagnostic tests for prion diseases require either the demonstration of protease-resistant PrPSc in brain or lymphatic tissue (19, 39) or the uncovering of hidden epitopes after denaturation (34, 35). More recently, PMCA has been shown to be more sensitive. This is important because experimentally infected animals tend to accumulate much higher levels of PrPSc. In 18 deer orally inoculated with a low dose of CWD PrPSc (1 gram of CWD agent-infected brain), one deer had disease-associated PrPSc aggregates at 84 days postinoculation. By 120 days, all inoculated deer had detectable PrPSc aggregates in their circulation. In a previous CWD pathogenesis study (36), PrPSc could be detected by immunohistochemical staining in lymph nodes at between 42 and 90 days postinoculation; however, these animals were inoculated with concentrations of PrPSc that were 10 times higher than concentrations used to inoculate the deer that we studied here. Therefore, it appears that Am-A-FACTT may be at least as sensitive as immunohistochemical staining in identifying preclinical infected deer.

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