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Experimental transmission of the chronic wasting disease agent to swine after oral or intracranial inoculation

Running Title: The chronic wasting disease agent transmits to swine

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Abstract

Chronic wasting disease (CWD) is a naturally occurring, fatal neurodegenerative disease of cervids. The potential for swine to serve as a host for the agent of chronic wasting disease is unknown. The purpose of this study was to investigate the susceptibility of swine to the CWD agent following experimental oral or intracranial inoculation. Crossbred piglets were assigned to one of three groups: intracranially inoculated (n=20), orally inoculated (n=19), or non-inoculated (n=9). At approximately the age at which commercial pigs reach market weight, half of the pigs in each group were culled ('market weight' groups). The remaining pigs ('aged' groups) were allowed to incubate for up to 73 months post inoculation (MPI). Tissues collected at necropsy were examined for disease-associated prion protein (PrP^{Sc}) by western blotting (WB), antigen-capture immunoassay (EIA), immunohistochemistry (IHC) and *in vitro* real-time quaking induced conversion (RT-QuIC). Brain samples from selected pigs were also bioassayed in mice expressing porcine prion protein. Four intracranially inoculated aged pigs and one orally inoculated aged pig were positive by EIA, IHC and/or WB. Using RT-QuIC, PrP^{Sc} was detected in lymphoid and/or brain tissue from one or more pigs in each inoculated group. Bioassay was positive in 4 out of 5 pigs assayed. This study demonstrates that pigs can support low-level amplification of CWD prions, although the species barrier to CWD infection is relatively high. However, detection of infectivity in orally inoculated pigs using mouse bioassay raises the possibility that naturally exposed pigs could act as a reservoir of CWD infectivity.

Importance

We challenged domestic swine with the chronic wasting disease agent by inoculation directly into the brain (intracranially) or by oral gavage (orally). Disease-associated prion protein (PrP^{Sc}) was detected in brain and lymphoid tissues from intracranially and orally inoculated pigs as early as 8 months of age (6 months post-inoculation). Only one pig developed clinical neurologic signs suggestive of prion disease. The amount of PrP^{Sc} in the brains and lymphoid tissues of positive pigs was small, especially in orally inoculated pigs. Regardless, positive results in orally inoculated pigs suggest that it may be possible for swine to serve as a reservoir for prion disease under natural conditions.

Introduction

The transmissible spongiform encephalopathies (TSEs) or prion diseases are fatal neurodegenerative diseases. Naturally occurring TSEs include chronic wasting disease (CWD) in cervids, scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and sporadic and familial prion diseases in humans.

The potential for swine to serve as a host for the agent of CWD is unknown. A naturally occurring TSE has not been reported in swine (1, 2). Intracranial challenge of swine with kuru, a human prion disease, was not successful (3), although, at the time of those studies, molecular tests for disease-associated prion protein (PrP^{Sc}) were not available. Pigs have been shown to be susceptible to bovine BSE following parenteral inoculation (simultaneous intraperitoneal, intravenous and intracranial routes), ovine BSE following intracranial inoculation (4), and ovine scrapie following intracranial

inoculation (5), but not to bovine BSE after oral challenge with large amounts of infected brain material (6-8).

The CWD agent has a wide host range amongst cervids and can be experimental transmitted to several other species. Naturally occurring CWD has been reported in cervids including mule deer (*Odocoileus hemionus*) (9-11), Rocky mountain elk (*Cervus elaphus nelson*) (11, 12), white-tailed deer (*Odocoileus virginianus*) (10, 11), moose (*Alces alces shirasi*) (13, 14), and reindeer (*Rangifer tarandus tarandus*) (15). In addition, Eurasian red deer (*Cervus elaphus*) (16), Eurasian fallow deer (*Dama dama*) (17), Asian muntjac (*Muntiacus reevesi*) (18), and reindeer (19, 20) have been shown to be susceptible to CWD following experimental inoculation. CWD has been experimentally transmitted to non-cervid species including sheep (21), cattle (22-25), domestic cats (26, 27), ferrets (28, 29), non-human primates (30-32), and laboratory rodents (reviewed in (33)).

Pigs could be exposed to CWD infectivity via two main routes: 1) Exposure of farmed or pet swine (*Sus scrofa domesticus*) to contaminated feed and 2) exposure of feral swine (*Sus scrofa*) to CWD infected carcasses or contaminated environments. In the US, feeding of ruminant by-products to ruminants is prohibited, but feeding of ruminant materials to swine, mink, and poultry still occurs. Therefore, it is possible that, if a CWD-affected cervid carcass entered the food chain through a commercial slaughter house, domesticated farmed and pet swine could be exposed to CWD infectivity in commercially prepared rations. As of 2015, feral pigs have been reported in 39 US states (34) and in 12 of these states CWD has been detected in free-ranging cervid populations (35). Environmental contamination with CWD infectivity in excreta or decomposing

carcasses contributes to horizontal transmission of CWD in mule deer (10). Prion infectivity has been shown to persist on the surface of contaminated plant leaves and roots (36) and in soil (37-39). Therefore, feral pigs could be exposed to infectivity through scavenging of CWD-affected carcasses, consumption of contaminated vegetation, and while rooting around in the soil during foraging. In this study we demonstrate that swine are susceptible to the CWD agent following oral or intracranial experimental inoculation and accumulate PrP^{Sc} in both brain and lymphoid tissues. Detection of PrP^{Sc} in brain and lymphoid tissues from orally inoculated pigs at 6 months after inoculation raises the possibility that naturally exposed pigs could potentially be a reservoir for CWD prions.

Results

Clinical presentation

All pigs culled at 6 MPI (8 months of age; n = 8 intracranially (IC) inoculated, n = 9 orally inoculated) were clinically normal with the exception of one pig (#35) that was noted to be limping on its left front and rear legs. Four IC inoculated pigs and 1 orally inoculated pig) developed intercurrent lameness from approximately 30 MPI usually beginning with the feet and legs and progressing to difficulty rising. At approximately 41 MPI 4 clinically normal pigs (n = 1 IC inoculated, n = 4 orally inoculated) were culled to reduce animal density in the containment space. Neurological signs were observed in one pig (animal #27, incubation period = 64 MPI) and included difficulty rising, and muscle fasciculations and tremors after rising. Pig #27 also had skin abrasions and/or ulceration

over pressure points and polyarthritis. All other pigs were found dead or culled due to intercurrent disease, most commonly lameness that was not responsive to treatment.

Detection of PrP^{Sc}

To determine if pigs inoculated with the agent of CWD accumulate misfolded prion protein in the central nervous system we assayed brainstem using western blot (WB), enzyme immunoassay (EIA), immunohistochemistry (IHC) and *in vitro* real-time quaking induced conversion (RT-QuIC). Results of screening of brainstem material from all pigs by WB and EIA, and results for additional testing of animals that were PrP^{Sc} positive by either screening test can be found in Table 1.

Western blotting

Using WB, PrP^{Sc} was detected in brain tissue from two intracranially inoculated pigs (#27, #28) necropsied at 64 and 73 MPI, respectively (Table 1).
The migration pattern of samples from pigs inoculated IC with the CWD agent were different from either the sample from a pig inoculated with classical bovine spongiform encephalopathy (BSE) or the original CWD inoculum (Figure 1). While the monoglycosylated (middle) band was most prominent in the sample from the pig inoculated with BSE, the diglycosylated (top) band was most prominent in the sample from the pig inoculated with the CWD agent and the original CWD inoculum.

Enzyme immunoassay

Using EIA, misfolded protein was detected in brain tissue from 1/10 IC inoculated market weight pigs, 5/10 IC inoculated aged pigs (42-73 MPI), 0/9 orally inoculated market weight pigs, and 1/10 orally inoculated aged pig (Table 1).

Real-time quaking induced conversion

Using RT-QuIC, PrP^{Sc} was detected in brainstem material from 3/6 IC inoculated market weight pigs, 7/7 IC inoculated aged pigs, 2/6 orally inoculated market weight pigs, and 5/6 orally inoculated aged pigs (Table 1, Figure 2). For each positive sample we quantified the seeding activity based on amyloid formation rate (AFR), which is the reciprocal of the time (h) that it takes for a reaction to reach the threshold (Ct), defined as the mean baseline fluorescence plus 5 standard deviations. For IC inoculated pigs (n = 10), the mean AFR for each animal ranged from 0.025-0.210. For orally inoculated pigs (n = 7) the range of mean AFRs was 0.010-0.029 (Table 1, Figure 2). Average RT-QuIC data, generated by calculating the mean of all replicates from all animals in each challenge group, can be found in Figure 3.

Differential proteinase K (PK) sensitivity of brainstem samples

To investigate possible biochemical properties of PrP^{Sc} that may have contributed to the variation in aggregation kinetics observed on the RT-QuIC assay, the EIA optical density was measured on matched samples with and without treatment with PK. The difference in optical density between non-PK treated and PK treated samples allows us to estimate the relative PK resistance of the PrP^{Sc} present in the brains of infected pigs (40).

158 PrP^{Sc} in EIA positive brain tissue from one IC inoculated market weight pig
159 (#15), one orally inoculated aged pig (#45), and one IC inoculated aged pig (#24) was PK
160 sensitive. PrP^{Sc} from the remaining 4 pigs with samples positive by EIA, all from the IC
161 inoculated aged pig group, was PK resistant (Table 1). Proteinase-K titration was
162 performed on all EIA positive samples and results were consistent across PK
163 concentrations of 0.4-50 μg/mL.
164 Six brain samples were EIA positive and RT-QuIC positive. Of these, the 4
165 samples that were PK resistant had higher AFRs (range = 0.17-0.21), while the 2 samples
166 that were PK sensitive had lower AFRs (0.01 and 0.03) (Figure 2).
167
168 **Detection of PrP^{Sc} in lymphoid tissues**
169 To determine if pigs inoculated with the CWD agent accumulate misfolded prion
170 protein in lymphoid tissues, EIA and RT-QuIC were applied to samples of the
171 retropharyngeal lymph node (RPLN), palatine tonsil, and mesenteric lymph node (MLN).
172 Full results for individual pigs can be found in Table 2.
173 All lymphoid tissues tested were PrP^{Sc} negative by EIA with the exception of pig
174 #37 (orally inoculated market weight pig) that had a positive MLN. Using the RT-QuIC
175 assay, PrP^{Sc} was detected in lymphoid tissues of the head (RPLN, palatine tonsil) in 3/6
176 IC inoculated market weight pigs, 5/7 IC inoculated aged pigs, 4/6 orally inoculated
177 market weight pigs, and 2/6 orally inoculated aged pigs. The MLN was positive in 5/6
178 orally inoculated market weight pigs, 3/4 orally inoculated aged pigs (samples were not
179 available for 2 pigs), 4/6 IC inoculated market weight pigs, 2/4 IC inoculated aged pigs.

Overall, the MLN was positive in 14/19 (74%) of samples examined, the RPLN in 8/18 (44%), and the tonsil in 10/25 (40%).

Histopathology and Immunohistochemistry

To determine if pigs inoculated with the CWD agent develop spongiform lesions or accumulate misfolded prion protein in the brain, coronal brain sections were examined by light microscopy after H&E staining and immunohistochemistry. Occasional neuropil vacuolation and white matter vacuolation were present in different brain sections of control and inoculated pigs. Small to medium-sized grey matter vacuoles were seen in the colliculus of at least one pig from each treatment group, including control pigs (Figure 4A, pig #7 and Figure 4B, #25). Vacuolation and PrP^{Sc} deposition in the colliculus was present in two pigs (#25, #26) from the IC inoculated aged pig group (Figure 4C, #25). Intraneuronal vacuolation was observed in large neurons of the dorsal motor nucleus of the vagus nerve (DMNV) in the medulla at the level of the obex (Figure 4E, #38). This type of vacuolation was present in pigs from all market weight treatment groups including non-inoculated control pigs, and in aged control pigs. PrP^{Sc} deposition in association with DMNV vacuolation was not observed in any pigs.

Positive PrP^{Sc} immunoreactivity was observed in samples from 4 pigs. In the brain, PrP^{Sc} immunoreactivity appeared as the intraneuronal type (coarse granular deposits of PrP^{Sc} in the neuronal perikarya surrounding the nucleus) in large neurons of the rostral medulla

reticular formation (#26), midbrain colliculus (#25, #26), midline thalamic nuclei and hypothalamus (#45, #28), or septal nuclei (#28) (Figure 4C).

PrP^{Sc} immunoreactivity was also seen in the retina of one pig (#26), granular to punctate immunoreactivity in the inner and out plexiform layers with occasional intraglial deposits (Figure 4F, #26). Disease-specific PrP^{Sc} immunoreactivity was not seen in any other tissues although non-specific immunolabeling was common (Figure 4D, brainstem; Figure 4G, retina).

Mouse bioassay

To determine if pigs inoculated with the CWD agent accumulate infectious material, brainstem material from selected pigs was bioassayed in Tg002 mice that express porcine prion protein at normal levels (5).

Pigs from the IC inoculated market weight (#18) and IC inoculated aged (#27, #28) groups, and the orally inoculated aged group (#48) produced positive bioassay results (Table 3). In mice inoculated with brain material from pig #18 (IC inoculated market weight pig) the average incubation period was 244 days post-inoculation (dpi) (2/28 mice). In mice inoculated with brain material from pig #27 (IC inoculated market aged pig group) the average incubation period was 167 dpi (3/29 mice, range 140-220 dpi). Two out of 27 mice were positive in the group inoculated with brain material from pig #28; one mouse was found dead at 314 dpi and the other was euthanized at the end of the study at 701 dpi. The highest attack rate resulted from the orally inoculated aged pig (#48), with 14/28 mice positive and an average incubation period of 263 dpi (range 111-621 dpi).

All pigs that produced a positive bioassay result also had a positive RT-QuIC result. In addition, pig #27 and #28 were positive by WB (both pigs), EIA (both pigs), and IHC (#28 only). Bioassay of brain tissue from pig #32 in the orally inoculated market weight group was unsuccessful (0/28 mice, study ended at 702 dpi) (Table 3), although PrP^{Sc} was detected in the brain of this pig using RT-QuIC (Table 1).

Discussion

We demonstrated that PrP^{Sc} can be detected in brain and lymphoid tissues from as early as 6 months post-inoculation in pigs inoculated orally or intracranially with the CWD agent. We show that pigs inoculated with CWD rarely develop neurologic signs suggestive of prion disease although PrP^{Sc} can be detected in brain samples. Furthermore, neuropathological changes are often equivocal and the amount of PrP^{Sc} present is generally low, so sensitive methods such as real-time quaking induced conversion (RT-QuIC) and bioassay were used for PrP^{Sc} detection.

Prion infection was subclinical in most pigs in this study; PrP^{Sc} was detected in brain from 18 pigs but neurologic signs suggestive of prion disease were observed in only one pig. This pig developed clinical signs of difficulty in rising and signs of tremor. Both these clinical signs have been reported previously in pigs challenged with bovine BSE (6) or sheep-passaged BSE (4). A number of pigs developed persistent recumbency with difficulty in rising, but these clinical signs were attributed to musculoskeletal lameness rather than neurological disease.

Similar to pigs with BSE (8), PrP^{Sc} accumulation was sparse and did not necessarily correlate with the degree of spongiform change. In addition to having a

restricted distribution, the range of morphological types of PrP^{Sc} was limited to just the intraneuronal type. Prominent intraneuronal immunolabeling is also a feature of scrapie in pigs (5). In contrast, a wider variety of PrP^{Sc} deposit types have been described in pigs challenged with bovine (6, 8) or sheep-passaged BSE (4).

Mild spongiform change was observed in the brain of both inoculated and non-inoculated pigs, suggesting that the presence of spongiform change in the brain should not be used as a sole diagnostic test for CWD in pigs. Similar to results reported by others, microscopic changes in negative control and inoculated pigs were limited to occasional scattered vacuoles in the neuropil or white matter throughout the brain (1), neuropil vacuolation of the superficial layers of the rostral colliculus (1, 8), and occasional neuronal vacuolation in the dorsal motor nucleus of the vagus nerve (1, 2, 8). Since the above microscopic changes can be observed in both non-inoculated control and inoculated pigs, when present in inoculated pigs they are considered equivocal, i.e. not related to prion disease. Co-localization of neuropil vacuolation and intraneuronal PrP^{Sc} deposits were present in the rostral colliculus of 2 pigs in our study but vacuolation did not extend to deeper layers of the rostral colliculi or to other areas of the brain (8), so was considered equivocal.

Limited microscopic and immunohistopathological changes observed in the brains of pigs with CWD compared to pigs inoculated with bovine or ovine-adapted BSE suggests that the species barrier for CWD to pigs is higher than for BSE to pigs. Despite this, pigs are able to accumulate misfolded prion protein and CWD infectivity.

Using standard diagnostic tests (western blot, enzyme immunoassay, or immunohistochemistry), PrP^{Sc} was detected in brain or lymphoid tissues from 8 pigs

from this study. The number of positive animals and tissues, in particular lymphoid tissues, was much higher when the RT-QuIC assay was used. Using RT-QuIC, PrP^{Sc} was detected in brain and lymphoid tissues that were PrP^{Sc} negative by all other tests. This is not surprising considering that RT-QuIC is reported to be at least as sensitive as bioassay (41) and 10,000 fold more sensitive than enzyme immunoassay and western blot assays for the detection of scrapie seeding activity in goat brain samples (42). With the exception of immunohistochemistry, diagnostic tests were performed on brainstem samples since this brain region is the preferred site for statutory diagnostic testing. Testing of additional brain regions may have revealed PrP^{Sc} accumulation elsewhere in the brain, as was observed on immunohistochemistry.

The RT-QuIC assay allows quantification of the seeding activity of prions in the samples based upon amyloid formation rate (AFR) values. The AFR is calculated as the reciprocal of the time taken for a reaction to reach the threshold (i.e. 1/(time to threshold in hours)). A higher AFR reflects a shorter time taken to threshold, which can also be termed a shorter 'lag phase'. Lag phases have previously been shown to be inversely correlated with seed concentration in RT-QuIC reactions (41, 43, 44). Since the AFRs of samples from IC inoculated aged pigs tended to be higher than those from orally inoculated aged pigs, it follows that the relative amount of PrP^{Sc} in the brain is higher in IC inoculated pigs. This seems logical considering that PrP^{Sc} in the inoculum was delivered directly into the brain in IC inoculated pigs, but delivered to peripheral tissues (oral cavity and gastrointestinal tract) in orally inoculated pigs.

We observed that the AFR of samples from positive animals that were determined to be PK sensitive was approximately one order of magnitude lower than the AFR of

294 samples that were PK resistant. Although the interpretation of these observations is
295 limited by the small sample size and the fact that samples were not normalized for total
296 protein content, it appears that there may be a relationship between AFR and PK
297 sensitivity.

298 One hypothesis is that larger seed particles present more seeding surfaces than
299 smaller particles and thus support faster RT-QuIC kinetics (45). In scrapie infected
300 hamsters, PK sensitive PrP^{Sc} molecules from low molecular weight aggregates are made
301 up of fewer PrP units (i.e. are smaller) than PK resistant PrP^{Sc} aggregates (46, 47).
302 Combining these observations with our own results, we hypothesize that the smaller
303 average seed particle size of PK sensitive PrP^{Sc} may result in slower RT-QuIC kinetics
304 and leads to lower AFRs and longer lag times. However, as stated above, this hypothesis
305 is based on a small number of samples.
306
307 The detection of PrP^{Sc} in lymphoid tissues from the head and gut of CWD-
308 infected pigs raises the possibility that pigs may be able to shed prions in excreta as has
309 been shown for saliva (48-51) and feces (52-54) from CWD-affected cervids.
310 Unfortunately, saliva and feces were not collected in the current study.
311 PrP^{Sc} was detected in brain and lymphoid tissues from orally inoculated pigs
312 killed at approximately market weight. These results suggest that, if they were to be
313 exposed to sufficient amounts of CWD infectivity, pigs in commercial swine production
314 systems have the potential to accumulate CWD prions by the time they reach market
315 weight.

316 In the case of feral pigs, exposure to the agent of CWD through scavenging of
317 CWD-affected cervid carcasses or through consumption of prion contaminated plants or
318 soil could allow feral pigs to serve as reservoirs of CWD infectivity. The range and
319 numbers of feral pigs is predicted to continue to increase due to the ability of pigs to
320 adapt to many climates, reproduce year-round, and survive on a varied diet (55). The
321 range of CWD-affected cervids also continues to spread, increasing the likelihood of
322 overlap of ranges of feral pigs and CWD-affected environments.
323
324 We demonstrate here that PrP^{Sc} accumulates in lymphoid tissues from pigs
325 inoculated intracranially or orally with the CWD agent, and can be detected as early as 6
326 months after inoculation. Clinical disease suggestive of prion disease developed only in a
327 single pig after a long (64 months) incubation period. This raises the possibility that
328 CWD-infected pigs could shed prions into their environment long before they develop
329 clinical disease. However, the low amounts of PrP^{Sc} detected in the study pigs combined
330 with the low attack rates in Tg002 mice suggest that there is a relatively strong species
331 barrier to CWD prions in pigs.

Materials and methods

Ethics statement

All animal experiments were reviewed and approved by the National Animal Disease Center's (NADC) Institutional Animal Care and Use Committee (protocol numbers 3510 (swine) and 2422 (mice)) and were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC) and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, Champaign, IL). Pigs were observed daily for clinical signs of disease and euthanized and necropsied at approximately 6 months post-inoculation, or when unequivocal signs of prion disease such as behavior changes, decreased feed intake, loss of body condition, ataxia, prolonged recumbency, or inability to rise were confirmed by a veterinarian, or when euthanasia was necessary due to intercurrent illness or injury that could not be remediated by veterinary care. Euthanasia was performed by intravenous injection of sodium pentobarbital according to the manufacturer's instructions.

Inoculum preparation

The pooled CWD inoculum was prepared from 3 brains from white-tailed deer that were inoculated intracranially with brain material from CWD-affected elk, white-tailed deer or mule deer (NADC Institutional Animal Care and Use Committee, protocol number 3347) (56). All donor deer were homozygous for glycine (G/G) at *PRNP* codon 96, and homozygous for serine (S/S) at codon 138. The brain tissue was ground in a

mechanical grinder and mixed with phosphate-buffered saline to produce a 10% weight per volume (w/v) homogenate.

Animal procedures

Crossbred piglets were inoculated at 8 weeks of age. Intracranially inoculated pigs (n = 20) received a single intracranial inoculation of 0.75 mL of 10% w/v CWD brain homogenate as described previously (57). Orally inoculated pigs (n = 19) received 15 mL of 10% w/v CWD brain homogenate by syringe with a soft feeding tube on four consecutive days (total dose 45 mL). Intracranially and orally inoculated pigs were housed in separate pens. At 2 weeks post-inoculation non-inoculated control pigs were introduced into the pens with the inoculated pigs.

At 6-7 months of age, approximately the time at which commercial pigs reach market weight, half of the pigs in each group were culled ('market weight' groups): n = 8 intracranially inoculated pigs, n = 9 orally inoculated pigs, and n = 2 control pigs. The remaining pigs ('aged' groups) were allowed to incubate for up to 73 months post inoculation (MPI) when the study ended. Swine were observed daily for the development of clinical signs.

Mouse bioassay

Infectivity in brain tissue from selected pigs was assayed via intracranial inoculation of *Tg002* mice that express porcine prion protein (GenBank porcine sequence accession no. GU595061) at approximately 1X the expression level of prion protein in FVB mice (5). Samples of brainstem at the level of the obex were prepared as 10% w/v

377 homogenates in PBS. Mice were inoculated intracranially with 20 µL of 10% w/v brain
378 homogenate as described previously (58). Mice were monitored daily and euthanized
379 when they displayed unequivocal neurological signs (difficulty moving, poor
380 coordination, unable to move, anorexia) or at the time of study termination
381 (approximately 700 days post-inoculation). Brain samples from mice were prepared as
382 10% w/v brain homogenates in phosphate buffered saline as described previously (59).
383 PrP^{Sc} was detected using enzyme immunoassay as described below.
384
385 **Sample collection**
386 A full necropsy was performed on all pigs including collection of two sets of
387 tissue samples. To minimize potential cross-contamination one pathologist collected
388 tissues from the head and a second pathologist collected tissues from the rest of the body.
389 Single use instruments were not used. One set of tissues included representative sections
390 of liver, kidney, spleen, skin, striated muscles (heart, tongue, diaphragm, masseter,
391 triceps, biceps femoris, psoas major), lymphoid tissues of the head (pharyngeal tonsil,
392 palatine tonsil, medial retropharyngeal lymph node), other lymph nodes (mesenteric,
393 hepatic, renal, popliteal, prescapular), nasal turbinates, lung, esophagus, small intestine,
394 cecum, colon, rectal mucosa, stomach, adrenal gland, pituitary gland, reproductive
395 tissues, peripheral nervous system (trigeminal ganglion, optic nerve, sciatic nerve, vagus
396 nerve), brain (hemisections of cerebral cortex, hippocampus, cerebellum, superior
397 colliculus and brainstem including obex) and eye (retina). Formalin–fixed tissues were
398 fixed in 10% neutral buffered formalin, moved to 70% ethyl alcohol after 48 hours,

399 embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (HE) for
400 light microscopy. The second set of tissues was frozen.
401
402 **Selection of animals and tissues for PrP^{Sc} detection**
403 Frozen brainstem from all pigs was screened for the presence of PrP^{Sc} using
404 antigen-capture enzyme immunoassay (EIA) and western blot (WB). Fixed tissues from
405 pigs that were positive on WB and/or EIA were examined by immunohistochemistry
406 (IHC). In addition, representative pigs from across the range survival times for each
407 group were also examined by IHC. Brainstem material from the pig with the longest
408 incubation period in each treatment group was bioassayed in *Tg002* mice. PrP^{Sc} detection
409 using quaking-induced conversion assay (QuIC) was applied to frozen brainstem and
410 lymphoid tissue from all pigs that were positive by any other test (EIA, WB, IHC,
411 bioassay) as well as additional animals so that 6-7 animals per group and across a range
412 of survival times were tested.
413
414 **Immunohistochemistry**
415 All paraffin-embedded tissues were immunostained by an automated
416 immunohistochemical method for detection of PrP^{Sc} as described previously (60) using
417 the anti-PrP monoclonal antibody L42.
418
419 **Antigen-capture enzyme immunoassay (EIA)**
420 Brain homogenates were homogenized in 1X PBS at a concentration of 20% w/v
421 and assayed with a commercially available EIA kit (HerdChek BSE-Scrapie Ag Test Kit,

EIA, IDEXX Laboratories, Westbrook, ME) as previously described (61). Assays were performed in accordance with the manufacturer's instructions. The EIA kit instructions indicated 3 protocols (standard, short, and ultrashort). The short protocol was used for testing of tissue samples in the present study. Each tissue sample homogenate was assayed in a single well along with negative and positive controls supplies with the kit. Two conjugate concentrate products were included with the kit: a conjugate concentrate intended for use with brain samples obtained from small ruminants (SRB-CC) and a conjugate concentrate intended for use with brain samples obtained from cattle or lymph node or spleen samples obtained from small ruminants (CC). In this study (SRB-CC) conjugate was used for testing the samples obtained from mice expressing pig prion protein. Absorbance was measured (SPECTRAmax 190, Molecular Devices, Sunnyvale, CA) at 450 nm with a reference wavelength of 620 nm. Cutoff values were established for each run according to kit instructions whereby 0.180 was added to the mean negative control value. Samples were interpreted as positive if their absorbance value at 450 nm minus the reference value at 620 nm was above the established cutoff value.

EIA-based proteinase-K sensitivity testing

Sensitivity to proteinase-K (PK) was determined using the EIA protocol described above but with the addition of a pre-testing PK-treatment step (40). Briefly, for each animal two 100 µL aliquots of 20% w/v brain homogenate were prepared: 5 µL of 1 mg/mL PK (USB Corporation, Cleveland, OH, USA) was added to one aliquot and 5 µL of PBS was added to the second aliquot. Both aliquots were incubated for 1 hour at 37 ^{o}C with shaking at 1000 rpm followed by the addition of 1.0 µL of 100mg/mL PK-inhibitor

(Pefabloc, Roche Diagnostics, Mannheim, Germany). The absorbance value for each sample was determined using EIA as described above. Samples for which the non-PK treated aliquot was EIA positive and the PK-treated aliquot was EIA negative were classified as PK sensitive. Samples for which the non-PK treated aliquot was EIA positive and the PK-treated aliquot was EIA positive were classified as PK-resistant.

Western blotting

Samples for WB were collected from the brainstem at the level of the obex and the midbrain between the optic and oculomotor nerves dorsal to the pituitary and performed as previously reported (57). Tissues were homogenized and enriched as described previously (22) with the following modifications: After the pellets were resuspended in 100 µL of water, samples were digested with proteinase K (PK) using a final enzyme concentration of 0.4 U/mL (8 µg/mL) at 37 ºC for 1 hr. The digestion was stopped by the addition of a serine protease inhibitor (Pefabloc SC, Roche Diagnostics GmbH, Mannheim, Germany) to a final concentration of 1 mg/mL. Western blots were developed using mouse anti-PrP monoclonal antibody L42, which targets to amino acids 145-163 of the ovine prion protein sequence (62), at 1:500 dilution (0.1 µg/mL). Due to the sparse PrP^{Sc} accumulation in the brains of inoculated pigs the blot in Figure 1 is a composite. The Pig CWD sample was enriched and loaded at 100 mg/eq. The Pig BSE positive control tissue was provided by the APHA Biological Archive (Addlestone, UK).

Expression and purification of the recombinant PrP substrate

468 The recombinant prion protein (rPrP) used in the RT-QuIC assay was expressed
469 and purified using a standard protocol from previous reports (41, 63). Briefly, rPrP
470 composed of Syrian hamster PrP residues 90-231 in the pET vector was transformed into
471 *Escherichia coli* Rosetta2 (DE3) cells and purified from inclusion bodies using fast
472 protein liquid chromatography as described previously (44, 64).
473
474 **Real-Time Quaking Induced Conversion (RT-QuIC) assay for brain and lymphoid**
475 **tissue samples**
476 We included brain and lymphoid tissue homogenates from clinical CWD-affected
477 white-tailed deer, age group matched non-inoculated pigs, and blank (buffer) as controls.
478 Samples were collected using a strict aseptic technique to minimize the risk of cross-
479 contamination. All of the samples were run using a blinded study design (N.K., S.M.).
480 Prior to testing, brain and lymphoid tissue samples were homogenized in 1X PBS
481 at a concentration of 20% w/v tissue, and then further homogenized using repeated
482 pipetting and sonication in a cup sonicator with two pulses of 30 seconds. The samples
483 were then further diluted to a concentration of 0.02% in sample dilution buffer (0.025%
484 SDS in 1X concentration of PBS).
485 The RT-QuIC assay was performed using previously published protocols (41, 65)
486 with slight modifications as described previously (64). All samples were run in
487 quadruplicate. The reactions consisted of 5 µg of protein from the brain and lymphoid
488 tissue homogenates that were used as seed in a 100 µL total reaction volume. A sample
489 was considered positive if the fluorescence intensity of at least half the replicate wells
490 crossed the threshold (Ct), which was calculated as the mean fluorescence of the negative

control sample plus 10 standard deviations (66-68). For each positive sample we quantified the seeding activity based on amyloid formation rate (AFR), which is the reciprocal of the time (h) that it takes for a reaction to reach the threshold (Ct), defined as the mean baseline fluorescence plus 5 standard deviations (41, 65). The AFR was calculated using all 4 replicates of each sample. Data analysis was performed using Biotek's Gen5 software version 2.07.17 and BMG's MARS software version 5.2.R8.

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References

1. Jahns H, Callanan JJ, Sammin DJ, McElroy MC, Bassett HF. 2006. Survey for transmissible spongiform encephalopathies in Irish pigs fed meat and bone meal. Vet Rec 159:137-42.
2. Köfler M, Seuberlich T, Maurer E, Heim D, Doherr M, Zurbriggen A, Botteron C. 2006. [TSE surveillance in small ruminants and pigs: a pilot study]. Schweiz Arch Tierheilkd 148:341-2, 344-8.
3. Gajdusek DC, Gibbs CJ, Jr., Alpers M. 1967. Transmission and passage of experimenal "kuru" to chimpanzees. Science 155:212-4.
4. Hedman C, Bolea R, Marin B, Cobriere F, Filali H, Vazquez F, Pitarch JL, Vargas A, Acin C, Moreno B, Pumarola M, Andreoletti O, Badiola JJ. 2016. Transmission of sheep-bovine spongiform encephalopathy to pigs. Vet Res 47:14.
5. Greenlee JJ, Kunkle RA, Smith JD, West Greenlee MH. 2016. Scrapie in swine: a diagnostic challenge. Food Safety 4:110-114.
6. Wells GA, Hawkins SA, Austin AR, Ryder SJ, Done SH, Green RB, Dexter I, Dawson M, Kimberlin RH. 2003. Studies of the transmissibility of the agent of bovine spongiform encephalopathy to pigs. J Gen Virol 84:1021-31.
7. Dawson M, Wells GA, Parker BN, Scott AC. 1990. Primary parenteral transmission of bovine spongiform encephalopathy to the pig. Vet Rec 127:338.
8. Ryder SJ, Hawkins SA, Dawson M, Wells GA. 2000. The neuropathology of experimental bovine spongiform encephalopathy in the pig. J Comp Pathol 122:131-43.

9. Williams ES, Young S. 1980. Chronic wasting disease of captive mule deer: a spongiform encephalopathy. J Wildl Dis 16:89-98.

10. Miller MW, Wild MA. 2004. Epidemiology of chronic wasting disease in captive white-tailed and mule deer. J Wildl Dis 40:320-7.

11. Spraker TR, Miller MW, Williams ES, Getzy DM, Adrian WJ, Schoonveld GG, Spowart RA, O'Rourke KI, Miller JM, Merz PA. 1997. Spongiform encephalopathy in free-ranging mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*) and Rocky Mountain elk (*Cervus elaphus nelsoni*) in North Central Colorado. J Wildl Dis 33:1-6.

12. Williams ES, Young S. 1982. Spongiform encephalopathy of Rocky Mountain elk. J Wildl Dis 18:465-71.

13. Baeten LA, Powers BE, Jewell JE, Spraker TR, Miller MW. 2007. A natural case of chronic wasting disease in a free-ranging moose (*Alces alces shirasi*). J Wildl Dis 43:309-14.

14. Kreeger TJ, Montgomery DL, Jewell JE, Schultz W, Williams ES. 2006. Oral transmission of chronic wasting disease in captive Shira's moose. J Wildl Dis 42:640-5.

15. Benestad SL, Mitchell G, Simmons M, Ytrehus B, Vikoren T. 2016. First case of chronic wasting disease in Europe in a Norwegian free-ranging reindeer. Vet Res 47:88.

16. Balachandran A, Harrington NP, Algire J, Soutyrine A, Spraker TR, Jeffrey M, Gonzalez L, O'Rourke KI. 2010. Experimental oral transmission of chronic

556 wasting disease to red deer (*Cervus elaphus elaphus*): early detection and late
557 stage distribution of protease-resistant prion protein. Can Vet J 51:169-78.
558 17. Hamir AN, Greenlee JJ, Nicholson EM, Kunkle RA, Richt JA, Miller JM, Hall M.
559 2011. Experimental transmission of chronic wasting disease (CWD) from elk and
560 white-tailed deer to fallow deer by intracerebral route: final report. Can J Vet Res
561 75:152-6.
562 18. Nalls AV, McNulty E, Powers J, Seelig DM, Hoover C, Haley NJ, Hayes-Klug J,
563 Anderson K, Stewart P, Goldmann W, Hoover EA, Mathiason CK. 2013. Mother
564 to offspring transmission of chronic wasting disease in reeves' muntjac deer.
565 PLoS One 8:e71844.
566 19. Moore SJ, Kunkle R, Greenlee MH, Nicholson E, Richt J, Hamir A, Waters WR,
567 Greenlee J. 2016. Horizontal transmission of chronic wasting disease in reindeer.
568 Emerg Infect Dis 22:2142-2145.
569 20. Mitchell GB, Sigurdson CJ, O'Rourke KI, Algire J, Harrington NP, Walther I,
570 Spraker TR, Balachandran A. 2012. Experimental oral transmission of chronic
571 wasting disease to reindeer (*Rangifer tarandus tarandus*). PLoS One 7:e39055.
572 21. Hamir AN, Kunkle RA, Cutlip RC, Miller JM, Williams ES, Richt JA. 2006.
573 Transmission of chronic wasting disease of mule deer to Suffolk sheep following
574 intracerebral inoculation. J Vet Diagn Invest 18:558-65.
575 22. Greenlee JJ, Nicholson EM, Smith JD, Kunkle RA, Hamir AN. 2012.
576 Susceptibility of cattle to the agent of chronic wasting disease from elk after
577 intracranial inoculation. J Vet Diagn Invest 24:1087-93.

578 23. Hamir AN, Cutlip RC, Miller JM, Williams ES, Stack MJ, Miller MW, O'Rourke
579 KI, Chaplin MJ. 2001. Preliminary findings on the experimental transmission of
580 chronic wasting disease agent of mule deer to cattle. J Vet Diagn Invest 13:91-6.
581 24. Hamir AN, Kunkle RA, Cutlip RC, Miller JM, O'Rourke KI, Williams ES, Miller
582 MW, Stack MJ, Chaplin MJ, Richt JA. 2005. Experimental transmission of
583 chronic wasting disease agent from mule deer to cattle by the intracerebral route. J
584 Vet Diagn Invest 17:276-81.
585 25. Hamir AN, Kunkle RA, Miller JM, Greenlee JJ, Richt JA. 2006. Experimental
586 second passage of chronic wasting disease (CWD(mule deer)) agent to cattle. J
587 Comp Pathol 134:63-9.
588 26. Mathiason CK, Nalls AV, Seelig DM, Kraft SL, Carnes K, Anderson KR, Hayes-
589 Klug J, Hoover EA. 2013. Susceptibility of domestic cats to chronic wasting
590 disease. J Virol 87:1947-56.
591 27. Seelig DM, Nalls AV, Flasik M, Frank V, Eaton S, Mathiason CK, Hoover EA.
592 2015. Lesion profiling and subcellular prion localization of cervid chronic
593 wasting disease in domestic cats. Vet Pathol 52:107-19.
594 28. Bartz JC, Marsh RF, McKenzie DI, Aiken JM. 1998. The host range of chronic
595 wasting disease is altered on passage in ferrets. Virology 251:297-301.
596 29. Sigurdson CJ, Mathiason CK, Perrott MR, Eliason GA, Spraker TR, Glatzel M,
597 Manco G, Bartz JC, Miller MW, Hoover EA. 2008. Experimental chronic wasting
598 disease (CWD) in the ferret. J Comp Pathol 138:189-96.

30. Marsh RF, Kincaid AE, Bessen RA, Bartz JC. 2005. Interspecies transmission of chronic wasting disease prions to squirrel monkeys (*Saimiri sciureus*). J Virol 79:13794-6.

31. Race B, Meade-White KD, Miller MW, Barbian KD, Rubenstein R, LaFauci G, Cervenakova L, Favara C, Gardner D, Long D, Parnell M, Striebel J, Priola SA, Ward A, Williams ES, Race R, Chesebro B. 2009. Susceptibilities of nonhuman primates to chronic wasting disease. Emerg Infect Dis 15:1366-76.

32. Race B, Meade-White KD, Phillips K, Striebel J, Race R, Chesebro B. 2014. Chronic wasting disease agents in nonhuman primates. Emerg Infect Dis 20:833-7.

33. Kurt TD, Sigurdson CJ. 2016. Cross-species transmission of CWD prions. Prion 10:83-91.

34. US Department of Agriculture National Feral Swine Mapping System. 2015. http://swine.vet.uga.edu/nfsms/index.jsp. Accessed Feb 13.

35. US Geological Survey National Wildlife Health Center. 2017. https://www.nwhc.usgs.gov/disease_information/chronic_wasting_disease/index.jsp. Accessed Feb 13.

36. Pritzkow S, Morales R, Moda F, Khan U, Telling GC, Hoover E, Soto C. 2015. Grass plants bind, retain, uptake, and transport infectious prions. Cell Rep 11:1168-75.

37. Johnson CJ, Phillips KE, Schramm PT, McKenzie D, Aiken JM, Pedersen JA. 2006. Prions adhere to soil minerals and remain infectious. PLoS Pathog 2:e32.

621 38. Johnson CJ, Pedersen JA, Chappell RJ, McKenzie D, Aiken JM. 2007. Oral
622 transmissibility of prion disease is enhanced by binding to soil particles. PLoS
623 Pathog 3:e93.
624 39. Seidel B, Thomzig A, Buschmann A, Groschup MH, Peters R, Beekes M, Terytze
625 K. 2007. Scrapie Agent (Strain 263K) can transmit disease via the oral route after
626 persistence in soil over years. PLoS One 2:e435.
627 40. Rodriguez-Martinez AB, Garrido JM, Zarranz JJ, Arteagoitia JM, de Pancorbo
628 MM, Atares B, Bilbao MJ, Ferrer I, Juste RA. 2010. A novel form of human
629 disease with a protease-sensitive prion protein and heterozygosity
630 methionine/valine at codon 129: Case report. BMC Neurol 10:99.
631 41. Wilham JM, Orru CD, Bessen RA, Atarashi R, Sano K, Race B, Meade-White
632 KD, Taubner LM, Timmes A, Caughey B. 2010. Rapid end-point quantitation of
633 prion seeding activity with sensitivity comparable to bioassays. PLoS Pathog
634 6:e1001217.
635 42. Dassanayake RP, Orru CD, Hughson AG, Caughey B, Graca T, Zhuang D,
636 Madsen-Bouterse SA, Knowles DP, Schneider DA. 2016. Sensitive and specific
637 detection of classical scrapie prions in the brains of goats by real-time quaking-
638 induced conversion. J Gen Virol 97:803-12.
639 43. Peden AH, McGuire LI, Appleford NE, Mallinson G, Wilham JM, Orru CD,
640 Caughey B, Ironside JW, Knight RS, Will RG, Green AJ, Head MW. 2012.
641 Sensitive and specific detection of sporadic Creutzfeldt-Jakob disease brain prion
642 protein using real-time quaking-induced conversion. J Gen Virol 93:438-49.

643 44. Kondru N, Manne S, Greenlee J, West Greenlee H, Anantharam V, Halbur P,
644 Kanthasamy A, Kanthasamy A. 2017. Integrated organotypic slice cultures and
645 RT-QuIC (OSCAR) assay: implications for translational discovery in protein
646 misfolding diseases. Sci Rep 7:43155.
647 45. Vascellari S, Orru CD, Hughson AG, King D, Barron R, Wilham JM, Baron GS,
648 Race B, Pani A, Caughey B. 2012. Prion seeding activities of mouse scrapie
649 strains with divergent PrPSc protease sensitivities and amyloid plaque content
650 using RT-QuIC and eQuIC. PLoS One 7:e48969.
651 46. Tzaban S, Friedlander G, Schonberger O, Horonchik L, Yedidia Y, Shaked G,
652 Gabizon R, Taraboulos A. 2002. Protease-sensitive scrapie prion protein in
653 aggregates of heterogeneous sizes. Biochemistry 41:12868-75.
654 47. Pastrana MA, Sajnani G, Onisko B, Castilla J, Morales R, Soto C, Requena JR.
655 2006. Isolation and characterization of a proteinase K-sensitive PrP(Sc) fraction.
656 Biochemistry 45:15710-15717.
657 48. Mathiason CK, Powers JG, Dahmes SJ, Osborn DA, Miller KV, Warren RJ,
658 Mason GL, Hays SA, Hayes-Klug J, Seelig DM, Wild MA, Wolfe LL, Spraker
659 TR, Miller MW, Sigurdson CJ, Telling GC, Hoover EA. 2006. Infectious prions
660 in the saliva and blood of deer with chronic wasting disease. Science 314:133-6.
661 49. Mathiason CK, Hays SA, Powers J, Hayes-Klug J, Langenberg J, Dahmes SJ,
662 Osborn DA, Miller KV, Warren RJ, Mason GL, Hoover EA. 2009. Infectious
663 prions in pre-clinical deer and transmission of chronic wasting disease solely by
664 environmental exposure. PLoS One 4:e5916.

50. Haley NJ, Seelig DM, Zabel MD, Telling GC, Hoover EA. 2009. Detection of CWD prions in urine and saliva of deer by transgenic mouse bioassay. PLoS ONE 4:e4848.

51. Henderson DM, Manca M, Haley NJ, Denkers ND, Nalls AV, Mathiason CK, Caughey B, Hoover EA. 2013. Rapid antemortem detection of CWD prions in deer saliva. PLoS One 8:e74377.

52. Haley NJ, Mathiason CK, Zabel MD, Telling GC, Hoover EA. 2009. Detection of sub-clinical CWD infection in conventional test-negative deer long after oral exposure to urine and feces from CWD+ deer. PLoS One 4:e7990.

53. Tamguney G, Miller MW, Wolfe LL, Sirochman TM, Glidden DV, Palmer C, Lemus A, DeArmond SJ, Prusiner SB. 2009. Asymptomatic deer excrete infectious prions in faeces. Nature 461:529-32.

54. Pulford B, Spraker TR, Wyckoff AC, Meyerett C, Bender H, Ferguson A, Wyatt B, Lockwood K, Powers J, Telling GC, Wild MA, Zabel MD. 2012. Detection of PrPCWD in feces from naturally exposed Rocky Mountain elk (*Cervus elaphus nelsoni*) using protein misfolding cyclic amplification. J Wildl Dis 48:425-34.

55. Wilcox J, Van Vuren D. 2009. Wild pigs as predators in oak woodlands of California. J Mammal 90:114-118.

56. Hamir AN, Richt JA, Miller JM, Kunkle RA, Hall SM, Nicholson EM, O'Rourke KI, Greenlee JJ, Williams ES. 2008. Experimental transmission of chronic wasting disease (CWD) of elk (*Cervus elaphus nelsoni*), white-tailed deer (*Odocoileus virginianus*), and mule deer (*Odocoileus hemionus hemionus*) to white-tailed deer by intracerebral route. Vet Pathol 45:297-306.

688 57. Greenlee JJ, Smith JD, Kunkle RA. 2011. White-tailed deer are susceptible to the
689 agent of sheep scrapie by intracerebral inoculation. Vet Res 42:107.
690 58. Smith JD, Nicholson EM, Greenlee JJ. 2013. Evaluation of a combinatorial
691 approach to prion inactivation using an oxidizing agent, SDS, and proteinase K.
692 BMC Vet Res 9:151.
693 59. Smith JD, Nicholson EM, Foster GH, Greenlee JJ. 2013. Exposure of RML
694 scrapie agent to a sodium percarbonate-based product and sodium dodecyl sulfate
695 renders PrPSc protease sensitive but does not eliminate infectivity. BMC Vet Res
696 9:8.
697 60. Greenlee JJ, Smith JD, West Greenlee MH, Nicholson EM. 2012. Clinical and
698 pathologic features of H-type bovine spongiform encephalopathy associated with
699 E211K prion protein polymorphism. PLoS ONE 7:e38678.
700 61. Smith JD, Greenlee JJ. 2014. Detection of misfolded prion protein in retina
701 samples of sheep and cattle by use of a commercially available enzyme
702 immunoassay. Am J Vet Res 75:268-72.
703 62. Hardt M, Baron T, Groschup MH. 2000. A comparative study of
704 immunohistochemical methods for detecting abnormal prion protein with
705 monoclonal and polyclonal antibodies. J Comp Pathol 122:43-53.
706 63. Atarashi R, Wilham JM, Christensen L, Hughson AG, Moore RA, Johnson LM,
707 Onwubiko HA, Priola SA, Caughey B. 2008. Simplified ultrasensitive prion
708 detection by recombinant PrP conversion with shaking. Nat Methods 5:211-2.
709 64. West Greenlee MH, Lind M, Kokemueller R, Mammadova N, Kondru N, Manne
710 S, Smith J, Kanthasamy A, Greenlee J. 2016. Temporal resolution of misfolded

prion protein transport, accumulation, glial activation, and neuronal death in the retinas of mice inoculated with scrapie. Am J Pathol 186:2302-9.

65. Orru CD, Groveman BR, Hughson AG, Zanusso G, Coulthart MB, Caughey B. 2015. Rapid and sensitive RT-QuIC detection of human Creutzfeldt-Jakob disease using cerebrospinal fluid. MBio 6.

66. Groveman BR, Orru CD, Hughson AG, Bongianni M, Fiorini M, Imperiale D, Ladogana A, Pocchiari M, Zanusso G, Caughey B. 2017. Extended and direct evaluation of RT-QuIC assays for Creutzfeldt-Jakob disease diagnosis. Ann Clin Transl Neurol 4:139-144.

67. Henderson DM, Davenport KA, Haley NJ, Denkers ND, Mathiason CK, Hoover EA. 2015. Quantitative assessment of prion infectivity in tissues and body fluids by real-time quaking-induced conversion. J Gen Virol 96:210-9.

68. Bongianni M, Orru C, Groveman BR, Sacchetto L, Fiorini M, Tonoli G, Triva G, Capaldi S, Testi S, Ferrari S, Cagnin A, Ladogana A, Poleggi A, Colaizzo E, Tiple D, Vaianella L, Castriciano S, Marchioni D, Hughson AG, Imperiale D, Cattaruzza T, Fabrizi GM, Pocchiari M, Monaco S, Caughey B, Zanusso G. 2017. Diagnosis of human prion disease using real-time quaking-induced conversion testing of olfactory mucosa and cerebrospinal fluid samples. JAMA Neurol 74:155-162.

731 **Tables**

732 **Table 1. Detection and characterization of disease-associated prion protein (PrP^{Sc}) from selected pigs.**

Animal number	Treatment group	Incubation period[a]	Overall result CNS	Overall result LRS	Antigen-capture enzyme immunoassay	Western blot	Immuno-histochemistry	Proteinase-K sensitivity	RT-QuIC result	RT-QuIC amyloid formation rate
1	Control market weight	0	-	nt	na	na	nt	nt	nt	nt
2		0	-	nt	-	-	nt	nt	nt	nt
3		0	-	nt	-	-	nt	nt	nt	nt
4		6	-	nt	-	-	nt	nt	nt	nt
5		6	-	-	-	-	-	na	-	0
6	Control aged	25	-	nt	-	-	nt	nt	nt	nt
7		41	-	nt	-	-	nt	nt	nt	nt
8		46	-	nt	-	-	nt	nt	nt	nt
9		73	-	-	-	-	-	na	-	0
10	Intracranially inoculated market weight	0	-	nt	-	-	nt	nt	nt	nt
11		0	-	nt	-	-	nt	nt	nt	nt
12		6	+	+	-	-	-	na	+	0.031
13		6	-	nt	-	-	nt	nt	nt	nt
14		6	+	+	-	-	-	na	+	0.025
15		6	+	+	+	-	-	sensitive	-	0
16		6	-	+	-	-	-	na	-	0
17		6	-	nt	-	-	nt	nt	nt	nt
18		6	+	+	-	-	-	na	+	0.120
19		6	-	-	-	-	-	na	-	0
20	Intracranially inoculated aged	30	-	nt	-	-	nt	nt	nt	nt
21		30	-	nt	-	-	nt	nt	nt	nt
22		30	+	+	-	-	-	na	+	0.080

23		30	-	nt	-	-	nt	nt	nt	nt
24		42	+	+	+	-	-	sensitive	+	0.030
25		45	+	+	+	-	+	resistant	+	0.180
26		56	+	+	+	-	+	resistant	+	0.190
27		64	+	-	+	+	-	resistant	+	0.170
28		73	+	+	+	+	+	resistant	+	0.210
29		73	+	+	-	-	-	na	+	0.050
32	Orally inoculated market weight	6	+	+	-	-	-	na	+	0.070
38		6	+	+	-	-	-	na	+	0.010
30		6	-	+	-	-	-	na	-	0
36		6	-	+	-	-	-	na	-	0
37		6	-	+	-	-	-	na	-	0
34		6	-	-	-	-	-	na	-	0
31		6	-	nt	-	-	nt	nt	nt	nt
33		6	-	nt	-	-	nt	nt	nt	nt
35		6	-	nt	-	-	nt	nt	nt	nt
39	Orally inoculated aged	19	-	+	-	-	-	na	-	0
40		41	-	nt	-	-	nt	nt	nt	nt
41		41	+	+	-	-	-	na	+	0.029
42		41	-	nt	-	-	nt	nt	nt	nt
43		45	+	+	-	-	-	na	+	0.020
44		55	+	+	-	-	-	na	+	0.030
45		64	+	-	+	-	+	sensitive	+	0.010
46		65	-	nt	-	-	nt	nt	nt	nt
47		65	-	nt	-	-	nt	nt	nt	nt
48		72	+	-	-	-	-	na	+	0.010

733 Incubation periods are expressed as months post-inoculation. RT-QuIC, real-time quaking-induced conversion assay; na, result not

734 applicable; nt, sample not tested.

735 **Table 2. Detection of disease-associated prion protein (PrP^{Sc}) in lymphoid tissues using antigen-capture enzyme immunoassay**

736 **(EIA) and real-time quaking induced conversion assay (RT-QuIC).**

Animal number	Treatment group	Incubation period[a]	Overall result	Retropharyngeal lymph node		Tonsil		Mesenteric lymph node	
				EIA	RT-QuIC	EIA	RT-QuIC	EIA	RT-QuIC
5	Non-inoculated controls	6	-	-	-	-	-	-	-
9		73	-	na	na	-	-	-	-
12	Intracranially inoculated market weight	6	+	-	-	-	-	-	+
14		6	+	-	+	-	+	-	-
15		6	+	-	+	-	+	-	+
16		6	+	-	+	-	+	-	+
18		6	+	-	-	-	-	-	+
19		6	-	-	-	-	-	-	-
22	Intracranially inoculated aged	30	+	-	-	-	-	-	+
24		42	+	na	na	-	+	na	na
25		45	+	-	+	-	-	-	-
26		56	+	-	+	-	+	na	na
27		64	-	na	na	-	-	na	na
28		73	+	na	na	-	+	-	+
29		73	+	-	+	-	-	-	-
30	Orally inoculated market weight	6	+	-	-	-	-	-	+
32		6	+	-	-	-	+	-	+
34		6	-	-	-	-	-	-	-
36		6	+	-	-	-	+	-	+
37		6	+	-	+	-	-	+	+
38		6	+	-	-	-	+	-	+
39	Orally inoculated aged	19	+	-	-	-	-	-	+
41		41	+	-	+	-	-	-	+
43		45	+	-	na	-	+	na	na
44		55	+	na	na	-	-	-	+
45		64	-	-	na	-	-	na	na
48		72	-	na	na	-	-	-	-

737 [a] Incubation periods are expressed as months post-inoculation. na, sample not available.

738 **Table 3. Results from bioassay of brain material from selected pigs in *Tg002* mice**

739 **that express porcine prion protein.**

740

Donor animal number	Donor treatment group (donor incubation period[a])	*Tg002* attack rate[b]	*Tg002* mean incubation period[c]
18	Intracranially inoculated market weight (6)	2/29	244
27	Intracranially inoculated aged (64)	3/29	167
28	Intracranially inoculated aged (73)	2/27	314, 701[d]
32	Orally inoculated market weight (6)	0/28	>700
48	Orally inoculated aged (72)	14/28	263

741

742 [a] Donor incubation period is expressed as months post-inoculation. [b] Disease-associated

743 prion protein in the brains of mice was detected using an antigen-capture enzyme

744 immunoassay (EIA). [c] Mouse incubation period is expressed as days post-inoculation.

745 Survival [d] Survival times for these 2 mice are so disparate that calculation of a mean

746 incubation period would not be meaningful.

747

Figure legends

Figure 1. Western blot analysis demonstrating a unique PrP^{Sc} profile in brain samples from pigs with CWD.

The positive brain sample from a pig inoculated with the CWD agent (Pig CWD) has a slightly higher migration relative to a brain sample from a pig inoculated with the agent of classical bovine spongiform encephalopathy (Pig BSE), and a much lower migration relative to the CWD inoculum (CWD Inoc). The diglycosylated band (top most band in each lane) is more prominent in the Pig CWD and CWD Inoc samples, while the monoglycosylated (middle) band is most prominent in the Pig BSE sample. Blot developed with monoclonal antibody L42. Note: due to the sparse PrP^{Sc} accumulation in the brains of inoculated pigs the blot in Figure 1 is a composite, see Materials and methods for details.

Figure 2. Amyloid formation rates (RT-QuIC) and proteinase-K sensitivity (EIA) of PrP^{Sc} from pig brain samples.

Treatment groups: animals 5 and 9, non-inoculated controls; 12-19, IC inoculated market weight pigs; 22-29, IC inoculated aged pigs; 30-38, orally inoculated market weight pigs; 39-48, orally inoculated aged pigs. Proteinase-K (PK) sensitivity: solid fill, PK sensitivity not determined (EIA negative); horizontal stripe fill, PK resistant; checked fill, PK sensitive.

Figure 3. Results of RT-QuIC assays of brain homogenate from inoculated and negative control pigs.

Average percent thioflavin T (ThT) fluorescence readings (thick lines) with standard deviations (thin lines) determined from all replicates (four replicate reactions per animal) from all pigs in each challenge group. Red, intracranially inoculated aged pigs (n = 7); blue, intracranially inoculated market-weight pigs (n = 6); purple, orally inoculated market-weight pigs (n = 6); orange, orally inoculated aged pigs (n = 6); green, non-inoculated control pigs (n = 2).

Figure 4. Vacuolar change and disease-associated prion protein in the brain and eye.

(A) Brainstem (Pig #7). Incidental, i.e. not related to prion disease, neuropil vaculation in the colliculus, * midline (hematoxylin and eosin, original magnification 4x). (B) Higher magnification view of (A) (original magnification 10x). (C) Brainstem (Pig #25). Intraneuronal PrP^{Sc} immunoreactivity (arrows) in neurons in the colliculus (monoclonal anti-PrP antibody L42, original magnification 20x). (D) Brainstem (Pig #8). Non disease-specific intraneuronal immunolabeling (arrows) in neurons in the colliculus from a non-inoculated control pig (monoclonal anti-PrP antibody L42, original magnification 40x). (E) Brainstem (Pig #38). Incidental intraneuronal vacuolation (*) in the dorsal motor nucleus of the vagus nerve (hematoxylin and eosin, original magnification 40x). (F) Retina (Pig #26). Granular to punctate PrP^{Sc} immunoreactivity in the inner and out plexiform layers with occasional intraglial deposits (arrow) (monoclonal anti-PrP

795 antibody L42, original magnification 40x). (G) Retina (Pig #4). Non disease-specific
796 immunolabeling in a non-inoculated control pig (monoclonal anti-PrP antibody L42,
797 original magnification 40x).