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Experimental Transmission of the Chronic Wasting Disease Agent to Swine after Oral or Intracranial Inoculation

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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 hosts for the agent of CWD 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 three groups, intracranially inoculated ($n = 20$), orally inoculated ($n = 19$), and noninoculated ($n = 9$). 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 postinoculation (mpi). Tissues collected at necropsy were examined for disease-associated prion protein (PrP^{Sc}) by Western blotting (WB), antigen capture enzyme 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. By RT-QuIC, PrP^{Sc} was detected in lymphoid and/or brain tissue from one or more pigs in each inoculated group. The bioassay was positive in four out of five 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 with a 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 postinoculation). 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 obtained with orally inoculated pigs suggest that it may be possible for swine to serve as a reservoir for prion disease under natural conditions.

KEYWORDS chronic wasting disease, prions, swine, transmissible spongiform encephalopathy

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.

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The potential for swine to serve as hosts for the agent of CWD is unknown. A naturally occurring TSE has not been reported in swine (1, 2). Intracranial challenge of swine with brain tissue from patients 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 BSE following parenteral inoculation (simultaneously by the intraperitoneal, intravenous, and intracranial routes), to ovine BSE following intracranial inoculation (4), and to ovine scrapie following intracranial inoculation (5) but not to BSE after an oral challenge with a large amount of infected brain material (6–8).

The CWD agent has a wide host range among cervids and can be experimentally 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 deer (*Muntiacus reevesi*) (18), and reindeer (19, 20) have been shown to be susceptible to CWD following experimental inoculation. CWD has been experimentally transmitted to noncervid species, including sheep (21), cattle (22–25), domestic cats (26, 27), ferrets (28, 29), nonhuman primates (30–32), and laboratory rodents (reviewed in reference 33).

Pigs could be exposed to CWD infectivity via two main routes, (i) exposure of farmed or pet swine (*Sus scrofa domesticus*) to contaminated feed and (ii) exposure of feral swine (*S. scrofa*) to CWD-infected carcasses or contaminated environments. In the United States, 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 slaughterhouse, 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 U.S. 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, by 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; eight intracranially [i.c.] inoculated, nine orally inoculated) were clinically normal, with the exception of one pig (no. 35) that was noted to be limping on its left front and rear legs. Four i.c. inoculated pigs and one 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, four clinically normal pigs (one noninoculated, three orally inoculated) were culled to reduce animal density in the containment space. Neurological signs were observed in one pig (no. 27; incubation period, 64 mpi) that 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 because of 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 the brain stem by

Western blotting (WB), enzyme immunoassay (EIA), immunohistochemistry (IHC), and *in vitro* real-time quaking-induced conversion (RT-QuIC). Results of screening of brain stem material from all pigs by WB and EIA and results of additional testing of animals that were PrP^{Sc} positive by either screening test are shown in Table 1.

WB. By WB, PrP^{Sc} was detected in brain tissue from two i.c. inoculated pigs (no. 27 and 28) necropsied at 64 and 73 mpi, respectively (Table 1).

The migration pattern of samples from pigs inoculated i.c. with the CWD agent was different from that of either the sample from a pig inoculated with classical BSE or the original CWD inoculum (Fig. 1). While the monoglycosylated (middle) band was most prominent in the sample from the pig inoculated with the BSE agent, the diglycosylated (top) band was most prominent in the sample from the pig inoculated with the CWD agent and the original CWD inoculum.

EIA. By EIA, misfolded protein was detected in brain tissue from 1/10 i.c. inoculated market weight pigs, 5/10 i.c. inoculated aged pigs (42 to 73 mpi), 0/9 orally inoculated market weight pigs, and 1/10 orally inoculated aged pigs (Table 1).

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

Differential PK sensitivity of brain stem samples. To investigate possible biochemical properties of PrP^{Sc} that may have contributed to the variation in aggregation kinetics observed in the RT-QuIC assay, the EIA optical density of matched samples was measured with and without treatment with proteinase K (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).

PrP^{Sc} in EIA-positive brain tissue from one i.c. inoculated market weight pig (no. 15), one orally inoculated aged pig (no. 45), and one i.c. inoculated aged pig (no. 24) was PK sensitive. PrP^{Sc} from the remaining four pigs with samples positive by EIA, all from the i.c. inoculated aged pig group, was PK resistant (Table 1). PK titration of all EIA-positive samples was performed, and the results were consistent across PK concentrations of 0.4 to 50 μg/ml.

Six brain samples were EIA and RT-QuIC positive. Of these, the four samples that were PK resistant had higher AFRs (range, 0.17 to 0.21), while the two samples that were PK sensitive had lower AFRs (0.01 and 0.03) (Fig. 2).

Detection of PrP^{Sc} in lymphoid tissues. To determine if pigs inoculated with the CWD agent accumulate misfolded prion protein in lymphoid tissues, EIA and RT-QuIC were applied to samples of the retropharyngeal lymph node (RPLN), palatine tonsil, and mesenteric lymph node (MLN). Full results for individual pigs are shown in Table 2.

All of the lymphoid tissues tested were PrP^{Sc} negative by EIA, with the exception of those of pig 37 (orally inoculated market weight pig), which had a positive MLN. By the RT-QuIC assay, PrP^{Sc} was detected in lymphoid tissues of the head (RPLN, palatine tonsil) in 3/6 i.c. inoculated market weight pigs, 5/7 i.c. inoculated aged pigs, 4/6 orally inoculated market weight pigs, and 2/6 orally inoculated aged pigs. The MLN was positive in 5/6 orally inoculated market weight pigs, 3/4 orally inoculated aged pigs (samples were not available from 2 pigs), 4/6 i.c. inoculated market weight pigs, and 2/4 i.c. inoculated aged pigs. Overall, the MLN was positive in 14/19 (74%) samples examined, the RPLN was positive in 8/18 (44%), and the tonsil was positive in 10/25 (40%).

TABLE 1 Detection and characterization of PrP^{Sc} from selected pigs

Treatment group and animal no.	Incubation period (mpi)	Overall result		Antigen capture EIA result	WB result	IHC result	PK sensitivity	RT-QuIC result	RT-QuIC AFR
		CNS[a]	LRS[b]						
Control market wt									
1	0	−	NT[c]	NA[d]	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
Control aged									
6	25	−	NT	−	−	NT	NT	NT	NT
7	41	−	NT	−	−	NT	NT	NT	NT
8	46	−	NT	−	−	NT	NT	NT	NT
9	73	−	−	−	−	−	NA	−	0
i.c. inoculated market wt									
10	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
i.c. inoculated aged									
20	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
Orally inoculated market wt									
32	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
Orally inoculated aged									
39	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

[a]CNS, central nervous system.
[b]LRS, lymphoreticular system.
[c]NT, sample not tested.
[d]NA, result not applicable.

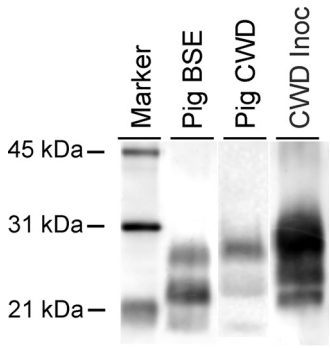

FIG 1 WB analysis demonstrating a unique PrP^{Sc} profile in brain samples from pigs with CWD. The positive brain sample from a pig inoculated with the CWD agent (pig CWD) has a slightly higher migration than the brain sample from a pig inoculated with the agent of classical BSE (pig BSE) and a much lower migration than the CWD inoculum (CWD Inoc). The diglycosylated band (topmost 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. The blot was developed with monoclonal antibody L42. Note that because of the sparse PrP^{Sc} accumulation in the brains of inoculated pigs, the blot shown is a composite; see Materials and methods for details.

Histopathology and IHC. 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 hematoxylin and eosin staining and by IHC.

Occasional neuropil vacuolation and white matter vacuolation were present in different brain sections of control and inoculated pigs. Small to medium-sized gray matter vacuoles were seen in the colliculus of at least one pig from each treatment group, including control pigs (Fig. 4A, pig 7, and B, pig 25). Vacuolation and PrP^{Sc} deposition in the colliculus were present in two pigs (no. 25 and 26) from the i.c. inoculated aged pig group (Fig. 4C, pig 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 (Fig. 4E, pig 38). This type of vacuolation was present in pigs from all of the market weight treatment groups, including noninoculated 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 four 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 (pig 26), midbrain colliculus (pigs 25 and 26),

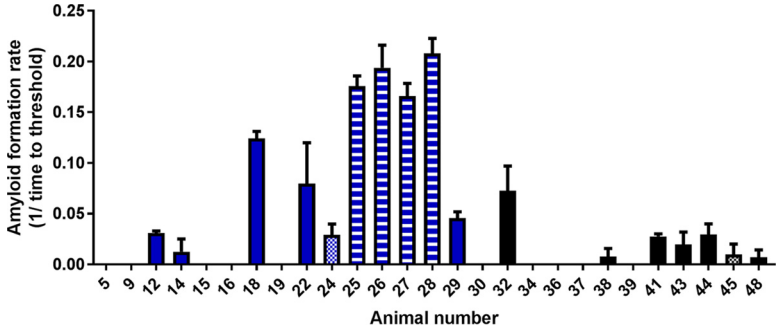

FIG 2 AFRs (RT-QuIC) and PK sensitivity (EIA) of PrP^{Sc} from pig brain samples. Treatment groups: animals 5 and 9, noninoculated controls; 12 to 19, i.c. inoculated market weight pigs; 22 to 29, i.c. inoculated aged pigs; 30 to 38, orally inoculated market weight pigs; 39 to 48, orally inoculated aged pigs. PK sensitivity: solid fill, PK sensitivity not determined (EIA negative); horizontal stripe fill, PK resistant; checked fill, PK sensitive.

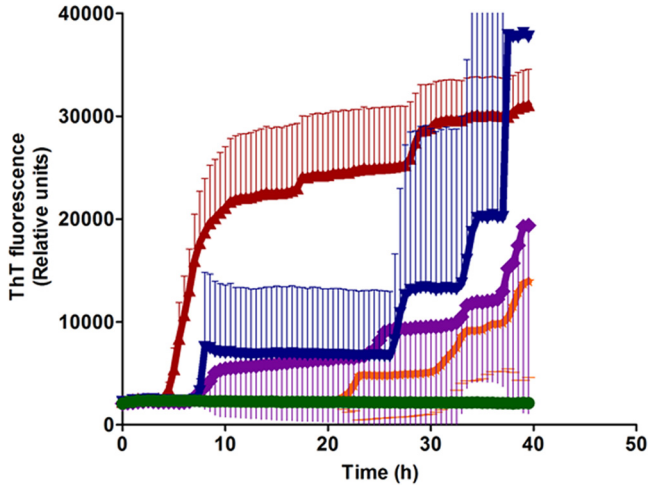

FIG 3 Results of RT-QuIC assays of brain homogenate from inoculated and negative-control pigs. Shown are the average percent thioflavin T (ThT) fluorescence readings (thick lines) with standard deviations (thin lines) determined from all replicates (four replicate reactions per animal) from all of the pigs in each challenge group. Red, i.c. inoculated aged pigs ($n = 7$); blue, i.c. inoculated market weight pigs ($n = 6$); purple, orally inoculated market weight pigs ($n = 6$); orange, orally inoculated aged pigs ($n = 6$); green, noninoculated control pigs ($n = 2$).

midline thalamic nuclei and hypothalamus (pigs 45 and 28), or septal nuclei (pig 28) (Fig. 4C).

PrP^{Sc} immunoreactivity was also seen in the retina of one pig, i.e., granular to punctate immunoreactivity in the inner and outer plexiform layers with occasional intraglial deposits (Fig. 4F, pig 26). Disease-specific PrP^{Sc} immunoreactivity was not seen in any other tissues, although nonspecific immunolabeling was common (Fig. 4D, brain stem, and G, retina).

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

Pigs from the i.c. inoculated market weight (pig 18) and i.c. inoculated aged (pigs 27 and 28) groups and the orally inoculated aged group (pig 48) produced positive bioassay results (Table 3). In mice inoculated with brain material from pig 18 (an i.c. inoculated market weight pig), the average incubation period was 244 days postinoculation (dpi) (2/28 mice). In mice inoculated with brain material from pig 27 (i.c. inoculated market aged pig group), the average incubation period was 167 (range, 140 to 220) dpi (3/29 mice). Two out of 27 mice were positive in the group inoculated with brain material from pig 28; 1 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 (no. 48), with 14/28 mice positive and an average incubation period of 263 (range, 111 to 621) dpi.

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

DISCUSSION

We demonstrated that PrP^{Sc} can be detected as early as 6 months postinoculation (mpi) in brain and lymphoid tissues of pigs inoculated orally or i.c. 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 RT-QuIC and bioassay were used for PrP^{Sc} detection.

TABLE 2 Detection of PrP^{Sc} in lymphoid tissues by antigen capture EIA and RT-QuIC assay

Treatment group and animal no.	Incubation period (mpi)	Overall result	RPLN[b]		Tonsil		MLN[c]	
			EIA result	RT-QuIC result	EIA result	RT-QuIC result	EIA result	RT-QuIC result
Noninoculated controls								
5	6	−	−	−	−	−	−	−
9	73	−	NA[a]	NA	−	−	−	−
i.c. inoculated market wt								
12	6	+	−	−	−	−	−	+
14	6	+	−	+	−	+	−	−
15	6	+	−	+	−	+	−	+
16	6	+	−	+	−	+	−	+
18	6	+	−	−	−	−	−	+
19	6	−	−	−	−	−	−	−
i.c. inoculated aged								
22	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	+	−	+	−	−	−	−
Orally inoculated market wt								
30	6	+	−	−	−	−	−	+
32	6	+	−	−	−	+	−	+
34	6	−	−	−	−	−	−	−
36	6	+	−	−	−	+	−	+
37	6	+	−	+	−	−	+	+
38	6	+	−	−	−	+	−	+
Orally inoculated aged								
39	19	+	−	−	−	−	−	+
41	41	+	−	+	−	−	−	+
43	45	+	−	NA	−	+	NA	NA
44	55	+	NA	NA	−	−	−	+
45	64	−	−	NA	−	−	NA	NA
48	72	−	NA	NA	−	−	−	−

[a]NA, sample not available.
[b]RPLN, retropharyngeal lymph node.
[c]MLN, mesenteric lymph node.

Prion infection was subclinical in most of the pigs in this study; PrP^{Sc} was detected in brain tissue from 18 pigs, but neurologic signs suggestive of prion disease were observed in only 1 pig. This pig developed clinical signs of difficulty in rising and signs of tremor. Both of these clinical signs have been reported previously in pigs challenged with the BSE agent (6) or the sheep-passaged BSE agent (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 has been described in pigs challenged with the cow-passaged (6, 8) or sheep-passaged (4) BSE agent.

A mild spongiform change was observed in the brains of both inoculated and noninoculated pigs, suggesting that the presence of a spongiform change in the brain should not be used as the 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,

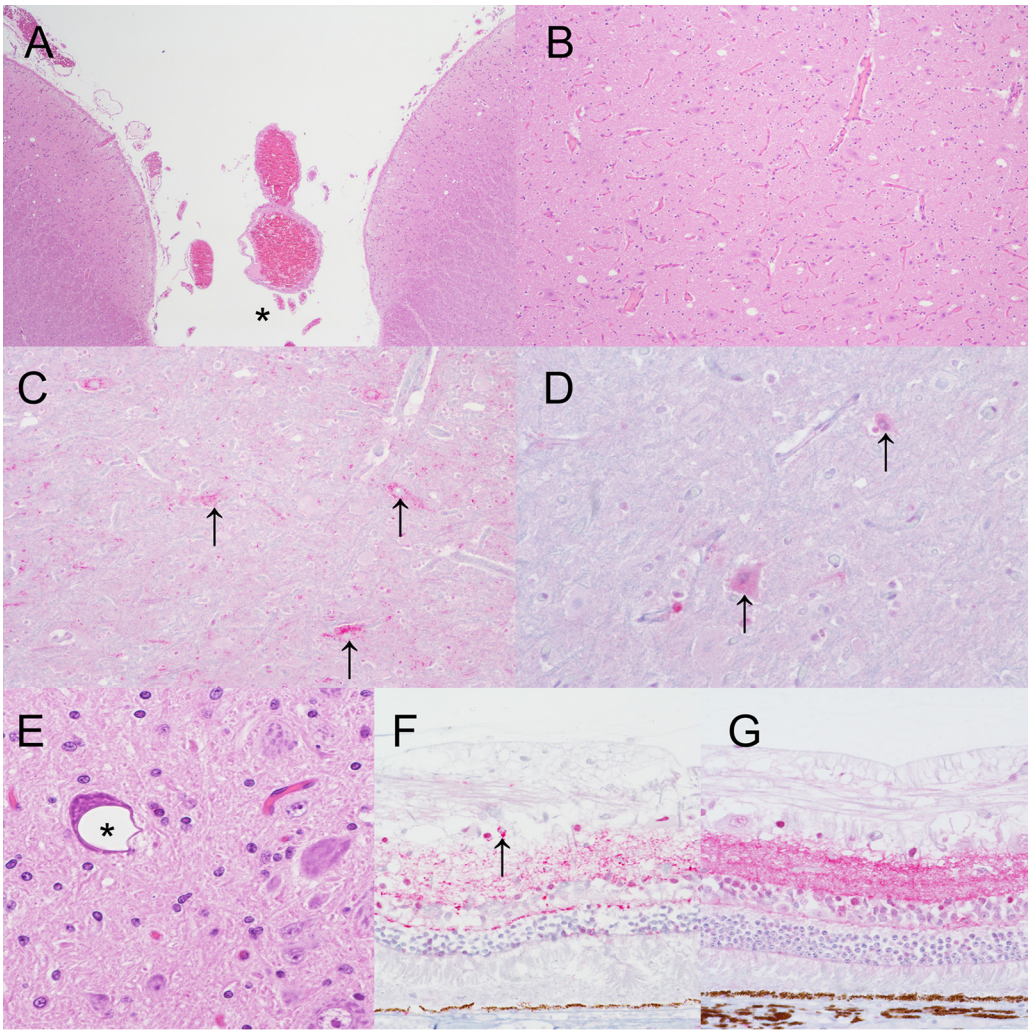

FIG 4 Vacuolar change and PrP^{Sc} in the brain and eye. (A) Brain stem of pig 7 showing incidental, i.e., unrelated to prion disease, neuropil vacuolation in the colliculus. *, midline (hematoxylin and eosin staining; original magnification, ×4). (B) Higher-magnification view of panel A (original magnification, ×10). (C) Brain stem of pig 25 showing intraneuronal PrP^{Sc} immunoreactivity (arrows) in neurons in the colliculus (anti-PrP monoclonal antibody L42; original magnification, ×20). (D) Brain stem of noninoculated control pig 8 showing non-disease-specific intraneuronal immunolabeling (arrows) in neurons in the colliculus (anti-PrP monoclonal antibody L42; original magnification, ×40). (E) Brain stem of pig 38 showing incidental intraneuronal vacuolation (*) in the dorsal motor nucleus of the vagus nerve (hematoxylin and eosin staining; original magnification, ×40). (F) Retina of pig 26 showing granular to punctate PrP^{Sc} immunoreactivity in the inner and outer plexiform layers with occasional intraglial deposits (arrow) (anti-PrP monoclonal antibody L42; original magnification, ×40). (G) Retina of noninoculated control pig 4 showing non-disease-specific immunolabeling (anti-PrP monoclonal antibody L42; original magnification, ×40).

8). Since the above microscopic changes can be observed in both noninoculated control and inoculated pigs, when present in inoculated pigs, they are considered equivocal, i.e., not related to prion disease. Colocalization of neuropil vacuolation and intraneuronal PrP^{Sc} deposits was present in the rostral colliculus of two 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 it was considered equivocal.

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

By standard diagnostic tests (WB, EIA, and IHC), PrP^{Sc} was detected in brain or lymphoid tissues from eight pigs in this study. The number of positive animals and

TABLE 3 Results of bioassays of brain material from selected pigs in Tg002 mice that express porcine prion protein

Donor animal no.	Donor treatment group (donor incubation period [mpi])	Tg002 mouse	
		Attack rate[a]	Mean incubation period (dpi)
18	i.c. inoculated market wt (6)	2/29	244
27	i.c. inoculated aged (64)	3/29	167
28	i.c. inoculated aged (73)	2/27	314, 701[b]
32	Orally inoculated market wt (6)	0/28	>700
48	Orally inoculated aged (72)	14/28	263

[a]PrP^{Sc} in the brains of mice was detected by an antigen capture EIA.

[b]The survival times of these two mice are so disparate that calculation of a mean incubation period would not be meaningful.

tissues, in particular lymphoid tissues, was much higher when the RT-QuIC assay was used. By 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 a bioassay (41) and 10,000 times as sensitive as EIA and WB for the detection of scrapie seeding activity in goat brain samples (42). With the exception of IHC, diagnostic tests were performed with brain stem samples since this brain region is the preferred site for statutory diagnostic testing. Testing of additional brain regions might have revealed PrP^{Sc} accumulation elsewhere in the brain, as was observed by IHC.

The RT-QuIC assay allows quantification of the seeding activity of prions in the samples on the basis of AFRs. The AFR is calculated as the reciprocal of the time it takes for a reaction to reach the threshold (i.e., 1/time to threshold in hours). A higher AFR reflects a shorter time taken to reach the 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 i.c. inoculated aged pigs tended to be higher than those of samples from orally inoculated aged pigs, it follows that the relative amount of PrP^{Sc} in the brain is larger in i.c. inoculated pigs. This seems logical, considering that PrP^{Sc} in the inoculum was delivered directly into the brain in i.c. 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 1 order of magnitude lower than the AFR of samples from positive animals that were PK resistant. Although the interpretation of these observations is limited by the small sample size and the fact that samples were not normalized for total protein content, it appears that there may be a relationship between AFR and PK sensitivity.

One hypothesis is that larger seed particles present more seeding surfaces than smaller particles and thus support faster RT-QuIC kinetics (45). In scrapie-infected hamsters, PK-sensitive PrP^{Sc} molecules from low-molecular-weight aggregates are made up of fewer PrP units (i.e., are smaller) than PK-resistant PrP^{Sc} aggregates (46, 47). Combining these observations with our own results, we hypothesize that the smaller average seed particle size of PK-sensitive PrP^{Sc} may result in slower RT-QuIC kinetics and lead to lower AFRs and longer lag times. However, as stated above, this hypothesis is based on a small number of samples.

The detection of PrP^{Sc} in lymphoid tissues from the heads and guts of CWD-infected pigs raises the possibility that pigs are able to shed prions in excreta, as has been shown for saliva (48–51) and feces (52–54) from CWD-affected cervids. Unfortunately, saliva and feces samples were not collected in the present study.

PrP^{Sc} was detected in brain and lymphoid tissues from orally inoculated pigs killed at approximately market weight. These results suggest that, if they were to be exposed to sufficient amounts of CWD infectivity, pigs in commercial swine production systems would have the potential to accumulate CWD prions by the time they reach market weight.

In the case of feral pigs, exposure to the agent of CWD through scavenging of CWD-affected cervid carcasses or through consumption of prion-contaminated plants or soil could allow feral pigs to serve as a reservoir of CWD infectivity. The range and numbers of feral pigs are predicted to continue to increase because of the ability of pigs to adapt to many climates, reproduce year-round, and survive on a varied diet (55). The range of CWD-affected cervids also continues to spread, increasing the likelihood of overlap of ranges of feral pigs and CWD-affected environments.

We demonstrate here that PrP^{Sc} accumulates in lymphoid tissues from pigs inoculated i.c. or orally with the CWD agent and can be detected as early as 6 months after inoculation. Clinical disease suggestive of prion disease developed only in a single pig after a long (64-month) incubation period. This raises the possibility that CWD-infected pigs can shed prions into their environment long before they develop clinical disease. However, the small amounts of PrP^{Sc} detected in our study pigs combined with the low attack rates in Tg002 mice suggest that there is a relatively strong species barrier to CWD prion transmission to pigs.

MATERIALS AND METHODS

Ethics statement. All of our animal experiments were reviewed and approved by the National Animal Disease Center (NADC) Institutional Animal Care and Use Committee (IACUC; protocols 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 mpi 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 because of intercurrent illness or injury that could not be remediated by veterinary care. Euthanasia was performed by intravenous injection of sodium pentobarbital in accordance with the manufacturer's instructions.

Inoculum preparation. The pooled CWD inoculum was prepared from three brains from white-tailed deer that were inoculated i.c. with brain material from CWD-affected elk, white-tailed deer, or mule deer (NADC IACUC protocol 3347) (56). All donor deer were homozygous for glycine (G/G) at *PRNP* codon 96 and serine (S/S) at codon 138. The brain tissue was ground in a mechanical grinder and mixed with phosphate-buffered saline (PBS) to produce a 10% (wt/vol) homogenate.

Animal procedures. Crossbred piglets were inoculated at 8 weeks of age. Pigs inoculated i.c. ($n = 20$) received a single dose of 0.75 ml of 10% (wt/vol) CWD brain homogenate as described previously (57). Orally inoculated pigs ($n = 19$) received 15 ml of 10% (wt/vol) CWD brain homogenate by syringe with a soft feeding tube on 4 consecutive days (total dose, 45 ml). Pigs inoculated i.c. and orally were housed in separate pens. At 2 weeks postinoculation, noninoculated control pigs were introduced into the pens with the inoculated pigs.

At 6 to 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) as follows: eight i.c. inoculated pigs, nine orally inoculated pigs, and two control pigs. The remaining pigs ("aged" groups) were allowed to incubate for up to 73 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 1× the expression level of prion protein in FVB mice (5). Samples of brain stem at the level of the obex were prepared as 10% (wt/vol) homogenates in PBS. Mice were inoculated i.c. with 20 μl of 10% (wt/vol) brain homogenate as described previously (58). Mice were monitored daily and euthanized when they displayed unequivocal neurological signs (difficulty moving, poor coordination, inability to move, anorexia) or at the time of study termination (approximately 700 dpi). Brain samples from mice were prepared as 10% (wt/vol) brain homogenates in PBS as described previously (59). PrP^{Sc} was detected by EIA as described below.

Sample collection. A full necropsy was performed on all pigs, including collection of two sets of tissue samples. To minimize potential cross-contamination, one pathologist collected tissues from the head and a second pathologist collected tissues from the rest of the body. Single-use instruments were not used. One set of tissues included representative sections of liver, kidney, spleen, skin, striated muscles (heart, tongue, diaphragm, masseter, triceps, biceps femoris, psoas major), lymphoid tissues of the head (pharyngeal tonsil, palatine tonsil, medial RPLN), other lymph nodes (mesenteric, hepatic, renal, popliteal, prescapular), nasal turbinates, lung, esophagus, small intestine, cecum, colon, rectal mucosa, stomach, adrenal gland, pituitary gland, reproductive tissues, peripheral nervous system (trigeminal ganglion, optic nerve, sciatic nerve, vagus nerve), brain (hemisections of cerebral cortex, hippocampus, cerebellum, superior colliculus, and brain stem, including obex), and eye (retina). Formalin-fixed tissues were fixed in 10% neutral buffered formalin, moved to 70% ethyl alcohol after 48 h, embedded in paraffin wax,

sectioned, and stained with hematoxylin and eosin for light microscopy. The second set of tissues was frozen.

Selection of animals and tissues for PrP^{Sc} detection. Frozen brain stem tissue from all pigs was screened for the presence of PrP^{Sc} by antigen capture EIA and WB. Fixed tissues from pigs that were positive by WB and/or EIA were examined by IHC. In addition, representative pigs from across the range of survival times in each group were also examined by IHC. Brain stem material from the pig with the longest incubation period in each treatment group was bioassayed in Tg002 mice. PrP^{Sc} detection by QuIC assay was applied to frozen brain stem and lymphoid tissues from all pigs that were positive by any other test (EIA, WB, IHC, bioassay), as well as additional animals, so that six or seven animals per group and across a range of survival times were tested.

IHC. All paraffin-embedded tissues were immunostained with anti-PrP monoclonal antibody L42 by an automated immunohistochemical method for detection of PrP^{Sc} as described previously (60).

Antigen capture EIA. Brain homogenates were homogenized in 1× PBS at a concentration of 20% (wt/vol) and assayed with a commercially available EIA kit (HerdChek BSE-Scrapie Ag Test; IDEXX Laboratories, Westbrook, ME) as previously described (61). Assays were performed in accordance with the manufacturer's instructions. The EIA kit instructions indicated three protocols (standard, short, and ultrashort). The short protocol was used to test tissue samples in the present study. Each tissue sample homogenate was assayed in a single well along with negative and positive controls supplied 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 to test samples obtained from mice expressing pig prion protein. Absorbance at 450 nm was measured (SpectraMax 190; Molecular Devices, Sunnyvale, CA) by using a reference wavelength of 620 nm. Cutoff values were established for each run in accordance with the kit instructions, whereby 0.180 was added to the mean negative-control value. Samples were interpreted as positive if the absorbance at 450 nm minus the reference value at 620 nm was above the established cutoff value.

EIA-based PK sensitivity testing. Sensitivity to PK was determined by the EIA protocol described above but with the addition of a pretesting PK treatment step (40). Briefly, for each animal, two 100-μl aliquots of 20% (wt/vol) 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 h at 37°C with shaking at 1,000 rpm, followed by the addition of 1.0 μl of 100 mg/ml PK inhibitor (Pefabloc; Roche Diagnostics, Mannheim, Germany). The absorbance of each sample was determined by 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.

WB. Samples for WB were collected from the brain stem at the level of the obex and the midbrain between the optic and oculomotor nerves dorsal to the pituitary 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 PK at a final enzyme concentration of 0.4 U/ml (8 μg/ml) at 37°C for 1 h. 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 with mouse anti-PrP monoclonal antibody L42, which targets amino acids 145 to 163 of the ovine prion protein sequence (62), at a 1:500 dilution (0.1 μg/ml).

Because of the sparse PrP^{Sc} accumulation in the brains of inoculated pigs, the blot in Fig. 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, United Kingdom).

Expression and purification of the recombinant PrP substrate. The recombinant prion protein (rPrP) used in the RT-QuIC assay was expressed and purified by a previously reported standard protocol (41, 63). Briefly, rPrP composed of Syrian hamster PrP residues 90 to 231 in the pET vector was transformed into *Escherichia coli* Rosetta2(DE3) cells and purified from inclusion bodies by fast protein liquid chromatography as described previously (44, 64).

RT-QuIC assay for brain and lymphoid tissue samples. We included brain and lymphoid tissue homogenates from clinical CWD-affected white-tailed deer, age group-matched noninoculated pigs, and a blank (buffer) as controls. Samples were collected by a strict aseptic technique to minimize the risk of cross-contamination. All of the samples were run by using a blinded study design (N.K., S.M.).

Prior to testing, brain and lymphoid tissue samples were homogenized in 1× PBS at a concentration of 20% (wt/vol) tissue and then further homogenized by repeated pipetting and sonication in a cup sonicator with two pulses of 30 s. The samples were then further diluted to a concentration of 0.02% in sample dilution buffer (0.025% SDS in 1× PBS).

The RT-QuIC assay was performed by previously published protocols (41, 65), with slight modifications as described previously (64). All samples were run in quadruplicate. The reaction mixtures consisted of 5 μg of protein from the brain and lymphoid tissue homogenates that were used as seed in a 100-μl total reaction volume. A sample was considered positive if the fluorescence intensity of at least half the replicate wells crossed the threshold, 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 on the basis of the AFR, which is the reciprocal of the time (in hours) that it takes for a reaction to reach the threshold, defined as the mean baseline fluorescence plus 5 standard deviations (41, 65). The

AFR was calculated by using all four replicates of each sample. Data analysis was performed with BioTek's Gen5 software version 2.07.17 and BMG's MARS software version 5.2.R8.

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