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Environmental Sources of Scrapie Prions▽

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Ovine scrapie and cervine chronic wasting disease show considerable horizontal transmission. Here we report that a scrapie-affected sheep farm has a widespread environmental contamination with prions. Prions were amplified by protein-misfolding cyclic amplification (sPMCA) from seven of nine environmental swab samples taken, including those from metal, plastic, and wooden surfaces. Sheep had been removed from the areas from which the swabs were taken up to 20 days prior to sampling, indicating that prions persist for at least that long. These data implicate inanimate objects as environmental reservoirs for prion infectivity that are likely to contribute to facile disease transmission.

Prion diseases are fatal neurological disorders. The archetypal prion disease is scrapie in sheep, and in the last few decades novel prion diseases have emerged in a range of species, including bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer, and variant Creutzfeldt-Jakob disease (vCJD) in humans. The "protein-only" hypothesis dictates that a pathological isoform, PrP^{Sc}, of the cellular prion protein (PrP^{C}) constitutes the infectious agent, or prion (13). A wide range of tissues from CWD- and scrapie-affected animals contain PrP^{Sc}, and affected animals have been shown to excrete or secrete prions in milk, saliva, urine, and feces (2, 3, 6, 7, 8, 10, 16). This finding led to the hypothesis that infectivity resides in the environment, thus explaining the facile transmission of CWD and scrapie. In support of this hypothesis, CWD infectivity has been transmitted from a combination of exposed bedding, water, and food of captive animals (9), and CWD PrP^{Sc} has been detected within a single environmental water sample (11).

Environmental prions are likely to be present at very low levels. The most sensitive method available for the detection of PrP^{Sc} is serial protein-misfolding cyclic amplification (sPMCA) (1). This technique was pioneered by Soto and colleagues (15), has been used for the amplification of scrapie (17), CWD (4), and vCJD (5) within their natural hosts, and has facilitated the detection of prions within ovine milk (6) and saliva (7) and within cervine urine (4). Here we investigated sources of environmental scrapie prions by applying sPMCA to samples taken from a range of surfaces that were accessible to animals on a farm where scrapie is endemic.

Environmental samples were taken from the Veterinary Laboratories Agency, United Kingdom, farm where natural scrapie is endemic, with a high incidence since 1996. Sheep within the flock were exposed to the scrapie agent by natural routes of transmission. Control samples were taken from a farm (ADAS, United Kingdom) that houses a New Zealand-derived scrapie-free flock kept under strict biosecurity conditions. Environmental swabs were taken by wetting foam swabs (Edson Electronics, Northumberland, United Kingdom) in sterile water and then gently swabbing five times in both directions across a surface approximately 10 cm by 2 cm. Two swabs were taken from each area, and all samples were stored at −80°C. A total of nine environmental samples from a scrapie-affected farm and a scrapie-free farm were analyzed by sPMCA.

Two swabs taken from each area were thawed to room temperature and placed in a single container to which 6 ml of 150 mM PO_4 buffer plus 0.5% (vol/vol) Nonidet P-40 and 0.5% (wt/vol) sodium deoxycholate were added. The container was rotated for 2 h. Prions released into this buffer were precipitated on silicon dioxide and then eluted in 200 μl of 0.1% (wt/vol) sodium dodecyl sulfate. Ten microliters of the eluate was amplified within PCR tubes by sPMCA exactly as previously described (7). Samples from both a scrapie-exposed environment and a non-scrapie-exposed environment were analyzed concurrently within the same run on the same sonicator. Each extract was amplified at least in triplicate within a single run and then analyzed by Western blotting (14).

Samples from four metal surfaces from an indoor pen occupied by sheep for a few days each week—a gate, a water trough, a feed trough, and penning—were analyzed. Samples from an outdoor environment that had contained sheep 20 days previously—a metal fence, a metal gate, a metal water trough, a plastic post where sheep frequently scratched, and a wooden fence post (Table 1)—were also analyzed. After 8 rounds of amplification, PrP^{Sc} was detected in all samples with the exceptions of the outdoor water trough and gate (Figure 1 and Table 1). Equivalent samples from a farm housing a scrapie-free flock were also analyzed, and PrP^{Sc} was not amplified even after 10 rounds of sPMCA. For indoor surfaces from the scrapie-affected farm, 83% of the sPMCA reactions

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TABLE 1. sPMCA of prions found in the environment[a]

Source of sample[b]	No. of positive tests/no. of total tests for samples from a farm where scrapie is endemic	PMCA round[c] that yielded PrP^{Sc}	No. of positive tests/no. of total tests for samples from a scrapie-free farm
Indoor environment			
Metal water trough	2/3	7	0/6
Metal gate	3/3	6	0/6
Metal penning	3/3	6	0/6
Metal feed trough	2/3	6	0/6
Outdoor environment			
Metal water trough	0/3	n/a	0/6
Metal gate	0/3	n/a	0/6
Metal fencing	1/3	7	0/6
Plastic scratching post	2/3	8	0/6
Wooden fence post	1/3	7	0/6

[a] Samples were taken from a farm where scrapie is endemic and one that is scrapie free and were subjected to 8 rounds of sPMCA.
[b] Metal surfaces were zinc-galvanized steel.
[c] Earliest round that yielded detectable proteinase K-resistant PrP^{Sc} from each scrapie-exposed sample. n/a, not applicable.

were positive ($n = 12$), and 0% were positive for equivalent samples from a scrapie-free farm ($n = 24$). Similarly, 27% of analyses were positive for samples from outdoor surfaces ($n = 15$), and again no prion was amplified from equivalent samples taken from a scrapie-free farm ($n = 30$). For comparison of the percentages of positive sPMCA reactions for different cohorts of samples, data were set up as 2 by 2 contingency tables, and Fisher's exact test (one-tailed) was applied to derive P values. Overall, prions were significantly more likely to be present in the scrapie-affected farm on indoor ($P < 0.001$) and outdoor ($P = 0.009$) surfaces. Analyses of all samples were carried out in two independent experiments that gave equivalent results.

The extracts from environmental swabs became positive for PrP^{Sc} after 6 to 8 rounds of PMCA (Table 1). In order to estimate the levels of prions within these extracts, the limit of detection of the amplification methodology was estimated by spiking a 10-fold dilution series of brain stem homogenate from a scrapie-affected sheep into the PMCA reaction. Following 6 rounds of amplification, PrP^{Sc} could be detected in extracts containing 1×10^{-11} g of brain material. The levels of total PrP and protease-resistant PrP within the brain material were estimated by enzyme-linked immunosorbent assay (ELISA) against a recombinant PrP standard curve, and the levels within 1×10^{-11} g of brain material equated to 0.24×10^{-15} g of protease-resistant PrP^{Sc} and 0.4×10^{-15} g of total PrP. A similar level of amplification was achieved from a one-tenth volume of a swab extract. These data indicate that a swab extract taken from a 20-cm^2 area of a farm surface contains approximately 2.4×10^{-15} g of protease-resistant PrP^{Sc} and 4×10^{-15} g of total PrP.

These data indicate that surfaces exposed to scrapie-infected animals can act as reservoirs of PrP^{Sc} and therefore have the potential to facilitate disease transmission. Prions were eluted from surfaces upon brief contact, indicating their availability for uptake by sheep through ingestion and/or skin scarification. Given the striking similarities between CWD and scrapie with regard to widespread *in vivo* prion dissemination, secretion of the disease agent, and facile disease transmission, it seems extremely likely that inanimate objects also serve as environmental reservoirs for CWD for both farmed and wildlife populations. For scrapie and CWD, it is likely that the widespread *in vivo* dissemination of infectivity facilitates the secretion and/or excretion of prions into the environment. It seems unlikely that most human prion diseases and BSE in cattle would display analogous excretion of prions, as there is limited *in vivo* spread of the infectious agent. However, vCJD, the human form of BSE, has widespread *in vivo* PrP^{Sc}, similar to CWD and scrapie (12). As data indicate that the causal agents of CWD (9, 11) and scrapie are maintained within the environment on a range of fomites, it should be a priority to determine whether vCJD prions follow similar dissemination routes.

The findings of the present study may well have a considerable impact on the understanding of the horizontal transmission of both scrapie and CWD and therefore on the management of farmed animals. The level of prions found on 20-cm^2 surfaces was similar to that detected in a milliliter of urine from scrapie-affected hamsters (2), a volume known to contain infectivity (3). However, at present it has not been confirmed that the low levels of prions on environmental surfaces are sufficient to cause disease in exposed sheep. As a first step in determining whether such prions are indeed capable of trans-

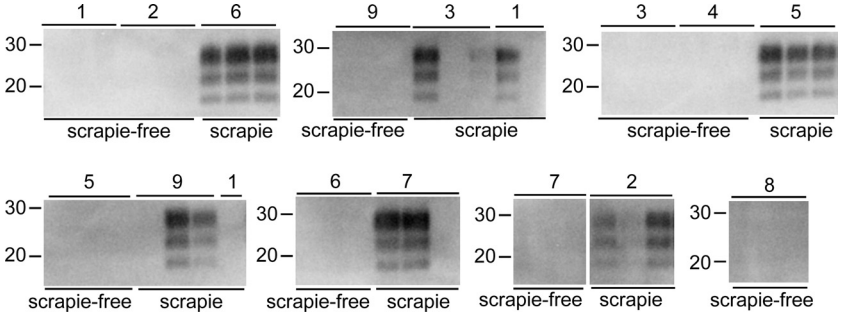

FIG. 1. Amplification of prions from environmental samples. Prions were extracted from swabs taken of surfaces from a scrapie-free farm or a farm where scrapie is endemic. Swabs were taken from a wooden post (1), a plastic scratching post (2), and the following metal surfaces: fencing (3), gate (4 and 6), pen (5), feed trough (7), and water trough (8 and 9). Samples 1, 2, 3, 4, and 8 were taken from outdoor surfaces and 5, 6, 7, and 9 from indoor surfaces. Extracts were used as seeds for 8 rounds of sPMCA. Products were digested with proteinase K and analyzed by Western blotting. PrP was detected with monoclonal antibodies SHA31 and P4. Molecular-weight markers are shown.

mitting disease in sheep, the concentrated extracts of the swabs will be inoculated into transgenic mice in order to determine the presence of infectivity.

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