The oral secretion of infectious scrapie prions occurs in pre-clinical sheep with a range of PRNP genotypes

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Abstract

Preclinical sheep with the highly scrapie-susceptible VRQ/VRQ PRNP genotype secrete prions from the oral cavity. In order to further understand the significance of orally available prions, buccal swabs were taken from sheep with a range of PRNP genotypes and analysed by protein misfolding cyclic amplification reaction (sPMCA). Prions were detected in buccal swabs from scrapie-exposed sheep of genotypes that are linked to high (VRQ/VRQ and ARQ/VRQ) and low (ARR/VRQ and AHQ/VRQ) lymphoreticular involvement in scrapie pathogenesis. For both groups, the levels of prion detection were significantly higher than that for the scrapie-resistant ARR/ARR sheep which were kept in the same farm environment and acted as sentinel controls for prions derived from the environment which might contaminate the oral cavity. In addition, sheep with no exposure to the scrapie agent did not contain any measurable prion within their oral cavity. Furthermore, prion was detected in sheep of a wide age range representing various stages of preclinical disease. These data demonstrate that orally available scrapie prions may be a common feature in sheep incubating scrapie, regardless of the PRNP genotype and any associated high level accumulation of PrPSc within lymphoreticular tissues. PrPSc was present in buccal swabs from a high proportion of sheep with PRNP genotypes associated with relatively low disease-penetrance; indicating that subclinical scrapie infection is likely to be a common occurrence. The significance of positive sPMCA reactions was confirmed by the transmission of infectivity in buccal swab extracts to Tg338 mice, illustrating the likely importance of orally available prions in the horizontal transmission of scrapie.
The transmissible spongiform encephalopathies (TSE) are a group of neurodegenerative diseases affecting many mammalian species, for which there is 100% mortality. These include chronic wasting disease (CWD) in deer, bovine spongiform encephalopathy (BSE) in cattle, variant Creutzfeldt-Jakob disease (vCJD) in humans and the prototype TSE, scrapie in sheep. Intensive research effort has been given to this group of diseases after the demonstration that the likely cause of variant CJD (vCJD) in humans is the consumption of BSE contaminated meat products (7). Central to the understanding of these diseases is the hypothesis that the infectious component is a conformer of a benign host protein known as the prion protein (PrP\textsuperscript{C}). It is the infectious conformer of this ubiquitously expressed protein (PrP\textsuperscript{Sc}) that is responsible for the conversion of further healthy PrP\textsuperscript{C} into PrP\textsuperscript{Sc} (21). This autocatalytic process leads to progressive neuronal loss and ultimately death, with the duration of disease incubation ranging from months in some rodent models to decades in some human TSEs (1). Whilst the levels of PrP\textsuperscript{Sc} accumulation are highest in the central nervous system (CNS), there can be appreciable accumulation in other tissue types. Within distinct TSE diseases, there are different levels of lymphoreticular system (LRS) involvement, which is dependent on both strain and host species. This is well illustrated for the BSE agent, in cattle there is little apparent involvement of the LRS with restriction of prion replication mainly to the CNS. However, in sheep experimentally infected with BSE and in human vCJD there is marked involvement of the LRS (10, 27). In the case of scrapie in sheep the genotype of the host can also have a strong influence on the tissue distribution of prion infectivity. In sheep with a V\textsubscript{136}R\textsubscript{154}Q\textsubscript{171}/A\textsubscript{136}R\textsubscript{154}R\textsubscript{171} PRNP genotype it has been reported that infection of the CNS can be seen without prior involvement of the LRS tissues, which is in contrast to disease pathogenesis within VRQ/VRQ animals where there is sustained LRS involvement in PrP\textsuperscript{Sc} accumulation during the development of disease, both before and after prion invasion of the CNS (12).
Epidemiological and experimental data for both ovine scrapie and cervine CWD have shown both direct horizontal transmission of disease as well as environmental reservoirs of infectivity (2,15,19). The dissemination of infectivity from infected sheep into the environment, and the direct animal-to-animal transmission of disease may be influenced by the levels of PrPSC within the LRS which in turn may dictate the levels of PrPSC within secretory/excretory sites; for example within skin (25), mammary glands (14), salivary glands (26) and nasal mucosa (11).

We recently demonstrated the detection of scrapie prions in buccal swabs taken from sheep naturally exposed to, and incubating scrapie. Prion was detected using an in vitro prion protein amplification technique known as serial protein misfolding cyclic amplification (sPMCA), a methodology conceptually analogous to nucleic acid amplification by the polymerase chain reaction (23). Amplifiable prion material was detected in buccal swabs from exposed sheep from 9 months of age to clinical disease in animals with a VRQ/VRQ PRNP genotype (17). It is likely that oral prion availability highlights an important factor in the horizontal spread of scrapie, both by direct sheep-to-sheep transmission of disease, and also via contribution to reservoirs of infectivity in the farm environment, including on fomites (15). Here, we analysed buccal swabs taken from sheep incubating scrapie and heterozygous for PrP alleles, representing different levels of LRS involvement during the infection process. Positive identification of prions within the oral cavity of sheep incubating scrapie was confirmed by the demonstration that PrPSC detected using this methodology correlated with biological infectivity transmitted to the Tg338 transgenic mouse line.

Materials and Methods
All animal procedures were performed under Home Office (United Kingdom) and local ethical review committee approval and compliance with the Animal (Scientific Procedures) Act 1986. Scrapie-exposed
sheep were from the AHVLA (UK) Ripley experimental scrapie sheep flock, which has had a high incidence of naturally acquired scrapie for the last 15 years. Samples were taken from sheep born into the flock with the scrapie susceptible genotypes ARR/VRQ, AHQ/VRQ, VRQ/VRQ and ARQ/VRQ. In addition, swabs were taken from ARR/ARR sheep associated with high resistance to scrapie infection and these served as controls for the presence of prions in the oral cavity that may have been taken up from the farm environment (15). Non-scrapie-exposed sheep with VRQ/VRQ, ARQ/ARQ and ARR/ARR genotypes were obtained from a scrapie-free New Zealand-derived flock kept under strict biosecurity conditions (ADAS UK). Buccal swabs taken from the scrapie exposed flock were from animals that were thought to be approaching the mid-point or were in the latter half of disease incubation (based on historical disease incubation times in this flock). Following exposure from birth, disease incubation periods for sheep with distinct PRNP genotypes are in the order VRQ/VRQ< ARQ/VRQ< AHQ/VRQ< ARR/VRQ.

Buccal swab samples were collected by gently rubbing foam swabs (Edson Electronics) across both inner cheeks of the animal. Four swabs were collected for each animal at single time-points and as such provide a snapshot of the likely presence of prion in the oral cavity. Two buccal swab samples were processed using a silicon dioxide (SiO2) enrichment step as previously described (17, 22). The SiO2 extracted material was then centrifuged for 3 min at 16,000 g and the supernatant diluted 1:10 into PMCA brain homogenate substrate (10% [wt/vol]) homogenate from a VRQ/VRQ PRNP genotype animal in PBS, 150 mmol/L NaCl, 4 mmol/L EDTA, pH 8.0, 1% [wt/vol] Triton X-100, and miniprotease inhibitor; Roche) to a final volume of 100 µL. All buccal samples were amplified in this single substrate as positive buccal swab extracts were most efficiently amplified within VRQ/VRQ rather than the homologous heterozygous substrate (data not presented). Samples contained in sealed 0.2 mL polymerase chain reaction tubes were placed in an ultrasonicating water bath (model S4000; Misonix) at 37 °C, and sonications were performed for 40 s at 200W. Sonications were repeated once...
every 30 min for 24 h (1 PMCA round) after which the amplified samples were diluted 1 in 3 with PMCA substrate in a final volume of 100 μL and the sample subjected to additional rounds of PMCA.

In the present study the brain homogenate substrate required between five and nine rounds of sPMCA to achieve the same levels of amplification that had previously been achieved at rounds three to four with a different brain substrate (17). This estimation in the level of amplification efficiency between substrates was carried out using buccal extracts from VRQ/VRQ sheep. During each sPMCA experiment, samples from both the scrapie exposed and unexposed cohorts were analysed at the same time, typically the sonicator horn contained 40% of one and 60 % of the other group in any given experiment.

Amplified samples were digested with 50 μg/mL proteinase K (PK), 0.045% (wt/vol) SDS for 1 h at 37°C before Western blot analysis using 12% (wt/vol) NuPAGE precast Bis-Tris gels, as described (22). A reaction was scored as a positive if there was a defined PK-resistant triplet (~18- 27kDa) visible on the Western blot, in each instance positive signals were determined to be at least 3X times that of the negative samples run on that particular western blot, as determined by Quantiscan software.

Murine bioassay was carried out in the Tg338 mouse line, which expresses ovine VRQ PrPC at high levels and is sensitive to low amounts of scrapie infectivity (13). Briefly, swab extracts from 12 VRQ/VRQ animals, (12 swabs), taken at 9 months post exposure to environmental scrapie, or 12 months of age from a scrapie free flock, were extracted as described for sPMCA. The extracts were pooled and PrPSc was recovered by ultracentrifugation at 100,000g for 1hour at 18 °C. Pelleted material was washed with sterile water and re-centrifuged three further times before resuspension of pelleted material in PBS. Ten mice were inoculated with swab extract from each swab pool. Each mouse received extract equivalent to half that extracted from a single swab as a 20 μL sample administered intra-cranially into the right hand side of the brain in phosphate buffered saline. Mice were monitored daily for signs of clinical disease. At post-mortem, brain and spleen tissues were taken and processed as
10% w/v homogenates as previously described (22). These samples were analysed by both sPMCA (up to 9 rounds) or by direct Western blot analysis of the proteinase K digested tissues.

Statistical analysis: In order to place significance on the data generated, amplification results were grouped in terms of numbers of positive and negative reactions per genotype. This data was analysed in 2x2 contingency tables and a one-tailed Fishers exact test was applied, comparing the number of positive reactions for samples from sheep with distinct genotypes with samples from either unexposed control animals or the scrapie-exposed ARR/ARR sentinel controls.

Results

PrPSc was detected in buccal swabs taken from sheep with VRQ/VRQ, ARQ/VRQ, ARR/VRQ and AHQ/VRQ genotypes (Table 1, Figure 1). These animals were all in the preclinical stages of scrapie incubation following natural exposure to the disease agent. No sample from the non-scrapie exposed control sheep amplified any PrPSc; this group included 27 individual animals of 3 homozygous genotypes and some 135 individual amplification reactions. All exposed sheep cohorts including the ARR/ARR sentinel controls had levels of positivity that were significantly above this background (Figure 2). Swabs from the ARR/ARR sentinel control animals, which were flock-mates of the scrapie-susceptible, exposed sheep and had therefore been housed within the same scrapie affected environment, showed a low level of prion amplification (3 out of 9 animals were positive, 5 out of 27 (19%) individual reactions). This is a measure of the levels of prion taken up from the farm environment and the figure correlates well with that previously reported for a similar control group from this same farm (17). The animals from those genotypes with the highest disease-penetrance and with the highest associated LRS involvement during infection, the VRQ/VRQ and ARQ/VRQ animals, both showed detectable prion positivity in buccal swab extracts significantly above samples from the sentinel control sheep (Figure 2). In total 78% of individual reactions from the VRQ/VRQ and 81% of individual
reactions from the ARQ/VRQ sheep were positive (representing 19 out of 20, and 7 out of 7 sheep respectively). Most ARQ/VRQ animals were estimated to be of an age representative of the latter half of disease incubation. Samples from VRQ/VRQ animals were taken at estimated mid-term incubation (11 animals) or were at time-points near to the end of disease incubation (9 animals). These data concur with our previous report where prions were readily detected in such samples taken from VRQ/VRQ sheep within the latter half of the disease incubation period. The ARR/VRQ and the AHQ/VRQ groups of sheep represent cohorts with associated lower disease-penetrance and little involvement of the LRS. For the small group of AHQ/VRQ animals, PrP\textsuperscript{Sc} was detected in swabs taken from all 3 animals with 44% of reactions being positive. However, whilst this was higher than the rate of prion amplification in the sentinel animals (19%), this was not significant (p=0.134). This is a likely reflection of the small sample size that was used in this instance. For ARR/VRQ animals, 8 out of 12 sheep contained amplifiable PrP\textsuperscript{Sc} in their oral cavity with 53% of the reactions being positive. This was significantly higher than that found in sentinel animals (p=0.005). When considering those genotypes associated with low (ARR/VRQ and AHQ/VRQ) and high (VRQ/VRQ and ARQ/VRQ) LRS accumulation of PrP\textsuperscript{Sc} as groups, both cohorts are significantly different to the control ARR/ARR exposed group (p=0.005 and 0.001 respectively). Interestingly, these two groups also differ from each other, with that associated with lower LRS involvement yielding significant less prion amplification (p=0.003).

In order to determine the presence of infectivity within sPMCA-positive buccal swab extracts, such samples were used to inoculate 10 Tg338 mice. 10 further mice were inoculated with identical extracts derived from non-scrapie-exposed sheep. At 176 days post challenge, one mouse receiving the positive buccal swab extract had shown clinical signs of disease. This mouse was negative by immunohistochemistry of the brain, however the left hand side of the brain and the spleen were used for further analysis by both sPMCA and direct Western blot. Such analyses were also carried out on tissues from an additional 4 animals receiving the scrapie exposed extract and 5 animals receiving the negative
control extract which were sacrificed at 310 days. Although IHC negative in the brain, the mouse showing clinical signs at day 176 displayed PK resistant PrP$^{Sc}$ in the spleen by Western blotting (figure 3), none of the other 4 mice sacrificed at 310 days were PrP$^{Sc}$ positive by Western blot in either brain or spleen (data not presented). However, both brain and spleen tissues were sPMCA positive for the clinical mouse, and 2 of the other 4 mice sacrificed at 310 days demonstrated a sPMCA positivity in the spleen (n=2) or brain (n=1), (table 2). All samples from the mice in the control cohort were sPMCA negative. The remaining 5 mice in each group are currently alive and not displaying any clinical signs (day 369).

Discussion

The data presented in this paper strongly suggest that the presence of PrP$^{Sc}$ in the oral cavity of sheep is a common feature of sheep scrapie, regardless of the genotype of the host and the associated level of LRS involvement in PrP$^{Sc}$ replication and dissemination. Sheep with genotypes associated with a ‘high LRS’ involvement in pathogenesis displayed higher levels of oral PrP$^{Sc}$ detection compared to animals with ‘low LRS’ involvement. It was previously speculated that the prion might enter the oral cavity from the reported presence of PrP$^{Sc}$ in the lumina of the salivary ducts (26). It is also possible however that the origin of the PrP$^{Sc}$ collected in the buccal swab results from the tissue of the buccal epithelium and possibly derives from innervation of the oral buccal surfaces (9). Another possibility is that this material is derived from a haematogenous origin (8). The contribution of the LRS to the secretion of prions into the oral cavity would seem to be supported by the current study, and the lower overall positivity of the ‘low LRS’ cohort. However, the lower level of positivity in ‘low LRS’ sheep could also be explained by the lower penetrance of scrapie in these genotypes, 20% of sheep of ARR/VRQ or AHQ/VRQ genotypes did not yield any amplifiable prion in their buccal swabs. This is much lower than for the VRQ/VRQ and ARQ/VRQ animals where PrP$^{Sc}$ was absent in samples from only 3% of animals.
In addition, the stage of scrapie incubation at the time of samples influences the levels of prion present within the oral cavity (17). Samples taken in the earlier stages of disease incubation may contain less prion than samples taken later, for example from the mid-point of disease incubation onwards (17).

Overall, almost all samples (13 of 15) were taken from the ‘low LRS’ group before the likely mid-point of disease incubation, whereas for the ‘high-LRS’ group most samples (16 of 27) were taken much later in disease incubation. However, what the data does establish is that PrPSc is present within the oral cavity in preclinical scrapie infected sheep with a range of PRNP genotypes, including those associated with limited LRS involvement in pathogenesis. This is in agreement with data showing the secretion of prion in the milk of sheep with these PRNP genotypes (16).

The observed presence of sPMCA amplifiable prion in the ARR/ARR sentinel control swabs may suggest that low level prion replication might occur in these highly scrapie resistant animals. There have been two published observations of ARR/ARR sheep with classical scrapie (4), such cases are extremely rare and this has never been observed in the studied flock. There is the possibility however that low level replication of prions within ARR/ARR animals could occur which never develops into clinical scrapie during the lifetime of these animals. Another explanation could be that the presence of prion in the oral cavity of these animals is due to uptake of the prion from the contaminated environment (17).

Interestingly, immunohistochemical or ELISA analysis of CNS and LRS tissues have shown that the incidence of scrapie in the AHVLA Ripley flock is low in ARR/VRQ and AHQ/VRQ animals: just 12% for the ARR/VRQ genotype and just 4% for the AHQ/VRQ genotype (unpublished observations). Here, we demonstrate far higher frequencies of PrPSc detection (52% of sheep of these genotypes) suggesting that there is low level replication of prion in sheep that do not develop clinical disease or accumulate measurable PrPSc by conventional testing. The very high sensitivity of sPMCA may well factor in the increased frequency of observation of positive animals; these data indicate that subclinical infections may be common in scrapie-exposed animals even for ARR and AHQ PRNP heterozygotes.
Preliminary data from a small number of transgenic mice inoculated with buccal swab extract demonstrates that the sPMCA positive material represents infectivity in the oral cavity. One animal in the bioassay exhibited clinical symptoms and was clearly Western blot positive for PrPSc in spleen tissue at 176 days, this animal was also sPMCA positive in both brain and spleen samples (figure 3). The sPMCA amplification was applied to brain material from all bioassay mice, 1 additional mouse of 5 tested was also positive at 310 days post challenge. These data imply that amplification from the brain tissue in two of these mice is the result of prion replication within the CNS. The presence of amplifiable prion within the spleen of 3 of the bioassay mice is further evidence for the propagation of scrapie in this bioassay. The analogous use of sPMCA to extend the sensitivity of prion bioassays has been described before in the context of CWD prions in white tailed deer (6). This methodology appears to detect very early signs of infection and is pertinent when studying very low amounts of infectivity via mouse bioassay; a scenario where incubation times to clinical disease is longer than the time limits of the bioassay.

Scrapie and CWD are examples of TSEs where it is known that environmental reservoirs of infectivity are involved in the horizontal transmission of disease. The contamination of the environment by infectious prion material from all sources represents a considerable problem for the effective management of these diseases. A number of publications now highlight the potential for CWD infectivity to be transmitted via saliva (5, 6, 18). Georgesson et al (2) document the survival of scrapie infectivity on a farm for a period of at least 16 years, and whilst this contamination will be from a variety of sources such as urine (3), faeces (24) and parturient material (20), it is likely that infectivity from the oral cavity is likely to contaminate items such as feed and water troughs, items of penning as well as potentially directly transmitting disease between sheep. A fuller understanding of all the routes of transmission of scrapie and CWD will greatly assist in the management of these diseases.
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Figure 1: Prion secretion into the oral cavity in sheep with a range of PRNP genotypes. Prion extracted from buccal swabs was used as seed for 9 rounds of sPMCA. Each sample was analysed in triplicate. Products were digested with PK and 10 μl applied to Western blots. PrP was detected with monoclonal antibodies SHA31 and P4; molecular mass markers (kDa) are shown. PRNP genotype, scrapie-exposure status and sheep ID are indicated, data are representative of sPMCA products produced throughout the study.

Figure 2: Comparison of prion secretion into the oral cavity between sheep of different PRNP genotypes. Reaction data were plotted as percentage positive reactions for each distinct PRNP genotype or for groups of genotypes associated with low (AHQ/VRQ and ARR/VRQ) or high LRS (VRQ/VRQ and ARQ/VRQ) accumulation of PrPSc. When comparing the percentage of positive sPMCA reactions for different cohorts of animals, data were set up as 2x2 contingency tables and Fisher's exact test (one-tailed) was applied to derive p-values. Each cohort was either compared to the VRQ/VRQ non-scrapie-exposed animals (*), or the ARR/ARR sentinel animals (#).

Figure 3 Panel A: Western blots of sPMCA products from murine bioassay brain and spleen tissue. Triplicate sPMCA analyses for brain and spleen samples from a scrapie challenged mouse displaying clinical symptoms and an unchallenged mouse are indicated as positive and negative respectively. Mouse 138615 was challenged with buccal swab extract and displayed clinical symptoms, was IHC negative, however this clearly shows sPMCA positivity in brain and spleen tissue. Panel B shows Western blot analysis of PK digested brain and spleen tissue from Tg338 challenged mice. + NaPTA corresponds to a NaPTA concentrated sample (27) (100 μL of 10% homogenate per lane), - NaPTA
corresponds to 7.5 µL of 10% digested homogenate analysed directly on the gel. Lane 1- clinical mouse 138615 brain sample, 2- clinical mouse 138615 spleen sample, 3- scrapie challenged positive control Tg338 clinical mouse brain, 4- scrapie challenged positive control Tg338 clinical mouse spleen.
Table 1: Presence of prion in buccal swabs, length of exposure of sheep to a scrapie-positive farm environment and sample collection times.

<table>
<thead>
<tr>
<th>Sheep ID (genotype)</th>
<th>Age at sample date (months)</th>
<th>Age at post mortem (months)</th>
<th>Post mortem scrapie status</th>
<th>Age if alive (months)</th>
<th>sPMCA positive/total reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>08-1215 (AHQ/VRQ)</td>
<td>34</td>
<td>N/A¹</td>
<td>N/A</td>
<td>41</td>
<td>2/3</td>
</tr>
<tr>
<td>08-1229 (AHQ/VRQ)</td>
<td>34</td>
<td>N/A</td>
<td>N/A</td>
<td>41</td>
<td>1/3</td>
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<tr>
<td>08-1230 (AHQ/VRQ)</td>
<td>34</td>
<td>N/A</td>
<td>N/A</td>
<td>41</td>
<td>1/3</td>
</tr>
<tr>
<td>06-1518 (ARQ/VRQ)</td>
<td>58</td>
<td>N/A</td>
<td>N/A</td>
<td>65</td>
<td>1/3</td>
</tr>
<tr>
<td>08-1206 (ARQ/VRQ)</td>
<td>34</td>
<td>39</td>
<td>Positive</td>
<td>N/A</td>
<td>1/3</td>
</tr>
<tr>
<td>08-1224 (ARQ/VRQ)</td>
<td>34</td>
<td>34</td>
<td>Positive</td>
<td>N/A</td>
<td>3/3</td>
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<tr>
<td>08-1257 (ARQ/VRQ)</td>
<td>34</td>
<td>37</td>
<td>Positive</td>
<td>N/A</td>
<td>3/3</td>
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<tr>
<td>08-1265 (ARQ/VRQ)</td>
<td>34</td>
<td>34</td>
<td>Positive</td>
<td>N/A</td>
<td>3/3</td>
</tr>
<tr>
<td>08-1266 (ARQ/VRQ)</td>
<td>34</td>
<td>34</td>
<td>Positive</td>
<td>N/A</td>
<td>3/3</td>
</tr>
<tr>
<td>08-1374 (ARQ/VRQ)</td>
<td>32</td>
<td>N/A</td>
<td>N/A</td>
<td>38</td>
<td>3/3</td>
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<tr>
<td>06-1500 (ARR/VRQ)</td>
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<td>N/A</td>
<td>N/A</td>
<td>65</td>
<td>0/3</td>
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<td>08-1240 (ARR/VRQ)</td>
<td>34</td>
<td>N/A</td>
<td>N/A</td>
<td>41</td>
<td>3/3</td>
</tr>
<tr>
<td>09-1229 (ARR/VRQ)</td>
<td>22</td>
<td>N/A</td>
<td>N/A</td>
<td>29</td>
<td>1/3</td>
</tr>
<tr>
<td>09-1209 (ARR/VRQ)</td>
<td>22</td>
<td>N/A</td>
<td>N/A</td>
<td>29</td>
<td>2/3</td>
</tr>
<tr>
<td>09-1189 (ARR/VRQ)</td>
<td>22</td>
<td>N/A</td>
<td>N/A</td>
<td>29</td>
<td>0/3</td>
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<tr>
<td>09-1184 (ARR/VRQ)</td>
<td>22</td>
<td>N/A</td>
<td>N/A</td>
<td>29</td>
<td>2/3</td>
</tr>
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<td>09-1337 (ARR/VRQ)</td>
<td>22</td>
<td>N/A</td>
<td>N/A</td>
<td>29</td>
<td>2/3</td>
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<td>09-1211 (ARR/VRQ)</td>
<td>22</td>
<td>N/A</td>
<td>N/A</td>
<td>29</td>
<td>3/3</td>
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<td>09-1218 (ARR/VRQ)</td>
<td>22</td>
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<td>N/A</td>
<td>29</td>
<td>3/3</td>
</tr>
<tr>
<td>09-1300 (ARR/VRQ)</td>
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<td>N/A</td>
<td>N/A</td>
<td>29</td>
<td>0/3</td>
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<td>09-1301 (ARR/VRQ)</td>
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<td>N/A</td>
<td>N/A</td>
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<td>0/3</td>
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<td>22</td>
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<td>N/A</td>
<td>29</td>
<td>3/3</td>
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<tr>
<td>Positive controls, n=20 (VRQ/VRQ)</td>
<td>9 to 22</td>
<td>12-26</td>
<td>Positive¹</td>
<td>29-30</td>
<td>47/60</td>
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<td>Sentinel controls, n=9 (ARR/ARR)</td>
<td>Mean 19 months exposure²</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>5/27</td>
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<tr>
<td>Non-exposed controls, n=9 (VRQ/VRQ)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>0/54</td>
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<tr>
<td>Non-exposed controls, n=9 (ARR/ARR)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>0/27</td>
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<tr>
<td>Non-exposed controls, n=9 (ARQ/ARQ)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>0/54</td>
</tr>
</tbody>
</table>

¹Post mortem analysis of scrapie status: immunohistochemical analysis of obex, cerebellum, ileum and/or retropharyngeal lymph node or and BioRad ELISA analysis of obex tissue.
²All samples were analysed in triplicate, prions were amplified for 9 rounds of sPMCA.
³N/A, non-applicable
⁴13 animals were culled and were positive at post mortem
⁵Animals retained within flock for 9-27 months, mean exposure time was 19 months
Table 2. Tg338 bioassay of mice challenged with buccal swab extracts from clinically normal, scrapie-exposed sheep with VRQ/VRQ PRNP genotype.

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>PM day</th>
<th>Brain Western</th>
<th>Spleen Western</th>
<th>Brain sPMCA</th>
<th>Spleen sPMCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>176</td>
<td>-</td>
<td>+</td>
<td>+ (3/3)</td>
<td>+ (3/3)</td>
</tr>
<tr>
<td>69</td>
<td>310</td>
<td>-</td>
<td>-</td>
<td>+ (1/3)</td>
<td>+ (1/3)</td>
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<tr>
<td>70</td>
<td>310</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>310</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ (1/3)</td>
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<tr>
<td>72</td>
<td>310</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

Western blots of tissue or sPMCA products were either positive (+) or negative (-). Number of positive reactions for sPMCA are recorded as number of positives in a triplicate analysis.
Figure 1: Prion secretion into the oral cavity in sheep with a range of PRNP genotypes. Prion extracted from buccal swabs was used as seed for 9 rounds of sPMCA. Each sample was analysed in triplicate. Products were digested with PK and 10 μl applied to Western blots. PrP was detected with monoclonal antibodies SHA31 and P4; molecular mass markers (kDa) are shown. PRNP genotype, scrapie-exposure status and sheep ID are indicated, data are representative of sPMCA products produced throughout the study.
Figure 2: Comparison of prion secretion into the oral cavity between sheep of different PRNP genotypes. Reaction data were plotted as percentage positive reactions for each distinct PRNP genotype or for groups of genotypes associated with low (AHQ/VRQ and ARR/VRQ) or high LRS (VRQ/VRQ and ARQ/VRQ) accumulation of PrPSc. When comparing the percentage of positive sPMCA reactions for different cohorts of animals, data were set up as 2x2 contingency tables and Fisher's exact test (one-tailed) was applied to derive p-values. Each cohort was either compared to the VRQ/VRQ non-scrapie-exposed animals (*), or the ARR/ARR sentinel animals (#).
Figure 3

Panel A: Western blots of sPMCA products from murine bioassay brain and spleen tissue. Triplicate sPMCA analyses for brain and spleen samples from a scrapie challenged mouse displaying clinical symptoms and an unchallenged mouse are indicated as positive and negative respectively. Mouse 138615 was challenged with buccal swab extract and displayed clinical symptoms, was IHC negative, however this clearly shows sPMCA positivity in brain and spleen tissue. Panel B shows Western blot analysis of PK digested brain and spleen tissue from Tg338 challenged mice. + NaPTA corresponds to a NaPTA concentrated sample (27) (100 µL of 10% homogenate per lane), - NaPTA corresponds to 7.5 µL of 10% digested homogenate analysed directly on the gel. Lane 1- clinical mouse 138615 brain sample, 2- clinical mouse 138615 spleen sample, 3- scrapie challenged positive control Tg338 clinical mouse brain, 4- scrapie challenged positive control Tg338 clinical mouse spleen.