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Running title: Prion detection in animal faeces

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Abstract

Faeces from infected animals have been suggested as a potential source of contamination and transmission of prion diseases in the environment. This work describes the development of a procedure for the detection of PrP^{res} in stools which is based on a detergent-based extraction and immunoprecipitation (IP). The procedure was evaluated by analyzing TSE-spiked sheep and mice faeces, and proved to be specific for PrP^{res} with sensitivities of 5-10 µg of infected brain tissue. In order to analyze the shedding of prions, we studied stools from orally-inoculated mice over 4-days post-inoculation and also stools from terminally-sick scrapie-infected mice. PrP^{res} was only

detected in stools shortly after the oral ingestion of TSE agents. The procedure described could be a useful tool for studying the excretion of prions and for evaluating potential environmental contamination by prions.

1. Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are characterized by the conversion of the normal cellular prion protein (PrP^{c}) to a partially protease-resistant prion isoform (PrP^{Sc}) which accumulates mainly in brain tissues forming readily detectable protein aggregates. Treating PrP^{Sc} with proteinase K (PK) removes its N-terminal part and the remaining C-terminal fragment is called PrP^{res}. PrP^{res} is considered the only direct molecular marker for TSE (Caughey et al., 1997; Prusiner et al., 1998). The tissue distribution of prions in affected individuals depends considerably on the type, dose and origin of the prion strain, as well as the genetic background of the host and the inoculation route. After oral administration, PrP^{res} is initially found in Peyer's patches and in gut-associated lymphoid tissues (GALT), especially in the distal ileum. Then, before reaching the brain tissue, the spleen and other lymph nodes are invaded. This pattern of prion deposition is valid for sheep scrapie (Andréoletti et al., 2000) and CWD (Sigurdsson et al., 1999), but not for BSE in cattle where PrP^{res} accumulation is almost entirely limited to CNS (Wells et al., 1998).

Much evidence suggests the existence of an environmental reservoir of infectivity that contributes to scrapie epizootics (Hourrigan, 1979; Pálsson, 1979) and to Chronic Wasting disease (CWD) epizootics (Miller et al., 2004). Scrapie-infected placenta could play as a source of contamination (Andréoletti et al., 2002). However, other sources such as urine and faeces must not be discarded. In a study of scrapie and BSE propagation by the oral route, Maignen et al. (1999) reported the presence of PrP^{res} from the stomach to the colon in the scrapie model only (including the ileum after removal of

the Peyer's patches). Deposition of extraneural PrP^{res} also occurs during CWD incubation in a manner that closely resembles the case of scrapie (Williams, 2005). This prion distribution -particularly in GALT- suggests that shedding of infectivity to the environment contributes to the horizontal transmission reported in natural conditions. Excretion of prions in urine has been demonstrated in mice (Seeger et al., 2005; Kariv-Inbal et al., 2006). Recently, application of the protein misfolding cyclic amplification (PMCA) technique to urine from scrapie-infected hamsters showed a high excretion (86%) of urinary PrP^{Sc} (Murayama et al., 2007). However, studies of prion excretion in faeces have been hampered so far by problems of insolubility and the volume of material to be tested (Maignen et al., 1999; Miller et al., 2004). Here we describe a specific methodology for the detection of PrP^{res} in mouse and sheep stool samples, and the subsequent analysis of different prion-infected animal faeces.

2. Material and methods

2.1. TSE-infected tissues and animal stool samples for methodological assays

For the development of a method for PrP^{res} detection in stool samples, scrapie-infected and BSE-infected, as well as non-affected, brain tissues were homogenized (20%, w/v) in 5% glucose using a TeSeE Precess (Bio-Rad). Also, a mouse brain infected by the scrapie Dawson strain (Stack et al., 1991) ($10^{7.5}$ intracerebral lethal dose 50 [$icLD_{50}$] g^{-1}) was used to study the sensitivity of the test. For the methodological assays, fecal samples (0.125 g) from healthy ewes and mice were typically spiked with 30 µl of 20% (w/v) brain homogenate and kept for 15 min at room temperature (RT) to allow the prions to adsorb to the matrix.

2.2. Extraction of PrP^{res} from stool samples

Different detergents as well as PK concentrations were assayed to extract PrP^{res} spiked into stool samples. Faecal material was 10% eluted with phosphate buffered saline

(PBS, pH 7.4) supplemented either with sodium dodecyl sulphate (SDS) or sarkosyl (SK) or a combination of both, in concentrations ranging 0.25% to 0.5% for SDS and 1% to 2% for SK. Solutions were complemented with proteinase K (concentrations ranging 0.1 to 0.4 mg ml^{-1}) and incubated for 10 min at 37ºC. Samples were homogenized, filtered through a 25-gauge needle and stored at -80°C.

2.3. Immunoprecipitation of PrP^{res} extracted from stool samples

For fecal samples, the IP protocol for brain homogenates developed by Morel et al. (2004) was modified. Briefly, PrP^{Sc} from brain tissue was immunoprecipitated using the mAb βS36 covalently coupled to magnetic beads. This mAb immunoprecipitates PrP^{Sc} but not PrP^{c} in human (CJD) and sheep (scrapie) brain homogenates (Féraudet et al., 2005). Magnetic beads were prepared as previously described (Morel et al. 2004).

For stool samples, 150 µl of sample concentrate was first diluted 10-fold in PBS pH 7.4 supplemented with 3mM Pefabloc (Roche). Then, 10 µl of magnetic beads coupled to the mAb βS36 (1 µl of beads for each 15 µl of sample) was added and the samples were incubated for 2 h at RT with gentle rotation. After 3 washes with PBS supplemented with Tween (1%), the beads were heated to 100ºC for 5 min with 50 µl of Laemmli buffer (LB) without reducing agents -for western blot (WB) analysis- or with 25 µl of C-reagent (from the Bio-Rad TeSeE-Purification kit) -for immunometric assays. However, in order to enhance the recovery efficiency, sample concentrates were also treated with increasing concentrations of microbeads (from 10 µl to 70 µl).

2.3. Sensitivity of the method developed

Once the most efficient conditions were selected (detailed in Fig. 1), the sensitivity of the test was determined for mouse and sheep stool samples spiked with 2-fold serial dilutions of 20% Dawson scrapie brain homogenate, so that amounts of brain homogenate corresponding to a range from 5 mg (25 µl) to 10 µg (0.05 µl) were added.

For BSE, sheep stool samples were also spiked with 2-fold serial dilutions of 20% BSE-infected non-titrated brain homogenate. All the samples were manipulated as described (Fig. 1), except for using 50 µl of beads (1 µl of beads for each 3 µl of sample) and finally denatured with 50 µl of Laemmli buffer for WB analysis.

2.4. Analysis of TSE-infected transgenic mice

BoPrP-Tg110 mice (Castilla et al., 2003) were intragastrically exposed to 200 µl (10%) of either BSE (n=3) or scrapie Langlade strain (n=3) infected brain homogenates. Intragastric inoculations were performed using a syringe connected to a silicon tube of 1-mm in diameter. Langlade strain is a naturally occurring scrapie case transmitted in natural controlled conditions through a sheep flock (Touzeau et al., 2006). Animals inoculated with the same agent were grouped in the same cage and faeces were collected from the cage at 24, 48, 72, and 96 h post-inoculation (hpi) and pooled. At 96 hpi, animals were culled and their intestinal tract tissues (duodenum, jejunum and ileum) were collected and stored at -80ºC. Stools from non-inoculated mice (n=3) and PrP-knock out mice (n=3) were also collected. Similarly, stools and intestinal tissues were collected from Tga20 transgenic mice (n=4) (Fischer et al., 1996) that had been intracerebrally inoculated with 20 µl of 10% brain homogenate of scrapie 22L strain (Kim et al., 1990). Mice were observed daily and their neurological status was assessed weekly as previously described (Castilla et al., 2003).

Faecal samples (0.2 g) were homogenized (10%, w/v) and 0.5 ml of the sample concentrate was immunoprecipitated as described after the addition of 1 volume of magnetic beads (1 µl of beads for each µl of sample). Immunoprecipitated proteins were finally eluted in 100 µl of LB without reducing agents and 25 µl was analyzed by WB. Intestinal tissues (60 mg ± 25.73) were eluted in 500 µl of 5% glucose. Scrapie-

associated fibrils (SAFs) from the tissue were prepared using the TeSeE-Purification kit (Bio-Rad) following the manufacturer's instructions.

2.5. Two-site immunometric assay

The immunometric assay was performed in 96-well maxisorb immunoplates coated with anti-PrP mAb 11C6. The mAb 11C6 epitope is undetermined, but it recognizes murine and ovine PrP (Féraudet et al., 2005). Samples were analyzed as previously described (Féraudet et al., 2005).

2.6. Western blot analysis

Typically, 12.5 µl of sample was loaded onto sodium dodecyl sulphate polyacrilamide gels (SDS-PAGE, 13.5%) and proteins were fractionated for 1 h at 200 V. The gel was blotted onto PVDF-membranes (Millipore) at 100 V for 1 h. Membranes were probed with the mAb SHa31 coupled to horseradish peroxidase, at a 1:2000 dilution. This mAb recognizes a linear epitope sequence (GNDYEDRY) located between residues 145 and 152 (GenBank accession number NP898902) (Morel et al., 2004). Immunoreactive bands were visualized using the ECL-Plus developer kit (GE Healthcare).

3. Results

3.1. Extraction and immunoprecipitation of PrP^{res} in animal stools

Buffers not supplemented with detergents failed to extract PrP^{res} previously spiked into animal stool samples in this study. We readily extracted PrP^{res} using either SDS or SK. However, no significant differences were observed using different concentrations. Significantly, stool samples spiked with unaffected brain homogenates and not treated with PK did not produce any signal, indicating that PrP^{c} is not detectable after 15 min in the faecal matrix. The high proteolytic activity presumably present in the samples was confirmed by the disappearance of the N-terminal domain of PrP^{Sc} molecules without the addition of PK. To analyze larger volumes of sample, βS36-mediated IP was

adapted to detect PrP^{res} extracted from sheep and mouse stools. Considering that variable concentrations of detergents showed no differences in extraction efficiency, those favouring PrP^{res} detection by βS36 were selected (Fig. 1).

For brain homogenates, the βS36-mediated IP proved to be more efficient (over 25%) than the SAF purification protocol (TeSeE Purification kit). For stool samples, we estimated the efficiencies of the method by the immunometric assay as 70% ($6.6\ 10^8$ beads ml^{-1}) for sheep and approximately 40% ($9.3\ 10^8$ beads ml^{-1}) for mouse stools.

3.2. Sensitivity of the method

The limit of detection was established in 18.75 µg of infected tissue for both scrapie- and BSE-infected homogenates in the sheep faecal matrix. In the mouse faecal matrix, the sensitivity was of 9.375 µg of Dawson infected tissue. Thresholds can be reduced by half by analyzing 25 µl in the blots (see Methods), and detection limits would thus be equivalent to 9.375 µg of Dawson tissue and 4.69 µg of BSE tissue for each 7.5 mg of matter tested. According to the infectious titre reported for the Dawson strain, these limits were estimated to be equivalent to $10^{2.47}$ mouse $icLD_{50}$ in sheep stool samples and $10^{2.17}$ mouse $icLD_{50}$ in mouse stool samples.

3.3 PrP^{res} excretion in stool samples

Excretion after oral inoculation of scrapie or Langlade scrapie was analyzed. In the case of scrapie, stool samples collected 24 hpi were shown to be positive, but those collected at 48 h, 72 h, or 96 hpi were not (Fig. 2). For BSE, PrP^{res} was detected in stool samples collected 24 and, to a lesser extent, 48 hpi. The prions identified consistently presented all three glycoforms without any sign of additional proteolytic modifications caused by the passage through the gastrointestinal tract. Additionally, intestines from each inoculated mouse did not show PrP^{res} accumulation.

In addition, the PrP^{res} excretion in terminally-sick scrapie-incubating mice was studied. As expected, PrP^{res} was detected in enteric tissues from all the inoculated animals with considerable variability. The presence of PrP^{res} in the intestinal tract raised the possibility of correlative PrP^{res} shedding in the stool samples of these mice. However, no prions were detected using the developed method (Fig. 2).

4. Discussion

In this study, a method for detection of PrP^{res} in mice and sheep stool samples has been developed. The specificity of the test was specifically considered taking into account the presumed abundance of proteins in this kind of sample. Three criteria were therefore set: resistance to PK, IP by mAb βS36 and, particularly, immunoreactivity with mAb SHa31 in WB, which was directly labelled to avoid the need for a secondary antibody giving cross-reactivity with mouse immunoglobulins. It also needs to be mentioned that the volumes used in the protocol may be a limiting factor for the investigations of faeces of bigger ruminants. Though the IP provides an effective concentration step and higher amounts of starting material could be used, it is still unclear if the method can be applied for the evaluation of PrP^{res} excretion in big ruminant animals.

We studied stools collected at moments where prion excretion was most plausible: after the ingestion of contaminated foodstuff and during the terminal stage of the disease when PrP^{Sc} is more disseminated. Shedding of PrP^{res} was observed 24-48 hours after the ingestion of both BSE and scrapie inocula. It is almost impossible to determine the doses of prion agents ingested by an animal in natural conditions. In cases such as BSE, very large doses of BSE-infected brain (100 g) have been used in pathogenesis studies, leading to a range of incubation periods shorter than those estimated to have occurred during the epidemic in Britain (Wells et al., 1998). The shedding of prions in these conditions does not seem unreasonable. However, a recent study indicates that the

single dose corresponding to the range of incubation periods actually observed in the field should be between 0.1 and 1 g (Wells et al., 2007) and it is not clear that such small doses can lead to any shedding of prions to the environment through faeces.

It has widely been suggested that environmental transmission explains apparently individual-to-individual contagion in some TSE such as scrapie and CWD. Contamination by carcasses or stools is considered the most probable explanation. The high dissemination in the peripheral tissues observed during the pathogenesis of scrapie and CWD strengthen this hypothesis. A recent time course experiment has demonstrated a high frequency of excretion (6 out of 7 animals) of urinary PrP^{Sc} during the terminal stage of the disease (Murayama et al., 2007). Complications (kidney dysfunction, bacterial urinary tract infection) at terminal stage would lead to lymphocyturia (leukocytes, renal epithelial cells). With respect to stools, potential excretion in faeces does not appear to involve the immune system and blood cells, rather gut epithelial cells. Thus, the relation between PrP^{res} in faeces and PrP^{res} in the gut needs to be specifically addressed. Data present in this study do not support a correlative shedding of PrP^{res} in stools from individuals showing PrP^{res} deposition along the intestinal tract. However, it is worth noting that some circumstances (abrasions, gastrointestinal disorders) could also play a determinant role for the excretion of prions to the environment and this reservoir may influence the natural transmission of TSE.

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Figure Legends

Fig. 1. Protocol of the developed method for PrP^{res} detection in stools. Volumes and steps are detailed as used for the analysis of faeces from mice orally inoculated with TSE. Reagent concentrations refer to final concentration in the sample. For abbreviations, see section Material and Methods.

Fig. 2. Analysis of stool samples from experimental mice: A, mice orally infected with BSE; B, mice orally infected with scrapie Langlade strain; C, faeces from mice at terminal stage of disease. Faeces from healthy BoTg mice (lanes BoTg) and Tga20 mice (lane Tga20), as well as PrP-knockout mice (lane $Prnp^{-/-}$), were also processed. Untreated non-affected brain homogenate (N) and gut-passaged Langlade inoculum (lane Lan) are also shown.

Fig. 1.

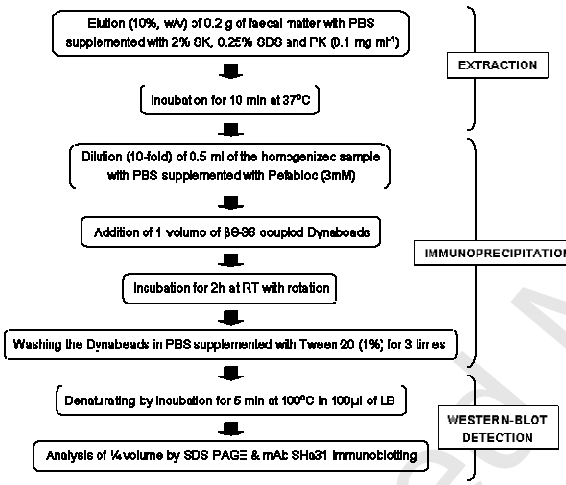

Fig. 2.