

## ANALYSIS OF FAECAL SAMPLES FROM SCRAPIE-INFECTED SHEEP AND BSE-INFECTED CATTLE FOR PrP<sup>RES</sup>

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### SUMMARY

Faecal samples from sheep and cattle, artificially infected with Scrapie or BSE, were examined for the presence of prion protein (PrP<sup>RES</sup>).

Sheep faecal samples, collected between day 1 and 5, and 37 days and 157 days post infection, and cattle faecal samples collected after 4, 7 and 8 months post infection were examined. A method was developed including several steps of chemical and physical enrichment and Western Blotting for the sensitive detection of prion proteins in faeces.

PrP<sup>RES</sup> was detectable in artificially spiked sheep and cattle faecal samples but not in any of the examined faeces samples of the infection experiments.

**Keywords:** TSE-agents, prion protein, faeces, enrichment, infection route, risk assessment

### ORIGINAL ASPECTS OF THE RESEARCH

So far there have been no findings on the possible excretion of TSE-agents in the faeces of TSE-infected animals nor has it been shown that faeces from these animals are contagious. These results have to be questioned due to the fact that only small quantities of faecal material had been analyzed.

In the present study faecal samples from sheep and cattle, artificially infected with Scrapie or BSE, were examined for the presence of prion protein (PrP<sup>RES</sup>).

The aim of the study was furthermore to identify possible infection routes of TSE-agents and to minimize or completely eliminate the risk of infectivity for the environment.

### METHODS

A total of 36 sheep and 6 cattle faecal samples were tested in two independent trials.

The collected faecal samples originated from infection experiments of three groups of sheep, which were orally infected with 4 g of homogenized brain material from Scrapie-infected sheep and two groups of cattle orally infected with BSE which had been fed brain material from BSE-positive cows. Sheep faecal samples collected between day 1 and 5, and 37 days and 157 days post infection, and cattle faecal samples collected 4, 7 and 8 months post infection were examined.

Ten grams of the faecal samples were dissolved in 30 ml Phosphate buffered saline. Three serial dilution steps were prepared. For isolation and enrichment of low amounts of prion protein in these faecal samples and the dilution steps a reisolation method was developed including several steps of chemical and physical enrichment.

To degrade rough particles in the faecal samples an acid hydrolysis using formic acid was performed. Dehydol 980, a detergent, was then added, and after an incubation time of 10 min at room temperature the samples were centrifuged for 5 min at 1,000 x g. The supernatant was transferred to another tube and L-Sarcosin was added to both, supernatant and pellet. Following a 30 min incubation time at room temperature the samples were centrifuged for 4 min at 2,000 x g. The pellets were discarded and the supernatants pooled. A third centrifugation for 10 min at 2,000 x g was carried out following 40 min incubation at 3°C with bovine serum albumin and ice-cold ethanol.

The supernatant was discarded and the remaining pellet was resuspended in Tris/HCl buffer with L-Sarcosin.

Following a proteinase K digestion (5 µg proteinase K in 30 ml for 45 min at 37°C) to unmask the epitope the enriched samples were tested for PrP<sup>res</sup> using Western Blot and immunodetection techniques.

For the separation of the precipitated proteins SDS-polyacrylamide gels (PAGE) were used. The concentration of the resolving gel was 15.0%.

9.0 µl of reducing loading buffer (RotiLoad) was added to the samples and heated for 5 min at 95°C. After the following centrifugation step for 2 min at 14,000 x g to remove undissolved proteins, the supernatant was separated using SDS-PAGE for 60 min at 150 V (electrophoresis buffer: 1.0 g SDS, 6.0 g Tris, 28.8 g Glycine in 1 l distilled water).

Scrapie and BSE specimens from the FLI (Friedrich Löffler Institut, Tübingen) served as a positive control. The molecular weight marker was purchased from Santa Cruz (Cruz Marker™ Molecular Weight Standards, sc-2035) and the protein marker from Peqlab (peqGold Protein-Marker IV prestained).

After electrophoresis the gel was removed and following a washing step in TBST (Tris-Buffered Saline Tween-20, 7.88 g Tris-HCl, 8.76 g NaCl, 1 ml Tween®20 in 1 l distilled water) for 10 min at 30 rpm (Rocking Shaker) incubated in transfer buffer (100.0 ml methanol, 2.9 g Tris, 14.6 g Glycine in 1 l distilled water) at room temperature.

The blotting paper and the PVDF (Immobilon-P-Polyvinylidene Difluoride) membrane which was moistened with 100% methanol were equilibrated in transfer buffer. After blotting at 80 mA for 1 hour the PVDF membrane was washed three times for 5 min each with TBST and incubated with blocking solution (3.0 g skim milk in 100 ml TBST) for 1 hour at 4°C on a rocking shaker at 30 rpm. Then it was washed again with TBST three times for 5 min for each washing step.

For the detection of prion protein an enzyme immunoassay (EIA) was performed.

To detect scrapie agent in the sheep samples the first antibody (POM 1, mouse anti-prion protein, FLI Tübingen) was diluted in blocking solution at a ration of 1:2,000 and the membrane was incubated with 10 ml of the POM1 antibody solution overnight at 4°C. The PVDF membrane was washed three times for 5 min each with TBST, incubated for 1 hour at room temperature with the second antibody (goat anti-mouse IgG-HRP sc-2031) which was diluted in blocking solution at a ratio of 1:1,000 and washed three times with TBST, once for 5 min with TBS.

Western blotting Luminol reagent solution was used to visualize the PrP<sup>res</sup> and the western blot was documented by photography (Hyperfilm ECL, Amersham Biosciences, 5 min).

To detect BSE-agent in the cattle samples EIA was performed as described above but monoclonal antibody L42 (Friedrich-Löffler-Institut Tübingen) in a dilution 1:500 was used as the primary antibody.

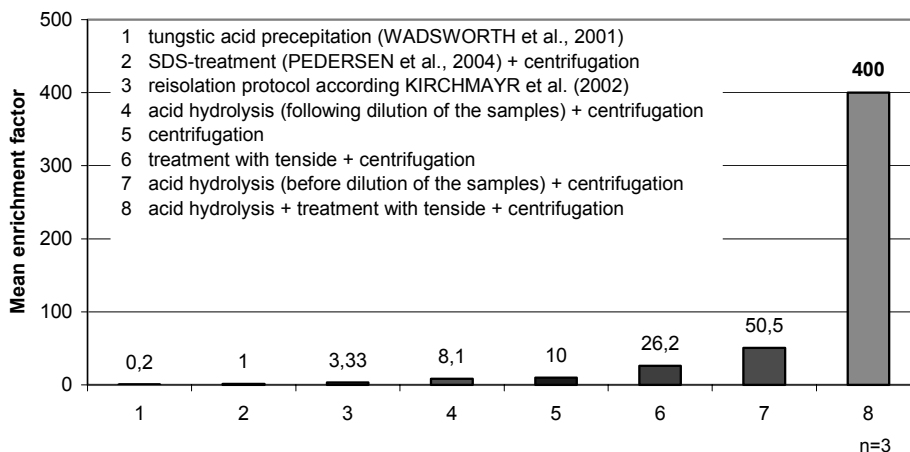
## RESULTS

PrP<sup>res</sup> was detectable in artificially spiked sheep and cattle faecal samples with a detection limit of 10<sup>4</sup> infectious units. There was no PrP<sup>res</sup> detectable in any of the examined faecal samples of the infection experiments (table 1) although the enrichment factor of the applied reisolation method is approx. 400 (figure 1).

**Table 1.** Detection of PrP<sup>res</sup> in faecal samples of orally infected sheep and cattle

	Sheep faecal samples: days post infection							Cattle faecal samples: months post infection		
	1	2	3	4	5	37	157	4	7	8
Detection PrP <sup>res</sup>	–	–	–	–	–	–	–	–	–	–

– : PrP<sup>res</sup> not detectable



**Figure 1.** Comparison of different reisolation protocols for the detection of PrP<sup>res</sup> in faecal samples

## CONCLUSIONS

The animals tested were all infected orally. We therefore expected a positive detection of PrP<sup>res</sup> in the first days after oral infection with a high infectious dose and even in a later phase of the infection.

The results indicate that if PrP<sup>res</sup> is excreted at all, only very low amounts (vestiges) of PrP<sup>res</sup> might be present in the faeces which are not detectable by the reisolation and detection method applied. It is possible that PrP<sup>res</sup> is adsorbed, resorbed, or degraded in the gastrointestinal tract, or it may be excreted at a later phase during the TSE pathogenesis. In further studies sheep faecal samples > 6 months and cattle faecal samples > 8 months post infection will be analyzed using the technique described and a bioassay will be performed in parallel to detect potential residual infectivity in faeces.