Ultra-sensitive detection of prion protein fibrils by flow cytometry in blood from cattle affected with bovine spongiform encephalopathy

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Abstract

Background: The definite diagnosis of prion diseases such as Creutzfeldt-Jakob disease (CJD) in humans or bovine spongiform encephalopathy (BSE) in cattle currently relies on the post mortem detection of the pathological form of the prion protein (PrPSc) in brain tissue. Infectivity studies indicate that PrPSc may also be present in body fluids, even at presymptomatic stages of the disease, albeit at concentrations well below the detection limits of currently available analytical methods.

Results: We developed a highly sensitive method for detecting prion protein aggregates that takes advantage of kinetic differences between seeded and unseeded polymerization of prion protein monomers. Detection of the aggregates was carried out by flow cytometry. In the presence of prion seeds, the association of labelled recombinant PrP monomers in plasma and serum proceeds much more efficiently than in the absence of seeds. In a diagnostic model system, synthetic PrP aggregates were detected down to a concentration of approximately 10⁻⁸ nM [0.24 fg/ml]. A specific signal was detected in six out of six available serum samples from BSE-positive cattle.

Conclusion: We have developed a method based on seed-dependent PrP fibril formation that shows promising results in differentiating a small number of BSE-positive serum samples from healthy controls. This method may provide the basis for an ante mortem diagnostic test for prion diseases.

Background

A group of fatal transmissible neurodegenerative diseases, including Creutzfeldt-Jakob disease (CJD), bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) and scrapie, is caused by an unusual infectious agent that has been termed prion [1]. Prions consist of an aberrant isoform (PrPSc) of the normal cellular prion protein (PrPc). PrPc is a cell surface glycoprotein expressed in neurons [2] and other cell types [3,4]. The precise physiological function of the cellular prion protein is not known yet. PrPSc differs from PrPc in its higher content of β-sheet structure [5,6], its partial resistance to protease digestion...
PrPSc propagates by converting the cellular prion protein to the PrPSc conformation [9]. PrPSc aggregates accumulate predominantly in the central nervous system (CNS), and definitive diagnosis of prion diseases currently relies on the post mortem detection of PrPSc in CNS tissue by immunohistochemistry, Western blotting, or ELISA [10]. Transmission studies indicate that prions may also be present in blood, potentially allowing for ante mortem diagnosis, but the sensitivity of the currently available analytical methods is insufficient for the detection of the extremely low prion titers that can be expected in body fluids [11].

Here, we report the development of a method based on kinetic differences between seeded and unseeded aggregation of prion protein that allows the detection of PrP aggregates in blood down to attomolar concentrations by flow cytometry.

**Results and discussion**

**Detection of synthetic prion protein aggregates in serum or plasma**

Kinetic differences between seeded and spontaneous polymerization of peptide monomers can be used for the detection of amyloid β-protein aggregates in the cerebrospinal fluid of Alzheimer's disease patients [15]. Here, we extend the principle of seeded polymerization to the detection of prion protein aggregates.

While trying to establish conditions for the labeling of synthetic prion protein aggregates with a fluorescently labeled prion protein probe, we observed that the formation of prion protein aggregates proceeds much less efficiently in serum or plasma (not shown) than in PBS (Fig. 1). This inhibition is probably caused by interactions of the prion protein probe with serum proteins.

Next, we found that the addition of preformed prion protein aggregates to plasma can partially overcome this inhibition (Fig. 2). The preformed aggregates presumably function as seeds that facilitate the formation of new aggregates in the inhibitory environment of plasma. The seeds stimulated the formation of prion protein aggregates at all concentrations tested, from 5 nM [120 ng/ml] to $10^{-8}$ nM [0.24 fg/ml] (Fig 2C). The average ratio of event counts in seeded samples to those in samples without seeds was 6.4. The number of events, however, was not proportional to the seed concentration, but remained relatively constant over the whole concentration range. Thus, the seed-dependent formation of prion protein aggregates can be used to detect extremely low amounts (down to the attomolar range) of spiked prion protein aggregates in blood.

![Figure 1](image)

**Figure 1**

Inhibition of PrP aggregation in serum. FITC-labeled recombinant bovine prion protein (concentration 10 nM) was incubated at 37°C for 20 h with continuous shaking, either in 150 µl PBS (left panel) or in the same volume of serum (right panel), followed by flow cytometry. The measurements are depicted in a Fluorescence 1 (FL1-H) vs. Fluorescence 2 (FL2-H) dot-plot. The number of counts in the area containing specific signals (R2) is given in the figures. Aggregate formation in serum is strongly inhibited.
Analysis of serum from clinical-stage, BSE-positive cattle

Studies demonstrating the transmission of prion diseases by blood transfusion suggest that prions are present in the blood of afflicted animals and people, even at pre-symptomatic stages of the disease [16-18]. We used the method of seed-dependent fibril formation to analyze serum from six confirmed cases of clinical-stage, BSE-positive cattle and four controls. Based on the spiking experiments described above, our hypothesis was that any PrPSc aggregates present in serum may act as seeds for the formation of easily detectable amounts of labeled PrP aggregates, whereas in the absence of seeds the formation of PrP aggregates would be inhibited. The serum samples from BSE-positive cattle and controls from healthy cattle were incubated with 10 nM of a FITC-labeled bovine PrP probe at 37°C for 20 h with continuous shaking, followed by analysis in a flow cytometer. All six BSE-samples could be clearly distinguished by a population of events that was absent in the controls (Fig. 3A–J, green dots in region R3; quantification in fig. 3K).

Conclusion

We have developed a method based on seed-dependent PrP fibril formation that shows promising results in differentiating a small number of BSE-positive serum samples from healthy controls. More samples need to be tested in order to validate its potential as an ante mortem diagnostic test for BSE and other prion diseases.

Methods

Biological fluids

Serum samples from six confirmed cases of BSE in cattle and four control animals were obtained from BFAV, Insel Riems, Germany. Control plasma was obtained from a blood bank.

Labeling of prion protein

Recombinant full-length bovine PrP was produced as described previously [12,13]. The purified protein was labeled with a FITC-labeling kit (Roche) according to the manufacturer’s instructions.

Preparation of fibrils from recombinant prion protein

25 µM of unlabeled bovine prion protein in PBS containing 0.2 % SDS was incubated for 10 min at room temperature, followed by a twentyfold dilution with PBS. For fibril formation, the diluted reaction mixture was incubated for 48 h at room temperature [14].

PrP fibril formation in serum or plasma

Recombinant FITC-labeled bovine prion protein was incubated in 150 µl serum or plasma at a concentration of 5 or 10 nM for 5–10 min. at 20°C, shaking at 550 rpm in an Eppendorf thermomixer, followed by an increase of the temperature to 37 °C h at constant shaking speed. The incubation was continued for 20 h. Samples were then analyzed by flow cytometry.

Flow cytometry

Analysis of the samples was carried out on a FACSVantage flow cytometer (BD Biosciences) at room temperature, measurement time was 30 sec per sample.

Authors’ contributions

LT participated in the design of the study, carried out the measurements and drafted the manuscript. ANS participated in the analysis of the data. EM prepared the recom-
**Figure 3**

**Analysis of serum from BSE-positive cattle.** FITC-labeled recombinant prion protein (10 nM) was incubated in 150 µl of the serum samples as described in the methods section and analyzed by flow cytometry. The measurements are shown in a Fluorescence 1 (FL1-H) vs. Side-Scatter (SSC) dot-plot. All six BSE-samples (A-F) can be differentiated from the controls (G-J) by a population of events in region R3 (green dots). Panel K: Quantification of measurements shown in panels A-J.
binant protein. KK and ST were also involved in protein expression and purification. HS participated in the design and coordination of the study. GB conceived of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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