Serum Nucleic Acids Directly Correlate to BSE Risk

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Abstract

Etiopathologic and immune responses to prions, the agents associated with the transmissible spongiform encephalopathy (TSE) diseases, such as scrapie in sheep and bovine spongiform encephalopathy (BSE) or mad cow disease remain unclear. Germ-line mutations in the prion coding region have been described in scrapie/sheep but yet to be identified in BSE/cows. Our analysis of microvesicular associated serum circulating nucleic acids (CNA) shows a distinct pattern of repetitive nucleic acids associated with prion-positive cattle and their associated feeding cohorts. Using a differential display PCR test of CNA, a study was conducted on confirmed prion positive cows (N=4), 15 at-risk feeding cohorts of confirmed prion positive cows (N=207) and randomly selected cattle from a local slaughterhouse (N=908). Gene amplification detected unique CNA profiles in all 15 confirmed prion positive associated cohorts with reactive patterns of at least 33% (mean= 65%) of the 207 total cohort animal sera tested. In contrast, only 0.55% of controls from normal herds and randomly selected cattle from slaughterhouse were reactive ($p<0.001$). The unique CNA profiles significantly associated with BSE at-risk cows support the approach of using a CNA PCR *ante mortem* test for identifying at-risk herds in the assistance of BSE eradication programs.

Methods

Cohorts and Controls.

The EU defines cohorts as all animals born or raised together with a BSE cow during ± 12 months of birthdate. Serum of cohort animals was withdrawn *ante mortem* at the time of culling. Table 1 shows the relative risk of cohort cattle to develop BSE. Sera of normal controls were drawn in a slaughterhouse. All cattle were subsequently shown to be negative in a prion rapid test.

TABLE 1

Official Data from BMVEL (2001 -2003)	Number of Cattle		
	PrP^{res} positive	PrP^{res} negative	% PrP^{res} positive
Slaughterhouse cattle	103	7.70×10^6	0.0013
Cohort cattle	8	5.58×10^3	0.14
ODDS ratio	-	-	107 (48.5 – 227)

Preanalytics. Blood samples were kept a 4 ºC until centrifugation for not more than 15 hours. For the Field Study this time was extended in two of the study groups.

Sample Preparation. Frozen samples were thawed at 4 °C in an ice-water bath and 250 µL were transferred into a 1.5 mL microcentrifuge tube. The tube was centrifuged at 20,000 x g for 30 min at 4 °C in a Model 5214 bench top centrifuge (Eppendorf, Hamburg, Germany). The supernatant was carefully removed and the pellet was used for further analyses.

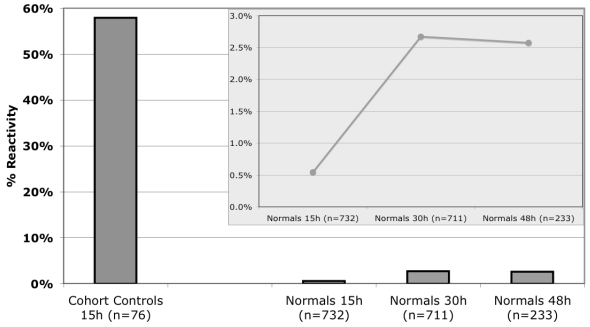
Nucleic acid extraction. 20,000 x g pellets were used with a standard silica based nucleic acid extraction (NucleoMag; Macherey & Nagel, Düren, Germany) according to the manufacturer's instructions. The resulting nucleic acid solutions were either used immediately or frozen at –80 °C until further use.

Diagnostic PCR. Three µL of the extracted CNA were used in a PCR in a total volume of 20 µL. Primers CHX-1F and CHX-1R (Chronix Biomedical GmbH, Göttingen, Germany), were used at 1 µM each using Advantage-2 PCR Kit (BD-Clontech, Heidelberg, Germany). After 30 cycles of 95 °C for 30 sec, 55 °C for 45 sec, 68 °C for 1 min, a SybrGreenI (Molecular Probes, Eugene, OR, USA) derived melting curve was recorded in a MX4000 PCR system (Stratagene, La Jolla, CA, USA). The area under the curve (AUC) of the derived melting function –d(F)/dT between 87 °C and 90 °C was used for analysis. Reactivity of each individual sample was calculated on the basis of an AUC above the detection limit, which is defined as mean + 5 standard deviations above baseline of non-template/normal controls.

Results (Figure 1)

- Serum should be separated within 15 hrs after collection (Fig. 1)
  - Whole blood should be kept at 4°C until centrifugation

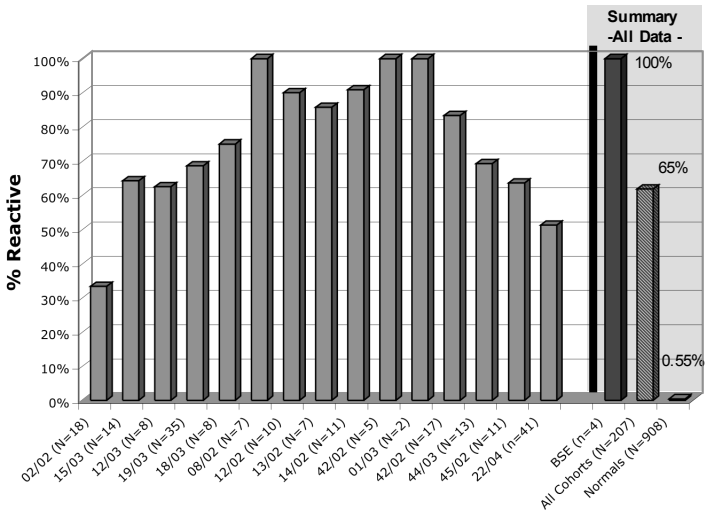
Figure 1: Field Study of GLT in Normal Cattle



Results (Figure 2):

- 15 of 15 Cohorts had GLT reactivities > 33% (mean: 65%)
- 4 of 4 PrP^{res} positive cattle were GLT reactive (100%)
- 5 of 908 normal control cattle showed GLT reactivity (0.55%)
  - $p < 0.001$ normals vs. cohorts

Figure 2: Serum Nucleic Acid in GLT: BSE - Cohorts - Normals



Summary and Conclusion

BSE is a progressive neurodegenerative disease in cattle. Current tests for BSE are performed only at necropsy (*post mortem*) and therefore limit efforts to eliminate BSE animals from living herds.

Epidemiologic efforts for controlling BSE will require robust *ante mortem* test procedures that identify and monitor at-risk individuals. To date, the confirmation test for animals with BSE is the *post mortem* direct detection of PrP^{res} in the brain. Because natural antibodies to PrP^{res} are rare, immunoassay tests for the detection of PrP^{res} antibodies in living animals (*ante mortem*) are not feasible. Another *ante mortem* epidemiologic approach is the detection of circulating nucleic acids (CNA). CNA are an effective laboratory means for monitoring events in chronic diseases (1-3). The Göttingen Living Test (GLT) for BSE-susceptibility was developed as a living test (*ante mortem*) for the detection of abnormal CNA profiles in cattle (4). The serum CNA sequences detected in the GLT are, in part, derived from what is referred to as the "repetitive" sequences that flank the majority of all genes including the prion gene. Nucleic acids are extracted from serum or plasma and amplified. The signal strength of the test sample is compared to healthy and BSE control samples. Samples that are more than five standard deviations (detection limit) above the normal healthy controls and non-template controls are considered reactive. In accordance with GLP procedures, reactive samples are rerun in duplicate to confirm that they are repeatedly reactive.

CNA profiles are an indication of a host's response to xenobiotic exposures. The data presented here show that BSE exposed cattle have abnormal serum CNA patterns. The high percentage of GLT reactivity (65%) of cohorts directly correlates with the significantly increased risk for developing BSE (Tab.1) (5).

Because BSE pathogenesis remains mysterious and there is a medically acknowledged risk of transmission to humans, GLT reactive samples should be handled as potentially infectious until further testing can confirm their handling status.

References

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