Antemortem Identification of Bovine Spongiform Encephalopathy from Serum Using Infrared Spectroscopy

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Since 1986, more than 180 000 clinical cases of bovine spongiform encephalopathy (BSE) have been observed in the U.K. alone. Most of these cases were confirmed by postmortem examination of brain tissue. However, BSE-related risk assessment and risk management would greatly benefit from antemortem testing on living animals. A serum-based test could allow for screening of the cattle population; thus, even a BSE eradication program would be conceivable. Here we report on a novel method for antemortem BSE testing, which combines infrared spectroscopy of serum samples with multivariate pattern recognition analysis. A classification algorithm was trained using infrared spectra of bovine sera from more than 800 animals (including BSE-positive, healthy controls and animals suffering from classical viral or bacterial infections). In two validation studies, sensitivities of 85 and 84% and specificities of 86 and 91% were achieved, respectively. The combination of classification algorithms increased the sensitivity and specificity of BSE detection to 96 and 92%, respectively.

Transmissible spongiform encephalopathies (TSEs) such as scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, or variant Creutzfeldt−Jakob disease (vCJD) in humans are a group of fatal neurodegenerative diseases caused by a family of unconventional pathogens. Conformational changes of the prion protein (PrP) play an important role in the pathogenesis of TSEs or "prion diseases" and are widely considered to be fundamentally involved in their aetiology.[1] Since zoonotic transmissions of TSEs may pose a considerable hazard to human health, immunological testing of brain stem homogenates from slaughtered cattle for the presence of the pathological prion protein PrP^{Sc} has been established as a routine procedure aiming at the identification of BSE-infected animals. However, the development of rapid and reliable diagnostic screening methods that permit the identification of TSEs at the clinical and preclinical stage remains a matter of utmost scientific, economic, and public health importance. Particularly, the screening of all living cattle would be valuable in assessing the prevalence and controlling the eradication of BSE in the cattle population. This would effectively contribute to the prevention of the spread of BSE to humans, thereby minimizing or even eliminating the risk of new primary vCJD infections. A noninvasive antemortem test for TSEs would possibly also allow another current challenge of public health to be addressed: the potential risk of a conceivable human-to-human transmission of vCJD via blood or blood products.

When living animals or humans are tested, blood or blood components such as plasma or serum are easily accessible and may therefore serve as an appropriate test material. However, despite considerable research efforts, none of the reported approaches[2−5] for the identification of TSE infection from blood or its components has been put into routine diagnostic practice.

To identify biochemical alterations in serum associated with the pathogenesis of BSE in cattle, we applied in the present study a combination of Fourier transform infrared (FT-IR) spectroscopy and advanced methods of pattern analysis. FT-IR spectroscopy is a structure-sensitive, reagent-free, and nondestructive technique, which has been increasingly applied to the characterization of complex biological matter such as microorganisms[6] eucaryotic cells,[7] biofluids,[8] cancer,[9] and brain tissue of TSE infected animals.[10] Infrared spectra of biological samples constitute highly characteristic, fingerprint-like signatures. These spectral patterns originate from the superposition of a plurality of various biomolecules in the samples. For a number of disease processes, it is found that the composition of the sample constituents is slightly, but characteristically altered. The disease-specific alterations can be revealed by FT-IR spectroscopy and are evaluated by advanced methods of data analysis. Recently, we have been able to apply

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this method to serum samples from experimentally infected scrapie hamsters.[11] In this feasibility study, the attained test sensitivity and specificity was 97 and 100%, respectively. Our experimental findings indicated the presence of distinct surrogate markers in the sera of scrapie-infected hamsters rather than the direct detection of the pathological prion protein. In this experimental hamster study, animals with a relatively homogeneous genetic background were fed the same food and were infected with the identical strain of scrapie agent (263K). Furthermore, all sera were taken by a limited number of persons exclusively at the precisely defined terminal stage of scrapie. These experimental conditions are in marked contrast to the important application of BSE testing of living cattle. Naturally, the situation in a field study is much more challenging since many parameters that may potentially influence the test results are difficult to control. In the present paper, we address the issue of whether the FT-IR method is capable of detecting BSE in field studies, i.e., under "*real life*" conditions in terms of stage of the BSE infection, gender, age, breed, sample acquisition, and alternative diseases.

EXPERIMENTAL SECTION

Samples. Serum samples from cattle were obtained from the Veterinary Laboratories Agency (VLA, Weybridge, U.K.), and from different sources in Germany. An overview of sample origins, sample numbers, and sample characterizations is given in Tables 1 and 2.

FT-IR Spectroscopy. A 2.6-μL aliquot of each serum sample was spread on a ZnSe window of a multisample cuvette and allowed to air-dry for 15 min at 37 °C. The cuvette was closed with a KBr window and then transferred to the spectrometer (IFS 28/B spectrometer, Bruker Optik GmbH). Spectra were recorded in the spectral range between 4000 and 500 cm^{-1}. Nominal physical resolution was 4 cm^{-1}. A zero filling factor of 4 was employed, yielding a point spacing in the spectra of 1 cm^{-1}. A total of 128 scans was averaged, and a Blackman−Harris three-term apodization function was applied before Fourier transformation. Each sample was measured in triplicate, i.e., at three different times. Further details are described in ref 11.

Data Pretreatment. Spectra were converted to second derivatives by applying a Savitzky−Golay algorithm with nine smoothing points. Subsequently, spectra were vector-normalized over the spectral range from 2820 to 2985 cm^{-1}. Finally, data reduction was performed by averaging three adjacent data points and by removing the spectral information in the spectral windows 500−700, 1500−1700, 1750−2800, and 3100−4000 cm^{-1}, respectively.

Feature Selection and Artifical Neural Network (ANN) Analysis. For ANN-based analysis, spectral feature selection was based on the calculation of the covariance of the spectral data points.[11] According to the covariance measure, the selected features were ranked in descending order and the best features were used as input for the ANN classifier. The selection of spectral features, network training, and ANN classification of the blinded sample spectra were carried out using Synthon's NeuroDeveloper software (Synthon KG, Heidelberg, Germany). Specifically, three-layer feed-forward networks with 80−140 input neurons, 4−20 hidden units, and 2 output units with the class assignment "BSE negative" or "BSE positive" were established. Resilient back-propagation (rprop) was used as the learning function.

Teaching Method. To teach the classification algorithms, the teaching data set was split into a subset for training and a subset for internal testing. Spectra of the three independent measurements on each sample were always grouped such that they appeared in only one of the respective subsets. Usually, a teaching subset contained spectra from 67% of the samples with known diagnosis and the testing subset 33%.

Feature Selection and Discriminant Analysis. When discriminant analysis was used, the feature selection was based on a stochastic search and optimization algorithm—a so-called genetic algorithm (GA). The advantage of this algorithm is the ability to work efficiently at search and optimization problems in large data sets. In the present study, we used the GA in combination with linear and quadratic discriminant analysis (LDA/QDA).[12] The advantage of LDA over QDA is the "robustness" of this method in relatively small data sets. QDA, on the other hand, delivers better classification results if the sample number is high. For LDA/QDA classification, the spectra were preprocessed as already described and divided into training and internal validation subsets. The GA was allowed to search the training data for the 20 most suitable spectral features. LDA/QDA classification of validation spectra was then carried out on these 20 features.

Decision Tree Used for Validation Study II ("Roche Study"). In this approach, we trained two different ANNs: The first one was specificity-optimized, i.e., trained to yield low numbers of false positive (FP) classifications within the teaching set. The second ANN was trained such that specificity and sensitivity were roughly *equal* (ANN_{EQ}). Furthermore, LDA and QDA were utilized in this approach. An individual animal of the validation set was assigned as BSE positive/negative if both ANNs made the same predictions. If not, the assignment was positive if the specificity-optimized ANN yielded a positive class assignment. The remaining animals were classified according to the LDA/QDA and ANN_{EQ} class assignment (in case of identical diagnoses) or as BSE negative (if the diagnoses by LDA/QDA differed from those made by ANN_{EQ}).

Unblinding Procedure. After establishing the classification results, a summary of the classifications, spectra, ANN parameters, and related documentation files were archived. The classification results were then sent to the Veterinary Laboratories Agency (VLA, Weybridge, U.K.) (validation study I) or Roche Diagnostics GmbH (validation study II), which in return made the original diagnoses available to the Robert Koch-Institut.

RESULTS AND DISCUSSION

The method of data analysis described in this paper exploits a data set of spectra for teaching a classification algorithm. When the teaching process is finished, the classifier is challenged by an independent validation data set. It is important to note that the validation data set is kept totally separate from the teaching procedure until the final validation has been performed.

A detailed description of the bovine serum samples employed for teaching the classification algorithm of validation study I ("VLA study") is given in Table 1. All BSE-positive serum samples were

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Table 1. Origins, Diagnoses, and Numbers of Serum Samples Used for Teaching

source of supply		diagnosis	no. of samples
Veterinary Laboratories Agency (VLA, Weybridge, U.K.)	1	confirmed BSE test positive	249
	2	clinical BSE suspect, but confirmed BSE test negative	34
	3	confirmed BSE negative	83
	4	clinically normal controls (animals not tested for BSE)	133
	5a	blind samples (VLA study) confirmed BSE positive[§]	48
	5b	blind samples (VLA study) confirmed BSE negative[§]	44
Federal Research Centre (FRC) for Virus Disease of Animals, Island of Riems, Germany	6	confirmed BSE negative[a]	45
	7	animals with specific viral infections, (animals not tested for BSE)[b]	22
Institute of Animal Nutrition (IAN), Federal Animal Research Centre, Braunschweig, Germany	8	control animals (animals not tested for BSE)[c]	100
Veterinary University (VU), Hannover, Germany	9	animals with nonspecific bacterial infectious diseases (animals not tested for BSE)[d]	35
Roche Diagnostics GmbH, Mannheim, Germany	10	confirmed BSE negative[e,f]	50

[a] Serum samples taken from slaughter cattle. [b] Sera from 6 animals infected with bovine leukemia virus (BLV), 5-weeks postinfection (p.i.); sera from 5 animals challenged with the bovine respiratory syncytial virus (BRSV); 6 sera from animals of a bovine virus diarrhoe study (BVD; −1, 14 and 28 days p.i.); sera from 5 animals infected with bovine herpes virus 1 (7 days p.i.). [c] Herd of cattle living under controlled conditions at Institute of Animal Nutrition, Federal Animal Research Centre (Braunschweig, Germany). [d] Serum samples from the Veterinary University Hannover ("*cow clinic*"), microbiology: bacterial infections not further specified, mostly from *E. coli*, Streptococci, Staphylococci, and Proteus. [e] These samples have only been included into the teaching set of the Roche study. [f] Serum samples from an abattoir in upper Bavaria.

obtained from animals that had been positively tested by histopathology or immunocytochemistry methods at the VLA and that were mostly in the late stage of the disease. Among the 250 BSE negative control samples from the VLA, we received 117 specimens with a confirmed negative BSE test. It is interesting to note that 34 of these 117 specimens were taken from animals showing symptoms ("clinical suspect for BSE") while the postmortem BSE test of these 34 animals revealed no evidence of the disease. Furthermore, 133 of the VLA control samples were taken from clinically inconspicuous animals (a BSE test was not performed on this group). Control samples were also obtained from sources in Germany: 45 serum samples from animals with a confirmed negative diagnosis for BSE were supplied by the Federal Research Center for Virus Disease of Animals, Island of Riems, Germany, and sera from 100 animals from the Institute of Animal Nutrition at the Federal Animal Research Center in Braunschweig, Germany. Within the scope of the VLA study we also included sera from animals suffering from a variety of "classical" viral or bacterial infectious diseases. A total of 35 sera from animals infected with nonspecific *Escherichia coli*, Staphylococci, Streptococci, or Proteus (mostly inflammation of claws and udder) were collected at the Veterinary University, Hannover, Germany. Furthermore, we received serum samples from animals experimentally infected with the bovine leukemia virus, the bovine respiratory syncytial virus, the bovine herpes virus-1, or the bovine diarrhea virus. For the blinded validation study II ("Roche study"; see below), the teaching sample set of the VLA study was augmented with 92 samples from the first VLA validation set (48 BSE-positive and 44 control samples) and from 50 confirmed BSE-negative cattle from a commercially run abattoir in upper Bavaria.

FT-IR spectroscopy was performed as described previously[11] and as detailed in the Experimental Section. Averages of all infrared spectra obtained from the sera of BSE-positive animals and BSE-negative controls are illustrated in Figure 1. To compensate for baseline drifts within the spectra, second derivatives of the original absorbance spectra were calculated. From the second-derivative difference spectrum between BSE-infected animals and controls (see trace 5 of Figure 1, magnified by a factor of 20) it is observed that infrared spectra of BSE-positive and BSE-negative sera differ at many distinct wavenumber positions. The magnitude in this difference spectrum at specific wavenumber positions is, however, not the only relevant parameter for assessing discriminative spectral features. Spectral information allowing the reliable differentiation of BSE-positive and BSE-negative animals should ideally exhibit an as high as possible interclass variance and as small as possible intraclass variances (i.e., high differences between the class mean spectra and low standard deviations within the classes). This relation of inter- and intraclass variances can be estimated from Figure 1, traces 6 and 7. These curves display the wavenumber-dependent standard deviation for the classes "BSE-positive" (trace 6) and "BSE-negative" (trace 7). Both standard deviation spectra (which are very similar in this case) clearly demonstrate that the differences between the class mean spectra are generally smaller than the intraclass standard deviations. Furthermore, this experimental finding clearly indicates the absence of distinct "BSE marker bands". As a consequence, a classification method that uses one or even a few spectral features only would generate insufficient results. Thus, an optimized FT-IR classification model for BSE identification should be multivariate, i.e., based on an as high as possible number of discriminative infrared spectral features.

After appropriate selection of the most discriminative spectral information (see ref 11), pattern recognition techniques were utilized for classification. We applied linear (LDA) and quadratic

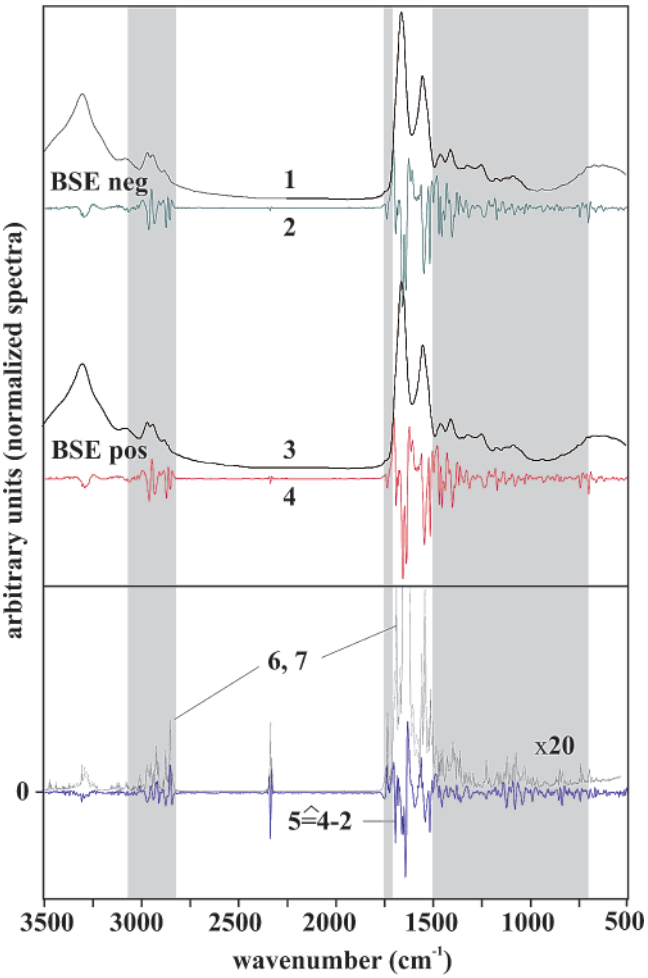

Figure 1. Typical infrared absorbance spectra (1, 3) and the corresponding second derivatives (2, 4) of sera originating from BSE-negative and BSE-positive animals. Traces 1−4 have been normalized and are calculated by averaging spectra obtained on 452 BSE-negative (1, 2) or 249 BSE-positive (3, 4) animals, respectively (VLA teaching set). Trace 5 (blue) represents the difference spectrum of the second derivatives (4, BSE positive; and 2, BSE negative). Traces 6 and 7 are the two standard deviation spectra of the classes BSE positive (6) and BSE negative (7) (which are nearly identical in this case). Note that the *y*-axis of difference spectrum 5 and the standard deviation spectra (6, 7) is magnified by a factor of 20 (see text for details).

(QDA) discriminant analysis as well as ANNs for classification. Furthermore, we chose a supervised approach for all classification schemes: first the classification algorithm underwent teaching based on the samples listed in Table 1. When the coefficients of the LDA/QDA and/or the weights of the ANNs had been determined, the classification algorithms were challenged with the blinded validation data sets described in Table 2.

Blinded validation samples were supplied to the Robert Koch-Institute from the VLA and from Roche Diagnostics within the scope of two independent studies:

Validation Study I (VLA Study). For the first validation study, the teaching process was based solely on ANNs. The FT-IR spectra of the teaching samples originated from 249 BSE-positive and 452 BSE-negative animals (cf. Table 1). To obtain optimal classification results, 20 different network architectures were tested, which varied in the numbers of input and hidden neurons.

Table 2. Characteristics of Samples Supplied to RKI for Blinded Validation

study	original source	diagnosis	no. of samples
VLA study	VLA	confirmed BSE positive	48
	VLA	confirmed BSE negative	44
Roche study	VLA	confirmed BSE positive	94
	VLA	BSE suspected, but confirmed BSE negative	17
	VLA	confirmed BSE negative from BSE-free farm	5
	abattoir (upper Bavaria)	confirmed BSE negative	151

Optimal teaching results were obtained for an ANN architecture of 85 input neurons, 5 neurons in the hidden layer, and 2 output neurons.

Initially, the validation set of serum samples of the VLA study contained 100 sera. For one of the samples, we were unable to obtain any FT-IR spectrum (not enough serum). Spectra from three other samples constantly failed in the test for spectral quality (see Experimental Section). Thus, we challenged the ANN by spectra obtained from 96 serum samples. Furthermore, after unblinding, it turned out that the validation set contained four serum duplicates (eight serum samples taken from four animals). Since the ANN classification of the duplicates yielded consistent class assignments, test parameters such as sensitivity or specificity were referenced to the number of animals (92).

Finally, the optimum ANN structure was challenged by the blinded validation set (see Table 2). The validation set exclusively contained FT-IR spectra of sera originating from animals from the U.K. It is important to note that blinded BSE-positive and BSE-negative animals were selected randomly by the VLA. Thus, the validation set comprised unmatched samples in terms of stage of BSE infection, age, breed, gender, and potentially, other diseases.

Upon unblinding the validation data set, we calculated the accuracy of the method: the number of true positive (TP) and true negative (TN) classifications are given in Table 3 with the number of false positive (FP) and false negative (FN) classifications. With the detection of 41 of the 48 BSE-positive samples and of 37 of the 44 BSE-negative samples, a sensitivity of 85.4% and a specificity of 84.1% were achieved. The weighted arithmetic mean of these two quantities is frequently called accuracy, which amounts to 84.8%.

Validation Study II (Roche Study). In the Roche study, the classification of the blind validation set was carried out by two different classification methods. While the first "pure ANN" approach was based on one optimized ANN, we used in the second classification trial a combination of two ANNs, one LDA, and one QDA. This "combined" approach gave the final classification for each individual animal by means of a decision tree (see Experimental Section). Prior to applying the trained classification algorithm and prior to unblinding the validation data, it was observed that 7 of the 267 samples repeatedly failed in the spectroscopic quality check. Thus, these seven samples were excluded from the further study.

Upon unblinding the validation data set, we determined for the first "pure ANN" classification approach a sensitivity and specificity of 86.5 and 90.6%, respectively. These numbers then

Table 3. Results of the Blinded Validation Studies[a]

study	no. of samples	TP	TN	FP	FN	SENS (%)	SPEC (%)	ACC (%)
VLA	92	41	37	7	7	85.4 (75.9−91.2)[b]	84.1 (73.7−91.2)[b]	84.8
Roche (ANN)	260	77	155	16	12	86.5 (79.6−91.6)[b]	90.6 (87.1−93.3)[b]	89.2
Roche (LDA/QDA/ANN)	260	85	158	13	4	95.5 (89.8−98.5)[b]	92.4 (89.4−93.9)[b]	93.5

[a] Results of the two validation studies upon unblinding the actual diagnosis. The FT-IR-based classifications were established by teaching classification algorithms with 701 (VLA study) or 843 serum samples (Roche study). The first row shows the results of a study carried out in collaboration with the VLA. Rows 2 and 3 depict the results of the Roche study (for details, see text). Abbreviations: TP, true positives; TN, true negatives; FP, false positives, FN, false negatives; SENS, sensitivity, SPEC, specifity, ACC, accuracy, where SENS = TP/(TP + FN), SPEC = TN/(TN + FP), and ACC = (TN + TP)/(TN + TP + FN +FP). [b] 95% confidence interval.

increased to 95.5 and 92.4%, respectively, when the "combined" classification method was applied (see Table 3). It should be mentioned at this point that these results could be achieved on experimental data containing "real" controls, i.e., animals showing clinical symptoms for BSE, but with a negative BSE test. Furthermore, we would like to point out that up to 96% of the BSE-positive animals were identified correctly by the classifiers although the training data set contained serum samples from cattle with viral or bacterial infections. This finding might be indicative for the existence of BSE-specific spectral features different from those of healthy controls and also from those of "classical" infectious diseases. The two validation data sets of blinded serum samples (see Table 2) did not contain sera from animals suffering from classical infections. We are therefore unable to present results for this group. However, internal tests carried out under nonblinded conditions clearly indicated that serum-based identification of viral or bacterial infections by IR spectroscopy is much more robust than the identification of BSE infection. This result might be not surprising, as the concentration of a number of serum constituents is known to be significantly altered upon "classical" infections, while similar unambiguous serum markers for BSE have not yet been identified.

In the present study we noticed that a high number of spectral features is suitable for classification. It turned also out that many of these spectral features are similarly discriminative; that is, the exclusion of a few features from further analysis did not significantly alter the quality of classification. Furthermore, we found a close relationship between the goodness of classification and the number of spectral features. Both findings clearly indicate that the IR method is not based on one unique serum marker. It seems rather plausible that the concentration of a plurality of serum constituents is slightly changed as suggested by the complex spectral difference pattern shown in trace 5 of Figure 1. A significant number of discriminative spectral features were found in the CH stretching region (2800−3100 cm^{-1}). We noticed that the accuracy of classification was reduced when this region was completely omitted from data analysis. It is known that serum lipids such as phospholipids, cholesterol, triglycerides, etc., significantly contribute to the absorptions in the CH stretching region. In an experimental effort to evaluate whether BSE is able to induce changes in the composition of serum lipids, 12 major serum lipid constituents such as cholesterol, triglycerides, or phospholipids were examined by a sensitive thin-layer chromatography-based technique.[13] These experiments revealed no or insignificant differences between lipids from sera of BSE-positive and -negative cattle (data not shown).

Given that FT-IR spectroscopy is able to unravel the existence of BSE-specific disease pattern in serum, the molecular nature of these alterations should be analyzed by more molecule-specific analytical techniques, such as various hyphenated mass or NMR spectroscopies (GC/MS, HPLC/MS). We have great confidence that the use of metabolomic or proteomic approaches is the way forward to identify TSE specific surrogate markers from blood or its components.

To put the IR methodology into practice, the classification accuracy should be improved. In the first place, the spectral database has to be further substantiated by enlarging the number of samples and by considering additional factors, which potentially influence the test results. Particularly the effects of age, breed, gender, or nutrition on the classification accuracy require further attention. Second, by investigating serum from experimentally infected cattle, the earliest point after infection that is possible to diagnose should be determined. Furthermore, due to the nature of the used classification systems it is easy to generate classifiers, which enhance either the sensitivity of the test (at the expenses of the specificity) or the specificity (at the expenses of the sensitivity). Although the inderdependency of sensitivity and specificity was not tested systematically, we could generate an ANN classifier, which yielded under blind conditions already a test sensitivity of 98% with a corresponding specificity of 83% (data not shown).

In summary, we presented the proof of principle of a new diagnostic tool for the diagnosis of BSE infection from serum. The methodology is based on FT-IR spectroscopy of serum samples combined with advanced methods of infrared spectral pattern analysis. Within the context of a field study, we achieved classification accuracies of up to 93.5%. Our findings suggest that further development of the method could lead toward a fully automatic, objective, and fast analytical tool for the antemortem diagnosis of BSE and possibly other TSEs.

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