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Identification of Scrapie Infection from Blood Serum by Fourier Transform Infrared Spectroscopy

Jürgen Schmitt,[†,⊥] Michael Beekes,[‡,#,⊥] Angelika Brauer,[§] Thomas Udelhoven,[†] Peter Lasch,[§] and Dieter Naumann*[,§]

FB VI, Spectroscopy Group, University of Trier, Universitätsring, 54286 Trier, Germany, P35 and P34, Robert Koch-Institut (RKI), Nordufer 20, 13353 Berlin, Germany

We describe a new Fourier transform infrared (FT-IR) spectroscopy-based diagnostic approach, which may provide for the first time a rapid, reliable, and inexpensive blood test for scrapie and related transmissible spongiform encephalopathies (TSE). Blood serum from 146 terminally ill Syrian hamsters infected with 263K scrapie via different routes of inoculation and from 166 healthy control animals was analyzed by FT-IR spectroscopy and artificial neural networks (ANN). This revealed characteristic molecular alterations in the serum of infected donors. Different ANN models were constructed and challenged with previously unknown samples in test runs in order to establish whether the new method is able to discriminate between normal and infected animals. Optimized ANNs consistently yielded test sensitivities and specificities of 97% and 100%, respectively. The predictive value of a positive (negative) test was 100% (98%). The proposed serum test circumvents substantial drawbacks of conventional TSE diagnostics and can be fully automated.

As a consequence of the BSE crisis in Europe, the development of diagnostic tests for transmissible spongiform encephalopathies (TSE) has become a matter of great scientific importance and public interest. So far, only a few approaches for the identification of TSE-infected individuals from blood samples have been reported.[1−5] Here, we describe a new method based on a combination of Fourier transform infrared (FT-IR) spectroscopy with advanced computer-aided pattern recognition techniques (artificial neural networks, ANNs), which may provide for the first time a rapid and reliable blood serum test for scrapie and other transmissible spongiform encephalopathies. The proposed test can be completely automated and potentially requires less than 15 min for taking the sample, acquiring the spectrum, and attaining the final diagnosis. This test is based on the analysis of the structural information and the total molecular composition of the serum samples by infrared spectroscopy. It is well-known that infrared spectra provide a molecular fingerprint with the numbers, frequencies, bandwidths, and intensities of vibrational bands characteristic for specific molecular structures. Using this technique, it is possible to obtain information on protein structure, nucleic acid conformation, membrane constitution, and lipid−protein interaction.[6] In addition, infrared spectra of biological samples, such as intact cells, tissues, and body fluids,[7,8] provide characteristic "signatures" of the respective specimens originating from the various biomolecules in the sample. The same principles apply to blood serum. If a disease process in animals or humans results in changes in the composition of serum, this will characteristically alter the spectral pattern obtained by infrared spectroscopy. In turn, changes of spectral serum features can be used for diagnostic purposes. However, this approach requires a high spectral quality and advanced methods of data analysis. Artificial neural networks represent sophisticated computer-aided pattern recognition techniques that can be applied to classify sample spectra based on disease-related features.

MATERIALS AND METHODS

Donors and Sampling of Serum. The following number of outbred female and male Syrian hamsters was used: 27 intracerebrally (i.c.), 90 intraperitoneally (i.p.), and 29 orally (p.o.) infected animals as well as 22 i.p. mock-, 31 p.o. mock-, and 113 noninfected controls. Hamsters for the i.c. series were taken from bioassay experiments and had received 50 μL inocula containing various amounts of 263 K scrapie infectivity. I.p. and p.o. infections were performed with 100 μL of a 10% 263K scrapie brain homogenate containing a dose of $1-3 \times 10^7$ LD_{50} i.c. as described elsewhere.[9] Controls were similarly i.p. and p.o. mock-infected with normal hamster brain homogenate or not challenged at all. Inocula were administered to the recipients at an age of 4−6 weeks. Incubation times of i.c. inoculated animals were in the range of 83-210 days. I.p. and p.o. infected hamsters showed mean

* Corresponding author. Phone: +4930 4547-2259. Fax: +49 30 4547-2606. E-mail: NaumannD@rki.de.

† University of Trier.

‡ P35, Robert Koch-Institut.

§ P34, Robert Koch-Institut.

Formerly P31 of the RKI.

⊥ These authors contributed equally to this work.

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Table 1. Results of the FT-IR Spectroscopic Test for Scrapie[a]

		scrapie infected	
		+	-
FT-IR test	+	77	0
	-	2	105

[a] FT-IR spectra acquired on 184 hamster serum samples (independent test data) were classified by an artificial neural net that has been trained and validated using spectra of another 128 serum samples. For the independent test set consisting of serum spectra from 79 scrapie infected hamsters (+) and 105 control animals (−) the following classification results were obtained: sensitivity, 97.5%; specificity, 100%; positive predictive value, 100%; negative predictive value, 98.1% (for more details see Table 2).

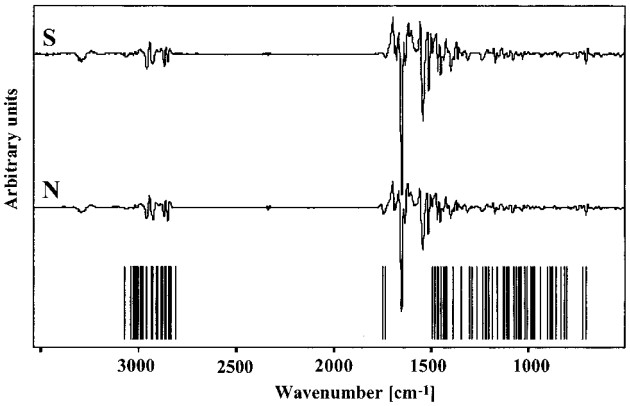

Figure 1. Representative FT-IR spectra (second derivatives) of hamster serum from a noninfected control animal (N) and an intraperitoneally (i.p.) infected animal in the terminal stage of scrapie infection (S). The spectral features (subregions) contributing most significantly to the separation of control and scrapie spectra into two different classes are shown at the bottom of the figure.

incubation periods of 118 ± 12 (SD) and 157 ± 9 (SD) days. All infected animals were sacrificed at the terminal stage of scrapie. I.p. mock-, p.o. mock-, and noninfected controls were sacrificed at an age of 140−180, 170−210, and 30−140 days, respectively. Euthanasia with CO_2, collection of blood, and isolation of serum were carried out as previously described.[10]

FT-IR Spectroscopy. A 2.6 μL portion of each serum sample was spread on a ZnSe optical plate in a multisample cuvette and air-dried to form a transparent film.[11] After incubation in an oven at 37 °C for 5 min, the multisample cuvette was transferred into the spectrometer. Spectra between 4000 and 500 cm^{-1} were recorded on an IFS 28/B spectrometer (Bruker Optik GmbH, Germany) equipped with a deuterated triglycine sulfate detector. Nominal physical resolution was set to 4 cm^{-1}, a Blackman/Harris 3-term apodization function was used for Fourier transformation, and a zerofilling factor of 4 was applied to yield an encoding interval of approximately one data point per wavenumber. Spectral data were collected using the data acquisition software OPUS 3.0 package supplied by Bruker.

Data Evaluation, Data Pretreatment. Conversion of the original absorbance spectra to second derivatives is a common practice in IR spectroscopy to enhance spectral details, such as shoulders of IR bands and to get rid of spectral baseline shifts. Second derivatives were calculated from absorbance spectra using a Savitzky-Golay 9-point smoothing algorithm. Spectra were subsequently vector-normalized over the spectral range from 2820 to 2985 cm^{-1}.

Training, Validation, and Test Data Set. Data were split into a training data set (used to estimate the network model parameters), a validation data set (used to check the generalization ability of the network), and a test data set. The three independent measurements on each serum sample were regarded as one sample and were distributed randomly to the respective groups of data sets. The training data set contained 89 openly labeled samples with 267 spectra; the validation data set, 39 openly labeled samples with 117 spectra; and the test data set, 184 blind samples with 552 spectra (see Table 1a).

Spectral Feature Selection. A spectral feature selection algorithm diminished the high degree of information redundancy over the entire spectral range. This generally improved the quality of the classifier considerably and reduced the complexity and dimensionality of the classification model. Prior to feature selection, data reduction was performed by averaging three data points, resulting in 1206 new data points out of 3620. The spectral windows for feature selection were predetermined between 700 and 1500 cm^{-1}, 1700 and 1750 cm^{-1}, and 2800 and 3100 cm^{-1}. Spectral feature selection was based on the calculation of the covariance of the spectral data points,[12] representing the variables (i.e., the spectral intensities at discrete wavenumber positions). Afterward, the selected features were ranked in descending order according to the covariance measure. The best 81 features selected by this procedure are shown in Figure 1. Blind serum samples from the test data set were diagnosed as positive or as negative when all or at least two of the corresponding spectra were classified as positive or as negative, respectively, by the neural network.

Artificial Neural Network Analysis. In this study, the Synthon NeuroDeveloper (Synthon KG, Gusterath, Germany) running on a Windows NT platform was used. A three-layer feed-forward network with 81 input neurons, 9 hidden units, and 2 output units allowing shortcut connections within the network was established. As the learning function, resilient back-propagation (Rprop) was used.[13,14]

RESULTS

In the present study, infrared spectra were acquired from a total of 312 hamster serum samples. All individual serum samples had been measured independently 3 times, generally in time intervals of days or weeks, sometimes even months. Representative second derivative spectra obtained from the serum of a healthy control and a terminally ill scrapie hamster are shown in Figure 1. The spectra of Figure 1 reflect the spectral information of all molecular groups present in the serum as well as their complex interactions. Because serum consists of several hundred sub-

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Table 2. Details of the Artificial Neural Network (ANN) Classification for the Training, Validation, and Independent Test Sets of FT-IR Spectra Acquired on Serum Samples from Scrapie Infected[a] (upper part) and Control[b] Hamsters[c] (lower part)

	number of samples and classification results								
	ANN training set			ANN validation set			ANN test set		
donors	N	True Positive	Sensitivity [%]	N	True Positive	Sensitivity [%]	N	True Positive	Sensitivity [%]
i.c.-inf.	15	15	100	5	5	100	7	7	100
i.p.-inf.	33	33	100	10	10	100	47	45	96
p.o.-inf.			n.a.	4	4	100	25	25	100
	N	True Negative	Specificity [%]	N	True Negative	Specificity [%]	N	True Negative	Specificity [%]
noninf.	38	38	100	13	13	100	62	62	100
ipm	3	3	100	1	1	100	18	18	100
pom			n.a.	6	6	100	25	25	100

[a] Upper part. [b] Lower part. [c] The ANN was trained, validated, and tested with spectral data from different origins: from serum samples of intracerebrally (i.c.), intraperitoneally (i.p.) or orally (p.o.) infected scrapie hamsters, and noninfected, intraperitoneally mock (ipm)-, or orally mock (pom)-infected control hamsters. As in Table 1, the number of animals tested positive or negative by the ANN is indicated.

stances, only some of which are known at this time, a direct transformation of the spectral signals to specific constituents on a molecular basis is impossible at present. To circumvent this problem and yet to discriminate between healthy and diseased individuals, we analyzed the FT-IR serum spectra by different combinations of feature selection methods and pattern analysis techniques. Among these, evaluations based on covariance analysis and artificial neural networks (ANN) turned out to be the methods of choice. In classification problems, a neural network provides nonparametric, nonlinear discriminant functions, which can be used to model probabilities of class membership. These functions are established during a learning process with selected training data and a predefined network architecture.

The Rprop learning function was used to optimize the training process.[12−14] The network architecture consisted of 81 input neurons corresponding to the number of spectral features, 9 hidden units, and 2 output units with the class assignment (I) "healthy" or (II) "infected". The classification problem was solved in the ANN-model with a high percentage of correct assignments in respect to the validation and the independent test data set. The percentage of correctly assigned individuals (accuracy) was 100% in the validation data set and 98.9% (182/184) in the test data set. Results from the test data can be considered as the best and strictest estimator for the generalization potential of the ANN-model, because they represent an external validation.[15] Nevertheless, the training and validation data also consisted of independent measurements and include information about the main sources of variance.

The test results of the serum samples combined in the independent test set are summarized in Table 1. Our test data contained IR spectra from serum samples obtained from 79 scrapie hamsters and 105 control animals, corresponding to a prevalence of scrapie in the test population of 42.9%. According to Table 1, 77 out of 79 (77/79) scrapie-infected animals were tested positive by the neural net. Furthermore, all (105/105) of the control animals were classified correctly. The overall sensitivity and specificity of the test were 97.5% and 100%, respectively. Accordingly, the predictive value of a positive test (PPV) is 100% and that of a negative test (NPV) 98.1%.

The first three rows of Table 2 display details of the ANN classification results obtained from serum samples of terminally ill scrapie infected animals (intracerebrally, i.c.; intraperitoneally, i.p.; or orally, p.o. infected). This part of the table also shows ANN classification results for the training, validation, and the independent test data set. Training of the ANN with scrapie specimens was performed with serum spectra from 15 i.c. infected and 33 i.p. infected hamsters. For validation, serum spectra from 5 i.c., 10 i.p., and 4 p.o. infected hamsters were used. Finally, spectra from blind serum samples of 7 i.c., 47 i.p., and 25 p.o. infected animals were included in the test data set. For 7/7 i.c. infected, 45/47 i.p. infected, and 25/25 p.o. infected hamsters, the test yielded a correct positive result. For the different routes of infection, this corresponds to test sensitivities of 100%, 96%, and 100%, respectively.

The last three rows of Table 2 show the classification results for serum samples of the control hamsters. For training of the ANN with control specimens, spectra from 38 noninfected and 3 i.p. mock-infected animals were used. Validation was performed with spectra from another 13 noninfected, one i.p. mock-infected and 6 p.o. mock-infected animals. Testing of blind samples was carried out with spectra from 62 noninfected, 18 i.p. mock-infected, and 25 p.o. mock-infected hamsters. 100% of the donor animals, either noninfected (62/62), i.p. mock-infected (18/18), or p.o. mock-infected (25/25) were correctly classified as negative.

DISCUSSION

The findings reported above, which are based on the examination of serum samples from 312 hamsters, suggest that analysis of FT-IR spectra from blood serum with artificial neural nets may provide for the first time a very rapid method for the ante mortem diagnosis of scrapie and possibly other TSEs.

However, with respect to the pathological alterations observed in the sera of scrapie hamsters, it needs to be addressed whether and to what extent they are specific for scrapie (and other TSEs). A comprehensive approach to assess the specifity of the FT-IR TSE test requires the analysis of serum samples from individuals with a broad spectrum of infectious (e.g., bacterial, viral, fungal,

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and parasitic) and noninfectious diseases. From a practical point of view, this panel of samples cannot be collected in the hamster model, nor should it be. Our report describes findings from a first "feasibility study" in rodents which urgently need to be transferred to cattle, sheep, and humans. Only in these species, will a large-scale "field study" be both feasible and diagnostically relevant. Thus, we think that only in these species will a further assessment of test parameters such as sensitivity, specifity, and accuracy be appropriate.

From the TSE literature, it is known that classical inflammatory or immunological responses to TSE infection are consistently absent during the preclinical phase and also at the clinically manifest stage.[16] This lack has been a key obstacle in previous attempts to develop a blood test for the noninvasive diagnosis of TSEs. It is also conceivable that the inoculation of brain tissue per se may cause an inflammatory/immunological response of the organism. However, serum spectra from mock-infected animals which had been challenged by intraperitoneal and intracerebral administration of normal brain homogenate could be clearly distinguished from those taken from scrapie hamsters. Additionally, an inflammatory response to exogenous brain tissue appears unlikely to persist until 80−210 days after inoculation.

The results of this feasibility study demonstrate that the terminal state of scrapie infection is accompanied by distinct compositional or structural alterations of serum constituents, independently of whether the animals were infected intracerebrally, intraperitoneally, or perorally. We could show that these changes can be detected by infrared spectroscopy and used for the identification of scrapie infected animals by ANN analysis. Nonetheless, the nature of the underlying disease-related processes remains unknown. FT-IR spectroscopy is a structure-sensitive technique, but for serum spectra, classical methods of assigning infrared bands to defined components cannot be applied. Bioorganic materials, such as tissues and serum, are composed of hundreds, if not thousands, of different types of biomolecules. Thus, although biomedical FT-IR spectroscopy is a very sensitive tool to detect structural or compositional changes in complex mixtures, that technique alone is not sufficient for the identification of specific serum constituents. To address this question, the use of analytical techniques other than FT-IR spectroscopy is required.

Thus, at present, we can only speculate about the nature of the alterations detected by the FT-IR technique. From different attempts of data analysis by ANNs, we learned that the classification accuracy is reduced if the C−H stretching region (2800−3050 cm^{-1}) is omitted from the ANN analysis. This may be indicative of differing amounts of lipids (phospholipids, sphingolipids, glycerol, etc.) in the sera of scrapie and control hamsters and needs to be addressed in future studies. Furthermore, it seems highly unlikely that any of the disease-related spectral alterations detected in the serum from scrapie hamsters result from pathological prion protein (PrP^{sc}).

Our observations and "the phantasmagoric immunology of transmissible spongiform encephalopathies"[16] strongly suggest that the FT-IR spectral changes observed in the serum of scrapie hamsters resulted neither from an nonspecific response to the inoculation of tissue homogenates nor from generalized inflammatory/immunological processes that are frequently associated with "conventional" infectious or nontransmissible diseases.

Petrich et al.[17] discussed the specifity of a disease pattern recognition of diabetes mellitus with infrared spectra from human serum samples. These authors reported a sensitivity and specifity of ≥ 80% and compared their new diagnostic approach with classical molecular parameters (glucose, triglycerides, cholesterol) from the identical samples measured by a clinical analyzer. Their classification results based on infrared spectra exhibited a superior correlation in the assignment of the true disease state, as compared to that achieved by a biochemical determination of specific biomolecules.

Although the precise nature of the FT-IR spectral alterations observed so far in the serum from individuals suffering from scrapie, diabetes mellitus, and other conditions remains unknown, the results of our and other IR spectroscopic investigations point to new diagnostic avenues opened by the combination of infrared spectroscopy and computational pattern recognition techniques.

At present, kinetic FT-IR studies on the serum of hamsters orally infected with scrapie are underway. These studies are expected to reveal whether and at which stage of incubation our diagnostic approach will also allow the preclinical detection of scrapie infection following uptake of agent via the gastrointestinal tract. To further evaluate its diagnostic potential, the test will be applied soon to serum samples from animals and humans with nonexperimental TSEs.

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