TABLE 1. Specimens analyzed in the MPD assay reported in this study

Species	Number of animals: Infected	Number of animals: Control	Route of inoculation	Infecting strain	Tissue
Sheep	13	1 pool of 25		Endemic	Serum
Mouse	1 pool of 75	1 pool of 12 and 1 pool of 5 knockout	IC*	Fukuoka-1	Plasma
Mouse	1	1 and 1 pool of 5 knockout	IC	Fukuoka-1	Brain
Human	5	5		Sporadic	Plasma
Monkey	8	4	IC	Human sporadic CJD	Plasma

* IC = intracerebral.

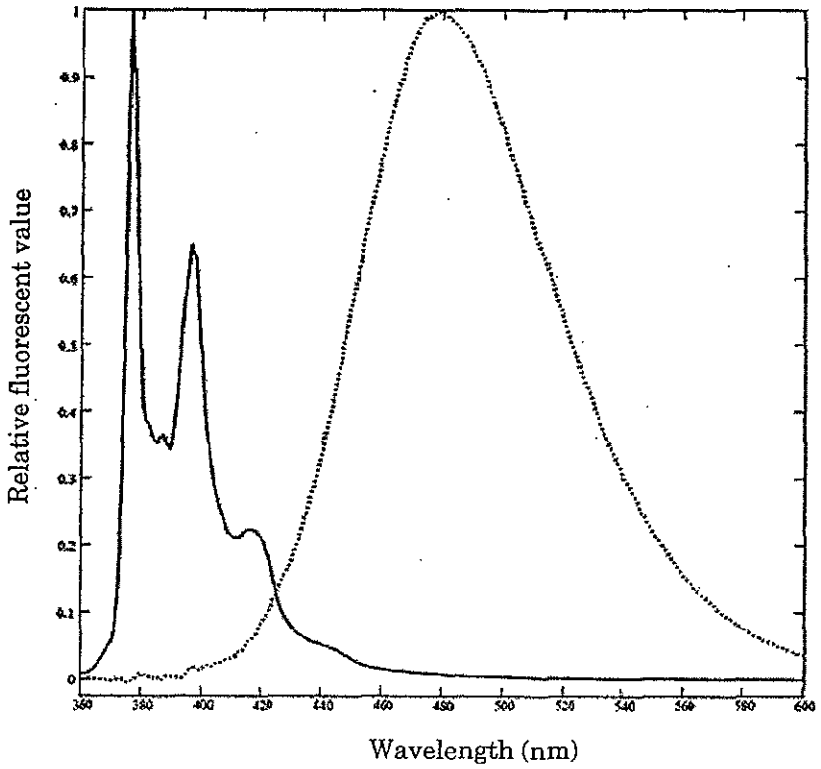

Fig. 1. Association of conformational change with fluorescence. Conformational change in the peptide is associated with a shift in fluorescence intensity from pyrene monomer to pyrene excimer emission. Monomeric pyrene molecule emission is characterized by the solid line spectrum with a maximum from 375 to 385 nm, whereas the excimeric form of pyrene, associated with β-sheet conformation, is portrayed by the dashed line spectrum with a maximum from 460 to 500 nm. The *x*-axis is wavelength (nm) and the *y*-axis is an arbitrary fluorescence scale.

Western blots were performed with primary antibody 6D11 (Signet Laboratories, Dedham, MA) at a 1:10,000 dilution, peroxidase-labeled goat anti-mouse immunoglobulin G (KPL, Gaithersburg, MD) at a dilution of 1:20,000, and chemiluminescent substrate (Super Signal West Pico, Pierce, Rockford, IL). The MPD assay used 10 µL of a 1:1,000 dilution of brain homogenate incubated with one of three different peptides, the PrP^{TSE} MPD peptide, the CTI, or a scrambled version of the peptide. Samples were treated with 50 µg per mL (final concentration) proteinase K (Sigma Aldrich) at 37°C for 1 hour, terminated by the addition of phenylmethylsulfonyl fluoride (Sigma) at a final concentration of 3 mmol per L.

Blood was obtained from 75 symptomatic mice that were euthanized at an advanced stage of disease 18 weeks after intracerebral inoculation with the Fukuoka-1 strain of GSS.[4] The blood was collected in citrate phosphate dextrose anticoagulant and pooled, and the plasma was separated and stored at −70°C. The infectious titer had previously been determined by bioassay to be 10 infectious doses (IDs) per mL. The MPD assay was performed on serial dilutions of an aliquot that had been stored at −70°C for 8 years, the small volume of which prevented replicate testing for statistical analysis. The MPD assay reactions were then fractionated on 2-mL Sepharose 2B (Sigma) columns to separate plasma components by size eluting with 0.5× PBS and collecting 100-µL fractions. Twenty-microliter aliquots of each fraction were analyzed in the MPD assay with preequilibrated peptide solution as described above. Two fractions showing the best discrimination of normal from infected were chosen for statistical evaluation.

Sporadic CJD primate model

Pooled brain homogenates were prepared from two human patients with sporadic CJD, and 0.1-mL volumes were inoculated intracerebrally at 10^{-1} or 10^{-3} dilutions into the left frontal cortex of eight squirrel monkeys.[18] Animals were kept under continuous clinical surveillance and regularly underwent standardized behavioral testing. The time to initial clinical signs ranged from 20.5 to 23 months after inoculation, with clinical signs lasting from 1 week to 7 months. Postmortem examinations revealed typical patterns of spongiform change and PrP^{TSE}

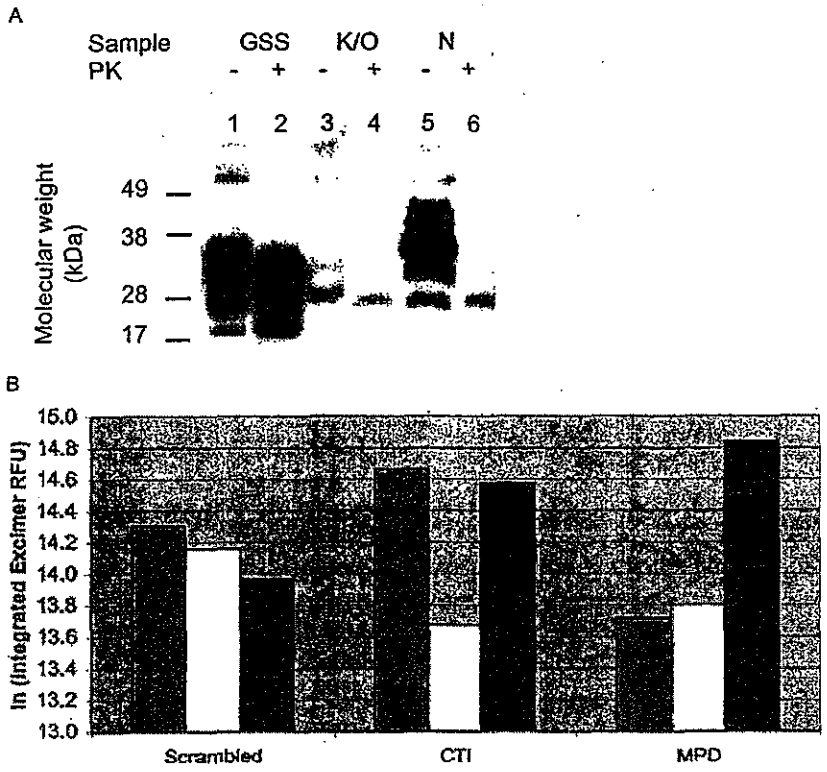

Fig. 2. MPD assay specificity. (A) Western blots of brain homogenates from a GSS-infected mouse, a PrP/KO mouse, and a normal mouse. Lanes 1 and 2 = GSS disease; Lanes 3 and 4 = PrP/KO; Lanes 5 and 6 = normal (N). Lanes 2, 4, and 6 = proteinase K (PK)-treated; Lanes 1, 3, and 5 = no PK treatment. See Materials and Methods for protocol details. (B) Brain homogenates from knockout (□), normal (▨), or GSS-infected (■) mice were evaluated with MPD, CTI, or scrambled peptides following the protocol described under Materials and Methods. Values are presented as the ln (integrated relative fluorescent value [RFU] for the excimer).

distribution similar to those seen in humans. Four uninoculated control animals were housed under identical conditions and bled at the same time as the inoculated animals. Fifty-microliter plasma aliquots from terminal bleeds were fractionated on 2-mL Sepharose 2B (Sigma) columns to separate plasma components by size eluting with 0.5× PBS and collecting 100-µL fractions. Twenty-microliter aliquots of the resulting fractions were analyzed in the MPD assay with preequilibrated peptide solution as described above under Materials and Methods.

Sporadic CJD in humans

Plasma samples obtained from five symptomatic neuropathologically verified and experimentally transmitted cases and five healthy individuals were stored at –70°C for periods of 10 to 20 years. One-hundred-microliter aliquots were fractionated on 2-mL Sepharose 2B (Sigma) columns to separate plasma components by size eluting with 0.5× PBS and collecting 100-µL fractions. Twenty-microliter aliquots of fractions were analyzed in the MPD assay with preequilibrated peptide solution as described above.

Endemic scrapie in sheep

Thirteen serum samples from neuropathologically and immunohistochemically confirmed sheep were obtained from the USDA Laboratory in Ames, Iowa. A pool of 25 normal sheep serum samples was purchased from Rockland Immunochemicals Inc. (Gilbertville, PA) and processed as the scrapie-free control. Serum glycoproteins were precipitated with phosphotungstic acid with a modification of a previously published method.[15] Two-hundred-microliter volumes were mixed by vortexing with an equal volume of 2× extraction buffer (20 mmol/L Tris, pH 7.4, 0.2 mol/L NaCl, 1.0% Igepal CA-360, 2% deoxycholic acid, 0.02% NaAz). Phosphotungstic acid (4%) was then added to a final concentration of 0.3 percent and the mixture was again vortexed, incubated for 1 hour with rocking, followed by centrifugation at 14,000 × g for 30 minutes. The resulting pellet was resuspended in PBS, and 20-µL aliquots were analyzed in the MPD assay as described above.

Statistical analysis

When adequate volumes permitted, samples were run in triplicate and standard deviations were calculated. Control samples from healthy animals or humans were run as a qualitative comparison in all cases. Peptide reagents (in the absence of samples) were routinely run in triplicate as a control for background peptide dynamics. Statistical analysis was used to discriminate positive from negative values whenever possible, with a t test or the binomial test when comparing a control pool to individual infected samples in the case of the sheep serum samples to generate the p values given with the figures.

RESULTS

Table 1 summarizes the samples evaluated in this study that spans a period of both sample processing and MPD assay method development as well as data analysis and presentation across a diverse set of blood samples.

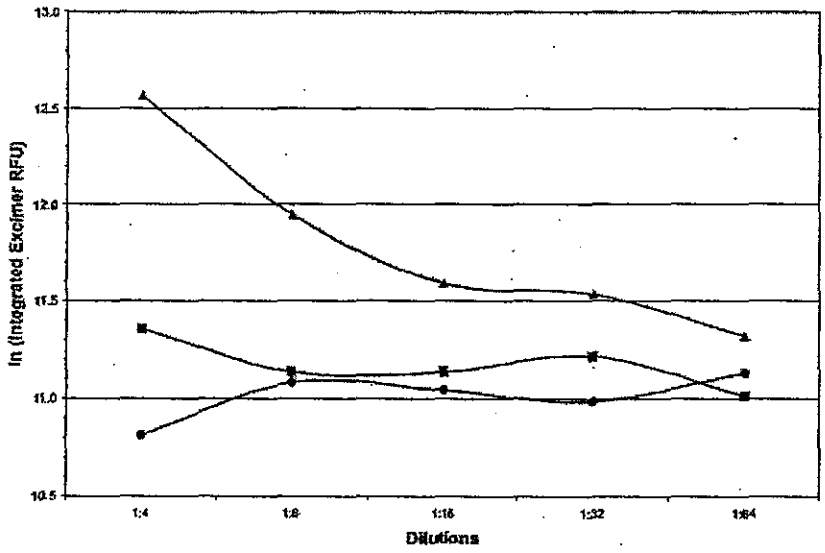

Fig. 3. MPD assay sensitivity. A dilution series of a previously titered terminal plasma pool from mice experimentally infected with the Fukuoka-1 strain of GSS (▲), a normal mouse plasma pool (■), and plasma from a PrP/KO mouse (●) were evaluated. Dilutions were made in PBS and read in a 200-μL reaction volume. The data are presented as the ln (integrated relative fluorescent value [RFU] for the excimer).

Fluorescence measurement of conformational change

Fluorophore-labeled peptide reagents were used to follow conformational change induced by the presence of sequence-specific misfolded PrP^{TSE} molecules. Figure 1 demonstrates the two distinct fluorescence spectra possible for the double pyrene-labeled peptides. Integrated peak values from 370 to 385 nm and from 430 to 530 nm correspond to monomer and excimer emission, respectively. The ratio of relative excimer fluorescence to relative monomer fluorescence emission provides a measurement of the presence of β-sheet-rich PrP^{TSE} target substrate.

Specificity of the MPD assay

To establish that the MPD assay detects PrP^{TSE}, we compared the results of MPD and Western blot assays of brain tissue from a GSS-infected mouse, a normal mouse, and a PrP/KO mouse. Western blots of these samples after treatment with proteinase K yielded the expected downward band shift of PrP^{TSE} in the GSS-infected brain and the absence of PrP^{C} bands in the normal and knockout specimens (Fig. 2A).

We also compared the MPD peptide reagent on the same three mouse brain homogenates with two MPD peptide variants, a scrambled version that produced no distinction across the three murine samples, and a CTI MPD peptide that distinguished both the normal and the GSS brain tissue from the KO mouse, but not the normal mouse, illustrating the importance of the correct structural sequence of the MPD peptide in distinguishing PrP^{TSE} from PrP^{C}. Only the MPD assay peptide, with both the proper sequence and the conformationally sensitive structure, was able to distinguish between the infected mouse brain sample and both uninfected samples (Fig. 2B).

Sensitivity of the MPD assay

Plasma from a pool of terminally ill GSS-infected mice was compared to plasma pools from normal and PrP/KO mice in a parallel series of twofold dilutions. The infected mouse plasma had been previously bioassayed and shown to contain 10 IDs per mL.[5] The positive signal at a 1:8 dilution and negative signal at a 1:16 dilution indicates a threshold of test sensitivity in the range of 1 ID per mL (Fig. 3).

Detection of PrP^{TSE} in blood: sporadic CJD primate model

A set of 12 coded plasma samples from the terminal bleeds from squirrel monkeys inoculated with a strain of human sporadic CJD and from uninoculated control monkeys was enriched in a fractionation protocol prior to evaluation in the MPD assay. Analysis of the data set with the natural log of the integrated excimeric peak (as done with all other experiments) is shown in Fig. 4A. Three of the 12 coded samples had recognizably "high" signals. A different analytic approach comparing the ratio of the excimeric signal to the monomeric signal in each of the different fractions revealed two fractions that gave a much cleaner separation of the samples into two groups: 8 samples with higher signals and 4 samples with lower signals. Based on signals recorded from one of these fractions, the two groups were scored as positive and negative before the sample identifications were "unblinded." The results showed an excellent correlation of high and low signals in inoculated and uninoculated animals, respectively, as shown in Fig. 4B. Statistical analysis yielded a p value of 0.002. This alternative data analysis was more appropriate for the protocol used in this experiment, which separated molecules present in the plasma samples, including the PrP^{TSE}, before introduction of the MPD peptide. The resulting fluorescence spectra differed from those produced by unfractionated samples that benefit from comparison of the excimeric peak to the monomeric peak.

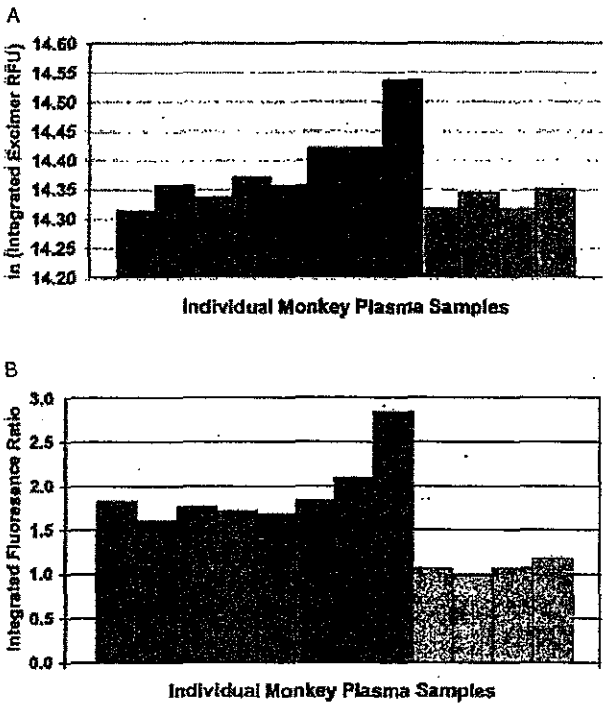

Fig. 4. MPD assay of sporadic CJD–infected primate plasma (blinded study). Coded terminal plasma samples from squirrel monkeys either inoculated with a human strain of GSS or uninoculated were evaluated. Thirteen samples fell into two distinct groups that upon breaking the code were revealed to correspond to infected (■) and uninfected (▦) animals. Data are presented in two formats: (A) Values are ln (integrated relative fluorescent value [RFU] for the excimer); (B) values are presented as the ratio of the integrated excimeric RFU to the monomeric RFUs (p = 0.002 by t test).

Detection of PrP^{TSE} in blood: endogenous infection

Human sporadic CJD. Blinded tests of a small number of plasma samples from human sporadic CJD patients showed modest but clearly increased signals from four of five CJD patients and a high signal from one of the five patients; all control samples were negative (Fig. 5). Statistical analysis of the patient and control groups yielded a p value of 0.03.

Sheep with natural scrapie. Thirteen serum samples from symptomatic sheep that on autopsy were histopathologically and immunohistochemically confirmed to have scrapie were compared to a pool of 25 serum samples from scrapie-free sheep (Fig. 6). Statistical comparison of the scrapie and control group yielded a p value of less than 0.001 with a binomial statistical test indicating that all 13 of the serum samples from scrapie animals were above the population mean of the pooled control serum sample. Individual control animal serum samples from the pool were not available to test.

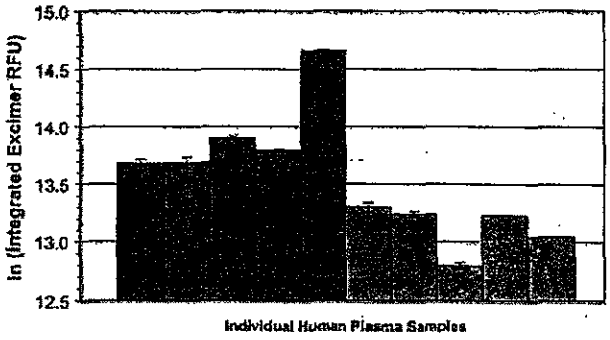

Fig. 5. MPD assay of human sporadic CJD plasma samples (blinded study). Five plasma samples from clinical sporadic CJD patients (■) and five normal human plasma controls (▦) were evaluated in the MPD assay. The data are presented as ln (integrated relative fluorescent value [RFU] for the excimer) (p = 0.03 with a two-sample t test).

DISCUSSION

One of the few undisputed facts about TSE is that the pathologic process depends on an α-helical coil to β-sheet molecular transformation of the normal cellular prion protein (PrP) into a proteinase-resistant amyloidogenic molecule (PrP^{TSE}), leading to deposition and accumulation of amyloid within the central nervous system and, to a lesser degree, lymphoreticular and other tissues. A variety of diagnostic tests have therefore been developed to detect the presence of PrP^{TSE} in these tissues and in circulating blood. We have used a small fluorophore-containing peptide, with homologous sequence specificity to a highly conserved region of PrP that is induced to undergo a similar conformational change in the presence of PrP^{TSE}. We present data showing that the MPD assay specifically detects PrP^{TSE}, with a threshold of sensitivity of approximately 1 ID per mL, and have applied the assay to the detection of PrP^{TSE} in the blood of experimentally infected mice and primates, endemic scrapie in sheep, and sporadic CJD in humans.

The signal generated in the assay is directly related to pyrene excimer formation induced by the peptide conformational change in the peptide. The thermodynamic conditions necessary to hold the peptide in an α-helical conformation, stabilizing the peptide monomer before addition of PrP^{TSE} as a reaction initiator, were the subject of previous studies and are important to the performance of the assay.[17] Monitoring of peptide activity through the use of synthetic substrates to induce maximal excimer formation is also critical to demonstrating the ability of the peptides to undergo a detectable conformational change induced by the presence of PrP^{TSE}.

Binding of tissue PrP^{TSE} to the MPD peptide, as well as peptide-to-peptide association, contribute to signal generation.[16] We have also considered the possibility that

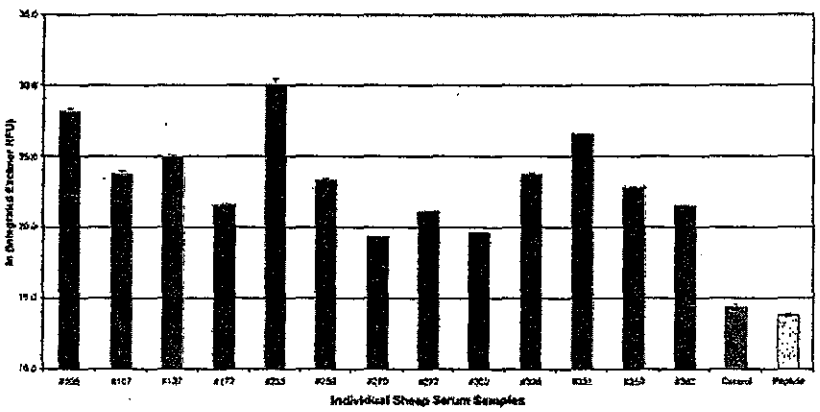

Fig. 6. MPD assay of scrapie-infected sheep serum. Values represent the mean of three replicated readings of individual scrapie sheep serum samples (■), a pool of 25 normal sheep serums (▨), and a MPD peptide only control (■); data are presented as ln integrated relative fluorescent value (RFU) for the excimer ($p < 0.001$ with a binomial test).

excimer formation could arise as a result of conformational changes induced by the presence of other β-sheet proteins or other prevalent proteins in blood, such as bovine serum albumin. The monitoring of peptide excimer formation in control samples indicates that pyrene excimer formation observed in the reaction was specific to PrP^{TSE}.

We previously reported a time point study of brain tissue from scrapie-infected hamsters in which the MPD signal became positive as early as 3 weeks after infection, well before the appearance of Western blot positivity,[16] a finding that invites similar evaluation of blood during the preclinical phase of TSE. Several other laboratories are currently working on methods that may be sufficiently sensitive and specific to detect the very low levels of PrP^{TSE} circulating in blood during the clinical, and in some cases, preclinical stages of TSE.[19-21] Different strategies have been devised that either concentrate or amplify the amount of PrP^{TSE} to detectable levels. These may involve the use of a "capture" antibody or chemical ligand that accomplishes enrichment of the molecular target.[22,23] Some methods use proteinase K to digest PrP^{C} or a denaturing agent that unfolds the PrP^{TSE} and exposes epitopes that were hidden within the interior of the unfolded molecular aggregate.[24] Other laboratories make use of an antibody that is specific for PrP^{TSE} without a preceding digestion or denaturation step.[24,25]

One interesting method utilizes the repeated sonication and amplification of PrP^{TSE} in blood with the addition of PrP^{C} substrate from brain tissue.[26-28] Although the assay method has succeeded in quantifying PrP^{TSE} in blood, it has the practical drawbacks of long cycle times of sonication and amplification (days) and amplification of infectious PrP^{TSE}. To the best of our knowledge, the MPD assay is unique in not using an antibody at any stage of the test, relying on a reactive synthetic peptide for both specificity and amplification. All of the published PrP^{TSE} detection methods have achieved successful detection of PrP^{TSE} "spikes" into blood, some have achieved success with blood collected from the clinical phase of disease in either animal or human TSEs, and a few have extended this success to include preclinical detection in animals. None has so far been able to reach the ultimate goal of identifying preclinical infection in humans. The MPD test has been previously shown to discriminate TSE-infected brain tissue from normal brain tissue,[15] and in this article we have expanded the spectrum of tissues to include blood in a variety of natural and experimental TSE infections.

The performance requirements for a practical method of blood detection for PrP^{TSE} depend on the diagnostic goal: whether as a confirmatory test of the clinical diagnosis in symptomatic animals and humans suspected of having TSE or as a test to identify infection during the preclinical phase of disease, that is, as a diagnostic screening test. A confirmatory diagnostic test requires a high degree of sensitivity, whereas a screening test mandates a high degree of specificity because of the need for an absolute minimum number of false-positive samples in the very large number of samples that would be screened.

Much recent research has focused on screening test applications, both as a tool to identify scrapie infections in animals that may then be culled from flocks to prevent continued spreading of disease (perhaps eventually also to identify chronic wasting disease of deer and elk) and as a way of identifying infected but apparently healthy blood donors to avoid secondary iatrogenic transmissions. We continue our efforts to optimize the conditions of the MPD assay to widen the margin of significance between positive and negative test values and to improve reproducibility and "packaging" as a practical test for use in blood collection centers.

ACKNOWLEDGMENTS

The authors thank Oksana Yakovleva for her excellent technical assistance in the Western blot analysis of murine brain homogenates. The authors acknowledge the valuable sample gifts of sheep serum from Michelle Crocheck DVM of the USDA, Ames, IA; human and murine samples from Larisa Cervenakova, MD, American Red Cross, Holland Laboratories, Rockville, MD, and Paul Brown, MD, Bethesda, MD; and squirrel monkey samples

from Susan Gibson, DVM University of South Alabama, AL; and Thomas R. Kreil, PhD, Baxter Bioscience, Vienna, Austria.

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医薬品　研究報告　調査報告書

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研究報告の概要

問題点（ドイツでのバベシア症の感染報告）
バベシア症は動物に共通した感染症であり、欧州において新興のダニ媒介人獣共通感染症として注目されている。
この論文は、ドイツで発症したヒトバベシア症の最初の報告である。
患者は 63 歳の男性のドイツ人で、結節性リンパ球優位性ホジキンリンパ腫が再発し脾臓摘出術を受けていた。
患者がリツキシマブ治療後、貧血とヘモグロビン尿による暗色尿のため入院した。
末梢血液塗抹標本（ギムザ染色）による診断で、梨状で変形体様の寄生虫が赤血球内で確認されたが、B. microti と B. divergenes の IgM、IgG 特異抗体は陰性であった。
PCR 法により 18S-rRNA をコードする rDNA 領域を増幅して、増幅された DNA を比較すると、Herwaldt らにより EU1 に指定されたバベシアと 99%以上のホモロジーを有していた。
患者はダニに刺された記憶がなく、入院前に輸血歴もない。
人獣共通感染能のあるバベシア症が特有な地方において、慢性的な感染者の輸血により感染することが知られている。

報告企業の意見	今後の対応
バベシア症は赤血球内にバベシア原虫が寄生するため発症するが、血漿分画製剤は血漿を原材料にしているため感染はないと考えられる。万一原料血漿に混入しても、製造工程で除去できると考えている。	今後とも新しい感染症に関する情報収集に努める所存である。

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First case of human babesiosis in Germany – Clinical presentation and molecular characterisation of the pathogen

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Abstract

Babesiosis is a common infection of animals and is gaining increasing attention as an emerging tick-borne zoonosis of humans in Europe. Here we report on the first case of human babesiosis in Germany in a 63-year-old splenectomised German patient with a relapse of nodular lymphocyte-predominant Hodgkin's lymphoma. After treatment with a chimeric anti-CD20 antibody preparation (Rituximab), the patient was hospitalised because of anaemia and dark urine from haemoglobinuria. Presumptive diagnosis of babesiosis was made based on piriform parasitic erythrocytic inclusions in peripheral blood smears and confirmed by *Babesia*-specific 18S rDNA PCR. Sequence analysis revealed a >99% homology of the amplicon with the recently described EU1 organism clustering within the *Babesia divergens/Babesia odocoilei* complex. Despite treatment with quinine and clindamycin the patient relapsed and developed chronic parasitaemia requiring re-treatment and long-term maintenance therapy with atovaquone before he eventually seroconverted and the parasite was cleared. Our findings suggest that human babesiosis occurs in Germany and can take a chronic course in immunocompromised individuals.

Keywords: Babesiosis; Germany; Immunocompromised patient; *Babesia* EU1

Introduction

Tick-borne infections are among the most important vector-borne infections in the northern hemisphere (Hunfeld and Brade, 2004). New infections have emerged all over Europe and the incidence of such diseases is rising steadily. Moreover, changes in the distribution and frequency of tick-borne diseases may be among the first impacts of global climatic changes on human health (Skarphedinsson et al., 2005). Babesiosis, which is caused by intraerythrocytic parasites of the protozoan genus *Babesia*, is one of the more common diseases of free-living animals worldwide and is gaining increasing attention as an emerging tick-borne zoonosis in humans (Herwaldt et al., 2003; Homer et al., 2000). The parasites are named after the Romanian scientist Victor Babes, who first identified pear-shaped, *Plasmodium*-like protozoan parasites as the causative agents of

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