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Prions in the Urine of Patients with Variant Creutzfeldt–Jakob Disease

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ABSTRACT

BACKGROUND

Prions, the infectious agents responsible for transmissible spongiform encephalopathies, consist mainly of the misfolded prion protein (PrPsc). The unique mechanism of transmission and the appearance of a variant form of Creutzfeldt–Jakob disease, which has been linked to consumption of prion-contaminated cattle meat, have raised concerns about public health. Evidence suggests that variant Creutzfeldt–Jakob disease prions circulate in body fluids from people in whom the disease is silently incubating.

METHODS

To investigate whether PrPsc can be detected in the urine of patients with variant Creutzfeldt–Jakob disease, we used the protein misfolding cyclic amplification (PMCA) technique to amplify minute quantities of PrPsc, enabling highly sensitive detection of the protein. We analyzed urine samples from several patients with various transmissible spongiform encephalopathies (variant and sporadic Creutzfeldt–Jakob disease and genetic forms of prion disease), patients with other degenerative or nondegenerate neurologic disorders, and healthy persons.

RESULTS

PrPsc was detectable only in the urine of patients with variant Creutzfeldt–Jakob disease and had the typical electrophoretic profile associated with this disease. PrPsc was detected in 13 of 14 urine samples obtained from patients with variant Creutzfeldt–Jakob disease and in none of the 224 urine samples obtained from patients with other neurologic diseases and from healthy controls, resulting in an estimated sensitivity of 92.9% (95% confidence interval [CI], 66.1 to 99.8) and a specificity of 100.0% (95% CI, 98.4 to 100.0). The PrPsc concentration in urine calculated by means of quantitative PMCA was estimated at 1×10⁻¹⁶ g per milliliter, or 3×10⁻²¹ mol per milliliter, which extrapolates to approximately 40 to 100 oligomeric particles of PrPsc per milliliter of urine.

CONCLUSIONS

Urine samples obtained from patients with variant Creutzfeldt–Jakob disease contained minute quantities of PrPsc. (Fundied by the National Institutes of Health and others.)
Prion diseases are fatal neurodegenerative disorders for which no therapy or definitive noninvasive intravitral diagnosis is available. These diseases affect humans and animals. Creutzfeldt–Jakob disease in humans and scrapie, bovine spongiform encephalopathy, and chronic wasting disease in animals are the most common forms of transmissible spongiform encephalopathies. The infectious agent in transmissible spongiform encephalopathies appears to be composed exclusively of the misfolded form of the prion protein (termed PrPSc), which self-propagates in the absence of nucleic acid. PrPSc replicates in infected persons by acting as a template for the misfolding of the cellular prion protein (PrPC). When exposed to the infectious agent, prions progressively replicate in the host body, and by the time clinical symptoms develop, large quantities of PrPSc have accumulated in the central nervous system. Although the disease is mostly confined to the central nervous system, small quantities of PrPSc are present in many tissues and body fluids even at early presymptomatic stages of the disease.

It is widely accepted that bovine spongiform encephalopathy has been transmitted to humans, generating a new disease termed variant Creutzfeldt–Jakob disease. Although the number of patients with this disease is fairly small (228 cases reported as of this writing), it is unclear how many people may carry infectious material due to a preclinical or subclinical condition. A recent retrospective study of archived surgically resected appendixes in the United Kingdom estimated that the prevalence of asymptomatic variant Creutzfeldt–Jakob disease infection in the U.K. population was approximately 1 case per 2000 persons; this suggests that approximately 30,000 people in the United Kingdom might be carriers of potentially infectious variant Creutzfeldt–Jakob disease prions. The potential existence of a large number of carriers raises the possibility of horizontal transmission of variant Creutzfeldt–Jakob disease from human to human, a course that has already led to hundreds of deaths in other human transmissible spongiform encephalopathies. Studies in animal models have shown that bovine spongiform encephalopathy infection often results in subclinical or carrier states, which on secondary transmission can produce the complete disease in a much more efficient way. This scenario is a concern, since cases of variant Creutzfeldt–Jakob disease have been linked to transfusion of blood donated by infected persons at the preclinical stage of the disease.

Noninvasive tests that can detect prions in samples of body fluids with high sensitivity and specificity could be useful for estimating the prevalence of variant Creutzfeldt–Jakob disease prion infection, reducing the risk of iatrogenic transmission, and facilitating prophylactic treatment of the disease. One of the most promising techniques for high-sensitivity detection of PrPSc is the protein misfolding cyclic amplification (PMCA) assay, which mimics in vitro the prion replication process occurring during the pathogenesis of prion disease. PMCA is a cyclic process that can detect the equivalent of a single oligomeric particle of PrPSc. This high level of sensitivity has permitted detection of PrPSc in the blood and urine of experimentally infected animals at both the symptomatic and presymptomatic stages of the disease. PMCA has been successfully used to detect PrPSc in affected species, including humans. We investigated whether PrPSc is present in the urine of patients with variant Creutzfeldt–Jakob disease and whether it can be detected by means of PMCA. Urine testing is the least invasive type of sampling for detection of a disease marker, and urine has been shown to carry small quantities of infectious PrPSc in experimental animal models.

**METHODS**

**PATIENTS AND STUDY OVERSIGHT**

We analyzed urine samples obtained from 68 patients with sporadic Creutzfeldt–Jakob disease and 14 patients with variant Creutzfeldt–Jakob disease (clinical information about these patients is provided in Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org), as well as 4 patients with genetic prion diseases, 50 patients with other neurodegenerative disorders (including Alzheimer’s disease, Parkinson’s disease, frontotemporal dementia, motor neuron disease, and progressive supranuclear palsy), 50 patients with nonneurodegenerative disorders (including cerebrovascular disease, multiple sclerosis, epilepsy, brain tumors, autoimmune encephalitis, meningitis,
myeloradiculopathy, and peripheral neuropathy), and 52 healthy persons. The diagnosis of human transmissible spongiform encephalopathy in all patients (except for 1 patient with variant Creutzfeldt-Jakob disease) was pathologically confirmed on autopsy or by means of biopsy of brain tissue, lymphoid tissue, or both. The diagnosis of the other neurologic diseases was established by means of clinical examination, as well as imaging and laboratory tests, when available. All patients and controls included in the study were homozygous for methionine at codon 129 of the gene encoding prion protein (PRNP) (PRNP genotyping is described in the Supplementary Appendix). Urine was collected at various times during the symptomatic period of the diseases and kept frozen at −80°C until analysis.

The methods of urine collection and genetic analysis were approved by the institutional review boards at the authors’ study centers, and all study participants provided written informed consent. The sponsors had no role in the study design, data collection and analysis, preparation of the manuscript, or decision to submit it for publication.

**URINE PROCESSING**

To attempt direct detection of PrPSc by means of Western blotting, 1 ml of each urine sample was concentrated by means of centrifugation at high speed (100,000×g for 1 hour at 4°C). The supernatant was discarded, and pellets were washed with phosphate-buffered saline. After washing, pellets were resuspended in 10 μl of phosphate-buffered saline. Samples were subjected to proteinase K digestion and Western blotting, as described below.

For PMCA, urine samples were processed as shown in Figure 1. Briefly, 1 ml of each sample was subjected to an initial low-speed centrifugation (5000×g for 20 minutes at 4°C) to precipitate debris. The supernatant was discarded, and pellets were washed with phosphate-buffered saline. After washing, pellets were resuspended in 10 μl of phosphate-buffered saline. Samples were subjected to proteinase K digestion and Western blotting, as described below.

PMCA was performed as previously described.22 As a substrate, we used brain specimens obtained from transgenic mice expressing human prion protein with methionine homozygosity at PRNP codon 129. Brains were harvested after perfusion, and 10% homogenate was prepared in conversion buffer (phosphate-buffered saline containing 150 mM sodium chloride and 1% Triton X-100) with the addition of protease inhibitors. To increase the efficiency of PMCA, 6 mM EDTA and 0.05% digitonin were added to the brain homogenate.

Urine samples, processed as described above, were mixed with 10% brain homogenate (from transgenic mice expressing human prion protein) in tubes containing three polytetrafluoroethylene (Teflon) beads and subjected to 96 cycles of PMCA with the use of a microsonicator (model Q700, Qsonica). Each cycle consisted of 29 minutes 20 seconds of incubation at 37 to 40°C, followed by a 40-second pulse of sonication set at a potency of 270 to 280 W. After one round of 96 PMCA cycles, an aliquot of the amplified material was diluted by a factor of 10 into fresh brain homogenate from transgenic mice and an additional round of PMCA cycles was performed. To avoid sample cross-contamination between rounds, thorough decontamination of instruments and equipment was performed with the
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Urine samples (1 ml)

Low-speed centrifugation (5000×g, 20 min)

Suspension in 1 ml of distilled water

Debris

High-speed centrifugation (100,000×g, 1 hr)

Suspension in brain specimens from transgenic mice (10% brain homogenate) (100 µl)

Supernatant

Pellet

Pellet

PrP Sc / (oligomeric seeds)

PrP C / (monomeric prion protein)

PMCA technique

Growth

Incubation

Sonication

Fragmentation

Repeat cycle

+ 

Debris

Suspension in 1 ml of distilled water

High-speed centrifugation (100,000×g, 1 hr)

Suspension in brain specimens from transgenic mice (10% brain homogenate) (100 µl)

Low-speed centrifugation (5000×g, 20 min)
Next, we used PMCA to detect PrP<sub>Sc</sub> in urine of patients with variant Creutzfeldt–Jakob disease. PMCA enabled PrP<sub>Sc</sub> detection in patients with variant Creutzfeldt–Jakob disease with an estimated sensitivity of 93% and an estimated specificity of 100%, and the positive and negative predictive values are close to 100% (Table 2). To investigate whether larger samples of urine from different control groups might be positive, we performed an experiment using samples of 10 ml of urine obtained from 10 patients with sporadic Creutzfeldt–Jakob disease, 10 patients with other neurodegenerative diseases, and 10 healthy controls. Samples were processed as described above, and all material obtained from the 10 ml of urine was used for the PMCA assays. As shown in Figure 3B, no signal was detected in any of these samples.

After proteinase K digestion, the electropho-
Figure 2. PrP<sub>Sc</sub> Detection in Urine by Means of PMCA.

As shown in Panel A, to optimize PrP<sub>Sc</sub> detection by means of PMCA, 1 ml of urine from healthy persons was supplemented with samples of variant Creutzfeldt–Jakob disease brain homogenate at distinct dilutions (10<sup>-5</sup> to 10<sup>-12</sup>). After processing by means of high-speed centrifugation, samples were subjected to various rounds of 96 PMCA cycles in the presence of 10% brain homogenate from transgenic mice expressing human prion protein, which was used as a substrate for PMCA. The PrP<sub>Sc</sub> signal was assessed by means of Western blot analysis after proteinase K digestion. NBH refers to the normal (healthy) brain homogenate. As shown in Panel B, urine samples obtained from patients with sporadic Creutzfeldt–Jakob disease MM1 (methionine homozygote, prion protein type 1) (lanes 1, 2, and 4), variant Creutzfeldt–Jakob disease (lane 3), and healthy controls (lanes 5, 6, and 7) were processed. Fractions corresponding to debris and supernatant were collected for each sample and subjected to serial rounds of 96 PMCA cycles. As a positive control, a urine specimen obtained from a healthy person (lane 9) was supplemented with a 10<sup>-5</sup> dilution of variant Creutzfeldt–Jakob disease brain homogenate. Lane 8 shows the molecular-weight marker, and lane 10 shows the normal brain homogenate, which was not treated with proteinase K (used as an electrophoretic migration marker). The result with the urine sample from one patient with variant Creutzfeldt–Jakob disease was representative of the 13 samples with positive test results. As shown in Panel C, urine samples obtained from patients who had other neurodegenerative disorders, including Alzheimer’s disease (lane 1), frontotemporal dementia (lanes 2 and 4), Parkinson’s disease (lane 3), and progressive supranuclear palsy (lane 5) were subjected to seven serial rounds of PMCA. The results for rounds 1, 3, 5, and 7 are shown, as well as the results with no amplification. Lane 6 shows a urine sample from a healthy control, lane 7 shows the molecular-weight marker, and lane 9 shows the normal brain homogenate not treated by proteinase K. Lane 8 shows samples of urine from a healthy control supplemented with a 10<sup>-5</sup> dilution of variant Creutzfeldt–Jakob disease brain homogenate. All samples were treated with proteinase K, except the NBH used as a PrP<sub>Sc</sub> migration control.
The retro characteristics of PrPSc in urine were the same in all the patients with variant Creutzfeldt–Jakob disease, regardless of their country of origin, and corresponded to the typical electrophoretic features of variant Creutzfeldt–Jakob disease (i.e., a type 2B mobility with predominance of the diglycosylated isoform) (Fig. 3A). All tests were repeated twice, and we never detected a false positive result in any of the 224 samples obtained either from patients with other diseases or healthy controls. To study whether PrPSc is detectable in the urine of patients with variant Creutzfeldt–Jakob disease at different times during the clinical phase of the disease, urine obtained at two distinct times from a single patient with variant Creutzfeldt–Jakob disease (vCJD, Patient 2 in Fig. 3A, and in Table S1 in the Supplementary Appendix) was analyzed by means of PMCA. As shown in Figure 3C, the 2 samples from this patient, collected 117 days apart, were both positive for PrPSc, with similar characteristics. However, the sample obtained later in the course of the clinical disease appeared to have a larger quantity of PrPSc. The estimation was based on a comparison of the PrPSc signal by means of Western blot analysis with a known concentration of recombinant prion protein (Fig. S2A in the Supplementary Appendix). Using this procedure, we calculated that the PrPSc concentration in the brain of this patient was approximately 100 μg per gram.

Thereafter, we supplemented urine from a healthy control with different concentrations of this brain homogenate and used these samples for PrPSc detection (Fig. S2B in the Supplementary Appendix). One round of PMCA enabled us to detect approximately 10−12 to 10−13 g of PrPSc per milliliter in urine. Two rounds of PMCA permitted detection of 10−15 to 10−16 g per milliliter, and three or more rounds of PMCA consistently detected 10−16 g per milliliter of PrPSc (Fig. S2B in the Supplementary Appendix), which is the last detectable quantity. Of the 13 urine samples obtained from patients with variant Creutzfeldt–Jakob disease in which PrPSc was detectable by means of PMCA, 1 was positive in the first round, 6 were positive in the second round, 3 in the third round, and 3 in the fourth round. Therefore, on average, PrPSc was detectable in urine in approximately 2.5 rounds of PMCA. Accordingly, our estimation is that the urine of patients with variant Creutzfeldt–Jakob disease contains a PrPSc concentration of approximately 1×10−16 g per milliliter, or 3×10−21 mol per milliliter (assuming the molecular weight of the monomer). This finding extrapolates to a concentration of approximately 2000 molecules of prion protein monomer per milliliter of urine. Since most estimations for the average size of the infectious PrPSc particle indicate that it contains between

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**Table 1. Urine Samples That Were Positive for PrPSc Detection by PMCA, According to Study Group.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Urine Samples in Which PrPSc Was Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with variant Creutzfeldt–Jakob disease</td>
<td>13/14</td>
</tr>
<tr>
<td>Patients with sporadic Creutzfeldt–Jakob disease</td>
<td>0/68</td>
</tr>
<tr>
<td>Patients with genetic prion disease (familial Creutzfeldt–</td>
<td>0/4</td>
</tr>
<tr>
<td>Jakob disease or Gerstmann–Sträussler–Scheinker syndrome)</td>
<td></td>
</tr>
<tr>
<td>Patients with other degenerative neurologic diseases</td>
<td>0/50</td>
</tr>
<tr>
<td>Patients with nondegenerative neurologic diseases</td>
<td>0/50</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>0/52</td>
</tr>
</tbody>
</table>

* PMCA denotes protein misfolding cyclic amplification, and PrPSc misfolded prion protein.
Figure 3. PrPSc Detection in Urine Samples Obtained from Patients with Variant Creutzfeldt–Jakob Disease.

Urine samples obtained from 14 patients with variant Creutzfeldt–Jakob disease (vCJD) were used to detect PrPSc by means of PMCA. Panel A shows the results obtained with the use of the supernatant fraction after three rounds of PMCA for four urine samples obtained from patients with vCJD, as well as samples obtained from persons with other neurodegenerative diseases and healthy controls (HC) (left). The dotted line indicates blot splicing. To compare the electrophoretic migration, aliquots of brain homogenate from patients with vCJD and patients with sporadic Creutzfeldt–Jakob disease MM1 (sCJD) were run in the same gel. Results obtained with the supernatant fraction of various nonvariant Creutzfeldt–Jakob disease urine samples (third round of PMCA) are shown on the right. NBH corresponds to the aliquots of brain homogenate from transgenic mice expressing human prion protein without proteinase K treatment, which were used as a migration control for PrPSc electrophoretic mobility. AD denotes Alzheimer’s disease, fCJD familial Creutzfeldt–Jakob disease, FTD frontotemporal dementia, GSS Gerstmann–Sträussler–Scheinker syndrome, PD Parkinson’s disease, and PSP progressive supranuclear palsy. As shown in Panel B, to determine whether PrPSc can be detected in larger volumes of urine in samples obtained from patients with nonvariant Creutzfeldt–Jakob disease, 10 ml of urine from 10 different patients with other neurologic diseases (ND), 10 patients with sCJD, and 10 healthy persons were processed without separating debris from supernatant. The entire content pelleted from the 10 ml was used for PMCA. The results obtained after four rounds of PMCA for urine samples obtained from 8 of the 10 persons in each group are shown. NBH refers to the normal brain homogenate not treated with proteinase K, which was used as a migration control. As shown in Panel C, urine samples (1 ml) obtained at two different times from the same patient (vCJD, Patient 2) were subjected to PMCA detection. The data in parentheses refer to the dates on which urine samples were obtained. The results obtained after three or four rounds of PMCA are shown. Control samples obtained from patients with Alzheimer’s disease (AD), patients with Parkinson’s disease (PD), and healthy persons were run simultaneously. The asterisks indicate faint signals from incomplete PrPSc digestion.
20 and 50 monomers of prion protein, we conclude that 1 ml of urine obtained from a patient with variant Creutzfeldt–Jakob disease has between 40 and 100 oligomer equivalents of PrPSc.

**DISCUSSION**

We found that small quantities of PrPSc are excreted into the urine of most patients with variant Creutzfeldt–Jakob disease. The one patient (Patient 8 in Table S1 in the Supplementary Appendix) with a urine sample that was found to be negative had a disease duration of 114 months and was receiving treatment with intracerebroventricular administration of pentosan polysulfate, an agent that has been studied for the treatment of variant Creutzfeldt–Jakob disease. Another patient with variant Creutzfeldt–Jakob disease (Patient 10), whose urine was positive for PrPSc, was receiving similar treatment; however, this patient had disease with a duration of 16 months (Table S1 in the Supplementary Appendix).

PrPSc was detectable only after extensive amplification by means of PMCA, suggesting that the concentration of the infectious protein in urine is small. Estimations by means of quantitative PMCA suggest that 1 ml of urine obtained from a patient with variant Creutzfeldt–Jakob disease contains as little as 40 to 100 oligomeric PrPSc particles. The results also indicate that urinary PrPSc has the ability to self-propagate in vitro by inducing the misfolding of normal human PrP. PrPSc was not detectable in any of the urine samples obtained from patients who had other human transmissible spongiform encephalopathies, suggesting that it is an exclusive feature of variant Creutzfeldt–Jakob disease. This result is not entirely surprising, considering that variant Creutzfeldt–Jakob disease is acquired by infection (probably by oral ingestion), and studies have shown more extensive peripheral involvement in variant Creutzfeldt–Jakob disease than in the sporadic or inherited forms of transmissible spongiform encephalopathies. Indeed, PrPSc is readily detectable by means of immunohistochemical or biochemical studies in lymph nodes, tonsil, spleen, portions of the intestinal tract, kidney, liver, pancreas, and skeletal muscle from patients with variant Creutzfeldt–Jakob disease.

In conclusion, the results of our study show that PrPSc can be detected in the urine of patients with variant Creutzfeldt–Jakob disease with high sensitivity and specificity. Further studies are needed to determine whether the detection of PrPSc in urine can be used diagnostically.

The views expressed in this article are those of the authors and not necessarily those of the U.K. Department of Health.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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