A Pitfall in Diagnosis of Human Prion Diseases Using Detection of Protease-resistant Prion Protein in Urine

CONTAMINATION WITH BACTERIAL OUTER MEMBRANE PROTEINS*

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Because a definite diagnosis of prion diseases relies on the detection of the abnormal isoform of prion protein (PrPSc), it has been urgently necessary to establish a non-invasive diagnostic test to detect PrPSc in human prion diseases. To evaluate diagnostic usefulness and reliability of the detection of protease-resistant prion protein in urine, we extensively analyzed protease K (PK)-resistant proteins in patients affected with prion diseases and control subjects by Western blot, a coupled liquid chromatography and mass spectrometry assay, and N-terminal sequence analysis. The PK-resistant signal migrating around 32 kDa previously reported by Shaked et al. (Shaked, G. M., Shaked, Y., Kariv-Inbal, Z., Halimi, M., Avraham, I., and Gabizon, R. (2001) J. Biol. Chem. 276, 31479–31482) was not observed in this study. Instead, discrete protein bands with an apparent molecular mass of ~37 kDa were detected in the urine of many patients affected with prion diseases and two diseased controls. Although these proteins also gave strong signals in the Western blot using a variety of anti-PrP antibodies as a primary antibody, we found that the signals were still detectable by incubation of secondary antibodies alone, i.e. in the absence of the primary anti-PrP antibodies. Mass spectrometry and N-terminal protein sequencing analysis revealed that the majority of the PK-resistant 37-kDa proteins in the urine of patients were outer membrane proteins (OMPs) of the Enterobacterial species. OMPs isolated from these bacteria were resistant to PK and the PK-resistant OMPs from the Enterobacterial species migrated around 37 kDa on SDS-PAGE. Furthermore, nonspecific binding of OMPs to antibodies could be mistaken for PrPSc. These findings caution that bacterial contamination can affect the immunological detection of prion protein. Therefore, the presence of Enterobacterial species should be excluded in the immunological tests for PrPSc in clinical samples, in particular, urine.

Prion diseases are a group of neurodegenerative disorders pathologically characterized by accumulation of an abnormal isoform of prion protein (PrPSc) in the central nervous system. A definite diagnosis of prion diseases relies on the detection of PrPSc (1). Concerning the link between bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease (CJD), the iatrogenic occurrence of prion diseases after dural transplantation, and the recent remarkable progress in therapeutic approaches have made it urgently necessary to establish a non-invasive in vivo test to enable a definite diagnosis of human prion diseases in the early or preclinical stage of the disease.

Diffusion-weighted magnetic resonance imaging of the brain is currently one of the most helpful techniques to detect abnormal high intensity lesions in the cerebral cortices and basal ganglia in the early stage of the disease (2). The detection of 14-3-3 proteins and measurement of phosphorylated tau protein in the cerebrospinal fluid has been found to be useful in supporting the clinical diagnosis of CJD (3,4). Although these tests are clinically useful, they are surrogate markers and therefore cannot provide direct evidence of the presence of PrPSc. Moreover, although a brain biopsy can reveal the deposition of PrPSc in the brain (5), it is highly invasive and is not suitable for preclinical screening or early diagnosis. Detection of PrPSc in body fluids such as blood and cerebrospinal fluid has been extensively investigated, but these tests still need a new technological device to increase the sensitivity (6).

As a potentially non-invasive diagnostic test, Shaked et al. (7) reported the presence of protease-resistant PrP in the urine (UPrPSc) of humans and animals affected with prion diseases. Their data suggests that UPrPSc will reflect the presence of PrPSc in the central nervous system and will also be a useful preclinical diagnostic test for prion diseases. In the present study, we have examined the urine protein of humans affected with prion diseases and controls using Western blot analysis to evaluate diagnostic usefulness and reliability of the UPrPSc assay in human prion diseases. A detailed analysis using coupled liquid chromatography and mass spectrometry (LC/MS) and N-terminal protein sequencing revealed that bacterial contamination might account for the misinterpretation in the interpretation of protease-resistant protein in urine.

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2 The abbreviations used are: CJD, Creutzfeldt-Jakob disease; PBS, phosphate-buffered saline; PK, protease K; OMP, outer membrane proteins.
**Bacterial Contamination and Prion Protein in Urine**

The abbreviations used are: GSS; Garstmann-Straussler-Scheinker syndrome; HDS-R; revised Hasegawa Dementia Rating Scale; MMSE; Mini-Mental State Examination; and MELAS; mitochondrial myopathy, lactic acidosis, and stroke-like episodes.

![Image](image.jpg)

**Table I**

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>No. of cases</th>
<th>Mean age</th>
<th>Mean clinical duration at examination</th>
<th>CSF 14-3-3 protein positive ratio</th>
<th>Brain DWI MRI positive ratio</th>
<th>Protease-resistant protein in urine positive ratio</th>
</tr>
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<tr>
<td><strong>Prion diseases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sporadic CJD</td>
<td>45</td>
<td>65.9</td>
<td>5.5 (1.5-18)</td>
<td>93.5 (29/31)</td>
<td>75.0 (15/20)</td>
<td>66.7 (30/45)</td>
</tr>
<tr>
<td>Dural graft-associated CJD</td>
<td>4</td>
<td>53.8</td>
<td>20.5 (6-48)</td>
<td>100 (3/3)</td>
<td>50 (1/4)</td>
<td>100 (4/4)</td>
</tr>
<tr>
<td>Familial CJD (E200K)</td>
<td>2</td>
<td>58.5</td>
<td>3.8 (3.5-4)</td>
<td>50 (1/2)</td>
<td>100 (2/2)</td>
<td>100 (2/2)</td>
</tr>
<tr>
<td>GSS (P102L)</td>
<td>3</td>
<td>57.3</td>
<td>45.3 (28-72)</td>
<td>NE</td>
<td>NE</td>
<td>66.7 (2/3)</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>58.9</td>
<td>9.58</td>
<td>91.7 (33/36)</td>
<td>69.2 (18/26)</td>
<td>70.4 (38/54)</td>
</tr>
</tbody>
</table>

**Note:**
- Determined by the presence of PK-resistant signal around 37 kDa.
- NE, not examined.
- NA, not available.
- Determined by the presence of PK-resistant signal around 37 kDa.
- NE, not examined.
- NA, data not available.
- Two patients were included in both groups.
- Identical patient.

## EXPERIMENTAL PROCEDURES

**Analysis of Human Urine**—Urine samples were collected from patients affected with prion diseases and control subjects under informed consent (Table I). A clinical diagnosis of prion disease was made by neurologists in order to follow the diagnostic criteria proposed by the World Health Organization (1). First morning urine samples were used whenever possible.

Protein was isolated from urine as previously described by Shaked et al. (7) with minor modifications. After dialysis and sedimentation by ultracentrifugation, the pellets obtained from 15 ml of urine were re-suspended in 30 µl of PBS (pH 7.4) containing 0.5% Nonidet P-40 and 0.5% sodium deoxycholate, instead of STE buffer containing 2% Sarkosyl, and digested with 40 µg/ml proteinase K (PK) (Roche Diagnostics) at 37 °C for 1 h. In some samples, urine protein was re-suspended in 2% Sarkosyl STE buffer prior to PK digestion as described by Shaked et al. (7).

Western blot analysis was performed using monoclonal antibodies 3F4 at 1:10,000 (Signet Laboratories), 6H4 at 1:5,000 (Prionics, Switzerland), or 3O8 at 1:1,000 (Cayman Chemical) followed by incubation with donkey-derived anti-mouse IgG (AP192A, Chemicon), goat-derived anti-rabbit IgG (BA1050, Promega), or the F(ab’)2 fragment of rabbit-derived anti-mouse IgG (710–4520, Rockland) and developed in a chemiluminescent substrate (CDP Star or ECL-Plus, Amersham Biosciences). Some blots were labeled with PrP2B, rabbit-derived polyclonal antibody raised against PrP-F89-103, followed by incubation with donkey-derived anti-rabbit IgG (AP182A, Chemicon). In some blots, incubation with primary antibody was omitted for the experimental purpose.

**Coupled Liquid Chromatography and Mass Spectrometry (LC/MS) Analysis of Protease-resistant Proteins**—A PK-resistant signal of 37 kDa on a SDS-polyacrylamide gel was cut out and transferred to a clean, siliconized Eppendorf tube. In-gel digestion was performed as previously described (8). After an overnight incubation of gels with trypsin at 37 °C, the digested protein was extracted twice with 50% acetonitrile, 50% trifluoroacetic acid and concentrated by vacuum centrifugation. An LC/MS analysis was performed using the QSTAR XL system (Applied Biosysytem) and MALDI 2002 liquid chromatography (Michrom BioResourse). The obtained protein masses were queried against entries for all species in the SwissProt data base using the Mascot Search program offered by Matrix Science.

N-terminal Protein Sequencing—PK-resistant protein was obtained from the urine of patients sCJD 4, 5, and 7 and dural graft-associated CJD-1 as described above, or from urine of other patients as described by Shaked et al. (7). After separation of the protein samples by 12% mini SDS-PAGE gels (Bio-Rad), proteins were transferred onto Immobilon-P (Millipore). PK-resistant bands visualized by Coomassie Brilliant Blue staining were cut out and stored at 4 °C until the sequencing procedure. N-terminal protein sequencing by automated Edman degradation was performed using the Procise 491LC protein sequencer (Applied Biosysytem), as previously described (9). N-terminal sequencing proceeded for 13 to 23 cycles. The obtained amino acid sequences were queried against entries for all species in the SwissProt data base using the FASTA search program offered by GenomeNet.

**Assays of Protease Resistance of Outer Membrane Proteins (OMP)—** OMPs were isolated from Klebsiella pneumoniae and Salmonella typhimurium as previously described (10) with minor modifications. In brief, cells harvested from overnight cultures in Super Broth medium were recovered by centrifugation. After washing with 10 mM Tris-HCl (pH 7.2), 5 mM MgCl2, cells were broken by sonication. Unbroken cells were eliminated and cell envelopes were recovered at 100,000 g for 1 h. After solubilization in 10 mM Tris-HCl (pH 7.2), 5 mM MgCl2, 0.5% Nonidet P-40, 0.5% deoxycholate for 30 min at 25 °C, insoluble OMPs were recovered by ultracentrifugation at 100,000 × g for 1 h. OMPs were resuspended in 0.5% Nonidet P-40, 0.5% deoxycholate in PBS (pH 7.4), or 2% Sarkosyl STE buffer and digested with 40 µg/ml of PK at 37 °C for 1 h, under the same conditions as urine proteins. After digestion, 15 µg of ovalbumin was digested with PK as a control.

**Binding of OMPs to F(ab’)2 Fragment of Immunoglobulin—** Isolated OMPs were separated by 12% SDS-PAGE and transferred onto the

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2 fasta.genome.ad.jp.
polyvinylidene difluoride membrane. To determine whether OMPs bind to antibodies via the Fc region of immunoglobulin, the membrane was incubated with the F(ab')2 fragment of anti-mouse IgG conjugated with alkaline phosphatase (710–4520, Rockland) after blocking with 5% nonfat milk. The membrane was then developed in a chemiluminescent substrate (CDP Star, Amersham Biosciences).

RESULTS

PK-resistant Protein in the Urine of Humans Affected with Prion Diseases Directly Reacted with Secondary Antibodies—

The results of urine protein examination and patient and control characteristics are summarized in Table I. We examined the PK sensitivity of urine proteins of patients affected with prion diseases (n = 54), healthy controls (n = 23), and disease control patients with (n = 20) and without dementia (n = 23). Clinical durations between disease onset and urine collection were 1.5 to 72 months in prion diseases. 14-3-3 protein in cerebrospinal fluid was frequently positive in sporadic CJD (93.5%, 29/31) and dural graft-associated CJD (100%, 3/3). Abnormal high intensity signals in the cerebral cortices or basal ganglia were observed in the majority of patients with sporadic CJD (75.0%, 15/20). Most patients carried methionine homozygosity at codon 129 in the prion protein gene (PRNP), except for one case that was affected with Gerstmann-Sträussler-Scheinker syndrome. No patients carried lysine polymorphisms at codon 219 in the PRNP.

Three kinds of signals migrating around 37, 28, and 22 kDa were observed after PK treatment of the urine. PK-resistant signals of 37 kDa were prominent and observed in all positive cases, whereas the other two signals were usually faint and not always observed in all the positive cases. The signals of 28 kDa were also observed in controls after digestion with PK, suggesting that it represented a nonspecific signal because of PK itself. PK-resistant signals around 32 kDa, detected by Shaked et al. (7) in CJD patients, were not observed in the present study. Therefore, we decided to utilize the 37-kDa signal as a PK-resistant protein in urine in this study. PK-resistant protein signals of 37 kDa were detectable in 70.4% (38/54) of the patients affected with prion diseases, whereas 3% (2/66) of the control subjects were positive for PK-resistant signals. The PK-resistant signal was not detectable in healthy controls or diseased controls with dementia (Table I and Fig. 1B).

Although PK-sensitive and -resistant signals were detectable by labeling with 3F4 (Fig. 1A, left panel), 6H4, 308, or PrP2B (data not shown), these signals were also detectable with anti-mouse IgG antibody alone, omitting the incubation with 3F4 (Fig. 1A, right panel). This phenomenon was observed in all cases (11 cases; sporadic CJD, one case; dura-associated CJD) tested and reproducible using three kinds of anti-mouse IgG antibodies (AP192A, Chemicon; S372B, Promega; and 710–4520, Rockland) and an anti-rabbit IgG antibody (AP182A, Chemicon) (data not shown).

To examine the possible influence of assay conditions on the detection of PK-sensitive or -resistant signals, urine proteins were re-suspended prior to PK digestion in 2% Sarkosyl STE buffer as described previously by Shaked et al. (7) or in 0.5% Nonidet P-40, 0.5% deoxycholate, PBS buffer. As shown in Fig. 1C, 37-kDa signals were similarly detectable in both assay conditions, indicating that the difference of the assay conditions did not influence the detection of these signals.

Contamination of Urine with Bacterial Outer Membrane Proteins—To characterize the PK-resistant protein of 37 kDa on Western blot analysis, the bands from the urine of three pa-
OMPs were isolated from overnight cultured *K. pneumoniae* in Super Broth medium. After digestion with PK, a considerable amount of OMPs remained undigested and migrated around 37 kDa on SDS-PAGE (Fig. 3A, *fourth and sixth lanes*), whereas ovalbumin was completely digested under the same conditions (Fig. 3A, *second lane*). The electrophoretic mobility of PK-resistant OMPs was similar to that of the PK-resistant urine protein isolated from a patient affected with sporadic CJD (Fig. 3A, *seventh lane*).

**OMPs Are Resistant to PK**—To evaluate PK sensitivity, OMPs were isolated from overnight cultured *K. pneumoniae* or *S. typhimurium* in Super Broth medium. After digestion with PK, a considerable amount of OMPs remained undigested and migrated around 37 kDa on SDS-PAGE (Fig. 3A, *fourth and sixth lanes*), whereas ovalbumin was completely digested under the same conditions (Fig. 3A, *second lane*). The electrophoretic mobility of PK-resistant OMPs was similar to that of the PK-resistant urine protein isolated from a patient affected with sporadic CJD (Fig. 3A, *seventh lane*).

**OMPs Reacted with the F(ab′)2 Portion of Immunoglobulins**—To evaluate if OMPs bind to the Fc region of immunoglobulins like protein A, a cell wall component of *Staphylococcus aureus*, one of the gels was blotted onto a polyvinylidene difluoride membrane to perform Western blot analysis. As shown in Fig. 3B, OMPs bound to the F(ab′)2 fragment of anti-mouse IgG, indicating that OMPs bind to immunoglobulins in a manner that is different from that of protein A with immunoglobulins. This observation as well as PK resistance of OMPs was not influenced by the difference in assay conditions (Fig. 3C).

**DISCUSSION**

In the present study, we found that the PK-resistant protein was frequently detected in the urine of patients affected with prion diseases. However, the LC/MS and N-terminal protein sequencing analysis revealed that the majority of PK-resistant proteins in the urine of patients, which migrated around 37 kDa on SDS-PAGE and reacted non-specifically with several secondary antibodies, comprised OMPs of bacteria such as *E. coli*, *K. pneumoniae*, and *S. typhimurium*, the popular causative agents for urinary tract infections. This finding indicated that bacterial contamination of urine might cause false-positive results in the assay for detecting UPrPSc.

It is known that urinary tract infections associated with urethral catheterization is the most common nosocomial infection and is often asymptomatic. In this study, the majority of patients affected with prion diseases were already bearing catheters because of severe deterioration of intellectual and motor functions as they were suspected to be suffering from prion diseases. Furthermore, two of the diseased control patients with positive PK-resistant urine protein were also bearing persistent or intermittent urethral catheters. One patient suffered from neurogenic bladder because of multiple sclerosis and another was long bedridden because of cerebral infarction. The signal intensity decreased after PK digestion in a patient affected with multiple sclerosis (Fig. 1B), whereas it was not significant in patients affected with sporadic CJD (Fig. 1A).

Differences in bacterial species or growth conditions in urine between the cases might cause such a variation in PK sensitivity of OMPs. On the other hand, all diseased controls affected with Alzheimer’s disease or cerebrovascular dementia were outpatients; therefore, they were thought to be at lower risk of urinary tract infections. These circumstances strongly supported that possible bacterial contamination resulted in the detection of confusing PK-resistant protein in urine.

OMPs are 36- to 39-kDa membrane spanning proteins that form channels in the outer membranes of Gram-negative bacteria. The primary and secondary structures of OMPs are well conserved in *Enterobacterial* species containing 16-stranded antiparallel β barrels to form channels (11). Biochemically,
Fig. 2. LC/MS analysis of the PK-resistant protein in the urine. Peptides from tryptic digestion of the PK-resistant protein in the urine of sCJD-3 were separated using MAGIC 2002 liquid chromatography and the eluate were analyzed by MS. A, base peak mass chromatogram of the 37-kDa protein, each peak is labeled with the retention time. B, molecular mass and amino acid sequence of each peak originating from the 37-kDa protein. All the determined amino acid sequences were identical to that of OMP of E. coli (OmpC precursor) based on the results of the data base search. R. Time, retention time; Mr(expt), molecular weight in the experiment; Mr(calc), molecular weight in calculation. C, amino acid sequence of OmpC of E. coli was shown. Bold style letters indicate sequences covered by the results of the LC/MS analysis. The sequence identified by N-terminal sequencing analysis is shown with an underline.
OMPs are heat modifiable and resistant to trypsin (12). In this study, we have confirmed that OMPs of *K. pneumoniae* and *S. typhimurium* were also resistant to PK and the resulting molecules migrated around 37 kDa on SDS-PAGE.

OMPs act as a determinant of the permeability of antimicrobial agents and affect the interaction between bacteria and host defense mechanisms (13). Whereas it is known that the OMPs of *K. pneumoniae* bind to C1q (14), there are no previous reports that indicate the binding between OMPs and IgG. We found that OMPs bound non-specifically to IgG (several kinds of antibodies) during the procedure of Western blot analysis.

Because protein A, a cell wall component of *S. aureus*, has been known to bind to the Fc region of IgG (15), we hypothesized that OMPs might also bind to IgG in the same manner. Contrary to our expectations, OMPs still reacted with the F(ab')\textsubscript{2} fragment of anti-mouse IgG, indicating that they bound to IgG.

**TABLE III**

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>N-terminal sequence</th>
<th>Protein identification and species</th>
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</thead>
<tbody>
<tr>
<td>Norwegian CJD</td>
<td>AEIYNKDGNK</td>
<td>OmpC, <em>K. pneumoniae</em></td>
</tr>
<tr>
<td>sCJD-1</td>
<td>AEIYNKDGNK</td>
<td>OmpC, <em>E. coli</em></td>
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<tr>
<td>sCJD-2</td>
<td>AEIYNKDGNK</td>
<td>OmpC, <em>E. coli</em></td>
</tr>
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<td>sCJD-5</td>
<td>AEIYNKDGNK</td>
<td>OmpC, <em>S. typhimurium</em></td>
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<tr>
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<td>sCJD-11</td>
<td>AEIYNKDGNK</td>
<td>OmpC, <em>E. coli</em></td>
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</table>

**Urine samples were re-suspended in STE buffer containing 2% Sarkosyl as described by Shaked et al. (7) or otherwise in PBS containing 0.5% Nonidet P-40 and 0.5% deoxycholate.**

**Fig. 3. PK sensitivity and immunoreactivity of OMPs of *K. pneumoniae* and *S. typhimurium*.**

A. OMPs isolated from *K. pneumoniae* and *S. typhimurium*, and protein isolated from 15 ml of urine of sCJD-1 patient were digested with PK. Proteins were re-suspended in PBS (pH 7.4) containing 0.5% Nonidet P-40 and 0.5% deoxycholate (DOC). An OMP homogenate containing 30 g of protein was applied in each lane. Fifteen micrograms of ovalbumin was used as a control. After the electrophoretic separation, the polyacrylamide gel was stained with Coomassie Brilliant Blue. B. After separation by SDS-PAGE, the gel was blotted onto a polyvinylidene difluoride membrane. Rabbit-derived F(ab')\textsubscript{2} fragment of anti-mouse IgG was used as a probe. C. OMPs isolated from *K. pneumoniae* were re-suspended in PBS (pH 7.4) containing 0.5% Nonidet P-40 and 0.5% deoxycholate or in STE buffer containing 2% Sarkosyl and digested with PK. The blot was incubated with the rabbit-derived F(ab')\textsubscript{2} fragment of anti-mouse IgG.
in a manner that is different from that of protein A with immunoglobulins. It might be suggested that accidentally acquired antibodies against bacterial OMPs in the serum of immunized animals might react with OMPs, resulting in protease-resistant signals. However, Western blot analysis using anti-mouse IgG produced by a phage-display method, for example, would be required to exclude this hypothesis.

Our findings were not consistent with those of a previous report by Shaked et al. (7). They showed that PK-resistant proteins in the urine of patients and animals affected with prion diseases were prion protein and termed them UPrPSc. The signal of UPrPSc showed a downward shift after PK digestion resulting in a 32-kDa fragment, whereas the majority of PK-resistant signals that we detected did not show a significant downward shift. Apart from the 37-kDa PK-resistant signal, a faint 22-kDa signal was observed in some patients and a 28-kDa signal was observed in both patients and controls. N-terminal sequencing revealed that these signals were fragments of OMPs and PK molecules, respectively. In this study, we did not observe any PK-resistant signals migrating around 32 kDa, which was detected by Shaked et al. (7) in the urine of patients. Therefore, the possibility that the PK-resistant molecule in this study might be a different molecule from UPrPSc, as demonstrated by Shaked et al. (7), was not excluded.

However, the high incidence of OMPs (37-kDa PK-resistant signals, non-specifically bind to immunoglobulins) in the urine of patients affected with prion diseases, irrespective of the assay conditions, indicated that bacterial contamination would always have to be considered in the application of a UPrPSc assay in the diagnosis of human prion diseases. Our findings suggest that PrPSc and PrPC may not always exist or could exist at a very low level in urine, and bacterial contamination may often cause false detection of a PK-resistant isoform of prion protein in urine and a misinterpretation of results.

We have also analyzed the urine protein of mice experimentally infected with a prion agent. The PK-resistant signals of 25 kDa were found in the urine of infected mice, but these signals were also detectable using a secondary antibody alone, omitting the labeling by a primary antibody (data not shown). Furthermore, N-terminal sequencing analysis revealed that these PK-resistant signals in mice urine were OMPs of Pseudomonas aeruginosa.

In conclusion, the detection of UPrPSc is not useful or reliable for ante-mortem, definite diagnosis of human prion diseases in the present situation. Further improvement in sensitivity and specificity of this assay may make it a powerful diagnostic tool for prion diseases in the future.

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