Enhanced prion detection in biological samples by magnetic particle extraction and real-time quaking-induced conversion

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Prions have been demonstrated in body fluids and excreta using bioassay, but at levels too low for detection by conventional direct-detection assays. More rapid and sensitive detection of prions in these clinically accessible specimens would be valuable for diagnosis and investigations of transmission, environmental impact, and interventions. In addition to very low concentrations of prions, in vitro amplification assays are challenged by the presence of inhibitors in these complex sources. Here, we leverage the prion attribute of avid metal binding with the versatile power of real-time quaking-induced conversion (RT-QuIC) to enhance and simplify detection of chronic wasting-disease prions in biological samples. Iron oxide particle binding and magnetic extraction combined with RT-QuIC permitted rapid analysis of the low concentrations of prions in saliva, urine, faeces, and cerebrospinal fluid. These methods are pertinent to ante-mortem detection, monitoring, and surveillance, and could conceivably be applicable to other protein-misfolding disorders.

Mammalian prion diseases include human Creutzfeldt-Jakob disease (CJD), sheep scrapie, bovine spongiform encephalopathy (BSE), cervid chronic wasting disease (CWD) and others. In varying degrees, as each disease progresses, low levels of infectious prions are present in peripheral tissues (especially lymphoid) (Sigurdson et al., 2001; Miller & Williams, 2002; Spraker et al., 2002; van Keulen et al., 1996) and may also be present in body fluids/excreta (blood, saliva, urine, faeces, nasal secretions) (Mathiason et al., 2006; Gonzalez-Romero et al., 2008; Safar et al., 2008; Orrú et al., 2014). Sensitive and rapid detection of low levels of prions in early stages of infection would aid substantially in understanding disease pathogenesis and epidemiology, and enable assessment of therapeutic and management strategies. There are, however, substantial challenges to this goal, including limits in assay sensitivity, specificity, and presence of assay inhibitors in body fluids/excreta (Chen et al., 2010; Atarashi et al., 2007).

While animal bioassays have demonstrated infectious prions in the above body fluids/excreta (Haley et al., 2009; Tamgüney et al., 2012; Mathiason et al., 2006), practical limitations of sensitivity, time, cost, and animal use have severely limited the real-time detection of prion disease and assessment of environmental prion contamination. In vitro amplification assays, notably protein-misfolding cyclical amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC), have vastly improved the detection of minute quantities of prions in tissues (Saborio et al., 2001; Atarashi et al., 2007). However, the detection of prion activity in body fluids/excreta has still proven difficult, even when concentration methods such as sodium phosphotungstic acid (NaPTA) precipitation are employed (Elder et al., 2013; Henderson et al., 2015b).

The binding of prions to metal surfaces has long been recognized (Bernoulli et al., 1977; Gibbs et al., 1994; Zobeley et al., 1999), and is presumably reflective of the metal-binding domains in the prion protein (Brown et al., 1997; Hornshaw et al., 1995). Metal-binding properties have been exploited to detect prions in brain using PMCA and in blood using enzyme-linked immunosorbent assay (ELISA) with or without cell-culture amplification (Miller & Supattapone, 2011; Lim et al., 2015; Edgeworth et al., 2011). In addition, Orrú et al. (2011) demonstrated that antibody-conjugated metal beads incubated with prion-positive brain and blood could bind prion-seeding activity detectable by RT-QuIC. Despite the above advances, a rapid, practical, consistent means of detecting and quantifying low levels of prions in tissues and body fluids/excreta is needed. In this respect, we describe a simplified,
inexpensive method for enhanced detection of CWD prions using iron oxide magnetic extraction coupled with real-time quaking-induced conversion (IOME-RT-QuIC) with versatility sufficient for application to brain, saliva, urine, faeces and cerebrospinal fluid with little variation in technical preparation. IOME-RT-QuIC may have potential to advance ante-mortem detection, enhance surveillance, and may lead to therapeutic strategies for multiple prion diseases.

The samples used in the present studies were collected from deer exposed to CWD by aerosol or oral inoculation of brain homogenate from a CWD-positive or -negative deer as previously described (Denkers et al., 2013; Goñi et al., 2015). All animal-related protocols and treatment procedures were approved by the CSU IACUC and adhered to.

The IOME protocol utilizes superparamagnetic iron oxide beads to extract amplification-competent prion amyloid from various sources. Superparamagnetic iron oxide beads (~9 μm; Bangs Laboratories, Indiana) were vortexed and 2 μl (49 mg ml^-1) were aliquoted, then washed twice with 0.5 ml 1× phosphate buffered saline (PBS) (20 mM NaPO_4, 150 mM NaCl, pH 7.4). Beads were separated and supernatants removed using a magnetic particle separator (MPS) (Pure Biotech, New Jersey), then resuspended in 0.5 ml of each sample, unless otherwise specified. Samples were incubated using an end-over-end rotator for 2 h at room temperature then set in the MPS for 5 min. Unless specified, supernatants were removed and beads resuspended in 10 μl of 0.1% sodium dodecyl sulphate (SDS). Two microlitres of beads from each extracted sample were added directly to quadruplicate wells containing RT-QuIC reaction mixture [380 mM NaCl, 20 mM NaPO_4, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 μM Thioflavin T, 0.1 mg ml^-1 Syrian hamster PrP (amino acids 90–231)]. No decrease in fluorescence signal was observed from the presence of 2 μl of beads but higher amounts of beads decreased fluorescence values (data not shown).

RT-QuIC reactions were performed as previously described (Henderson et al., 2013). Reactions were deemed positive if the raw fluorescence value was greater than the mean initial fluorescence value plus 5 standard deviations of that value (~11 000–15 000 fluorescence units). The inverse (1/time to threshold) of the time in hours when the sample crossed the threshold, is the amyloid formation rate (AFR). Importantly, use of iron oxide beads did not increase or affect the false-positive rate in RT-QuIC assays. Only one of the CWD-negative brain homogenates analysed by IOME-RT-QuIC or un-extracted serial dilutions produced positive reactions (1/128 replicates) (data not shown).

In order to determine the increase in sensitivity achieved with the use of iron oxide particles, we analysed serial dilutions (10⁻²–10⁻¹⁰) of CWD-positive and -negative brain as either 2 μl of each dilution per RT-QuIC well (un-extracted) or 500 μl of each serial dilution extracted with IOME. To display quantitative differences between IOME-RT-QuIC and un-extracted samples, we displayed the AFR.

**Fig. 1.** IOME increases the detection level of prions in brain samples by two orders of magnitude. (a) AFRs of serially diluted prion brain samples (10⁻⁵–10⁻¹⁰) either IOME (blue) or un-extracted (black). Blue or black symbols indicated mean AFR and error bars indicate standard deviation. (b) Individual replicates for 10⁻⁷–10⁻¹⁰ for un-extracted (black) and IOME-RT-QuIC (blue). IOME decreases range/variance and increases sensitivity (more positive replicates) at lower dilutions. (c) Increased sensitivity is observed when larger volumes of lower dilutions (10⁻⁹–10⁻¹⁰) are analysed by IOME-RT-QuIC. Black indicates un-extracted 10⁻⁹–10⁻¹⁰ dilutions of CWD-positive brain. Maroon indicates increasing volumes of 10⁻⁸ IOME samples. Gold indicates increasing volumes of 10⁻¹⁰ IOME samples.
of the analysed brain samples. RT-QuIC AFR (mean and standard deviation) show that IOME of brain-homogenate dilutions of $10^{-9}$ and $10^{-6}$ produced results similar to un-extracted samples; however, IOME performed on dilutions of $10^{-7}$–$10^{-10}$ yielded faster rates and more positive replicates than unenriched samples (Fig. 1a). Analysis of brain-homogenate dilutions demonstrated that IOME not only increases sensitivity, as indicated by more positive replicates and at a faster rate at higher dilutions, but narrowed the range/variance versus un-extracted serial dilutions (Fig. 1b). Both the $10^{-7}$ and $10^{-8}$ dilutions in the IOME samples have a narrower and statistical variance than the un-extracted samples; difference in variance was determined by $f$-test.

In order to maximize sensitivity potential, we performed IOME on larger volumes (0.5–2.0 ml) of the higher brain-homogenate dilutions ($10^{-9}$ and $10^{-10}$). We observed an increased number of positive replicates and increased reaction rates, demonstrating that extraction of larger volumes of dilute prion sources can increase detection (Fig. 1c). Overall, it appeared that IOME-RT-QuIC extraction enhanced detection approximately 100-fold and offered the greatest advantage in those samples/dilutions with lower prion concentration. Given this framework, we proceeded to test other biological samples wherein CWD prions are present in much lower concentration than in brain or lymphoid tissues.

To expand upon the findings of increased sensitivity shown with diluted brain material, we subjected urine and saliva samples to IOME-RT-QuIC. We used 29 blinded saliva (100 µl saliva/400 µl 1× PBS) and 15 blinded urine (500 µl neat) samples previously assayed by RT-QuIC with NaPTA enrichment (Henderson et al., 2015b) and they were analysed via IOME-RT-QuIC. We tested samples that were sorted into three groups by NaPTA results obtained previously: strongly positive (>40 % positive replicates), intermediately positive (>25 % positive replicates) and negative, as well as unoinoculated negative control samples (Fig. 2a–c). In all three groups, the samples analysed by IOME-RT-QuIC had levels of detection equivalent to our previous results using NaPTA precipitation (Fig. 2a–c). Thus, IOME-RT-QuIC successfully discriminated known positive from negative samples, and confirmed that varying levels of prions are present in individual samples, as suggested by previous work (Henderson et al., 2015b). Likewise, samples from CWD-inoculated deer that were previously negative by NaPTA precipitation were also negative by IOME-RT-QuIC (Fig. 2c).

From the above experiments, it appeared that IOME-RT-QuIC was adept at extracting very low seeding potential from dilute biological fluids. We therefore tested cerebrospinal fluid (CSF) due to its exemplar applicability as a diagnostic sample. Earlier work with cervid CSF assayed by RT-QuIC produced a 50 % correlation rate with immunohistochemistry (IHC) on the brainstem of the same CWD-infected animal (Haley et al., 2013). To expand upon that study, we assayed blinded CSF samples from five terminal CWD-positive and two terminal CWD-negative deer, using either 10 µl (un-extracted) or 10 µl (diluted 1:50) with IOME. All CSF samples from CWD-inoculated deer examined using IOME-RT-QuIC were robustly positive and yielded approximately the same results as un-extracted samples (Fig. 3a). One replicate from the un-extracted, CWD-negative controls generated a false-positive replicate (Fig. 3a).

Longitudinal analysis of CSF throughout the disease course of CWD has not been performed. To simulate an earlier state of disease wherein prion levels are likely to be at lower concentration, we diluted three different terminal CSF samples to a $10^{-3}$ dilution. To investigate whether extraction from larger fluid volumes with IOME-RT-QuIC would enhance sensitivity, each CSF dilution was first assayed directly as 2 µl of un-extracted sample and then as a $10^{-3}$ dilution using IOME-RT-QuIC and increasing volumes of each dilution (0.05–1.0 µl). In the first CSF sample, 1/8 replicates were positive in both the un-extracted sample and the extracted 50 µl sample. As the volume was increased to either 0.5 or 1.0 µl and assayed by IOME-RT-QuIC, all the replicates were positive (blue circles) (Fig. 3b). The second sample had three positive replicates for the un-extracted analysis of the $10^{-3}$ dilution and also increased to 100 % positive replicates by increasing volume and IOME-RT-QuIC (red circles) (Fig. 3b). The third sample had very low seeding activity at a $10^{-3}$ dilution and was marginally better when 1.0 µl was assayed by IOME-RT-QuIC (black circles) (Fig. 3b). IOME-RT-QuIC analysis of larger volumes of negative samples did not result in any false-positive replicates (data not shown).

Next, we applied IOME-RT-QuIC to faeces from terminal CWD-infected and control deer. Faecal samples have traditionally been difficult to assay by any method due to the complexity of the material present (Pulford et al., 2012; Krüger et al., 2009; Saborio et al., 2001). Blinded faecal samples from six CWD-positive and three CWD-negative deer were analysed, with or without IOME-RT-QuIC. Faecal samples were made into a 10 % wt/vol. homogenate with 1× PBS and 500 µl was applied to 2 µl of iron oxide beads. Beads were washed one time with 1× PBS prior to being resuspended in 10 µl 0.1 % SDS and loaded into 4 wells of a RT-QuIC reaction mixture. A total of 16 replicates from each blinded sample were analysed in four independent experiments and AFRs were calculated. Five of six (83 %) samples from CWD-infected deer were positive (*$P<0.05$) when compared with the negative controls as determined by non-parametric Mann-Whitney analysis. Negative controls had a low false-positive rate of 6.25 % (3/48) (Fig. 3c). By comparison, only one of six (16.6 %) un-extracted faecal samples from these animals demonstrated positive reaction rates with no false positives (Wilcoxon-signed rank test with a assumed value of 0 for negative controls) (Fig. 3d). Thus, IOME-RT-QuIC is the first demonstration of RT-QuIC-based detection of CWD prions in faecal samples.
Fig. 2. IOME enables detection of prions in saliva and urine. (a) Highly positive saliva and urine samples were analysed by IOME-RT-QuIC. (b) Intermediately positive saliva and urine samples identically assayed as above. (c) Urine and saliva were analysed by RT-QuIC from either uninfected animals (grey-shaded regions) or infected, but previously assayed as negative animals. Dotted lines represent thresholds of 20, 40 and 60 h, for reference. Circles represent the AFRs for individual replicates. PO, per os; A, aerosol (+); AN, aerosol negative.
Rapid, sensitive, specific detection of low levels of prions in tissues and body fluids/excreta in the early or carrier stages of infection would facilitate a better understanding of prion disease pathogenesis and therapy research. The propensity for prions to bind to metal surfaces has been well known dating to the iatrogenic transmission of CJD through contaminated stainless steel surgical instruments, even after sterilization (Gibbs et al., 1994; Bernoulli et al., 1977). The present work builds on that of other prion researchers who have employed antibody-conjugated and untreated steel or iron beads for prion activity amplification (Edgeworth et al., 2011; Orrú et al., 2011; Miller & Supattapone, 2011). It should also be noted that other investigators have observed that the use of protein coating and PrP-specific antibodies on beads may inhibit binding and prion amplification (Müller-Schiffmann et al., 2009). Phosphotungstic acid precipitation has also been shown as effective for concentrating prions from brain, peripheral tissues and excreta prior to detection via RT-QuIC (Elder et al., 2013; Henderson et al., 2015b; Wadsworth et al., 2001); however, NaPTA precipitation has limitations with more complex matrices such as faeces. In the present study, we report that iron oxide paramagnetic bead extraction combined with RT-QuIC increased prion detection sensitivity up to 100-fold in higher dilutions of brain, but offered perhaps its greatest advantage in enriching prions from clinically relevant fluid/excreta samples. We also demonstrate the first RT-QuIC-based method for the detection of CWD prions in faeces with the IOME-RT-QuIC approach.

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Fig. 3. IOME-RT-QuIC increases detection sensitivity in CSF and faecal samples from CWD prion-infected deer. (a) CSF samples from terminally positive CWD-infected deer were analysed either un-extracted (2 µl; blue) or with IOME-RT-QuIC (10 µl diluted in 500 µl of PBS; black). Each technique was similarly effective. (b) Three terminally positive CSF samples were diluted to 10⁻³ and analysed either un-extracted (2 µl) or IOME (2 µl of beads incubated with 0.05 ml, 0.50 ml or 1.0 ml of the indicated dilutions). An increase in sensitivity was seen as a larger volume was extracted, as previously observed with brain dilutions. (c) Terminal faecal samples were processed by IOME and analysed by RT-QuIC for six positive (A1-A6) and three uninfected deer (pooled in the negative column) (blue). AFRs are plotted for each of 16 replicates (*P<0.05). A total of 3 of 48 replicates from negative animals were positive by IOME-RT-QuIC (6.25% false-positive rate; two from one animal, one from one, zero from one). (d) 2 µl of un-extracted 10% faecal homogenates were analysed by RT-QuIC for the same samples as above (black). Two of six animals were positive by the same statistical analysis (*P<0.05).
Our previous studies have shown that prions are present at very low levels in body fluids/excreta and thus present significant challenges for comparison with analysis of nervous tissues (Haley et al., 2009; Elder et al., 2015; Henderson et al., 2015a). In the present work, we found that the advantage offered by IOME-RT-QuIC had to do with the ability to simply and efficiently extract prions from the inhibiting activities commonly present in excreta samples. In comparing IOME-RT-QuIC with NaPTA-RT-QuIC analyses of saliva and urine samples from CWD-infected deer, we found the results from these two assay protocols were concordant, with similar sensitivity, but that IOME improved assay consistency (reduced range among replicates). These results could infer that IOME may preferentially bind prions from complex matrices, perhaps reducing potential assay inhibitors. We tested this theory on faeces, which has traditionally presented significant problems for in vitro prion-amplification assays (Pullord et al., 2012; Tamgâney et al., 2009). Despite higher false-positive rates compared with other excreta assays, the IOME protocol resulted in a statistical significance difference between faecal samples from CWD-positive versus -negative deer. While additional studies to expand this test population and improve sensitivity and specificity are in process, these findings are the most successful we have experienced for detection prions in faeces by in vitro methods.

CSF is potentially one of the most important clinically relevant samples for early detection of protein-misfolding diseases. Our previous work on CSF from CWD deer and elk yielded less than 50% correlation between IHC positivity in the brainstem and CSF positivity by PMCA or RT-QuIC (Haley et al., 2013). By contrast, IOME-RT-QuIC produced 100% concordance of CSF with brain IHC results but on a limited sample size. To simulate early infection, CSF samples were serially diluted and assayed at increasing volumes to yield a substantial increase in detection sensitivity. As CSF represents an immuno-privileged site, fewer assay inhibitors may be present. Therefore, very low prion concentrations may be enriched from larger sample volumes, potentially making IOME useful as an early disease-state diagnostic assay.

Finally, in light of the recent detection of amyloid seeding activity in nasal swabs from CJD patients (Orrù et al., 2014), we suggest that IOME may enhance the sensitivity of prion detection in such samples. Further research regarding use for nasal swabs and other biological samples will be needed to assess increases in sensitivity that may be achieved with IOME-RT-QuIC.

In summary, IOME-RT-QuIC offers the utility of a simple, versatile method that can detect and quantify prion seeding activity in an array of complex biological samples containing very low concentrations of prions. This method may thereby be useful in diagnostic and investigational contexts for prion, and potentially other protein-misfolding disorders.

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References


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